Molecular basis of ice-binding and cryopreservation activities of type III antifreeze proteins

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ABSTRACT
Antifreeze proteins (AFPs) can inhibit the freezing of body fluid at subzero temperatures to promote the survival of various organisms living in polar regions. Type III AFPs are categorized into three subgroups, QAE1, QAE2, and SP isoforms, based on differences in their isoelectric points. We determined the thermal hysteresis (TH), ice recrystallization inhibition (IRI), and cryopreservation activity of three isoforms of the notched-fin eelpout AFP and their mutant constructs and characterized their structural and dynamic features using NMR. The QAE1 isoform is the most active among the three classes of III AFP isoforms, and the mutants of inactive QAE2 and SP isoforms, QAE2ACT and SPACT, displayed the full TH and IRI activities with respect to QAE1 isoform. Cryopreservation studies using mouse ovarian tissue revealed that the QAE1 isoform and the active mutants, QAE2ACT and SPACT, more effectively preserved intact follicle morphology and prevented DNA double-strand break damage more efficiently than the inactive isoforms. It was also found that all active AFPs, QAE1, QAE2ACT, and SPACT, formed unique H-bonds with the first 310 helix, an interaction that plays an important role in the formation of anchored clathrate water networks for efficient binding to the primary prism and pyramidal planes of ice crystals, which was disrupted in the inactive isoforms. Our studies provide valuable insights into the molecular mechanism of the TH and IRI activity, as well as the cryopreservation efficiency, of type III AFPs.

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1. Introduction
Antifreeze protein (AFP) was first isolated from an Antarctic fish in 1969 [1] and since then has been found in various organisms living in polar regions including fish, insects, plants, bacteria, and fungi [2–6]. The primary indicator for the antifreeze activities of AFPs is thermal hysteresis (TH), which gives rise to a difference between the freezing point (Tf) and melting point (Tm) [2–7]. The mechanism of AFP’s TH activity involves binding to ice crystals and inhibiting their growth, and thus AFP is called an ice-binding protein (IBP); such activity results in ice recrystallization inhibition (IRI) [8]. Ice recrystallization produces large ice crystals by growth of small ice crystals, and this is fatal to cells. However, AFPs prevent the recrystallization process through IRI activity, resulting in a cryoprotective effect especially from cell membrane damages [9–11].

Despite the beneficial effects of TH activity, most studies about AFPs have focused on the basic principles of the TH activity rather than its biological applications. Moreover, only a few studies have been conducted on the role of AFPs in cryopreservation of red blood cells, oocytes, embryos, and ovaries [11–14]. Preservation of fertility after cancer treatment has become an important health

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Zoarces elongatus Kner could be converted into active mutants: alanine-rich surfaces, which bind to the pyramidal and primary prism planes of AFP (residues 9–21 and 41–44 in Fig. 1A) consists of two adjacent human and others [19–21]. Oocyte, embryo, and ovarian tissue (OT) cryopreservation are clinically available options, but improvement of the survival and function of cryopreserved cells and tissue is still necessary. Three previous studies reported beneficial effects of AFPs on ovarian tissue quality after the vitrification-warming process in mouse models [13–15]. However, the exact mechanism and molecular basis of the protective effects have not been fully investigated.

Ovarian tissue cryopreservation has been widely used to preserve fertility in women. It can be applied by either slow freezing or vitrification. In the slow freezing, tissue is slowly frozen to approximately –80 °C followed by the storage of the tissue in liquid nitrogen at −196 °C [16–18]. Slow freezing shows a limitation which has potential risk for ice crystal formation in the ovarian tissue that may occur mechanical damage to the cells. On the other hand, vitrification is an instant solidification of the solution as a result of increased viscosity during cooling with higher concentrations of cryoprotectants compared with that of used in slow freezing. Notably, vitrification does not make an ice crystal theoretically. Many studies have demonstrated that vitrification is a useful option to cryopreserve ovarian tissue in mouse, sheep, human, and others [19–21].

Fish AFPs are grouped into four types on the basis of their structures: α-helical type I AFPs, cysteine-rich type II AFPs, ~7-kDa globular type III AFPs, and glutamate/glutamine-rich α-helical type IV AFPs [2–6]. Type III AFPs are compact globular proteins [22] and have been categorized into two subgroups quaternary-amino-ethyl (QAE), further divided into QAE1 and QAE2 subgroups, and sulfopropyl (SP) sephadex-binding isoforms based on differences in their isoelectric points. The QAE1 isoform, which exhibits TH and IRI effects similar to those of its paralogue QAE1 (QAE1ACT), has a much higher TH activity than QAE2 and QAE1INACT, which bears the substitutions V9Q/V19L/G20V or V9Q/V19L/G20V/I42V, was structurally compared with QAE1 and QAE2 [25]. Vice versa, the inactive mutants of the QAE1 isoform (QAE1INACT), which bears the substitutions V20G, L19V/V20G, or Q9V/L19V/V20G, had no TH activity [27]. The active QAE1 isoform and QAE2ACT mutant showed unusual intermolecular hydrogen bonding (H-bonding) interactions between the T18 side-chain and the surface water molecule, whereas this interaction was not observed in the inactive QAE2 isoform and QAE1INACT mutants [26,27]. It has been suggested the anchored clathrate water (ACW) mechanism in which the AFP exhibited H-bonding interactions with an array of surface waters that three-dimensionally match to both the pyramidal and primary prism planes of the ice crystal [28]. Recently, Mahatabuddin et al. reported that in type III AFP, the two ice-like water clusters also exhibited a space-match to the primary prism and pyramidal ice planes like ACW [29]. In the ACW mechanism, the AFP–water interaction can be assisted by the hydrophobic interaction via side-chain groups of IBS, which might regulate the TH and IRI activities of AFPs [28,29]. However, the detailed molecular mechanism by which AFPs exhibit cryopreservation effects through TH and/or IRI activities is not well understood and requires not only further biophysical characterization but also further studies of its biological applications.

NMR is a powerful technique for the study of structure, dynamics, and folding of biomolecules such as nucleic acids [30] and proteins [31]. Hydrogen exchange measurement using NMR provides residue-specific information on the thermodynamics and kinetics of H-bonded secondary structure and exposure to solvent water. NOE cross-peaks of the exchangeable protons with water resonance can be used to determine their chemical exchange with solvent water on the s–ms timescale. Recently, in AFPs, NOE data of the amide protons suggested the existence of unusual intermolecular H-bonding interactions between the AFPs and the surface water molecule [26,27].

To understand the molecular basis of the physical and biological function of type III AFPs, we have compared three wild-type (wt) AFPIII isoforms from Zoarces elongatus Kner (notched-fin eelpout) (nfeAFP) with their mutants bearing triple mutations of IBS residues.
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For the functional assays, the purified proteins were dissolved in a buffer containing 10 mM sodium phosphate (pH 6.0) and 0.1 M NaCl, USA. For the functional assays, the purified proteins were dissolved in a buffer containing 10 mM sodium phosphate (pH 6.0) and 0.1 M NaCl. The concentrations of all proteins were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Purified protein solutions were standardized (with each residue number; nfeAFP8, N23 – A88; nfeAFP11 N23 – A88; nfeAFP6, G23 – K88.

of their structural and dynamical properties studied by NMR provide valuable insights into the molecular mechanisms of the TH and IRI activity as well as cryopreservation efficiency of type III AFPs.

2.2. TH measurements

Details of TH measurements have been described in a previous report [27]. The TH of the wt and mutant nfeAFPs was measured using a nanoliter osmometer (Otago, New Zealand) attached to the stage of a conventional microscope (Zeiss AxioLab, Germany), and then were flash-frozen at −30 °C for 5 min. After complete freezing, the temperature was increased up to −6 °C and held for 30 min and 240 min to allow recrystallization. The coverslips were viewed on the stage using an attached microscope (×100).

Five-week-old B6D2F1 female mice (Orient Co., Seongnam, South Korea) were housed under a 12-h light/dark cycle at 22 °C and fed ad libitum. Retrieved OTs from the mice (n = 139) were randomly allocated into a sham control (vitrification and warming of OTs without any AFPs) and six experimental groups (wt AFPs nfeAFP6, nfeAFP8, nfeAFP11; mutant forms nfeAFP6_tri, nfeAFP8_tri, and nfeAFP11_tri). All experimental procedures were performed under the approval of the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital (Approval no.: BA1707-227/062-01).

The IRI of the wt and mutant nfeAFPs was measured using the following equation: 10 mg/ml) was placed between two circular 12 mm diameter, coverslips, which were gently blotted dry to remove any excess fluid on the surface. The coverslips were placed on the center of a temperature-controlled freezing stage of a nanoliter osmometer (Otago, New Zealand) attached to the stage of a conventional microscope. The TH activity of the wt and mutant nfeAFPs was measured using a nanoliter osmometer (Otago, New Zealand) attached to the stage of a conventional microscope (Zeiss AxioLab, Germany), and then were flash-frozen at −30 °C for 5 min. After complete freezing, the temperature was increased up to −6 °C and held for 30 min and 240 min to allow recrystallization. The coverslips were viewed on the stage using an attached microscope (×100).

2.4. Experimental animals and ethics statement

In accordance with the optimal mouse OT vitrification protocol, vitrification was performed by two-step procedures as previously described [14,37–40]. For the first step, OTs were placed in an equilibration solution consisting of 7.5% (v/v) ethylene glycol (EG; Sigma-Aldrich, St. Louis, MO, USA) and 7.5% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich) in Dulbecco’s phosphate-buffered saline (D-PBS, WelGene, Daegu, Korea) with 20% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) for 10 min at room temperature (RT). In the second step, equilibrated OTs were moved to the vitrification solution containing 20% EG, 20% DMSO, and 0.5 M sucrose (Sigma-Aldrich) in D-PBS with 20% FBS and placed for 5 min at room temperature. To improve heat conductivity, the OTs were loaded onto an electron microscopic copper grid and the residual solution was removed as previously described [38,41]. Then the OTs were put into 1.8 ml cryovials (Nunc, Roskilde, Denmark) and directly plunged into liquid nitrogen. The OTs were cryopreserved for more than one day. To warm the OTs, tissues were immersed into four steps of serially diluted sucrose media (1.0 M, 0.5 M, 0.25 M, and 0 M sucrose in D-PBS with 20% FBS) in a step-wise manner at RT for 5 min. For each experimental group, 10 mg/mL of the selected nfeAFP was added to the vitrification solution (2nd step of vitrification) and the first step of the warming solution (1 M sucrose solution) as previously described [14].
2.6. Ovarian follicle classification and morphological analysis

All OTs were fixed with Bouin’s solution (Sigma-Aldrich) for more than one day, and the OTs of each group were embedded into paraffin blocks. The blocks were serially sectioned at 5-μm thickness. For hematoxylin and eosin (Merck, Darmstadt, Germany) staining, sections at 100-μm intervals were stained and used for ovarian follicle classification and apoptosis detection as described in previous studies [16,42].

The developmental stages of ovarian follicles were classified as follows: 1) primordial, a single layer of flattened pre-granulosa cells; 2) primary, a single layer of granulosa cells, one or more of which is cuboidal; 3) secondary, two or more layers of cuboidal granulosa cells, without the antrum; or 4) antral, multiple layers of cuboidal granulosa cells with the antrum [37,41,43,44].

The morphological integrity of the follicle was graded as follows: 1) primordial/primary follicle: grade 1 (G1), spherical with even distribution of the granulosa cells; grade 2 (G2), granulosa cells are pulled away from the edge of the follicle, but the oocytes are still spherical; and grade 3 (G3), pyknotic nuclei, misshapen oocytes, or vacuolation; and 2) secondary/antral follicle: G1, intact spherical follicle with evenly distributed granulosa and theca cells, small spaces, and spherical oocytes; G2, intact theca cells, disrupted granulosa cells, and spherical oocytes; and G3, disruption and loss of granulosa and theca cells, pyknotic nuclei, and missing oocyte. Under light microscopy (Nikon, Tokyo, Japan) at 400× magnifications, only follicles with visible oocytes were counted and classified to avoid miscounting. Since primordial follicles are an important indicator of ovarian reserve, their integrity was assessed separately. In the present study, we conducted this experiment at least five times to evaluate the statistical significance.

2.7. Evaluation of follicle apoptosis

Apoptosis of ovarian follicles was evaluated by TUNEL assays using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) as previously described [39]. Briefly, the tissue sections were deparaffinized, rehydrated and incubated with ready-to-use proteinase K solution (Dako, Hovedstaden, Denmark) for 15 min at room temperature. Following incubation, the slides were treated with TUNEL reaction mixture in a humidified dark chamber for 1 h at 37 °C. After that, the slides were mounted using the VECTASHIELD® Mounting Medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and examined under the inverted Zeiss AX10 microscope (Carl Zeiss, Oberkochen, Germany) at 400× magnification. The cells that had fragmented DNA emitted green fluorescence, while normal cells emitted blue fluorescence. Follicles with over 30% of apoptotic-positive cells or oocyte nuclei (emitting a green signal) were regarded as follicles with DNA DSBs. In the same way, follicles containing at least one Rad51 (+) nucleus were regarded as follicles with DNA repair [14,40,45,46].

2.9. Statistical analysis

The chi-square test was performed for follicle integrity, apoptosis and DNA DSB/repair ratios using the SPSS version 12.0 software (SPSS Inc., Chicago, IL, USA). The results were considered significantly different when the P value was < 0.05.

2.10. NMR experiments

For NMR experiments, the purified proteins were concentrated to 1 mM in a 90% H2O/10% D2O buffer containing 10 mM sodium phosphate (pH 6.0) and 100 mM NaCl. All of the 1H, 13C, and 15N NMR experiments were performed on an Agilent DD2 700-MHz spectrometer (GNU, Jinju, Korea) or a Bruker Avance-III 800-MHz spectrometer (KBSI, Ochang, Korea) equipped with a triple-resonance cryogenic probe. All three-dimensional (3D) triple-resonance experiments were carried out with 1 mM 13C, 15N-labeled wt nfeAFP11 and wt nfeAFP6. All 2D 1H/11N-HSQC, 3D NOESY-1H/15N-HSQC and 3D TOCSY-1H/15N-HSQC spectra for wt and mutant nfeAFPs were obtained using a 1 mM 15N-labeled protein samples. 2D and 3D data were processed with NMRPIPE [47] and analyzed with SPARKY [48].

The 1H, 13C, and 15N backbone resonance assignments for wt nfeAFP11 and wt nfeAFP6 were obtained from the following 3D experiments: CACB(CO)NH, HNCACB, HNCO, NOESY-1H/15N-HSQC, and TOCSY-1H/15N-HSQC. The 1H and 15N backbone resonance assignments for nfeAFP11_tr and nfeAFP6_tr were obtained from the 3D NOESY-1H/15N-HSQC and TOCSY-1H/15N-HSQC experiments. The 1H/15N chemical shift changes (Δδavg) were calculated using the following equation:

\[
\Delta \delta_{\text{avg}} = \sqrt{(\Delta \delta_H)^2 + (\Delta \delta_N/5.88)^2}
\]

where ΔδH and ΔδN are the 1H and 15N chemical shift changes between the wt and mutant nfeAFPs, respectively. Backbone dynamics parameters, including longitudinal R1 relaxation rates, transverse R2 relaxation rates, and 1H-15N heteronuclear NOEs were measured using 15N-labeled wt and mutant nfeAFPs at 5 °C. R1 values were measured in a series of spectra with relaxation delays of 50, 100, 150, 200, 250, 300, 400, 500, and 600 ms. R2 measurements were taken with relaxation delays of 10, 30, 50, 70, 90, 110, 130, 150, 190, and 230 ms. For evaluation of 1H-15N heteronuclear NOE values, three different data sets with and without an initial proton saturation (2.5-s period) were measured.

3. Results

3.1. TH activity of nfeAFPs

Previous study [27] reported that an QA2 isoform (wt nfeAFP8) from Zoarces elongatus Kner shows full TH activity (Fig. 2). A QA2E8K mutant of the QA2E isoform (nfeAFP11_tr), which bears substitution V9Q/V19L/G20V (Fig. 1C), exhibits TH and IRI effects similar to those of its parologue QA1E (Fig. 2) [25]. In this study, a triple mutant (SP57T) of the inactive SP isoform (nfeAFP6_tr) was prepared by P19, A20, and G42 on the IBS with the corresponding residues (L19, V20, and S42) of the QA1E isoform (Fig. 1D). Interestingly, this mutant exhibits remarkable TH activity, similar to that of wt nfeAFP8 (Fig. 2). As the temperature decreased below Tm, the crystal growth of the ice bipyrarnid in nfeAFP6_tr solution
was strongly inhibited (the sixth row of the third column in Fig. 2A). However, wt nfeAFP11 and wt nfeAFP6 are incapable of stopping the growth of an ice bipyramid below $T_m$ (Fig. 2A). The bursting elongation of wt nfeAFP11 and wt nfeAFP6 was accompanied by rapid crystal expansion (the third and fifth rows of the third column in Fig. 2A). Thus, these two isoforms had much smaller TH values ($= |T_m - T_{burst}|$) than wt nfeAFP8, nfeAFP11_tri, and nfeAFP6_tri, because $T_m$ ~ $T_{burst}$, where $T_{burst}$ is the bursting temperature. The difference in the IBS is that the inactive isoforms, wt nfeAFP11 and wt nfeAFP6, have V/V/G/S and Q/P/A/G at positions 9/19/20/42, respectively, but the active AFPs, wt nfeAFP8, nfeAFP11_tri, and nfeAFP6_tri have Q/L/V/S. These results indicate that the sequence of the IBS is very important for the ACW network formation that is related to the TH activity of type III AFPs.

3.2. IRI activity of nfeAFPs

Fig. 3 shows IRI activities of the three wt isoforms of nfeAFP, nfeAFP8, nfeAFP11, and nfeAFP6, and their triple mutants, nfeAFP8_tri, nfeAFP11_tri, and nfeAFP6_tri. The first row of Fig. 3 indicates the polycrystalline ice created by rapid freezing at $-30^\circ$C; this is the expected result of IRI activity. Because the coverslips were flash-frozen at a higher temperature than $-70^\circ$C of previous report [35] ice crystals exhibited leaf shapes rather than round or bipyramidal shapes in contrast to the TH results as shown in Fig. 2A. At $-6^\circ$C, compared with the BSA control, all AFP samples displayed IRI over the first 30 min (second row of Fig. 3). However, after 4 h at $-6^\circ$C, the wt nfeAFP11, wt nfeAFP6, and nfeAFP8 (inactive mutant of nfeAFP8) demonstrated lower IRI activity with some ice crystal growth, whereas wt nfeAFP8 and the nfeAFP11_tri and nfeAFP6_tri mutants still exhibited significant IRI effects (third row of Fig. 3).

3.3. Morphological evaluation of ovarian follicles

In order to demonstrate that AFPs preserve tissue quality by preventing structural and genetic disruption, we added the different AFPs to the vitrification and warming solutions as indicated in Fig. 4A. Following the vitrification-warming process, the ovarian tissues were histologically analyzed. A total of 22,286 mouse ovarian follicles were evaluated for the proportion of morphologically intact (G1) follicles, both for all collected follicles and for the subset of primordial follicles. In terms of primordial follicles, the vitrified-warmed control showed the lowest proportion of intact follicles (54.1%); however, the proportions were significantly increased to a range of 61 – 70% in the AFP-treated groups, except for the nfeAFP8_tri group (58.7%) (Fig. 4A). Primordial follicles in OTs incubated with AFPs with high TH and IRI activity, i.e., wt nfeAFP8, nfeAFP6_tri, and nfeAFP11_tri, had significantly higher proportions of intact follicles than those incubated with inactive AFPs (wt nfeAFP8, wt nfeAFP11, and nfeAFP8_tri) (Fig. 4A).

Similarly, the proportion of total follicles that were morphologically intact was higher in the AFP-treated samples, with a range of 52 – 63%, whereas the proportion of intact follicles in the vitrified-warmed control was only 37.5% (Fig. 4A). As with the subset of primordial follicles, the proportion of intact total follicles was highest when treated with AFPs with high TH and IRI activity and lowest when treated with the inactive AFPs (Fig. 4A).

3.4. Evaluation of apoptotic follicle ratios

To evaluate the efficiency of cryopreservation of ovarian follicles, we assessed the ability of AFPs to prevent ovarian follicular apoptosis. Exposure to AFPs significantly decreased the proportion of apoptotic follicles (17 – 27%) compared with the vitrified-
warmed control (31.7%) as shown in Fig. 4B. Notably, the nfeAFP8-
treated OTs had the lowest proportion of apoptotic follicles
(Fig. 4B). In addition, the OTs treated with the QAE2ACT (nfeAFP11_tri)
and SPACT mutants (nfeAFP6_tri) showed significant differences from
the other nfeAFP-treated samples, which had no TH and IRI activity
(Fig. 4B).

3.5. Evaluations of DNA DSBs and repair

Next, we assessed the number of DNA DSBs, as indicated by
detection of γH2AX, and DNA repair activity, as indicated by
Rad51 staining, in the follicles of OTs treated with AFPs. The
nfeAFP8- and nfeAFP11_tri-treated samples had lower proportions
of γH2AX–positive follicles (43.6% and 48.9%, respectively), indi-
cating a relatively lower level of DSBs than the other groups (52 – 57%) (Fig. 4C). The nfeAFP8-treated group had a significantly
lower proportion of Rad51-positive follicles (43.0%) than the other
groups (49 – 53%) (Fig. 4D). Representative images of hematoxylin
and eosin staining, terminal deoxynucleotidyl transferase dUTP
nick end labelling (TUNEL) assays, and immunohistochemistry
for γH2AX and Rad51 are shown in Supplementary Data
Fig. S1.
3.6. Assignment of amide backbone resonances of wt and mutant nfeAFPs

To investigate the structural features of the AFP isoforms that give rise to their differing cryopreservative characteristics, NMR studies of wt nfeAFP8, nfeAFP8_tri, wt nfeAFP11, nfeAFP11_tri, wt nfeAFP6, and nfeAFP6_tri were performed to compare the effects of mutations in the IBS on the 3D structures of AFPS. Amide backbone resonance assignments for wt nfeAFP11 and wt nfeAFP6 were made by heteronuclear NMR experiments [NOESY-1H/15N-HSQC, TOCSY-1H/15N-HSQC, HNCACB, and CACB(CO)NH]. The amide backbone resonances of nfeAFP11_tri and nfeAFP6_tri were assigned by comparing their 3D NOESY-1H/15N-HSQC and TOCSY-1H/15N-HSQC spectra with those of wt nfeAFP11 and wt nfeAFP6, respectively. Amide backbone resonance assignments for wt nfeAFP8 and nfeAFP8_tri were previously reported [27]. Chemical shifts of all the assigned backbone resonances for wt nfeAFP8, nfeAFP8_tri, wt nfeAFP11, nfeAFP11_tri, wt nfeAFP6, and nfeAFP6_tri are listed in Supplementary Data Tables S1 – S3.

Fig. 5. Chemical shift differences between wt and mutant nfeAFPs. (A) The weighted average 1H/15N chemical shift changes (ΔDavg) between the wt and mutant nfeAFP8, nfeAFP11, and nfeAFP6. # symbols indicate the disappearance of the peak upon mutation. 3D structures of (B) nfeAFP8 (PDB id = 1HG7), (C) nfeAFP11 (PDB id = 2LX2), and (D) nfeAFP6 (PDB id = 1OPS) are shown. Dark purple color indicates the mutated residues in each nfeAFP isoform. Colors used to illustrate 1H/15N chemical shift changes (ΔDavg) between the wt and mutant nfeAFPs: red, blue, and green, >0.12 ppm; magenta, cyan, and yellow, 0.08–0.12 ppm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7. NOESY-1H/15N-HSQC spectra of wt nfeAFP6 and nfeAFP6_tri

Fig. 6 depicts the expansion of NOESY-1H/15N-HSQC spectra showing the NOE cross-peak of the amide protons of the first 3_10 helix and β2 strand in wt nfeAFP6 and nfeAFP6_tri. In the inactive wt nfeAFP6, the A20 – M22 amide protons showed NOE cross-peaks with the solvent water resonance but had no NOE interactions with the T18-H1 resonance (Fig. 6A). However, the active nfeAFP6_tri mutant displayed the T18-H1 resonance at 5.04 ppm, which showed significant NOE cross-peaks with the amide protons of T18 – M22 (Fig. 6B). A previous study found that the T18 – M22 amide protons in wt AFP8 showed significant NOE cross-peaks with the T18-H1 resonance at 5.09 ppm [27]. A similar result was observed for the active mutant of nfeAFP11, nfeAFP11_tri [26]. However, like nfeAFP6, the inactive nfeAFP11 isoform [26] and the nfeAFP8_tri mutant [27] do not have these NOE interactions. Instead, in these AFPS, the T18 amide protons showed NOE cross-peaks with a solvent water resonance [26,27]. These results indicate that the T18 side-chain of the active QAE1 isoform exhibits unique H-bonding interactions, which are disrupted in the inactive QAE2 and SP isoforms.

In wt nfeAFP6, the F34 side-chain showed strong NOE cross-peaks with the amide protons of residues C24 and K25 of the β2 strand (Fig. 6C). In addition, this side-chain also exhibited weak...
NOEs with the E23 amide proton (Fig. 6C). However, in nfeAFP6_tri, the E23 amide proton had no NOE cross-peak with the F34 side-chain (Fig. 6D). These results indicate that the F34 side-chain has different orientation in the nfeAFP6_tri compared to wt nfeAFP6. In addition, in wt nfeAFP6, the F34 and G24 amide protons showed the NH...NH NOE with the S4 and V6 amide protons, respectively (Fig. 6C), whereas nfeAFP6_tri did not show these NOEs (Fig. 6D). These NOE data indicate that the wt nfeAFP6 displays a distinct conformation of the first 3_10 helix and b2 strand compared to the active QAE1 isoform of nfeAFP6; these features are correlated with the unusual chemical shift changes of nfeAFP6 upon the P19L/A20V/G42S mutations described above. Taken together, this distinct conformation explains why the SP isoform exhibits a lower TH and IRI activity than the QAE1 isoform.

### 3.8. Backbone dynamics of wt and mutant nfeAFPs

Next, in order to understand why the QAE2 isoform displays low TH and IRI activity, even though its IBS is similar in conformation to the QAE1 isoform, we determined the longitudinal \( R_1 \) relaxation rates, transverse \( R_2 \) relaxation rates, and \( ^{1}H\)\(^{15}N \) heteronuclear NOEs for wt and mutant nfeAFPs (Supplementary Data Fig. S4). Interestingly, in the inactive isoform, wt nfeAFP11, the \( R_2 / R_1 \) ratio of G20 is significantly larger than the average value (Fig. 7A). We further analyzed the products \( R_1 R_2 \) to clarify the structural flexibility, because the \( R_2 / R_1 \) ratio has both the structural flexibility and anisotropic motion information [49]. The wt nfeAFP11 has much larger \( R_1 R_2 \) value for G20 with an NOE value of 0.86 ± 0.03 than the average value at a magnetic field strength

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**Fig. 6.** NOESY-\(^{1}H\)/\(^{15}N\)-HSQC spectra of the nfeAFP6s. Strip plots of the NOESY-\(^{1}H\)/\(^{15}N\)-HSQC spectra (mixing time: 160 ms) in (A, C) wt nfeAFP6 and (B, D) nfeAFP6_tri at 5 °C. The data show the cross-peaks aligned to the (A, B) T18, L19, A20/V20, M21, and M22 and (C, D) M22, E23, G24, K25, and F34 amide resonances. Wat indicates the resonance originating from the free water molecules. Rectangles in D show where cross-peaks are lost after the mutations are introduced.
of 16.4 T (Fig. 7B), indicating conformational flexibility of the first 3₁₀ helix on the µs – ms timescale. Similarly, the inactive mutant of nfeAFP8, nfeAFP8_tri, which was previously studied [27] also had a significantly large $R_1R_2$ value of G20 with an NOE value of 0.85 ± 0.04 at a field strength of 16.4 T (Fig. 7B). These results indicate that both the wt nfeAFP11 and nfeAFP8_tri exhibit conformational flexibility in the first 3₁₀ helix, which might explain their lower TH and IRI activity.

The inactive nfeAFP6 isoform does not display this kind of flexibility (Fig. 7A), perhaps because the P19 residue introduces backbone rigidity into the first 3₁₀ helix. In addition, the active mutants, nfeAFP11_tri and nfeAFP6_tri, also show no clear backbone conformational flexibility of the first 3₁₀ helix (Fig. 7).

4. Discussion

The type III AFPs are classified as QAE1, QAE2, and SP isoforms [23,24]. The QAE1 isoform is able to bind to both the primary prism and pyramidal planes of ice [50]. This isoform is capable of stopping the growth of ice crystals and exhibits full TH and IRI activity (Figs. 2 and 3). However, the QAE2 and SP isoforms can bind to only the pyramidal plane [50] and are incapable not only of stopping ice growth but also of inhibiting ice recrystallization (Figs. 2 and 3). Thus, the ability to bind to the primary prism plane of ice crystals is thought to be the very important for the TH and IRI activity.

The QAE2 isoform nfeAFP11 differs from the QAE1 isoform nfeAFP8 by four residues positioned at the prism plane binding...
surface (residues 9, 19, 20, and 41) (Fig. 1). A previous study revealed that replacement of residues 9, 19, and 20 in nfeAFP8 with the corresponding residues of nfeAFP11 converted the active isoform into an inactive AFP (called a QAE1\textsuperscript{INACT}) [27]. This QAE1\textsuperscript{INACT} (nfeAFP8\textsubscript{tri}) lost both TH and IRI activity (Figs. 2 and 3). Conversely, the inactive QAE2 isoform can be converted to a fully active AFP (called a QAE2\textsuperscript{ACT}) by mutation of the same three residues [25]. In the case of the SP isoform nfeAFP6, the prism plane binding surface has three residues (19, 20, and 42) that differ from the corresponding plane of nfeAFP (Fig. 1). The current study found that replacement of these residues in nfeAFP6 with the corresponding residues of nfeAFP8 converted it to a fully active SP\textsuperscript{ACT} (Figs. 2 and 3).

In order to connect the TH and IRI activities of AFPs and their biological effects, we also studied the effects of the wt and mutant nfeAFP8 on mouse OT cryopreservation. As expected, the three active AFPs (nfeAFP8, nfeAFP11\textsubscript{tri}, and nfeAFP6\textsubscript{tri}) preserved significantly higher proportions of intact ovarian follicles than the QAE2 and SP isoforms or the control solution (Fig. 4A). A similar pattern was also observed in the apoptosis data of ovarian follicles incubated with AFPs (Fig. 4B). This means that the specific mutations made to the inactive nfeAFP11 and nfeAFP6, which converted them into active AFPs, could not completely recover the protective capacity of wt nfeAFP8. For better understanding of the effect of AFPs on follicular apoptosis, more systematic biophysical and cryopreservation studies are required.

The mechanism that AFPs protect ovarian tissue during vitrification is not fully known. In the present study, mutants that has higher TH and IRI activity showed better cryoprotective results. TH activity is not the main mechanism of cryoprotection because ice formation can occur when temperature is decreased to below TH gap. During vitrification, theoretically ice formation does not occur thus ice recrystallization during warming is the main cause of cryodamage. Previous study showed that IRI during the warming procedure is critical to the cryoprotective outcomes of the vitrification-warming of ovarian tissue [15]. Other previous studies demonstrate that TH activity was not necessarily translated into improvements in the cryopreservation efficiency of biological samples [51,52]. Interaction with membrane is another possible cryoprotective mechanism of AFPs. One mechanism of cell damage at low temperature is thermotrophic phase transition partly due to weakened hydrophobic interactions. When the transition from liquid crystalline to gel phase occurred, cell membranes become leaky, resulting in cell death [53]. Rubinsky et al. proved AFPs protect cell membrane during hypothemic storage [54].

Cryodamage involves mechanical and osmotic stress and induces DNA DSBs followed by severe or lethal mutations of cells [55,56]. In this study using mouse OT, the proportions of γH2AX (+) follicles were significantly decreased in the active AFP-treated samples compared with the sham control-treated and the inactive AFP-treated samples. This study demonstrated that nfeAFP8 (high TH and IRI) was superior in protecting follicles form DNA damage, leading to decreased need for DNA repair during cryopreservation of OT. In the case of the nfeAFP6\textsubscript{tri} and nfeAFP11\textsubscript{tri} mutants, they had high TH and IRI activities but did not improve DNA damage/repair as much as nfeAFP8, and this could be due to the limitations of mutant AFPs. Only wt nfeAFP8 with high TH and high IRI activities showed remarkably improved outcomes in all evaluations in this study when compared to the vitrified-warmed control. These results suggest that supplementation of the vitrification and warming media for cryopreservation of OT with the native QAE1 isoform, especially nfeAFP8, would be a promising approach for patients who need to preserve their fertility.

In the present study, ovarian tissues were exposed to high concentration of cryoprotective agents (CPAs) which induce an inevitable osmotic shock in cells and tissue. Increased ROS generation is associated with osmotic stress in a variety of somatic cells as well as in sperm and may serve as a signaling mechanism responsible for the cell adaptive response [57,58]. Notably, cryopreservation process also creates abundant amount of ROS in oocytes, spermatozoa, somatic cells and tissues [12,58,59]. In our previous study, vitrification media supplemented with different types of AFPs decrease the ROS production in mouse oocyte and it may result in a reduction of DSBs [12]. Collectively, AFPs in cryopreservation and warming media might modulate osmotic injuries during ovarian tissue cryopreservation and eventually show the protective effects in terms of intracellular DNA damage. However, further studies how AFPs decrease ROS production would be required.

As described above, for the efficient cryopreservation of mouse OT achieved by supplementation of AFPs, the AFPs must stop the growth of ice crystals and efficiently inhibit ice recrystallization. The QAE1 isoform, which has high TH and IRI activities, can bind to both the primary prism and pyramidal planes of hexagonal ice crystals, whereas the QAE2 and SP isoforms bind solely to the pyramidal plane (see Fig. 8) [25,50]. Comparison of the conformation and dynamics of the QAE1 isoform with the inactive QAE2 and SP isoforms can explain the strong binding affinity of the QAE1 isoform to the prism plane of ice crystals (summarized in Fig. 8). First, in the QAE1 isoform, the L19 and V20 amide protons and the T18 γ- OH of the QAE1 isoform play an important role in anchoring water molecules (#78, #80, and #81 in Fig. 8), which might be considered the ACW cluster that space-match to the primary prism plane of the ice crystal. The same H-bonding interaction was also observed in the QAE2\textsuperscript{ACT} tetra-mutant (V90/V191/L20V/I41V) [25]. This structural feature is consistent with the observation of the T18-Hy1 resonance and absence of NOEs of the L19 and V20 amide protons with the solvent water resonance in wt nfeAFP8 [27]. In addition, the QAE2\textsuperscript{ACT} (nfeAFP11\textsubscript{tri}) [26] and SP\textsuperscript{ACT} mutants (nfeAFP6\textsubscript{tri}) (Fig. 6B) displayed the same NOE pattern as the wt nfeAFP8. In contrast to wt nfeAFP8, in several QAE1\textsuperscript{INACT} mutants of nfeAFP8 (V20G, L19V/V20G, and nfeAFP8\textsubscript{tri}), the T18-Hy1 resonance is absent and the NH of residues 19 and 20 showed significant NOEs with the solvent water [27]. Similar results were observed for the inactive QAE2 [26] and SP isoforms (Fig. 6A). This NOE pattern is consistent with the structural feature of the QAE2 isoform, in which the amide protons of residues V19 and G20 exhibited H-bond interaction with surface water molecule rather than the ACW network [25]. Recently, it has been reported that the efficiency of TH and IRI activities was determined by the binding affinity of AFPs to a wider area of ice crystal [60]. Taken together, it has been suggested that the disruption of ACW networks in the inactive QAE2 and SP isoforms as well as QAE1\textsuperscript{INACT} mutant reduces the AFP-binding area of ice crystal, resulting in loss of TH and IRI activity.

Second, in the inactive AFPs, QAE2 and QAE1\textsuperscript{INACT}, the first 3\textsubscript{10} helix, which constitutes the prism plane binding surface, showed a flexible backbone conformation compared to that of the QAE1 isoform (Fig. 8). The inactive AFPs, QAE1\textsuperscript{INACT} (nfeAFP8\textsubscript{tri}) and QAE2 (wt nfeAFP11) had a significantly larger R\textsubscript{1}/R\textsubscript{2} value for G20 than the average value (Fig. 7B), indicating conformational flexibility. The structural study revealed that, in the QAE2 isoform, the amide protons of residues V19 and G20 exhibited H-bond interaction with surface water molecule rather than the ACW network [25]. The larger R\textsubscript{1}/R\textsubscript{2} value of G20 can be explained by the chemical exchange between the surface water-bound and -unbound conformations. However, the active AFPs, such as QAE1 and QAE2\textsuperscript{ACT}, exhibited the unique H-bonding interaction with two ACW networks which space-match to the primary prism and pyramidal ice planes [25]. Thus, the QAE1 isoform as well as the active mutants, QAE2\textsuperscript{ACT} (nfeAFP11\textsubscript{tri}) and SP\textsuperscript{ACT} (nfeAFP8\textsubscript{tri}), show no clear backbone conformational flexibility of the first 3\textsubscript{10} helix.
The inactive SP isoform (wt nfeAFP6) does not display this kind of flexibility (Fig. 7B) because the P19 residue introduces backbone rigidity into the first 310 helix. It was concluded that, in the inactive AFPs, QAE1\textsuperscript{INACT} and QAE2, the flexible structure of the first 310 helix interferes with the H-bonding interaction between the IBS and the prism plane of ice crystals, thereby preventing full TH and IRI activity.

Third, the inactive SP isoform displays unusual backbone conformational differences in the \(\beta_1\) and \(\beta_2\) strands and the second 310 helix, which constitutes the prism plane binding surface, compared to the active QAE1 isoform (Fig. 8). In nfeAFP6\textsubscript{tri}, residues of the \(\beta_1\) and \(\beta_2\) strands and the second 310 helix presented significant chemical shift changes compared to wt nfeAFP6, even though these residues are far from the mutated residues (Fig. 5D). However, in the case of nfeAFP8\textsubscript{tri} and nfeAFP11\textsubscript{tri}, the triple mutations caused significant chemical shift changes in the residues located close to the mutated residues (Fig. 5). Taken together, it was concluded that the inactive SP isoform exhibits a distinct overall backbone conformation from the active QAE1 isoform, which disrupts the H-bonding interaction of the IBS with the prism plane of ice crystals for full TH and IRI activity.

5. Conclusions

In this study, we have studied the TH, IRI, and cryopreservation activities as well as the structural and dynamic features of the three isoforms of type III nfeAFPs and their IBS mutants. We found that, like the active QAE1 isoform, the QAE2\textsuperscript{ACT} and SP\textsuperscript{ACT} mutants of the inactive isoforms displayed the full TH and IRI effects. Cryopreservation studies of mouse OT revealed that the supplementation of preservation and warming media with the QAE1 isoform and QAE2\textsuperscript{ACT} and SP\textsuperscript{ACT} mutants showed more effective preservation of intact follicles and prevented DNA DSB damage more effectively than other inactive isoforms. NMR studies showed that the QAE1 isoform, including the QAE2\textsuperscript{ACT} and SP\textsuperscript{ACT} mutants, formed unique H-bonds within the first 310 helix that played an important role in the formation of ACW networks for efficient binding to the primary prism plane of ice crystals for full TH and IRI activity.
prism and primary planes of ice crystals, which was disrupted in the inactive isoforms. Our results suggest that the unique conformation of the IBS in the type III AFPs is modulated by specific mutations in the IBS and thus determines the degree of TH, IRI, and cryopreservation activity.

CRediT authorship contribution statement

Seo-Ree Choi: Conceptualization, Data curation, Formal analysis, Writing - original draft. Jaewang Lee: Data curation, Formal analysis, Writing - original draft. Yeo-Jin Seo: Conceptualization, Data curation, Formal analysis, Writing - original draft. Hyun Sun Kong: Data curation, Formal analysis. Minjae Kim: Data curation, Formal analysis. EunSeon Jin: Data curation, Formal analysis. Jung Ryool Lee: Conceptualization, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. Joong-Hwa Lee: Conceptualization, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] DeVries AL, Wohlschlag DE. Freezing resistance in some Antarctic fishes. [2] Lee HK, Lee HJ, Kim HK, Lee JH, Ko Y, Kim SM, et al. Effects of antifreeze proteins on the vitrification of mouse oocytes: comparison of three different antifreeze proteins. Hum Reprod 2015;30:2110–9. [3] Lee SM, Youm HW, Lee SJ, Park JH, Kim SY, CS, SM, CS. SH. Effect of antifreeze protein on mouse ovarian tissue cryopreservation and transplantation. Yonsei Med J 2015;56:778–84. [4] Lee J, Kim SK, Youm HW, Kim JH, Lee JR, Suh CS, et al. Effects of three different types of antifreeze proteins on mouse ovarian tissue cryopreservation and transplantation. PLoS One 2015;10:e0126252. [5] Kong HS, Kim EJ, Youm HW, Kim SK, Lee JR, Suh CS, Kim SH. Improvement in ovarian tissue quality with supplementation of antifreeze protein during warming of vitrified mouse ovarian tissue. Yonsei Med J 2018;59:331–6. [6] Gandolfi F, Palfoni A, Papassobrambilla B, Bonetti S, Brevini T, Ragni G. Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. Fertil Steril 2006;85:1150–6. [7] Jensen AK, Macklon KT, Fedder J, Ernst E, Humaidan P, Andersen CY. 86 successful births and 9 ongoing pregnancies worldwide in women transfused with frozen-thawed ovarian tissue: focus on birth and perinatal outcome in 40 of these children. J Assist Reprod Genet 2017;34:325–36. [8] Lee S, Ryu KY, Kim B, Kang D, Kim YY, Kim T. Comparison between slow freezing and vitrification for human ovarian tissue cryopreservation and xenotransplantation. Int J Mol Sci 2019;20:3346. [9] Amorim CA, Curaba M, Van Langendonckt A, Dolmans MM, Donnez J. Vitrification as an alternative means of cryopreserving ovarian tissue. Reprod Biomed Online 2011;23:160–86. [10] Fujihara M, Kameko T, Inoue-Murayama M. Vitrification of canine ovarian tissues with polyvinylpyrrolidone preserves the survival and developmental capacity of primordial follicles. Sci Rep 2019;9:3970. [11] Suzuki N, Yoshioka N, Takase S, Sugishita Y, Tamura M, Hashimoto S, et al. Sucrose-based fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency. Hum Reprod 2015;30:608–15. [12] Antson AA, Smith DJ, Roper DL, Lewis S, Caves LSD, Verma CS, et al. Understanding the mechanism of ice binding by type III antifreeze proteins. J Mol Biol 2001;305:875–89. [13] Dew CL, Wang NC, Joshi S, Fletcher GL, Scott GK, Hayes PH, et al. Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, Macrozoarces americanus. J Biol Chem 1988;263:12049–52. [14] Nishimaya Y, Sato R, Takamichi M, Miura A, Tsuda S. Co-operative effect of the isoforms of type III antifreeze protein expressed in Notch-fed eelpout, Zoarces elongatus Kner. FEBS J 2005;272:482–92. [15] Garnham CP, Nishimaya Y, Tsuda S, Davies PL. Engineering a naturally inactive isoform of type III antifreeze protein into one that can stop the growth of ice. FEBS Lett 2005;586:8376–81. [16] Kumeta H, Ogura K, Nishimaya Y, Miura A, Inagaki F, Tsuda S. NMR structure note: a defective isoform and its activity-improved variant of a type III antifreeze protein from Zoarces elongatus Kner. J Biol Chem 2013;255:225–30. [17] Choi S-R, Seo Y-J, Kim M, Eo Y, Ahn H-C, Lee A-R, et al. NMR study of the antifreeze activities of active and inactive isoforms of a type III antifreeze protein. FEBS Lett 2016;590:4202–12. [18] Garnham CP, Campbell RL, Davies PL. Anchored clathrate waters bind antifreeze proteins to ice. Proc Natl Acad Sci U S A 2011;108:7363–7. [19] Mahatubuddin S, Fukami D, Ara T, Nishimaya Y, Shimizu R, Shibazaki C, et al. Polynuclear-ice-like water networks emerge solely in an activated variant of ice-binding protein. Proc Natl Acad Sci U S A 2018;115:5456–61. [20] Choi S-R, Kim-N H, Jin H-S, Seo Y-J, Lee J-H. Base-pair opening dynamics of nuclear acids in relation to their biological function. Comput Struct Biotechnol J 2015;10:797-804. [21] Redfield C. Using nuclear magnetic resonance spectroscopy to study molten globule states of proteins. Methods 2004;34:121–32. [22] Kobashigawa Y, Nishimaya Y, Miura A, Ohgichi S, Miura A, Tsuda S. A part of ice nucleation protein exhibits the ice-binding ability. FEBS Lett 2005;579:1493–7. [23] Bravo LA, Griffith M. Characterization of antifreeze activity in Antarctic plants. J Exp Bot 2005;56:1189–96. [24] Gwak GS, Lee Jung W, Kim HJ, Kang S-H, Jin E. Antifreeze protein in Antarctic marine diatom, Chaetoceros neogracile. Mar Biotechnol 2010;12:972–37. [25] Jia Z, Davies PL. Antifreeze proteins: an unusual receptor–ligand interaction. Trends Biochem Sci 2002;27:101–6. [26] Cheung RCT, Ng TR, Wong JH. Antifreeze proteins from diverse organisms and their applications: an overview. Curr Protein Pept Sci 2017;18:262–83. [27] Bar Dolev M, Draskovsky I, Davies PL. Ice-binding proteins and their function. Annu Rev Biochem 2016;85:515–42. [28] Garnham CP, Gilbert JA, Hartman CP, Campbell RL, Laybourn-Parry J, Davies PL. A Ca²⁺-dependent bacterial antifreeze protein domain has a novel beta-helical ice-binding fold. Biochem J 2008;411:171–80. [29] Raymond JA, Christner BC, Schuster SC. A bacterial ice-binding protein from the Voskot ice core. Extremophiles 2008;12:713–7. [30] Lee SG, Koh HY, Lee JH, Kang S-H, Kim HJ. Cryopreservative effects of the recombinant ice-binding protein from the arctic yeast leucosporidium sp. on red blood cells. Appl Biochem Biotechnol 2012;167:824–34. [31] Lee HH, Lee HK, Kim JH, Lee JH, Ko Y, Kim SM, et al. Effects of antifreeze proteins on the vitrification of mouse oocytes: comparison of three different antifreeze proteins. Hum Reprod 2015;30:2110–9. [32] Lee SM, Youm HW, Lee SJ, Park JH, Kim SY, CS, SM, CS. SH. Effect of antifreeze protein on mouse ovarian tissue cryopreservation and transplantation. Yonsei Med J 2015;56:778–84. [33] Lee J, Kim SK, Youm HW, Kim JH, Lee JR, Suh CS, et al. Effects of three different types of antifreeze proteins on mouse ovarian tissue cryopreservation and transplantation. PLoS One 2015;10:e0126252.
Lundy T, Smith P, O’Connell A, Hudson NL, McNatty KP. Populations of granulosa cells in small follicles of the sheep ovary. Reproduction 1999;115:251–62.

Lee J, Lee JR, Youm HW, Suh CS, Kim SH. Effect of preoperative simvastatin treatment on transplantation of cryopreserved-warmed mouse ovarian tissue quality. Theriogenology 2015;83:285–93.

Youm HW, Lee J, Kim EJ, Kong HS, Lee JR, Suh CS, et al. Effects of Angiopoietin-2 on Transplanted Mouse Ovarian Tissue. PLoS One 2016;11:e0166782.

Youm HW, Lee JR, Lee J, Jee BC, Suh CS, Kim SH. Transplantation of mouse ovarian tissue: comparison of the transplantation sites. Theriogenology 2015;83:854–61.

Kong HS, Lee J, Youm HW, Kim SK, Lee JR, Suh CS, et al. Effect of treatment with angiopoietin-2 and vascular endothelial growth factor on the quality of xenografted bovine ovarian tissue in mice. PLoS One 2017;12:e0184546.

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax Ad. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR 1995;6:277–93.

Goddard TD, Kneller DG. SPARKY 3. San Francisco, CA: University of California; 2003.

Kneller JM, Lu M, Bracken C. An effective method for the discrimination of motional anisotropy and chemical exchange. J Am Chem Soc 2002;124:1852–3.

Takamichi M, Nishimiya Y, Miura A, Tsuda S. Fully active QAE isoform confers thermal hysteresis activity on a defective SP isoform of type III antifreeze protein. FEBS J 2009;276:1471–9.

Dijlje LLC, Meister K, DeVries AL, Duman JG, Guo S, Bakker HJ, et al. Blocking rapid ice crystal growth through nonbasal plane adsorption of antifreeze proteins. Proc Natl Acad Sci U S A 2016;113:3740–5.