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Original Research Article

Antagonist of sphingosine 1-phosphate receptor 3 reduces cold injury of rat donor hearts for transplantation

Eisho Kanemitsu [1,2], Xiangdong Zhao [1], Keiko Iwaisako [1,3], Asuka Inoue [4], Akihide Takeuchi [2,5], Shintaro Yagi [1,6], Hidetoshi Masumoto [7], Hiroaki Ohara [2], Motoyasu Hosokawa [2], Tomonari Awaya [2], Junken Aoki [4], Etsuro Hatano [1], Shinji Uemoto [1,8], Masatoshi Hagiwara [2,*]

[1] Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[2] Department of Anatomy and Developmental Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[3] Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan
[4] Laboratory of Molecular and Cellular Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Miyagi, Japan
[5] Department of Developmental Biology and Functional Genomics, Ehime University Graduate School of Medicine, Ehime, Japan
[6] Department of Surgery, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan
[7] Clinical Translational Research Program, RIKEN Center for Biosystems Dynamics Research (BDR), Kobe, Japan
[8] Shiga University of Medical Science, Otsu, Shiga, Japan

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ABSTRACT

Cold storage is widely used to preserve an organ for transplantation; however, a long duration of cold storage negatively impacts graft function. Unfortunately, the mechanisms underlying cold exposure remain unclear. Based on the sphingosine-1-phosphate (S1P) signal involved in cold tolerance in hibernating mammals, we hypothesized that S1P signal blockage reduces damage from cold storage. We used an in vitro cold storage and rewarming model to evaluate cold injury and investigated the relationship between cold injury and S1P signal. Compounds affecting S1P receptors (S1PR) were screened for their protective effect in this model and its inhibitory effect on S1PRs was measured using the NanoLuc Binary Technology (NanoBiT)-β-arrestin recruitment assays. The effects of a potent antagonist were examined via heterotopic abdominal rat heart transplantation. The heart grafts were transplanted after 24-hour preservation and evaluated on day 7 after transplantation. Cold injury increased depending on the cold storage time and was induced by S1P. The most potent antagonist strongly suppressed cold injury consistent with the effect of S1P deprivation in vitro. In vivo, this antagonist enabled 24-hour preservation, and drastically improved the beating score, cardiac size, and serological markers. Pathological analysis revealed that it suppressed the interstitial edema, inflammatory cell infiltration, myocyte lesion, TUNEL-positive cell death, and fibrosis. In conclusion, S1PR3 antagonist reduced cold injury, extended the cold preservation time, and improved graft viability. Cold preservation strategies via S1P signaling may have clinical applications in organ preservation for transplantation and contribute to an increase in the donor pool.

Introduction

Organ transplantation was developed as a treatment for fatal organ dysfunction. The duration of transplanting organs from the donor to recipient is critical to minimize cellular damage and preserve organ function. Since early times, the conventional method of cold preservation (at approximately 4°C) has been adopted as a gold standard owing to its simplicity and ability to reduce oxygen and energy consumption

Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; βarr, β-arrestin; BSA, bovine serum albumin; CK, creatinine kinase; DMEM, Dulbecco's modified Eagles medium; D-PBS, Dulbecco's phosphate-buffered saline; ECGS, endothelial cell growth supplement from bovine neural tissue; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GPCR, G protein-coupled receptors; HBSS, Hanks' balanced salt solution; HE, hematoxylin & eosin; HEK293, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPF, high power fields; HUEhT-2, HUVEC established by electroporation of pIRES-hTERT-hygr; HUVEC, human umbilical vein endothelial cell line; IVC, inferior vena cava; LDH, lactate dehydrogenase; LEW, Lewis rats; LgBiT, large biT fragment; MT, Masson's trichrome; NanoBiT, NanoLuc Binary Technology; PCR, polymerase chain reaction; PPI, protein-protein interaction; PV, pulmonary veins; qPCR, quantitative PCR; RT-PCR, reverse transcription–PCR; S1P, sphingosine-1-phosphate; S1PR, S1P receptors; SmBiT, small bit fragment; SPHK1, sphingosine kinase 1; SVC, superior vena cava; TUNEL, terminal deoxynucleotidyl transferase dUTP nick and labeling; UW, University of Wisconsin

* Reprint requests: Masatoshi Hagiwara, Department of Anatomy and Developmental Biology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho Sakyo-ku, Kyoto 606-8501, Japan

E-mail address: hagiwara.masatoshi.8c@kyoto-u.ac.jp (M. Hagiwara).

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At A Glance Commentary

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Background

For organ transplantation, the cold storage time of a harvested heart is limited, that is, only 4–6 hours. This leads to a decrease in the utilization of the donor graft.

Translational Significance

We focused on sphingosine-1-phosphate (S1P) involved in the cold tolerance of hibernating mammals and identified a novel S1P receptor 3 antagonist that protected the rat graft heart from cold injury. This antagonist could be effective as an organ preservation solution that extends the cold storage time of graft organs and improves the outcome of organ transplantation therapy.

levels.[1,2] Integrated approaches technically based on cold storage have been developed; these include cryopreservation, subzero cooling,[3,4] and various ranges of low temperature with perfusion.[5-7] However, despite efforts to maintain graft function, long cold exposure inevitably causes adverse effects such as dysfunction of the membrane and organelles.[8] Therefore, cellular damage increases in a time-dependent manner and organs can be kept viable only for a limited time under cold conditions. While organ transplantation remains the best treatment method for patients with advanced organ failure and the number of candidates is increasing worldwide, the supply of organ donors cannot keep pace with this demand. In particular, the shortage of hearts is worse owing to logistical limitations between the donor and recipient, which leaves many untreated patients struggling with poor quality of life and limited life expectancy. Because the preservation time for graft organs is limited from the 4–6 hours for heart to 18–24 hours for kidneys, the donor graft and recipient must be matched over relatively short geographic distances and time periods; this often results in the use of grafts that are immunologically not well-matched to recipients. This time constraint restricts donor organ usage and contributes to a high rate of graft discard of up to 26%–27% for abdominal organs and 70%–80% for thoracic organs of over 10,000 organ offers in the United States, many of which could have been successfully transplanted under the right circumstances.[2,9] Thus, innovative methods that decrease the adverse effects of cold storage and enable longer preservation are highly desirable. However, because graft damage during cold storage is often recognized as "(cold) ischemia reperfusion injury" shown after rewarming and inseparable from rewarming injury, scientific evidence of the protective effect of cold exposure and the disruptive adverse effect, called "cold injury," has received insufficient attention. New clues to elucidate the regulation of cold adaptation that reduces cold injury as well as a solution for longer preservation are required.

To this end, we focused on mammalian hibernation, which is known as a seasonal state of dormancy characterized by a controlled reduction in body temperature, reduced cellular metabolism, and decreased oxygen and energy consumption levels leading to "cold tolerance."[10] Small hibernators are able to survive under hypothermia equal to ambient temperature for a few days and occasional rewarming; thus, they are ideal models to elucidate the mechanism of cold tolerance for organ preservation.[11,12] Molecular mechanisms of cold tolerance to adapt to the cold and protect hibernators from cold injury are still largely unknown; however, recent studies have revealed a close association between cold tolerance and sphingolipids, which are a class of lipids containing a backbone of sphingoid bases, a set of aliphatic amino alcohols that includes sphingosine.[13-15] Sphingosine-1-phosphate (S1P) is a type of sphingolipid that plays an essential role in neurogenesis and lymphocyte trafficking through G protein-coupled receptors (GPCRs) of S1PRs (S1PR1-5)[16] as well as in the modulation of an array of prosurvival cellular processes.[17] Recent studies indicate that S1P levels decrease in a temperature-dependent manner when mammalian hibernators enter into torpor, leading to lymphopenia, which is one of the hallmarks of hibernation,[13,14] and the administration of a S1PR antagonist, W146, induces lymphopenia in mice.[15] These data suggest that the deprivation of S1P or administration of S1PR antagonist is a key regulator to induce cold tolerance and protect from cold injury. Here, we attempted to reduce cold injury in human cells and isolated rat hearts by focusing on S1PR signaling to extend the cold preservation time and achieve improved graft function.

Materials and Methods

Reagents and compounds

MCDB131 medium, FBS, Glutamax, HEPES, and Opti-MEM were purchased from ThermoFisher (Waltham, MS). Dulbecco's modified Eagle's medium (DMEM, 1.0 g/L glucose), penicillin-streptomycin mixed solution, protease inhibitor, phosphatase inhibitor cocktail, and Blocking One were purchased from Nacalai Tesque (Kyoto-city, Kyoto, Japan). Heparin sodium was purchased from Mochida Pharmaceutical Co. Ltd. (Shinjuku, Tokyo, Japan). Belzer UW cold storage solution was purchased from Bridge to Life Ltd. (Columbia, SC). S1P, VPC 23019, CYM 5541, TY52156, MP-A08, and SKI II were purchased from Tocris Bioscience (Bristol, UK). Fingolimod, SEW2871, W146, JTE-013, and CAY10444 were purchased from Cayman Chemical (Ann Arbor, MI). Endothelial cell growth supplement from bovine neural tissue (ECGS), CYM 5520, CYM 50358, and CelLytic M were purchased from Sigma-Aldrich (St. Louis, MO). CYM 50308 was purchased from Tronto Research Chemicals (North York, ON, Canada). AlamarBlue Cell Viability Reagent was purchased from Invitrogen Corporation (Carlsbad, CA). Cytotoxicity Detection Kit (LDH) was purchased from Roche Diagnostic GmbH (Basel, Switzerland).

Cell culture

The human hepatoma cell line HepG2 (JCRB1054) was cultured in DMEM (1.0 g/L glucose) supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. The immortalized human umbilical vein endothelial cell (HUVEC) line established by electroporation of pIRES-hTERT-hygr HUEhT-2 (JCRB1459) was cultured in MCDB131 medium supplemented with 10% FBS, 0.03 g/L ECGS, 5 mg/L heparin sodium, and 5% Glutamax. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

In vitro cold storage and rewarming model

Experiments were performed with cells grown to 70%–80% confluence. Exposure to cold storage was performed by replacing warm media with complete media containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 4°C. Chemical compound was added concurrently with cold media, and cells were subjected to cold storage for each time-course at 4°C in a refrigerated incubator. To stimulate rewarming after cold storage, cold media was replaced with warm complete media and the cells were again incubated at 37°C for 1 hour.

Cell viability assay

The alamarBlue Cell Viability assay was performed according to the manufacturer's instructions. Briefly, alamarBlue Cell Viability Reagent was added to the cells, absorbance at 570 nm was measured after 1 hour, and cell viability was expressed as a percentage, divided by the absorbance measured for cold storage 0-hour control cells. The absorbance of the control cells was measured at the same time when cold storage exposure began for the experimental groups.

LDH release assay

LDH release was measured according to the manufacturer's instructions. Briefly, 100 μL/well supernatant was carefully removed into a clear 96-well flat bottom microplate and centrifuged at 250 × *g* for 5 minutes. To determine the LDH activity, 100 μL reaction mixture was added to each well, incubated for up to 30 minutes at +15 to +25°C (plates were protected from light), and the absorbance at 490 nm was measured. Control cells were treated with 1% TritonX-100, 1 hour prior to medium collection and were not cooled.

NanoLuc Binary Technology (NanoBiT)-β-arrestin recruitment assay

Cell culture

Human Embryonic Kidney A (HEK293A) cells (Thermo Fisher Scientific) were cultured in complete DMEM.

Plasmid construction for the NanoBiT-β-arrestin recruitment assay

In NanoBiT, NanoLuc luciferase is split into Small BiT fragment (SmBiT) and Large BiT fragment (LgBiT) subunits and the fused proteins are used for detecting intracellular protein-protein interaction (PPI).[18] For the assay, a receptor construct was designed to fuse the SmBiT of the NanoBiT complementation luciferase to the C-terminus of human $S1P_{1\text{-}5}$ with a 15-amino acid flexible linker (GGSGGGGSGGSSSGG), whose sequences were recommended by the manufacturer (Promega). The construct (HA-$S1P_{1\text{-}5}$-SmBiT) was assembled and inserted into a pCAGGS mammalian expression plasmid (a kind gift from Dr. Junichi Miyazaki, Osaka University) at the KpnI-XhoI site using the NEBuilder HiFi DNA Assembly system (New England Biolabs, Ipswich, MS). A β-arrestin construct was generated by fusing the LgBiT whose nucleotide sequences were gene-synthesized with mammalian codon optimization (Genscript), to the N-terminus of human β-arrestin1 or β-arrestin2 (βarr1 or βarr2, respectively) with the 15-amino acid linker. To increase βarr recruitment signal, alkaline phosphatase (AP)-2-binding-deficient mutations were introduced in βarr1 (R393E and R395E) and βarr2 (R393E and R395E).[19] The mutant LgBiT-βarr1 and LgBiT-βarr2 were inserted into the KpnI- and XhoI-digested pCAGGS plasmid.

NanoBiT-β-arrestin recruitment assay

HEK293 cells were seeded in a 10-cm dish at 2×10^6 cells in 10 mL of complete DMEM and cultured for 1 day in a CO_2 incubator. The cells were transfected with a mixture of $S1P_{1\text{-}5}$-SmBiT plasmid (1 μg) and the LgBiT-βarr plasmid (LgBiT-βarr1 or LgBiT-βarr2; 500 ng) by diluting in 500 μL of Opti-MEM, and combining 25 μL of 1 mg/mL PEI reagent diluted in 500 μL of Opti-MEM. As a negative control, the pCAGGS plasmid was used instead of the $S1P_{1\text{-}5}$-SmBiT plasmid. Twenty-four hours after addition of the transfection solution, the cells were harvested with 5 mL of a 0.53 mM ethylenediaminetetraacetic acid (EDTA)-containing Dulbecco's phosphate-buffered saline (D-PBS), followed by rinsing with 5 mL of Hanks' balanced salt solution (HBSS) containing 5 mM HEPES (pH 7.4). The cells were centrifuged at 190 × *g* for 5 minutes and suspended in 10 mL of 0.01% bovine serum albumin (BSA)-containing HBSS. The cell suspension was seeded in a 96-well white plate at a volume of 80 μL per well and loaded with 20 μL of 50 μM coelenterazine (Carbosynth, Compton, Berkshire, UK) diluted in the BSA-HBSS. After incubation at room temperature (approximately 23°C) for 2 hours, background luminescent signals were measured using a luminescent microplate reader (SpectraMax L equipped with 2 detectors, Molecular Devices, San Jose, CA). Test compounds at 6X concentration (20 μL) were manually added to the cells. Five minutes after ligand addition, luminescent signals were measured for 5 minutes with a 20-second interval. For each well, luminescent signal was normalized to the initial count and fold-change values over 5–10 minutes after ligand stimulation were averaged.

Data analysis

Combined data of 3–5 independent experiments, each performed in duplicates, are presented. For fitting sigmoidal concentration–response curve, fold-change luminescent signals (NanoBiT-βarr assay) were fitted to a 4-parameter sigmoidal curve using the Prism 7 software (GraphPad Prism).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR)

Total RNA was extracted from cells and heart tissue using RNeasy Mini Kit (Qiagen, Toronto, Ontario, Canada). A total of 1 μg of total RNA was used to synthesize cDNA using PrimeScript RTase (Takara). Gene expression of S1PR1, S1PR2, S1PR3, and GAPDH was detected by qPCR using the SYBR Green method. Primers used for PCR were: S1PR1 (F: 5′-TCTGCTGGCAAATTCAAGCGA-3′ and R: 5′-GTTGTCC CCTTCGTCTTTCTG-3′), S1PR2 (F: 5′-ATCGTGCTAGGCGTCTTTATCG-3′ and R: 5′-AGTGGGCTTTGTAGAGGATCG-3′), S1PR3 (F: 5′-GGATGTGCTGGCTCATTGC -3′ and R: 3′- CAGGATGGTAGAGCAGT-CAGG -5′), GAPDH (F: 5′-ACAACTTTGGTATCGTGGAAGG-3′ and R: 3′-GCCATCACGCCACAGTTTC-5′). qPCR was conducted in a 10 μL PCR 1 × SYBR Green mixture (Bio-Rad), 10 μM primers, 1 μL of cDNA, with the following thermal profiling: an initial activation step was carried out at 95°C for 10 minutes, followed by 45 cycles of: 95°C for 15 seconds, 64°C for 1 minute. Expression levels between groups were quantitatively compared using the ΔΔCt method with GAPDH as the endogenous control from RNA expression.

Animals

Inbred line of Lewis (LEW) rats (male, 7-weeks old, weight: 180–240 g) were purchased from the Oriental Bio Service (Koto, Japan), and used as both donors and recipients. The rats were fed water and standard rat chow without fasting, and were housed at a density of 3 per cage and maintained on a 12-hour light–dark cycle at 21°C. At the end of each procedure and experiment, rats were euthanized by exsanguination according to AVMA Guidelines for the Euthanasia of Animals under anesthesia with 5% isoflurane inhalation through a facial mask. The experiment was approved by the Animal Research Committee of Kyoto University (Med Kyo 19178), and all animals received humane care, in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Academy of Science and the National Institutes of Health.

Experimental design

Donor and recipient rats (inbred line of Lewis) matched in age and weight were randomly assigned to 10 μM TY52156 or the vehicle group. The transplantation maneuvers were standardized and performed at the same time of day by the same microsurgeon (X.Z.). The grafts were perfused with plain University of Wisconsin (UW) cold preservation solution (fresh control) or UW solution supplemented with compound (S1PR antagonist, TY52156) or vehicle and preserved for 24 hours at 4°C in the same solution. After 24-hour hypothermic preservation, the grafts were flushed with Ringer solution and transplanted. Fresh control was transplanted immediately after donation with 30-minute cold storage and perfusion with Ringer solution.

Heterotopic abdominal heart transplantation

Heterotopic abdominal heart transplantation was performed as previously reported.[20] Briefly, the rat hearts were surgically removed under anesthesia with 5% isoflurane inhalation through a facial mask. After anesthetization, sodium heparin (1000 U/kg) was administered from the dorsal vein of the penis to avoid thrombus formation. The chest of the donor rat was opened using the median sternotomy approach to expose the heart. The superior vena cava (SVC), inferior vena cava (IVC), and pulmonary veins (PV) were tied with silk 6.0 sutures, and ascending aorta and pulmonary artery were cut at 3–4 mm from their

origin. The heart was then perfused in situ with 4°C plain UW solution (fresh control) or UW solution with compound (S1PR antagonist, TY52156) or vehicle by retrograde perfusion through ostia of coronary artery in the ascending aorta stump and venous return (majority through the coronary sinus in the right atrium, some through the auxiliary opening in the right ventricle, and via the Thebesian veins into the cavity of the left ventricle), rapidly excised, and preserved in the same solution as perfusion. Heart grafts were preserved at 4°C for 24 hours. After the same anesthesia as donor rats, recipient rats underwent a mid-line abdominal incision. The heart graft was flushed with 4°C Ringer solution along the same route as the harvest, and then the ascending aorta and pulmonary artery of the graft were anastomosed with the infra-renal abdominal aorta and IVC using 9-0 suture thread, respectively. The median warm ischemic time was 32.5 minutes (range: 36−27 minutes).

Graft evaluation

Primary endpoints were the effect on cardiac graft function and histology at 7 days, and analyses were performed by a trained individual who was blinded to the study. One rat in the fresh control group died of anastomosis rupture caused by thin suture thread and was thus excluded from statistical analyses. Beating score of the graft was evaluated visually at 10 minutes and 7 days after reperfusion by direct inspection, and at 24 hours by palpitation using the Stanford Cardiac Surgery Laboratory graft scoring system: 0 = no contraction; 1 = contraction barely visible or palpable; 2 = obvious decrease in contraction strength, but still contracting in a coordinated manner, with rhythm disturbance; 3 = strong, coordinated beat, but noticeable decrease in strength or rate; 4 = strong contraction of both ventricles, at regular rate.[21] The horizontal and vertical lengths of pretransplant grafts were measured by caliper ex vivo. Graft sizes were expressed as multiplication of horizontal and vertical lengths. At 7 days after reperfusion, rats were subjected to laparotomy to examine the graft survival. Wall motion and beat, post-transplant size in vivo at diastolic phase were measured, and the rats were then sacrificed. Graft enlargements were expressed as ratio of the post-transplant size to the pretransplant size. Grafts excised at 7 days were fixed in 10% formalin-PBS, embedded in paraffin, and stained with hematoxylin & eosin (HE), Masson's trichrome (MT), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, In Situ Apoptosis Detection Kit, TaKaRa, Kusatsu, Shiga, Japan). Interstitial edema was measured in randomly selected coronary arteries (at least 5/heart) and expressed as a median diameter. Perivascular edema (S1) and the vascular area (S2) were measured. The edema index was determined as the ratio of S1 subtracted S2 to S2 and graded as follows: 0 = index $\leq$ 0; 1 = index 0.1 to $\leq$0.5; 2 = index 0.6 to $\leq$1.0; 3 = index > 1.0.[22] The grade of inflammatory cell infiltration in the myocardium: 0 = no infiltration; 1 = mild (focal, perivascular) infiltration; 2 = moderate (multifocal); 3 = extensive (most dense and diffuse), and Myocyte lesion: 0 = no lesion; 1 = vacuolization (myocyte becomes vacuolized); 2 = focal myocyte necrosis (irregular border, fragmented sarcoplasm, debris, myocyte dropout); 3 = extensive myocyte necrosis (interstitial hemorrhage and eosinophil infiltration) were also evaluated.[22] TUNEL-positive cells were counted in 5 randomly selected high-power fields (HPF, magnification 400 ×) adjacent to the necrotic area, and were expressed as the average number of TUNEL-positive cells per single HPF. The fibrotic areas in MT were expressed as ratio to the total heart area. Images were obtained using a fluorescence microscope BZ-X710 (Keyence, Osaka, Japan), and processed using computerized planimetry software (KEYENCE). The fibrotic areas were expressed as ratio to the total heart area. Grafts fixed in 10% formalin-PBS at the end of storage were immersed in 30% sucrose, embedded in OCT compound, frozen in liquid nitrogen, and stained with HE. Blood was drawn from the IVC at 7 days after reperfusion and the rats were euthanized. Serum was obtained by centrifugation and aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) levels, and creatinine kinase (CK) activity were measured by standard enzymatic methods (JSCC for AST, ALT, and CK; IFCC for LDH). All measurements were performed in the central laboratory of the Nihon Rinsho and according to international standards.

Statistical analysis

All in vitro data presented were confirmed in at least 3 or 4 independent experiments. An unpaired Student's *t*-test was performed for cell death and cell viability assay between groups and one-way ANOVA was performed at $P = 0.05$, with post hoc Dunnett's test for comparisons of time-course assays. Curve fittings for EC_{50} values in concentration-dependent curve were determined using nonlinear regression analysis GraphPad (Prism). One-way ANOVA was performed at $P = 0.05$, with post hoc Tukey's multiple comparison test for comparisons between different preservation groups over time. All in vitro and in vivo values are expressed as means ± SEM.

Results

Long cold exposure causes irreversible damage to human cells in a time-dependent manner

To evaluate cold injury of the cells derived from major target organs in transplantation therapy, we generated an in vitro model of cold injury (Fig 1, A) using a HepG2 cell line. Cell integrity was gradually lost during incubation at 4°C and morphological changes were observed, which appeared more severe after rewarming than before rewarming (Fig 1, B). During cold storage, LDH release increased in a time-dependent manner (Fig 1, C), indicating that cold exposure time-dependently impairs cells. Cell viability after the indicated cold storage time points followed by 1-hour rewarming decreased in a time-dependent manner in inverse proportion to LDH release (Fig 1, D), indicating that cell viability after rewarming is inversely correlated with cold injury. Cold storage for 8 hours led to a 50% mortality in HepG2 cells after 1-hour rewarming, and the cells almost completely died after 24-hour cold storage followed by 1-hour rewarming. To achieve longer preservation and better cell viability after rewarming, we attempted to reduce cold injury.

Deprivation of S1P protects human cells from cold injury

To confirm that S1P is a key factor for inducing cold injury, we examined the effect of deprivation of S1P during cold storage on cell viability after rewarming. HUVECs are endothelial cells isolated from human umbilical vein. Endothelial cells, which commonly exist in target organs for transplantation, are the main source of circulating S1P secreted after intracellular S1P synthesis,[23] and are known to be susceptible to hypothermic conditions.[24] Thus, we used HUEhT-2 cells, which are immortalized HUVECs, established by the electroporation of pIRES-hTERT-hygr, which have sufficient and stable capacity to secrete S1P into the extracellular space. S1P is generated from plasma membrane lipids and its catalytic process in the intracellular space is mediated by 1 of 2 sphingosine kinases (sphingosine kinase 1 (SPHK1) or SPHK2) resulting in increased intracellular S1P level or secretion into extracellular space and acting in an autocrine or a paracrine manner.[16] For S1P deprivation, we used 2 different SPHK inhibitors, MP-A08 and SKI II. Charcoal stripped lipid-free fetal bovine serum (FBS) was used to completely deprive S1P. When HUEhT-2 cells were incubated with SPHK inhibitors and lipid-free serum in normothermic conditions for 24 hours, cell viability decreased in a dose-dependent manner (Fig 2, A and B), indicating that S1P is necessary for survival during normothermia.[16] When HUEhT-2 cells were subjected to cold storage and rewarming, cell viability decreased in a time-dependent manner after the indicated cold storage time points followed by 1-hour rewarming (Fig 2, C), similar to that observed in HepG2 cells, although the durations were different. Cold storage for 3 days followed by 1-hour rewarming resulted in 80% mortality in HUEhT-2 cells, and HUEhT-2 cells almost completely died after 5 days of cold storage followed by 1-hour rewarming. Next, we added

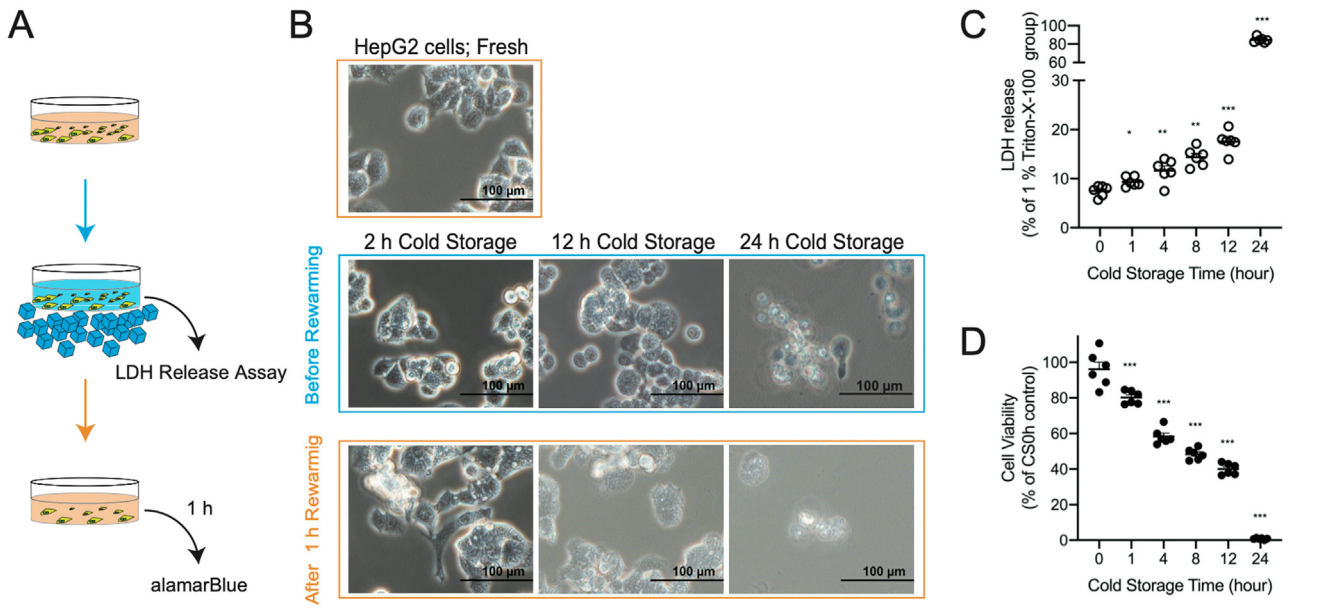

Fig 1. Long cold exposure causes irreversible damage to human cells in a time-dependent manner. (A) Scheme of in vitro study of cold injury. (B) Representative image ($n = 4$) of phase differential image at physiological temperature and indicated cold storage time points before and after 1-hour rewarming in HepG2 cells. Scale bar, 100 μm (400 ×). (C and D) Cytotoxicity induced by cold storage at 4°C before rewarming was evaluated by periodic measurement of LDH release (C), and cell viability was expressed using alamarBlue assay after indicated cold storage time points followed by rewarming for 1 hour in HepG2 cells (D). For C and D, $n = 6$ for each time point. *n.s.* $P > 0.05$, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the corresponding baselines of cold storage 0-hour calculated using one-way ANOVA followed by post hoc Dunnett's test. In all statistical plots, data are shown as the mean ± SEM.

compounds into cold media at indicated concentrations during cold storage and removed it by washing out at rewarming. When HUEhT-2 cells were grown in media supplemented with SPHK inhibitor and lipid-free serum and subjected to cold storage for 5 days followed by 1-hour rewarming, the decrease in cell viability after 1-hour rewarming was substantially alleviated in a dose-dependent manner (Fig 2, D and E), indicating that S1P deprivation reduces cold injury. To determine whether the effect of SPHK inhibitor was specifically caused by the deprivation of S1P, we added exogenous S1P and examined whether it could nullify the effect of S1P deprivation. With treatment of 25 μM MP-A08 or 5 μM SKI II using lipid-free serum during cold storage for 3 days (80% mortality after 1-hour rewarming), the addition of exogenous S1P decreased cell viability after 1-hour rewarming in a S1P dose-dependent manner (Fig 2, F and G). These data indicate that S1P induces cold injury in a dose-dependent manner and the deprivation of S1P reduces cold injury.

S1PR antagonists protect human cells against cold injury

To examine whether the inhibition of S1P receptors (S1PR) could reduce cold injury in human cell lines, we used compounds affecting S1PRs to reduce the potential adverse effects of S1P deprivation during normothermic rewarming (Fig 2, A and B).[17] Using the 24-hour cold storage and 1-hour rewarming model (100% mortality; Fig 1, D) in HepG2 cells that were confirmed to express S1PR 1, 2, and 3 (Fig 3, A), we screened compounds affecting S1PRs and found that TY52156 and JTE-013 induced a substantial increase in cell viability in a dose-dependent manner (Fig 3, B). Furthermore, TY52156 was found to be the most potent, with an effect of up to 89.8 ± 1.1% of viability in the 100% mortality model.

Next, we tried to confirm the antagonistic effect of TY52156 on S1PR1−5 using the NanoLuc Binary Technology (NanoBiT)-β-arrestin recruitment assay system (Fig 3, C). We found that the activated GPCRs were uncoupled from G-proteins, resulting in signal desensitization by recruitment of β-arrestin-1 and -2.[25] One of the $S1PR_{1-5}$-SmBiT plasmids was transiently cotransfected with the LgBiT-βarr1/βarr2 plasmid into parental cells, and the recruitment of β-arrestin to the plasma membrane upon S1P stimulation was quantified using NanoBiT luminescence. This background demands an extremely high transfection efficiency in NanoBiT assay, and HEK293 cells, which have high transfection efficiency,[18,19] were used. The administration of S1PR antagonists inhibited the recruitment of β-arrestin, as observed through lost luminescence. The administration of S1P as a positive control induced luciferase activity in all S1PR1−5 receptors (Fig 3, D), indicating that S1P-signaling stimulation was detected through β-arrestin-1 and -2 recruitment. In particular, TY52156 had stronger antagonizing effects on S1PR3, compared to that on the other receptors, in both β-arrestin-1 and -2 recruitment (Fig 3, E and F). We examined the time duration for which the most potent compound, TY52156, could maintain its protective effect during extended cold storage in HepG2 and HUEhT-2 cells. HUEhT-2 cells also expressed S1PR 1, 2, and 3, and showed stronger expression of S1PR1 and 3 mRNAs compared to HepG2 cells (Fig 3, A). HepG2 and HUEhT-2 cells treated with 10 μM TY52156 remained viable even after 48 hours and 14 days of cold storage followed by 1-hour rewarming, respectively (Fig 3, G and H). TY52156 extended the cold storage time more than twice in HepG2 cells and more than 3-fold in HUEhT-2 cells. These results indicate that S1PR antagonists maintain cellular viability during cold storage, similar to the result observed after S1P deprivation, which reduced cold injury (Fig 2, D and E). Furthermore, in contrast to the finding that S1P deprivation showed potential adverse effects in normothermia (Fig 2, A and B), we found that TY52156 neither inhibited cell proliferation nor reduced cell viability in normothermic conditions in both HepG2 (Fig 3, I) and HUEhT-2 cells (Fig 3, J). These results indicate that the inhibition of S1PRs is a viable solution for cold preservation in human cells.

TY52156 treatment reduces heart graft damage and improves transplanted graft function after prolonged cold preservation in vivo

To confirm whether the effect of TY52156 could apply for the organ preservation, heterotopic abdominal rat heart transplantation was

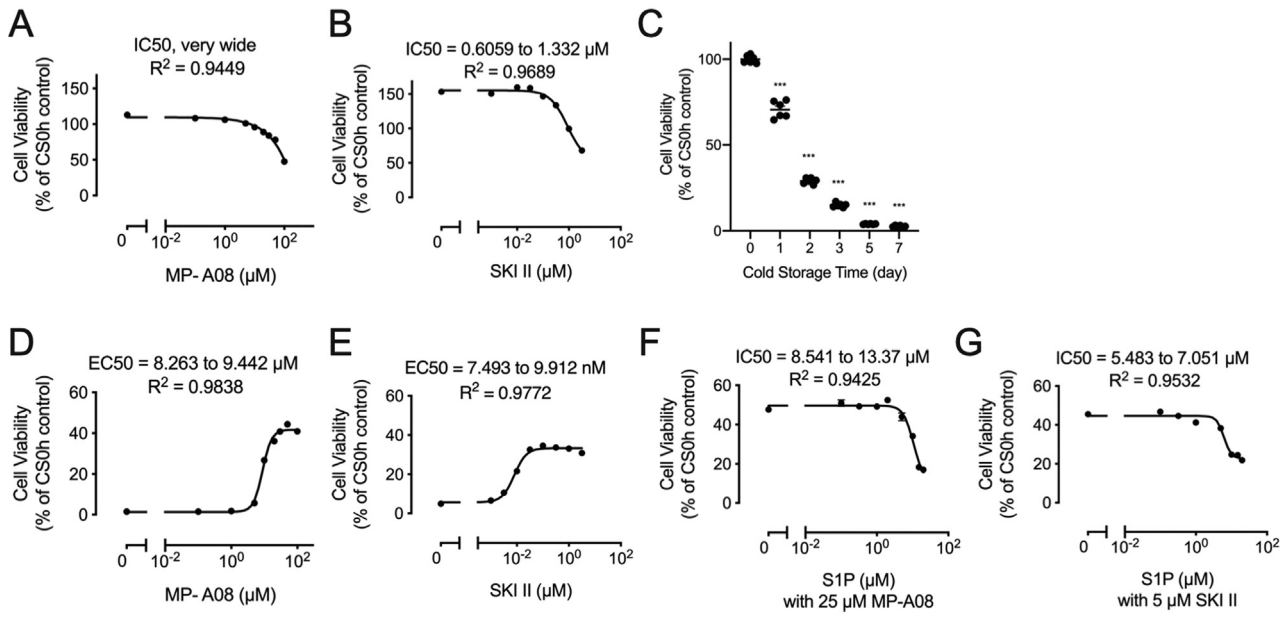

Fig 2. Deprivation of S1P protects human cells from cold injury. (A and B) Cell viability after 24-hour incubation in normothermic condition supplemented with MP-A08 (A) and SKI II (B) in HUEhT-2 cells. For A and B, charcoal stripped lipid-free fetal bovine serum (FBS) was used to deprive S1P completely ($n = 6$ for each concentration point). Curve fitting for IC_{50} values were determined using nonlinear regression analysis. (C) Cell viability was expressed using alamarBlue after indicated cold storage time points followed by 1-hour rewarming in HUEhT-2 cells. For C, $n = 6$ for each time point. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the corresponding baselines of cold storage 0-hour calculated using one-way ANOVA followed by post hoc Dunnett's test. (D and E) Cell viability after 5-day cold storage and 1-hour rewarming (almost 100% mortality) with MP-A08 (D) and SKI II (E) of HUEhT-2 cells. For A and B, charcoal stripped lipid-free fetal bovine serum (FBS) was used to deprive S1P completely ($n = 6$ for each concentration point). Curve fitting for IC_{50} values were determined using nonlinear regression analysis. (F and G) Cell viability after 3-day cold storage and 1-hour rewarming (80% mortality) of HUEhT-2 cells given 25 μM MP-A08 (F), or 5 μM SKI II (G) supplemented with exogenous S1P at indicated concentrations. At cold storage, compounds were added into cold media at indicated concentrations and removed by washing out at rewarming. For D–G, charcoal stripped lipid-free FBS was used to deprive S1P completely and $n = 6$ for each concentration point. Curve fitting for EC_{50} and IC_{50} values were determined using nonlinear regression analysis. In all statistical plots, data are shown as the mean ± SEM.

examined. With reference to conventional methods indicating that the time limit for rat heart preservation in the University of Wisconsin (UW) cold storage solution is 12 hours,[26] isolated rat hearts were preserved 24 hours prior to transplantation (Fig 4, A). To evaluate the graft at the end of cold storage, hematoxylin & eosin (HE) staining of graft section were taken (Supplementary Fig 1). While normal heart (Supplementary Fig 1A) and fresh control (cold storage for 30 minutes, Supplementary Fig 1B) group showed comparable normal tissue structure, vehicle group (cold storage for 24 hours with vehicle, Supplementary Fig 1C) showed disruption of myofibril architecture and endothelial edema. In contrast, grafts treated with TY52156 (cold storage for 24 hours with TY52156, Supplementary Fig 1D) showed a little structural disruption but not as much the graft treated with vehicle. Next, we evaluated the graft at the 7 days after transplantation. In the fresh control group, grafts showed a normal shape, no distension, healthy wall motion (Supplementary Video 1A), and high beating score (10 minutes, 3.8 ± 0.20; 24 hours, 4.0 ± 0; 7 days, 4.0 ± 0). However, in the grafts of the vehicle group, distension, loss of coordination, and hypokinetic wall motion were detected (Supplementary Video 1B) and beating score was low (10 minutes, 1.4 ± 0; 24 hours, 0.8 ± 0; 7 days, 1.6 ± 0). In contrast, grafts treated with TY52156 showed no apparent distension, well-maintained wall motions (Supplementary Video 1C), and significantly higher beating scores ($P <$ 0.001 at each time point) than those of the vehicle group (10 minutes, 3.4 ± 0.40; 24 hours, 3.8 ± 0.20; 7 days, 3.6 ± 0.24; $P < 0.001$ at each time point, Fig 4, B), which were similar to the fresh control group levels. To further evaluate the graft, histopathological analysis was carried out. Interstitial edema, inflammatory cell infiltration, and myocardial lesions were evaluated by HE staining (Fig 4, C). The vehicle group showed clear perivascular edema; less edema was observed in the fresh control and TY52156 groups. The extent of interstitial edema in the vehicle group (2.0 ± 1.2) was significantly higher than that of the fresh control group (0.2 ± 0.5, $P < 0.001$) and TY52156 group (0.6 ± 0.9, $P <$ 0.001, Fig 4, D). The inflammatory cell infiltration and myocyte lesions were also extensive and diffuse in the vehicle group but lesser in the fresh control or TY52156 group (Fig 4, C). The vehicle group showed significantly severe inflammatory cell infiltration and myocyte lesions (2.6 ± 0.6 and 2.4 ± 0.8) compared to the fresh control group (1.0 ± 0.4 and 0.2 ± 0.4, $P < 0.001$ for each) or TY52156 group (1.9 ± 0.7 and 1.4 ± 1.0, $P < 0.001$ for each, Fig 4, D). Next, irreversible cellular damage leading to cell death, measured using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Fig 4, C), showed only a small number of TUNEL-positive cells in both the fresh control group (0.5 ± 0.4 counts/HPF) and TY52156 group (4.5 ± 4.1 counts/HPF, n.s. for fresh control vs TY52156). In contrast, the vehicle group showed a significantly larger number of TUNEL-positive cells (23.4 ± 12.0 counts/HPF) than the fresh control group or TY52156 group ($P < 0.001$ and $P < 0.01$ for vehicle vs fresh control and vehicle vs TY52156, respectively, Fig 4, E). These results indicated that TY52156 significantly minimized TUNEL-positive cardiomyocyte cell death caused by cold storage followed by transplant. Graft enlargement and fibrosis expressed in Masson's trichrome (MT) staining were examined to determine the quantity of distension and fibrosis of myocardium (Fig 4, C). Compared to that in the fresh control group, the vehicle group showed significant enlargement of the graft (fresh control group 97.0 ± 9.6 %, vehicle group 139.2 ± 23.6%, $P < 0.01$ for fresh control vs vehicle, Fig 4, F). Enlargement of the graft was not detected in the TY52156 group compared to that in the fresh control group (95.6 ± 6.4%, n.s. for fresh control vs TY52156), indicating that TY52156 treatment significantly improved cardiac distention. The vehicle group (55.6 ±13.5%) showed significantly larger fibrotic areas than the fresh control group (13.5 ± 3.5%, $P < 0.001$ for vehicle vs fresh control, Fig 4, G). In the TY52156 group, the fibrotic area was significantly diminished compared to that in the vehicle group

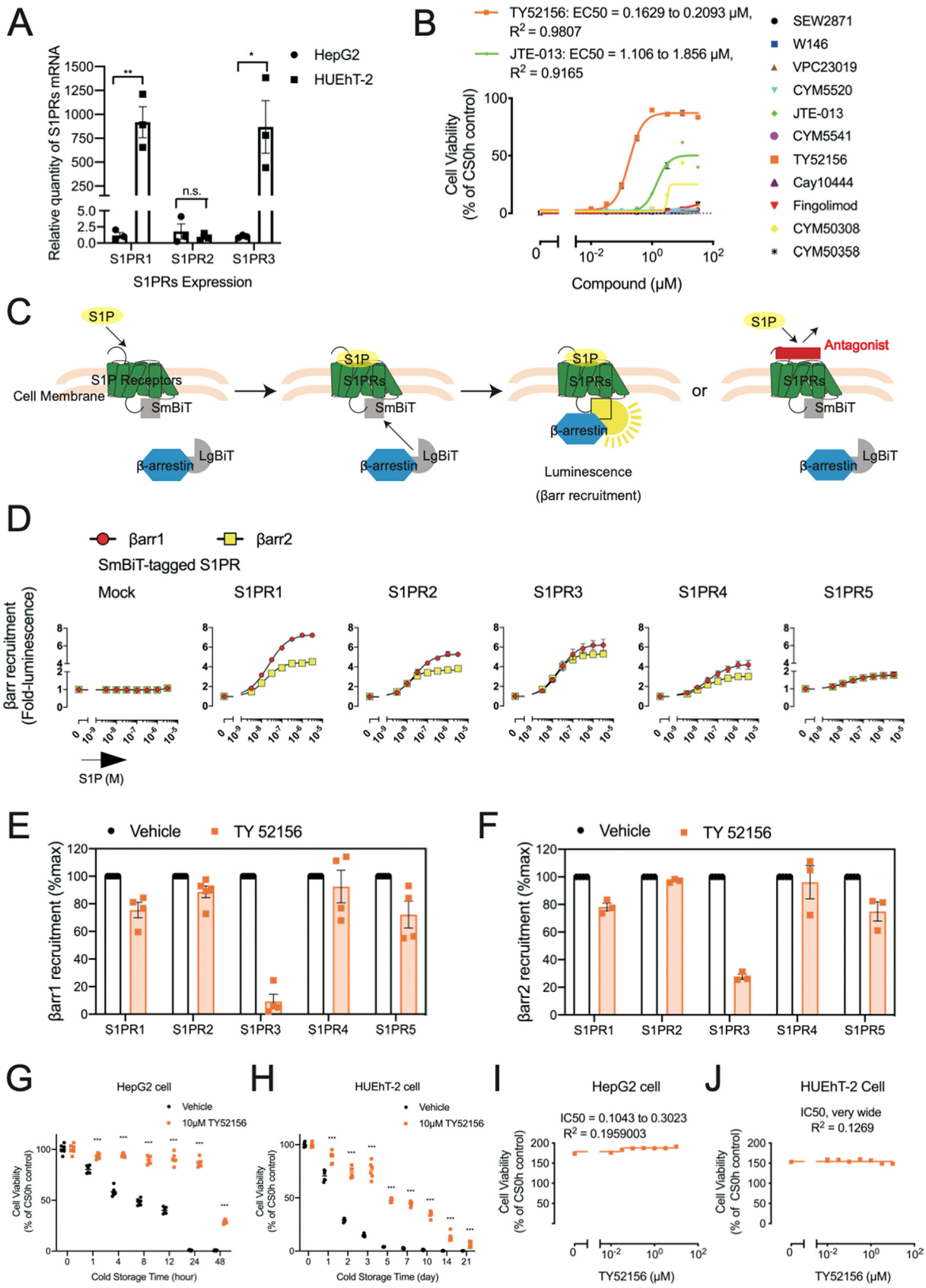

Fig 3. S1PR antagonists protect human cells against cold injury. (A) Relative quantification of S1PR1, 2, and 3 mRNA comparing HUEhT-2 cells with HepG2 cells. For A, $n = 3$ for each group. *n.s.* $P > 0.05$, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to each S1PR group calculated using 2-sided Student's *t*-test. (B) Effect of the compounds affecting S1PRs after 24-hour cold storage and 1-hour rewarming in HepG2 cells (100% mortality). For B, $n = 6$ for each concentration point. (C) Scheme of NanoLuc Binary Technology (NanoBiT)-β-arrestin recruitment assay system. (D) Dose–response curves for NanoBiT-β-arrestin recruitment of S1PR1-5 upon S1P stimulation. (E and F) Inhibitory effect of vehicle, 10 μM TY52156 on β-arrestin1 (E) and β-arrestin2 (F) recruitment to S1PR1-5 under 1 μM S1P. For

(TY52156 group: 35.7 ± 8.5%, $P < 0.05$ for vehicle vs TY52156, Fig 4, G), indicating that cold injury introduced fibrosis of myocardium, and TY52156 significantly minimized this damage. Finally, the serological markers, aspartate aminotransferase (AST, Fig 4, H), alanine aminotransferase (ALT, Fig 4, I), lactate dehydrogenase (LDH, Fig 4, J), and creatinine kinase (CK, Fig 4, K), that show extent of graft injury, were measured. The vehicle group showed significantly increased serum AST, ALT, LDH, and CK (1162 ± 80 U/L, 94 ± 4 U/L, 4042 ± 194 U/L, and 10482 ± 600 U/L) compared to the fresh control group (122 ± 19 U/L, 42 ± 2 U/L, 1626 ± 111 U/L, and 1963 ± 185 U/L, $P < 0.001$ for each) or TY52156 group (478 ± 156 U/L, 53 ± 10 U/L, 2474 ± 388 U/L, and 5104 ± 1062 U/L, $P < 0.01$ for each), indicating that serological markers of graft injury were significantly improved in TY52156 group compared to vehicle group. These results clearly demonstrated that TY52156 treatment reduced cold injury in vivo and protected the cardiac graft from damage caused by 24-hour cold storage, doubling the preservation time compared with those of the conventional cold preservation methods.

Discussion

In cold preservation, there is an inverse relationship between the duration of cold storage and graft function.[27] The maximum preservation time of rat heart is a little longer than that of the human heart, which is limited to 4–6 hours in SCS; however, there is a consensus that rat cardiac graft function preserved in UW solution can be maintained and recovered for up to 12-hour preservation, but not 18-hour preservation or longer.[26] TY52156 treatment reduced TUNEL-positive cell death and graft fibrosis, maintained cardiac graft viability, and dramatically improved graft function in 24-hour cold preservation, doubling the preservation time compared with that of the conventional cold storage method. Each year in the United States, thousands of organs are discarded, and approximately 10% of the number of donor hearts currently discarded is equivalent to the number of waiting-list patients who die or become too sick for a transplant before receiving graft.[9] Extension of preservation time could enable the transport of organs over longer distances, which could reduce the graft discard rate. Furthermore, it could benefit low-priority recipients and provide more appropriate allocation in donor-recipient matching that could decrease graft rejection and the need for immunosuppression and finally improve the outcome of heart failure patients. S1PR1 to 3 are reported to be expressed in both human and rat heart.[28] Thus, rat heart models demonstrate tremendous potential for translation into clinical studies. In this study, we found that S1P induced cold injury in a dose-dependent manner during cold exposure. On the contrary, deprivation of S1P and using S1PR3 antagonists reduced cold injury, a state that differently mimicked cold tolerance in human cells and rat heart. Owing to its dramatic protective effect against cold injury, we termed TY52156 as "TOACH" for Tolerant Adaptation toward Cooling as Hibernation. To further extend the proof-of-concept of this treatment in small animal studies toward humans, we are currently planning experiments with another rodent organ transplant model. The future plan will involve large animal transplant models and subsequent clinical trials.

S1P is generated from the phosphorylation of sphingosine, a product of ceramide hydrolysis derived from plasma membrane lipids.[16] Cold exposure was reported to alter the metabolism of sphingolipids and composition of sphingolipid classes to increase levels of ceramide and sphingomyelin, which are precursors of S1P, and to decrease the breakdown of these to S1P.[29] Until now, the functional consequence and exact roles of changes in sphingolipid composition induced by cold exposure had not been determined,[29,30] and only recent reports suggested that S1P decrease causes lymphopenia in hibernators.[13,14] However, we demonstrated that S1P induced cold injury, and deprivation of S1P and using S1PR3 antagonists reduced cold injury or mimicked cold tolerance, which are consistent with the findings in other organisms.[31-35] A defect in the negative regulation of S1P synthesis in *Aspergillus fumigatus* causes hypersensitivity to cold,[35] and the cells resistant to the inhibitor of S1P synthetic enzyme shows deteriorated growth at lower temperatures.[33] Recent studies also indicate close associations between the species of sphingolipid and cold tolerance in plants[31,34] and fish.[32] These results suggest that a decrease in S1P biosynthesis is related to cold tolerance to adapt to cold exposure. Potential signaling roles of sphingolipid including S1P altered in cold exposure remain to be explored and the signaling role of S1P in cold tolerance should be elucidated in future studies.

The simple SCS method is widely used in clinical settings to preserve isolated organs for up to 18–24 hours for kidneys, 12 hours for livers, and 4–6 hours for thoracic organs.[2] To further extend preservation time and improve donor graft conditions, several cold preservation methods have been developed; these include subnormo-, mid-, to hypo-thermic perfusion,[5-7] and subzero cooling.[3,4] Hypothermic perfusion has already been adopted for clinical transplantation and shows improved graft condition compared to SCS.[36,37] An alternative cold preservation method is subzero cooling, which uses cooling devices with cryoprotective agents. Subzero cooling has drastically extended liver and heart preservation times, with up to 3–4-fold increase in small animal experiments.[3,4] S1P is generated by SPHK, secreted into extracellular space, and acts in an autocrine or a paracrine manner. Although these hypothermic perfusion methods have been technically developed based on SCS and generally superior to SCS, wash out of S1P or some harmful metabolites from the preservation solution might partially contribute to the protection. To further elucidate this regulation, perfusion experiment to evaluate amount of S1P and metabolites should be performed in future studies.

In our experiment, endothelial cells were protected from cold injury in vitro and interstitial edema and inflammatory cell infiltration were attenuated in vivo, which suggests that protection for endothelial cells is most important in our model. This is reasonable for the previous reports explaining that cold exposure injures the endothelium and increases the myocardial ET-1 secreted by vascular endothelial cells, which leads to cardiovascular dysfunction,[38] or the endothelium damage occurring during cold preservation represents the initial factor leading to ischemia reperfusion injury (IRI) finally causing parenchymal cell death in liver.[39] Furthermore, Cold stress is thought to induce compromised endothelial function through the decrease of eNOS activity,[40] and induce oxidative stress represented by reactive oxygen species (ROS) and is suggested to damage the myocardium through ROS production in the myocardium.[38] S1P signaling is known to play a protective role in warm ischemia reperfusion injury (IRI) through S1PR2 or 3-mediated activation of Akt and nitric oxide synthesis in not only endothelium but also cardiomyocyte.[41,42] In endothelium, S1PR3 was shown to activate endothelial nitric oxide synthase (eNOS) via an Akt-mediated pathway resulting in vasorelaxation[43] but promote leukocyte infiltration via Gi, PLC, and Ca^{2+} following to inflammation.[44] In contrast, while SPHK1a overexpression in mice was indicated to induce cardioprotective effect of myocardium in warm IRI, chronic activation of S1P signaling induces myocardial fibrosis via S1PR3 mediated ROS production at

inhibition experiments, compounds were pretreated for 30 minutes before stimulation with 1 μM S1P. D–F are combined data of 3–5 independent experiments with each performed in duplicates. (G and H) Cell viability at the indicated cold storage time points followed by rewarming for 1 hour in HepG2 (G) and HUEhT-2 cells (H) treated with vehicle or 10 μM TY52156. For G and H, $n = 6$ at each time point. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the corresponding time points of vehicle group calculated using 2-sided Student's *t*-test. (I and J) Cell viability after 24-hour incubation at normothermic condition supplemented with TY52156 of HepG2 cells (I) and HUEhT-2 cells (J). For I and J, $n = 6$ for each concentration point. For B, D, I, and J, fitting for concentration curve was determined using nonlinear regression analysis. In all statistical plots, data are shown as the mean ± SEM.

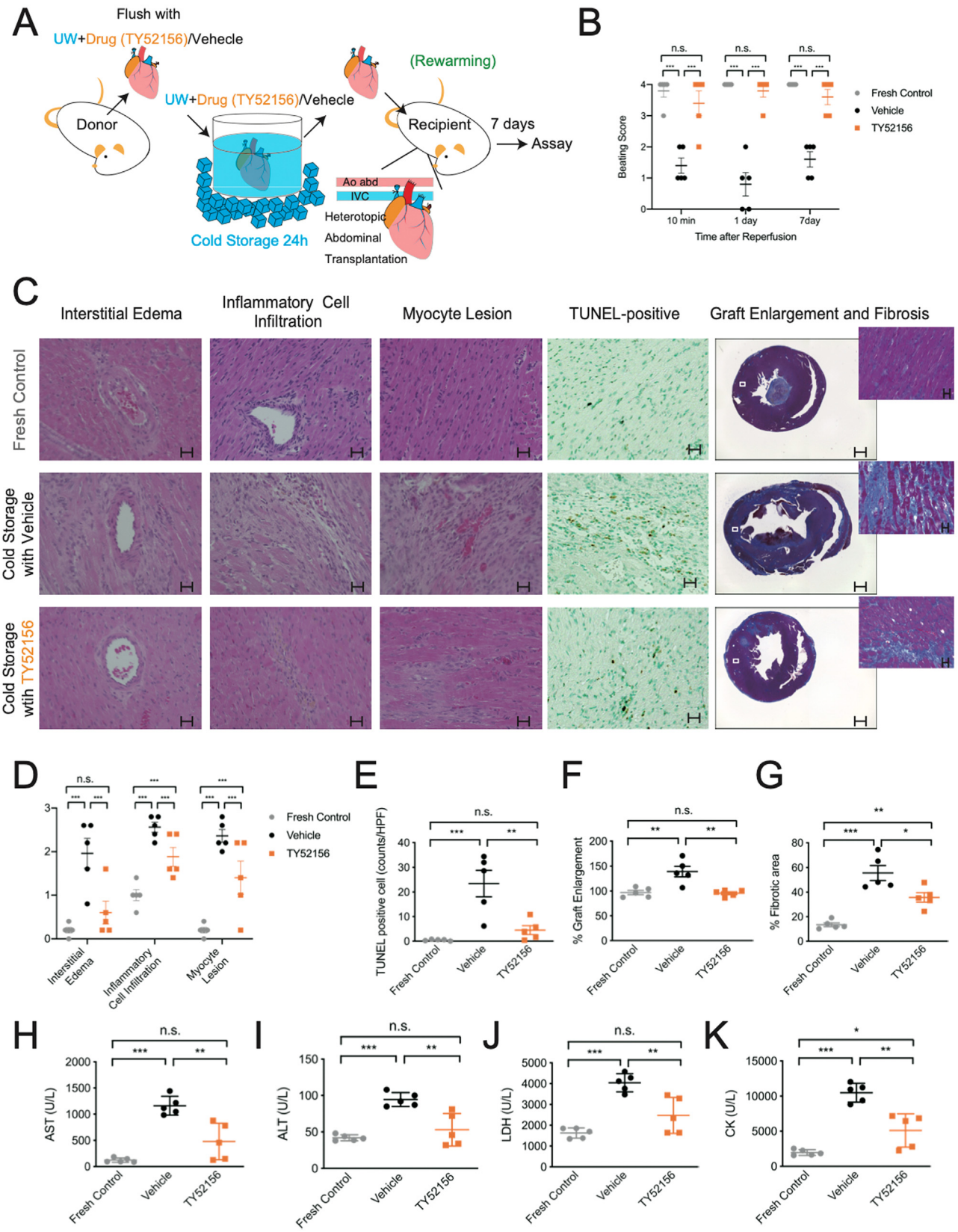

Fig 4. TY52156 treatment reduces heart graft damage and improves transplanted graft function after prolonged cold preservation in vivo. (A) Scheme of in vivo study of cold injury using heterotopic cardiac transplantation model. (B) Beating score in heart graft at 10 minutes, 24 hours, and 7 days after reperfusion. (C) Representative images of hematoxylin & eosin (HE, 400 ×) staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, 400 ×) staining, graft enlargement (20 ×), and Masson's trichrome (MT, 400 ×) staining of heart sections at 7 days after transplantation in fresh control group and treated with vehicle or TY52156 treatment during cold storage for 24 hours. (D) Quantification of interstitial edema, inflammatory cell infiltration, and myocyte lesion at 7 days after heterotopic cardiac transplantation. (E) Quantification of TUNEL-positive myocytes at 7 days after heterotopic cardiac transplantation. (F and G) Quantification of measured cardiac graft enlargement (F) and fibrotic area (G) at 7 days after heterotopic cardiac transplantation. The graft enlargement was expressed as the product of horizontal and vertical lengths. (H

normothermia.[45] Summarizing the previous reports above, S1PR3 inhibition is assumed to induce unfavorable decrease of nitric oxide synthesis and eNOS activity resulting in decreased protection of endothelium and myocardium, whereas it induces favorable inhibition of leukocyte infiltration in endothelium and amelioration of fibrosis through the decrease of ROS production in myocardium. It is plausible that S1PR3 inhibition during cold storage potentially inhibited leukocyte infiltration in endothelium and ameliorated myocardium fibrosis through the decrease of ROS production while eNOS activity might be attenuated both in endothelium and myocardium in our in vivo model. However, decreased myocardial lesions, TUNEL-positive cardiomyocyte, and myocardium fibrosis in our data remains to be determined whether the effect is primally acquired or comes from the protection of endothelial cells resulting in a decrease of infarction. Further, although we used heterotopic abdominal heart transplantation models as the most simple- and easiest-to-use approach to apply an in vitro proof-of-concept for small animal studies, cardiomyocyte and endothelial cells need to be used to find the most effective compound in protecting rat heart from cold injury. To further extend the study and find the most optimized compound for cardiac preservation, we should define the target structure, elucidate the mechanism, and screen the compound optimized for the heart by using cardiomyocyte and endothelial cells in future study.

Cold injury leads to a loss in the integrity of the cellular membrane, causing its collapse and the dysfunction of membrane lipid and enzymes. These undesired changes subsequently cause swelling of cells, accumulation of calcium, disruption of cytoskeleton, and mitochondrial dysfunction resulting in the loss of ATP.[8,46] However, the molecular or signaling mechanism underlying cold injury-induced changes are still unknown,[2] and associations between these undesired changes and S1P or S1PR have not been revealed. Currently, S1P and S1PRs are considered as ideal targets for selective intervention in many diseases.[16] Pleiotropic biological activities of S1P are explained by the relative cellular expression levels of its 5 S1P receptor subtypes or coexpression of partner G-proteins, which perform redundant functions between the S1PRs. Owing to such complexities, physiological functions of such S1P receptors are still in the process of elucidation.[17] Furthermore, although β-arrestins were originally discovered for their inhibitory effect on GPCRs, they have been reported to also trigger endocytosis and kinase activation leading to activation of specific G protein-independent signaling pathways.[47,48] These new insights into β-arrestin-dependent signaling make the mechanism even more complicated. Elucidating the molecular mechanisms of S1P signaling regulation by several types of S1PRs, binding of diverse G protein signals to S1PRs, β-arrestin-inhibition, β-arrestin-dependent signals, and how or whether these relate to cold injury and cold tolerance would bring greater benefits not only to clinical applications but also to thermal biology research.

There are some limitations to our study. First, although we performed the transplantation procedure between isogenic rats (Lewis-to-Lewis), which does not result in immunologic rejection, human organ transplantation is almost always performed between allogenic individuals under aggressive immunosuppression to prevent rejection.[49] The main contributor to human graft loss is allograft rejection; additionally, allogenic rat heart transplantation causes various degrees of rejection contributing to cardiac graft injury.[50,51] To apply TOACH treatment for clinical graft preservation, immunological allogenic transplant models should be performed in future studies. Second, the heterotopic abdominal heart transplantation model results in remodeling of the graft myocardium due to volume unloading,[52] which maintains graft function only for short periods up to 2–4 weeks.[53-55] Therefore, long-term graft survival and graft function should be confirmed in future studies using other transplant model composed of physiological blood flow. However, because better graft condition during preservation decreases the incidence of immunogenic rejection and maintains better graft function after transplantation,[56] we expect that TOACH treatment would achieve long graft survival and not exacerbate rejections. Third, although we evaluated the graft viability at the end of cold storage and detected the difference in the disruption of tissue structure (Supplementary Fig 1), changes between the groups were less remarkable than that of at 7 days after transplantation (Fig 4C). The histological change and graft ATP content of the organ at the end of cold storage are rarely measurable,[57] and another alternative would be needed to perform a short normothermic (not hypothermic) perfusion to evaluate the clinical state of the graft at the end of storage.

In conclusion, our study provides significant evidence for the improvement of cold organ preservation in transplantation as well as further insight into the unknown mechanism of cold tolerance.

Authors' Contributions

Conceptualization, E.K.; Methodology, E.K.; Investigation, E.K., X.Z., A.I., HO, M.H., and TA; Writing – Original Draft, E.K.; Writing – Review & Editing, K.I., A.T., S.Y., H.M., E.H., and M. Hagiwara; Funding Acquisition, X.Z., K.I., A.I., J.A., S.U., and M. Hagiwara; Resources, X.Z., K.I., H. M., J.A., E.H., S.U., and M. Hagiwara; Supervision, K.I., S.U., and M. Hagiwara.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2022.11.003.

References

1. Guibert EE, Petrenko AY, Balaban CL, et al. Organ preservation: current concepts and new strategies for the next decade. Transfus Med Hemother 2011;38:125–42.
2. Senior M. Beating the organ clock. Nat Biotechnol 2018;36:488–92.
3. Berendsen TA, Bruinsma BG, Puts CF, et al. Supercooling enables long-term transplantation survival following 4 days of liver preservation. Nat Med 2014;20:790–3.

–K) Serum AST (H), ALT (I), LDH (J), and CK release (K) at 7 days after heterotopic cardiac transplantation. For B to K, $n = 5$ rats for each group. Scale bar, 50 μm for 400× and 1000 μm for 20×. *n.s.* $P > 0.05$, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ calculated using one-way ANOVA followed by post hoc Tukey's test. In all statistical plots, data are shown as mean ± SEM.

4. Que W, Hu X, Fujino M, et al. Prolonged cold ischemia time in mouse heart transplantation using supercooling preservation. Transplantation. 2020;104:1879–89.
5. Spetzler VN, Goldaracena N, Echiverri J, et al. Subnormothermic ex vivo liver perfusion is a safe alternative to cold static storage for preserving standard criteria grafts. Liver Transpl 2016;22:111–9.
6. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant 2014;14:1400–9.
7. Qayumi AK, Jamieson WR, Rosado LJ, et al. Preservation techniques for heart transplantation: comparison of hypothermic storage and hypothermic perfusion. J Heart Lung Transplant 1991;10:518–26.
8. Breton S, Brown D. Cold-induced microtubule disruption and relocalization of membrane proteins in kidney epithelial cells. J Am Soc Nephrol 1998;9:155–66.
9. Giwa S, Lewis JK, Alvarez L, et al. The promise of organ and tissue preservation to transform medicine. Nat Biotechnol 2017;35:530–42.
10. Geiser F. Metabolic rate and body temperature reduction during hibernation and daily torpor. Annu Rev Physiol 2004;66:239–74.
11. Cerri M. The central control of energy expenditure: exploiting torpor for medical applications. Annu Rev Physiol 2017;79(79):167–86.
12. Ou J, Ball JM, Luan Y, et al. iPSCs from a hibernator provide a platform for studying cold adaptation and its potential medical applications. Cell 2018;173:851–63. e16.
13. Bouma HR, Kroese FG, Kok JW, et al. Low body temperature governs the decline of circulating lymphocytes during hibernation through sphingosine-1-phosphate. Proc Natl Acad Sci U S A 2011;108:2052–7.
14. Nelson CJ, Otis JP, Carey HV. Global analysis of circulating metabolites in hibernating ground squirrels. Comp Biochem Physiol Part D Genom Proteom 2010;5:265–73.
15. Tarrason G, Auli M, Mustafa S, et al. The sphingosine-1-phosphate receptor-1 antagonist, W146, causes early and short-lasting peripheral blood lymphopenia in mice. Int Immunopharmacol 2011;11:1773–9.
16. Proia RL, Hla T. Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. J Clin Invest 2015;125:1379–87.
17. Maceyka M, Harikumar KB, Milstien S, et al. Sphingosine-1-phosphate signaling and its role in disease. Trends Cell Biol 2012;22:50–60.
18. Dixon AS, Schwinn MK, Hall MP, et al. NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. ACS Chem Biol 2016;11:400–8.
19. Vrecl M, Jorgensen R, Pogacnik A, et al. Development of a BRET2 screening assay using beta-arrestin 2 mutants. J Biomol Screen 2004;9:322–33.
20. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225.. +.
21. Tanaka M, Terry RD, Mokhtari GK, et al. Suppression of graft coronary artery disease by a brief treatment with a selective epsilonPKC activator and a deltaPKC inhibitor in murine cardiac allografts. Circulation 2004;110:II194–9.
22. Wu K, Turk TR, Rauen U, et al. Prolonged cold storage using a new histidine-tryptophan-ketoglutarate-based preservation solution in isogeneic cardiac mouse grafts. Eur Heart J 2011;32:509–16.
23. Venkataraman K, Lee YM, Michaud J, et al. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circ Res 2008;102:669–76.
24. Hidalgo MA, Shah KA, Fuller BJ, et al. Cold ischemia-induced damage to vascular endothelium results in permeability alterations in transplanted lungs. J Thorac Cardiovasc Surg 1996;112:1027–35.
25. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. Science 2005;308:512–7.
26. Masters TN, Fokin AA, Schaper J, et al. Changes in the preserved heart that limit the length of preservation. J Heart Lung Transpl 2002;21:590–9.
27. Kayler LK, Magliocca J, Zendejas I, et al. Impact of cold ischemia time on graft survival among ECD transplant recipients: a paired kidney analysis. Am J Transplant 2011;11:2647–56.
28. Ahmed N, Linardi D, Decimo I, et al. Characterization and expression of sphingosine 1-phosphate receptors in human and rat heart. Front Pharmacol 2017;8:312.
29. Xu Z, You W, Zhou Y, et al. Cold-induced lipid dynamics and transcriptional programs in white adipose tissue. BMC Biol 2019;17:74.
30. Fabri J, de Sa NP, Malavazi I, et al. The dynamics and role of sphingolipids in eukaryotic organisms upon thermal adaptation. Prog Lipid Res 2020;80:101063.
31. Li HX, Xiao Y, Cao LL, et al. Cerebroside C increases tolerance to chilling injury and alters lipid composition in wheat roots. PLoS One 2013;8:e73380.
32. Palmerini CA, Mazzoni M, Giovinazzo G, et al. Blood lipids in Antarctic and in temperate-water fish species. J Membr Biol 2009;230:125–31.
33. Randez-Gil F, Prieto JA, Rodriguez-Puchades A, et al. Myriocin-induced adaptive laboratory evolution of an industrial strain of *Saccharomyces cerevisiae* reveals its potential to remodel lipid composition and heat tolerance. Microb Biotechnol 2020;13:1066–81.
34. Takahashi D, Imai H, Kawamura Y, et al. Lipid profiles of detergent resistant fractions of the plasma membrane in oat and rye in association with cold acclimation and freezing tolerance. Cryobiology 2016;72:123–34.
35. Zhai P, Song J, Gao L, et al. A sphingolipid synthesis-related protein OrmA in *Aspergillus fumigatus* is responsible for azole susceptibility and virulence. Cell Microbiol 2019;21:e13092.
36. Schold JD, Kaplan B, Howard RJ, et al. Are we frozen in time? Analysis of the utilization and efficacy of pulsatile perfusion in renal transplantation. Am J Transplant 2005;5:1681–8.
37. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. Am J Transplant 2015;15:161–9.
38. Kong X, Liu H, He X, et al. Unraveling the mystery of cold stress-induced myocardial injury. Front Physiol 2020;11:580811.
39. Peralta C, Jimenez-Castro MB, Gracia-Sancho J. Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu. J Hepatol 2013;59:1094–106.
40. Li Y, Hu XX, Fu L, et al. Time window is important for adenosine preventing cold-induced injury to the endothelium. J Cardiovasc Pharmacol 2017;69:382–8.
41. Means CK, Xiao CY, Li Z, et al. Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2007;292:H2944–51.
42. Theilmeier G, Schmidt C, Herrmann J, et al. High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P(3) lysophospholipid receptor. Circulation 2006;114:1403–9.
43. Nofer JR, van der Giet M, Tolle M, et al. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. J Clin Invest 2004;113:569–81.
44. Nussbaum C, Bannenberg S, Keul P, et al. Sphingosine-1-phosphate receptor 3 promotes leukocyte rolling by mobilizing endothelial P-selectin. Nat Commun 2015;6:6416.
45. Takuwa N, Ohkura S, Takashima S, et al. S1P3-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species. Cardiovasc Res 2010;85:484–93.
46. Stefanovich P, Ezzell RM, Sheehan SJ, et al. Effects of hypothermia on the function, membrane integrity, and cytoskeletal structure of hepatocytes. Cryobiology 1995;32:389–403.
47. Kang DS, Tian X, Benovic JL. Role of beta-arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. Curr Opin Cell Biol 2014;27:63–71.
48. Shukla AK, Singh G, Ghosh E. Emerging structural insights into biased GPCR signaling. Trends Biochem Sci 2014;39:594–602.
49. Mueller XM. Drug immunosuppression therapy for adult heart transplantation. Part 1: immune response to allograft and mechanism of action of immunosuppressants. Ann Thorac Surg 2004;77:354–62.
50. Szabo G, Bahrle S, Sivanandam V, et al. Immunomodulatory effects of poly(ADP-ribose) polymerase inhibition contribute to improved cardiac function and survival during acute cardiac rejection. J Heart Lung Transplant 2006;25:794–804.
51. Bedi DS, Riella LV, Tullius SG, et al. Animal models of chronic allograft injury: contributions and limitations to understanding the mechanism of long-term graft dysfunction. Transplantation 2010;90:935–44.
52. Benke K, Sayour AA, Matyas C, et al. Heterotopic abdominal rat heart transplantation as a model to investigate volume dependency of myocardial remodeling. Transplantation 2017;101:498–505.
53. Brinks H, Tevaearai H, Muhlfeld C, et al. Contractile function is preserved in unloaded hearts despite atrophic remodeling. J Thorac Cardiovasc Surg 2009;137:742–6.
54. Welsh DC, Dipla K, McNulty PH, et al. Preserved contractile function despite atrophic remodeling in unloaded rat hearts. Am J Physiol Heart Circ Physiol 2001;281:H1131–6.
55. Ito K, Nakayama M, Hasan F, et al. Contractile reserve and calcium regulation are depressed in myocytes from chronically unloaded hearts. Circulation 2003;107:1176–82.
56. Nair RV, Morris RE. Immunosuppression in cardiac transplantation: a new era in immunopharmacology. Curr Opin Cardiol 1995;10:207–17.
57. Wakayama K, Fukai M, Yamashita K, et al. Successful transplantation of rat hearts subjected to extended cold preservation with a novel preservation solution. Transpl Int 2012;25:696–706.