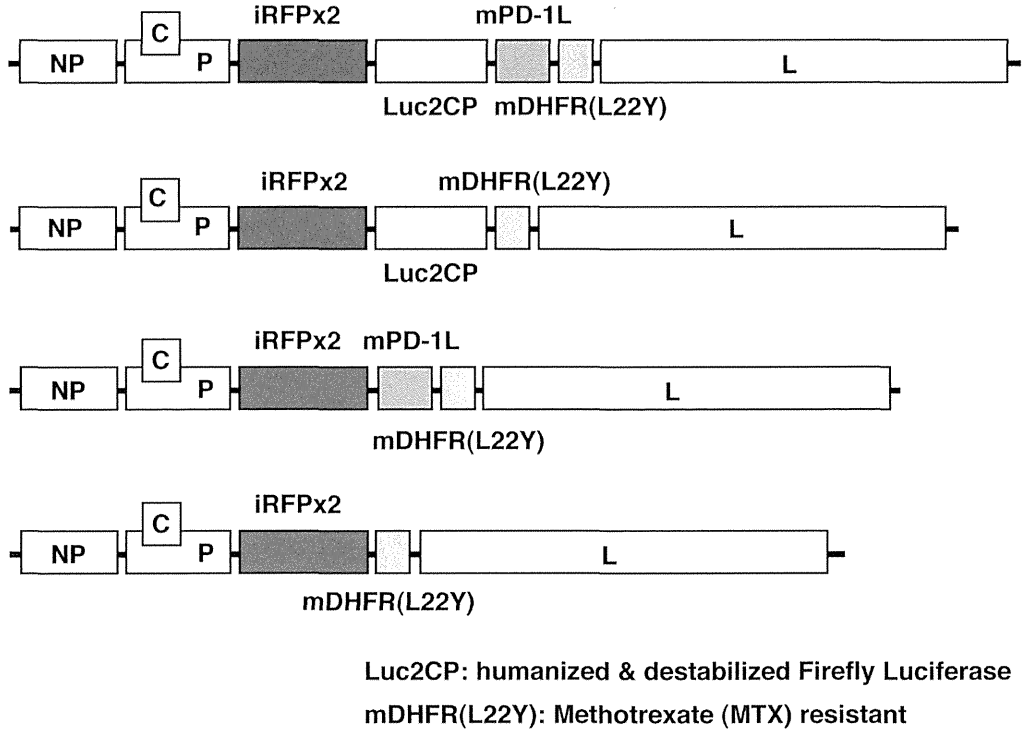


図１

遠赤外蛍光タンパク質 iRFP 遺伝子を搭載したステルス型 RNA ベクターのゲノム構造

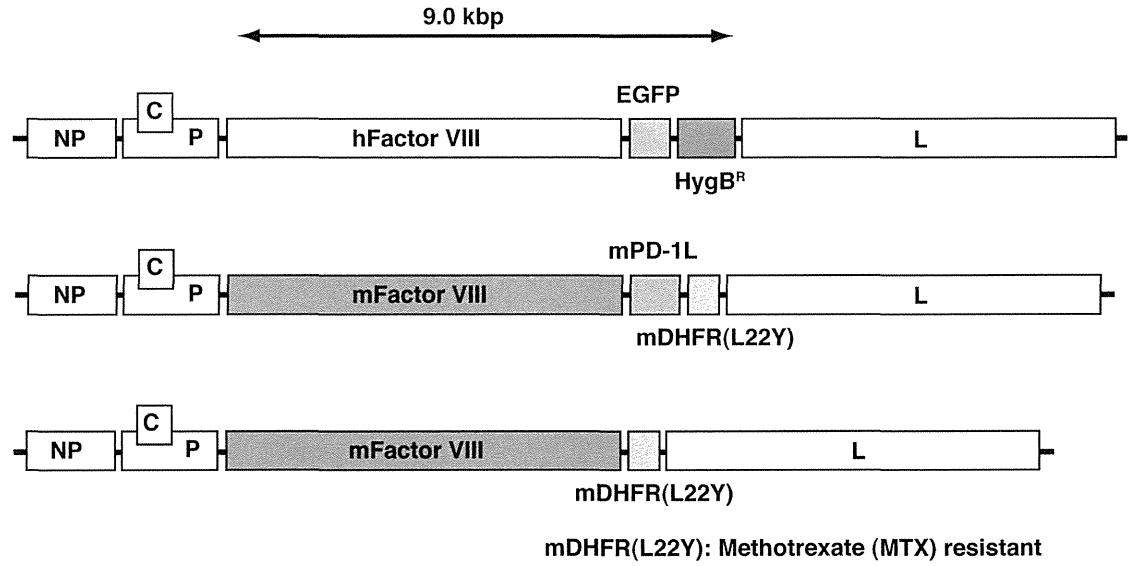


図２　血液凝固第Ⅷ因子遺伝子を搭載したステルス型 RNA ベクターのゲノム構造

厚生労働科学研究委託費（再生医療実用化研究事業）
委託業務成果報告書（業務項目）

高性能の新規RNAベクターによる血友病遺伝子治療の開発
血液凝固因子抗原および活性評価系の開発およびベクターの性能評価

担当責任者　伊藤　昌史　（エーザイ株式会社筑波研究所）
同　福島　敬　（筑波大学医学医療系）
研究協力者　八牧　愉二　（筑波大学附属病院）

研究要旨

新規RNAベクターの性能評価の準備として、脂肪細胞から分泌される血液凝固因子の活性および抗原を測定するアッセイ系を検討することを目的とした。本実用化研究で使用する第VIII血液凝固因子の３つのタイプのcDNAを、PiggyBac Transposon System (System Biosciences, Inc.)を使用してHEK293細胞株に導入し、発現させた培養上清中の、第VIII血液凝固因子の活性は、Coatest SP4 Factor VIII (Chromogenix)を用いて、抗原はVisuaLize FVIII Antigen Kit (Affinity Biologicals)を用いて定量することが可能であった。血友病マウスに対する治療効果を判定するためには、マウス凝固因子のアッセイ系が必要であり、継続検討課題である。

A．研究目的

ベクターの性能評価の準備として脂肪細胞から分泌される血液凝固因子の活性および抗原を測定するアッセイ系を検討する。

一般的な培養細胞(HEK293 細胞)を用いて、第VIII 血液凝固因子(FVIII)を発現させて、発現した組換体が活性を有するかの確認を行う。培養上清中の FVIII の抗原定量系(ELISA)と活性測定系(合成基質法)によるアッセイ系を構築し、今後の実験のプラットホームを確立する。

B．研究方法

概要を資料１に示すが、PiggyBac Transposon System (System Biosciences, Inc.)を用いて、野生型(H1)、B ドメイン欠損型(H1_Bdel)、および野生型と比較して分泌効率が約 10 倍まで増大するように作成された変異型FVIII である226N6 変異型FVIII(H1_N6) の３種類のヒトFVIII cDNA をHEK293 細胞に遺伝子導入した。細胞培養液中に分泌されたFVIII抗原量はVisuaLize FVIII Antigen Kit (Affinity Biologicals)を用いて、ELISA 法で測定した。細胞培養液中に分泌されたFVIII の活性は Coatest SP4 Factor VIII (Chromogenix)を用いて、合成基質法で測定した。

（倫理面への配慮）

筑波大学臨床研究倫理審査委員会の審査を受け、附属病院長による承認を得て、共同研究契約に基づいて実施した（II 4 資料1,2参照)。

C．研究成果

１．抗原定量（ELISA)

測定結果を資料２に示す。FL（完全型)、BDD（B ドメイン欠失型)、N6R の３種類の cDNA に由来するFVIII 分子（蛋白）の定量値には若干の差異がみられた。

２．活性定量（合成基質法)

測定結果を資料 3-1、3-2 に示す。FL(完全型)、BDD（B ドメイン欠失型)、N6R の３種類の cDNA に由来するFVIII 分子（蛋白）の定量値には若干の差異がみられた。

D．考察

HEK293細胞において組換えFVIIIの発現に関してはPiggyBac システムで十分な発現が確認された。Ogata ら（Gene Therapy 2004）およびMatsui ら（Plos One 2014）の報告によれば、PiggyBac

システムを使用したFVIII遺伝子導入発現実験における培養上清中の定量値は、大きな差異はなく、スクリーニングでの使用は可能であると考えられた。しかし、培養上清中のFVIIIの比活性は血漿や市販の凝固因子製剤に比べて低かった。蛋白質転換酵素であるfurinによる細胞内プロセッシングの促進要否、培地への添加物等、今後の検討が必要である。

FVIII の抗原量や活性の評価は、これまでの遺伝子治療に関する報告（PLoS One. 9(8): e104957 (2014)、Molecular Therapy. 19(4): 723-730 (2011)、PLoS One. 8(12): e83280 (2013)）と同様の方法で行った。FVIII の定量系（ELISA 法）は、今回のキットで問題ないと考えられたが、コントロールとして血漿や凝固因子製剤をどのように使用していくかについてはさらに検討が必要である。特に、N6R または BDD を測定する際の Standard curve 作成の際には BDD 型 FVIII 製剤を用いるのがより適切であるかどうかの検討が必要である。

FVIII の活性測定系（合成基質法）については、活性の高いサンプルに関しては今回のキットで問題なく測定できたが、活性の低い（<5%）サンプルに関しては、希釈率、検量線の引き方等に関しての検討が必要である。

今後は脂肪細胞培養上清を用いて同様の検討を行う。また、マウスを用いた実験を実施できるようにマウスFVIIIについてもアッセイ系を構築し、今回使用した測定キットがマウスFVIIIに交差するかの検討を行う。

E．結論

1．ヒトFVIIIのうち、完全型、N6、およびBドメイン欠失型の3種のcDNAに由来するものにおいては、市販キットを複数利用することによって、活性および抗原の定量は可能である。

2．マウスのFVIII活性・抗原定量系については、今後検討が必要である。

F．健康危険情報　　該当せず

（委託業務成果報告（業務項目）には記入せずに、委託業務成果報告（総括）にまとめて記入）

G．研究発表

1．論文発表　　なし

2．学会発表

（発表誌名巻号・頁・発行年も記入）

なし

H．知的財産権の出願・登録状況

1．特許取得

遺伝子治療用初代脂肪細胞

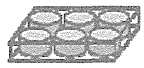
発明人：齋藤康（千葉大学）、伊藤昌史（エーザイ株式会社）、出願番号：PCT/JP03/07721、特許番号：JP 4879867、US 7,820,438 B2、EP 1541674 B1　（日・米・欧、オーストラリア、韓国、中国、他40カ国特許成立済み）

2．実用新案登録　　なし

3．その他　　なし

資料１　HEK293EBNAへの遺伝子導入

HEK293EBNA

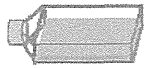


Medium 2ml

↓ Sampling@24hr

↓ Sampling@48hr

↓ Expand



Medium 20ml

↓ Selection Puro(10ug/ml)

Medium 12ml

↓

Sup. (-80℃)

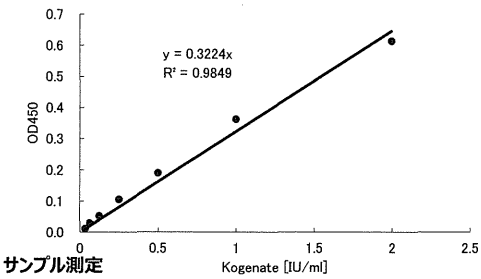
Day0 transfection (6well-plate)
Day1 sampling _24hr -80℃ stock
Day2 sampling _48hr -80℃ stock
Day3 Expand to F75 flask
　　Puro selection (10ug/ml)start

Day7 medium change (without Puro)
Day10 medium change (～80%confluent)
Day11 sampling -80℃ stock

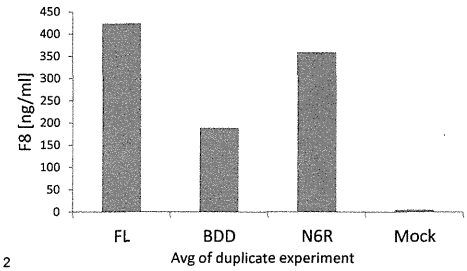
1

資料２　FVIII ELISA (stable expression)

Standard curve



サンプル測定



Standard control
Kogenate FS (バイエル)
1IU/ml=250ng/mlとして換算

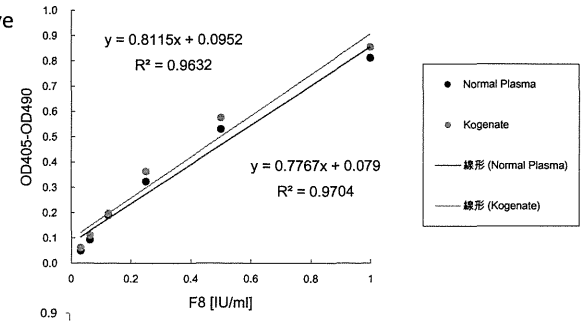
Medium change at 70-80%confluent
F75/12ml medium
24hr>sampling

BDDとN6RはReFactoをコントロールとするべき

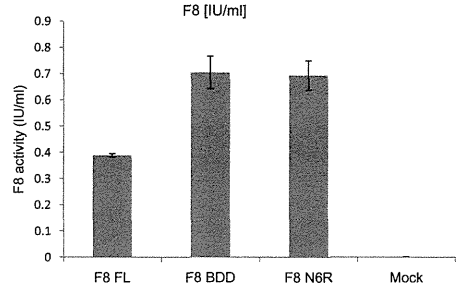
2

資料3-1 FVIII活性測定(stable expression)

Standard curve



サンプル測定



3

資料3-2 活性測定結果(293EBNA piggyBac stable)

	antigen(ng/ml)	activity(IU/ml)	specific activity IU/mg	SEM
FL	424.4141	0.388632	915.6911	±14.7262
BDD	189.4531*	0.704251	3717.286	±324.6848
N6R	359.1797*	0.693161	1929.844	±154.7334
Kogenate	250	1	4000	

The specific activity of KOGENATE Bayer is approximately 4000 IU/mg protein
The specific activity of ReFacto AF is 7,600-13,800 IU/mg protein

＊定量値は、コントロールにReFactoを用いないと正確でない

4

業務報告書(伊藤ら)資料3-1,3-2

厚生労働科学研究委託費（再生医療実用化研究事業）
委託業務成果報告書（業務項目）

高性能の新規 RNA ベクターによる血友病遺伝子治療の開発

遺伝子導入脂肪細胞を開発対象としたGMP生産・非臨床試験計画（予備的毒性試験）の構築に向けた規制対応研究の計画の共同立案

担当責任者　　麻生 雅是　（セルジェンテック株式会社）

研究要旨

血友病A治療用の第Ⅷ凝固因子遺伝子導入脂肪細胞を開発対象としたGMP生産・非臨床試験計画（予備的毒性試験）の構築に向けて、行政機関等からの情報に基づく規制対応研究の計画の非臨床試験の準備を進めた。

Ａ．研究目的

遺伝子導入脂肪細胞を開発対象としたGMP生産・非臨床試験計画（予備的毒性試験）の構築に向けた規制対応研究の計画の共同立案筑波大学と密に連絡を取りながら、非臨床試験の準備を進める。

遺伝子導入脂肪細胞を開発対象とした場合の非臨床試験について、行政機関等からの情報を踏まえ、合理的な方法を検討する。最短での遺伝子改変脂肪細胞GMP生産ライン確立を念頭において検討を進める。

Ｂ．研究方法

１．GMP 生産

脱脂肪のヒト脂肪細胞を用いた GMP(GCTP)製造の検討を行っており、ヒト生物由来原材料の規制に留意し、不純物含有試験、無菌やウイルス否定試験等の品質試験方法の再確認試験を実施する。

２．非臨床試験計画

ヒト第 VIII 血液凝固因子（FⅧ）遺伝子導入脂肪細胞の非臨床試験計画の立案を筑波大学と連携を以て行う

（倫理面への配慮）

本研究開発は以下の原則、法令、省令及び倫理指針に遵守し実施する。

移植用細胞製造・再生医療等の安全性の確保等に関する法律（平成 26 年 11 月 25 日施行）、遺伝子治療臨床研究に関する指針（平成平成２６年１１月２５日一部改正）

Ｃ．研究成果

１．GMP 生産

脱脂肪のヒト脂肪細胞を用いた GMP(GCTP)製造の検討を行っており、ヒト生物由来原材料の規制に留意し、不純物含有試験のバリデーション方法、無菌やウイルス否定試験等の品質試験方法の確認試験の妥当性を

検討中である。

２．非臨床試験計画

凝固因子遺伝子導入脂肪細胞の非臨床試験計画の立案を筑波大学と連携し行っており、脂肪細胞を用いたfeasibility試験（基礎的POCの確立）のための血友病Aモデルマウス（完全欠損や部分欠損マウス）の飼育などの調査活動を実施中である。

Ｄ．考察

１．GMP生産

ヒト生物由来原材料の規制に留意し、不純物含有試験、無菌やウイルス否定試験等の品質試験方法の確認試験を実施しているが、遺伝子導入の際使用する凝固因子遺伝子搭載の新規 RNA ベクターの由来の安全性・品質管理およびが重要となる。また発現凝固因子の培養中の力価測定方法の開発に今後取り組む。

２．非臨床試験計画

血友病マウスで実現可能性を調査するにあたって、凝固因子遺伝子導入脂肪細胞の凝固因子発現能と移植可能細胞数から導入コピー数の決定を行い、血友病マウスへの移植を行う。またその際の抗体発現などのモニターシステムの開発が必要となる。

Ｅ．結論

１．GMP生産

凝固因子遺伝子搭載の新規 RNA ベクターの由来の不純物試験や安全性・品質管理試験以外は、PMDAの指導に従って、ヒト生物由来原材料の規制に留意し、不純物含有試験、無菌やウイルス否定試験等の品質試験方法の確認試験を実施している。

２．非臨床試験計画

凝固因子遺伝子導入脂肪細胞の凝固因子発現能と移植細胞数の推定が急務であること、またその発現持続性や細胞形質や特性について広く情報取集が必要である。

Ｆ．健康危険情報　　該当せず

（委託業務成果報告（業務項目）には記入せずに、委託業務成果報告（総括）にまとめて記入）

Ｇ．研究発表

１．論文発表　　なし

２．学会発表　　なし

（発表誌名巻号・頁・発行年も記入）

Ｈ．知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　　なし

2. 実用新案登録　　なし

3. その他　　なし

様式第１９

学　会　等　発　表　実　績

委託業務題目「高性能の新規RNAベクターによる血友病遺伝子治療の開発」
機関名　国立大学法人　筑波大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
近赤外非侵襲蛍光イメージング技術の開発と応用（招待講演）	三輪佳宏	横浜（第27回日本動物実験代替法学会）	2015年3月	国内
ステルス型RNAベクターの開発と再生医療への応用	中西真人	東京（第14回日本再生医療学会総会）	2015年3月	国内
Feeder-free及びxeno-free条件下でのヒト末梢血由来単球からのiPS細胞誘導	大高真奈美、久保陽子、西村健、佐野将之、中西真人	東京（第14回日本再生医療学会総会）	2015年3月	国内
SeVdpベクターを利用した細胞リプログラミングにおけるマイクロRNA検出系の評価	佐野将之、大高真奈美、飯島実、中西真人	東京（第14回日本再生医療学会総会）	2015年3月	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Novel mitochondria-targeted heat-soluble proteins identified in the anhydrobiotic tardigrade improve osmotic tolerance of human cells.	Tanaka S, Tanaka J, Miwa Y, Horikawa DD, Katayama T, Arakawa K, Toyoda A, Kubo T, Kunieda T.	Plos One	2015 Feb 12; 10(2):e0118272. doi: 10.1371/journal.pone.0118272. eCollection 2015	国際誌
Biosynthesis of Ribosomal RNA in Nucleoli Regulates Pluripotency and Differentiation Ability of Pluripotent Stem Cells.	Watanabe-Susaki, K., Takada, H., Enomoto, K., Miwata, K., Ishimine, H., Intoh, A., Ohtaka, M., Nakanishi, M., Sugino, H., Asashima, M. Kurisaki, A.	Stem Cells	32: 3099-3111, 2014	国際誌

Manipulation of Klf4 expression generates paused iPSCs stalled at distinct stages of reprogramming.	Nishimura, K., Kato, T., Oinam, L., Shiomitsu, E., Ayakawa, D., Ohtaka, M., Fukuda, A., Nakanishi, M. Hisatake, K.	Stem Cell Reports	3: 915-929,2014	国際誌
Advanced Feeder-Free Generation of iPSC Directly From Blood Cells.	Trokovic, R., Weltner, J., Nishimura, K., Ohtaka, M., Nakanishi, M., Salomaa, V., Jalanko, A., Otonkoski, T. and Kyttälä, A.	STEM CELLS Translational Medicine	3: 1402-1409,2014	国際誌
Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes.	Takayama, K., Morisaki, Y., Kuno, S., Nagamoto, Y., Harada, K., Furukawa, N., Ohtaka, M., Nishimura, K., Imagawa, K., Sakurai, F., Tachibana, M., Sumazaki, R., Noguchi, E., Nakanishi, M., Hirata, K., Kawabata, K. Mizuguchi, H	Proc. Natl. Acad. Sci. USA	111: 16772-16777,2014	国際誌

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

PLOS | ONE

RESEARCH ARTICLE

Novel Mitochondria-Targeted Heat-Soluble Proteins Identified in the Anhydrobiotic Tardigrade Improve Osmotic Tolerance of Human Cells

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Abstract

Tardigrades are able to tolerate almost complete dehydration through transition to a metabolically inactive state, called "anhydrobiosis". Late Embryogenesis Abundant (LEA) proteins are heat-soluble proteins involved in the desiccation tolerance of many anhydrobiotic organisms. Tardigrades, *Ramazzottius varieornatus*, however, express predominantly tardigrade-unique heat-soluble proteins: CAHS (Cytoplasmic Abundant Heat Soluble) and SAHS (Secretory Abundant Heat Soluble) proteins, which are secreted or localized in most intracellular compartments, except the mitochondria. Although mitochondrial integrity is crucial to ensure cellular survival, protective molecules for mitochondria have remained elusive. Here, we identified two novel mitochondrial heat-soluble proteins, RvLEAM and MAHS (Mitochondrial Abundant Heat Soluble), as potent mitochondrial protectants from *Ramazzottius varieornatus*. RvLEAM is a group3 LEA protein and immunohistochemistry confirmed its mitochondrial localization in tardigrade cells. MAHS-green fluorescent protein fusion protein localized in human mitochondria and was heat-soluble *in vitro*, though no sequence similarity with other known proteins was found, and one region was conserved among tardigrades. Furthermore, we demonstrated that RvLEAM protein as well as MAHS protein improved the hyperosmotic tolerance of human cells. The findings of the present study revealed that tardigrade mitochondria contain at least two types of heat-soluble proteins that might have protective roles in water-deficient environments.

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Introduction

Tardigrades are microscopic invertebrates comprising the phylum Tardigrada. Although all tardigrade species are primarily aquatic and require water to grow and reproduce offspring, some terrestrial species can tolerate almost complete desiccation by entering an ametabolic dehydrated state, referred to as anhydrobiosis [1]. Dehydrated tardigrades exhibit extreme tolerance against various environmental stresses [2,3]. The molecular mechanism of anhydrobiosis in tardigrades, however, remains largely unknown.

Anhydrobiotic ability is observed in selected invertebrate species belonging to a few animal phyla, such as tardigrades, nematodes, arthropods, and rotifers. Trehalose accumulates extensively in nematodes and arthropods in a dehydrated state, and is suggested to play an important role in their anhydrobiotic ability [4–6]. In contrast, low or no trehalose accumulation is detected in tardigrades and rotifers, suggesting that anhydrobiosis is achieved by another mechanism [7,8].

Another candidate molecule is late embryogenesis abundant (LEA) protein. LEA protein was originally detected as an abundantly accumulated protein in desiccating plant seeds and later in various anhydrobiotic animals in the phyla mentioned above [9]. In principle, LEA protein expression is significantly induced by desiccation in most anhydrobiotic animals [10,11]. LEA proteins are highly hydrophilic and intrinsically unstructured proteins [12], and maintain solubility even after boiling in water (heat-soluble) [11,13]. *In vitro* studies demonstrated that LEA proteins weakly interact with other macromolecules to prevent undesirable aggregation of proteins [14,15] or conformational changes of lipid membranes by acting as a 'molecular-shield' [15]. LEA proteins are also proposed to act as ion-scavengers and anti-oxidants, but their precise functions are not completely understood. Consistent with their protective activity *in vitro*, introduction of LEA proteins to stress-sensitive cells like human cells improves tolerability against various water stresses, such as dehydration, hyperosmotic treatment, and freezing [16–18]. Several LEA proteins potentially localize in specific subcellular compartments, like mitochondria, cytosol, or endoplasmic reticulum, and likely protect macromolecules in the respective compartments [16–19].

Although LEA-like sequences are found in EST or TSA databases of tardigrades [20–22], our previous heat-soluble proteome study revealed that two novel protein families other than LEA proteins are the predominant heat-soluble proteins in the anhydrobiotic tardigrade *Ramazzottius varieornatus*: cytoplasmic abundant heat soluble (CAHS) proteins and secretory abundant heat soluble (SAHS) proteins [23]. CAHS proteins localize in the cytoplasm and occasionally in the nucleus, whereas SAHS proteins are mainly secreted to the extracellular space and a minor population is detected in secretory organelles. Subcellular localization analyses of these proteins using green fluorescent protein (GFP)-fused protein in culture cells clearly demonstrated that mitochondria lack both of these heat-soluble proteins [23]. Other potent heat-soluble proteins like LEA proteins or potential protective molecules have not yet been identified in tardigrade mitochondria.

In the present study, we identified two novel mitochondrial heat-soluble proteins from the anhydrobiotic tardigrade *R. varieornatus*. One of the identified proteins, RvLEAM, is the first mitochondrial LEA protein identified in tardigrades. The other identified protein, Mitochondrial Abundant Heat Soluble (MAHS) protein, is not an LEA protein, and rather forms a novel mitochondrial heat-soluble protein family that is conserved in tardigrades, but not found in other phyla. Furthermore, we demonstrated that either RvLEAM or MAHS protein could confer hyperosmotic tolerance to human cells. The presence of MAHS protein, together with previously identified CAHS and SAHS proteins, indicates that tardigrades possess a unique repertoire of heat-soluble proteins covering most cellular compartments, including the

mitochondria. The identified repertoire of tardigrade-unique heat-soluble proteins will provide important clues to the desiccation tolerant mechanism in tardigrades.

Materials and Methods

Animals

For all experiments, YOKOZUNA-1 strain *R. varieornatus* was used. The strain was established from a single individual in a previous study [24] and was maintained on water-layered agar plates by feeding algae, as previously described [24]. Tardigrades lay eggs at intervals of approximately 4 to 5 days. Embryos with eggshells were collected at multiple developmental stages and collectively subjected to immunohistochemical analyses.

Sequence analysis

Three independent programs, Wolf PSORT [25], TargetP [26,27], and MitoProt2 [28], were used to predict the subcellular localization of candidate proteins. The PORTER program was used to predict the secondary structures of RvLEAM (LC002821) and MAHS (LC002822) [29]. Similarity searches were performed by BLASTP or TBLASTN using NCBI non-redundant (nr), EST and TSA databases. A Kyte-Doolittle hydropathy plot was analysed with Genome Consortium for Active Teaching Genomics Tools (http://gcat.davidson.edu/DGPB/kd/KD.html) and a grand average of hydropathy (GRAVY) score was calculated by Sequence Manipulation Suite Protein GRAVY (http://www.bioinformatics.org/sms2/protein_gravy.html) [30]. Motif search and de-novo motif discovery were performed by InterProScan (http://www.ebi.ac.uk/interpro/) [31] and MEME program (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) [32], respectively. Multiple alignments and Sequence Logo analysis were performed using Clustalx and CLC Main Workbench 6 (CLC bio).

Subcellular localization of GFP-fused protein

Expression of GFP-fused protein and analyses of its subcellular localization were performed essentially as described previously [23]. Briefly, expression constructs were prepared by inserting coding sequences of candidate proteins into the pAcGFP1-N1 vector (Clontech) to fuse GFP at the C-terminus of target proteins. The constructs were transfected to human HEp-2 or HEK293T cells using X-tremeGENE 9 reagent (Roche). The parental cell lines were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. After 24-h incubation, nuclear DNA and mitochondria were stained with Hoechst 33342 (Lonza) and MitoTracker Red CMXRos (Lonza), respectively. Fluorescent images were obtained using a confocal microscope, LSM710 (Carl Zeiss).

Immunohistochemistry

Collected embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. After rinsing the samples with PBS, they were incubated overnight in 30% sucrose in PBS and embedded in 2% agar block. The ager blocks were trimmed and embedded in O.C.T compound (Tissue-Tek). Frozen sections were cut at a 10-μm thickness using a cryostat and collected on glass slides. The slides were rinsed with 0.1% Tween-20 in Tris-buffered saline (TBS) and microwaved in 0.1 M sodium citrate buffer (pH 6.0) for antigen activation. Anti-RvLEAM rabbit antiserum was raised against bacterially expressed and purified full-length RvLEAM protein. To visualize the mitochondria, we used anti-ATP5A [15H4C4] antibody (Abcam). After incubation with blocking buffer (2% goat serum and 0.1% Tween-20 in TBS), anti-ATP5A mouse antibody solution was added at a dilution of 1/250. After three washes with

0.1% Tween-20 in TBS, the specimens were reacted with secondary anti-mouse IgG antibody labelled with Alexa-Fluor 568 in blocking buffer for 1 h at room temperature. After three washes with 0.1% Tween-20 in TBS, anti-RvLEAM antiserum solution was added at a dilution of 1/500. After three washes with 0.1% Tween-20 in TBS, the specimens were reacted with secondary anti-rabbit IgG antibody labelled with Alexa-Fluor 488 in blocking buffer for 1 h at room temperature. Prior to observation with a confocal microscope, DNA was stained with Hoechst 33342 (Lonza).

Heat-solubility assay

The heat-solubility assay was performed as described previously [23] with slight modification. Recombinant proteins tagged with 6xHis at the N-terminus were expressed in *Escherichia coli* BL21(DE3) using the pET system (Novagen) for RvLEAM and MAHS, and the pCold-I system (Takara) for ATPM1 (Actively transcribed Transcript encoding Potential Mitochondrial 1, LC002823). Predicted mitochondria-targeting peptides were not included in the construct for MAHS and ATPM1. After inducing proteins according to the manufacturer's protocol, the bacterial pellets were lysed by sonication in PBS containing protease inhibiter cocktail, Complete EDTA-free (Roche). His-tagged proteins were captured with Ni-NTA Superflow (Qiagen) and eluted with 250 mM imidazole in PBS after extensive washes with PBS. Imidazole was removed by dialysis against PBS using a micro-dialyzer, TOR-14K (Nippon Genetics). Prior to heat treatment, the protein solutions were centrifuged at 10,000 rpm for 30 min at 4°C and supernatants were used as a starting material for the heat-solubility assay. After heat treatment at 92°C for 15 min, samples were cooled to room temperature and centrifuged at 10,000 rpm for 30 min to separate the supernatants and precipitates as heat-soluble fractions and insoluble fractions, respectively. Protein fractions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining.

Osmotic tolerance assay

We utilized an Epstein-Barr virus-based vector system to efficiently establish stably transfected cells [33]. HEp-2 cells were used as parental cells and were essentially maintained in Eagle's minimal essential medium (EMEM; 286–317 mOsm/kg; Sigma-Aldrich M4655) containing 10% fetal bovine serum (FBS; 305 mOsm/kg; Corning 35–010-CV, lot. 35010109). Coding sequences of target proteins were inserted downstream of the CAG promoter of the pEB6-CAGMCS a/p vector and the resultant constructs were transfected into HEp-2 cells using Lipofectamine LTX & Plus Reagent (Invitrogen). Transfected cells were selected by 4-μg/ml puromycin treatment over 7 d. Cells were seeded at a density 4.0×10^4 cells in 24-well plates 24 h before stress exposure. For exposure to hyperosmotic stress, the medium was replaced with freshly prepared hyperosmotic EMEM containing 10% FBS and various amounts of additional sucrose (final 100–350 mM; theoretical upshifts of osmolality are 100–350 mOsm/L) and cells were incubated for 48 h. After hyperosmotic exposure, the metabolic activities of treated cells were measured by replacing the medium with serum-free Opti-MEM (280–320 mOsm/kg, Invitrogen) containing the cell viability indicator PrestoBlue (Molecular Probes). PrestoBlue is a cell permeable resazurin-based reagent, which is converted to highly fluorescent resorufin by reducing power of living cells and thus metabolic activities can be quantitatively measured by detecting fluorescence. After incubation for 30 min, fluorescent signals were measured as an indication of cell viability with a Gemini EM Microplate Reader (Molecular Devices). Independent three wells were examined for each condition and relative cell metabolic activity was calculated by dividing each value by the mean value of the same cell strain at 0 mM additional

sucrose. Improvements of cell viability were statistically analysed using one-sided upper *Dunnett*'s test against HEp-2 control cells.

Results

Identification of a putative mitochondrial LEA protein, RvLEAM, in *R. varieornatus*

As candidate proteins involved in protecting mitochondria from desiccation stress, we detected an LEA protein potentially targeted to the mitochondria from our transcriptome database of *R. varieornatus*. We designated this protein RvLEAM. RvLEAM showed sequence similarity with nematode LEA proteins in the BLAST search (e-value < 1e-05) and matched with LEA-RELATED motif (PTHR23241) of PANTHER database in InterProScan search. Motif discovery program MEME and manual inspection revealed 9 LEA-like 11-mer motifs in the RvLEAM sequence (Fig. 1a-b, S1 Fig.). Seven of them formed three regions composed of contiguously repeated LEA-like motifs (Fig. 1a). Contiguous repeats of the 11mer LEA motif is characteristics of group 3 LEA proteins and its consensus sequence, ΨΩΨΦXΩΦΦΩXΦ [34], matched well with all motifs found in RvLEAM (Fig. 1b; Ψ, basic residue; Ω, acidic or amide residue; Φ, aliphatic residue; X, non-conserved residue). Similar to most LEA proteins, RvLEAM is highly hydrophilic (GRAVY score, -0.94) and a hydropathy plot showed no clear hydrophobic region over the entire sequence, suggesting that RvLEAM localized in the mitochondrial matrix rather than integrating into the mitochondrial membrane (Fig. 1a). Another characteristic of group 3 LEA proteins is their amphipathic helical structure under water-deficient conditions. The secondary structure prediction program predicted long helical regions that contained nine LEA-like motifs, and, in particular, a region containing two contiguously repeating LEA motifs (3 and 4) showed a typical amino-acid distribution forming putative amphipathic helix like LEA proteins (Fig. 1a, 1c).

We tested three subcellular localization prediction programs, TargetP, WoLF PSORT, and MitoProt2, and all three programs predicted that the RvLEAM protein localized in the mitochondria with high scores (S1 Table). TargetP also predicted a 31-mer mitochondria-targeting peptide at the N-terminus (Fig. 1a, S1 Fig.). To experimentally examine the mitochondrial-targeting potential of RvLEAM, we analysed the subcellular localization of RvLEAM in cultured mammalian cells by fusing GFP at the C-terminus. RvLEAM-GFP showed condensed localization in cells that was well co-localized with mitochondria stained by MitoTracker Red CMXRos (Fig. 1d). GFP alone was dispersed throughout cytoplasm, indicating that the mitochondrial localization of RvLEAM-GFP was due to the targeting potential of RvLEAM (Fig. 1d). Together, these findings suggested that RvLEAM is a mitochondria-targeted LEA protein, the first report of such a protein in tardigrades.

RvLEAM protein localized in tardigrade mitochondria

To clarify the intact subcellular localization of RvLEAM in tardigrade cells, we performed immunohistochemical analysis using frozen sections of tardigrades. To clearly visualize subcellular localization, we chose early embryonic stage because embryonic cells are much larger than adult cells and the embryos also possess anhydrobiotic ability as adults do [24]. The sections of the tardigrade embryos were reacted with newly raised antiserum against RvLEAM. For simultaneous visualization of the mitochondria, the sections were co-stained with anti-ATP synthase (ATP5A) antibody as a mitochondrial marker. The fluorescent signals of RvLEAM were detected in almost all cells and their localization was mostly merged with those of ATP5A, indicating mitochondrial localization of RvLEAM in tardigrade cells (Fig. 2). In sections reacted with control antibodies, no signals were detected inside the eggshells (S2 Fig.),

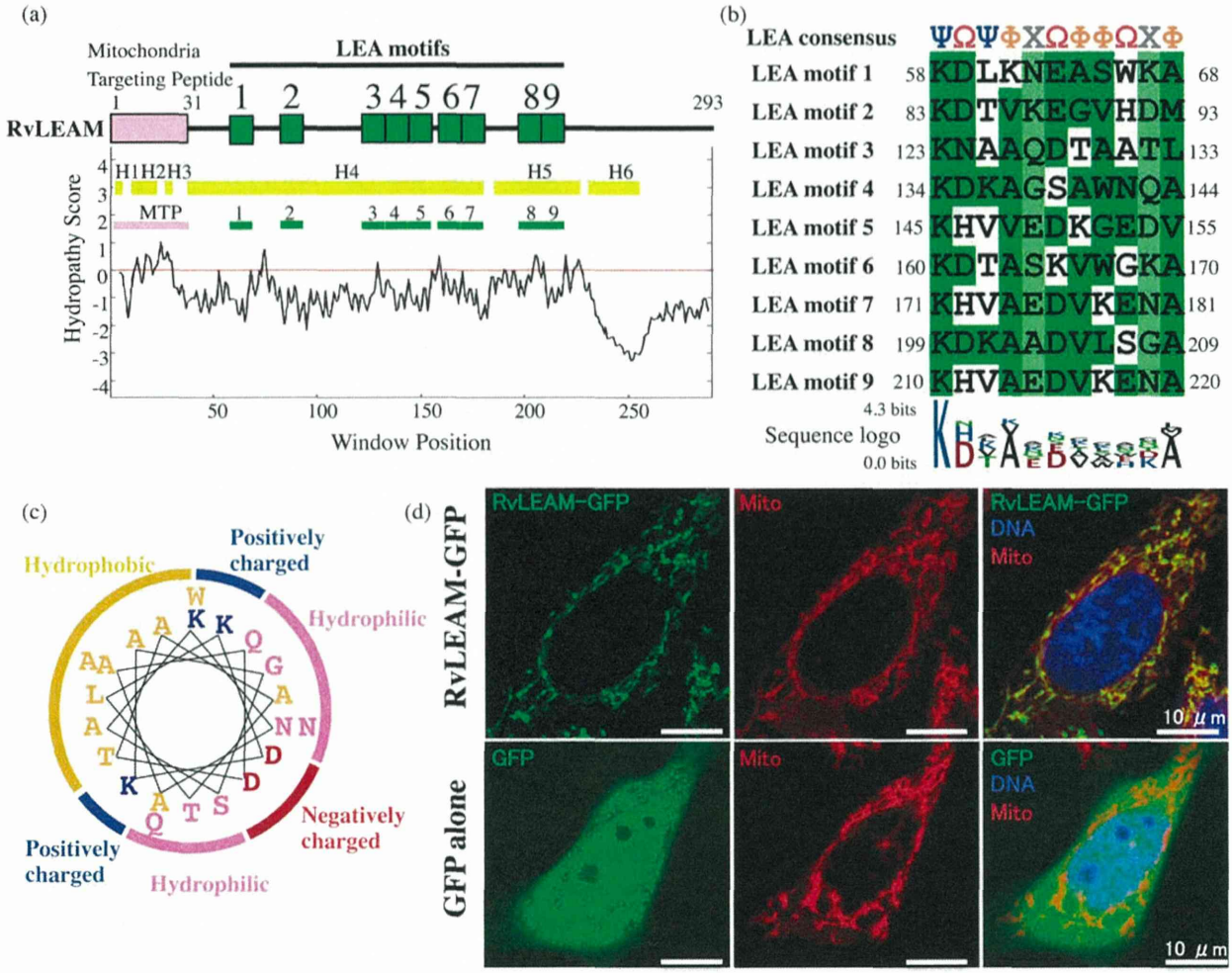

Fig 1. Protein structure and mitochondria-targeting potential of RvLEAM. (a) Schematic representation of RvLEAM protein structure with hydropathy plot. Pink box indicates Mitochondria Targeting Peptide (MTP) at the N-terminus and the nine green boxes indicate LEA motifs found in RvLEAM protein. The six yellow bars in the hydropathy plot indicate predicted helix regions (H1–6) (b) Alignment of LEA motif sequences of RvLEAM with consensus. Greek characters in the consensus sequence indicate the following: Ψ, basic residue; Ω, acidic or amide residue; Φ, aliphatic residue; X, non-conserved residue. Residues consistent with the consensus are highlighted in deep green, and amino acids at non-conserved positions are shown in light green. Sequence logo created from nine LEA motifs of RvLEAM is shown below the alignment. (c) Amphipathic distribution of amino acids in predicted helical region of repeating LEA motifs 3 and 4. Amino acids are indicated as hydrophilic (pink), negatively-charged (red), positively-charged (blue), and hydrophobic (yellow) residues. (d) Subcellular localization of RvLEAM-GFP fusion protein (top) and GFP alone (bottom) in human HEp-2 cells. GFP signals (left). Mitochondria stained with Mito-Tracker (centre). Merged images (right).

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confirming the specific staining of RvLEAM and ATP5A in Fig. 2. This is the first immunohistochemical evidence for organelle-selective localization of identified LEA proteins in tardigrades. These findings are consistent with the RvLEAM-GFP localization in human cells (Fig. 1d) and validate the subcellular localization analysis using GFP-fusion protein in mammalian cells for tardigrade proteins. Tardigrades likely share at least a partially common mechanism with human cells to deliver proteins to the mitochondria.

Heat-solubility of RvLEAM protein

LEA proteins are highly hydrophilic and maintain their solubility even after heat treatment. To determine whether RvLEAM has biochemical properties similar to those of other LEA proteins,

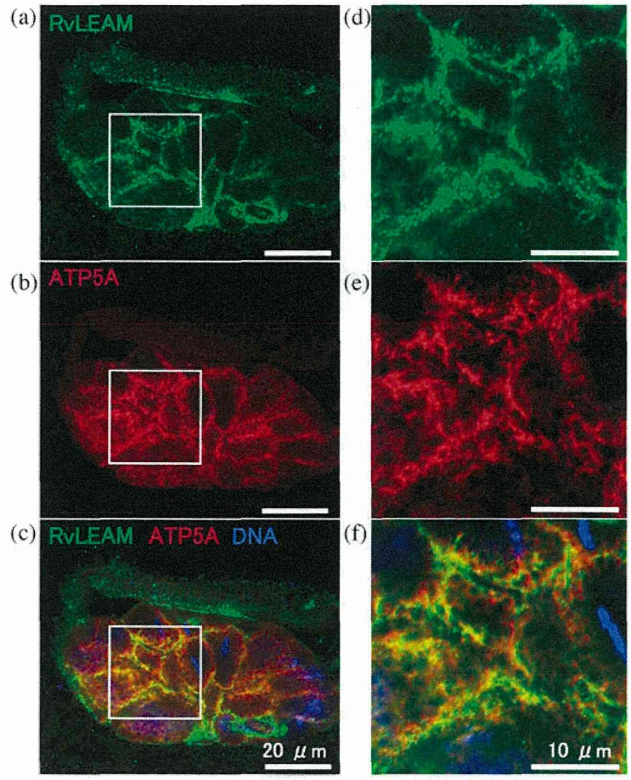

Fig 2. Mitochondrial localization of RvLEAM protein in tardigrade embryo. Tardigrade embryos were collected with eggshells and immunohistochemical analysis was performed using 10-μm frozen sections. Images show a representative section that was simultaneously reacted with anti-RvLEAM antiserum (a, d) and anti-ATP5A (mitochondrial ATP synthase) antibody (b, e). (c, f) Merged images. (d-f) Magnified images corresponding to the white boxes in (a-c).

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we examined the heat-solubility of RvLEAM protein. RvLEAM protein was bacterially expressed and subjected to heat treatment as either a bacterial lysate or purified protein solution. Heat treatment of the bacterial lysate resulted in the precipitation of most bacterial proteins, whereas RvLEAM protein was mostly recovered in the supernatant even after heat treatment (Fig. 3a), indicating a heat-soluble property of RvLEAM proteins. Furthermore, we confirmed that purified RvLEAM protein alone exhibited heat-solubility (Fig. 3b), indicating that the heat-solubility of RvLEAM is a characteristic of the protein itself and requires no other macromolecules like sugars or polyamines. These results confirmed that RvLEAM protein belongs to the LEA protein family based on both its primary sequence and biochemical properties.

MAHS, a novel mitochondrial heat-soluble protein unique to tardigrades

A previous heat-soluble proteome study revealed that the most abundant heat-soluble proteins in tardigrade are not LEA proteins, but tardigrade-unique proteins, such as CAHS and SAHS proteins [23]. Neither CAHS nor SAHS is localized in the mitochondria, and RvLEAM is currently the only known mitochondrial heat-soluble protein in tardigrades. LEA proteins work synergistically with trehalose to protect macromolecules or cells from water stress [17,35–37]. In tardigrades, however, there is low or no accumulation of trehalose in the dehydrated state [8], and thus there could be other protective molecules in addition to LEA proteins in tardigrades. Tardigrade-unique heat soluble proteins, such as CAHS and SAHS proteins, are

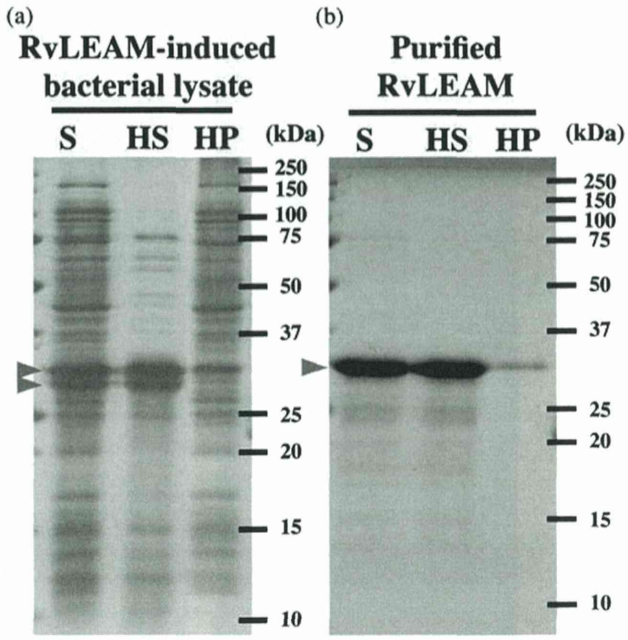

Fig 3. Heat-soluble property of RvLEAM protein. Heat-solubility of RvLEAM protein is shown using either RvLEAM-induced bacterial lysate (a) or purified RvLEAM protein (b). 'S' indicates starting sample before heat treatment. After heat treatment, the proteins were separated into a soluble fraction (HS) and insoluble precipitate (HP). Arrows indicate RvLEAM protein.

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possible candidates. In analogy with CAHS and SAHS proteins, we postulated that there could be novel tardigrade-unique heat-soluble proteins in the mitochondria. To evaluate this possibility, we identified six genes from abundantly expressed genes in our transcriptome data that are unique to tardigrades and are predicted to localize in the mitochondria. We examined the mitochondria-targeting potential of these candidate gene products by expressing GFP-fused protein in mammalian culture cells. Two of them, MAHS and ATPM1, showed mitochondrial localization (Fig. 4a, S3 Fig.). Comparison of the localization pattern of GFP-fusion protein with predicted score values of the three prediction programs showed that MitoProt2 provided good estimates regarding mitochondrial localization of the examined proteins (S1 Table). MitoProt2 would be a suitable bioinformatic tool for predicting mitochondrial localization for the tardigrade proteome in future studies. A heat-solubility assay of these two proteins demonstrated that only one protein had a heat-soluble property similar to that of RvLEAM protein, either in bacterial lysate or as a purified protein (Fig. 4b, S3b Fig.). We designated this protein MAHS protein in analogy with CAHS and SAHS proteins. Although purified MAHS protein was recovered in the soluble fraction after heat treatment, the heat-treated MAHS protein showed relatively slower migration in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Fig. 4b), indicating a possible conformational change induced by heat treatment.

The MAHS protein contained a predicted long mitochondrial targeting peptide at the N-terminus and the resultant putative mature form was highly hydrophilic (GRAVY score of -0.77) like RvLEAM protein (Fig. 4c). A BLASTP search in non-redundant (nr) database retrieved no sequences, and TBLASTN searches in EST/TSA databases retrieved only two sequences of other tardigrades (e-value < 1); one from the EST database of *Hypsibius dujardini* and the other from the TSA database of *Milnesium tardigradum*. No LEA proteins were retrieved in the search and also no LEA-like motif was found by either a InterProScan search or

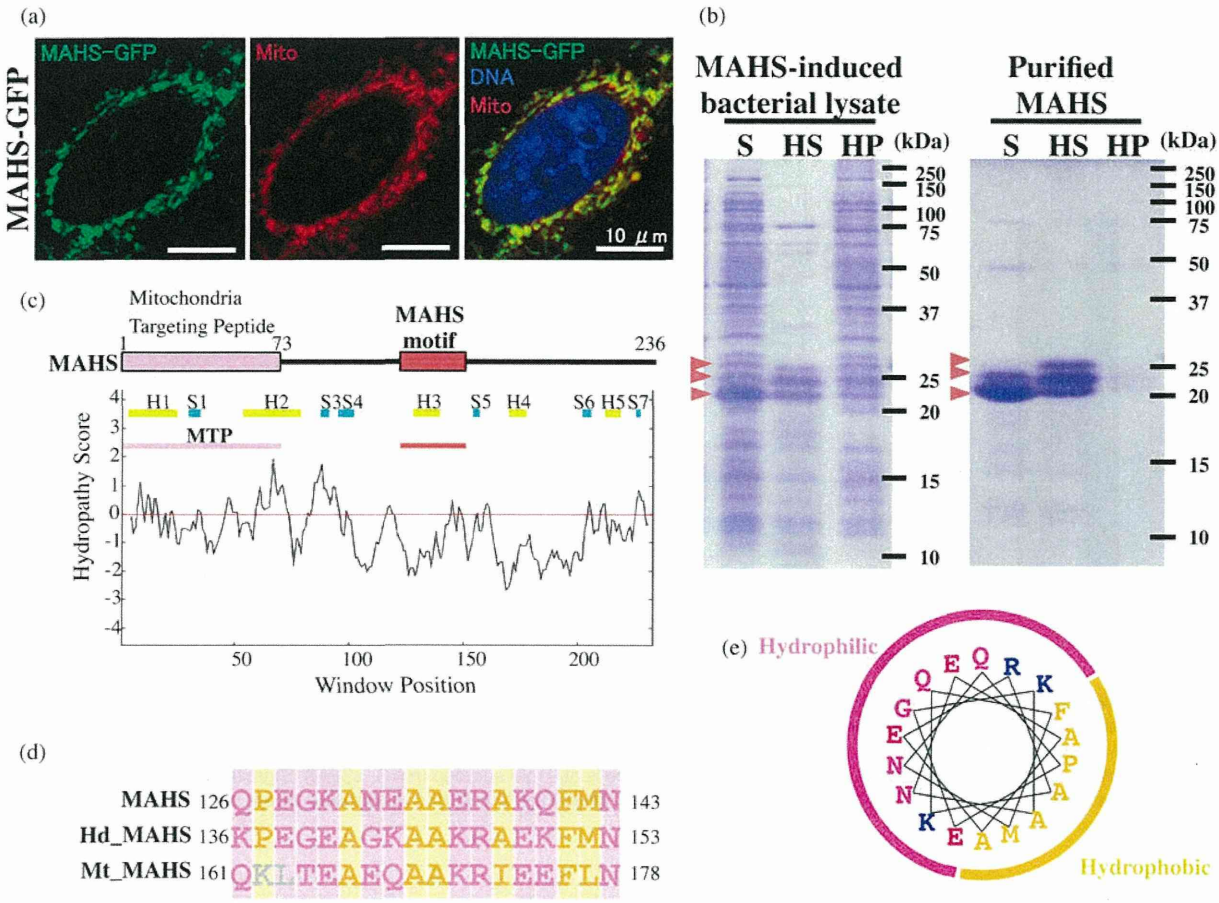

Fig 4. Subcellular localization of a novel mitochondrial heat-soluble MAHS protein. (a) Subcellular localization of MAHS-GFP fusion protein in human HEp-2 cells. (b) Heat-solubility of MAHS protein using bacterial lysate (left) or purified protein (right). Starting samples before heat treatment (S), soluble fraction after heat treatment (HS), and insoluble precipitate (HP) are shown. Red arrows indicate MAHS protein. (c) MAHS protein structure with hydrophathy plot. Mitochondria Targeting Peptide and conserved MAHS motif are indicated by the pink box and red box, respectively. Yellow and blue bars in the hydropathy plot indicate predicted helix regions (H1–5) and strand regions (S1–7), respectively. (d) Sequence alignment of conserved MAHS motifs among three tardigrade species, *R. varieornatus*, *H. dujardini* (Hd), and *M. tardigradum* (Mt). Pink and yellow indicate hydrophilic and hydrophobic residues, respectively (d) Amphipathic nature of predicted helix in MAHS motif. Amino acids are coloured as described in Fig. 1c.

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manual inspection. These findings suggested that the MAHS protein forms a novel heat-soluble protein family targeted to the mitochondria, which is unique to and conserved in tardigrades. Sequence comparison among putative tardigrade MAHS proteins revealed a conserved region in the middle of the protein (S4 Fig.), and this region was partially predicted to form an alpha-helix by PORTER predication software (Fig. 4c-d), potentially with an amphipathic property (Fig. 4e), implying that MAHS proteins have a role similar to that of LEA proteins in anhydrobiosis.

RvLEAM and MAHS proteins improved osmotic tolerance of human cells

LEA proteins are thought to play an important role in protecting macromolecules from desiccation stress in anhydrobiotic animals [9]. Introduction of LEA proteins to non-anhydrobiotic animal cells, like human cells, improved cellular viability and cellular integrity in water-deficient conditions such as dehydration or hyperosmotic treatment [16–18]. In these assays, the

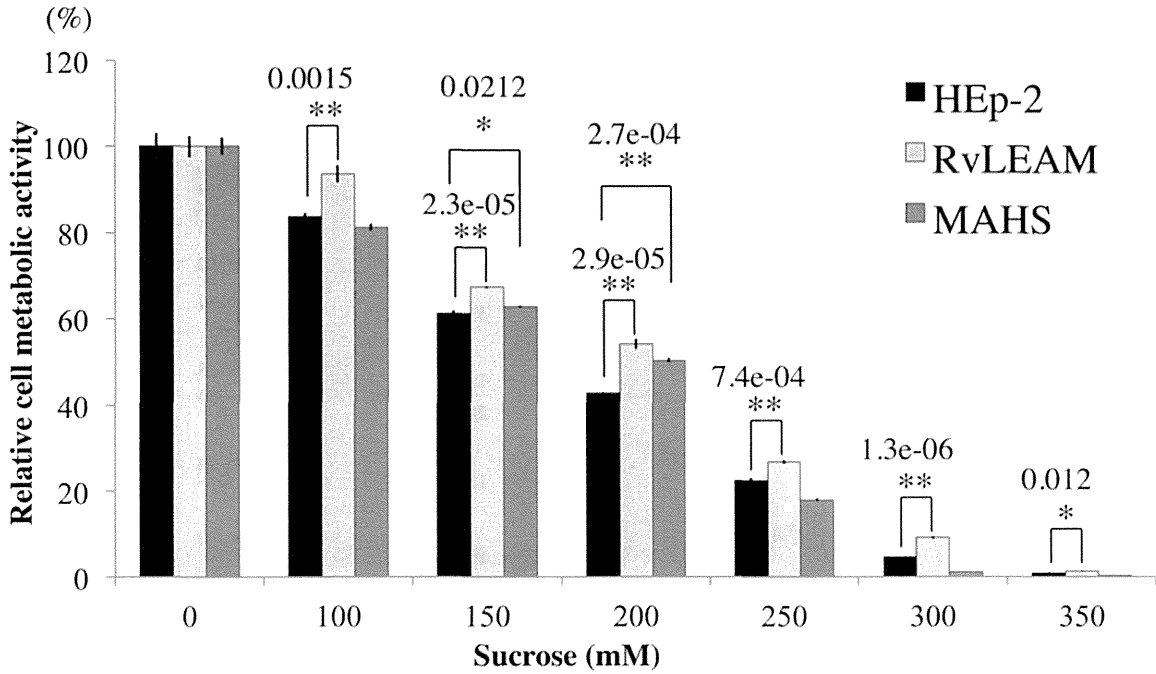

Fig 5. Effects of RvLEAM and MAHS proteins on metabolic activity of human cells under hyperosmotic stress. Human HEp-2 cells (black), and HEp-2 cells expressing RvLEAM (light grey) or MAHS (dark grey) were exposed to medium supplemented with defined concentrations of sucrose for 48 h. After replacement of the medium with serum-free isotonic medium, cell metabolic activities were analysed using PrestoBlue reagents. Metabolic activities at each sucrose concentration are shown as a percentage of the mean activity observed in the cells at 0 mM sucrose (n = 3, ±SEM). Statistical significance against untransfected HEp-2 cells was determined by *Dunnett*'s test (*, $P<0.05$; **, $P<0.01$). *P*-values are shown above asterisks where statistically significant.

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hyperosmotic treatment was relatively mild water stress compared to dehydration, enabling more sensitive detection of the protection ability of the introduced genes. In accordance, we tested whether the tardigrade mitochondrial heat-soluble proteins also improve the tolerability of human cells against hyperosmotic stress. First, we introduced RvLEAM or MAHS to human HEp-2 cells and established stably transfected cells that constitutively expressed either RvLEAM or MAHS under the control of the CAG promoter. The cells were shocked by exposure to hyperosmotic medium containing supplemented sucrose (final 100 mM to 350 mM) for 2 d, and the retained metabolic activities were assessed by measuring reducing power of cells using cell viability indicator in isotonic medium. Cells expressing RvLEAM had significantly higher metabolic activities, compared to untransfected control cells, at all examined sucrose concentrations (Fig. 5). Metabolic activity was increased two-fold by RvLEAM at 300 mM sucrose and exceeded that of untransfected cells by at least 10% in any examined condition. In addition, cells expressing MAHS also had significantly increased metabolic activities at 150 mM and 200 mM sucrose. The best improvement by MAHS (~20%) was observed at 200 mM sucrose, which is close to the EC50 value (179 mM) of untransfected cells (Fig. 5). The results suggested that mitochondrial heat-soluble proteins of tardigrades, even non-LEA protein like MAHS, improve the tolerability of human cells to hyperosmotic stress.

Discussion

In the present study, we identified two novel heat-soluble proteins targeted to mitochondria, RvLEAM and MAHS, from the anhydrobiotic tardigrade *R. varieornatus* (Figs. 1, 4). RvLEAM is a group 3 LEA protein family member, whereas MAHS protein shows no sequence similarity

to known protein families, including LEA proteins, nor known motifs. MAHS—like sequences were found in EST/TSA databases of other tardigrades, indicating that MAHS protein comprises a novel heat-soluble protein family conserved in tardigrades (Fig. 4). Despite differences in the primary structures between MAHS and RvLEAM, both proteins are highly hydrophilic and contain potential amphipathic helical regions (Figs. 1, 4). Furthermore, transfection of RvLEAM or MAHS improved cell viability against hyperosmotic stress in cultured human cells (Fig. 5). In LEA proteins, the amphipathic helix is suggested to be important for loose interactions with other macromolecules to prevent undesirable aggregation or conformational changes of proteins and liposomes, so-called 'molecular shielding' [38,39]. In MAHS protein, an apparently conserved region, the MAHS motif, has the potential to form an amphipathic helix (Fig. 4), consistent with the probably protective role of the amphipathic helix.

Introduction of RvLEAM improved the metabolic activity of human cells 2-fold (100% increase) at 300 mM sucrose (upshift of ~300 mOsm). Two studies have examined the effects of animal LEA proteins to improve osmotic tolerance in non-anhydrobiotic animal cells. In one study, a cytosolic LEA protein of the anhydrobiotic nematode transfected to human T-REx293 cells improved metabolic activity by 20% to 70% compared to uninduced cells after 2-d exposure to hyperosmotic medium (400 mOsm upshift with various solutes) [16]. In the second study, mitochondrial LEA protein of the anhydrobiotic crustacean Artemia improved the membrane integrity of insect cells by 20% to 60% in 50 to 200 mM sucrose. Although there are many differences in the cell types, hyperosmotic solutes, and methods used to measure cellular viability, improvements by RvLEAM were comparable to, or even better than, those by other animal LEA proteins in previous reports. Thus, the newly identified tardigrade mitochondrial LEA protein, RvLEAM, also improves the stress tolerability of sensitive cells. Introduction of MAHS protein induced significant improvements in the hyperosmotic tolerance of human cells (Fig. 5). The improvement, however, was only observed within a particular osmotic range of 150 to 200 mM sucrose (upshift of 150~200 mOsm) with up to a 20% increase. In tardigrades, both heat soluble proteins, MAHS and RvLEAM, likely localize in the mitochondria. MAHS protein might require other molecules, like RvLEAM, for efficient protection, as MAHS protein alone provided significant, but comparatively less, improvement of cellular activity in the hyperosmotic condition.

It is unclear why tardigrades contain at least two types of mitochondrial heat-soluble proteins. A possible explanation is that MAHS and RvLEAM protect different types of macromolecules; for example, one may protect proteins and the other lipids. The anhydrobiotic rotifer *Adineta ricciae* has two LEA proteins, ArLEA1A and ArLEA1B [15]. While ArLEA1A protects proteins from undesirable aggregation, ArLEA1B enhances protein aggregation, interacts with lipids preferentially, and is potentially involved in membrane protection. This could also be the case for RvLEAM and MAHS proteins. Another possible explanation is that simultaneous expression of two proteins contributes to increase the availability of heat-soluble proteins in mitochondria. Identification of the targets of RvLEAM and MAHS in future studies will be useful for distinguishing between these two explanations.

Based on the mitochondria-selective localization of RvLEAM and MAHS proteins (Figs. 1d, 2, 4a), they likely protect macromolecules inside the mitochondria rather than the whole cell content. How RvLEAM and MAHS proteins confer osmotic tolerance to cells, however, remains mysterious. Mitochondria are the energy centre of eukaryotic cells and produce ATP through oxidative phosphorylation, which simultaneously generates reactive oxygen species (ROS), and thus mitochondria are a major production source of ROS. In normal environments, ROS detoxification mechanisms in mitochondria are sufficient to counteract the generated ROS [40]. Water stress enhances ROS accumulation [41–43], however, which could exceed the detoxification capacity of mitochondria under stressed conditions. Thus, mitochondrial