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− 162 −

TERT attenuated ER stress-induced cell death

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ABSTRACT

Tumor cells are frequently encountered in nutrient-deprived areas, though the mechanisms underlying their survival are unclear. In the present study, we found that depriving cells of glucose caused endoplasmic reticulum stress (ER stress) in a breast cancer cells line, MCF-7, and that specific activation of ER stress increased telomerase reverse transcriptase (TERT) expression. TERT expression would function in counteracting against the stress because over-expression of TERT diminished ER stress-induced cell death. Therefore, the results provide evidence for the underlying mechanisms of tumor progression in stressed conditions, highlighting that ER stress induces TERT expression to withstand environmental stress, a mechanism which we termed the "ER stress-TERT axis".

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1. Introduction

Because of their enhanced glucose consumption, cancer cells are frequently encountered in areas deprived of nutrients. Moreover, solid tumors are often located for from blood vessels and not supplied with sufficient glucose [1]. Although normal cells would die in such situations, cancer cells do not. However, the mechanisms by which cancer cells survive these conditions are not well understood. One possible explanation for these observations would be due to the increased level of surviving branch of unfolded protein response (UPR), which is induced in endoplasmic reticulum (ER)-stressed cells, in tumors [2]. However, the mechanisms of the survival in tumor cells by activating UPR induction are enigma.

The endoplasmic reticulum (ER) plays an important role in calcium homeostasis and protein folding. However, when cells are exposed to stressed-environment such as glucose deprivation, it causes ER disruption which results in ER stress. Disruption of ER functions leads to the accumulation of unfolded proteins. Cells then activate an unfolded protein response (UPR) to alleviate such

stress by: (1) increasing folding capacity, (2) inhibiting general protein translation, and (3) promoting the degradation of misfolded proteins [3]. Three major ER-resident proteins have been identified as sensors for ER stress; inositol-requiring protein-1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). The activation of these stress-sensor proteins leads to the expression of UPR-related factors such as glucose regulated protein 78 (GRP78) or CCAAT/enhancer-binding protein homologous protein (CHOP). Increasing evidence has suggested that ER stress is involved in diseases such as Alzheimer's disease, Parkinson's disease, diabetes, obesity and cancer [4,5]. In the present study, we hypothesized that increased levels of ER stress-induced activation of UPR to be involved in the resistance of tumor cell death under stressed conditions.

The abnormal proliferation which occurs among cancer cells is usually accompanied by an increase in telomerase activity. Telomerase adds 5′-TTAGGG-3′ at the 3′-end of DNA strands, resulting in a 15–10 Kbp telomeric DNA sequence being repeated at the ends of eukaryotic chromosomes [6]. Therefore, activation of telomerase has been suggested to function in avoiding telomere shortening. Consistent with this model, most of cancer cells have high level of telomerase activity [7]. Telomerase is a huge complex, composed of the genetic template of the telomere (teromere RNA component; TR), a reverse transcriptase (telomerase reverse transcriptase; TERT), and other catalytic subunits [8]. Interestingly, in addition to TERT's role in extending telomerase activity, recent evidence suggest that it stimulates the proliferation of mouse hair-follicle stem cells [9] or regulates cell survival [10].

Based on these observations and that tumor cells are frequently encountered with stressed environment, we hypothesized that TERT regulates ER stress-induced cell death. We investigated the possible involvement of UPR in cancer cell resistance under stressed conditions. In the present study, we found a link between ER stress and TERT expression, i.e. ER stress induced TERT, which prevented cell death. The results suggest that TERT is a novel factor involved in regulating UPR. This "ER stress-TERT axis" may play a key role in preventing the death of cancer cells in a stressed environment.

2. Materials and methods

2.1. Materials and reagents

Tunicamycin and thapsigargin were obtained from Wako Pure Chemical Industries, Ltd. (Japan).

2.2. Cell culture

MCF-7 human breast cancer cell line was a kind gift from Nari-aki Fujimoto (Research Institute for Radiation Biology and Medicine, Hiroshima University, Japan) [11]. MCF-7 and TIG-3 cell lines were cultured in DMEM with 10% FCS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B (nacalai tesque, Japan). The cells were kept at 37 °C in 5% CO_2/ 95% air.

2.3. Transduction of hTERT by a retroviral method

pMSCV-puro-hTERT retroviral constructs were transfected into the PT67 packaging cell line (Takara Bio USA, Madison, WI, USA) with the FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany). The supernatant was collected and passed through a 0.45-μm filter (Millipore, Billerica, MA, USA) after polybrene was added at a final concentration of 8 μg/ml. The filtered supernatant was then used to infect the target cells. Supernatant from pMSCV-puro-hTERT was used to infect human MCF-7 cells. After 24 h of incubation with the virus, the medium was replaced with fresh complete medium containing puromycin (1 μg/ml). A hTERT-transduced clone (mixed clone) was obtained and several single clones isolated.

2.4. Western blotting analysis

Western blotting was performed as described previously [12]. Cells were washed with ice-cold PBS and lysed in a buffer containing 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% NP-40 for 20 min. The lysate was centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatant was collected. The samples were boiled with laemmli buffer for 3 min, fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred at 4 °C to nitrocellulose membranes. The membranes were incubated with anti-KDEL (StressGen; 1:1000), CHOP (Santa Cruz; 1:500), anti-PERK (cell signaling; 1:1000), anti-phospho (Ser51)-eIF2α (cell signaling; 1:1000), anti-phospho (Ser724)-IRE1α (Novus Biologicals; 1:1000) and anti-GAPDH (Chemicon; 1:1000) antibodies, followed by an anti-horseradish peroxidase-linked antibody. Peroxidase was detected using an enhanced chemiluminescence system.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRI Reagent (Sigma–Aldrich, St. Louis, MO) or TriPure Isolation Reagent (Roche Molecular Biochemicals, Indianapolis, IN). RT-PCR was performed as described previously [13]. Specifically, cDNA was synthesized from the total RNA by reverse transcription using 100 U of Superscript III Reverse Transcriptase (Invitrogen) and the Oligo (dt)$_{12-18}$ primer (Invitrogen) in a 20-μl reaction mixture containing Superscript buffer (Invitrogen), 1 mM dNTP mix, 10 mM dithiothreitol (DTT), and 40 U of RNase inhibitor. The total RNA and Oligo (dt)$_{12-18}$ primer were incubated at 70 °C for 10 min prior to reverse transcription. After incubation for 1.5 h at 46 °C, the RT reaction was terminated by denaturing the reverse transcriptase for 15 min at 70 °C. For PCR amplification of GRP78, p23, c-Myc and GAPDH, 1.2 μl of cDNA was added to 10.8 μl of a reaction mix containing each primer, dNTP mix, Taq polymerase (Expand High Fidelity; Roche), and reaction buffer. For PCR amplification of TERT, 1.2 μl of cDNA was added to 10.8 μl of a reaction mix containing each primer, dNTP mix, Taq polymerase (LA Taq polymerase; Takara, Japan), and reaction buffer (GC buffer). PCR was performed in a DNA Thermal Cycler (MJ Research, PTC-220). The following primers were used: TERT upstream, 5'-tct ttg ggg tct tgc ggc tga a-3'; TERT downstream, 5'-gcg tct ggg ctg tcc tga gtg a-3'; GRP78 upstream, 5'-tgc ttg atg tat gtc ccc tta-3'; GRP78 downstream, 5'-cct tgt ctt cag ctg tca ct-3'; p23 upstream, 5'-tga aaa atc caa act tac at-3'; p23 downstream, 5'-atc atc tgc tcc atc tac tt-3'; c-Myc upstream, 5'-cca gga ctg tat gtg gag cg-3'; c-Myc downstream, 5'-ctt gag gac cag tgg gct gt-3'; XBP-1 upstream, 5'-cct tgt agt tga gaa cca gg-3'; XBP-1 downstream, 5'-ggg gct tgg tat ata tgt gg-3'; GAPDH upstream, 5'-aaa ccc atc acc atc ttc cag-3'; and GAPDH downstream, 5'-agg ggc cat cca cag tct tct-3'. The PCR products (10 μl) were resolved by electrophoresis in an 8% polyacrylamide gel in TBE buffer. The gels were stained with ethidium bromide or gel red, and then photographed under ultraviolet light.

2.6. Measurement of telomerase activity

Telomerase activity was measured by Stretch PCR with a Telo Chaser kit (TOYOBO, Japan)

2.7. Lactate dehydrogenase leakage assay

The viability of cells was estimated by the lactate dehydrogenase (LDH) leakage method using a cytotoxicity detection kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. LDH activity was measured as the optimal density at 492 nm.

2.8. Statistics

Results are expressed as the mean ± S.E. Statistical analyses were performed using a Paired *t*-test.

3. Results

3.1. Glucose-deprivation caused ER stress in MCF-7 cancer cells

Nutrient deprivation, which induces ER stress, is frequently encountered in tumor cells [14]. GRP78 has been reported to be up-regulated in MCF-7 human breast cancer cells compared with normal human breast epithelial cell lines at basal levels [15]. Glucose-regulated proteins were identified to be regulated by glucose [16,17]. We therefore examined whether glucose-deprivation would further cause ER stress in MCF-7 cells. We treated the cell
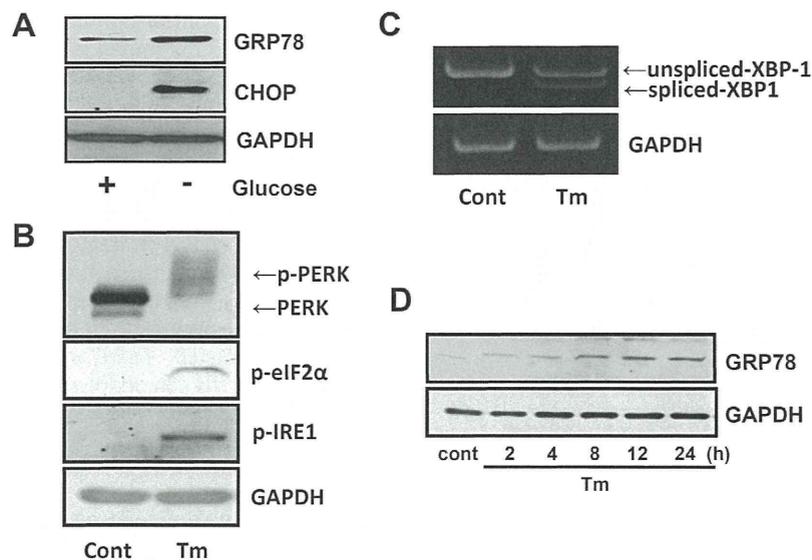
Fig. 1. Glucose depletion or specific inducer of ER stress activated the unfolded protein response in MCF-7 cells. (A) MCF-7 cells were treated with or without 4.5 g/l D-Glucose in serum free media for 24 h. The cells were lysed with lysis buffer and the levels of GRP78, CHOP and GAPDH were detected by Western blotting. (B) MCF-7 cells were treated with tunicamycin (Tm: 10 μg/ml) for 4 h and phosphorylation levels of PERK, eIF2α and IRE1α were analyzed by Western blotting. (C) MCF-7 cells were treated with tunicamycin (Tm: 10 μg/ml) for 4 h and XPB-1 splicing was detected by RT-PCR. (D) MCF-7 cells were treated with tunicamycin (Tm: 2 μg/ml) for 2–24 h and levels of GRP78 and GAPDH were detected by Western blotting.
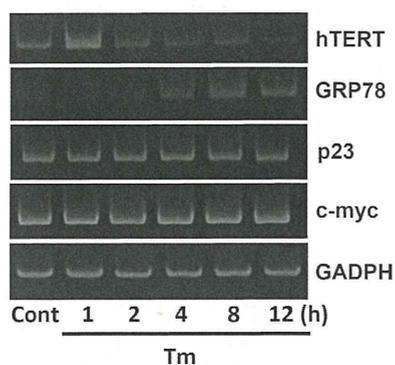
Fig. 2. ER stress increased expression level of TERT. MCF-7 cells were treated with tunicamycin (Tm: 10 μg/ml) for 1–12 h and RT-PCR was performed using specific primers for hTERT, GRP78, c-myc, p23 or GAPDH mRNA. ER stress specifically increased hTERT levels.

line with glucose-free medium for 24 h and analyzed levels of GRP78 and CHOP, an UPR-regulated gene product. As shown in Fig. 1A, we observed a marked increase in GRP78 and CHOP, indicating that glucose-deprivation activates UPR in MCF-7 cells. To specifically induce ER stress, we used tunicamycin, which interferes with protein glycosylation. Treatment with tunicamycin increased GRP78 levels and the activation of UPR branches such as PERK, eIF2α, IRE1 and XBP-1, confirming that tunicamycin can induce ER stress under the conditions (Fig. 1B–D). Overall, these results suggest that ER stress is enhanced by in glucose-deprivation or tunicamycin-treatment in MCF-7 cancer cells.

3.2. ER stress increased TERT expression

To find out the mechanisms of resistance to the stress, we next investigated the possible link between ER stress and telomerase, both activated in cancer cells [2,7]. We observed an increase in GRP78 mRNA levels on tunicamycin-treatment, confirming ER stress (Fig. 2). Thus, we next analyzed levels of TERT, p23 and c-myc in ER stressed cells. As shown in Fig. 3, TERT levels increased
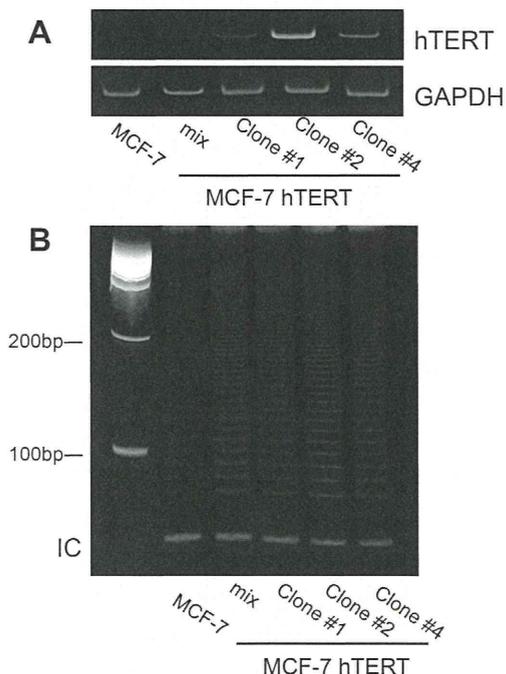
Fig. 3. Characteristics of hTERT-transfected MCF-7 cells. (A) RT-PCR analysis of MCF-7 cells and MCF-7 cells stably transfected with the hTERT gene (MCF-7-hTERT: mix, clone#1, clone#2, and clone#4). Levels of hTERT mRNA were increased in the MCF-7-hTERT cell line. (B) The telomerase activity in MCF-7 and MCF-7-hTERT cells was analyzed by Telochaser (TOYOBO, Japan). IC: internal control. PCR cycle: 24 cycles.

1–2 h after tunicamycin-treatment, which decreased thereafter. There was no increase in c-myc or p23 levels in the tunicamycin (1–12 h)-treated cells (Fig. 2). These findings indicate that ER stress can specifically induce TERT expression.

3.3. TERT prevented ER stress-induced cell death

As TERT levels were increased by ER stress, we were next interested in the role of TERT in ER-stressed tumor cells. To this end, we
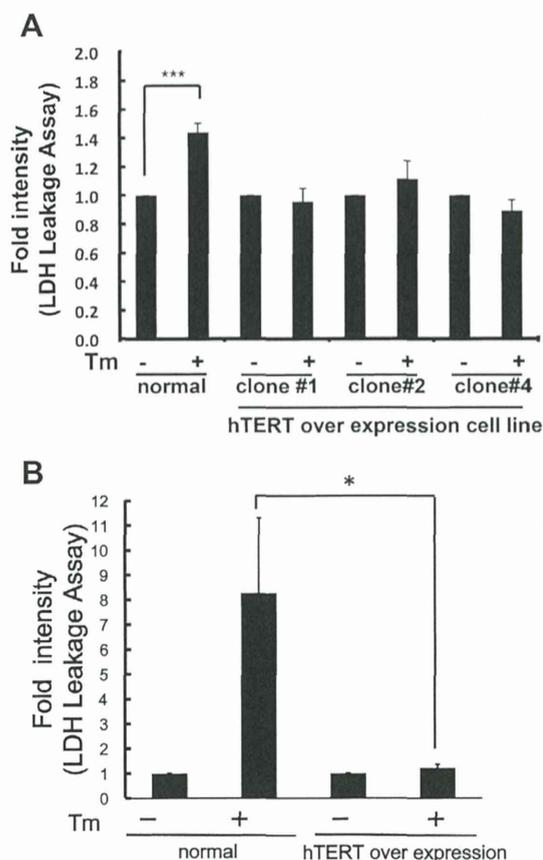
Fig. 4. TERT protected ER stress-induced cell death. (A) MCF-7 cells or MCF-7-hTERT cells (clone#1, clone#2, and clone#4) were treated with tunicamycin (Tm: 10 μg/ml) for 72 h and LDH activity was measured as an indicator of cytotoxicity. ***$p<0.001$. $n = 7$. ER stress increased cell death in MCF-7 cells but not in MCF-7-hTERT cells. (B) TIG-3 cells or TIG-3-hTERT cells were treated with tunicamycin (Tm: 1 μg/ml) for 48 h and LDH activity was measured as an indicator of cytotoxicity. *$p < 0.05$. $n = 8$. ER stress increased cell death in TIG-3 cells but not in TIG-3-hTERT cells.

established a TERT-transfectant and analyzed cell viability under ER-stress. TERT was introduced by a retroviral method to obtain MCF-7-hTERT cells (MCF-7-hTERT mix). In addition, we also isolated single clones (clone#1, #2, and #4) from the MCF-7-hTERT mix cells. The levels of TERT and telomerase activity were drastically increased in these clones (Fig. 3). Thus, we subsequently investigated possible roles of TERT against ER stress-induced cell death. Tunicamycin-treatment caused an increase in death among wild-type MCF-7 cells (Fig. 4). However, the MCF-7-hTERT cell lines (clones #1, #2 and #4) were resistant to ER stress-induced cell death (Fig. 4A). We further investigated whether TERT can also protect against ER stress-induced cell death in normal cells. We used TIG-3 cell line, the human embryonic fibroblasts [18]. ER stress increased cell death in TIG-3 cells. However, the cell death was significantly inhibited in TIG-3-hTERT cell line, which over express hTERT (Fig. 4B). Thus, TERT appears to play a key role in protecting against ER stress-induced cell death.

4. Discussion

Tumor cells are frequently encountered under condition which induce ER stress such as hypoxia, nutrient deprivation, and changes in pH [14]. The mechanisms by which tumor cells survive in such a stressed environment are unclear. In the present study, we investigated the role of UPR on cancer cell survival and found that TERT was involved in this process. We observed that TERT was up-regulated in response to short-term exposure to ER stress.

Then, what is the physiological significance of this observation? It has been reported that increased expression of TERT was observed in tumor cells, which is involved in cell survival [7]. Therefore, it is possible that tumor cells are maintaining survival by inducing TERT expression at the stressed environment. Indeed, we found that ER stress induced TERT expression, which subsequently protected cell death. Thus, the high levels of TERT in tumor cells would be associated with resistance to ER stress-induced cell death, highlighting possible novel strategy in the treatment of cancer. At present, it is unknown whether this linkage would also exist on other types of tumors, which would be an interesting subject. On the other hand, with long-term exposure to ER stress, TERT expression was down-regulated. Therefore, when stress exposure is prolonged, which cells can no longer survive, cells would down-regulate TERT expression. Overall, from these observations, we can speculate possibility that TERT is monitoring the ER stress-mediated cell status. Further studies are needed to examine this hypothesis.

Overall, we found novel mechanism of tumor progression; i.e. "ER stress-TERT axis", to play an important role in resistance under stressed conditions, which cancer cells frequently encounter. The present findings provide a basic understanding of the mechanisms of survival of cancer cells, which would offer a new strategy for treating tumor.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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Databases and ontologies

EVpedia: a community web portal for extracellular vesicles research

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1

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Abstract

Motivation: Extracellular vesicles (EVs) are spherical bilayered proteolipids, harboring various bioactive molecules. Due to the complexity of the vesicular nomenclatures and components, online searches for EV-related publications and vesicular components are currently challenging.
Results: We present an improved version of EVpedia, a public database for EVs research. This community web portal contains a database of publications and vesicular components, identification of orthologous vesicular components, bioinformatic tools and a personalized function. EVpedia includes 6879 publications, 172 080 vesicular components from 263 high-throughput datasets, and has been accessed more than 65 000 times from more than 750 cities. In addition, about 350 members from 73 international research groups have participated in developing EVpedia. This free web-based database might serve as a useful resource to stimulate the emerging field of EV research.
Availability and implementation: The web site was implemented in PHP, Java, MySQL and Apache, and is freely available at http://evpedia.info.
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1 Introduction

Almost all living organisms on earth shed extracellular vesicles (EVs) into their microenvironment. EVs are spherical bilayered proteolipids with an average diameter of 20–1000 nm (Ellen *et al.*, 2009; Lee *et al.*, 2008; Théry *et al.*, 2009). Cells release EVs either constitutively or in a regulated manner and these vesicles harbor a specific subset of proteins, mRNAs, miRNAs, lipids and metabolites reflecting their originating cell types and conditions (Bellingham *et al.*, 2012; Choi *et al.*, 2014; de Jong *et al.*, 2012; Duperthuy *et al.*, 2013; Mayr *et al.*, 2009; Raimondo *et al.*, 2011; Simpson *et al.*, 2008; Subra *et al.*, 2007; Wai *et al.*, 2003). EVs are also found in various biological fluids, such as amniotic fluid, ascites, breast milk, plasma, saliva, semen, serum and urine (Asea *et al.*, 2008; Caby *et al.*, 2005; Cheng *et al.*, 2014a, b; Dai *et al.*, 2008; George *et al.*, 1982; Lässer *et al.*, 2011; Poliakov *et al.*, 2009; Raj *et al.*, 2012; Witwer *et al.*, 2013). Recent advances in this fast growing field (Fig. 1) have facilitated several insights: (1) EVs play multifaceted functions in intercellular communication (Cocucci *et al.*, 2009;

Lee *et al.*, 2008; Simons and Raposo, 2009; Théry *et al.*, 2009); (2) EV-mediated intercellular communication is an evolutionarily conserved phenomenon (Deatherage and Cookson, 2012; Lee *et al.*, 2008, 2009); (3) EVs are rich sources of biomarkers for non-invasive diagnosis and prognosis of various human diseases (Chaput *et al.*, 2005; Choi *et al.*, 2013a; D'Souza-Schorey and Clancy, 2012; Mullier *et al.*, 2013; Sarlon-Bartoli *et al.*, 2013; Shedden *et al.*, 2003; Simpson *et al.*, 2009); and (4) Diverse therapeutic approaches have been pursued to utilize EVs and their mimetics for vaccine, chemotherapeutic drug and siRNA delivery (Alvarez-Erviti *et al.*, 2011; Chaput *et al.*, 2005; Jang *et al.*, 2013; Kordelas *et al.*, 2014; Lai *et al.*, 2010; Lee *et al.*, 2012; Simpson *et al.*, 2009; Sun *et al.*, 2010).

Publications on EVs have grown rapidly during the last several years, indicating that the field of EVs is expanding intensively (Fig. 1). The identification of vesicle-specific cargos could help us to unravel the molecular mechanisms underlying the cargo sorting and biogenesis of EVs. In addition, this will lead to better comprehension of the pathophysiological functions of EVs, and discovery of EV-based potential biomarkers of human diseases. Therefore, many

investigators have focused on categorizing these complex vesicular components by various high-throughput technologies, such as mass spectrometry-based proteomics and lipidomics as well as micro-array- and next-generation sequencing-based transcriptomics (Barry *et al.*, 1997; Choi *et al.*, 2013b; Koh *et al.*, 2010; Utleg *et al.*, 2003; Valadi *et al.*, 2007). Together with conventional biological approaches, these multiomics-based analyses of EVs derived from various cell types and body fluids have identified several thousands of different vesicular components.

EV secretion and EV-mediated intercellular communication are evolutionarily conserved (Biller *et al.*, 2014; Deatherage and Cookson, 2012; Lee *et al.*, 2008, 2009). Researchers in this field have coined dozens of different names for EVs, especially for more complex eukaryotic cell-derived EVs as listed in Box ('Extracellular Vesicles: Diverse Nomenclature') (Choi *et al.*, 2014; Gould and Raposo, 2013; Kim *et al.*, 2013). Nevertheless, there is progress toward a single nomenclature, since the different names in use make it difficult to follow the progress in the field. In addition, most vesicular components identified by multiomics-based high throughput analyses are presented in the supplementary information of published articles. Taken together, online searches for EV-related publications and vesicular components are currently challenging, especially for start-up researchers in this field. Therefore, a comprehensive public repository of EV-related publications and vesicular

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Extracellular Vesicles: Diverse Nomenclature

Prokaryotes
Archaea
  Membrane Vesicles
Gram-negative Bacteria
  Extracellular Vesicles, Membrane Blebs, Outer Membrane Blebs, Outer Membrane Vesicles
Gram-positive Bacteria
  Extracellular Vesicles, Membrane Vesicles

Eukaryotes
  Argosomes, Blebbing Vesicles, Budding Vesicles, Dexosomes, Ectosomes, Exosome-like Vesicles, Exosomes, Exovesicles, Extracellular Membrane Vesicles, Extracellular Vesicles, Matrix Vesicles, Membrane Particles, Membrane Vesicles, Microparticles, Microvesicles, Nanovesicles, Oncosomes, Prominosomes, Prostasomes, Shedding Microvesicles, Shedding Vesicles, Tolerosomes

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components will help the community of EV research to understand various aspects of these complex extracellular organelles.

2 Databases that store EV data

The explosion of EV data has justified the need for databases that catalog proteins, nucleic acids and lipids associated with EVs. Currently, three databases exist for EV research including ExoCarta (Simpson *et al.*, 2012), EVpedia (Kim *et al.*, 2013) and Vesiclepedia (Kalra *et al.*, 2012). These existing databases have made large-scale bioinformatics analyses feasible and provide an ideal platform for EV-based biomarker studies. EVpedia provides additional benefits compared with ExoCarta and Vesiclepedia. EVpedia is the only resource that contains data on both prokaryotes and eukaryotes. In addition, EVpedia allows for Gene Ontology enrichment analysis, network analysis of vesicular proteins and mRNAs, and set analysis of vesicular datasets by ortholog identification. Other databases do not have any such embedded analysis tools.

3 Launch of EVpedia

EVpedia (http://evpedia.info) was first launched in January 2012 (Kim *et al.*, 2013). To construct this public web-based database, we first collected publications on prokaryotic and eukaryotic EVs through a combination of NCBI PubMed searches (http://www. ncbi.nlm.nih.gov/pubmed) for text-mining solutions and manual curation using all nomenclatures assigned to EVs described in Box (see also Kim *et al.*, 2013). Based on these EV-related publications, we constructed the comprehensive and integrated database of proteins, mRNAs, miRNAs, lipids and metabolites for systematic analyses of prokaryotic and eukaryotic EVs.

4 Overview of current EVpedia

Since the launch of EVpedia, we have improved this database by continually collecting additional EV-related publications and datasets, by adding more tools for systematic analyses of EVs, and by supplementing the menu bars for 'Top 100+ EV markers', 'User forum' as well as 'My EVpedia'. Through closed and open beta tests, we built an 'EVpedia Community' (about 350 world-wide EV researchers) and updated EVpedia most recently in May 2014.

The updated EVpedia has five functional modules for systematic analyses of EVs derived from prokaryotic and eukaryotic cells (Fig. 2): (i) a database of publications and principal investigators, (ii) a database of vesicular proteins, mRNAs, miRNAs, lipids and
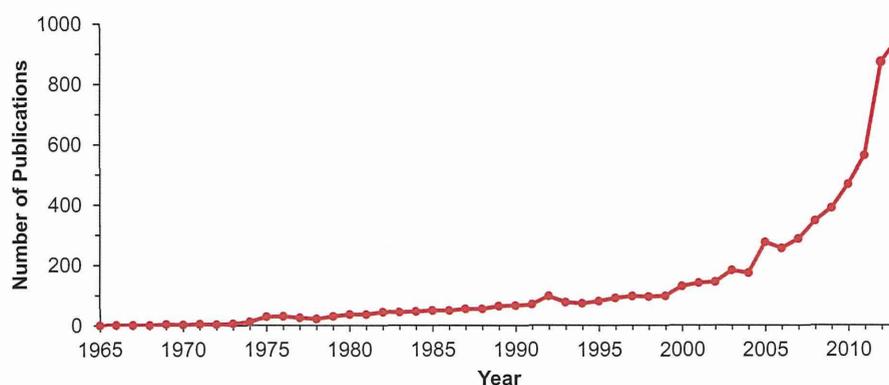

Fig. 1. Publication trend of EV studies. This graph shows the number of publications on EVs per year, indicating that the field of EVs is expanding rapidly