Biochemistry and Biophysics Reports 37 (2024) 101613

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Exploring an immortal *Turritopsis sp.* as a less conventional natural system for study of aging

Shuang Liu [a,*], Erika Takemasa [a], Yasuyuki Suzuki [a,b,c], Masaki Mogi [a]

[a] Department of Pharmacology, Ehime University Graduate School of Medicine, Tohon, Ehime, Japan
[b] Department of Anesthesiology, Saiseikai Matsuyama Hospital, Matsuyama, Japan
[c] Research Division, Saiseikai Research Institute of Health Care and Welfare, Tokyo, Japan

ARTICLE INFO

Keywords:
Aging
Immortal jellyfish
Regeneration
RNA-seq
Turritopsis sp.

ABSTRACT

Aim: Given its excellent capability of escaping from unavoidable harm and death, *Turritopsis sp.* (*T. sp.*) has captured the attention and fascination of scientists as a less conventional tool for aging research. The current study introduces a method for establishment of a research model and comprehensive transcriptomic analysis to reveal the structural and functional diversity of *T. sp.*

Methods: T. sp. medusae collected from the Pacific Ocean near Japan were reared using a common laboratory setting. Tissues of the gastrovascular cavity part (GP) and nerve ring part (NP) were collected, and total RNA was extracted. Bulk RNA-seq was performed to compare the different transcriptome landscapes between GP and NP.

Results: The GP fragment could be utilized for studies related to stress response and systemic senescence, while the NP fragment could be used to explore system rejuvenation, self-repair and regeneration.

Conclusions: As a less conventional system for aging research, by employing the most recently developed tools and techniques in the genomic revolution, comprehensive elucidation of the composition, development, and functions of *T. sp*. enabled us to explore the underlying mechanisms of the response to environmental stress and rejuvenation.

1. Introduction

As a representative of hydrozoans within the phylum Cnidaria, *Turritopsis,* including *Turritopsis dohrnii* and *Turritopsis sp.* (*T. sp.*), commonly known as the "immortal jellyfish", is a species of small, bell-shaped jellyfish. They display the greatest adaptability with respect to behaviors in response to environmental stress and their antiaging strategy. When encountering aging, injury, or other adverse environmental conditions, the medusa form shrinks, loses its swimming ability and undergoes retrograde transformation to a protective, poorly differentiated cyst-like structure, which eventually gives rise to a preceding juvenile morph, the polyp [1]. These behaviors thus result in effectively bypassing death and allowing it to restart its life cycle, which is known as “rejuvenation” [2].

The basic *Turritopsis* life cycle proceeds from the adult medusa to the planula larva, and to the polyp, which is an asexual and often colonial stage [3]. The structure of the adult medusa includes a gastrovascular cavity with a set of manubrium, oral lobes, radical canal, and gonad inside, and a velum surrounded by a nerve ring with tentacles [4,5]. The body wall of the gastrovascular cavity consists of an outer epidermis, outer mesoglea, endoderm, inter mesoglea with the formation of a gastral cavity, radial canals and ring canal. A layer of mononucleated, striated muscle cells and a smooth muscle layer are also observed in the mature medusa. A polyp could not regenerate from an isolated manubrium and tentacles alone, whereas excised manubrium and tentacles additionally containing the tissue/cells around the velum with nerve ring maintained the capability of stolon formation.

Given its excellent capability of escaping from unavoidable harm and death, *Turritopsis* has captured the attention and fascination of scientists as a less conventional tool for examining factors governing regeneration, aging, and immortality. By employing the most recently developed tools and techniques in the genomic revolution, such as next-generation sequencing, single-cell/molecular sequencing, spatial transcriptomics, multi-omics integration, and CRISPR-Cas9 genome editing [6,7], it has been possible to comprehensively elucidate the composition, development, and functions of *Turritopsis*, enabling us to explore the underlying mechanism of the response to environmental stress and rejuvenation. In the current study, a basic method for comparative transcriptome

* Corresponding author.
E-mail address: liussmzk@m.ehime-u.ac.jp (S. Liu).

https://doi.org/10.1016/j.bbrep.2023.101613
Received 25 October 2023; Received in revised form 5 December 2023; Accepted 11 December 2023
2405-5808/© 2023 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

analysis of the upper half of the medusa without the nerve ring and the lower half of the medusa including the nerve ring is introduced. The transcriptomic landscape revealed the structural and functional diversity of different fragments of *T. sp.*, a *Turritopsis* medusa which was collected from the Pacific Ocean near Japan, in particular focusing on the study of aging.

2. Methods

2.1. Rearing

About 100 *T. sp.* medusae were collected on August 2022 in Tanabe Bay, Japan (33.68'E, 135.36'N) and reared in the laboratory at 25 °C in a 500 ml beaker and fed every other day with newly hatched *Artemia salina.* One hour after feeding, half of the seawater was removed and replaced with freshly prepared seawater.

Long-term rearing for more than 1 month can be challenging and requires additional specialized water circulation equipment. A gentle circular flow pattern is required to prevent damage to the *T. sp.* and is able to create a gentle current and promote water exchange in a small tank. A combination of sponge filters, protein skimmers and activated carbon filter was used for the rearing systems. Proper lighting is crucial for the development of *T. sp.* and rejuvenates. An LED light with blue spectrum predominance was adjusted to mimic natural sunlight, and the lighting cycle simulated day and night periods with regular intervals.

2.2. Total RNA extraction and library preparation

Whole medusae were anesthetized in 3.5 % $MgCl_2$ (w/v in seawater) and dissected into the upper half containing the manubrium, oral lobes, and radical canal, and the lower half containing the velum with nerve ring and tentacles, under a stereomicroscope. The tissues were frozen on dry ice and stored at −80 °C for subsequent RNA extraction.

Total RNA was isolated using a NucleoSpin RNA Plus XS kit (TaKaRa, Tokyo, Japan) and quantified on a Qubit 4 fluorometer (ThermoFisher Scientific, Tokyo, Japan). After RNA quality assessment on a Bioanalyzer 2100 system (Agilent Technologies, Tokyo, Japan), samples with RIN value of 7.0 or higher were chosen for subsequent library preparation. Nanopore sequencing, which utilizes a next generation portable long-reading MiNION sequencer which runs off la laptop computer with conventional laboratory settings, was carried out using a ligation-based cDNA-PCR sequencing kit (Oxford Nanopore Technologies (ONT), Didcot, UK) according to the manufacturer's protocol. Briefly, using the strand-switching protocol, full-length cDNA from 50 ng total RNA was prepared using Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific). LongAmp Taq 2x Master Mix (New England Laboratories, Tokyo, Japan) was used for amplifying full-length transcripts, and the PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The adaptor-ligated libraries were sequenced using a MinION sequencer with FLO-MIN 106 flow cells and R9.4 chemistry (ONT).

2.3. Data acquisition and bioinformatics analysis

Basecalling was performed using a Guppy basecaller (version 6.4.6+ae70e8f) by translating ionic signals into a nucleotide sequence. Subsequently, Minimap2 software (version 2.24-r1122) was employed to align the FASTQ files to SAM files with the reference genome generated from Genome assembly TUR_r1.0 (WGS project No: BQMF01) [8]. The SAM files were then converted to BAM files, sorted, and indexed using Samtools software (version 1.15.1). Finally, FeatureCounts (version 2.0.4) was utilized to perform read counting, and functional annotation was performed using Diamond Blast function equipped in OmicsBox 3.0 (Biobam Bioinformatics, Cambridge, MA, USA). The bioinformatic procedures were computed utilizing SHIROKANE, a high-performance computing apparatus situated at the Human Genome Center.

Annotated raw bead counts were obtained and processed using a web portal for pathway analysis (iDEP.95/iDEP1.1; http://bioinformatics.sdstate.edu/, accessed on 7 June 2023) [9]. Identification of differentially expressed genes (DEGs) was performed by extraction with an FDR cutoff of 0.1 and min-fold change of 2 as the default setting. A hierarchical clustering was performed as input the Euclidean distance computed between genes and samples. A heatmap of all DEGs was subsequently generated in which numerical values of points were represented by a range of colors. All DEGs Pathway analysis was performed using Parametric Gene Set Enrichment Analysis (PGSEA) with gene sets from the Gene Ontology Molecular Function and all other available databases. The cutoff for significance was set at 0.2.

3. Results

3.1. Diversity of structure and function of T sp.

Understanding the complexity and organization of *T. sp*. requires uncovering the tissue and functional diversity, which now can be predicted using bulk sequencing, in addition to traditional histological and ultrastructural observation. The medusae were dissected into the upper part and lower part as shown in Fig. 1a. The manubrium, oral lobes, and radical canal are included in the upper part (gastrovascular cavity part, GP), while the tentacles and velum with the nerve ring and ring cannel are included in the lower part (nerve ring part, NP). Within these organs, the tissue surrounding the nerve ring has been considered to be an important contributor to the rejuvenation process. By employing comprehensive transcriptome sequencing, heatmap analysis extracted the top 1000 up-regulated and down-regulated genes in comparison between GP and NP (Fig. 1b). By exploring the database of Co.Expression.Tissue.Protein.Expression.from.Human.Proteome.Map, the results of PGSEA predicatively revealed that heart-, adrenal-, prostate- and kidney-like tissues are contained in the GP fraction, while retina-like and placenta-like tissues are contained in the NP fraction (Fig. 1c). These genes were functionally identified as belonging to four clusters. Functional enrichment revealed that the genes in cluster 2 (green circle) are related to metabolic processes, while the genes in cluster 3 are initially related to negative regulation of metabolic processes and transferase activity, cell projection assembly, rhythmic processes and phagocytosis. The network of these enriched pathways is shown in Fig. 1d. These results systemically indicate the potential structural and functional diversity of *T. sp*. medusae.

3.2. GP fragment dominates process and response to external and internal stress

GO pathway enrichment analysis of differentially expressed genes (DEGs) revealed that pathways including cellular responses to external or internal stress, such as DNA damage, abiotic stimulus, radiation, and reactive oxygen species, are dominant in the GP fragment compared to the NP fragment. Functions related to regulation of sensory function, central neuronal system development and response to hypoxia are significantly activated in the NP fragment (Fig. 2a). DEGs related to the response to the oxidative stress pathway were extracted and shown in Fig. 2b. Overexpression of genes related to the stress response, including *TPO*, *GSS*, *BTK*, *SLOX5*, *PKD2*, and *PLA2R1*, was observed in the GP fragment. In DEGs collected from the hypoxia response pathway, which has been considered a major initialization factor of activation of stem cells, higher activation was confirmed in the GP fragment compared to the NP fragment. Genes encoding proteins involved in transcription-related genes (*TGFB1*, *ERCC3*, and *FAXO3*), fertilization and development-related genes (*SRC*, *EP300*, *ADORA1*, and *ADAM17*), cell cycle-related genes (*ATM* and *NPEPPS*), and structure formation-related genes (*ANGPT4*, *OPRD1*, *MMP2*, *THBS1*, and *HSP90B1*) were extracted.

Taken together, these results suggest that the GP fragment is

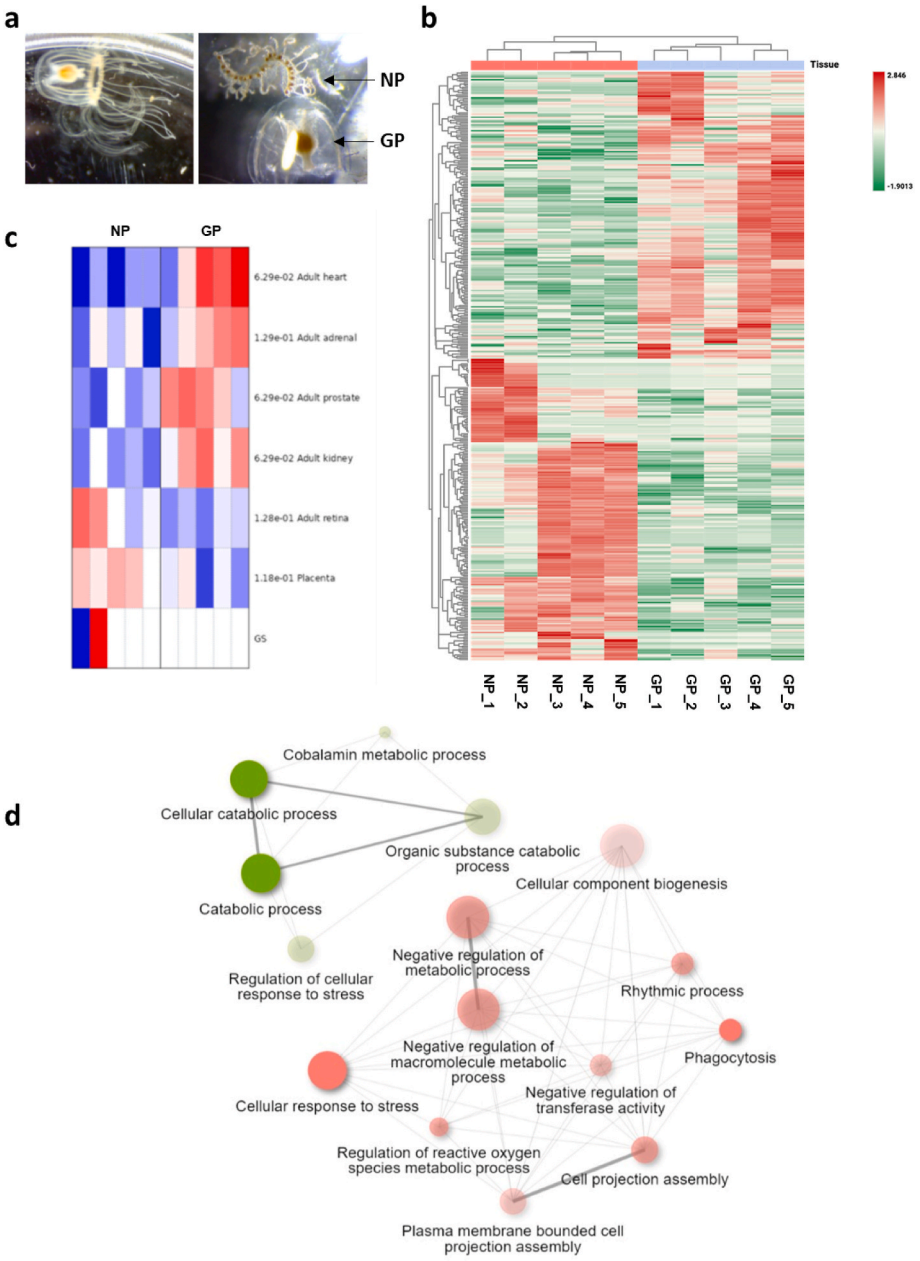

Fig. 1. Predicated structural and functional diversity of *T. sp.* (a) The medusae were dissected into gastrovascular cavity part (GP) and nerve ring part (NP). Left panel: Mature medusa under anesthetic condition. Right panel: The medusa was dissected into GP and NP fragments. (b) Heatmap analysis extracted the top 1000 up-regulated and down-regulated genes in comparison between GP and NP. Downregulated genes are highlighted in green; upregulated genes are highlighted in red. (c) Functional predication of tissues by PGSEA analysis with exploration of the database of Co.Expression.Tissue.Protein.Expression.from.Human.Proteome.Map. Downregulated pathways are highlighted in blue; upregulated pathways are highlighted in red. (d) GO enrichment networks of four clusters. GO enrichment used padj <0.05 as the threshold criterion for significant enrichment. Enriched pathways in cluster 2 (green circle) and cluster 3 (red circle) are highlighted. No enrichment was extracted from cluster 1 and cluster 4. The size of circles indicates the number of included genes.

functional dominant in the regulation of cellular responses to external or internal stress, whereas the NP fragment may contribute to developmental initialization processes in *T. sp.*

3.3. NP fragment may decide fate of rejuvenation

We then explored all available databases equipped in the PGSEA module, and the top 30-hit pathways were summarized (Fig. 3a). With particular interest in exploring contributors to the response to rejuvenation, partial DEGs involved in HMGIY target genes aroused our attention. The expression of genes encoding regulating factors involved in the initialization process of development and proliferation, such as *PPP1R15B*, *ATF5*, *HEATR4*, *ROR1*, *FUT8*, and *EIF4H*, was significantly higher in the NP fragment compared to the GP fragment (Fig. 3b). Also, enhanced expression of *PEF1* and *TM7SF3*, which encode factors related to programmed cell death, was observed. Parallel to the observation of previous studies, genes encoding proteins that are expected to be contributors involved in the neuronal network, including *AOC3*, *ZFYVE26*, *RAB39B*, *CLCN3*, and *GABRD,* were enriched in the NP fragment.

Together, these findings show that the NP fragment indeed serves as a central nervous-like system in *T. sp.* In view of the possibility of stem cell existence as a placenta-like tissue contained in NP fragments, the enrichment of factors involved in the initialization process of development and programmed cell death strongly suggests that the NP fragment

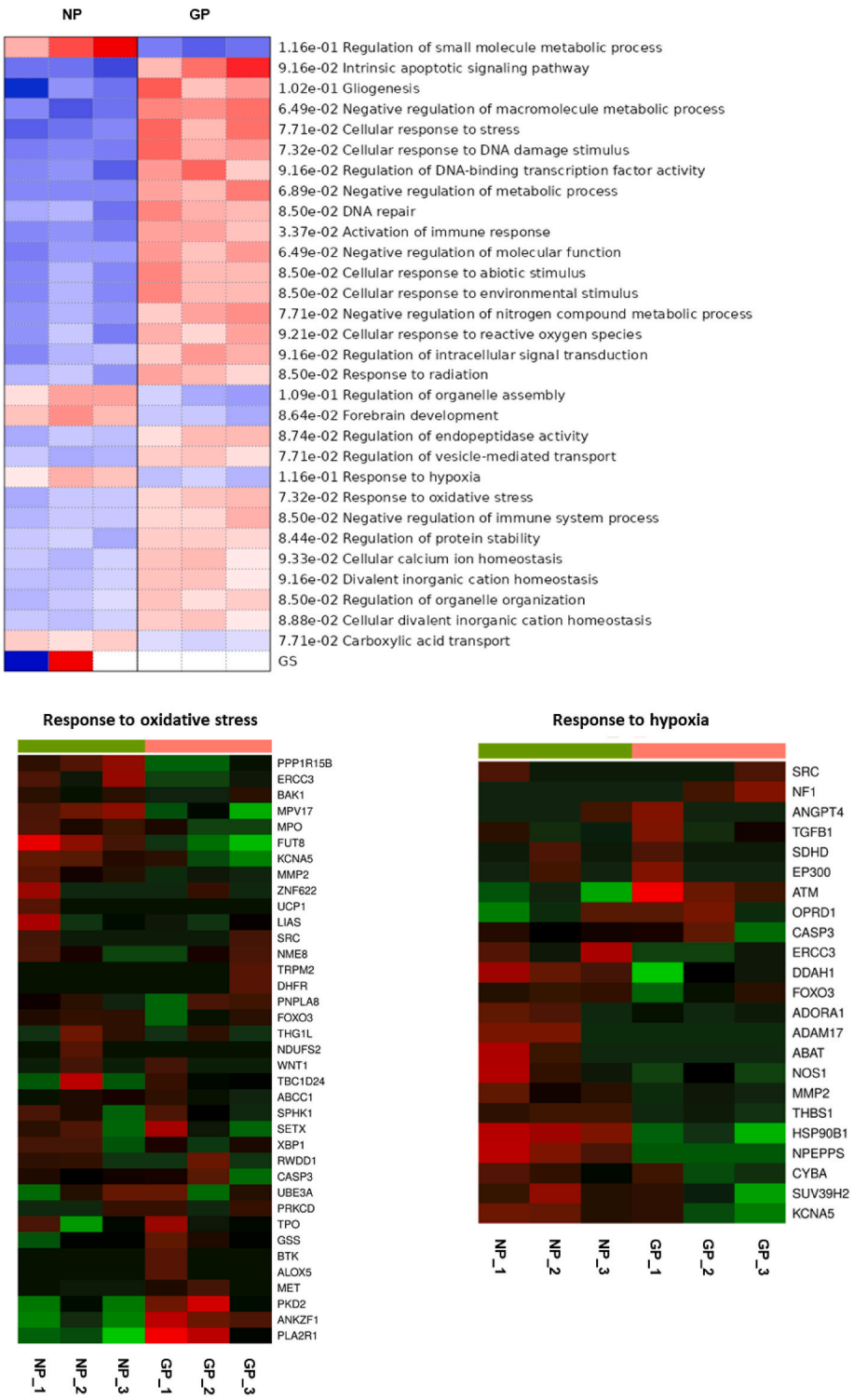

Fig. 2. Results of GO biological process pathway analysis in GP and NP fragments. (a) Functional prediction of differentially expressed genes was performed by PGSEA analysis by exploring the database of GO biological process. A default value of *FDR* < 0.2 served as the threshold criterion for significant cutoff. The 30 GO pathways with the most significant enrichment are shown in the heatmap. Downregulated pathways are highlighted in blue; upregulated pathways are highlighted in red. Normalized expression of genes included in representative pathways, including (b) response to oxidative stress and (c) response to hypoxia, was visualized in both the GP and NP samples with green-black-red heatmaps.

may play a crucial role in the fate of the rejuvenative process in *T. sp.*

4. Discussion

The answers to fundamental questions about the evolutionary and mechanistic response of age-related morphological and functional deterioration leading to death have been mainly explored in a limited number of experimental systems, including mammals (*Mus musculus*, *Homo sapiens*) and invertebrates (*Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*) [1]. In fact, the spectrum of

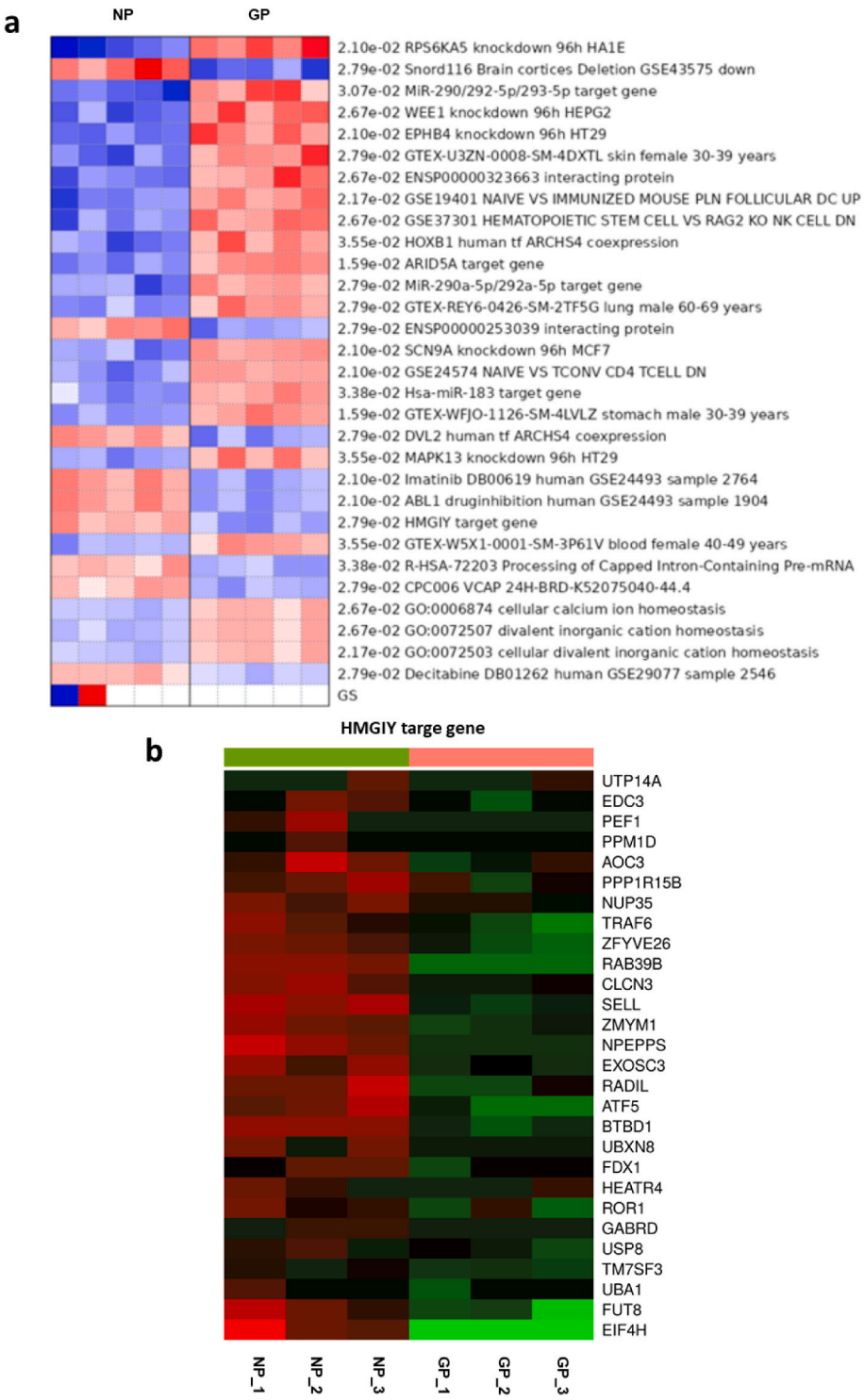

Fig. 3. Expression of HMGIY-targeted genes in GP and NP fragments. (a) Functional prediction of differentially expressed genes was performed by PGSEA analysis by exploring all available databases currently equipped in iDEP1.1. A default value of *FDR* < 0.2 served as the threshold criterion for significant cutoff. The top 30 pathways with the most significant enrichment are shown in the heatmap. Downregulated pathways are highlighted in blue; upregulated pathways are highlighted in red. (b) Normalized expression of genes included in the HMGIY pathway was visualized in both GP and NP samples with a green-black-red heatmap.

valuable systems for studying aging is much more extensive, which allows us to look at this phenomenon using a holistic rather than a reductionist approach. In the current study, with a particular focus on one of the aging permutations, immortality (neverending existence), the contribution of tissue diversity to the response to environmental stress and rejuvenation was dissected in a less conventional hydrozoan system, *T. sp.*, by comparison of tissue fragments with and without the nerve ring.

Compared to sessile polyps, the prominent nerve ring in free-swimming medusae has been considered a much higher level of centralized nervous system, containing a well-developed optical system that can be integrated into sophisticated light and gravity-sensing organs [10,11]. The structure of the nerve ring contributes to the control of swimming behaviors such as the avoidance of obstacles or escape from

predators, by generating Ca^{2+}-dependent or Na^{+}-dependent depolarization potentials [12]. In current study, a retina-like structure was also confirmed in the NP fragment. Beyond that, an unexpected potential association between the NP structure and system development was identified. Compared to the GP fragment, the NP fragment is more dominant in the regulation of the systemic response to hypoxia, regulation of organelle assembly, control of developmental initialization and programmed cell death. As a featured signaling pathway, but not limited to this, up-regulated HMGIY genes could be one of the initialization factors of transcriptional activation of stem cells in the NP fragment as the existence of breakpoints outside the gene. These transcriptome characteristics are closely related to functional activity, such as proliferation and differentiation of stem cells. On the other hand, the GP fragment is more responsible for the systemic response to different kinds of stimuli, such as DNA damage, oxidative stress and radiation. Less response to harmful external stimuli in NP could be protective for the retrograde cyst and contribute to a high propensity to tolerate manipulations within environmental variations. Therefore, we assume that the GP fragment could be mainly responsible for systemic senescence, whereas the NP fragment may be fundamental for the rejuvenation process. The report of Piraino et al. supports our assumption [3]. They dissected *Turritopsis nutricula*, another hydrozoan which was assumed not to undergo organismic death, into small fragments. Under an *in vitro* culture condition, the transdifferentiating rates of fragments were evaluated. The results indicated that the structure surrounding the nerve ring, including exumbrella epidermic, velum, tentacle bulbs, and partial tentacle, which was defined as the NP fragment in the current study, was critical for destabilization and stolon formation in the polyp. For aging studies using the *T. sp.* system, the GP fragment could be utilized for study related to stress response and systemic senescence, while the NP fragment could be explored for self-repair and regeneration approaches. Identification of specific genes or pathways associated with stress response and rejuvenation in *T. sp.* provides potential targets for therapeutic interventions. This knowledge may contribute to the development of pharmaceutical interventions aimed at modulating stress response and promoting regenerative processes in human tissues. Moreover, the study sheds light on the mechanisms underlying the response to environmental stress. Such knowledge has broader implications for understanding how organisms, including humans, adapt to environmental challenges. Insights into stress response mechanisms can have applications in developing strategies to mitigate the impact of environmental stressors on human health.

While *T. sp.* possesses unique biological features that make it an intriguing research subject, there are certain limitations and challenges associated with using it as a research tool. First of all, the mature medusae of immortal *T. sp.* are notably sensitive to alterations in water quality, temperature and other environmental factors. Maintaining stable and optimal conditions for their survival and growth can be demanding, requiring precise control of parameters such as water chemical composition, temperature and feeding regimen. Secondly, while *T. sp*. has fascinating regenerative abilities, its biological mechanisms may not directly translate to other organisms, including humans. Its genomic resources are also relatively limited compared to other well-established model organisms. Extending broad conclusions or generalized findings from *T. sp.* to other species requires caution and further comparative studies across different organisms. Finally, the collection and manipulation of *T. sp.* specimens need to be conducted responsibly and in accordance with ethical guidelines. Ensuring the welfare and humane treatment of *T. sp.* during research is of utmost importance.

Is there any possibility of applying the rejuvenation process, an exceptional ability to revert back to an earlier developmental stage through transdifferentiation, to humans? Or are *homo sapiens* equipped with this fantastic ability but it has not yet been noticed? Is it possible that aging-related tumor development or death could just be a precursor for the transdiffentiation process, similar to what is observed in *T. sp.*? Maybe extraordinary advances in the technological challenges of storing and reviving a deceased human body while preserving its structural integrity and stem cell viability could help us to answer these questions. In any case, achieving true rejuvenation and reversing the effects of aging in humans, as seen in *T. sp.*, remains firmly in the realm of science fiction and further exploration is required for essential elucidation of the fundamental biological differences between *T. sp.* and humans.

Source of funding

S.L. and M.M. were funded by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (No. 22K08564). Y.S. was funded by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (No. 21K16549).

CRediT authorship contribution statement

Shuang Liu: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Erika Takemasa:** Methodology, Investigation, Formal analysis. **Yasuyuki Suzuki:** Methodology, Formal analysis. **Masaki Mogi:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

We are sincerely grateful to Mr. Kubota Shin for his valuable comments and support with the establishment of animal models.

References

[1] J. Mikuła-Pietrasik, M. Pakuła, M. Markowska, P. Uruski, L. Szczepaniak-Chicheł, A. Tykarski, K. Książek, Nontraditional systems in aging research: an update, Cell. Mol. Life Sci. 78 (2021) 1275–1304, https://doi.org/10.1007/s00018-020-03658-w.

[2] Y. Hasegawa, T. Watanabe, M. Takazawa, O. Ohara, S. Kubota, De novo assembly of the transcriptome of Turritopsis, a jellyfish that repeatedly rejuvenates, Zool. Sci. 33 (2016) 366–371, https://doi.org/10.2108/zs150186.

[3] S. Piraino, F. Boero, B. Aeschbach, V. Schmid, Reversing the life cycle: medusae transforming into polyps and cell transdifferentiation in Turritopsis nutricula (Cnidaria, Hydrozoa), Biol. Bull. 190 (1996) 302–312, https://doi.org/10.2307/1543022.

[4] A. Garm, P. Ekström, M. Boudes, D.E. Nilsson, Rhopalia are integrated parts of the central nervous system in box jellyfish, Cell Tissue Res. 325 (2006) 333–343, https://doi.org/10.1007/s00441-005-0134-8.

[5] O. Koizumi, S. Hamada, S. Minobe, K. Hamaguchi-Hamada, M. Kurumata-Shigeto, M. Nakamura, H. Namikawa, The nerve ring in cnidarians: its presence and structure in hydrozoan medusae, Zoology (Jena) 118 (2015) 79–88, https://doi.org/10.1016/j.zool.2014.10.001.

[6] B.A.M. Bouwman, N. Crosetto, M. Bienko, The era of 3D and spatial genomics, Trends Genet. 38 (2022) 1062–1075, https://doi.org/10.1016/j.tig.2022.05.010.

[7] N. Liu, L. Zhou, J. Qu, S. Yao, Recent advances in CRISPR/Cas9 directed base editing, Curr. Gene Ther. 21 (2021) 327–337, https://doi.org/10.2174/1566523221666210322090638.

[8] Y. Hasegawa, T. Watanabe, R. Otsuka, S. Toné, S. Kubota, H. Hirakawa, Genome assembly and transcriptomic analyses of the repeatedly rejuvenating jellyfish Turritopsis dohrnii, DNA Res. 30 (2023), https://doi.org/10.1093/dnares/dsac047.

[9] S.X. Ge, E.W. Son, R. Yao, iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data, BMC Bioinf. 19 (2018) 534, https://doi.org/10.1186/s12859-018-2486-6.

[10] R. Petie, A. Garm, D.E. Nilsson, Visual control of steering in the box jellyfish Tripedalia cystophora, J. Exp. Biol. 214 (2011) 2809–2815, https://doi.org/10.1242/jeb.057190.

[11] A. Garm, M. Oskarsson, D.E. Nilsson, Box jellyfish use terrestrial visual cues for navigation, Curr. Biol. 21 (2011) 798–803, https://doi.org/10.1016/j.cub.2011.03.054.

[12] R.A. Satterlie, Cnidarian nerve nets and neuromuscular efficiency, Integr. Comp. Biol. 55 (2015) 1050–1057, https://doi.org/10.1093/icb/icv067.