Texture-Based Screening to Identify Genes Involved in Reproductive Aging in *Caenorhabditis elegans*

Momoko Imakubo¹,², Koji Kyoda², Hiroya Itoga², Jun Takayama³, Shuichi Onami¹,²,³*

¹ Graduate School of System Informatics, Kobe University, Kobe, Japan.
² RIKEN Center for Biosystems Dynamics Research, Kobe, Japan.
³ RIKEN Quantitative Biology Center, Kobe, Japan.

* Corresponding author. Tel.: +81-78-306-3441; email: sonami@riken.jp
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**Abstract:** Reproductive capacity declines with aging, thereby increasing errors in fertilization, chromosome segregation, and embryonic cleavage. Age-related changes in oocytes involve not only function but also appearance. We previously showed that the texture of the cytoplasm in *Caenorhabditis elegans* oocytes varies with their age. We quantitatively characterized this change by evaluating two image features: Mm Value (mean difference between the maximum and minimum intensities within each moving window) and Correlation (COR), which is based on the Gray Level Co-occurrence Matrix (GLCM). However, the association between genetics and changes in cytoplasmic texture with aging has not been clarified. Genes that cause texture changes similar to those observed during oocyte aging are candidates for involvement in the aging pathway. Here, to examine whether gene knockdown of specific genes can change the Mm Value or COR, we analyzed the cytoplasmic texture in images from the Worm Developmental Dynamics Database 2. This database stores differential interference contrast microscopy images of early *C. elegans* embryos with genes silenced by RNA-mediated interference. Of 316 target genes screened, we identified five genes (*smc-4*, *F10C2.4*, *tin-44*, *let-754*, *hmp-2*) whose knockdown decreased the Mm Value and one gene (*csr-1*) whose knockdown increased the COR. The five genes included genes involved in age-related function, such as chromosome segregation and mitochondrial function, and some genes that are not known to be related to aging. The gene that increased the COR is also involved in chromosome segregation. Our findings indicate that these six genes could potentially be involved in reproductive aging.

**Key words:** *Caenorhabditis elegans*, image texture analysis, Nomarski DIC microscopy, RNAi screening, statistical image processing, reproductive aging.

**1. Introduction**

Reproductive capacity declines with aging, thereby increasing errors in fertilization, chromosome segregation, and embryonic cleavage [1]-[3]. However, the mechanisms regulating reproductive aging remain incompletely understood.

*Caenorhabditis elegans* (*C. elegans*) is a leading model for studying aging because of its short lifespan (~3 weeks) and the evolutionary conservation of longevity pathways from *C. elegans* to humans [4]. In particular, *C. elegans* has been developed as a model for studying age-related decline in fertility [5].

Age-related changes are found not only in oocyte function but also in oocyte appearance [2], [5], [6]. We previously showed that the texture of the cytoplasm in *C. elegans* oocytes varies with age [7]. We
quantitatively characterized this change by measuring the Differential Interference Contrast (DIC) image features of Max-min Value (Mm Value) and Correlation (COR) (Fig. 1a and Fig. 1b). The Mm Value is defined as the mean of the difference between the maximum and minimum intensities within successive moving windows and is a measure of texture roughness [7]. The COR, which is based on Gray Level Co-occurrence Matrix (GLCM) [8], measures the gray level linear dependencies of pixels at specified positions relative to each other. To calculate the COR, we first define a spatial relationship by using the parameters distance $d$ and angle $\theta$. We then calculate the second-order joint probability $P(i, j | d, \theta)$ of two pixels with gray levels $i$ and $j$ ($0 \leq i < G$, $0 \leq j < G$). To calculate $P(i, j | d, \theta)$, we sum the number of pixels with paired intensities $(i$ and $j)$ in the defined spatial relationship. We then extract the image feature using GLCM [7].

When the cytoplasm of 1-day-old and 3-day-old C. elegans oocytes are compared, the Mm Value decreases with aging whereas the COR, at several levels of parameters, increases with aging [7]. However, the relationship between variances in texture and biological and genetic processes has not been clarified. We consider that genes whose knockdown causes texture changes similar to those seen in aging oocytes are candidates for involvement in the aging pathway, and that identification of such genes may lead to understanding of the mechanisms underlying oocyte aging.

Worm Developmental Dynamics Database 2 (WDDD2, https://wddd.riken.jp), an update of WDDD [9], shares four-dimensional DIC microscopy images of 33 wild-type and 1728 RNAi-treated C. elegans embryos. RNAi is a phenomenon in which specific genes are inactivated by introduction of double-stranded RNA with complementary sequences [10]. The RNAi-treated embryos represent the 316 embryonic genes that exhibited an embryonic lethal phenotype in 100% of offspring when silenced in the previous genome-wide screen [11].

RNAi screening using computational image processing or automatic screening system is one of the state-of-the-art technologies in biomedical research [12], [13]. RNAi screening based on morphological features from image processing is unbiased and high-throughput approach. Recently, we proposed a method to quantify the age-associated changes in the cytoplasmic texture of C. elegans oocytes [7]. This method provides a new opportunity to apply the computational image processing-based RNAi screening to studies of aging in C. elegans oocytes.

Here, to examine whether gene knockdown can decrease the Mm Value or increase the COR (as seen in oocytes with aging), we analyzed the cytoplasmic texture in the early embryo images in WDDD2. Morphological defects in oocytes correlate with a decreased embryo-hatching rate [5], [6]. We assumed that the cytoplasmic texture properties in the oocytes are reflected in the early embryo and screened for genes whose knockdown decreased the Mm Value or increased the COR in cytoplasmic regions of early embryos. We identified five genes whose knockdown resulted in a decreased Mm Value, and one gene whose knockdown led to increased COR, suggesting that the genes are involved in reproductive aging.

The remainder of this paper is organized as follows. We provide details of the methods in Section 2. In Section 3, we describe the results. First, we overview our computational experiments (3.1). Then, we screen genes using the Mm Value (3.2), investigate the interactions among the candidate genes identified by the Mm Value (3.3). Finally, we screen genes using the COR (3.4). In Section 4, we discuss the implementation details and the candidate genes. Finally, in Section 5, we conclude this work.

2. Methods
2.1. WDDD2

Four-dimensional DIC microscope images in the WDDD2 were recorded in multiple focal planes and at multiple time points (0.102 µm per pixel). In the RNAi screen, young adult worms were separately injected with each double-stranded RNA, and embryos were dissected from the worms immediately after fertilization.
2.2. Calculation of Image Features

We used the images at the first time point in embryo development in WDDD2 and at the focal planes where the Mm Value in the entire image was maximum. To detect the cytoplasmic region, we used local image entropy [14]. To calculate image features, random regions of 80 × 80 pixels were extracted from the cytoplasmic region. Image features of individual embryos were defined as the mean of those in the extracted four regions.

The Mm Value was calculated by moving the local window except for the border in the same way as in the previous study of oocytes [7]. We set the size of the moving window to 3 × 3 pixels. COR, a second-order statistical feature based on GLCM, was calculated by using the following equation and the co-occurrence matrix \( P(i, j | d, \theta) \) of two pixels with gray levels \( i \) and \( j \) \((0 \leq i < G, 0 \leq j < G)):\n
\[
\text{COR} = \frac{\sum_{i=0}^{G-1} \sum_{j=0}^{G-1} ijP(i, j) - \mu_x \mu_y}{\sigma_x \sigma_y}
\]

(1)

where \( G \) is the number of gray levels, \( \mu_x, \mu_y, \sigma_x \) and \( \sigma_y \) are the means and standard deviations in the \( x \) and \( y \) direction given by \( P(i, j | d, \theta) \).

\[
\mu_x = \sum_{i=0}^{G-1} \sum_{j=0}^{G-1} iP(i, j), \mu_y = \sum_{i=0}^{G-1} \sum_{j=0}^{G-1} jP(i, j)
\]

and

\[
\sigma_x^2 = \sum_{i=0}^{G-1} \sum_{j=0}^{G-1} P(i, j)(i - \mu_x)^2, \sigma_y^2 = \sum_{i=0}^{G-1} \sum_{j=0}^{G-1} P(i, j)(j - \mu_y)^2
\]

The co-occurrence matrix defined is symmetric.

3. Results

3.1. Overview of Our Experiments

To identify genes whose expression might affect Mm Values and/or the COR in aging oocytes, we examined the differences in image features between RNAi-treated and wild-type embryos in WDDD2 (Fig. 1c). We used 33 datasets from wild-type embryos and 1728 data sets from RNAi experiments, where 316 embryonic essential genes were individually silenced.

3.2. RNAi Screening Using the Mm Value

Of the 316 genes tested, we detected five genes, F35G12.8 (smc-4), F10C2.4, T09B4.9 (tin-44), C29E4.8 (let-754) and K05C4.6 (hmp-2), whose knockdown significantly reduced the Mm Value compared with that of the wild type (Fig. 2), suggesting that they are involved in reproductive aging. No genes were found to significantly increase the Mm Value compared with that of wild type.

The smc-4 gene encodes an ortholog of human SMC4 (structural maintenance of chromosomes 4) protein, a mitotic condensing subunit that acts with MIX-1 to enable chromosome segregation [15]. Chromosomal abnormalities are a major cause of age-related decline in oocyte quality [2]. In C. elegans mutants of the TGF-β Sma/Mab pathway, reproductive aging is delayed [16]. The smc-4 gene is upregulated in the TGF-β mutant oocytes [13]. The condensin SMC declines in both mouse and human oocytes with aging [17], [18], suggesting that chromosome segregation is a key process determining oocyte quality that is conserved between worms.
and mammals [13]. Knockdown of smc-4 degrades germline and oocyte morphology and increases the rate of unfertilized oocytes and unhatched embryos [13]. In WDDD2, almost all smc-4 RNAi worms exhibited embryonic lethality and some of them produced unfertilized oocytes.

The F10C2.4 gene encodes the DNA polymerase delta complex subunit and is required for normal chromosome segregation [19]. Reduced expression of this gene may affect reproductive aging due to chromosome segregation errors, similar to smc-4.

The tin-44 gene functions in transporting proteins into the inner mitochondrial membrane and matrix [20], [21]. Mitochondria have a pivotal role not only in energy metabolism but also in regulation of the rate of aging.
tin-44 RNAi worms exhibited a shorter lifespan than wild type [23], suggesting that mitochondrial dysfunction caused by RNAi of tin-44 possibly affects aging.

The genes let-754 encodes adenylate kinase [24], [25] and hmp-2 encodes β-catenin and is required for proper cell migration [26]. Neither protein has been directly linked to aging. The genes let-754 and hmp-2 may be related to the aging pathway or may cause abnormalities in cytoplasmic textures via alternative pathways.

![Fig. 2. Genes identified using the Mm value. When the five genes displayed were silenced in C. elegans embryos, the cytoplasmic region showed a significantly lower Mm Value than was seen in wild-type embryos. Red bars indicate mean values; error bars indicate SEM. Asterisks indicate statistical significance versus wild type (*P < 0.05, **P < 0.01; Dunnett’s test).](image)

### 3.3. Interaction among the Candidate Genes Identified by the Mm Value

We then automatically predicted the interactions (genetic interactions, co-expression, and physical interactions) among all five gene candidates identified by the Mm Value by using GeneMania software (http://www.genemania.org) [27]. In particular, we examined whether let-754 and hmp-2 interact with the three candidate genes that have known age-related functions (i.e., smc-4, F10C2.4, tin-44). From the network shown in Fig. 3, three genes, hcp-3, zfp-1, and mut-7 have predicted simultaneous interactions with smc-4, F10C2.4, and hmp-2. Although tin-44 was predicted to interact with hmp-2 via two genes, the interaction was relatively weak. We found no association between let-754 and the genes identified by the Mm Value. The results suggest that smc-4, F10C2.4, and hmp-2 have high genetic interactions and tin-44 and let-754 have less interactions with other genes.

We then examined whether the knockdown of the five candidate genes shares any of the phenotype categories annotated by Piano et al. [28], which are available in the RNAi Database (http://www.rnai.org) [29]. We focused on cytoplasmic phenotypes and found that knockdown of smc-4, F10C2.4, and hmp-2 was included in the category “Multiple cavities”, which is described as “multiple vesicles, vacuoles, or cavities are seen during early embryogenesis”. Both the shared phenotype category and the predicted gene interactions mentioned above suggest that smc-4, F10C2.4, and hmp-2 have similar properties in terms of function and phenotype; therefore, hmp-2 may function in an aging pathway related to chromosome segregation like smc-4 and F10C2.4. In addition, out of the 27 genes in the “Multiple cavities” category, 13 genes were not included in WDDD2. These 13 genes might include gene(s) whose knockdown decreases Mm Value and have a related
function to smc-4, F10C2.4, and hmp-2.

The five genes whose knockdown reduced the Mm Value can be divided into three groups: a chromosome abnormality group (smc-4, F10C2.4, and hmp-2), mitochondrial function group (tin-44), and unknown group (let-754), based on the results of the gene interaction and phenotype category analyses. Therefore, multiple pathways may contribute to reductions in Mm Value.

3.4. RNAi Screening Using the COR

We then screened the RNAi-treated embryos’ images in WDDD2 to find RNAi-treated embryos whose cytoplasmic regions displayed significantly higher COR than in wild type. The angle parameter of GLCM was set to $\theta = 0, 45, 90$, and $135$ degrees. For the 316 genes tested, the COR when the angle was set to 0, 45, or 135 degrees did not differ significantly between RNAi and wild-type embryos (data not shown). However, for two genes, csr-1 and gbp-1, knockdown significantly increased and decreased the COR, respectively, compared with wild type when $\theta = 90$ (Fig. 4).

The csr-1 gene encodes CSR-1, an Argonaute protein that is essential for chromosome segregation and embryo viability [30]. CSR-1 is a core component of P granules and acts with P granules to maintain germline integrity [31]. Because the COR increased when csr-1 was silenced, csr-1 is a candidate for involvement in oocyte aging.

Conversely, knockdown of gbp-1 decreased the COR. The gbp-1 gene encodes the heterotrimeric G protein $\beta$ subunit, which is required for proper spindle orientation and regulation of egg laying [32], [33], but has not yet been shown to be directly related to reproductive aging. Further experiments are needed to clarify the associations between the decline of the COR and gene functions.
Fig. 4. Genes identified using the COR. Correlation (COR) is displayed as a function of distance $d$ for two genes whose knockdown (in RNAi embryos) resulted in significantly increased or decreased COR compared with wild type. Error bars indicate SEM. Asterisks indicate statistical significance between the RNAi embryos and wild-type embryos (*$P < 0.05$, Dunnett’s test).

4. Discussion

In our previous study, the Mm Value and the COR in oocytes changed simultaneously with aging [7]. However, in the current study, knockdown of specific genes changed the image features independently, suggesting that the image features can be used to characterize different properties of the age-associated changes in the cytoplasmic texture. In other words, texture changes caused by RNAi knockdown can be divided into two patterns, one that decreases the Mm Value and one that increases the COR, and the associated genes might function in different pathways involved in aging. The insulin/insulin-like growth factor pathway, a well-known pathway controlling the lifespan of C. elegans, and the TGF-β Sma/Mab pathway are considered to regulate reproductive lifespan independently [16], [34], [35]. Our results show that knockdown of smc-4, which functions in the TGF-β Sma/Mab pathway, decreased the Mm Value in cytoplasmic regions of C. elegans embryos, implying that the texture characterized by the Mm Value is involved in the TGF-β pathway. In contrast, the texture characterized by the COR is possibly involved in the insulin/insulin-like growth factor pathway although there is no direct experimental evidence to support this. The texture characterized by the Mm Value reflects the changing contrast in the DIC granules, which may be due to chemical modification or a difference in content quantity of the granules. A change in the COR reflects a change in the size of cytoplasmic granules [7]. Further experiments are needed to understand the association between gene function and the morphology of the cytoplasmic granules. Elucidation of this association may be helpful for understanding the aging pathways and mechanisms.

5. Conclusion

By screening images of RNAi-treaded embryos in WDDD2, we identified genes whose knockdown changes the cytoplasmic texture in early C. elegans embryos, mirroring the changes in oocytes with aging. Of 316 target genes, knockdown of five genes was found to significantly decrease the Mm Value and knockdown of one gene significantly increased the COR. These genes could potentially be involved in reproductive aging.

Conflict of Interest

The authors declare no conflict of interest.
Author Contributions
Momoko Imakubo, Jun Takayama and Shuichi Onami designed research; Momoko Imakubo and Shuichi Onami wrote the paper; Momoko Imakubo performed computational experiments; Koji Kyoda and Hiroya Itoga managed and prepared datasets; all authors had approved the final version.

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Momoko Imakubo received her Ph.D. from Kobe University in 2021. She has been working toward a Ph.D. degree at the Kobe University and RIKEN Center for Biosystems Dynamics Research. Currently, she is working at Sysmex Crop., Japan. Her current research interests include computational biology, systems biology, and bioimage informatics.

Koji Kyoda received his Ph.D. from Keio University in 2005. He is now a research scientist at RIKEN Center for Biosystems Dynamics Research. His research interests include systems biology, bioimage informatics, high-throughput biological data analysis, and data formats for biological dynamics.

Hiroya Itoga received his Ms. and Ph.D. of engineering degrees from University of Tsukuba in 1997 and 2002, respectively. He was a research associate, an assistant professor, and an associate professor at Department of Computer Science, Faculty of Information Science and Engineering, Ritsumeikan University, Japan, from 2003 to 2018. He was a technical staff from 2018 and has been a technical scientist since 2019 at RIKEN Center for Biosystems Dynamics Research. He has interest in computer science, software engineering, and computational biology.

Jun Takayama received his Ph.D. from University of Tokyo in 2009. He has been working at RIKEN Quantitative Biology Center. He is now an associate professor at Tohoku University Graduate School of Medicine. His research interests include systems biology, genome informatics, and human genetics.

Shuichi Onami received his bachelor degree from the University of Tokyo in 1994, and his Ph.D. from the Graduate School for Advanced Studies in 1998. He was an associate professor at Keio University from 2002 to 2006 and joined RIKEN as a senior scientist at Genomic Sciences Center in 2006. He is currently a team leader at RIKEN Center for Biosystems Dynamics Research and a visiting professor at Graduate School of System Informatics, Kobe University. His current research interests include mathematical modeling of animal development and its application to medicine.