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Germ cells are specialized cells that can transmit genetic materials from one generation to the next in sexual reproduction. All of the other cells of the body are somatic cells. This separation of germ and somatic cells is one of the oldest problems in developmental biology. In many animal groups, a specialized portion of egg cytoplasm, or germ plasm, is inherited by the cell lineage which gives rise to germ cells. This cell lineage is called germline. The germline progenitors eventually migrate into the gonads, where they differentiate as germline stem cells (GSC) to form eggs and sperm when the organisms are physically matured. Our laboratory aims to find the molecular mechanisms regulating germline segregation and GSC niche function in *Drosophila*.

I. Role of maternal Ovo protein in the germline of *Drosophila* embryos

It has been proposed that germline-specific gene expression is initiated by the function of maternal factors that are enriched in the germ plasm. However, such factors have remained elusive. We have done a genome-wide survey of maternal transcripts that are enriched in the germ plasm and encode transcription factors for germline-specific gene expression of *vasa* and/or *nanos*. We finally identified 6 transcripts required for germline-specific gene expression by knockdown experiments using RNA interference (RNAi). Among the 6 transcripts, we focused on *ovo*. The *ovo* gene encodes a DNA-binding, C2H2 Zn-finger protein that is involved in oogenesis and in epidermal development. The *ovo* gene produces at least three alternate isoforms. Ovo-A and Ovo-B function as a negative and a positive transcriptional regulator in the germline, respectively. Ovo-Svb is expressed in the epidermal cells and is required for their differentiation. We found that Ovo-B is the major isoform expressed in PGCs during embryogenesis. To understand its function, we over-expressed the Ovo-A repressor only in the primordial germ cells (PGCs), and examined their developmental fate. Our data shows that the reduction in maternal Ovo-B activity results in a decrease in the number of primordial germ cells during post-embryonic stages. Thus, maternal Ovo-B has an essential role in germline development in both sexes. Experiments for identifying the downstream genes regulated by Ovo-B in germline are now on-going.

II. Germline Functional Genomics and Systems Biology using a *Bombyx mori* cell line

Conserved mechanisms regulating germline properties have remained largely unknown. Despite extensive use of *vasa* expression as a marker for germline in various animal species, systematic analyses on regulators for *vasa* expression have not been accomplished. With availability of genomic information and development of various *omics* technologies, functional genomics and/or systems biology approaches have been becoming more powerful to elucidate complex regulatory systems. However, such approaches require parallel data collection for many samples on many parameters to extract information on the systems, which is prohibitively difficult to perform using germline cells isolated from animals.

BmN4-SID1, a cell line derived form a *Bombyx mori* ovary, is known to express a germline gene, *piwi* (Kawaoka *et al*. 2009) and carries a nuage-like structure with *Vasa* protein (Tatsuke *et al*. 2010). Without any feeder cells, BmN4-SID1 can easily be propagated for large-scale experiments. Furthermore, a knockdown system by RNAi in the cell line has been established by introducing *C. elegans* SID1 (Mon *et al*. 2012). These properties are suitable to obtain parallel and scalable data collection from genetically manipulated germline cells. Therefore, we aimed to establish resources using BmN4-SID1 for functional genomics and systems approaches on germline properties. First, approximately 375 million reads of 50bp paired-end sequences were obtained by RNA-seq. Since the genetic background of BmN4 is unclear and gaps exist in the reference *B. mori* genome sequence, the obtained reads were used to build transcript contigs by the *de novo* assembler Trinity. Fifty thousand eight hundred eight contigs fully or partially matching the current gene models of *B. mori* were obtained. These contigs include *piwi* and *vasa* as well as the genes involved in sex differentiation and meiosis. With the structural information of the BmN4-SID1 transcripts, we carried out *in silico* dsRNA designed to efficiently knockdown each of the transcripts. These resources provide a platform enabling genome-wide perturbations and system-level measurements.

We have initiated RNAi screening for regulators of *vasa* and/or *piwi* expression. Among 300 regulatory genes which are conserved between *D. melanogaster* and *B. mori* and are involved in fertility of *D. melanogaster*, 7 genes including *ovo* were identified to regulate *vasa* and/or *piwi* expression. In addition, we have initiated elucidation of topology of regulatory network for germline gene expression (Figure 1). In this analysis, we also aimed to identify regulatory relationships between the genes known to be involved in germline development and the ones in other developmental processes. Since our transcriptome analyses on *D. melanoster* and *M. musculus* germline indicated that most of genes encoded by the genomes were dynamically regulated

during their development, it is intriguing to examine functional association between the genes involved in germline development and in biological processes that were not known to be involved in germline development. We are now performing RNAi-based knockdown of the genes including the 7 genes which are required for vasa expression in the cell line. The RNA was isolated from the dsRNA-treated BmN4-SID1, and then was analyzed by RNA-seq. The transcriptome data was used to infer regulatory relationships among the genes perturbed.

Although BmN4-SID1 does not fully maintain germline properties such as the ability to produce gametes, it has advantages on performing high-throughput and/or massive phenotyping on germline gene expression signatures to build testable hypotheses. Further studies using comparative functional analyses with model organisms such as *D. melanogaster* that are feasible for *in vivo* studies would identify developmental roles of genes and/or regulatory networks identified using BmN4-SID1.

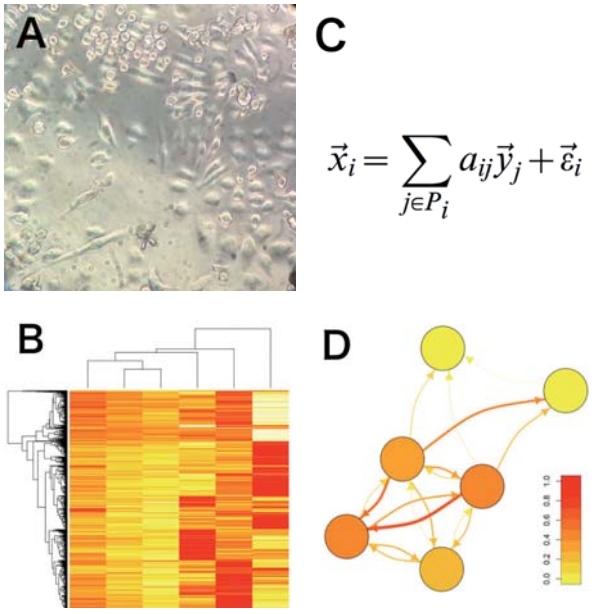

Figure 1. The workflow of modeling the gene regulatory network in BmN4-SID1. (A) BmN4-SID1 soaked in the culture media containing dsRNA. (B) Collection of network states obtained by transcriptome analyses. RNA-seq analyses were performed to collect mRNA expression differences between control and RNAi-treated cells, as inputs for network modeling. (C) EdLEGG for network modeling. The EdLEGG algorithm was applied to the mRNA expression difference data. $\bar{x}_i$ and $\bar{y}_j$ are gene expression differences caused by knockdown of gene *I* and *J*, respectively. In the linear combination, regulatory relationships between gene *I* and *J* are estimated by coefficients a_{ij}. (D) The resultant network model obtained so far. Nodes and links represent the genes targeted by RNAi and regulatory relationships between these genes, respectively.

III. The role of HSPGs in germline stem cell niche of *Drosophila*

Stem cells posses the remarkable capacity to generate daughter cells that retain a stem-cell identity and others that differentiate. Stem cells reside in dedicated cellular microenvironments termed stem-cell niches. These niches dictate stem-cell identity, maintain the stem cell population, and coordinate proper homeostatic production of differentiated cells. The GSC niche in *Drosophila* gonads is a useful model system for studying the stem-cell niche, because the cellular components of this niche have been characterized and the signaling pathways, such as BMPs and JAK/STAT which are essential for GSC maintenance, are known. Ligands for these signaling pathways (niche signals) are secreted from the niche cells, and are received by GSCs to activate the pathway responsible for GSC maintenance. Thus, the GSC niche is defined as the specialized region retaining a sufficient amount of niche signals for GSC maintenance. However, it is not well understood how the distribution of the niche signals is precisely controlled in the GSC niche.

To address this question, we have been investigating the function of Heparan Sulfate Proteoglycans (HSPGs) in the GSC niche. HSPGs are an evolutionally conserved family of sugar modified protein, which is an essential component of the extracellular matrix. One of the important functions of HSPGs during animal development is to regulate distribution of growth factors in extracellular space by binding to them. Thus, we speculated that HSPGs could retain a sufficient amount of niche signals for GSC maintenance. We found that Glypcan, a membrane-associating type of HSPG, is an essential component of the GSC niche both in female and male gonads. Glypican was highly expressed in niche cells both in ovary and testis, and its mutations caused a significant reduction in GSC number. In the GSC of the mutant ovary, the signaling pathway activated by Dpp (a BMP homologue acting as a niche signal) was impaired. Conversely, ectopic expression of Glypican in female gonads caused an increase in the number of GSCs with Dpp signaling. These results strongly suggest that Glypican defines the female GSC niche by regulating distribution of Dpp.

The question of whether the other HSPGs have functions in the GSC niche remains unclear. Since disrupting biosynthesis of all HSPGs by knockdown of NDST gene caused more severe GSC-loss phenotype than Glypican mutant, the other types of HSPGs could have functions in the GSC niche. We found that Syndecan and Perlecan, two evolutionally conserved groups of HSPGs, are essential in the GSC niche. These HSPGs were highly expressed in female GSC niche cells, and reduction of their function in niche cells caused a decrease in GSC number. Surprisingly, in these ovaries, ectopic GSC-like cells were also observed. This phenotype has not been observed in Glypican mutants. Thus, we speculated that Syndecan and Perlecan could regulate Dpp distribution in the GSC niche, in a way distinct from Glypican. We have succeeded to visualize Dpp protein distribution in the female GSC niche (Figure 2A). When Glypican was ectopically expressed in female gonads, Dpp distribution was ectopically observed (Figure 2B). We are now trying to visualize Dpp distribution in Syndecan and Perlecan mutant ovaries. Furthermore, we have also succeeded to visualize the GSC niche signal in male gonads (Figure 2, C, D). This enables us to study HSPGs function in the male GSC niche.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

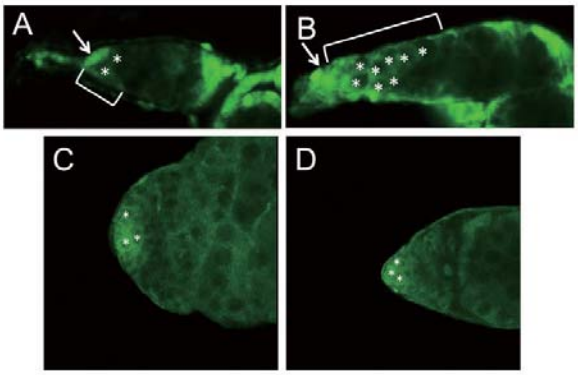

Figure 2. Distribution of GSC niche signals.
(A,B) Dpp distribution in distal tip region (germarium) of normal ovary (A) and of ovary expressing Glypican in the somatic cells throughout germarium (B). Green signal indicates Dpp protein (Brackets). Dpp distribution is expanded in *dally*-expressing ovary, compared to that observed in normal ovary. Arrows show niche cells, which are the source of Dpp. Asterisks indicate GSCs. (C, D) Distribution of male GSC niche signals, Upd (JAK/ STAT ligand, Green, C) and Gbb (BMP ligand, Green, D). Distribution of both niche signals were limited within the male GSC niche. Asterisks indicate GSCs.

Publication List

〔Original papers〕

- Dejima, K., Takemura, M., Nakato, E., Peterson, J., Hayashi, Y., Kinoshita-Toyoda, A., Toyoda, H., and Nakato, H. (2013). Analysis of Drosophila glucuronyl C5-epimerase: implications for developmental roles of heparan sulfate sulfation compensation and 2-O-sulfated glucuronic acid. J. Biol. Chem. 288, 34384-34393.
- Hira, S., Okamoto, T., Fujiwara, M., Kita, H., Kobayashi, S., and Mukai, M. (2013). Binding of Drosophila maternal Mamo protein to chromatin and specific DNA sequences. Biochem. Biophys. Res. Commun. 438, 156-160.

〔Original paper (E-publication ahead of print)〕

- Lim, R., Anand, A., Nishimiya-Fujisawa, C., Kobayashi, S., and Kai, T. Analysis of Hydra PIWI proteins and piRNAs uncover early evolutionary origins of the piRNA pathway. Dev. Biol. 2013 Dec. 16.