LABORATORY OF MOLECULAR GENETICS FOR REPRODUCTION

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Laboratory Scope

Our laboratory aims to reveal the molecular mechanisms of the formation of the gonads and sex differentiation. We are using medaka fish (*Oryzias latipes*) for these purposes.

Medaka has been recently established as a model vertebrate. The entire genome sequence was determined and a variety of inbred strains with a large polymorphic genome are available, which allows us to investigate biological phenomena by the means of molecular genetics. Furthermore, an exogenous gene can be introduced into medaka genome (transgenic medaka) and cells can be transplanted to host medaka to generate chimera medaka.

With these advantages, we have been generating transgenic medaka enabling us to identify the different cell lineages by fluorescence and to analyze the process of gonad formation and sex differentiation in vivo. Additionally, in order to identify the genes essential for gonadogenesis, we carried out a mutational screening of medaka with a defect in gonads and are performing a positional cloning. With these two unique analytical methods (visualising cells and mutants), we are attempting to unveil the fundamental mechanisms of sex differentiation common to many organisms.

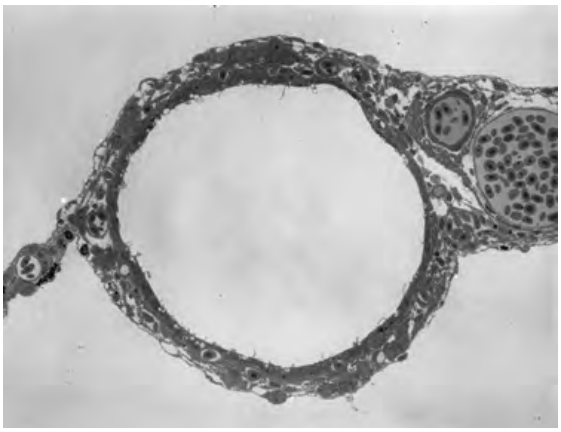

Figure 1. The germ cell-deficient gonad exhibits a single fundamental structure common to both ovary and testis. A large lumen is located in the middle and a single layer of supporting-like cells encloses the lumen, which is separated by a basement membrane from an outer stromal region.

Ⅰ. Germ cells are essential for sexual dimorphism of the gonads

The nature of somatic cells and germ cells is a topic of broad and long standing interest. Many studies have therefore explored the interaction between germ cells and gonadal somatic cells. The view resulting from the previous studies is that germ cells do not significantly affect the sex differentiation of gonads.

We generated medaka that completely lack germ cells in the gonad by impairment of PGC migration. The morphology of the gonad in the germ cell-less medaka exhibits a tube-like structure which is composed of a lumen in the middle surrounded by a single layer of inner cells separated by a basement membrane from an outer stromal layer (Figure 1). This structure is common to the unit that constitutes ovarian follicles and testicular tubules, indicating that germ cells are essential for organizing sex-specific structures in the gonads.

This medaka also exhibits another interesting phenotype, male secondary sex characteristics irrespective of the genetic sex. Novel *aromatase*-expressing theca cells that we identified in ovary did not develop properly in the germ cell-deficient gonads and female supporting cells that control gametogenesis began to express male-specific genes as development of the gonad proceeded in the absence of germ cells, suggesting trandifferentiation from female to male supporting cells. The cells producing male sex steroid hormone persisted in the germ cell-deficient medaka. All lines of the data indicated that the gonadal somatic cells are predisposed to adopting male development and the production of male steroid hormone is responsible for male secondary sex characteristics. Thus, we demonstrated that, contrary to accepted thinking, germ cells are essential for sexually dimorphic gonads (Kurokawa *et al*., 2007, PNAS *104*, 16958-16963).

Ⅱ. Successful identification of a mutated gene that causes male to female sex reversal (*hotei* mutant)

In collaboration with the SORST Kondoh team, we have been screening mutants affecting the development of primordial germ cells and the formation of gonads. The screening has been performed in such a way that particular attention is paid to the presence, the number and the distribution pattern of germ cells at a somitogenesis stage and at ten days post hatching (10 dph). Nine mutants (19 alleles) and twelve mutants (14 alleles) were identified for PGCs and gonads, respectively.

One mutant, *hotei*, is of particular interest because of the excessive number of germ cells that are arrested in the early development of follicle growth and because male to female sex reversal occurs irrespective of their genetic sex. As a result of the positional cloning, we have successfully identified a candidate gene as the gene for a type Ⅱ receptor of anti-Müllerian hormone (*amhrⅡ*). This result reveals that amhr Ⅱ, together with its ligand, anti-Müllerian hormone (amh), regulates the proliferation of germ cells. Both *amh* and *amhrⅡ* are expressed in the gonadal somatic cells. It is interesting to note that *amhrⅡ* is not detected in the absence

of germ cells, suggesting that a reciprocal cross talk between germ cells and gonadal somatic cells is present during the term of sex differentiation. These results also support our contention that the proliferation of germ cells is closely related to sex differentiation of the gonads. (Morinaga *et al*., 2007, PNAS *104*, 9691-9696).

Another mutant, *zenzai*, is a good contrast with the *hotei* mutant in that germ cells are not maintained in the gonad (Figure 2). Inheritance of the phenotype indicates that the allele is recessive. We again identified one possible candidate gene for the phenotype of *zenzai* mutant.

We are also characterizing other mutants in another category, namely the irregular distribution of germ cells in gonads. These mutants include, *hadare, mizore, hyou* and *arare*.

Figure 2. Blue staining shows PGCs in the gonad. Left: *zenzai* mutant that cannot maintain gerrn cells. Middle: wild type. Right: *hotei* mutant that shows overproliferation of germ cells.

Ⅲ. Modes of Germ Cell Proliferation are important components of gonadal sex differentiation

In order to investigate the mode of germ cell proliferation, a small number of germ cells are labeled by fluorescence and monitored in the developing gonads of living medaka embryos. These observations reveal that two modes of proliferation are taking place, one type (type Ⅰ) with intermittent division found in both male and female developing gonads, and another type (type Ⅱ) with successive and synchronous division, leading to cystic germ cells and subsequent meiosis and oogenesis, which is only detected in the female (Figure 3).

Since type Ⅰ-proliferation is impaired in *zenzai* mutants, resulting in the depletion of germ cells, type Ⅰ is responsible for the maintenance of germ cells and type Ⅱ is the mode of germ cell proliferation which commits to gametogenesis. Our results also demonstrate that transition from type Ⅰ to type Ⅱ is regulated in a sex-dependent manner (Saito *et al*., 2007, Dev. Biol. *310*, 280-290).

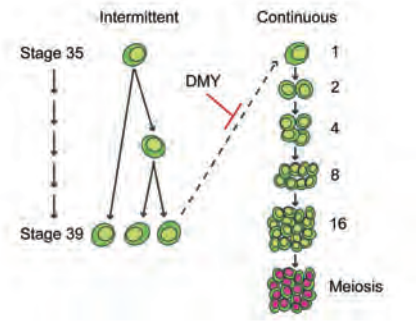

Figure 3. Two types of germ cell proliferation are important components of sexual dimorphism of medaka developing gonads.

Ⅳ. Generation of transgenic medaka to identify the cell lineages that constitute the gonads

To clarify the cell types that constitute the gonad, we are generating transgenic fish to visualize the cell lineages. We have established several lines of transgenic fish that allow us to analyze how they build up the gonad during the course of development. Crossing the transgenic fish with different colors enabled us to successfully reconstitute novel units of structure that have not been reported yet in the gonad.

An attempt to monitor the process of development of each lineage has also been made in living embryos and larva using timelapse movies. In order to solve the difficulties in visualizing the cells located in deep positions in the embryos and larva, confocal microscopy and SPIM have been applied to the transgenic embryos and larva. This attempt is still in progress in collaboration with EMBL's Jochen Wittbrodt Lab.

Publication List

〔Original papers〕

- Hano, T., Oshima, Y., Kinoshita, M., Tanaka, M., Mishima, N., Ohyama, T., Yanagawa, T., Wakamatsu, Y., Ozato, K., and Honjo, T. (2007). Quantitative bioimaging analysis of gonads in olvas-GFP/ST-II YI medaka (transgenic *Oryzias latipes*) exposed to ethinylestradiol. Environ. Sci. Tech. *41*, 1473-1479.
- Kurokawa, H., Saito, D., Nakamura, S., Katoh-Fukui, Y., Ohta, K., Aoki, Y., Baba, T., Morohashi, K., and Tanaka, M. (2007). Germ cells are essential for sexual dimorphism in the medaka gonad. Proc. Natl. Acad. Sci. USA *104*, 16958-16963.
- Morinaga, C., Saito, D., Nakamura, S., Sasaki, T., Asakawa, S., Shimizu, N., Mitani, H., Furutani-Seiki, M., Tanaka, M (Corresponding Author), and Kondoh, H. (2007). The *hotei* mutation of medaka in the anti-Mullerian hormone receptor causes the dysregulation of germ cell and sexual development. Proc. Natl. Acad. Sci. USA *104*, 9691-9696.
- Saito, D., Morinaga, C., Aoki, Y., Nakamura, S., Mitani, H., Furutani-Seiki, M., Kondoh, H., and Tanaka, M. (2007). Proliferation of germ cells during gonadal sex differentiation in medaka: insights from germ cell depleted mutant *zenzai*. Dev. Biol. *310*, 280-290.
- Takamatsu, N., Kurosawa, G., Takahashi, M., Inokuma, R., Tanaka, M., Kanamori, A., and Hori, H. (2007). Duplicated Abd-B class genes in medaka hoxAa and hoxAb clusters exhibit different expression patterns in pectoral fin buds. Dev. Genes Evol. *217*, 263-273.