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 use medaka fish (*Oryzias latipes*) for these purposes and have been generating transgenic medaka (Figure 1) enabling us to identify 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 defective gonads and are performing a positional cloning. With these two unique analytical methods (visualizing cells and mutants), we are attempting to unveil the fundamental mechanisms of sex differentiation and plasticity common to many organisms.

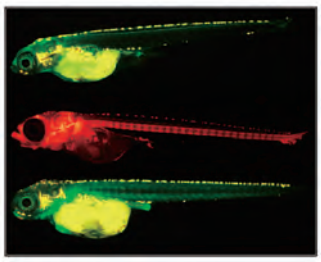

Figure 1. Various transgenic medaka.

I. Canalization of germ cells to feminization vs. male predisposition of somatic cells

We have been analyzing the role of germ cells during the course of sex differentiation of the gonads and have revealed an essential function of germ cells on the development of ovaries.

In wild type medaka, sex is determined genetically in an XY manner. Without germ cells, however, both genetically female and male medaka exhibit male secondary sex characteristics and gonadal somatic cells are masculinized in terms of male-specific gene expression and production of a male steroid hormone. This can be explained by the hypothesis that the female character of germ cells antagonizes the autonomous masculinization of gonadal somatic cells and that balancing between germ cells and gonadal somatic cells is essential for both sex differentiation and maintenance of sex.

In support of this claim, medaka mutants that display a germ cell-hypertrophic phenotype, *hotei*, exhibit female secondary sex characteristics in approximately half of genetic male *hotei* mutants. In the germ cell-deficient *hotei* mutants, none of them show any sex reversal of secondary sex characteristics. In addition, female-specific gene expression seen in genetic male *hotei* mutants is also abolished in the germ cell-depleted mutants. These results indicate that feminization of the gonad in the mutant is a consequence of hypertrophic germ cells but not of a gonadal somatic cell-autonomous event.

The gene responsible for this phenotype is the type II receptor gene for anti-Mullerian homone (*amhrII*). We found that both *amhrII* and its ligand, *amh*, are expressed in gonadal somatic cells (supporting cells) but not in germ cells, indicating that Amh signaling acts autonomously of supporting cells.

II. Amh signaling is involved in sex differentiation by modulating reciprocal interaction between germ cells and somatic cells

The results from both germ cell-deficient medaka and germ cell-hypertrophic mutants (*hotei*) suggest an intrinsic mechanism of sex differentiation that is independent of genetic sex determination. We propose that, irrespective of genetic sex, germ cells have an intrinsic character that canalizes feminization of the gonad (*canalization to female*) while gonadal somatic cells are predisposed to male development (*male predisposition*). According to this proposal, the possible function of the medaka testis determination gene on the Y chromosome is enhancement of masculinization that conquers canalization towards ovary by germ cells. The close interaction has to be present, thereby reciprocal signals between germ cells and somatic cells acting to form proper sex differentiation. Since the complete defect of Amh signaling results in improper sex differentiation, Amh signaling is very likely to modulate the reciprocal signals and balance the two intrinsic signals.

The balancing of the two opposing characters may be a conserved cellular interaction among vertebrates. In fact, there have been several reports of the masculinization of mice ovaries following the depletion of germ cells by genetic or physical manipulations. Even in an undifferentiated gonocholist, zebrafish, which firstly develops ovaries before some of the population turn into males, prior to the development of testis the germ cell number decreases by apoptosis. The suppression of the germ cell number at the early stage of medaka gonadal sex differentiation and the decrease in germ cells in zebrafish can be viewed as different ways of achieving the same result (Tanaka et al., 2008 DGD: Saito et al., Sex. Dev. 2009).

III. Estrogen-producing cells – the earliest female-specific somatic cells develop in a germ cell-dependent manner

As mentioned above, germ cells and the surrounding somatic cells (supporting cells) are important players in the gonads. But there are many other functionally unknown somatic cells in the gonads. Transgenic medaka with GFP fluorescence are powerful tools to analyze them because they provide fine structure and organization of the gonad at single cell levels. We have generated transgenic medaka that enable us to keep track of *cyp19a1* (*aromtatase*)-expressing cells in

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2009. The former title is indicated by an asterisk (*).

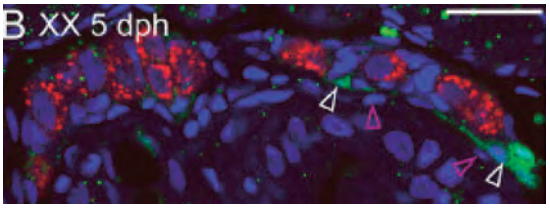

Figure 2. The *cyp19a1*-expressing theca cells (green) are one of the earliest female-specific cells in the female developing gonad. blue: nuclea, red: germ cells. Picture from Nakamura et al (2009).

the gonad by the established method of using medaka BAC (bacterial artificial chromosome) (Nakamura et al., 2008 DGD). The *cyp19a1*-GFP transgenic medaka mimics endogenous *cyp19a1* expression by GFP fluorescence (Nakamura et al., 2009).

Once germ cells enter meiosis, they form the follicular structure with both inner and outer somatic cells surrounding oocytes. The aromatase (product of the gene, *cyp19a1*), which catalyzes a precursor steroid, testosterone, to produce female-specific hormone, estrogen, has generally been used as a good marker of the inner somatic cells called granulosa cells.

The expression of *cyp19a1* in medaka has been observed as early as 5 days post hatching (5dph) in female developing gonads (Figure 2) but not male developing gonads. The *cyp19a1*-expressing cells first appear in the most ventral side of a stromal region adjacent to the epithelium of the female gonad. Immediate after appearance of the *cyp19a1*-expressing cells they were found on the outer layer of follicles but not on the inner layer. This result indicates that the *cyp19a1*-expressing cells are not the granulose cells but theca cells. Very interestingly, another theca cell marker, *P450c17*, is exclusive to *cyp19a1* expression, demonstrating that there are two different types of theca cells on the follicles. We often observe that *cyp19a1*-expressing cells on the ventral stromal region extend their cellular process towards follicles. This observation suggests that the *cyp19a1*-expressing cells in the ventral region are the precursors of one of the theca cells (Figure 3).

Consistent with our result, in the absence of germ cells, female-specific *cyp19a1*-expressing cells are not maintained in the developing ovary (Nakamura et al 2009).

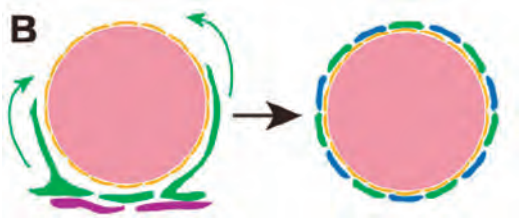

Figure 3. Development of *cyp19a1*-expressing theca cells (green). These cells are different from another type of theca cell expressing *P450c17* (blue). The *cyp19a1*-expressing theca cells first arise near the epithelium (purple) and surround oocytes (pink) cells. Illustration from Nakamura et al (2009)

IV. Expression and syntenic analysis of medaka *nanos* gene family

Nanos is known to be essential for germ cell development. Availability of medaka genomic information allows us to successfully identify four different *nanos* genes on the medaka genome (*nanos 1a, 1b*, *2,* and *3*). *Nanos3* is most conserved among vertebrates at the levels of amino acid sequence and synteny. *Nanos1a* and *1b* are expressed in neuronal cells in common, *nanos2* in gonial cells and *nanos3* specifically in germ cell-lineage (Aoki et al., 2009)

Publication List

〔Original papers〕

- Aoki, Y., Nakamura, S., Ishikawa, Y., and Tanaka, M. (2009). Expresssion and syntenic analyses of four *nanos* genes in medaka. Zool. Sci. *26*, 112-118.
- Hano, T., Oshima, T., Kinoshita, M., Tanaka, M., Wakamatsu, Y., Ozato, K., Nassef, M., Shimazaki, Y., and Honjo, T. (2009). In ovo nanoinjection of nonylphenol affects embryonic development of a transgenic see-through medaka (*Oryzias latipes*), *olvas-GFP*/STII-YI strain. Chemosphere *77*, 1594–1599.
- Herpin, A., Nakamura, S., Wagner, T., Tanaka, M., and Schartl, M. (2009). A highly conserved *cis*-regulatory motif directs differential gonadal synexpression of *Dmrt1* transcripts during gonad development. Nucleic Acids Res. *35*, 1510–1520.
- Nakamura, S., Kurokawa, H., Asakawa, S., Shimizu, N., and Tanaka, M. (2009). Two distinct types of theca cells in the medaka gonad: Germ cell-dependent maintenance of *cyp19a1*-expressing cells. Dev. Dyn. *238*, 2652-2657.

〔Review articles〕

- Kinoshita, M., Murata, K., Naruse, K., and Tanaka, M. (eds) (2009). Medaka – Biology, Management, and Experimental Protocols. 419 pages, Iowa, USA. Wiley-Blackwell. ISBN:978-0-813-818511.
- Saito, D., and Tanaka, M. (2009). Comparative aspects of gonadal differentiation in medaka: a conserved role of developing oocytes in sexual canalization. Sex. Dev. *3*, 99-107.