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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Analysis of a novel gene, *Sdgc*, reveals sex chromosome-dependent differences of medaka germ cells prior to gonad formation

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ABSTRACT

In vertebrates that have been examined to date, the sexual identity of germ cells is determined by the sex of gonadal somatic cells. In the teleost fish medaka, a sex-determination gene on the Y chromosome, *DMY*/*dmrt1bY*, is expressed in gonadal somatic cells and regulates the sexual identity of germ cells. Here, we report a novel mechanism by which sex chromosomes cell-autonomously confer sexually different characters upon germ cells prior to gonad formation in a genetically sex-determined species. We have identified a novel gene, *Sdgc* (sex chromosome-dependent differential expression in germ cells), whose transcripts are highly enriched in early XY germ cells. Chimeric analysis revealed that sexually different expression of *Sdgc* is controlled in a germ cell-autonomous manner by the number of Y chromosomes. Unexpectedly, *DMY*/*dmrt1bY* was expressed in germ cells prior to gonad formation, but knockdown and overexpression of *DMY*/*dmrt1bY* did not affect *Sdgc* expression. We also found that XX and XY germ cells isolated before the onset of *DMY*/*dmrt1bY* expression in gonadal somatic cells behaved differently *in vitro* and were affected by *Sdgc*. *Sdgc* maps close to the sex-determination locus, and recombination around the two loci appears to be repressed. Our results provide important insights into the acquisition and plasticity of sexual differences at the cellular level even prior to the developmental stage of sex determination.

KEY WORDS: Sexual identity, Germ cell, Sex chromosome, Medaka

INTRODUCTION

In vertebrates, the master sex-determination genes identified so far are expressed in the gonadal somatic cells (supporting cells) (Hattori et al., 2012; Kamiya et al., 2012; Koopman et al., 1991; Matsuda et al., 2002; Smith et al., 2009; Yoshimoto et al., 2010). Therefore, it is generally accepted that sex determination first occurs in the gonadal somatic cells during development. Following this step, under the influence of the somatic cells, the sex of germ cells is determined, and these cells are then fated to develop into either sperm or eggs.

In mice, when germ cells are isolated from XY gonads at 11.5 days post-coitum (dpc) and reaggregated with XX somatic cells from urogenital ridges, the XY germ cells behave like XX female germ cells and enter meiosis. By contrast, germ cells from XY gonads at 12.5 dpc mitotically arrest as prospermatogonia in response to the same treatment (Adams and McLaren, 2002; McLaren, 2000). This observation indicates that by 12.5 dpc, germline sex is largely determined by the action of the sex-determination gene *Sry* in the gonadal somatic cells.

Somatic cells also exert a strong influence on germ cell sex determination in teleost fish, as demonstrated by the reciprocal transplantation of testicular and ovarian germ cells. In rainbow trout and zebrafish, isolated oogonia and spermatogonia from mature gonads can recolonize the undifferentiated gonads of embryos and produce either sperm or eggs, depending on the sex of the surrounding somatic cells (Okutsu et al., 2006; Wong et al., 2011; Yoshizaki et al., 2010).

In medaka (*Oryzias latipes*), expression of the sex-determination gene on the Y chromosome, *DMY*/*dmrt1bY* (Matsuda et al., 2002; Nanda et al., 2002), has so far been reported to begin in gonadal somatic cells immediately after the gonadal primordium forms at stage 33 (st.33); it suppresses the shift of germ-cell proliferation modes from a self-renewal type of division (type I division) towards a gametogenesis-committed cystic type of division (type II division) at st.35. The shift from type I to type II division is crucial for ovary formation (Nakamura et al., 2012a,b; Saito et al., 2007; Tanaka et al., 2008).

Taken together, the evidence enumerated above indicates that, in vertebrates, the gonadal somatic cells control the sex of germ cells and determine whether they develop into spermatids or oocytes. In this study, we found that medaka germ cells exhibit sexually different characters even before the formation of the gonadal primordium. We found that the differences at this early stage are controlled in a germ cell-autonomous manner by the number of Y chromosomes, but not by the expression of the sex-determination gene protein DMY/dmrt1bY. Thus, our findings reveal a novel mechanism by which sexually different characters arise at the cellular level.

RESULTS

Germ cell-specific expression of a novel transcript: *Sdgc*

To study gene expression in germ cells and gonadal somatic cells during gonadal sex differentiation, we used a *sox9b*-DsRed/*olvas*-

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EGFP transgenic medaka (Nakamura et al., 2010), and isolated EGFP-positive germ cells and DsRed-positive supporting cells from XX and XY embryos by fluorescence-activated cell sorting (FACS) at st.33 and st.35. Microarray expression profiling revealed that several transcripts exhibited sexually different expression levels in germ cells by st.33. Among them, a novel transcript, *olte54h09* (NCBI accession: FS527139; named *Sdgc* after its function, sex chromosome-dependent differential expression in germ cells), was highly enriched in XY germ cells relative to XX germ cells (supplementary material Fig. S1A,B). Quantitative PCR (qPCR) analysis of *Sdgc* and other gonadal markers confirmed that *Sdgc* was already upregulated in XY embryos by st.33 (supplementary material Fig. S1C). This observation raised the possibility that germ cells exhibit sexually different gene expression even before the expression of *DMY*/*dmrt1bY* begins in gonadal somatic cells (supplementary material Fig. S1D).

To confirm this possibility, we performed qPCR and *in situ* hybridization for *Sdgc* in st.30 embryos. At st.30, the gonad is not yet formed, and the germ cells are located lateral to the forming hindgut; from this position, they subsequently move towards the prospective gonadal area on the dorsal side (Nakamura et al., 2006). *Sdgc* expression was detected as clusters on the lateral sides, and was stronger in XY embryos than in XX embryos (Fig. 1A-D). The expression levels of *Sdgc* were more than twofold higher in XY embryos than in XX embryos (Fig. 1I). When endogenous germ cells were removed by inhibiting germ-cell migration using *nanos3*/*cxcr4*-morpholinos (Kurokawa et al., 2006, 2007), the *Sdgc* signal totally disappeared (Fig. 1E-I). Together with microarray data showing higher enrichment in isolated germ cells, these observations indicate that *Sdgc* is expressed in germ cells.

The *Sdgc* transcript encodes a putative protein of 142 amino acids (supplementary material Fig. S2). Eleven repeats of QGPPAQ(E)GR constitute the majority of the protein, and no known domains were found in the sequence. The 5′UTR was found on three different scaffolds, but none of those scaffolds had yet been mapped to medaka linkage groups (LG). When we performed qPCR analysis of *Sdgc* in a different strain, Hd-rR-II1, expression was also upregulated in XY embryos (supplementary material Fig. S3). Therefore, upregulation of *Sdgc* in XY germ cells is not specific to the OKcab strain.

We then examined *Sdgc* expression in adult testes and ovaries. *Sdgc* was highly enriched in testes when compared with ovaries (supplementary material Fig. S4A). In testes, the signal was more abundant in type B spermatogonia than in type A spermatogonia, but was absent in spermatocytes (supplementary material Fig. S4B,C). In ovaries, the transcripts were present only at extremely low levels in early stage oocytes (supplementary material Fig. S4D). No expression was detected in oogonia (supplementary material Fig. S4E), consistent with the sexually different expression in germ cells at the early embryonic stages.

Expression of *Sdgc* depends on Y chromosome number in germ cells

Next, we examined the expression levels of *Sdgc* using medaka with different Y-chromosome constitution and found that *Sdgc* expression levels increased with the copy number of the Y chromosome (Fig. 1J). We then asked whether the upregulation of *Sdgc* was due to the presence of a Y chromosome in germ cells or in somatic cells. To address this issue, we generated chimeric medaka by transplanting XY germ cells into XX hosts and vice versa (Nakamura et al., 2012b), using *sox9b*-DsRed/*olvas*-EGFP transgenic line as hosts and non-transgenic strains as donors. Prior to manipulation, the host germ cells with EGFP fluorescence were ablated by *nanos3*/*cxcr4*-morpholino injection. *Sdgc* expression levels in XY hosts with XX germ cells resembled that of XX hosts with XX germ cells. By contrast, the expression of *Sdgc* in both XX and XY hosts with XY germ cells was more than twofold higher than in hosts with XX germ cells (Fig. 1K). The sex of the host did not correlate with any statistically significant difference in the *Sdgc* expression level in germ cells. These results clearly indicate that upregulation of *Sdgc* depends on the presence of a Y chromosome in germ cells, but is independent of the genetic sex of

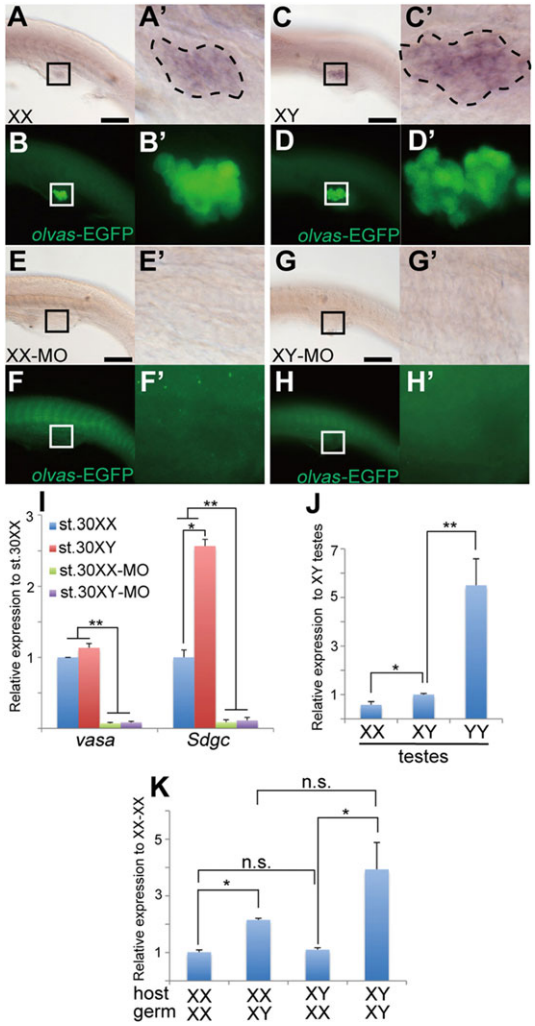

Fig. 1. *Sdgc* is expressed specifically in germ cells and upregulated depending on Y chromosome number in germ cells. (A-D′) Expression analysis of *Sdgc* by *in situ* hybridization and immunohistochemistry with anti-EGFP using *olvas*-EGFP transgenic embryos at st.30. Purple signals (A′ and C′, dotted lines) are observed in clusters of germ cells (B′ and D′, *olvas*-EGFP: green) lateral to developing hindgut. (E-H′) When germ cells were ablated by morpholinos, the *Sdgc* signal disappears (E,G) along with the germ cell marker (F,H). Scale bars: 100 µm. (I) qPCR analysis of *Sdgc* using embryos with and without (MO) germ cells at st.30. The *y*-axis indicates the expression levels relative to those observed in st.30 XX control embryos. The expression levels were normalized to β-*actin* (n=2). (J) *Sdgc* expression levels increased in parallel with the number of Y chromosomes in testes. The *y*-axis indicates the expression levels of *Sdgc* in XX and YY testes relative to those observed in XY testes; signals were normalized to *olvas* (n=3). (K) qPCR analysis of chimeric embryos at st.30. The *y*-axis indicates the expression levels relative to those observed in XX embryos with XX germ cells (n=2). The expression levels of *Sdgc* are not affected significantly by the sex of the host. *P<0.05, **P<0.01, Student's *t*-test. Values are expressed as mean±s.e.m.

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the surrounding somatic cells, suggesting that sexually different expression occurs as a germ cell-autonomous event.

DMY/*dmrt1bY*-independent expression of *Sdgc* in germ cells

The only differences identified to date in the two medaka sex chromosomes reside at the sex-determination locus, leading us to speculate that *DMY*/*dmrt1bY* may upregulate the *Sdgc* gene in XY germ cells in a germ cell-autonomous manner. Interestingly, *DMY*/*dmrt1bY* transcripts were detected in XY germ cells at both st.33 and st.35 by microarray (supplementary material Fig. S1D). The germ-cell expression of *DMY*/*dmrt1bY* was further confirmed by *in situ* hybridization in st.30 embryos. The *DMY*/*dmrt1bY* signal was also detected, only in XY embryos, as single clusters on both lateral sides of the forming hindgut, where it was colocalized with expression of OLVAS, a marker of germ cells (Fig. 2A-C). When endogenous germ cells were removed by morpholino treatment, the *DMY*/*dmrt1bY* signal disappeared (Fig. 2D). To further investigate *DMY*/*dmrt1bY* expression in germ cells, we generated a *DMY*/*dmrt1bY*-EGFP reporter transgenic medaka, in which *DMY*/*dmrt1bY* expression could be visualized by EGFP driven by regulatory elements of *DMY*/*dmrt1bY*. *DMY*/*dmrt1bY* reporter expression in germ cells was first detected as early as st.28 and persisted until around st.31 (supplementary material Fig. S5A-C). Together, these data indicate that *DMY*/*dmrt1bY* is expressed in germ cells before formation of the gonadal primordium.

To determine whether *Sdgc* is directly regulated by DMY/dmrt1bY protein, we knocked down *DMY*/*dmrt1bY* using synthetic antisense oligonucleotides (gripNA). Specifically, we used a *dmy*-gripNA described previously (Paul-Prasanth et al., 2006), for which the specificity had already been confirmed by *in vitro* translational suppression and *in vivo* analysis. We also confirmed that this gripNA effectively decreased green fluorescence in embryos of *DMY*/*dmrt1bY*-EGFP reporter transgenic medaka (Fig. 2E,F). Although the reporter fluorescence was dramatically reduced, *Sdgc* expression levels did not change in gripNA-injected embryos relative to normal embryos at st.30 (Fig. 2G). Overexpression of *DMY*/*dmrt1bY* also had no effect on the expression level of *Sdgc* (supplementary material Fig. S6A,B). These results demonstrate that upregulation of *Sdgc* is not due to a direct effect of DMY/dmrt1bY protein expression, but rather is crucially dependent on the presence of a Y chromosome in germ cells.

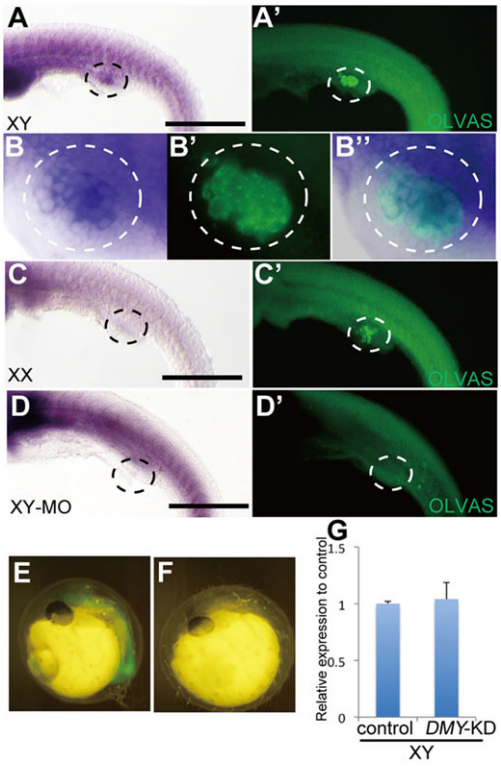

Fig. 2. *DMY*/*dmrt1bY* is expressed in germ cells before the formation of the gonadal primordium. (A-D′) Lateral views of embryos at st.30. The anterior region is on the left. (A,A′) *In situ* hybridization for *DMY*/*dmrt1bY* and immunohistochemistry to detect OLVAS expression using XY embryos at st.30. *DMY*/*dmrt1bY* signal (purple; dotted line) is detected at the lateral sides of the intestine where germ cells are located at st.30. (B-B′) The *DMY*/*dmrt1bY* signal colocalizes with the germ cells marker OLVAS (green; dotted line). (C,C′) The *DMY*/*dmrt1bY* signal is not detected in the OLVAS-positive germ cells (green; dotted line) of XX embryos. (D,D′) When germ cells are ablated by the morpholinos (XY-MO), the *DMY*/*dmrt1bY* signal disappears. Scale bars: 200 µm. (E,F) Injection of gripNA targeting *DMY*/*dmrt1bY* (*dmy*-gripNA) into *DMY*/*dmrt1bY*-EGFP embryos. (E) Uninjected controls show EGFP fluorescence in a whole body (EGFP-positive: *n*=27, EGFP-negative: *n*=0). (F) EGFP fluorescence level is reduced by *dmy*-gripNA injection (EGFP positive, *n*=3; EGFP negative, *n*=31). (G) qPCR analysis of XY embryos injected with *dmy*-gripNA (*DMY*-KD, *n*=3). The expression level of *Sdgc* does not differ between *dmy*-gripNA injected embryos and control embryos. Values are expressed as mean±s.e.m.

Sdgc is mapped closely to the *DMY*/*dmrt1bY* locus on the sex chromosomes

We suspected that upregulation of *Sdgc* may have been due to a conformational difference or presence of single-nucleotide polymorphisms (SNPs) between the X and Y chromosomes. If this is true, then *Sdgc* should be located on the sex chromosomes. To test this possibility, we mapped *Sdgc* using the M-markers (Kimura et al., 2012) (supplementary material Fig. S7 and Table S1). We found that both *Sdgc* and a sex-linked marker (*SL1*) flanking *DMY*/*dmrt1bY* (Matsuda et al., 2002; Nanda et al., 2002) are located between the *MID0122* and *MID0123* linkage markers (Fig. 3). Thus, *Sdgc* maps near the region where *DMY*/*dmrt1bY* resides on the sex chromosomes (LG1). Interestingly, in the Kaga strain, 11 SNPs found in the intronic region of *Sdgc* were heterozygous only in XY specimens (supplementary material Fig. S7), further supporting the idea that the gene is located near *DMY*/*dmrt1bY*, where homologous recombination should occur less frequently. Because *Sdgc* expression was detected in XX germ cells, we reasoned that *Sdgc* must be present on both the X and Y chromosomes, but that the two alleles have different expression.

A cell-autonomous and *DMY*/*dmrt1bY*-independent sex difference in the mitotic activity of isolated germ cells

Finally, we investigated whether germ cells behaved differently between the two sexes, using an *in vitro* culture system. First, germ cells dissociated from embryonic tissue at st.30 were plated onto culture dishes together with dissociated somatic cells. Then, the mitotic activity of germ cells was examined in the two different media by monitoring incorporation of EdU (supplementary material Fig. S8A and Table S2). Under both culture conditions, the rate of EdU incorporation in XY germ cells was higher than in XX germ cells (supplementary material Fig. S8C,D). To exclude the possibility that the sex of the dissociated somatic cells might affect the mitotic activity of germ cells, we isolated XX or XY germ cells at st.30 by FACS and cultured them on XX or XY feeder cells, respectively, derived from somatic cells of the tail region at st.30.

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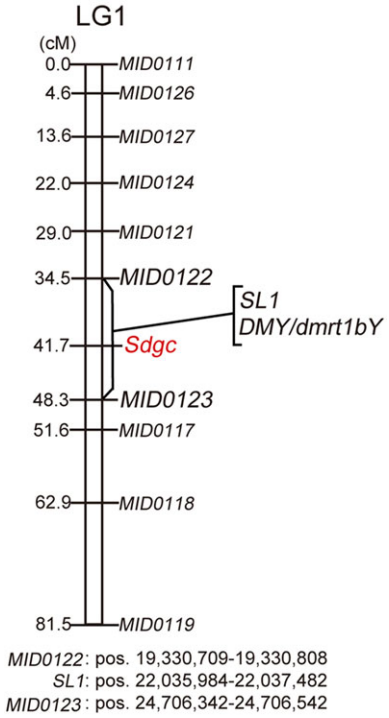

Fig. 3. Linkage analysis of *Sdgc*. *Sdgc* was mapped between *MID0122* and *MID0123* on LG1, which are the sex chromosomes (X and Y) in medaka. The physical map revealed that the sex-linked marker *SL1*, which closely maps to *DMY*/*dmrt1bY*, is also located between the same *MID* markers [see the position (pos.) below]. Thus, *Sdgc* resides near the *DMY*/*dmrt1bY* locus on the sex chromosomes. *DMY*/*dmrt1bY* and *SL1* are not used in the present linkage analysis, and the order of *Sdgc*, *SL1* and *DMY*/*dmrt1bY* is not determined.

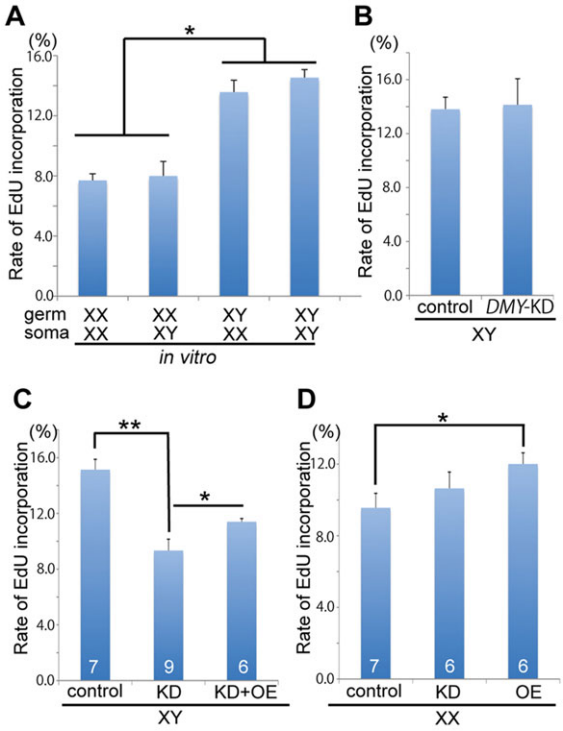

Fig. 4. Sexually different mitotic activity observed in cultured germ cells. (A) An *in vitro* analysis of EdU-positive germ cells reveals that XY germ cells incorporate EdU at a higher rate when compared with XX germ cells, irrespective of the sex of the somatic cells (Germ-Soma; XX-XX versus XY-XX, P=0.00286; XX-XY versus XY-XX, P=0.00404; XX-XX versus XX-XY, P=0.794, XY-XX versus XY-XY; P=0.369, Student's t-test, n=3). (B) Knockdown of *DMY*/*dmrt1bY* by injection of 0.5 mM *dmy*-gripNA (*DMY*-KD) does not affect the mitotic activity of germ cells *in vitro*. As a control, 0.1 mM human-CREB gripNA was injected (n=4). (C,D) Knockdown of *Sdgc* by 0.25 mM *Sdgc*-gripNA (KD) significantly decreased EdU incorporation in XY germ cells (C) but not in XX germ cells (D). Overexpression of *Sdgc*, achieved by injecting 200 ng/µl mRNA of *FLAG*:*Sdgc*:*2A*:*mCherry*:*olvas*-3′UTR (the expression of *Sdgc* can be localized in germ cells, OE) in XX germ cells (D), and the rescue experiment (KD+OE), achieved by injecting both *Sdgc*-gripNA and the mRNA in XY germ cells (C), significantly increased the EdU incorporation. Each number in the bar indicates the number of culture dishes examined. Student's t-test, *P<0.05, **P<0.01. All values (%) are expressed as mean±s.e.m.

After 24 h in culture, EdU treatment was performed for an additional 24 h to observe the mitotic activity of the germ cells (supplementary material Fig. S8B). Again, the rate of EdU incorporation in XY germ cells was significantly higher than in XX germ cells, and this difference was not dependent on the sex of the somatic cells (Fig. 4A). This observation suggests that an intrinsic sexual difference within germ cells contributes to the difference in germ-cell mitotic activity.

To examine the relationship between cell-autonomous gene expression and sexually different mitotic activity, we knocked down *DMY*/*dmrt1bY* and *Sdgc* and performed culture experiments on the resultant cells. *DMY*/*dmrt1bY* knockdown had no effect on the mitotic activity of XY germ cells (Fig. 4B), whereas knockdown of *Sdgc* significantly reduced mitotic activity (Fig. 4C), an effect that could be rescued by overexpression of *Sdgc* (Fig. 4C). Knockdown of *Sdgc* had no effect on XX germ cells (Fig. 4D), but *Sdgc* overexpression increased the mitotic activity of XX germ cells (Fig. 4D). Collectively, these results suggest that *Sdgc* confers higher mitotic activity upon XY germ cells relative to XX germ cells *in vitro*. This observation further supports the idea that the cell-autonomous acquisition of sexually different characters in germ cells is independent of DMY/dmrt1bY protein expression.

DISCUSSION

In this study, we have examined the gene expression and behavior of germ cells before gonadal primordium formation in medaka. We found that *Sdgc* and the sex-determination gene *DMY*/*dmrt1bY* were expressed in germ cells at this early stage. However, although *Sdgc* expression was upregulated only when a Y chromosome is present in germ cells, it was unaffected by *DMY*/*dmrt1bY* knockdown or overexpression. This sexually different expression was induced in a germ cell-autonomous manner. In addition, early XX and XY germ cells exhibited different mitotic activities *in vitro*. Together, these data show that XX and XY germ cells possess different characters even before gonadal primordium formation and the onset of sex determination by gonadal somatic cells.

The master male sex-determining gene evolved relatively recently (5-10 million years ago) from a duplicated copy of *dmrt1* (Kondo et al., 2003; Matsuda et al., 2003). This evolutionary process was associated with the insertion of a transposable element called *Izanagi* that confers a novel expression pattern upon *DMY*/*dmrt1bY* (Herpin et al., 2010). Unexpectedly, in our study, sexually different characters of germ cells were not impaired by knockdown and overexpression of *DMY*/*dmrt1bY*. Instead, upregulation of *Sdgc* and increased mitotic activity of germ cells *in vitro* were dependent on the presence of the Y chromosome in germ cells. In addition, *Sdgc* mapped close to *DMY*/*dmrt1bY* on sex chromosomes and produced higher mitotic activity in XY germ cells than in XX germ cells *in vitro*. Although the possibility that the transcriptional level

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of *DMY*/*dmrt1bY* affects *Sdgc* expression cannot be completely ruled out, our findings suggest two possible models for inducing cell-autonomous, sexually different characters of germ cells.

First, the sexually different characters may be caused at the chromosomal level. Previous studies (Kondo et al., 2004, 2006; Takeda, 2008) have shown that the male sex-determining gene *DMY*/*dmrt1bY* was inserted into one homologue of a chromosome pair that became the X and Y. Outside the inserted fragment and where *Sdgc* is located, X and Y are largely homologous and have the same gene content. Thus, *Sdgc* was most likely already on this chromosome pair, and both copies might have been expressed at the same level prior to the insertion event. It is likely that insertion of *DMY*/*dmrt1bY* and/or subsequent changes are related to restriction of recombination near the *Sdgc* locus (Kondo et al., 2001; Takeda, 2008), resulting in accumulation of DNA fragments such as repetitive sequences. Recent analyses suggest that these elements affect the structural change of chromatins through a small RNA-related mechanism and/or epigenetic regulation (e.g. Slotkin and Martienssen, 2007). The structural changes caused by these fragments may have facilitated and maintained the expression difference between the X and Y copies, leading to sexually different characters of germ cells. As for development of *Sdgc*, however, we cannot exclude the possibility that a high-expression version of *Sdgc* was inserted near *DMY*/*dmrt1bY*, and that this linkage has been conserved.

Second, infrequent recombination may result in single-nucleotide polymorphism (SNP) accumulation in the intergenic or non-coding region near *Sdgc* and *DMY*/*dmrt1bY* locus. This effect could be due to the possibility of either genetic hitchhiking around the *DMY/dmrt1bY* locus or the *DMY/dmrt1bY* insertion process itself. Both cases can cause sex-specific differences in the X and Y chromosomsal regions around the insertion site and consequently in different transcriptional activity between the X and Y chromosome copies of *Sdgc*. Consistent with this, we identified sex-specific SNPs around *Sdgc*. Importantly, in either model, structural changes of the chromosome or different SNPs, *Sdgc* could potentiate sexually different cell behavior, as evidenced by the reduction of mitotic activity of XY germ cells by *Sdgc* knockdown *in vitro*.

Regarding the events that occur differently on the X and Y chromosome prior to gonad formation in vertebrates, X-chromosome reactivation is initiated during the development of primordial germ cells in mice (Sugimoto and Abe, 2007). However, it is still not known whether this reactivation occurs in a germ cell-autonomous manner or instead is affected by surrounding somatic cells. Our chimeric analysis provides good evidence that germ cells in medaka exhibit cell-autonomous, sexually different characters that are independent of somatic cells.

No obvious effect of *Sdgc* knockdown on germ cells was observed during late developmental stages of sex differentiation *in vivo*. One possibility is that mitotic activity of germ cells is predominantly regulated by somatic cells at that stage, thus cell-autonomous effects may be overridden. It is also possible that the *Sdgc*-gripNA, which was injected at the one- or two-cell stage, is not sufficiently active by the sex differentiation stage, which occurs late during development. Further investigation will be necessary to resolve this issue.

Knockdown of *DMY*/*dmrt1bY* did not affect expression of *Sdgc*. *DMY*/*dmrt1bY* expression was also detected in the *Sdgc*-knockdown XY embryos at the onset of sex determination by somatic cells (supplementary material Fig. S9A,B). Thus, given that *DMY*/*dmrt1bY* functions as a protein, *Sdgc* seems to function in a pathway independent of *DMY*/*dmrt1bY*.

Our findings provide insight into the relationship between sex chromosome differentiation and sexual plasticity at the cellular level. Many studies indicate that the medaka (*Oryzias latipes*) sex chromosome arose relatively recently (Herpin et al., 2010; Kondo et al., 2004; Myosho et al., 2012; Takehana et al., 2007) and is not as differentiated as in mammals, in which sexual plasticity is much reduced in germ cells (e.g. Lavery et al., 2011; Koopman et al., 1991). However, our results indicate that the newly arisen sex chromosomes can confer cell-autonomous, sexually different characters upon early stage germ cells. These results may indicate that the medaka sex chromosomes are on the evolutionary path to developing genes that influence sexually different characters of germ cells. These results also indicate that even in medaka, in which sex is determined by a specific gene, additional cell-autonomous mechanisms may affect sexual plasticity.

MATERIALS AND METHODS

Animals

All the treatments of animals in this research followed the guideline of National Institute for Basic Biology and were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences. The OKcab strain and *sox9b*-DsRed/*olvas*-EGFP transgenic medaka (Nakamura et al., 2010) were used in this study. To produce all XX progeny, XX males, which had been treated with 100 ng/ml 11-ketotestosterone during embryonic development, were crossed with XX females. To produce all XY progeny, first, XY females were generated by treatment with 200 ng/ml estradiol and were crossed with XY males. As a consequence, ~25% of the progeny were YY males, which were subsequently crossed with XX females to generate all XY progeny. All XX and XY progenies were used for the isolation of germ and somatic cells by FACS, *in situ* hybridization, cell culture and qPCR experiments as described below. The Hd-rR-II1 strain was also used for qPCR experiment. To ablate germ cells, we injected 2000 ng/µl *cxcr4*-morpholino (MO) and 1000 ng/µl *nanos3*-MO into one- or two-cell stage embryos (Kurokawa et al., 2006, 2007).

Generation of *DMY*/*dmrt1bY* reporter transgenic medaka lines

A bacterial artificial chromosome (BAC) transgenic method that uses homologous recombination was employed to generate a *DMY*/*dmrt1bY*-*EGFP*-3′UTR reporter construct as previously described (Nakamura et al., 2008). Specifically, the targeting DNA fragment for recombination was prepared to include the *DMY*/*dmrt1bY*-3′UTR downstream of the EGFP open reading frame. After homologous recombination, this fragment was inserted immediately downstream of the translation initiation site of the *DMY*/*dmrt1bY* gene in BAC clone DMY H1. Microinjection of BAC clone DNA was performed as previously described (Nakamura et al., 2008). For early germ cell tracking, one-cell stage embryos were injected with *mCherry*:*nos3*-3′UTR (mCherry ORF fused to the 3′UTR of *nanos3*) capped RNA as previously described (Herpin et al., 2007).

Fluorescence-activated cell sorting (FACS) and microarray analysis

All XX and all XY progeny of *sox9b*-DsRed/*olvas*-EGFP transgenic medaka were used in this study. Approximately 500 gonadal fragments, which included intestines, gonads and body trunks, were dissected from st.33 and st.35 embryos and were digested with 0.2% collagenase (Worthington, CLS4) and 1% trypsin (Worthington, TRL) in Leibovitz's L15 for 2 h at 29°C. The cell suspension was the resuspended with L15 supplemented with 10% FBS to stop the digestion reaction, and was filtered through 35 µm cell strainers.

olvas-EGFP= and *sox9b*-DsRed-positive cells were isolated by FACS (Beckman Coulter, Coulter Epics Altra) according to fluorescence intensity and size. Total RNA was extracted from 8000-10,000 cells using an RNaqueous-Micro Kit (Ambion, AM1931), followed by cRNA labeling using a Low Input Quick Amp Labeling Kit (Agilent, 5190-2305) according to the manufacturer's instructions. Hybridization of cRNA targets was

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performed on a 60-mer oligo microarray (Agilent, Design ID: 027381). The microarray experiments were carried out using three independent biological replicates. Scanned data were processed by Feature Extraction (Agilent) and further analyzed by the Subio platform. The microarray data have been deposited at the NCBI Gene Expression Omnibus (GEO) under accession number GSE59521.

In situ hybridization and immunohistochemistry

Whole-mount *in situ* hybridization and immunohistochemistry were performed as previously described (Nakamura et al., 2006). A cDNA clone for *Sdgc* (clone name: olte54h09) was obtained from NBRP medaka (http://www.shigen.nig.ac.jp/medaka/). For immunohistochemistry, anti-OLVAS (medaka Vasa antibody, 1:100, rat) (Aoki et al., 2008) or anti-GFP (1:100, mouse; Clontech, 632381) was used as a primary antibody that was detected with Alexa 488-conjugated secondary antibodies (1:100, goat; Life Technologies, A11029).

Chimeric analysis

sox9b-DsRed/*olvas*-EGFP transgenic embryos (Nakamura et al., 2010) were used as hosts, and OKcab embryos were used as donors. To eliminate the host's germ cells, 2000 ng/µl *cxcr4*-MO and 1000 ng/µl *nanos3*-MO were injected into one- or two-cell stage embryos (Kurokawa et al., 2006, 2007). The transplantation procedure was performed as previously described (Nakamura et al., 2012b).

Quantitative PCR (qPCR)

Germ cells are located at the lateral sides of the intestine in st.30 embryos. In each experiment, approximately 50 tissue fragments containing germ cells were dissected from XX and XY embryos, and total RNA was extracted using Isogen (Nippon Gene, 317-02503). For the experiment using *cxcr4*/*nanos3*-MO-treated embryos and chimeric embryos, 20-30 *olvas*-EGFP-negative embryos (no endogenous germ cells) were used. For extracting RNA from adult gonads, three testes and one ovary were used in each experiment. After total RNA extraction, cDNA was produced using SuperScript III (Invitrogen, 18080-044) or ReverTra Ace qPCR RT Kit (Toyobo, FSQ-101). Quantification was performed with the ABI StepOnePlus Real-Time PCR system (Applied Biosystems) and SYBR Green (Toyobo, QPS-201). The primers used in this study are shown in supplementary material Table S3. The amount of target gene normalized to the β-*actin* or *olvas* (for chimeric analysis) expression and relative to the XX gonads was calculated using the $\Delta\Delta C_T$ method. All qPCR reactions were performed in triplicate. Three independent experiments using independent pools of normal gonads and two independent experiments using independent pools of morpholino-treated gonads and chimera gonads were performed.

Sequence analysis and identification of SNPs in the *Sdgc* genomic region

To identify SNPs in the genomic region of the *Sdgc* gene, the genomic fragments were amplified and sequenced using DNA from OKcab (XX: n=4, XY: n=4) and Kaga (XX: n=4, XY: n=6) inbred lines. From OKcab DNA, 535 bp genomic fragments, including an intron, were amplified and sequenced by q-Sdgc-F and -R primers (see supplementary material Table S3). From Kaga DNA, 346 bp genomic fragments were amplified and sequenced using gSdgc-F and q-Sdgc-R primers. As a result, 14 SNPs were observed in the 346 bp amplicon (supplementary material Fig. S7).

Linkage analysis of *Sdgc*

For single nucleotide polymorphism (SNP) genotyping, XX Kaga and XY Hd-rR-II1 were crossed to generate the F1 progeny, which were subsequently intercrossed to obtain F2 progeny (Kimura et al., 2012). Genomic DNA from the F2 progeny (n=94) was amplified using gSdgc-F and q-Sdgc-R. The PCR products were then diluted 100-fold and used as templates for nested PCR, the products of which were further genotyped via High resolution melting analysis using a LightScanner (Idaho Technology) (supplementary material Table S1). Locus order was determined together with M-markers (Kimura et al., 2012) by AntMap (http://lbm.ab.a.u-tokyo.ac.jp/~iwata/antmap/).

Knockdown of *DMY*/*dmrt1bY* and *Sdgc* by gripNA

GripNA (Active Motif) was designed to target the translation initiation site of *DMY*/*dmrt1bY* and *Sdgc* at the sequence CATGTCAGCCCGGGGAGC (Paul-Prasanth et al., 2006) and CTCGGGTTGGTTTTGCAT, respectively. The underlined CAT regions are complementary to the ATG start codon. For qPCR analysis and cell culture experiments, 0.5 mM *DMY*/*dmrt1bY*-gripNA and 0.25 mM *Sdgc*-gripNA were injected into one- or two-cell stage embryos. Injection of 0.1 mM human-CREB gripNA was used as a control.

Overexpression and rescue experiments of *Sdgc*

Sdgc ORF was amplified using FLAG-tagged primers and inserted into a *Sal*I site of the pBLSK+ PTV1-2A-mCherry vector (Nakamura et al., 2012b). *olvas*-3′UTR was inserted into downstream of the mCherry by InFusion (Clontech, 639649) to generate *FLAG*:*Sdgc*:*2A*:*mCherry*:*olvas*-3′UTR. Capped RNA was synthesized with T7 promoter using mMESSAGE mMACHINE kit (Ambion, AM1344). For overexpression, 200 ng/µl of the mRNA was injected into XX embryos. For the rescue experiment, 200 ng/µl of the mRNA and 0.25 mM *Sdgc*-gripNA were injected into XY embryos. The subsequent culture experiments are described below.

Overexpression of *DMY*/*dmrt1bY*

The construction of *EGFP*:*nos3*-3′UTR (EGFP ORF fused to the 3′UTR of *nanos3*) has been described previously (Kurokawa et al., 2006). Based on the *EGFP*:*nos3*-3′UTR construct, *DMY*/*dmrt1bY* ORF was inserted into downstream of *EGFP* by In-Fusion (Clontech) to generate *EGFP*:*DMY*:*nos3*-3′UTR. Capped RNA was synthesized with SP6 promoter using mMESSAGE mMACHINE kit (Ambion, AM1340). 50 ng/µl of each construct was injected into one- or two-cell stage embryos.

Mitotic activity of germ cells in culture

Approximately 200 tissue fragments containing germ cells at st.30 were dissociated using 0.1% trypsin and 0.1% collagenase in PBS at 29°C for 1 h. The cells were resuspended in L15 containing 10% FBS to stop the enzymatic activity. Next, GFP-positive germ cells were isolated by FACS (Beckman Coulter, Coulter Epics Altra) using the enrich mode. Approximately 5.0×10^3 XX and 7.8×10^3 XY germ cells were successfully isolated. For the preparation of somatic cells, tails from XX and XY embryos were separately dissociated using trypsin and collagenase under the same conditions described above. Germ cells and somatic cells were divided into two tubes and mixed in the following combinations: germ XX-soma XX, germ XX-soma XY, germ XY-soma XX and germ XY-soma XY. The cell mixtures were divided into three portions and plated on collagen-coated cover slips that had been placed in a six-well dish. After a 2 h incubation, 2 ml of Medium #1 (see supplementary material Table S2) was added to each dish. After a 24 h incubation at 26°C, the culture medium was changed to include 25 µM EdU, and cells were cultured for an additional 24 h, then fixed in 4% paraformaldehyde and analyzed by immunohistochemistry. For knockdown and overexpression experiments, 30-40 tissue fragments containing germ cells at st.30 were dissociated as described above. The cell suspension was then divided into two portions and plated on collagen-coated cover slips. Cell culture and EdU treatment were performed as described above.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.N. and M.T. designed the experiments and wrote the manuscript. T.N., A.H., T. Kimura, I.H., S.N. and Y.Y. performed the experiments. A.H. and M.S. generated *DMY*/*dmrt1bY*-EGFP transgenic medaka and wrote the corresponding section. T. Kimura, I.H. and K.N. analyzed the mapping experiment. T. Kawasaki and N.S. participated in the *in vitro* experiment. T.L.S., J.Y., S.M., T.T. and S.S. designed the

medaka microarray platform. S.K. and M.S. discussed/commented on the result and edited the manuscript. All authors read and approved the manuscript.

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Supplementary material
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