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Temperature, Genes, and Sex: a Comparative View of Sex Determination in *Trachemys scripta* and *Mus musculus*

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Sex determination, the step at which differentiation of males and females is initiated in the embryo, is of central importance to the propagation of species. There is a remarkable diversity of mechanisms by which sex determination is accomplished. In general these mechanisms fall into two categories: Genetic Sex Determination (GSD), which depends on genetic differences between the sexes, and Environmental Sex Determination (ESD), which depends on extrinsic cues. In this review we will consider these two means of determining sex with particular emphasis on two species: a species that depends on GSD, *Mus musculus,* and a species that depends on ESD, *Trachemys scripta*. Because the structural organization of the adult testis and ovary is very similar across vertebrates, most biologists had expected that the pathways downstream of the sex-determining switch would be conserved. However, emerging data indicate that not only are the initial sex determining mechanisms different, but the downstream pathways and morphogenetic events leading to the development of a testis or ovary also are different.

Key words: gonad, mouse, sex determination, turtle.

Abbreviations: *AMH*, anti-Müllerian hormone; *DMRT1*, *Drosophila Doublesex* and *C. elegans Mab-3* related transcription factor 1; GSD, genetic sex determination; TSD, temperature-sensitive sex determination; *Sry*, sex-determining region of the Y chromosome; *SOX8*, *Sry*-like HMG-box protein 8; *SOX9*, *Sry*-like HMG-box protein 9; *SF1*, steroidogenic factor 1; *WT1*, Wilm's tumor gene 1; *Wnt4*, wingless-related MMTV integration site 4; *Bmp2*, bone morphogenic protein 2; *FoxL2*, forkhead box L2.

1. Mechanisms of sex determination

In GSD, sex is determined by genetic differences between individuals in the population. These may involve heteromorphic sex chromosomes or chromsomal regions, dosage differences of chromosomes or genes, or the cumulative effect of multiple variable alleles in the genome. The most familiar example of heteromorphic sex chromosomes is the case in humans, where males carry one X and one Y chromosome, and females carry two X chromosomes. This is the system employed by all mammals with the exception of a few species (*1*). In mammals it is clear that a single gene on the Y chromosome, *Sry*, is responsible for male sex determination (*2*, *3*) (Fig. 1A). In humans the occurrence of viable and recognizable XO and XXY individuals with sex chromosome aneuploidy has made it clear that the dosage of the X chromosome does not influence sex determination. XO individuals are Turner's syndrome females, who have recognizable characteristics and are infertile, yet are phenotypically female (*4*). XXY individuals are Klinefelter males—also with typical but less obvious physical characteristics and infertility, yet are phenotypically male (*5*). These cases clearly indicate that it is not the dosage of the X that is important for determining sex, but the presence or absence of the Y.

Birds also utilize a system of heteromorphic sex chromosomes. However, in contrast to mammals, female birds are the heterogametic sex, carrying one W and one Z chromosome, while males are homogametic (ZZ) (*6*). In birds no genetic locus regulating sex determination is yet known. Because investigations of birds with sex chromosome aneuploidy have been inconclusive, it is not clear whether sex determination results from the presence of two Z chromosomes or the absence of a W chromosome. In fact evidence suggests that it might be a combination of both mechanisms (*7*, *8*). In other species, where heteromorphic chromosomal regions have not been identified between the sexes, it has been proposed that sex is a multigenic trait that is controlled by the cumulative effect of a number of allelic variants segregating in the population at different loci. This is the case in the housefly, *Musca* (*9*).

Many animals depend on extrinsic factors to determine their sex. Environmental sex determination (ESD) can depend on a wide range of influences including temperature, visual cues, population cues, or hormone activities (*10–12*). These mechanisms would not work for mammals where both sexes develop under constant temperature and hormonal conditions in the uterus. Dependence on ESD mechanisms permits a rapid adaptation of the sex ratio to a changing environment. On the other hand, an ESD population is dangerously vulnerable to extrinsic changes that lead to significant changes in the sex ratio.

Most turtles, all crocodilians, and some lizards depend on temperature dependent sex determination (TSD), in which the incubation temperature of the egg determines sex. Sex inducing temperature varies between species

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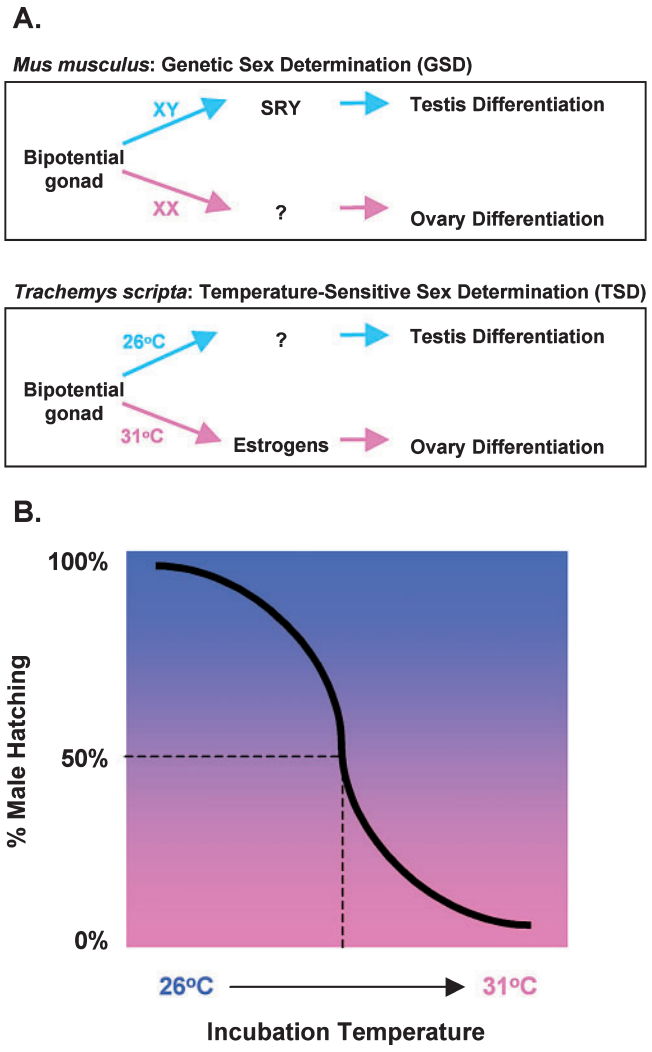

Fig. 1. **A: Current sex determination paradigms for mouse (*Mus musculus* with a genetic sex determination mechanism) and red-eared slider turtle (*Trachemys scripta* with a temperature-sensitive sex determination mechanism). B: Diagram of TSD in *T. scripta*: Nearly 100% of *T. scripta* eggs incubated at 26°C develop as males; whereas incubation at 31°C produces nearly 100% females.** At median temperatures ~50% develop as male, and 50% as female.

such that female differentiation can either occur at the high temperature, the low temperature, or the intermediate temperature (*13*). In *Trachemys scripta*, incubation at 31°C yields nearly 100% female offspring, while incubation at 26°C produces nearly 100% males—regardless of normal genetic differences that must exist between eggs in an outbred population. Interestingly, when eggs are incubated at temperatures between male- and female-inducing temperatures, some develop as males and some as females, but rarely as intersexes (although intersexes are more frequent in TSD populations than they are in mammals; Fig. 1, A and B) (*14*). This suggests that sex determination is a process that hangs in the balance of competing signals, but once the decision is made, sex-determining pathways are strongly canalized such that the entire organism is recruited to male or female differentiation.

For many reptilian species, the period of sensitivity to temperature has been defined by shifting eggs from one temperature to the other at different stages of development. The period of sensitivity to temperature is always the period immediately prior to the time when morphological differentiation of the gonad is initiated, consistent with the fact that sex determination in all vertebrates occurs at the point where the fate of the gonad is determined. For *T. scripta*, the temperature sensitive window for ovarian development extends from stage 16.5–19.0, and from stage 17.0–21.0 for testis development (*15*).

Perhaps it is worth pointing out that constant incubation conditions in the laboratory do not simulate conditions in the wild where significant fluctuations in temperature occur within the day/night cycle and over a temperature sensitive period. TSD can be demonstrated in the laboratory in some species that have heteromorphic sex chromosomes. It is likely that in the wild these species respond to a combination of both GSD and TSD mechanisms. At temperature extremes, TSD mechanisms may overbalance genetic ones; whereas, at median temperatures, segregating genetic elements may dominate the decision to develop as a male or female (*16*).

2. Sex determination at the molecular level

In all vertebrates, male and female gonads are morphologically indistinguishable before sex determination occurs. Although vertebrates use diverse mechanisms to trigger sex determination, most species develop adult gonads with striking structural and functional similarities: a testis consisting of testis cords in which Sertoli cells surround germ cells undergoing spermatogenesis, or an ovary with typical follicular structures containing developing oocytes. Based on these morphological similarities, it was hypothesized that conserved molecular and cellular mechanisms would be found downstream of the sex determination switch to regulate sexually dimorphic development of the gonads. In mice, considerable progress has been made in the identification and characterization of genes with critical roles in the testis determination pathway downstream of *Sry* (*17–19*). Comparative studies have identified orthologous genes in other vertebrates, including chickens (*6*), alligators (*20*), and turtles (see below), that are expressed in gonads during the developmental period of sex determination. Although some genes show similar expression patterns between mammals and other vertebrates, many do not. In the red-eared slider turtle, six of these genes (Steroidogenic factor 1, *SF1*; Wilm's tumor gene 1, *WT1*; *Drosophila Doublesex* and *C. elegans Mab 3* Related Transcription factor 1, *DMRT1*; *Sry*-related HMG-box protein 9, *SOX9*; *Sry*-related HMG-box protein 8, *SOX8*, and anti-Müllerian hormone, *AMH*) have been characterized in relation to the temperature sensitive period. Some of these have remarkably divergent expression patterns in the gonad. To illustrate this point, we present a comparative view of the expression of these six genes during gonadal sex determination in turtles and mice.

Sf1 and *Wt1* belong to a network of transcription factors that are essential for initial gonad formation in mice and humans. Mutation or loss of either gene leads to gonadal agenesis (*21*, *22*). In mice, *Sf1* and *Wt1* are expressed in a similar pattern in XX and XY gonads

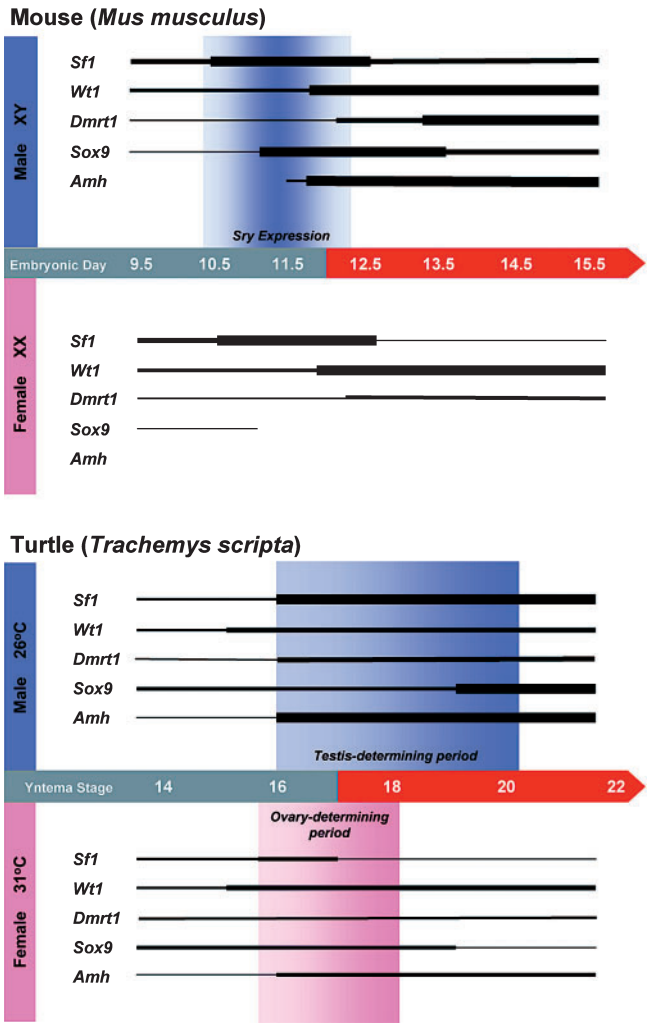

Fig. 2. **Diagrammatic expression profiles of genes critical for sex determination during embryogenesis in mouse (*Mus musculus*) and red-eared slider turtle (*Trachemys scripta*).** Relative expression is indicated by the thickness of lines (thicker lines indicate stronger expression and *vise versa*). It is valid only to compare expression levels of a given gene between sexes, not between genes or species. Red bars that highlight the developmental stage indicate the appearance of morphological differences in gonads between sexes. Blue and pink shaded areas represent gene or temperature sensitive periods. anti-Müllerian hormone, *AMH*; *Drosophila Doublesex* and *C. elegans Mab-3* Related Transcription factor 1, *DMRT1*; genetic sex determination, GSD; temperature-sensitive sex determination, TSD; Sex-determining region of the Y chromosome, *Sry*; *Sry*-like HMG-box protein 8, *SOX8*; Sry-like HMG-box protein 9, *SOX9*, Steroidogenic factor 1, *SF1*; Wilm's tumor gene 1, *WT1*.

before sexually dimorphic structures arise (Fig. 2). Following *Sry* expression and the onset of divergent morphogenesis, *Sf1* is down-regulated in the XX gonad and becomes XY-specific (*23*), whereas *Wt1* expression persists in both sexes (*24*). Interestingly, profiles of *SF1* and *WT1* expression are comparable in turtle gonads. Before the onset of the temperature sensitive period, all turtle gonads express *SF1* and *WT1* similarly. While gonads from both sexes continue to express *WT1* (*25*, *26*), only gonads incubated at the male-producing temperature maintain *SF1* expression (*27*, *28*). Similar *SF1* and *WT1* expression patterns in both sexes of turtles and mice before the onset of sex determination suggest conserved roles of these two transcription factors in the initial formation of the gonads. Later male-specific *SF1* expression in response to the sex-determining switch (*Sry* or temperature), suggests that SF1 also plays a sex-specific role in the testis pathway. In the mouse, SF1 takes part in transcriptional regulation of the *Amh* gene (*29–33*), a testis-specific factor in vertebrates that causes regression of the Müllerian duct, the anlagen of the female genital ductal system. Later testis-specific roles for WT1 are also known in mammals (*34*). No loss of function studies have been reported in turtles.

Sox9 has been proposed to be the "master" testis-determining factor downstream of *Sry* in mammals. In mice, *Sox9* expression is up-regulated in XY gonads immediately after *Sry* expression begins (*35*). Inactivation of *Sox9* in XY embryos leads to male to female sex reversal (*36*) and furthermore, exogenous expression of *Sox9* in XX gonads can induce testis formation in the absence of *Sry* (*37*, *38*). These studies suggest that in mammals, *Sox9* can act as the downstream effector of *Sry*. SOX9 has been shown *in vivo* and *in vitro* to participate in the initiation of *Amh* expression in Sertoli cells (*30*, *39*) (Fig. 2). Because of its high sequence homology and testis-specific expression patterns in other vertebrate species, it was expected to play similar roles. However, evidence from alligators and chickens was the first to suggest that *SOX9* does not play a conserved role as the "master" testis effector gene. In contrast to mouse, *SOX9* does not become testis-specific in chicken and alligator until testicular structures are established. Furthermore, *SOX9* upregulation occurs later than male-specific *AMH* expression begins in both alligator and chicken gonads (*40*, *41*). These studies indicate that SOX9 cannot control the primary up-regulation of *AMH* expression in these species as it does in mice. In red-eared slider turtles, *SOX9* expression follows a pattern similar to chickens and alligators. Turtle gonads from both sexes express a low level of *SOX9* before and during the temperature sensitive period (Stage 16 to 20), and male-specific *SOX9* expression only begins at Stage 20, when testis structures are already apparent (*26*). *Sox8*, another HMG-type DNA binding protein closely related to *Sox9*, shows a testis-specific expression pattern similar to *Sox9* in mice, and appears to act redundantly with *Sox9* in mammalian systems (*42*). It was proposed that *SOX8* might take over the primary role in turtles and other non-mammalian systems; however, expression of *SOX8* is not sex specific in red-eared slider turtles (*43*). These studies lead to the conclusion that neither *SOX9* nor *SOX8* occupies a key position in the sex determination pathway in *T. scripta*, unlike the case in mammals.

However, in the sea turtle *Lepidochelys olivacea*, male-specific *SOX9* expression precedes testis organization, more closely resembling the pattern in mice (*44*, *45*). In non-mammalian vertebrates, *SOX9* is consistently associated with the male pathway, but may or may not play the central role in morphological organization of the testis. Differences in the pattern of *SOX9* expression could possibly be related to different mechanisms involved in testis morphogenesis across species (see below).

Another gene that may have conserved roles in sex determination is *DMRT1*. *DMRT1* shares high homology and exhibits gonad-specific expression in species from metazoans to vertebrates (*46*). In mice, *Dmrt1* expression is similar in both sexes before and during the initial divergence of testis and ovary development (Fig. 2). It is not until 14.5 dpc, 48 h after structural divergence of the ovary and testis, that *Dmrt1* expression is upregulated in testes (*47*). Normal formation of testes in *Dmrt1* null mice further indicates that *Dmrt1* does not act as a testis-determining factor in mice as originally proposed. In red-eared slider turtles, male-specific *DMRT1* expression starts at the beginning of the temperature sensitive period (Stage 17) and is ongoing during testis development (*48*, *49*). This expression pattern is similar to the pattern reported in chickens where the gene resides on the Z chromosome (*50–53*). Although *Dmrt1* is not involved in testis determination in mice, its involvement in non-mammalian temperature-dependent sex determination remains to be determined. *DMRT1* is strongly implicated as a major sex determination gene in medaka (*54*), but does not seem to be the primary sex determinant in zebrafish (*55*).

Based on expression profiles of these six key regulators (*Sf1, Wt1, Sox8, Sox9, Amh,* and *Dmrt1*), red-eared slider turtles seem to utilize conserved genes (*SF1* and *WT1*) for initial gonad formation, similar to other vertebrate species. However, molecular events downstream of the testis-determining switch in different species are highly divergent, as evidenced by the species-specific regulation of the *SOX* genes, *AMH*, and *DMRT1*. Even within the turtle family, different patterns of gene expression appear to regulate downstream events.

In mammals, hormones are not produced in embryonic gonads until after sex determination occurs. The earliest difference between male and female gonads is the expression of *Sry* in XY gonads, which initiates the male pathway. In contrast, in the red-eared slider turtle, an early effect of incubation at the female-producing temperature is the expression of aromatase and the production of estrogens (*56*). Blocking estrogen synthesis by aromatase inhibitors causes female to male sex reversal at the female-producing temperature (*57*). In turtles, estrogens not only promote ovarian fate, but also can override the testis pathway. Estrogen treatments of turtle eggs incubated at the male-producing temperature lead to variable feminization of the embryo (*14*). In fact environmental estrogens and estrogen mimics are of considerable concern in populations of alligators breeding in heavily populated areas, where a high incidence of intersex young has been reported (*58*, *59*).

The ovary-determining influence of estrogens appears to be conserved in most egg-laying species such as alligators (*60*), fish (*61*, *62*), and birds (*63*). However, in most mammals, where embryos are exposed to estrogens from the maternal circulation, sex determination is not sensitive to estrogen levels. In eutherian mammals, male to female sex reversal of genetic XY gonads has never occurred in cases of pharmacological exposure of estrogens and its agonists *in utero* (*64*) or when XY gonads are cultured with estrogenic compounds during the sex-determining period (*65*). Furthermore, inactivation of the estrogen pathways in aromatase or estrogen receptor double knockout mice has no effect on initial ovary formation, providing conclusive evidence that estrogen is not involved in primary sex determination in eutherian mammals (*66*, *67*). Interestingly, marsupials appear to be intermediate in this respect as estrogen exposure at the time of gonadal differentiation can lead to partial XY to XX sex reversal (*68*, *69*). Several genes have recently been identified in the female pathway in mice including wingless-related MMTV integration site 4, *Wnt4* (*70–73*), follistatin (*Fst*) (*72*), bone morphogenic protein 2, *Bmp2* (*72*), and forkhead box L2, *FoxL2* (*74–76*). However, expression profiles for these genes in the turtle gonad in relation to temperature and estrogen treatments remains to be established.

3. Sex determination at the cellular level

Remarkable similarities among vertebrates in the structure and function of adult testes and ovaries led investigators to hypothesize that conserved cellular mechanisms regulate the morphogenesis of these two organs. Adult testes are all organized into testis cords in which germ cells are enclosed inside long tubes of epithelialized somatic cells that extend throughout the organ. In females, individual germ cells are surrounded by a layer of somatic cells to form ovarian follicles that reside in the cortex of the ovary.

As in all vertebrates, mouse and turtle gonads arise as bipotential organs that are initially indistinguishable in male and female embryos. However, distinct structural differences exist at this stage between turtle and mouse gonads. In mouse, somatic and germ cells are intermixed throughout the bipotential gonad prior to sex determination. In contrast, gonads of most reptiles, birds, and alligators have a different arrangement of somatic and germ cells at the earliest stages. In *T. scripta*, primitive sex cords composed of epithelialized somatic cells are present throughout the gonad from its earliest formation, and germ cells are initially restricted to the coelomic epithelium where they are found between somatic cells (*15*, *77*). Antibody staining with laminin in the turtle indicates that the basal laminae of primitive sex cords is continuous with the basal membrane of the coelomic epithelium [Fig. 3 (*77*)]. The existence of primitive sex cords in undifferentiated gonads of nonmammalian species has been reported histologically for many years (*13*, *78*). It is not clear what accounts for this initial organizational difference between mammals and other vertebrates. One possibility is that it is related to different mechanisms by which germ cells arrive and populate the gonad.

The difference in the initial location of germ cells between turtle and mouse gonads may be important for the process of morphogenesis of the testis and ovary. Germ cells in the mouse are located in the interior of the gonad rather than at the coelomic surface as in *T. scripta* (Fig. 3). This may be the consequence of the different paths of germ cell entry into gonads. In mouse, germ cells migrate through the hindgut mesentery, traverse the mesonephros, and migrate into the interior of the genital ridges between 10.5–11.5 dpc (*79*). The migration path of turtle germ cells is unknown, but migration of germ cells in chick is well studied. Chick germ cells circulate through the vascular system, extravagate from small vessels, and then enter the neighboring thickened coe-

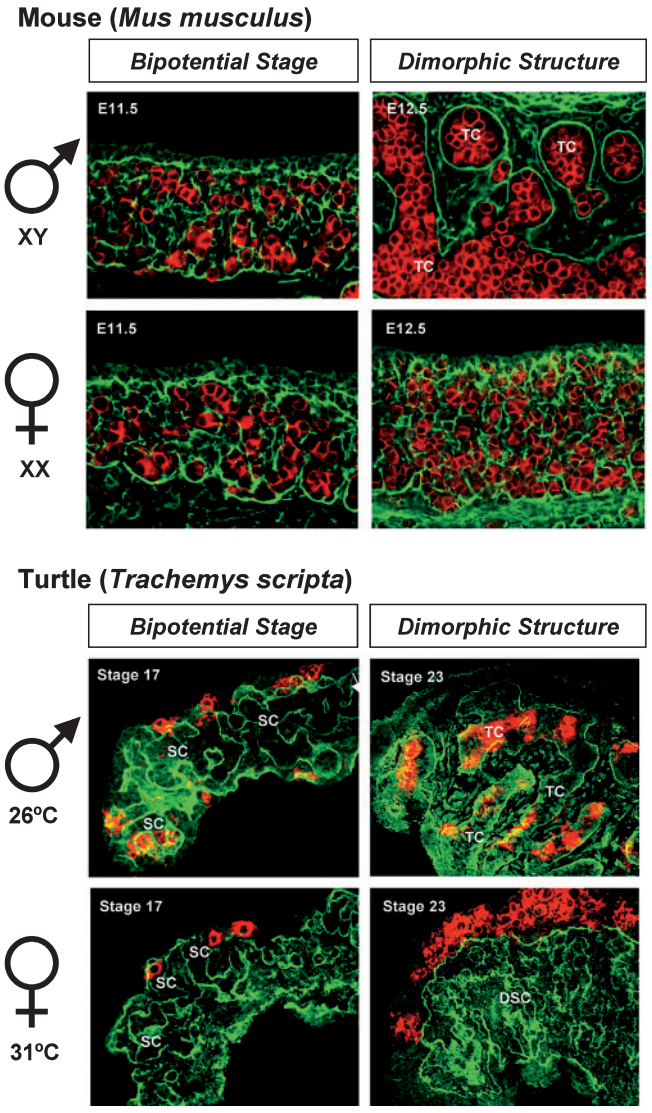

Fig. 3. **Morphological differences between mouse (*Mus musculus*) and red-eared slider turtle (*Trachemys scripta*) at and after bipotential stages.** In mouse gonads, primordial germ cells were highlighted by an anti–E-cadherin antibody (red; Zymed) and testis cords (TC) were outlined by an anti-laminin antibody (green). In turtle gonads, primordial germ cells were highlighted by an anti-VASA antibody (red) and sex cords (SC) and testis cords (TC) were outlined by an anti-laminin antibody (green). Images for turtles were adopted from Yao *et al.* 2004 (*77*) with permission from the publisher. DSC: degenerated sex cord.

lomic epithelium, which becomes the definitive gonadal anlagen (*80*). The close association of turtle germ cells with the coelomic epithelium suggests that their path of entry into the gonad may be very similar to chick. Although the initial location of germ cells in turtle gonads is different from mouse, the outcome after morphogenesis of the testis and ovary is the same. In female turtle gonads, germ cells remain on the surface of the gonad as the primitive sex cords retract to the medulla. In contrast, as male turtle gonads develop, the primitive sex cords gradually envelop the germ cells and differentiate into typical testis cords (*77*).

The structural differences between turtle and mouse undifferentiated gonads present different starting points for the morphological organization of the testis and ovary. This disparity leads to differences in cellular mechanisms required to reach a similar endpoint of testis or ovary morphology. In *T. scripta*, the male-producing temperature serves to maintain and elaborate the existing sex cords whereas the female-producing temperature leads to the retraction of sex cords. Because no primitive sex cords are present in the mouse gonad at the bipotential stage, *Sry* must activate the *de novo* assembly of testis cords.

In the mouse, organization of adult type testis cords occurs between 36–48 h after *Sry* expression is initiated. The role of *Sry* is to specify the Sertoli cell lineage, which, in turn, orchestrates the transformation of the bipotential gonad into a testis. Some of the critical cellular processes downstream of *Sry* in the mouse gonad have come to light (*18*, *81*). For example, proliferation of somatic cells is rapidly up-regulated soon after *Sry* expression begins (*82*, *83*). An increase in proliferation is also seen in turtle gonads at the male producing temperature (*84*), and, in fact, is associated with the male pathway in all species examined so far (*85*).

Contribution of the coelomic epithelium to different somatic cell lineages in gonads is a critical event during sex determination. Lineage tracing experiments were performed by labeling proliferating cells in the coelomic epithelium and following their fate. In mouse gonads of both sexes, some of these labeled cells delaminated from the coelomic epithelium, moved into the gonad, and differentiated as Sertoli cells (*86*). Similar lineage tracing experiments were performed on *T. scripta* gonads (*77*). Regardless of the incubation temperatures, the turtle coelomic epithelium gave rise to somatic cells in primitive sex cords and probably to other somatic lineages outside the cords. However, in contrast to mouse gonads where individual labeled coelomic epithelial cells divided, delaminated, and moved into the gonad, labeled cells in turtle gonads remained in close proximity to the original labeled populations in the coelomic epithelium. This suggests a mechanistic difference in how the coelomic epithelium contributes to formation of sex cords. In XY mouse gonads, testis cords form by *de novo* assembly of individual cells; whereas, in turtle gonads, primitive cords appear to form in undifferentiated gonads by invagination of the coelomic epithelium.

Besides contribution from the coelomic epithelium, assembly of testis cords in mice requires migrating cells from the adjacent mesonephros. Migration of these cells is triggered by expression of *Sry* in the XY gonad, and they are believed to participate in formation of the basal lamina of the epithelialized testis cords (*87*, *88*). Xenograft experiments to test the ability of male turtle gonads to induce mesonephric cell migration were performed in *T. scripta*. EGFP mouse mesonephroi were cultured apposed to male or female turtle gonads and assayed for migration of EGFP mouse mesonephric cells into the turtle gonad (*77*). These experiments revealed no evidence for mesonephric cell migration in the turtle. However, this negative result was difficult to interpret because of potential incompatibility of tissues and proteins between the two species. Moreno-Mendoza and col-

leagues cultured the sea turtle (*Lepidochelys olivacea*) gonad separated from the mesonephros during the bipotential period and showed that testis cord development does not require the presence of the mesonephros (*45*). Given the similar structure and development of the testis in these two turtle species, it seems likely that mesonephric cell migration is not required in the turtle gonad. Furthermore, because primitive sex cords are present in both male and female turtle gonads before the onset of the temperature sensitive period, if mesonephric migration is involved in sex cord formation in turtles, it must be regulated by a non-sex specific mechanism and it must occur at the beginning of the bipotential period.

4. Conclusion

The conservation among vertebrates of many of the same genes, as well as testis and ovary structure, suggested that mechanisms of morphogenesis downstream of the sex determination switch would be similar between turtles and mice. However, accumulating evidence suggests that there is surprising diversity of mechanisms employed in the organogenesis of the testis, even though a similar repertoire of genes seems to be involved. A comparison of the molecular and cellular events during sex determination in the mouse and the turtle will reveal how these genes are deployed under different circumstances to elicit changes in morphology and the differentiation of testis and ovary cell types in different vertebrates.

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