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Blurring the Edges in Vertebrate Sex Determination

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Summary of recent advances

Sex in vertebrates is determined by genetic- or environmentally-based signals. These signals initiate molecular cascades and cell-cell interactions within the gonad that lead to the adoption of the male or female fate. Previously, genetic- and environmentally-based mechanisms were thought to be distinct, but this idea is fading as a result of the unexpected discovery of coincident genetic and thermal influences within single species. Together with accumulating phylogenetic evidence of frequent transitions between sex-determining mechanisms, these findings suggest that genetic and environmental sex determination actually represent points on a continuum rather than discrete categories, and that populations may shift in one direction or the other in response to mutations or changing ecological conditions. Elucidation of the underlying molecular basis of sex determination in mice has yielded a bistable model of mutually antagonistic signaling pathways and feedback regulatory loops. This system would be highly responsive to changes in the upstream primary signal and may provide a basis for the rapid evolution of and transitions between different methods of sex determination.

Introduction

Across vertebrates, primary sex determination is defined as the decision within the bipotential gonad to develop as a testis or an ovary. Within the past decade, the traditional view of this process, in which a strict division is drawn between species that employ genetic mechanisms (genetic sex determination, GSD; e.g., mammals, birds) to determine sex and those that use environmental mechanisms (such as temperature-dependent sex determination, TSD; e.g., crocodiles), has been supplanted by the theory that GSD and TSD actually represent points on a continuum along which populations can and do shift, under selective pressure [1,2,3••]. This novel perspective is strengthened by the recent accumulation of empirical data suggesting that genetic elements influence systems that use TSD, and that functional or vestigial temperature sensitivity is present in organisms that employ GSD, even some with heteromorphic sex chromosomes [4•,5•,6•,7••]. Additionally, many of the cellular processes, transcription factors and signaling pathways involved in sex determination and gonadogenesis are conserved across vertebrates, implying that the underlying machinery may be similar despite modifications in the dominant upstream signal used. This perspective will be discussed in the present review, in the context of converging ideas about how sex-specific development of the gonads is initiated.

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Rapid evolutionary transitions between sex-determining mechanisms

The methods of sex determination (e.g., GSD (XX/XY, ZZ/ZW, or homomorphy), TSD, polygenic, or density-dependent) used by many species of reptile, amphibian and fish have been elucidated in recent years (Figure 1)[3••,8-10]. When these are plotted onto a phylogenetic map, the evolutionary lability of sex determination is apparent within several major branches of the tree, where numerous transitions must have occurred to achieve the present diversity [3••]. Most of these inferred transitions remain unexplained, but two studies provide definitive examples of rapid and recent transitions between different GSD mechanisms.

Investigation of a species of Japanese frog, *Rana rugosa*, demonstrated the presence of both ZZ/ZW and XX/XY heterogamety in neighboring populations, with homology between the Z-Y and W-X chromosomes. This evidence suggests that population intermixing may have driven the transitions between chromosomal complements in this case [11•].

In two closely related medaka species, a master male sex-determining gene, *DMY*, derived from the conserved male-promoting gene *DMRT1*, was identified on the Y chromosome [12, 13]. However, this gene is not present in the genome of the other three species of this genus, which must, therefore, use another method to determine sex [14,15]. In a relationship akin to that of murine *Sry* and *Sox9* (discussed below), DMY appears to preempt the female pathway by advancing expression of a *DMRT1* ortholog in the XY medaka gonad, thus promoting testis development. This finding implies that gene duplication can initiate rapid transitions to new forms of GSD.

Overriding GSD by hormones and temperature

Eutherian mammals, with XX/XY male heterogamety, comprise one of the most strictly GSD groups in the animal kingdom. Classic work in the mouse and human systems demonstrated that expression of the Y-chromosome-linked *Sry* gene in the supporting cell lineage leads to their differentiation as Sertoli cells and the adoption of the testis fate [16,17]. In the absence of this upstream signal, ovarian development ensues. The evolution of both viviparity and endothermy in eutherian mammals required a mechanism not based on temperature or environment. The accumulation of fertility factors on the Y chromosome and the low viability of XY oocytes [18,19] operate to fix the GSD mechanism in mammals. However, viviparity and TSD coexist in several reptiles where pregnant females can influence offspring sex ratio through their thermoregulatory behavior [20,21].

In contrast to eutherian mammals, sex determination in other vertebrate species that employ GSD is sensitive to exogenous hormones (Figure 1). Both marsupials, which share the *Sry*-dependent XX/XY system of eutherian mammals [22,23] (though sex determination occurs after birth [24]), and birds, which exhibit ZZ/ZW female heterogamety with an unknown upstream signal [25], are sensitive to the application of hormones [8,9]. Susceptibility to hormone treatment is shared by most reptiles, including numerous species traditionally classified as either GSD or TSD [26-28].

Sex in several reptilian GSD species is also affected by incubation at temperatures toward the limit of the viable range. A species of scincid lizard, *Bassiana duperreyi*, with heteromorphic sex chromosomes (XX/XY) shows both GSD and TSD mechanisms operating within a single population [5•,29]. Incubation at a low temperature can sex-reverse this lizard from female to male [29]. While incubation temperature has been previously shown to override genetic sex in several amphibian and fish species [30-32], this is the first report of a sensitive heterogametic species. In this system, the observed unidirectional temperature-induced sex reversal (female to male) would produce XX males, not XY females. Fitness-compromised YY individuals will

therefore not arise in the next generation, and both mechanisms might be simultaneously maintained without significant risk.

Similarly, high egg incubation temperatures can also induce discordant sexual phenotypes in a ZZ/ZW agamid lizard, *Pogona vitticeps* [4•]. Here, the converse argument applies. Sex reversal in this female heterogametic system also appears to be functionally unidirectional (male to female) yielding ZZ females rather than ZW males. Potentially disadvantaged individuals with a WW genotype will not be generated. Together, these studies suggest that limited thermal sensitivity within a GSD system may have adaptive significance (see below), or serve as raw material for an evolutionary transition to TSD under a new set of selective pressures.

Thermosensitive gene expression in a GSD turtle

In TSD species, many genes known to be involved in sex determination or gonadogenesis show temperature-specific expression patterns during the temperature-sensitive period of development, before sex has been determined; however, the functional significance of these findings is not clear [e.g. 33,34,35•,36-38]. For example, expression of *DMRT1* in the red-eared slider turtle, *Trachemys scripta*, is elevated at the male-producing temperature as compared to the higher female-producing temperature at stages when sex is still labile [39]. Gonadal expression levels of the *WT1* gene were compared between related TSD and GSD turtle species to determine whether any vestigial thermal sensitivity is present in the gene regulatory network underlying gonad development in the GSD species [6•]. When incubated at the temperature that produces 100% males in the TSD species, gonads of both species exhibited higher *WT1* levels. However, artificially elevated *WT1* levels did not override the genetic mechanism in the GSD species and induce sex reversal [6•]. Nonetheless, this study illustrates the subtle thermal sensitivity that may exist within the underlying genetic network. In these systems, a single mutation leading to thermosenstivity of one or more key genes could modify a genetic mechanism and initiate a transition from GSD towards TSD (Figure 2).

Geographic pressures on TSD

The pivotal temperature (T_P) for a TSD species is defined as the range of incubation temperatures that produce a 1:1 male to female sex ratio. In many species, temperatures below the T_P will yield an increasingly male-biased ratio, and higher temperatures will generate more females (MF), though this pattern is often reversed (FM). While only one T_P has been observed in many species, others exhibit a FMF pattern with two distinct transition points [7••]. It has been proposed that the existence of a single T_P may reflect viability constraints at one end of the temperature range, rather than a fundamental difference between the underlying temperature-sensitive mechanisms [4•]. The T_P(s) for a given species has been shown to shift according to the local climate and latitude of individual populations, suggesting the presence of selective pressure to maintain a balanced sex ratio and inherent flexibility in the thermosensitivity of the system [7].

Evolutionary advantage of TSD

As TSD has been documented in a wide array of species, it must carry selective advantages in particular environments. Unfortunately, the longevity and delayed sexual maturity characteristic of most reptilian TSD species have made direct evaluations of reproductive fitness impractical. The Charnov-Bull model predicts that TSD should be favored if the fitness of (either or both) males and females is enhanced by development under a particular set of environmental conditions [40]. In their study of the TSD agamid lizard *Amphibolurus muricatus*, Warner and Shine have provided the first empirical evidence in reptiles that supports this model [41••]. This species of lizard matures rapidly, allowing its reproductive output to

be tracked in the laboratory and genetically confirmed. Females are produced at both high and low temperatures, while intermediate temperatures yield a 1:1 male to female ratio. The authors generated males at three representative temperatures by treating developing embryos with a hormone inhibitor, and then compared the lifetime fecundity of male individuals produced at the natural (intermediate) male-producing temperature to that of males from temperatures that normally produce only females. Females generated at the intermediate temperature were also compared to females from eggs incubated at high and low temperatures. The results show that the fecundity of each sex was maximized by development at the temperature that naturally produces that sex. For example, males incubated at the intermediate temperature had higher reproductive outputs than sex-reversed individuals from female-producing temperatures, in accord with the predictions of the Charnov-Bull model.

Antagonistic pathways: a plastic system for evolutionary adaptation

The decision to develop as a male or female depends on whether the gonad develops as a testis or an ovary. In mammals, this decision rests on the fate of the supporting cell lineage, which either initiates differentiation as Sertoli (male) or follicle (female) cells. *Fgf9* and *Wnt4* act as mutually antagonistic signals that converge on *Sox9* during sex determination to regulate the fate of the supporting cell lineage in mice [42•]. Both *Fgf9* and *Wnt4* are expressed in the bipotential gonad. *Sry* triggers the up-regulation of *Sox9*, which then up-regulates *Fgf9* in a feed forward loop that is required to sustain *Sox9* expression, establish Sertoli cell differentiation and repress *Wnt4*. In the absence of *Fgf9*, or its receptor, FGFR2 [43,44], *Sox9* is down-regulated. *Wnt4* gains control of the fate of gonadal cells and initiates follicle cell (ovary) development. Surprisingly, in *Wnt4* mutant XX gonads, *Sox9* expression is transiently elevated in a manner reminiscent of the initiation of the male pathway, and although sex-reversal is incomplete, gonads undergo morphological changes characteristic of testis development [42•]. Importantly, this occurs in XX gonads that lack *Sry*. Conversely, although *Sry* is the dominant switch in mammals, it is now clear that the male pathway can be over-ridden by *β-catenin* expression in XY gonads [45]. Both these and other lines of evidence [46-49] argue strongly for regulation of sex determination through antagonistic signaling pathways in mammals.

So far, evidence for this model has been demonstrated only in mice and humans. However, mechanisms of sex determination based on antagonistic signaling could be highly labile in evolutionary terms, and might easily be regulated in diverse ways. For example, minor shifts in the timing, level, or activity of a single factor could trigger an imbalance in the system (Figure 2). In some cases a factor with a major influence may have evolved (i.e., *DMY* in medaka), whereas in others, several factors may have a cumulative influence on the outcome over a relatively long bipotential period. In either case, the canalizing effect of feedback regulation, both within and between cells, acts to stabilize one pathway and force commitment to either testis or ovary fate.

Although both testis/ovary morphology and expression of many of the genes (other than *Sry* itself) involved in sex determination in mammals are conserved in all vertebrates tested to date [e.g. 35•,50-52], the order of morphological events and the sequence and timing of gene expression are not fully conserved. This suggests that there are multiple entry points into the regulatory loops that establish testis or ovary development (Figure 3). While it is clear that the supporting cell lineage is the cell type where primary commitment occurs in mammals, this is not clear for other vertebrates, where germ cells or steroidogenic precursors may be the focal point of the sex-determining decision. Nonetheless, consideration of the diversity and rapid variation of sex determination systems in the context of this model may be instructive.

Conclusions

In contrast to the high conservation of most developmental pathways across species from *Drosophila* to man, a stunning variety of mechanisms of sex determination exist within the animal kingdom. The distinction between genetic- and environmentally-based primary signals is beginning to blur as a result of the discovery of coincident thermal and genetic influences within single species, as well as the high degree of conservation in the genes governing gonadogenesis. The model of antagonistic signaling and feedback reinforcement that has emerged in mammals provides a possible explanation for the rapid evolution of and transitions between different sex determination mechanisms seen among vertebrates. Although the entry points into and the order of the pathways governing sex determination seem to be different, this general model may be valuable in the quest to understand these highly diverse processes. Surveys for novel differentially expressed genes specific to the avian, reptilian, amphibian, or fish systems, as well as development of methods to test the functional involvement of these genes, will be important to make significant progress toward understanding the molecular basis of sex determination in non-mammalian species.

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Abbreviations

GSD
genetic sex determination

TSD
temperature-dependent sex determination

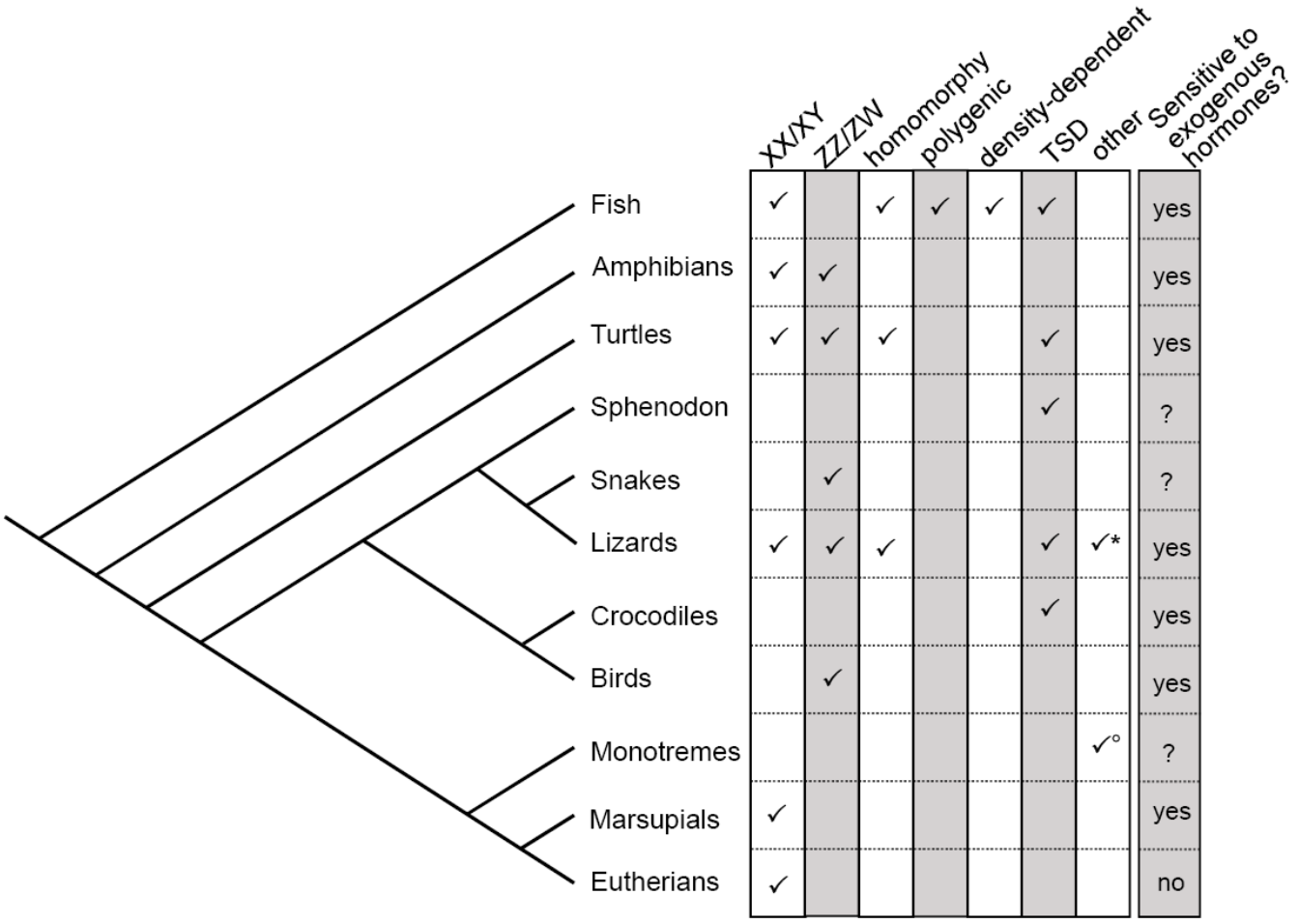

Figure 1.

Sex determination in extant vertebrates. Fish, amphibians, turtles, and lizards each exhibit more than one method of sex determination, which fall into genetic (GSD) and environmentally based categories. XX/XY and ZZ/ZW refer to male and female heterogametic systems, respectively, while homomorphy refers to GSD in the absence of differentiated sex chromosomes. Polygenic and density-dependent sex determination are characteristic of a subset of fish, and many reptiles and fish determine sex according to the incubation temperature of the egg (TSD). With the exception of eutherian mammals, most vertebrate embryos are susceptible to exogenous hormone-induced sex reversal. *Co-occurrence of TSD and GSD has been noted in several species of lizards. °Monotremes (i.e., platypus and echidna) have a complex arrangement of X and Y sex chromosomes, which assemble into a chain during meiosis.

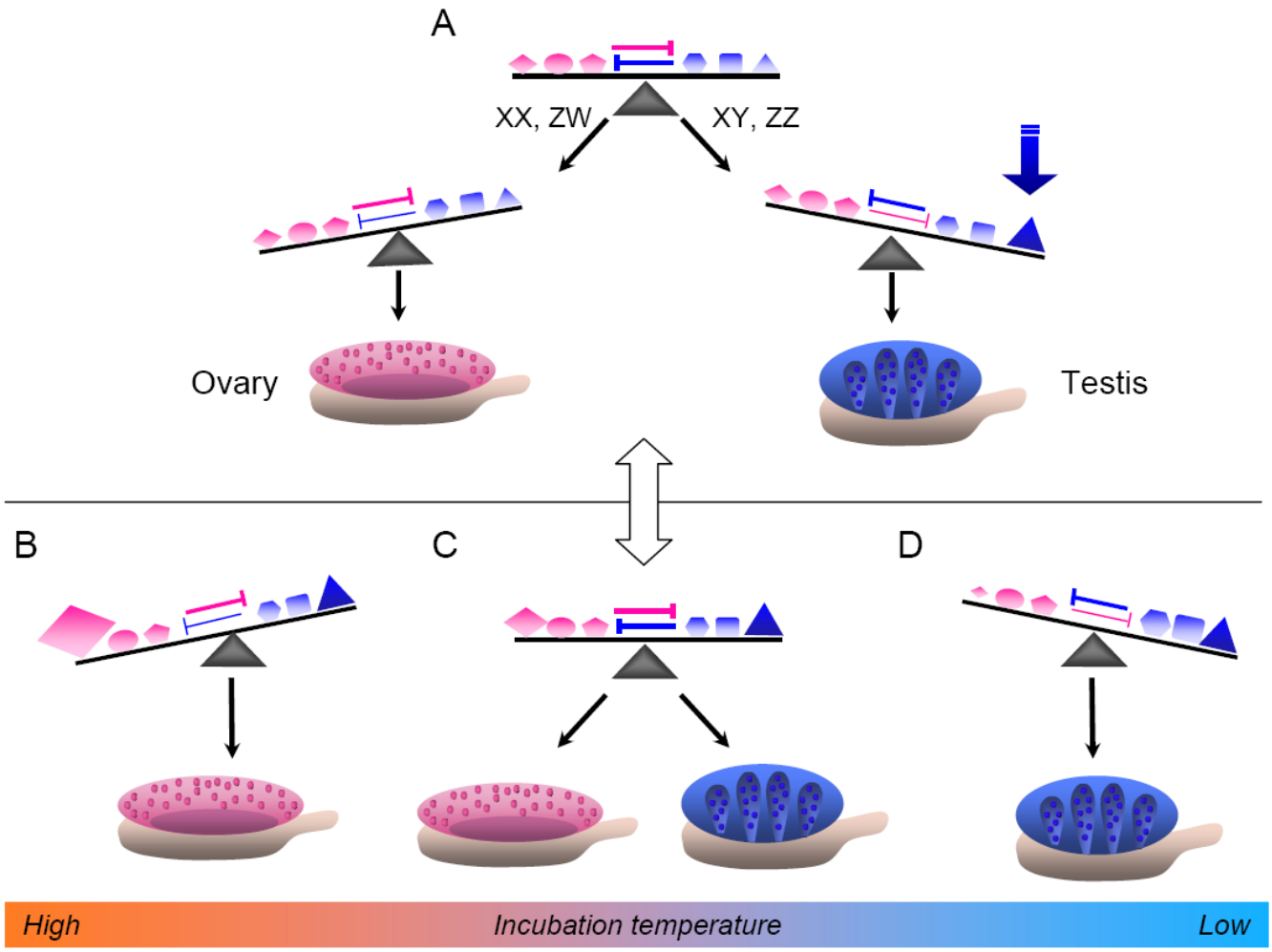

Figure 2.

Hypothetical evolutionary transition between GSD and TSD systems. **A**, Based on the mammalian model, the bipotential gonad is initially balanced between alternative fates by mutually antagonistic male and female factors. The appearance of a segregating, dominant allele of a key gene(s) in either the male or female pathway can fix a genetic mechanism. This may occur by gene duplication and evolution of a sex chromosome (as in the case of *Sry* in mammals or *Dmy* in medaka). **B**, Subsequent acquisition of a temperature-sensitive mutation in a female gene (e.g., *aromatase*) that increases expression or activity at a high temperature, may override the male factor and shift the balance towards the female pathway. **C**, At intermediate temperatures, the pathways will remain balanced, so that males and females are produced stochastically at equal frequencies. **D**, At a low temperature, the mutated female factor will be far from its thermal optimum, activity will drop, and the balance will shift towards the male pathway. Depending on the strength of the determining factor, the system may be more or less stable to perturbation by the appearance of new mutations. Selective pressure may favor changes that maintain a balanced sex ratio.

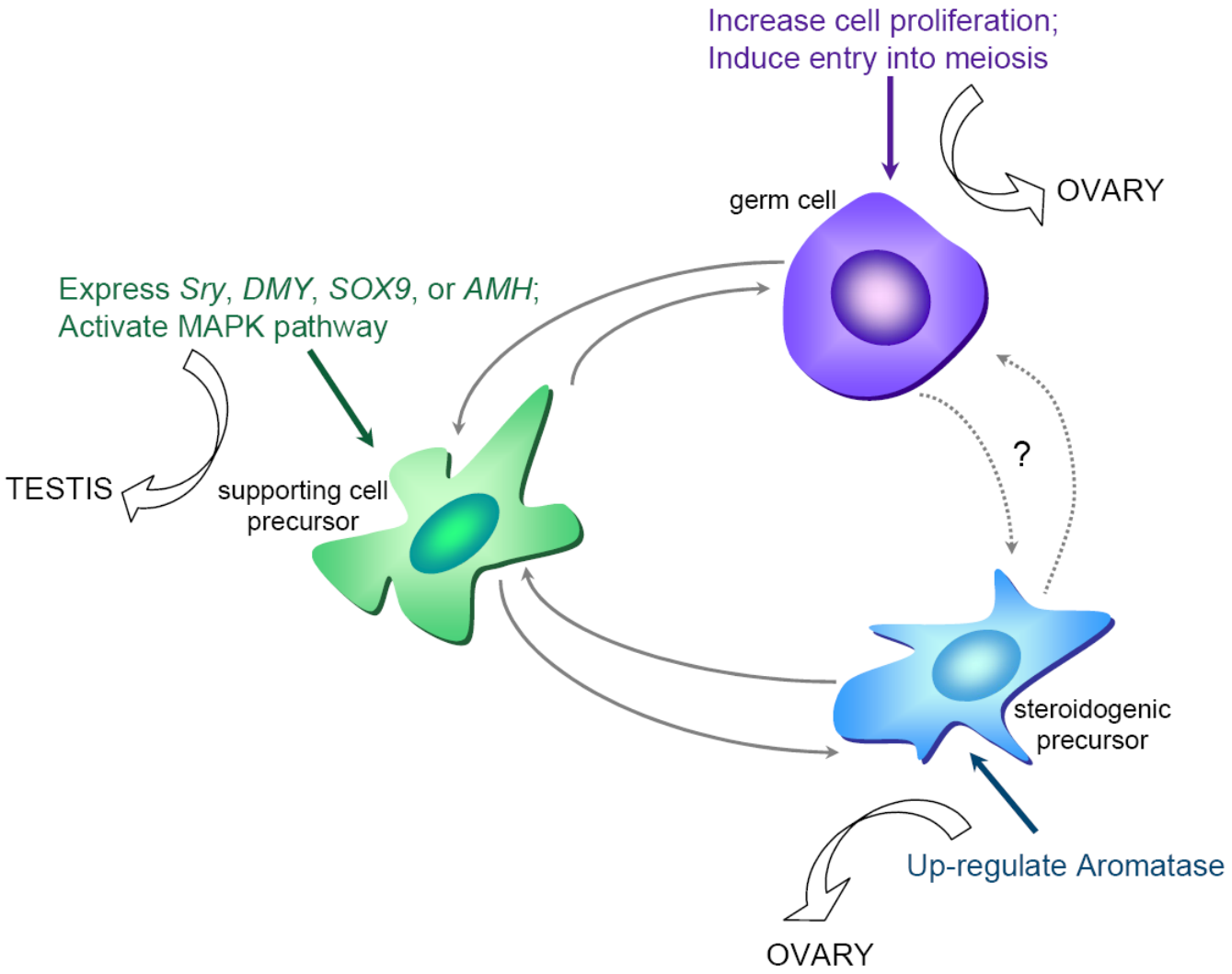

Figure 3.

The decision to develop as a testis or ovary may originate in distinct gonad cell types in different vertebrates. For example, *Sry* is expressed in mammalian supporting cell precursors, leading to their differentiation as Sertoli cells, which then direct germ cells to enter mitotic arrest and steroidogenic precursors to differentiate as Leydig cells [17]. This cascade of cell-cell interactions and differentiation might also be initiated by expression of other male factors in the supporting cell precursors [12,43,53,54]. Alternatively, in other vertebrates, the decision might be made in other cell lineages. Upregulation of *aromatase*, a steroidogenic enzyme that converts testosterone to estrogen, in the steroidogenic precursors may allow these cells to induce ovarian development by a hormonal mechanism [reviewed in 55]. Similarly, mitotic proliferation of germ cells or entry into meiosis [56] could induce changes in the steroidogenic and supporting cell precursors that together initiate adoption of the female fate. Solid and dotted arrows represent demonstrated and putative cell-cell interactions, respectively.