Advanced Review

The development of sexual dimorphism: studies of the *Caenorhabditis elegans* male

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Studies of the development of the *Caenorhabditis elegans* male have been carried out with the aim of understanding the basis of sexual dimorphism. Postembryonic development of the two *C. elegans* sexes differs extensively. Development along either the hermaphrodite or male pathway is specified initially by the X to autosome ratio. The regulatory events initiated by this ratio include a male-determining paracrine intercellular signal. Expression of this signal leads to different consequences in three regions of the body: the nongonadal soma, the somatic parts of the gonad, and the germ line. In the nongonadal soma, activity of the key Zn-finger transcription factor TRA-1 determines hermaphrodite development; in its absence, the male pathway is followed. Only a few genes directly regulated by TRA-1 are currently known, including members of the evolutionarily conserved, male-determining DM domain Zn-finger transcription factors. In the somatic parts of the gonad and germ line, absence of TRA-1 activity is not sufficient for full expression of the male pathway. Several additional transcription factors involved have been identified. In the germ line, regulatory genes for sperm development that act at the level of RNA in the cytoplasm play a prominent role.

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INTRODUCTION

Like most animal species, nematodes have two sexes, male and female, and reproduce sexually. Research focused on the male of *Caenorhabditis elegans*, the extensively studied genetic model nematode, has provided important insights into the common process whereby the genome specifies not one body plan and sexual form but two, and chooses for each individual one of two alternative developmental pathways. In the case of *C. elegans*, an androdioecious species, the two sexes are male and hermaphrodite. But the hermaphrodite is really a modified female that generates sperm during a brief period in development. With these sperm, it can produce about 300 self-progeny. However, when mated with a male, the male sperm preferentially fertilize the oocytes and the hermaphrodite can produce many more progeny.[1]

Collections of *C. elegans* from the wild yield predominantly selfing hermaphrodites, but outcrossing does occur and the genomes of most *C. elegans* strains carry the genes necessary for development of fully functional and fertile males.[2,3] These observations suggest that the male sex is maintained by natural selection.[3,4] Indeed, experiments have shown that a small amount of outcrossing appears to provide a selective advantage in variable and challenging environments.[5] Among species of the genus *Caenorhabditis*, *C. elegans* is unusual, though not unique. Most species of the genus are gonochoristic, having females and males.[6] Gonochoristic and androdioecious species have been found living together on a single rotting fruit.[7] What the particular selective force is that explains why androdioecy occasionally arises and persists (though probably not for long) is not established, but may have to do with an advantage in colonizing patchy resources.

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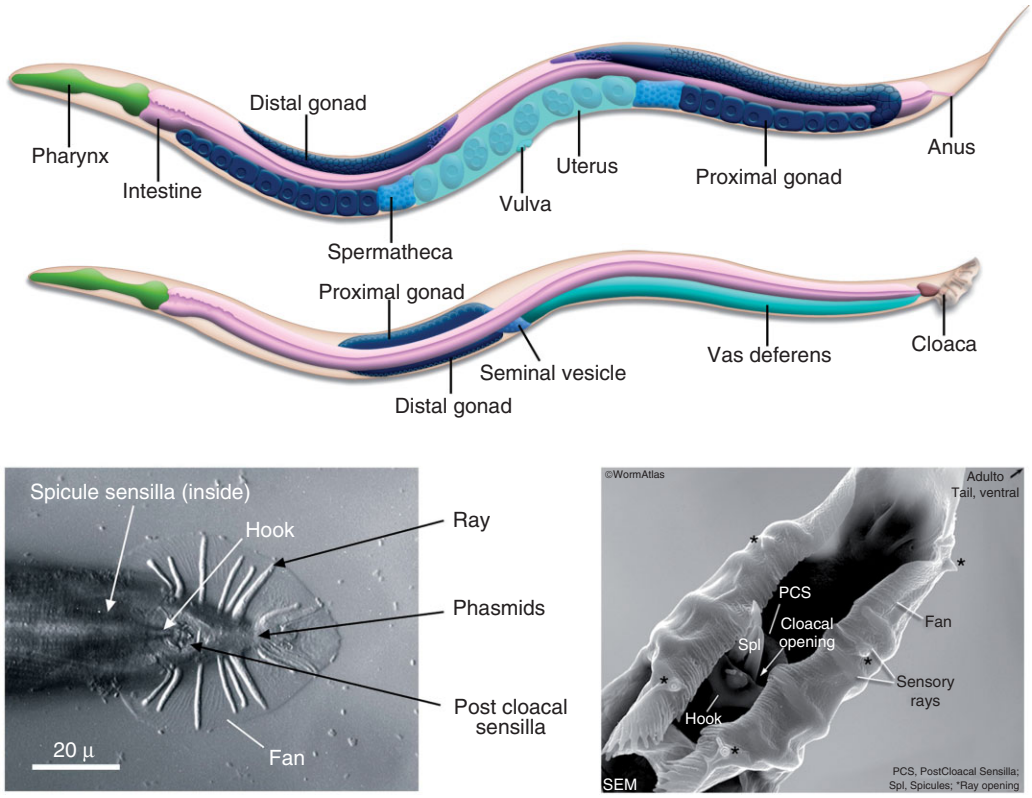

FIGURE 1 | The two adult sexual forms of *Caenorhabditis elegans*, hermaphrodite (top) and male. The male body is smaller and thinner, has specialized copulatory structures in the tail with male-specific muscles that control them, and expresses sex-specific behaviors, most notably copulation. The hermaphrodite gonad has two U-shaped, reflexed arms, arrayed symmetrically about the central vulva, whereas the single-armed male gonad opens at the cloaca. In both sexes, germ line stem cells reside in the distal gonad. In the hermaphrodite, mature oocytes in the proximal gonad are fertilized as they pass through the spermatheca and embryos begin to develop in the uterus. In the male, sperm mature in the proximal gonad, and are stored in the seminal vesicle before ejaculation in fluids generated by the vas deferens. Bottom two images: the male tail is specialized for copulation. Left: Nomarski image, ventral view, showing the five types of sensilla. The fan is an acellular fold of the outer layer of the cuticle. Right: Scanning EM, ventral view. The spicules, structures that are inserted into the hermaphrodite vulva during mating, can be seen protruding from the cloaca. (Source: http://www.wormatlas.org)

The self-fertilizing capability of the hermaphrodite has facilitated genetic studies focused on this sex.[8] However, with an eye toward understanding sexual dimorphism as well as the genetic specification of behavior, the *C. elegans* male has been studied alongside the hermaphrodite from the earliest days of *C. elegans* research.[9,10] John Sulston and coworkers determined the postembryonic cell lineages and described the ultrastructural anatomy of the male-specific structures and tissues.[11,12] Jonathan Hodgkin examined the effect on the male of the many mutations that had been isolated up to that time in studies of the hermaphrodite and began the isolation of mutations focusing specifically on the male, identifying genes denoted *mab*, for male abnormal.[13] The connectivity of the male neural circuits that govern mating behavior has been described, and reconstruction of the head circuitry has recently been completed, yielding the complete wiring diagram of the male nervous system to go along with that of the hermaphrodite[14,15] (this laboratory, unpublished). Thus male anatomy and development is as fully described as that of the hermaphrodite.

These studies have documented many and extensive differences between the two sexual forms, but the list no doubt remains incomplete (Figure 1). Of course, a major aspect of the male is differentiation of germ cells exclusively as sperm and development of the somatic structures of the gonad to support this process and expel sperm-containing seminal fluid through the cloaca during mating. But in addition, the male body is somewhat smaller and thinner than the hermaphrodite, has specialized copulatory structures in the tail with male-specific muscles that control them, and expresses sex-specific behaviors that go well beyond copulation. The sex-specific characteristics of both sexes arise primarily during postembryonic development, embryonic development appearing to

be nearly identical in the two sexes. Postembryonic somatic cell lineages and the resulting cellular composition of the body differ (including an additional 89 neurons in the male), common cells differentiate in a sex-specific manner, common tissues express different genes, neurons wire up differently and support novel behaviors. A detailed description of male anatomy is available at WormAtlas.org. Previous reviews that cover development include Hodgkin,[9] Emmons and Sternberg,[16] Emmons,[17] Portman,[18] and Wolff and Zarkower.[19]

SPECIFICATION OF MALE FATE BY THE SEX DETERMINATION PATHWAY

Sexual differentiation is one of those processes common enough in biology, examples being metamorphosis in many species and, in worms, choice of alternative larval forms, that requires a large proportion of the cells in the body to make a uniform binary developmental choice. An advantage in unraveling this process in the case of sexual differentiation is that the primary signal is often known. In *C. elegans*, sex is determined by the ratio of autosomes to sex chromosomes: one-to-one dictates hermaphrodite choice (naturally AA, XX), two-to-one dictates male choice (naturally AA, XO).[20,21] How this ratio inaugurates pathways leading to specification of sexual phenotype has been studied for many years by a number of laboratories and elucidated in considerable molecular detail.[22–24]

A crucial step is expression by cells with AA, XO genotype of a secreted protein, HER-1. Binding of HER-1 to its receptor TRA-2 dictates male cellular fate by preventing a feminizing activity of TRA-2. In *tra-2* loss-of-function mutants, AA XX individuals are transformed toward male development, while in wild type, AA, XX individuals, which lack HER-1, the feminizing activity of TRA-2 results in hermaphrodite development.

HER-1/TRA-2 binding is both necessary and sufficient for male fate at the single-cell level. In an animal mosaic for *her-1(+)*, a cell of *her-1(−)* XO genotype can nevertheless take male fate due to *her-1(+)* expression in other cells.[25] This paracrine mechanism is no doubt responsible at least in part for insuring that a uniform developmental choice is made by all the tissues of the body. However, the HER-1/TRA-2 interaction is not completely determinative. When the feminizing activity of *tra-2* is eliminated by mutation, an animal of AA, XX chromosomal composition is only incompletely transformed to a male, while an animal of AA, XO chromosomal composition is fully male.[26] The explanation for this observation, suggesting that the X to autosome ratio can influence sexual phenotype even in the absence of the TRA-2 receptor, is unknown.

The intracellular events downstream of TRA-2 differ in three regions of the body—the nongonadal soma, the somatic parts of the gonad, and the germ line (Figure 2). In the nongonadal soma, HER-1 binding to TRA-2 results in the ubiquitin pathway-mediated degradation of TRA-1, a Zn-finger transcription factor, the protein product of the *tra-1* gene, which is transcribed in both sexes.[27–29] For the somatic body, TRA-1 is the single ultimate necessary and sufficient feminizing activity downstream of TRA-2. TRA-1 activity results in hermaphrodite somatic development, while absence of TRA-1 activity results in male somatic development. Mutation of *tra-1* results in the complete masculinization of the nongonadal somatic tissues of an XX individual. Thus, the sex determination pathway exerts its influence on differentiation of the nongonadal somatic tissues through the transcriptional regulation of subsets of genes.

This *C. elegans*, indeed nematode, somatic sex-determination pathway appears to be a derivative of the hedgehog signaling pathway.[30] In other animals, the hedgehog pathway plays a widespread role in developmental patterning of tissues. TRA-1 is the *C. elegans* homolog of Ci/GLI-1, the Zn-finger transcription factor regulated by the hedgehog pathway, whereas TRA-2 is related to the hedgehog receptor Patched.[27,31,32] Like Ci/GLI and Patched, both TRA-1 and TRA-2 are present in multiple protein isoforms that are subject to complex pathways of proteolytic processing and degradation.[28,32,33] The HER-1 ligand, while not homologous to hedgehog or sonic hedgehog, is a protein of similar size (175 aa for HER-1; 174 aa for sonic hedgehog).

Sexual systems are highly derived and widely varied among species of both plants and animals. Their evolution is rapid and intimately involved in the process of evolution and speciation itself. A possible explanation for the diversity of sexual systems is that they have evolved independently to regulate a primordial mechanism originally consisting of a single, dominant sex determining, possibly male-determining, gene.[34] In the case of nematodes, it appears that the hedgehog pathway has been recruited for this purpose and its role in pattern formation dispensed with. Possible candidates for male-determining factors regulated by the sex-determination pathway through TRA-1 are discussed below.

The flexibility of sexual differentiation illustrated by this origin of the *C. elegans* sex determination pathway may help us to understand sex

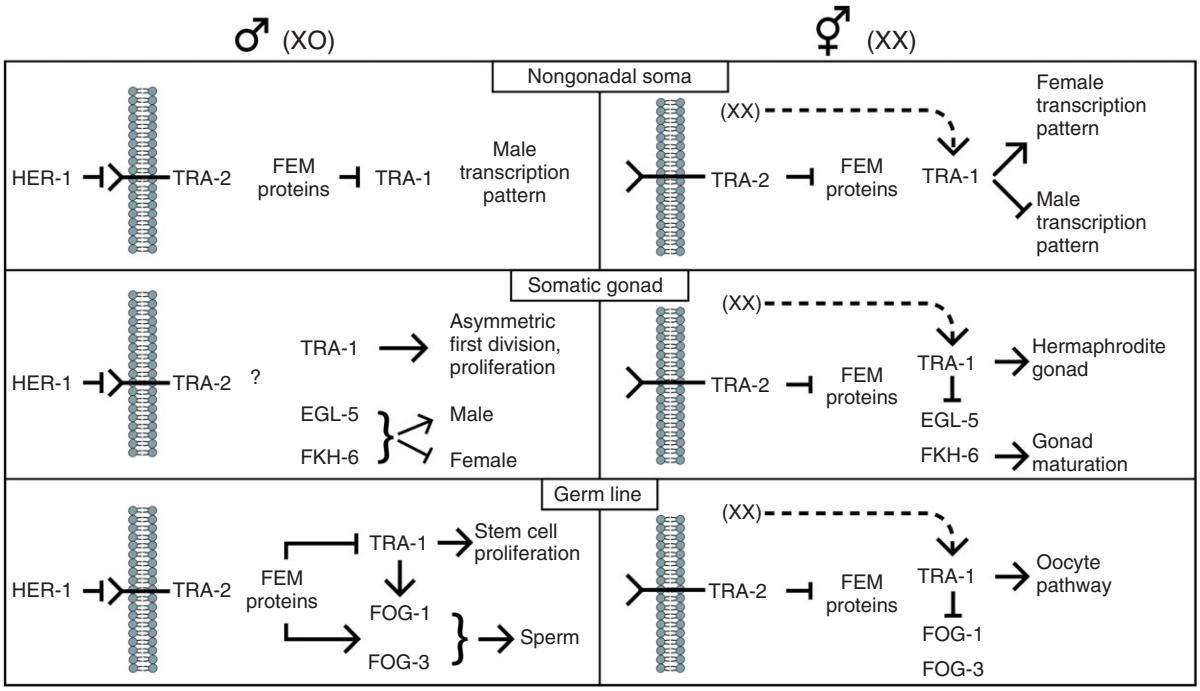

FIGURE 2 | Events in sex determination differ in three parts of the body: the soma apart from the gonad, the somatic parts of the gonad, and the germ line. In each region, HER-1 binding to the TRA-2 receptor dictates male fate. In the somatic body, this results in elimination of the activity of the female-pathway determining Zn-finger transcription factor TRA-1 by the ubiquitin pathway *fem* genes. In XX individuals, TRA-2 inhibits the FEM proteins. In addition, an unknown signal possibly independent of the HER-1/TRA-2/*fem* pathway promotes some TRA-1 activity (the dotted line). In the somatic parts of the gonad, similar events occur, but TRA-1 retains some activity in the male and additional transcription factors contribute to male versus hermaphrodite pathways. In the germ line, two cytoplasmic proteins, FOG-1 and FOG-3, are essential for sperm differentiation and require the FEM proteins for their activity. In the adult male, TRA-1 promotes FOG-1 and FOG-3 activity for continued sperm production. For further details of these pathways, see text.

determination in the remaining two regions of the *C. elegans* body, the somatic parts of the gonad and the germ line (Figure 2(b) and (c)). In these regions, sex determination is less sharply delineated from other developmental processes. While TRA-1 still functions and tends to dictate female fate, its state of activity is not completely determinative—*tra-1* null mutants are not fully sex-transformed in these tissues. TRA-1 is required for early cell patterning events in the cell lineages of the somatic gonad in both sexes. Meanwhile, additional transcription factors participate in dictating male versus female pathways. Finally, in the germ line, cytoplasmic regulatory events play a more prominent role in determining gene activity patterns. Two genes at the top of the hierarchy for sperm development, *fog-1* and *fog-3*, function in the cytoplasm at the level of mRNA and translation. However, TRA-1 still retains a role. It is required for proliferation of germ line stem cells in both sexes, and during later adulthood it dictates male fate, the continuous differentiation of germ cells as sperm rather than oocytes. These pathways, outlined in greater detail below, are still incompletely understood.

SEXUAL DIFFERENTIATION OF THE NONGONADAL SOMA

Sex-Specific Differentiation of Cells Present in Both Sexes

Sex-Specific Gene Expression in a Tissue: the Gut; Discovery of the DM Domain Transcription Factor Family

While mutations affecting the so-called global sex determination pathway upstream of and including *tra-1* uncouple sexual fate from chromosomal composition and affect the sexual differentiation of the entire animal, mutations in genes acting downstream of *tra-1* may affect the characteristics of only a single tissue or subset of tissues. This consideration led to a focus on a gene required to prevent expression of yolk protein by the male gut, a gene expression pattern appropriate to the hermaphrodite.[35,36] Cloning of this gene, *mab-3*, revealed that it is homologous to a gene known from studies of sex determination in *Drosophila*, *doublesex* (*dsx*).[37] *Drosophila dsx* also regulates yolk protein expression, and both *mab-3* and *dsx* have additional

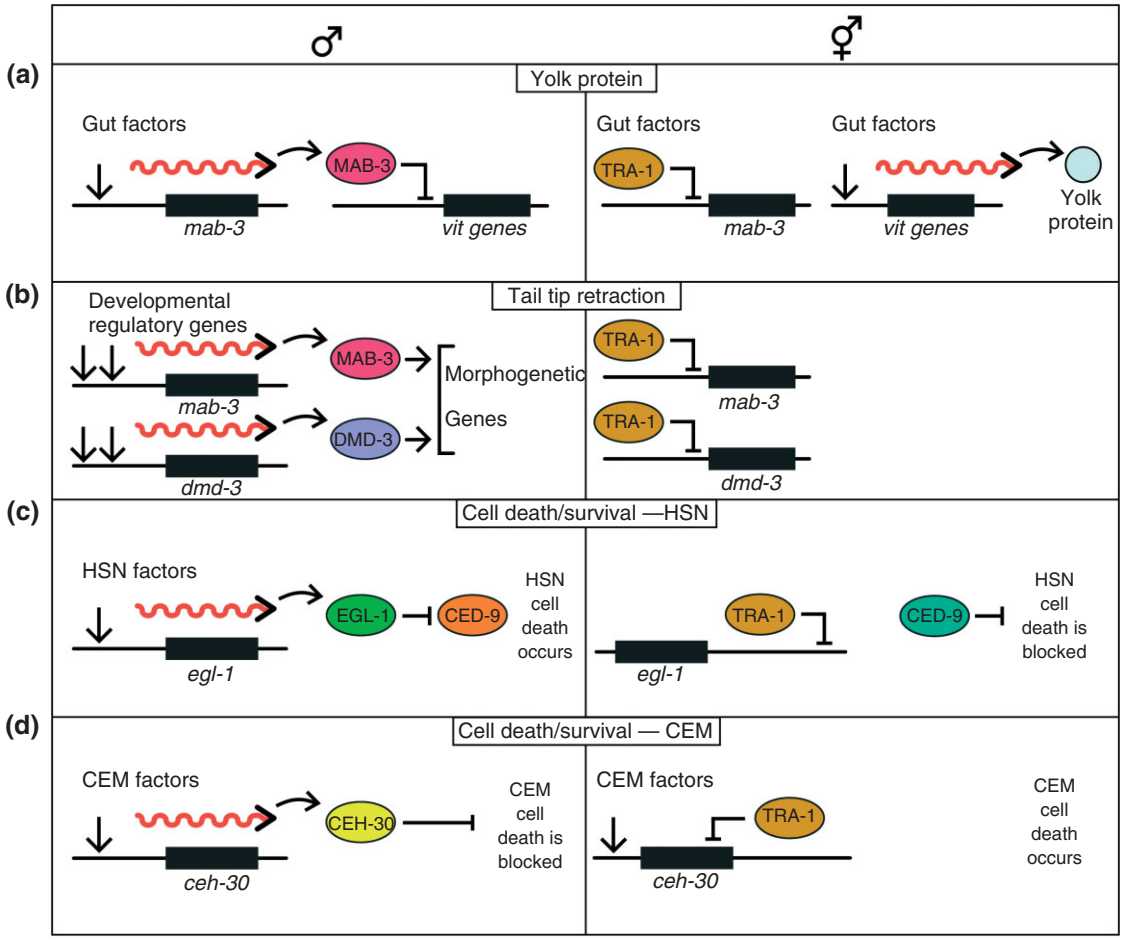

FIGURE 3 | Four cases where regulatory transcription factors specifying male differentiation have been identified. Transcription of these genes is repressed in hermaphrodites by TRA-1 DNA binding upstream (*mab-3*, *dmd-3*), downstream (*egl-1*), or within an intron (*ceh-30*).

effects on sexual differentiation, including the sexual fate of certain neuroblasts.

Before this finding, the genes identified in sex determination pathways were always unique to particular species—this was the first evidence of a conserved gene involved in specifying sexual fate. Subsequent studies revealed that *mab-3* and *dsx* are two representatives of a gene family, named the DM domain family after a DNA-binding Zn-finger motif shared by DSX, MAB-3, and the other family members. The DM domain family is now thought to play a role in sexual development, predominantly male development, throughout metazoa.[38] Possibly it comprises the descendants of the postulated primordial male-determining gene mentioned above.[34]

The mechanism by which *mab-3* controls yolk protein gene expression has been determined and is straightforward (Figure 3(a)). In males, MAB-3 is expressed in the gut, binds sequences in the promoter regions of vitellogenin (yolk protein) genes, and represses their transcription.[39] In hermaphrodites, transcription of *mab-3* in the gut is repressed by TRA-1 binding at a site in its promoter, thus allowing vitellogenin transcription and yolk protein production to proceed.[40] Thus a complete chain of molecular events has been established between the X/A ratio and this sex-specific phenotype.

Male-Specific Morphogenesis of a Tissue: the Hypodermis

The most evident distinction between a *C. elegans* male and hermaphrodite, apart from the gonad, involves the morphology of the body, which is thinner and somewhat smaller in the male and highly modified in the tail (Figure 1). The tail copulatory structures are formed in a morphogenetic episode at the end of the last larval stage that results in fusion and retraction of four tapering tail tip hypodermal cells, a general anterior movement of cells, the generation of an acellular fan (a fold in the outer layer of the cuticle) containing sensory structures known as rays, and additional changes to the hindgut region to form the cloaca and

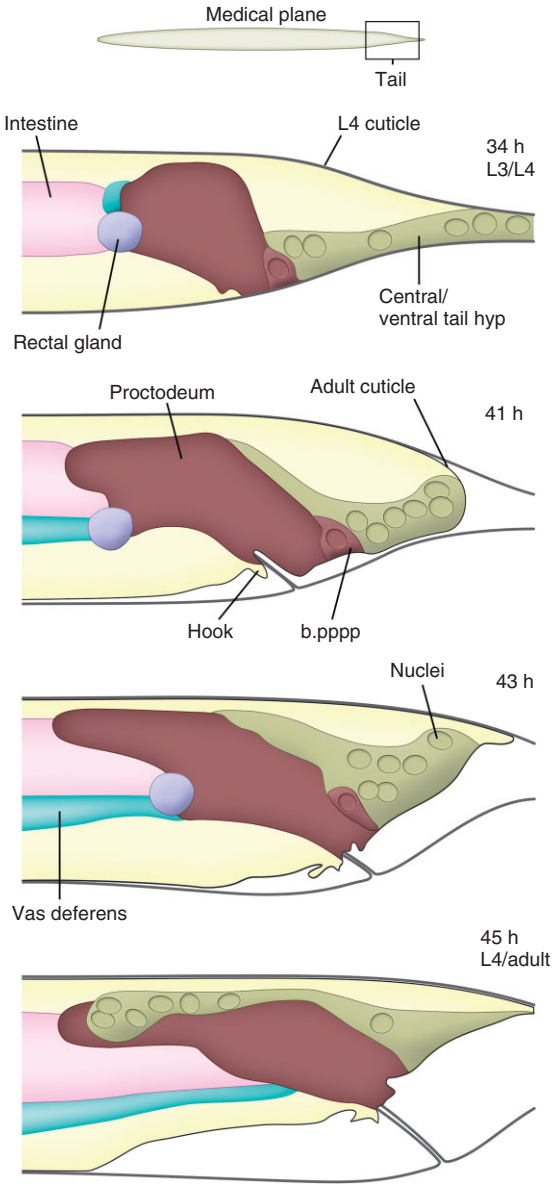

FIGURE 4 | Morphogenesis of the male tail during L4. This medial section shows the movement of the nuclei of the tail tip hypodermal cells as the cells fuse and move anteriorly. At the same time the rays extend in the acellular fan (a fold in the outer cuticle), the rectal epithelium forms the proctodeal chamber and spicules, and the extending vas deferens connects to the proctodeum. Elongation of the spicules requires their muscle attachments, whereas general retraction of the body is driven in part by pressure in the liquid-filled space that increasingly separates the nascent adult cuticle from the overlying L4 cuticle[11] (S.W.E unpublished). (Source: http://www.wormatlas.org)

the chamber known as the proctodeum, containing the spicules[11,41] (Figure 4).

While some of the cells involved in this morphogenetic event arise from male-specific cell lineages (see below), others, including the four hypodermal cells of the tail tip, are present in both sexes. Fusion and retraction of the tail tip cells have been studied in some detail. Two DM domain genes cooperate to orchestrate this process, along with the overall anterior withdrawal of additional cells in the tail.[42] In *dmd-3* mutants, the tail tip cells do not fuse or retract, resulting in a leptoderan tail, so-named because of the similarity to leptoderan nematode species having males with pointed, unretracted tail tips. In *dmd-3*; *mab-3* double mutants, the morphogenetic defect is enhanced, so that the male tail retains an overall morphology nearly identical to that of the hermaphrodite.

mab-3 and *dmd-3* are expressed in the tail tip hypodermal cells in the male only. As with *mab-3* in the gut, in the hermaphrodite *dmd-3* expression is directly repressed by TRA-1 binding to the promoter (Figure 3(b)). If the TRA-1 target site in the *dmd-3* promoter is removed, *dmd-3* is expressed in the hermaphrodite and the tail tip undergoes retraction. Thus *dmd-3*, playing the major role, together with *mab-3* controls remodeling of the tail tip.

Consistent with this central role, an extensive analysis of additional genes involved in tail tip retraction places *mab-3* and *dmd-3* at the center of a regulatory network with 'bow tie' architecture.[43] Multiple regulatory pathways conveying the various developmental coordinates—cell type, time, position, sex—converge on the DM genes. These in turn divergently regulate multiple genes that bring about the morphological changes—genes involved in vesicular trafficking, membrane fusion, and rearrangement of the cytoskeleton (Figure 3(b)).

The DM Domain Transcription Factor Family in C. elegans

The *C. elegans* genome contains eight additional DM domain family genes, only one of which has been studied in detail, *mab-23*. This gene too has a male-specific function. One allele of *mab-23* was identified in a wild population of worms that gave abnormal (Mab), infertile males, and a second was recovered in a screen for misexpression of dopamine by male ray sensory neurons.[2,44] In addition to this neural defect, *mab-23* mutants have defects in several male structures, including the male-specific diagonal muscles and attachment of the gonad to the proctodeum, but no apparent defects in hermaphrodites. All of these defects are shared with *dmd-3* mutants, and *mab-23 dmd-3* double mutants are additive, expressing the most severe defects of each single.[45] Thus, just as with tail tip retraction, two DM domain transcription factors cooperate to generate these male-specific structures.

Despite having no observable function in hermaphrodites, *mab-23* is expressed in both sexes

in several tissues (several neurons in the head, ventral body wall muscles, phasmid neurons, hindgut, and tail spike) and therefore is apparently not regulated by TRA-1. Moreover, it is expressed in hermaphrodite-specific cells involved in egg laying (ventral uterus, spermatheca, and HSN neurons), although there is no evident egg-laying defect. If *mab-23* has any function in hermaphrodites, it could be redundant with another gene, possibly another DM domain family member. Alternatively, other factors present only in the male might be necessary for *mab-23* activity to have any effect, a possible alternative explanation discussed further below.

If the DM domain family of proteins is indeed descended from a primordial male-determining gene, it might be expected that the eight additional family members in the *C. elegans* genome would also be involved in aspects of male development, a possibility so far unexplored. Apart from genes that encode proteins unique to sperm and semen, the DM domain transcription factor family is the only known family of genes that appears, at least so far, to have a role uniquely dedicated to specifying male characteristics. With these exceptions, male-specific development utilizes the same developmental toolkit as hermaphrodite development. This commonality of gene function has been seen when the transcriptomes of the two sexes are compared: very few genes with sex-limited expression have been found so far.[46–48] However, neither the male transcriptome nor male genetics has been thoroughly explored. There are many genes observed to have no obvious phenotype in global RNAi screens. As these screens only examined the hermaphrodite, these genes could have functions limited to the male.

Male-Specific Differentiation of Shared Neurons

Along with the gut and hypodermis, additional examples of sex-specific differentiation of cells present in both sexes are found within the nervous system. The behavior of *C. elegans* males and hermaphrodites differ in many ways. Copulation itself is primarily the responsibility of the male, hermaphrodites being largely indifferent or even resistant.[49,50] Additional sex differences affect general behaviors not directly related to copulation. Males swim with tighter and faster body bends. In the absence of hermaphrodites, adult males, apparently in order to find mates, will leave a food source to explore their environment, something adult hermaphrodites and juveniles of both sexes almost never do.[51] The two sexes respond differently to pheromones and make different choices in olfactory-preference tests.[52–58]

While each sex has its own exclusive set of neurons, six in the hermaphrodite controlling the vulva and egg laying and 89 in the male involved in mating and pheromone sensing, some of the sex-specific differences in general behaviors have been traced to sex-specific properties of neurons in the common set of 294 neurons. Differential responses to odorants and pheromones, even including male-specific attraction to hermaphrodite pheromones, have been traced to the functions of common sensory neurons in the head.[52,54–56,58] Two genes encoding proteins of the serpentine receptor class are expressed in shared sensory neurons in the male only.[18,59] Such sex-specific expression of chemoreceptors could underlie some of the observed differences in chemotactic behavior.

Reconstruction of synaptic connectivity in the male head has revealed that sex-specific differences in general behaviors do not come about through extensively different wiring. Connectivity among head neurons, including the circuits involved in navigation, is nearly identical in the two sexes (this laboratory, unpublished). The situation is different in the male posterior nervous system. Examples of extensive remodeling of shared neurons and neuronal connectivity are found in the tail ganglia. In the male tail, 144 neurons create the circuits that control copulation.[14] Of these, 81 are neurons present only in the male, while the remaining 63 neurons are present in both sexes. The shared sets are intimately involved along with the male-specific neurons in creating the neural network that guides mating. In this role, they engage in many more synaptic connections in the male than they do in the hermaphrodite. Some grow out extensive new branches in order to engage with other neurons in the network (Figure 5(a)). One prominent pair of interneurons changes its role entirely, having a completely different set of synaptic partners in each sex (Figure 5(b)).

Are these sex-specific phenotypes of shared neurons dictated cell autonomously by *tra-1* activity in the same manner as differentiation of other somatic cell types? Initial results in explorations of this issue are consistent with this conclusion. By manipulating the sex-determination pathway in individual cells it is possible to change the activity state of *tra-1* independently of chromosomal composition.[54] Using this approach, Lee and Portman[52] showed that a hermaphrodite in which head sensory neurons were transformed to the *tra-1 OFF* male state displayed male-specific olfactory preferences. White and Jorgensen[58] have shown that the growth factor TGFβ prevents hermaphrodite attraction to a hermaphrodite pheromone. Expression

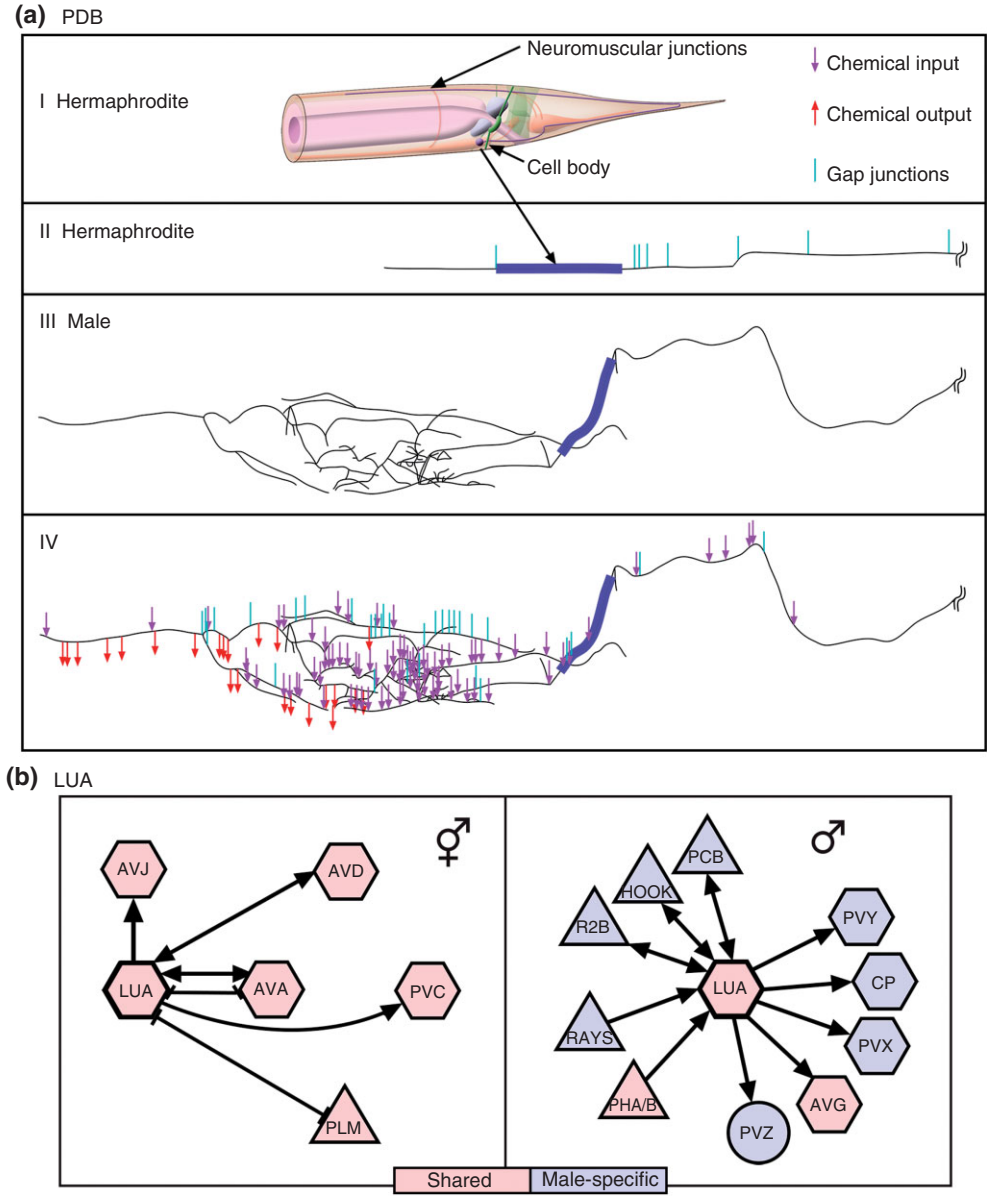

FIGURE 5 | Examples of sexual dimorphism of shared neurons in the preanal ganglion. (a) PDB. I. PDB is a motor neuron with cell body in the preanal ganglion and a process that runs around to the dorsal side and makes neuromuscular junctions with dorsal body wall muscles. II. In the hermaphrodite preanal ganglion, PDB makes a small number of gap junctions with other neurons. III. In the male, the anterior process from the cell body is extensively grown out and branched. IV. The male-specific branches synaptically interact with other neurons in the male mating circuits. PDB is likely to be involved in controlling the ventrally-arched posture of the male posterior region during mating. (b) LUA. In the hermaphrodite, the LUA interneuron likely functions in the posterior touch circuit, as it connects the PLM touch neuron to the locomotion command interneurons AVA, AVD, and PVC (Chalfie et al., 1985). In the male, LUA plays a similar role mediating connectivity between male-specific sensory neurons and interneurons (see Ref 14).

of TGFβ occurs in both sexes. For a male to escape its action, allowing attraction, both sensory neurons and their interneuron targets in a chemotaxis circuit must have male fate. In all these examples of sexual dimorphism of neurons, the targets of *tra-1* action, and whether they include one or more members of the DM domain family, are not yet known.

Sex-Specific Cell Fate: Programmed Cell Death

In two cases, one in the hermaphrodite and one in the male, differences between the male and hermaphrodite nervous systems come about through sex-specific cell deaths. Numerous cells in the *C. elegans* embryonic and postembryonic cell lineages undergo programmed cell death, which is considered a specific cell fate.

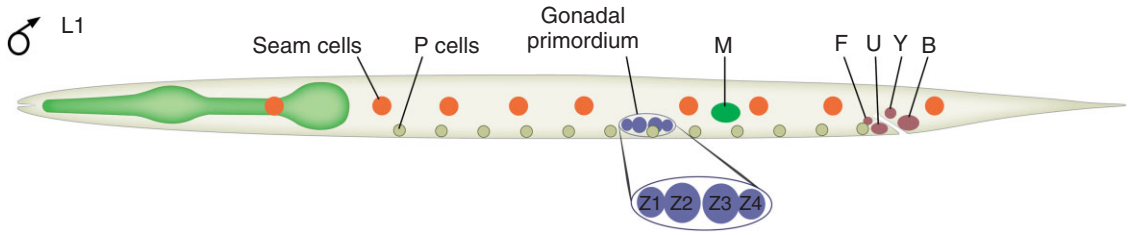

FIGURE 6 | The five classes of postembryonic blast cells in the L1 larva. The bilateral rows of nine hypodermal seam cells (H0, H1, V1–V6, T) give new hypodermal cells and a sensory structure, the postdeirid; in the male, also the rays. The single row of ventral P cells (P1–P12) give rise to sex-specific motor neurons as well as sexual structures—the vulva in the hermaphrodite, the hook in the male. Hypodermal B, Y, U, and F divide only in the male, giving many sex-specific structures and neurons, including spicules, gubernaculum, and proctodeal chamber. The mesoblast cell M gives body wall muscles and sex-specific muscles in both sexes. Z1 and Z4 generate the somatic structures of the gonad; Z2 and Z3 are the primordial germ cells. (Source: http://www.wormatlas.org)

Thus sex-specific cell deaths represent sex-specific assignment of cell fate. In both examples, the action of *tra-1* has been studied. In these instances it does not involve a DM protein.

In the first of these examples, two cells destined to become hermaphrodite-specific neurons (HSN), serotonergic motorneurons that innervate muscles controlling the vulva, die during embryogenesis in the male. This occurs because male HSN cells express the pro-apoptotic gene *egl-1*. *egl-1* causes cell death by inhibiting the antiapoptotic BCL-2 protein CED-9. In the hermaphrodite, transcription of *egl-1* in the HSN's is repressed by TRA-1, and so these cells survive[60] (Figure 3(c)). Repression is via direct binding of TRA-1 to a regulatory site 5.6 kb 3′ of the *egl-1* transcriptional unit.

In the second example, four cells destined to become the CEM head sensory neurons in the male die in the hermaphrodite. In this case, the situation is similar but reversed—the target of TRA-1 repression is an antiapoptotic gene, *ceh-30*[61,62] (Figure 3(d)). In hermaphrodites, TRA-1 binds a sequence within a *ceh-30* intron to inhibit its expression, allowing cell death. Expression of *ceh-30* in the male CEM's inhibits cell death and allows their survival. It might be thought that *ceh-30* would protect CEM's by blocking the action of *egl-1*, but this is not the case. Survival of cells in the absence of *egl-1* function generally requires *ced-9*, as it does in the case of the HSN's, but Schwartz and Horvitz[61] concluded that the survival of the CEM's in the male does not. Either the *ced-9* alleles used retained residual activity, or there is a second cellular pathway for regulation of cell death.

These diverse modes of regulation of the sex-specificity of cell death illustrate the flexibility with which the TRA-1-mediated somatic sex determination pathway can target cellular functions other than DM domain family genes to bring about sex-specific differentiation.

Male Structures Generated by Male-Specific Cell Lineages

Many adult cells and structures in both sexes are generated postembryonically from cell divisions that occur throughout larval development (Figure 6). Some of these cell lineages occur in both sexes but are sex-specifically modified. Others are unique to one sex or the other. The vulva, for example, is generated from cell lineages that occur only in the hermaphrodite. The most significant male-specific somatic structures arise both by modification and in some cases extensive elaboration of cell lineages that occur in both sexes, and also by unique lineages generated by blast cells that divide only in the male.

Events that initiate these sex-specific cell lineages are clearly under the control of *tra-1*, but at precisely which points has in most cases not been investigated in detail. Once a sex-specific developmental program has been initiated, subsequent events utilize the same myriad of factors that function in diverse combinations throughout development in both sexes: heterochronic genes that specify developmental time, Hox genes that pattern cell fates along the anteroposterior body axis, neurogenic genes that define neuronal fate, cell signaling pathways such as the EGF, TGFβ, *lin-12*/Notch and Wnt pathways that influence cell fates according to their positions and neighbors. After a developmental program is initiated in one sex only, it is not clear whether *tra-1* has any further role to play.

Rays

The rays provide examples of male-specific structures that arise from sex-specific modification of common cell lineages. Each of the 18 rays consists of the processes of two sensory neurons of different types (designated type A and type B) along with the process of a glial-like structural cell that surrounds the sensory endings and attaches the structure to an opening in the cuticle and a hypodermal sheath. Each set of three

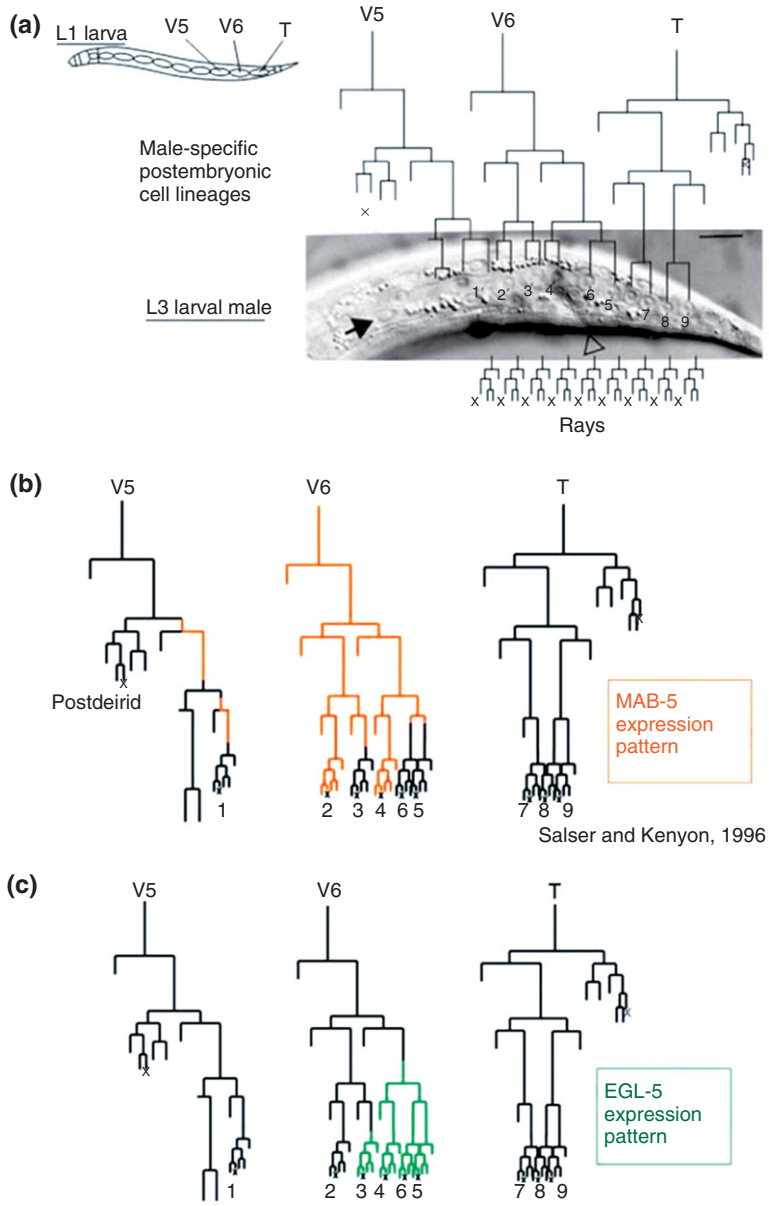

FIGURE 7 | The postembryonic cell lineages leading to the rays. Each ray has a unique identity affecting morphogenesis, neurotransmitter and neuropeptide expression. The identities of rays 1–6 are defined by expression of the Hox genes *mab-5* and *egl-5*. In the absence of EGL-5, *mab-5* expression is not selectively turned off and all the V6 rays take the identity of ray 2.[63]

ray cells is generated by a seam cell specified as a ray precursor cell. This cell initiates a stereotyped neurogenic cell sublineage during the L3 larval stage (Figure 7(a)).[11]

Sexual dimorphism in the seam cell lineages begins well before specification of the ray precursors themselves. A key factor resulting in ray generation in the male is the Hox gene *mab-5* (Figure 7(b)). Initially, *mab-5* is expressed in both sexes, but its expression is soon turned off in the hermaphrodite. How *mab-5* expression comes under TRA-1 regulation has not been established. *mab-5* expression, which undergoes complex regulation during the male V5 and V6 cell lineages, induces male-specific divisions within the V6 cell lineage, activates a second Hox gene, *egl-5*, required for generation of the ray precursor cell for ray 6 and for patterning ray differences, and induces expression during the L3 larval stage of the key proneural bHLH transcription factor gene *lin-32* (Figure 7(c)).[63–65]

lin-32 functions together with a second bHLH gene *hlh-2*.[66] *lin-32* is the homolog of *Drosophila atonal*, which initiates similar neurogenic sublineages in the fly, while *hlh-2* is the worm homolog of the ubiquitous bHLH cofactor gene E/*daughterless*. When *lin-32* is expressed during L2 in the V5 lineage in both sexes, it initiates generation of a related sensory structure, the postdeirid. Only in the male seam is *lin-32* expressed during L3, and this initiates the ray sublineage.

Interestingly, if L3-fates are expressed during the second larval stage, in a precocious *lin-28* heterochronic gene mutant background, the sublineage generated at the normal position of the postdeirid appears to be a ray sublineage.[67] *lin-28* is a conserved, cytoplasmic, RNA-binding protein required for events specific to the second larval stage. Whether this unexpected sex-transformation occurs via regulation of *tra-1*, or by an independent pathway, is not known. Nor is it known what genes define the sex-specific characteristics of these sublineages.

The early events leading up to sex-specific ray development are not known to require DM domain family genes. However, these genes do play a role in development of the rays themselves. In the V5 and V6 lineages, *mab-3* is required along with *mab-5* for expression of *lin-32* by ray precursor cells, and hence for generation of rays 1–6. It apparently acts by repressing the bHLH gene *ref-1*, which in turn represses *lin-32*.[68] *mab-23* and *dmd-3* are required for patterning certain ray-specific properties, such as expression of dopamine and acetylcholine neurotransmitters.[44,45] Unlike their functions in regulating yolk protein expression or tail-tip retraction described above, their sex-specific action in this instance is not due to sex-specific expression. *mab-3* is expressed throughout the seams in both sexes, yet it promotes the generation of rays only in descendants of V5 and V6 and only in the male.[40] This illustrates how, as suggested above for *mab-23*, sex-specific function of a gene can arise not because of a sex-specific expression pattern but because of the sex-specific context in which expression occurs.

Although rays 7–9, which are generated by the posterior-most seam cell, T, arise from the same sublineage and have similar structure as rays 1–6, the V-rays, and require *lin-32* for sublineage expression, other aspects of their development differs. They do not require *mab-5* or *mab-3* and do require *mab-19*, a gene whose protein product remains unknown (Sutherlin and Emmons, 1994). Studies of the asymmetric divisions within the T lineage and their orientation lead to important initial elucidation of the role of the Wnt pathway in generation of an oriented, asymmetric cell division.[69] In the tail, an important orienting source of the Wnt signal is the tail tip cells.[70]

The P Cells

In a second example of sex-specific modulation of a postembryonic cell lineage, several male-specific neurons and the hook copulatory structure situated at the cloacal opening arise from a set of common postembryonic blast cells known as P cells (Figure 6). P-cell lineages contribute neurons and other neuronal and hypodermal structures in both sexes, but, as with the seam cells, division patterns and cell fates in certain lineage branches are sex-specific. Differentiation of the descendants of the P cells has been studied in some detail and illustrates nicely how developmental functions used by both sexes are deployed in sex-specific programs.

During the L1 larval stage, the P cells migrate from two bilateral rows into the ventral midline where they interdigitate to form a line of 12 cells denoted P1–P12, from anterior to posterior. During L1, P1–P12 divide once. The anterior daughter of this cell division, Pn.a, goes on in both sexes to generate a stereotyped cell sublineage that produces new body wall motor neurons. Certain details of these motorneuron sublineages differ along the anteroposterior body axis, and some of these details are sex-specific.[11,12] In addition, both anterior and posterior branches generate sex-specific cells (Figure 8). Patterning of the P-cell lineages along the anteroposterior body axis is specified by the Hox genes *lin-39*, *mab-5*, and *egl-5*.[71–74] The domains of Hox gene expression are determined by complex mechanisms and are dynamic.[64,75] The interface between

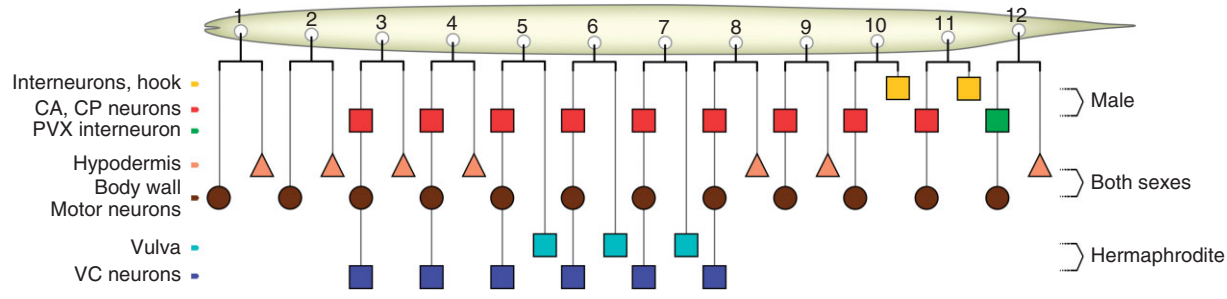

FIGURE 8 | Sex-specific cells generated by the P-cell lineages. CA and CP neurons innervate the gonad and body wall muscles, as well as each other and additional neurons; VC neurons innervate vulval muscles. While the details are different, note the similarities: in both sexes the anterior P-cell daughters generate neurons that control the genital structures, while more posterior daughters generate hypodermal cells that differentiate the genital opening.

the axial patterning genes and the sex determination pathway that endows the Hox genes with sex-specific expression patterns and downstream effects have not been explored. In the male, studies of the fate of the posterior-most cell, P12, showed that this requires *egl-5*, whose expression is induced by EGF and Wnt pathway signals.[76]

Together with P3.p, P4.p, and P8.p in the hermaphrodite and P9.p in the male, the vulval and hook precursor cells shown in Figure 8 form equivalence groups.[77] Cells in an equivalence group, as the name implies, have the same developmental potential. The possible alternative fates open to them are allocated by cell-cell interactions. The details of the genetic and molecular mechanisms underlying this process have been studied in great detail in the vulval equivalence group (reviewed in Sternberg[78]). These studies have made a major contribution to developmental biology, contributing to our understanding of how conserved developmental genes, including ras and additional components of the ras pathway, the EGF receptor, and *lin-12*/Notch, function in the assignment of cell fates. The vulva pathway includes an inductive EGF signal from another tissue, a *lin-12*/Notch pathway signal to allocate alternative fates to adjacent cells, and a role of the Wnt pathway in specifying the polarization of developing vulval cells. In the male, these same patterning mechanisms, EGF, *lin-12*/Notch, Wnt, are used to pattern the P9–P11 equivalence group with subtle differences and of course a male-specific outcome.[79,80]

Muscles

A major difference between the adult *C. elegans* male and hermaphrodite is the presence of sex-specific muscles. In the male there is a complement of 40 male-specific sex muscles that control various actions in the mating program, including holding the arched posture of the posterior body region, opening the cloaca, and driving the spicules (Figure 9(a)). In the hermaphrodite, a set of 16 muscles control the vulva and uterus.[11,12] Again, as we have seen above, these sex-specific cells are generated from the same blast cells, but these cells generate postembryonic cell lineages that differ between the two sexes. Unlike the seam and P-cell lineages, the mechanisms that pattern cell fates in the lineages giving rise to the sex muscles have not been the focus of significant study.

During embryogenesis, the same muscles are generated in both sexes. Postembryonically, a single mesoblast, M, divides during the L1 larval stage to produce new body wall muscles and the sex muscles (Figure 6). The sex muscles are generated from sex mesoblasts arising from specific lineage branches, two in the hermaphrodite and the same two plus four additional ones in the male. Thus *tra-1* represses male character and promotes hermaphrodite character for two cells, while inhibiting the division of the other four cells.

In both sexes, the sex mesoblasts divide three times.[11,12] The pattern of cell fates they generate is complex. Among the 40 sex muscles generated in the male (18 bilateral pairs and 4 that lie on the midline), there are at least nine types that can be identified based on their similar structure, innervation, and the motion they induce (Figure 9(a)). Within each type, members have distinct attachment points on the body wall and to other muscles, so the number of possible separate cell types is likely greater than nine. These sex muscle cell lineages are a remarkable example of a developmental program that, like clockwork, generates a complex pattern of cell fates through a reproducible cell lineage. As with cells generated by neuronal cell lineages, each separate muscle cell is endowed with the capacity of making specific attachments at precise sites to other cells or body structures through recognition processes that remain largely unknown. Given their larger size and structures less intricate than neurons, the muscle cells present a potentially rewarding opportunity for future research into the problem of cell fate allocation and cell–cell recognition in the assembly of the animal body.

In addition to these sex-specific lineage events, two muscles present in both sexes undergo remarkable reorganization at the end of male larval development[11] (Figure 9(b)). The intestinal sphincter loses its ventral attachments and pulls the intestinal opening into the proctodeum closed and up during ejaculation. The anal depressor reorients its muscle fibers 90° from dorsoventral to anterior–posterior so as to open the cloaca. It becomes part of the circuit connecting the postcloacal sensillum to the spicule muscles[14,81]

Cells That Divide Exclusively in the Male—B, F, U, Y

A significant fraction of the male sexual structures in the tail arise from cells that do not divide at all in the hermaphrodite. Four male-specific hypodermal blast cells lining the larval rectal canal undergo extensive cell lineages that create structures that include the postcloacal and spicule sensilla along with the spicules themselves, male-specific neurons, and the hypodermal structures that generate the proctodeum, the chamber where the intestine joins the distal end of the gonad and that also accommodates the spicules, and its overlying gubernaculum (Figure 6). Beginning during the L1 larval stage, these four cells generate lineages of up to seven cell divisions, creating at least 14

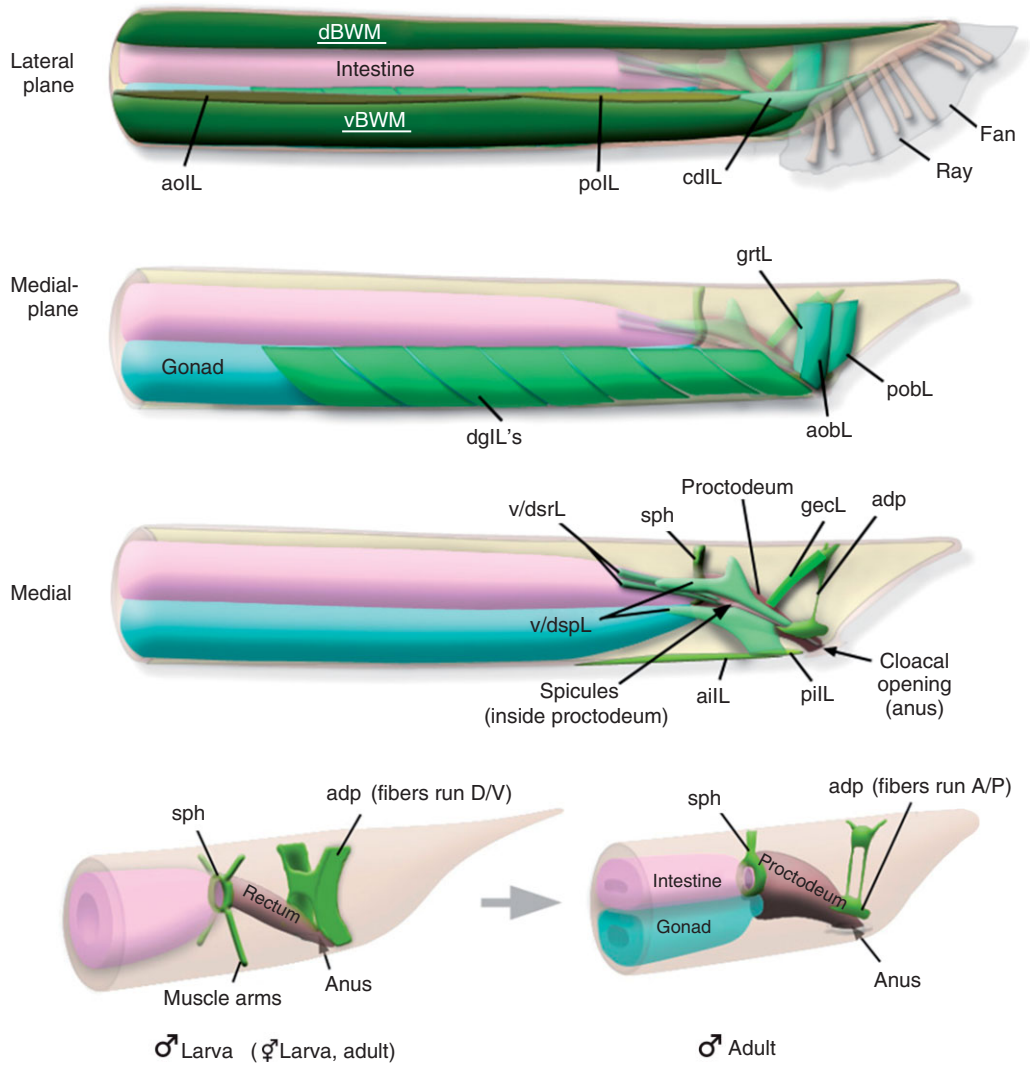

FIGURE 9 | The muscles of the male tail (left side for left/right pairs, denoted by 'L'): dBWM, dorsal body wall muscle; vBWM, ventral body wall muscle; aol, anterior outer longitudinal; pol, posterior outer longitudinal; cdl, caudal longitudinal; dgl, diagonal; grt, gubernacular retractor; gec, gubernacular erector; aob, anterior oblique; pob, posterior oblique; v/dsr, ventral/dorsal spicule retractor; v/dsp, ventral/dorsal spicule protractor; adp, anal depressor; ail, anterior inner longitudinal; pil, posterior inner longitudinal. Bottom two diagrams: the sex shared sphincter (sph) and anal depressor (adp) muscles reorient in the adult male. (Source: http://www.wormatlas.org)

different cell types including neurons and cells associated with sensilla plus a similar number of specialized hypodermal cells.

A description of the anatomy of this complex region and the structures generated, first described by Sulston et al.,[11] may be found in WormAtlas.[82] Not surprisingly, the underlying developmental program utilizes numerous regulatory transcription factors and cell signaling pathways. Since these cells ultimately generate much of the male tail, mutations affecting their identities and development generate a Mab (male abnormal) phenotype easily visible in the dissecting microscope. The contribution of several regulatory factors to male development, some previously known from their effects in the hermaphrodite, has been identified on this basis, including a T-box transcription factor, *mab-9*, a PAX type transcription factor, *egl-38*, and a Zn-finger transcription factor, *lin-48*, as well as the Hox genes *mab-5* and *egl-5*.[83–88]

The first division of the largest of the four cells, the B cell, is asymmetric, giving rise to a larger anterior daughter and a smaller posterior daughter. Studies of mutants defective in this division identified a role for the *C. elegans* Pax6 homolog, *vab-3*, and the Wnt pathway, in specification of an asymmetric cell division and cell fate.[69,89,70,90] As the lineage unfolds, a complex set of cell–cell interactions contributes to the allocation of cell fates (Figure 10). These were

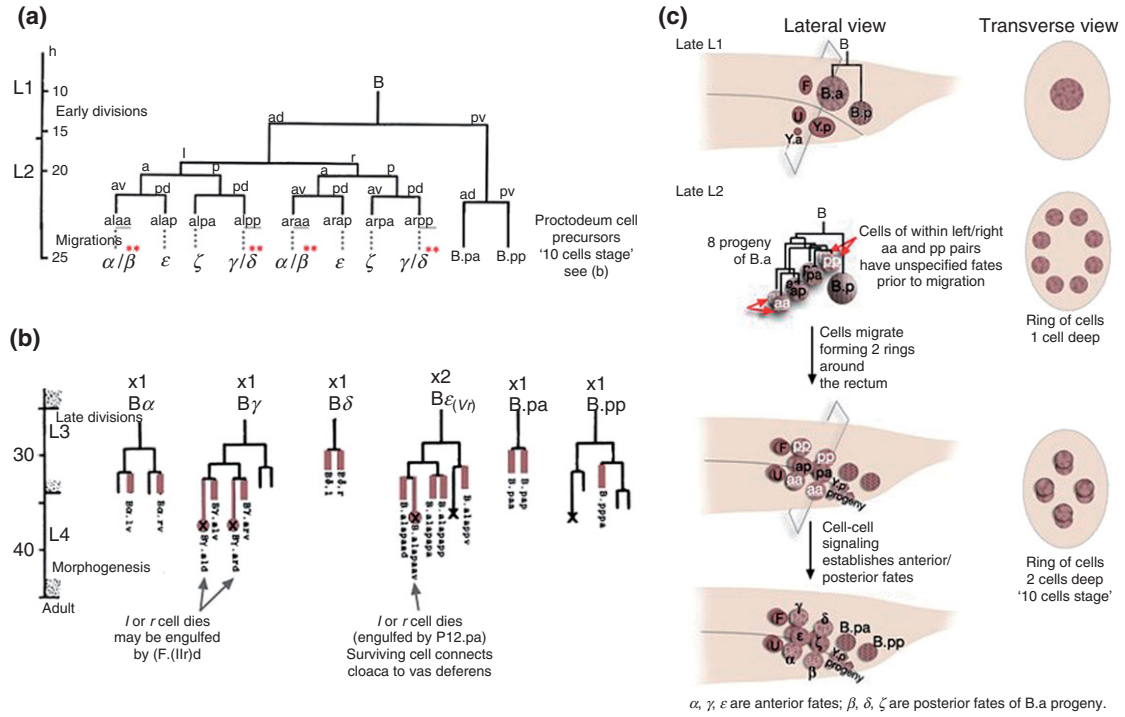

FIGURE 10 | Allocation of cell fates by cell–cell interactions in the cell lineages leading to development of the male hindgut structures. (a and b) Several of the fates of intermediate cells in the B lineage branches generated by a left/right divisionare specified by final cell position rather than lineage ancestory. (c) Arrangement of the cells as the lineage proceeds. Some of the cells in left right branches migrate to the midline, where their fates are set by signals with neighbors. Their order along the a/p axis is not predetermined, leading to the indeterminancy mentioned above. (Source: http://www.wormatlas.org). (Reprinted with permission from Ref 91. Copyright 1993 The Company of Biologists Limited)

dissected in elegant studies using the technique of cell ablation with a laser microbeam, first by Sulston and White,[77] and later by Chamberlin and Sternberg.[91] Cell fate allocation along the anteroposterior axis is influenced by an EGF signal emanating from two of the cells in this set of four blast cells.[92] Symmetry on the left/right axis is broken by cell competition involving the *lin-12*/Notch pathway.[80]

Development of the Male Nervous System

Perhaps the most complex sex-specific structure generated during the postembryonic maturation of the male is the neural network that controls male copulation.[14] The stereotyped series of actions that this network controls is the most intricate behavior of *C. elegans* (Figure 11).

The 81 male-specific neurons, which, along with 63 shared neurons, create the neural network, are born during a 10-hour interval in the L3 and early L4 larval stages.[11] They are generated by cell lineages originating from all three sets of hypodermal blast cells—seam cells, P-cells, and rectal epithelial blast cells B, Y, U, and F. Eventually these neurons populate a series of enlarged ganglia in the tail, but some of them, notably the ray sensory neurons, do not take up their final positions until the time of the L4 to adult molt, when they are swept up in the morphological changes that generate the fan (Figures 4 and 12). Meanwhile, they send processes into the pre-anal ganglion where most of the nearly 8000 chemical and electrical synapses are made.[93] It is not until 16 hours into adulthood that a male mates efficiently (unpublished observations). Hence an intense period of synaptogenesis occurs during the L4 larval stage and into the first hours of adulthood. Altogether, some 3200 connected cell pairs are generated, including neuromuscular junctions, comparable in number to the entire hermaphrodite nervous system.[94] The genetic underpinnings of this process, particularly the synapse specificity determinants that create the desired connections, are essentially unknown.

SEXUAL DIFFERENTIATION OF THE SOMATIC STRUCTURES OF THE MALE GONAD

Superficially, the gonads of males and hermaphrodites appear dissimilar (Figure 1). The hermaphrodite gonad consists of a mirror-symmetrical pair of

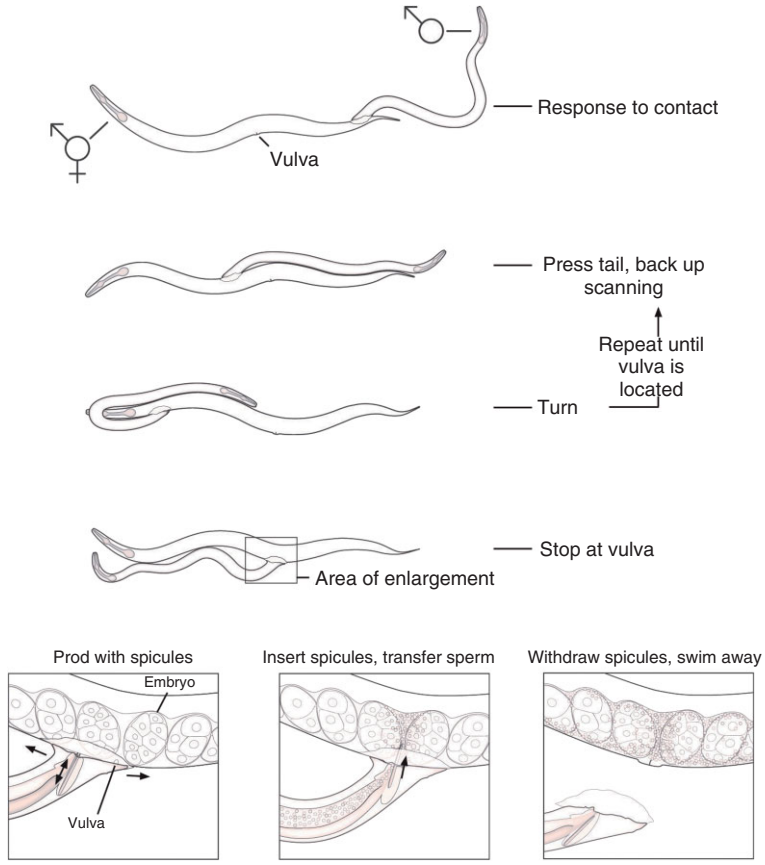

FIGURE 11 | The steps of male mating. When a competent, well-fed adult male encounters a hermaphrodite, it responds by scanning its tail along the hermaphrodite body in order to locate the vulva. Scanning continues on both sides of the body until this is achieved. At the vulva, it prods with its spicules. When the spicules breach the vulval opening, prodding ceases and the spicules remain protracted while seminal fluid passes into the uterus. Finally, the male retracts its spicules and swims away. This stereotyped program is flexible. Steps can be skipped over or the program backed up as appropriate to achieve the final goal. (Reprinted with permission from Ref 14. Copyright 2012 AAAS)

reflexed arms, each containing a germ cell developmental series that starts distally with a mitotic stem cell population, progresses through a syncytial ovary where oocyte components are synthesized in a cytoplasmic central core called the rachis, followed by a reflexive turn where single oocytes are separated from the rachis, mature, pass through a spermatheca, where they are fertilized, and enter the proximal uterus.[95] Fertilized eggs start to develop in utero and are laid through the vulva located in the ventral central body. In the male, by contrast, the gonad is one-armed and exits at the cloaca in the tail. But the testis similarly contains a germ cell series starting with a distal stem cell population and progressing through a region where the germ cells mature into sperm and are stored in a seminal vesicle, followed by a region differentiated to expel the gametes.[95–97]

The similar organization of the two forms is reflected in the similarity of their developmental origins—they are clearly versions of a common developmental program. Like the sex-specific muscles that are generated postembryonically in both sexes by the same mesoblast cell, the somatic structures of the gonad arise from the same two blast cells, Z1 and Z4 (Figure 6). Moreover, the programs initiated by Z1 and Z4 make common use of some of the same genes. How they are caused to diverge in the two sexes is not fully understood (Figure 2). Although XX *tra-1(0)*

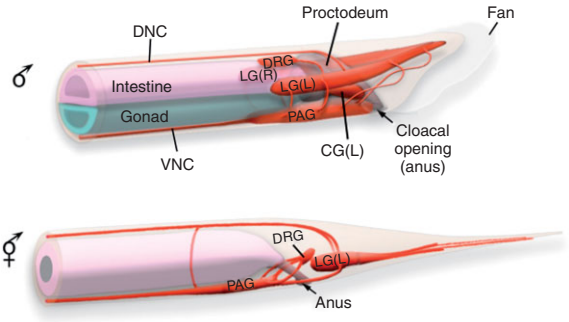

FIGURE 12 | The posterior ganglia are much enlarged in the male, due to an additional 81 male-specific neurons. The 89 neurons present in the bilateral lumbar and single dorso-rectal and pre-anal ganglia in the hermaphrodite are also present in the male. The lumbar ganglia are enlarged mainly by the addition of 18 ray neurons and 9 ray support cells on each side, while the remaining male-specific neurons populate the other two ganglia plus a bilateral pair of cloacal ganglia. CG(L/R), cloacal ganglia; DRG, dorsorectal ganglion; LG(R/L), lumbar ganglia; PAG, pre-anal ganglion; VNC, ventral nerve cord; DNC, dorsal nerve cord. (Source: http://www.wormatlas.org)

animals have a recognizably male gonad, this is not fully normal. TRA-1 is expressed in Z1 and Z4 in both sexes and is required initially for their correct placement within the gonad primordium, for the polarity of the asymmetrical first division of Z1, and for the normal proliferation of the somatic gonad cell lineage.[98–100] TRA-1 carries out this function in conjunction with a second Zn-finger protein, ENH-3.[99] In *tra-1 enh-3* double mutants, Z1 and Z4 fail to proliferate almost entirely in both sexes.[99] Later, the level of TRA-1 declines in males. How *tra-1* escapes for a time repression by the sex determination pathway in the male gonad is not known. Possibly sequestration of the two somatic gonad precursor cells together with the two embryonic germ cells in the gonad primordium, surrounded by a basement membrane, shields these cells sufficiently from the influence of the HER-1 ligand generated by the somatic cells of the body to influence the level of *tra-1* gene function.

Additional transcription factors, along with TRA-1, appear to contribute to defining the male versus hermaphrodite character of somatic gonad differentiation. A forkhead class transcription factor, FKH-6, plays an important role in development of the male gonad.[101] In *fkh-6* mutants, the male gonad is severely abnormal and genes normally only expressed by the male gonad are expressed in the hermaphrodite, suggesting a sex transformation. However, like *tra-1*, the function of *fkh-6* is not exclusive to one sex. While in the hermaphrodite expression of *fkh-6* is repressed in late L1 by *tra-1*, expression recurs in the L3 and is necessary for completing development of the hermaphrodite gonad, which in *fkh-6* mutants has morphological abnormalities and fails to produce viable embryos.[101]

The Hox gene *egl-5* also plays a role in discriminating the two sexual choices. As in other animals, *C. elegans* Hox cluster genes specify cell fates predominantly on a regional basis. *egl-5* is essential for correct specification and differentiation of many tail cells and structures in both sexes.[72] However, in the gonad, *egl-5* is required for development of male characteristics in XO animals and is necessary to prevent expression of hermaphrodite-appropriate genes.[102] *egl-5* reporter gene expression is severely reduced in *fkh-6* mutants and *egl-5* functions downstream of both *tra-1* and *fkh-6*. Since the XO gonad presumably lacks TRA-1, but an *egl-5* XO mutant expresses genes normally restricted to the hermaphrodite, hermaphrodite gene expression in this instance does not appear to require TRA-1.[103]

Interestingly, EGL-5 homologs in the AbdB gene family in other animals also function in development of the male gonad, possibly reflecting these gonadal structures as primordial abdominal structures.[104–106] Thus we see that the line between the sex determination pathway and other developmental pathways is blurred in the somatic gonad. In this organ, many essential aspects of sexual differentiation remain to be understood.

SEXUAL DIFFERENTIATION OF THE MALE GERM LINE

The somatic gonad is filled with germ line stem cells and developing and mature gametes. These cells arise from the remaining two embryonic cells in the gonad primordium, Z2 and Z3 (Figure 6). Z2 and Z3 proliferate indiscriminately during larval development. In the male, spermatocytes first appear during mid-L4. Spermatocytes undergo two rounds of meiosis and unequal cell divisions in which most of the spermatocyte cytoplasm is left in a residual body and four spermatids are produced, which then differentiate into sperm (Figure 13). Nematode sperm differ from sperm of most species in not having a flagellum. They are amoeboid cells that move by extending a pseudopod and crawling over a surface.[107] *C. elegans* sperm development has been studied extensively, at the levels of genetics, cell biology, and gene expression. For a review of this work, see L'Hernault.[108]

While the morphological transformations that accompany germ cell differentiation into gametes are as drastic as those undergone by any somatic cell, there is an important distinction between germ line and soma. Unlike differentiation of somatic cells, differentiation of the gametes is temporary and reversible. This may explain why regulation of gene activity in the germ line differs from the mechanism in the soma. Whereas in somatic cells, regulation of gene activity occurs largely at the level of transcription initiation, regulation of gene expression in germ cells as they undergo meiosis and progress toward gamete differentiation appears to rely on posttranscriptional regulation via sequences in the 3′ untranslated regions (3′-UTR) of messages. For a selection of transgenes examined, promoter sequences induced expression throughout the germ line, whereas 3′UTR's gave the restricted expression patterns of the native genes.[109]

An important exception to this generalization was found in the same study for those genes tested that were sperm-specific in their expression patterns. For these genes, promoter sequences and not 3′-UTRs provided expression specificity. This transcriptional regulation will account for the large number of sex-specific transcripts, including sperm-specific transcripts, identified in studies of gene expression in the germ line.[48,110] Two transcription factors responsible in part

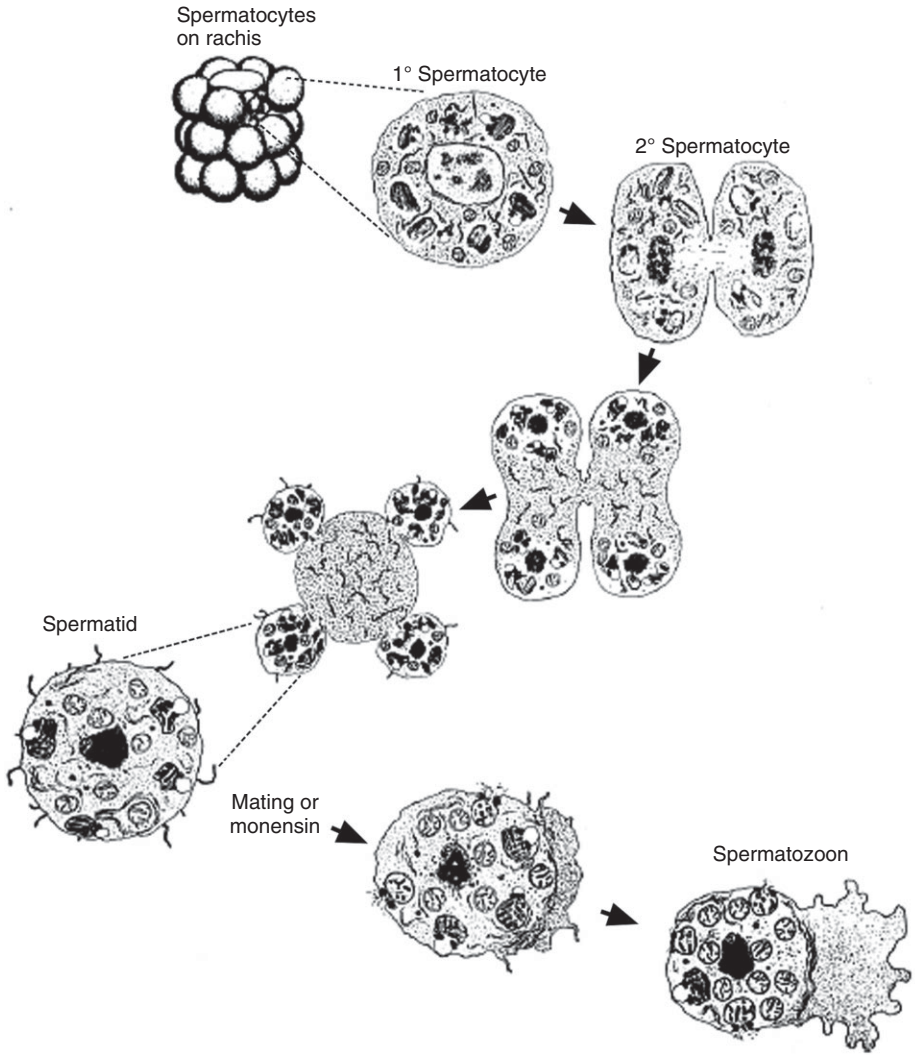

FIGURE 13 | Differentiation of amoeboid sperm from germ cells. Most of the cytoplasmic contents are left in a residual body as four spermatids are produced. (Reprinted with permission from Ref 107. Copyright 1981 Rockefeller University Press)

for this sperm-specific transcription have been identified, ELT-1[111] and SPE-44.[112] Transcription of many of the genes that encode proteins specific to sperm is likely to be induced by these transcription factors.

Sex-specific properties of the germ line are apparent from the earliest stages of germ cell development. In both sexes, the gametes differentiate from a self-renewing population of stem cells residing at the distal end of the gonad. These stem cells divide throughout adulthood. While not overtly sexually differentiated, they nevertheless express sexually dimorphic features, including a somewhat different arrangement and a shorter cell cycle time in the male.[96] The choice made by the progeny of the stem cells to differentiate either as sperm or oocyte must be continuously reinforced. It can be reversed at any time in the mature adult, for example, by blocking an essential gene by RNAi or by raising the temperature of a temperature sensitive mutant.[113]

Just as for the somatic tissues, the male choice is induced by HER-1 activity (Figure 2). This activity can come from somatic tissues. For example, expression of HER-1 in muscle or the gut induces sperm production in XX individuals.[25,114] This indicates that the basement membrane surrounding the gonad primordium is not impermeable to HER-1. Thus the sex of the germ line is determined in part by the sex of the soma. As in the somatic tissues, HER-1 binds the TRA-2 receptor, releasing TRA-2 inhibition of the FEM proteins, which in turn result in degradation of TRA-1.

Consistent with the observation that much regulation of gene activity in the germ line occurs post-transcriptionally, two key proteins at the top of the sperm differentiation pathway are cytoplasmic.

TABLE 1 | Summary of Sex Differences

	Male	Hermaphrodite
Embryogenesis		
	HSN cell death	CEM cell death
Hypodermis		
Seam cells V5, V6, T	Ray neurons, rays, fan	
Ventral hypodermis, Pn.a	CA, CP neurons	VC neurons
Ventral hypodermis, Pn.p	Hook neurons, hook	Vulva
Rectal epithelium B, Y, U, F	38 Neurons and neuronal support cells, proctodeum, spicules	
Gut		
		Synthesize yolk proteins
Muscles		
	40 Sex muscles	16 Vulval and uterine muscles
Nervous system		
Anterior nervous system	Some male-specific synapses, gene expression	VC neurons HSN neurons
	CEM neurons	
Posterior nervous system	81 Additional neurons Extensive new synaptogenesis, including with shared neurons	Some hermaphrodite-specific connections
Somatic gonad		
	Single-armed, exit at cloaca	Dual-armed, exit at vulva
Germ line		
	Produces exclusively sperm	Produces sperm, then oocytes

FOG-1 is a cytoplasmic polyadenylation element binding (CPEB) protein, while FOG-3 encodes a protein of the Tob/BTG family, which has poorly characterized functions in both transcriptional and translational regulation.[113,115] Each of these two proteins is necessary, in both the male and the hermaphrodite, for differentiation of germ cells as sperm. In loss of function mutants, germ cells differentiate as oocytes, while there are no other apparent effects on the soma, including the gonad.[116,117]

In XX animals, transcription of *fog-1* and *fog-3* is repressed by TRA-1.[118] However, unlike in the soma, in the sperm differentiation pathway the *fem* genes do not promote male fates only by inhibiting TRA-1. In *fem tra-1* XO double mutants, germ cells differentiate unexpectedly as oocytes, and this is because *fog-1* and *fog-3* gene activities are lost, even though *fog-3* transcripts are present.[118,119] Details of why the *fem* pathway is necessary for *fog* gene function have not been elucidated. FOG-1 and FOG-3 appear to be necessary for expression of the key transcription factors ELT-1 and SPE-44, but here again, details are not known.

In addition to its role early in development of the germ line stem cells, TRA-1 is required for their continued proliferation to normal levels. Further, later in adult life it is required for differentiation of the gametes as sperm instead of oocytes.[98,100] This may come about by transcriptional activation of *fog-3*.[118] This mysterious reversal of the role of TRA-1 from repressor to activator and from female-determining to male-determining remains to be explained. Summaries of these complex and incompletely understood pathways may be found in several reviews.[120–122]

SUMMARY AND PROSPECTS

Virtually every tissue in the male differs in some way from the corresponding tissue of the hermaphrodite. Those discussed in this review are summarized in Table 1. Despite an early interest in how these differences arise, for only a small subset can we say we have a reasonably complete understanding. This gap in our knowledge seems remarkable given the large amount of information about the male we do have and the number of studies that have been carried out focused on sex determination and male development.

For the nongonadal somatic tissues, it would seem that the problem ought to be straightforward,

as the choice is specified by the activity or inactivity of a single Zn-finger transcription factor, TRA-1. It might be thought that sex-specific developmental pathways could be identified simply by determining what genes are directly regulated by TRA-1 binding. However, remarkably, this connection has been made only for yolk production (*mab-3*), male tail morphogenesis (*dmd-3*), and programmed cell death (*egl-1*, *ceh-30*). (In the germ line, the additional gene *fog-3* also appears to be a direct target of TRA-1[118]). As is the case for almost all transcription factors, the target sequence of TRA-1 is too short and ambiguous to be identified unequivocally in the genome sequence, and in any case, it may be located far from the transcription unit it regulates (5.6 kb 3′ in the case of *egl-1*, where it was identified by a fortuitous mutation[60]).[123,124] Chromatin immunoprecipitation (ChIP-seq) experiments identified 184 genomic regions bound by TRA-1, but for only 6 of 35 of these regions examined could male-specific transcriptional activity be demonstrated.[125] For the somatic parts of the gonad and the germ line, the situation is further complicated because the state of activity or inactivity of TRA-1 is not fully determining and therefore additional genes must be involved. More fully characterizing these less well known pathways is an important goal for future studies.

The lack of knowledge of TRA-1 target genes seems particularly critical in the cases where the two sexes differ in their expression of complex developmental programs. In these instances, involving expression or transformation of entire cell lineages, sex-specific patterns of gene expression give rise to cells of entirely different types or expressing different versions of a common fate. How does the sex determination pathway engage the cell-division cycle? Does TRA-1 regulate expression of one or a small number of key regulatory genes at the beginning of each program and then become irrelevant? Or does TRA-1 influence expression of many genes throughout the realization of these programs, including terminal-stage differentiation genes? The lack of gene mutations that bring about wholesale sexual transformations of entire cell lineages suggests the latter possibility is more likely. This question is addressable through examination of TRA-1 mosaics. Intriguingly, among the genes bound by TRA-1 identified in the ChIP-seq experiments cited above were six heterochronic genes. Taken together with the apparent sex transformations of the postdeirid lineage and the male tail tip cells seen in heterochronic mutants, this observation suggests there may have been an evolutionary recruitment of the timing pathway for purposes of sexual differentiation, a speculation worthy of investigation.[43,67]

Comparative studies may help us understand these complexities of sex-specific differentiation, especially the blurred sex-determination lines in the somatic gonad and germ line. It is often the case in biology that a current mechanism can only be understood in the context of its evolutionary origin. Species comparisons have revealed surprisingly divergent mechanisms of hermaphroditism in *C. elegans* and related nematodes.[126,127] An early role for TRA-1 in sex determination in the nematode lineage is suggested by the finding of a *tra-1* homolog in *Pristionchus pacificus*, 300 million years distant from *C. elegans*.[30] As in *C. elegans*, mutations in this gene cause virtually complete sex transformation in the soma but only partial transformation in the germ line. Yet the two genes share almost no sequence similarity outside the Zn-finger regions, in keeping with the observation of rapid evolution of sex determination genes generally. Further explorations in this direction may locate the origin of this mechanism in the hedgehog pathway. Comparative studies will be particularly important to trace the history of the DM domain transcription factor family and its role in specifying the male phenotype.

There are a number of important questions of significance in both sexes where the male lends itself particularly well for experimental analysis. The complex male-specific cell lineages, as they are postembryonic and nonessential, may be fruitful targets for understanding cell fate specification within a cell lineage. The male sex-muscle program may reveal how cells may be of a single type yet have different morphologies and attachment points with other cells and tissues. Are there roles for the remaining DM domain genes in specifying these male-specific fates? The question of genetic specification of cell contacts appears particularly acute in the male nervous system, where thousands of synaptic relationships are set up during the L4 larval stage. To find their targets, male-specific neurons must extend out branching processes within the volume of an established neuropil containing many potential targets. This contrasts with synaptogenesis during embryogenesis, when connectivity is established between largely unbranched processes running in restricted neighborhoods with limited numbers of cell contacts. Male mating behavior has evolved among related nematode species. It is interesting to consider the role evolution of synaptic specificity may have played in this behavioral evolution.

Finally, an outstanding issue that remains concerns the question whether there is still a large cohort of male-specific genes yet to be identified. A significant fraction of the annotated *C. elegans* genes are associated with no GO terms and yielded no

discernable phenotype in global RNAi screens. These screens invariably examined hermaphrodites only, and hence would have missed functions for genes acting exclusively in males. A similar caveat holds for the global expression studies that have been performed. As an example, there are a large number of genes with similarity to carbohydrate-binding C-type lectins (*clec* gene class) that are strongly enriched in males and have no known function[47] (this laboratory, unpublished). Until we learn the functions of the thousands of genes in this 'dark' category, we will likely not have a complete understanding of *C. elegans* development and the manner in which it produces two sexual forms.

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