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REVIEW

Developmental mechanisms of sex differences: from cells to organisms

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ABSTRACT

Male-female differences in many developmental mechanisms lead to the formation of two morphologically and physiologically distinct sexes. Although this is expected for traits with prominent differences between the sexes, such as the gonads, sex-specific processes also contribute to traits without obvious male-female differences, such as the intestine. Here, we review sex differences in developmental mechanisms that operate at several levels of biological complexity – molecular, cellular, organ and organismal – and discuss how these differences influence organ formation, function and whole-body physiology. Together, the examples we highlight show that one simple way to gain a more accurate and comprehensive understanding of animal development is to include both sexes.

KEY WORDS: Sex difference, Cell death, Cell proliferation, Stem cell, Organ homeostasis, Cell signaling

Introduction

Male-female differences in many developmental processes lead to the formation of two morphologically and physiologically distinct sexes (Arnold, 2017, 2019). The prominent nature of some morphological differences between males and females has motivated studies aimed at uncovering the developmental mechanisms underlying the formation of these highly dimorphic traits. For example, an elegant body of work across many species has provided in-depth knowledge of the distinct genetic and hormonal mechanisms that contribute to the development of sex-specific structures such as the gonads (Arnold, 2017; Capel, 2000; Hubbard and Greenstein, 2000; Matson and Zarkower, 2012; Nef and Parada, 2000; Sekido and Lovell-Badge, 2013; Swain and Lovell-Badge, 1999; Whitworth et al., 2012; Williams and Carroll, 2009; Zarkower, 2001, 2013). Studies on sex-limited traits such as pigmentation, weapons and ornaments have similarly identified mechanisms that contribute to trait development in only one sex. For example, horns are observed only in male adult *Onthophagus taurus* beetles due to female-specific cell death during development (Kijimoto et al., 2010). This mechanism also explains the development of functional wings in adult male, but not female, winter moths (*Nyssiodes lefuarius*) (Niitsu et al., 2014). In contrast, sex-specific cell proliferation explains why males from some *Xiphophorous* fish species have a larger sword than females (Offen et al., 2008; Powell et al., 2021; Schartl et al., 2021). Sex-specific developmental mechanisms therefore contribute to traits with conspicuous differences between males and females.

In addition to highly dimorphic traits, it has become increasingly clear that sex differences exist in many cell types, tissues and organs that are shared between the sexes. For example, there are sex differences in neuron number, dendritic projections and size of specific brain regions in many animals (Jazin and Cahill, 2010). Although the physiological significance of these differences has not been fully elucidated, sex differences in specific neuronal populations contribute to male-female differences in diverse phenotypes such as body size, physiology and behavior (Jazin and Cahill, 2010). There are also differences between males and females across species, including humans, in the number of pancreatic insulin-producing β-cells (Marchese et al., 2015; Parchami and Kusha, 2015; van der Kroon et al., 2017), in whole-body fat storage and distribution (Karastergiou et al., 2012), in nephron number (Ecelbarger, 2016) and in conducting airways (Martin et al., 1987). Because β-cell mass is greater in females, which show a smaller average pancreas volume and duct size than males (Wang et al., 2021), this difference cannot be fully explained by the sex difference in organ size. Similarly, fat storage is ∼10% higher in female mammals despite a lower average body weight (Karastergiou et al., 2012), and the sex difference in conducting airways remains even after correcting for male-female differences in lung size (Martin et al., 1987). Thus, for many cell types, tissues and organs, sex differences cannot be fully explained by a model in which one sex has more of a specific cell type because the corresponding organ is larger. Despite sex differences in these and additional cell types and tissues (Arnold, 2019), knowledge of the underlying developmental mechanisms that establish sexual dimorphism in shared cell types, tissues and organs remains incomplete.

One reason for this knowledge gap is the frequent use of mixed- and single-sex animal groups when studying shared traits. When development is studied in a single sex, there is a significant risk that the contribution of a gene or cellular process to organ formation will be overlooked if that gene or process does not operate in the sex under investigation. Single-sex studies also carry the risk of inaccurately estimating the capacity of an organ for repair following a developmental insult, or the ability of an animal to withstand environmental fluctuations. Mixed-sex studies carry the risk of disregarding the contributions of important genes and cellular processes to the development and function of an organ because a sex-limited effect did not achieve statistical significance. Studying both sexes, and including sex as a variable in the analysis, therefore provides two simple ways for researchers to obtain a more accurate, mechanistic and detailed understanding of development. The importance of this task is shown by mounting evidence that males and females differ in many aspects of development, and in the incidence and progression of diseases related to the dysregulation of fundamental developmental processes (e.g. cell proliferation, cell death, organ repair and growth restriction) (Arnold, 2017, 2019;

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Clocchiatti et al., 2016; Gannon et al., 2018; Morrow, 2015; Ober et al., 2008).

One illustration of why both sexes must be included when studying development comes from high-throughput bulk and single-cell sequencing studies across multiple tissues. Gene expression differences have been documented between the sexes in many animals across tissue types (Mank, 2017; Mayne et al., 2016; Melé et al., 2015). Indeed, up to 40% of genes are differentially expressed between males and females in somatic tissues (Oliva et al., 2020). Interestingly, the degree of gene expression differences between the sexes amplifies over development, and is most pronounced in adults (Emlen et al., 2012; Mank et al., 2010; Perry et al., 2014). This suggests that gene regulatory differences between the sexes in larval, juvenile or other immature stages may be the most crucial, at least in some cases, to manifest sex differences in adults. Because development ultimately relies upon differential gene regulation, this profound male-female difference in gene expression has major implications for studies aimed at revealing genes that influence development (Lopes-Ramos et al., 2020). Given that transcriptional responses to genetic and environmental manipulations also differ between the sexes (Camus et al., 2019; Graze et al., 2018; Honjoh et al., 2017; Mitchell et al., 2016), it is clear that studying one sex does not adequately capture, or allow the prediction of, basal and induced gene expression responses in both sexes.

The overall goal of this Review is to communicate the importance of including both sexes in studies of the fundamental mechanisms underlying development. To achieve this goal, we will use specific examples from studies in a range of model and non-model animals to make general points about the benefits associated with studying both sexes. First, we discuss how sex differences at the genetic and molecular level influence developmental outcomes. We next describe how male-female differences in fundamental cellular processes shape tissue and organ properties. Third, we examine how sex differences in stem cell behavior impact organ size and capacity for repair. Finally, we highlight how sex differences in cell signaling pathways influence development at the organismal level. When taken together, the convergent patterns observed repeatedly across many animals demonstrate that lessons learned from studies in a mixed- or single-sex animal group cannot always be broadly applied to both sexes. A more accurate and mechanistic understanding of development will therefore emerge from studies that include both sexes.

Sex differences at the genetic and molecular level influence development

A mechanistic understanding of development requires the identification of genes and gene networks that contribute to phenotypic outcomes. Emerging literature shows that the number of genes that affect a phenotype, and the relative contribution of each gene to the phenotype, often differs between the sexes. This suggests that the genetic architecture, which refers to the number and effect sizes of genetic variants that influence a given phenotype, differs between males and females for many traits (Karp et al., 2017). Although this might be expected for highly dimorphic traits (e.g. gonads, pigmentation), sex differences in genetic architecture exist even in traits without obvious male-female differences (see Table 1) (Van der Bijl and Mank, 2021).

Sex-biased effects of sex chromosome-associated genes on development

Sex and sex differences in many species are determined entirely by environmental cues (Bachtrog et al., 2014), and in these species there are no genetic differences between females and males. However, in many species, sex is associated with sex chromosomes. Although the environment can play an important role in sex differences even in species with sex chromosomes (Badyaev, 2002), the sex chromosome complement of each sex means that by definition, some genes will affect only a single sex (Fig. 1). For example, in species with the XY sex determination system, Y chromosome genes only contribute to traits in males. Indeed, the control of pigmentation by Y chromosome genes in *Poecilia parae* perfectly illustrates this sex-specific phenotypic effect (Fig. 1).

In contrast, in many species, the X chromosome is present in two copies in females and one in males. Many species lack complete X chromosome dose compensation (Mank, 2013), and the difference in chromosome dose leads to higher expression on average in females for X-linked genes. Many other species have complete X chromosome dose compensation mechanisms, which equalize expression between the sexes (Arnold, 2017; Balaton and Brown, 2016; Deng et al., 2014), although many genes escape these mechanisms (Balaton and Brown, 2016). Because the X chromosome is present in each sex, it may influence development in males and females; however, predicting the developmental effects of X chromosome-associated genes is difficult. For example, in *Drosophila*, fat body loss of a growth-promoting gene called

Table 1. International Mouse Phenotyping Consortium: traits with significant measures of sexual dimorphism

Trait category	Trait name
Morphology	Fat mass, bone mineral content (excluding skull), heart weight, spleen weight and bone area
Physiology	Fasted blood glucose concentration, initial response to glucose challenge and area under glucose response curve
	Sodium, potassium, creatinine, albumin, phosphorus and iron levels
	Alanine aminotransferase and alkaline phosphatase levels
	Total cholesterol, HDL (high density lipoprotein) cholesterol, triglycerides, fructosamine, lactate dehydrogenase, alpha-amylase, UIBC (unsaturated iron binding capacity), LDL (low density lipoprotein-cholesterol) and free fatty acids
	Heart rate, RR (R wave to R wave) interval, PQ (P wave to QRS complex) interval, PR (P wave to R wave) interval, QRS (Q, R and S waves) complex, ST(QRS complex to T wave) segment, corrected QT (Q wave to T wave) interval, heart rate variability, correction QT dispersion, mean SR (S wave to R wave) amplitude and mean R wave amplitude
	Forelimb grip strength measurement mean and forelimb and hindlimb grip strength measurement mean
	White blood cell count, red blood cell count, platelet count, neutrophil cell count, lymphocyte cell count, monocyte cell count, eosinophil cell count and large unstained cell (LUC) count
Immunology (refers to cell number)	NKT cells, DN T cells, CD4 NKT cells, DN NKT cells, CD8 CD25+ T cells, CD8 CD25+ NKT cells, DN CD25− NKT cells, CD4 CD44+CD62L− T cells, DN CD44+CD62L− T cells, DN CD44−CD62L+ T cells, DN CD44-CD62L− T cells, CD4 CD44+CD62L− NKT cells, DN CD44−CD62L+ NKT cells, eosinophils, NKT cells, cDC cells and pDC cells
Behavior	Whole arena average speed, periphery distance traveled, center distance traveled, locomotor activity, center average speed and distance traveled – total

Data taken from from van der Bijl and Mank (2021). For each category, traits with significant sexual dimorphism are shown.

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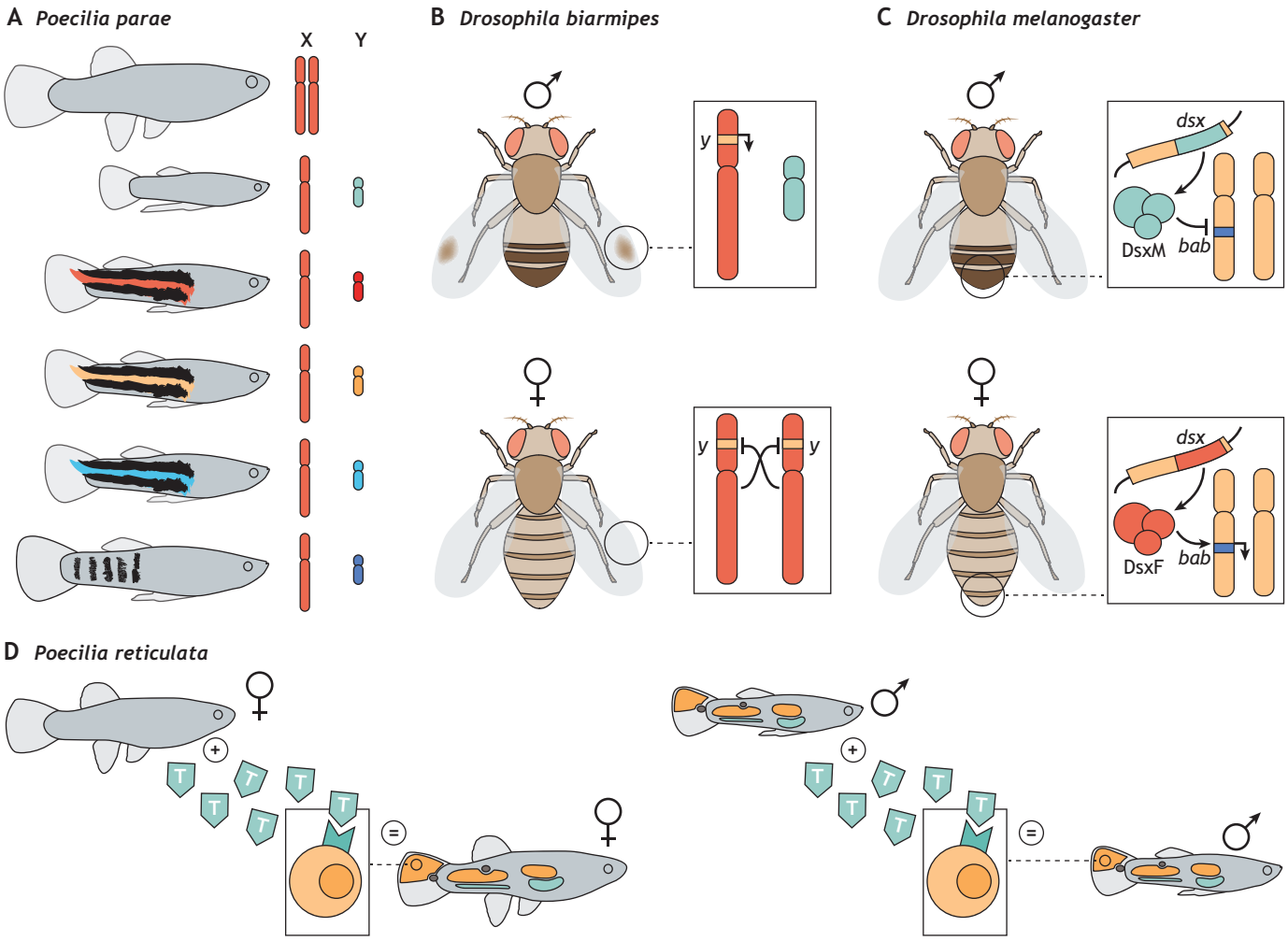

Fig. 1. Sex chromosome and sex hormone effects on sex-specific trait development. (A) The sex chromosomes, in species that have them, often provide the initial genetic trigger for phenotypic sex differences. This occurs through several different routes. First, the Y chromosome presents a male-specific region of the genome, and some dimorphisms are encoded on Y-linked genes. For example, there are five male morphs in the fish *Poecilia parae*, each of which displays distinct differences in color, behavior and physiology. These five morphs are each associated with a different Y chromosome, meaning that the Y ultimately encodes the male-specific traits as females of this species carry two X chromosomes (Sandkam et al., 2021). However, the coding content of the Y chromosome is very restricted in many species, and some species, in which sex is determined by X chromosome dose, have lost the Y chromosome entirely (Bachtrog et al., 2014). (B) The X chromosome differs in dose between the sexes, and although dose compensation systems in some systems equalize expression for most X-linked genes between males and females, the presence of two X chromosomes in females and one in males can play a role in sex differences. For example, male-specific wing pigmentation in *Drosophila biarmipes* is thought to be ultimately encoded by an X-linked gene, and transgene analysis in *D. melanogaster* indicates that the regulation of the gene is ultimately controlled by whether an individual has one X chromosome or two, and is independent of the sex-determination pathway itself (Galouzis and Prud'homme, 2021). Although further functional validation of this mechanism in *D. biarmipes* is needed, these results suggest there is a repressive interaction between the encoding gene (*yellow*, *y*) and the corresponding allele on the homologous X chromosome in females, a phenomenon known as transvection. Males have only one copy of the X chromosome, and so lack a homologous X to repress *yellow*. (C) Sex chromosome complement also initiates the sex-determination pathway, where some dimorphisms are controlled by sex-determination genes. For example, abdominal coloration in male *Drosophila* is controlled by *bric-à-brac* (*bab*), which is regulated by the *Drosophila* sex-determining locus *doublesex* (*dsx*) (Kopp et al., 2000). The female isoform of *dsx* is called DsxF and activates *bab* transcription, whereas the male *dsx* isoform, DsxM, represses *bab*. (D) The sex-determination cascade also leads to differences in sex hormone levels. Testosterone (T) treatment of females (which normally lack coloration) is sufficient to produce male coloration patterns in many fish (e.g. *Poecilia reticulata*; Gordon et al., 2012), birds (Ardia et al., 2010) and reptiles (Cox et al., 2005), indicating that sex hormones regulate these traits.

stunted, an X-linked gene, decreases body size in females but not in males (Millington et al., 2021a). In contrast, adult flies lacking the coding sequence for the X-linked gene *Drosophila insulin-like peptide 6* show reduced adult weight in males but not females (Millington et al., 2021b). Both sexes must therefore be included when studying the phenotypic effects of X-linked mutations, as the size and direction of the effect cannot be predicted in advance. Indeed, the X chromosomes make a larger contribution to sex differences than expected based on their size or total gene content (Mank, 2009).

Genome-wide association studies reveal sex differences in genetic architecture of sex-specific and non-sex-specific traits

Autosomal genes also differentially affect development in males and females (Ober et al., 2008). Although the autosomes are shared equally between sexes, their vastly greater coding content makes a larger overall contribution to sex differences in the genetic architecture of development. This suggests that both sexes must be considered even in studies on developmental phenotypes associated with autosomal genes. One line of evidence supporting this suggestion emerges from genome-wide association studies

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Box 1. Sex differences in disease

Evidence that a large proportion of human diseases have sex differences in prevalence, age of onset, severity and/or response to treatment has been building for some time (Ober et al., 2008). Recent institutional calls to balance biomedical and clinical studies with regard to sex (Clayton and Collins, 2014) have led to a tidal wave of evidence for sex differences in disease and medically relevant phenotypes (Karp et al., 2017; Khramtsova et al., 2019; Morrow, 2015; Richardson et al., 2021).

Some of these differences are due to the X chromosome and the fact that males have an increased incidence of some X-linked diseases due to their single copy. Examples include X-linked color blindness (Xie et al., 2014) and muscular dystrophy (Towbin et al., 1993). However, the sex chromosomes do not explain the vast majority of sex differences observed in disease traits (Ge et al., 2017; Traglia et al., 2017), such as the incidence of psychiatric disorders (Bogetto et al., 1999; Mandy et al., 2012; Tükel et al., 2005) and cardiovascular disease (Haast et al., 2012; Lawlor et al., 2001). The sex chromosomes also do not explain the fact that the sexes show major differences in the pharmacokinetics for many drugs (Soldin and Mattison, 2009). Failure to account for sex differences in pharmacokinetics has led to some dangerous dosing guidelines, exemplified by the case of Ambien (zolpidem), a sleep aid where differences in metabolic clearance rates between the sexes left a significant percentage of women with lingering sedation after the intended 8 h dose (Greenblatt et al., 2000).

Moreover, detailed genetic case-control comparisons have revealed many differences in the genetic architecture of disease risk and heritability (Duncan et al., 2018; Ge et al., 2017; Traglia et al., 2017). These differences underscore the need to account for sex in clinical and pre-clinical research, and the need to develop sex-informed treatment plans for patients.

(GWAS). GWAS are genomic scans looking for a correlation between genetic variants (e.g. single-nucleotide polymorphisms) and phenotypic traits, and represent a powerful big-data method to characterize the genetic architecture of specific phenotypes based on large sample numbers.

GWAS have identified sex differences in autosomal genetic architecture for traits with pronounced dimorphism, such as fat deposition (Shungin et al., 2015), body size and shape (Heid et al., 2010; Randall et al., 2013; Winkler et al., 2015), neurological traits (Lu and Cantor, 2012; Matoba et al., 2019) and disease (Box 1). These associations occur despite little evidence for allele frequency differences in genetic variants for autosomal loci between the sexes (Boraska et al., 2012). In phenotypes for which there is no obvious or known sex difference, evidence for sex differences in genetic architecture has also accumulated through sex-stratified GWAS (Graham et al., 2019; Khramtsova et al., 2019). In most cases, the sex difference in genetic architecture is due to variants that are statistically associated with a trait in one sex or the other (sex specific), despite being present in the genomes of both sexes, as discussed in the following section. Fewer genetic architecture differences arise from variants having opposing effects in the sexes (sex discordance) (Harper et al., 2020 preprint), suggesting that, although genetic architecture may differ, it does not often act in fundamentally different ways between the sexes.

Large-scale phenotyping studies show sex-biased and sex-specific requirements for many genes in regulating development

Like GWAS, large-scale phenotyping studies also indicate widespread and unpredictable sex differences in genetic architecture for both sex chromosome- and autosomal-linked genes. For example, the International Mouse Phenotyping Consortium (IMPC) aims to map out the function of every protein-coding gene in the mouse genome (Dickinson et al., 2016). Male and female knockout mutants are subjected to a phenotyping pipeline that spans developmental, cellular, physiological, behavioral and morphological traits. Because the phenotypic effects of knockout mutations are measured in males and females, it is possible to determine the underlying differences in genetic architecture for specific traits (Karp et al., 2017).

Although previous studies mapped the effects of mutants to sex-specific phenotypes such as fertility and sterility (Connallon and Clark, 2011; Tweedie et al., 2009), the IMPC effort was the first large-scale agnostic phenotyping analysis of sex differences across a broad range of somatic traits. The results showed that not only was sexual dimorphism prevalent in phenotypes for which there was no obvious explanation (Karp et al., 2017), with up to 31% of somatic traits showing significant sex differences (Table 1) (van der Bijl and Mank, 2021), but even the variability of many traits differs between males and females (Zajitschek et al., 2020). Most importantly, roughly 17% of autosomal genes had sex-biased or sex-specific phenotypic effects (Karp et al., 2017). Of knockouts with different effects between the sexes, about 75% produced a phenotype in one sex or the other, 20% produced effects with opposing directions between the sexes and 5% produced effects in the same direction but of a different magnitude. Of note, these sex-biased and sex-specific effects were not limited to highly sex-specific organs such as the gonad. Rather, they include but are not limited to organs such as the gut, adipose tissue, the heart and the pancreas, organs where sexual dimorphism is far less obvious and highly understudied (Table 1) (Karp et al., 2017).

Together, these lines of evidence demonstrate the importance of including both sexes in studies aimed at revealing the genetic and molecular mechanisms underlying development. This principle is equally important when studying both sex chromosome- and autosomal-linked genes, and when studying developmental traits that may or may not show prominent differences between the sexes. One important risk of not including both males and females in studies is overlooking profound phenotypic consequences caused by loss of gene function. This occurs when the size of the effect does not achieve statistical significance in a mixed-sex population, or when the unaffected sex is studied in a single-sex population. In contrast, studying both sexes separately provides the opportunity to discover new genes associated with developmental outcomes that were missed in single- and mixed-sex studies.

Sex differences in fundamental cellular processes influence tissue and organ development

Understanding development also requires knowledge of mechanisms that operate at the level of individual cells to specify tissue and organ properties. A rapidly growing body of evidence has identified sex differences in many fundamental cellular processes, and shown that these differences contribute to final tissue size and shape, and to organ size, anatomy and cell composition. This suggests that the cellular processes that govern final tissue and organ properties may not be shared between the sexes. Notably, these differences are not limited to reproductive organs.

Sex differences in fundamental cellular processes exist in tissues and organs without obvious sexual dimorphism

It is widely appreciated that traits with prominent sexual dimorphism (e.g. sex-limited weapons and ornaments, pigmentation and coloration) will be associated with sex-specific developmental mechanisms. Beyond these obviously dimorphic traits, however, a growing body of evidence shows that male-female differences in fundamental cellular processes exist in many organs

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and tissues that are shared between the sexes. In flies and other invertebrates, male-female differences have been reported in the size, shape and cell composition of the fat, wing, leg, central nervous system (CNS), gut and muscle (Stokes et al., 1994; Demir and Dickson, 2005; Rideout et al., 2010; Robinett et al., 2010; Rideout et al., 2015; Hudry et al., 2016; Regan et al., 2016; Ahmed et al., 2020; Wat et al., 2020). Importantly, many of these differences are functionally significant: sex-specific neural circuits in *Drosophila* promote male courtship behaviors (Demir and Dickson, 2005; Rideout et al., 2010; Robinett et al., 2010), whereas sex differences in the intestine influence reproductive output and lifespan responses to dietary nutrients (Hudry et al., 2016; Regan et al., 2016). In vertebrates, including humans, sex differences have been reported in the brain, heart, fat, pancreas, kidney and lungs (de Simone et al., 1995; Ecelbarger, 2016; Jazin and Cahill, 2010; Karastergiou et al., 2012; Legato and Legha, 2004; LoMauro and Aliverti, 2018; Marchese et al., 2015; Parchami and Kusha, 2015; Taylor et al., 2010; van der Kroon et al., 2017). Although these sex differences are largely descriptive in nature, and the underlying mechanisms have not been fully elucidated, male-female differences in phenotypes related to these organs have been noted. For example, in alignment with a higher number of pancreatic β-cells in women (Marchese et al., 2015; van der Kroon et al., 2017), glucose-stimulated insulin secretion is higher in women than in men in the context of equivalent insulin sensitivity (Basu et al., 2006, 2017; Horie et al., 2018). At least some differences noted in descriptive studies may therefore be relevant for male-female differences in development, physiology and behavior – a possibility that requires further investigation.

Of the tissues and organs without prominent sexual dimorphism, the CNS represents one of the best-studied examples of how sex differences in fundamental cellular processes shape organ properties (Ampatzis and Dermon, 2007; Jazin and Cahill, 2010; Portman, 2007; Sulston et al., 1983). For example, in *Drosophila melanogaster*, sex-specific programmed cell death occurs in many regions of the developing CNS (Garner et al., 2018; Kimura et al., 2008; Sanders and Arbeitman, 2008; Taylor and Truman, 1992), including the 'neurons medially located, just above antennal lobe' (mAL) (Kimura et al., 2005). Normal male flies have ∼30 mAL neurons, whereas females have ∼5 mAL neurons (Kimura et al., 2005). In males, these mAL neurons extend neurite projections bilaterally, whereas the smaller number of female mAL neurons have only contralateral projections (Fig. 2A). Remarkably, sex differences in both the number and projections of mAL neurons are largely due to sex-specific programmed cell death, as genetic inhibition of apoptosis in females increases the number of mAL

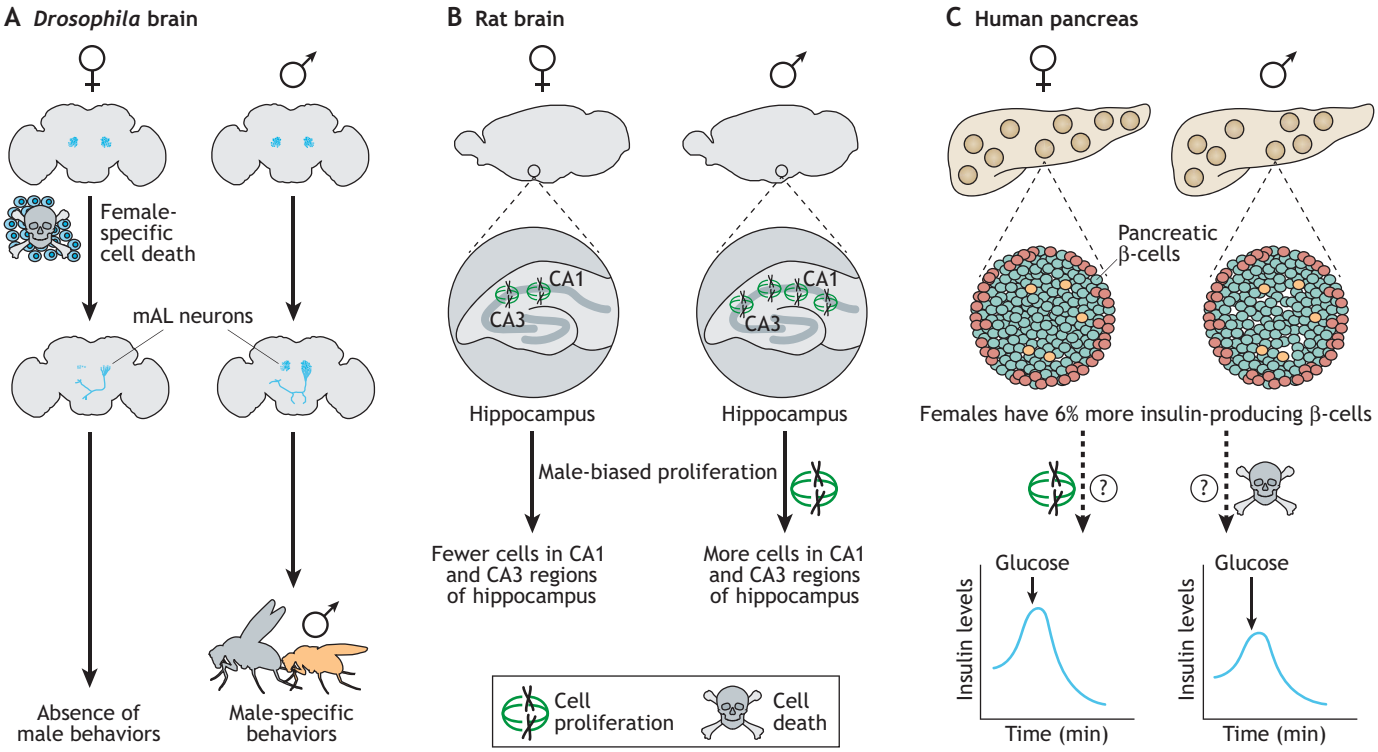

Fig. 2. Sex differences in fundamental cellular processes shape specific cell populations within organs. (A) In *Drosophila melanogaster*, a sex difference in programmed cell death occurs in the 'neurons medially located, just above antennal lobe' (mAL) (Kimura et al., 2005). In females, cell death leads to fewer mAL neurons, and surviving mAL neurons have different neurite projections from males. In combination with other male-specific neuronal populations, the sex difference in mAL neurons supports normal courtship and reproductive behaviors in males. Although this figure displays a sex difference in cell death in *Drosophila* neurons, sex-specific neuronal cell death occurs in many animals (e.g. worms, frogs and birds) (Forger and de Vries, 2010; Kay et al., 1999; Kirn and DeVoogd, 1989). (B) In newborn rats, there is higher proliferation in the CA1 and CA3 regions of the hippocampus in males than in females (Bowers et al., 2010; Zhang et al., 2008). This increased proliferation may contribute to the male-biased increase in neuron number in these regions of the hippocampus (Hilton et al., 2003). Although this figure displays a sex difference in proliferation in rat neurons, sex-specific proliferation occurs in the CNS of many animals (e.g. flies, worms and fish) (Ampatzis and Dermon, 2007; DeWulf and Bottjer, 2002; Ross et al., 2005; Taylor and Truman, 1992). (C) Pancreatic islets derived from human female donors contain a slightly higher proportion of insulin-producing β-cells than males; however, the fundamental cellular processes that contribute to this difference remain unknown. This modest difference in β-cell mass may contribute to the increase in glucose-stimulated insulin release that has been observed in females compared with males in the context of equivalent insulin sensitivity between the sexes (Horie et al., 2018); however, a functional link between higher β-cell mass and increased glucose-stimulated insulin secretion in females has not yet been established.

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neurons and specifies male-like bilateral projections in 'rescued' mAL neurons. In the case of mAL neurons (Kimura et al., 2005) and many other neuronal populations in *Drosophila* (Billeter et al., 2006; Demir and Dickson, 2005; Garner et al., 2018; Kimura et al., 2008; Rideout et al., 2007, 2010; Sanders and Arbeitman, 2008; Shirangi et al., 2016; Taylor and Truman, 1992), genes in the sex determination pathway have been shown to establish and maintain this sex difference in neuron number.

Sex differences in cell proliferation also exist in the CNS. For example, in newborn rats, gonadal hormones contribute to a sex difference in cell proliferation in several hippocampal regions (Fig. 2B), with newborn male rats showing higher proliferation in these regions than females (Bowers et al., 2010; Zhang et al., 2008). Because there was no accompanying increase in cell death (Zhang et al., 2008), cell proliferation and not cell survival likely explains why young male rats have an increased number of neurons in these regions (Hilton et al., 2003). Indeed, sex differences in cell proliferation and death extend broadly within the CNS, occurring both during development and throughout life (Forger and de Vries, 2010). Although these differences in CNS development may seem modest, it is important to note that even small differences in cell number or dendrite projections are functionally significant.

Sex-specific neuronal clusters in several animals have been linked with diverse behaviors and physiological parameters (Barr et al., 2018; Billeter et al., 2006; Brenowitz and Arnold, 1986; Cahill, 2006; Hellier et al., 2018; Narayan et al., 2016; Nottebohm and Arnold, 1976; Pavlou et al., 2016; Portman, 2007; Rezával et al., 2014; Rideout et al., 2007; Sammut et al., 2015; Shirangi et al., 2016; Wang et al., 2018). For example, in *C. elegans*, one pair of male-specific MCM neurons mediates the ability of male worms, but not hermaphrodites, to undergo a switch in chemosensory behavior after exposure to a potential mate, a form of sexual conditioning (Sakai et al., 2013; Sammut et al., 2015). Similarly, 30 TN1 interneurons present in the male, but not the female, ventral nerve cord in *Drosophila* are required for the production of sine song, a key component of the male courtship ritual (Rideout et al., 2007; Sanders and Arbeitman, 2008; Shirangi et al., 2016). In mice, sexual dimorphism in POMC neuron number and activity contribute to the male-female difference in diet-induced obesity (Wang et al., 2018). Although the male-specific MCM and TN1 neurons in *C. elegans* and *D. melanogaster*, respectively, are produced through the action of sex determination genes (Rideout et al., 2007; Sammut et al., 2015; Shirangi et al., 2016), the mechanisms underlying the sexual dimorphism in POMC number remain unclear. Together, these findings and others demonstrate that even small differences in neuron number between the sexes can influence behavior and physiology.

These examples of sex-specific cell proliferation and death illustrate why studying both sexes helps to understand the fundamental mechanisms that shape organ and tissue properties. This is equally important in organs with and without obvious sexual dimorphism (Fig. 2C), as sex differences are found across many organs. Benefits arising from studying both sexes also apply to studies on processes in addition to cell proliferation and death, as multiple cellular mechanisms may contribute to differences in tissue and organ mass (e.g. cell proliferation, growth, death, competition and migration). Indeed, there is a significant risk of overlooking a fundamental cellular process that shapes tissue and organ size when using single- or mixed-sex populations. Furthermore, without certainty about whether a group of cells is present in both sexes or not, incorrect conclusions may be drawn about the functional significance of that cell type. Studying both sexes, and including biological sex as a variable during data analysis, will therefore allow a more accurate understanding of how fundamental cellular processes influence tissue and organ development, and of how these organs function within the body.

Sex differences in stem cells impact tissue and organ homeostasis

Knowledge of mechanisms that operate at the level of whole organs to maintain tissue and organ homeostasis is also required for a complete understanding of development. In some organs (e.g. intestine and skin), maintaining homeostasis requires cells of the correct type to be replaced in a regulated manner according to cell loss (Biteau et al., 2011; Fuchs, 2008; Jiang and Edgar, 2011). Although cell loss may occur under normal physiological conditions, it also occurs following mechanical, biological and chemical insults (e.g. injury and infection). In many animals, sex differences have been reported in both tissue and organ homeostasis, and in their capacity for repair, suggesting the mechanisms underlying these processes differ between the sexes.

Sex differences in stem cell behavior influence organ plasticity, repair and aging

One example of a sex difference in organ homeostasis and repair emerges from studies on intestinal stem cells (ISC) in the *Drosophila melanogaster* gut (Ahmed et al., 2020; Hudry et al., 2016; Regan et al., 2016). Normally, ISCs divide asymmetrically to produce a daughter cell called an enteroblast (EB) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). EBs are transient progenitors that differentiate into enterocytes (EC) and enteroendocrine cells (EE), two important cell types in the adult intestine (Jiang and Edgar, 2012; Lemaitre and Miguel-Aliaga, 2013). ISC proliferation therefore maintains intestinal homeostasis under normal physiological conditions (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) and plays a crucial role in repairing damage due to mechanical and biological insults (Amcheslavsky et al., 2009; Buchon et al., 2009; Jiang et al., 2009).

Several studies now show that, under normal physiological conditions, the proliferation of female ISCs is ∼400-800% higher than the proliferation of male ISCs (Ahmed et al., 2020; Hudry et al., 2016; Regan et al., 2016). This difference exists after flies ingest a detergent to damage the intestinal epithelium (Ahmed et al., 2020; Hudry et al., 2016) and after oral infection with *Erwinia carotovora* or *Pseudomonas entomophila* (Ahmed et al., 2020; Regan et al., 2016), and persists throughout life (Regan et al., 2016). The sex difference in proliferation is due to intrinsic ISC sexual identity, and is further enhanced in females following a mating-induced increase in the steroid hormone ecdysone (Ahmed et al., 2020; Reiff et al., 2015; Zipper et al., 2020). Importantly, the sex difference in ISC proliferation has physiological consequences: higher ISC proliferation in females leads to a larger intestine (Hudry et al., 2016) and may contribute to increased female resistance to infection-related insults (Regan et al., 2016).

Despite these benefits, however, females suffer more age-related damage to the intestinal epithelium (Regan et al., 2016) and increased susceptibility to genetically induced tumors (Ahmed et al., 2020; Hudry et al., 2016). A sex difference in stem cell proliferation therefore not only has important consequences for normal physiology, but also influences the risk of disease. Although less is known in other models about sex differences in ISCs and intestinal homeostasis, a recent study documented higher ISC proliferation in female mice (Zhou et al., 2018). Future studies will need to resolve whether this difference leads to male-female

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differences in gut size, plasticity and capacity for repair in mammals, as it does in flies. Because the sex difference in ISC proliferation was not driven by a direct effect of estrogen, it will also be important to determine the mechanism underlying increased female ISC divisions.

An established literature on sex differences in hematopoietic stem cells (Nakada et al., 2014) similarly suggests that more work is needed to understand how stem cells contribute to male-female differences in the regulation and function of the immune system (Grossman, 1984; Klein and Flanagan, 2016). Given that sex differences also exist in adrenal stem cells (Grabek et al., 2019), neural stem cells (Bramble et al., 2019; Mahmoud et al., 2016), adipose-derived stromal cells (Ogawa et al., 2004), multipotent mesenchymal stromal cells (Bragdon et al., 2015) and muscle-derived stem cells (Deasy et al., 2007), there is currently an unmet need for knowledge of how sex differences in stem cell behavior impact organ homeostasis and repair throughout the body.

Exploiting sex differences in stem cell behavior to optimize outcomes in tissue engineering and regenerative medicine

In addition to studying tissue and organ homeostasis, sex differences in stem cell behavior also impact the production and function of stem cells for downstream applications, including regenerative medicine and tissue engineering. For example, muscle-derived stem cells isolated from female mice are more efficient at regenerating skeletal muscle than male stem cells when implanted into a mouse model of Duchenne muscular dystrophy (Fig. 3A) (Deasy et al., 2007). The increased regeneration ability of female muscle-derived stem cells is largely due to a sex difference in myogenic differentiation upon cell stress in the implantation environment, where increased post-implant differentiation in male muscle-derived stem cells potentially depletes the stem cell pool available for regeneration.

Another example is a sex difference in the ability of male- and female-derived stem cells to form self-organized three-dimensional organ or tissue cultures called organoids (Fig. 3B). Stem cell-derived organoids reproduce some aspects of organ or tissue structure and gene expression, and in some cases may recapitulate functional properties of the organ or tissue from which the stem cells were isolated. One recent study showed that colonoids cultured from colon crypts of females were not only more numerous when compared with males, but female colonoids also had a significantly larger size (Noguerol et al., 2019).

Beyond considerations of donor sex in stem cell studies, both sexes must also be included as recipients of stem cells and stem cell-derived tissues. The importance of this point is shown by the effect of recipient sex on the maturation of stage 4 (S4) human embryonic stem cell-derived pancreatic progenitors into glucose-responsive insulin-secreting cells (Fig. 3C) (Saber et al., 2018). S4 pancreatic progenitors mature into glucose-responsive insulin-producing cells when implanted into a host and restore glucose control when implanted in mice with impaired insulin function

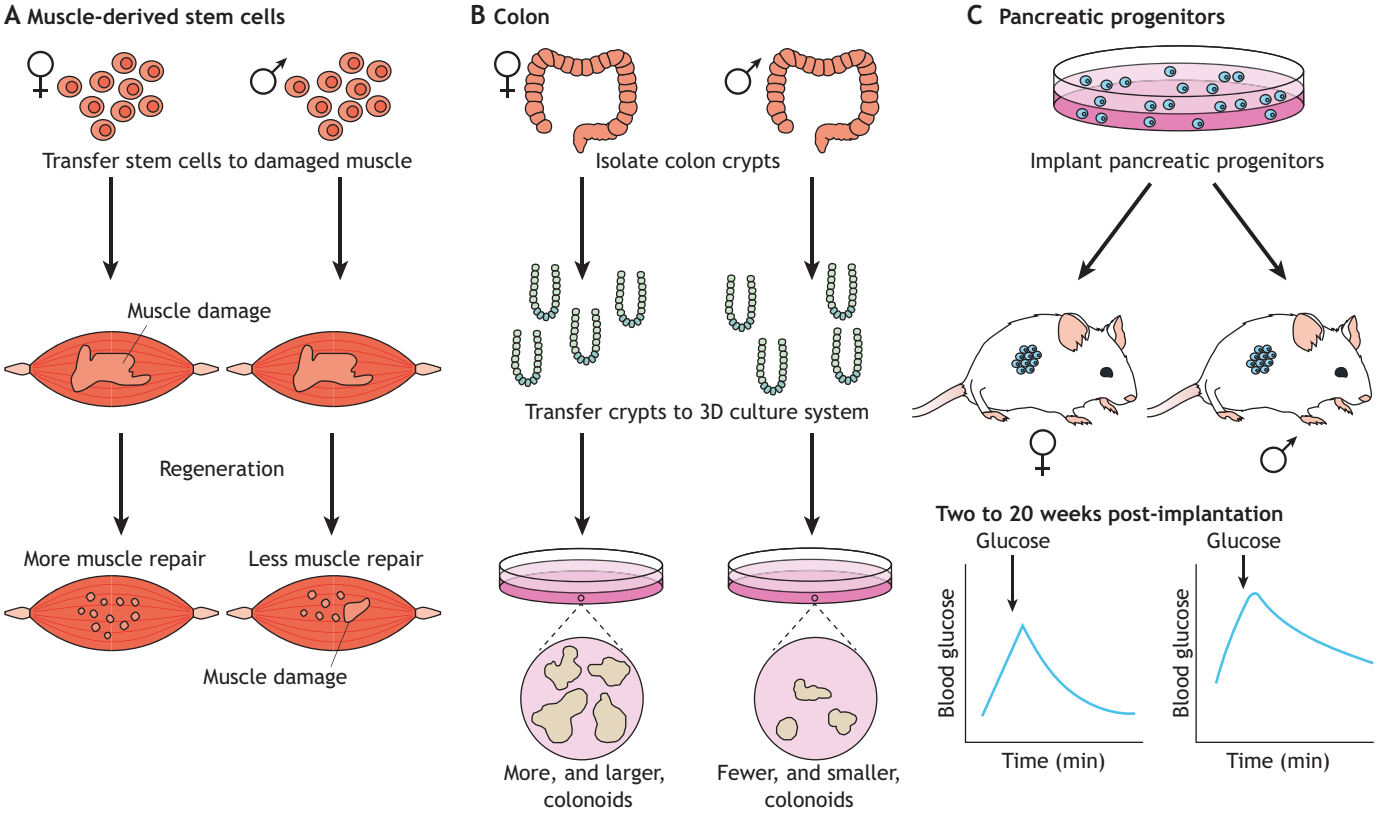

Fig. 3. Sex differences in stem cell behavior influence tissue regeneration and organoid formation, and host-derived factors impact maturation of implanted progenitor cells. (A) Muscle-derived stem cells isolated from female donors support higher muscle regeneration in a mouse model of Duchenne muscular dystrophy (Deasy et al., 2007). (B) Colon crypts isolated from female donors produced colonoids larger in size and greater in number than colonoids generated from male crypts (Noguerol et al., 2019). (C) Stage 4 (S4) human embryonic stem cell-derived pancreatic progenitors implanted into female recipients demonstrated significant glucose responsiveness weeks earlier than S4 pancreatic progenitors implanted into males (Saber et al., 2018). As a result, blood glucose levels were lower in female mice than in males following a glucose challenge between 2 and 20 weeks post-implant, suggesting earlier maturation of S4 cells in females improved implant function.

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(Bruin et al., 2013; Rezania et al., 2012). Interestingly, S4 pancreatic progenitors implanted into females demonstrated significant glucose responsiveness weeks earlier than pancreatic progenitors implanted into males (Saber et al., 2018). Because glucose responsiveness is an important attribute of pancreatic progenitors intended for therapeutic purposes, it is biologically significant that progenitors implanted into females achieve this goal sooner.

Together, these studies highlight why both sexes must be included when investigating how stem cell behavior influences organ homeostasis and repair. This applies to stem cell studies in the context of living animals and also to studies aimed at using stem cells for applications such as regenerative medicine or tissue engineering. When both sexes are not studied, there is a risk of over- or underestimating the capacity of an organ for repair and an individual's ability to survive loss of organ function. Conversely, accounting for sex provides benefits such as identifying organoids with the most desirable characteristics, and uncovering the stem cells most likely to regenerate lost or damaged tissue. In addition, when stem cells are implanted into both male and female recipients, the functional properties of implanted cells within a host will be described with more accuracy.

Sex differences in cell signaling pathways contribute to whole-body development

A complete mechanistic understanding of development also requires knowledge of mechanisms that operate at the level of the whole organism to control whole-body phenotypes. In all animals, signals sent between tissues and organs, such as growth factors and hormones, play a key role in regulating development at the level of the whole organism (Leopold and Perrimon, 2007). These signals may be released locally to act upon neighboring cells, or into the circulation to impact distant cells. On target cells, these signals interact with receptors to activate cell signaling pathways, triggering an intracellular response. The coordinated activation of cell signaling pathways in multiple tissues and organs supports development at the whole-organism level. Importantly, sex differences in cell signaling pathways and in phenotypes regulated at the organismal level have been detected in multiple animals (Millington and Rideout, 2018).

Sex differences in signaling pathways affect development under normal physiological conditions

One example of how sex differences in cell signaling pathways affect development is the insulin/insulin-like growth factor signaling pathway (IIS). In all animals, IIS plays a key role in regulating growth during development (Grewal, 2009; Oldham and Hafen, 2003; Teleman, 2010). In both invertebrates and vertebrates, binding of IIS ligands to a receptor triggers the activation of an intracellular signaling cascade in target tissues to augment cell and tissue growth, leading to a larger body size. IIS activity is therefore a key determinant of final body size across multiple species. Notably, studies in diverse animals report male-female differences in IIS regulation and function.

In *Drosophila melanogaster*, IIS activity is higher in late third-instar female larvae than in age-matched males (Rideout et al., 2015; Millington et al., 2021a). Several factors likely contribute to increased IIS activity in females, including increased secretion of *Drosophila* insulin-like peptide 2 (Dilp2) (Rideout et al., 2015). Females also have higher levels of factors that stimulate Dilp2 secretion (Millington et al., 2021a) and lower levels of proteins that interact with Dilp2 to inhibit binding with the insulin receptor (Arquier et al., 2008; Honegger et al., 2008; Millington et al., 2021b). Thus, in *Drosophila* larvae, there is a robust difference in IIS activity between the sexes due to sex-specific regulation of IIS ligands and factors that influence ligand-receptor interactions. Although the mechanisms underlying this sex-specific IIS regulation remain incompletely understood, the function of the sex determination gene *transformer* contributes to the male-female difference in Dilp2 secretion (Millington et al., 2021a; Rideout et al., 2015).

In mammals, it is difficult to generalize about a sex difference in IIS activity due to multiple receptors and tissue-specific requirements for IIS in regulating body size; however, sex differences in IIS ligands have been reported (Gatford et al., 1998). In rats, mice, pigs, sheep and cattle, levels of the IIS ligand insulin-like growth factor 1 (IGF1) are higher in males than in females, although IGF1 levels are higher in female primates (Gatford et al., 1998). Circulating levels of another IIS ligand, insulin, are also higher in female humans than in males (Basu et al., 2006, 2017), whereas a sex difference in IGF2 levels remains unclear (Geary et al., 2003; Yu et al., 1999). As in flies, sex differences in IGF-binding and -interacting proteins have also been observed (Geary et al., 2003; Yu et al., 1999), suggesting sex-specific IIS regulation exists across diverse species.

Although IIS activity is only 20% higher in *Drosophila* females compared with males (Rideout et al., 2015; Millington et al., 2021a), this difference is physiologically significant: adult females are normally 30% larger than adult males, a difference that is strongly reduced or abolished upon decreased IIS activity (Testa et al., 2013; Millington et al., 2021b). In mammals, although total IGF1 levels are only modestly higher in males than in females, this difference allows males to achieve a heavier body weight than females (Ashpole et al., 2017; Baker et al., 1993; Bikle et al., 2001; Liu et al., 1993; Powell-Braxton et al., 1993). Thus, even minor differences in cell signaling activity between males and females cause visible differences in development.

Although we focused on the sex-specific regulation and function of IIS in this section in order to illuminate many ways in which a pathway may differ between the sexes, it is essential to note that multiple pathways have been implicated in regulating male-female differences in development. For example, studies have identified sex-specific requirements for different ligands of the bone morphogenetic protein (BMP) signaling pathway in regulating gametogenesis and reproductive processes in many animals (reviewed by Lochab and Extavour, 2017; Shah and Rogers, 2018), and sex hormones regulate BMP family members across many cell types (reviewed by Shah and Rogers, 2018). Similarly, male-specific production of a janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway ligand by the somatic gonad early in development activates pathway activity in the germline to promote male germ cell behavior (Wawersik et al., 2005). Indeed, there are clues from multiple animals that many signaling pathways (e.g. Notch, Hedgehog and Wnt) are sex-specifically regulated during gonad development (DeFalco et al., 2003; Deshpande et al., 2016; Franco and Yao, 2012; Liu et al., 2009; Penn and Schedl, 2007; Sanchez et al., 2001; Windley and Wilhelm, 2016), findings that may be relevant for other aspects of development and physiology regulated by these important pathways (e.g. stem cell proliferation, metabolic regulation and body size).

Sex differences in cell signaling pathways in response to environmental and life history changes

Changes in environmental conditions (e.g. temperature and nutrient availability) and life history (e.g. reproduction) also modulate the activity of specific cell signaling pathways. These context-dependent

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changes to cell signaling stimulate metabolic and physiological changes required to maintain homeostasis, to survive and to reproduce. The ability of an individual to coordinate pathway activity with environmental cues or life history changes is therefore an important determinant of stress resistance, survival and reproduction. Studies in multiple contexts now show that males and females differ in the coordination of cell signaling activity with environmental stimuli and life history events. For example, in rhinoceros beetles, there is sex-limited coupling between nutrients and horn size (Emlen et al., 2012). Normally, male beetles have horns and females do not. In nutrient-rich conditions, male beetle horns grow significantly larger, whereas a nutrient-poor diet causes the development of smaller horns. Female beetles, in contrast, do not show any nutrient-dependent changes in horn size. Interestingly, these effects on horn size were mediated by male-specific changes to IIS activity, demonstrating sex-limited coupling between an environmental condition and IIS regulation.

Similar sex-biased coupling between nutrients and cell signaling pathways has been reported in *Drosophila melanogaster*, where females have an increased ability compared with males to adjust body and trait size in response to several nutrients (Shingleton et al., 2017; McDonald et al., 2020; Millington et al., 2021a). In the case of dietary protein, female larvae reared on a protein-rich diet display a greater increase in body size than males (Millington et al., 2021a). Interestingly, the protein-rich diet triggers a female-biased increase in IIS activity, suggesting that IIS activity is more tightly coupled with protein intake in females than in males (Millington et al., 2021a). This sex-biased coupling between environmental cues and cell signaling extends to the activation of the Toll pathway following infection (Duneau et al., 2017), activation of cellular responses to oxidative stress in *Drosophila* (Pomatto et al., 2017) and to neurons that mediate behavioral responses to chemosensory cues (e.g. diacetyl and sodium dodecyl sulphate) in *C. elegans* (Bayer et al., 2020; Lawson et al., 2020; Ryan et al., 2014). Because similar differences have been recorded with other signaling pathways and animals (Baar et al., 2016; Clegg et al., 2003; Duneau et al., 2017; Havel et al., 1996; Klein and Flanagan, 2016; Saad et al., 1997; Testa and Dworkin, 2016; Tower et al., 2020), sex differences in the coupling between environmental factors and cell signaling pathways are found across many species. Beyond sex-specific coordination between environmental cues and cell signaling, sex-specific life history events contribute to male-female differences in cell signaling. This is exemplified by mating-dependent changes to female physiology and behavior (Avila et al., 2011; Chapman, 2001; Gillott, 2003; Pitnick et al., 2009; Poiani, 2006; Rodríguez-Martínez et al., 2011; Wolfner, 1997; Adams and Wolfner, 2007; Ameku and Niwa, 2016; Apger-MdGlaughon and Wolfner, 2013; Avila and Wolfner, 2009; Carvalho et al., 2006; Cognigni et al., 2011; Hadjieconomou et al., 2020; Harshman et al., 1999; Heifets and Wolfner, 2004; Mattei et al., 2015; Rubinstein and Wolfner, 2013; Sieber and Spradling, 2015; Vargas et al., 2010; Ribeiro and Dickson, 2010; Walker et al., 2015). Importantly, these mating-induced phenotypes have been shown to depend on specific hormonal and cell signaling pathways, indicating that a sex difference in life history events such as mating can be an important way to achieve a sex difference in cell signaling.

Sex differences in cell signaling are often subtle; however, there are important consequences for development, metabolic regulation, survival and lifespan. For example, although changes to nutrient quantity and IIS activity in *Drosophila* females significantly influence body size and lifespan, nutrient- and IIS-dependent changes to these phenotypes are less severe or absent in males (Clancy et al., 2001; Giannakou, 2004; Magwere et al., 2004; Millington et al., 2021a,b; Tatar et al., 2001; Woodling et al., 2020). Changes to nutrients and IIS activity often, but not always, also have female-biased effects on lifespan in other animals (Bokov et al., 2011; Holzenberger et al., 2003; Honjoh et al., 2017; Mao et al., 2018; Mitchell et al., 2016; Templeman et al., 2017; Xu et al., 2014).

When taken together, these studies highlight how including both sexes in studies on cell signaling pathways provides an opportunity to gain a deeper mechanistic understanding of how these pathways affect development. This principle holds when studying development in a normal physiological context, and when investigating how development is affected by diverse environmental conditions and life history events. Using a single- or mixed-sex population risks overlooking profound phenotypic consequences caused by changes to cell signaling activity if the affected sex does not comprise a large enough proportion of the experimental population. Similarly, there is a risk of making inaccurate predictions about the resilience of each sex to environmental variation, and about which pathways mediate these effects (Noiret et al., 2020).

Conclusions

Sex differences in developmental mechanisms are found across species and at each level of biological complexity. Although it is easy to predict that different mechanisms will operate in males and females for traits with visible differences between the sexes, distinct mechanisms also operate in the large number of organs with less conspicuous differences. Because we are likely aware of only a minority of sex differences within organs due to extensive use of single- and mixed-sex animal groups, as a community, we should normalize the inclusion of males and females in developmental biology studies. Successful and widespread inclusion of both sexes will provide several important benefits. First, we will gain a more-complete and accurate understanding of the genetic and molecular mechanisms underlying many aspects of development, behavior and physiology. When both sexes are not studied separately, the contribution of a specific gene to development may be obscured in mixed-sex groups or completely overlooked in single-sex groups. Second, we will gain a better understanding of the fundamental mechanisms that govern tissue and organ size control and the capacity of tissues and organs for repair. This is essential, as both size and capacity for repair are related to how well an organ performs its vital functions within the body. Third, we will improve our understanding of the cell signaling pathways that regulate development and maintain homeostasis in each sex. Given that signaling pathways regulate multiple aspects of development, influence organismal phenotypes (e.g. stress resistance, successful reproduction, lifespan) and contribute to disease, including males and females in developmental biology studies will provide insights relevant for a very broad scientific community.

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Competing interests

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