Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease

P Bermejo-Alvarez, D Rizos¹, P Lonergan² and A Gutierrez-Adan¹

Bond Life Sciences Center, University of Missouri, Columbia, Missouri 65211, USA, ¹Departamento de Reproducción Animal y Conservación de Recursos Zootécnicos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid 28040, Spain and ²School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Dublin, Ireland

Correspondence should be addressed to A Gutierrez-Adan; Email: agutierr@inia.es

Abstract

In adult tissues, sexual dimorphism is largely attributed to sex hormone effects, although there is increasing evidence for a major role of sex chromosome dosage. During preimplantation development, male and female embryos can display phenotypic differences that can only be attributed to the transcriptional differences resulting from their different sex chromosome complements. Thus, all expressed Y-linked genes and those X-linked genes that totally or partially escape X-chromosome inactivation at each specific developmental stage display transcriptional sexual dimorphism. Furthermore, these differentially expressed sex chromosome transcripts can regulate the transcription of autosomal genes, leading to a large transcriptional sexual dimorphism. The sex-dependent transcriptional differences may affect several molecular pathways such as glucose metabolism, DNA methylation and epigenetic regulation, and protein metabolism. These molecular differences may have developmental consequences, including sex-selective embryo loss and sex-specific epigenetic responses to environmental hazards, leading to long-term effects. This review discusses transcriptional sexual dimorphism in preimplantation embryos, its consequences on sex ratio biases and on the developmental origin of health and disease, and its significance for transcriptional studies and adult sexual dimorphism.

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Introduction

Sex is the individual characteristic which has the largest impact on mammalian phenotype and disease (Ober *et al.* 2008). In adult tissues, although there is increasing evidence for chromosome-led hormone-independent sexual dimorphism, most sexual dimorphism is believed to be caused by hormonal differences (both organizational and activational hormonal effects). However, before gonadal formation occurs, and therefore before any sex-related hormonal difference appears, the differences in sex chromosome dosage lead to a transcriptional sexual dimorphism that has been detected even before implantation (Kobayashi *et al.* 2006, Bermejo-Alvarez *et al.* 2010a). This phenomenon is responsible for the differences in metabolism, cell number and survival under suboptimal *in vitro* conditions or following cryopreservation observed for preimplantation embryos in several species, which may result in sex-specific mortality rates affecting sex ratio or lead to sex-specific long-term effects (reviewed in Gutierrez-Adan *et al.* (2006)). In this manuscript, we

review the current knowledge of the phenomenon and the implications for sex ratio skews, long-term consequences, transcription in the early embryo and adult sexual dimorphism.

Transcriptional sexual dimorphism in preimplantation embryos

Before gonadal differentiation, male and female embryos only differ in their sex chromosome content. This difference results in a sex bias in the expression level of sex chromosome-encoded genes, which may also affect the expression of autosomal genes. Y-linked genes are exclusively expressed in males and *SRY* transcription has been reported in male embryos in mice (Boyer & Erickson 1994), cattle (Gutierrez-Adan *et al.* 1997) and humans (Fiddler *et al.* 1995). More recently, global transcriptional studies (Kobayashi *et al.* 2006, Bermejo-Alvarez *et al.* 2010a) have reported the expression of other Y-linked genes, such as *EIF2S3Y*, *DDX3Y*, *KDM5D* and *YZRSR2*, in male embryos. On the other hand, X-linked genes are present in a double dose in females, but dosage

compensation by random X-chromosome inactivation (XCI) ensures an equal transcription level of most X-linked genes for both sexes in adult tissues. However, during the preimplantation period, X inactivation is a reversible dynamic process; both X-chromosomes are active after embryonic genome activation and XCI is not fully accomplished during early development, which leads to a higher expression of X-linked genes in female embryos (Kobayashi *et al.* 2006, Bermejo-Alvarez *et al.* 2010a). In this sense, a higher expression of selected X-linked genes such as *XIST*, glucose-6-phosphate dehydrogenase (*G6PD*), *HPRT1*, *PGK*, *XIAP* and *MAOA* was reported for mouse (Kay *et al.* 1994, Hartshorn *et al.* 2002), bovine (Gutierrez-Adan *et al.* 2000, Peippo *et al.* 2002, Wrenzycki *et al.* 2002, Jimenez *et al.* 2003, Morton *et al.* 2007) and human (Taylor *et al.* 2001) embryos. The developmental stage at which XCI is accomplished is not clear and may differ greatly between species (Okamoto & Heard 2009), but two global transcriptional studies reported that many X-linked genes are upregulated in female blastocysts in mice (Kobayashi *et al.* 2006) and especially in cattle, where most of them (~90%) are highly expressed in females (Bermejo-Alvarez *et al.* 2010a). Furthermore, imprinting mechanisms affecting X-linked genes, causing a unique or preferential expression of the paternal allele, may have a role in sex-related transcriptional differences, as male embryos lack the paternally inherited X-chromosome. In this sense, some X-linked genes such as *RHOX5*, *BEX1*, *BEX2*, *CAPN6*, *SRPX2* and *UBE2A* had been reported to be partially imprinted during preimplantation development (Kobayashi *et al.* 2006, Bermejo-Alvarez *et al.* 2010a), suggesting a synergistic effect of double X dosage and imprinting mechanisms on sex-related transcriptional differences.

The sex chromosome transcripts differentially expressed between the sexes can regulate the transcription of autosomal genes. X-linked genes may play a major role in this transcriptional regulation over autosomal genes, as they are more numerous than Y-linked genes, and it has been observed that the accomplishment of XCI is accompanied by a reversion of the transcriptional sexual dimorphism of autosomal genes (personal unpublished data). Indirect evidence for sexual dimorphism in autosomal genes were provided by antiviral assays, which suggested that interferon-tau (IFNT2) was produced to a greater extent in female embryos in ruminants (Larson *et al.* 2001). Later, different studies have reported sex-related differences in the transcription of candidate autosomal genes such as *SLC2A3*, *HSP70*, *DNMT3A*, *DNMT3B*, *HMT1*, *ILF3*, *GSTM3* and *PGRMC1* at the blastocyst stage (Morton *et al.* 2007, Bermejo-Alvarez *et al.* 2008b, 2010b). Two global gene expression studies at the blastocyst stage not only enlarged greatly the list of genes known to display transcriptional sexual dimorphism but also allowed an estimation of the extent of this phenomenon and a

deeper knowledge of the molecular pathways involved. In the mouse model, 591 transcripts were reported to display sex specific differences (Kobayashi *et al.* 2006). Among the transcripts upregulated in females, one-fourth were X-linked, with the rest being autosomal. In cattle, the extent of the sexual differences amounted to almost one-third of the transcripts expressed (2921 out of 9322), with most of them (2702) being autosomally encoded (Bermejo-Alvarez *et al.* 2010a). These two studies provide evidence for a large sex chromosome-lead transcriptional regulation of autosomal genes. The different extension of the transcriptional sexual dimorphism between both studies may be caused by the larger power to detect transcriptional differences in the bovine study due to the larger sample size. On the other hand, there may be differences among species, as the timing of XCI may differ greatly (Okamoto & Heard 2009). In this sense, the percentage of X-linked genes with a higher expression in females was greater in bovine (Bermejo-Alvarez *et al.* 2010a) than in mouse (Kobayashi *et al.* 2006) embryos, which may explain the differences at the autosomal level.

Molecular pathways involved

Transcriptional sexual dimorphism can lead to sex differences in molecular pathways, which may lead to different susceptibilities to environmental stressors and to sex-specific mortality rates or long-term effects in the offspring. One of the most discussed phenotypic sexual differences is that found in the speed of development, a controversial issue as different studies report contradictory results. Recent studies involving novel techniques such as reliable early embryo sexing and sex-sorted semen (Bermejo-Alvarez *et al.* 2010b) suggest that sex-related differences in speed of development appear only under suboptimal conditions such as high glucose concentration (Bredbacka & Bredbacka 1996), as a result of the transcriptional dimorphism (Gutierrez-Adan *et al.* 2006; Figs 1 and 2).

Glucose metabolism has been largely proposed to differ between male and female embryos. Total glucose metabolism was reported to be twofold higher in males compared with females, and the activity of the pentose-phosphate pathway (PPP) is four times greater in female than in male bovine blastocysts (Tiffin *et al.* 1991). Higher pyruvate and glucose uptake were also found for male human embryos (Ray *et al.* 1995). Transcriptional studies have observed a higher expression of the X-linked gene *G6PD* in bovine (Gutierrez-Adan *et al.* 2000, Wrenzycki *et al.* 2002, Jimenez *et al.* 2003) and human (Taylor *et al.* 2001) embryos. *G6PD* is the first and key PPP regulatory enzyme and therefore may be responsible for these metabolic differences, which may be responsible for altered sex ratio, as discussed below. The autosome-encoded facilitated glucose transporter *SLC2A3* was reported to be more highly expressed in

Figure 1 The effect of environmental hazards on the embryo may have no consequences, generate epigenetic alterations or affect embryo survival. Due to transcriptional sexual dimorphism, male and female genomes may react differently to environmental stress, leading to sex-specific long-term effects, as epigenetic marks may be altered in a sex-specific way, or altering sex ratio, as embryo survival rates may depend on sex.

bovine male blastocysts than in female blastocysts (Morton *et al.* 2007). However, gene ontology analysis of global transcriptional differences did not detect major alterations in glucose metabolism (Bermejo-Alvarez *et al.* 2010a), and the fold change of different transcripts involved in either anaerobic glycolysis or PPP did not show a clear pattern of expression (Fig. 3). However, the plasticity of early embryos could increase the differences in gene expression between male and female embryos only when maternal or *in vitro* culture environment affects embryo physiology (Table 1).

On the other hand, the higher expression of *XIAP* and *HPRT1* in female blastocysts could be related with the potential of female embryos to exhibit decreased apoptosis in high glucose environments (Gutierrez-Adan *et al.* 2001), and this could have implications for their enhanced survival under hyperglycemic conditions. The increased expression of X-linked genes may also explain the observation that male embryos are more sensitive to heat stress-induced oxidative damage than female embryos (Perez-Crespo *et al.* 2005). A further X-linked gene of potential interest is O-linked N-acetyl glucosamine transferase (*OGT*), which is involved in the

metabolism of UDP-N-acetyl glucosamine, and is more highly expressed in female bovine blastocysts (Bermejo-Alvarez *et al.* 2010a). The changes in the activity of *OGT* have been related to altered protein function, which in turn can affect the cellular phenotype. Interestingly, gene ontology analysis of global transcription have showed differences between male and female embryos in translation, proteolysis and protein transport (Bermejo-Alvarez *et al.* 2010a).

Epigenetic regulation, and particularly DNA methylation, can also differ among sexes during the pre-implantation stages. Particularly, it has been reported that both *de novo* DNA methyltransferases (*DNMT3A* and *DNMT3B*) and two genes related with histone methylation (*HMT1* and *ILF3*) are highly expressed in male bovine blastocysts compared with their female counterparts (Bermejo-Alvarez *et al.* 2008b). The same study observed a higher methylation level in one of the five sequences analyzed, in agreement with the transcriptional data, suggesting that sex-related differences in methylation are not a genome-wide but a sequence- or genomic region-specific phenomenon. Another recent study showed that the methylation level

Figure 2 Putative secondary sex ratio (i.e. at birth) control mechanisms. Before conception, primary sex ratio could be altered by either spermatozoa selection by the COC (which has been refuted (Bermejo-Alvarez *et al.* 2008a)) or by unequal chances to reach the site of fertilization between X- or Y-bearing spermatozoa. After conception, sex ratio can be skewed by a sex-specific embryo loss that may occur before maternal recognition due to the preimplantation sexual dimorphism.

Figure 3 Fold changes between male and female embryos for different transcripts expressed in bovine blastocysts involved in pentose-phosphate pathway PPP (left) or glycolysis (right) based on RTPCR (*G6PD*; Gutierrez-Adan *et al.* 2000) or array probe intensity (Bermejo-Alvarez *et al.* 2010a). Different substrates are framed; the transcripts detected in blastocysts are in italics, with those exhibiting sexual differences in bold letters with their fold change: positive values are upregulated in females and negative in males.

of one differentially methylated region (DMR) of the *IGF2* gene was lower in female than in male blastocysts (Gebert *et al.* 2009). The gene ontology analysis of the transcriptional sexual dimorphism in bovine blastocysts suggested a higher global transcriptional level in female embryos, in agreement with the lower DNA methylation (Bermejo-Alvarez *et al.* 2010a). These differences in epigenetic regulation may have consequences in the epigenetic alterations that can lead to adult disease, as addressed below.

Finally, protein metabolism and mitochondrial activity have been also suggested to differ among sexes. The gene ontology analysis in our bovine study (Bermejo-Alvarez *et al.* 2010a) observed that translation, proteolysis and protein transport were over-represented in the genes

upregulated in males, suggesting a more active protein metabolism. This finding may be related with the sex differences in amino acid turnover observed in bovine blastocysts (Sturme *et al.* 2010). Mitochondria have also been proposed to play a role in sex-related metabolic differences (Mittwoch 2004). In this sense, it has been observed that mtDNA copy number is higher in male bovine blastocysts than in female blastocysts (Bermejo-Alvarez *et al.* 2008b), and the cellular components 'mitochondria' and 'mitochondrial inner membrane' were over-represented in the upregulated genes in male embryos (Bermejo-Alvarez *et al.* 2010a).

Implications for sex ratio skews

It has been suggested that some maternal parameters such as body condition score, diet, glucose levels, dominance status, testosterone level, stress or exposure to environmental hazards may have an effect on sex ratio (Gutierrez-Adan *et al.* 2006). However, these observations lack convincing evidence for an underlying biological mechanism, and nowadays, only three methods (flow cytometrically sorted semen, embryo biopsy and transgenic models) have proved their efficiency in selecting the sex of the offspring. It is worth noting that due to its importance and ease of recording, sex ratio is one of the most common parameters analyzed in any animal population, and therefore, within such a wide number of observations, false skews are expected, emphasizing the need for mechanistic studies.

Mechanisms responsible for skewed sex ratios may occur before or after fertilization (Fig. 2; Gutierrez-Adan *et al.* 1999). The first scenario would imply either a putative spermatozoa selection mechanism occurring at the site of fertilization by the cumulus oocyte complex (COC) or unequal chances to reach the fertilization site between X- or Y-bearing spermatozoa due to differences in motility, capacitation or survival. However, reported spermatozoa selection mechanisms by the COC (Grant & Chamley 2007) have been refuted (Bermejo-Alvarez *et al.* 2008a) and, to date, it seems that there are no differences in motility or survival between X- or Y-bearing spermatozoa, at least in simple salt solutions (Penfold *et al.* 1998). The second scenario involves a sex-selective embryo/fetal loss, which requires the existence of sexual dimorphism. These postconceptional mechanisms are linked to a reduction in fertility, in contrast to the preconceptional mechanisms. The effect of embryo/fetal mortality on the reproductive success depends on the species and the stage of development. Thus, in polytocous species, a moderate embryo loss may not affect pregnancy and only lead to a reduction in litter size or even normal litter size, as ovulation rate is usually higher than litter size. In contrast, in monotocous species, the embryo loss occurring after maternal pregnancy recognition implies a pregnancy failure, whereas before

Table 1 Molecular pathways which may be affected by transcriptional sexual dimorphism in preimplantation embryos according to different molecular evidence, and their developmental consequences.

Molecular pathway	Evidences for sexual dimorphism	Sex-specific consequences
Glucose metabolism	Transcription: <i>G6PD</i> (Gutierrez-Adan <i>et al.</i> 2000, Taylor <i>et al.</i> 2001, Wrenzycki <i>et al.</i> 2002, Jimenez <i>et al.</i> 2003) and <i>SLC2A3</i> (Morton <i>et al.</i> 2007) Not clear in gene ontology (Bermejo-Alvarez <i>et al.</i> 2010a) Metabolism studies (Tiffin <i>et al.</i> 1991, Ray <i>et al.</i> 1995)	Speed of development (Bredbacka & Bredbacka 1996) Embryo survival (Gutierrez-Adan <i>et al.</i> 2001, Jimenez <i>et al.</i> 2003, Kimura <i>et al.</i> 2005)
DNA methylation and epigenetics	Transcription: <i>DNMT3A</i> , <i>DNMT3B</i> , <i>HMT1</i> and <i>ILF3</i> (Bermejo-Alvarez <i>et al.</i> 2008b) Gene ontology in embryos (Bermejo-Alvarez <i>et al.</i> 2010a) and differentiated cells (Wijchers <i>et al.</i> 2010) DNA methylation levels in embryos (Bermejo-Alvarez <i>et al.</i> 2008b, Gebert <i>et al.</i> 2009), ES (Zvetkova <i>et al.</i> 2005) and differentiated cells (Tsai <i>et al.</i> 2009, Wijchers <i>et al.</i> 2010)	Long-term effects for DNA methylation-related disorders (Fernandez-Gonzalez <i>et al.</i> 2004, Sjoblom <i>et al.</i> 2005, Weksberg <i>et al.</i> 2005) Long-term effects of periconceptual methyl-deficient maternal diet (Sinclair <i>et al.</i> 2007)
Protein metabolism	Gene ontology (Bermejo-Alvarez <i>et al.</i> 2010a) Amino acid turnover (Sturmey <i>et al.</i> 2010)	Long-term effects of periconceptual low-protein maternal diet (Kwong <i>et al.</i> 2000, Watkins <i>et al.</i> 2008)

that point (i.e. during preimplantation development), embryo loss represents a similar expenditure of energy and time for the mother to a non-fertile insemination. Thus, from an evolutionary point of view, postconceptional mechanisms are more likely to occur earlier than later, in agreement with the observation that the majority of pregnancy losses occur before maternal recognition (Dunne *et al.* 2000).

Sex-specific mortality has been demonstrated under suboptimal conditions. In humans, embryo transfer at early stages did not skew the sex ratio (Lansac *et al.* 1997), whereas several reports suggest a sex ratio skew in the offspring when embryos are transferred at the blastocyst stage (Tarin *et al.* 1995, Quintans *et al.* 1998, Menezo *et al.* 1999). In relation to the described differences in glucose metabolism between male and female embryos, glucose was proposed to accelerate the development of males and slow down the development of female bovine embryos (Bredbacka & Bredbacka 1996), and a moderate glucose concentration was reported to cause a preferential loss of female bovine embryos (Gutierrez-Adan *et al.* 2001). In agreement, glucose concentration during bovine *in vitro* culture (IVC) was positively associated with the percentage of males, which could be reversed by G6PD inhibition (Kimura *et al.* 2005). Surprisingly, the sex ratio changes were not accompanied by a sex-specific embryo loss, as survival rates were not altered (Kimura *et al.* 2005). In contrast, higher glucose concentrations during bovine and murine IVC reduced the percentage of males (Jimenez *et al.* 2003), and maternal diabetes in humans was suggested to result in a higher proportion of daughters (reviewed in Gutierrez-Adan *et al.* (2006)). Furthermore, the optimal glucose concentration may depend largely on the basal medium composition (Biggers & McGinnis 2001), which may confound any interpretation of its putative sex-specific effects. Taken together, the effect of glucose on sex ratio remains controversial.

Implications for the developmental origins of health and disease

Early preimplantation development is characterized by precise epigenetic events that completely reprogram the mammalian genome and establish epigenetic marks which will persist after birth. Because of global reprogramming (Santos & Dean 2004), these early stages are especially susceptible to epigenetic alterations caused by suboptimal environmental conditions, which are known to lead to long-term consequences during adulthood (Fernandez-Gonzalez *et al.* 2004). The sex-related differences in the transcription of epigenetic-related genes and on DNA methylation previously reported (Bermejo-Alvarez *et al.* 2008b, 2010a, Gebert *et al.* 2009) imply that male and female embryonic genomes may react quite differently to environmental stress, which provides a molecular basis for sex-specific long-term effects originating during preimplantation development. In this sense, most of the studies which report a developmental origin of health and disease (DOHaD) indicate that the effects depend on sex, including those limited to alterations during the preimplantation period such as assisted reproductive technologies (Klemetti *et al.* 2005). One of the most studied long-term effects is the large offspring syndrome (LOS) in bovine and ovine models, which is caused by epigenetic alterations caused by suboptimal *in vitro* culture conditions and which is characterized by a disproportionate growth of the fetus accompanied by a reduction in its viability. The presence of serum in the culture media of mouse embryos is known to produce long-term effects similar to the LOS, which differ between male and female embryos; in particular, increased body weight was only found in females and several behavioral abnormalities also depended on sex (Fernandez-Gonzalez *et al.* 2004). In the same animal, but using another model (presence or absence of a growth factor in the embryo culture medium), another

study associated suboptimal *in vitro* culture conditions with increased body weight and decreased relative brain size in males, but not in females (Sjoblom *et al.* 2005). In humans, Beckwith–Wiedemann syndrome (BWS) is an overgrowth syndrome demonstrating heterogeneous epigenetic alterations of two imprinted domains on chromosome 11p15, which originate during the pre-implantation period, and it has been related with altered methylation patterns in the DMRs regulating a cluster of imprinted genes including some involved in IGF2 signaling. A high frequency in monozygotic twins has been reported; with few exceptions, these twins are discordant for BWS and females (Weksberg *et al.* 2005).

Dietary alterations during the periconceptual period have also been reported to result in sex-specific long-term effects. In line with the sexual dimorphism in DNA methylation, a methyl-deficient maternal diet during the periconceptual period in sheep leads to long-term effects in the offspring by affecting methylation levels of some loci in a sex-specific manner: over half of the loci whose methylation was altered by the diet were only affected in males, which was consistent with a greater clinically relevant phenotypic effect for that sex (Sinclair *et al.* 2007). Regarding sexual dimorphism in protein metabolism (Bermejo-Alvarez *et al.* 2010a, Sturmey *et al.* 2010), a low-protein diet during the periconceptual period leads to cardiovascular and behavioral diseases in the offspring, and these phenotypes are sex-specific in mice and rats (Table 1; Kwong *et al.* 2000, Watkins *et al.* 2008).

Implications for transcriptional studies and adult sexual dimorphism

Sexual transcriptional dimorphism in adult tissues can be mediated by sex hormone effects or by sex chromosome complement (Bermejo-Alvarez *et al.* 2010a). The preimplantation period constitutes an ideal model to study the latter. In the field of embryology, the elevated number of genes exhibiting sexual dimorphism at these stages has obvious implications for transcriptional studies in individual embryos: data from male and female embryos should be analyzed separately, especially when a limited number of embryos per experimental group are used, as a stochastic excess of males or females is likely to occur in small numbers. The same can be said for nuclear transfer experiments, where cloned embryos for one sex are compared with a mix of male and female embryos. Furthermore, the sex chromosome-driven sexual dimorphism impacts transcription beyond the preimplantation period (Gabory *et al.* 2009).

In agreement with the situation observed in embryos, murine embryonic stem (ES) cells, which are directly derived from blastocysts, XX cell lines were reported to be hypomethylated compared with XY lines

(Zvetkova *et al.* 2005), and it has been suggested that the X-chromosome may encode a modifier locus whose product represses *de novo* DNA methyltransferases (Zvetkova *et al.* 2005). In differentiated cellular models, sexual dimorphism at the epigenetic level has also been found in neonatal mouse brains, with higher levels of both histone methylation and acetylation in males (Tsai *et al.* 2009). Interestingly, the higher level of histone acetylation seemed to be caused by testosterone exposure, but the higher level of histone methylation could not be reached in females exposed to testosterone (Tsai *et al.* 2009). Similarly, in sex reversal mouse models, sex chromosome complement rather than sex *per se* determined the extent of heterochromatic silencing, which was greater in XY lymphocytes than in XX lymphocytes, irrespective of their sex (Wijchers *et al.* 2010), in agreement with the situation observed in bovine blastocysts (Bermejo-Alvarez *et al.* 2008b). Furthermore, the effect was due to the X-chromosome complement, rather than the presence or absence of the Y-chromosome, which leads to a transcriptional regulation affecting over 1000 autosomal genes between XY and XX males or XX and XY females with 369 represented in both comparisons. Gene ontology of these sex-complement-sensitive genes highlighted differences in regulation of gene expression and RNA processing (Wijchers *et al.* 2010), similar to the bovine microarray study (Bermejo-Alvarez *et al.* 2010a). Taken together, it seems that while sex hormones undoubtedly play an important role in adult sexual dimorphism, sex chromosome dosage may exert a great influence and determine sexual differences in environmental epigenetic programming (Gabory *et al.* 2009).

Conclusion

Sex chromosome complement, irrespective of sex hormone exposure, may lead to a large transcriptional dimorphism affecting both sex chromosome- and autosome-encoded genes. Transcriptional sexual dimorphism affects different molecular pathways, resulting in functional consequences and varying susceptibility to environmental stressors. From an embryo perspective, this situation can lead to sex ratio skews by sex-selective embryo loss or to a sex-specific epigenetic response that culminates in sex-dependent long-term effects in the offspring. Researchers often assume that basic physiological processes are similar in female and male embryos; however, the sex of the experimental embryos should be considered a variable in the research. Furthermore, the preimplantation embryo constitutes an ideal model to study hormone-independent transcriptional sexual dimorphism, which may play a major role in adult sexual dimorphism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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