Sex-related physiology of the preimplantation embryo

David K. Gardner*, Mark G. Larman, and George A. Thouas

Department of Zoology, University of Melbourne, Melbourne, Victoria 3010, Australia

*Correspondence address. Tel: +61-3-8344-6259; Fax: +61-3-8344-7909; E-mail: david.gardner@unimelb.edu.au

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ABSTRACT: Male and female preimplantation mammalian embryos differ not only in their chromosomal complement, but in their proteome and subsequent metabolome. This phenomenon is due to a finite period during preimplantation development when both X chromosomes are active, between embryonic genome activation and X chromosome inactivation, around the blastocyst stage. Consequently, prior to implantation male and female embryos exhibit differences in their cellular phenotype. Manifestations of such differences include altered total activity of specific X-linked enzymes and the metabolic pathways they regulate. Subsequently, one would expect to be able to determine differences in the rate of consumption and utilization of specific nutrients between male and female embryos. Data to date on animal models support this, with sex-specific differences in glucose and amino acid utilization being reported for the mouse and cow blastocysts. Such differences in metabolic phenotype may logically be involved in the reported differences in growth rates between preimplantation embryos of different sex. As the fields of proteomics and metabolomics are being increasingly applied to human assisted conception it is prudent to consider how such technologies may be applied to identify sex differences in the human embryo. Such data would have implications far beyond current invasive technologies used to identify the sex of an embryo conceived in vitro for the diagnosis of X-linked diseases.

Key words: clinical assisted conception / embryo metabolism and metabolomics / embryo selection / preimplantation embryo physiology / sex-specific development

Introduction

Historically, in order to determine the sex of the preimplantation embryo, blastomere biopsy at either the cleavage or blastocyst stage has been performed, followed by the identification of the sex chromosomes through either fluorescence in situ hybridization (FISH) (Munne et al., 1993), polymerase chain reaction (PCR) (Martin-hago et al., 2010,) or more recently through single-nucleotide polymorphism (SNP) arrays (Treff et al., 2010) or comparative genomic hybridization (CGH) (Fragouli et al., 2008). However, such approaches are invasive and time-consuming (the issue of relative costs will not be addressed here). An alternative approach would be to consider the fundamental biological differences that exist between male and female embryos. It is evident that for a set period of time, spanning embryonic genome activation up to X chromosome inactivation, female embryos have two active X chromosomes (Fig. 1). The consequence of this is that the proteome of a female embryo is actually different from that of a male (with such differences persisting for some time after X chromosome inactivation) (Epstein et al., 1978). If such differences in the proteome can be quantitated non-invasively, either directly by analysis of the secretome (proteins released into the surrounding medium), or indirectly by quantitating cellular functions under the influence of X-linked genes/proteins, theoretically one could develop a non-invasive means of determining the sex of an embryo.

Over the past few years, there has been a resurgence of interest in the physiology of the human embryo, both at the proteomic (Katz-Jaffe et al., 2006a, b; Dominguez et al., 2010) and metabolic/metabolomic levels (Gardner et al., 2001; Seli et al., 2007), with the intent of using such technologies to better understand the embryo in order to develop more suitable culture systems (Gardner et al., 2000; Houghton et al., 2002) and cryopreservation technologies (Gardner et al., 2007), and to develop quantitative methods of embryo selection (Gardner and Leese, 1993). However, these highly sensitive technologies could also be used to assist in confirming, or otherwise, that male and female human embryos can be identified in the culture dish through their sex-specific cellular phenotype. Certainly, there exists data in animal models to support this proposition.

In the following sections, we wish to place differences in sex-specific embryo phenotype into a biological and evolutionary perspective. We shall also introduce data to show how the environment can affect the sex ratio at birth. One key factor in the environment that reflects its suitability to sustain reproduction is the level and quality of nutrition. Parental diet has a direct impact on the quality of the gametes produced,
and in the case of the mother, alters the environment within the reproductive tract, changing the levels of specific nutrients to which the preimplantation embryo is exposed. The relevance of this to human IVF is evident, given that the immediate environment of the embryo is determined by the composition and the nature of the supplements added to the media.

**Biological aspects of embryo sex**

**Environmental effects**

Environmental factors can affect maternal health by impacting directly on reproductive success and offspring survival, with sex selection acting as a key adaptative physiological response (Cameron, 2004), although to date this trend has proven incredibly complex and difficult to correlate with global environmental and ecological factors. The selection process is believed to be manifested in the adjustment of sex ratios during pregnancy, as seen in polygynous species such as primates and rodents, whereby the sex with greater reproductive success is favoured, as hypothesized by Trivers and Willard (1973). Changes in the abundance and availability of food are likely to represent key environmental factors, mainly in terms of female nutrition effects on reproductive physiology and behaviour. Sex-ratio skewing, the proportion of males to females in a cohort, is an important and repeated observation as a result of these environmental and nutritional effects, with most data emanating from mammalian studies, particularly rodent and livestock models. Temporarily malnourished female mice have been observed to deliver litters comprising significantly more females (Rivers and Crawford, 1974; Meikle and Drickamer, 1986). Similar evidence of skewing towards female offspring in malnourished females has been observed in the golden hamster (Huck et al., 1986, 1988). Conversely, female mice fed a diet very high in saturated fats produced significantly more males (Rosenfeld et al., 2003; Rosenfeld and Roberts, 2004). Despite the relative paucity of human data, skewing of the sex ratio has been observed in malnourished Ethiopian women, in a study finding that expectant mothers gave birth to more females than males (Andersson and Bergstrom, 1998).

Environmental variations in reproductive efficiency and sex ratio according to seasonal variability are also possible. For example, mice have been observed with decreased fertility, fecundity and offspring sex ratios skewed towards males in summer and females in winter months, supposedly to promote male survival during territory searching in the lead up to winter when females would be more abundant (Drickamer, 1990). Although in cold climate animals like the Siberian hamster, this trend was markedly reversed (Navara et al., 2010), due to shortened photoperiods. Interestingly, a seasonal trend has been observed in humans. A retrospective study of North American women revealed a skewing towards more females at birth, when the mother was herself born during colder months (Nonaka et al., 1999), perhaps representing a population-wide effect. It remains to be determined whether similar trends occur conclusively in women according to global factors (e.g. famine, war, geography, cultural background) or in selected subpopulations (e.g. women undergoing assisted conception).

**Maternal effects**

A variety of nutritional regimens have been shown to result in a skewing of the sex ratio, including high levels of sodium and potassium, and even caffeine (Rosenfeld and Roberts, 2004). Glucose has received considerable attention regarding fertility, where compromised embryo survival has been reported in association with maternal diabetes (Beebe and Kaye, 1991; Moley et al., 1991; Petry and Hales, 2000) with some evidence of skewed offspring sex ratios (Machado et al., 2001; James, 2006).

Recently, an offspring sex skewing in response to modifications in maternal consumption of lipids has been demonstrated in a sheep model (Green et al., 2008). It appears that sex-ratio skewing may represent a physiological response of the organism to changes in the intrauterine environment, mirrored by changes in maternal physiology, in the same way that compromised embryo viability has been observed following artificial induction of hyperlipidaemia in the same model (Leroy et al., 2010). Observations from other mammalian studies have also demonstrated that the internal environmental...
conditions of the female tract are directly affected by systemic conditions in the female (Bazer and Roberts, 1983). Rosenfeld et al. have furthermore shown quite dramatic differences in sex ratios in rodent offspring when mothers were supplied with variable lipid- or glucose-rich feed stocks (Rosenfeld et al., 2003).

In terms of specific nutrients, Jaszczak et al. have demonstrated that the amino acid complement of the blastocoele fluid in rabbit blastocysts largely matches that of the uterine fluid (Jaszczak et al., 1972), which may have some relevance to the way blastocysts exhibit sex-specific differences in amino acid exchange (Sturmey et al., 2010).

It has been suggested that maternal control of gestation is subject to several levels of environmental and physiological regulation (Linklater, 2007). Adjustment of the metabolism and growth rate of the preimplantation embryo is perhaps the best described point of physiological control of embryo development, implantation potential and intrauterine survival. Other maternal–foetal interactions are also likely to have an important impact on sex ratios at birth, especially in polyparous mammals. Marked sex-specific differences have been observed in bovine blastocysts produced in vitro following insemination with mechanically sorted populations of X- and Y-bearing sperm, possibly due to sex-specific sperm damage (Bermejo-Alvarez et al., 2008). Similarly, such differences between X- and Y-bearing sperm subpopulations to survive in utero may be influenced by the changing physical and biochemical milieu of the maternal tract, resulting in a maternal selection pressure on sex-skewing during fertilization (Pratt et al., 1987; Mitra and Chowdhury, 1989) (Fig. 2). This effect was also shown definitively in the golden hamster in relation to correlations between offspring sex-ratio and acidity of vaginal secretions (Pratt et al., 1987). To what degree such sex-specific differences occurring at the level of the embryo influence the sex-ratio of live born offspring, aside from maternal influences remains a point of controversy in any mammalian species.

## Sex differences in preimplantation embryo physiology

### X chromosome effects

The female embryo possesses two active X chromosomes until the blastocyst stage, as demonstrated in the mouse (Epstein et al., 1978), although the precise timing of X-inactivation has yet to be fully characterized in the human (van den Berg et al., 2009). In livestock species, complete X-inactivation is yet to occur by this stage, with widespread persistence of up-regulated X-linked gene expression (Okamoto and Heard, 2009). Some species-specific differences have also been reported in the tissue specificity and parental origin of the inactivated X chromosome (Harper et al., 1982; Goto et al., 1997). Regardless of the timing, it is clear that a sex-specific proteome would be functional for several hours, or days (as yet to be determined) after the corresponding genes on the X chromosome are inactivated. This state persists in the embryo post-implantation through to adulthood, however, the inactivated X chromosome is no longer structurally intact, becoming the perinuclear Barr-body (Lyon, 1972). Developmental differences between male and female embryos may also be associated with the persistence of mRNAs transcribed in a dosage-dependent manner from both X chromosomes before X-inactivation.

In a landmark study, Epstein et al. (1978) demonstrated that female cleavage-stage mouse embryos contain double the active form of the X-linked enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) compared with male embryos. This amazing piece of work was facilitated by using half embryos (created by splitting embryos at the 2-cell stage) and seeing half of the resultant blastocysts, while analysing enzyme activity in the other half. Epstein showed that a bimodal pattern of enzyme activity existed, reflecting two distinct pools of embryos within

![Figure 2](https://example.com/figure2.png) **Figure 2** Biological mechanisms during fertilization and preimplantation embryo development that could promote sex ratio skewing. (A) The ability of the reproductive tract to preclude or retard the progression of one sex-specific population of sperm could influence the outcome of fertilization (diagrammatically in the case of female (X-bearing) sperm, allowing the oocyte access to larger proportions of male (Y-bearing) sperm. For example, vaginal pH has been shown to influence offspring sex ratio. Also, the occurrence of sex-specific sperm damage may further influence the proportions of X- or Y-bearing sperm populations. (B) The oocyte may preferentially select one population of sperm during sperm attachment, further influencing the outcome of fertilization. For example, the time of ovulation (in relation to insemination) may promote preferential binding of sperm, as represented diagrammatically for female sperm. (C) The ability of a particular population of embryo to undergo selective implantation. For example, preimplantation male embryos have been shown to develop faster than female embryos, depicted here as reaching blastocoele formation sooner, thereby enabling them to survive for longer periods in the uterine environment.
the population studies, i.e. males and females. These data were subsequently confirmed by Kratzer and Gartler (1978) who showed that a bimodal distribution of HGPRT activity was evident from the 8-cell stage to the blastocyst. Analysis of human embryos through PCR analysis revealed no sex-related difference in HGPRT activity after 2 days in vitro, but that on Day 3, female embryos had higher expression than males (Taylor et al., 2001).

Other X-linked genes such as glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase (PGK) in the mouse embryo exhibit higher expression and enzyme activity in female mouse embryos than male ones, prior to X-inactivation at the blastocyst stage (Krietsch et al., 1982; Williams, 1986). Similarly in the bovine, HGPRT and G6PD are up-regulated in female blastocysts compared with their male counterparts (Gutierrez-Adan et al., 2000). It has also been shown that female embryos possess more mRNA for the X-linked inhibitor of apoptosis protein (XIAP) (Jimenez et al., 2003). Since the locus for the XIAP gene is closely upstream of the HGPRT gene, it is possible that female embryos have the potential for decreased apoptosis in high glucose environments (Gutierrez-Adan et al., 2001), which has implications for their enhanced survival under hyperglycemic conditions. The increased expression of such X-linked genes may also explain the observation that female mouse embryos are less sensitive to oxidative induced heat stress than male 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 (Kimura et al., 2008). Changes in the activity of OGT have been related to altered protein function, which in turn can affect the cellular phenotype.

More recently, whole genome array technology has been used to screen embryos after they have been sexed, providing important new insights into the role of the X chromosome, totalling ~1000 genes (Ross et al., 2005). Female embryos exhibit higher levels of expression of X-linked genes, compared with their male siblings. Differential expression of around 600 X-linked genes by the blastocyst stage has been reported in the mouse (Kobayashi et al., 2006), and a similar pattern is observed in the bovine, with up-regulation of nearly 90% of the 218 detected X-linked genes (Bermejo-Alvarez et al., 2010). Interestingly, both studies showed that a substantial number of autosomal genes are co-expressed with X-linked genes in a sex-specific manner, with the latter study showing up-regulation of more than 2900 autosomes (Bermejo-Alvarez et al., 2010). Therefore, as well as direct effects on the embryo phenotype, through an increased expression of specific X-linked genes, sex chromosomes also indirectly modulate dimorphisms in phenotype through their actions on other chromosomes. Conversely, these co-expressed autosomal genes may also be viewed as pivotal in the epigenetic regulation of balanced X-linked gene expression, as the zygotic genome becomes transcriptionally active and differentiation ensues.

**Y chromosome effects**

There is less evidence in relation to how the Y chromosome influences embryo developmental and implantation competence, following establishment of chromosomal sex in the developing preimplantation embryo. The Y chromosome contains several highly mutable genes whose loss of function has been associated with a range of defects in sperm structure and production (Krausz et al., 2003). Notably, the Y chromosome contains the sex-determining SRY gene, responsible for male gonadal differentiation in the developing fetus, however this occurs during post-implantation development (Haqq et al., 1994). Earlier on, there are morphological differences evident after fertilization and X-inactivation, such as smaller sperm asters in bovine XY embryos compared with XX embryos, following IVF using fluorescence-activated cell sorting (Beyhan et al., 1999). So it is plausible that genes expressed on the Y chromosome, or with associated changes in epigenetic reprogramming during the preimplantation period, have an influence on embryo development. In line with this, Burgoyne described developmental differences in euploid mouse XY cleavage stage embryos compared with XX embryos, including increased morula cell number and more advanced blastocoeal formation, which is reversed upon replacement of the Y chromosome with that from a less permissive strain, using inter-specific crossing (Burgoyne, 1993).

**Sex differences in epigenetic reprogramming**

Epigenetic reprogramming represents an important functional mechanism that is likely to mediate the transcription changes of sex-linked and autosomal genes to sex-specific differences in embryo development and physiology. Specific regions of chromatin-containing variable levels of methyl or acetyl side-groups are known to mediate the structural conformation and packaging of chromatin, as well as to block binding of specific transcription factors and other gene products, thereby causing transcriptional silencing of a gene. This can occur in some autosomes (e.g. H19 and Igf2), where one parental copy is more methylated than the other, the so-called parental ‘imprint’, in the sex chromosomes, the same process of differential methylation regulates the onset of X-inactivation (Monk, 1995).

Epigenetic modifications can occur in embryos in response to poor culture conditions (Doherty et al., 2000) or in vivo, as a side-effect of superovulation on oocyte development (Sato et al., 2007); in both cases, developmental compromise of the embryo can result. Such modifications are potentially serious, since they are heritable, sensitive to dynamic environmental changes and may persist in utero to affect post-implantation embryogenesis and maternal-fetal interactions (Mao et al., 2010). It is therefore logical that sexual dimorphisms in both the developmental competence and onset of imprinted gene methylation defects can occur simultaneously. This has been shown in the bovine, where sex differences in the onset and repair of imprinted gene methylation have been reported following IVF (Wrezyncki et al., 2002; Gebert et al., 2009).

**Sex differences in rates of embryo development**

Although the sex ratio of mouse blastocysts flushed from the uterus is 50:50 (Vickers, 1967) such types of analyses of in vivo-derived embryos will not reflect the speed at which embryos reached the blastocyst stage. Interestingly, Tsunoda et al. reported that the sex ratio of live young could be significantly skewed by transfer of mouse blastocystcs according to their blastocele formation times in vitro, with a shift towards male pups derived from fast-developing blastocysts (Tsunoda et al., 1985). Gardner and Leese (1987) also reported a shift towards male offspring when mouse embryos were selected for transfer based on their developmental capacity.
Sex-related physiology of the preimplantation embryo

Metabolomics and sex identification

Metabolic biomarkers of embryo sex and development

These sex-specific differences in developmental kinetics are plausibly mediated by embryo metabolism. The metabolic control of embryo development is pivotal to embryo physiology, providing energy for homeostasis and the mechanisms of differentiation and cell division. Individual preimplantation embryos are able to regulate development by adjustment of uptake and secretion of metabolites (the metabolicome), nutrient utilization (metabolism or metabolic rate) and regulation of metabolic proteins, such as enzymes (metabolic proteome). Historically, this is based on evidence from animal and human studies investigating carbohydrate and amino acid metabolism (Gardner et al., 2000), showing that developmental competence and viability are closely linked.

Glucose metabolism appears to be a pivotal metabolic biomarker, especially at the blastocyst stage (Gardner and Leese, 1987; Lane and Gardner, 1996). In one of the original studies to relate the utilization of glucose by individual mouse blastocysts to their subsequent viability (Gardner and Leese, 1987), it was observed that glucose uptake by female embryos (4.92 pmol/embryo/h, n = 13) was elevated compared with males (4.37 pmol/embryo/h, n = 25). In a follow-up study to determine whether metabolism could be used as a prospective criterion for embryo selection, it was again observed that female embryos exhibited an elevated glucose uptake (6.94 pmol/embryo/h, n = 9) compared with males (5.47 pmol/embryo/h, n = 13; P < 0.07) (Lane and Gardner, 1996). In both cases, the trend to higher glucose consumption was consistent with two important points; firstly female embryos possess higher levels of the X-linked enzyme G6PD (a rate limiting enzyme in the pentose phosphate pathway; PPP) (Williams, 1986), and secondly that female embryos exhibit a higher relative activity of the PPP (Tiffin et al., 1991). The two studies on the mouse (above) had both determined embryo sex through embryo transfer of individual blastocysts after their metabolism had been determined non-invasively through ultramicrofluorescence. Consequently, the numbers of embryos analysed was relatively small. Subsequently, our laboratory set out to investigate the relationship between the metabolism of the mouse blastocyst and its sex through the use of a male mouse with an X-linked fluorescent transgene to facilitate non-invasive sex determination (Hadjianakis et al., 1998). Males of this strain possess an X-linked enhanced green fluorescent protein (eGFP) (The Jackson Laboratory: tg (ACTB-EGFP)D4 Nagy/J), permitting the identification of subsequent sexed blastocysts which express adequate eGFP levels for detection by epifluorescence from the 8-cell embryo stage onward. Once the sex of each embryo had been diagnosed, non-invasive analysis of glucose utilization was determined (Leese and Barton, 1984). Using this approach a sample of 93 sexed blastocysts had their metabolism quantitated (Fig. 3). Consistent with the two earlier studies, female embryos exhibited a significantly higher glucose uptake than their male counterparts. Although the data in Fig. 3 show that a significant difference between glucose uptakes in male and female embryos exists, the magnitude of the increase involved (~12%) indicate that it may be difficult to use this metabolic parameter in isolation as a definitive prospective selection criterion. It is proposed that such analysis may best be done concomitantly with other substrates, i.e. amino acids (discussed below).

Such data on glucose metabolism are in agreement with previous studies in the bovine using radio labelled tracer substrates to quantify the fate of glucose, with the remarkable finding that female bovine embryos exhibit a 4-fold higher activity of the PPP, compared with male embryos (Tiffin et al., 1990, 1991). Interestingly, the increased...
glucose consumption may appear paradoxical, considering the female mouse embryo potentially develops at a slower rate; however, this effect can perhaps be explained by the observations discussed earlier of the differential effects of high glucose on sex ratios (Bredbacka and Bredbacka, 1996; Larson et al., 2001). Since bovine embryo development in the presence of elevated glucose is improved by the addition of antioxidants (Iwata et al., 1998) it has been proposed that the increase in embryonic glucose metabolism could lead to reactive oxygen species generation. Overall, based on its regulation at multiple biochemical and physiological levels, glucose appears a logical candidate for one of the embryo sex biomarkers (Rieger, 1984).

However, the only clinical data to date on sex differences was reported by Ray et al. (1995) who showed that pyruvate uptake was higher in male embryos from Day 2 to Day 5, with glucose uptake and lactate production significantly higher in male embryos on Days 4–5. Clearly, further analysis of human carbohydrate metabolism with respect to sex in the human embryo is required.

Amino acid metabolism represents another important biomarker of embryo development, and potentially sex. The collective effects of amino acids have been demonstrated conclusively to be beneficial to embryo development in several species (Gardner, 2008), and therefore make a logical nutrient to analyse with respect to subsequent embryo viability. In relation to sex, glutamine has been shown, again using single substrate metabolomics, to influence PPP metabolism of glucose to a greater extent in female bovine embryos, as an alternative energy source for carbohydrates (Tiffin et al., 1991). Newer non-invasive metabolic approaches have recently revealed differences in amino acid flux with embryo development and viability (Houghton et al., 2002; Brison et al., 2004). A recent study by Sturmeys et al. (2010) using HPLC has demonstrated a difference in the uptake of seven amino acids between male and female bovine blastocysts cultured in vitro. Interestingly, only two of those amino acids were significantly different between male and females when the blastocysts developed in vivo. For blastocysts developed in vitro, it was observed that female embryos consumed more arginine, glutamate and methionine, and produced more glycine, while male embryos consumed more phenylalanine, tyrosine and valine. No biological explanations for these differences were prescribed.

**Conclusions**

Sex differences are evident at the level of preimplantation embryo physiology, as shown by extensive studies in animal models. The basis of these differences remains the subject of intense investigation, with effects at multiple levels of regulation, including genetic and epigenetic, biochemical and metabolic, physiological, the maternal environment and in a broader sense at the level of conserving environmental and evolutionary trends. Detection of embryo sex using new technologies such as non-invasive metabolic analysis, are building upon the capabilities of ART to improve the treatment of infertility and reproductive health. While sex can already be detected using a number of established methods (Handyside et al., 1990), newer metabolomics technologies may provide an opportunity to generate predictive information based on sex-related differences in embryo physiology. The degree of manifestation of such sex-specific phenotypes appears to be affected by the environment in vitro.

Although the precise time of complete X chromosome inactivation has yet to be fully established in the mammalian embryo (including the human) (Cheng and Distechte, 2004; van den Berg et al., 2009), it is evident that at specific times during the preimplantation period expression of certain X-linked genes is higher in females (Taylor et al., 2001; Kobayashi et al., 2006; Bermejo-Alvarez et al., 2010). Furthermore, specific transcripts and resultant proteins can remain functional after X chromosome inactivation commences. Conservatively, female embryos during the period from the 8-cell to the early blastocyst stage, will exhibit a different cellular phenotype from males (Epstein et al., 1978). The evolutionary significance of this may be the ability of the mother to regulate sex-specific embryo development, through the composition of the fluids of the reproductive tract, which in turn reflect environmental conditions.

It is envisaged that ongoing and future studies will help to elucidate specific proteomic and metabolomic differences between male and female embryos. Glucose and certain amino acids make for promising candidates for direct analysis, and together with other metabolomic platforms, algorithms should be forthcoming on differences between male and female human embryos conceived through IVF.

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Sex-related physiology of the preimplantation embryo

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