Minireview

Sex-specific gene expression in preimplantation mouse embryos

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Abstract

The 3.5-day-old blastocyst-stage mouse embryo consists of two tissues and contains approximately 60 cells. This tiny structure has now been observed to express nearly 600 genes in a sex-specific fashion, including at least one gene (*Rhox*/*Pem*) expressed only in females from their paternal X chromosome.

The daily deluge of marketing advertisements might have us believe that our entire lives are occupied with sex. Recent work from Kobayashi and colleagues [1] suggests that this may not be too far from the truth. It has long been observed that gene-expression differences correlated with genetic sex exist at the preimplantation stages. This was first observed 30 years ago, when it was noticed that 50% of eight-cell-stage mouse embryos expressed the histocompatibility Y (Hya) antigen. At the time these were correctly presumed to be the male embryos [2]. Later work [3,4] confirmed their prediction. Since then, several studies have confirmed the existence of a handful of genes that appear to be expressed in a sex-specific fashion in the preimplantation embryo (reviewed in [5]). Kobayashi *et al*. [1] recently increased that handful of genes by two orders of magnitude when they observed 591 transcripts that appeared to be differentially expressed in either male or female blastocysts.

To study sex-specific expression, they analyzed RNA derived from more than 1,000 sexed mouse blastocysts on a DNA microarray representing 20,000 transcripts [1]. Although gene arrays have been used before with staged preimplantation embryos and have identified a complicated milieu of signaling pathways [6], they have not been used to look at differences between the sexes. To overcome the technical problem of correctly sexing so many embryos, Kobayashi *et al*. [1] used a strain of transgenic mice harboring a constitutively expressed X-linked green fluorescent protein (GFP) transgene (X^{GFP}) [7]. By breeding $X^{GFP}Y$ transgenic males to non-transgenic (XX) females, female progeny could be distinguished at preimplantation stages from male littermates by their green fluorescence, which results from the zygotic expression of the paternally inherited GFP transgene. Male blastocysts, which have only a maternally inherited X chromosome, were identifiable by the lack of fluorescence.

While the identification of such a large number of genes with possible sexually dimorphic expression will provide much grist for the sex-determination field, this study will also provide material to pursue the mechanisms of autosomal and sex-chromosome imprinting - the preferential inactivation of genes according to maternal or paternal inheritance.

Gene expression from the inactive paternal X chromosome

To test the validity of the assay, Kobayashi *et al*. [1] used reverse transcription PCR (RT-PCR) to confirm the sex-specific expression of the 11 genes that showed the greatest difference in expression between the sexes. Four of the 11 genes proved to be expressed in a sex-specific fashion, with between-sex expression differentials ranging from 2.5- to 14.8-fold. Although a number of autosomal loci were implicated as being differentially expressed in the study, the confirmation of expression patterns was carried out only for genes residing on the sex chromosomes.

Perhaps the most interesting aspect of the recent work by Kobayashi *et al.* [1] is that on further study, it became evident that one of the X-linked genes, *Rhox5/Pem* (reproductive homeobox 5/placenta and embryos gene), was apparently being expressed in females preferentially from their paternal X chromosome, which is preferentially inactivated in the preimplantation embryo. As a means of achieving dosage compensation in females, only one X chromosome is believed to be generally active in any given cell. This is true for the entire body, but the mechanism by which this occurs is different at different developmental stages. In later postimplantation stages, inactivation is random in the embryo proper (which will give rise to the fetus and resulting animal), that is, there is no preference for whether the maternal or paternal X chromosome is inactivated. This is not the case in the preimplantation embryo, however, nor in extraembryonic tissues after implantation: in both of these stages the paternal X chromosome exhibits preferential, imprinted, inactivation. [8,9] Furthermore, a body of evidence [10-14] is beginning to form suggesting that the choice of which X chromosome to inactivate during the preimplantation stages is not a singular decision and may be governed by cumulative dynamic changes (Figure 1).

In the diploid primordial germ cells of females, X inactivation occurs randomly [15], whereas in males both sex chromosomes undergo temporary transcriptional inactivation during meiosis [16,17]. After fertilization, it is likely that these initial imprints are modified coincident with the onset of zygotic transcription at the two- to four-cell stage (Figure 1b). The emerging molecular model holds that two separate pathways enact this modification [10,12,18]. First, it appears likely that the paternal X chromosome is delivered to the unfertilized egg in a partially inactivated state (Figure 1a). This is supported by the observation that two-cell embryos have partially inactivated paternal X chromosomes [12]. At this time a second pathway initiates a wave of *de novo* imprinting that acts upon the inactivated X through to the eight-cell stage to further impair its transcriptional activity (Figure 1c) [11]. This inactivation is correlated with spreading of the *Xist* RNA, accumulation of hypermethylated histones, and localization of *Polycomb* group proteins along the inactivating X chromosome [13]. *Xist* RNA is produced from the inactive X-specific transcripts (*Xist*) locus on inactivating X chromosomes and contributes to inactivation by binding progressively along the chromosome. Whether this *de novo* imprinting occurs after partial or complete removal of the imprints added in the sperm, or is simply layered on top of them, is uncertain. Thus, the level of inactivation of the paternal X chromosome increases through early development, possibly as a function of proximity of chromosomal regions to the paternally expressed *Xist* locus [12].

As the embryo begins to develop into a blastocyst, a new round of modifications occurs on the paternal X chromosome of those cells allocated to the inner cell mass [14], the precursor tissue of the embryo proper (Figure 1d). In these cells, the paternal X is reactivated, and dosage compensation is re-established in a random fashion. The trophectoderm cells, which have an extraembryonic fate, maintain paternal X inactivation.

This model does not rule out the presence of transcripts expressed from the paternal X chromosome and does not provide a mechanism for repression of the maternal allele of those transcripts. Thus, the finding by Kobayashi *et al.* [1] that *Rhox5/Pem* is expressed as a solely male transcript places it in a relatively rare class of genes including, most notably, *Xist* [5]. An additional 16-20 genes that are expressed only from the allele on the paternal X chromosome have been putatively identified by gene-array screens comparing midgestation parthenogenetic and androgenetic embryos (which are engineered to contain either two maternal or two paternal genomes, respectively) [19]. So far, however, the imprinted expression of *Xist*, and now *Rhox5/Pem,* are the only experimentally confirmed preimplantation examples of X-linked paternally expressed genes.

Rhox5/Pem is a member of the newly identified reproductive homeobox (Rhox) gene cluster. Like their better known Hox cluster cousins, the temporal and spatial order of expression of members of the Rhox cluster during gametogenesis correlates with their order on the X chromosome [20]. *Rhox5/Pem*-deficient mice display male subfertility phenotypes [20,21] consistent with the proposed role of Rhox proteins in gametogenesis. Given the imprinted expression of *Rhox5/Pem*, it will be interesting to examine whether other members of the Rhox cluster are expressed similarly in preimplantation embryos. As *Rhox5/Pem* is expressed during spermatogenesis, when the paternal X chromosome is acquiring its initial imprints, a detailed profile of *Rhox5/Pem* expression dynamics during spermatogenesis will undoubtedly be useful in determining the nature of the imprinting mechanism at this locus. In addition, as *Xist*-mediated inactivation occurs in a progressive manner, it will be important to look for correlations between the *Rhox5/Pem* expression dynamics in the preimplantation embryo and the expression of other genes between the *Rhox5/Pem* and *Xist* loci.

Towards defining the breadth of sex-specific differences

A couple of issues may cast a cloud over the observations made by Kobayashi and colleagues [1]. As they note, in mice, male preimplantation-stage embryos develop more rapidly than females [22,23]. Thus, some of the differentially expressed transcripts observed in the blastocysts sampled may represent artifacts produced by the slightly different developmental stages of the male and female embryos. Another complication could arise from the fact that the experiments were performed on *in vitro* cultured embryos.

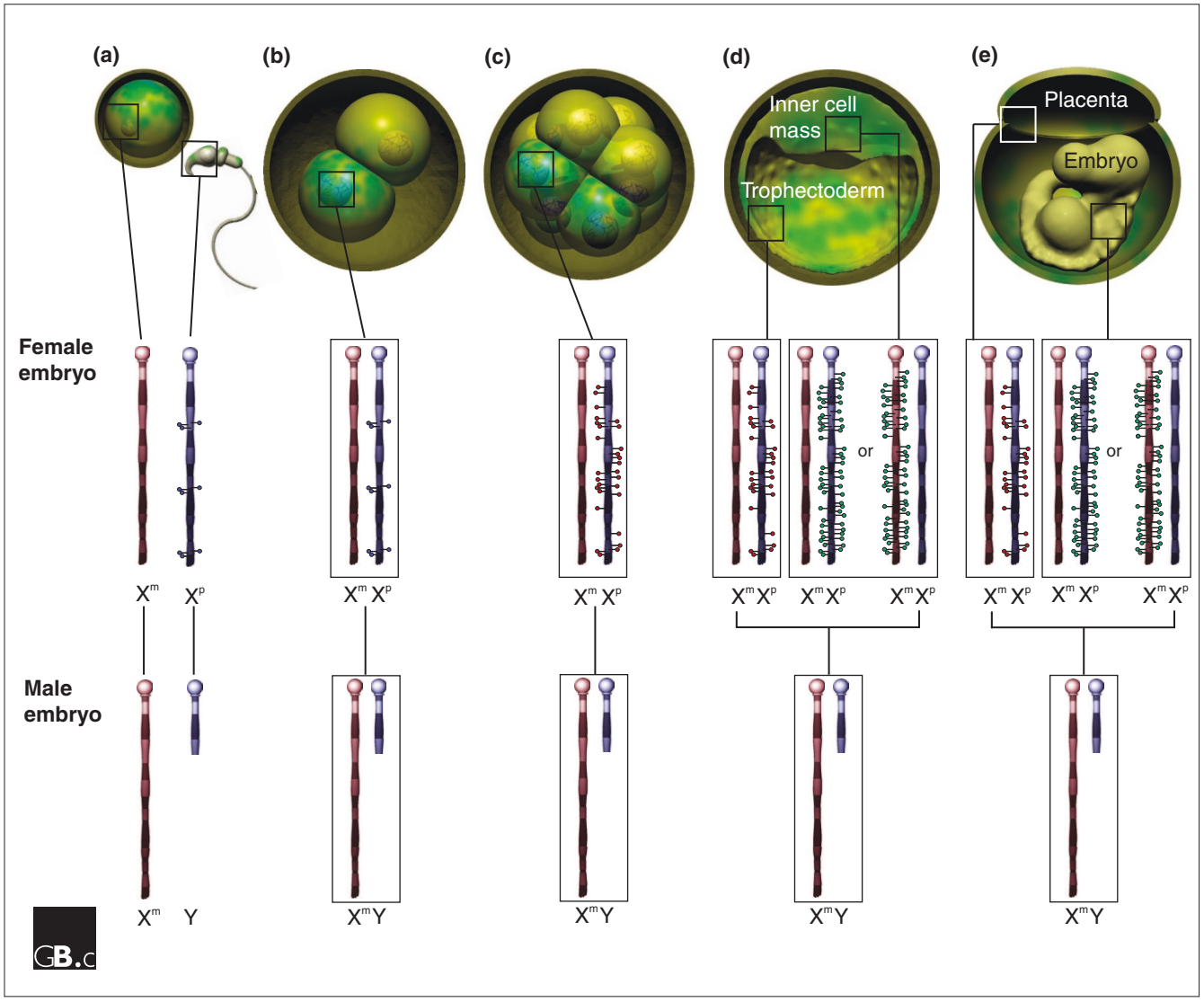

Figure 1
Model for X inactivation in the female mouse embryo. **(a)** Sperm bearing an X chromosome (the paternal X or X^p) have epigenetic marks (blue flags), leading to transcriptional repression of a portion of the chromosome. **(b)** In the female embryo (XX) these marks carry over through fertilization and the first cleavages. **(c)** From the two-cell to the eight-cell stage, a separate mechanism establishes additional marks (red flags) on the paternal X, repressing transcription at most loci. The maternal X chromosome (X^m) is unaffected. **(d)** In the blastocyst, repression of the paternal X is maintained in trophectoderm cells that will go on to form the trophoblast (an extraembryonic lineage) but is lifted in cells of the inner cell mass (ICM) (which are fated to form the embryo proper), where X inactivation now occurs randomly (green flags). **(e)** This state is maintained through later development, in which the paternal X is inactive in extraembryonic lineages, but random X inactivation occurs in the embryo proper. In male (XY) embryos, which inherit only a maternal X chromosome, no silencing occurs through these mechanisms.

Previous work has established that imprinting may be perturbed during some *in vitro* conditions. For example, 65% of mouse embryos cultured in Whitten's medium, a commonly used mouse embryo culture medium, inappropriately expressed transcripts from the autosomal *H19* locus, which is normally expressed only from the maternal allele, as well as from the X-linked *Xist* locus [24-26]. This aberrant imprinting was observed to persist through gastrulation [26], the last developmental stage tested. In fact, Kobayashi and colleagues [1] note that when the number of PCR cycles in the RT-PCRs were increased, a small amount of both *Xist* and *Rhox5/Pem* could be detected in male embryos (which do not contain a paternal X chromosome). The authors did control for this by looking at RT-PCR products from uncultured non-transgenic blastocysts; these blastocysts are, however, still subject to the same sex-related aging biases. Although the use of potassium simplex optimized medium (KSOM) in the present study probably avoids most of the artifactual gene expression seen with Whitten's medium, the expression signatures of individual genes will undoubtedly need to be

confirmed in subsequent studies using embryos that have developed *in vivo*.

As often is the case, we are left with many open questions. As the Rhox cluster maps distantly from *Xist*, it is uncertain whether the imprinting of *Rhox5/Pem* is correlated with coating of this region by *Xist* RNA or whether it is repressed by another mechanism. From a developmental standpoint, the results of Kobayashi and colleagues [1] beg the question as to exactly which cells express *Rhox5/Pem*. Are the cells of the blastocyst expressing *Rhox5/Pem* from the paternal allele the ones fated to form specific tissues (such as the trophectoderm), where *Rhox5/Pem* will be expressed predominantly from the maternal chromosome? One intriguing observation [1] is that *Rhox5/Pem* is expressed specifically in extraembryonic tissues, the ones that are fated to inactivate their paternal X chromosome. Even so, the expression switch reported by Kobayashi and colleagues is intriguing, and may indeed present a model for looking at X-inactivation dynamics near the Rhox cluster in peri-implantation embryos.

This work has given us yet another glimpse of the breadth of genetic differences between the sexes, and in doing so has revealed an intriguing early developmental imprint to a gene known for its role in gametogenesis. Clearly much work remains to be done, but as the putatively differentially expressed genes are individually tested, it will be interesting to determine whether these sex-specific transcripts have sex-specific roles in these earliest of embryos. One might be tempted to fantasize that within their DNA array, Kobayashi and colleagues [1] may have at hand information to identify loci influencing our own sex-specific traits. But, sadly, we still have to wait for the final word on what differentiates blastocysts from their 'blasto-sisters'.

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