Status of genomic imprinting in mouse spermatids

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The advent of human round spermatid microinjection (ROSI) into oocytes as a treatment for severe male infertility raises the question of whether spermatids have undergone all of the maturation processes necessary for normal development. It is particularly important to know whether spermatids have undergone correct genomic imprinting, which results in the parent-of-origin-specific expression of only one allele of a gene. We assessed the imprinting status of three maternally and three paternally expressed genes in interspecific hybrid embryos generated by injecting Mus castaneus spermatids into Mus musculus oocytes. We used the single nucleotide primer extension (SNuPE) assay to measure the relative expression of maternal and paternal alleles on the basis of sequence polymorphisms in the transcripts. Expression of imprinted genes in mouse embryos derived by ROSI did not differ from controls, indicating that paternal genes have undergone proper imprinting by the round spermatid stage.

Key words: genomic imprinting/infertility/ROSI/spermatids

Introduction

Genomic imprinting results in non-equivalent expression of the paternal and maternal alleles of certain genes in mammals (Stewart et al., 1997). Accordingly, mouse embryos reconstructed from either two female (parthenogenetic) or two male (androgenetic) pronuclei develop abnormally (McGrath and Solter, 1984; Surani et al., 1984). While the nature of the imprint that marks the parental alleles remains unclear, it must be capable of being erased and then reset sometime during gametogenesis so that oocytes carry the maternal imprint and spermatozoa carry the paternal imprint. The chromatin of the male gamete undergoes dramatic changes during the transition from spermatid to mature spermatozoon including the replacement of histones with protamines (Braun et al., 1995). The impact of the chromatin changes on imprinting is currently unknown. Current hypotheses suggest that methylation of imprinting control regions is the mark that differentiates the parental chromosomes (Tilghman et al., 1998). However, the exact timing of the erasure and subsequent establishment of the parent-specific imprint is an unresolved question. In contrast to their mono-allelic expression in most somatic tissues, imprinted genes are bi-allelically expressed in germ cells from the time that primordial germ cells (PGC) colonize the fetal gonad (E11.5) until oocyte meiotic maturation in the female and until the spermatid stage in the male (Szabo and Mann, 1995; Villar et al., 1995). This bi-allelic expression indicates either that the imprints are erased during germ cell development, or that imprints are present but not recognized. Therefore, transcriptional analysis of germ cells does not actually reveal the timing of erasure and re-establishment of the imprint. Because the imprint is not manifested in germ cells, determining whether imprinting is correctly established in spermatids requires the analysis of embryos resulting from fertilization by spermatids (Tycko et al., 1997).

In mice, offspring have been reported following fertilization of oocytes with spermatids (Ogura et al., 1994; Kimura and Yanagimachi, 1995). Initially the efficiency of producing liveborn offspring was low when the spermatids were fused to the oocytes, but this was dramatically increased when the spermatids were injected directly into the oocyte using a piezoelectric pipette-driving unit (Kimura and Yanagimachi, 1995). Mice derived in this way develop normally and are fertile. Subsequent generations of mice originally derived from spermatids have been born and show no deleterious effects (Y. Kimura and R. Yanagimachi, unpublished observations).

Given that the mice generated by spermatid injection appear normal, does it follow that imprinting in these mice is normal? Recent observations suggest that loss of genomic imprinting may be compatible at least with normal fetal and postnatal development. Chimeras created by the injection of Mus musculus/Mus spretus hybrid embryonic stem (ES) cells into Mus musculus blastocysts resulted in phenotypically normal chimeras that expressed imprinted genes bi-allelically in the hybrid cells (A.J. Villar, J. Gold, K. Mate, J. Meneses, J. McLaughlin, R. Pedersen, unpublished observations). Similarly, chimeras created with DNA methyltransferase −/− ES cells that had been rescued with a DNA methyltransferase transgene, resulted in healthy mice even though the contributing ES cells showed no imprinting (Tucker et al., 1996). In both of these cases, the ES cells constituted a high proportion of the chimeric tissues but no harmful effects from the presence of these abnormally imprinted ES cells were seen. Thus, absence of imprinting in the chimeric descendants of ES cells (which are known to contribute to fetal tissues but not to extra-embryonic endoderm and trophectoderm) appears to be compatible with normal development. By analogy, the
apparently normal phenotype of spermatid-derived mice may not be informative about imprinting in the paternal gamete.

The injection of spermatids for the fertilization of human oocytes has recently been used as a treatment for severe male infertility, specifically in cases of incomplete maturational arrest of spermatogenesis leading to azoospermia (Tesarik and Mendoza, 1996; Tesarik et al., 1996; Antinori et al., 1997; Vanderzwalmen et al., 1997). In order to evaluate the clinical relevance of spermatid injection it is important to address the imprinting status of spermatids in mammals. In addition to prenatal effects of any abnormalities in imprinting, there could be longer term effects. In humans some genetic diseases and tumour formation are associated with abnormal imprinting be longer term effects. In humans some genetic diseases and tumour formation are associated with abnormal imprinting.

Materials and methods

Animals

Mus musculus B6D2F1 females were oocyte donors and Mus castaneus Ei males were spermatid and sperm donors. C57BL/6J.CAST/Ei (N2) (F6), which are homozygous for the Mus castaneus Ei (N2) (F6), which are homozygous for the Mus castaneus Ei alleles at the distal 7 (Igf2, H19, Mash2), proximal 7 (Snrpn), and proximal 17 (Igf2r) regions were used for natural mating controls (gift from Jeff Mann).

Spermatid injections

Spermatid and sperm injections were carried out as described by Kimura and Yanagimachi (Kimura and Yanagimachi, 1995a,b). Briefly, oocytes were isolated from the oviducts of superovulated B6D2F1 females 16 h post-human chorionic gonadotrophin (HCG) injection, cumulus cells removed with hyaluronidase treatment and kept in CZB medium (Chatot et al., 1989, 1990; Kimura and Yanagimachi, 1995) at 37.5°C under 5% CO2 in air. Before spermatid injection, oocytes were activated with Ca2+-free CZB containing 5 mM SrCl2 for 30 min, then rinsed and cultured in CZB for 15 min. Round spermatids and epididymal spermatozoa were isolated from the seminiferous tubules of the testes and epididymis of Mus castaneus males (Jackson Laboratories) and suspended in HEPE–CZB containing 8% polyvinylpyrrolidone (PVP) respectively. Injections were performed in HEPE–CZB on a cooled (17–18°C) stage using a piezoelastic pipette-driving unit (Model PMM-10, Prada Me Packers, Tsuchiura Japan). A single spermatid nucleus was injected into an activated oocyte 15 to 45 min after SrCl2 treatment. As a control, an epididymal sperm head separated from the tail by application of a few piezo pulses was injected into an unactivated (metaphase II) oocyte. Injected oocytes were cultured in CZB. Oocytes containing two pronuclei were transferred to the oviducts of pseudopregnant recipients at the pronuclear or 2-cell stage and allowed to develop to mid-gestation stages or to term.

Isolation of RNA

Recipient females were killed and their embryos dissected at 10, 12 and 13 days of embryonic development. Embryos and extraembryonic tissue (including the amnion, yolk sac, allantoidos, and a portion of the chorion) were dissected in cold phosphate-buffered saline (PBS) and transferred to RNAsol B (Telsest Inc., Friendswood, TX, USA) on ice. Pools of four to 10 embryos or extraembryonic portions were frozen at −50°C in 3 ml RNAsolB. RNA was isolated following manufacturer’s instructions and resuspended in diethyl pyrocarbonate (DEPC)-treated H2O.

SNuPE reaction

SNuPE reactions were carried out as described (Singer-Sam et al., 1992; Szabo and Mann, 1995). To quantify allele-specific expression, reverse transcription of the embryonic and extraembryonic RNA samples was done as follows: reaction mix (1 µg RNA, 2.5 mM lower primer, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 1 mM dNTP) was placed at 4°C for 10 min, 42°C for 15 min, 2.5 U/µl reverse transcriptase (RT; Superscript II, Gibco BRL) was added and the reaction continued at 42°C for 15 min. The reaction was stopped by heating to 99°C for 5 min, and finally held at 65°C. Controls without RT were done for each RNA sample to ensure that DNA was not amplified. Two microliters of cDNA were then added to a polymerase chain reaction (PCR) mix (20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 mM dNTP, 0.5 mM lower and upper primers, 2.5 U/Rxn Taq DNA polymerase (Gibco, BRL). The reaction conditions were as follows: 94°C for 30 s, 42°C for 30 s, 72°C for 2 min (40 cycles), 72°C for 10 min followed by a 45°C soak for H19, Igf-2, Snrpn and Igf-2r, and 94°C for 45 s at 45°C for 15 min (40 cycles), 72°C for 15 min (40 cycles), 72°C for 10 min followed by a 45°C soak for Peg1 and Mash2. PCR products were purified with Prep-A-Gene DNA purification Kit (BioRad). Approximately 10 ng of PCR product were added to the SNUPE reaction (20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 2 µCi of the specific [32P]dNTP, 1 mM SNUPE primer, 0.75 U Taq DNA polymerase) and the reaction proceeded as follows: 4°C for 30 s, 42°C for 30 s, 72°C for 1 min. Electrophoresis through 12% polyacrylamide (BioRad) gels allowed SNUPE reaction visualization by autoradiography and quantification with a PhosphorImager. Specific [32P]dNTP added to each reaction were as follows: Peg1 maternal (M)-dTTP, paternal (P)-dCTP, Snrpn M-dGTP, P-dATP; Igf-2 M-dCTP, P-dTTP; Igf-2r M-dATP, P-dGTP; H19 M-dATP, P-dCTP; Mash2 M-dATP, P-dGTP.

RT–PCR primers

The PCR primers used to produce a 197 bp fragment of Peg1 were as follows: upper primer (U), 5'-GCTGGGGAAGTAGTCAGT-3'; lower primer (L), 5'-TTTCTTTTATGCAAGGGC3-'; SNuPE
Table I. Status of oocytes 3–5 h after injection with spermatid nuclei

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<th>Series</th>
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<th>No. of oocytes survived</th>
<th>% of injected oocytes</th>
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PN = pronuclei.

Table II. Status of oocytes 3–5 h after injection with spermatozoa nuclei

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<th>No. of oocytes survived</th>
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<tr>
<td>Total</td>
<td>113</td>
<td>96</td>
<td>85.0</td>
<td>82.7</td>
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</table>

PN = pronuclei.


Results

Round spermatid injections

Round spermatid nuclei were injected in 234 oocytes over six replicates. Of these 234 oocytes, 221 oocytes (94.4%) survived the injection. The remaining oocytes lysed during or shortly after the injection. Oocytes were evaluated for the presence of pronuclei 3–5 h after the injection. A total of 168 embryos were normally fertilized as indicated by the appearance of two normal-sized pronuclei (76% of surviving oocytes). In 48 oocytes (22%) one normal-sized pronucleus and one small pronucleus were seen as has been previously described (Kimura and Yanagimachi, 1995) (Table I).

Of the 83 embryos transferred to oviducts of pseudopregnant females at the pronuclear or 2-cell stage, 37 (45%) developed to term. Fifty-nine of 80 embryos (74%) transferred at the pronuclear stage implanted and developed to at least day 10 at which time the pregnancies were terminated to collect fetal tissue.

Spermatozoa injections

As a control for the injection procedure, oocytes in two replicates were injected with spermatozoa. Of the 113 oocytes injected, 89 (92.7%) were normally fertilized as indicated by two equally sized pronuclei. When these embryos were transferred at the pronuclear stage, 65 (73%) implanted and developed to at least day 10 at which time the pregnancies were terminated to collect fetal tissue (Table II).

Normal matings

As a control for the normal imprinting status of the genes investigated, superovulated B6D2F1 females were mated to C57BL/6J.CAST/Ei (N2) (F6) males. RNA was isolated and analysed from 10 concepti at day 10, seven at day 12 and six at day 13.

Analysis of paternally expressed genes

We investigated the allele-specific expression of the paternally expressed genes Snrpn, Igf-2 and Peg-1 in day 10, day 12 and
Genomic imprinting in mouse spermatids

Figure 1. Allele-specific expression of paternally expressed genes from embryos derived from spermatid injection. Single nucleotide primer extension (SNuPE) assay of (A) Snrpn, (B) Igf-2, and (C) Peg1 cDNA was made from 1 µg of total RNA from day (d) 10, day 12 and day 13 hybrid embryos and their extraembryonic (exemb) tissues, and for controls, from 1 µg total RNA from C57BL6 and castaneus day 15 embryos, or a mixture of the two. Reverse transcriptase–polymerase chain reaction (PCR) was performed on 10% of the resulting cDNA. Approximately 10 ng of the isolated PCR products was used in each SNuPE reaction. The numbers below each lane indicate the percentage of the total amount of transcript from the maternal and paternal alleles determined by PhosphorImager analysis. Percentages were corrected for the number of nucleotides added to the primer and for background determined by the quantitation of the control lanes which were 100% C57BL6 RNA or 100% Mus castaneus RNA. Expression of Snrpn and Igf-2 in embryos derived from spermatid injection is compared to embryos from natural matings, while expression of Peg1 is compared to embryos derived from spermatozoa injection. B6 = C57BL6 mice; Cast = Mus castaneus.

Snrpn and Igf-2 are both located on mouse chromosome 7, but the Peg-1 gene is located on mouse chromosome 6. Since Mus castaneus chromosome 6 is not present in C57BL6/J.CAST/Ei (N2) (F6) males which are homozygous for the Mus castaneus alleles at the distal 7 (Igf2 etc), proximal 7 (Snrpn etc), and proximal 17 (Igf2r) regions, Snrpn and Igf-2 are both located on mouse chromosome 7, but the Peg-1 gene is located on mouse chromosome 6. Since Mus castaneus chromosome 6 is not present in C57BL6/J.CAST/Ei (N2) (F6) males, we injected Mus castaneus spermatozoa into B6D2 F1 oocytes as an additional control. The sperm injection served as both a control for the injection procedure and gave us Mus castaneus/Mus musculus hybrid embryos at specific stages which are difficult to time with natural matings. Paternally expressed genes Snrpn, Igf-2 and Peg-1 were all found to be expressed exclusively from the paternal allele in embryos made by spermatid injection as well as control embryos from a normal mating, or in the case of Peg-1, from control embryos derived from sperm injection (Figure 1).

Analysis of maternally expressed genes

We investigated the allele-specific expression of the maternally expressed genes Mash-2, Igf-2r and H19 in day 10, day 12 and day 13 spermatid-derived embryos and their extraembryonic tissues. Mash-2 and H19 are both located on distal mouse chromosome 7 and Igf-2r gene is located on mouse chromosome 17. All are represented by the Mus castaneus alleles in

1053
Figure 2. Allele-specific expression of Igf-2r and Mash2 from embryos derived from spermatid injection. SNuPE assay of (A) Igf-2r and (B) Mash2. Analysis of the levels of transcription as noted in Figure 1 legend. Spermatid-injected embryos are compared to naturally mated controls for both genes. The numbers below each lane denote the percentage of transcripts from each allele. For abbreviations see Figure 1.

Figure 3. Allele-specific expression of the H19 gene from embryos derived from spermatid injection. In the case of H19 both naturally mated and sperm-injected controls were used. Analysis of the levels of transcription as noted in Figure 1 legend. The numbers below each lane denote the percentage of transcripts from each allele. For abbreviations see Figure 1.

The expression of H19 was variable; in spermatid-derived embryos, a substantial fraction (9–30%) of H19 transcripts were paternal in extraembryonic samples and to a much lesser extent (1–3%) in embryonic samples. By contrast, H19 expression was exclusively maternal when assayed in the RNA from natural mating controls. To determine whether this difference was due to in-vitro manipulation as previously observed for the H19 gene expression in preimplantation embryos (Sasaki et al., 1995), we assayed H19 expression in sperm-injected controls. Paternal expression of H19 was also detected at low levels (1–14%) in the sperm-injected control extraembryonic tissues and to a lesser extent in the embryos proper (1–2%). In both tissue classes, paternal expression of H19 was lower than in concepti derived from spermatid injection (Figure 3).

Discussion

Round spermatids have been injected into oocytes in order to determine the developmental potential of the male gamete (mouse) and as a treatment for severe male infertility (human). The results with mice have been impressive with normal pups being born, but few pregnancies have yet been obtained in humans. In neither mice nor humans has it been determined whether genomic imprinting is properly established by the spermatid stage. As previously noted, the status of the imprints...
in the immature gamete cannot be determined by assaying expression in the germ cells, which exhibit bi-allelic expression (Szabo and Mann, 1995; Villar et al., 1995), but must instead be determined by assaying imprinting in the resulting conceptus where the transacting factors or other products necessary for imprint recognition are functional. If the imprints from the previous generation were not erased, half of the spermatids would carry the maternal imprint. This would result in no expression of the paternal genes and bi-allelic expression of the maternal genes in 50% of the embryos. If the imprints were erased but not yet re-established, we might expect bi-allelic expression by analogy with the DNA methyltransferase knock-out experiments of Li et al. (1993). If the parental-specific imprints were properly established, mono-allelic expression would be expected.

By using the SNuPE assay to measure the expression of maternal and paternal alleles of imprinted genes, we have shown that imprinting appears normal for most imprinted genes tested in embryos derived from round spermatid injection. The three paternal genes, Igf-2, Snrpn and Peg-1, showed normal imprinting in the spermatid-injected embryos. This result was expected if the expression of the maternal copy of these genes had been repressed in the mature oocytes used. The maternal genes Igf-2r and Mash-2 both showed slightly leaky expression from the paternal alleles; however, this was also seen to the same extent in the controls. If the paternal genome had not been properly imprinted in spermatids we would have expected one of two results depending on whether the imprint was activating or repressing the genes being studied. In the case of repression, we would have expected completely bi-allelic expression (i.e. 50% of transcripts) of maternal genes in the absence of imprinting, but this was not observed. If the imprint resulted in activation of paternal genes, we would have expected to see an absence of paternal gene expression in the absence of imprinting. Again, this was not observed. Unlike the paternal expression of Igf-2r and Mash2, the leaky paternal expression of H19 seen with spermatozoa and round spermatid-injected embryos was not seen in embryos from normal matings. It has been shown in previous studies that H19 loses imprinting during in-vitro manipulations (Sasaki et al., 1995), which is consistent with our results. Only in one sample (day 12 extraembryonic tissue) did paternal H19 transcript levels even approach bi-allelic expression. In the remainder of samples, paternal H19 expression like that of Igf-2r and Mash2 was leaky rather than bi-allelic.

In principle, improperly imprinted genes in some of the embryos might have been masked by the pooling of the embryos. There was 10–20% leakage seen in the maternal genes which could have been due to a single embryo with improper imprinting. However, this leaky paternal expression was also seen in the controls to a similar degree. In addition, our pools of embryos were small; pools of seven and 11 at day 10 and pools of five at day 12 and four at day 13. The sensitivity of the assay was such that even a single improperly imprinted embryo at day 12 and day 13 would have been detected, as well as detecting bi-allelic expression. Absence of expression of Igf-2 from improper imprinting would have resulted in abnormally small embryos (DeChiara et al., 1990, 1991), and bi-allelic expression of Igf-2 results in larger embryos (Leighton et al., 1995), neither of which was seen. However, overexpression of H19 and Snrpn has no phenotype (Pfeifer et al., 1996). Mice with paternal disomy of the region of mouse chromosome 7 containing Snrpn appear normal (Cattanach et al., 1992). All round spermatid-derived embryos at day 10, 12 and 13 appeared normal and of equal size.

Whereas expression of both maternal and paternal genes is seen in spermatids when assessed directly using the SNuPE method (Szabo and Mann, 1995), they are nevertheless properly imprinted as shown by correct parental-specific expression of the genes in the resulting embryos. Our results show that during normal gametogenesis, the correct paternal imprint is largely or entirely established by the spermatid stage, even though it may be labile in the case of H19. The ethical implications and possible consequences of the clinical use of ROSI to treat male infertility associated with severe testicular dysfunction continues to engender debate (reviewed by Aslam et al., 1998; Sofikitis et al., 1998). Potential clinical applications of spermatic injection in human oocytes will depend on whether imprinting will be normal in spermatids derived from males with abnormal spermatogenesis. Our results do not address the status of imprinting in the spermatids of the infertile male; this question therefore requires further study.

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References


