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Short communication

Sexual differences of imprinted genes' expression levels

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ABSTRACT

In mammals, genomic imprinting has evolved as a dosage-controlling mechanism for a subset of genes that play critical roles in their unusual reproduction scheme involving viviparity and placentation. As such, many imprinted genes are highly expressed in sex-specific reproductive organs. In the current study, we sought to test whether imprinted genes are differentially expressed between the two sexes. According to the results, the expression levels of the following genes differ between the two sexes of mice: *Peg3*, *Zim1*, *Igf2*, *H19* and *Zac1*. The expression levels of these imprinted genes are usually greater in males than in females. This bias is most obvious in the developing brains of 14.5-dpc embryos, but also detected in the brains of postnatal-stage mice. However, this sexual bias is not obvious in 10.5-dpc embryos, a developmental stage before the sexual differentiation. Thus, the sexual bias observed in the imprinted genes is most likely attributable by gonadal hormones rather than by sex chromosome complement. Overall, the results indicate that several imprinted genes are sexually different in terms of their expression levels, and further suggest that the transcriptional regulation of these imprinted genes may be influenced by unknown mechanisms associated with sexual differentiation.

1. Introduction

In mammals, a subset of genes are functionally different between two alleles due to an epigenetic mechanism called genomic imprinting, by which an allele of a given gene is repressed (imprinted) depending upon its parental origin (Bartolomei and Ferguson-Smith, 2011). About 100 autosomal genes are known to be imprinted, and these imprinted genes are usually expressed in early-stage embryos, placenta and brains (Bartolomei and Ferguson-Smith, 2011). According to the results from human and mouse genetic studies, mutations on these imprinted genes have very similar functional outcomes, such as changes in fetal growth rates and perturbations in animal behaviors that are associated with reproduction and social interaction (Bartolomei and Ferguson-Smith, 2011). Genomic imprinting is also found only in eutherian mammals, which have a very unusual reproduction scheme that involves viviparity and placentation (Renfree et al., 2013). Thus, genomic imprinting is believed to have co-evolved with this unusual reproduction scheme of placental mammals to control the dosage of a subset of genes that play critical roles in the reproduction-related physiology and behaviors (Feil and Berger, 2007; Ivanova and Kelsey, 2011; Keverne, 2013; Renfree et al., 2013).

In mammals, sex is determined first by chromosome, more specifically by the testis-determining factor on Y chromosome (*Sry*), which is responsible for the formation of two different reproductive organs, testis and ovary. The subsequent sexual differentiation of developing brains is further triggered by the high levels of testosterone released from neonate's testis, which eventually determines animal sexual behaviors for reproduction (McCarthy and Arnold, 2011). As an outcome of a series of sexual determination and differentiation, males and females display quite different morphological and behavioral traits although both have almost identical genome sequences. Recent surveys further demonstrate that a large number of autosomal genes are sexually biased or dimorphic in terms of their expression levels in various tissues (Mozhui et al., 2012; van Nas et al., 2009; Yang et al., 2006). As described earlier, the overall biological impetuses of genomic imprinting are closely associated with the reproduction strategy of eutherian mammals, and thus it is likely that some of imprinted genes might also be sexually biased or dimorphic in terms of their function. In that regard, it is interesting to note that many imprinted genes are known to control fetal growth rates, variations in which could easily lead to different body sizes, a well-known sexual dimorphism in mammals. Also, many imprinted genes are highly expressed in the hypothalamus, which is one of the main targets within brains that are differentially organized by gonadal hormones between the two sexes (McCarthy, 2009; McCarthy and Arnold, 2011). Thus, it is feasible to predict that genomic imprinting may interact functionally with sexual differentiation for the successful reproduction of eutherian mammals.

Abbreviations: Peg3, paternally expressed gene 3; Zim1, zinc finger, imprinted 1; Usp29, ubiquitin carboxyl-terminal hydrolase 29; Zac1, zinc finger protein 1 regulating apoptosis and cell cycle arrest; Igf2, insulin-like growth factor 2; Grb10, growth factor receptor-bound protein 10; Dlk1, protein delta homolog 1 isoform 1 precursor; Snrpn, small nuclear ribonucleoprotein-associated; Ube3a, ubiquitin-protein ligase E3A isoform 2; Ndn, necdin; Nnat, neuronatin isoform alpha; Rasgrf1, ras-specific guanine nucleotide-releasing factor; Esr1, estrogen receptor 1; Adamts2, A disintegrin and metalloproteinase with thrombospondin motifs 2 isoform 1 preproprotein; Prlr, prolactin receptor precursor; Mest, mesoderm-specific transcript protein; H19, H19 mRNA; Gtl2, gene target locus 2.

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According to recent studies, some of imprinted genes are indeed either controlled by or at least affected by sexual differentiation although it is currently unknown at what level and to what extent sexual differentiation influences the function of these genes. During the gestation period of the mouse, the expression levels of several imprinted genes were shown to respond dramatically to a temporary mal-nutrition condition (starvation), and yet these responses were sex-specific. In particular, the expression level changes of *Peg3* were detected only in males, but not in females, whereas those of *Zac1* and *Igf2* were more pronounced in females (Radford et al., 2012). Similarly, a mutagenesis experiment deleting the imprinting control region of the *Peg3* domain, Peg3-DMR (Differentially Methylated Region), also derived unusual sex-specific effects: male pups tend to be more severely affected by the deletion than female pups in terms of body weight reduction and embryonic lethality (Kim et al., 2012). In the current study, we performed a series of expression analyses using various-stage mouse tissues to measure to what extent genomic imprinting is influenced functionally by sexual determination and differentiation. According to the results, several imprinted genes are indeed variable between two sexes in terms of their expression levels in developing brains.

2. Materials and methods

2.1. Mouse strain and breeding

The mouse strain with B6/129-mixed background mice was obtained from the Jackson lab and has been maintained at the animal care facility of Louisiana State University according to the animal care protocol. All the experiments related to mice were performed in accordance with the National Institutes of Health guidelines for care and use of animals, and also approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC), protocol #10-071. All animals were kept in a temperature-controlled room (22 °C) with 3–4 mice per cage at a 12-hour light/dark cycle and they were given *ad libitum* access to food and water. Embryos with different developmental stages were harvested through time-mated breeding schemes.

2.2. Sex determination of embryos

Genders of embryos were determined by PCR using the Maxime PCR premix kit (Intron Biotech). The Primer set was mouse *Sry* gene; mSry-F (5-GTCCCGTGGTGAGAGGCACAAG-3) and mSry-R (5-GCAGCTCTACTC CAGTCTTGCC-3). The PCR conditions were 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Genomic DNA was prepared from the amnion sac of harvested embryos. Each tissue was incubated overnight at 65 °C in the lysis buffer (0.1 M Tris-Cl, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl, pH 8.0, 20 μg/ml Proteinase K). This lysed extract was diluted 30 times with distilled water and 1 μl of the diluted extract was used in each PCR reaction.

2.3. RNA isolation and quantitative RT-PCR analyses

Total RNA was isolated using a commercial kit Trizol (Invitrogen) as per the manufacturer's protocol. The isolated total RNA (5μg) from each of the samples was reverse-transcribed using M-MLV kit (Invitrogen). The subsequent cDNA was used as a template for quantitative real time PCR. This analysis was performed using the iQ SYBR Green Supermix (Bio-Rad) using the iCycler iQTM multicolor real-time detection system (Bio-Rad). All qRT-PCR reactions were carried out for 40 cycles under the standard PCR conditions and two genes (*β-actin* and *28S*) were used as the internal control. Results of the qRT-PCR were analyzed based on the threshold (Ct) value. The experiments were performed in triplicate for each imprinted gene. At first a ΔCt value was calculated through subtracting the Ct value of that given replicate of a particular gene from the average Ct value of the internal control (*β-actin*). After that, the fold difference was determined for each replicate by raising 2 to the ΔΔCt powers (Winer et al., 1999). The relative expression level of an individual sample was further calculated through dividing its raw value with the average raw value of a given litter. One-tailed Student's *t*-test was used for statistical comparison of four male and four female samples ($p < 0.05$). Primer sequences and PCR conditions are available as Supplemental Table 3.

2.4. Box plot and data analysis

Box plot (also known as box and whisker diagram) was used to present the groups of numerical values in the current study. The data obtained from qRT-PCR was shown in their five number summaries, such as lower quartile (Q1), median (Q2), upper quartile (Q3), smallest observation (sample minimum), and largest observation (sample maximum). In this graphical representation, the first segment of the stacked column is invisible and ends where the lower boundary of the second quartile begins. The next segment consists of second quartile, which is calculated as: median − first quartile. The third segment is the third quartile and calculated as: third quartile − median. The maximum and minimum values are represented by the length of the whiskers and calculated as: first quartile − minimum and maximum − third quartile, respectively.

3. Results

3.1. Expression level differences of imprinted genes in 14.5-dpc fetuses

To detect potential sexual differences associated with genomic imprinting, we decided to measure expression level differences of imprinted genes between the two sexes of mice. Imprinted genes are usually expressed in brains and placenta during embryonic stages (Bartolomei and Ferguson-Smith, 2011), and thus we harvested two litters of 14.5-dpc (days post coitum) fetuses that had been derived from the mouse strain with 129/B6-mixed background. The gender of each fetus was first determined with PCR using DNA isolated from the amnion sac. From each litter, two males and females were selected for the isolation of total RNA from embryo head and placenta. The isolated RNA was individually reverse-transcribed and used for quantitative PCR analyses. Two internal controls (*β-actin* and *28S*) were used for normalization of the expression level of each imprinted gene. The relative expression levels of a given gene between individual embryos were represented as percentile scores by dividing each embryo's expression value with the average value of a given litter (Table 1). Two values were further calculated from the initial set of percentile scores: F/M ratio (the ratio of female to male values) and STD ratio (the ratio of standard deviation values of the entire set to each gender group). The actual formula for the STD ratio is as follows: the standard deviation value of the entire set divided by the average value of two standard deviation values from female and male groups. The STD ratio was used to evaluate the degree to which gender difference is accountable for the overall variation in a given gene: the greater values indicate the variations driven more by gender difference rather than stochastic individual differences.

The expression levels of 15 imprinted genes and one non-imprinted gene (*Esr1*, estrogen receptor 1) were initially analyzed using the total RNA isolated from the embryos and placentas of two harvested litters (Supplemental material 1). As expected, the majority of imprinted genes displayed high levels of expression in both embryo head and placenta based on the Ct (threshold cycle) values: the Ct values of imprinted genes ranged from 15 to 25 while the Ct values of the two internal controls were 14 through 15. Out of the initial set of imprinted genes tested, the following 5 genes displayed consistently different levels of expression between two sexes in the head of 14.5-dpc embryos: *Peg3*, *Zim1*, *Igf2*, *H19* and *Zac1* (Table 1 & Fig. 1A). The expression levels of these genes were greater in males than in females