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Germ-Line Chimerism and Paternal Care in Marmosets (Callithrix Kuhlii)

Corinna N. Ross
Texas A&M University-San Antonio, corinna.ross@tamusa.edu

J. A. French

G. Ortí

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The formation of viable genetic chimeras in mammals through the transfer of cells between siblings in utero is rare. Using microsatellite DNA markers, we show here that chimerism in marmoset (Callithrix kuhlii) twins is not limited to blood-derived hematopoietic tissues as was previously described. All somatic tissue types sampled were found to be chimeric. Notably, chimerism was demonstrated to be present in germ-line tissues, an event never before documented as naturally occurring in a primate. In fact, we found that chimeric marmosets often transmit sibling alleles acquired in utero to their own offspring. Thus, an individual that contributes gametes to an offspring is not necessarily the genetic parent of that offspring. The presence of somatic and germ-line chimerism may have influenced the evolution of the extensive paternal and alloparental care system of this taxon. Although the exact mechanisms of sociobiological change associated with chimerism have not been fully explored, we show here that chimerism alters relatedness between twins and may alter the perceived relatedness between family members, thus influencing the allocation of parental care. Consistent with this prediction, we found a significant correlation between paternal care effort and the presence of endothelial chimerism, with males carrying chimeric infants more often than nonchimeric infants. Therefore, we propose that the presence of placental chorionic fusion and the exchange of cell lines between embryos may represent a unique adaptation affecting the evolution of cooperative care in this group of primates.

Results

We examined the prevalence of chimerism in tissues derived from different embryonic origins by analyzing genotypes of microsatellite loci with a probability of detecting chimerism of 98% based on parental genotypes for these loci. A total of 92 intergenerational individuals that included 36 twin sets of Callithrix kuhlii (Wied’s black tufted-ear marmosets) and their parents were assessed. The samples were genotyped in an appropriate blind fashion such that the identity of the individual and the tissue type were unknown. All alleles were noted for each locus, and samples were identified as potentially chimeric if they contained three or four allelic variants at a single locus. The samples were then matched to identity, and twins were noted to be chimeric at a tissue only if the alleles were found to match both the parents as well as their twin. Further, a majority rule approach was used to assign alleles as “self” (i.e., diploid and inherited vertically from the parents) and “sibling” (inherited horizontally from the twin in utero) [see example in supporting information (SI) Fig. 3]. Of the 36 twin sets surveyed, 26 (72.2%) were determined to carry chimeric tissues. Exchange of alleles
Table 1. The number of Callithrix kuhlii individuals chimeric for each tissue type

<table>
<thead>
<tr>
<th>Samples from deceased animals</th>
<th>Tissue type</th>
<th>Genotyped, no.</th>
<th>Chimeric, no.</th>
<th>Chimeric, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>H</td>
<td>7</td>
<td>7</td>
<td>100.0</td>
</tr>
<tr>
<td>Blood</td>
<td>H</td>
<td>2</td>
<td>2</td>
<td>100.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>H</td>
<td>28</td>
<td>14</td>
<td>50.0</td>
</tr>
<tr>
<td>Liver</td>
<td>H</td>
<td>39</td>
<td>15</td>
<td>38.5</td>
</tr>
<tr>
<td>Heart</td>
<td>S</td>
<td>30</td>
<td>7</td>
<td>23.3</td>
</tr>
<tr>
<td>Hair</td>
<td>S</td>
<td>35</td>
<td>6</td>
<td>17.1</td>
</tr>
<tr>
<td>Lung</td>
<td>S</td>
<td>30</td>
<td>4</td>
<td>13.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>S</td>
<td>33</td>
<td>4</td>
<td>12.1</td>
</tr>
<tr>
<td>Gonad</td>
<td>G</td>
<td>21</td>
<td>2</td>
<td>9.5</td>
</tr>
<tr>
<td>Skin</td>
<td>S</td>
<td>36</td>
<td>2</td>
<td>5.6</td>
</tr>
<tr>
<td>Brain</td>
<td>S</td>
<td>31</td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>S</td>
<td>34</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td>Samples from living animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm</td>
<td>G</td>
<td>7</td>
<td>4</td>
<td>57.1</td>
</tr>
<tr>
<td>Saliva</td>
<td>S</td>
<td>31</td>
<td>16</td>
<td>51.6</td>
</tr>
<tr>
<td>Blood</td>
<td>H</td>
<td>45</td>
<td>22</td>
<td>48.9</td>
</tr>
<tr>
<td>Hair</td>
<td>S</td>
<td>50</td>
<td>13</td>
<td>26.0</td>
</tr>
<tr>
<td>Fecal</td>
<td>S</td>
<td>22</td>
<td>2</td>
<td>9.09</td>
</tr>
</tbody>
</table>

H, hematopoietic; S, other somatic; G, germ line.

between twins was not always bidirectional. In 14 twin sets, only one twin was found to have chimeric tissue types, whereas the other 12 chimeric twin sets revealed chimerism in both twin’s tissues.

Chimerism was found to be present in every tissue that was analyzed, and the occurrence of chimerism in tissues harvested from marmoset cadavers differed significantly across liver, spleen, kidney, heart, lung, brain, muscle, skin, and hair (“Samples from deceased animals” in Table 1; n = 25, Cochran’s Q = 51.6, df = 8, P < 0.001). To determine which tissue type accounted for the variance among the tissues, the tissues were ranked according to percentage of occurrence of chimerism and then grouped. A comparison of liver and spleen revealed a nonsignificant difference between the tissue types (n = 25, Q = 4, df = 1, P = 0.15). The addition of hair samples revealed a significant difference between the tissue types (n = 25, Q = 14.3, df = 2, P < 0.001). The grouping of all other tissues (heart, hair, lung, kidney, skin, brain, and muscle) resulted in no significant difference between the tissue types for the presence of chimerism (n = 25, Q = 10.3, df = 6, P > 0.1). Hematopoietic tissues were significantly more likely to be chimeric than all other tissue types (χ² = 4.88, df = 1, P < 0.05). The assessment of chimerism in tissues collected from living marmosets revealed nonsignificant differences between the tissue types (“Samples from living animals” in Table 1).

The presence of sibling-derived alleles in multiple tissues suggested that all embryonic cell lineages in C. kuhlii might be affected by chimerism, including germ tissue. In fact, gonadal tissue was found to be chimeric (2/21), and sperm samples were also chimeric (4/7). Additionally, the 36 twin sets analyzed for chimerism comprised multiple generations within 15 family lines. We determined that individuals in 5 of the 15 families passed on alleles to their offspring that represented gene lineages inherited horizontally from the sibling (see examples in Fig. 1 and SI Fig. 4). One breeding female, whose uterine twin was a male, produced offspring that inherited her sibling’s alleles. This documents the possibility that an XY primordial germ cell is capable of maturing and producing viable eggs in a female, a phenomenon that has not been documented for primates. Although we are not currently able to document the fate of the Y chromosome during development of the female’s oocytes, our data suggest the intriguing possibility that a female may pass on a Y chromosome to her offspring.

The presence of cells derived from different lineages within an individual may impact behavioral decisions. Genetic chimerism may give rise to genomic conflict such that an individual’s decision to cooperate within a group and care for members of the group may depend on the true, or perceived, genetic relatedness between the individuals (12, 16). To illustrate this with a simple example, we consider the increased proportion of shared alleles, because of genetic chimerism, between male twins produced by nonchimeric parents. A chimeric individual’s coefficient of relatedness to his twin could increase from the expected fraternal twin value of r = 0.5 to as much as r = 1 in certain tissues. Based on the prevalence of chimerism, the proportion of cells within a tissue that carry sibling alleles, and the probability of the direction of exchange obtained from our data, we estimate that male twins are on average related by r = 0.574 (see SI Text for calculations). More specifically, in a case of unidirectional exchange in which the soma of the donating twin is nonchimeric, he is related to the sperm of the recipient twin by an average r of 0.625 (see SI Text). The relatedness calculations suggest that chimeric marmoset siblings are more closely related to each other than typical nonchimeric mammalian siblings. Calculations of relatedness under more complex scenarios and involving parental chimerism are beyond the scope of this report; thus, at this stage, it is not known how parental–offspring relatedness may be affected by chimerism.
were found due to parity ($F_{1,28} = 2.124$, not significant) or family ($F_{8,28} = 1.865$, not significant). An intriguing possibility is that chimeric offspring might match the father at more kin recognition alleles, elevating the perceived relatedness and thus, the confidence of paternity in fathers. An alternative, but not mutually exclusive explanation for this behavior is that mothers actively avoid chimeric offspring. Although the behavioral data do not allow us to discriminate between these two hypotheses, they demonstrate a significant correlation between the chimeric status of offspring and altered patterns of maternal and paternal care in marmosets.

Discussion
This study thoroughly characterizes the extent and distribution of genetic chimerism throughout the tissues of callitrichid primates, which has not been done at this level previously. Hematopoietic chimerism in callitrichids was unambiguously documented in the 1960s (2), but it was unknown whether callitrichids displayed chimerism in tissues other than blood-derived tissues. Using highly variable genetic markers, we found that all tissue types sampled contain sibling alleles inherited via horizontal cell exchange. Perhaps most importantly, the germ line is also chimeric. Molecular genotyping analyses revealed that sperm can be genetically chimeric, and genealogical analyses demonstrated that marmosets can pass on sibling alleles, acquired \textit{in utero} from their twin, to their offspring.

Several lines of evidence were used to determine that the chimerism noted in nonhematopoietic tissues was not simply due to contamination of tissues with blood products. First and foremost, if chimerism was limited to blood products, then the sperm samples that were genotyped after they were separated from the ejaculate material should not have been chimeric. Additionally, the presence of hematopoietic chimerism alone cannot account for the finding of transmission of chimeric cell lineages across generations. Further, tissues known to be rich in blood supply such as the heart and lung tissue, as well as those likely to have high white blood cell counts because of immune function such as lungs, saliva, and skin, should have had equal intensity and prevalence of chimerism as the blood samples, which they did not. Finally, 12 animals were chimeric for nonhematopoietic tissues, yet they were not chimeric for hematopoietic tissues.

Although chimerism in other mammals, such as cows, cats, and humans, usually leads to sterility and appears to be selected against (1, 21), marmosets exhibit high rates of placental fusion and genetic chimerism. The delay in embryonic development at the time of chorionic fusion (6, 7) increases the chance that stem cell exchange between twin embryos occurs before advanced differentiation of embryonic tissues, thereby facilitating genetic exchange between the twins. All species in the subfamily Cal-
litrichinae share, as a derived character, a high investment of males in infant care, alloparental care, and obligate fraternal twinning (22, 23). Potential effects of chimerism for a marmoset include an increase in self-matching phenotypes between offspring and family members, which may lead to greater investment by other group members in offspring.

Chimerism may help to explain the unusual attraction of males to infants in callitrichids, although chimerism itself may not explain cooperative breeding and paternal care in other organisms. Genetic chimerism may serve as a genetic determinant influencing behavioral decisions involving cooperation and conflict in callitrichids, either through genomic conflict or direct and indirect fitness. The quantification of chimerism in callitrichids provides the basic knowledge to develop future field and captive studies to examine reproductive success, kin recognition systems, genomic conflict, and the impacts of relatedness on social behavior.

Materials and Methods

Study System. The only North American breeding colony of Callithrix kuhlii (Wied’s black-tufted-ear marmosets) was established in 1991 at the University of Nebraska at Omaha (UNO) by Jeffrey French. The colony at UNO provided a complete known breeding history, and multiple tissue samples were available for the majority of individuals because all carcasses of deceased animals were archived. We identified twin sets with known parentage, using colony breeding histories. We selected families in which a single female and male were housed together, with no subordinate males of breeding age, to ensure known paternity of the offspring. Thirty-six twin sets were available that fit these criteria, and all 36 twin sets and their parents (15 breeding pairs) were available for DNA sampling for this study.

Genetic Analysis. All samples were assigned a random letter/number combination to perform all genotyping analyses under an appropriate experimental blind. DNA extractions were done by using proteinase K digestion, phenol/chloroform purification, and ethanol precipitation. All samples were resuspended in water. Hair, saliva, sperm, and blood samples were extracted by using a QIAamp DNA Easy extraction kit (Qiagen, Valencia, CA) to ensure high quality and quantity DNA. Fecal samples were extracted by using the QIAGEN DNEasy stool kit. A GeneQuant II spectrophotometer was used to quantify DNA from the extracted sample. All DNA was then diluted to 20 ng/μl. DNA was PCR-amplified by using markers CJ1, CJ6, CJ13, and CJ14 (26), as well as species-specific marker CK2 developed for this project. Amplification products were analyzed with an ABI310, and genotypes were scored by using GeneScan software (Applied Biosystems, Foster City, CA). To determine the individual’s self genotype for each locus a majority rule analysis was applied. In cases where tissue genotypes varied within an individual, it was assumed that the diploid genotype found most prevalently across tissues most likely represented the alleles present in the individual because of vertical inheritance from the parents (self), rather than horizontal transfer from the twin (sibling alleles). In fact, there were no cases in which two different diploid genotypes were found among tissues in a single individual; all cases of chimerism involved three or four alleles in the tissue samples. Chimerism was verified for an individual when the blind was lifted and the putative sibling alleles were shown to be the majority rule genotype of the individual known to be the twin, and all alleles noted were also found in the parents of the twins. Heterozygous and chimeric genotypes were amplified at least three times and were confirmed as chimeric if all genotypes matched. Additionally, duplicate samples were collected for tissues such as the liver; all duplicate sample genotypes matched in 100% of the replicates. The allele frequencies for the microsatellite loci CJ1, CJ6, CJ13, CJ14, and CK2 are shown in Table 2.

Behavioral Data. Carrying data were collected daily via scan samples recorded throughout the day during the first 2 weeks of life for each infant. Each infant was identified by using unique markings, such as white stripes on the tail, and was assigned a unique name. The identity of the individual and the family member carrying the individual were noted at the time of observation. These scores were then tallied and the average carrying effort of each family member was calculated.

Statistical Analyses. Cochran’s Q test was used to evaluate whether there were significant differences between tissues that were identified as chimeric. Cochran’s Q provides a method for testing whether three or more matched frequencies or nominal data differ significantly among themselves (27). χ² tests were used to further examine the differences between the prevalence of chimerism in tissue types. Carrying effort of the caregivers of thirty infants that had been genetically assessed for chimerism were analyzed by using an analysis of variance, specifically 2 × (time: week1/week2) × 2 (chimeric/nonchimeric). Parity effects on care giving behaviors was assessed by a 2 (parity: multiparous/primiparous) × 2 (time: week1/week2) × 2 (carrier: dam/sire) ANOVA. Carrying differences between family groups was assessed with a 2 (time: week1/week2) × 2 (carrier: dam/sire) × 9 (family group) ANOVA.

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