Evolutionary genetics and implications of small size and twinning in callitrichine primates

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Edited by Alan F. Dixson, Victoria University of Wellington, Wellington, New Zealand, and accepted by the Editorial Board November 30, 2013 (received for review August 26, 2013)

New World monkeys (NWMs) are characterized by an extensive size range, with clawed NWMs (subfamily Callitrichinae, or callitrichines) such as the common marmoset manifesting diminutive size and unique reproductive adaptations. Perhaps the most notable of these adaptations is their propensity toward multiple gestations (i.e., dichorionic twins and trichorionic triplets). Indeed, with the exception of Goeldi's monkey (*Callimico*), callitrichine singleton pregnancies rarely occur. Multiple gestations seem to have coevolved with a suite of reproductive adaptations, including hematopoetic chimerism of siblings, suppression of reproduction in nondominant females, and cooperative alloparenting. The sequencing of the common marmoset (*Callithrix jacchus*) genome offers the opportunity to explore the genetic basis of these unusual traits within this primate lineage. In this study, we hypothesized that genetic changes arising during callitrichine evolution resulted in multiple ovulated ova with each cycle, and that these changes triggered adaptations that minimized complications common to multiple gestations in other primates, including humans. Callitrichine-specific nonsynonymous substitutions were identified in *GDF9*, *BMP15*, *BMP4*, and *WFIKKN1*. *WFIKKN1*, a multidomain protease inhibitor that binds growth factors and bone morphogenetic proteins, has nonsynonymous changes found exclusively in common marmosets and other tested callitrichine species that twin. In the one callitrichine species that does not produce twins (*Callimico*), this change has reverted back to the ancestral (nontwinning) primate sequence. Polymorphisms in *GDF9* occur among human cohorts with a propensity for dizygotic twins, and polymorphisms in *GDF9* and *BMP15* are associated with twinning in sheep. We postulate that positive selection affected NWM growth patterns, with callitrichine miniaturization coevolving with a series of reproductive adaptations.

reproductive biology | primate evolution

Callitrichines (Callitrichinae), also referred to as clawed New World monkeys (NWMs), are a subfamily of South and Central American NWMs composed of marmosets (*Callithrix*, *Cebuella*, *Mico*, and *Callibella*,), tamarins (*Saguinus*), lion tamarins (*Leontopithecus*), and Goeldi's monkey (*Callimico*), a monophyletic genus most closely related to the marmosets (Fig. 1). The taxonomic classification of Callitrichinae as a subfamily of Cebidae is a revision (1) of the original classification of these genera in the family Callitrichidae (callitrichids) (2). Although this revision is not universally accepted, we use the Callitrichinae classification in this paper. Callitrichines make up a distinctive primate lineage that is characterized by unique reproductive adaptations and small body size, ranging from 116 to 600 g based on species averages (3), with some individual tamarins exceeding 600 g. The lineage also displays other traits that are unusual in anthropoid primates and that have previously been linked to the reduction in body size that occurred in the callitrichine lineage (4). These traits include the presence of claw-like nails associated with apical pads on all digits except the hallux; the reduction or loss of the third molar, except in *Callimico*; and dentition and gut specializations for tree gouging and consuming tree exudates in marmosets. Callitrichines are notable for their dependence on secondary growth, disturbed forest or forest edges and can be grouped into a diverse diet type that includes gums, fruits, and insects (5–7).

Although other anthropoid primates occasionally produce twins and rarely higher-order litters, callitrichines are the only anthropoid primates having an "obligate multiples phenotype" resulting from ovulation of multiple ova per cycle (8, 9). Dichorionic, dizygotic (DZ) twins comprise the predominant litter size in the wild, although triplets have been reported (10), and twin, triplet, and quadruplet litters are frequently reported in captive colonies. The callitrichine species *Callimico goeldii*, which is most closely related to the marmosets, is an exception in that they produce singleton births. The production of singletons, along with the presence of a third molar that is missing in the other callitrichines, contributed to an earlier view that *Callimico* was ancestral in the callitrichine lineage (1, 11). However, more recent taxonomic classifications based upon DNA sequence place *Callimico* as most closely related to marmosets of the genus *Callithrix*

Significance

New World monkeys (NWMs) are characterized by an extensive size range, and clawed NWMs (callitrichines) such as marmosets manifest diminutive size and unique reproductive adaptations such as twinning. Evolutionary explanations have been proposed for these traits, and with the common marmoset genome assembly the genetic underpinnings of these traits can now be explored. Callitrichine-specific nonsynonymous substitutions were identified in *GDF9*, *BMP15*, *BMP4*, and *WFIKKN1*. We postulate that positive selection affected NWM growth patterns, with callitrichine miniaturization coevolving with a series of reproductive adaptations that bear benefit when gestating multiples. Given the high rate of morbidity and mortality with human twins, future studies into callitrichine genomic adaptations will undoubtedly lead to unique insights of benefit to their human counterparts.

Author contributions: R.A.H., S.D.T., and K.M.A. designed research; T.V. and K.M.A. performed research; S.D.T., T.V., D.E.W., K.C.W., and K.M.A. contributed new reagents/analytic tools; R.A.H., T.V., J.N.R., J.R., and K.M.A. analyzed data; and R.A.H., S.D.T., and K.M.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. A.F.D. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1316037111/-/DCSupplemental.

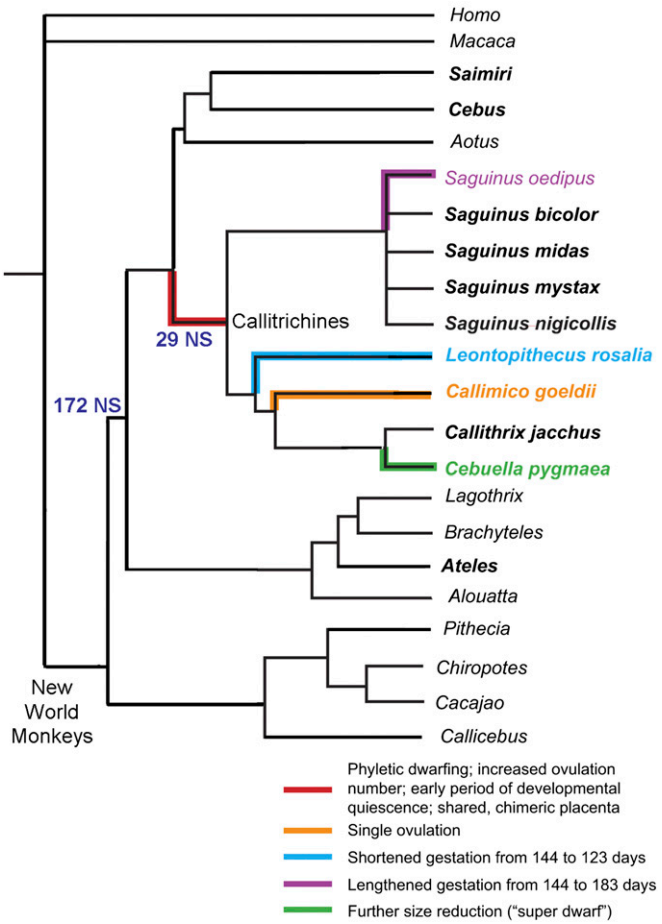

Fig. 1. Phylogeny of NWMs along with branches in which callitrichine reproductive phenotypes evolved. Annotated are unique traits likely critical to reproductive success and adaptation to multiple gestations, including phyletic dwarfing, increased ovulation of ova per cycle (manifesting as twins or higher-order multiple litters), early periods of developmental quiescence, and a shared chimeric placenta. Similarly depicted are single ovulations (*C. goeldii*), altered gestational lengths (*S. oedipus* and *Leontopithecus rosalia*), and further diminutive size (*Cebuella pygmaea*). Genes from NWM taxa in bold were sequenced as part of this study. Branch length does not represent phylogenetic distance. The number of lineage-specific NSs identified in callitrichines (Table S4 and eight GH/IGF substitutions) and NWMs (172 GH/IGF substitutions) are shown in blue. Phylogeny based on the work of Wildman et al. (82).

and not as an outlying or ancestral form (12–15). Given their phylogenetic position within the callitrichine clade, this is considered to be a derived (reversed) phenotype arising from an ancestor that twinned (16) (Fig. 1). *Callimico*, therefore, provides an interesting natural experiment from which the genetic basis of twinning can be further explored (9).

Concomitant with the high fecundity of twinning callitrichines are behavioral and physiologic adaptations suited to successful rearing of multiple offspring. These include a cooperative social organization of multiple males and females, in which generally a single female is reproductively active and reproduction is suppressed in nondominant females, either through behavioral suppression or physiological suppression of ovulation (17, 18). In the wild, adult males, nondominant females, and subadults share the responsibility of caring for the offspring, whereas in captivity juveniles also care for younger siblings. Possibly the most remarkable adaptation to twinning is their hematopoietic chimerism whereby the DZ twins that share a placenta also share placental circulation and exchange hematopoietic stem cells in a process that produces adult callitrichines whose circulating leukocytes consist of a mixed population of cells from all littermates. The reader is referred to Ross et al. (19) and Sweeney et al. (20) for recent observations.

It has been proposed that production of multiple gestations in twinning callitrichine primates is the result of selection pressures stemming from their small body size. Originally thought to be an ancestral trait (21), with characterization of the ancestral NWM it is now evident that callitrichines underwent an evolutionary reduction in body size (22). NWMs range in size from the pygmy marmoset (0.116 kg) to the wooly spider monkey (9 kg) (23), which is 78 times larger (3). This is a much wider size range than seen in Old World monkeys, where the mandrill is 18 times larger than the talapoin (3). Size evolution in NWMs is linked with dietary diversification, and Marroig and Cheverud (24, 25) propose that the dramatic differences in adult body size among NWMs are the result of complex adaptations to different dietary niches and diminished in utero growth velocity, and not a longer gestational length per se (26). However, diminutive maternal size in primates bears risk because it may result in fetal or maternal demise or morbidity, such as that arising from passage of a relatively large-brained infant through a small pelvic outlet (27). Adaptations such as a capacity to condense growth velocity in the fetal interval (akin to fetal growth restriction, but applied at a species-wide level) or a predilection for multiple gestations would successfully overcome such limitations. Similarly, it has been suggested that selection favors multiple births owing to factors such as increased predation of smaller animals (28), and others have argued that cooperative breeding followed selection for multiple gestations (29). Whereas most models of callitrichine evolution proposed to date have suggested that miniaturization in size preceded selection for increases in litter size, there has been very little analysis of the genetic basis of either small callitrichine body size, birth weight, or their pattern of obligate twinning to either substantiate or refute this supposition.

The sequencing of the common marmoset (*Callithrix jacchus*) genome offers the opportunity to explore the genetic basis of these unusual traits within this primate lineage. We hypothesized that genetic changes arising during callitrichine evolution resulted in multiple ovulated ova with each cycle, and that this change triggered a series of adaptations that minimized complications common to multiple gestations in other primates, including humans. We used the common marmoset genome to interrogate candidate genes that may regulate multiple ovulations. Based on our findings described herein, we propose a refined model of NWM, notably callitrichine, evolution.

Results

We investigated 63 candidate genes chosen because previous studies suggested a potential role for these loci in the number of ova produced each cycle and/or control of growth and body size (Table S1). Exons containing nonsynonymous substitutions (NSs) with a potential effect on protein function were identified based on marmoset alignments to other mammals (Fig. S1 and Tables S2 and S3). Exon sequencing in callitrichine ($n = 7$) and noncallitrichine ($n = 3$) NWMs yielded 21 callitrichine-specific NSs in 12 genes (Table S4, Fig. 2, and Dataset S1) presumptively involved in ovulatory regulation. Both sorting intolerant from tolerant (SIFT) (30) and PolyPhen (31) identified NS changes that likely alter protein function in three of these genes [bone morphogenetic protein 4 (*BMP4*), *FSTL4*, *WFIKKN1*], whereas SIFT alone scored a mutation in growth differentiation factor 11 (*GDF11*) as affecting protein function. UniProt (32) protein annotations were used to identify protein regions based on the orthologous human amino acid positions (Fig. 2 and Table S4). UniProt annotation identified these noted functional alterations: N163S in *GDF9* disrupting a glycosolyation site (Fig. 2*A*) and *FSHR* NS occurring in an extracellular topological domain that binds *FSH* (33). It is particularly interesting to note that the chr12:642862 NS in *WFIKKN1* is present in all callitrichines except *C. goeldii*, which is the only callitrichine species that regularly produces singletons rather than twins (Fig. 2*D* and Table S4). Given the phylogenetic position of *C. goeldii*, it is highly likely

to have reverted back to singleton births from an ancestral state that exhibited twinning. This amino acid change in WFIKKN1 is therefore a strong candidate for a role in the origin of twinning in callitrichines.

Likelihood ratio tests for positive selection (34) were performed to identify genes positively selected in marmosets compared with other primates with sequenced genomes. Among the genes showing significant evidence of positive selection were five genes in the growth hormone/insulin like growth factor (GH/IGF) axis with potential effects on perinatal growth velocity and diminutive body size: *GHSR* ($P = 0.034$), *IGF2* ($P = 0.00065$), *IGF1R* ($P = 0.0014$), *IGFBP2* ($P = 0.023$), and *IGFBP7* ($P = 0.0499$). Exons from these genes were also sequenced in other callitrichines and noncallitrichine NWMs. The marmoset sequences for these five genes were mapped to the squirrel monkey genome (*Saimiri boliviensis boliviensis*, Broad Institute, SaiBol1.0) and the resulting squirrel monkey sequences were aligned to the genes from other primate assemblies. The combination of targeted sequencing and comparison with the squirrel monkey genome allowed us to determine lineage specificity for 139 of the 162 NSs seen in comparisons of the marmoset genome to other primates with 8 callitrichine-specific and 131 NWM-specific NSs identified (Dataset S2). Callitrichine-specific NSs in *GHSR* and *IGFBP7* were predicted as possibly affecting protein function by PolyPhen (Dataset S2). SIFT or PolyPhen analysis predicted one protein affecting NS in *IGF2*, two proteins affecting NS in *GHSR*, and three proteins affecting NS in *IGF1R* at sites of NWM-specific NS. A protein affecting NS in *IGFBP7* showed undetermined lineage specificity (Dataset S2). Additional NWM-specific NSs were identified in other genes in the GH/IGF axis that were not identified as undergoing positive selection: *IGF2BP3* (1), *IGF2R* (1), *IGFALS* (14), *IGFBP4* (17), *IGFBP5* (2), and *IGFBP6* (6) (Dataset S3).

UniProt was used to identify the protein regions in which NS occurred. The callitrichine-specific NS in *GHSR* with a possible effect on protein function occurred in a cytoplasmic topological domain and the NS in *IGFBP7* occurred in an Ig-like C2-type domain (Dataset S2). NWM-specific NSs in *IGFBP2* were present in the IGF-binding protein (IGFBP) N-terminal and thyroglobulin type-1 domains. NWM-specific NSs observed in *IGF2* were present in the E domain of the precursor molecule, which is typically cleaved to produce the 7.5-kDa *IGF2* protein. In *IGF1R*, we observed 46 NWM-specific NSs likely to result in changes in binding specificity (Dataset S2). Specifically, NWM-specific NSs were present within the L1 and L2 domains of the α chain crucial for binding insulin-like molecules (35). A striking sequence of NSs within the Cys-rich region (CR1) essential for binding specificity of the ligand was also observed. The callitrichine-specific NS at chr6:8994565 occurred in the CR1 region and may have an effect on ligand binding affinity.

Discussion

Callitrichines are characterized by three distinctive and likely related traits (Fig. 1). The first is a reproductive biology characterized by obligate multiple gestations and associated adaptations in physiology, such as hematopoietic chimerism. In addition, adaptations in social organization, such as alloparenting and suppression of reproduction in subordinate females, similarly are observed. Second, callitrichine primates display a relatively small adult body size that seems to be the result of miniaturization following divergence from a larger NWM ancestor. Third, they have taken advantage of a dietary niche (forest edge habitats) that includes substantial insectivory and exudativory. The marmoset genome assembly in conjunction with targeted sequencing of ovulation- and growth-related genes in other callitrichines and noncallitrichine NWMs has enabled us to examine the molecular genetic basis of these traits.

The underlying biological mechanism for DZ twinning is clearly the release and fertilization of multiple oocytes, and both animal models and human data suggest multifactorial inheritance (36–38). In support of this being a polygenic trait in humans, spontaneous multiovulatory events in a single cycle leading to DZ twins has long been noted to occur more often in specific familial cohorts and ethnic groups, the highest incidence being in the Central African region (39); no single genetic locus has been identified to date (37). Similarly, we now report that the callitrichine obligate twinning phenotype is likely a polygenic trait resulting from the interaction of multiple genes, and we have identified callitrichine-specific NSs in 13 genes with a previously described role in regulating ovulation (Table S4).

BMP15 and *GDF9* are prime candidates for a role in callitrichine twinning based on numerous studies in other species, notably sheep breeds that frequently twin. A single callitrichine-specific NS was identified in the signal peptide of BMP15 (Fig. 2*C*). In sheep, five polymorphisms (40–42) and one deletion (43, 44) in BMP15 similarly associate with the twinning phenotype (Fig. 2*C*). *GDF9* contains three callitrichine-specific NSs in the propeptide region, including one that disrupts a glycosylation site (Fig. 2*A*). The propeptide region is cleaved before the production of the mature protein, but it serves a function in establishing the structure of the mature protein (38). In human case-control cohort analysis, four *GDF9* polymorphisms, including two in the propeptide region and a 2-bp deletion introducing a premature stop codon (aa position 433), show higher frequencies in mothers of DZ twins, and the proportion of mothers of DZ twins carrying any variant is significantly increased. An additional 1-bp insertion (C392-393insT) was identified in the propeptide region of a mother of twins causing a premature stop codon at amino acid position 143 but was not genotyped in other individuals. In twinning sheep breeds, two SNPs have been identified in the mature protein region (42, 45). Recently it has been shown that heterodimers of BMP15 and GDF9 are more biologically active than homodimers of either protein (46). Peng et al. (46) engineered and produced purified recombinant human and murine GDF9 and BMP15 and demonstrated both species specificity and 1,000- to 3,000-fold increased bioactivity of the human GDF9:BMP15 heterodimer in granulosa and cumulus cell expansion assays. Consistent with the notion that these molecules were selected for in callitrichines, GDF9:BMP15 heterodimeric preference was ~10^2- to 10^3-fold higher in humans than heterodimer-driven expansion in mice. This suggestion of functional species-specific heterodimeric preference lends further support to several decades of genetic observations in humans (46).

Aside from *BMP15* and *GDF9*, two other strong candidates for a role in callitrichine obligate twinning were identified. *BMP4* (Fig. 2*C*) contained two NSs, one of which was identified as affecting protein function. In Hu sheep, *BMP4* mRNA expression in the ovary is positively correlated with ovulation number (47). *WFIKKN1* (Fig. 2*D*), which has an inferred role in ovulation (48), exhibited four NSs, more than any other ovulation-related gene examined, including one with a likely effect on protein function. Interestingly, the chr12:642862 NS in *WFIKKN1* was not present in *Callimico*, the only callitrichine that does not twin. The nucleotide in *Callimico* was the same as that in nontwinning primates, suggesting it has reverted back to the ancestral sequence and may be involved in the loss of obligate twinning in this species.

Our findings pertaining to the role of the GH/IGF axis in relation to both ovulation and growth in callitrichines is supported from the following considerations. IGF is an essential regulator of fetal and placental growth, and data from a variety of mammalian models suggest a link between regulation of *IGF1*, *IGF2*, and the IGF-binding proteins in ovarian folliculogenesis and as determinants of fetal growth and later adult body size (49, 50). Marroig and Cheverud (4) previously proposed that the miniaturization of callitrichines could be accomplished almost entirely in utero given their strikingly different prenatal growth pattern (51–54). This timing of the development of the placenta and organogenesis is unusual, with the embryo being quiescent until around day 40, such that organogenesis lags behind that observed in other primates by about 3 wk (55). The smallest of all primates, dwarf and mouse lemurs (Cheirogaleidae), are characterized by multiple births but combined with a primitive horned uterus. This group seems to have undergone body-size reduction owing to reduced duration of growth phases (56). We propose that

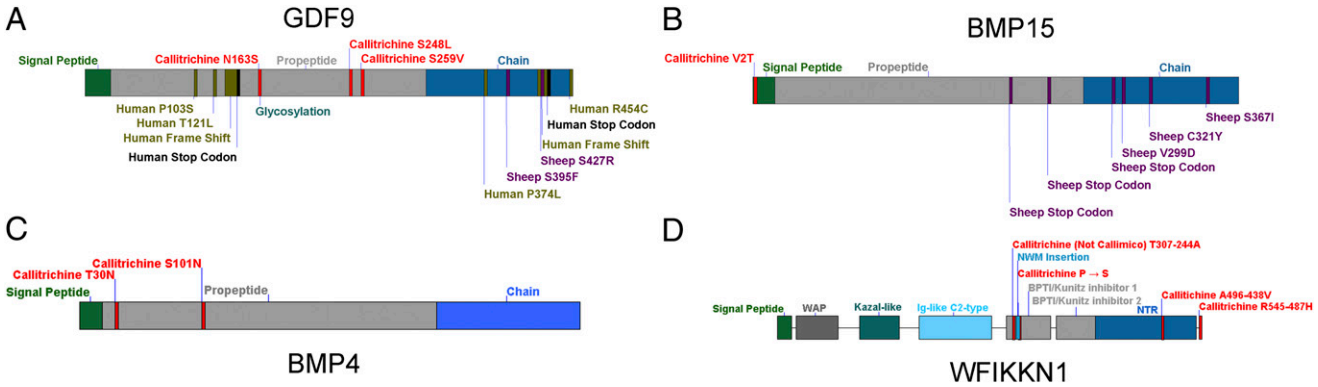

Fig. 2. Callitrichine-specific amino acid changes (annotated in red) in four genes with a likely role in regulating the number of ova and hence multiple gestations (*A–D*). In addition to NS substitutions in callitrichines (annotated as Callitrichine in red), polymorphisms found in both human populations (annotated as Human in black for stop codons and olive green for other mutations) and sheep breeds (annotated as Sheep in purple) that demonstrate a propensity toward twinning are shown. Protein regions and domains are based on UniProt annotations. The WFIKKNA T307A mutation absent in *Callimico goeldi*, the only nontwinning species, is noted in panel *D*.

the distinctive combination of obligate twinning within a simplex uterus and shared placentation created selection pressures that may have led to reduced prenatal growth in the callitrichines. The twinning callitrichine bidiscoid placenta is produced by trophoblasts contributed from both conceptuses. Within a week of implantation, the blastocyst has rapidly expanded and fused into a common chorion. Not dissimilar to human twin–twin transfusion in monochorionic gestations, extensive vascular connections develop within each disk, forming a single exchange unit (57). However, distinct from human monochorionic gestations, at day 61 hematopoietic foci begin to develop within the placenta, peaking in mass at around day 100 then declining so that few are present at delivery around day 143. These hematopoietic foci within the chimeric placenta are the source of hematopoietic cells for both embryos, resulting in hemaotopoietic chimerism. The benefit of such an early developmental lag and timing of hematopoietic foci would be to protect against common discordant growth and placental pathologies known among other primates to be associated with gestating litters in a simplex uterus (58).

The variation observed in gestation lengths among the callitrichines is further not explained allometrically (27). Whereas the majority of callitrichines, including *Callimico*, have a gestation period of around 140–145 d, with the early developmental lag as described above, two groups, the cotton-top tamarins (*Saguinus oedipus*) and the lion tamarins (*Leontopithecus*), deviate from this pattern. *S. oedipus* has an unusually long gestation period of 183 d (59), and ultrasound results suggest that this lengthening of gestation is due to a lengthening of the lag period (55). In contrast, the largest of the tamarins, *Leontopithecus*, have a significantly shorter gestation period of ∼122 d (60). The fact that these two nonallometric changes are seen within the group points to the possibility of interesting evolutionary change in this trait (61).

Targeted sequencing of the positively selected genes along the GH/IGF axis together with comparison to the squirrel monkey genome revealed that many of the NS contributing to their identification as positively selected are shared with non-callitrichine NWM. Only eight callitrichine specific NS were identified among the genes, but 131 NWM NS were present (Dataset S4). This raises the possibility that positive selection acted to globally alter growth patterns across all NWM, and specific changes, such as the callitrichine specific NS *GHSR* and *IGFBP7*, fine-tuned the diversity of sizes seen across the NWM. In support of this, 22 NS specific to single species of NWM and 5 NS specific to subsets of NWM were identified in *GHSR*, *IGF2*, *IGF1R* and *IGFBP2* (Dataset S5). Numerous *IGF1R* mutations are associated with both late prenatal and early postnatal growth restriction in humans (62, 63), and mutations of *IGF1R* in mice have been demonstrated to slow embryonic growth (64). *IGF2* is the primary growth factor controlling placental development and growth and is also critical in early embryonic growth (65–68). The IGFBPs are a family of proteases that bind with high affinity to the IGFs, serving to prolong their half-life and modulate availability and function with notable impacts on developmental growth and metabolism (49). With respect to later postnatal and juvenile growth, over a dozen mutations in the *GHSR* gene have been identified in humans with short stature (69–71), and increased concentrations of ghrelin have been reported in infants who were small for their gestational age (72, 73).

We have incorporated our findings herein with the observations of others into a schematic representation of the evolutionary ecology of the small NWMs, emphasizing the unusual but highly adaptive aspects of callitrichine biology (Fig. 3). The dietary specializations shown in Fig. 3 are in addition to frugivory, which is shared by all NWMs. Although it was originally proposed that callitrichines' diminutive size was an ancestral state (21) that preceded twinning, integration of ecological, behavioral, reproductive, and genetic characteristics of this primate subfamily (Callitrichinae) into a positive feedback model accounts for multiple elements within the combination of features seen in callitrichines. Extending beyond a general evolutionary background, our genetic evidence suggests that positive selection of genes in the GH/IGF axis occurred at the stem of the Cebidae and Atelidae families (and/or the common ancestor of all NWMs), which may have laid the genomic background for callitrichine-specific nonsynonymous substitutions affecting growth. It has been proposed that ancestral callitrichines were able to exploit the small primate ecological niche that is filled by prosimians in Africa and Asia, where Old World monkeys are excluded from this ecological niche (74). Marmosets share with prosimians such as the eastern fork-marked lemur (*Phaner furcifer*) some dental adaptations to exudativory, including lower anterior dentition with short canines and incisors roughly equal in length to the canines that are somewhat similar to prosimian tooth combs (75), lending to the notion that the development of these dietary specializations may have coevolved with miniaturization. Additional refinement in size among callitrichines could have been achieved by further functional genetic substitutions and reinforced both by diet and delayed early placental and embryonic development, resulting in the extremely diminutive size of modern callitrichines.

If the developmental growth delay and timing of hematopoietic foci is tied to protection of the fetuses against common pathologies known to be associated with gestating litters in a simplex uterus, then litter production—the driving force for the delay—would precede developmental delay—the driving mechanism behind smaller size (Fig. 3). This outcome raises the question of whether

increased litter size might, itself, have been an incident force for producing continually smaller monkeys. The initial ecological move toward exploitation of insects, and later adaptations to exudativory primarily in marmosets, in disturbed or edge habitats may have provided additional selective advantage to individuals capable of rapidly producing multiple offspring once they disperse into newly disturbed, and thus empty, microhabitats. This would reinforce the selective advantage of twinning over singleton births, as well as loss of lactational anovulation. In captive marmoset populations there is a significant positive correlation between the number of ovulations (one or two versus three or four) and increased maternal weight when examined by logistic regression (8). Because obligate twinning and shortened interbirth intervals would increase anabolic metabolic demands on breeding females, we, like others have in the past (76, 77), view the preadaptation for paternal care concomitant with alloparenting by siblings and reproductive suppression of subordinates as highly effective. We suggest that this collective suite of traits coevolved with twinning as the callitrichine solution to altered maternal metabolic demands. It would logically follow that the suppression of subordinate females would similarly impart a demographic advantage of facilitating immediate increases in the number of breeding females, as fresh edge microhabitats appear and subsequently allow formation of new social groups. Our findings on the genetic underpinnings of twinning and diminutive size in callitrichines resolves the potential need for a persistent metabolic trade-off between intergestational and interbirth intervals. In sum, callitrichines are optimally able to more fully exploit edge habitats by virtue of their encoded and imparted reproductive adaptations, including successful gestation and rearing of multiples (Fig. 3).

The potential applications of this work in reproductive medicine are multifold. First, it reveals a well-defined set of candidate loci to be further characterized in human populations with a higher heritability for twinning. In an era of reproductive biology wherein as high as 1% of infants born in the United States are conceived using assisted reproductive technology (78), identification of those at risk for multiple ovulatory events in a cycle is of inherent value. Second, it opens the opportunity to explore the genetic basis of callitrichine adaptations to reduce complications of multiple gestations. Future studies into callitrichine genomic adaptations will undoubtedly lead to unique insights of benefit to their human counterparts.

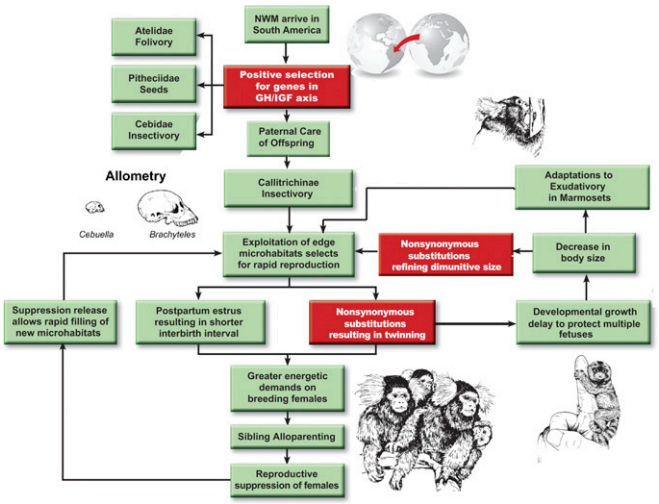

Fig. 3. Proposed model of callitrichine evolution, emphasizing associations among ecological, behavioral, reproductive, and genetic characteristics of this primate subfamily. Our evidence suggests that positive selection of genes in the GH/IGF axis in the stem NWM laid the groundwork for further callitrichine-specific NSs affecting growth. Whereas callitrichines were able to exploit the small primate ecological niche of insectivory, with additional dental adaptations to exudativory in marmosets, functional genetic substitutions including those resulting in twinning were reinforced both by diet and developmental growth delay, and collectively resulted in the extremely diminutive size of modern callitrichines. Because these adaptations are tied to the protection of the fetuses against common pathologies known to be associated with gestating litters in a simplex uterus, it follows that callitrichines are optimally able to more fully exploit edge habitats by virtue of their unique reproductive adaptations, including successful and efficient gestation and rearing of multiples. Red boxes indicate supportive genetic data presented in this study. New World monkey allometry drawings are proportional to skull sizes as initially reported by Marroig and Cheverud (25).

Materials and Methods

Further details as to the genomic science and computational approaches used can be found in *Supporting Information*. Briefly, we compiled a list of 63 candidate genes based on a literature search for genes implicated in twinning phenotypes in mammals that normally have singletons or in growth restriction phenotypes (Table S1). NSs in marmosets were identified in the candidate genes using the 33-way eutherian mammal Enredo Pecan Ortheus (EPO) alignments generated by Ensembl (Fig. S1). These NSs were then compared with the other haplorhine primates in the EPO alignments (human, chimpanzee, gorilla, orangutan, rhesus macaque, and tarsier) and only those marmoset NSs conserved among all of the other haplorhines were retained. Manual curation using the NCBI nucleotide database and ENCODE was used to remove substitutions present in other NWMs or for which there was conflicting evidence (Table S2). Overlapping amplicons were assembled using Velvet (79) and the resulting contigs were mapped to the marmoset assembly using Blat (80). The contigs that did map to sequencing target regions were aligned, together with orthologous regions from human, chimpanzee, gorilla, orangutan, rhesus macaque, and marmoset assemblies based on EPO alignments using MAFFT (81).

ACKNOWLEDGMENTS. We acknowledge the assistance of Muthuswamy Raveendran, David Rio Deiros, and Natalie M. Jameson in this study. This work was funded by National Institutes of Health Grant DP2120OD001500-01 (to K.M.A.), National Institute of Child Health and Human Development/National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK080558-01 (to K.M.A.) and R01-DK077639 (to S.D.T.), National Institute of Child Health and Human Development and the National Institutes of Health Office of Research on Women's Health K12HD055892 (J.R.), and Grant P51-OD011133 to Southwest Regional Primate Research Center, a Burroughs Welcome Fund Preterm Birth Initiative grant (to K.M.A.), National Science Foundation Grant BCS-0751508 (to D.W.), and European Community Seventh Framework Programme Grant VEGA 1/1085/12 (to T.V.).

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