Effects of maternal nuclear genome on the timing of puberty in mice offspring

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Abstract

The timing of puberty is a complex trait which is regulated by environmental and genetic factors, but the detailed regulatory mechanism remains elusive. Maternal nutrition administration during late gestation in rats revealed that the time of onset of puberty in daughter rats was influenced by the mother's nutritional and physiological status during the embryonic development period. In this study, the potential effects of the maternal nuclear genome on the timing of puberty of offspring were investigated. Two inbred strains of mice (C3H/HeJ (C3H) and C57BL/6J (B6)) were used to set up two pedigrees (direct and reciprocal crosses), and the timing of puberty in all these mice (parent, F1 and F2) was recorded (the females were assessed by vaginal opening (VO) and the males by balano preputial separation (BPS)). The results from data of 822 mice showed that: 1) in female mice, the heritability of the timing of puberty in direct and

reciprocal crosses is 68.51% and 63.97% respectively; 2) in female mice, a significant difference in the timing of puberty is observed between B6 and C3H ($P=3.7 \times 10^{-13}$) mice as well as between direct and reciprocal F1 hybrids ($P=5.4 \times 10^{-3}$), but not between direct and reciprocal F2 hybrids ($P=0.0941$); 3) in male mice, direct and reciprocal F1 hybrids differ significantly from each other in the timing of BPS ($P=2.7 \times 10^{-7}$), while such differences vanish in their male progenitor and progeny. The significant discrepancy between direct and reciprocal crosses in F1 but not in either cross of F2 hybrids reveals that the maternal nuclear genome has effects on the timing of puberty in mice progeny, probably through imprinting genes or the genes associated with intra-uterine physiological status.

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Introduction

Puberty is an important developmental stage and has a pervasive relationship with many other aspects of physiology and pathology (Meyer *et al.* 1991, Loesch *et al.* 1995, Rockhill *et al.* 1998, Magnusson *et al.* 1999, Wattigney *et al.* 1999, Wyatt *et al.* 1999). The onset of puberty is one of the key characteristics of this event (Palmert & Boepple 2001, Palmert & Hirschhorn 2003, Sisk & Foster 2004, Francis 2005). The calculated heritability in previous studies suggested that up to 50 to 80% of the variance in the timing of pubertal onset may be genetically controlled (Meyer *et al.* 1991, Loesch *et al.* 1995, Palmert & Boepple 2001, Ibanez *et al.* 2006). Genetic analysis indicated that familial central precocious puberty was an autosomal dominant transmission with an incomplete, sex-dependent penetrance (Vries *et al.* 2004). Clinical investigation also provided evidence that the X-chromosome was involved in precocious puberty (Jarvela *et al.* 1993, Grosso *et al.* 1999, Talaban *et al.* 2005). *GPR54*, *KISS-1* and *CYP3A4* have been nominated as puberty genes by some researchers, as variations in them may cause pubertal abnormality (Kadlubar *et al.* 2001, 2003, Roux *et al.* 2003,

Seminara *et al.* 2003). Quantitative trait loci (QTL) on chromosome 6 and 13 have been shown to regulate the timing of vaginal opening (VO) in mice chromosome substitution strains (CSSs) generated by A/J and C57BL/6J mice (Krewson *et al.* 2003). However, our understanding of the detailed regulatory mechanism of the timing of puberty is still far from complete (Sisk & Foster 2004).

On the other hand, more and more evidence has indicated that maternal effects on the timing of puberty onset in offspring cannot be ignored (Cooper *et al.* 1996, Lumey & Stein 1997, Magnusson *et al.* 1999, Adair 2001). A maternal diet low in protein administered during pregnancy and lactation delayed the onset of VO, while a maternal diet high in n-6 polyunsaturated fats administered during pregnancy induced precocious puberty in female rat offspring (Hilakivi-Clarke *et al.* 1997). The correlation between the age of puberty onset of the mother and the age at which her son or daughter attained pubertal milestones demonstrated that the timing of puberty onset was genetically regulated, especially by the maternal genome (Garn & Bailey 1978, Persson *et al.* 1999). In effect, single nucleotide polymorphism (SNP) rs2297660 in the maternal *LRP8* gene was found to be

associated with fetal growth (Wang *et al.* 2006). All these pieces of evidence indicate that, as well as their own genome, the maternal nuclear genome also has a part to play in the regulation of puberty onset in the progeny.

In order to investigate the potential effects of the maternal genomic background on the timing of puberty in the progeny, two inbred strains of mice (C3H/HeJ and C57BL/6J) were used to produce two pedigrees (direct and reciprocal crosses) because the female individuals differ significantly from each other in the timing of VO ($P < 0.05$; Nelson *et al.* 1990). Data on the time of puberty onset in three generations of both genders were recorded and analyzed (the females were assessed by VO and the males by balano preputial separation (BPS)). From the data of 822 mice in total, significant discrepancies were shown between the direct and reciprocal crosses in F1 but not in F2 hybrids of both genders, which revealed that the maternal nuclear genome had effects on the timing of puberty in the progeny. This was supported by the results of fostering experiments. Heterosis was evaluated in two ways: fecundity in female mice (litter size) and puberty advance in male mice.

Materials and Methods

Animals and housing

All mice used in the study were first generation offspring bred in our colony. Parent C57BL/6J (B6) and C3H/HeJ (C3H) mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, P.R.China). The two inbred strains (B6 and C3H), direct crosses of F1 (B6 female $\times$ C3H male (B6C3H F1)) and F2 (B6C3H F2) hybrids, and reciprocal crosses of F1 (C3H female $\times$ B6 male (C3HB6 F1)) and F2 (C3HB6 F2) hybrids were studied.

All mice used in this study were housed in standard polysulfone microisolator cages with hardwood chips (SLAC Laboratory) and were allowed unlimited access to water and food (SLAC Laboratory). During mating, pregnancy and lactation, mice were fed a breeder diet.

Animals were maintained on a 12 h light:12 h darkness schedule (lights on at 0600 h) at a mean ambient temperature of 23–25 °C. For breeding, individual males were placed in a cage with 2–3 females until each female was obviously pregnant. To minimize the exposure of female pups to male pheromonal and hormonal signals, each female breeder was then isolated in a clean cage containing a sterile cotton nestlet for the remainder of the pregnancy, and the date of birth was designated as the day pups were observed. Pups were weighed daily and weaned between the ages of 20 and 21 days, and males and females were then housed separately. No more than 5 female pups were housed per cage to ensure that access to food and water was unfettered.

Animals in this study were housed one genotype per shelf; cages containing males were interspersed between cages containing females to equalize pheromonal exposures, and

genotypes were rotated weekly on the shelves to avoid placement bias. All animal procedures were conducted in accordance with the Experimental Animal Management Ordinance of the People's Republic of China (1988).

Fostering experiment

Twenty C3H and B6 female mice (10 of each inbred strain) were mated with males of the alternative strain. C3H $\times$ B6 F1 and B6 $\times$ C3H F1 progeny were separated from their mothers on the day after their birth and fed randomly by lactating B6 female mice until weaning. Thus hybrid progeny of both crosses were fostered by maternal parents with the B6 genetic background. Litter size was curtailed to 6–8 pups. Another group of female mice mated with homologous males were manipulated in the same way for comparison. Pups were weighed daily until all the mice of the same cross and gender attained pubertal onset.

Assessment of pubertal onset

Beginning on the day of weaning, mice were examined daily between 0800 and 1100 h and the dates of VO for females and BPS for males were recorded together with the concurrent body weights of the animals.

Statistical analysis

Data were recorded as the mean, the standard deviation and the variation. Mean two-tailed, nonparametric tests for independent variables (Mann–Whitney U tests) were used for comparisons of time of puberty onset because of the non-normal distribution of the timing of puberty in mice. Differences with $P \leq 0.05$ were considered statistically significant.

Heritability was calculated as

$$h^2 = \frac{[V_{F2} - 1/3(V_{P1} + V_{P2} + V_{F1})]}{V_{F2}}$$

V_{F2} , V_{P1} , V_{P2} and V_{F1} are the computed variances (equal to the square of the standard deviation) for the F2, parental (P) 1, P2 and F1 populations respectively. Analyses were performed using SPSS software (Chicago, IL, USA).

Results

Distribution of timing of puberty in different generations of direct and reciprocal pedigrees

The timing of puberty onset in female and male mice is described in Figs 1 and 2. Only the data from litters of normal size were analyzed because litter size may influence the trait (evaluated by distribution of litter size in each generation). In our study, data from 414 out of 564 female mice and 408 out of 549 male mice were used.

Figure 1 Comparison of age at VO between maternal strains, direct and reciprocal crosses in F1 and F2 hybrids of female mice. Box plots indicate the median (dark bar), 25th and 75th percentiles (borders of boxes) and 12.5th and 87.5th percentiles (whiskers). Open circle represents the outlying value. Significant difference in timing of VO existed between B6 and C3H maternal strains ($P < 10^{-13}$), and B6C3H F1 and C3HB6 F1 hybrids ($P < 0.01$), but not between B6C3H F2 and C3HB6 F2 hybrids ($P > 0.5$).

The comparison of the timing of puberty between direct and reciprocal crosses

In female mice, VO occurred significantly earlier in C3H compared with B6 mice ($P = 3.7 \times 10^{-13}$) and such statistical discrepancy also existed between C3HB6 F1 and B6C3H F1 ($P = 5.4 \times 10^{-3}$) hybrids, but the difference vanished between B6C3H F2 and C3HB6 F2 hybrids ($P = 0.941$; Fig. 1). In male mice, a significant difference in the timing of BPS was only observed between the direct and reciprocal crosses in F1 hybrids ($P = 2.7 \times 10^{-7}$), and not in paternal strains ($P = 0.149$) or in F2 hybrids ($P = 0.216$; Fig. 2).

Figure 2 Comparison of age of BPS between paternal strains, direct and reciprocal crosses in F1 and F2 hybrids of male mice. Box plots indicate the median (dark bar), 25th and 75th percentiles (borders of boxes) and 12.5th and 87.5th percentiles (whiskers). Open circles and stars represent the outlying values and extreme outlying values respectively. Significant difference in timing of BPS existed between B6C3H F1 and C3HB6 F1 hybrids ($P < 10^{-6}$), but not between B6 and C3H paternal strains ($P > 0.1$) or B6C3H F2 and C3HB6 F2 hybrids ($P > 0.1$).

Comparison of body weight variation between the two crosses

A similar discrepancy was also reflected in concurrent body weight. Although C3H female mice weighed less than their B6 counterparts at VO (as VO is triggered much earlier in C3H mice than in B6 mice), C3H and C3HB6 F1 female mice weighed more than B6 and B6C3H F1 mice at the same age ($P = 4.8 \times 10^{-7}$ and 0.018 respectively), while direct and reciprocal F2 hybrids had similar body weight at VO ($P = 0.614$; Table 1).

The body weight of C3H and C3HB6 F1 male mice at BPS was much greater than that of B6 and B6C3H F1 mice ($P = 2.2 \times 10^{-4}$ and 9.7×10^{-8} respectively), while the difference in body weight at BPS vanished in F2 hybrids ($P = 0.313$; Table 2).

C3H female mice were heavier than their B6 counterparts at birth ($P < 0.005$). Female F1 hybrids with a C3H maternal parent were heavier at birth than those with B6 mothers ($P < 0.001$), although both had the same genotype and they had the same birth weight as their mothers. Similarly, the male hybrid progeny from C3H females were heavier than those from B6 females.

Fostering experiment

The differences in the time of puberty onset and the concurrent body weight between the reciprocal crosses of F1 hybrids could be caused by many factors apart from genetic background as all the F1 hybrids had the same genotype. In order to exclude the effects of post birth nutrition and maternal behavior/mothering on this trait, a fostering experiment on another group of F1 hybrids was carried out. The results of the fostering experiment were described by growth curves of the parental strains and the F1 hybrid progeny. The growth curves indicated that C3H female mice grew faster than their B6 counterparts, and their F1 hybrid female progeny had a similar difference in growth rate (Fig. 3). C3H male mice grew at a similar rate as B6 male mice, while their hybrid male progeny had a significantly different growth rate (Fig. 4). The puberty onset status of these animals was similar to that of their counterparts fostered by their own mothers (data not shown) and the difference between the two reciprocal crosses of F1 hybrids continued.

Heterosis

Heterosis was defined as the difference between the traits of interest in the hybrid progeny compared with those of their inbred parents. In this study, heterosis was evaluated by fecundity in female mice (shown by litter size) and puberty advance in male mice.

The assumption of heterosis of fecundity was assessed by analysis of variance (ANOVA), with the maternal gene background as the independent variable and litter size as the dependent variable. The discrepancy was highly significant between mothers with an F1 heterogenic background and

Table 1 Comparison of body weight at birth and at vaginal opening (VO) between parent, direct and reciprocal crosses in F1 and F2 hybrids of female mice

	Number	Weight at birth (g; mean±s.d.)	Weight at VO (g; mean±s.d.)	Probability values Mann–Whitney
Gene background				
Parental strain				
B6	37	1.40±0.15*	13.48±1.57	4.8×10 ^{−7}
C3H	36	1.66±0.23 [†]	11.29±1.48	
F1 hybrids				
B6×C3H	56	1.30±0.16 [‡]	13.36±1.59	0.018
C3H×B6	34	1.69±0.28 [§]	14.45±1.84	
F2 hybrids				
B6×C3H	104		12.80±1.96	0.614
C3H×B6	147		13.10±2.14	

Superscripts following means indicate Tukey groupings for significantly different means.

mothers with a B6 ($F_{1,107}=7.314$, $P=8.0\times10^{-3}$) or C3H ($F_{1,101}=30.805$, $P=2.3\times10^{-7}$) homogenic background (Fig. 5).

On the other hand, BPS of male mice occurred earlier in F1 and F2 hybrids than in the parental strains. As shown in Fig. 6, timing of BPS in F1 hybrids (28.7 ± 1.3 days in B6C3H F1 and 27.2 ± 1.2 days in C3HB6 F1) is earlier than the minimum threshold in the parental strains (29.6 ± 1.5 days in B6 and 29.2 ± 1.8 days in C3H). BPS was triggered significantly earlier in all F2 progeny compared with parental strains ($P<0.001$).

Heritability

Heritability was evaluated only in female mice and was calculated using the following formula:

$$h^2 = \frac{V_G}{V_T} = \frac{V_{F2} - V_E}{V_{F2}} = \frac{[V_{F2} - 1/3(V_{P1} + V_{P2} + V_{F1})]}{V_{F2}}$$

where V_G is the variance for genetic effect in the F2 population, V_T is the total variance in the F2 population, V_E is the total variance for the environmental effect in the F2 population, and V_{F2} , V_{P1} , V_{P2} and V_{F1} are the computed

variances for the F2, P1, P2 and F1 populations respectively (Table 3).

For B6C3H F2, the computed heritability of timing of VO is

$$h^2 = \frac{[V_{F2} - 1/3(V_{P1} + V_{P2} + V_{F1})]}{V_{F2}}$$

$$= \frac{[11.56 - 1/3(1.44 + 3.24 + 6.25)]}{11.56} = 0.6851$$

For C3HB6 F2, the computed heritability of timing of VO is

$$h^2 = \frac{[V_{F2} - 1/3(V_{P1} + V_{P2} + V_{F1})]}{V_{F2}}$$

$$= \frac{[8.41 - 1/3(1.44 + 3.24 + 4.41)]}{8.41} = 0.6397$$

The heritability of timing of puberty is 68.51% and 63.97% in direct and reciprocal crosses respectively.

Table 2 Comparison of body weight at birth and at balano preputial separation (BPS) between parent, direct and reciprocal crosses in F1 and F2 hybrids of male mice

	Number	Weight at birth (g; mean ± s.d.)	Weight at BPS (g; mean ± s.d.)	Probability values Mann–Whitney
Gene background				
Parental strain				
B6	27	1.43 ± 0.12*	17.54 ± 0.19	2.2 × 10 ^{−4}
C3H	28	1.53 ± 0.15†	18.62 ± 1.06	
F1 hybrids				
B6 × C3H	76	1.27 ± 0.19‡	17.07 ± 0.73	9.7 × 10 ^{−8}
C3H × B6	30	2.05 ± 0.28§	18.94 ± 1.86	
F2 hybrids				
B6 × C3H	98		18.45 ± 1.31	0.313
C3H × B6	149		18.60 ± 1.52	

Superscripts following means indicate Tukey groupings for significantly different means.

Figure 3 Growth curves of two inbred strains of female mice and the direct and reciprocal F1 crosses. *N* indicates the number of pups included.

Discussion

The onset of puberty, which involves the activation of hypothalamic function and maturation of the pituitary–gonadal axis, is a complex trait affected by both genetic and environmental factors. The calculated heritability from our experiments on inbred mice indicated that up to 63–70% of the variance in the pubertal timing of female mice is genetically determined, consistent with previous results from human population groups.

For a quantitative trait which is determined only by the nuclear genome, the phenotype distribution of hybrid progeny from two inbred strains is as follows: there is no distributional difference between direct and reciprocal crosses of F1 hybrids as their genotype is the same; the distribution of F2 hybrids is between those of the parental strains, with the mean varying according to the hereditary model. Our experiments revealed, for the first time, that there was significant discrepancy in the timing of puberty onset and the concurrent body weight increase between direct and reciprocal crosses in F1 but not in F2 hybrid mice of both genders. The discrepancy between F1 progeny with the same genotype could be ascribed to maternal effects as well as to mitochondrial haplotype if such a discrepancy continued in

Figure 4 Growth curves of two inbred strains of male mice and the direct and reciprocal F1 crosses. *N* indicates the number of pups included.

Figure 5 Comparison of litter size among three generations of female mice in direct and reciprocal crosses. Direct cross: B6 × C3H, reciprocal cross: C3H × B6. Letters at the top of the columns indicate Tukey groupings for significantly different means.

F2 hybrids, because mitochondria of direct and reciprocal F2 progeny were derived from different progenitors. Although genotype differed in the F2 hybrids, a large number of individuals would still be able to reflect an effect of the mitochondrial haplotype if such an effect existed. But in fact, VO and BPS occurred at the same age in either cross of F2 progeny, which meant that different mitochondrial haplotypes had not affected this trait. Therefore, it is reasonable to suppose that the maternal nuclear genetic background may account for the observed discrepancy between direct and reciprocal F1 progeny. In order to avoid the influence of post birth nutrition and maternal behavior on the timing of pubertal onset in offspring, fostering experiments were carried out on a complementary group of F1 hybrids. The results showed that even when fostered by mothers with the same genetic background, F1 hybrids of a contrasting cross grew at a different rate and pubertal onset occurred at a different age, even though they had the same genotype; this suggests that such a difference was brought about prenatally rather than postnatally.

The maternal nuclear genome may impact on the timing of puberty of offspring in both direct and indirect ways; the former may be through genes associated with physiological status during pregnancy that influence the development of the fetus in the uterus. The latter may be through imprinting genes which can be differentially expressed when inherited from either the maternal or the paternal parent to affect growth and development of offspring.

Research in many organisms has shown that maternal hormones (Eising *et al.* 2001), and maternal mRNA in eggs (Nagler 2000) can influence embryonic development. Androgens, corticosteroids and thyroid hormones are among the maternally derived hormones with significant roles during embryonic development in vertebrates. Previous studies showed that ingredient variations in the maternal diet during pregnancy and lactation could affect the pubertal status of female progeny (Cooper *et al.* 1996, Lumey & Stein 1997, Magnusson *et al.* 1999, Adair 2001, Leonhardt *et al.* 2003,

Figure 6 Comparison of age at BPS among three generations of male mice in direct and reciprocal crosses. Direct cross: B6×C3H, reciprocal cross: C3H×B6. Letters at the top of the columns indicate Tukey groupings for significantly different means.

Guzman *et al.* 2006). Experiments on rats showed that maternal exposure to a high level of serum estradiol during pregnancy advanced VO in female offspring (Hilakivi-Clarke *et al.* 1997). In essence, Wang *et al.* (2006) conducted a candidate gene association study on birth weight and fetal growth restriction in two independent samples and provided consistent evidence that SNP *rs2297660* in the maternal *LRP8* gene was associated with fetal growth. These experiments suggested that maternal gene polymorphisms might result in trait variations related to fetus development perinatally. Identification of maternal genes associated with intra-uterine physiological status may help to unravel the maternal genetic specification of and the physiological basis for the timing of puberty in progeny.

A number of loci related to growth and development have been shown to be imprinted (including insulin-like growth factor (IGF)-II, IGF-II receptor, Insulin 1 (Ins1), and Ins2) and cause the imprinting effect associated with identified QTL (Van Laere *et al.* 2003). Overweight children, especially girls, tend to mature earlier than lean children, which implies that the degree of body fatness may trigger the neuroendocrine events that lead to the onset of puberty (Shalitin & Phillip 2003), while the parent-of-origin effects, perhaps including genomic imprinting, play a role in human obesity (Dong *et al.* 2005). Such correlations may suggest some association between imprinting genes and pubertal development. Further studies on the imprinting pattern of GnRH (gonadotropin releasing hormone) neuron of different developmental stages may help to clarify the mechanism of puberty about the mechanism of this trait. Correlation between the ages at which a mother and her son or daughter attained various pubertal milestones provide extra evidence of maternal genetic regulation of the timing of puberty (Garn & Bailey 1978).

The parental strain of mice (C3H and B6) with a homogeneous allele at each genetic locus may be physiologically weaker than their hybrid progeny, as they have lower fertility and viability, defined as heterosis. Heterosis in the

Table 3 Standard deviations and computed variances of timing of vaginal opening (VO) in female mice

	Timing of VO (day)		Variance (s.D. ²)
	Average	s.D.	
Gene background			
Parental strain			
B6	27·6	1·2	1·44
C3H	22·6	1·8	3·24
F1 hybrids			
B6×C3H	27·0	2·5	6·25
C3H×B6	25·6	2·1	4·41
F2 hybrids			
B6×C3H	24·0	3·4	11·56
C3H×B6	23·9	2·9	8·41

fecundity of female mice (evaluated by litter size) could be supported by the larger litter size of hybrid progeny compared with that of their progenitor. F1 hybrid female mice, which were genetically heterogeneous at each locus, could procreate more progeny than their maternal progenitor. Heterosis may improve the mother's intra-uterine physiological status, which may be an important factor for reproductive function and pubertal development. Furthermore, inbred female mice give birth to more hybrid than inbred progeny (litter size: 7.5 vs 7.0 for B6 and 6.8 vs 5.0 for C3H; Fig. 5), which could be the result of stronger vitality of the hybrid embryo than that of the inbred one *in utero*.

Heterosis can also be exhibited in male mice by earlier timing of BPS in F1 and F2 progeny compared with that of their paternal progenitor. In our experiment, a significantly different BPS onset time was observed between direct and reciprocal F1 hybrid males, but not between the two parental strains. Hybrid progeny from C3H mothers grew faster than their inbred counterparts, and they triggered earlier BPS than the latter. Maternal effects were reflected significantly in F1 hybrids, but concealed in parental strains due to their physiological weakness, as male hybrid progeny from C3H mothers triggered BPS earlier than those from B6 females while the inbred paternal mice had the same BPS onset time. The physiological weakness of the inbred strains could be manifested as later BPS timing than their hybrid progeny. The discrepancy between direct and reciprocal F1 hybrids may be interpreted by their improved physiological status, so as to show the maternal effects on this trait.

From our data it would seem that puberty onset time was associated with growth velocity in mice as, in general, the faster the mice grew, the earlier they attained the onset of puberty. The relationship was more significant in the male than in the female. However, vaginal opening in female C3H mice was triggered much earlier than in their B6 counterparts but C3H mice were much lighter than the latter (Table 1), suggesting that the onset of puberty was not solely dependent on body weight.

Most of the genes and pathways are shared between mice and humans and a growing number of examples demonstrate that the investigation of variation among inbred mouse strains can be applied to analysis of the genetic model of human traits and diseases (Bedell *et al.* 1997, Darvasi 1998, Jansen 2003). Our studies reveal that the maternal nuclear genome, probably through imprinting genes and the genes associated with intra-uterine physiological status, plays a role in determining the timing of puberty in mice. It is reasonable to suspect that such an effect of the maternal nuclear genome also exists in humans. In clinical practice, there are many patients suffering from puberty disorders, and genetic analysis is regarded as a particularly suitable way to identify allelic variants of candidate genes that are associated with variations in the timing of puberty. Our results indicate that integration of the effects of the maternal nuclear genome on the timing of puberty may help to unravel the genetic specification of and physiologic basis for this trait.

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