Genetic dissection of trabecular bone structure with mouse inter-subspecific consomic strains

Taro Kataoka*, †, Masaru Tamura*, ‡, Akiteru Maeno*, Shigeharu Wakana† and Toshihiko Shiroishi*, †

*Mammalian Genetics Laboratory, Genetic Strains Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan
†Department of Genetics, The Graduate University for Advanced Studies (SOKENDAI), Mishima, Shizuoka 411-8540, Japan
‡Technology and Development Team for Mouse Phenotype Analysis, RIKEN BioResource Center, Tsukuba, Ibaraki 305-0074, Japan
Short running title:

Novel QTLs affecting bone structure

Five key words:

- Trabecular bone structure
- C57BL/6J
- MSM/Ms
- QTL
- Consomic mouse strains

Corresponding author: Toshihiko Shiroishi, Mammalian Genetics Laboratory, National Institute of Genetics, Yata-1111, Mishima, Shizuoka, 411-8540, Japan, TEL: +81-55-981-6818, E-mail: tshirois@nig.ac.jp
ABSTRACT

Trabecular bone structure has an important influence on bone strength, but little is known about its genetic regulation. To elucidate the genetic factor(s) regulating trabecular bone structure, we compared the trabecular bone structure of two genetically remote mouse strains, C57BL/6J and Japanese wild mouse-derived MSM/Ms. Phenotyping by X-ray micro-CT revealed that MSM/Ms has structurally more fragile trabecular bone than C57BL/6J. Toward identification of genetic determinants for the difference in fragility of trabecular bone between the two mouse strains, we employed phenotype screening of consomic mouse strains in which each C57BL/6J chromosome is substituted by its counterpart from MSM/Ms. The results showed that many chromosomes affect trabecular bone structure, and that the consomic strain B6-Chr15\textsuperscript{MSM}, carrying MSM/Ms-derived Chromosome 15, has the lowest values for the parameters BV/TV, Tb.N and Conn.D and the highest values for the parameters Tb.Sp and SMI. Subsequent phenotyping of sub-consomic strains for Chromosome 15 (Chr15) mapped four novel trabecular bone structure-related QTLs (Tbsq1-4) on mouse Chr15. These results collectively indicate that genetic regulation of trabecular bone structure is highly complex, and that even in the single Chr15, the combined action of the four
*Tbsq* controls the fragility of trabecular bone. Given that *Tbsq4* is syntenic to Human Chr 12q12-13.3, where several bone-related SNPs are assigned, further study of *Tbsq4* should facilitate our understanding of the genetic regulation of bone formation in human.
INTRODUCTION

Bone is an important tissue that not only supports the body but also has the function of storing mineral salts such as calcium and phosphorus. Homeostasis of bone tissue is maintained by bone remodeling. An excess of bone resorption in imbalanced bone remodeling manifests as reduced bone mineral density (BMD), and microstructural deterioration of trabecular bone eventually causes bone diseases such as osteoporosis (Feng and McDonald 2011). BMD and the trabecular bone structure are important factors for determining bone strength (Nazarian et al. 2008). The defect of these factors increases risk of fracture, and affects the quality of life. Thus far, many genome-wide association studies (GWAS) of BMD have been performed using dual-energy X-ray absorption scanning, because this method is used as the clinical standard for diagnosing osteoporosis, and it affords high-throughput assay for BMD. Through this approach, numerous single-nucleotide polymorphisms (SNPs) associated with BMD have been reported in human, as reviewed recently (Richards et al. 2012).

In model animals, a number of BMD-related QTLs have been found by genetic crosses of laboratory mouse strains, indicating that BMD is a typical complex trait and controlled by many genes (Ackert-Bicknell et al. 2010). The majority of these QTLs
are found in mouse syntenic regions of human BMD-related loci detected by GWAS (Rivadeneira et al. 2009; Ackert - Bicknell et al. 2010; Cho et al. 2009; Xiong et al. 2009; Zhang et al. 2010). These observations suggest that the mouse is a good model system to find the genetic factor(s) contributing to the skeletal fragility and homeostasis of bone tissue.

In contrast to BMD, information about genetic factors and QTLs that affect trabecular bone structure is severely limited. To analyze trabecular bone structure, X-ray micro computed tomography (micro-CT) analysis is essential. Image data obtained by this method provide indispensable information about trabecular bone structure, such as bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.D) and structure model index (SMI). Of these, BV/TV is the most important parameter, because the level of BV/TV is positively correlated with trabecular bone strength and stiffness (Nazarian et al. 2008). In human, osteoporotic trabecular shows fewer connectivity and thinner rod-like structure than normal trabecular, indicating that value of Conn.D is positively and value of SMI is negatively correlated with trabecular bone strength (Brandi 2009). A drawback of this method is that it is not suitable for high-throughput assay, particularly
for humans. Therefore, it is challenging to find the genetic factors responsible for trabecular bone structure in human. On the other hand, X-ray micro-CT analysis has been successfully applied in mouse. For example, it has been reported that age-related change of trabecular and cortical bone structures in male mice is similar to that in human (Halloran et al. 2002). Moreover, several QTLs that affect trabecular bone structure have been found by genetic crosses of laboratory strains (Bouxsein et al. 2004; Bower et al. 2006; Beamer et al. 2012). However, in these genetic studies, standard laboratory mouse strains, namely C57BL/6 (hereafter abbreviated as B6) and C3H, whose genomes are mainly derived from the single subspecies Mus musculus domesticus, were used, and our knowledge of genetic factors and QTLs that confer phenotypic difference in trabecular bone structure remains limited.

We previously reported B6-MSM consomic mouse strains (B6-ChrN\textsuperscript{MSM}), in which each chromosome of the chromosome host strain B6 is replaced by its counterpart from the chromosome donor strain MSM/Ms (hereafter abbreviated as MSM), an inbred strain established from the Japanese wild mouse M. m. molossinus (Moriwaki et al. 2009). As a consequence of a high degree of genome divergence from B6, MSM appeared to have unique complex traits that had never been observed in the
standard laboratory strains (Yonekawa et al. 1980; Moriwaki 1994; Yonekawa 1994; Moriwaki et al. 1999; Takada et al. 2008). Moreover, the whole genome sequence of MSM was determined, and more than ten million SNPs between MSM and B6 have been identified thus far (Takada et al. 2013). Information about the MSM genome and the SNPs for B6 is now freely available on an NIG Mouse Genome Database named NIG_MoG (http://molossinus.lab.nig.ac.jp/msmdb/index.jsp) (Takada et al. 2015).

Taking advantage of these developments, the consomic strains B6-ChrN\textsuperscript{MSM} have been used for genetic studies of a variety of complex traits, elucidating phenotypic effects of individual chromosomes (Takada et al. 2008; Takahashi et al. 2008a; Takahashi et al. 2008b; Takahashi et al. 2010; Nishi et al. 2010).

In this study, capitalizing on the unique genetic status of MSM, we first used X-ray micro-CT to investigate the trabecular bone structure of MSM, focusing on the parameters, BV/TV, Tb.N, Conn.D, Tb.Sp, SMI and Tb.Th, in comparison with those of B6. We found significant strain differences, with MSM having lower values of BV/TV, Tb.N and Conn.D, and higher Tb.Sp and SMI, than those of B6. Subsequently, we carried out a genetic dissection of the phenotypic effects of individual chromosomes with the full set of B6-ChrN\textsuperscript{MSM} mouse strains. The results revealed that trabecular bone
structure is indeed a highly polygenic trait, with many individual chromosomes each having a significant phenotypic effect. Notably, substantial epistasis was found among the individual chromosomes, because the sum of the individual effects often far exceeded the difference between the two parental strains B6 and MSM.

Next, we addressed the phenotypic effects within a single chromosome focusing on Chromosome 15 (Chr15), because consomic strain B6-Chr15	extsuperscript{MSM}, carrying MSM-derived Chr15, had the lowest values of BV/TV, Tb.N and Conn.D, and the highest Tb.Sp and SMI, among the full panel of consomic strains, which were almost the same measurement values as MSM. To genetically dissect the phenotypic effects of Chr15, we generated sub-consomic strains, in which only a part of the Chr15 is derived from MSM while the rest of Chr15 and all other chromosomes originate from B6. X-ray micro-CT measurement of the trabecular bone structure of these sub-consomic strains revealed that multiple genes control the phenotypic effects on Chr15. Finally, we found four novel QTLs affect trabecular bone formation and bone strength.
MATERIALS AND METHODS

Animals

The Animal Care and Use Committee of the National Institute of Genetics (NIG) approved all of the animal experiments. Development of a full set of consomic strains was reported previously (Takada et al. 2008). Briefly, each consomic strain has the C57BL/6J (B6) genome except for one chromosome that is replaced by the corresponding chromosome of MSM/Ms (MSM). The full set of consomic strains, denoted the consomic panel, was established in a collaboration between NIG and the Tokyo Metropolitan Institute of Medical Science, and is available from NIG and RIKEN BioResource Center. B6 was purchased from CLEA Japan and maintained at NIG. According to the consomic nomenclature, each strain was named B6-ChrN^{MSM}, where N is the number of the chromosome transferred from MSM. All animals were maintained under a 12-h light/dark cycle (light period, 06:00-18:00; dark period, 18:00-06:00) in a temperature- (23 ± 2°C) and humidity-controlled (50 ± 10%) room in a specific pathogen-free area. All mice were weaned after 4 weeks of age and housed individually in standard plastic cages on wood chips, and fed a standard diet, CE-2 (CLEA Japan).
Construction of sub-consomic strains

Sub-consomic strains possessing subdivided MSM-derived chromosome 15 (Chr15) were generated by crossing B6 and B6-Chr15\textsuperscript{MSM} (hereafter abbreviated as C15). The F\textsubscript{1} hybrid mice of B6 and C15 were then backcrossed to B6, and the resultant progeny were genotyped for SNP marker loci; heterozygous mice with an appropriate recombinant breakpoint were intercrossed to obtain homozygotes of the recombinant Chr15 on the B6 genetic background. Established sub-consomic strains that harbor various lengths of MSM-derived fragments of Chr15 were named B6-Chr15\textsuperscript{MSM}-SubX (hereafter referred to as Sub-X), and maintained as homozygous lines. All sub-consomic strains generated in this study are available from the Genetic Strains Research Center at NIG.

Genotyping

Genotyping of mice was carried out using the Mass ARRAY system (SEQUENOM) according to the manufacturer’s instructions. The DNA markers we used to assign detailed recombinant breakpoints in Chr15 between B6 and MSM in the sub-consomic strains are listed in Table S1. For determining fine borders of B6 and MSM
chromosomal fragments in the sub-consomic strains, we designed primer sets to detect
size differences in PCR-amplified products that resulted from structural variation such
as indels between B6 and MSM genomes.

X-ray micro computed tomography (micro-CT) analysis

All mice were sacrificed at 6 or 10 weeks of age. Bone samples were dissected and
fixed in 10% formalin in PBS (-) for 24 h and then transferred to PBS (-). Bone
structure in the metaphysis of the proximal tibia was scanned by micro-CT. Analyses of
trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), connectivity
density (Conn.D), trabecular separation (Tb.Sp), structure model index (SMI) and
trabecular thickness (Tb.Th) were conducted by TRI/3D-BON software (RATOC
System Engineering). Bone samples of 6-week-old B6, C15 and sub-consomic mice
were scanned using a ScanXmate-L090 micro-CT machine (Comscantecno). The image
size was set at 1024 x 1024 pixels. Scans were made using the following parameters:
tube voltage peak of 75 kVp, tube current of 52 μA, 360° rotation angle and 1200
projections. The region of interest (ROI) was 2 mm width from 0.35 mm below the
growth plate. Bone samples of 10-week-old mice were scanned with a
ScanXmate-E090S micro-CT scanner (Comscantecno). The image size was set at 992 x 992 pixels. Scans were made using the following parameters: tube voltage peak of 60 kVp, tube current of 130 μA, 360° rotation angle and 600 projections. The ROI of all mouse strains except for MSM was 1 mm width from 0.36 mm below the growth plate; that of MSM was 0.5 mm width from 0.25 mm below the growth plate, due to the difference in bone size between MSM and other strains. In all bone imaging experiments, BMD calibration of the micro-CT scanner was carried out every day with a phantom standard provided by the manufacturer. Micro-CT parameters that we used in this study are defined as follow (Bouxsein et al. 2010). BV/TV is ratio of the segmented trabecular bone volume to the total volume of the region of interest. Tb.N is measure of the average number of trabecular per unit length. Conn.D is measure of the degree of connectivity of trabeculae normalized by total volume of the interest. Tb.Sp is mean distance between trabeculae. SMI is indicator of shape of trabeculae: it is close to 0 if the trabecular network is mainly composed of parallel plates, and near 3 if cylindrical rods dominating. Tb.Th is mean thickness of trabeculae.

Statistical analysis
All data are expressed as mean ± SE. For phenotype screening of the consomic strains at 10 weeks of age, all consomic strains were compared with B6 as control, and in statistical analysis Dunnett’s test was performed using EZR (Kanda 2013). Significance was declared when \( P < 0.05 \). All relationships between two traits were assessed by Spearman’s rank correlation coefficient. Spearman’s rho (r) values and their significance were calculated using EZR. Significance was declared when \( P < 0.05 \). In comparisons of sub-consomic strains, Student’s t-test was performed with Welch’s correction. Significance was declared when \( P < 0.005 \) (\( P \) of 0.05/10 multiple comparisons).

Data availability

The C57BL/6J strain is commercially available from CLEA Japan. MSM/Ms, and all consomic strains and sub-consomic strains are available upon request. The DNA markers we used to assign detailed recombinant breakpoints in Chr15 between B6 and MSM in the sub-consomic strains are listed in Table S1. Table S2 and File S1 contain detailed micro-CT data for consomic strains. Table S3 and File S2 contain detailed micro-CT data for sub-consomic strains. Phenotype data for physiological parameters,
body weight and body length are available from the NIG phenotype database (http://molossinus.lab.nig.ac.jp/phenotype/index.html). The NIG Mouse Genome Database NIG-MoG (http://molossinus.lab.nig.ac.jp/msmdb/index.jsp) was used to determine the SNP information between B6 and MSM for each candidate gene.
RESULTS

Phenotype screening of trabecular bone structure for the B6-MSM consomic panel

We obtained X-ray micro-CT images for the proximal metaphyseal region of the tibia of B6 and MSM mice at 10 weeks of age, and measured six parameters: BV/TV, Tb.N, Conn.D, Tb.Sp, SMI and Tb.Th. We compared the measurement values of MSM with those of B6 (Figure 1 and Table S2), and found that the values of BV/TV, Tb.N and Conn.D of MSM were significantly lower than those of B6, whereas the values of Tb.Sp and SMI of MSM were significantly higher than those of B6. Although a statistically significant difference in the values between B6 and MSM was not observed for Tb.Th, MSM tended to have a lower Tb.Th value than B6.

Next, we obtained micro-CT images at the proximal metaphyseal region of the tibia of the full set of the B6-ChrN\textsuperscript{MSM} consomic panel, and assessed the same six parameters. The results showed large variation in the measurement values of the parameters among the consomic strains (Table S2). We aligned all 26 consomic strains as well as the parental strains, B6 and MSM, in ascending order of the measurement values (Figure 2). With regard to BV/TV and Tb.N, MSM showed the lowest values and those of all the consomic strains were distributed within the range between MSM and
B6 (Figure 2A and B). A similar strain distribution was observed for Conn.D (Figure 2C), although four consomic strains showed lower values than MSM. Interestingly, most of the consomic strains, including those with the Y chromosome and mitochondrial genome of MSM, showed significantly lower values for BV/TV, Tb.N and Conn.D than those of B6. Moreover, an inverse strain distribution was observed for the values of Tb.Sp and SMI (Figure 2D and E). B6 and MSM strains showed extremely low and high values, respectively, and almost all consomic strains were distributed between these parental strains. By contrast, with regard to Tb.Th, there was no statistically significant difference between the parental strains, and the values for many consomic strains exceeded the range between MSM and B6 strains (Figure 2F). In particular, consomic strain C14, which harbors Chromosome 14 of MSM, showed a significantly lower value than B6.

Among all consomic strains, B6-Chr15\textsuperscript{MSM} (C15), which has MSM-derived Chr15, showed the lowest values of BV/TV, Tb.N, and Conn.D, and the highest values of Tb.Sp and SMI; their values were almost the same as those in MSM (Figure 2). These results implied that mouse Chr15 contains QTL(s) with strong effects on trabecular bone structure, and that Chr15 of MSM tends to decrease BV/TV, Tb.N and
Conn.D, and to increase Tb.Sp and SMI. Notably, C15 has almost the same body size and body weight as B6 (http://molossinus.lab.nig.ac.jp/phenotype/index.html) (Takada et al. 2008), suggesting that the trabecular phenotype of C15 is not attributable to secondary effects of the shorter body length and lower body weight of MSM mice.

In the B6-ChrN\textsuperscript{MSM} consomic strains, the ascending orders for BV/TV, Tb.N and Conn.D are very similar, implying that these parameters correlate with each other. To confirm this correlation, and to establish which parameters are associated with BV/TV and which is the most important parameter for determining the fragility and stiffness of trabecular bone, we investigated correlations for all pairs of BV/TV, Tb.N, Conn.D, Tb.Sp, SMI and Tb.Th, using the measurement values of all individual samples of the consomic panel. We assessed the correlation coefficients and \(P\)-values among them (Figure 3). A very strong positive correlation was observed between all pairs of BV/TV, Tb.N and Conn.D. We also found a strong positive correlation between Tb.Sp and SMI, and these two were negatively correlated with BV/TV, Tb.N and Conn.D. Between any pair of these five parameters, the absolute \(r\)-value was more than 0.77. By contrast, Tb.Th was correlated with none of the other parameters (the absolute \(r\)-value was less than 0.25).
Genetic dissection of trabecular bone structure with C15-derived sub-consomic strains

To investigate whether a single major gene is responsible for the C15 phenotype or multiple genes confer the phenotype, we generated sub-consomic strains that harbor various fragments of MSM-derived Chr15. In total, we successfully established eight sub-consomic strains (Figure 4), which are fully fertile and have no reproductive deficiency (data not shown). We obtained X-ray micro-CT images at the proximal metaphyseal region of the all sub-consomic strains, and measured three parameters, BV/TV, Tb.N and Tb.Th, to narrow down the genetic region(s) responsible for trabecular bone structure. Since the difference in BV/TV and Tb.N between B6 and C15 was observed at as early as 6 weeks of age, we carried out phenotyping of these sub-consomic strains at 6 weeks of age (Figure 4).

To assign chromosomal fragments that contain QTL(s) responsible for the differences in the values of BV/TV, Tb.N and Tb.Th between B6 and C15, we aligned the eight sub-consomic strains as well as B6 and C15 in order to minimize the difference in the length of the MSM-derived chromosomal fragment between two
neighboring strains (Figure 4). Comparison of the measurement values between each pair of neighboring strains showed a statistically significant difference in four of the 10 pairs of strains. This result indicated that four QTLs affecting trabecular bone structure exist in mouse Chr15. Each pair of two neighboring strains defined 10 separate chromosomal fragments. We numbered these chromosomal fragments from Block1 to Block10 (Figure 5, gray and black chromosomal segments). The four QTLs are contained in Block2, Block6, Block8 and Block10, which are defined by comparison between two sub-consomic strains, namely Sub-26 and Sub-25, C15 and Sub-5, Sub-8 and Sub-9, and Sub-10 and B6, respectively (Figure 5, black chromosomal segments). We named these QTLs trabecular bone structure quantitative locus 1 to 4 (Tbsq1-4).

Tbsq1 resides in Block2, which is located at the centromeric region of Chr15, and the MSM allele at this locus increases trabecular thickness (Tb.Th). Tbsq2 in Block6 affects BV/TV, and the MSM allele at this locus increases the value of BV/TV. Block6 includes Block2 that harbors Tbsq1, but no significant difference in Tb.Th was observed between Sub-5 and C15. Tbsq3 in Block8 affects both BV/TV and Tb.N, and the MSM allele at this locus decreases the value of the above two parameters. Tbsq4 in Block10, located at the telomeric region of Chr15, affects both BV/TV and Tb.N, and
the MSM allele at this locus significantly decreases the value of the above two parameters.
DISCUSSION

In this study, mouse inter-subspecific genome differences between the standard laboratory strain B6 and the Japanese wild mouse-derived strain MSM allowed us to dissect genetic determinants that regulate trabecular bone structure. As a result, we found that trabecular bone structure regulation is extensively polygenic in mouse.

Phenotyping with the B6-ChrN\textsuperscript{MSM} consomic strains revealed pervasive QTLs that affect the parameters BV/TV, Tb.N, Conn.D, Tb.Sp and SMI on the mouse genome. BV/TV is known to be the most important parameter for determining the fragility of trabecular bone (Nazarian et al. 2008). The present study revealed that roughly two thirds of the chromosomes or chromosomal regions harbored QTLs affecting BV/TV, indicating that a large portion of mouse chromosomes contributes to the physical strength of trabecular bone (Figure 2). Notably, we also showed unequivocally that the Y chromosome and the mitochondrial genome possess QTLs affecting trabecular bone strength, which has not been reported before.

Recently, QTLs affecting trabecular bone structure in mice were reported based on the genetic cross of B6 and another laboratory strain, C3H/HeJ (C3H) (Beamer et al. 2012). Using nested congenic mouse strains, at least 10 QTLs were assigned at the
mid-distal region of Chr4 that affect the bone-related traits measured by peripheral quantitative computed tomography (pQCT) and/or micro-CT. Our study also showed that the consomic strain C4, which harbors MSM-derived Chr4, had the second-lowest values for BV/TV, Tb.N and Conn.D, following consomic C15, and this result indicated that Chr4 has the second-largest phenotypic effect on trabecular bone strength. It is possible that the causative genome variation(s) responsible for the reduced trabecular bone strength of C3H originated from the Japanese subspecies *M. m. molossinus.*

As a striking feature of the gene regulation involved in trabecular bone structure, we found extensive non-additive phenotypic effects on trabecular bone structure. With regard to the measurement values of BV/TV and Tb.N, summation of the phenotypic effects of individual chromosomes far exceeded the difference between the two parental strains, B6 and MSM. For example, summation of the phenotypic effects of 22 strains that showed a statistically significant difference in the BV/TV value from that of B6 yielded 1,390% of the parental difference. A similar result was also found for Tb.N, where the sum of the phenotypic effects was 1,291% of the parental difference. Such strong epistatic effects have often been reported in phenotyping of mouse consomic strains for many other complex traits (Shao et al. 2008; Takada et al.)
With respect to Tb.Th, only sub-consomic strain C14 demonstrated significantly lower trabecular thickness than B6. This suggests that disruption of an epistatic gene interaction between the MSM allele in Chr14 and B6 gene(s) in other chromosome(s) gives rise to the phenotype.

In this study, we investigated the correlation coefficients among six parameters, all of which are related to trabecular bone structure. We observed strong positive correlations in every pair of BV/TV, Tb.N, and Conn.D, and between Tb.Sp and SMI. The former three parameters showed negative correlations with the latter two. Considering these positive and negative correlations among the parameters, lower value of BV/TV shows not only fewer trabecular bones, but is also associated with morphological features such as rod-shaped trabecular bones and disconnected trabecular bones. The observed correlations between the five parameters suggest that they are regulated by common genetic factor(s). On the other hand, the parameter of trabecular thickness (Tb.Th) did not correlation with any of the other parameters. Therefore, the genetic factors contributing to Tb.Th are independent of those contributing to the other parameters.
It has been reported that values of BV/TV, Tb.N and Conn.D in mice peak at around six weeks of age, and gradually decrease with age, while, conversely, Tb.Sp and SMI increase with age. By contrast, Tb.Th does not change significantly with age. The decrease of BV/TV, Tb.N and Conn.D, and the increase of Tb.Sp and SMI, occur linearly with age, and the values of the parameters at the early phase (6-10 weeks of age) are important for predicting of trabecular strength in the later life of mice (Halloran et al. 2002). We inferred that the genetic factors contributing to BV/TV, Tb.N and Conn.D could be involved in the formation of trabecular bones at early stage of the life span, rather than in the regulation of homeostasis of bone remodeling.

This study showed that Chr15 has the strongest genetic influence on the trabecular bone structure of mice, and that it contains four novel trabecular bone structure-related QTLs (Tbsq1-4). The MSM alleles at two of these loci, Tbsq3 and 4, decrease of the BV/TV, reflecting the phenotype of the original consomic strain C15. The MSM allele at Tbsq2 acts in the opposite way to increase the BV/TV. The MSM allele at Tbsq1 increases the Tb.Th, although the original consomic strain C15 does not show a significant difference of Tb.Th compared to B6. We searched public databases and previous reports for candidate genes for Tbsq1-4. As a result, we identified a total of 20
candidate genes in the genomic regions encompassing the four QTLs (Table 1). Among these, twelve have non-synonymous SNPs between the B6 and MSM genomes, and nine have been reported to be involved in bone formation or homeostasis by in vivo assays. Although these nine genes are good candidates for the QTLs, other genes that have only synonymous SNPs or no SNPs in their coding sequence cannot be excluded from the list of candidate genes. If these genes had SNPs in their cis-regulatory elements, such as the promoter and enhancer, gene expression could be altered, and the SNPs and other structure variants could eventually cause the phenotype. The Block10 region that contains Tbsq4 is syntenic to human Chr12q12-13.3, where several bone-related SNPs have been assigned from GWAS (Bezerra et al. 2008; Gentil et al. 2007; Kim et al. 2007; Pérez et al. 2008; Bedrač et al. 2009; Dundar et al. 2009; Gentil et al. 2009; Mencej-Pluskiewicz et al. 2009; Timpson et al. 2009; Zmuda et al. 2009). Identification of the causative gene(s) for Tbsq4 should facilitate our understanding of the genetic regulation of bone structure in humans. In any case, further studies are needed to reveal the causative genes for Tbsq1-4. Collectively, the results of this study demonstrate that the mouse genome encodes numerous genetic factors regulating trabecular bone structure. Considering the
phenotypic effects of the four QTLs identified in Chr15, many other QTLs may have modest effects on bone phenotypes. It would be very difficult to detect such QTLs using linkage analysis by general outcross experiments, F\textsubscript{1} intercross and backcross. Thus, this study has also revealed the marked complexity of genetic architecture that controls trabecular bone structure in mouse, and demonstrated that analysis with consomic and sub-consomic strains has considerable power to extract each of numerous QTLs, even if its phenotypic effect is modest.
ACKNOWLEDGEMENTS

The authors would like to thank F. Murofushi, T. Aoki, S. Fujii and all animal facility members for maintaining the mouse strains; K. Masuyama for excellent technical assistance; T. Takada, T. Amano and all members of the mammalian genetics laboratory for helpful discussions. This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.
29

LITERATURE CITED

2 Ackert - Bicknell, C.L., D. Karasik, Q. Li, R.V. Smith, Y.H. Hsu et al., 2010

3 Mouse BMD quantitative trait loci show improved concordance with

4 human genome - wide association loci when recalculated on a new,


6 (8):1808-1820.

7 Ajima, R., T. Akiyama, M. Usui, M. Yoneda, Y. Yoshida et al., 2008

8 Osteoporotic bone formation in mice lacking tob2; involvement of Tob2

9 in RANK ligand expression and osteoclasts differentiation. FEBS

10 Letters 582 (9):1313-1318.

11 Akhter, M., D. Cullen, and L. Pan, 2006 Bone biomechanical properties in


14 al., 2012 Multiple quantitative trait loci for cortical and trabecular

15 bone regulation map to mid-distal mouse chromosome 4 that shares

16 linkage homology to human chromosome 1p36. Journal of Bone and

Bennett, C.N., K.A. Longo, W.S. Wright, L.J. Suva, T.F. Lane et al., 2005

Regulation of osteoblastogenesis and bone mass by Wnt10b.

Proceedings of the National Academy of Sciences of the United States of America 102 (9):3324-3329.


Mencej-Bedrač, S., J. Preželj, T. Kocjan, K. Teskač, B. Ostanek et al., 2009

The combinations of polymorphisms in vitamin D receptor, osteoprotegerin and tumour necrosis factor superfamily member 11 genes are associated with bone mineral density. *Journal of Molecular Endocrinology* 42 (3):239-247.

Moriwaki, K., 1994 Wild mouse from a geneticist's viewpoint. *Genetics in wild mice*.


Moriwaki, K., N. Miyashita, A. Mita, H. Gotoh, K. Tsuchiya et al., 2009 Unique inbred strain MSM/Ms established from the Japanese wild mouse. *Experimental Animals* 58 (2):123-134.


Takada, T., T. Ebata, H. Noguchi, T.M. Keane, D.J. Adams et al., 2013 The ancestor of extant Japanese fancy mice contributed to the mosaic


Timpson, N.J., J.H. Tobias, J.B. Richards, N. Soranzo, E.L. Duncan *et al.*, 2009 Common variants in the region around Osterix are associated


Yang, J., S. Murthy, T. Winata, S. Werner, M. Abe et al., 2006 Recq1 haploinsufficiency in mice leads to defects in osteoblast progenitors: Implications for low bone mass phenotype. *Biochemical and Biophysical Research Communications* 344 (1):346-352.


Yonekawa, H., K. Moriwaki, O. Gotoh, J. Watanabe, J.-I. Hayashi et al., 1980 Relationship between laboratory mice and the subspecies Mus musculus domesticus based on restriction endonuclease cleavage


Table 1 Proposed candidate genes for four QTLs in mouse Chr15: physical region, candidate genes, biological effects and SNP information between B6 and MSM.

<table>
<thead>
<tr>
<th>QTL</th>
<th>Genetic region (Mb)</th>
<th>Gene symbol</th>
<th>Gene function in bone</th>
<th>Information about SNPs and indels&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbsq1</td>
<td>6.64-21.75</td>
<td>Rictor</td>
<td>Skeletal growth and bone anabolism (Chen et al. 2015).</td>
<td>7 / 2 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osmr</td>
<td>Promotion of bone formation (Walker et al. 2010).</td>
<td>6 / 8 / 1 / 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lifr</td>
<td>Osteoclast number (Ware et al. 1995).</td>
<td>12 / 6 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cdh6</td>
<td>Osteoclast maturation (Mbalaviele et al. 1998).</td>
<td>12 / 2 / 0 / 0</td>
</tr>
<tr>
<td>Tbsq2</td>
<td>0-32.35</td>
<td>Ghr</td>
<td>Bone growth (Sjogren et al. 2000).</td>
<td>3 / 3 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ptger4</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; receptor. Bone formation (Akhter et al. 2006).</td>
<td>3 / 0 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myo10</td>
<td>Osteoclast bone resorption &lt;em&gt;in vitro&lt;/em&gt; (McMichael et al. 2010).</td>
<td>26 / 4 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ank</td>
<td>Ossification (Ho 2000).</td>
<td>7 / 0 / 0 / 0</td>
</tr>
<tr>
<td>Tbsq3</td>
<td>71.63-84.21</td>
<td>Ptk2</td>
<td>Osteoblast mechanotransduction &lt;em&gt;in vitro&lt;/em&gt; (Castillo et al. 2012).</td>
<td>6 / 3 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ly6a</td>
<td>Age-dependent osteoporosis (Bonyadi et al. 2003).</td>
<td>0 / 0 / 0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recql4</td>
<td>Osteoprogenitor proliferation (Hoki et al. 2003; Yang et al. 2006).</td>
<td>3 / 4 / 0 / 0</td>
</tr>
<tr>
<td>Gene</td>
<td>Function and References</td>
<td>SNPs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>--------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pdgfb</td>
<td>Bone metabolism (Xie et al. 2014).</td>
<td>0 / 0 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atf4</td>
<td>Osteoblast differentiation (Yang et al. 2004).</td>
<td>3 / 0 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mchr1</td>
<td>Cortical BMD (Bohlooly et al. 2004).</td>
<td>1 / 0 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tob2</td>
<td>Rankl expression and osteoclast differentiation (Ajima et al. 2008).</td>
<td>2 / 1 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scube1</td>
<td>Early cranial bone formation (Tu et al. 2008).</td>
<td>18 / 6 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tbsq4</td>
<td>Bone homeostasis (Yoshizawa et al. 1997; Yamamoto et al. 2013) and human GWAS</td>
<td>8 / 9 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col2a1</td>
<td>Endochondral ossification (Li et al. 1995).</td>
<td>10 / 1 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt10b</td>
<td>Osteoblast differentiation (Bennett et al. 2005) and human GWAS (Zmuda et al. 2009).</td>
<td>6 / 0 / 0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp7</td>
<td>Osteoblast differentiation (Nakashima et al. 2002) and human GWAS (Timpson et al. 2009).</td>
<td>0 / 0 / 0 / 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. a No. of synonymous SNPs / non-synonymous SNPs / insertions / deletions.
FIGURE LEGENDS

Figure 1 Bone morphology of B6 and MSM at 10 weeks of age. A to D, representative micro-CT images of the proximal region of trabecular bone of B6 (A and C) and MSM (B and D) mice at 10 weeks of age. A and B, sagittal cross-section images; C and D, axial cross-section images. Each ROI is 1 mm in width from 0.36 mm below the growth plate in B6 (C), and 0.5 mm in width from 0.25 mm below the growth plate in MSM (D). Scale bars are 1 mm. E, measurement values of the six parameters, BV/TV, Tb.N, Conn.D, Tb.Sp, SMI and Tb.Th, of B6 and MSM. Student’s t-test with Welch’s correction was performed for statistical analysis. Significance is declared when *P < 0.01.

Figure 2 Screening of trabecular bone features among B6, MSM and the consomic strains. B6, MSM and the consomic strains are aligned in ascending order of each measurement value for the micro-CT results. All measurement values of the parameters BV/TV (A), Tb.N (B), Conn.D (C), Tb.Sp (D), SMI (E) and Tb.Th (F) were obtained from 10-week-old males. The consomic strain B6-ChrN^{MSM} is abbreviated as CN, where N is the number of the chromosome transferred from MSM. CNC and CNT (e.g.,
C13C and C13T) denote consomic strains that harbor the centromeric and telomeric half of MSM-derived chromosomes. Y and Mt denote consomic strains that harbor the Y chromosome and mitochondrial genome of MSM, respectively. The measurement values of each consomic strain were compared with those of B6. Dunnett’s test was performed for statistical analysis. Significance is declared when *$P < 0.05$ (vs. control B6).

**Figure 3** Correlation among six trabecular bone traits in the consomic strains.

Correlations between all pairs of the six parameters were examined using data for all individuals in the consomic panel. The cells above and to the right of the name of each parameter display scatterplots for each pair of parameters. Those below and to the left show the corresponding Spearman’s rank correlation coefficients with $P$-values. Significance is declared when $P < 0.05$ and n.s. means not significant.

**Figure 4** Measurement values of three micro-CT parameters of the sub-consomic strains. In the left panel, B6, C15 and the all sub-consomic strains are aligned such that two neighboring strains have the minimum difference in the C15 (MSM)-derived
chromosomal fragments. The recombination breakpoints that define the borders between B6 and MSM chromosomal fragments in the sub-consomic strains are indicated by genetic markers (e.g., C15_6.364 and C15_102.254) at the top of the strain alignment. The genomic information about the DNA markers used for determining the recombinant breakpoints is summarized in Table S1. To the right, the values of BV/TV, Tb.N and Tb.Th are shown. Significance is declared when *$P < 0.005$ (0.05 / 10 comparisons).

Figure 5 Chromosomal blocks and four trabecular bone structure-related QTLs ($Tbsq1$ to $4$). Ten chromosomal blocks (gray and black segments) are defined by the difference of chromosomal composition between the neighboring two strains. Among the 10, four black segments contain QTLs, named $Tbsq1$ to $4$. The parameters affected by these QTLs are shown at the right side of the blocks.

Figure S1 Full dataset of the micro-CT measurement values of the sub-consomic strains. Full data for the three parameters, Conn.D, Tb.Sp and SMI, of B6, C15 and the
sub-consomic strains are shown. Significance is declared when \(*P < 0.005\) (0.05 / 10 comparisons).
Figure 1

- A: Microarchitecture of bone tissue
- B: Bone remodeling
- C: Bone density measurement
- D: Bone structure

E: Bar charts showing bone parameters:
- BV/TV: Bone Volume/Total Volume
- Tb.N: Number of trabeculae
- Conn.D: Connec
tion Density
- Tb.Sp: Trabecular Separation
- SMI: Structural Model Index
- Tb.Th: Trabecular Thickness

Comparison between B6 and MSM strains.
Figure 2

A

B

C

BV/TV (%) vs. Tb.N (1/μm) vs. Conn.D (1/mm^3)

Tb.N (1/μm)

Conn.D (1/mm^3)
Figure 2

D

Tb.Sp (μm)

E

SMI

F

Tb.Th (μm)
Figure 3

- BV/TV (%): $r = 0.915$, $P < 0.001$
- Tb.N (1/μm): $r = -0.840$, $P < 0.001$
- Conn.D (1/mm³): $r = -0.910$, $P < 0.001$
- Tb.Sp (μm): $r = 0.919$, $P < 0.001$
- SMI: $r = 0.187$, $P < 0.05$
- Tb.Th (μm): $r = -0.112$, n.s.
- Tb.N (1/μm): $r = -0.069$, n.s.
- Conn.D (1/mm³): $r = -0.243$, $P < 0.05$
- SMI: $r = -0.062$, n.s.
Figure 5

Genetic markers:

- Block1
- Block2
- Block3
- Block4
- Block5
- Block6
- Block7
- Block8
- Block9
- Block10

Chr15: 0 Mb - 104 Mb

Tbsq1, Tb.Th

Tbsq2, BV/TV

Tbsq3, BV/TV and Tb.N

Tbsq4, BV/TV and Tb.N