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Perspective Article

Genetic regulation of bone mineral density in mice

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Abstract

Peak bone mass is a major determinant of risk of osteoporotic fracture. Family and twin studies have found a strong genetic component to the determination of bone mineral density (BMD). However, BMD is a complex trait whose expression is confounded by environmental influences and polygenic inheritance. The number, locations and effects of the individual genes contributing to natural variation in this trait are all unknown. The extreme difficulty of dissecting out environmental factors from genetic ones in humans has motivated the investigation of animal models. Genetically distinct animal strains raised under strict environmental control are critical tools for defining genetic regulation. The availability of inbred strains, combined with its relative fecundity, has established the mouse as the best model system for the study of mammalian genetics and physiology. Importantly, genes identified in murine analyses can usually be readily mapped to particular human chromosomal regions because of the high degree of synteny that exists between the mouse and human genomes. We employed quantitative trait locus (QTL) analysis to examine peak BMD in 24 recombinant inbred (RI) mouse strains, derived from a cross between C57BL/6 (B6) and DBA/2 (D2) progenitors (BXD RI). The distribution of BMD values among these strains clearly indicated the presence of strong genetic influences, with an estimated narrow sense heritability of 35%. The differences in peak whole body BMD in the BXD strains were integrated with a large database of genetic markers previously defined in the RI BXD strains to generate chromosome map sites for QTL locations. This QTL analysis provisionally identified a number of chromosomal sites linked to BMD. In the second phase of our BMD QTL mapping efforts, we used three independent mouse populations (all derived from B6 and D2 progenitor strains) to confirm and narrow the genetic locations of 4 QTLs (on chromosomes 1, 2, 4, and 11) that strongly influence the acquisition of peak BMD in mice. Using a novel, fine-mapping approach (recombinant inbred segregation testing), we have succeeded in narrowing two of the BMD-related chromosomal regions and in the process eliminated a number of candidate genes. The homologous regions in the human genome for each of these murine QTLs have been identified in recent human genetic studies. In light of this, we believe that findings in mice should aid in the identification of specific candidate genes for study in humans.

Keywords: Quantitative Trait, Inbred Strain, Osteoporosis, Bone Mineral Density, Heredity, Genetics

Introduction

Peak bone mass is a major determinant of risk of osteoporotic fracture. Family and twin studies have found a strong genetic component to the determination of bone mineral density (BMD)[1]. BMD is a quantitative trait. That is BMD *per se* shows continuous variation, and discrete phenotypes are not generally discernible by studying the frequency distribution for bone density. Under controlled environmental conditions, continuous variation is the consequence of the effects of genes (alleles) at multiple chromosomal loci that influence bone mass. Mapping the loci that specify quantitative traits (quantitative trait loci, or QTLs) is a formidable challenge, because the effect of each QTL may account for only a fraction of the trait variance. However, the development of high-density genetic maps and powerful statistical algorithms offers the possibility of precise QTL mapping, ultimately allowing the positional cloning of underlying genes[2].

To overcome problems plaguing genome-wide searches for complex disease, it is necessary to reduce the impact of other factors surrounding the effect of individual genes. Workers investigating determinants of bone mass in humans have limited ability to intervene in the genetics, personal environment, or skeletal biology of their subjects. In a complex disorder such as osteoporosis, experimental approaches that can either manipulate or hold constant biological variables

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that determine BMD provide a crucial opportunity to systematically examine the pathophysiologic processes that contribute to osteoporosis vulnerability. Animal research can help to elucidate possible roles of genetic and environmental constituents in the regulation of bone mass that might be otherwise difficult to untangle. While the genetic basis for some extreme phenotypes might be due to the deletion of a gene or to an inactivating mutation, it is much more likely that extremes of BMD are due to subtle changes in gene expression, perhaps developmental genes early in life or possibly arising from allelic differences in untranslated regions of the genome that contain sequences controlling gene expression. Consequently, it will be very hard to devise a way of proving that a candidate gene really does underlie the phenotype. There may be no discernible sequence or expression difference to identify the gene, and even if there is, its presence does not prove etiological significance. While association studies can go some way to implicating a particular genetic locus, they can never be proof of a causal relation. For this a functional assay is needed: a way to alter the genetic sequence and see whether this modification results in a different phenotype. Such experiments are possible only in animals and may be the sole way to understand how genetic differences result in individual variation in bone mass[3].

Recombinant inbred strains

Genetically distinct animal strains raised under strict environmental control are critical tools for defining genetic regulation. The availability of inbred strains, combined with its relative fecundity, has established the mouse as the best model system for the study of mammalian genetics and physiology[4]. Importantly, genes identified in murine analyses can usually be readily mapped to particular human chromosomal regions because of the high degree of synteny that exists between the mouse and human genomes[5,6]. Recent studies indicate that phenotypically normal inbred strains of mice exhibit marked differences in various parameters of skeletal integrity[7-10]. Since these genetically distinct strains of mice were raised under controlled environmental conditions, the observed differences are likely the result of genetic variation.

The substantial allelic variability that can exist between laboratory strains has been accentuated further by the introduction of recombinant inbred (RI) strains, which are derived by systematic inbreeding starting from a cross between two inbred strains known to differ at some characteristic of interest (Fig. 1). They are called RI strains because the parental chromosomes are recombined several times per chromosome during their development, resulting in a unique pattern of recombinations of the two initial parental genomes in each RI strain. The starting points are two inbred genotypes that are used to produce a group of F_1 hybrids. Brother x sister pairs of F_1 hybrids are mated to create an F_2 generation, in which all genes now segregate independently. Following the production of an F_2 generation

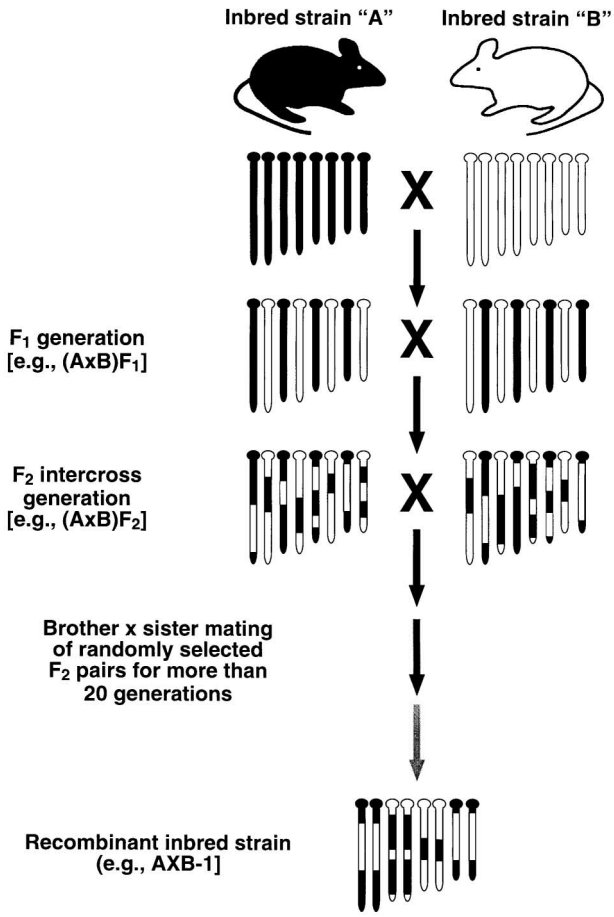

Figure 1. Generation of recombinant inbred strains. Only four of the 19 autosome pairs from parental inbred strains "A" and "B", and the assortment of chromosomes in the subsequent crosses derived from these strains, are shown. F_1 hybrids are genetically identical to each other, but individuals in the subsequent F_2 generation are not because of recombination events. RI strains also harbor recombinations but are homozygous at all loci as a result of the extensive inbreeding involved in their production.

from this interstrain cross, 20 or more different brother-sister pairs of F_2 individuals are mated. In each subsequent generation, only a single male and female from each pair are mated. After 20 generations, one has many inbred lines that differ from each other due to random differences in gene segregation, a process begun with the F_2. All the RI lines contain only those genes that were present in one or another of the parental strains. RI lines have been very useful in genetic mapping of traits that differ between inbred strains.

The RI strains were originally developed as a tool for detecting and mapping major gene loci[11,12]. Over the years, the RI strains have been characterized in respect to many genetic markers with known location on different chromosomes. The influence of a single major gene on a given trait can be inferred when RI strain means for the trait are found to fall in a bimodal distribution (i.e., all the RI strains with one allele are in one phenotypic group and all those with the other allele are in the other group). Comparison of the strain

distribution pattern (SDP) for that trait can be made with the SDPs for known marker loci previously mapped to a particular chromosome region. A close match in SDPs between the unknown locus and a marker locus would thus allow provisional mapping to a chromosome region of the latter[12,13]. Recent advances in statistics have succeeded in tailoring this experimental approach to a broader range of phenotypes, including continuously distributed traits without apparent major gene effects.

We have recently examined peak whole body BMD in mice from a panel of 24 RI BXD strains, derived from a cross between C57BL/6J (B6) and DBA/2J (D2) progenitors[14]. The distribution of BMD values among the BXD RI strains clearly indicated the presence of strong genetic influences, with an estimated narrow sense heritability of 35%. The pattern of differences in peak whole body BMD in the BXD strains were integrated with a large database of genetic markers previously defined in the RI BXD strains to generate chromosome map sites for trait locations. After correction for redundancy among the significant correlations, analysis of the BXD RI strain series provisionally identified seventeen chromosomal sites linked to peak bone mass development in mice.

An especially important feature of the RI method is the fixed nature of the genotypes of each of the RI strains. This means that any new hypothesis about a physiological mechanism underlying a trait can be assessed by making only observations on the new variable, and relating the outcome to the database already established[15]. For example, epidemiological studies have clearly demonstrated that body weight is a very strong predictor of BMD[16-19]. However, the mechanism underlying the strong association of weight with BMD is poorly understood. The coincidence of increased body weight with increased BMD could stem from environmental factors such as complementary nutritional effects on body composition and skeletal mass or the association could largely be the result of mechanical loading[20]. In addition to environmental causes, body weight and BMD may be modulated by linked genes or perhaps even the same genes. In our BXD RI experiment[14], 4 genetic loci for body weight were also identified. All 4 of these loci had been previously identified by Keightley et al.[21] in a prior analysis of mouse lines divergently selected for body weight from a base population derived from the same B6 and D2 parental strains. Interestingly, one locus that was linked to body weight was also strongly linked to inherited variation in BMD in the BXD RI strain set. These findings raise the intriguing possibility that body weight and peak BMD may be influenced by linked genes or perhaps by common genes with pleiotropic effects. Furthermore, they demonstrate the increasing value of a RI series as data, both about phenotypes and about genotypes, are gathered from all of the laboratories utilizing them.

There are two additional aspects of the RI approach which deserve comment. First, only a few inbred strains are represented in the existing RI sets (e.g., the BXD RI set is the largest and it currently is composed of only 26 separate strains) and it is not easy to construct new sets. Inasmuch as the strain means are the units of analysis, the statistical power of the RI method is directly related to the number of RI strains within a given set. Thus, genetic associations only above a certain effect size will be discernible with this experimental method. For osteoporosis research purposes, this limitation is only a modest one, as the current objective is simply to identify any relevant genetic associations in either animal models or humans. A second, and perhaps more serious, disadvantage of the RI method is that some genetic correlations of marker and phenotype are likely to be fortuitous. Because of the large number of statistical tests performed (e.g., over 1500 informative genetic markers have been genotyped in the BXD RI strains), the type I error rate relative to a single correlation similarly increases. One way to reduce the chance of such errors is to increase the required significance level and consider only those correlations that are significant at a very high probability. However, in choosing this level of stringency, one risks not considering QTLs that may be important (i.e., type II error). A useful compromise is to use a moderately stringent alpha level and regard correlational analysis in RI strains as a preliminary screen for genetic associations to be confirmed using other techniques, such as verification in an F_2 population.

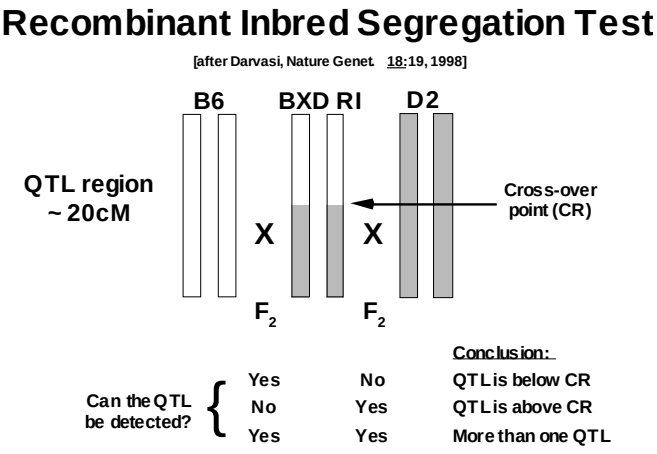

Figure 2. The recombinant inbred (RI) segregation test strategy. A RI strain that possesses a recombination or cross-over point in the region of a QTL is used to generate two F_2 populations - one with each parental strain. Analysis of the two populations will detect the population on which the QTL is segregating and accordingly locate the QTL above or below the recombination point. In the B6 cross shown here, linkage between phenotype and markers below the cross-over point is examined. If marker genotype correlates with phenotype in this population then the QTL must reside below the recombination point. Conversely, in the D2 cross, linkage between phenotype and markers above the cross-over point is examined. If marker genotype correlates with phenotype in this population, then the QTL must reside above the recombination point. If linkage is observed in both F_2 populations, then two closely linked QTLs are likely to be present.

BMD QTL confirmation

Virtually all proven QTL mapping strategies for polygenically determined (quantitative) traits have four common features[22-24]. First, a population is tested for the trait of interest and genotyped at many marker loci distributed throughout the genome (genome-wide scan). Second, the population is divided into genotypic classes at each marker locus (marker-based approach). Alternatively, the population can be divided into two phenotypic classes based on the highest and lowest scoring animals for a trait (trait-based approach). Third, a statistical test (e.g., correlation, regression, χ^2, t, F, LOD) is used to determine if the genotypic classes differ with respect to the phenotype, or if the phenotypic classes differ with respect to genotype (or allele) frequency. Fourth, if the statistical test is significant (at least $p < 0.0001$ or LOD 3.3, df = 1), it is concluded that a QTL exists in the same chromosomal region as the marker, i.e., the marker and QTL are linked.

The technology to carry out a genome-wide scan is a powerful capability, but it comes at a rather high statistical price – an increased likelihood of Type I errors (false positives) arising from the large number of markers required[2,25]. The standard remedy for this problem is to use more stringent levels (reduce acceptable Type I error risk), but this approach may result in an unacceptable level of false-negative outcomes. Multi-stage research programs have been proposed to address this issue, suggesting initial nominations in one study population with a relaxed a-level, and subsequent confirmation tests in other populations under stringent conditions. The most common form of multi-step QTL mapping is to use recombinant inbred (RI) strains as an initial screen of the genome to identify provisional QTLs. This initial step usually requires no genotyping since most RI sets have been genotyped for hundreds of markers throughout the genome. However, the limited number of genotypes (i.e., strains) in existing mouse RI sets is generally too small to map all but the largest QTLs unequivocally. Therefore, the RI QTL results must be followed by at least a second step-confirmation testing in other independent populations derived from the same progenitors.

We have now used three independent mouse populations – an F_2 intercross, short-term phenotypically-selected lines and short-term genotypically-selected lines (all derived from B6 and D2 progenitor strains) – to confirm and narrow the genetic locations of 4 QTLs that strongly influence the acquisition of peak BMD in mice (Table 1)[26,27]. Although, the false-positive rate for the initial BXD RI analysis was high (76%), it was not unexpected given the numerous comparisons being made[28]. Comparative gene mapping in humans and rodents has revealed evidence for substantial conservation of gene order during mammalian evolution. Shown in Table 1 are the homologous sites in the human genome for the 4 BMD QTLs. It is of note that each of these homologous regions has also been identified by QTL analysis of human populations. It is important to point out though, that these kinds of linkage studies can only identify relatively broad chromosomal regions that may contain QTLs regulating BMD and should therefore be regarded with caution. However, the possibility that the four identified murine QTLs contain genes that regulate bone mass acquisition can now be directly pursued by examining whole body BMD in congenic strains of mice that are genetically identical except for selected segments of this chromosome[12].

Conclusion

The systematic analysis of inbred strain databases is beginning to reveal important aspects of the genetic regulation of bone mass acquisition and maintenance. The recent advances in genetic mapping of complex traits, such as QTL mapping, are especially promising. A major strength of this approach is that it enables the provisional identification of candidate genes in the absence of any prior hypothesis about the mechanism by which the phenotype is expressed. The identification of those chromosomal regions where marker allelic and trait variation significantly covary is now a straightforward (although large-scale) enterprise. QTL mapping offers an attractive interface between forward and reverse genetics. Molecular cloning has shown that almost all genes in mice have homologues in humans, and vice versa[6]. Thus, identification and mapping of genes in the mouse offers immediate hope for extrapolation to the human genome. For the future, more molecularly based techniques are likely to be on the leading edge of progress. As candidate genes are identified as having important skeletal functions, the tools of molecular biology will allow the genetic diversity underlying their expression and function to be more fully

Chromosome	*p values*				*Combined*		Human Region
	Marker	F_2	S_3	RIST-F_2	P	LOD *df*=2	
1	*D1Mit 291*	1.8×10^{-4}	2.9×10^{-3}	5.0×10^{-3}	5.6×10^{-7}	6.2	1q21-q43
2	*D2Mit94*	4.8×10^{-6}	3.1×10^{-4}	1.3×10^{-5}	1.0×10^{-11}	11.0	2q21-q32/11p-q12
4	*D4Mit312*	2.6×10^{-9}	2.1×10^{-2}	2.0×10^{-6}	4.9×10^{-13}	12.3	1p36.3-p34.1
11	*D11Mit349*	9.0×10^{-8}	8.9×10^{-5}	1.9×10^{-2}	7.1×10^{-11}	10.1	5q31-q35/17p13-q22

Table 1. Summary of evidence for QTLs involved in peak whole body BMD

examined. Discoveries made with animal models can often set the stage for skeletal research in human subjects to augment the results from animals and confirm their relevance to our own species. Perhaps the most versatile aspect of animal model systems is in their use as a proving ground for hypotheses regarding the genetic as well as the epigenetic basis of osteoporosis. Old ideas regarding disease mechanisms can now be rigorously tested *in vivo*, and what is more important, provocative new concepts can emerge.

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