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PERSPECTIVES

A Thematic Overview of Some Recent Advances in Skeletal Genetics

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It has now been 10 years since Morrison *et al*. (1) reported an association between vitamin D receptor (VDR) genotype and bone mineral density (BMD), thus spurring the hope that it would be possible to identify specific alleles of a small number of "bone genes" that would account for observed population variability of fragility fracture risk. Although many scientists, myself included, doubt that the reported VDR alleles play an important role in bone biology, the authors nevertheless succeeded in raising awareness in the bone community about genetic methods and the contribution of hereditary factors in determining clinically important bone properties. Because a great deal of progress has been made in the intervening decade, providing an encyclopaedic review of work in the field is not possible. This essay reviews *some* of the most important findings in a thematic fashion, with apologies to investigators whose research is not cited.

Identification of Molecular Signaling Pathways Whose Roles in Skeletal Health Were Previously Unappreciated

Rare diseases, the genetics of which follow a simple Mendelian pattern of inheritance, offer a powerful tool for understanding some of the molecular mechanisms that operate in establishing the size and shape of bones and the properties of the matrix within bones. Sometimes, identifying the mutated gene offers limited new insight, as in the case of osteogenesis imperfecta. It surprises no one that mutations affecting the major structural protein in bone matrix result in skeletal fragility of varying severity. This is not the case for several other Mendelian disorders, in which identifying the responsible genes has revealed previously unsuspected biology.

The importance of the *WNT* signaling pathway in establishing bone mass is perhaps the best known example of an unexpected biological mechanism underlying a bone disease, but it is not the only one. Loss of function mutations of the low-density lipoprotein receptor-related protein 5 gene (*LRP5*) cause osteoporosis-pseudoglioma syndrome (2-4), whereas gain of function mutations in the gene result in the high bone mass phenotype (5-7). The *WNT* pathway was well known prior to the discovery that it contributes to bone mass regulation, dating back to early work on the *Drosophila* mutation *wingless*. Investigation of the precise roles of *LRP5* and the other genes involved in the *WNT* pathway in bone is an active research area that is informed by an understanding of *WNT* biology in a variety of other settings.

Positional cloning has also been central in establishing that a phosphate regulatory mechanism operates in conjunction with parathyroid hormone and vitamin D. Once again, uncommon hereditary diseases with simple inheritance patterns -- X-linked hypophosphatemic rickets (XLH) (8,9) and autosomal dominant hypophosphatemic

rickets (ADHR) -- were the starting points (10). The determination that fibroblast growth factor 23 (FGF-23) is phosphaturic, a phosphate-regulating endopeptidase on the X chromosome (PHEX) substrate, and resistant to proteolytic degradation when an ADHR mutation is present establishes a mechanistic link between the two diseases (11,12). Subsequently, high circulating levels of FGF-23 have also been found in patients with tumor-induced osteomalacia (13,14), extending the scope of the pathway's actions to acquired disorders.

The PHEX/FGF-23 story is more complex, however. Both matrix extracellular phosphoglycoprotein (15,16) and frizzled-related protein 4 (17,18) have been proposed as phosphaturic factors. Moreover, some investigators have failed to find elevations of FGF-23 in XLH or PHEX-mediated degradation of FGF-23 *in vitro*, whereas others have proposed that PHEX may affect phosphate regulation and bone matrix mineralization through mechanisms other than proteolysis of putative phosphaturic factors (19,20). Further complexity arises from FGF-23 and PHEX in the kidney; relationships to both renal phosphate transport and vitamin D 1-hydroxylation have been demonstrated, but not yet fully characterized (*e.g.,* 21-23). These conflicting observations support the existence of multiple important phosphaturic factors, and ongoing research seeks to better define the roles of each in mediating phosphaturia and bone matrix mineralization. The key point in the present context -- that investigation of rare genetic diseases has spurred discovery of novel and unexpected biology -- is strengthened by current efforts to reconcile seemingly inconsistent data.

In both examples cited above, fruitful new hypotheses have emerged because the identities of disease-causing genes were surprising. None of these genes would have been considered attractive candidates in the absence of compelling linkage data, and our efforts to assimilate the implications of the linkage data have clearly advanced our understanding of bone mass and phosphate homeostasis.

Insightful Use of Animal Models

Animal models allow investigators to measure more relevant and more precisely defined phenotypes than is possible in clinical studies. Indeed, in the examples above, the focus of ongoing research has shifted from informative families to either naturally occurring mutant mice or genetically engineered mice (24-27).

Mouse and rat models are now so well integrated into bone research that there is no need to belabor the methods by which they are generated or the range of studies to which they have been applied. However, it is worth noting that several general-purpose model animals have been developed, such as those expressing reporter genes or Cre recombinase in a tissue-restricted fashion (*e.g.*, 28,29).

Animal models remain a particularly powerful resource for identifying genes that affect bone properties, particularly in the context of identifying quantitative trait loci (QTLs). The concept underlying QTL genetics is that alternative alleles of multiple genes each contribute incrementally to a trait of interest. Furthermore, segregation of the various QTL alleles is a major source of variation in the trait among members of a population. Although human families are enormously useful in identifying genes and pathways involved in diseases with transparent inheritance patterns, human samples are poorly suited for determining the basis of differences in bone properties among members of a population. Population-level differences in bone properties are much more subtle than those encountered in disease states and are modified by environmental factors and age. Moreover, human alleles that are identical by state (*i.e.,* according to the detection method, such as microsatellite size or restriction fragment length polymorphism) might not be identical by descent (*i.e.,* ancestral origins may differ and therefore they may be truly different in a genetic sense). Experimental crosses in animals obviate some of the problems attending investigation of population variation. Invasive phenotypes that cannot be studied in humans can be investigated in model

organisms. Husbandry can be controlled and animals studied at the same age, thus limiting environmental sources of variability. Because inbred strains are available for study, one can limit the number of alleles segregating at each locus. Use of inbred strains also eliminates the problem of distinguishing identity by descent from identity by state. In experimental crosses, it is straightforward to determine both linkage (*i.e.* location of a gene) and association (*i.e.* which allele favors a high value of the trait being studied). These advantages are considerable, but entail some costs. There is no assurance that bone loci found in an experimental cross will prove important in humans. Moreover, a cross can only identify a locus if the parental strains harbor different alleles and the effect size is sufficiently large to allow detection. Finally, there is considerable work between mapping a locus and identifying the responsible gene. Limitations notwithstanding, experimental crosses have moved from being a promising strategy to a proven method for identifying genes that control bone properties.

In at least one case thus far, a QTL mapping study in mice has led to the identification of a gene -- *Alox15* -- and not just a chromosomal region (30). This study, conducted by Klein and colleagues, is the culmination of research started in the late 1990s (31,32). The recently reported findings include the validation of a chromosome 11 QTL in congenic strains; recognition of *Alox15* as a positional candidate gene, based on the strength of a microarray gene expression analysis, demonstrating an approximate 35-fold difference in *Alox15* expression between C57BL/6 (low) and DBA/2 (high); and experimental demonstration of the impact of *Alox15* on femoral BMD and biomechanical performance in three distinct and independent experimental tests. Based on the known ability of *Alox15* to metabolize arachidonic and linoleic acids to peroxisome proliferator-activated receptor gamma agonists, the investigators hypothesized that high *Alox15* activity favors differentiation of mesenchymal stem cells along the adipocyte lineage, in preference to the osteoblast lineage. As in the cases of *LRP5*, *FGF-23*, and *PHEX*, this work reveals a metabolic pathway that was previously not suspected of having important effects on bone. Adding further to the interest of this story is the prior detection of a human BMD QTL in the region harboring *ALOX15* (33).
The path from validated QTL (*i.e.,* the preservation of the effect in a congenic strain) to identification of the responsible gene is not always as rapid and straightforward as in the case of *Alox15*. Sometimes, on further breeding, a linkage peak proves not to be a single locus, but a group of linked genes. The bone group in the Jackson Laboratory has demonstrated precisely this phenomenon on mouse chromosome 1 (34), and the same may be true of other QTLs segregating in the C57BL/6J x C3H/HeJ cross (35). Furthermore, the number of positional candidate genes -- even if isolated within a congenic segment -- remains large, and testing these systematically is an arduous task.

Of interest, human allele association studies can be a powerful tool in the endgame of identifying which gene in a candidate interval is responsible for the phenotypic effect. The ability to undertake genetic studies involving multiple species is made possible by the existence of comparative genetic maps *(e.g.*, the mouse-human comparative map is available at http://www.ncbi.nlm.nih.gov/Homology/Davis/). This hybrid strategy is being pursued to find an X-linked gene identified as contributing to postmaturity BMD change in mice and lumbar BMD in postmenopausal women, narrowing the search to one of two genes (36). The short distances over which linkage disequilibrium persists and the complex pattern of population mixing among humans facilitate dissection of a chromosome region that is too small for crossovers to be helpful in a mouse model. Thus, some of the very features that make human populations unattractive for genome-wide scans make them ideal for fine-scale mapping of an established positional candidate.

Although identifying the responsible genes remains the primary goal of experimental crosses, congenic strains constructed to confirm linkage are valuable in a number of

ways that are independent of their utility in identifying the gene(s) underlying a QTL. Unlike the unique animals generated in a cross, congenics are inbred, allowing the study of multiple genetically matched individuals. In a cross, investigators often choose to sacrifice the biological depth of the traits being measured to gain either precision, ease of measurement, or both -- deferring more detailed and biologically informative phenotypic studies until congenics are available. Thus, for example, although a QTL on chromosome 4 was mapped because of its effect on femoral volumetric BMD, experiments with the resulting congenic strain have demonstrated that there is a mechanical responsiveness gene within the donor segment (37). Congenic strains are also powerful resources in studies addressing interactions among QTLs (38), between QTLs and environment (39), and between QTLs and sex (40,41). Such experiments exploit the advantages of animal models, and the study of interactions is an area in which research with experimental animals will continue to provide more insight than can be gained from clinical studies.

Human Genetic Methodology

Genome-wide screens for bone QTLs in humans, by either linkage or association methods, are potentially problematic, but recent methodological advances promise to help overcome two of the limitations inherent in human quantitative trait genetics. Genome-wide scans in humans for quantitative bone traits are generally underpowered, regardless of whether linkage or association methods are used. Underpowered genetic studies lead to two related errors: failure to identify QTLs and overestimation of the effect sizes of identified QTLs (42). Cross-sectional allele association studies are subject to false-positive results that arise from population stratification. Research addressing each of these problems is briefly noted here.

The Framingham investigators have applied principal components analysis to extract synthetic uncorrelated principal component phenotypes from a larger number of interdependent bone-related raw data measurements, including multiple dual-energy x-ray absorptiometry and quantitative heel ultrasound parameters (43). The approach is conceptually simple and can be performed with standard statistical analysis software. The multiple raw measurements are first combined into a composite measure and then broken down into a minimal set of orthogonal vectors (*i.e.*, the principal components). All lumbar spine, femoral, and calcaneal data from the Framingham study can be represented as two principal components that accounted for 66% and 24% of the composite phenotype variability. In the case of hip data alone, a single principal component was extracted that accounted for 90% of the variability. Mapping the principal components identified several regions suggestive of linkage, but none of the regions achieved genome-wide statistical significance. It is important to note, however, that the principal component heritabilities exceeded those of the individual underlying measurements, thus demonstrating that they are more robust for mapping studies. Therefore, despite the failure to achieve significant linkage, this paper is important because of the methodological innovation.

Using data from the Indiana Sisters Study, the bone genetics group at Indiana University has measured the effects of population stratification on the rate of false-positive associations (44). If a population is stratified, association can arise artifactually -- as a consequence of coincidental differences in allele frequencies between two population groups that also differ with regard to the trait being studied, rather than as a consequence of a true biological connection between the phenotype being studied. A trivial example illustrates the point: although the HBB^S allele is associated with skin pigmentation, all recognize that this association reflects the higher prevalence of sickle cell hemoglobin in individuals of African descent, rather than an inherent causal relationship between hemoglobin genotype and skin color. The problem of population stratification is common (*i.e.*, most populations are stratified), and the degree of stratification is often difficult or impossible to quantify. The investigators found that increasing

admixture increases the rate of false-positives in association studies and that one can nevertheless address admixture by including unlinked "negative control" polymorphisms to estimate the magnitude of the admixture effect. Their proposed inclusion of unlinked loci to control for admixture provides a simple, practical approach that can be used as an alternative to the more difficult alternative of performing association studies using the two-generation transmission disequilibrium test.

A Philosophical Reflection on Complex Trait Genetics

Ultimately, as a community, we face the task of achieving a philosophical advance to match the scientific advances of recent years. As scientists, we are deeply committed to seeking and understanding the causes underlying natural phenomena. Yet, our notions of causality are both inadequate and imprecise. We generally agree about acknowledging the “causality” of factors that are either *necessary* or *sufficient* for a specified outcome. Neither of these criteria of causality seems to be adequate for helping us understand complex trait genetics, because genes that contribute to bone properties may be neither necessary nor sufficient to determine biologically important features. Similarly, simply quantifying the effect of a particular allele is also inadequate, as effect sizes can vary among populations (both human and model organisms) and according to environmental variables. One of our conceptual challenges in the future will be to develop useful ways of ascertaining and describing conditional effect sizes. Current treatments of interactions (gene x sex, gene x gene, and gene x environment) provide a starting point, but we have a long, challenging, and exciting road ahead.

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