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Editorial

Hylonome

An integrated approach to assess structure-to-function relationships in the skeleton

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Abstract

In general, the disciplines of biomechanics, morphology, densitometry, biochemistry, cell biology and molecular biology have advanced independently of one another. In spite of this fragmentation, there have been incremental increases in our understanding of the organization, mechanical properties, growth, remodeling and repair of the tissues comprising the skeleton. As a practical application, this increased knowledge has greatly improved our capabilities for early diagnosis of bone loss and has proven similarly useful in determining the efficacy of interventions to prevent osteoporosis. This approach, however, has been much less successful in countering several other important musculoskeletal disorders, including arthritis. In the immediate future, a major emphasis will be placed on tissue regeneration (engineering) to restore lost mechanical function to a compromised skeleton. To accomplish this goal, it will be necessary to employ much more sophisticated approaches toward evaluating the structure-to-function relationships, ones which will include integration of the respective contributions of gene expression, cell number and activity, matrix composition and architecture to achieve adequate tissue function.

Keywords: Gene Discovery, Structure to Function, Bone Formation, Bone Resorption, Signal Transduction

Introduction

Musculoskeletal complaints are the most common reason that patients seek medical attention[1]. While arthritis and osteoporosis are examples of common disabling musculoskeletal conditions having limited effective treatment options, there are numerous less common but equally devastating musculoskeletal diseases. Chronic musculoskeletal diseases have in common a loss of biomechanical function.

This perspective will focus on restoring structure-to-function relationships in the skeleton because merely preventing further deterioration is inadequate to effect a cure.

The skeleton is a complex organ system composed of a wide assortment of specialized tissues. Although this discussion will emphasize bone, the principles we discuss are intended to be equally applicable to soft as well as mineralized tissues.

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Structure-to-function relationships within the skeleton

It has long been recognized that structure and function are intimately related in biology, and that the skeleton is no exception.

However, structure-to-function relationships in the skeleton cannot be precisely calculated with our current understanding of physical laws. Instead, they are viewed as a largely trial and error process that has been driven by evolutionary pressures. Attempts to describe skeletal development, repair and remodeling based on mechanical considerations alone (e.g., "Wolff's Law") are at best useful approximations. This is likely because a skeleton is created by the activities of living cells which are subject to biologically based constraints. For this reason, optimization of the mechanical function of the skeleton competes with other functions. As an example, the critical need of the individual to maintain blood calcium within narrow parameters can cause changes in bone metabolism which over the long run can compromise the integrity of the skeleton's mechanical function.

The relationships between the individual processes that

promote a specific function are illustrated in Figure 1. The arrows indicate the direction of control. Briefly, the successful functioning of a tissue is dependent on its structure, which in turn is determined by the activity of cells. The behavior of these cells is mediated by the expression of specific genes. As a consequence of these relationships we conclude that genes determine function. Thus, purposeful manipulation of the genes which regulate the activity of cells might be used to alter structure to affect an improvement in function.

The relationships shown in Figure 1 are applicable to bone, cartilage and other connective tissues. We are emphasizing the mechanical function of these tissues, in part, because any loss in mechanical function has an immediate visible negative impact. As a consequence, it is reasonable to assume that adaptation of the skeleton to optimize its mechanical properties has been an important driving force in evolution. On the other hand, it is also reasonable that other physiological functions of the skeleton (e.g. regulation of mineral homeostasis and reproduction) are governed by independent sets of relationships between genes, cells, structure and function. Thus, the composite skeletal structure represents the weighted average of competing sets of relationships.

The concept of improving bone mechanical properties by the purposeful regulation of gene expression is attractive. But, to fully exploit this principle it will be necessary to have a far more comprehensive knowledge than is presently available as to how to recapitulate developmental processes, in adults, which lead to self assembly of structures that are mechanically competent.

A starting point in this endeavor is to develop tools that will allow us to begin the process and at the same time be able to access our level of success in attempting to mimic nature. The remainder of this perspective will be directed toward discussing these tools.

Bone mass does not a complete story make

A low bone mass can be diagnostic of osteoporosis, but increases in bone mineral density (BMD) following successful treatment of osteoporosis have been reported to account for less than 20% of the reduction in fracture rate[2]. These findings suggest that a deterioration in bone quality, which is not measured by BMD, contributes to osteoporotic fractures. Furthermore, bone mass measurements provide minimal insight into mechanisms of action. In spite of these limitations, bone mass is often the only end point related to bone structure that is measured by investigators. Progress in developing more effective therapies for restoring normal function to a compromised skeleton will be more rapid when the structure-to-function relationships of the skeleton are understood. To accomplish this goal, it will be necessary to consider the respective contributions of genes, cells, tissue mass, architecture, and material properties to achieving optimal mechanical function.

Bone mechanical properties are determined by only three variables: mass, composition and architecture. Mass is self-explanatory; it represents the total amount of bone and is easily measured. A deficiency in bone mass itself can lead to an impaired mechanical property. As an example, a reduced bone mass contributes to the increased fracture risk associated with postmenopausal osteoporosis[3].

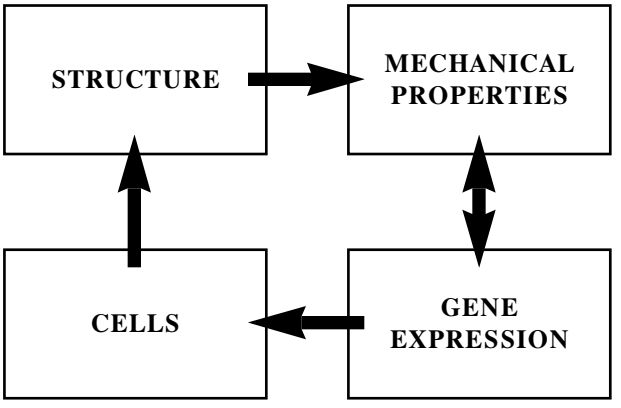

Figure 1. Structure to function relationships in the skeleton.

Composition refers to the content and organization of the chemical components in bone and is difficult to measure because of its complexity. The organic and mineral phases of bone determine the material properties of bone. Defects in mineralization and collagen structure are responsible for the compromised mechanical properties associated with rickets and osteogenesis imperfecta, respectively. Repetitive mechanical loading can result in accumulation of microdamage with little or no change in bone mass which, if unrepaired, can result in deterioration of bone mechanical properties. Additionally, cell signaling molecules such as cytokines and growth factors in bone matrix that had been deposited by osteoblasts play a critical role coupling bone formation to bone resorption during bone remodeling[4]. Bone mass and architecture can vary greatly between species and even between bones in the same individual. These radical structural differences contrast with the relatively homogeneous chemical composition of the organic and mineral phases of bone matrix which has been highly conserved during evolution. The critical importance of matrix chemistry is dramatically illustrated by natural experiments; a point mutation in the type 1 collagen gene resulting in a single amino acid substitution can result in a drastic deterioration in bone material and mechanical properties[5]. There is considerable evidence that the mechanical properties of cartilage and connective tissues are just as sensitive to matrix chemistry. Thus, attempts to restore mechanical function to a compromised skeleton using either gene therapy or conventional approaches require careful attention to the quality of the matrix produced.

The non-structural signaling molecule component is likely to contribute little to the material properties of bone matrix. However, changes in the concentration of these proteins might influence future changes in bone mass and architecture because of their essential role in regulating the bone remodeling cycle. Additionally, these factors are critical to the osteoinductive capacity of bone matrix and as a

consequence are essential for normal bone repair following a fracture[6]. There is very limited knowledge concerning the skeletal distribution and function of specific signaling molecules. This deficiency represents a potential enormous challenge for gene therapy. At present, we do not know how cells are programmed to secrete appropriate concentrations of the various signaling molecules into tissue matrices to fulfill local needs. Advances in our ability to localize and measure these proteins are greatly needed.

Architecture and function

Architecture refers to the three-dimensional organization of a tissue and is a major determinant of its mechanical properties. Two bones with identical mass and material properties could have drastically different mechanical properties because of differences in spatial organization (Figure 2).

Bone densitometry is the most common method of evaluating human bone and measurements made using this method are often the primary end point for animal models as well. This approach has an important advantage over many others in that it is relatively non-invasive. As was discussed, this method is unfortunately largely uninformative regarding two of the variables which determine bone quality, composition and architecture. However, if composition is unaltered, as is believed to be the case with osteoporosis, and deterioration in mechanical properties parallels the reduction in bone mass associated with the syndrome then bone mass measurements are sufficient to evaluate the status of bone function.

There is evidence, however, that improvements in bone quality can have greater impact on bone mechanical properties than increases in bone mass. The practical implications of this finding is that the efficacy of an intervention to treat osteoporosis cannot be accurately determined using measurement of bone mass as the exclusive surrogate for fracture rate. As a consequence, relying entirely on bone mass measurements could result in rejection of effective drugs which improve bone quality without resulting in overall increases in bone mass and has resulted in selection of agents (fluoride) which result in large but mainly unproductive increases in bone mass.

Rat Vertebrae-Role of Bone Growth-Enhancing Drugs
Micro-CT. 20μm voxels

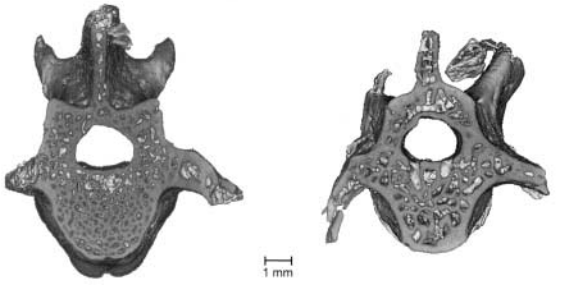

Figure 2. μCT images of vertebrae showing bones from two rats treated with different bone growth-enhancing drugs. The bones are nearly identical in mass but have different architectures. One bone has a small number of very thick trabeculae, whereas in the other trabeculae are more numerous but are much smaller in cross section. These bones having different mechanical properties could not be distinguished by measuring BMD.

Arterial Blood Supply of Rat Tibia
Micro-CT. 20μm cubic voxels, PbCr0, Microfil in arteries

Figure 3. μCT image of a rat long bone showing the associated vasculature. The imaging method has been described in detail[7]. Skeletal tissues function in collaboration with other organs including the vasculature (see Figure 6) whose normal function is essential to normal skeletal function.

Without validated non-invasive technologies for assessing bone quality, it is likely that human investigation will continue to depend primarily upon bone mass measurement as a surrogate for fracture risk. In animal models, however, some routinely performed invasive procedures can be used to detect change in bone quality. The combination of morphological measurements and mechanical testing can provide an accurate depiction of bone quality. Conventional histomorphometry is primarily used to measure regional bone mass as well as rates of bone growth and turnover and it can be used to detect changes in bone architecture. This method can be used to distinguish bone modeling from remodeling and is especially useful for investigating the cellular mechanisms underlying changes in bone architecture. Histomorphometry is a destructive procedure. As a consequence, mechanical testing must be performed in another bone specimen. Another disadvantage of histomorphometry is that it is difficult to accurately extrapolate two-dimensional histological measurements of anisotropic structures to three-dimensional architecture. Finally, histomorphometry is largely uninformative regarding bone composition.

The limitations of conventional histomorphometry for evaluating bone quality can be improved upon by scanning methods such as high resolution (micro) computed tomography (μCT). These emergent technologies can reveal changes in the three-dimensional architecture of bone as well as in bone density. Because the method is non-destructive, it is practical to perform mechanical testing on the bones following scanning. In the future it may be possible to non-destructively load bones in the μCT to evaluate bone material and mechanical properties[7]. Scanning methods can also be used to visualize connective tissues as well as the vasculature[8] (Figure 3).

Measurement of cell number, turnover and activity

Histomorphometry provides the most commonly used method for measuring cell number and activity. Fluorochrome labeling has been used with great success for estimating osteoblast activity and bone mineralization. Osteoblasts, lining cells, osteocytes, and osteoclasts can be recognized by their respective morphologies and in some cases by expression of characteristic marker proteins (e.g. acid phosphatase for osteoclasts and osteocalcin for osteoblasts). Osteoblast and osteoclast turnover can be studied by labeling cells in S-phase of their cell cycle to measure proliferation and detecting apoptotic cells to measure cell death. Similar methods can be used for measuring the number and activities of cartilage and connective tissue cell differentiation and turnover. However, many gaps in our knowledge remain. In part, this is because most studies employ few of the available methods for evaluating cells. Also, we have no direct measurements for the activities of some cell types. Our inability to measure bone resorption in a histological section in a manner analogous to bone formation is a conspicuous example. However, there are other important deficiencies such as the inability to reliably identify osteoblast and osteoclast precursors. It is clear that improvements in our assays are needed to fully understand bone cell kinetics. Such an understanding is essential if we are to obtain a complete understanding of the cellular mechanism for changes in tissue mass and/or architecture. A bone fracture is due to a failure at the local level and the local behavior of bone cells is fundamental to maintaining normal function.

Method	Number of genes typically measured	RNA typically measured
Northern Analysis	1-3	5-30 μg
PCR	1 or 2	1 ng
Multi-probe RPA	Up to 10	10-20 μg
cDNA Microarray and Gene Chip Technology	Up to 10,000	1 μg
PCR: Polymerase chain reaction, RPA: RNase protection assay		

Table 1. Comparison of methods used to measure gene expression.

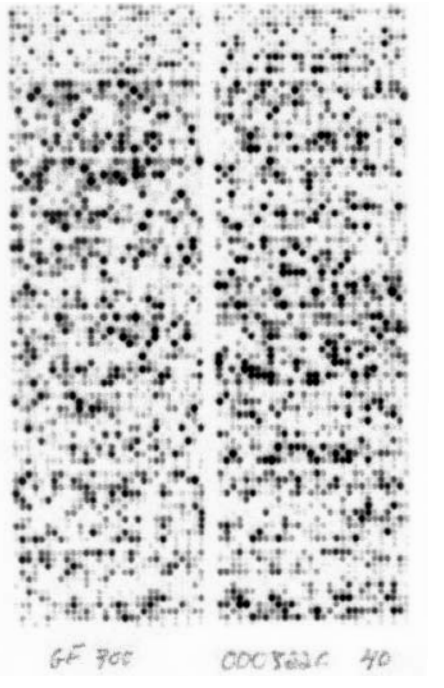

Figure 4. Phosphoimage of a cDNA microarray filter having sequences for over 5,000 rat genes following hybridization. The signal intensity is related to the abundance for each gene specific mRNA extracted from the tissue, which for this example was from rat tibial metaphysis.

Gene expression

The behavior of cells is genetically determined. Knowledge of the pathways of action can lead to an understanding of the molecular mechanisms for impaired tissue function and lead to the rational development of interventions to reverse the detrimental changes.

The most common method used to measure gene expression in the skeleton is the candidate gene approach. Basically, this method requires the investigator to make an educated guess as to which gene(s) play(s) an important role in the process under investigation. Gene expression is then analyzed by measuring protein and/or mRNA levels. The limitations of this approach are obvious. The investigator may choose the wrong gene because of an incomplete understanding of the underlying mechanisms or, alternatively, may choose only one of several critical genes. It is possible that the relevant gene has not been sequenced, and as a result, there are no probes for that gene available. However, the candidate gene approach has provided valuable information because gene changes precede phenotypic changes. For example, anabolic agents such as parathyroid hormone have been shown to result in coordinated increases in mRNA levels for various bone matrix proteins prior to a detectable change in bone formation which in turn increases prior to measurable increases in bone mass[8]. These findings provide strong evidence that the rate of bone matrix synthesis is regulated at the transcriptional level.

A second approach is to assay a battery of genes. Gene microarray and gene chips are examples of technologies that

allow the simultaneous analysis of the expression of a very large number of genes (Table 1). There are over 10,000 sequenced genes in the rat and this number is increasing rapidly. This large number of known genes greatly increases the likelihood of detecting important components of any relevant signaling pathway (Figure 4).

A significant technical problem associated with gene microarrays is defining the magnitude of change which constitutes a meaningful difference in expression[9]. It has been the authors' experience with Northern analysis that assay variability is cDNA dependent. Additionally, the magnitude of the interanimal variation differs considerably between genes. Great effort is being expended to understand how to cope with the enormous volume of data which is created by microarrays. These data, however, represent an incredible resource which can be mined to reveal the biochemical relationships which provide the key to understanding tissue function at incremental increases in sophistication.

Commercial gene microarrays were not designed specifically for bone, cartilage and connective tissues. They do not necessarily include all of the genes of interest to a particular investigator. For this reason, the development of custom gene arrays and gene chips for a particular project would be valuable.

No single method for identifying genes involved in mediating physiological processes in bone will be 100% comprehensive. Thus, to increase the probability of identifying the most important genes it will be prudent to employ more than one method. The gene microarray/gene chip-based approach offers a straightforward and rapid approach for the identification of differentially expressed genes. However, the genome size in animal models is comparable to humans. The commercially available rat and mouse microarrays/gene chips represent at most 25% of the number of genes expected to be coded by the rat and mouse genomes. Far fewer genes are described in other commonly used animal models (e.g. dogs, rabbits, birds, etc.). Nothing is known about what fraction of the genes represented in the microarray/gene chips are specific to bone. An approach that is completely dependent upon such microarray-based strategy will fail to identify all of the genes expressed in bone. Clearly, other methods, not dependent upon prior knowledge of genes will need to be employed.

Differential display and sequential analysis of gene expression (SAGE) have been used to identify previously unknown genes. An even newer approach has been termed preferential amplification from coding sequences (PACS) and employs amplification of mRNA sequences by specifically designed primers to anchor at "ATG" containing sequences and other primers placed at PCR-amplifiable distance from the ATG-containing primers[10]. Thus, PACS preferentially identifies differentially expressed coding sequence tags (dCSTs). An important advantage of this method is that dCSTs can be used for homology searches to identify human homologs. Additionally, dCSTs can be used to construct custom microarrays to further characterize regulation of their expression.

A schematic which outlines an example of a protocol to discover "new" genes related to skeletal function is shown in Figure 5. First, RNA is isolated from the tissue of interest. The expression of known genes can be evaluated using commercial cDNA gene microarrays. These will detect many genes with full length sequences as well as numerous uncharacterized expressed sequence tags (ESTs). The PACS-based approach can be used to identify novel genes. Once full length cDNAs are recovered and human homologs identified it will be possible to investigate the function of these newly identified genes. The construction of custom gene arrays containing cDNAs from known and "new" genes that are expressed in the skeleton can then be used as a tool to help characterize regulatory pathways.

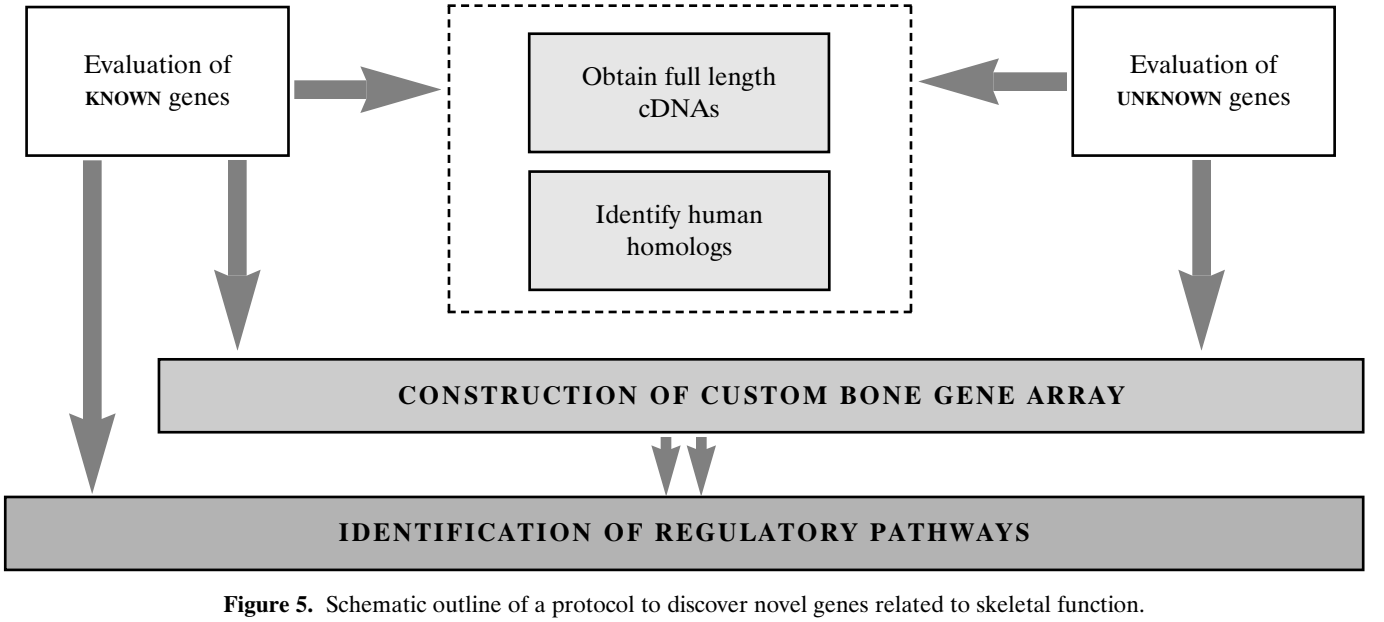

Figure 5. Schematic outline of a protocol to discover novel genes related to skeletal function.

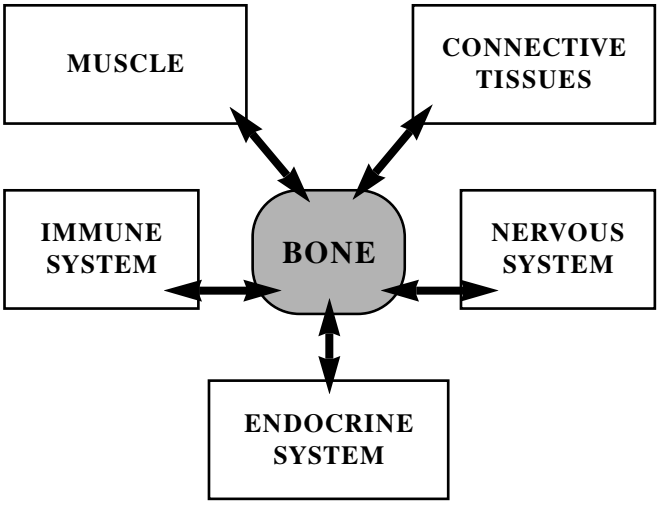

Figure 6. The relationship of bone function to selected other tissues.

Higher levels of organization

As illustrated in Figure 6, the skeleton functions in close collaboration with other organs. As a consequence of interactions between these organs, it is unlikely that successful regeneration and restoration of normal function of a skeletal tissue can be accomplished if abnormalities in another organ system persist that contributed to the original problem. Some of these relationships are obvious. The mechanical loading of bone by muscle through connective tissue attachments has been long recognized. Similarly, the important role of the endocrine system, immune system and cartilage are appreciated. However, the precise relationships between bone metabolism and vasculature, nervous system and many components of bone marrow are largely uninvestigated.

Summary

Gene therapy, stem cell replacement, tissue engineering and new classes of anabolic drugs may each be useful, alone and in combination, to promote regeneration of skeletal tissues. The safe and effective application of these methods will require major advances in our understanding of the regulation of proliferation, differentiation, death and activity of numerous cell types in the context of the specific tissues being generated. On the positive side, many of the tools for obtaining this knowledge are presently available.

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