NEWS

A New Skeletal Role for an Immune System Protein

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A collaborative effort between Boston-area immunology and bone researchers has resulted in an important breakthrough in the understanding of adult bone formation (1). Immunology investigators from a laboratory led by Laurie Glimcher at the Harvard School of Public Health, in cooperation with skeletal biology researchers based in a lab helmed by Melvin Glimcher, Laurie's father, at Harvard Medical School and Children's Hospital, have discovered that Schnurri-3 regulates bone mass in adult mice. This protein, first identified as one necessary for the recombination of immune system genes, exerts its impact by enhancing the ability of an enzyme called WWP1 to target the crucial transcription factor Runx2 for degradation.

"This work reveals another level of osteoblast regulation and increases our understanding and appreciation of how powerful Runx2 is in terms of its functioning as a master regulatory protein," says Jane Lian, Professor of Cell Biology at the University of Massachusetts Medical School. Most tantalizing of all, the findings thrust into the spotlight a new potential target for future efforts to develop drugs for the treatment of bone diseases such as osteoporosis. However, additional experiments, including those that assess the quality of bone present in strikingly increased amounts in Schnurri-3 knockout mice, are first necessary to determine if the current research can in fact be translated successfully into the therapeutic realm.

An Unexpected Phenotype

The original aim of the study, published in the May 26th issue of Science, was to learn more about the function of Schnurri-3 in the immune system by generating knockout mice missing the gene encoding the Schnurri-3 protein. The appearance of the knockouts, however, compelled a shift to a focus on bone. "The knockouts do exhibit defects in the immune system, but these defects are nowhere near as profound as the skeletal phenotype," says research fellow Dallas Jones, lead co-author of the current study along with MD-PhD candidate Marc Wein, both of whom work as part of Laurie Glimcher's team. In fact, the knockout mice displayed striking increases in the postnatal bone mass of their long, calvarial and vertebral bones. Most notable of all: the mice continued to gain bone as they aged. "We think there are very few genes known to play a role in the regulation of bone formation by osteoblasts during adult skeletal remodeling," says Wein. "This is probably the reason why we're most excited about these mice, because they develop perfectly normally, but then as they age, instead of losing bone, they gain it."

Wein and Jones had turned to the skeletal expertise of Melvin Glimcher's laboratory for help in characterizing the Schnurri-3-deficient mice. In addition to the high bone mass, the knockouts exhibited other striking changes. "The mice showed increases in volumetric bone mineral density, as well as thicker and an increased number of trabeculae," according to Jochen Hofstaetter, a co-author of the current paper who was working as a postdoctoral research fellow in Melvin Glimcher's lab at the time. "We also found a huge amount of trabecular bone in places, such as in the diaphysis of the femur, where normally there is no bone at all."

In order to explain these remarkable changes, the researchers examined the osteoclasts and osteoblasts of the knockout mice. Experiments showed that the osteoclasts appeared normal in vivo, and in vitro work revealed that osteoclasts...
differentiated from the bone marrow of knockout mice appeared similar in number and function to osteoclasts differentiated from the bone marrow of normal mice. In addition, attempts to replenish the osteoclasts of knockout mice, whose bone marrow had been experimentally destroyed by radiation, with bone marrow from normal animals did not affect the appearance of the knockouts, who still exhibited high bone mass, suggesting that osteoclast defects were not responsible for this altered phenotype. The histomorphometric finding that the knockouts exhibited an increased rate of bone formation in vivo, along with studies that replicated this finding in vitro, really focused attention on the bone-forming osteoblasts. "When we did our in vitro cultures and compared wild type and knockout osteoblasts, we saw that the knockout osteoblasts in vitro, just like in vivo, made more extracellular matrix." Wein says. "This really drove home to us the idea that Schnurri-3 is an inhibitor of matrix mineralization by osteoblasts."

**Seeking a Mechanism**

If Schnurri-3 acts in this fashion, what is the mechanism that allows it to do so? It seemed reasonable to hypothesize that Schnurri-3 inhibited osteoblasts through an effect on Runx2 because osteoblasts from Schnurri-3 knockouts exhibited an increase in the activity of genes known to be targets of this key osteoblast differentiator. Experiments designed to test this reasoning revealed that osteoblasts from the knockout mice exhibited increased amounts of Runx2 protein, as well as increased levels of Runx2 binding to DNA. Additional in vitro studies showed that Schnurri-3 could decrease the amount of Runx2 protein in cells and could enhance the tagging of Runx2 with ubiquitin, a protein that marks other proteins like Runx2 for degradation. Further work showed that Schnurri-3 physically interacted with Runx2 and in doing so decreased its stability.

While it seemed possible that Schnurri-3 itself added the ubiquitin tag to Runx2, the evidence did not support this contention, in part because Schnurri-3 lacks the key characteristics of such proteins, termed ubiquitin ligases. "There are motifs—sequences of amino acids—that are found in proteins that have ubiquitin ligase function, and none of these motifs were found in Schnurri-3," Wein notes. The investigators also observed that Schnurri-3 was unable to promote the ubiquitination of Runx2 in vitro, suggesting that another protein served this function. They wondered if perhaps Schnurri-3 interacted with this as yet unspecified ubiquitin ligase and in doing so helped facilitate the ubiquitination of Runx2.

Experiments along these lines found that a ubiquitin ligase called WWP1 appeared in increased amounts when osteoblasts were coaxed to differentiate in in vitro studies and that Schnurri-3 associated with it. A model began to take shape: maybe Schnurri-3 interacted with WWP1 and improved the ability of this ubiquitin ligase to target the Runx2 protein for degradation. Consequently, the researchers designed experiments to characterize cells engineered to produce increased amounts of WWP1 and to measure the impact that the addition of Schnurri-3 might have on such cells. These in vitro studies found that not only does Runx2 interact with WWP1, but also that the addition of Schnurri-3 enhances this interaction. Cells with elevated levels of WWP1 also showed decreases in levels of Runx2 protein. Finally, while such cells exhibited only low levels of Runx2 ubiquitination, the addition of Schnurri-3 led to far greater amounts of this key cellular activity.

In addition to the above experiments, the investigators also designed knockdown studies, using RNA interference technology, that measured the effects of reducing WWP1 levels in cells. Using primary osteoblasts, they found that reducing WWP1 levels resulted in increased levels of proteins that Runx2 targets, as well as increased levels of the Runx2 protein itself. These osteoblasts also exhibited increased numbers of mineralized matrix nodules. "When we knocked down WWP1 in the primary osteoblasts using RNA interference and saw that those osteoblasts looked very similar to the Schnurri-3 knockout osteoblasts, this really solidified our working
model," Wein says. In this model, Schnurri-3,
WWP1 and Runx2 join in a complex that
inhibits the activity of Runx2. The role of
Schnurri-3 in this complex is to make WWP1
better able to target Runx2 for degradation.

A New Drug Target?

The astonishing high bone mass phenotype
of the Schnurri-3 knockouts, and the
identification and characterization of the
forces responsible for generating it, naturally
turn thoughts to diseases, like osteoporosis,
characterized by low bone mass. Can the
results of the current study be translated into
the therapeutic realm for such conditions?
The investigators certainly think so, and they
are beginning to pursue collaborative
studies with Gregory Petsko, a biochemist at
Brandeis University, to discover compounds
that might inhibit WWP1. Unlike Schnurri-3,
a large, non-enzymatic protein, WWP1
looms as a very attractive target for drug
development because of its status as an
enzyme. "Inhibiting an enzyme is much
easier than, say, activating a transcription
factor, or blocking protein-DNA interactions,
or disrupting a protein-protein complex,"
Petsko explains. "Since an enzyme has a
substrate, there is a chemical structure that
you already know binds to it, and you also
already know the site that you need to attack,
the active site, where the chemistry occurs."
Quite fortunately for the investigators, the
crystal structure of WWP1 had already been
determined from previous, unrelated studies,
so they do indeed know where to focus their
efforts.

Petsko notes that while a pharmaceutical
company might start by looking at hundreds
of thousands of compounds and by
performing real assays measuring whether
any of them could actually inhibit WWP1, the
computational approach that he is pursuing
for the Glimcher team, a line of attack more
feasible for academic labs with more limited
resources interested in translating basic
research findings into the clinic, has distinct
advantages in the search for inhibitors. "The
good news about doing this work
computationally is that you can cast your net
infinitely broadly, you can dock any kind of
structure, from any kind of database, to this
WWP1 protein," he emphasizes. Indeed,
from a database of approximately 3.5 million
compounds, Petsko will narrow the list down
to about 36,000 compounds representative
of the major pharmaceutical classes and will
then, with computer assistance, "dock" each
of these compounds—test if they fit into the
active site of the enzyme, a process Petsko
likens to a much simpler task. "It seems like
a daunting procedure, but it’s actually the
computational equivalent of what a child
would do, taking a block and trying different
ways to fit it into holes on a board. Here, the
holes in the board are the active site of the
enzyme, and the drug is the block that will fit
the shape of the holes precisely." Once a
manageable list of potential inhibitors has
been generated in this way, Wein, Jones
and colleagues will then perform real assays
that will test in vitro and then in vivo whether
any of the candidates can actually bind to
WWP1 and generate increases in bone
mass.

Identifying a viable new inhibitor would be
welcome news since current treatments for
osteoporosis have a significant failing.
Indeed, while bisphosphonates are currently
used to treat osteoporosis and are safe and
effective agents, they do not build new bone,
but rather work by inhibiting the resorption
of bone by osteoclasts. The availability of
drugs that could actually stimulate the
synthesis of new bone—options are limited
now in this regard—would provide physicians
with a new way to attack the problem of low
bone mass.

Questions for the Future

While the scientists hope their work may one
day help meet an important medical need,
they must first address key questions before
they can prove the feasibility of WWP1 as a
drug target. Perhaps the most important
issue is whether the bone found in the
Schnurri-3 knockout mice is actually high
quality bone of the kind one would actually
want to increase. "We know these mice
show a very large increase in bone mass,
but we first have to check whether the bone
these mice produce is actually normal
bone," stresses Dr. Hofstaetter, who is now
working on bone quality studies along with
colleagues at the Ludwig Boltzmann Institute of Osteology in Vienna, Austria.

In addition to these studies, the biological significance in organisms of the Schnurri-3/WWP1/Runx2 interaction, as well as timing details regarding when that interaction is called into play, remain uncertain. "We know the interaction is important from the phenotype of the mouse," Dr. Lian explains, "but when does that interaction take place? Does it take place in an early osteoprogenitor or stem cell, is it a way of regulating Runx2 levels so that you don't make a bone cell or a differentiated phenotype, unless the timing is correct in development and during the stages of osteoblast differentiation? When and why these interactions become operative, and for what purpose, is not clear."

Jones and Wein are also now pursuing more detailed molecular biological studies. They are particularly interested in learning in much greater detail precisely how Schnurri-3 regulates WWP1. "Very little is known about how ubiquitin ligases like WWP1 are regulated at the level of protein-protein interaction," Jones observes. In addition, they are also curious to know whether there are other signaling mechanisms operating in osteoblasts that govern the interaction between Schnurri-3 and WWP1.

As work unfolds in these areas, the future continues to brighten for those potentially interested in collaborative scientific work in two fields whose connections seem to grow stronger each day. "This research on Schnurri-3 is a nice example of how immunologists can interact with bone researchers," notes Joseph Lorenzo, Professor of Medicine and Director of Bone Biology Research at the University of Connecticut Health Center. Opportunities for these kinds of interactions seem natural considering the close relationship between the immune and skeletal systems. "We know that osteoclasts are a derivative of immune cells and are related to macrophages and myeloid dendritic cells, that osteoblasts are critical for maintaining hematopoiesis and immune cells in the bone marrow, and that immune cells produce a number of factors that regulate bone cells, so there is a lot of crosstalk between the systems," adds Lorenzo. Though the current study does not show a direct connection between the immune and skeletal systems, it does offer additional evidence that many of the same molecules are involved in each system, and augurs well for future collaborative work.

References