SUPPLEMENT

Schnurri-3 is an essential regulator of osteoblast function and adult bone mass

Dallas C Jones, Marc N Wein, Laurie H Glimcher

Ann Rheum Dis 2007;**66**(Suppl III):iii49–iii51. doi: 10.1136/ard.2007.078352

See end of article for authors' affiliations

Correspondence to: Laurie H Glimcher, Department of Immunology and Infectious Diseases, Harvard School of Public Health, 651 Huntington Ave, FXB 205, Boston, MA 02115, USA; lglimche@hsph.harvard.edu

Accepted 29 June 2007

Skeletal remodelling is a cyclical process where under normal physiological conditions, bone formation occurs at sites where bone resorption has previously taken place. Homeostatic remodelling of the skeleton is mediated by osteoclasts, giant multinucleated cells of haematopoietic origin that are responsible for bone resorption and osteoblasts, which originate from mesenchymal stem cells, and synthesise the matrix constituents on bone-forming surfaces.[1] Proliferation, differentiation and bone remodelling activities of these cells involve a complex temporal network of growth factors, signalling proteins and transcription factors. Dysregulation of any one component may disrupt the remodelling process and contribute to the pathogenesis of common skeletal disorders, like osteoporosis and Paget's disease. Rare single gene disorders resulting in elevated bone mass due to osteoclast defects are collectively termed osteopetrosis. Rarer still are single gene disorders, collectively termed osteosclerosis, in which elevated bone mass is due to intrinsically elevated osteoblast activity.[2] While we have learned much about the molecular control of skeletal formation and remodelling from these mutations, additional genes that regulate bone mass have yet to be characterised.

The current lack of adequate treatments for bone loss associated with certain skeletal diseases will present an expanding source of morbidity and mortality as the population in the USA ages. Further elucidation of the molecular pathways that regulate the differentiation and activity of osteoblasts may yield promising therapeutic targets for the treatment of these various skeletal disorders.

MESENCHYMAL STEM CELLS AND OSTEOGENESIS

Formation and patterning of the skeleton involve numerous genes that are temporally and spatially regulated throughout embryogenesis.[3] This intricate network of gene regulation is necessary for individual skeletal elements to be generated in a specific location with the correct morphology. A number of essential molecules that regulate skeletogenesis were identified in mice that contain spontaneous or engineered single gene mutations that resulted in skeletal dysmorphogenesis.[4] These skeletogenic regulatory factors are frequently involved in controlling lineage differentiation of mesenchymal stem cells (MSCs). Given that MSCs can differentiate into chondrocytes and osteoblasts, as well as adipocytes and other lineages, mutations in this stem cell population can affect both endochondral and intramembranous ossification. A complete lack of these ossification processes in mice deficient for Runx2, an essential regulator of osteoblastogenesis, validates this concept.[5 6]

In addition to the essential role of MSCs during embryologic skeletogenesis, these cells have an equally important function in regulating bone mass postnatally. The high rate of osteoblast apoptosis following the bone formation phase requires that these cells be continually replenished from the MSC compartment to maintain skeletal integrity. An age-associated impairment in the MSC's ability to maintain the osteoblast population has been put forward as one explanation for the aetiology of osteoporosis.[7 8] The differentiation of MSCs into a specific lineage requires the presence of certain molecules that promote one cell fate while repressing other possible fates. Therefore, molecules that are anti-osteogenic may be dysregulated in MSCs during the progression of osteoporosis.[9] A detailed molecular understanding of the key determinants that regulate both MSC population maintenance and the differentiation of these cells into the osteoblast lineage could lead to new drug targets for the treatment of numerous skeletal disorders, including senile osteoporosis.

SCHNURRI-3 AND POSTNATAL SKELETAL FORMATION

Analysis of skeletal patterning and remodelling in mice bearing single gene mutations has established a dichotomy of factors that function to regulate osteoblast differentiation and activity. The first set, including Runx2,[10 11] Twist,[12] and Osterix,[13] function during skeletogenesis to regulate the differentiation of MSCs to the osteoblast lineage. The second set, including the Wnt coreceptor LRP5[14] and the transcription factor ATF4,[15] function later in mature osteoblasts to regulate their synthetic function during bone remodelling. Although Runx2 is clearly required for early commitment of MSCs into osteoprogenitors, it also functions later in osteoblast differentiation to regulate extracellular matrix formation.[16]

We have recently identified Schnurri-3 (Shn3), a member of the ZAS family of zinc finger proteins, as an essential regulator of postnatal skeletal remodelling that we propose belongs in the second group of factors that regulates mature osteoblast activity. Shn3 is one of three mammalian homologues of Drosophila *Shn*, a protein that acts during embryogenesis as an essential nuclear cofactor for signalling by Decapentaplegic (Dpp), the Drosophila homologue of BMP/TGFβ.[17] In contrast to the elegant work defining the biological processes regulated by Drosophila *Shn*, the physiological relevance of the mammalian Shn proteins was until recently unknown. However, a number of in vitro and in vivo studies have lately suggested that these large proteins may regulate the differentiation and function of cells derived from MSCs, including chondrocytes, adipocytes and osteoblasts.[18–20]

To gain further insight into the biological relevance of Shn3, we have generated mice bearing a null mutation in Shn3. As stated above, our analysis of homozygous Shn3 mutant

Abbreviations: HTS, high-throughput screening; MSCs, mesenchymal stem cells; WT, wild type

(Shn3–/–) mice revealed a pronounced high bone mass phenotype, due to augmented osteoblast synthetic activity and bone formation.[19] Analysis of primary osteoblasts derived from calvariae of newborn Shn3–/– and wild type (WT) mice verified dysregulated osteoblast activity, characterised by increased levels of bone sialoprotein and osteocalcin mRNA but similar levels of alkaline phosphatase mRNA compared to WT osteoblasts. Osteoblasts isolated from Shn3–/– mice also exhibited increased extracellular matrix production and mineralised nodule formation when compared to WT control osteoblasts. Collectively, these results demonstrate that Shn3 regulates the expression of genes important in bone formation and mineralisation.

The genes overexpressed in Shn3–/– osteoblasts are all direct Runx2 targets,[10 15] suggesting that Shn3 might inhibit osteoblast activity through Runx2. While Runx2 mRNA levels were comparable between Shn3–/– and WT osteoblasts, Runx2 protein levels were elevated in Shn3–/– osteolbasts. We demonstrated that this incongruity between Runx2 transcripts and protein was the result of Shn3's physical association with Runx2 that promoted its degradation and led to decreased Runx2 protein levels.

The degradation of Runx2 was associated with its ubiquitination. While Shn3 promoted the ubiquitination of Runx2, Shn3 itself contains no canonical E3 ubiquitin ligase domains. We therefore hypothesised that Shn3 may associate with a known E3 ubiquitin ligase to promote Runx2 ubiquitination. Indeed, we found that Shn3 could readily co-immunoprecipitate with WWP1, a member of the Nedd4 family of E3 ubiquitin ligases. We observed that WWP1 promoted low levels of Runx2 ubiquitination when overexpressed in 293T cells. However, when WWP1 was coexpressed with Shn3, the two synergistically acted to promote Runx2 ubiquitination.

Taken together, these data suggest a model in which the formation of a multimeric complex between Runx2, Shn3 and the E3 ubiquitin ligase WWP1 in mature osteoblasts inhibits Runx2 function. Shn3 is an integral adaptor protein in this complex, as it enhances the ability of WWP1 to promote Runx2 polyubiquitination and proteasome-dependent degradation.

WWP1 AND E3 UBIQUITIN LIGASES AS DRUG TARGETS

The initial paradigm of protein ubiquitination suggested that this post-translational modification only functioned to target proteins for degradation by the 26S proteosome. However, ubiquitination is now recognised as an important modification that regulates other aspects of protein function, including protein sorting and transcriptional activity.[21 22] Therefore, E3 ubiquitin ligases that catalyse the conjugation of ubiquitin to target proteins have an essential role in almost all known eukaryotic biological processes.[23] The Nedd4 family of E3 ligases, which includes WWP1, belongs to the HECT class of E3 ubiquitin ligases. All members of the Nedd4 family contain a catalytic HECT domain in the C-terminus that mediates the transfer of ubiquitin to the target substrate. The two other modulatory domains that are conserved in all Nedd4 family members are the C2 and WW domains, which function in regulating subcellular localisation and substrate binding. The WW domain mediates substrate interaction through the binding of a PPxY motif in the target protein.[24] Given that all Nedd4 family members utilise identical protein domains to recognise a common binding motif in a target substrate, it is likely that redundant functions may exist within this protein family. Indeed, multiple Nedd4 family members can regulate the same biological function through the ubiquitination of a shared substrate. However, individual Nedd4 proteins have also developed specialised functions through the targeting of unique substrates.

Our studies suggested that WWP1 regulates osteoblast biology by promoting the ubiquitination of Runx2 and possibly additional substrates. Given the scarcity of drugs that function as anabolic agents to promote bone formation, WWP1 is an attractive target for drug discovery. However, the very high cost of high-throughput screening (HTS) for inhibitors, a time and labour-intensive process, has generally prohibited academic or non-profit groups from serious drug discovery programmes. Advances in structural biology and computer-based modelling have recently provided a set of new tools to probe the interaction between proteins and various molecules (including other proteins or potential small molecule inhibitors). Thus, it is now feasible to undertake HTS for drug candidates primarily in silico by modelling the binding of proteins to a docked library of compounds. This can greatly enrich the pool of candidate inhibitors to be tested in vitro and in vivo.

An in silico HTS is a computer simulation of an actual experimental drug screen in which a very large library of candidate compounds can be screened by docking models of their structures into the active site of the target and then calculating the energy of interaction between the compound and the protein to identify those molecules that show a good fit for the target site. Whereas an actual HTS directly selects on the basis of the presence of a biological activity, for example, enzyme inhibition, an in silico method merely screens for hypothetically active compounds; hence, in silico screening requires subsequent in vitro and in vivo testing of the identified compounds. Importantly, however, rather than testing millions of compounds in vitro, the in silico screen can reduce the number of candidates to a few dozen or, perhaps at most, a few hundred, a much more manageable number.[25] Experience suggests that, properly applied, in silico screening can greatly increase the hit rate of actual screening. An HTS usually has hit rates of 0.01% or less. Thus a set of 200 000 or more compounds may have to be screened to find 20 hits. In silico screening can produce a list of 100 compounds from which one may find 20–30 that actually bind to the target protein with reasonable affinity, a hit rate of about 25%.

CONCLUSIONS

Osteoporosis afflicts an estimated 10 million Americans over the age of 50, with 34 million Americans at risk. Osteoporosis-induced fractures occur in approximately 1.5 million individuals per year with serious health consequences. Indeed, 20% of patients with osteoporosis who suffer a hip fracture will die within the year. The increasing risk of fractures with age coupled with the ageing of the American population leads to the prediction that the rate of hip fractures may triple by the year 2020 unless we seek to improve the prevention, diagnosis and treatment of bone disease. Currently, agents available for treatment of osteoporosis mainly function through an anti-resorptive mechanism to inhibit osteoclast-mediated bone loss. Ideally, treatment for osteoporosis would couple antiresorptive therapy with anabolic agents that promote new bone formation. The high bone mass phenotype observed in the Shn3–/– mice that arises through an increased bone formation rate suggests that small molecules that target this protein would be ideal anabolic agents to treat osteoporosis. However, until the structure and functional domains of this large protein are more fully characterised, the development of agents that antagonise Shn3's activity are highly unlikely. Conversely, the solved crystal structure of WWP1 makes this E3 ligase a more suitable molecule for HTS to identify selective inhibitors that may augment osteoblast function and potentially stimulate bone formation.

Authors' affiliations

Dallas C Jones, Marc N Wein, Laurie H Glimcher, Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, USA

Funding: Supported by NIH grants AI29673 (LHG), AR46983 (LHG), postdoctoral fellowships from the Arthritis Foundation (DJ), and the Medical Scientist Training Program at Harvard Medical School (MW).

Competing interests: None declared.

REFERENCES

1 **Karsenty G**, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2002;**2**:389–406.
2 **Whyte MP**. *Sclerosing bone disorders*, 5th edn. Washington: The American Society for Bone and Mineral Research, 2003.
3 **Mariani FV**, Martin GR. Deciphering skeletal patterning: clues from the limb. *Nature* 2003;**423**:319–25.
4 **Horton WA**. Skeletal development: insights from targeting the mouse genome. *Lancet* 2003;**362**:560–9.
5 **Otto F**, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, *et al*. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 1997;**89**:765–71.
6 **Komori T**, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, *et al*. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997;**89**:755–64.
7 **Bonyadi M**, Waldman SD, Liu D, Aubin JE, Grynpas MD, Stanford WL. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. *Proc Natl Acad Sci USA* 2003;**100**:5840–5.
8 **Stolzing A**, Scutt A. Age-related impairment of mesenchymal progenitor cell function. *Aging Cell* 2006;**5**:213–24.
9 **Lecka-Czernik B**, Suva LJ. Resolving the two ''bony'' faces of PPAR-gamma. *PPAR Res* 2006;**2006**:27489.
10 **Stein GS**, Lian JB, van Wijnen AJ, Stein JL, Montecino M, Javed A, *et al*. Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. *Oncogene* 2004;**23**:4315–29.
11 **Ducy P**, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 1997;**89**:747–54.
12 **Bialek P**, Kern B, Yang X, Schrock M, Sosic D, Hong N, *et al*. A twist code determines the onset of osteoblast differentiation. *Dev Cell* 2004;**6**:423–35.
13 **Nakashima K**, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, *et al*. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 2002;**108**:17–29.
14 **Gong Y**, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, *et al*. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 2001;**107**:513–23.
15 **Yang X**, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, *et al*. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* 2004;**117**:387–98.
16 **Ducy P**, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, *et al*. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev* 1999;**13**:1025–36.
17 **Affolter M**, Marty T, Vigano MA, Jazwinska A. Nuclear interpretation of Dpp signaling in Drosophila. *Embo J* 2001;**20**:3298–305.
18 **Tanaka K**, Matsumoto Y, Nakatani F, Iwamoto Y, Yamada Y. A zinc finger transcription factor, alphaA-crystallin binding protein 1, is a negative regulator of the chondrocyte-specific enhancer of the alpha1(II) collagen gene. *Mol Cell Biol* 2000;**20**:4428–35.
19 **Jones DC**, Wein MN, Oukka M, Hofstaetter JG, Glimcher MJ, Glimcher LH. Regulation of adult bone mass by the zinc finger adapter protein Schnurri-3. *Science* 2006;**312**:1223–7.
20 **Jin W**, Takagi T, Kanesashi SN, Kurahashi T, Nomura T, Harada J, *et al*. Schnurri-2 controls BMP-dependent adipogenesis via interaction with Smad proteins. *Dev Cell* 2006;**10**:461–71.
21 **Dhananjayan SC**, Ismail A, Nawaz Z. Ubiquitin and control of transcription. *Essays Biochem* 2005;**41**:69–80.
22 **Bonifacino JS**, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Ann Rev Biochem* 2003;**72**:395–447.
23 **Varshavsky A**. The ubiquitin system. *Trends Biochem Sci* 1997;**22**:383–7.
24 **Chen HI**, Sudol M. The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc Natl Acad Sci USA* 1995;**92**:7819–23.
25 **Kitchen DB**, Decornez H, Furr JR, Bajorath J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat Rev Drug Discov* 2004;**3**:935–49.