

Contributed Mini Review

Proteomics approaches for the studies of bone metabolism

Ji-Hyun Lee & Je-Yoel Cho*

Department of Veterinary Biochemistry, BK21 and Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea

Bone is an active tissue, in which bone formation by osteoblast is followed by bone resorption by osteoclasts, in a repeating cycle. Proteomics approaches may allow the detection of changes in cell signal transduction, and the regulatory mechanism of cell differentiation. LC-MS/MS-based quantitative methods can be used with labeling strategies, such as SILAC, iTRAQ, TMT and enzymatic labeling. When used in combination with specific protein enrichment strategies, quantitative proteomics methods can identify various signaling molecules and modulators, and their interacting proteins in bone metabolism, to elucidate biological functions for the newly identified proteins in the cellular context. In this article, we will briefly review recent major advances in the application of proteomics for bone biology, especially from the aspect of cellular signaling. [BMB Reports 2014; 47(3): 141-148]

PROTEOMICS STRATEGIES

Mass spectrometry (MS)-based proteomics has become a common discovery tool for biological functional analyses, and is applied for verification and validation types of studies (1-3). Generally, in an LC/MS/MS, which is the combination of mass spectrometry and liquid chromatography separation (LC), whole proteins from complex biological samples are digested to peptide fragments by enzyme, and LC/MS is then used to identify the amount of proteins in the biological samples. These samples can be the protein elution of cells and tissues, serum, plasma, urine, etc. (4, 5).

Mass Spectrometry (MS)-based proteomics studies were routinely analyzed by 2-dimensional gel electrophoresis (2-DE-MS), LC-MS/MS, and more recently by quantitative MS approaches. MS-based expression screening proteomics con-

tribute to the identification of targeted molecules and their characterization; whereas, quantitative MS approaches will help to measure the protein's relative or absolute quantity in the identification step, and further quantitative measurement of target protein by MRM, etc. (6). Stable isotope labeling techniques have become very popular in recent years, to perform quantitative mass spectrometry experiments with high precision, and the elucidation of molecular mechanism in multiple biological samples (7). In contrast to label-free approaches, multiplexed isotopically labeled samples can be simultaneously analyzed, resulting in increased reproducibility and accuracy for the quantification of peptides and proteins from different biological states (8). In SILAC, two groups of cells are grown in equal culture media except one: the first group contains the "light", and the second contains the "heavy" isotope labeled amino acids (e.g. L-leucine or deuterated L-leucine). Thus, during cell doubling time, the amino acids are replaced, to give a light or heavy form close to 100% (1, 9). Each pep-

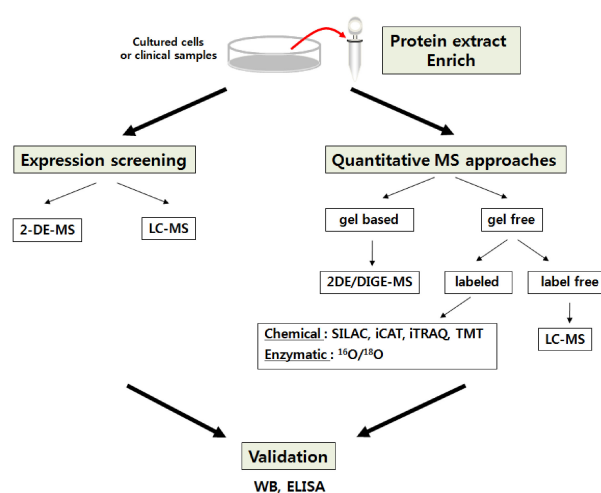


Fig. 1. Proteomics technologies used in publications to study bone metabolism. General proteomics approaches are described to study cellular signaling, secretory proteins, and other analysis of cellular proteins. 2-DE, two-dimensional gel electrophoresis; LC, liquid chromatography; MS, mass spectrometry; DIGE, differential in gel electrophoresis; SILAC, stable isotope labeling with amino acids in cell culture; iCAT, isotope-coded affinity tag; iTRAQ, isobaric tags for relative and absolute quantification; TMT, tandem mass tags.

*Corresponding author. Tel: +82-2-880-1268; Tel: +82-2-886-1268; E-mail: jeycho@snu.ac.kr

<http://dx.doi.org/10.5483/BMBRep.2014.47.3.270>

Received 2 December 2013, Revised 16 December 2013,
Accepted 4 January 2014

Keywords: ECM, Growth factors, MSC, Osteoblast differentiation, Proteomics

tide peak shows a difference in mass of 6 Da in mass spectrometry analysis, and the relative peak intensities indicate the abundance ratios. In iTRAQ labeling, each specimen is labeled with a different amine-specific isobaric tag. In ¹⁶O/¹⁸O labeling, one specimen is mixed with “light” water, containing oxygen in its natural isotopic state (mostly ¹⁶O); and a second specimen with “heavy” water, which contains mostly water molecules with the ¹⁸O isotope that has two extra neutrons (10). The general proteomic techniques used to study biological signal transduction are summarized in Fig. 1.

BONE METABOLISM

Bone is comprised by cells and an extracellular matrix, which

becomes mineralized by the deposition of calcium hydroxyapatite, giving the bone rigidity and strength. Bone has three major cell types: osteoblasts, osteoclasts, and osteocytes, which are osteoblasts tied up within the lacunae of the bone (11). Osteoblasts release bone matrix proteins, such as type I collagen, the most abundant extracellular matrix protein of bone, and are also responsible for mineralization of the tissue (12). Osteoclasts are multinucleated cells derived from the monocyte/macrophage hematopoietic lineage that differentiate and adhere to bone matrix, then secrete acid and enzymes that degrade it in a specialized, extracellular composition (13). Bone is continuously being remodeled, in an energetic progress, where osteoblasts are responsible for bone formation, and osteoclasts for its resorption (14). Proteomic approach can

Table 1. Proteomic approaches for cell line and tissues in bone metabolism

Cell/Tissue	Source of proteins		Proteomic strategy	Findings	Ref.
	Origin	Fraction			
MSC	Human	Whole cell	LC-MS/MS	Identified the phosphorylation of TRIM16, ATF6B, MECP2, THRAP3, PTRF, NACA, and KANK2, during osteogenic differentiation	(21)
MSC/OST	Human	Whole cell	2D LC-MS/MS	Expression of extracellular matrix proteins collagen VI, fibronectin, vitronectin and thrombospondin may be hallmarks of osteogenic differentiation in hMSC	(22)
MSC	Human	Secreted proteins	SILAC, LC-MS/MS	STC2 stimulates osteoblast differentiation of hMSC, in an autocrine fashion	(23)
MSC	Human	Whole cell	2DE MALDI-TOF-MS	Most of the cytoskeletal proteins identified, such as Vimentin, PI4K2β, GAPDH, TPM1, MnSOD, CALR, and TPD54	(26)
C2C12	Mouse	Whole cell	SILAC, LC-MS/MS	The inactivation of Nedd4 and activation of Uchl-3 might improve BMP2-induced osteoblast transdifferentiation	(36)
A375	Human	Whole cell	SILAC, LC-MS/MS	PKN1 inhibits WNT3A-dependent phosphorylation of LRP6	(42)
BMSC	Human	Whole cell	SILAC, LC-MS/MS	BMP2 modulates the Wnt/β-catenin signal via the PI3K pathway in hBMSC	(27)
RAW264.7	Mouse	Whole cell	LC-MS/MS	PDGF BB is a key factor in bone remodeling	(55, 56)
RAW264.7	Mouse	Secreted proteins	2DE, ICAT, LC-MS/MS	All detected cathepsins were down-regulated as to collagenolytic activity	(57)
BMSC	Human	Secreted proteins	LC-MS/MS	Identified SMOC1 as a putative regulator of osteoblast differentiation of BMSCs	(65)
MSC	Human	Secreted proteins	LC-MS/MS	Annexins A1 and A2 were upregulated, whereas PAI-1 and cystatin C were downregulated, during osteogenesis	(69)
MSC	Human	Secreted proteins	SILAC, LC-MS/MS	ECM proteins modulated by activin A	(70)
Saos-2	Human	Secreted proteins	LC-MS/MS	Novel MV residents were detected, such as inorganic pyrophosphatase 1, SLC4A7	(71)
Ulna midshafts	Rat	Whole tissue	LC-MS/MS	Drugs targeting on Raf1 and PDCD8 may regulate bone metabolism, via prevention of osteocyte apoptosis	(72)
MC3T3-E1	Mouse	Whole cell	LC-MS/S	Tanshinone can be a good marker compound, to explain the antiosteoporotic function of SM	(73)

TRIM16: tripartite motif-containing 16, ATF4: activating transcription factor 4, MECP2: methyl CpG binding protein 2, THRAP3: thyroid hormone receptor-associated protein 3, PTRF: polymerase I and transcript release factor, NACA: nascent polypeptide-associated complex α subunit, KANK2: KN motif and ankyrin repeat domain-containing protein 2, hMSC: human mesenchymal stem cell, STC2: stanniocalcin 2, PI4K2β: phosphatidylinositol 4-kinase type 2 beta, GAPDH: D-glyceraldehyde-3-phosphate dehydrogenase, TPM1: tropomyosin alpha-1 chain isoform 3, MnSOD: manganese superoxide, CALR: calreticulin precursor variant, TPD54: tumor protein D54 isoform b, Nedd4: neural precursor cell expressed developmentally down-regulated protein 4, Uchl-3: ubiquitin C-terminal hydrolase L3, BMP2: bone morphogenetic protein 2, PKN1: Protein Kinase N1, WNT3A: wingless-type MMTV integration site family, member 3A, PI3K: phosphatidylinositol 3-kinases, hBMSC: human bone marrow stem cell, PDGF BB: platelet-derived growth factor BB homodimer, SMOC1: SPARC-related modular calcium-binding protein 1, BMSCs: bone marrow derived mesenchymal stem cells, PAI-1: plasminogen activator inhibitor-1, ECM: extracellular matrix, MV: matrix vesicle, SLC4A7: Solute carrier family 4, sodium bicarbonate co-transporter, member 7, Raf1: RAF proto-oncogene serine/threonine-protein kinase, PDCD8: programmed cell death 8, SM: salvia miltiorrhiza.

be an effective means of the elucidation of novel regulatory mechanisms of bone metabolism and therapeutic strategies, and biomarkers for bone-related diseases. Table 1 summarizes the proteomic studies that have been performed up to date, and those proteins related to bone metabolism that were found by each approach.

APPLICATION OF PROTEOMIC TECHNOLOGY TO BONE METABOLISM

Bone recovery with MSCs analyzed by proteomics approaches

Osteoblasts and osteoclasts are assigned to bone remodeling, with a balance between bone formations. However, in some circumstances, bone regeneration exceeds bone self-repair capacities. This is notably often the case after bone fractures (15). Accordingly, bone tissue engineering with autologous or allogenic mesenchymal stem cells (MSCs) has been widely developed. However, the fine control of the differentiation process into specific cell lineage is not guaranteed, as the basic molecular pathway underlying this process has hardly been solved.

MSCs are fibroblast-like and non-hematopoietic stem cells in the bone marrow, and are able to differentiate along multiple lineages, including osteoblast, adipocyte, chondrocyte and muscle cells, under proper culture conditions (16-19). MSC differentiation into osteoblasts can be achieved by adding vitamin D₃, ascorbic acid and β -glycerophosphate to the culture medium (20).

Protein phosphorylation plays a crucial role in the signaling transduction network. In an attempt to discover the signaling proteins in osteogenic differentiation, phosphoproteomic profiling was performed on hMSCs, during the early stage of osteogenic differentiation (21). These phosphorylated peptides corresponded to 698, 534, 549, and 534 different proteins in hMSC, after 0, 1, 3, and 7 days of induction, respectively, with an overlap of 183 proteins. The authors revealed a dramatic loss of protein phosphorylation level, after 1 day of osteogenic induction. During osteogenic differentiation, differentially expressed phosphoproteins demonstrated dynamic alterations in cytoskeleton, at the early stages of differentiation. Comparative proteome analyses between human bone marrow stromal cells (hBMSCs) and osteoblasts (hOSTs) individuals have been performed (22). Of the 755 differential proteins identified, two sets of 247 and 158 proteins were found only in hMSCs and hOST cells, respectively. The authors showed the application of gene ontology (GO) analysis of the protein expression profiles in hMSCs and hOST cells. The authors suggest that the expression of MAP4 and Fillamin C may serve as new markers of hOSTs. Likewise, the expression of extracellular matrix proteins collagen VI, fibronectin, vitronectin, and thrombospondin were found to be features of osteogenetic differentiation in hMSC. Kristensen *et al.* (23) identified 466 secreted proteins using SILAC labeling, and STC2 enhances os-

teoblastic differentiation of hMSC, suggesting a role as an autocrine/paracrine factor, in the bone marrow microenvironment.

MSCs from dental tissues shared similar hallmarks of expression and multilineage potential, with MSCs derived from bone marrow (24, 25). In the study by Patil *et al.* (26), the authors compared the expression profile of three dental MSC derived from follicle, pulp and papilla tissue, and identified 19 proteins that were commonly expressed, such as Vimentin, PI4K2 β , GAPDH, TPM1, CALR, MnSOD and TPD54. The results presented that the cellular characteristics and multilineage potential were not sufficient to differentiate the MSCs of follicle, pulp and papilla; but the proteomic analysis, at least in part, indicated interchangeable uses in cell therapy.

Growth factors stimulating osteoblast differentiation analyzed by proteomics approaches

Osteoblast differentiation is regulated by various growth factors, such as bone morphogenetic proteins (BMPs), and Wnt (27, 28). BMPs are growth factors that belong to the transforming growth factor β (TGF β) superfamily, and induce the formation of both cartilage and bone through the Smad pathway (29-31). BMP signals are mediated by type II and type I receptors, and their downstream molecules, regulatory Smads (Smad 1, 5 and 8). Phosphorylated Smad 1, 5 and 8 form a complex with Smad 4, and then are translocated into the nucleus, where they interact with various transcription factors, including Runx2 (32, 33). Runx2 is considered as the principal osteoblast transcription factor, and regulates many genes that determine the osteoblast phenotype (34, 35). This Smad pathway is inhibited by Smad 6 and Smad 7, which block the phosphorylation of R-Smads (15).

Our previous studies showed activated phospho-proteins and protein expressions, in the process of BMP2-induced trans-differentiation of murine C2C12 cells to osteogenic lineage cells, using SILAC-based proteomics analysis (36). Expression profile analyses revealed that the upregulation of Neuronal precursor cell expressed, developmentally downregulated 4 (Nedd4), by BMP2 stimulation. Nedd4 is a 115 kDa E3 protein-ubiquitin ligase (37). The Nedd4 interacted with p-Smad1, and ubiquitinated it to degrade, and thus mitigate the p-Smad1 signaling, which suggests the potential significance of Nedd4 as a modulator, in the activities of the two BMP2 and TGF β 1 pathways. It was previously known that both BMP2 and TGF β 1 achieve overall balanced expression levels of their specific target genes, by concurrently driving their major Smads pathways. But, these proteomics studies revealed that both BMP2 and TGF β 1 can reciprocally activate the non-canonical Smads pathways; in addition to the classical roles of these molecules in linear Smads pathways, BMP2 also activated Smad2/3, and TGF β 1 also activated Smad1/5/8. Interestingly, the study also showed that Nedd4 was activated about one hour after the TGF β 1 stimulation, and thus can eliminate the p-Smad1 signaling, while maintaining TGF β 1's own p-Smad2 signal. Therefore, the proteomics approaches for the BMP2/

TGFβ1 signaling studies could reveal that Nedd4 is an important cellular signal modulator of p-Smad1 activation, in the stimulation of BMP2 or TGFβ1 (Fig. 2) (38).

Secreted Wnts are glycoproteins that play key roles during development and tissue homeostasis. The Wnt/β-catenin pathway is usually indicated as the canonical pathway, leading to GSK3β inhibition, β-catenin stabilization, translocation into the nucleus, and regulation of TCF/LEF transcription activity (39, 40). Day *et al.* (41) established that the canonical Wnt pathway is necessary for bone development. James *et al.* (42) investigated the functions of the Wnt/β-catenin signaling pathway in melanoma. These authors revealed that the protein kinase N1 (PKN1) inhibits Wnt/β-catenin signaling, and sensitizes melanoma cells to cell death, stimulated by WNT3A. Several studies have been published about the cross talk between the effects of BMP and Wnt/β-catenin signals, during osteoblast differentiation. Silverio *et al.* (43) performed stabilization of β-catenin, by WNT3A preventing BMP2-mediated induction of osteoblast differentiation in SVF4 cells. On the other hand, Zhang (44) demonstrate that the Wnt/β-catenin signaling pathway is an upstream activator of BMP2 expression in osteoblasts, offering novel aspects of the nature of functional cross talk, integrating the BMP and Wnt/β-catenin pathways in osteoblastic differentiation and the maintenance of bone homeostasis. Our previous SILAC-based quantitative proteomics study revealed that BMP2 treatment to human bone marrow stem cells (hBMSCs) can upregulate β-catenin, a canonical Wnt effector molecule, and this regulation of BMP2 to β-catenin signaling was through the PI3K pathway; and also revealed that this novel pathway plays a role in BMP2-induced osteoblast differentiation of hBMSCs (27).

Osteoclast function-related proteins identified by proteomics approaches

Osteoclasts, derived from hematopoietic cells of mono-

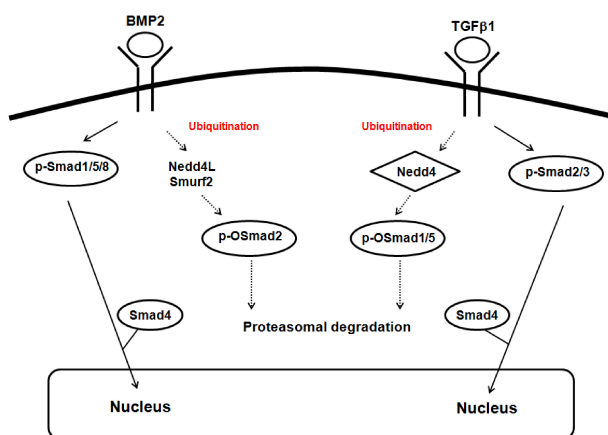


Fig. 2. Illustration of phosphorylated opposite Smads regulation by Nedd4 in BMP2/TGFβ1 signaling pathways.

cyte/macrophage lineage, are multinucleated cells; whereas osteoblasts are like fibroblasts, and are derived from mesenchymal lineage (45-47). RANKL and OPG are both produced by osteoblasts, and have an important role in regulating osteoclast (48, 49). RANKL is a member of the Tumour Necrosis Factor (TNF) superfamily of cytokines, and is produced as a membrane bound protein in osteoblasts (50, 51). OPG is a secreted protein, and disturbs osteoclastogenesis from osteoclast precursors to mature osteoclasts, which can resorb bone (52-54). Thus, the balance between RANKL and OPG decides the formation and activity of osteoclasts.

Kubota *et al.* (55, 56) identified the regulatory factors of osteoclasts involved in the differentiation of osteoblasts. In the studies, 2DE and ICAT-based quantitative proteomics approaches have been used to screen for the secreted proteins, during osteoclast differentiation (57). The authors identified 8 proteins in 2DE, and 40 proteins in ICAT analysis, such as Cathepsins, GILT, HE1, Legumain, MIP-1α, Osteopontin, and other proteins. Among the proteins, the authors showed that the platelet-derived growth factor homodimer BB (PDGF BB), secreted by the osteoclasts stimulated by RANKL, directly inhibited the osteoblastic differentiation. Therefore, this finding by a proteomic approach revealed that PDGF BB is a significant factor in bone homeostasis.

Extracellular matrix (ECM) in osteoblast differentiation

The extracellular compartment is essential for bone, because it determines most of the bone quality properties, such as its strength, stability, and integrity (58, 59). ECM proteins in bone are composed of type I collagen (60, 61), and various non-collagenous proteins, such as osteocalcin, osteonectin, bone sialoprotein, and proteoglycans (62), which interact with their receptors, such as AnxA5 (63), or Integrins present at the matrix vesicles (MV) (64). ECM proteins might also mediate regulate osteoblast differentiation, by interacting with growth factors.

Recently, several proteomics approaches for secretome analysis were performed, to find ECM proteins of mesenchymal stem cells (MSCs), in the course of osteoblast differentiation by a label-free LC-MS/MS quantitative approach. Choi *et al.* (65) detected 64 secreted proteins in the hBMSCs, and demonstrated the role of SPARC-related modulator calcium-binding protein 1 (SMOC1) in osteoblastic differentiation. SMOC1 (66) is a member of the secreted protein and rich in cysteine (SPARC) family that belongs to the matricellular ECM proteins (67, 68). Based on the results, our group demonstrated that SMOC1 is an important modulator in the regulation of osteoblast differentiation, but does not appear to promote mineralization. In a second study (69), a similar approach also revealed that a total 315 proteins were identified, 177 of which increased, and 88 of which decreased, during osteogenesis. Importantly, calcium homeostasis-related proteins were upregulated; whereas, stem cell proliferation-related proteins were downregulated, during osteoblast differentiation. Our recent results in the BMSC secretome analysis also re-

vealed that SERPIN E2 plays an important role in the differentiation of osteoblast from BMSC, especially at an early time, when the two signals of TGF β 1 and BMP2 are changed in their main roles (Park *et al.*, in preparation).

SILAC-based quantitative proteomics disclosed that Activin A treatment resulted in intricate protein composition alterations of ECM, including the changed expression of collagen XII and osteonectin (70). The authors found that the activation of Activin A signaling during bone formation has detrimental effects on the ECM production and maturation phase. Comparative proteome analyses between matrix vesicle (MV) and microvilli individuals have been performed (71). Among the 451 microvillar and 282 MV proteins, 262 were common to both fractions. These studies identified new proteins that may regulate PP_i and P_i homeostasis (inorganic pyrophosphatase 1), Ca²⁺ ion homeostasis (voltage dependent Ca²⁺ channel and sorcin), intracellular pH (vacuolar H⁺-ATPase and SLC4A7), or lipid composition of MV membrane (sphingomyelin phosphodiesterase 3).

PROTEOMIC APPROACHES TO DISCOVER THERAPEUTIC TARGETS IN BONE DISEASE

Comparative proteomic analysis, to discover potential therapeutic targets in bone disease, was published (72). The protein profiles of isolated proteins from ulna midshafts and fatigue loaded rats were compared, using LC-MS/MS. The authors found that a combination of decreased anti-apoptotic factor Raf1, and increased pro-apoptotic factor PDCD8, resulted in numerous increases in the number of apoptotic osteocytes, following fatigue loading. Therefore, it was suggested that development of small molecule activators of Raf1 and PDCD8 may find broad therapeutic applications. In another study by Kim *et al.* (73), the authors showed the correlation of *salvia miltiorrhiza* (SM), a well-known traditional Chinese herbal medicine, and its components, such as tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone, with osteoporosis and osteoclast function. SM is commonly used to treat various cardiovascular diseases (74). The authors suggested tanshinone can be a good marker compound to explain the antiosteoporotic function of SM.

CONCLUSION

The progression of proteomics, remarkably ameliorated sample preparation and enrichment techniques, has allowed the more inclusive characterization of the proteins. The proteins identified, and their crosstalk, appears to confer the altered status of the osteoblast differentiation. These proteomics approaches have provided us with various bone metabolism related signaling details, secreted activating proteins, potential therapeutic targets etc., which can be used as new diagnostic markers, and to search for potential drug targets of bone diseases, including osteoarthritis, bone metabolic dysfunctions

and neoplasias. Because the primary cells at the stage of differentiation, including osteoblasts, osteocyte, chondrocyte and osteoclasts from bone tissues, have high heterogeneity, and there is still limitation for the LC-MS/MS technology to detect the low abundant proteins, current elucidation of complex mechanisms during skeletogenesis *in vivo* by proteomics still has great limitations. If these several limitations are overcome by more sensitive and finer proteomics technology development, proteomics may provide better understanding of the bone development-related signal networks, and develop better biomarkers and therapeutic targets for bone diseases.

ACKNOWLEDGEMENTS

This work was supported by the Bio & Medical Technology Development Program, of the National Research Foundation (NRF), funded by the Ministry of Science, ICT & Future Planning (No. 2012M3A9C6049716 & No. 20110019355).

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