PTH, Calcium, & Phosphate Goals in CKD?

Balancing Arteriosclerosis, Renal Function, & Bone Integrity

Part 1: Pathophysiology

Disclosures: none

Objectives: Introduction to arterial osteogenesis

1. To discuss the mechanisms for osteogenesis, bone maintenance, & calcium-phosphate homeostasis
2. To identify the hormonal, cellular, & mineral modulators of arterial calcification
3. To propose possible therapies to reduce CV events & renal dysfunction without adversely affecting bone

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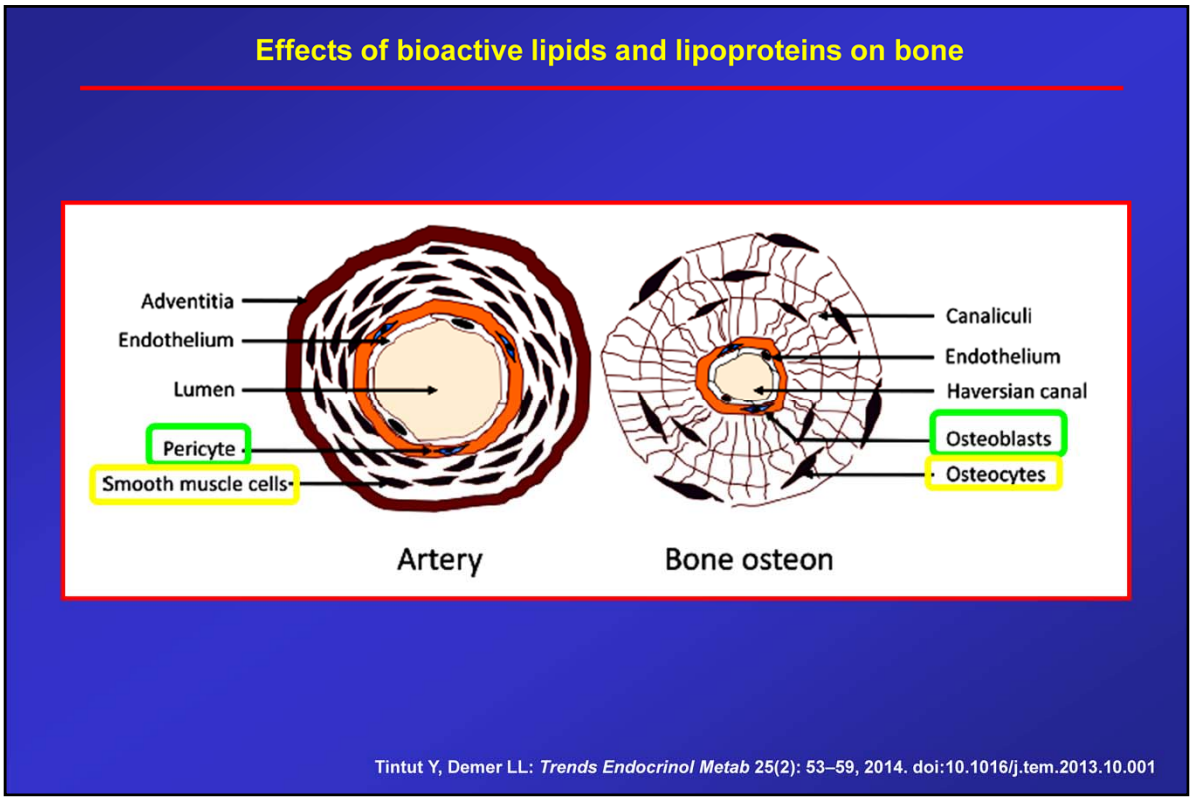


Figure 1. Schematic comparing the anatomic structure of an artery and bone osteon

Both the artery and osteon are centered on a blood lumen, which is surrounded by a single layer of endothelium. This, in turn, is surrounded by a basement membrane housing immature mesenchymal cells. In arteries, the immature cells are pericytes and/or smooth muscle cells, whereas, in bone, they are pericytes and/or preosteoblasts.

(Modified from Parhami et al., Arteriosclerosis, Thrombosis, and Vascular Biology, 1997; 17:680–687).

Arteriosclerosis & Bone Physiology

Arteriosclerosis: arterial mechanical stiffening from any cause

1. Atherosclerosis: Eccentric intimal–medial atherosclerotic plaques with calcification, fibrosis, and cholesterol-laden lipoprotein deposition
2. Concentric medial & adventitial fibrosis with medial calcification, elastinolysis, & mural thickening (DM & CKD)

Bone Physiology: development and maintenance of bone

1. Osteogenesis → *de-novo* development of bone, both normal & pathological (arteries)

   Osteoblasts & BMP-2 (bone morphogenetic protein)

2. Bone Maintenance & Integrity → repairs damage & responds to changing forces

   Osteocytes & PTHrp

3. Calcium & Phosphate Homeostasis → osteocytes, osteoclasts, osteoblasts, & circulating PTH

   Sacrifices bone for serum calcium

Arteriosclerosis & Bone Physiology

Cellular Growth:

Proliferation

Migration

Invasion

Survival (Anti-apoptosis)

Necrosis

Cellular Differentiated Function:

Differentiation → stop dividing

Adhesion → maintain location

Perform Secretory or Structural function

Pro-apoptosis → remove damaged or unneeded cells

Leptin

WNT (promoters: PTH / PTHrp)

TGF-β (promoter: Leptin)

BMP-2 (promoters: PTH, WNT)

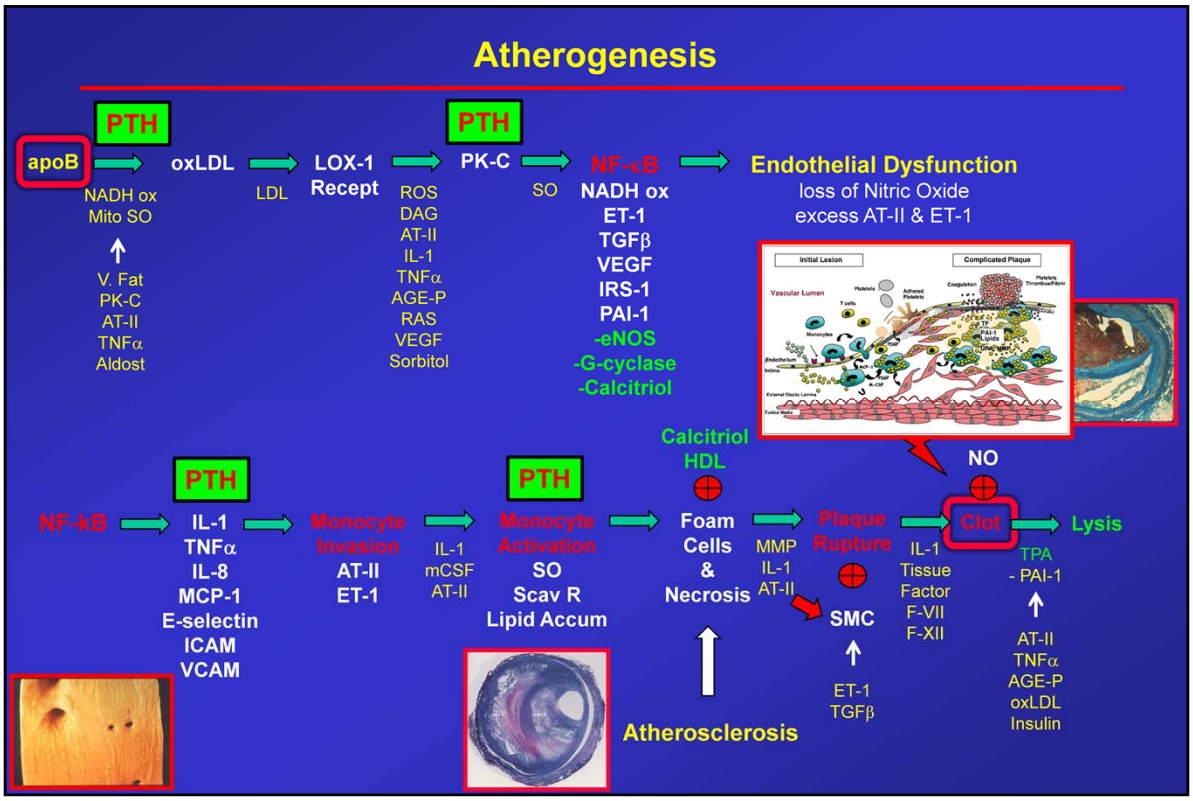
NF-κB (promoters: TAK-1, cytokines)

Adiponectin

PPARγ

Vitamin D (inhibits NF-kB, NFAT, PTH)

Shift to Growth → Arteriosclerosis, Nephropathy, Osteoporosis, Hyper-cellularity, Fibrosis, Cancer, etc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VEGF
Sorbitol
PK-C
SO
NF-κB
NADH ox
ET-1
TGFβ
VEGF
IRS-1
PAI-1
-eNOS
-G-cyclase
-Calcitriol
Endothelial Dysfunction
loss of Nitric Oxide
excess AT-II & ET-1
Initial Lesion
Complicated Plaque
Vascular Lumen
Platelets
T cells
Monocytes
Endothelium
Lesion
Adhered Platelets
Coagulation
Platelets Thrombus/Fibrin
PAI-1
Lipids
Smooth Muscle
Calcitriol
HDL
NO
NF-kB
PTH
IL-1
TNFα
IL-8
MCP-1
E-selectin
ICAM
VCAM
Monocyte Invasion
AT-II
ET-1
IL-1
mCSF
AT-II
PTH
Monocyte Activation
SO
Scav R
Lipid Accum
Foam Cells & Necrosis
MMP
IL-1
AT-II
Plaque Rupture
SMC
ET-1
TGFβ
IL-1
Tissue Factor
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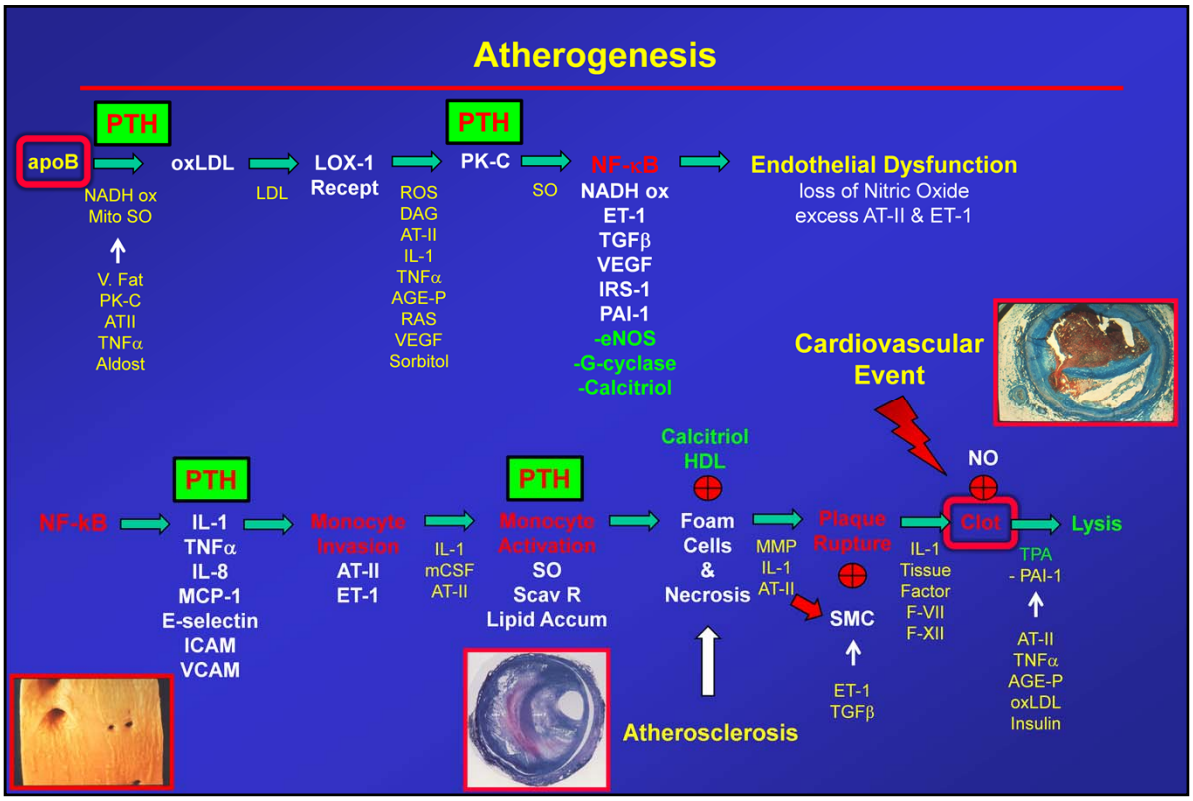
oxLDL
Insulin
Atherosclerosis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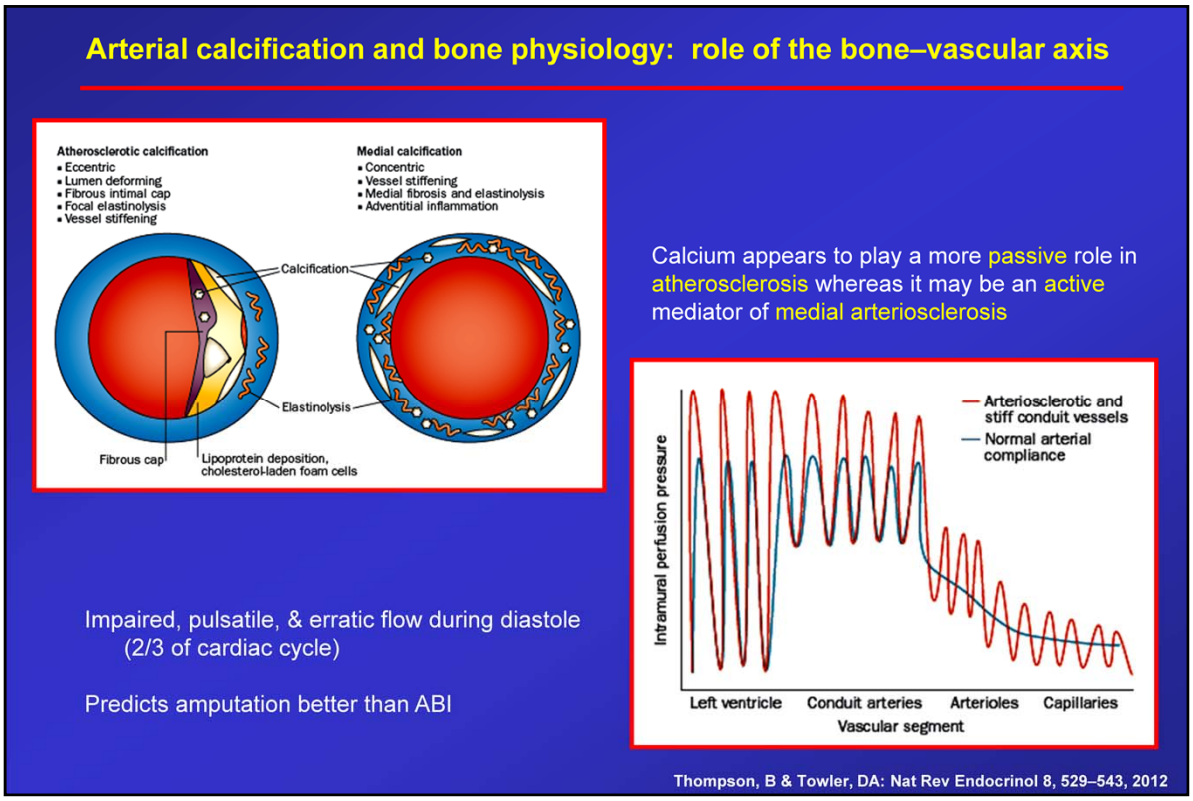
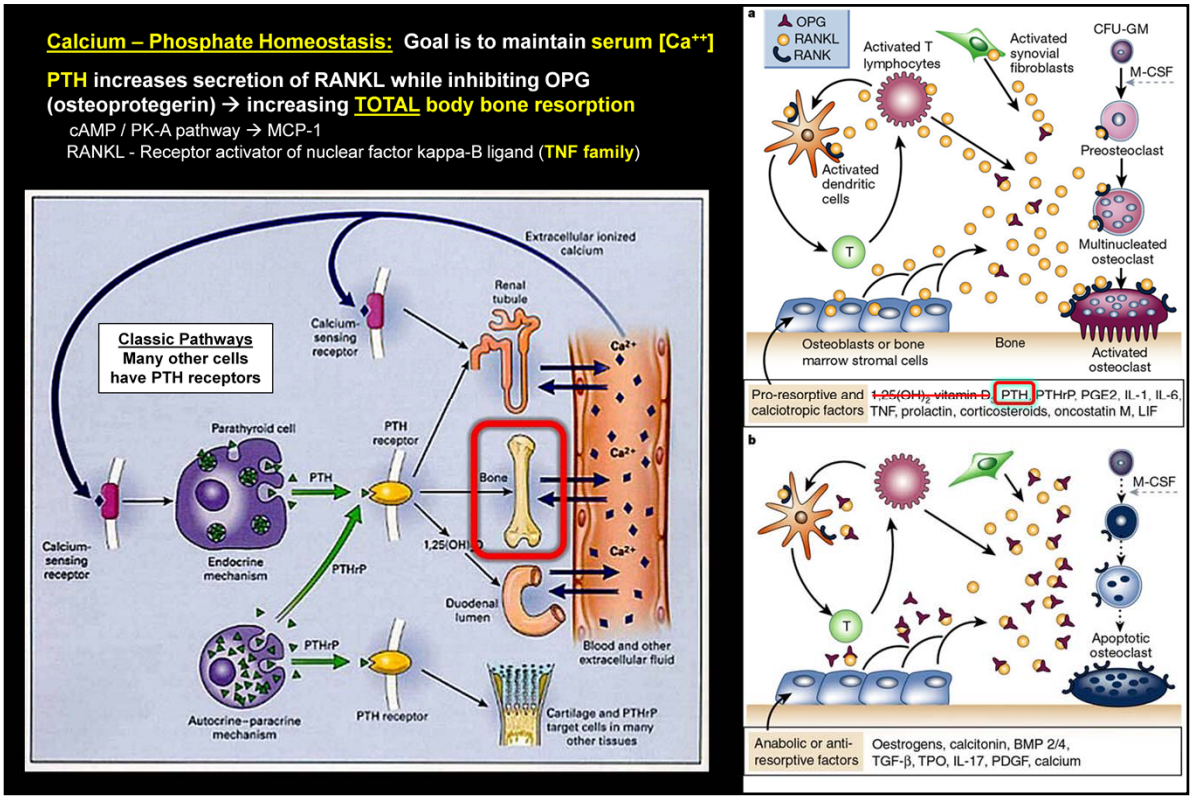


Figure 1 | Consequences of arterial stiffening and impaired Windkessel physiology. During systole, some kinetic energy is stored as potential energy in the elastic conduit arteries. This stored energy permits not only coronary perfusion but also smooth distal capillary perfusion during diastole (blue tracing). With arteriosclerotic stiffening (red tracing), less potential energy is stored during systole, giving rise to impaired, pulsatile and erratic flow during diastole (two-thirds of the cardiac cycle). Systolic blood pressure is also increased.



Osteoclasts are detrimental to bone integrity in this setting but osteoblasts control this osteoclastic activity so they are actually the enablers of this injurious action.

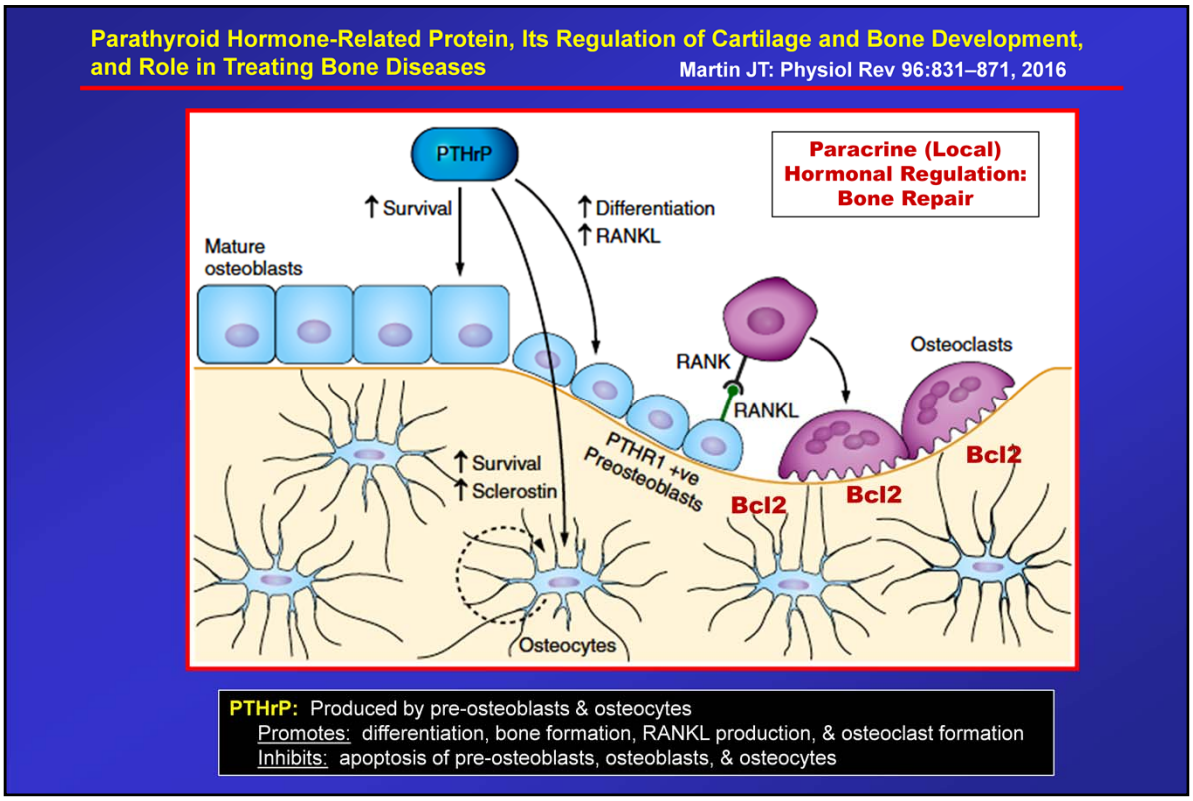


FIGURE 12. Paracrine actions of PTHrP in bone remodeling. PTHrP produced by cells early in the osteoblast lineage acts on cells of the lineage that have differentiated to the stage of possessing the PTH1R, promoting their differentiation and therefore bone formation, as well as increasing production of RANKL and osteoclast formation. PTHrP also inhibits apoptosis of mature osteoblasts, of earlier cells, and of osteocytes.

Osteoclasts are integral components of the bone repair process so their actions in this setting are beneficial to bone integrity. Again, osteoblasts and osteocytes control the osteoclastic activity.

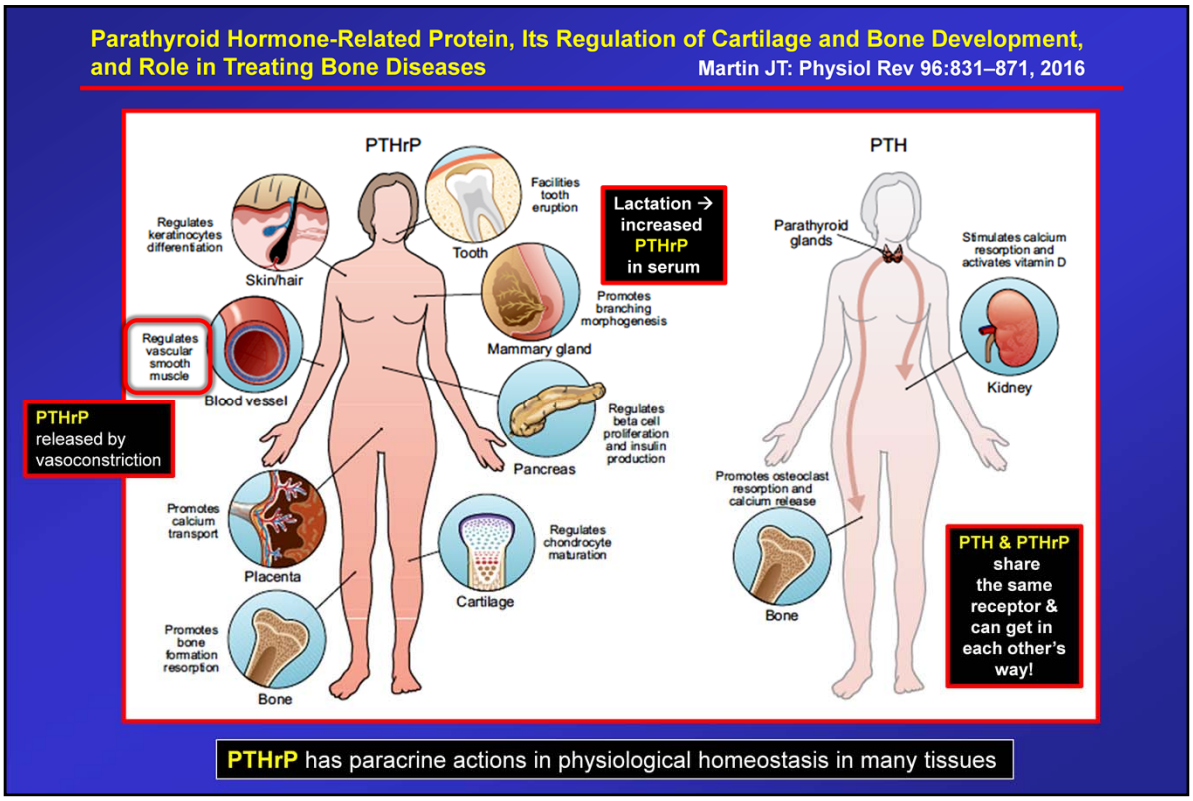


FIGURE 8. Paracrine actions of PTHrP and endocrine actions of PTH. PTHrP has paracrine actions in physiological homeostasis in many tissues, including keratinocytes/hair follicles, cartilage, vascular smooth muscle, bone, mammary gland development, tooth eruption, and pancreas, whereas PTH has relatively fewer physiological actions through its role as a circulating hormone. The summary diagram omits important details such as the role of PTHrP in lactation

Vitamin D: Systemic & Paracrine Effects on Bone & Vessels

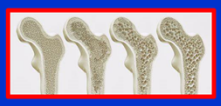
Nagpal et al: Endocrine Reviews 26:662-687, 2005

1,25-$(OH)_2$D3 binds to its receptor (VDR) dimerized to RXR **(3% of all genes)**

Co-activators: SRC, DRIP205, CBP/p300, TAFII

Most cells (not small intestine) have 1a-hydoxylase → not dependent on circulating calcitriol

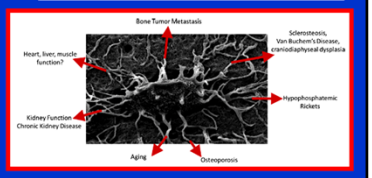
Some genes with Vitamin D response elements: (**Empty** VDR is a repressor on some genes)



Activates: (+ response element): *May be activated by 25-Vitamin D in MSCs

Bone Remodeling:	Osteocalcin*, RANKL (weak), TNAP*, NPP1 (+PPi)
Ca Binding:	**Trpv6**, Atp2b1, Calbindin-9k (S100g) (Intestinal Ca^{++} transport)
Metabolism:	Cyp3A4, Cyp3A1, Cyp3A11, 24-Hydroxylase → reduces calcitriol
Adhesion:	β-3 integrin (FGF-23 inhibitor?)
Anti-proliferation:	p21, IGFBP-3
Differentiation:	Involucrin, Phospholipase C gamma1

TNAP: Tissue Non-specific Alk Phos
NPP1: ATP → AMP + PPi



Inhibits: Probably the dominate Bone effect

NFAT, NF-kB (VDR competes with co-activators) →

Anti-inflammatory:	IL-2, IL-12, TNFa, IFNg, GM-CSF
Anti-proliferative:	EGF-R, c-myc, K16

Negative Response elements: PTH, PTHrP, Rel B (transcription activator)

NFAT: Nuclear Factor of activated T-Cells

S.M. Lee, J.W. Pike / Journal of Steroid Biochemistry & Molecular Biology 164 (2016) 265–270

The vitamin D receptor functions as a transcription regulator in the absence of 1,25-dihydroxyvitamin D3

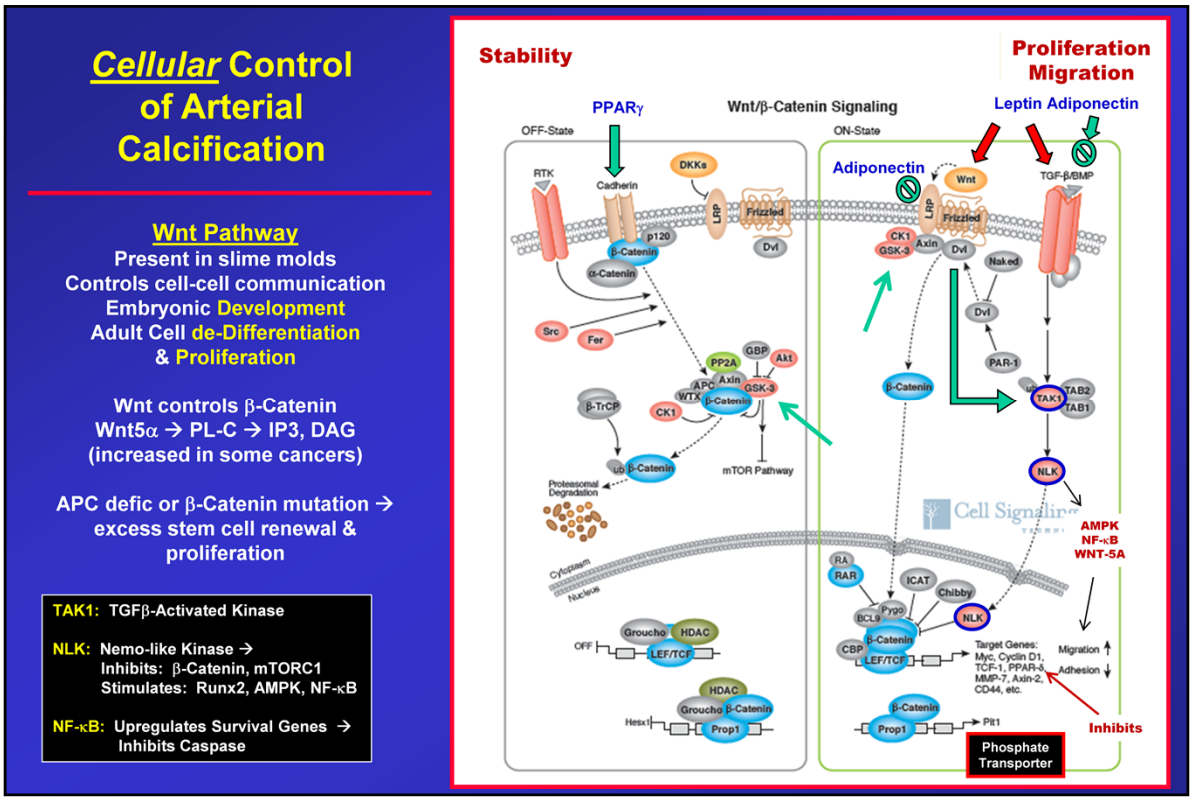
25-Hydroxyvitamin D3 induces osteogenic differentiation of human mesenchymal stem cells; Yan-Ru Lou, Tai Chong Toh, Yee Han Tee, & Hanry Yu

Scientific Reports | 7:42816 | DOI: 10.1038/srep42816 2017

Wikipedia: Nuclear factor of activated T-cells (**NFAT**) is a general name applied to a family of transcription factors shown to be important in immune response. One or more members of the NFAT family is expressed in most cells of the immune system. NFAT is also involved in the development of cardiac, skeletal muscle, and nervous systems. The NFAT transcription factor family consists of five members NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5. NFATc1 through NFATc4 are regulated by calcium signaling. Calcium signaling is critical to NFAT activation because calmodulin (CaM), a well-known calcium sensor protein, activates the serine/threonine phosphatase Calcineurin (CN). Activated CN rapidly dephosphorylates the serine-rich region (SRR) and SP-repeats in the amino termini of NFAT proteins, resulting in a conformational change that exposes a nuclear localization signal, resulting in NFAT nuclear import. Nuclear import of NFAT

proteins is opposed by maintenance kinases in the cytoplasm and export kinases in the nucleus. Export kinases, such as **PKA and GSK-3β, must be inactivated for NFAT nuclear retention**.

NFAT proteins have weak DNA-binding capacity. Therefore, to effectively bind DNA, NFAT proteins must cooperate with other nuclear resident transcription factors generically referred to as NFATn. This important feature of NFAT transcription factors enables integration and coincidence detection of calcium signals with other signaling pathways such as ras-MAPK or PKC. In addition, this signaling integration is involved in tissue-specific gene expression during development. A screen of ncRNA sequences identified in EST sequencing projects discovered a 'ncRNA repressor of the nuclear factor of activated T cells' called NRON.



TAK1: A Molecular Link Between Liver Inflammation, Fibrosis, Steatosis, and Carcinogenesis

Front. Cell Dev. Biol., 14 October 2021

Chronic insult and persistent injury can cause liver inflammation, fibrosis, and carcinogenesis; it can also be associated with metabolic disorders. Identification of critical molecules that link the process of inflammation and carcinogenesis will provide prospective therapeutic targets for liver diseases. Rapid advancements in gene engineering technology have allowed the elucidation of the underlying mechanism of transformation, from inflammation and metabolic disorders to carcinogenesis. Transforming growth factor-β-activated kinase 1 (TAK1) is an upstream intracellular protein kinase of nuclear factor kappa-B (NF-κB) and c-Jun N-terminal kinases, which are activated by numerous cytokines, growth factors, and microbial products. In this study, we highlighted the functional roles of TAK1 and its interaction with transforming growth factor-β, WNT, AMP-activated protein kinase, and NF-κB signaling pathways in liver inflammation, steatosis, fibrosis, and carcinogenesis based on previously published articles.

Adiponectin in renal fibrosis

AGING 12(5):4660-4672, 2020

Renal fibrosis is an inevitable consequence of parenchymal scarring and is the common final pathway that mediates almost all progressive renal diseases.

Adiponectin, a hormone produced by adipose tissue, possesses potent anti-insulin, anti-inflammatory, and anti-fibrotic properties. Reportedly, adiponectin serves as an important messenger that facilitates complex interactions between adipose tissue and other metabolically related organs. In recent years, a growing body of evidence supports adiponectin involvement in renal fibrosis. These studies provide a deeper understanding of the molecular mechanism of action of adiponectin in renal fibrosis and also offer a potential preventive and therapeutic target for renal fibrosis. In this review, the physiological role of adiponectin is briefly introduced, and then the mechanism of adiponectin-mediated renal fibrosis and the related signaling pathways are described. Finally, we summarize the findings regarding the clinical value of adiponectin in renal fibrotic diseases and prospected its application potential.

Adiponectin inhibits vascular smooth muscle cell calcification induced by beta-glycerophosphate through JAK2/STAT3 signaling pathway

J Biosci 44:86,1-19, 2019

Vascular calcification is a common problem in the elderly with diabetes, heart failure and end-stage renal disease. The differentiation of vascular smooth muscle cells (VSMCs) into osteoblasts is the main feature, but the exact mechanism remains unclear. It is not clear whether adiponectin (APN) affects osteogenic differentiation of VSMCs. This study aims to explore the effect of APN on vascular calcification by using a cell model induced by beta-glycerophosphate (b-GP). VSMCs were isolated and treated with b-GP and APN in this study. The alkaline phosphatase (ALP) activity and expression levels of Runx2, BMP-2, collagen type I and osteocalcin were determined. The expression levels of STAT3 and p-STAT3 in nucleus and cytoplasm of VSMCs were analyzed. The results showed that APN significantly inhibited the expression of ALP, Runx2, BMP-2, collagen I, osteocalcin and the formation of the mineralized matrix in VSMCs induced by b-GP. APN reduces the osteogenic differentiation of VSMCs induced by b-GP and down-regulates the expression of the osteogenic transcription factor osterix by inhibiting STATS3 phosphorylation and nuclear transport. APN may be one of the potential candidates for clinical treatment of vascular calcification.

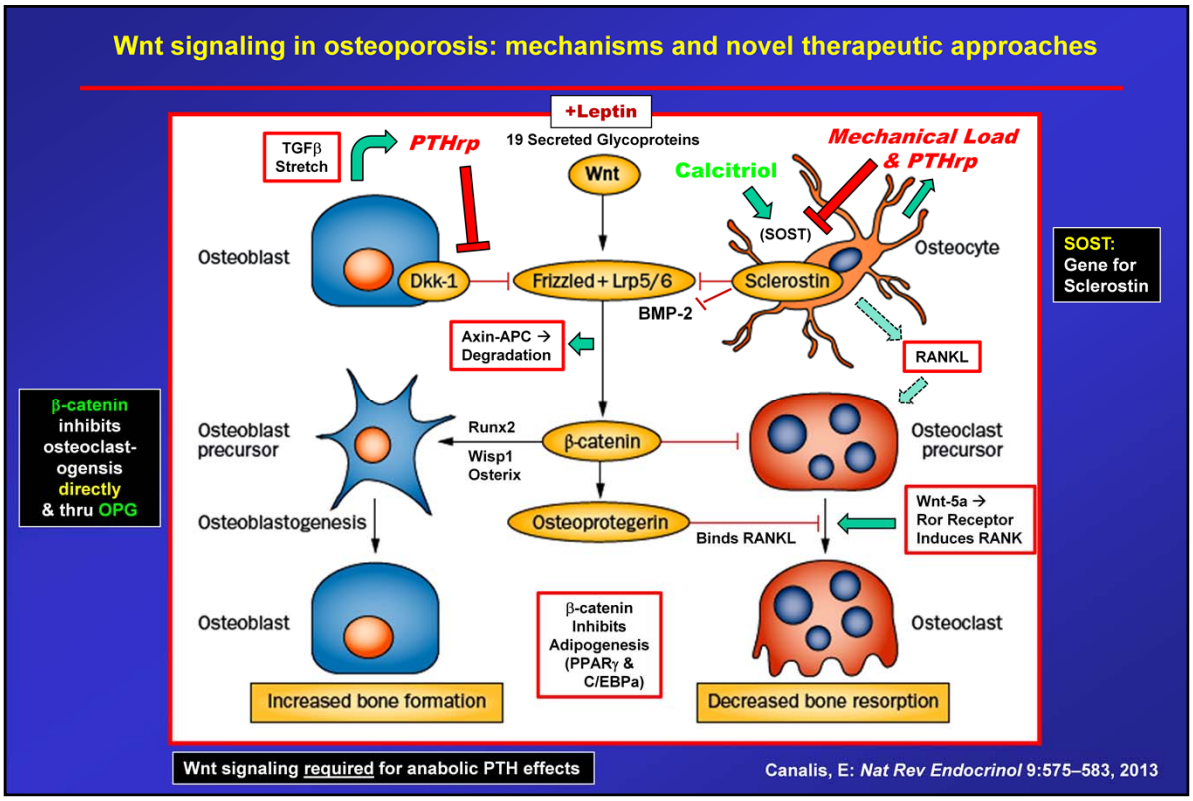


Figure 1 | Canonical Wnt signalling and bone remodelling. Wnt induces osteoblastogenesis and thereby enhances bone formation. Canonical Wnt signalling suppresses osteoclastogenesis by inducing osteoprotegerin. In addition, Wnt signalling suppresses bone resorption by an osteoprotegerin-independent mechanism acting directly on osteoclast precursors. The dual effect of Wnt on cells of the osteoblast and osteoclast lineage results in an increase in bone mass. Sclerostin and Dkk-1 bind to Wnt co-receptors and thereby prevent Wnt-receptor interaction and signalling.

Abbreviations: Dkk-1, Dickkopf-related protein 1; LRP-5, LDL receptor related protein co-receptor 5; LRP-6, LDL receptor related protein co-receptor 6. Ror: receptor tyrosine kinase-like orphan receptor

Osteoporosis is a skeletal disorder characterized by bone loss, which results in architectural deterioration of the skeleton, compromised bone strength and an increased risk of fragility fractures. Most current therapies for osteoporosis stabilize the skeleton by inhibiting bone resorption (antiresorptive agents), but the development of anabolic therapies that can increase bone formation and bone mass is of great interest. Wnt signalling induces differentiation of bone-forming cells (osteoblasts) and suppresses the development of bone-resorbing cells (osteoclasts). The Wnt pathway is controlled by antagonists that interact either directly with Wnt

proteins or with Wnt co-receptors. The importance of Wnt signalling in bone formation is indicated by skeletal disorders such as sclerosteosis and van Buchem syndrome, which are caused by mutations in the gene encoding the Wnt antagonist sclerostin (SOST). Experiments in mice have shown that downregulation or neutralization of Wnt antagonists enhances bone formation. Phase II clinical trials show that 1-year treatment with antisclerostin antibodies increases bone formation, decreases bone resorption and leads to a substantial increase in BMD. Consequently, Wnt signalling can be targeted by the neutralization of its extracellular antagonists to obtain a skeletal anabolic response.

PTHrp is made by all SMCs in response to vasoconstriction (stretch) & causes relaxation → anti-AT-II

PTHrp is made by osteoblasts in response to TGFβ, EGF, calcitriol, cortisol, & stretch

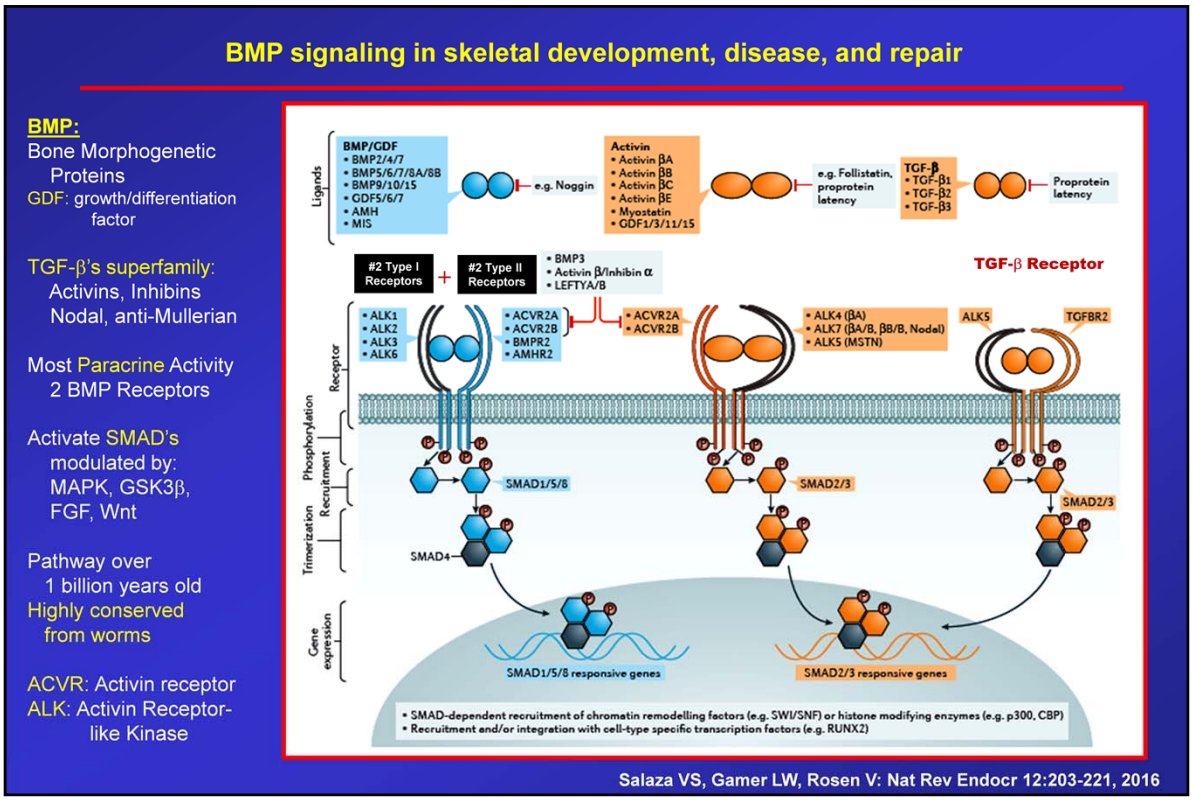


Figure 2 | **Fundamental mechanisms of canonical BMP superfamily signalling.** Over 30 bone morphogenetic protein (BMP) superfamily ligands have been discovered in humans. Most are secreted as mature disulfide-linked dimers, with the exception of TGF-β1, TGF-β2 and TGF-β3, which can be secreted in a latent form and require proteolytic activation. BMPs signal through a multimeric cell surface complex consisting of two type I receptors and two type II receptors. Type I and type II BMP receptors are single pass transmembrane proteins with an intracellular serine/ threonine kinase domain. After ligand binding, type II receptors phosphorylate (P) the type I receptors. Activated type I receptors recruit and phosphorylate pathway-specific R-SMADs (SMAD1, SMAD5 and SMAD8 (blue pathway), and SMAD2 and SMAD3 (orange pathway)), which can form trimers with SMAD4 and translocate to the nucleus. SMADs have intrinsic DNA-binding activity and are able to regulate gene expression by recruitment of chromatin-remodelling machinery and integration with tissue-specific transcription factors. SMAD8 is also known as SMAD9. The pathway can be antagonized by many mechanisms including neutralization of ligands by secreted traps such as noggin or follistatin, secretion of latent ligands bound to their propeptides, or via titration of receptors by non-signalling ligands such as BMP3, activin β/inhibin α dimers or LEFTY monomers.

ACVR, activin receptor; ALK, activin receptor-like kinase; AMH, anti-Müllerian hormone; AMHR2, AMH receptor 2; BMPR, BMP receptor; GDF,

growth/differentiation factor; TGF, transforming growth factor; TGFBR, TGF-β receptor.

Since the identification in 1988 of bone morphogenetic protein 2 (BMP2) as a potent inducer of bone and cartilage formation, BMP superfamily signalling has become one of the most heavily investigated topics in vertebrate skeletal biology. Whereas a large part of this research has focused on the roles of BMP2, BMP4 and BMP7 in the formation and repair of endochondral bone, a large number of BMP superfamily molecules have now been implicated in almost all aspects of bone, cartilage and joint biology. As modulating BMP signalling is currently a major therapeutic target, our rapidly expanding knowledge of how BMP superfamily signalling affects most tissue types of the skeletal system creates enormous potential to translate basic research findings into successful clinical therapies that improve bone mass or quality, ameliorate diseases of skeletal overgrowth, and repair damage to bone and joints. This Review examines the genetic evidence implicating BMP superfamily signalling in vertebrate bone and joint development, discusses a selection of human skeletal disorders associated with altered BMP signalling and summarizes the status of modulating the BMP pathway as a therapeutic target for skeletal trauma and disease.

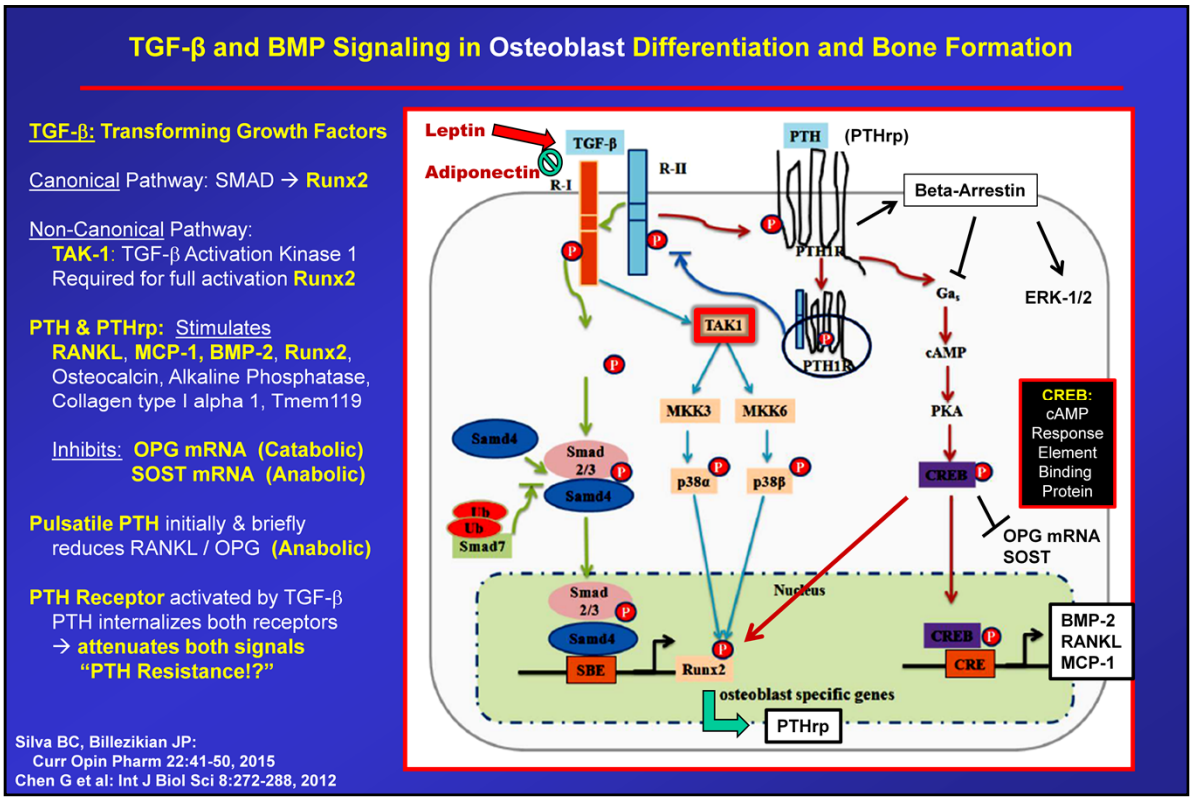


Figure 1. **TGF-β signaling and negative regulation in bone formation.**

Canonical Smad-dependent TGF-β signaling first binds to receptor type II (R-II) and receptor type I (R-I), and then signaling transduces to their Smads. Activated Smads form a complex with Smad4 and then translocate into the nucleus where they interact with other transcription factors to trigger target gene expression. Smad7 disrupts the activated Smad2/3 to form a complex with Smad4. The non-Smad-dependent TAK1 signaling pathway also regulates bone formation. PTH binding activates PTH1R to stimulate several downstream effectors. PTH binding also drives internalization of PTH1R-TGFβRII complex, which attenuates both TGF-β and PTH signaling on bone development. Transcriptional factor cAMP response element binding protein (CREB) mediates PTH signaling in osteoblasts.

Parathyroid hormone: anabolic and catabolic actions on the skeleton.

Parathyroid hormone (PTH) is essential for the maintenance of calcium homeostasis through, in part, its actions to regulate bone remodeling. While PTH stimulates both bone formation and bone resorption, the duration and periodicity of exposure to PTH governs the net effect on bone mass, that is whether it is catabolic or anabolic. PTH receptor signaling in osteoblasts and osteocytes can increase the RANKL/OPG ratio, increasing both osteoclast recruitment and osteoclast activity, and thereby stimulating bone resorption. In contrast, PTH-induced bone formation is explained, at least in part, by its ability to downregulate SOST/sclerostin

expression in osteocytes, permitting the anabolic Wnt signaling pathway to proceed. The two modes of administration of PTH, that is, continuous vs. intermittent, can regulate, in bone cells, different sets of genes; alternatively, the same sets of genes exposed to PTH in sustained vs. transient way, will favor bone resorption or bone formation, respectively. This article reviews the effects of PTH on bone cells that lead to these dual catabolic and anabolic actions on the skeleton.

TGF-β and BMP signaling in osteoblast differentiation and bone formation.

Transforming growth factor-beta (TGF-β)/bone morphogenic protein (BMP) signaling is involved in a vast majority of cellular processes and is fundamentally important throughout life. TGF-β/BMPs have widely recognized roles in bone formation during mammalian development and exhibit versatile regulatory functions in the body. Signaling transduction by TGF-β/BMPs is specifically through both canonical Smad-dependent pathways (TGF-β/BMP ligands, receptors and Smads) and non-canonical Smad-independent signaling pathway (e.g. p38 mitogen-activated protein kinase pathway, MAPK). Following TGF-β/BMP induction, both the Smad and p38 MAPK pathways converge at the Runx2 gene to control **mesenchymal precursor cell differentiation**. The coordinated activity of Runx2 and TGF-β/BMP-activated Smads is critical for formation of the skeleton. Recent advances in molecular and genetic studies using gene targeting in mice enable a better understanding of TGF-β/BMP signaling in bone and in the signaling networks underlying osteoblast differentiation and bone formation. This review summarizes the recent advances in our understanding of TGF-β/BMP signaling in bone from studies of genetic mouse models and human diseases caused by the disruption of TGF-β/BMP signaling. This review also highlights the different modes of cross-talk between TGF-β/BMP signaling and the signaling pathways of MAPK, Wnt, Hedgehog, Notch, and FGF in osteoblast differentiation and bone formation.

P: phosphorylation; Ub: ubiquitination.

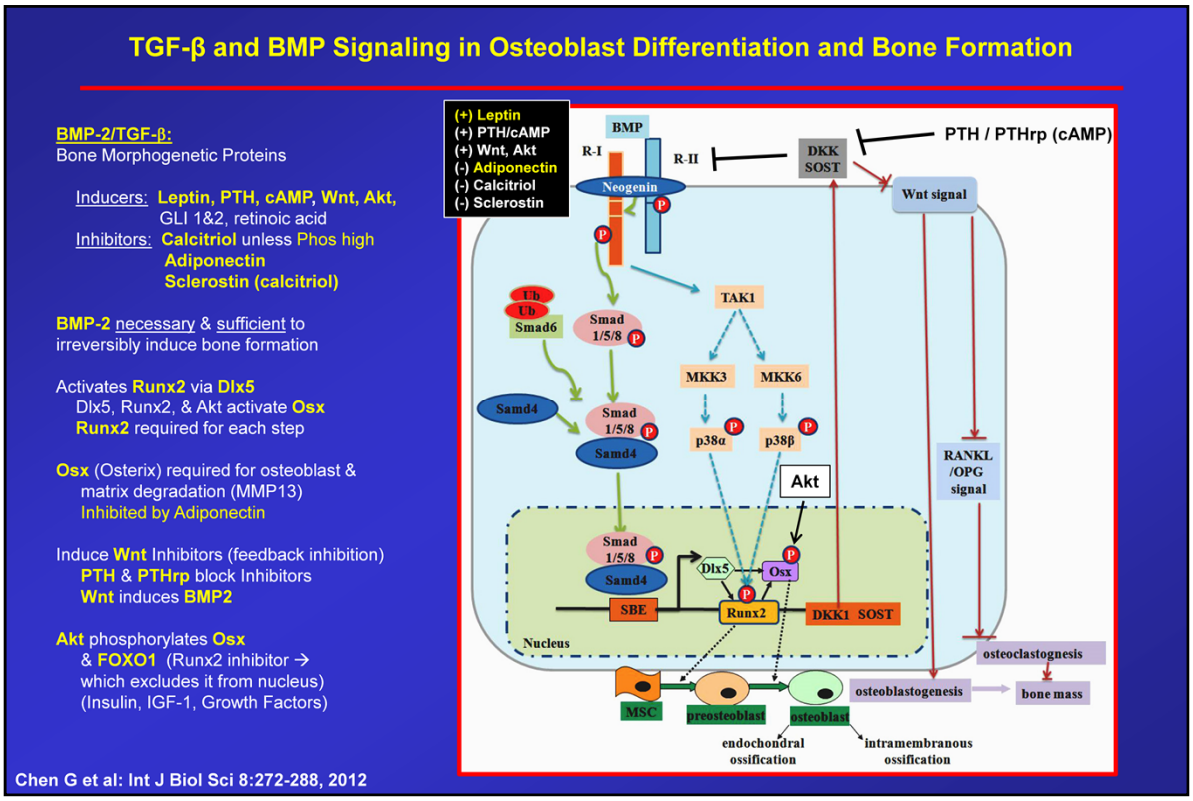
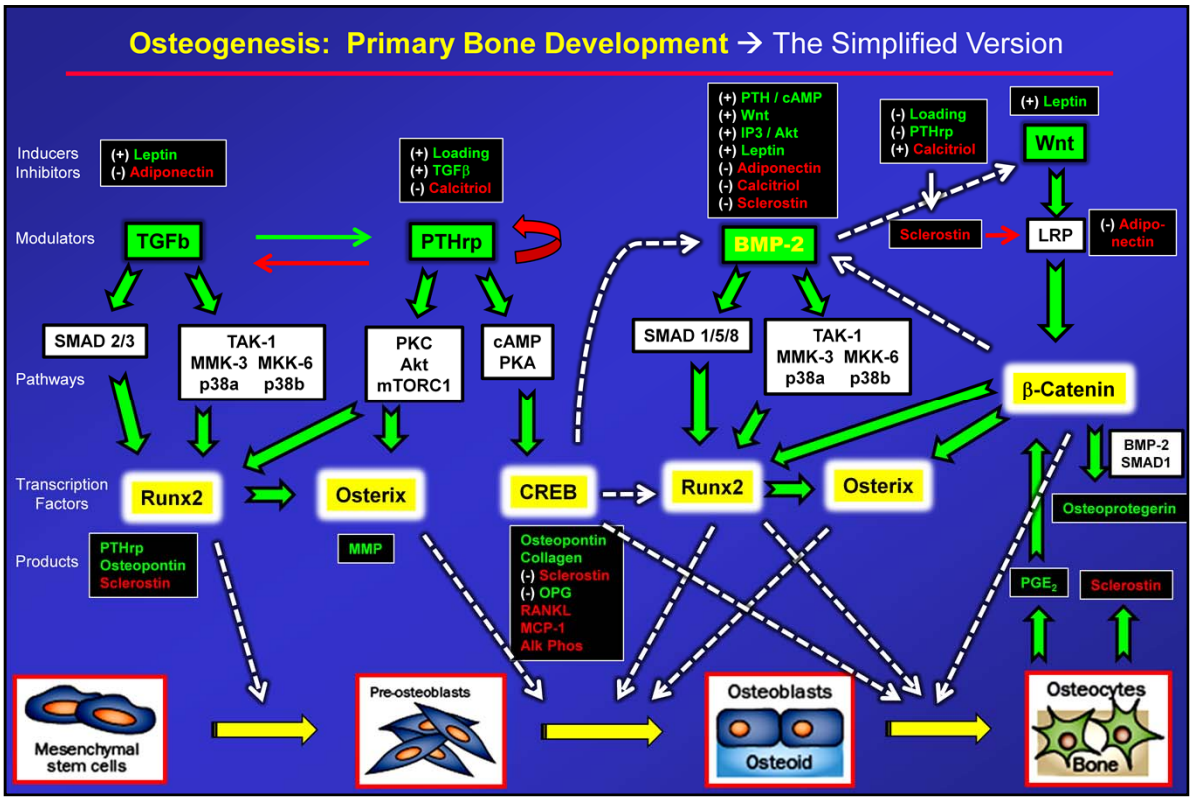
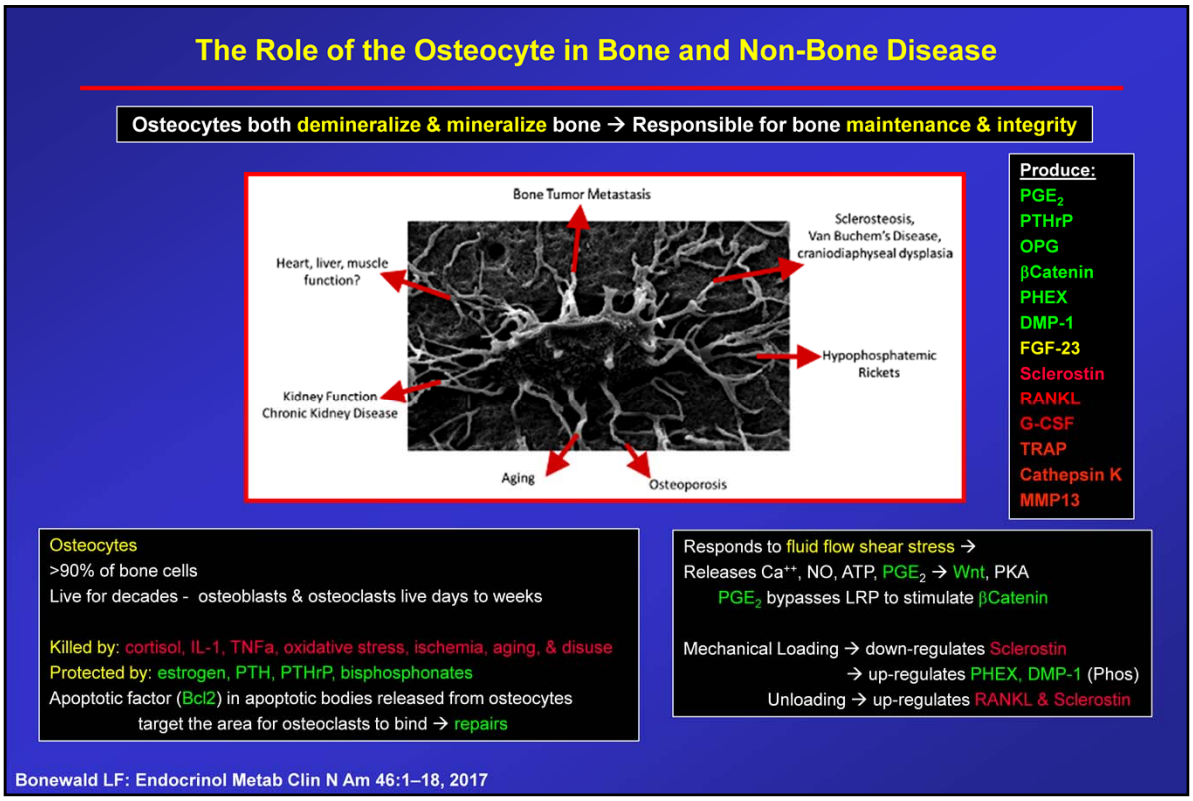


Figure 2. **BMP signaling and negative regulation in bone formation.**

Smad-dependent-BMP signaling binds to receptor type II (R-II) and receptor type I (R-I) and then the signaling transduces to their Smads. Activated Smads form a complex with Smad4 and then translocate into the nucleus where they interact with other transcription factors to trigger target gene expression. Neogenin regulates BMP receptor association and Smad1/5/8 signaling. Activated Smads regulate expression of transcriptional factors and transcriptional coactivators important in osteoblasts (Dlx5, Runx2 and Osx). Smad6 binds type I BMP receptor and prevents Smad1/5/8 to be activated. Non-Smad-dependent TAK1 signaling pathway also regulates bone formation. The interplay between BMPs and Wnt signaling affects bone formation (99). BMPR1a signaling upregulates Sost expression primarily through Smad-dependent signaling, while it upregulates DKK1 through Smad-dependent and non-Smad-dependent signaling. Both Sost and DKK1 inhibit canonical Wnt signaling, leading to a decrease in bone mass. P: phosphorylation; Ub: ubiquitination.


Osteogenesis: Primary Bone Development → The Simplified Version
Inducers Inhibitors
(+) Leptin
(-) Adiponectin
(+) Loading
(+) TGFβ
(-) Calcitriol
(+) PTH / cAMP
(+) Wnt
(+) IP3 / Akt
(+) Leptin
(-) Adiponectin
(-) Calcitriol
(-) Sclerostin
(-) Loading
(-) PTHrp
(+) Calcitriol
(+) Leptin
Wnt
Modulators
TGFb
PTHrp
BMP-2
Sclerostin
LRP
(-) Adipo-nectin
Pathways
SMAD 2/3
TAK-1 MMK-3 MKK-6 p38a p38b
PKC Akt mTORC1
cAMP PKA
SMAD 1/5/8
TAK-1 MMK-3 MKK-6 p38a p38b
β-Catenin
Transcription Factors
Runx2
Osterix
CREB
Runx2
Osterix
BMP-2 SMAD1
Osteoprotegerin
Products
PTHrp Osteopontin Sclerostin
MMP
Osteopontin Collagen
(-) Sclerostin
(-) OPG
RANKL
MCP-1
Alk Phos
PGE2
Sclerostin
Mesenchymal stem cells
Pre-osteoblasts
Osteoblasts
Osteoid
Osteocytes
Bone



Fluid flow shear stress activates the Wnt/β-catenin signaling pathway through the rapid release of prostaglandin, which acts through EP receptors to bypass LDL receptor-related protein activation. Components of the β-catenin pathway are essential for osteocyte viability, mechano-sensation, transduction, and release of important factors essential for bone homeostasis. The central molecule through which all molecules must go is b-catenin. β-Catenin regulates expression of both the positive activators of this pathway, the Wnts, and the negative regulators of this pathway, sclerostin and Dkk1 (for a review see 16). Global deletion of β-catenin is embryonically lethal, but deletion in osteocytes using the Dmp1-Cre results in dramatic bone loss characterized by perforated cortises. Interestingly, deletion of only 1 allele in osteocytes results in mice with a normal skeleton but a completely abrogated response to anabolic loading. β-Catenin plays an important role in bone integrity, osteocyte communication, and osteocyte viability, but also in bone response to loading. This role extends to other components of this signaling pathway.

Before osteocytes were recognized as active essential bone cells necessary for bone health, it was assumed that all the action took place on the bone surface and not within the bone. Osteoblasts and osteoclasts were the major players, osteoblasts making bone and osteoclasts resorbing bone to maintain bone homeostasis. It was assumed that osteoblasts and osteoclasts were regulated by external factors such as parathyroid hormone (PTH) or 1,25 dihydroxyvitamin D3, and other external

regulatory factors. It has also been proposed that osteoblasts make factors that regulate osteoclast activity and, conversely, that osteoclasts make factors that could regulate osteoblast activity. Therapeutics were generated that would target either osteoclasts or osteoblasts. Osteocytes were left out of the picture.

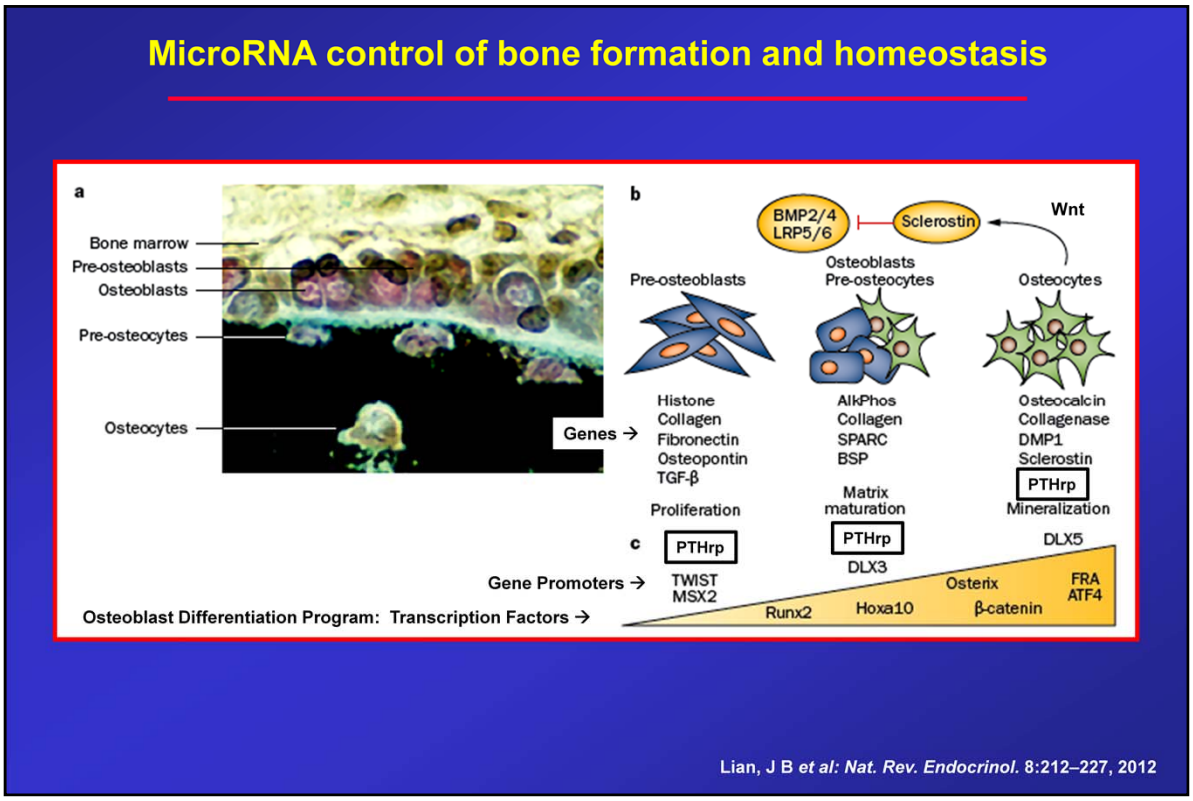


Figure 2 | The osteoblast differentiation program.

a | *In vivo*: bone surface shows organization of indicated osteoblast lineage cells (black, mineralized tissue). Mesenchymal stem cells and osteoprogenitor cells cannot be seen.

b | *In vitro*: stages of differentiation of committed preosteoblast cells isolated from newborn rodent calvarium or bone marrow stromal cells. Peak expression of genes that are markers for the three major stages are shown. At mineralization, a feedback signal from sclerostin secreted by osteocytes inhibits BMP and Wnt osteogenic-mediated bone formation by regulating the number of cells entering the osteoblast lineage.

c | Examples of transcription factors regulating osteoblast differentiation and *in vivo* bone formation are shown. Within the triangle are those that increase during differentiation, whereas those above the triangle are functional on gene promoters at the indicated stages of maturation.

Abstract MicroRNAs (miRNAs) repress cellular protein levels to provide a sophisticated parameter of gene regulation that coordinates a broad spectrum of biological processes. Bone organogenesis is a complex process involving the

differentiation and crosstalk of multiple cell types for formation and remodeling of the skeleton. Inhibition of mRNA translation by miRNAs has emerged as an important regulator of developmental osteogenic signaling pathways, osteoblast growth and differentiation, osteoclast-mediated bone resorption activity and bone homeostasis in the adult skeleton. miRNAs control multiple layers of gene regulation for bone development and postnatal functions, from the initial response of stem/progenitor cells to the structural and metabolic activity of the mature tissue. This Review brings into focus an emerging concept of bone-regulating miRNAs, the evidence for which has been gathered largely from in vivo mouse models and in vitro studies in human and mouse skeletal cell populations. Characterization of miRNAs that operate through tissue-specific transcription factors in osteoblast and osteoclast lineage cells, as well as intricate feedforward and reverse loops, has provided novel insights into the supervision of signaling pathways and regulatory networks controlling normal bone formation and turnover. The current knowledge of miRNAs characteristic of human pathologic disorders of the skeleton is presented with a future goal towards translational studies.

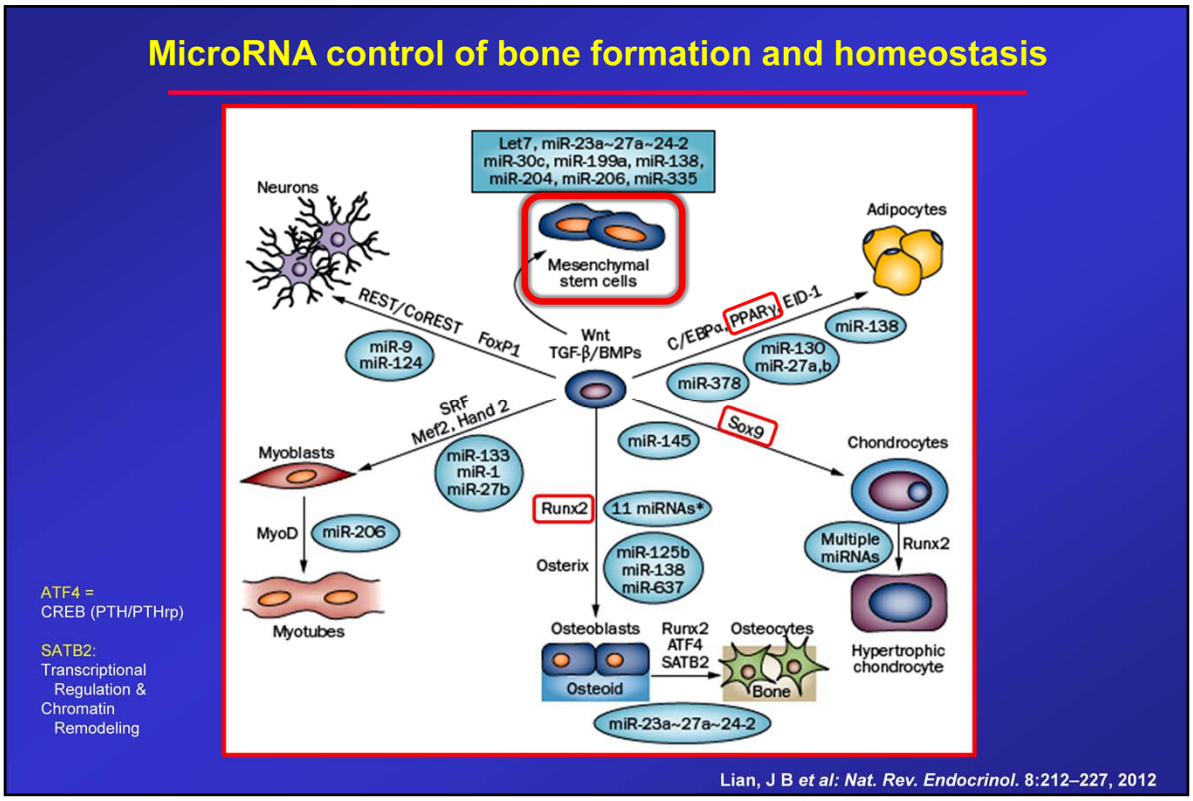


Figure 6 | Allocation of mesenchymal stem cells (MSCs) to lineage-specific phenotypes by transcription factors and microRNAs. Schematic illustration of MSC lineages directed by cell-type specific transcription factors (arrows). Selected miRNAs highly expressed in MSCs are shown because they are downregulated during differentiation into phenotype-committed cells. Cell-type-related miRNAs targeting the transcription factors or their coregulatory proteins are indicated. Although the transcription factors are attenuated by miRNAs at different stages of maturation, they are critical for regulating a normal program of differentiation and for cell specification within a tissue. Relevant references to support this concept for the indicated tissues are as follows: muscle, nerve, fat, bone, and cartilage (Table 1). *Many miRNAs repress Runx2 directly (shown in Figure 7), and to date three miRNAs have been shown to repress osterix: miR-125b140 in vascular smooth muscle cells prevents calcification and miR-138 in MSCs retains stemness, and miR-637 is expressed in adipocytes.

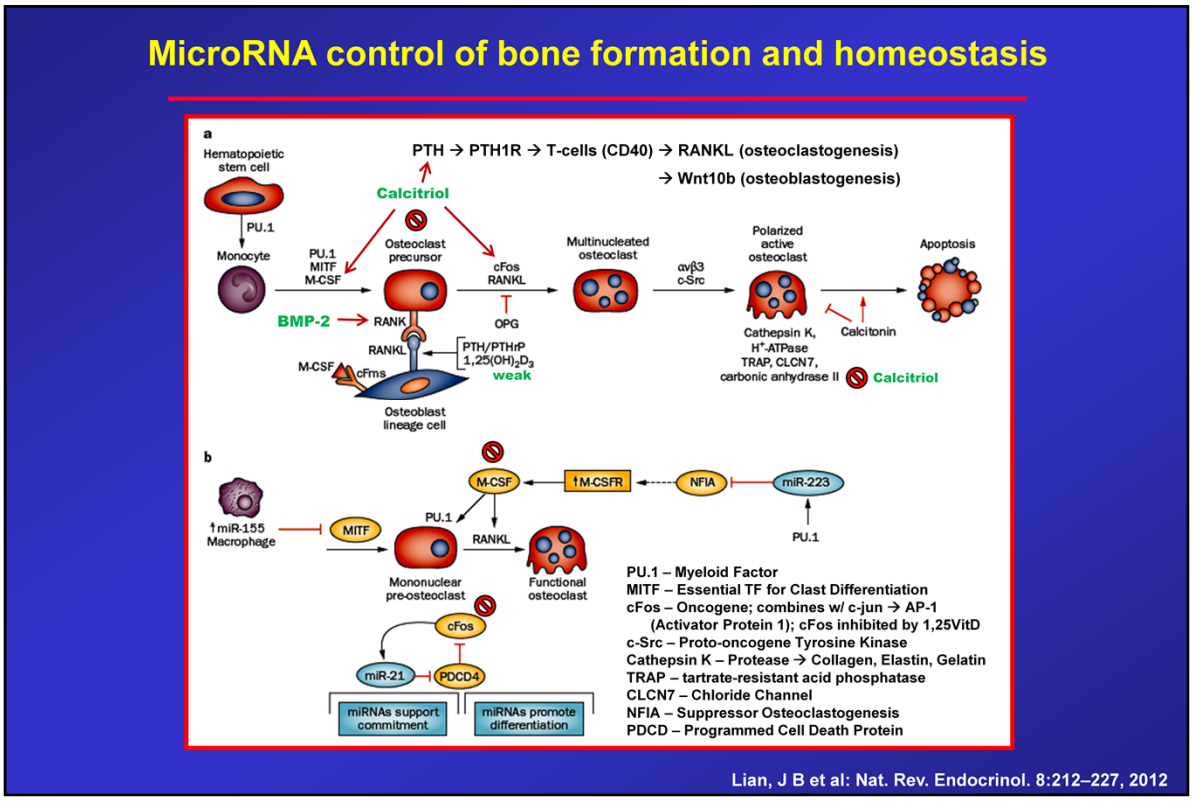
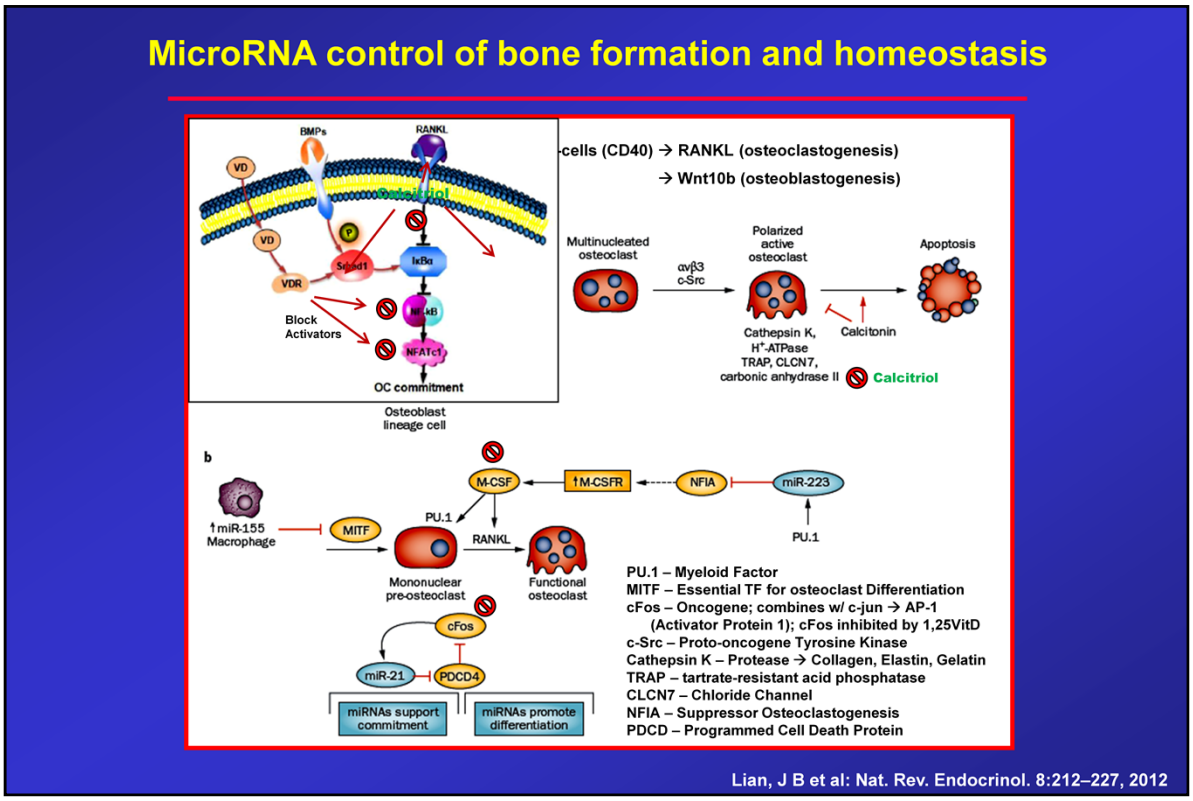


Figure 3 | Osteoclast differentiation sequence and effect of microRNAs.

a | Stages in the differentiation of the multinucleated osteoclast from its hematopoietic precursor are illustrated with key transcription factors and regulatory proteins established as critical for progression to the activated osteoclast. These include the RANK–RANKL interaction regulated by the indicated hormones and the inhibitor of RANK signaling osteoprotegerin. Integrin (αvβ3) mediates attachment of osteoclasts to bone surface and c-Src signaling induces polarization of the osteoclast and formation of the characteristic ruffled border for active bone resorption.

b | MicroRNAs regulating commitment to osteoclastogenesis have been identified. Indicated are three different mechanisms. miR-155 functions as an inhibitor of osteoclastogenesis, being highly expressed in macrophages to support robust expression of this phenotype by inhibiting MITF, essential for preosteoclast differentiation. PU.1 initiates a feedforward mechanism increasing miR-223 which downregulates an inhibitor of osteoclast differentiation, NFIA, resulting in an increase in M-CSFR and thereby M-CSF functional activity. Also shown is a regulatory loop between miR-21 and cFos (AP-1), which activates many osteoclast genes essential for multinucleated cell formation and promotes resorptive activity.

Abbreviations: αvβ3, integrin αvβ3; CLNC7, chloride channel; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase.



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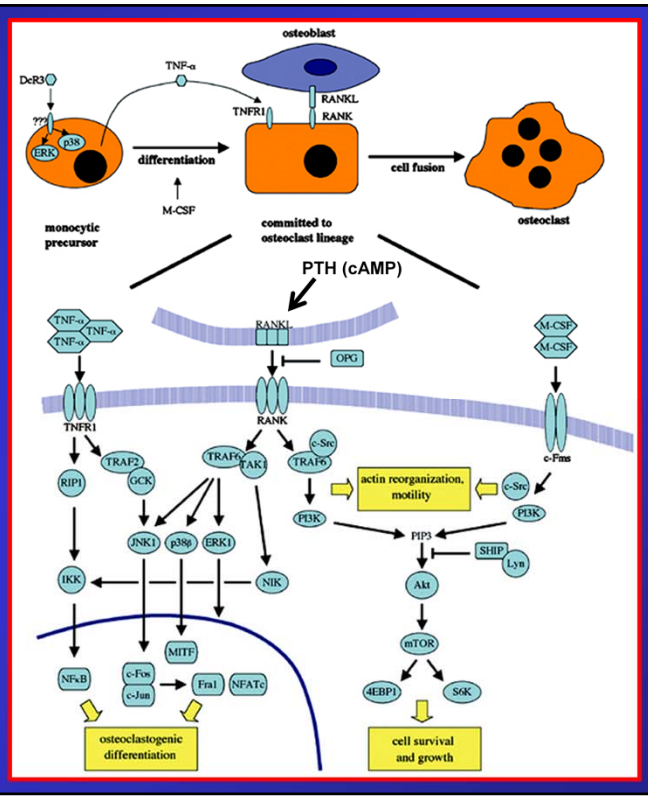
Bone Metabolism

Osteoclast Activation

TNFa + RANKL + mCSF

RIPk1
TRAF
TAK1
Akt

Similar to Foam Cell Development!



Mineral Control of Arterial Calcification

Promotors:

- Hyperphosphatemia
- Hypercalcemia or Hypocalcemia??

- FGF-23?
- PTH / PTHrp
- BMP
- Wnt
- Osteopontin
- High or Low Bone Turnover

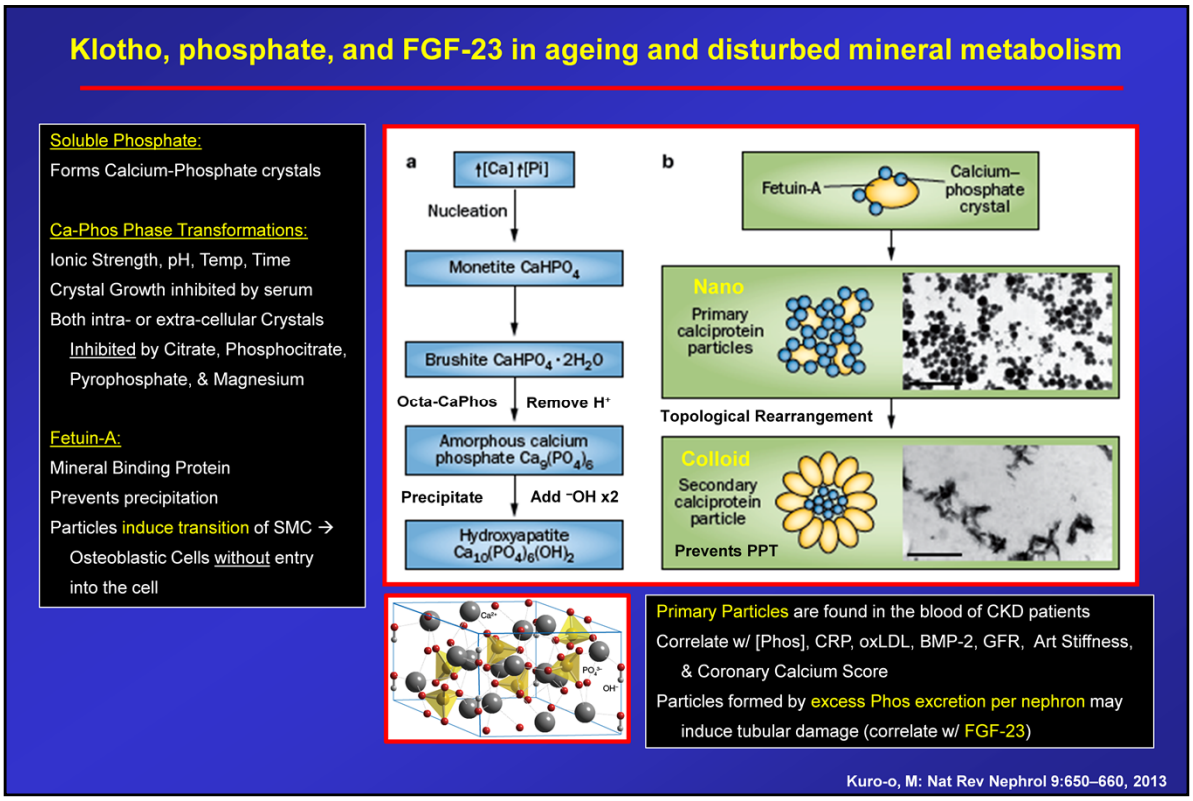
- Hyperlipidemia & Hypertension
- Super Oxide & H_2O_2
- Oxysterols (oxLDL)
- IL-1 & TNFa
- Angiotensin II (RAAS)
- Glucose (RAGE)
- Hypoxia
- Leptin

- Indoxyl Sulfate (renal toxins)

Inhibitors:

- Magnesium (high normal)
- Citrate
- Pyrophosphate (PPi)
- Phospho-citrate
- Acidosis

- 25 Vitamin D & 1-25 Vitamin D (20Vitamin D?)
- Fetuin-A ??
- mGP (matrix gamma-carboxyglutamic acid protein)
- αKlotho – increased by:
  - Pioglitazone (PPARγ)
  - ARB's
  - Statins
- Adiponectin



Klotho, phosphate and FGF-23 in ageing and disturbed mineral metabolism. Kuro-o, M. *Nat. Rev. Nephrol.* 9, 650–660 (2013)

Figure 2 | The formation of calciprotein particles.

a | When the concentration of free calcium and phosphate exceed the concentration of the formation product, calcium–phosphate crystals are generated by nucleation. Calcium–phosphate crystals are transformed from monetite to hydroxyapatite through different phases and eventually precipitate.

b | In the presence of serum, calcium–phosphate crystals bind to fetuin-A and form colloidal nanoparticles. The calcium–phosphate-crystal-laden fetuin-A molecules aggregate to form nanoparticles (50–100 nm diameter; scale bar 500 nm), which are called primary calciprotein particles. Primary calciprotein particles undergo topological rearrangement to form a stable structure, in which a densely packed fetuin-A monolayer covers a mineral core, thereby preventing further crystal growth.[83,84] These particles are referred to as secondary calciprotein particles and are 100–200 nm diameter.

The image in panel b is republished with permission of the American Society of Nephrology, from © Nanoparticle-based test measures overall propensity for calcification in serum. Pasch, A. *J. Am. Soc. Nephrol.* 10, 1744–1752 (2012);

Abstract High concentrations of extracellular phosphate are toxic to cells. Impaired urinary phosphate excretion increases serum phosphate level and induces a premature-ageing phenotype. Urinary phosphate levels are increased by dietary phosphate overload and might induce tubular injury and interstitial fibrosis. Extracellular phosphate exerts its cytotoxic effects by forming insoluble nanoparticles with calcium and fetuin-A; these nanoparticles are referred to in this Review as calciprotein particles. Calciprotein particles are highly bioactive ligands that can induce various cellular responses, including the osteogenic transformation of vascular smooth muscle cells and cell death of vascular endothelial cells and renal tubular epithelial cells. Calciprotein particles are detected in the serum of animal models of kidney disease and in patients with chronic kidney disease (CKD) and might be associated with a (mal)adaptation of the endocrine axes mediated by fibroblast growth factors and Klothos that regulate phosphate homeostasis and ageing. These observations raise the possibility that calciprotein particles contribute to the pathogenesis of CKD. This theory, if verified, is expected to provide novel diagnostic markers and therapeutic targets in CKD.

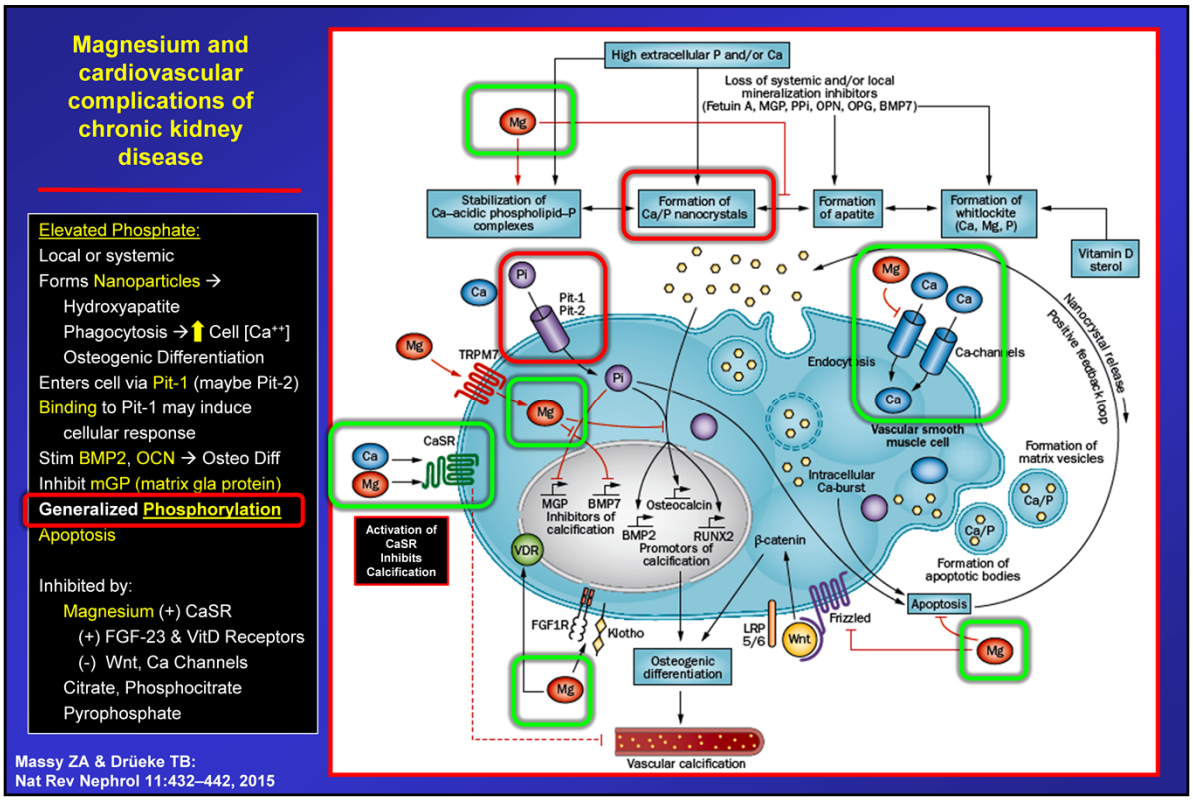


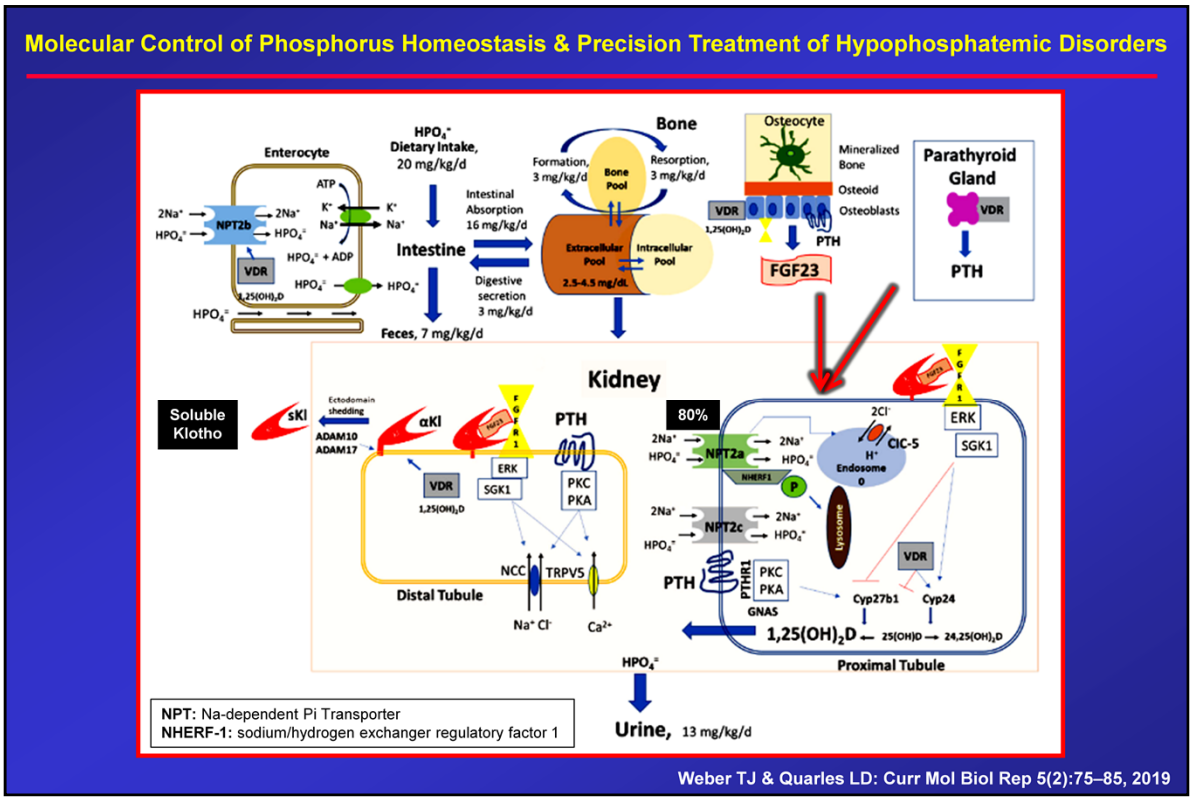
Figure 1 | The putative inhibitory effects of magnesium on the process of vascular calcification. Abnormalities in mineral metabolism, particularly hyperphosphataemia, and loss of inhibitors of mineralization leads to the formation and deposition of Ca/P nanocrystals, which are taken up by VSMCs. Lysosomal degradation of the endocytosed crystals results in intracellular release of Ca and Pi. In addition, Pi accumulates in the cell via uptake through Pit-1 and probably also Pit-2. To compensate for excess Ca/P, VSMCs form matrix vesicles loaded with Ca/P products and the mineralization inhibitors. The intracellular Ca-burst induced by endocytosed nanocrystals and Pi uptake triggers apoptosis, resulting in the formation of Ca/P-containing apoptotic bodies. Matrix vesicles and apoptotic bodies cause a positive feedback loop through nanocrystal release into the surrounding milieu, thus amplifying the calcification process. Furthermore, Ca/P nanocrystals and Pi induce the expression of genes that promote the calcification–mineralization process and repress the expression of factors that inhibit calcification, resulting in transdifferentiation of VSMCs to osteoblast-like cells and, ultimately, vessel calcification.

Mg interferes with the process of vascular calcification by inhibiting transformation of amorphous Ca/P to apatite and by forming Mg-substituted whitlockite crystals, which result in smaller, more soluble deposits. Secondly, Mg functions as a Ca-channel antagonist and thus inhibits the entry of Ca into the cells. Thirdly, Mg enters the cell via TRPM7 and restores the balance between expression of

calcification promoters and inhibitors by neutralizing phosphate-induced inhibition of MGP and BMP7 and enhanced expression of RUNX2 and BMP2. These effects prevent osteoblastic conversion and calcification of VSMCs. In addition, Mg acts on the CaSR; activation of this receptor by calcimimetics has been shown to inhibit VSMC calcification but the molecular mechanisms have not yet been identified.

Abbreviations: BMP, bone morphogenetic protein; Ca, calcium; CaSR, calcium-sensing receptor; FGF1R, fibroblast growth factor receptor-1; LRP 5/6, LDL receptor-related protein 5/6; Mg, magnesium; MGP, matrix gla protein; OPG, osteoprotegerin; OPN, osteopontin; Pi, inorganic phosphate; Pit, sodium-dependent phosphate transporter; PPi, pyrophosphate; RUNX2, runt-related transcription factor 2; TRPM7, transient receptor potential cation channel subfamily M member 7; VDR, vitamin D receptor; VSMC, vascular smooth muscle cell. Permission obtained from Oxford University Press © Massy, Z. A. & Drüeke, T. B. *Clin. Kidney J.* 5 (Suppl. 1), i52–i61 (2013).

Abstract Cardiovascular complications are the leading cause of death in patients with chronic kidney disease (CKD). Abundant experimental evidence suggests a physiological role of magnesium in cardiovascular function, and clinical evidence suggests a role of the cation in cardiovascular disease in the general population. The role of magnesium in CKD-mineral and bone disorder, and in particular its impact on cardiovascular morbidity and mortality in patients with CKD, is however not well understood. Experimental studies have shown that magnesium inhibits vascular calcification, both by direct effects on the vessel wall and by indirect, systemic effects. Moreover, an increasing number of epidemiologic studies in patients with CKD have shown associations of serum magnesium levels with intermediate and hard outcomes, including vascular calcification, cardiovascular events and mortality. Intervention trials in these patients conducted to date have had small sample sizes and have been limited to the study of surrogate parameters, such as arterial stiffness, vascular calcification and atherosclerosis. Randomized controlled trials are clearly needed to determine the effects of magnesium supplementation on hard outcomes in patients with CKD.



Abstract

Purpose of Review—Serum phosphorus is maintained in a narrow range by balancing dietary phosphate absorption, influx and efflux of phosphorus from bone and intracellular stores, and renal reabsorption of filtered phosphate. Acute hypophosphatemia, typically caused by transient increases in cellular uptake, can lead to severe complications such as cardiopulmonary dysfunction and rhabdomyolysis that can warrant parenteral phosphate repletion. Chronic hypophosphatemia, however, generally represents true phosphate deficiency and may result in long-term metabolic and skeletal complications, particularly in children due to the critical importance of phosphorus to skeletal mineralization and longitudinal growth.

Recent Findings—In addition to the well characterized roles of vitamin D and parathyroid hormone (PTH), a new bone-kidney axis has been discovered that regulates phosphate homeostasis through the bone-derived hormone Fibroblast Growth Factor 23 (FGF23) and its phosphaturic actions that are mediated by activation of fibroblast growth factor receptors (FGFRs) complexed with α-Klotho in renal tubules. Chronic hypophosphatemia can now be classified as FGF23 dependent or independent.

Summary—In cases of FGF23 dependent hypophosphatemia, traditional non-

specific treatments with elemental phosphorus and 1,25(OH)2 vitamin D (calcitriol) can now be replaced with a targeted approach by using an FGF-23 blocking antibody (Burosumab).

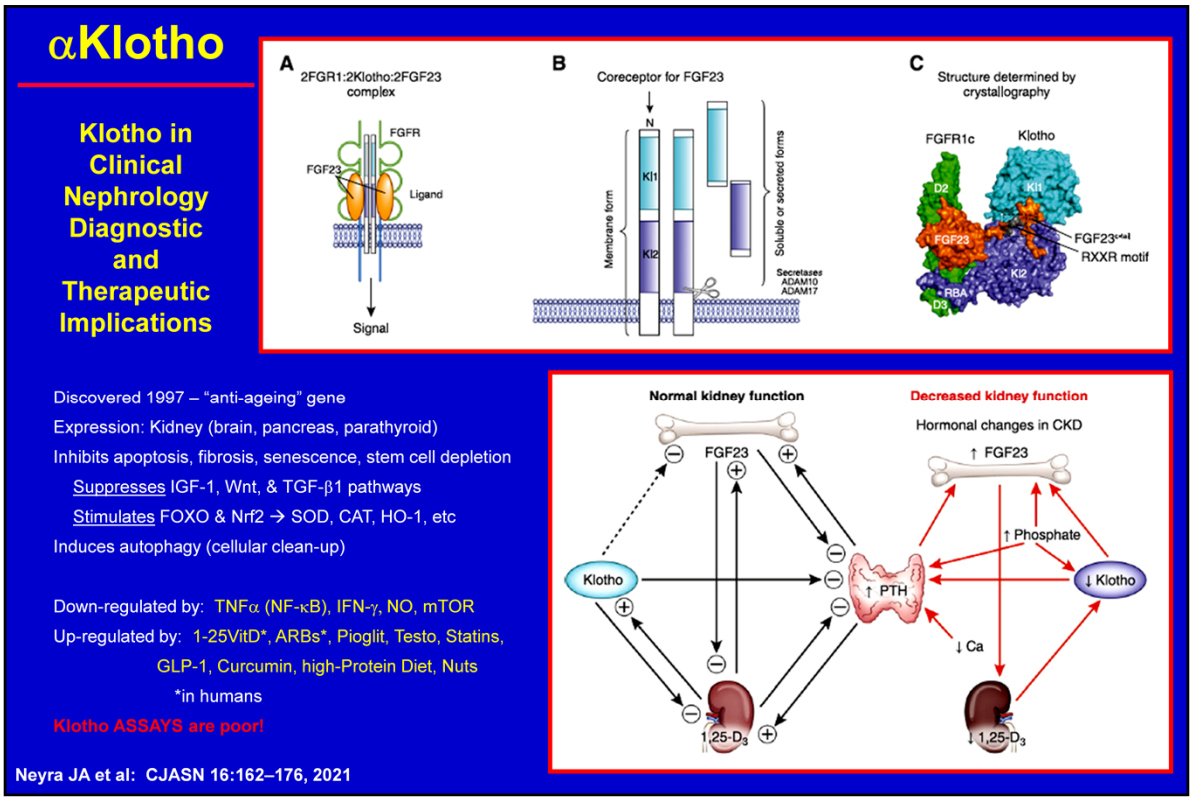
Intestinal absorption of phosphate.

The recommended dietary allowance of phosphate is 700 mg/day, and the maximal tolerated intake is 4,000 mg/day in individuals with normal renal function. In the general population in the United States, phosphorus intakes are above the recommended amounts, with average daily intakes of 1,602 mg in men and 1,128 mg in women [7]. Nearly 70% of dietary phosphate is absorbed, resulting in transient postprandial increases in serum phosphate concentrations. The bioavailability of phosphorus in foods is variable, with phytate phosphorus found in plants being poorly absorbed because we lack phytases that release soluble phosphate (Figure 1).

Dietary phosphate is actively transported transcellularly in intestinal cells through a sodium dependent transporter SLC34a2 (**Npt2b**) [8] and passively absorbed through paracellular pathways. Npt2b-mediated active transport is important in phosphate deficient states. Npt2b expression is increased by 1,25(OH)2D and low dietary phosphate and decreased by **nicotinamide**. The non-essential role of Npt2b in regulating phosphate homeostasis is revealed by the absence of hypophosphatemia in individuals with inactivating Npt2b mutations, which have pulmonary alveolar microlithiasis [9]. Malnutrition, malabsorption states and phosphate binding medications may lead to hypophosphatemia through reduction in intestinal phosphate absorption. Niacin, which inhibits Npt2b, had small effects to reduce serum phosphate (i.e. 0.08 mg/dL (0.03 mmol/L) reduction per year of treatment) in patients with CKD [10].

Renal Handling of Phosphate

Renal reabsorption and excretion of phosphate is the principal mechanism regulating phosphate homeostasis. Phosphate is filtered by the glomerulus and reabsorbed in the proximal tubule (Figure 1). **NPT2a** (SLC34a1) and **NPT2c** (SLC34a3) located in the luminal brush border membrane of the PT reabsorb **80%** and **20%** of the total urinary phosphorus, respectively. The sodium/hydrogen exchanger regulatory factor 1 **NHERF-1** binds to NPT2a and is an important regulator of membrane expression [11]. PTH and FGF23 lead to phosphorylation of NPT2a causing translocation to lysosomes and inhibition of phosphate reabsorption. CLCN5 gene encodes an endosomal H+/2Cl antiporter that regulates endosomal acidification and internalization of NPT2a. **α-Klotho**, which is predominantly expressed in the distal convoluted tubule, is released into the circulation as a soluble Kl1+Kl2 biologically active fragment (sKl) by ADAM10 and ADAM17 sheddases, may also be filtered by the glomerulus and regulate NPT2 membrane localization in the PT [12].



Abstract

aKlotho (called Klotho here) is a membrane protein that serves as the co-receptor for the circulating hormone fibroblast growth factor 23 (FGF23). Klotho is also cleaved and released as a circulating substance originating primarily from the kidney and exerts a myriad of housekeeping functions in just about every organ. The vital role of Klotho is shown by the multi-organ failure with genetic deletion in rodents, with certain features reminiscent of human disease. The most common causes of systemic Klotho deficiency are AKI and CKD. Preclinical data on Klotho biology have advanced considerably and demonstrated its potential diagnostic and therapeutic value; however, multiple knowledge gaps exist in the regulation of Klotho expression, release, and metabolism; its target organs; and mechanisms of action. In the translational and clinical fronts, progress has been more modest. Nonetheless, Klotho has potential clinical applications in the diagnosis of AKI and CKD, in prognosis of progression and extra-renal complications, and finally, as replacement therapy for systemic Klotho deficiency. The overall effect of Klotho in clinical nephrology requires further technical advances and additional large prospective human studies.

Figure 1. | Overview of Klotho protein.

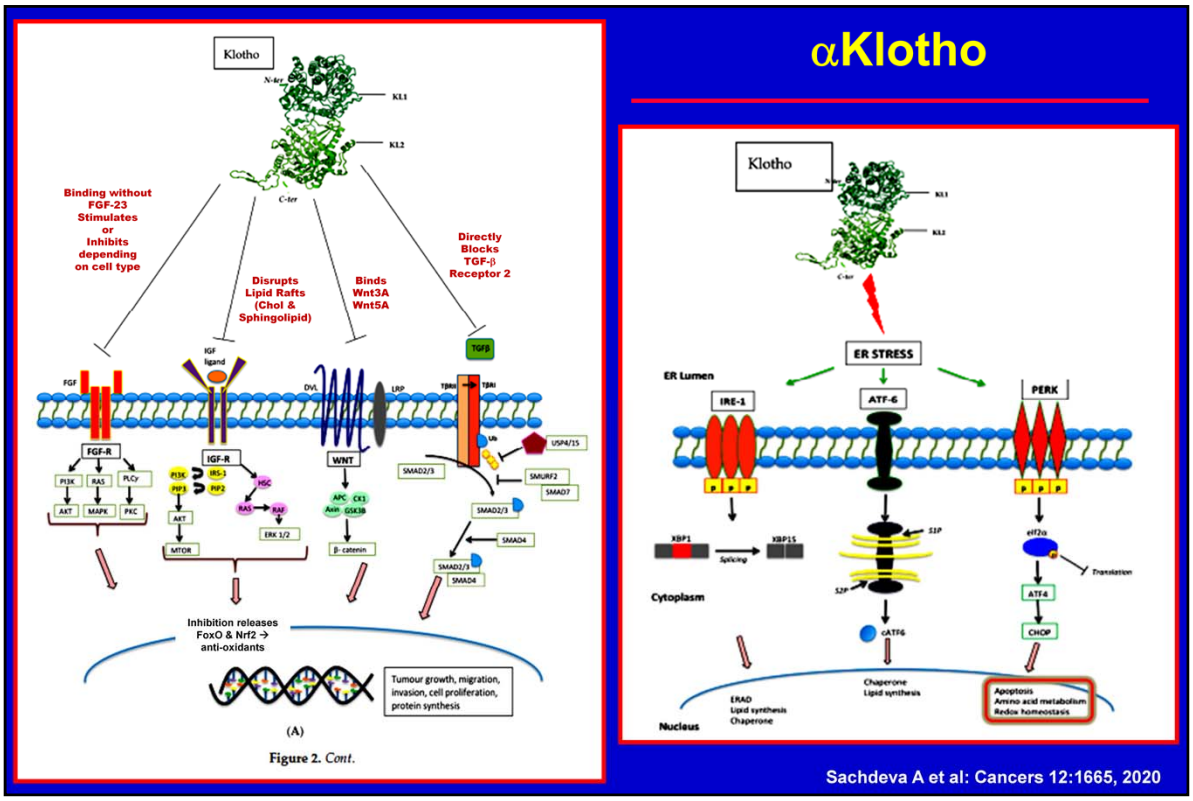
(A) Fibroblast growth factor 23 (FGF23) engages the fibroblast growth factor receptor (FGFR)-Klotho co-receptor complex that triggers cellular signaling.

(B) Transmembrane Klotho with its Kl1 and Kl2 domains and the generation of soluble circulating Klotho by secretases (ADAM10 and ADAM17).

(C) Structure of FGFR1c, Klotho, and FGF23 (one molecule each) as determined by Chen et al. (12). RXXR motif, proteolytic cleavage motif.

Figure 2. | Physiologic and pathophysiologic role of Klotho in mineral metabolism with preserved and decreased kidney function. The dashed line indicates putative action on the basis of experimental or clinical data; no evidence supports direct effect yet. PTH, parathyroid hormone.

Nuclear factor erythroid 2-related factor 2 (**Nrf2**) regulates redox-homeostasis and chemo-resistance in cells. Nrf2 induces antioxidant proteins such as superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (Prx), glutathione reductase (GR), thioredoxin reductase (TR), heme oxygenase-1 (HO-1), and NAD(P)H quinone oxidoreductase 1 (NQO).



Abstract: Klotho was first discovered as an anti-ageing protein linked to a number of age-related disease processes, including cardiovascular, renal, musculoskeletal, and neurodegenerative conditions. Emerging research has also demonstrated a potential therapeutic role for Klotho in cancer biology, which is perhaps unsurprising given that cancer and ageing share similar molecular hallmarks. In addition to functioning as a tumour suppressor in numerous solid tumours and haematological malignancies, Klotho represents a candidate therapeutic target for patients with these diseases, the majority of whom have limited treatment options. Here, we examine contemporary evidence evaluating the anti-neoplastic effects of Klotho and describe the modulation of downstream oncogenic signalling pathways, including Wnt/-catenin, FGF, IGF1, PIK3K/AKT, TGF, and the Unfolded Protein Response. We also discuss possible approaches to developing therapeutic Klotho and consider technological advances that may facilitate the delivery of Klotho through gene therapy.

Figure 2.

(**A**) Klotho inhibits FGF, IGF, WNT, and TGF-a signalling pathways, resulting in reduced tumour growth, tumour migration, tumour invasion, cell proliferation, and protein synthesis.

(**B**) Klotho activates the Unfolded Protein Response, causing activation of PERK-elfF2a pathway and the subsequent increase in pro-apoptotic signals.

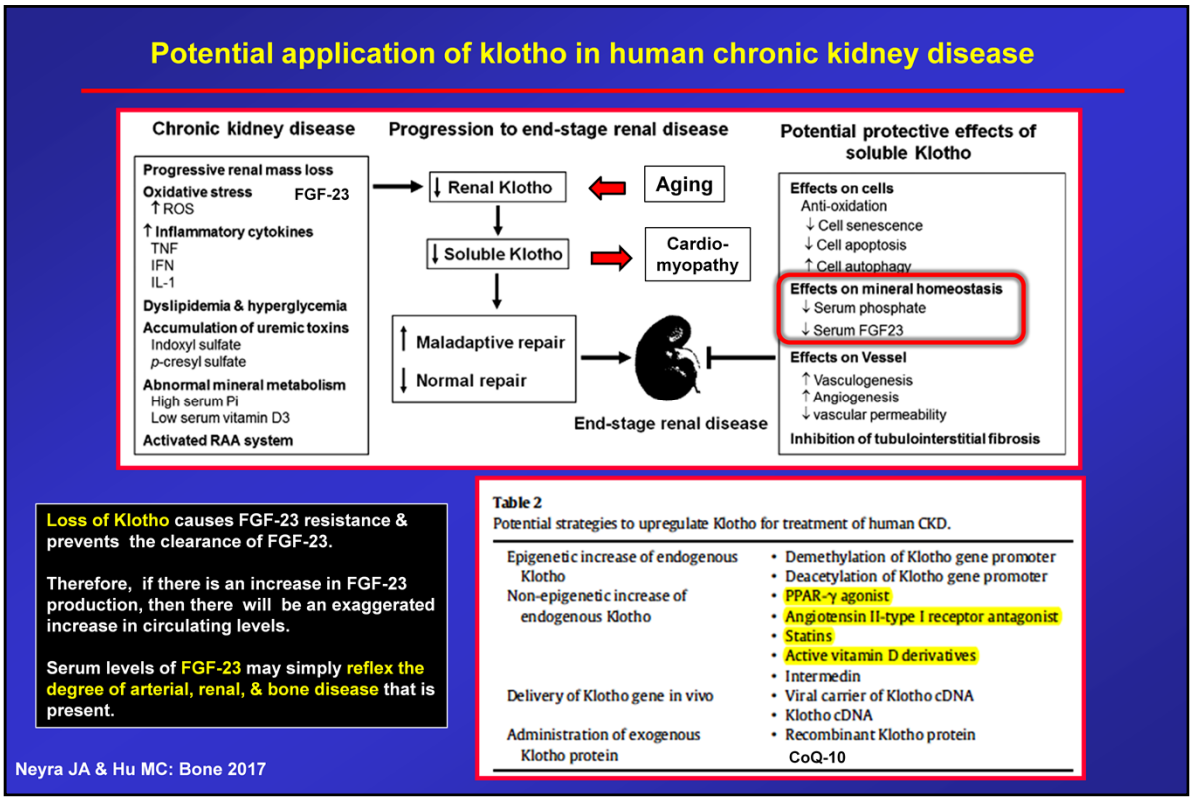
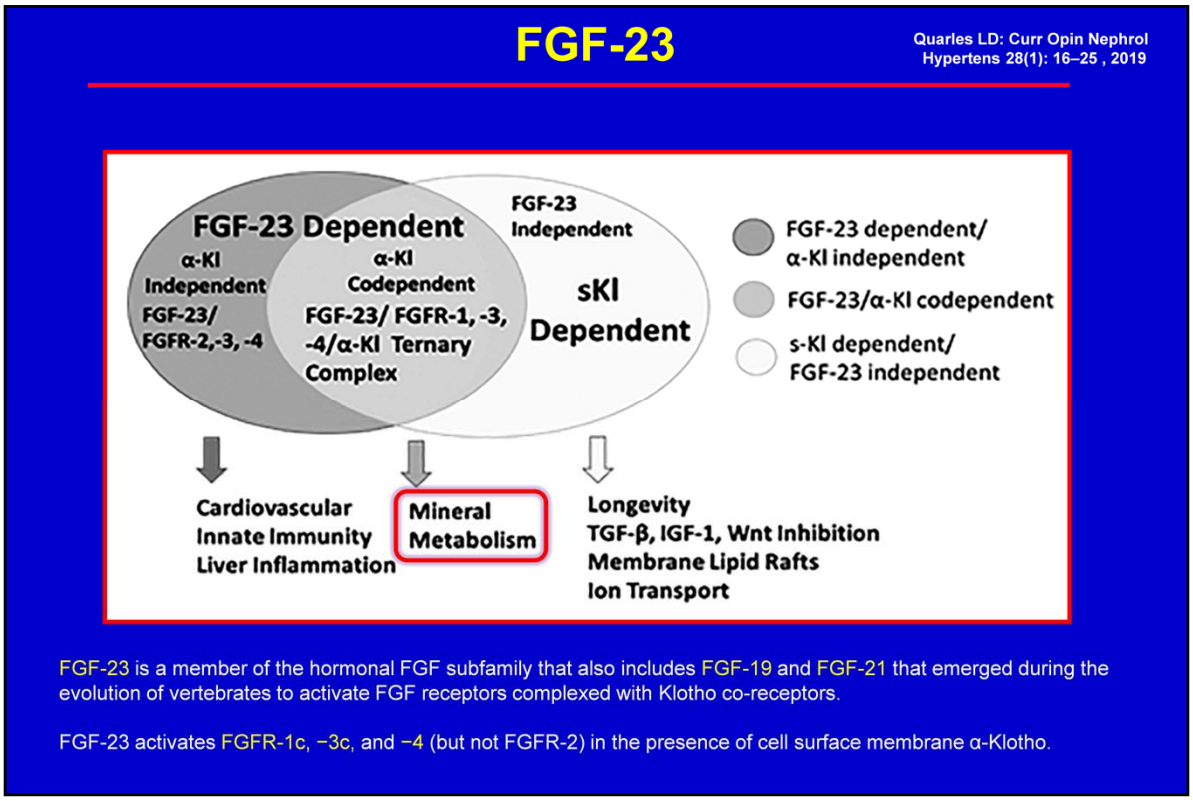


Fig. 2. Potential mechanisms of Klotho downregulation in CKD, and beneficial effects of soluble Klotho on CKD. Left panel: Loss of renal mass, over production of reactive oxygen species (ROS) as well as pro-inflammatory cytokines including tumor necrosis factor (TNF), interferon (IFN) and interleukin 1 (IL-1), dyslipidemia and hyperglycemia, and elevation of uremic toxins including indoxyl sulfate and p-cresyl sulfate may contribute to or participate in downregulation of renal Klotho. Furthermore, high serum phosphate and FGF23 as well as low serum 1,25-Vit.D3 inhibit renal Klotho expression. Low serum 1,25-Vit.D3 not only reduces Klotho expression, but also stimulates renin-aldosterone-angiotensin (RAA) system which further suppresses Klotho production. Middle panel: Reduced Klotho expression in the kidney would lead to endocrine Klotho deficiency in CKD. Low soluble Klotho promotes CKD progression to ESRD through impaired normal renal repair process and induction of maladaptive repair process. Right panel: Supplementation of soluble Klotho protein retards CKD progression through multiple biologic actions: (1) cytoprotection via anti-oxidation, reduction of cell senescence and apoptosis, and upregulation of autophagy, hence accelerating renal tubule regeneration; (2) correction of high serum phosphate and FGF23; (3) maintenance of peritubular capillary formation and function; and (4) inhibition of tubulo-interstitial fibrosis.

Abstract The extracellular domain of transmembrane alpha-Klotho (αKlotho, hereinafter simply called Klotho) is cleaved by secretases and released into the circulation as soluble Klotho. Soluble Klotho in the circulation starts to decline

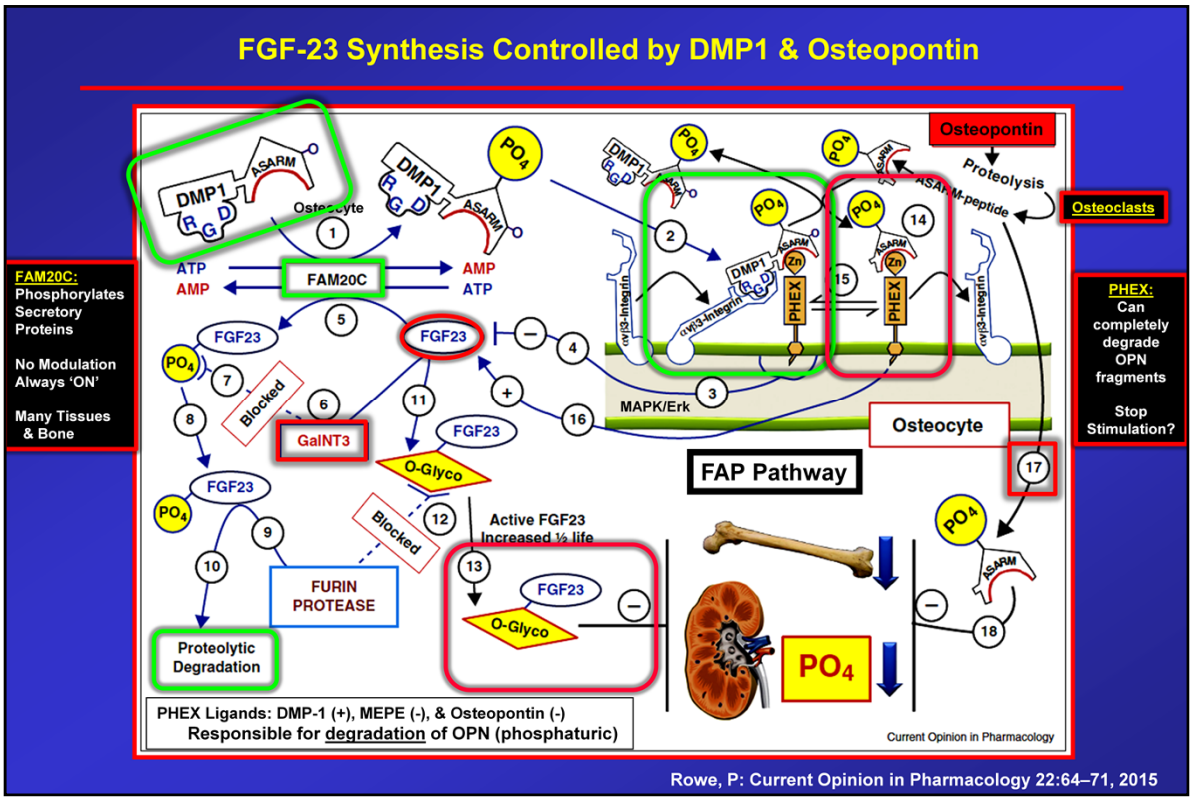
early in chronic kidney disease (CKD) stage 2 and urinary Klotho possibly even earlier in CKD stage 1. Therefore soluble Klotho could serve as an early and sensitive marker of kidney function decline. Moreover, preclinical animal data support Klotho deficiency is not just merely a biomarker, but a pathogenic factor for CKD progression and extra-renal CKD complications including cardiovascular disease and disturbed mineral metabolism. Prevention of Klotho decline, re-activation of endogenous Klotho production or supplementation of exogenous Klotho are all associated with attenuation of renal fibrosis, retardation of CKD progression, improvement of mineral metabolism, amelioration of cardiomyopathy, and alleviation of vascular calcification in CKD. Therefore Klotho is not only a diagnostic and/or prognostic marker for CKD, but the treatment of Klotho deficiency may be a promising strategy to prevent, retard, and decrease the burden of comorbidity in CKD.



FGF-23 is a member of the hormonal FGF subfamily that also includes FGF-19 and FGF-21 that emerged during the evolution of vertebrates to activate FGF receptors complexed with Klotho co-receptors [1]. FGF-23 activates FGFR 1c, −3c, and −4, but not FGFR-2, in the presence of cell surface membrane α-Klotho [2].

α-Klotho is a single-pass transmembrane cell surface protein with extracellular KL1 (Glu-34 to Phe-506) and KL2 (Leu-515 to Ser-950) domains [3]. The N-terminus of FGF-23 and the KL2 domain of α-Klotho interact with FGFRs and the C-terminus of FGF-23 binds to a pocket created by the KL1 and KL2 domains to form the active ternary receptor complex [3]. This canonical FGF-23/FGFR/α-Klotho co-dependent signaling limits FGF-23 effects to tissues expressing α-Klotho (Figure 1, middle circle) [2].

α-Klotho orthologues with a single KL1-like domain emerged during evolution before FGF-23, indicating the presence of FGF-23 independent functions [4]. In mammalians, α-Klotho undergoes ectodomain shedding and subsequent proteolytic cleavage to generate soluble KL1 and KL2 (sKl) circulating protein fragments. Circulating sKl imparts a multitude of purported FGF-23 independent functions [5] (Figure 1, left circle). Recent studies suggest that FGF-23 can directly activate FGFs in the absence of membrane α-Klotho [6] (Figure 1, right circle).



Scheme illustrating the ASARM-model and the FAM20C-kinase link to the FAP pathway: the numbers highlighted in the circles refer to the explanations in the following text. The interactions depicted on the osteocyte cell-surface **between DMP1, PHEX, integrin, and ASARM-peptides are dynamic and competitive** occurring on the extracellular cell-surface. Arrows linking other factors represented in the cartoon illustrate positive and negative effector relationships (paracrine, autocrine, allosteric, signal transduction or gene expression):

(1) FAM20C-kinase phosphorylates the DMP1 C-terminal ASARM-motif

(2) phosphorylation of the DMP1–ASARM motif is required for binding to PHEX and the RGD motif of DMP1 binds to a5b3 integrin to form a [PHEX–DMP1–a5b3-integrin] trimeric complex. This interaction occurs on the cell surface of the osteocyte where PHEX and a5b3-integrin have an intramembranous domain, a short intracellular domain, and a large extracellular domain

(3 and 4) formation of the [PHEX–DMP1–a5b3 integrin] trimeric complex initiates a signaling pathway (MAPK/Erk) that suppresses FGF23 expression

(5) FAM20C-kinase also phosphorylates FGF-23 (Ser180)

(6 and 7) FAM20C phosphorylation of Ser180 inhibits O-glycosylation of FGF-23 by polypeptide N-acetylgalactosaminyltransferase 3 (GalNT3)

(8–10) the under-glycosylated and phosphorylated FGF-23 is targeted for Furin degradation and proteolysis

(6 and 11) in contrast, non-phosphorylated FGF-23 is O-glycosylated by GalNT3

(12 and 13) O-glycosylation of FGF-23 increases resistance to Furin degradation and increases ½ life of full length active FGF-23. Of relevance, mutations in GalNT3 are responsible for reduced circulating full-length FGF-23 resulting in hyperphosphatemia and tumoral calcinosis [48,78,79]. This is the opposite phenotype to high FGF-23 or hypophosphatemia.

In summary, FAM20C is responsible for suppressing FGF-23 expression via the [PHEX–DMP1–a5b3-integrin] trimeric complex and decreasing full-length active FGF-23 by targeting the hormone for furin degradation. This is consistent with the high circulating active FGF23, increased FGF23 mRNA expression, rickets and hypophosphatemia (ARHR 2) reported in mice that are null for bone expressed FAM20C [45 ,46];

(14 and 15) the binding of PHEX to the DMP1–ASARM-motif is also competitively regulated by free ASARM-peptide. Specifically, free ASARM-peptide can directly bind to PHEX preventing the binding to DMP1 and thereby disrupting the [PHEX–DMP1–a5b3-integrin] trimeric complex;

(16) this in turn results in increased expression of FGF23;

(17 and 18) also, free ASARM-peptide inhibits mineralization and renal phosphate uptake. In X-linked and autosomal recessive rickets there are high levels of circulating ASARM-peptides. Both in vitro and in vivo (bolus and infusion) administration of ASARM-peptides and transgenic mice over expressing ASARM-peptides causes mineralization defects and hypophosphatemia [4,8–10,16–18,22,80]. The bimodal effects of SPR4-peptide administration are not shown in the scheme but are discussed in detail in recent publications [10,13,14]. The dual pharmacological effects depend on phosphate diet and mode of administration (bolus or continuous fusion). Briefly, SPR4-peptide binds to either the DMP1–ASARM motif or free ASARM-peptide. Binding to the DMP1–ASARM motif results in increased expression of FGF-23 whereas binding to ASARM-peptides decreased FGF-23 expression.

Abstract The beginning of the millennium saw the discovery of a new bone-matrix protein, **Matrix Extracellular PhosphoglycoprotEin (MEPE)** and an associated **C-terminal motif called ASARM**. This motif and other distinguishing features **occur in a group of proteins called SIBLINGs**. These proteins include dentin matrix protein 1 (**DMP1**), **osteopontin**, dentin-sialophosphoprotein (DSPP), statherin, bone sialoprotein (BSP) and **MEPE**. MEPE, DMP1 and ASARM-motifs regulate expression of a phosphate regulating cytokine FGF23. Further, a trimeric interaction between phosphate regulating endopeptidase homolog X-linked (PHEX), DMP1, and **α5β3-integrin** that occurs on the plasma-membrane of the osteocyte mediates FGF23 regulation (FAP pathway). ASARM-peptides competitively inhibit the trimeric complex and increase FGF23. A second pathway involves specialized structures, matrix vesicles pathway (MVP). This review will discuss the FAP and MVP pathways and present a unified model for mineral and energy metabolism.

Journal of Bone and Mineral Research, Vol. 28, No. 3, March 2013, pp 688–699 Barros NMT et al

We report that OPN is a full-length protein substrate for PHEX. Degradation of OPN was essentially complete, including hydrolysis of the ASARM motif, resulting in only very small residual fragments. Western blotting of Hyp (the murine homolog of human XLH) mouse bone extracts having no PHEX activity clearly showed accumulation of an 35 kDa OPN fragment that was not present in wild-type mouse bone. Immunohistochemistry and immunogold labeling (electron microscopy) for OPN in Hyp bone likewise showed an accumulation of OPN and/or its fragments compared with normal wild-type bone. Incubation of Hyp mouse bone extracts with PHEX resulted in the complete degradation of these fragments. In conclusion, these results identify full-length OPN and its fragments as novel, physiologically relevant substrates for PHEX, suggesting that accumulation of **mineralization-inhibiting OPN fragments** may contribute to the mineralization defect seen in the osteomalacic bone characteristic of XLH/HYP.

J Bone Miner Res 30(3):449–454, 2015 Linberg I et al

A new ubiquitously distributed secretory kinase, **FAM20C**, has recently been identified in **many tissues**, including bone. Because it is the only kinase identified thus far that **contains a signal peptide**, FAM20C is thought to be responsible for **most, if not all, phosphorylation of secretory proteins** at serines located within S-X-E/D phosphorylation consensus sites. The direct phosphorylation of many acidic secreted bone proteins in the SIBLING protein family is efficiently accomplished by this kinase, and mutations in this kinase are known to result in a human bone disease. Interestingly, FGF23 itself contains several consensus sites for FAM20C phosphorylation. In this report, we present data showing that the carboxy-terminal fragment of FGF23 is multiply phosphorylated by an endogenous kinase in bone cells, likely FAM20C.

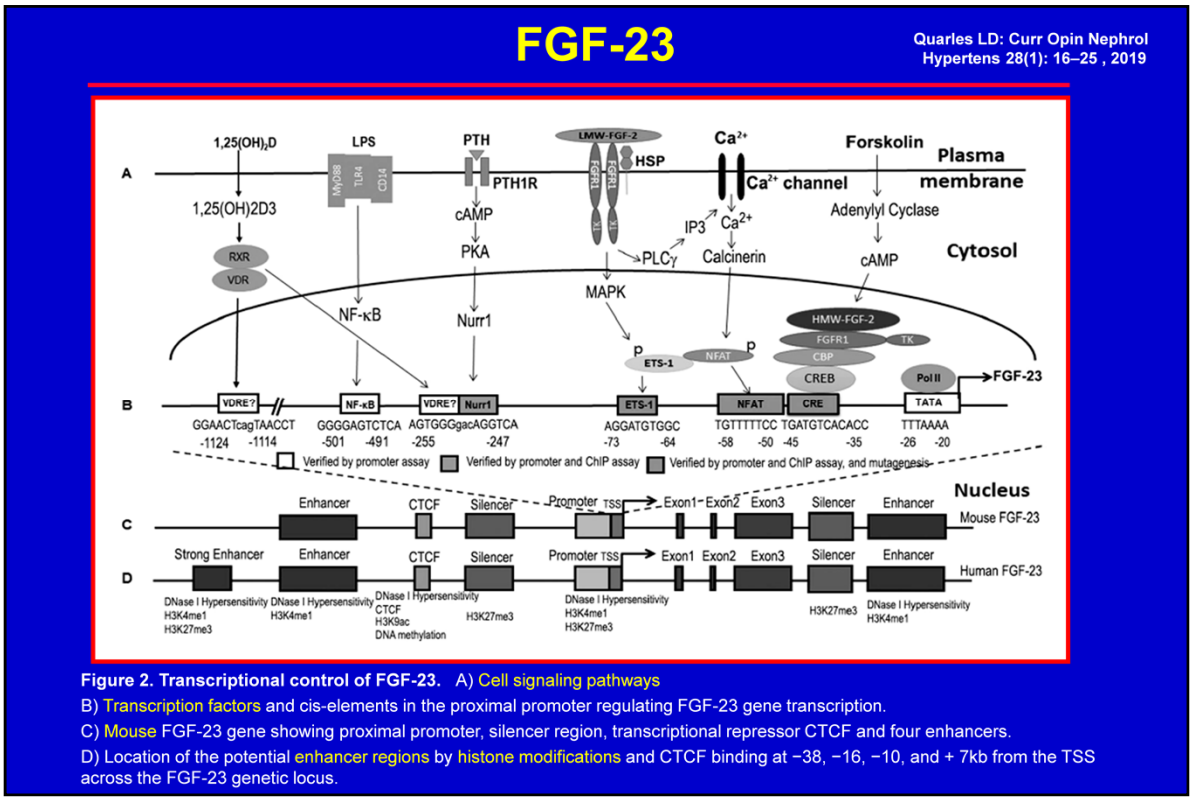


Figure 2. Transcriptional control of FGF-23. A) Cell signaling pathways
B) Transcription factors and cis-elements in the proximal promoter regulating FGF-23 gene transcription.
C) Mouse FGF-23 gene showing proximal promoter, silencer region, transcriptional repressor CTCF and four enhancers.
D) Location of the potential enhancer regions by histone modifications and CTCF binding at −38, −16, −10, and + 7kb from the TSS across the FGF-23 genetic locus.

Purpose of review: This review examines what is known about the FGF-23/α-Klotho co-dependent and independent pathophysiological effects, and whether FGF-23 and/or α-Klotho are potential therapeutic targets.

Recent findings: FGF-23 is a hormone derived mainly from bone, and α-Klotho is a transmembrane protein. Together they form a trimeric signaling complex with FGFRs in target tissues to mediate the physiological functions of FGF-23. Local and systemic factors control FGF-23 release from osteoblast/osteocytes in bone, and circulating FGF-23 activates **FGFR/α-Klotho complexes** in kidney proximal and distal renal tubules to regulate renal phosphate excretion, 1,25(OH)2D metabolism, sodium and calcium reabsorption, and ACE2 and α-Klotho expression. The resulting bone-renal-cardiac-immune networks provide a new understanding of bone and mineral homeostasis, as well as identify other biological effects FGF-23. Direct FGF-23 activation of FGFRs in the absence of α-Klotho is proposed to mediate cardiotoxic and adverse innate immune effects of excess FGF-23, particularly in chronic kidney disease, but this FGF-23, α-Klotho independent signaling is controversial. In addition, circulating soluble Klotho (sKl) released from the distal tubule by ectodomain shedding is proposed to have beneficial health effects independent of FGF-23.

Summary: Separation of FGF-23 and α-Klotho independent functions has been

difficult in mammalian systems and understanding FGF-23/α-Klotho co-dependent and independent effects are incomplete. Antagonism of FGF-23 is important in treatment of hypophosphatemic disorders caused by excess FGF-23, but its role in chronic kidney disease is uncertain. Administration of recombinant sKl is an unproven therapeutic strategy that theoretically could improve the healthspan and lifespan of patients with α-Klotho deficiency.

Bone and mineral metabolism.

One established function of the FGF-23/FGFR/α-Klotho ternary complex in all vertebrates from fish to humans is to coordinate **bone mineral metabolism** with renal handling of phosphate. FGF-23 production in bone is regulated by factors involved in extracellular matrix mineralization [17], including mutations in **Phex**, dentin matrix protein 1 (**Dmp1**), **Enpp1**, and **FAM20C**.

Multiple systemic factors also converge to regulate FGF-23 gene transcription. **1,25(OH)2D**, extracellular **calcium**, and **PTH/cAMP** dependent signaling pathways, but surprisingly not phosphorus, stimulate FGF-23 production in Obs/Ocys [38]. The **store operated Ca2+ entry (SOCE)** also regulates FGF-23 expression in osteocytes [20]. The **PTH receptor and GαS signaling** regulates FGF-23 transcription in osteocytes [39].

FGF-23 targets **FGFR/α-Klotho complexes** in both the proximal and distal tubule. Recent studies show that conditional deletion of α-Klotho in the proximal tubules results in loss of FGF-23 effects on inhibit Npt2a and Npt2c sodium-dependent phosphate co-transporters and suppression of Cyp27b1 and upregulation of Cyp24a1 [43]. **FGFR3** and **FGFR4** preferentially regulate **vitamin D** metabolism in the proximal tubule [44], whereas **FGFR1** is most important in mediating the effects of FGF-23 on **phosphate and calcium transport** in the proximal and distal tubules [45].

Physiologically, local factors linked to mineralization of extracellular matrix in conjunction with PTH, 1,25(OH)2D and circulating calcium, regulate FGF-23, which targets the kidney to match the phosphate buffering capacity/mineralization of bone [46]. There is also a **FGF-23 vitamin D counter-regulatory loop**, whereby **1,25(OH)2D stimulates FGF-23** and **FGF-23 suppresses 1,25(OH)2D** levels by inhibiting Cyp27B1 and stimulating Cyp24A1 in the renal proximal tubule [47]. In addition, there is a **calcium-FGF-23 endocrine loop**, whereby **calcium stimulates FGF-23 in bone** and **FGF-23 stimulates calcium reabsorption in the distal tubule** [45,48].

Originally it was proposed that PTH stimulates FGF-23 in bone and FGF-23 suppresses PTH production/secretion in the PTG to create a feed-back loop [49]. Recent studies that conditionally deleted FGFRs and α-Klotho in the PTG found that **FGF-23 stimulates PTH secretion and cell proliferation** [50]. Clinical disorders of excess FGF-23 are typically characterized by elevated PTH.

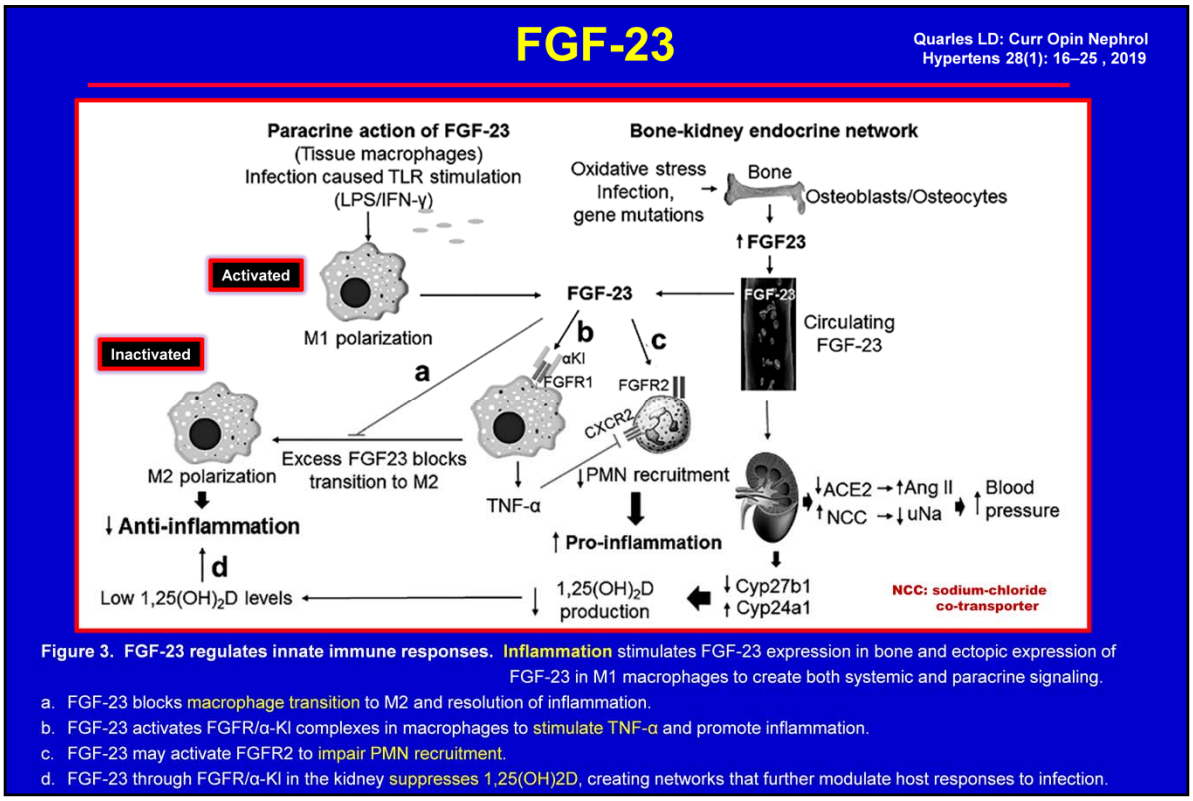


Figure 3. FGF-23 regulates innate immune responses. **Inflammation** stimulates FGF-23 expression in bone and ectopic expression of FGF-23 in M1 macrophages to create both systemic and paracrine signaling.

a. FGF-23 blocks macrophage transition to M2 and resolution of inflammation.
b. FGF-23 activates FGFR/α-Kl complexes in macrophages to stimulate TNF-α and promote inflammation.
c. FGF-23 may activate FGFR2 to impair PMN recruitment.
d. FGF-23 through FGFR/α-Kl in the kidney suppresses 1,25(OH)2D, creating networks that further modulate host responses to infection.

Immunity and inflammation

Elevated FGF-23 is linked to inflammation and adverse infection outcomes in CKD [57]. **FGF-23 is proposed to impair host responses to infection and impair innate immune responses** [58,59]. FGF-23 is expressed in **endothelial cells** in the venous sinusoid of bone marrow, as well as in the thymus and spleen [9]. The function of FGF-23 in these sites is not clear, but this location implicates a role of FGF-23 in regulating macrophage, neutrophils, T-cell and/or B-cell functions. FGF-23 **activates immune cells** in the inflammatory milieu [7,18] may lead to adverse outcomes associated with bacterial infections.

A paracrine role for FGF-23 in regulation of innate immune responses has been described that consists of the **ectopic production of FGF-23 and α-Kl** in activated **macrophages** to reconstitute canonical FGFR/α-Klotho signaling in the inflammatory milieu [7,18] (Figure 3). Resting macrophages do not normally express either FGF-23 or α-Klotho. **Inflammation stimulates the production of FGF-23 locally in M1 activated macrophages** [18]. As noted above, circulating FGF-23 is stimulated in both animal models and humans in response to infection, inflammation, and oxidative stress. **TNFα** also stimulates FGF-23 gene transcription in **osteoblasts** in vitro [18].

Systemic elevations of FGF-23 may indirectly impact innate immune responses

through suppression of 1,25(OH)2D production by the kidney. **Low vitamin D and high FGF-23 serum levels are associated with infectious and cardiac deaths** in a large cohort of patients with end stage renal disease (ESRD) [60,61]. Microbiota induced inflammation in the germ free mice inhibits FGF-23 and suppresses TNF-α to modulate vitamin D homeostasis [62].

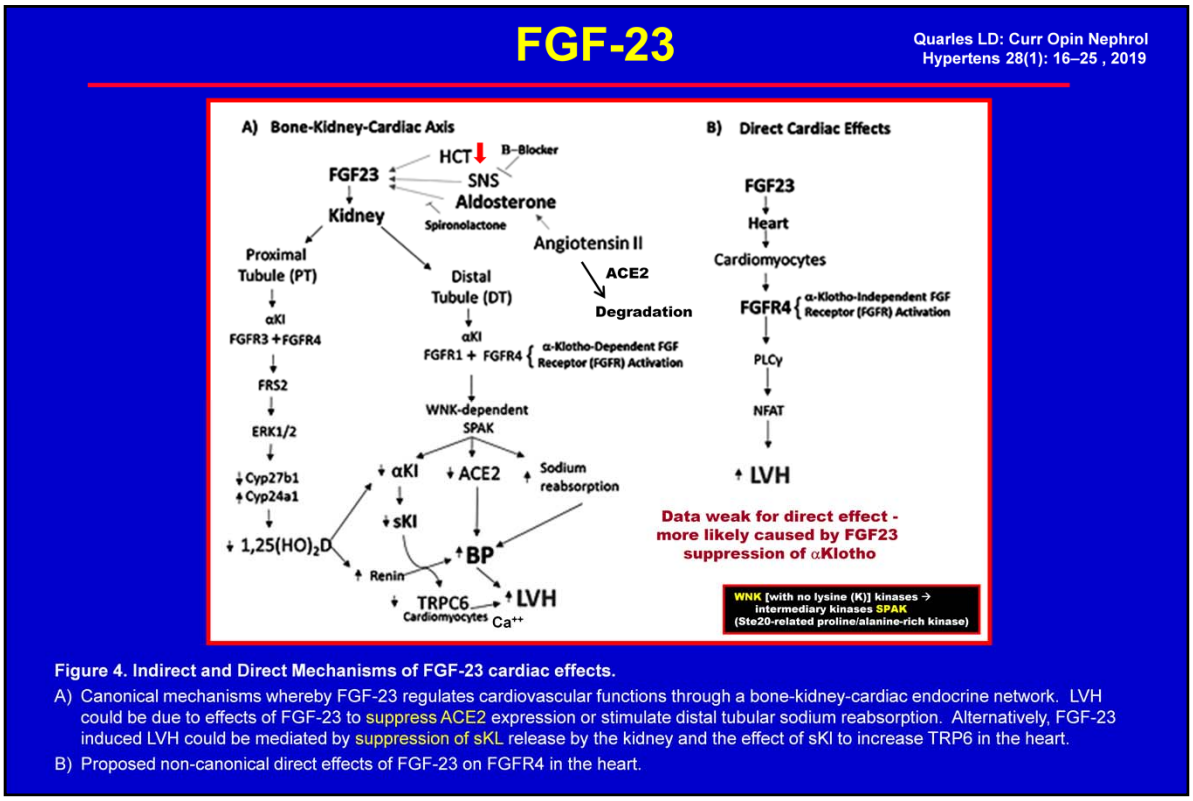


Figure 4. Indirect and Direct Mechanisms of FGF-23 cardiac effects.

A) Canonical mechanisms whereby FGF-23 regulates cardiovascular functions through a bone-kidney-cardiac endocrine network. LVH could be due to effects of FGF-23 to suppress ACE2 expression or stimulate distal tubular sodium reabsorption. Alternatively, FGF-23 induced LVH could be mediated by suppression of sKL release by the kidney and the effect of sKl to increase TRP6 in the heart.

B) Proposed non-canonical direct effects of FGF-23 on FGFR4 in the heart.

Cardiovascular homeostasis.

Chronic elevations of FGF-23 are linked to increased mortality and cardiovascular disease in CKD and in the normal population [63]. The cardiac effects of FGF-23 are potentially explained by a bone-renal-cardiac axis created by FGF-23 "on-target" activation of FGFR/α-Klotho binary complexes in the kidney. The afferent limb of this bone-renal-cardiac axis consists of **aldosterone, Ang II, and β-adrenergic pathways stimulate FGF-23** gene expression in bone [19,20]. The efferent limb consists of FGF-23 activation of FGFRs/α-Kl in renal tubules through at least four mechanisms (Figure 4). First, FGF-23 could **activate RAAS** through either **suppression of 1,25 (OH)2D**, which would **increase renin expression** [64], and/or **reduction of ACE2** expression. Both RAAS activation and ACE2 insufficiency are linked to cardiac hypertrophy and myocardial fibrosis, and deletion of ACE2 in the renal proximal tubule (PT) in mice results in HTN. Ang II and excess FGF-23 have additive effects on induction of LVH in mouse models [19,65]. Second, FGF-23 administration to mice induces HTN and LVH through stimulation of **sodium-chloride cotransporter (NCC)** in the renal distal tubule (DT) leading to sodium retention [66]. Third, **FGF-23** and **α-Klotho**, which are not expressed in the normal heart, are **ectopically expressed** in the **stressed myocardium** [67], which may potentially reconstitute canonical signaling in a paracrine manner. Fourth, FGF-23 **suppresses** kidney expression of **α-Kl** [65], which may lead to LVH through loss of soluble Klotho (sKl) cardioprotective effect (i.e., α-Klotho independent effects, vide infra) [68].

FGF-23 effect independent of α-Klotho (non-canonical effects of FGF-23).

Several groups have proposed that FGF-23 can activate FGFRs in the absence of α-Klotho. High concentrations of FGF-23 do activate FGFR signaling in the absence of α-Kl in vitro, under some conditions, but the effect is small compared to FGF-23 activation of FGFRs in the presence of α-Klotho [77]. Non-canonical effects of FGF-23 have been reported in the heart, PMNs and liver that do not normally express α-Klotho. **Several studies suggest that FGF-23 directly activates FGFR4 in the myocardium to stimulate LVH [6,77] and in hepatocytes to stimulate cytokine production [78], and FGFR2 in PMNs to impair host response to infection** [58].

Direct activation of FGFRs by FGF-23 in the absence of α-Klotho is inconsistent with the functional and structural data regarding the obligate requirement of α-Klotho for FGF-23 tissue restricted activation of FGFRs [3]. It is difficult to conceive how non-specific activation of FGFRs by FGF-23 would be limited to only a few tissues. Low expression of α-Klotho, below the detection limit of immunohistochemistry, has been shown to impart canonical signaling in the proximal tubule and osteoblasts, and confounds attributing FGF-23 to direct activation of FGFRs [43,79]. It is also difficult to exclude α-Klotho dependent effects due to the ectopic expression of α-Klotho in the heart and immune cells and because sKl130 may be able to substitute for membrane α-Klotho in FGF-23 dependent FGFR activation [3,18].

The evidence that **FGFR4 is directly activated by FGF-23** in the myocardium and liver **lacks rigor** [77,78]. There are weaknesses in study designs, including failure to conditionally ablate FGFR4 in the heart or hepatocytes; and reliance on global FGFR4−/− mice, which could have effects due to loss of FGFR4 in the kidney or other tissues. With regard to the cardiac effects, existing studies did not administer rFGF-23 to FGFR4−/− mice to test if loss of FGFR4 prevents FGF-23 induced LVH; rather studies used dietary phosphate loading to secondarily elevate FGF-23 levels without controlling for cofounding effects of hyperphosphatemia [77]. Circulating concentrations of FGF-21 are also increased in CKD, yet activation of FGFR4 signaling through FGF-21 is purported to have cardioprotective effects [80]. It is not clear why FGF-23, but not FGF-21, activation of FGFR4 would have different direct effects on the myocardium. If FGFR2 and FGFR4 activation by FGF-23 can occur in the absence of membrane α-Kl and/or presence of circulating s-Kl, new concepts are needed to explain the observed tissue restrictive functions of FGF-23 [2].

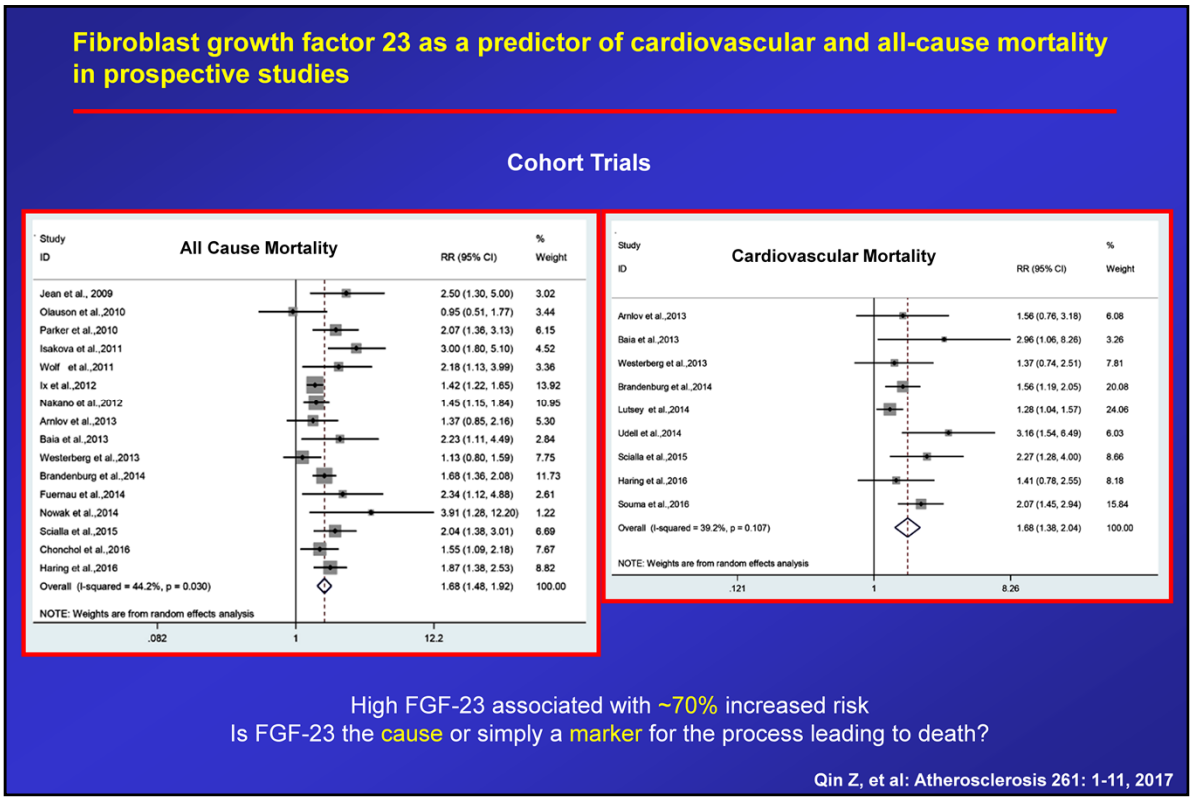
α-Klotho-independent effects.

α-Klotho orthologues emerged earlier in evolution than FGF-23, indicating the presence of FGF-23 independent functions. C50F7.10 and E02H9.5, the Klotho orthologues present in Caenorhabditis elegans, encode only the K1 domain and lacks the structural features necessary to form binary complexes with FGFRs. Klotho orthologues in these invertebrates

inhibit DAF-2, which is **homologous to IGF1 signaling** in mammals. There is compelling evidence that α-Klotho has effects independent of FGF-23. For example, transgenic **overexpression of α-Kl is associated with increased longevity** [5], a phenotype distinct from FGF-23 excess [4]. Moreover, injection of sKl130 elicits biological responses distinct from FGF-23. **Enhancing Klotho levels in the brain are proposed to enhance cognition and prevent age-associated neurodegenerative, demyelinating** [76].

α-Klotho is highly expressed in the distal tubule of the kidney; where its level of expression is more than what is needed to impart FGF-23 signaling, indicating that the distal tubule of the kidney is an endocrine organ that releases sKl into the circulation [12]. α-Klotho undergoes ADAM 10 and 17 dependent ectodomain shedding to release soluble Kl1+Kl2 proteins (sKl130). In addition, a 176Arg-His-Thr-Arg179 proteolytic cleavage site allows genesis of distinct Kl1 (Glu-34 to Phe-506) and Kl2 (Leu-515 to Ser-950) fragments. There is also an alternatively spliced α-Klotho mRNA that may not be translated into a protein in mammalian systems [81,82]. The relative concentrations of the sKl130 (sKl1+ Kl2) and sKl65 (sKl1) proteins and their respective biological contributions remain unclear [83]. **Insulin stimulates the cleavage and release of the extracellular domain of Klotho** by ADAM10 and ADAM17 [84]. α-Klotho gene transcription in the kidney is also **stimulated by 1,25(OH)2D** [85] and suppressed by FGF-23 [65]. Reductions in sKl are observed in CKD [86].

There are several possible mechanisms mediating the FGF-23 independent effects of α-Klotho. **The sKl soluble isoforms may act as a decoy receptor to disrupt IGF1, Wnt and TGF-β signaling** [87]. sKl is reported to exert cardioprotective effects by **downregulating TRPC6** channels in cardiomyocytes [88], and to inhibit **RAAS** and normalize blood pressure in CKD mouse models [89]. Other FGF-23 independent functions are likely due to α-Klotho sialidase activity that **stabilizes membrane expression** of transient receptor potential cation channel, subfamily V, member 5 (**TRPV5**) [90,91] and renal outer medullary **potassium channel 1 (ROMK1)** [92]. Finally, sKl is reported to bind to membrane lipid rafts to disrupt lipid organization and down-regulate raft-dependent **PI3K/Akt signaling** [93]. The biological effects of sKl differ from FGF-23. Moreover, **FGF-23 mediated suppression of sKl**, rather than direct effects of FGF-23 on FGFR4, may account for the cardiotoxic effects of FGF-23 on the heart [94].



Abstract BACKGROUND AND AIMS: The prognostic value of fibroblast growth factor 23 (FGF23) for mortality remains controversial. We performed a meta-analysis of cohort studies to examine the controversial relationship between FGF23 and mortality.

METHODS: PubMed, EMBASE, the Cochrane Library databases and reference bibliographies were searched through September 2016 to identify prospective cohort studies with relative risks (RRs) and 95% confidence intervals (CIs) for FGF23 and mortality. A random effects model was used to pool the risk estimates. A dose-response analysis of the risk for all-cause mortality associated with FGF23 was conducted using the generalized least squares trend estimation method.

RESULTS: Nineteen prospective cohort studies were eligible for inclusion in this meta-analysis, of which 16 reported all-cause mortality and 9 reported cardiovascular mortality. During the follow-up periods ranging from 1 to 18.6 years, 5606 deaths occurred among 22,805 participants and 2458 cardiovascular deaths occurred among 28,845 participants. Elevated FGF23 was associated with an increased risk of all-cause mortality (RR 1.68; 95% CI 1.48-1.92) and cardiovascular mortality (RR 1.68; 95% CI 1.38-2.04) with moderate heterogeneity. These associations were not markedly modified by the geographic location, follow-up length, patient predisposition, FGF23 measurement or study quality. A sensitivity

analysis yielded a similar effect on the pooled risk estimate. Evidence of a nonlinear relationship between FGF23 and all-cause mortality was observed in the dose-response analysis, with the risk gradually increasing as FGF23 increased.

CONCLUSIONS: This meta-analysis showed that individuals with increased plasma FGF23 levels might suffer a higher risk of all-cause mortality and cardiovascular mortality.

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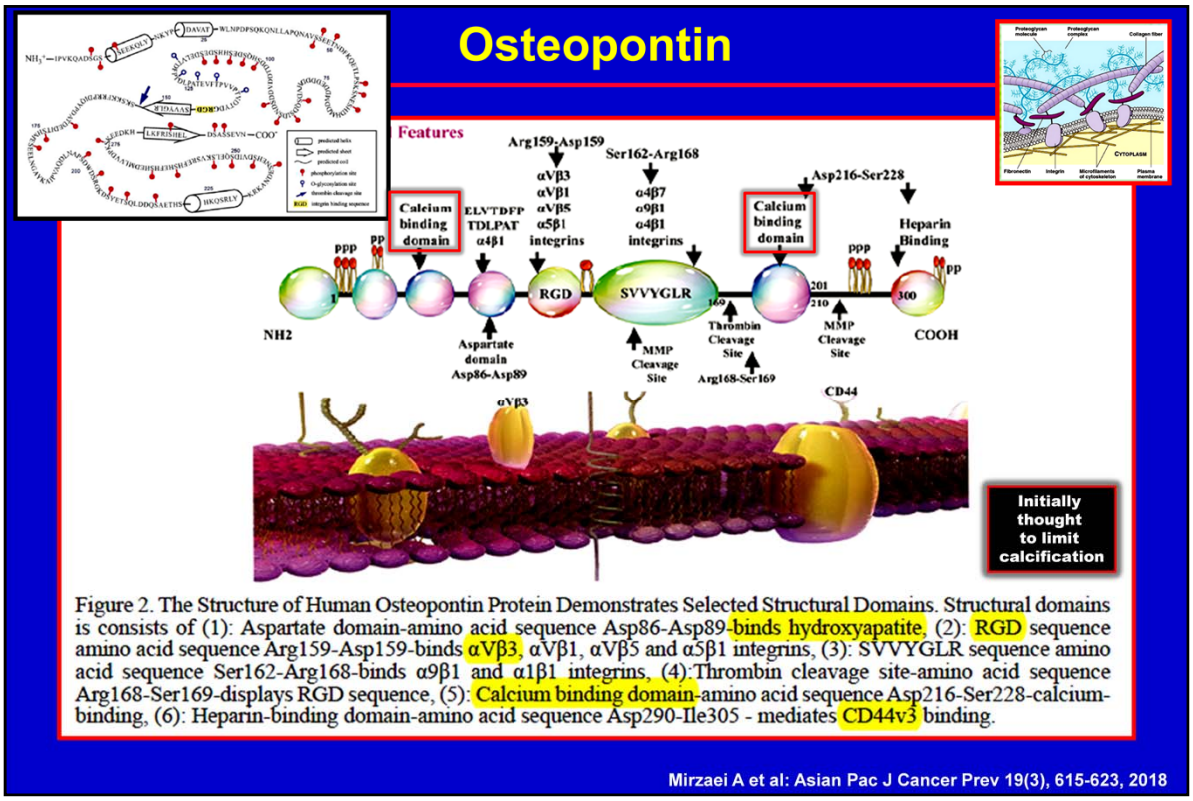


Figure 2. The Structure of Human Osteopontin Protein Demonstrates Selected Structural Domains. Structural domains is consists of (1): Aspartate domain-amino acid sequence Asp86-Asp89-binds hydroxyapatite, (2): RGD sequence amino acid sequence Arg159-Asp159-binds αVβ3, αVβ1, αVβ5 and α5β1 integrins, (3): SVVYGLR sequence amino acid sequence Ser162-Arg168-binds α9β1 and α1β1 integrins, (4):Thrombin cleavage site-amino acid sequence Arg168-Ser169-displays RGD sequence, (5): Calcium binding domain-amino acid sequence Asp216-Ser228-calcium-binding, (6): Heparin-binding domain-amino acid sequence Asp290-Ile305 - mediates CD44v3 binding.

Abstract

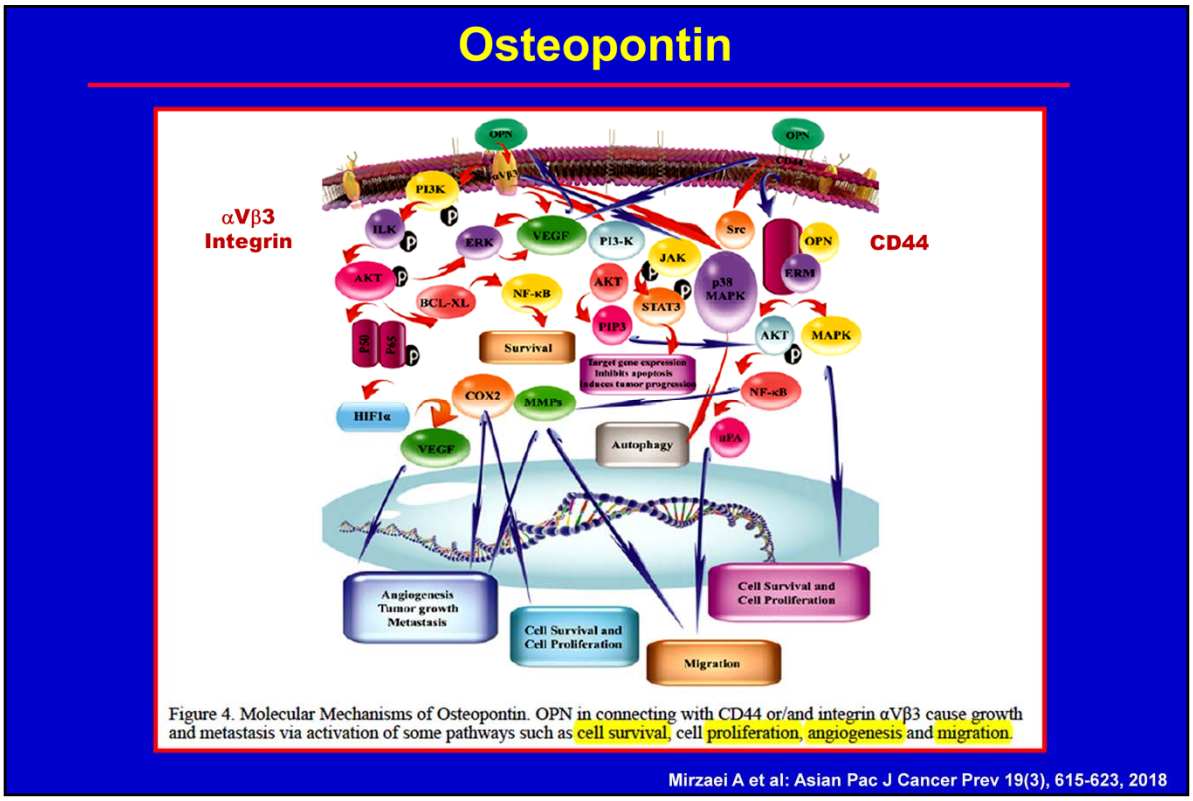
Osteopontin (OPN) is a glycoprotein involved in regulation of various influences on tumor progression, such as cellular proliferation, apoptosis, angiogenesis, and metastasis. Vascular endothelial growth factor (VEGF) is a secreted molecule supporting angiogenesis in various cancers through activation of the PI3K/AKT/ERK1/2 pathway. OPN and VEGF have a number of isoforms with various activities. In spite of the well-defined association between OPN and VEGF isoform expression and cure rate for solid tumors, there is a scarcity of information as to any association in leukemia. Based on the critical role of OPN in cell survival, it seems reasonable to hypothesize that OPN and VEGF isoform expression levels may impact on chemo-resistance and relapse in leukemia the same as in solid tumors. Hence, the aim of our review was to explain relationships between OPN and VEGF isoforms and angiogenesis and related pathways in chemo-resistance of leukemia and solid tumors. Our findings demonstrated that OPN-b and OPN-c alongside with VEGF isoforms and other gene pathways are involved in angiogenesis and also might promote chemo-resistance and even recurrence in leukemia and solid tumors. To sum up, targeting OPN isoforms, particularly b and c, might be a novel therapeutic strategy for the treatment of leukemia as well as solid tumors.

Osteopontin Structure

Osteopontin (OPN) is a glycoprotein with 314 amino acids which was investigated in osteoblasts for the first time. OPN is also recognized as BPP, BSPI, TAP, ETA-I, TSP1, USP, and SPP1 terms. It is expressed in multiple tissue types and the secreted form can be involved in cell attachment and signal.

The Human OPN gene is encoded by a single-copy gene on chromosome 4q21-q25 with seven exons (Young et al., 1990) (Figure1). It contains numerous highly conserved extracellular structural domains, including; aspartate domain, **RGD sequence**, **SVVYGLR sequence**, thrombin cleavage site, calcium-binding domain, and heparin interconnecting domain. Likewise, protease cleavage sites are probably pivotal in adjusting its activity (Figure 2).

It is well proved that OPN assists as a substrate for thrombin and matrix metalloproteinases like MMP2, MMP3, MMP7, MMP9, and MMP12. Therefore, it can bind to the extracellular matrix proteins, fibronectin, and collagen. OPN was recognized with seven protein interactions: ITGAV, IGFBP5, PDLIM7, CD44, ITGA5, CTNNBL1, and SGTA. It binds to cell surface receptors through integrins such as vitronectin receptor **αVβ3**, α4β1, α9β1, **CD44** as well as to extracellular matrix components such as **collagen** and **matrix metalloproteinase**. Integrin receptors identified with both the RGD and the SVVYGLR motifs.



Our previous study in leukemia cells showed that OPN plays a role in activation the signal pathway of the **PI3K/Akt**, following the binding to avβ3 integrin or CD44. Furthermore, OPN causes **mTOR** phosphorylation in Ser-2448. Previously in some studies, we reported that in parallel with OPN knockdown by using siRNA, **AKT/mTOR/ PTEN/β-catenin** expression levels were significantly decreased in AML cell lines and also enhanced apoptosis in AML blasts and CD34+/CD38-/ CD123+ leukemic stem.

Soutto and Servetas studies revealed that TFF1 (tumor suppressor) could inhibit β-catenin and NF-κB via negative regulation of Akt/GSK-3β. OPN protein can trigger MEK/ERK1/2 pathway in cancer in order to stimulate growth and metastasis in the tumor.

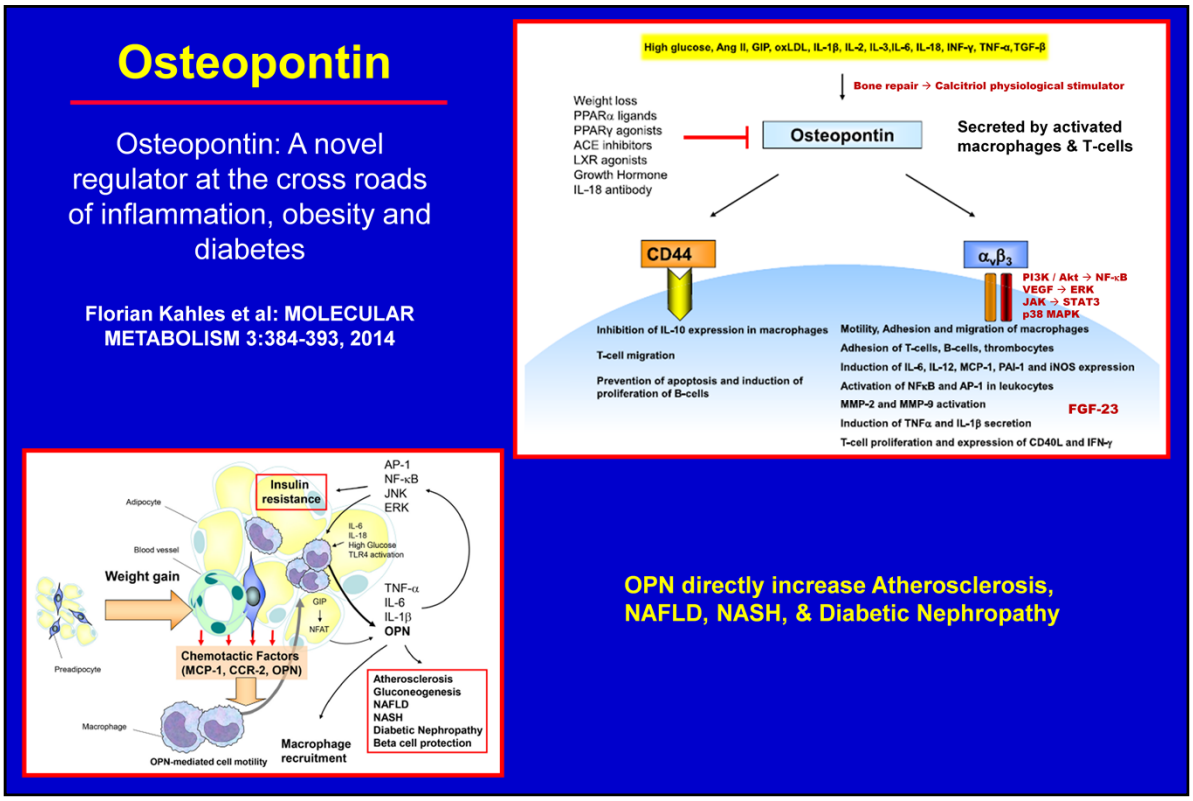
The MMP family has an essential role in tumor invasion and metastasis. OPN promoted metastasis through MMP-2, MMP-7, MMP-9 and VEGF, and uPA. There is a positive relationship between OPN, VEGF, and COX-2, and they are able to induce angiogenesis and metastasis in cancer. COX-2 inhibitors have antitumor activity via OPN down-regulation, with occlusion of OPN regulatory network.

VEGF and OPN are two markers that have recently been identified as a biomarker for prognosis in various cancers. OPN and VEGF are involved in proliferation,

invasion, tumor progression and finally in angiogenesis of cancer cells. There is a synergistic effect between VEGF and OPN. For example, OPN can stimulate VEGF by the integrin αvβ3 in umbilical artery cells.

Chakraborty et al., (2008) confirmed that OPN stimulates tumor progression and angiogenesis by activation of some pathways such neuropilin-1/Brk/ NF-κB/ATF-4 signaling in various grades of cancer. Moreover, they indicated that OPN via using some mechanisms stimulates VEGF-dependent cancer progression and angiogenesis.

VEGF can stimulate integrin αvβ3, OPN, and thrombin which leads to accelerated migration activity through endothelial cells at some point of angiogenesis. In endothelial cells, OPN stimulates angiogenesis and induces VEGF via three main pathways including, phosphorylation and activation of PI3K/AKT and ERK1/2 cascades and mutually, VEGF induces OPN with integrin αvβ3 and thrombin.



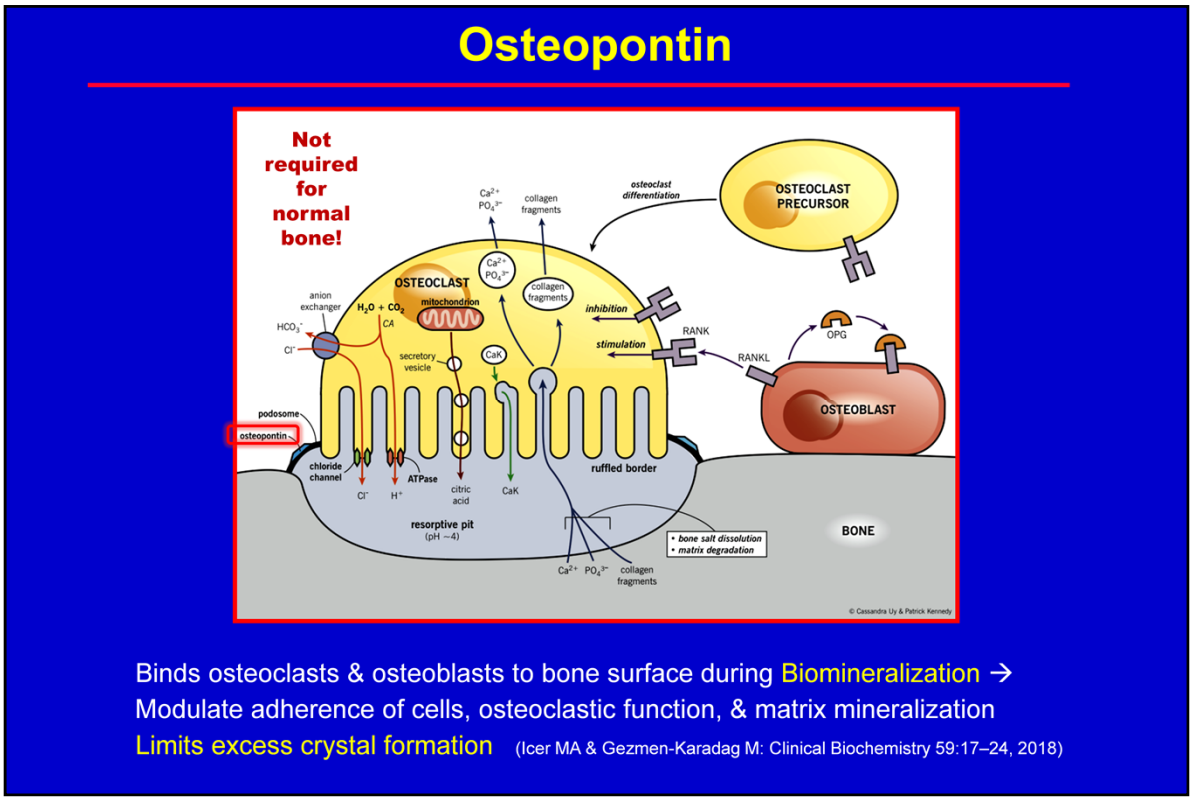
ABSTRACT

Since its first description more than 20 years ago osteopontin has emerged as an active player in many physiological and pathological processes, including **biomineralization**, **tissue remodeling**, and **inflammation**. As an extracellular matrix protein and proinflammatory cytokine osteopontin is thought to facilitate the recruitment of monocytes/macrophages and to mediate cytokine secretion in leukocytes. Modulation of immune cell response by osteopontin has been associated with various inflammatory diseases and may play a pivotal role in the development of adipose tissue inflammation and insulin resistance. Here we summarize recent findings on the role of osteopontin in metabolic disorders, particularly focusing on diabetes and obesity.

Figure 1: OPN is secreted by activated macrophages and T-cells and has been shown to be an important component of early cellular immune responses and inflammation. These proinflammatory effects of OPN are mediated through engagement of a number of receptors. Of particular interest are the integrin receptor avb3 and the CD44 receptor. Ligation to these receptors results in important proinflammatory functions allowing OPN to mediate the recruitment and activation of leukocytes at sites of inflammation.

Figure 2: During diet-induced weight gain OPN is upregulated and mediates

macrophage infiltration into adipose tissue. OPN expression in adipose tissue macrophages is enhanced by high glucose, TLR4 activation, IL-6 and IL-18. In adipocytes GIP increases OPN secretion. OPN itself activates several inflammatory signaling pathways leading to adipose tissue insulin resistance and type 2 diabetes. Furthermore, **OPN was shown to directly increase atherosclerosis, NAFLD, NASH, and diabetic nephropathy**.



3.2. Osteopontin and biomineralization

As mentioned before, OPN is released in mineralized tissues including bones and teeth and produced by osteoclastic and osteoblastic cells at high levels [20,34]. Osteopontin synthesis stimulation is performed by calcitriol which is known as trigger of bone destruction. Furthermore, OPN which is one of the most strongly linked non-collagen proteins is considered to assist binding of the osteoclasts onto the bone surface. Due to the effects mentioned above, OPN has 3 major functions during biomineralization phase including modulation of adherence of osseous cells, modulation of osteoclastic function, and modulation of matrix mineralization.

Osteopontin increases the adhesion of bone cells by concentrating in mineralized collagen matrix during formation of the bone tissue. Furthermore, OPN increases adhesion of osteoblast and osteoclast cells. However, there was not any kind of abnormality on bone and tooth development processes of the rats in the studies conducted on the OPN-null mutant rats. These studies pointed out that other bone matrix proteins such as osseous sialoprotein or fibronectin would perform the adherence and signal functions required for osseous cells.

Osteopontin takes a role in bone tissue destruction by two basic mechanisms. The first one is providing identification and adherence of osteoclastic cells by integrin αvβ3-mediated OPN; and the second is modulation of osteoclastic function via

integrin αvβ3. Moreover, it is reported that OPN acts as a physical limiter limiting crystal formation in bones and teeth.

In line with such data, OPN which has a significant importance in suppression of over-mineralization may be considered to reduce the risk of bone fractures through the aforesaid mechanisms.

Icer MA & Gezmen-Karadag M: Clinical Biochemistry 59:17–24, 2018

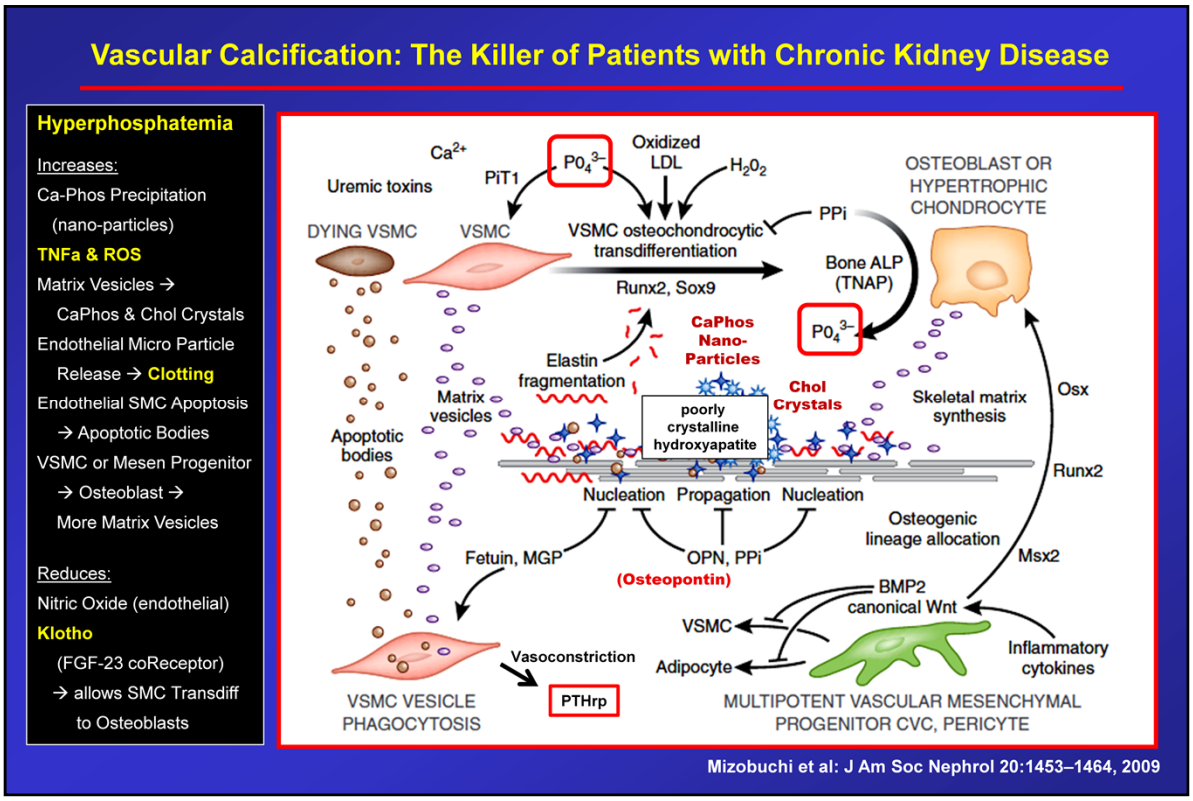


Figure 1. Cell fate, function, and phenotype in vascular calcification. In response to uremic toxins or elevated calcium and phosphate levels, VSMCs elaborate lipidaceous vesicles from apoptotic cells or produce matrix vesicle. The latter, approximately one third the diameter of apoptotic bodies, are much more efficient in nucleating mineral deposition. These vesicles nucleate calcium deposition in the form of a poorly crystalline hydroxyapatite, associated with the elastin-rich extracellular matrix of arteries. The process of elastinolysis not only creates sites for vesicle, mediated nucleation, but also released EDPs that promote osteochondrogenic "transdifferentiation" of VSMCs. This latter process is stimulated by oxidized LDL and ROS, *viz*., hydrogen peroxide. With osteochondrogenic differentiation, gene expression profiles change dramatically, with induction of bone ALP, production of a highly collagenous extracellular matrix, and elaboration of matrix vesicle. Bone ALP locally degrades inorganic pyrophosphate, an important inhibitor of mineralization and transdifferentiation. In addition, multipotent vascular mesenchymal progenitors called calcifying vascular cells (CVC) or pericytes can yield cells of the osteoblast and chondrocyte lineage. This occurs through paracrine BMP and canonical Wnt signals that "shunt" these proliferating progenitors away from other fates, such as the mature VSMC, and toward osteogenic lineages. Inflammatory cytokines such and TNF play critical roles. Of note, as shown by Shanahan's group, the phagocytic clearance of matrix vesicles by VSMCs is critical in limiting the number of sites that nucleate mineral deposition. In severely advanced atherosclerotic lesions, cholesterol crystals have also been shown to nucleate calcium phosphate deposition as well.

Abstract Cardiovascular complications are the leading cause of death in patients with chronic kidney disease (CKD). Vascular calcification is a common complication in CKD, and investigators have demonstrated that the extent and histo-anatomic type of vascular calcification are predictors of subsequent vascular mortality. Although research efforts in the past decade have greatly improved our knowledge of the multiple factors and mechanisms involved in vascular calcification in patients with kidney disease, many questions remain unanswered. No longer can we accept the concept that vascular calcification in CKD is a passive process resulting from an elevated calcium-phosphate product. Rather, as a result of the metabolic insults of diabetes, dyslipidemia, oxidative stress, uremia, and hyperphosphatemia, "osteoblast-like" cells form in the vessel wall. These mineralizing cells as well as the recruitment of undifferentiated progenitors to the osteochondrocyte lineage play a critical role in the calcification process. Important transcription factors such as Msx 2, osterix, and RUNX2 are crucial in the programming of osteogenesis. Thus, the simultaneous increase in arterial osteochondrocytic programs and reduction in active cellular defense mechanisms creates the "perfect storm" of vascular calcification seen in ESRD. Innovative clinical studies addressing the combined use of inhibitors that work on vascular calcification through distinct molecular mechanisms, such as fetuin-A, osteopontin, and bone morphogenic protein 7, among others, will be necessary to reduce significantly the accrual of vascular calcifications and cardiovascular mortality in kidney disease. In addition, the roles of oxidative stress and inflammation on the fate of smooth muscle vascular cells and their function deserve further translational investigation.

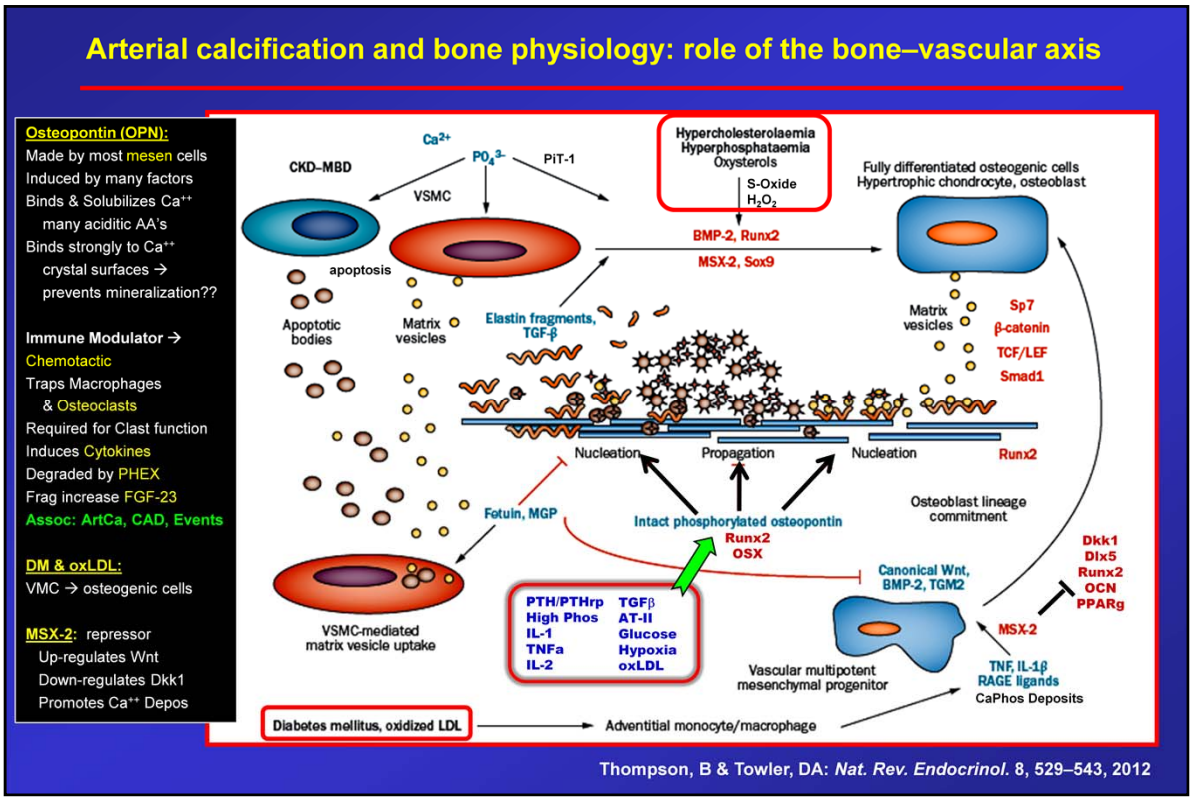


Figure 3 | Vascular osteogenic cell origins, functions and phenotypes in arterial calcification. Vascular mineralization is regulated by processes overlapping yet distinct from those that control skeletal bone formation. Osteogenic progenitors can arise from 'transdifferentiation' of VSMCs, or osteogenic lineage allocation of multipotent mesenchymal progenitors. Healthy VSMCs also have an important role in limiting vascular calcium accrual via fetuin-dependent and MGP-dependent pinocytotic uptake of matrix vesicles. Metabolic and inflammatory insults induce vascular changes that impair normal VSMC function and viability and induce osteogenic differentiation of vascular mesenchymal cells. Not shown are the circulating osteoprogenitors that may contribute to the 'vascular ossification'—true bone formation replete with marrow elements—that can be seen in ~15% of calcified vascular segments. Extracellular factors are blue and intracellular transcriptional regulators are red.

Abstract Bone never forms without vascular interactions. This simple statement of fact does not adequately reflect the physiological and pharmacological implications of the relationship. The vasculature is the conduit for nutrient exchange between bone and the rest of the body. The vasculature provides the sustentacular niche for development of osteoblast progenitors and is the conduit for egress of bone marrow cell products arising, in turn, from the osteoblast-dependent haematopoietic niche. Importantly, the second most calcified structure in humans after the skeleton is the vasculature. Once considered a passive process of dead and dying cells, vascular

calcification has emerged as an actively regulated form of tissue biomineralization. Skeletal morphogens and osteochondrogenic transcription factors are expressed by cells within the vessel wall, which regulates the deposition of vascular calcium. Osteotropic hormones, including parathyroid hormone, regulate both vascular and skeletal mineralization. Cellular, endocrine and metabolic signals that flow bidirectionally between the vasculature and bone are necessary for both bone health and vascular health. Dysmetabolic states including diabetes mellitus, uraemia and hyperlipidaemia perturb the bone-vascular axis, giving rise to devastating vascular and skeletal disease. A detailed understanding of bone-vascular interactions is necessary to address the unmet clinical needs of an increasingly aged and dysmetabolic population.

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Osteopontin: OPN is a highly negatively charged, extracellular matrix protein that lacks an extensive secondary structure. Osteopontin is biosynthesized by a variety of tissue types including cardiac fibroblasts, preosteoblasts, osteoblasts, osteocytes, odontoblasts, some bone marrow cells, hypertrophic chondrocytes, dendritic cells, macrophages, smooth muscle, skeletal muscle myoblasts, endothelial cells, and extra-osseous (non-bone) cells in the inner ear, brain, kidney, deciduum, and placenta. Synthesis of osteopontin is stimulated by calcitriol. OPN expression in bone predominantly occurs by osteoblasts and osteocytes (bone-forming cells) as well as osteoclasts (bone-resorbing cells). Runx2 (aka Cbfa1) and osterix (Osx) transcription factors are required for the expression of OPN. Runx2 and Osx bind promoters of osteoblast-specific genes such as *Col1α1*, *Bsp*, and *Opn* and upregulate transcription. There is a high-specificity vitamin D response element (VDRE) in the OPN gene promoter. Extracellular inorganic phosphate (ePi) has also been identified as a modulator of OPN expression. Stimulation of OPN expression also occurs upon exposure of cells to pro-inflammatory cytokines, classical mediators of acute inflammation (e.g. tumour necrosis factor α [TNFα], infterleukin-1β [IL-1β]), angiotensin II, transforming growth factor β (TGFβ), and parathyroid hormone (PTH) although a detailed mechanistic understanding of these regulatory pathways are not yet known. Hyperglycemia and hypoxia are also known to increase OPN expression.

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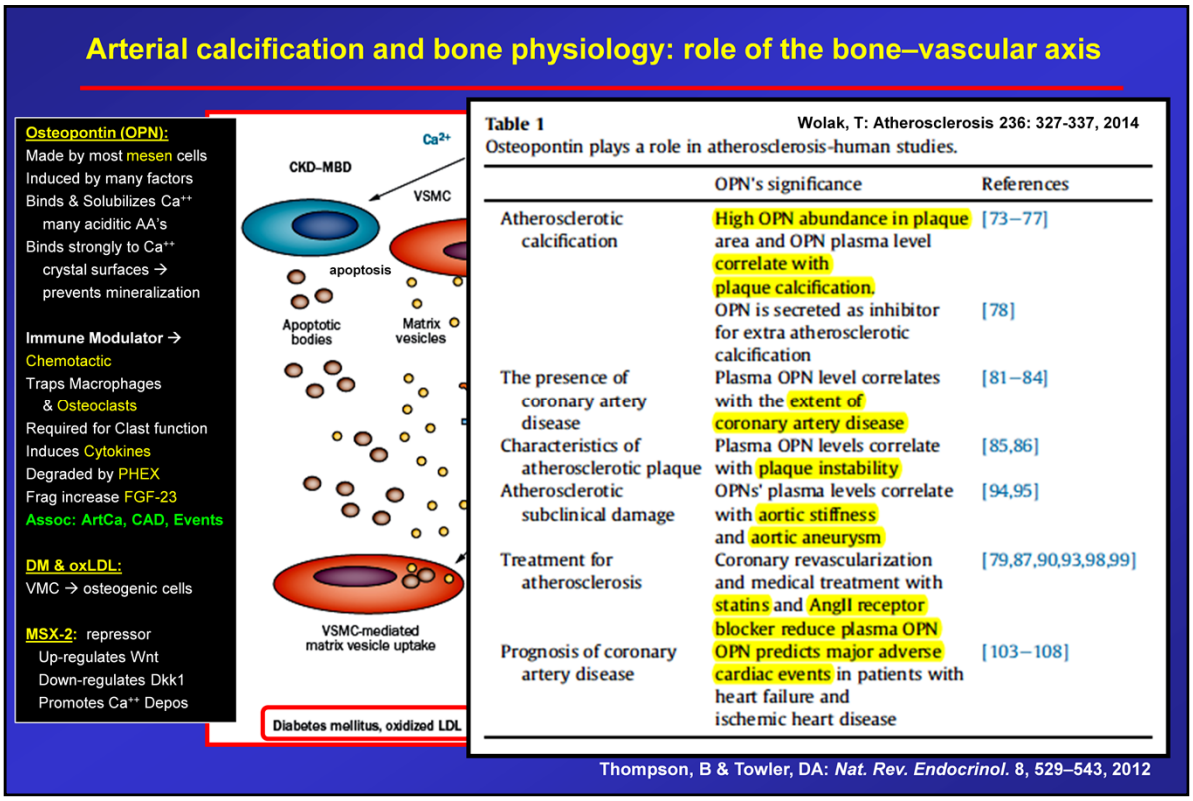

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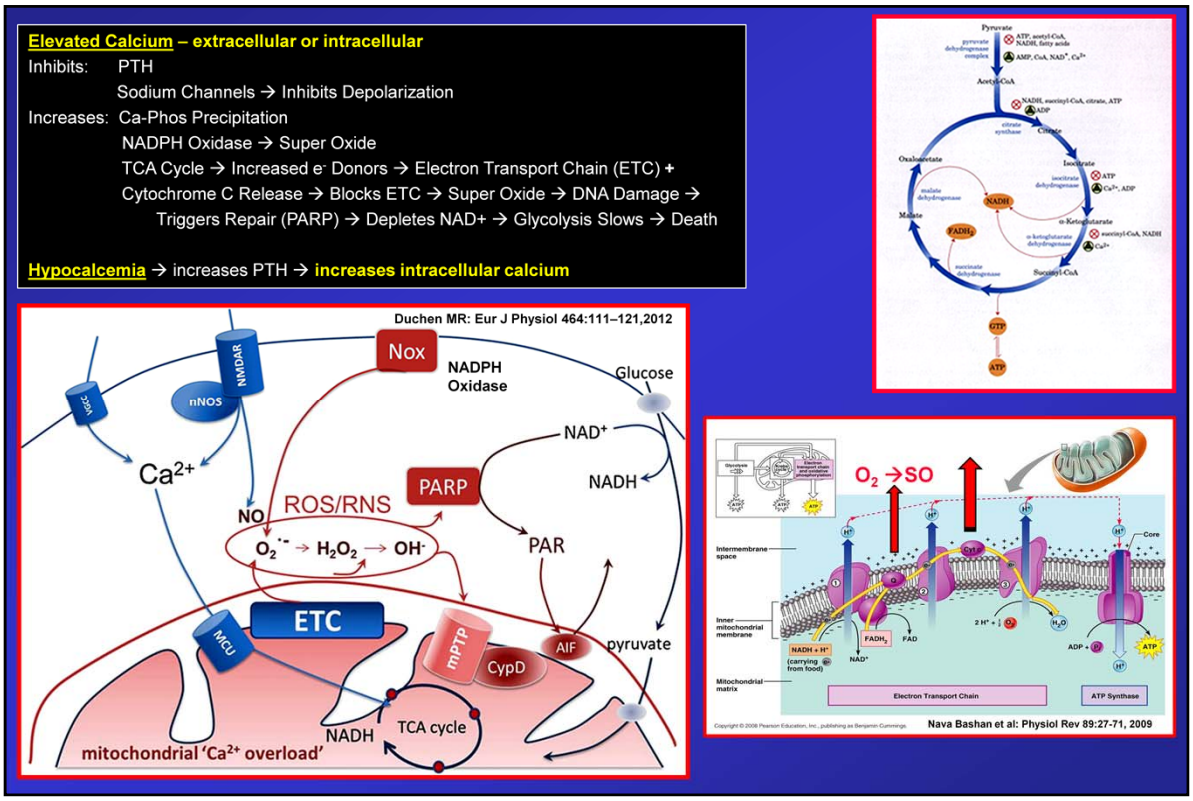

Mitochondria, calcium-dependent neuronal death and neurodegenerative disease. Eur J Physiol 464:111–121, 2012. Michael R. Duchen

Fig. 3 Scheme of pathways involved in glutamate-induced excitotoxicity. Calcium influx through voltage-gated or NMDAR-gated channels is followed by mitochondrial Ca2+ influx through the mitochondrial calcium uniporter (MCU). While the physiological consequence of raised intra-mitochondrial [Ca2+] is an increased activity of the three rate limiting enzymes of the TCA cycle, pathological and prolonged Ca2+ influx leads to mitochondrial Ca2+overload. NMDAR mediated Ca2+ influx is closely coupled to the generation of NO by nNOS; raised Ca2+ may activate the NADPH oxidase (Nox), while mitochondrial Ca2+ overload may also increase generation of superoxide by the electron transport chain (ETC). Nitrosative or oxidative stress arising either from the ETC or from Nox activation may cause over activation of PARP. PARP consumes NAD+ to form PAR polymers, causing depletion of NAD+, failure of glycolysis and so failure of mitochondrial substrate supply. This culminates in the loss of $\Delta\psi m$, ATP depletion and cell death. The PAR polymers generated by PARP may also cause release of AIF which amplifies cell death following its translocation to the nucleus.

Abstract Understanding the mechanisms of neuronal dysfunction and death represents a major frontier in contemporary medicine, involving the acute cell death

in stroke, and the attrition of the major neurodegenerative diseases, including Parkinson's, Alzheimer's, Huntington's and Motoneuron diseases. A growing body of evidence implicates mitochondrial dysfunction as a key step in the pathogenesis of all these diseases, with the promise that mitochondrial processes represent valuable potential therapeutic targets. Each disease is characterized by the loss of a specific vulnerable population of cells--dopaminergic neurons in Parkinson's disease, spinal motor neurons in Motor neuron disease, for example. We discuss the possible roles of cell type-specific calcium signalling mechanisms in defining the pathological phenotype of each of these major diseases and review central mechanisms of calcium-dependent mitochondrial-mediated cell death.

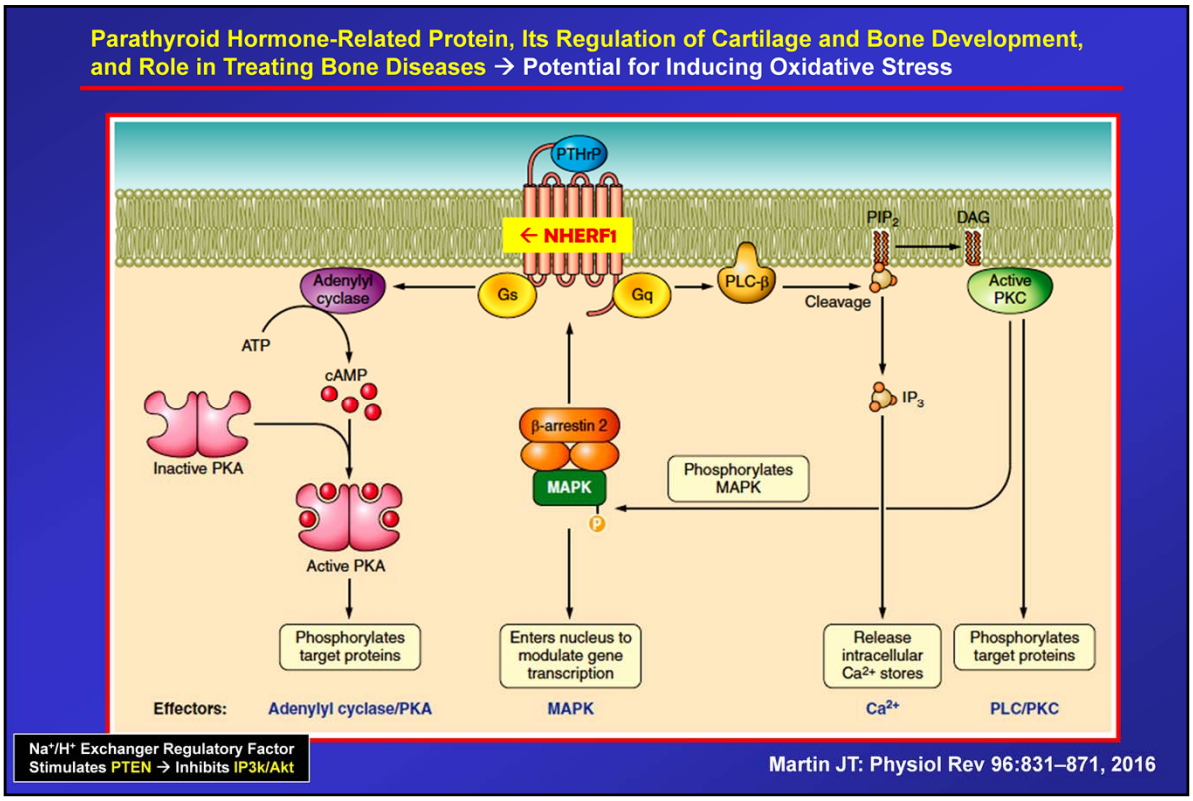

FIGURE 5. Signal transduction pathways in ligand-induced activation of PTHR1. Ligand binding leads to association with Gs subunit and adenylyl cyclase activation, or with Gq that activates phospholipase C-(PLC-). MAPK can be involved through interaction of PTHR1 with the MAPK scaffolding protein-arrestin 2.

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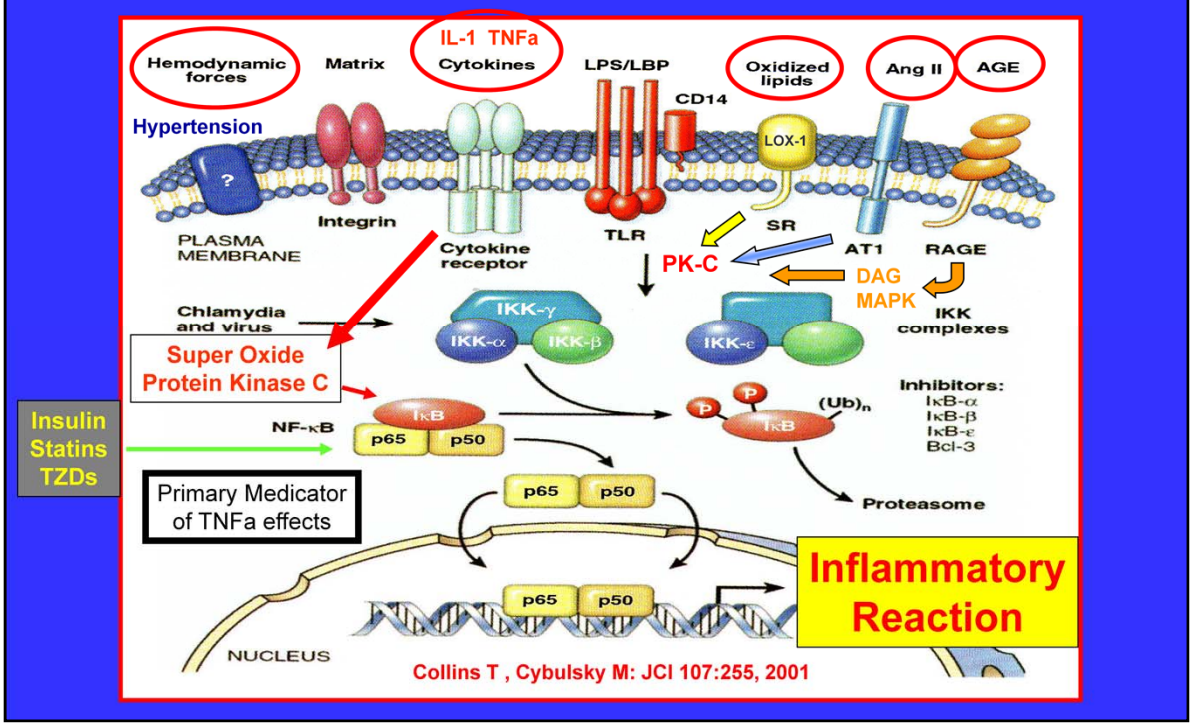
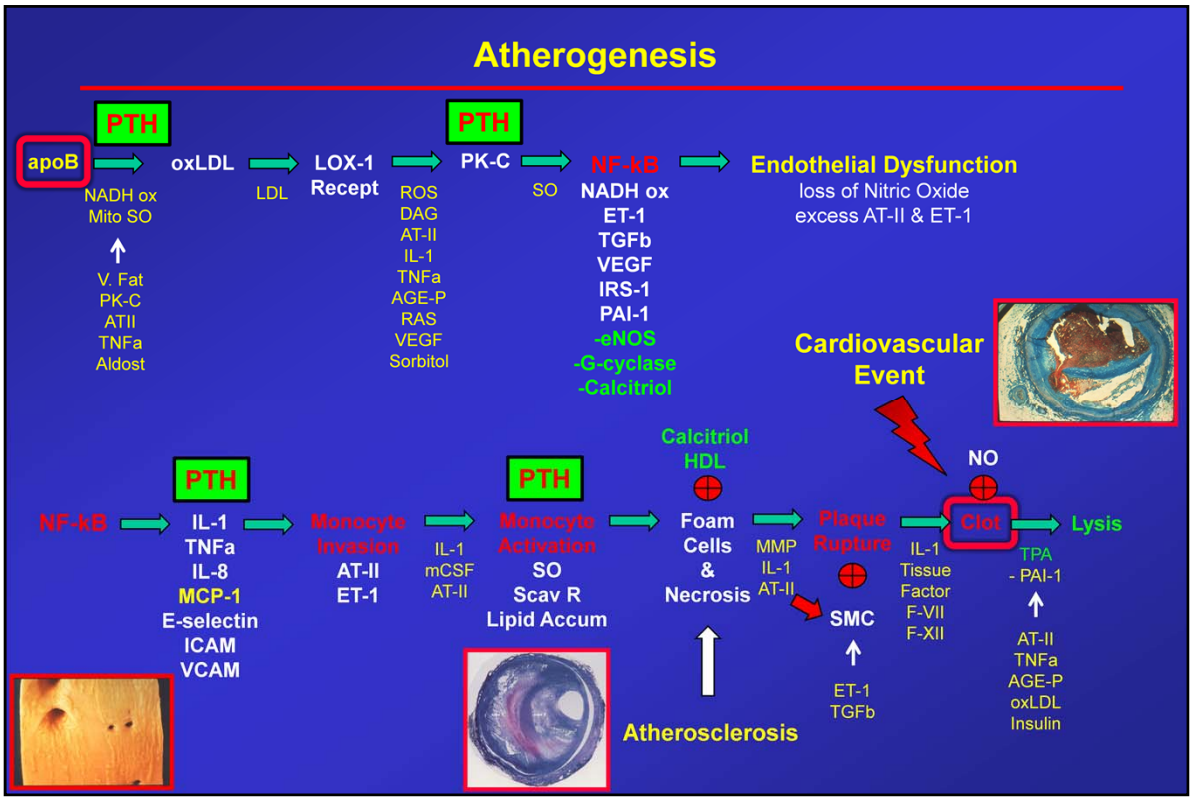
important physiological mediator of bone remodeling postnatally. Thus the use of intermittent injection of PTH as an anabolic therapy for bone appears to be a pharmacological application of the physiological function of PTHrP. There is much current interest in the possibility of developing PTHrP analogs that might enhance the therapeutic anabolic effects.

N Engl J Med 359:1128-1135, 2008:

Impaired renal phosphate reabsorption, as measured by dividing the tubular maximal reabsorption of phosphate by the glomerular filtration rate (TmP/GFR), increases the risks of nephrolithiasis and bone demineralization. Data from animal models suggest that sodium–hydrogen exchanger regulatory factor 1 (NHERF1) controls renal phosphate transport. We sequenced the *NHERF1* gene in 158 patients, 94 of whom had either nephrolithiasis or bone demineralization. We identified three distinct mutations in seven patients with a low TmP/GFR value. No patients with normal TmP/GFR values had mutations. The mutants expressed in cultured renal cells increased the generation of cyclic AMP (cAMP) by parathyroid hormone (PTH) and inhibited phosphate transport. These NHERF1 mutations suggest a previously unrecognized cause of renal phosphate loss in humans.

Nuclear Factor kB (NF-kB) Activation




Atherogenesis
PTH
apoB
NADH ox
Mito SO
V. Fat
PK-C
ATII
TNFa
Aldost
oxLDL
LDL
LOX-1
Recept
PTH
ROS
DAG
AT-II
IL-1
TNFa
AGE-P
RAS
VEGF
Sorbitol
PK-C
SO
NF-kB
NADH ox
ET-1
TGFb
VEGF
IRS-1
PAI-1
-eNOS
-G-cyclase
-Calcitriol
Endothelial Dysfunction
loss of Nitric Oxide
excess AT-II & ET-1
Cardiovascular Event
NO
Clot
Lysis
TPA
- PAI-1
AT-II
TNFa
AGE-P
oxLDL
Insulin
Calcitriol
HDL
Foam Cells & Necrosis
MMP
IL-1
AT-II
Plaque Rupture
SMC
ET-1
TGFb
IL-1
Tissue Factor
F-VII
F-XII
Atherosclerosis
NF-kB
PTH
IL-1
TNFa
IL-8
MCP-1
E-selectin
ICAM
VCAM
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AT-II
ET-1
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mCSF
AT-II
PTH
Monocyte Activation
SO
Scav R
Lipid Accum

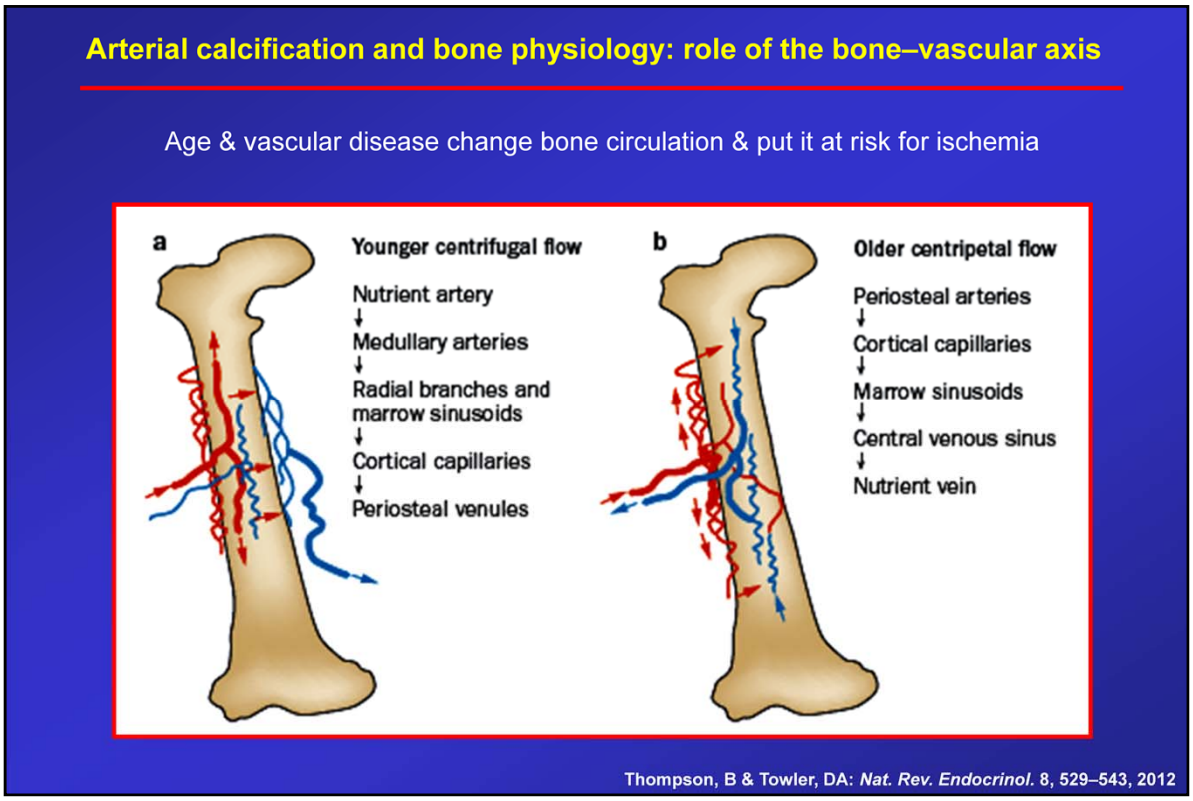


Figure 5 | Age-dependent changes in cortical blood flow of long bones.

a | Healthy cancellous bone has a marrow flow of about 20 ml/min/100 g via the nutrient, ascending and descending medullary arteries; this flow helps maintain a fairly high intramedullary pressure that drives centrifugal flow through cortical bone (~5 ml/min/g).

b | With aging and arteriosclerosis, perfusion is altered, with blood supply to the aging cortex increasingly provided from periosteal conduit vessels. Age-related and exercise-related changes in vasodilatation responses of nutrient arteries can also affect the extent of centrifugal versus centripetal cortical blood flow, as the nutrient arteries are more responsive to vasoconstrictors.

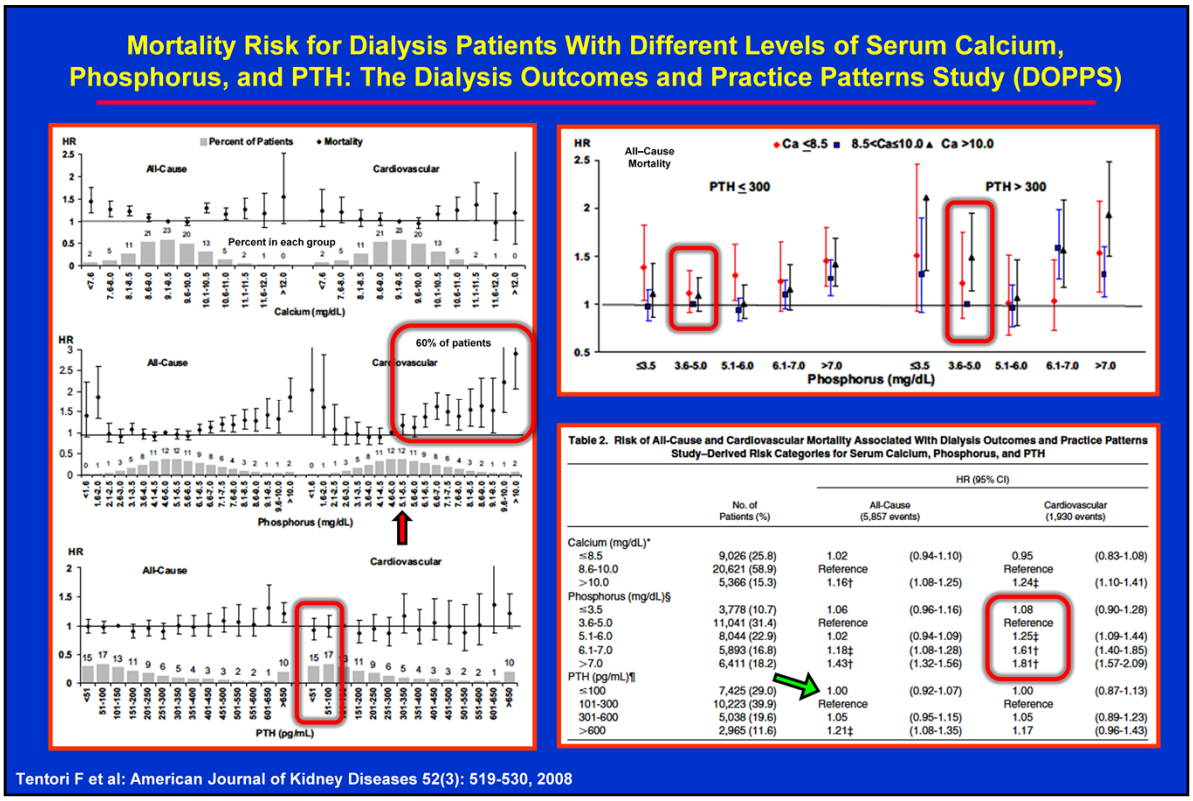


Figure 2. Risk of all-cause and cardiovascular mortality associated with categories of baseline serum calcium, phosphorus, and parathyroid hormone (PTH) levels. Participants, n=25,529. Hazard ratios (HRs) and 95% confidence intervals (whiskers) for all-cause (events, n=5,857) and cardiovascular mortality (events, n=1,930). Models were stratified by study phase and region and adjusted for facility clustering effect, baseline age, sex, race, body mass index, time with end-stage renal disease, and 13 comorbid conditions. Grey bars indicate percentage of patients in each category at baseline.

To convert serum calcium or albumin-corrected calcium (calciumAlb) in mg/dL to mmol/L, multiply by 0.25; serum phosphorus in mg/dL to mmol/L, multiply by 0.323; PTH levels expressed in pg/mL and ng/L are equivalent.

Figure 4. Risk of all-cause mortality associated with combinations of baseline serum phosphorus and calcium (Ca; mg/dL) categories by parathyroid hormone (PTH) level (pg/mL). Participants, n 25,529. Hazard ratios (HRs) and 95% confidence intervals (whiskers) for all-cause mortality (5,857 events). Models were stratified by study phase and region and adjusted for facility clustering effect, baseline age, sex, race, body mass index, time with end-stage renal disease, and 13 comorbid conditions. Separate models were run for patients with baseline PTH levels of 300 or less and greater than 300 pg/mL. To convert serum calcium or albumin-corrected calcium (calciumAlb) in mg/dL to mmol/L, multiply by 0.25; serum phosphorus in mg/dL to mmol/L, multiply by 0.323; PTH levels expressed in

pg/mL and ng/L are equivalent.

Abstract BACKGROUND: Abnormalities in serum calcium, phosphorus, and parathyroid hormone (PTH) concentrations are common in patients with chronic kidney disease and have been associated with increased morbidity and mortality. No clinical trials have been conducted to clearly identify categories of calcium, phosphorus, and PTH levels associated with the lowest mortality risk. Current clinical practice guidelines are based largely on expert opinions, and clinically relevant differences exist among guidelines across countries. We sought to describe international trends in calcium, phosphorus, and PTH levels during 10 years and identify mortality risk categories in the Dialysis Outcomes and Practice Patterns Study (DOPPS), an international study of hemodialysis practices and associated outcomes.

STUDY DESIGN: Prospective cohort study.

PARTICIPANTS: 25,588 patients with end-stage renal disease on hemodialysis therapy for longer than 180 days at 925 facilities in DOPPS I (1996-2001), DOPPS II (2002-2004), or DOPPS III (2005-2007).

PREDICTORS: Serum calcium, albumin-corrected calcium (Ca(Alb)), phosphorus, and PTH levels.

OUTCOMES: Adjusted hazard ratios for all-cause and cardiovascular mortality calculated using Cox models.

RESULTS: Distributions of mineral metabolism markers differed across DOPPS countries and phases, with lower calcium and phosphorus levels observed in the most recent phase of DOPPS. Survival models identified categories with the lowest mortality risk for calcium (8.6 to 10.0 mg/dL), Ca(Alb) (7.6 to 9.5 mg/dL), phosphorus (3.6 to 5.0 mg/dL), and PTH (101 to 300 pg/mL). The greatest risk of mortality was found for calcium or Ca(Alb) levels greater than 10.0 mg/dL, phosphorus levels greater than 7.0 mg/dL, and PTH levels greater than 600 pg/mL and in patients with combinations of high-risk categories of calcium, phosphorus, and PTH.

LIMITATIONS: Because of the observational nature of DOPPS, this study can only indicate an association between mineral metabolism categories and mortality.

CONCLUSIONS: Our results provide important information about mineral metabolism trends in hemodialysis patients in 12 countries during a decade. The risk categories identified in the DOPPS cohort may be relevant to efforts at international harmonization of existing clinical guidelines for mineral metabolism.

Arterial Calcification, Bone Physiology, & Renal Function

Summary

Need to consider 3 processes: **Arteriosclerosis, Osteodystrophy, & Nephropathy (A-O-N)**

Osteogenesis is a "normal" function of arteries & BMP-2 (PTHrp) is required for this function

All of the modulators for osteogenesis are available in arteries (TGFβ, PTHrp, BMP-2, Wnt)

Osteogenesis is triggered by many of the same factors that trigger atherosclerosis

Leptin, Free Radicals, Inflammation, Oxy-sterols, Hypertension, Hyperglycemia

Phosphate appears to be the dominate promotional mineral

Magnesium may be the dominate inhibiting mineral

As bone becomes "non-responsive" it is less able to buffer changes in Calcium & Phosphate

PTH's primary purpose is to maintain serum [Ca++], not maintain bone integrity & strength

PTHrp & Wnt are the primary mediators for maintaining bone integrity

Osteoblasts, BMP-2, OPN, FGF-23 & excess phosphate are not good things to have in arteries

Mg, αKlotho, Adiponectin, & Vitamin D may be the best protectors against **A-O-N**

Arterial Calcification, Bone Physiology, & Renal Function

Potential Therapies → Future Discussions

Therapy → prevent or reverse: **Arteriosclerosis, Osteodystrophy, & Nephropathy**

Established therapies that reduce inflammation, oxidative stress, fibrosis, and arterial calcification:

- Control Lipids – Statins (atorvastatin reduces proteinuria), niacin, fibrates, omega-3, sequestrants
- Control BP – RAAS (ACE-I/ARB & spironolactone), Verapamil (proteinuria), anti-Adrenergic (β-blocker, clonidine)
- Control Glucose – Pioglitazone & Metformin (inhibit Leptin, mTORC1 & NF-κB; increase adiponectin & Klotho)
- Replace Vitamin D → high normal (~60 to 90)

Prevent **Calcification**

Monitor: Renal Panel (Ca^{++}, Phos, & Alb), Mg^{++}, 25VitD, PTH, BSAP, mAlb; DEXA & Coronary Calcium Score

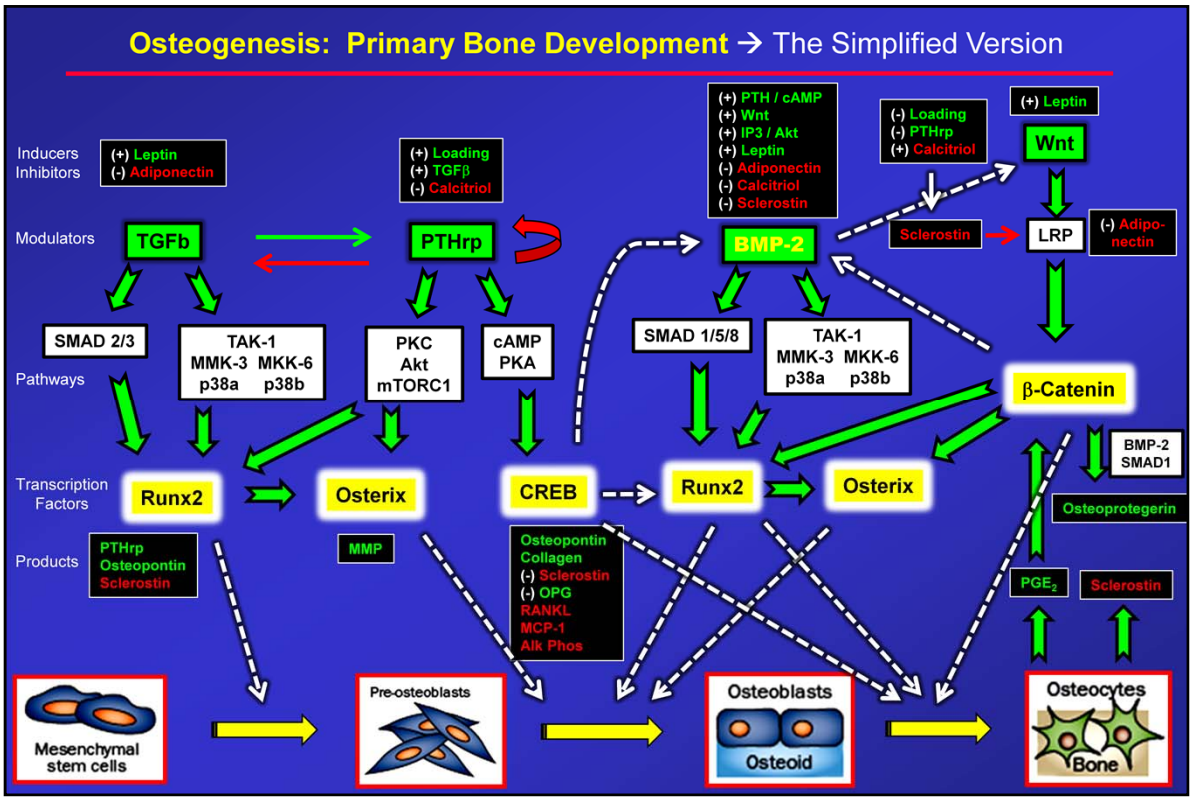
- Maintain Serum Phosphate 3 → 4 & Urine Phos ?? → Diet, Vitamin D, Sevelemer, MagCitrate?, Niacinamide
- Maintain corrected Ca^{++} within normal range → Diet , Vitamin D, Calcitriol, & Calcium Citrate (restrict dairy)
- Prevent Crystallization → Mg^{++}, Citrate, Klotho (Pioglitazone, ARB's, & Statins)
  Fetuin-A & Osteopontin (OPN) probably not good choices

Maintain or reduce **PTH** to <50 if possible without causing hypercalcemia; works best early in disease process

- Calcium Citrate, Vitamin D, Calcitriol, Mg^{++}, citrate, cinacalcet, Klotho
- Is it possible to induce intermittent lowering of PTH? → Prevent PTH resistance which also impacts PTHrp

Osteodystrophy prevention: estradiol, testosterone, DHEA, bisphosphonates, PTH?, denosumab, calcitonin

- Prevent bone ischemia


Osteogenesis: Primary Bone Development → The Simplified Version
Inducers Inhibitors
(+) Leptin
(-) Adiponectin
(+) Loading
(+) TGFβ
(-) Calcitriol
(+) PTH / cAMP
(+) Wnt
(+) IP3 / Akt
(+) Leptin
(-) Adiponectin
(-) Calcitriol
(-) Sclerostin
(-) Loading
(-) PTHrp
(+) Calcitriol
(+) Leptin
Wnt
Modulators
TGFb
PTHrp
BMP-2
Sclerostin
LRP
(-) Adipo-nectin
Pathways
SMAD 2/3
TAK-1 MMK-3 MKK-6 p38a p38b
PKC Akt mTORC1
cAMP PKA
SMAD 1/5/8
TAK-1 MMK-3 MKK-6 p38a p38b
β-Catenin
Transcription Factors
Runx2
Osterix
CREB
Runx2
Osterix
BMP-2 SMAD1
Osteoprotegerin
Products
PTHrp Osteopontin Sclerostin
MMP
Osteopontin Collagen
(-) Sclerostin
(-) OPG
RANKL
MCP-1
Alk Phos
PGE2
Sclerostin
Mesenchymal stem cells
Pre-osteoblasts
Osteoblasts
Osteoid
Osteocytes
Bone