Chapter 3

Regulatory Loops Consisting of Transcription Factors and microRNA Species Determine the Mineralizing Characteristics of Cell Phenotypes — Implications for Bone Engineering and Prevention of Soft Tissue Mineralization

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1. Introduction

1.1. Regulation of mineralization in bone and soft tissue

The first stages of cell mediated tissue bio-mineralization involve the activation of various signaling pathways, such as those activated by growth factors and hormones like BMPs, TGFs, PTH, Leptin, Vitamin D and many others.

One major signaling pathway responsible for bio-mineralization is the Wnt-pathway. Wnt-mediated transduction occurs via both β-catenin dependent and independent signaling. In β-catenin dependent signaling, extracellular Wnt ligands bind to the LRP5-Frizzled (Frz) complex, subsequently inhibiting an intracellular cluster of molecules comprised of axin, glycogen synthase kinase 3 (GSK3), and the adenomatosis polyposis coli (APC) protein. This complex then inhibits the cytosolic degradation of β-catenin, which accumulates and subsequently enters the nucleus to heterodimerize with two important transcription factors, LEF and TCF, conferring the Wnt-effect on gene transcription. Via the β-catenin independent signaling pathway, a similar complex forms between Wnt, Frz, and Ror2, which stimulate the synthesis of secondary messengers [1, 2].

Another important pathway is the one mediated via Hedgehog. The Hedgehog (HH) ligand precursor is subjected to a series of modifications before reaching an active, multimeric form. This process releases the transcription factor Smoothened (SMO), which in turn activates the Gli2/3 complex, which goes on to promote gene expression of Gli1, while repressing the transcriptional repressor Gli3 [3].

A third pathway is the NELL-1 signaling pathway. NELL-1 is a secretory osteoinductive protein, binding to the cell surface receptors Integrinβ1&3. A multitude of intracellular signaling pathways are activated due to NELL-1 stimulation. These include MAPK, Hedgehog, and β-catenin dependent Wnt-signaling. Despite the fact that these pathways are still undefined, NELL-1 activation enhances Runx2 transcription and phosphorylation, and development of an osteogenic phenotype [4].

The last stage of bio-mineralization encompasses the precipitation of hydroxyapatite crystals, which spontaneously results in supersaturated or metastable salt solutions. Whether bio-mineralization will take place, is determined by the genetic programming of precursor cells into a mineralization-competent state (as in physiological bone formation=osteogenesis) or to pathological mineralization (i.e. ectopic mineralization or calcification) [5]. Hence, all tissues which are not meant to minerealize, should be actively "protected" or inhibited from precipitation of mineral. Fetuin-A is a circulating protein produced by the liver, directly inhibiting ectopic mineralization. Monomeric fetuin-A protein binds small clusters of both calcium and phosphate. This interaction results in the formation of prenucleation cluster-laden fetuin-A mono-and polymers, calciprotein monomers, and considerably larger soluble aggregates of protein and mineral calciprotein particles of colloid (soluble) nature [6, 7].

In this manner, fetuin-A serves as a mineral carrier protein and a systemic inhibitor of pathological mineralization, i.e. mineralization not brought about by bone residing osteoblasts, but by fibroblast like cells demonstrating mineralizing phenotypic characteristics.

1.2. Untoward calcification of soft tissues; some model systems

Vascular calcification inevitably afflicts the aging and dysmetabolic population. Modern concepts state that this process has emerged as a highly regulated form of bio-mineralization organized by collagenous and elastin extracellular matrices. Paracrine osteogenic signals, mediated by potent morphogens of the bone morphogenetic protein (BMPs) and wingless-type integration site family member (Wnt) superfamilies, are also active in the programming of arterial osteoprogenitor cells during vascular and heart valve calcification. Inflammatory cytokines, as well as reactive oxygen species, and oxylipids (which are more active within the clinical settings of atherosclerosis, diabetes, and uremia) elicit the ectopic vascular activation of osteogenic morphogens. Specific inhibitors (e.g. MGP, CV2, COMP2, Noggin, Gremlin, Chordin, and Folliastatin) of bone BMP-Wnt signaling have been identified, contributing to the modulation of osteogenic mineralization during development and disease. These inhibitory pathways and their regulators afford therapeutic strategies to prevent and treat valve and vascular sclerosis [1, 3].

In this context, the expression of vascular BMP-2 and BMP-4 (mostly elaborated by endothelial cells) are actively enhancing [Smad-DNA]-binding, thus trans-activating a cascade of factors like Msx2, Runx2, NFATc1 and Osterix (SP7), deemed necessary in the mineralizing process [1, 3]. The downstream pro-osteogenic signaling cascades initiated through heterodimer BMP receptors is held in check by a panoply of extracellular BMP binding proteins and intracellular transcriptional inhibitors (I-Smads, i.e. Smad6 and Smad7) [1, 3].

Calcific aortic valve stenosis (CAVS) is an increasing health problem affecting aging societies. The discovery of osteoblast-like and osteoclast-like cells in the heart have forced a paradigm shift where CAVS is considered to be actively regulated. It has also been shown that valvular fibrosis, as well as calcification, plays an important role in restraining cusp movement. Furthermore, CAVS should probably be regarded as a fibro-calcific ailment [8, 9].

It has been speculated what type of precursor cells are being able to turn into osteoblast-like cells in aortic valves, giving rise to human calcific aortic valve stenosis. Activated myofibroblasts are likely to come from either quiescent valvular interstitial cells (VICs) or from a subpopulation of endothelial cells that undergo an "endothelial to mesenchymal transformation" (EMT) [1, 8, 10]. Lastly, it has been asserted that circulating osteoprogenitor cells (positive for OPN and ALP) may enter the active side of the valve from the circulation [9]. Hence, it should be noted that a lack of "bone-homing" of osteoblast-precursors, as seen in the elderly population, may lead to the development of CAVS. It is also noteworthy that monocytes may enter the valve tissue, transform into osteoclast-like cells and/or inflammatory Th-cells, and thus affect the above mentioned osteoprogenitor cells to start a mineralization process due to their response to TNFα and interleukins [1, 8, 9].

Blood vessels are the first to form in the developing embryo and build extensive networks supplying all cells and organs with nutrients and oxygen. An ageing blood vessel often becomes abnormal in structure and function, thus contributing to a plethora of age-related diseases like ischemic heart&brain-disease, neurodegeneration, and/or cancer. The first regulators to be linked to the aging process were the Forkhead box "O" (FOXO) transcription factors and sirtuin (Sirt) deacetylases [9, 11]. They are now emerging as key regulators of the vascular development and disease. The integration of FOXO-and Sirt-family members into the aspect of vessel maintenance, offers new perspectives on mechanisms of aging, which is the most important risk factor for diseases of vascular system [9, 10].

The FOXO transcription factors control a plethora of cellular responses, encompassing apoptosis, DNA-repair, metabolism, as well as ROS detoxification and cell proliferation. On stimulation of PI3K/AKT-signaling by growth factors, AKT phosphorylates FOXOs on conserved residues with ensuing cytoplasmic sequestration and inactivation [1, 10]. Of specific interest for the precipitation of atherosclerotic plaques is FOXO3A, however, FOXO1 and FOXA3 should also be mentioned, since they has been shown that they hetero-dimerize with the transcription factor SXR, known to bind vitamin K2 [12-14].

The Sirts (especially Sirt1) are involved in the modulation of key genes involved in regulating lifespan and health span, including AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), and insulin-like growth factor 1 (IGF-1), and their roles modulating

cardiovascular health status [15, 16]. It is to be noted that the FOXO class of transcription factors affect the levels of sirtuins (including Sirt1), thus enabling the body's cell-and tissue armamentarium to respond to the energy state (NAD^+/NADH-ratio) in a proper manner to preserve organ function and longevity [10, 15]. In this context, the FOXOs and sirtuins are both instrumental for the heart and vasculature to avoid the numerous detrimental alterations during ageing.

Finally, the present report focusses on altered gene expression leading to proper or disturbed calcification of bone and soft tissues, as a result of perturbed transcription or posttranscriptional control. Particularly, certain transcription factors (TFs) and microRNAs (miRNAs) will be described [17, 18], which are members of so-called regulatory loops [19].

Interestingly, several groups or clusters of miRNAs, targeting several hundred mRNA species encoding TFs, as well as tissue specific or non-specific genes, have been shown to impact conditions like cardiac hypertrophy and cardiac fibrosis [17, 18].

1.3. MicroRNAs in atherosclerogenesis

The dysregulation of cholesterol homeostasis is one of the underlying causes of atherosclerosis. One regulatory factor in cholesterol metabolism includes the sterol regulatory element-binding protein (SREBP) family, which regulates the expression of a plethora of cholesterogenic genes [20]. The microRNA species 33b and 33a, respectively, have been shown to target the ATP-binding cassette A1 cholesterol transporter [21], which mediates intracellular cholesterol efflux from cells to form HDL that is protective against atherosclerosis [22, 23]. Upon injection of antisense miR-33 into western diet-fed mice, serum HDL levels were significantly elevated, and treatment of LDL-receptor deficient animals with anti-miR-33, resulted in augmented levels A1 in both liver and macrophages, with a net increase in blood HDL-levels along with reduced plaque size and inflammation-related gene expression [23].

However, other microRNA species, such as the miR-145/143 cluster, miR-133, and miR-221, exert a more direct role in atherogenesis. The miR-145/miR-143 cluster is enriched in visceral and vascular smooth muscle cells (VSMC) from early embryonic days and throughout adulthood [24-26]. Genomic deletion of this cluster yielded a mild vascular phenotype with no cardiac abnormalities, however, smooth muscle cells were smaller and exhibited an increase in rough endoplasmic reticulum (RER) and a decrease in actin stress fibers, resulting in thinner tunica media [26]. The failure to detect a consistent increase in VSMC proliferation or apoptosis indicates the miRNA cluster is involved in the ability of VSMC to differentiate in response to contractile demands [25].

Krupple-like factor-5, first identified as a transcription factor inducing the gene expression of smooth muscle myosin heavy chain, is involved in smooth muscle proliferation by stimulating cyclin D1 expression [27, 28]. MiR-133 is known to be enriched in the heart and skeletal muscle, but one report asserts that it is also expressed in smooth muscle cells [29]. Over-expression of miR-133 in the carotid artery has been shown to halt VSMC proliferation and prevent neointimial hyperplasia [17]. Interestingly, the level of miR-133 in this tissue exhibits a strong inverse correlation with its targets, one of which is the transcription factor SP1, and it may be asserted

that SP1 inhibition leads to a deactivation of Krupple-like factor-5, which in turn prevents down-regulation of Myh11 (myosin heavy chain gene family), and hence VSMC phenotype switching and proliferation [17].

In contrast to miR-145 and miR-133, miR-221, but not the co-clustered miR-222, positively regulates smooth muscle proliferation [30]. It is believed to be induced by platelet-derived growth factor (PDGF), which is known to stimulate VSMC switching and proliferation during angiogenesis and neo-intimal formation [31]. In gain-and-loss-of function experiments, it was demonstrated that miR-221 was required for mediating the effects of PDGF on the suppression of the cell-cycle inhibitors p27Kip1&2, and c-kit [30, 31]. The effects of miR-221 are reinforced by the concurrent up-regulation of miR-21 in neointimal lesions, and miR-21 was shown to inhibit PTEN, which acts as a dual-specificity protein phosphatase, dephosphorylating tyrosine-, serine- and threonine-phosphorylated proteins [32-34], antagonizing the PI3K-AKT/PKB signaling pathway [35]. In this manner, miR-21 inversely modulates apoptotic VSMC death and reduces neo-intimal thickness. Consequently, neo-intimal formation is brought about by a combinatorial effect of changes in the expression of several miRs and their targets, with ensuing regulation of VSMC differentiation, proliferation, and survival.

However, the scope of the present book chapter, is to focus on microRNA species being involved in the process of matrix mineralization, whether or not they are intrinsic miRs residing within osteoblasts or smooth vascular cells or "imported" miRs via exosomes shedded by immune cells (like macrophages and Th-cells) invading the vasculature due to inflammatory processes. One may assert that microRNA species down-regulated in mineralizing osteoblasts should be up-regulated in healthy, non-calcifying soft tissues like blood vessels and heart valves. However, this is a simplification of the issue, since the acquired osteoblastic or mineralizing phenotype is secured by a balanced impact of microRNAs targeting osteogenic markers (or modulators) like BMP-2, SMADs, Runx2, Osterix (SP7), Dkk-1, and RANKL and others, a loss of calcification inhibitors like MGP and Fetuin-A, and finally a loss of smooth muscle cell markers like α-actin and certain MHC-class of antigens, as shown by Goettsch and co-workers [18]. This major compilation of published literature indicates that several microRNAs well known to be down-regulated in osteoblasts (like the miR-species 23a, 24, 27a, 29a, 34c, 133a, 135a, 149, 204, and 328) [36-39] are not necessarily down-regulated in "mineralizing" or "calcifying" vascular cells.

Interestingly, it has been shown that Th17-cells produce exosomes containing high levels of microRNAs (like miR-16, -24, -27a ,-27b, -125b, and -586) known to be low in developing and mature osteoblasts [36, 37], while also carrying high levels of, amongst many; miR-21, -22, -221, -222, and -520 (data not shown). According to Goettsch et al. [18], miR-21 is up-regulated in atherosclerotic arteries, while all the other mentioned miRNAs are down-regulated in arteries, plaques or bicuspid aortic valves. It is therefore necessary to define a cluster of minimal and sufficient microRNAs, which may represent a signature for soft tissue calcification. Hitherto, we do not see that such a cluster has been suggested, nor validated.

It is therefore of major interest to seek help from bioinformatics software to define regulatory loops, consisting of microRNAs, transcription factors and marker genes, known to be sensitive and prone to alterations in precursor cells, but resilient to changes in defined phenotypes. It

is therefore exciting to learn that miR-133, already known to affect SP1 [40] seems to be players in regulatory loops, consisting of a selection of microRNA species and transcription factors, where miR-22, -27, -29, -133, -149, and -328, are connected to FOS, ETS1, SP1, SP3, RUNX1, FOSB, and EGR2, in a reciprocal feed-forward and feed-back stabilizing network.

2. The histopathology of vascular calcification

Vascular calcium deposition can be usefully organized into four histoanatomic variants. As outlined, each type of vascular calcification is associated with a characteristic spectrum of vascular disease processes. Moreover, in the setting of the calcified atherosclerotic plaque and senile calcific aortic sclerosis, the initial dystrophic calcification process evolves during vascular injury and remodeling into endochondral, non-endochondral, or mixed ossification mechanisms.

2.1. Atherosclerotic calcification

Atherosclerotic calcification is a dystrophic calcification characterized by cellular necrosis, inflammation, and the presence of lipoprotein and phospholipid complexes [41, 42]. The lipid complexes, originating from cellular membranes, thrombo-fibrinoid complexes, and circulating lipoproteins precipitate calcium in association with atherosclerotic plaques. The ensuing endothelial cell dysfunction provides a thrombogenic surface coated with fibrin and phospholipids, which drive additional lipid deposition. Oxidized lipid products provide several signals that recruit and activate macrophages and T-cells [43]. The calcification appears first in the lipid core of the fibro-calcific plaques, juxtaposed to inflammatory cell infiltrates and necrotic areas. Eventually, calcified cartilage formation follows the degenerative tissue calcification via a vascular remodeling process, leading to the deposition of endochondral bone [43].

2.2. Calcification of cardiac valves

Cardiac valve calcification occurs following mechanical stress and inflammation, leading to dystrophic mineralization and non-endochondral ossification. Degenerative lipid accumulation, fatty expansion of the *valvular fibrosa*, and interstitial calcium deposition are rapidly followed by the invasion of macrophages and T-cells [44, 45]. Hence, valve calcification of the young and elderly, is initiated via overlapping mechanisms, but distinct from calcification observed within atherosclerotic plaques [46-49]. However, during progression of the calcification process, histological and molecular analyses indicate that a secondary phase of active, osteogenic mineral deposition perpetuates the vascular calcium accumulation, but via non-endochondral processes [50, 51].

2.3. Medial artery calcification

Calcification of the medial artery is a non-endochondral ossification process of the arterial *tunica media*, occurring frequently in patients suffering diabetes and end-stage renal disease

[52]. The mineralization pattern resembles intra-membranous calvarial bone formation and odontogenesis [53], driven by the BMP-2/Msx1&2-dependent signaling pathway in the absence of any cartilaginous precursor [54-56]. Many hypotheses of the major molecular determinants of medial calcification vs atherosclerotic calcification with cartilage metaplasia, have been launched, however, these determinants remain elusive. Two model systems may render some clues, though: the characteristics of vascular calcification responses of the (LDLR$^{-/-}$) and (apoE$^{-/-}$) mice [55-57]. Both animal model systems develop atheroma; however, LDLR$^{-/-}$ mice calcify valves and tunica media via a non-endochondral osteogenic process, while apoE$^{-/-}$ mice calcify vessels via cartilage metaplasia.

It has been shown that a myofibroblast cell population, activated by diet-induced diabetes, is diverted to the osteoblast lineage by Msx2-dependent transcriptional programming [54-56]. It was therefore asserted that this migratory myofibroblast population, which responds to vascular smooth muscle cell (VSMC) osteopontin (OPN) production [56, 58, 59], contributes to vascular remodeling and the medial calcification of diabetes. Interestingly, both hyperglycemia [56, 58] and hyperphosphatemia [60] induce OPN expression, which *per se* is a consistent and predicted feature of medial calcification [61-63]. Furthermore, the absence of the osteoprotegerin (OPG) gene expression in mutant mice also results in medial calcification with ensuing vascular T-cell infiltration [64]. Noteworthy is the observation that blood levels of OPG are increased in diabetic humans [65] and diabetic (LDLR$^{-/-}$) mice [55], which may indicate a state of resistance to OPG, namely inhibition of RANK-L signaling. It has been shown that the OPG/RANK-L secretion ratio is enhanced in carotid plaques compared to femoral plaques, which explains why carotid plaques contain less calcium and number of macrophages and T-cells [43].

2.4. Vascular calciphylaxis (soft tissue calcification)

Vascular calciphylaxis occurs when the calcium phosphate solubility threshold is exceeded [66]. When the serum calcium-phosphate product exceeds this threshold, widespread soft tissue deposition of amorphous calcium phosphate will occur. To prevent this happening, an array of mineralization inhibitors have evolved, encompassing fetuin-A, tissue pyrophosphate generating systems, as well as tissue OPN production [67].

Fetuin-A, which is an abundant serum glycoprotein, has been shown to limit organ and soft tissue calcification, including vascular calcium deposition [67]. Pyrophosphate inhibits nucleation and epitaxial calcification and also up-regulates the expression of OPN [68, 69]. The generation of tissue pyrophosphate is obtained via ectonucleotide pyrophosphatases/phosphodiesterases (ENPPs). ENPP1 (or PC-1) is instrumental in limiting calcification in "soft tissues", like blood vessels, skeletal muscle fibers, ligaments and tendons [68, 69].

2.5. Vascular calcification and cartilage metaplasia

In vertebrates, bone formation occurs via both endochondral and non-endochondral mechanisms [53]. In endochondral ossification, avascular cartilage is subjected to vascular invasion, cartilage calcification, remodeling by osteoclasts, and eventually deposition of bone by

osteoblastic cells. Endochondral bone formation and neovascularization depend heavily on the expression of Runx2, acting in concert with Sox9 [53, 70]. The array of molecular players in endochondral ossification is also associated with the progression of untoward, degenerative atherosclerotic calcification [71], as shown in models of heritable vascular calcification [72, 73]; matrix Gla protein (MGP) knockout mice and apoE knockout mice, which both develop arterial cartilage metaplasia.

In this context, MGP functions as a noggin-like inhibitor of vascular BMP-2 mediated signaling [74]. Hence, in the absence of MGP, enhanced BMP-2 signaling may promote vascular cartilage metaplasia. Loss of the intracellular BMP-R2 signaling inhibitor Smad6 also provokes arterial cartilage metaplasia and medial endochondral bone formation [75]. Noteworthy is that BMP-2 signaling can drive both chondrogenic and osteogenic differentiation of multipotent mesenchymal precursor cells [76]. However, it is important to emphasize that there is a finely tuned balance between sustained Msx2 expression, which will promote osteogenic differentiation at the expense of adipocyte and chondrocyte development [54, 63] and "intermittent" Msx2 expression. The former will secure functional bone remodeling, while the latter is meant to prohibit mineralization of soft tissues [43]. Unfortunately, this balance may be tilted in both directions, resulting in less mineralized bone tissue and untoward calcification of soft tissues.

2.6. The modulatory effect of vitamin K2

Vitamin K1 and the VCOR activation cycle is apparently of great importance for the status of bone health through the influence of bone derived carboxylated osteocalcin (OC) on several other organ systems, like pancreas (insulin secretion), adipose tissue (adiponectin secretion) and testis (testosterone production), which all form reciprocally interacting organs in a homeostatic organ cross-talk system [77-80]. However, since vitamin K2 (MK-7 and/or MK-4) has been shown to be a ligand for the steroid and xenobiotic receptor SXR also designated pregnane X receptor PXR, orphan nuclear receptor PAR1, and NR1/2 [81], the knowledge of which genes are transcribed with the aid of MK-7 or MK-4 in different organ systems or cells [12-14, 82, 83], is vital to understand how vitamin K2 status affects organ homeostasis in general, and especially bone health and the regulation of detrimental mineralization of soft tissues in particular [1, 8-10, 84].

Apart from the up-regulation of mRNAs for OC, BMP-2, and RANK-L, and alleviation of TNFα-mediated suppression of pro-bone SMAD expression in osteoblasts [13, 14], it has been shown that MK-7/4 in bone acts as a general transcriptional regulator of extracellular matrix-related genes that are involved in the collagen assembly (indicated by gene ontology analyses). Hence, MK-7/4 synergizes with calcitriol (active vitamin D=1,25$(OH)_2D_3$) in maintaining bone growth and homeostasis throughout life. However, it seems that MK-7/4 deserve a closer scrutiny, since there are a plethora of additional modulatory hormone-like effects exerted by this "vitamin", as envisaged by its modulatory effect on gene expression in osteoblastic cells. Some interesting features are summarized in the table underneath:

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Gene	*Biological effect related to biomineralization processes*
CSNK1A (casein kinase 1) (see Gene Card)	Modulates Wnt and hedgehog (HH) signaling pathways, which play a major role in the differentiation of stem cells to osteoblasts, and contributes to the features of mineralizing osteoblasts
FETUB, fetuin B (see Gene Card)	Enables super-saturation of free Ca-P_i molecules, thus prohibiting precipitation of calcium-hydroxyapatite crystals in soft tissues
MAPK9 (MAP Kinase 9) (see Gene Card)	When activated, promotes β-catenin (CTNNB1) degradation and inhibits the canonical Wnt signaling pathway
SMAD2 (SMAD family member 2) (see Gene Card)	SMAD2 is involved as a transcription factor contributing to the activity of SMAD2/ SMAD3:SMAD4 heterotrimer and down-regulation of TGF-β receptor signaling. Involved in the regulation of osteoblastogenesis and acquisition of mineralizing properties of cells in general
JUN (c-JUN, JUNB, JUND) (see Gene Card)	Part of the dimeric AP1 complex. JUN associates with FOS, thereby affecting differentiation of osteoblasts, osteoclasts, as well as immune cells (e.g. T-cells)
RYK (Receptor-Like Tyrosin Kinase) (see Gene Card)	May be a co-receptor along with FZD8 of Wnt proteins, such as WNT1, WNT3, WNT3A and WNT5A (of which WNT3A&5A are important for osteoblastogenesis and mineralization)
CRSP3 (Mediator complex subunit 23) (see Gene Card)	**The protein encoded by this gene is a subunit of the CRSP complex, necessary for efficient activation by SP1**

Table 1. Genes affected by Vitamin K2 (MK-7 or MK-4), as described by "Gene Card" on the Web. The genes tabulated are involved in biomineralization processes as described by Slatter and co-workers [85].

In short, vitamin K2, without any doubt, heavily affects the phenotypic characteristics of osteoblastic cells, i.e. both differentiation of stem cells to mature osteoblasts, as well as their mineralizing capacity. Furthermore, it seems that vitamin K2 is involved in the steady state level of SP1, the transcription factor, shown to be implicated in reciprocal regulatory loops with several microRNA species known to be discriminators of osteoblast differentiation and function [1, 3, 36-38]. Hence, it may be postulated that vitamin K2 may serve as an important modulator of the process of bio-mineralization, stabilizing cell phenotypes within a narrower span of features. In other words, vitamin K2 may optimize mineral deposition in bone, while blocking untoward mineralization in soft tissues.

3. Putative problems, and future directions

The interaction between hormonal, metabolic, inflammatory, and mechanical stressor molecules, as well as passive mineralization inhibitors determines the ultimate "phenotype" of osteoblasts mediating mineralization in bone, as well as vascular progenitors regulating the deposition of calcium in soft tissues. However, several issues have yet to be resolved concerning the regulation of vascular calcification. Firstly, a detailed comprehension of the origins of

the chondro-and osteo-progenitors is still elusive [43]. Secondly, the relative contribution of trans-differentiating VSMCs and migratory myofibroblasts to vascular calcification responses is not fully understood [43]. Additionally, a "homing response" induced by a vascular injury will probably recruit circulating marrow skeletal progenitors [86] and contribute to damage progression, including the development of ectopic vascular bone marrow in association with vascular osteogenesis [43]. And finally, a better (i.e. minimal, but necessary and/or exhaustive) set cell surface markers of the mesenchymal cell lineage seem to be required.

Furthermore, the impact of T-cells and macrophages in vascular calcification is not fully understood, but may explain the reciprocal relationships between bone turnover and vascular calcification. One interesting phenomenon is the shedding of microRNA-containing exosomes from these immune cells [87]. Hence, future efforts in identifying which of these miRNA species and other factors, which may "alter" the phenotype of the fibroblast like cells in vessels and heart valves, are welcomed.

In a clinical setting, once vascular osteogenic tissue has acquired the ability to form mature bone, there is a possibility that anabolic bone-building hormones intended to treat osteoporosis may also augment mineral deposition in soft tissues via coupled matrix turnover processes. It should also be taken into consideration that diseases, like end stage renal disease (ESRD), hyperglycemia, hypertension, hypercholesterolemia, hyperphosphatemia, PTH-resistance, and iatrogenic calcitriol excess may all contribute to the vascular calcium load [88-90].

Hence, it seems that the reciprocally regulated calcification of various body organs/tissues leaves little "space" for non-overlapping phenomena: calcification in bone with no soft tissue mineralization, and decalcification of soft tissues without losing bone mass. To solve the problematic issue of these tied and reciprocal phenomena, on may resort to apply the fine-tuning ability of bio-molecular "players" like vitamin K2. This vitamin, with its hormonal actions through the transcription factor SXR, seems to enable the body to control both bone mineralization and strength [91-93] and counteract soft tissue calcification in an optimal fashion [81, 94].

4. Materials and methods

In general, the description of materials and methods used is depicted in Figure 1. The results presented in this chapter are based on the following:

Materials: Human mesenchymal stem cells (hMSCs), human fibroblasts/HUVECs were either from in-house stock strains, or obtained from commercial sources. Human MSCs were differentiated to osteoblasts and heart valvular interstitial cells (VICs) according to standard procedures [95], while the fibroblasts/HUVECs were used directly on demand. The cells were exposed to appropriate media and differentiation protocols described elsewhere [96]. Furthermore, bovine calf bone chips were obtained fresh from the slaughterhouse and incubated with or without PBMCs differentiated to osteoclasts, according to standard procedures [97-99].

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Methods (incubations, manipulations and analyses): The data presented herein mainly emanate from manipulation of differentiated cells (transfected with vectors expressing various transcription factors, i.e. SP1, ETS1, and RUNX1) in control and mineralizing media (see above) with siRNAs, pre-miRNAs and/or antago-miRNAs (corresponding to miR-149 and miR-328) in the presence or absence of either cytokines (TNFα, IL-1, IL-6, and IL-17A) or vitamin K2 (MK-7). End point analyses of results obtained were performed using mRNA isolation techniques (Quiagen), Q-PCR analyses of pertinent gene transcripts according to the literature [100-102], ELISA-based quantification of secreted cell marker growth factors/cytokines and others (e.g. osteocalcin, IL-10, TGFβ, OPG, and RANK-L). Finally, mineralization surface was measured using the Alizarin red S dye on cells in monolayers [103].

Bioinformatics: Interactions between microRNA-species, transcription factors (TFs) and cell phenotype "specific" marker genes were emulated using the Mir@nt@n algorithm [19]. On the charts, genes and microRNAs are visualized like this: TFs in red boxes; miRNAs in orange rectangles.

Statistics: Mean values were considered significantly different from controls when $p < 0.05$ (non-parametric testing, n=9) (refs). Differences compared to controls are marked with a star *.

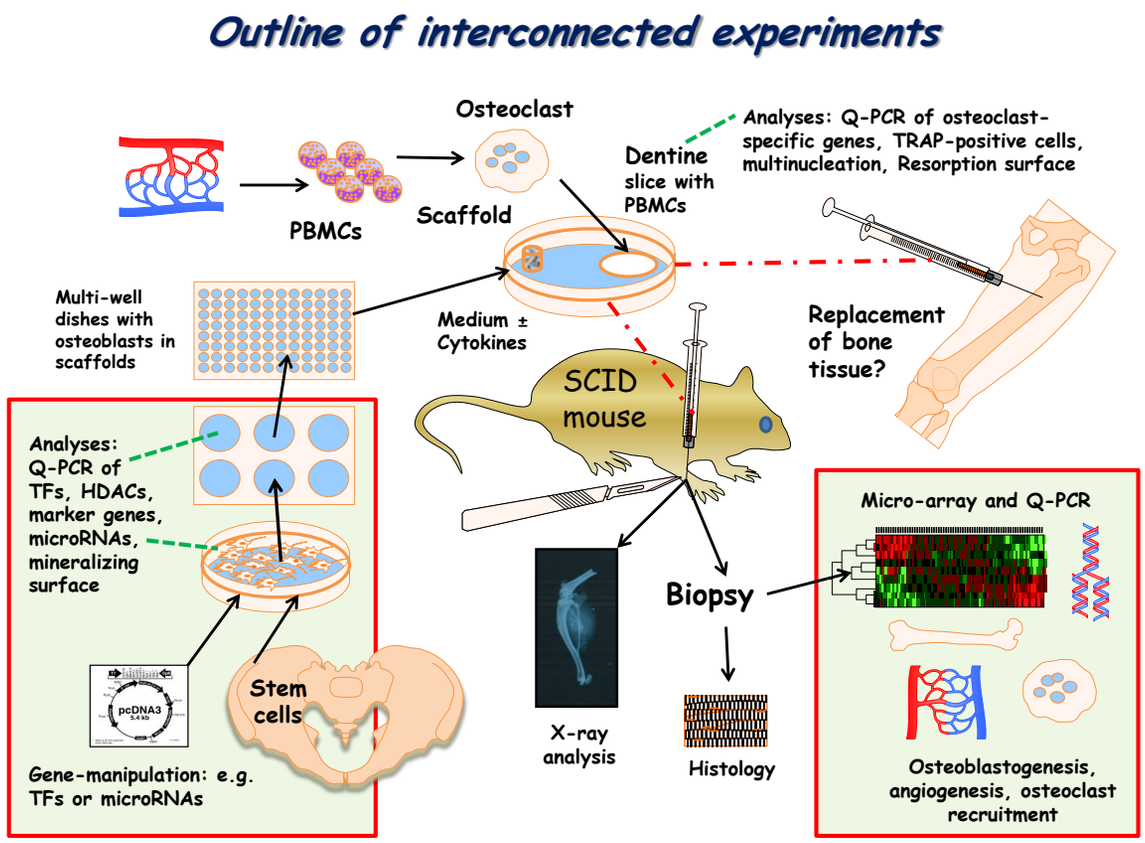

Figure 1. Illustration of interconnected experiments contained within a differentiation scheme of stem cells towards osteoblasts, manipulation of cellular phenotypes in the absence and presence of inflammatory cells or their cytokines, as well as analyses of parameters pertaining to matrix deposition and its calcification.

5. Results

5.1. Bioinformatics analyses using the Mir@nt@n algorithm

Figure 2, which is based on available osteoblast transcriptome data, featuring modulated transcription factor (TF) mRNAs and microRNAs (miRs), shows that ETS1, SP1, and RUNX1 (encircled in green) are involved in feed-forward/feed-back regulatory loops with several miRs included within the Stein [38] and Gordeladze [37] "miR-signatures" for osteoblastic cells. The emulation was performed with the highest possible stringency. Other TFs within the same network system are FOS, FOSB, SP3, and EGR2.

Transcription factors	MicroRNA species «connected»
SP1	Let-7f, 18b, 24, 27a, 27b, 29a, 29c, 31, 96, 135b, 141, 149, 182, 200a, 377, 522, 597
ETS1	19a, 34a, 125a-5p, 133b, 135b, 148b, 206, 222, 328, 377, 492, 522
FOSB	23a, 23b, 27a, 27b, 152, 182, 200a, 204, 220c, 224, 637, 638
RUNX1	17, 20a, 20b, 23a, 27a, 27b, 30a, 91, 93, 106a, 141, 494
JUNB	Let-7a,c,d,f, 15a, 93, 326, 494, 597
EGR2	20b, 23a, 25, 106a, 137
SPI1	34a, 155, 326, 663
FOS	29a, 29c, 149, 597
SP3	133b, 135b, 182, 191
JUN	9, 200b, 522, 637
STAT1	20a, 20b
SOX9	206

Figure 2. Tabulation of transcription factors (TFs) targeted by various microRNA species (either predicted by the Mir@nt@n algorithm or directly shown in experiments with reporter constructs). TFs and microRNAs focused on in this chapter are highlighted in yellow and red (the Gordeladze osteoblast-"signature") or green (the Stein-"signature"), respectively.

Figure 3 shows the result of a Mir@nt@n emulation with low stringency, indicating that SP1, ETS1, FOSB, and RUNX1, are the TFs putatively binding the largest number of microRNA species. It should be emphasized that the "Stein-signature" of miRs (highlighted in green) are involved with more TFs than the "Gordeladze-signature" (highlighted in red), indicating that the former is a better marker and/or predictor of the osteoblast phenotype.

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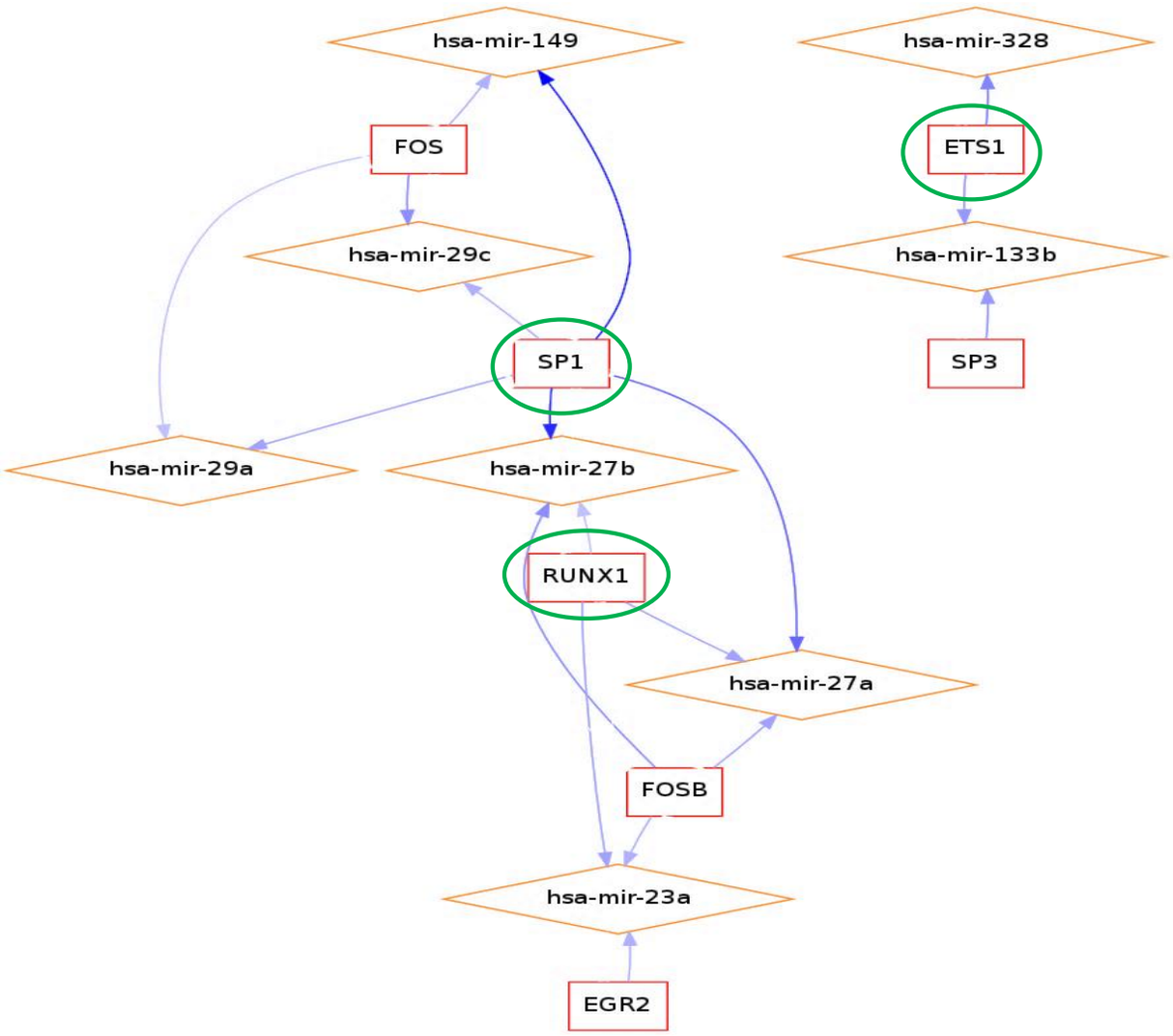

Figure 3. High stringency emulation of feed-forward and feed-back ("reciprocal") regulatory loops encompassing transcription factors and microRNA species using osteoblast transcriptomes and microRNA species known to be involved in the differentiation and/or function of mineralizing osteoblastic cells [19].

Figure 4 shows the network of interacting TFs and miRs of the "Gordeladze-signature" including histone deacetylaces (HDACs) emphasizing the important feed forward/feed-back loops involving SP1 and ETS1 with the microRNA species 149 and 328. Many of the "Gordeladze-signature miRs are interacting with sirtuins (e.g. SIRT1, one HDAC subspecies), which indicates that the regulatory TF-miR system is influenced by the osteoblast's energy status, since it is known that the NAD^+/NADH-ratio is influencing the activity of HDACs [10].

Figure 5a&b shows Q-PCR-data on transcription factor, marker gene and microRNA levels, as well as mineralizing surface (%) in osteoblasts exposed to cytokines (cfr**. Methods**) transfected with vectors containing SP1, ETS1 or RUNX1, or TF+cytokines (average of all three TFs from separate experiments). In essence, the two panels indicate that the cytokine mix is detrimental to the osteoblasts, and that over-expression of the transcription factors or antago-

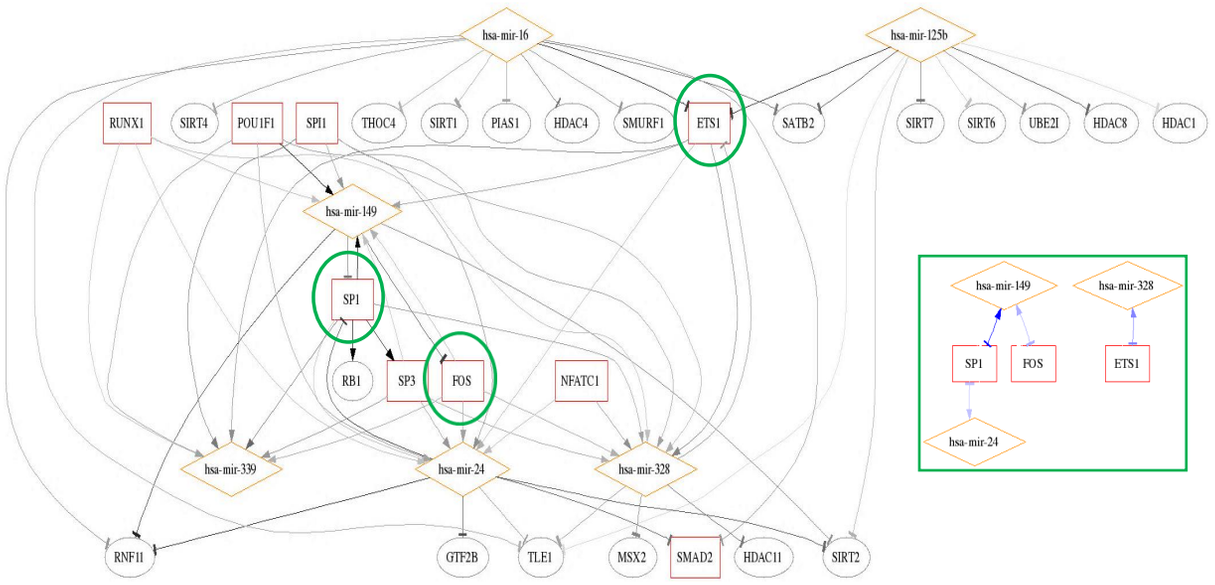

Figure 4. High stringency emulation of microRNA species interacting with transcription factors (TFs), functional marker genes and the complete published "Gordeladze mini-signature" of microRNAs in osteoblasts, including histone deacetylases (HDACs and Sirtuins=Sirts). Insert: Resiprocal regulatory loops involving the miR-24,-149, and-328, and the TFs SP1, FOS, and ETS1.

miRs counteract the negative impact of the cytokines on the osteoblast phenotype. Sections of Alizarin red S colored osteoblasts are depicted underneath the tables.

Figure 6 a&b indicates the same type of analyses/data as Figure 5. Here, Q-PCR-data and per cent mineralizing surface are presented. DKK1, which is an inhibitor of osteoblastogenesis is, inversely regulated within the various treatment groups, compared to the other markers. However, the data set grossly indicates that cytokine exposure "deprives" the osteoblasts of their phenotype markers, while overexpression of either transcription factor (SP1, ETS1 or RUNX1) in the presence of all cytokines "reinstates" the osteoblast phenotype characteristics.

Figure 7 shows one Mir@nt@n-emulation of regulatory network lattices consisting of osteoblast related transcription factors and microRNA species. The insert shows that the vitamin K2 receptor SXR (also known as NR1I2) associates with miR-760, which is tied to JUNB in a reciprocal looping system, connecting vitamin K2 to a network resembling the one featured in Fig. 2. However, vitamin K2 apparently needs miR-597 to connect to this looping system. The significance of miR-597 is discussed in **Section 6.3**.

Figure 8 depicts the effect of the vitamin K2 analogue MK-7 (menaquinone-7) on the secretory function of osteoblasts embedded in bovine bone slices exposed to either, MK-7 (10 ng/ml), SXR-siRNA or pre-miR-760. Clearly, MK-7 exposure of the bone chips for 7 days enhances the secretory profile (osteocalcin, IL-10, TGFβ, OPG and RANKL) measured with ELISA-kits. Furthermore, concomitant incubations with either SXR-siRNA or pre-miR-760 obliterate the effect of MK-7.

Figure 9 shows the impact of the transcription factors FoxA3 and FoxO1 on Runx2 expression in osteoblastic cells (left). Clearly, siRNA directed against either transcription factor obliterates

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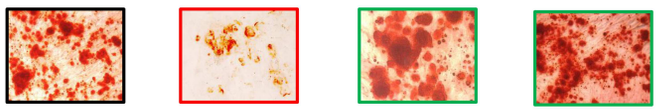
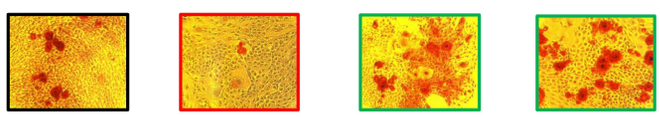
Osteoblast Parameters	Control = 100%	+ Cytokines	+ TF	+ TF + Cytokines
TF = SP1, ETS1 or RUNX1	100	31*	544*	389*
Runx2	100	17*	432*	324*
Collagen1a1	100	47*	145*	133*
Osteocalcin	100	23*	345*	288*
Osterix (SP7)	100	18*	182*	171*
Mineralizing «surface»	100	22*	234*	198*
OPG/Rank-L ratio	100	435*	27*	31*
MiR-149	100	546*	34*	75
MiR-328	100	465*	37*	58*
MiR-27b	100	388*	31*	36*
MiR-29a	100	481*	28*	30*
MiR-204	100	376*	27*	38*
MiR-211	100	422*	38*	41*



(a)

Osteoblast Parameters	Control = 100%	+ Cytokines	+ Antago-miRs	+ Antago-miRs + Cytokines
TF = SP1, ETS1 or RUNX1	100	39*	573*	366*
Runx2	100	45*	389*	319*
Collagen1a1	100	36*	319*	272*
Osteocalcin	100	27*	321*	251*
Osterix (SP7)	100	31*	301*	199*
Mineralizing «surface»	100	47*	247*	167*
OPG/Rank-L ratio	100	378*	21*	271*
MiR-149	100	449*	17*	69
MiR-328	100	577*	21*	48*
MiR-27b	100	466*	18*	31*
MiR-29a	100	521*	21*	33*
MiR-204	100	488*	33*	18*
MiR-211	100	469*	21*	25*



(b)

Figure 5. Tabulation of osteoblast-related parameters (transcription factors=TFs, microRNAs and mineralizing surface) measured in osteoblasts differentiated from hMSCs, in the presence or absence of MK-7, cytokines or both. All values are given as a percentage of controls (=100%). All results are means of separate experiments with the TFs indicated. Figures marked by stars (*) indicate p-values < 0.01.

Osteoblast GENE expression	Control = 100%	+ Cytokines	+ TF	+ TF + Cytokines
SP1, ETS1 or RUNX1	100	41*	17*	11*
MSX2	100	17*	32*	11*
WNT3A	100	21*	15*	23*
DKK1	100	329*	225*	451*
OPN(SPP1)	100	22*	21*	17*
MMP9	100	31*	17*	17*
RUNX2	100	12*	22*	11*
OPG/RANKL-ratio	100	21*	30*	15*
COL1a1	100	28*	29*	18*
PIT-1	100	21*	26*	18*
Mineralizing «surface»	100	18*	27*	15*
MiR-149	100	345*	367*	622*
MiR-29a	100	421*	304*	607*

(a)

Fibr./HUVEC/VIC GENE expression	Control = 100%	+ Cytokines	+ TF	+ TF + Cytokines
SP1, ETS1 or RUNX1	100	431*	21*	89
MSX2	100	288*	23*	78
WNT3A	100	365*	31*	104
DKK1	100	27*	245*	81
OPN(SPP1)	100	433*	12*	67*
MMP9	100	369*	23*	87
RUNX2	100	489*	27*	81
OPG/RANKL-ratio	100	363*	34*	65
COL1a1	100	478*	37*	78
PIT-1	100	387*	31*	88
Mineralizing «surface»	100	416*	68*	135
MiR-149	100	23*	227*	138
MiR-29a	100	18*	238*	97

(b)

Figure 6. Tabulation of osteoblast-related parameters (transcription factors=TFs, microRNAs and mineralizing surface) measured in osteoblasts (fig. a) or fibroblasts/HUVECs/VICs (fig. b) isolated from human tissues or differentiated from hMSCs, in the presence or absence of cytokines and transcription factors (SP1, ETS1 or RUNX1) expressing constructs. All values are given as a percentage of controls (=100%). All results are means of separate experiments with the TFs indicated. Figures marked by stars (*) indicate p-values < 0.01.

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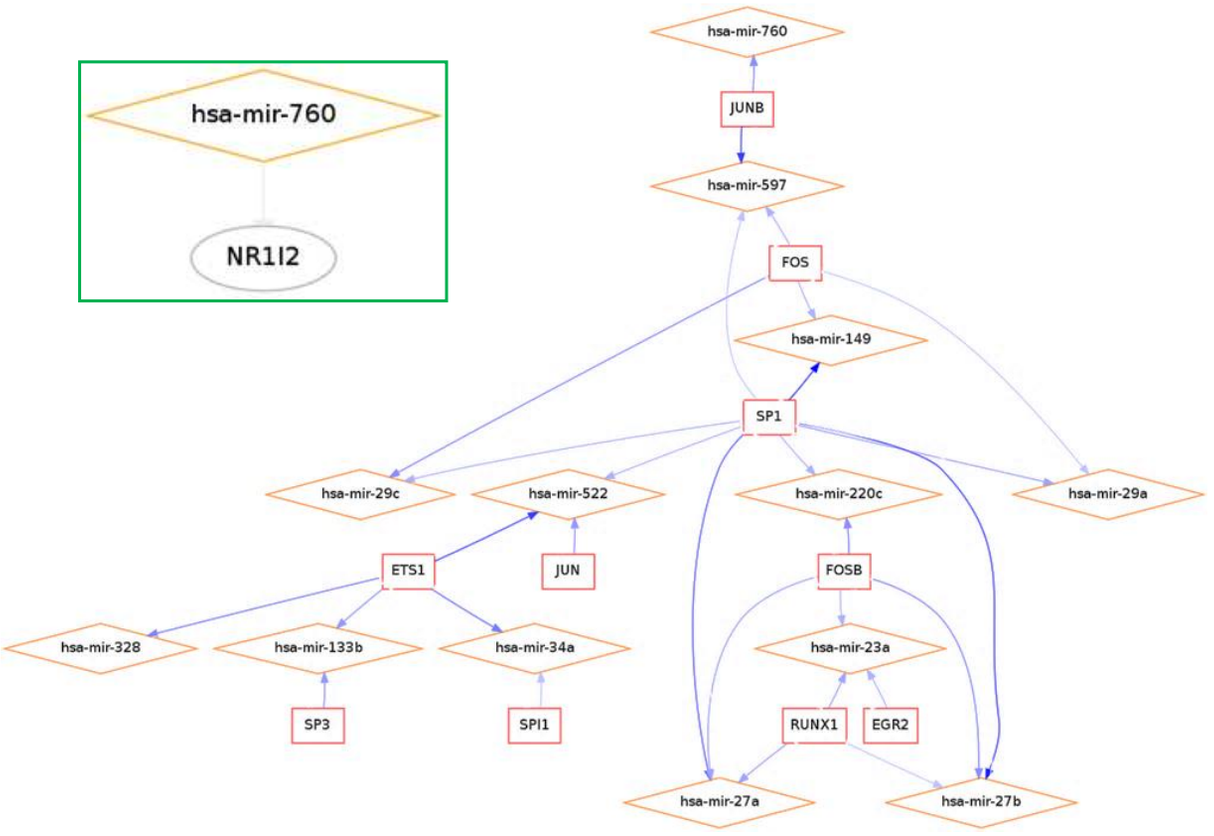

Figure 7. Mir@nt@n emulation [19] of osteoblast transcription factors=TFs and microRNAs, including miR-760, which was shown to putatively couple to NR1I2=SXR with the highest binding characteristics (the Sanger microRNA Database), known to be a receptor for vitamin K2 (i.e. MK-7 and/or the metabolite MK-4.

the stimulatory effect of MK-7 on Runx2 mRNA. It is well known that insulin and various growth factors modulate the activity of both FoxA and FoxO species via the PI3-Kinase/Akt/PKB signaling pathway, indicating that vitamin K2 is able to modulate hormonal or growth factor mediated impact on osteoblasts and possibly also other cell phenotypes.

Figure 10a&b depicts how osteoblasts (a) or fibroblasts/HUVECs/VICs (b) transfected with empty vectors, respond to the exposure of MK-7, a cytokine mixture (see text-Fig 8) or MK-7+cytokines in terms of transcription of genes and microRNAs, modulating mineral deposition in their surrounding matrix.

Figure 11 summarizes the effect ("correction") of transcription factors (TFs), microRNA species and vitamin K2 on the phenotypic characteristics of osteoblasts, fibroblasts, HUVECs and VICs. By manipulating different cell phenotypes with TFs and miRs engaged in reciprocal regulatory loops, in the presence of vitamin K2, it may be possible to stabilize and/or correct cell phenotypes to restrict mineralization of extracellular matrix to organs where mineral deposition is wanted (bone) and inhibit calcification of soft tissues like blood vessels and heart valves. Pay special attention to the reciprocal regulation of Runx2/SP7/BGP vs Matrix GLA protein (MGP) in osteoblasts and fibroblasts/ HUVECs/VICs.

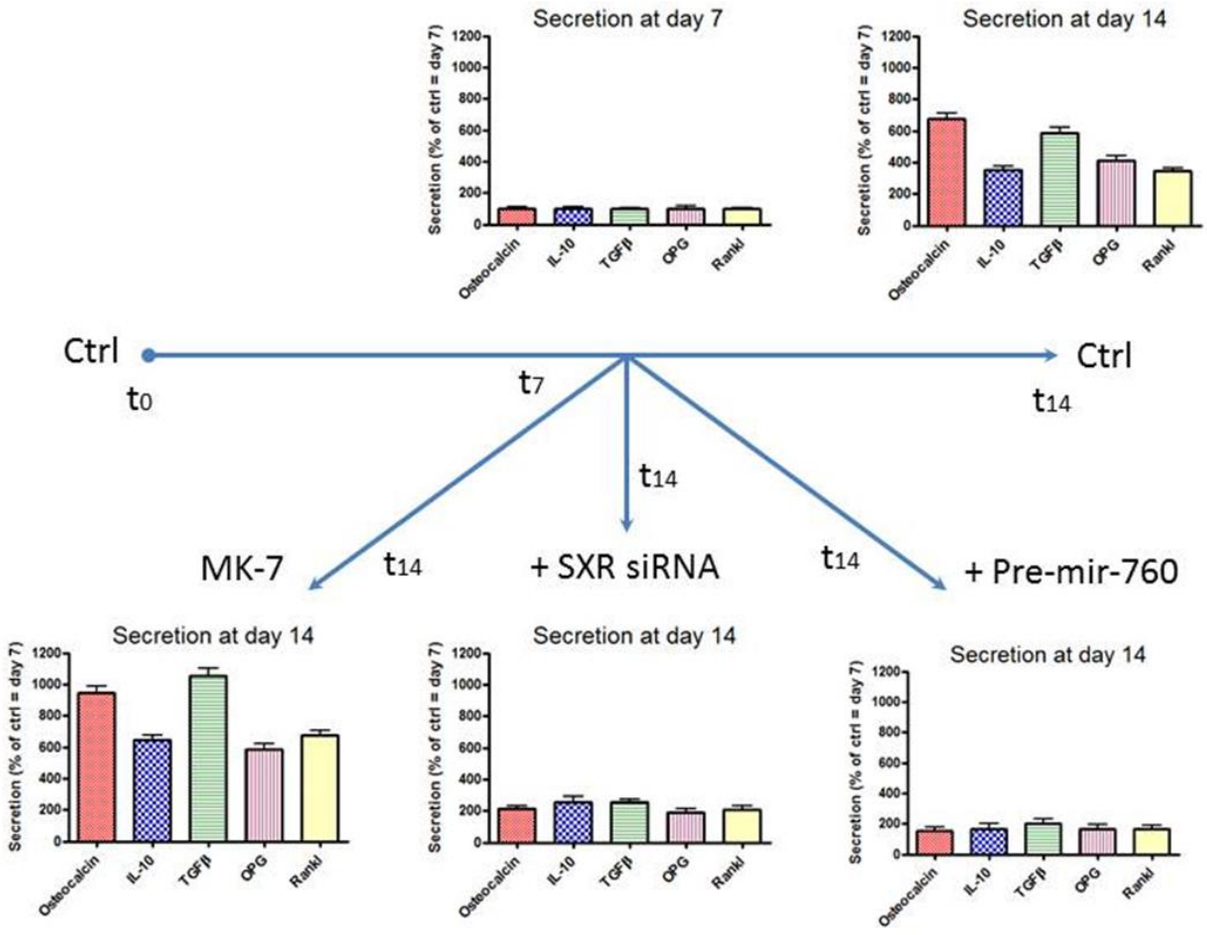

Figure 8. Secretory profile (osteocalcin, IL-10, TGFβ, OPG, and RANK-L) of mature osteoblasts within freshly isolated bovine bone chips for 7 days (days 8-14) after being pre-conditioned for 7 days (day 1-7) with either MK-7 (10 ng/ml), siRNA against SXR, or pre-miR-760.

6. Discussion

6.1. The importance of regulatory loops for the control of cellular phenotype

Feed-forward and feed-back regulatory loops consisting of transcription factors (TFs) and microRNAs are parts of an epigenetically "stabilizing" machinery [104]. Three categories of signals are proposed to operate in establishing a resilient, heritable epigenetic state. An extracellular signal designated the ''epigenator'', conveyed from the environment, triggers the start of the epigenetic pathway. The ''epigenetic initiator'' receives the signal from the ''epigenator'' and determines the precise chromatin location and/or DNA environment for establishing the epigenetic pathway. The ''epigenetic maintainer'' sustains the chromatin environment in both the initial and succeeding generations. Persistence of the chromatin milieu may require cooperation between the initiator and the maintainer. Hence, microRNAs and TFs, once they have attained an interactive "steady state", function as "maintainers" of a given cell phenotype with defined characteristics.

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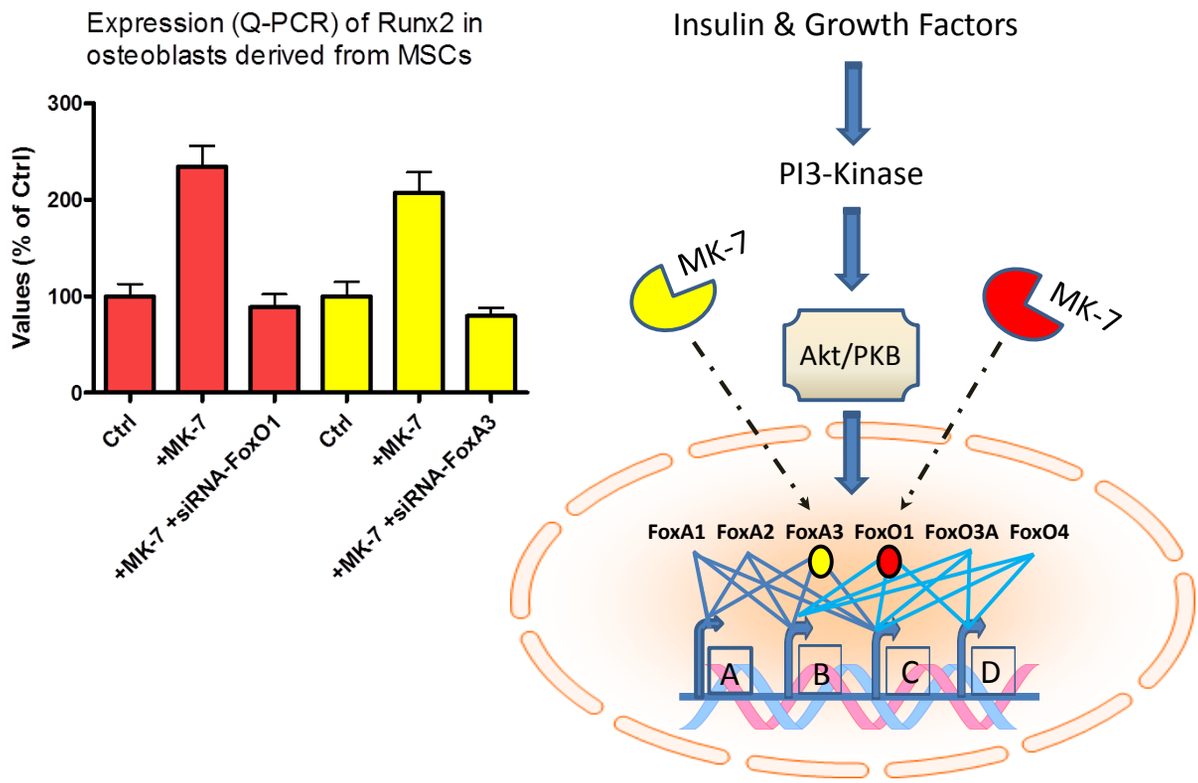

Figure 9. Left: Expression of Runx2 (Q-PCR) in osteoblasts differentiated from hMSCs in the absence (controls) or presence of MK-7 (10 ng/ml), MK-7+either FoxO1 -or FoxOA3-siRNAs (featuring two separate experiments). Right: Schematic representation of the impact of MK-7 on the signaling systems driven by insulin and growth factors (TGFs/BMPs) on the transcription of genes via PI3-kinase and the Akt/PKB-system, as described in the literature [108].

From a literature search (PubMed) on "SP1 transcription factor and osteoblasts", SP1 is somehow interfering with the effect of Runx2, SP7 (Osx), FIAT (inhibitor of ATF4), ETS-like TFs, MZF1 (myeloid zinc finger), JunB, and also directly affecting the transcription of marker genes like Col1α1, Col5α1, Col5α3, Col11α2, Fibromodulin, Osteocalcin, MGP (matrix-gla protein), RANKL, Pit phosphate transporter, Integrin β5, and TGFβ-R1. Furthermore, a similar search on "FosB and osteoblasts" revealed that FosB is interfering with the effect of BMPs and TGFs on the expression of downstream signaling molecules like Smads, TCF/LEF, as well as c-myc, and fra-1, but also directly modulating the transcription of IL-11 (which suppresses DIKK1 & DIKK2, thus enhancing Wnt-signaling), stimulating Pref-mediated dedifferentiation of adipocytes, relaying stretch-mediated osteoblast differentiation, counteracting the negative effect of Notch1 (a decrease in the expression of Col1α1, Osteocalcin, and ALP) by obliterating its negative effect on the Wnt/β-catenin pathway. Finally, the recent literature describes the positioning of ETS1 in the differentiation of osteoblasts in this way: Leptin is a strong inducer of osteoblast differentiation working through Stat3 (ref), and it was shown that ETS1, along with Stat1, Stat3, and VDR were induced by Calcitriol ($1,25(OH)_2D_3$) in UMR-106 osteoblast like cells, and that ETS1 is essential for connective tissue factor (CTGF/CCN2) induction by TGF-β1 in osteoblasts, synergizing with Smad3.

Osteoblast GENE expression	Control = 100%	+ MK-7	+ Cytokines	+ MK-7 + Cytokines
	100	143	47*	121
CSNK1A1	100	433*	47*	121
MSX2	100	356*	65	97
RUNX2	100	476*	33*	88
Osterix (SP7)	100	389*	27*	122
Osteocalcin (BGP)	100	489*	34*	143*
Matr. GLA pr. (MGP)	100	87	47*	112
PIT-1	100	432*	30*	143*
Mineralizing «surface»	100	188*	29*	88
MiR-149	100	17*	188*	78
MiR-328	100	22*	211*	111
Mir-29a	100	11*	251*	123
MiR-133b	100	18*	217*	71*

(a)

Fibr./HUVEC/VIC GENE expression	Control = 100%	+ MK-7	+ Cytokines	+ MK-7 + Cytokines
	100	108	167*	87
CSNK1A1	100	87	321*	122
MSX2	100	54*	188*	91
RUNX2	100	61*	354*	127
Osterix (SP7)	100	31*	449*	86
Osteocalcin (BGP)	100	53*	376*	132
Matr. GLA pr. (MGP)	100	387*	31*	378*
PIT-1	100	78	321*	87
Mineralizing «surface»	100	88	567*	132
MiR-149	100	465*	23*	367*
MiR-328	100	388*	17*	421*
Mir-29a	100	456*	24*	337*
MiR-133b	100	477*	31*	411*

(b)

Figure 10. Tabulation of recently recognized "discriminating" (for comparison, see Figures 6a&b) osteoblast-related parameters (transcription factors=TFs, microRNAs and mineralizing surface) measured in osteoblasts (fig. a) or fibroblasts/HUVECs/VICs (fig. b) isolated from human tissues or differentiated from hMSCs, in the presence or absence of cytokines and transcription factors (SP1, ETS1 or RUNX2) expressing constructs. All values are given as a percentage of controls (=100%). All results are means of separate experiments with the TFs indicated. Figures marked by stars (*) indicate p-values < 0.01.

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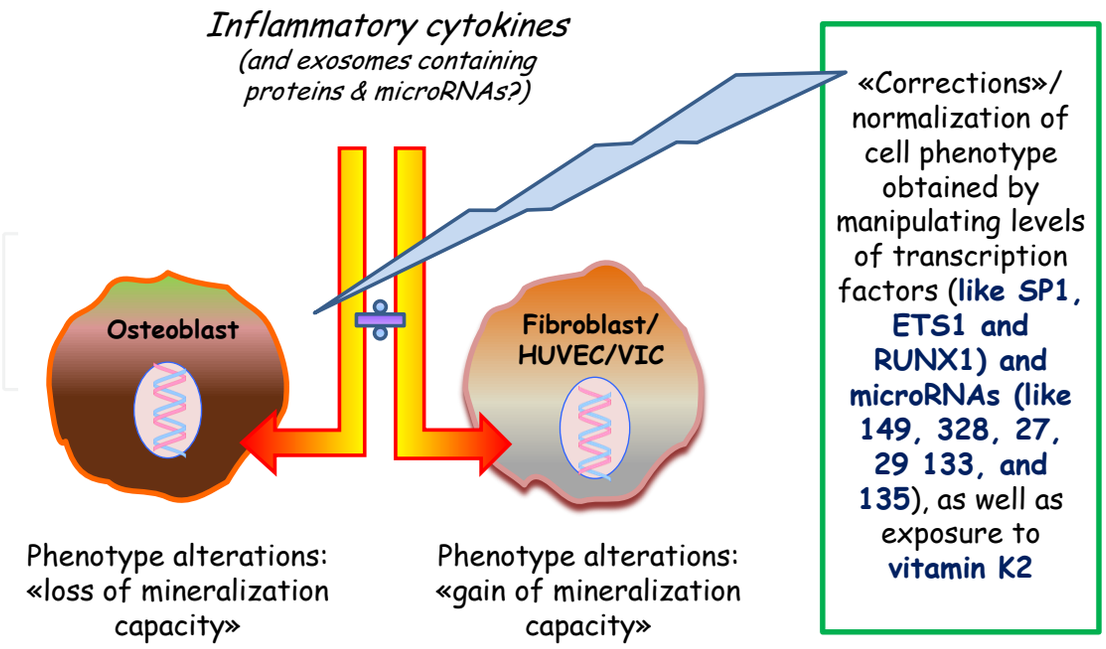

Figure 11. Schematic representation (summary) of the impact of transcription factors (like SP1, ETS1, and RUNX1), microRNA species possibly constituting a minimal and sufficient "osteoblast"-like signature (miR-149,-328,-27,-29,-133, and-135), and vitamin K2 (e.g. MK-7) on the stability of the osteoblast and fibroblast/HUVEC/VIC phenotype (i.e. mineralizing facilitating and mineralization prohibiting) development and/or maintenance.

Furthermore, BMP2 stimulation of pre-osteoblasts is mediated through ETS1, which transactivates Osteopontin, Runx2, PTHrP, and Col1α1 genes. The most compelling evidence for the importance of ETS1 (together with SP1 and SP7=Osx) on conferring the osteoblast phenotype of differentiating cells, is that the Runx2 P1 promoter in mesenchymal cells is co-stimulated at purine-rich DNA sequences (Y-repeats). It has also been shown that mechano-stimulation of MSCs enhances ETS1, Runx2, and ALP transcription and translation in a sequential manner, and that RNF11, also deemed necessary for osteoblastogenesis, in fact, is regulated by ETS1. Finally, the AJ18 gene, which is as a novel KRAB/C82/H(2) gene implicated in the differentiation of osteogenic cells, displays several response elements for proteins like ETS1 and SP1, as well as Runx2, Smads and NFκB.

It may be asserted that the larger number of microRNAs involved in the modulation of the activity of a certain cell's TFs, the more stable the cellular phenotype will become, and the more finely tuned its functions will be. Hence, it may be postulated that the alteration of the steady state levels of players contained within reciprocal regulatory loops in osteoblasts, may also determine whether the cells in other organs than bone may acquire mineralizing characteristics or not. Our results clearly state that this is the case, since it was possible to fortify the mineralizing phenotype of osteoblasts, and weaken the mineralizing properties of fibroblasts, HUVECs and VICs by manipulating the levels of SP1, ETS1, and RUNX1, or the levels of osteoblast-"specific" signature-microRNAs, like miR-149 and miR-328 [36, 37]. Suppression of miR-149 enhances the mRNA level of target proteins associated with the differentiation of osteoblasts (Runx2, APC2, RNF11, and SP1). At the same time, a reduction in miR-149 will

enhance the transcription of factors (cEBPA, ATF3, Stat3, and PIAS1) known to counteract chondrocyte development induced by TGFβ-R and Sox9. Enhancing the level of miR-149 (i.e. the corresponding pre-miR) will favor chondrogenesis at the expense of osteoblast differentiation. Hence, miR-149 may be defined as a "switch-miR".

In an excellent review, Goettsch et al, [18] list up-and down-regulated microRNA species in coronary artery disease, aortic stenosis and arteriosclerosis obliterans, of which several, e.g. 29a, 125b, 135b, and 204, having been shown targeting osteogenic markers (like BMP2, DKK1, RANKL, Osterix, Runx2, SMADs, and BMP-2), and 335-5p and 155, known to target calcification inhibitors (like MGP and Fetuin-A). However, this is the first time it has been shown that microRNAs involved in reciprocal regulatory loops with transcription factors are able to "determine" whether a cell type will be mineralizing or not.

6.2. Gene expression profiles as markers for osteoblast and fibroblast/HUVEC/VIC analyses

The tables showing the impact of vitamin K2, and vitamin K2, and cytokines, list various marker gene expression profiles, as well as mineralizing surface obtained with Alizarin red S in 2D-cell cultures. The parameters are selected from a set of articles featuring the characteristics of mineralizing osteoblasts, as well as calcifying soft tissue fibroblast like cells with emphasis on CSNK1A1, WNT3A, DKK1 (signaling molecules), MSX2, Runx2, Osterix=SP7 (transcription factors), collagen1α1, osteocalcin, matrix GLA protein (matrix structural proteins), MMP9, OPN=SPP1 (extracellular bioactive molecules=proteinase, hydroxyapatite binder). PIT-1=POU1F1 (Na^+-phosphate transporter), and RANKL (activator of NFκB) and OPG (osteoclast inhibitory factor), as well as microRNA species contained within various miR-"signatures" [36, 38] of osteoblasts, where they inhibit factors responsible for osteoblastogenesis and expression of biomolecules necessary for mineralization of the cellular matrix. As pointed out elsewhere (under results), the pattern of parameter modulation of osteoblasts and fibroblasts/HUVEC/VIC genes and microRNA expression is compatible with a strengthening of the osteoblast phenotype when the transcription factors SP1, ETS1, or RUNX1 are reinforced, or when microRNAs (e.g. mir-149, -328 (as well as -204, -211,- 27b, and -133b; data not shown in this chapter) are targeted in particular.

6.3. The impact of vitamin K2 on the mineralizing properties of cells

Vitamin K2 (MK-7 (or its metabolite MK-4) was shown to be involved in the regulatory loops consisting of microRNAs and transcription factors (TFs), as stated above. MK-7/MK-4, by binding to the transcription factor SXR/PXR/NR1I2 is able to connect to the looping system via miR-760, JUNB, and miR-597. The microRNA species 597 thus putatively targets (as envisaged by the Mir@nt@n and TargetScan algorithms): APC2, BMP-1,CD44, CTNNA1, CTNNB1, CTNNBL1, FOS, FOXA2, FOXA3, FOXO3, FOXP3, FOXP4, GATA6, GATAD2A, HES1, IL-17D, IRF4, JUNB, MSX1, NFATC4, NOTCH2, RORC, RUNX1, RUNX2, RUNX3, SMAD2, SMAD3, SMAD4, SMAD7, SOCS2, SOX4, SOX6, SOX9, SOX11, SP1, SP2, SP4, SP6, SP8, SPRY1, SPRY3, STAT1, TGFB-2, VLDLR, WNT2, and WNT9B.

These genes are all heavily involved in different signaling pathways determining the development of mineralizing properties, as well as the differentiation of Th-2 and Th-17 cells from precursor immune cells, i.e. T-regulatory cells [105]. It may therefore be asserted that vitamin K2 plays an important modulatory role in: 1) the homeostasis of mineral deposits, i.e. calcification of bone and decalcification of soft tissues, as well as 2) the process of soft tissue infiltration by immune cells, which eventually leads to untoward calcification.

However, when searching various databases for putative interactions between microRNA-species involved in reciprocal regulatory loops with transcription factors (i.e. miR-149, -328, -29, -27, -23, -34a, -133, -220c, -597, -522) for putative interference with the transcription/translation of NFKB1, NFKB2, and/or with their subunits RELA/RELB, known to be important for the differentiation of inflammatory macrophages and Th-cells, no interactions could be predicted. Hence, the well-known modulatory effect of vitamin K2 on osteoclasts and Th-cells related to bone mass and inflammation, respectively, most certainly are not determined directly by the subject reciprocal regulatory loops, as suggested by the coupling of SXR=NR1I2 to FOS, via the miR-7/JUNB/miR-597 axis.

6.4. The overall modulatory effect of reciprocal regulatory loops involving transcription factors and microRNAs, and interference from vitamin K2

From the data presented here, it may be hypothesized that certain transcription factors and microRNA species are heavily involved in determining whether a given cell type will express mineralizing properties or not. In this context, it would be beneficial to exploit this knowledge to directly reinforce mineralization of bone (via osteoblasts) and block the calcification of soft tissues (induced by fibroblasts/HUVECs/VICs in blood vessels and heart valves) due to senescence or active inflammatory processes involving macrophages and T-cells, through a gene therapy program.

However, by optimization of tissue exposure to vitamin K2, one may both directly and indirectly obtain similar results, since vitamin K2 (MK-7 or MK-4 or both) affects the reciprocal regulatory loops, reinforcing both the osteoblast and fibroblast/HUVEC/VIC phenotypes, both in the absence and presence of cytokines derived from active, inflammatory Th-cells.

7. Summary and future perspectives

The present text features the dynamic interaction between important biological player molecules, determining the spectrum of features expressed by different cell types exposed to the same ambient environmental factors (e.g. oxygen, nutrients and hormones/growth factors). Osteoblasts and soft tissue cells, like fibroblasts/HUVECs/VICs, respond to the same bioactive molecules, however, osteoblasts mineralize bone matrix, while the others do not normally calcify soft tissues. We have shown that regulatory looping systems consisting of microRNAs and transcription factors (TFs) may determine whether mineralization is going to take place or not, and that the cell/organ homeostasis is disrupted in the presence of cytokines from inflammatory cells (Th-cells).

By manipulating these loops, containing several members of the epigenetic machinery, one may strengthen wanted cell phenotypes (e.g. osteoblasts in patients with osteoporosis) and weaken unwanted characteristics (e.g. calcifying fibroblast like cells in the elderly or patients suffering from hypertension, diabetes, end-stage renal disease, hyperlipidemia and other diseases). If gene therapy is not warranted or wanted, one may resort to optimal "vitamin K2 therapy", since MK-7 was shown to normalize and strengthen the mineralizing osteoblast phenotype, and weaken the mineralizing "fibroblast" phenotype in the presence of inflammatory cytokines.

We have previously shown that the regulatory looping system also contains histone deacetylaces (HDACS, including the Sirtuin class, the latter responding to the energy state of an organ) (see Fig. 4). Sirtuins may respond to small activator molecules [106, 107], such as plant polyphenols, which confer their activating potential through FOXO3 binding to the Sirt1 mRNA. Sirt1 transcription is activated in mature osteoblasts (where miR-16 levels are diminished), compared to other cell types. Hence, the FoxA/FoxO gene activating system, also driven by vitamin K2, may be further stimulated (synergistically?) by Sirt activators found in natural foods, thus conserving the osteoblast phenotype better during ageing and or inflammatory processes. It should not surprise anyone, if soft tissue cells like fibroblasts/HUVECs/VICs will respond in an opposite manner to the same polyols, reinforcing the anti-calcifying properties of vitamin K2 on soft tissues like blood vessels and heart valves.

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References

[1] Bostrom, K.I., N.M. Rajamannan, and D.A. Towler, *The regulation of valvular and vascular sclerosis by osteogenic morphogens.* Circ Res, 2011. 109(5): p. 564-77.

[2] Fukuyo, S., et al., *IL-6-accelerated calcification by induction of ROR2 in human adipose tissue-derived mesenchymal stem cells is STAT3 dependent.* Rheumatology (Oxford), 2014. 53(7): p. 1282-90.

[3] Fuchs, S., E. Dohle, and C.J. Kirkpatrick, *Sonic Hedgehog-mediated synergistic effects guiding angiogenesis and osteogenesis.* Vitam Horm, 2012. 88: p. 491-506.

[4] Zhang, X., et al., *The role of NELL-1, a growth factor associated with craniosynostosis, in promoting bone regeneration.* J Dent Res, 2010. 89(9): p. 865-78.

[5] Kirsch, T., *Biomineralization--an active or passive process?* Connect Tissue Res, 2012. 53(6): p. 438-45.

[6] Brylka, L. and W. Jahnen-Dechent, *The role of fetuin-A in physiological and pathological mineralization.* Calcif Tissue Int, 2013. 93(4): p. 355-64.

[7] Jahnen-Dechent, W., et al., *Fetuin-A regulation of calcified matrix metabolism.* Circ Res, 2011. 108(12): p. 1494-509.

[8] Miller, J.D., R.M. Weiss, and D.D. Heistad, *Calcific aortic valve stenosis: methods, models, and mechanisms.* Circ Res, 2011. 108(11): p. 1392-412.

[9] North, B.J. and D.A. Sinclair, *The intersection between aging and cardiovascular disease.* Circ Res, 2012. 110(8): p. 1097-108.

[10] Oellerich, M.F. and M. Potente, *FOXOs and sirtuins in vascular growth, maintenance, and aging.* Circ Res, 2012. 110(9): p. 1238-51.

[11] Novack, D.V., *The FOX(O1) blasts off.* Cell Metab, 2010. 11(3): p. 175-6.

[12] Ichikawa, T., et al., *Steroid and xenobiotic receptor SXR mediates vitamin K2-activated transcription of extracellular matrix-related genes and collagen accumulation in osteoblastic cells.* J Biol Chem, 2006. 281(25): p. 16927-34.

[13] Igarashi, M., et al., *Vitamin K induces osteoblast differentiation through pregnane X receptor-mediated transcriptional control of the Msx2 gene.* Mol Cell Biol, 2007. 27(22): p. 7947-54.

[14] Katsuyama, H., et al., *Menaquinone-7 regulates gene expression in osteoblastic MC3T3E1 cells.* Int J Mol Med, 2007. 19(2): p. 279-84.

[15] Pucci, B., et al., *Sirtuins: the molecular basis of beneficial effects of physical activity.* Intern Emerg Med, 2013. 8 Suppl 1: p. S23-5.

[16] Sanchez-Fidalgo, S., et al., *Sirtuin modulators: mechanisms and potential clinical implications.* Curr Med Chem, 2012. 19(15): p. 2414-41.

[17] Abdellatif, M., *Differential expression of microRNAs in different disease states.* Circ Res, 2012. 110(4): p. 638-50.

[18] Goettsch, C., J.D. Hutcheson, and E. Aikawa, *MicroRNA in cardiovascular calcification: focus on targets and extracellular vesicle delivery mechanisms.* Circ Res, 2013. 112(7): p. 1073-84.

[19] Le Bechec, A., et al., *MIR@NT@N: a framework integrating transcription factors, microRNAs and their targets to identify sub-network motifs in a meta-regulation network model.* BMC Bioinformatics, 2011. 12: p. 67.

[20] Vallett, S.M., et al., *A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene.* J Biol Chem, 1996. 271(21): p. 12247-53.

[21] Najafi-Shoushtari, S.H., et al., *MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis.* Science, 2010. 328(5985): p. 1566-9.

[22] Brooks-Wilson, A., et al., *Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency.* Nat Genet, 1999. 22(4): p. 336-45.

[23] Rayner, K.J., et al., *Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis.* J Clin Invest, 2011. 121(7): p. 2921-31.

[24] Xin, M., et al., *MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury.* Genes Dev, 2009. 23(18): p. 2166-78.

[25] Boettger, T., et al., *Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster.* J Clin Invest, 2009. 119(9): p. 2634-47.

[26] Elia, L., et al., *The knockout of miR-143 and-145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease.* Cell Death Differ, 2009. 16(12): p. 1590-8.

[27] Watanabe, N., et al., *BTEB2, a Kruppel-like transcription factor, regulates expression of the SMemb/Nonmuscle myosin heavy chain B (SMemb/NMHC-B) gene.* Circ Res, 1999. 85(2): p. 182-91.

[28] Suzuki, T., et al., *Kruppel-like factor 5 shows proliferation-specific roles in vascular remodeling, direct stimulation of cell growth, and inhibition of apoptosis.* J Biol Chem, 2009. 284(14): p. 9549-57.

[29] Torella, D., et al., *MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo.* Circ Res, 2011. 109(8): p. 880-93.

[30] Davis, B.N., et al., *Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype.* J Biol Chem, 2009. 284(6): p. 3728-38.

[31] Liu, X., et al., *A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia.* Circ Res, 2009. 104(4): p. 476-87.

[32] Ji, R., et al., *MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation.* Circ Res, 2007. 100(11): p. 1579-88.

[33] Meng, F., et al., *Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines.* Gastroenterology, 2006. 130(7): p. 2113-29.

[34] Meng, F., et al., *MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer.* Gastroenterology, 2007. 133(2): p. 647-58.

[35] Martini, M., et al., *PI3K/AKT signaling pathway and cancer: an updated review.* Ann Med, 2014: p. 1-12.

[36] Gordeladze, J.O., et al., *Concerted stimuli regulating osteo-chondral differentiation from stem cells: phenotype acquisition regulated by microRNAs.* Acta Pharmacol Sin, 2009. 30(10): p. 1369-84.

[37] Gordeladze, J.O., et al., *From stem cells to bone: phenotype acquisition, stabilization, and tissue engineering in animal models.* ILAR J, 2009. 51(1): p. 42-61.

[38] Lian, J.B., et al., *MicroRNA control of bone formation and homeostasis.* Nat Rev Endocrinol, 2012. 8(4): p. 212-27.

[39] van Wijnen, A.J., et al., *MicroRNA functions in osteogenesis and dysfunctions in osteoporosis.* Curr Osteoporos Rep, 2013. 11(2): p. 72-82.

[40] Qiu, T., et al., *MiR-145, miR-133a and miR-133b inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer.* FEBS Lett, 2014. 588(7): p. 1168-77.

[41] Bostrom, K., et al., *Bone morphogenetic protein expression in human atherosclerotic lesions.* J Clin Invest, 1993. 91(4): p. 1800-9.

[42] Demer, L.L., *Vascular calcification and osteoporosis: inflammatory responses to oxidized lipids.* Int J Epidemiol, 2002. 31(4): p. 737-41.

[43] Vattikuti, R. and D.A. Towler, *Osteogenic regulation of vascular calcification: an early perspective.* Am J Physiol Endocrinol Metab, 2004. 286(5): p. E686-96.

[44] Otto, C.M., et al., *Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies.* Circulation, 1994. 90(2): p. 844-53.

[45] Wallby, L., et al., *T lymphocyte infiltration in non-rheumatic aortic stenosis: a comparative descriptive study between tricuspid and bicuspid aortic valves.* Heart, 2002. 88(4): p. 348-51.

[46] Hisar, I., et al., *Aortic valve calcification: its significance and limitation as a marker for coronary artery disease.* Angiology, 2002. 53(2): p. 165-9.

[47] Ortlepp, J.R., et al., *Cardiovascular risk factors in patients with aortic stenosis predict prevalence of coronary artery disease but not of aortic stenosis: an angiographic pair matched case-control study.* Heart, 2003. 89(9): p. 1019-22.

[48] Stewart, B.F., et al., *Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study.* J Am Coll Cardiol, 1997. 29(3): p. 630-4.

[49] Yamamoto, H., et al., *Valvular and thoracic aortic calcium as a marker of the extent and severity of angiographic coronary artery disease.* Am Heart J, 2003. 146(1): p. 153-9.

[50] Mohler, E.R., 3rd, et al., *Bone formation and inflammation in cardiac valves.* Circulation, 2001. 103(11): p. 1522-8.

[51] Rajamannan, N.M., et al., *Human aortic valve calcification is associated with an osteoblast phenotype.* Circulation, 2003. 107(17): p. 2181-4.

[52] Chen, N.X. and S.M. Moe, *Arterial calcification in diabetes.* Curr Diab Rep, 2003. 3(1): p. 28-32.

[53] Karsenty, G., *Minireview: transcriptional control of osteoblast differentiation.* Endocrinology, 2001. 142(7): p. 2731-3.

[54] Cheng, S.L., et al., *MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors.* J Biol Chem, 2003. 278(46): p. 45969-77.

[55] Shao, J.S., et al., *Teriparatide (human parathyroid hormone (1-34)) inhibits osteogenic vascular calcification in diabetic low density lipoprotein receptor-deficient mice.* J Biol Chem, 2003. 278(50): p. 50195-202.

[56] Towler, D.A., et al., *Diet-induced diabetes activates an osteogenic gene regulatory program in the aortas of low density lipoprotein receptor-deficient mice.* J Biol Chem, 1998. 273(46): p. 30427-34.

[57] Davies, M.R., R.J. Lund, and K.A. Hruska, *BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure.* J Am Soc Nephrol, 2003. 14(6): p. 1559-67.

[58] Bidder, M., et al., *Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein-1 activities.* J Biol Chem, 2002. 277(46): p. 44485-96.

[59] Li, G., et al., *Estrogen attenuates integrin-beta(3)-dependent adventitial fibroblast migration after inhibition of osteopontin production in vascular smooth muscle cells.* Circulation, 2000. 101(25): p. 2949-55.

[60] Chen, N.X., et al., *Phosphorus and uremic serum up-regulate osteopontin expression in vascular smooth muscle cells.* Kidney Int, 2002. 62(5): p. 1724-31.

[61] Moe, S.M., et al., *Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins.* Kidney Int, 2002. 61(2): p. 638-47.

[62] Shanahan, C.M., et al., *Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification.* Circulation, 1999. 100(21): p. 2168-76.

[63] Takemoto, M., et al., *Enhanced expression of osteopontin in human diabetic artery and analysis of its functional role in accelerated atherogenesis.* Arterioscler Thromb Vasc Biol, 2000. 20(3): p. 624-8.

[64] Bucay, N., et al., *osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification.* Genes Dev, 1998. 12(9): p. 1260-8.

[65] Browner, W.S., L.Y. Lui, and S.R. Cummings, *Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women.* J Clin Endocrinol Metab, 2001. 86(2): p. 631-7.

[66] Qunibi, W.Y., C.A. Nolan, and J.C. Ayus, *Cardiovascular calcification in patients with end-stage renal disease: a century-old phenomenon.* Kidney Int Suppl, 2002(82): p. S73-80.

[67] Schafer, C., et al., *The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification.* J Clin Invest, 2003. 112(3): p. 357-66.

[68] Johnson, K., et al., *Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression.* J Bone Miner Res, 2003. 18(6): p. 994-1004.

[69] Rutsch, F., et al., *PC-1 nucleoside triphosphate pyrophosphohydrolase deficiency in idiopathic infantile arterial calcification.* Am J Pathol, 2001. 158(2): p. 543-54.

[70] Zelzer, E., et al., *Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2.* Mech Dev, 2001. 106(1-2): p. 97-106.

[71] Tyson, K.L., et al., *Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification.* Arterioscler Thromb Vasc Biol, 2003. 23(3): p. 489-94.

[72] Luo, G., et al., *Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein.* Nature, 1997. 386(6620): p. 78-81.

[73] Matsui, Y., et al., *Osteopontin deficiency attenuates atherosclerosis in female apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2003. 23(6): p. 1029-34.

[74] Zebboudj, A.F., M. Imura, and K. Bostrom, *Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2.* J Biol Chem, 2002. 277(6): p. 4388-94.

[75] Schneider, M.D., V. Gaussin, and K.M. Lyons, *Tempting fate: BMP signals for cardiac morphogenesis.* Cytokine Growth Factor Rev, 2003. 14(1): p. 1-4.

[76] Galvin, K.M., et al., *A role for smad6 in development and homeostasis of the cardiovascular system.* Nat Genet, 2000. 24(2): p. 171-4.

[77] Hamidi, M.S., O. Gajic-Veljanoski, and A.M. Cheung, *Vitamin K and bone health.* J Clin Densitom, 2013. 16(4): p. 409-13.

[78] Feigh, M., et al., *Oral salmon calcitonin enhances insulin action and glucose metabolism in diet-induced obese streptozotocin-diabetic rats.* Eur J Pharmacol, 2014. 737: p. 91-6.

[79] Oury, F., et al., *Osteocalcin regulates murine and human fertility through a pancreas-bone-testis axis.* J Clin Invest, 2013. 123(6): p. 2421-33.

[80] Riddle, R.C. and T.L. Clemens, *Insulin, osteoblasts, and energy metabolism: why bone counts calories.* J Clin Invest, 2014. 124(4): p. 1465-7.

[81] Suhara, Y., et al., *[Structure-activity relationship of novel vitamin K analogues as steroid and xenobiotic receptor (SXR) agonists].* Yakugaku Zasshi, 2012. 132(8): p. 881-6.

[82] Tabb, M.M., et al., *Vitamin K2 regulation of bone homeostasis is mediated by the steroid and xenobiotic receptor SXR.* J Biol Chem, 2003. 278(45): p. 43919-27.

[83] Yamaguchi, M. and M.N. Weitzmann, *Vitamin K2 stimulates osteoblastogenesis and suppresses osteoclastogenesis by suppressing NF-kappaB activation.* Int J Mol Med, 2011. 27(1): p. 3-14.

[84] Zhou, C., S. Verma, and B. Blumberg, *The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism.* Nucl Recept Signal, 2009. 7: p. e001.

[85] Slatter, J.G., et al., *Microarray-based compendium of hepatic gene expression profiles for prototypical ADME gene-inducing compounds in rats and mice in vivo.* Xenobiotica, 2006. 36(10-11): p. 902-37.

[86] Kuznetsov, S.A., et al., *Circulating skeletal stem cells.* J Cell Biol, 2001. 153(5): p. 1133-40.

[87] de Candia, P., et al., *Serum microRNAs as Biomarkers of Human Lymphocyte Activation in Health and Disease.* Front Immunol, 2014. 5: p. 43.

[88] Ishimura, E., et al., *Different risk factors for peripheral vascular calcification between diabetic and non-diabetic haemodialysis patients--importance of glycaemic control.* Diabetologia, 2002. 45(10): p. 1446-8.

[89] Goodman, W.G., et al., *Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis.* N Engl J Med, 2000. 342(20): p. 1478-83.

[90] El Asmar, M.S., J.J. Naoum, and E.J. Arbid, *Vitamin k dependent proteins and the role of vitamin k2 in the modulation of vascular calcification: a review.* Oman Med J, 2014. 29(3): p. 172-7.

[91] Iwamoto, J., et al., *Bone quality and vitamin K2 in type 2 diabetes: review of preclinical and clinical studies.* Nutr Rev, 2011. 69(3): p. 162-7.

[92] Iwamoto, J., T. Takeda, and Y. Sato, *Menatetrenone (vitamin K2) and bone quality in the treatment of postmenopausal osteoporosis.* Nutr Rev, 2006. 64(12): p. 509-17.

[93] Tanana, I. and H. Oshima, *[Vitamin K2 as a potential therapeutic agent for glucocorticoid-induced osteoporosis].* Clin Calcium, 2006. 16(11): p. 1851-7.

[94] Horie-Inoue, K. and S. Inoue, *Steroid and xenobiotic receptor mediates a novel vitamin K2 signaling pathway in osteoblastic cells.* J Bone Miner Metab, 2008. 26(1): p. 9-12.

[95] .Huang, W., et al., *Fn14 promotes differentiation of human mesenchymal stem cells into heart valvular interstitial cells by phenotypic characterization.* J Cell Physiol, 2014. 229(5): p. 580-7.

[96] Campillo-Fernandez, A.J., et al., *Analysis of the biological response of endothelial and fibroblast cells cultured on synthetic scaffolds with various hydrophilic/hydrophobic ratios: influence of fibronectin adsorption and conformation.* Tissue Eng Part A, 2009. 15(6): p. 1331-41.

[97] Davies, C.M., et al., *Mechanically loaded ex vivo bone culture system 'Zetos': systems and culture preparation.* Eur Cell Mater, 2006. 11: p. 57-75; discussion 75.

[98] David, V., et al., *Ex Vivo bone formation in bovine trabecular bone cultured in a dynamic 3D bioreactor is enhanced by compressive mechanical strain.* Tissue Eng Part A, 2008. 14(1): p. 117-26.

[99] Fritz, V., et al., *Bone-metastatic prostate carcinoma favors mesenchymal stem cell differentiation toward osteoblasts and reduces their osteoclastogenic potential.* J Cell Biochem, 2011. 112(11): p. 3234-45.

[100] James, A.W., *Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation.* Scientifica (Cairo), 2013. 2013: p. 684736.

[101] Persy, V. and P. D'Haese, *Vascular calcification and bone disease: the calcification paradox.* Trends Mol Med, 2009. 15(9): p. 405-16.

[102] Jensen, E.D., R. Gopalakrishnan, and J.J. Westendorf, *Regulation of gene expression in osteoblasts.* Biofactors, 2010. 36(1): p. 25-32.

[103] Gordeladze, J.O., et al., *Transient down-regulation of cbfa1/Runx2 by RNA interference in murine C3H10T1/2 mesenchymal stromal cells delays in vitro and in vivo osteogenesis, but does not overtly affect chondrogenesis.* Exp Cell Res, 2008. 314(7): p. 1495-506.

[104] Vrtacnik, P., J. Marc, and B. Ostanek, *Epigenetic mechanisms in bone.* Clin Chem Lab Med, 2014. 52(5): p. 589-608.

[105] Pateras, I., et al., *NF-kappaB signaling at the crossroads of inflammation and atherogenesis: searching for new therapeutic links.* Expert Opin Ther Targets, 2014. 18(9): p. 1089-101.

[106] Cho, S.Y., et al., *Identification of a small molecule activator of SIRT1 gene expression.* Aging (Albany NY), 2013. 5(3): p. 174-82.

[107] Allard, J.S., et al., Dietary activators of Sirt1. Mol Cell Endocrinol, 2009. 299(1): p. 58-63.

[108] Czech, M.P., Insulin's expanding control of forkheads. Proc Natl Acad Sci U S A, 2003. 100(20): p. 11198-200.