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The Influence of DNA Methylation on Bone Cells

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Abstract: DNA methylation in eukaryotes invokes heritable alterations of the of the cytosine base in DNA without changing the underlying genomic DNA sequence. DNA methylation may be modified by environmental exposures as well as gene polymorphisms and may be a mechanistic link between environmental risk factors and the development of disease. In this review, we consider the role of DNA methylation in bone cells (osteoclasts/osteoblasts/osteocytes) and their progenitors with special focus on *in vitro* and *ex vivo* analyses. The number of studies on DNA methylation in bone cells is still somewhat limited, nevertheless it is getting increasingly clear that this type of the epigenetic changes is a critical regulator of gene expression. DNA methylation is necessary for proper development and function of bone cells and is accompanied by disease characteristic functional alterations as presently reviewed including postmenopausal osteoporosis and mechanical strain.

Keywords: Bone, Osteoblast, DNA methylation, Osteoporosis, Wnt, Osteoclast, Epigenetics.

INTRODUCTION

Although all cells contain the same genes, their expression and function may differ widely from one tissue to another. The expression of tissue and cell specific genes is regulated by complex interactions involving tissue and cell specific transcription factors, extracellular signals, chromatin packing and epigenetic changes including DNA methylation of cytosine residues.

DNA is methylated by transfer of a methyl group from S-adenosyl methionine (SAM) to the cytosine residue of a CpG dinucleotide in DNA. This reaction is catalyzed by DNA methyltransferases (DNMT), including DNMT1, DNMT3a and DNMT3b. The latter two, being *de novo* methyltransferases, methylate previously unmethylated CpG dinucleotides, while DNMT1 is a maintenance methyltransferase [1]. CpG dinucleotides are underrepresented in the genome, but tend to be clustered in CpG islands of length >200 bp (on average 1000 bp) having a higher than expected number of CpG dinucleotides [2].

DNA methylation is a key regulator of gene transcription. Highly methylated promoter regions often lead to reduced transcription due to hampered binding of transcription factors or recruitment of methyl-CpG binding proteins that in turn attach to chromatin modifier complexes, causing subsequent chromatin condensation and gene silencing [3]. On the other hand, methylation within the transcribed DNA region have been associated with increased expression of the affected gene, possibly due to reduced use of spurious intergenic promoters [4, 5]. Also, as reviewed [6], binding of transcription factors to a promoter region may promote or inhibit DNA methylation depending of the properties of that factor.

To study the effect of DNA methylation on expression of distinct genes, cultured cells are often treated with the DNA methyltransferase (DNMT) inhibitors 5-azacytidine or its deoxyribose analogue 5-aza-2´deoxycytidine (5AzadC) to cause global demethylation. DNA demethylation of the entire genome may lead to erroneous interpretation of the consequences on a given gene, since accompanying demethylation of other genes may play a pivotal role. Furthermore, these substances are cytotoxic, causing DNA damage and apoptosis at low concentrations, e.g. as shown in gastric cancer BGC-823 cells [7]. 5-azacytidine is mainly incorporated into RNA, affecting RNA synthesis and stability, and thereby also protein synthesis, and part of 5-azacytidine is reduced to 5AzadC which forms DNA adducts, causing mutations, double strand breaks and apoptosis, as reviewed [8]. Thus, premature conclusions have been made regarding the influence of DNA methylation, e.g. for the gene Wnt inhibitory factor 1 (*WIF1*) in osteosarcoma cell lines. A recent paper showed that 5AzadC treatment probably had activated *WIF1* expression indirectly by inducing maturation of the OS cell lines rather than having a primary direct effect on *WIF1* [9]. Some of the global and toxic effects of 5AzadC could be avoided using e.g. siRNAs which have been used successfully for targeting *DNMT1* mRNA in various cell lines [10, 11]. Alternative small molecules attacking the DNMT1 enzyme are also being developed [12]. We have, however, not found studies where these alternative methods have been used on bone cells.

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In addition to transcript levels, DNA methylation is also associated with features like histone modifications, nucleosome positioning and gene heterogeneities. Grundberg *et al.* [13] showed that 10.5% of CpGs were associated with nearby (± 100 kb) single nucleotide polymorphisms (SNPs) applying a conservative threshold for significance. Since SNPs are inborn, these methylations must be secondary to gene heterogeneities. As reviewed [4], some reports indicate that DNA methylation is also influenced by other genomic changes, such as histone modifications and nucleosome mobility and positioning. However, a most recent study [14], indicate that nucleosome assembly and positioning is preceded and facilitated by DNA methylation, which promotes chromatin packaging and inaccessibility to the transcriptional machinery. In any case, DNA methylation being a potentially reversible event [15], which ranges from being genome-wide to local gene-specific, is an important marker affecting transcription and is experimentally easier to study than the higher levels of DNA organization.

MAJOR SIGNALING PATHWAYS IN OSTEOBLASTS AFFECTED BY DNA METHYLATION

The ligands of the wingless/int-1 class (WNTs) and Bone Morphogenetic Proteins (BMPs) activate essential signaling pathways that are central for osteoblast function and differentiation. Mutations in components of these pathways are associated with variation of bone mineral density, increased fracture risks as well as with other human skeletal disorders [16]. In the canonical Wnt pathway (Fig. **1**) a Wnt extracellular protein binds to a Frizzled (fzd) transmembrane receptor and one of the coreceptors, low-density lipoprotein receptor-related protein (LRP)-5 or LRP6, causing phosphorylation of the intracellular protein disheveled (Dvl). The phosphorylated form of Disheveled (Dvl) then inhibits glycogen synthase kinase 3 β (GSK3 β) from phosphorylating cytosolic β catenin, thus preventing its degradation. Unphosphorylated β catenin stabilizes, translocates to the nucleus and binds to the transcription factors T-cell factor-1/lymphoid enhancing factor-1(TCF/LEF), causing transcriptional activation of target genes. The non-canonical Wnt signaling involves fzd signaling but does not require co-receptor LRP5/6 and has alternative routings from Dvl, independent of β -catenin [17] (Fig. **1**). The critical role of LRP5/6 in signaling is illustrated by the fact that mutations in these Wnt co-receptors may cause loss- or gain-of-function and hence an osteoporotic or a high bone density phenotype, respectively [18]. The Wnt pathway is inhibited by several extracellular antagonists, like sclerostin (gene product of *SOST*) and dickkopf WNT signaling pathway

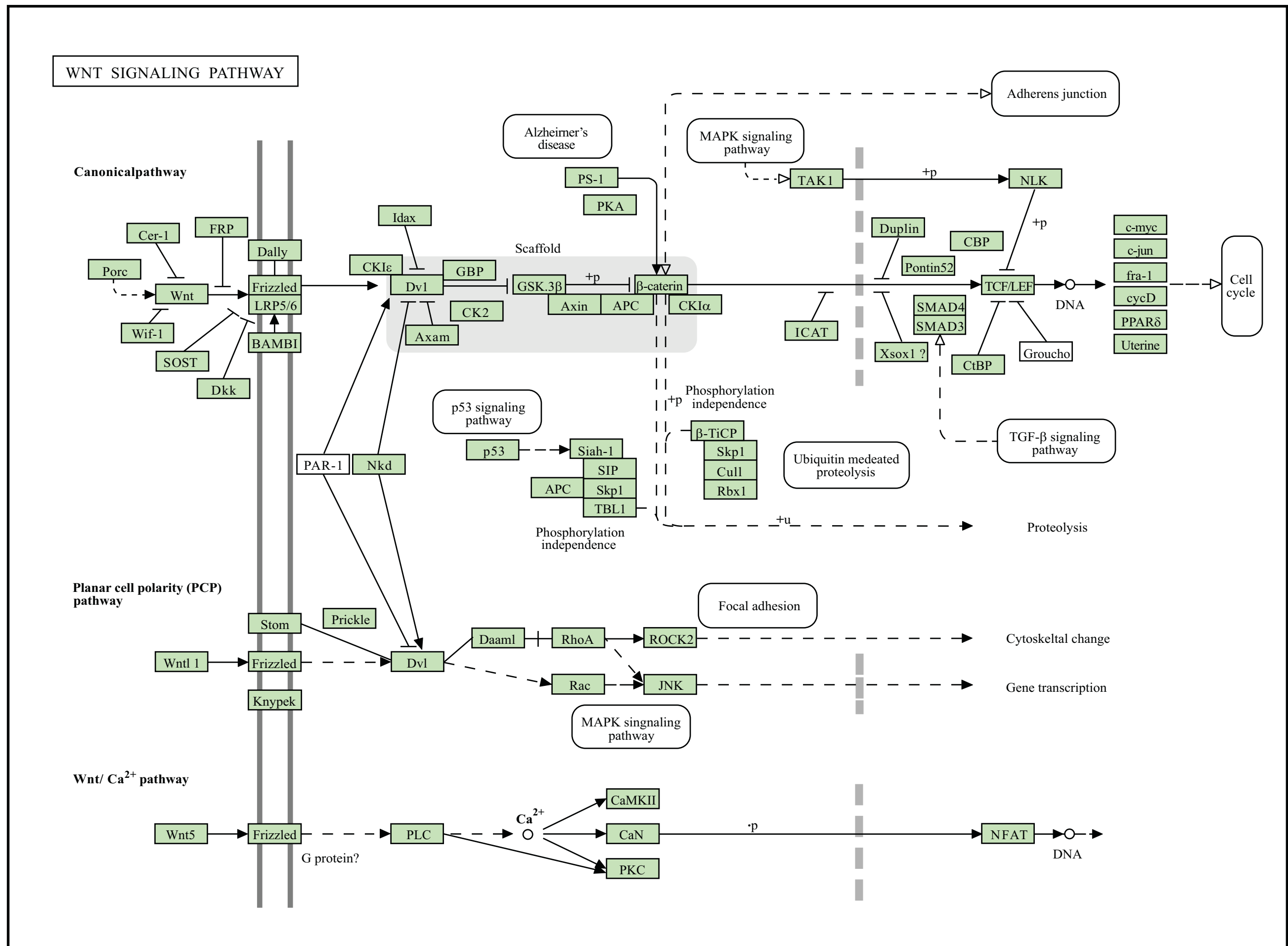

Fig. (1). Wnt signaling pathway from KEGG. A detailed description is found at the KEGG web site: http://www.genome.jp/dbget-bin/www_bget?pathway:map04310.

inhibitor 1 (DKK1) which bind LRP5/6 [19]. Wnt signaling is also inhibited by Secreted frizzled-related proteins (sFRPs) and WNT inhibitory factor 1 (WIF1) which bind to Wnt ligands or Frizzled receptors [20]. Several of the Wnt signaling genes have been subject to DNA methylation analyses and the results show marked functional implications as described later.

BMPs are ligands of heteromeric complexes of the bone morphogenetic protein receptor, type I and II (BMPRI and BMPRII), which are serine/threonine kinase transmembrane receptors. Upon ligand binding, BMPRI phosphorylates specific Smad proteins (Smad-1, -5 or -8), which may form complexes with the common key mediator Smad-4. The Smad-4 complex is then transported to the nucleus and it regulates transcription of specific target genes. The Wnt and BMP signaling pathways are cross-regulated, but the nature of this regulation is complex and can be synergistic or antagonistic, depending on the cellular context [16]. Also, components of BMP signaling are regulated by DNA methylation. 5AzadC up-regulated the expression of several BMPs, including BMP-2, BMP-4 and BMP-6, as well as several BMP downstream targets [21]. The web tool DAVID was used for functional annotation analysis of the most upregulated genes following the 5AzadC treatment. The upregulated genes were preferentially overrepresented in the categories “regulation of cytokine production”, “positive regulation of multicellular organism process”, and “positive regulation of cytokine production”.

GLOBAL METHYLATION PATTERNS OF GENES ARE HIGHLY ASSOCIATED WITH BMD IN POSTMENOPAUSAL WOMEN

As an attempt to characterize the DNA methylation pattern of genes with transcripts highly associated with BMD in 80 postmenopausal women, we analyzed >480 000 CpG methylation sites in bone biopsies [22] (Reppe *et al.*, submitted). We have initially focused on the 100 genes whose transcripts were most significantly correlated to BMD [23]. CpG methylation levels in these 100 genes were cross-correlated with their transcript levels. The four transcripts (*MEPE, SOST, WIF1* and *DKK1*) that correlated to the highest number of CpG methylations (n>100, r < -0.4, 5% FDR) in many different genes, are all well-known inhibitors of bone metabolism. In addition, other genes previously not recognized to be associated with BMD showed correlation between transcript levels and degree of methylation. We also identified 63 CpGs that differed in methylation level between osteoporotic and healthy controls at 10% FDR. Five of the 63 CpGs were significant at 5% FDR: RAD23 homolog B (cg14919562, *RAD23B*), peroxisomal biogenesis factor 14 (cg14170597, *PEX14*), tenascin XB (cg03822479, *TNXB*), solute carrier family 25 (mitochondrial iron transporter), member 37 (cg26617611, *SLC25A37*), phosphofurin acidic cluster sorting protein 2 (cg08105005, *PACS2*)) (Reppe *et al.*, submitted). The RAD23 CpG showed reduced methylation in osteoporotic women, while methylation levels at the other 4 were increased. Using linear regression on all bone donors with T-score (total hip BMD) as response and the 5 methylations at 5% FDR as covariates, we found from R^2, that these methylations explained 19% of the variation in BMD. Thus, our results demonstrate that in bone a relationship exists between DNA methylation and level of transcription of genes highly associated with BMD and therefore may explain part of the missing heritability component in genome-wide association studies (GWAS).

MEDIATION OF OSTEOBLAST SPECIFIC EXPRESSION BY DNA METHYLATION

An important feature of DNA methylation is enabling cell and tissue specific regulation in health and disease. The Wnt signaling inhibitor protein sclerostin, encoded from *SOST* gene, is predominantly expressed in osteocytes [24], and has emerged as an important bone modulator following experiments showing that monoclonal antibodies increases bone formation and bone mass, both in experimental animals and in humans [25, 26]. DNA methylation of *SOST* and its effect on expression have been extensively studied in osteoblastic cells and bone. Delgado-Calle *et al.* [27] observed that the experimentally demethylated proximal promoter region of *SOST* increased its transcription while hypermethylation had a repressive effect. They also showed that the *SOST* proximal promoter region was hypomethylated in osteocytes from human femoral bone but not in primary osteoblasts and bone-lining cells, indicating that the differential *SOST* expression between these cell types may at least be partly regulated by DNA methylation. This conclusion was further supported by electromobility shift assay (EMSA), which showed that DNA methylation affected protein binding to the core of the proximal *SOST* promoter. The affected *SOST* CpG methylations localized close to or at putative binding sites for transcription factors (SOST (A) in Fig. **2**) important for bone formation near the transcription start site (TSS). DNA methylation could effectively interfere with the binding of these bone modulatory transcription factors, thus leading to reduced transcription of *SOST*.

The question then arises how differential DNA methylation is mediated and if the extent of methylation also is modulated by a variety of external stimuli exerted on the mature osteocyte. Recently Delgado-Calle *et al.* [28] reported that the bone cell line HOS treated with 5AzdC and exposed to PFF has reduced expression of *SOST*, independently of *SOST* methylation in the proximal region. Nitric oxide (NO) is known to be increased upon mechanical stimulation of bone, and the authors showed that PFF in-

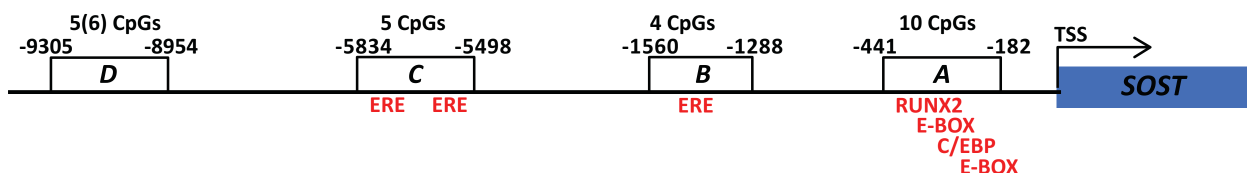

Fig. (2). SOST upstream region with CpG rich sequences with putative transcription factor binding sites (red text). TSS: transcription start site ERE: estrogen response element.

creased NO independently of the DNA methylation level in the cell line. PFF in presence of a NO inhibitor (1400 W) failed to reduce *SOST* expression. Furthermore, reduced expression of *SOST* upon incubation of static bone cells in growth medium from cells subjected to PFF indicated that the signal from NO is mediated via soluble factors independent of DNA methylation.

Further insight has been gained by *ex vivo* studies of DNA methylation in iliac bone biopsies. A study by Reppe *et al.* [29] compared DNA methylation levels in *SOST* upstream regions (A, B, C and D in Fig. **1**), initially in 4 healthy and 4 osteoporotic postmenopausal women of similar age and BMI, and identified differential methylation levels in region A. The results were replicated in independent cohorts of healthy (n = 36) and osteoporotic women (n = 27). Bone *SOST* mRNA and serum sclerostin levels were measured in all women and correlated positively with age-adjusted and BMI-adjusted total hip BMD ($r = 0.47$ and $r = 0.43$, respectively; both $p < 0.0005$), and inversely to serum bone turnover markers. Thus the *in vitro* discovery of Delgado-Calle *et al.* [27], that *SOST* proximal promoter DNA methylation was inversely associated with *SOST* expression levels, were confirmed by *ex vivo* experiments. The, at first glance, surprising positive association between bone *SOST* mRNA and serum sclerostin levels with BMD was explained by an adaptive homeostatic mechanism involving increased *SOST* DNA methylation in the osteoporotic women as an attempt to halt further bone loss and activate bone formation by increasing Wnt signaling.

Recently, sclerostin super-producer SaOS-2 clones that had reduced CpG methylation in the *SOST* proximal promoter have been identified. The comparison of these with “normal” SaOS-2 cells may provide further insight into how DNA methylation of this specific region is regulated [30]. However, it is important to point out that SAOS2 is an osteosarcoma cells line and global hypomethylation is a generally recognized feature of oncogenesis.

The case with receptor tyrosine kinase-like orphan receptor 2 (ROR2) is another example of DNA methylation as an important regulator of Wnt signaling as well as DNA methylation having an important role in osteoblastic differentiation. ROR2 is a specific receptor or co-receptor for WNT5A. Its promoter is increasingly demethylated during *in vitro* differentiation of human bone marrow stem cells (hBMSC) to osteoblasts [31]. In another study the expression of *Ror2* mRNA was shown to increase 300-fold upon differentiation of pluripotent stem cells to preosteoblasts but was absent in osteocytes [32]. The induced expression of *ROR2* by lentivirus promoted differentiation of MSC to the osteoblastic phenotype [33], thus lending further support to the above findings. It is important to emphasize that, as mentioned above for *WIF1*, not all Wnt regulatory genes are regulated by DNA methylation [9].

Cho *et al.* [34] showed that Wnt3a was only able to activate *Bmp2* and alkaline phosphatase (*Alpl)* expression in cells destined to osteoblastic differentiation. Non-osteogenic cells had highly methylated *Bmp2* and *Alpl* promoters which prevented their respective expression. Demethylation with 5AzadC permitted expression of *Bmp2* and *Alpl* upon treatment with Wnt3A. In line with these observations, these authors showed reduced CpG methylation in the promoter areas of these genes in osteoinducible cell lines and increased methylation in non-inducible cell lines. Similar results were shown with methylation of the distal-less homeobox 5 (*DIx5)* and Osterix (*Osx)* promoters, which were unmethylated in osteogenic cell lines expressing these genes but methylated in non-osteogenic cell lines where these genes were silenced [35]. Furthermore, demethylation with 5AzadC increased the expression of *DIx5 and Osx*. Similar results were obtained with multipotent dental pulp cells, having the capacity to differentiate to osteoblastic cells capable of carrying out mineralization, and showing increased activity of alkaline phophatase and increased expression of *DLX5*, *OSX* and *RUNX2* upon 5AzadC mediated demethylation [36]. The treatment of mouse MSC with another DNMT inhibitor, 5-azacytidine, also down-regulated the methylation levels in the promoter of the *Dlx5* gene, resulting in osteogenic differentiation [37].

Interestingly, BMP-2 stimulation of the myoblastic cell line C2C12 for 6 hours resulted in hypomethylation of the *Dlx5* promoter and thereby induced *Dlx5* expression and osteogenic differentiation [35]. These findings indicate that situations with increased expression of BMPs leads to altered DNA methylation levels at the Dlx5 promoter.

A recent paper indicates that hypoxia also may affect DNA methylation. Although likely effects on DNA were not studied, the stimulation of the osteoblastic cell line HOS using hypoxia mimetic deferoxamine was shown to down-regulate DNMT3A, the enzyme that carries out genome-wide *de novo* DNA methylation [38]. Several histone modifying enzymes were also affected, which is consistent with increased chromatin condensation.

As expected a select group of genes are silenced as MSC differentiate to the osteoblastic phenotype. The transcription factor Brachyury, also known as transcription factor T, showed reduced expression and increased promoter methylation upon transition to osteoblast, while other stem cell related genes (developmental pluripotency associated 5 (*DPPA5*), fibroblast growth factor 4(*FGF4*), forkhead box D3 (*FOXD3*), lin-28 homolog A (*LIN28*), nestin (*NES*) and zinc finger protein 42 (*ZFP42*)) showed minor changes from the unmethylated stage [39].

MECHANICAL REGULATION OF DNA METHYLATION IN OSTEOGENIC CELLS

Skeletal loading has been shown to be a key regulator of bone metabolism controlling bone turnover, growth and mineralization [40, 41]. In cell culture experiments, exposure to PFF is widely used to mimic stress experienced by cells within bone. An example by Hum *et al.* [42] illustrates how mechanical stress can mediate expressional regulation via DNA methylation. With PFF the tyrosine kinases Pyk2 and c-src are activated and translocated to the nucleus. Upon accumulation, they associate with Methyl-CpG-binding domain protein 2 (MBD2) which binds methylated CpG islands in promoters. The authors suggested that this complex functions as an “off switch” to suppress the anabolic response of bone subjected to mechanical load in osteocytes. This hypothesis is supported by increased bone mass in *Pyk2* and *Src* null mice [43, 44]. However, we found no studies report-

ing effects of Pyk2 and c-src deficiency on expression of osteoclast activating proteins.

When mouse bone marrow progenitor cells were subjected to PFF for 3 hours, subsequent DNA methylation and mRNA expression analysis showed that the methylation level of a CpG in the Osteopontin promoter was reduced 1.5 fold while the mRNA increased 2.3 fold. No change was seen for Osteocalcin or Collagen I [45]. Although only one CpG was tested in each of the three promoter regions, this study showed that mechanical loading has the potential to initiate altered DNA methylation and osteogenic differentiation affecting distinct genes.

In humans, expression of the neurological transcriptional factor zinc finger of the cerebellum 1 (*ZIC1*) has been shown to be markedly increased in loaded (vertebral) bone as compared to unloaded (iliac) bone [46, 47]. In a paper in prep by H.Datta and K.M.Gautvik *et al.* we show that increased *ZIC1* expression in loaded bone is associated with reduced methylation in several CpGs in the *ZIC1* promoter region. The basal *ZIC1* expression in the human bone osteosarcoma cell line SAOS2 is much higher (>10 fold) than the level in normal stromal cells, i.e. human skin fibroblasts. The differences in the *ZIC1* expression mirrored closely the degree of *ZIC1* promoter methylation in the two cell types, being considerably lower in SaOS2 cells than in the fibroblast. When SAOS2 were subjected to PFF there was a significant further rise in *ZIC1* expression, which could be demonstrated despite relatively high preexisting basal expression. The increased *ZIC1* expression in the fibroblast induced by PFF was accompanied by *ZIC1* promoter demethylation.

To our knowledge, no previous study has reported specific genome-wide effects on DNA methylation on osteoblastic cells caused by PFF, although the publications of Hum and Arnsdorf *et al.* [42, 45] clearly indicate that this type of experiment is called for.

OTHER DNA METHYLATIONS AFFECTING BONE QUALITY

The examples of methylation affecting bone quality include promoter methylation of lysyl oxidase (*Lox*). A paper by Thaler *et al.* [48] demonstrated that homocysteine could inhibit the collagen cross-linking enzyme lysyl oxidase (Lox) in osteoblasts by increasing its proximal promoter methylation. The authors suggested that the stimulation was mediated via increased expression of interleukin 6 (IL-6), followed by activation of Janus kinase 2 (Jak2) which in turn upregulated expression of the transcription factor Friend leukemia virus integration 1 (*Fli1*) and subsequently *Dnmt1* expression, which mediated methylation of the LOX promoter.

Alkaline phosphatase is important for providing inorganic phosphate (Pi) for the mineralization of bone as well as for removing the mineralization inhibitor PPi [49]. Analysis of *ALPL* showed that methylation in a 1415 bp CpG island flanking both sides of the TSS was inversely associated with the transcript levels of *ALPL* in the osteoblastic cell line MG-63 and the non osteoblastic cell line MCF-7 [50]. The same study demonstrated high methylation (>90%) in dissected human osteocytes while dissected bone lining cells showed an intermediate level of methylation (58±13%). Although mRNA was not assessed in that study, a previous study [51] showed low expression of *ALPL* mRNA in microdissected osteocytes from frozen cortical bone as compared to primary osteoblasts. In addition, demethylation with 5AzadC induced a strong increase in *ALPL* expression in MG-63 cells, accompanied by a parallel increase in alkaline phosphatase activity, while 5AzadC did not affect alkaline phosphatase levels in primary cultures of hOBs that were already hypomethylated. Together these results demonstrate that DNA methylation is regulating *ALPL* expression. Also osteocalcin, which is implicated in bone mineralization and calcium ion homeostasis, showed increased expression and reduced promoter methylation during osteoblast differentiation, but this was perhaps not a direct effect from methylation because key transcription factors bound to the promoter elements irrespective of the methylation levels [52].

Upon loss of estrogen production at menopause a sharp drop in BMD occurs, presumably due to altered activity of the estrogen receptor alpha (*ESR1*) in bone cells. In osteoblastic cells, expression of *ESR1* is primarily regulated by the promoter F, and its methylation levels may thus be associated with bone metabolism. Penolazzi *et al.* [53] showed that DNA methylation at 4 CpGs distal within the examined ~700 bp promoter F region tended to correlate inversely with expression in osteoblastic (SaOS2 and MG-63) breast cancer cell lines (MDA-MB-231 and MCF-7) and osteoblasts isolated from bone biopsies. The affected methylation sites were close to binding sites for transcription factors activator protein 1(AP-1) and RUNX2.

DNA METHYLATION AFFECTING OSTEOCLAST ACTIVITY

Osteoclasts are giant multinuclear cells that are responsible for degradation of the bone matrix, prior to formation of new bone. These cells differentiate from the monocyte/macrophage lineage upon stimulation by the two cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) produced in osteoblasts as described below [54]. A defective NF-κB signaling pathway results in the suppression of osteoclastogenesis often leading to osteopetrosis. Upon binding of RANKL to RANK, the signal may be routed via various pathways which converge on nuclear factor-activated T cells c1 (NFATc1), considered as a master regulator of osteoclast differentiation. NFATC1 controls expression of genes like Tartrate-Resistant Acid Phosphatase (*TRAP, ACP5*), cathepsin K (*CTSK*), calcitonin receptor (*CALCR*), and osteoclast-associated receptor *(OSCAR*) through cooperation with microphthalmia-associated transcription factor (MITF) and c-Fos [55]. A soluble decoy receptor, osteoprotegerin (OPG), inhibits osteoclast activity.

Since RANKL and M-CSF are produced in osteogenic cells, DNA methylation of these genes has the potential to regulate osteoclast function. The mouse stromal/osteoblastic lineage ST2 has been shown to support osteoclastogenesis at early passages, but this support ceased with increasing number of culture passages concomitantly with increased DNA methylation of a CpG island around the *Rankl* transcriptional start site (-66 to +357) and reduced mRNA levels [56, 57]. In

a later comprehensive follow up paper [58] the same authors showed that especially a CpG three bases upstream of the *Rankl* TATA box, showed increased methylation in ST2 cells at higher passage numbers. Electromobility shift assays (EMSA) with a oligonucleotide methylated at this site bound CpG binding protein (MeCP2) while the unmethylated oligonucleotide bound TATA-box binding protein (TBP) as shown by supershift analysis with antibodies against the respective proteins. This assay along with chromatin immunoprecipitation (ChIP) assay indicated that MeCP2 and TBP could not co-occupy the site. Furthermore, transfected ST2 cells with reporter constructs having methylated or unmethylated *Rankl* upstream regions, showed increased expression from the unmethylated constructs. Thus, DNA methylation levels of this region alone (in presence of vitamin D) is sufficient for regulation of *Rankl* expression. The authors speculate that generalized accumulation of methylation at the CpG island in the mouse *Rankl* promoter may result in the recruitment of histone deacetylase and chromatin condensation leading to the stable epigenetic silencing of *Rankl*.

Methylation of *RANKL* and *OPG* has also been studied in human HEK-293 cells [59] that have a low basal level expression of these ligands, and the DNA demethylating agent 5AzadC produced a 170-fold induction of *RANKL* and a 20-fold induction of *OPG* mRNA expression. Furthermore, methylation-specific PCR (qMSP) and pyrosequencing of CpG islands close to the TSS demonstrated inverse degrees of DNA methylation and mRNA levels when comparing the cell lines MG-63, HOS-TE85, HEK-293 and primary cultured osteoblasts. However, although *RANKL* mRNA and *RANKL*:*OPG* mRNA ratio were significantly higher in femoral bone from patients with fractures than in surrogate controls with osteoarthritis, no evidence for differential methylation across patient groups were found.

Several papers have shown various types of epigenetic regulation in osteoclastic cells (as reviewed [60]), but few have dealt with DNA methylation. However, a recent comprehensive study by de la Rica *et al.* [61] compared the global DNA methylation profiles (>480 000 CpGs) of human monocytes and their derived osteoclasts following stimulation with M-CSF and RANKL. Both hypomethylation and hypermethylation changes were observed and, interestingly, the changes occurred in the virtual absence of DNA replication, suggesting the participation of active mechanisms. Hypomethylation was seen at most of the genes necessary for osteoclast function, including *ACP5*, *CTSK* and dendrocyte expressed seven transmembrane protein (*TM7SF4, DCSTAMP)* concomitant with increased expression. Furthermore, regions undergoing DNA methylation changes were enriched for binding motifs for AP-1, NF-kB, and PU.1, which are key transcription factors in osteoclastogenesis. The authors showed that PU.1 has a central role in driving DNA methylation changes during osteoclast differentiation. ChIPseq showed that PU.1 bound to both hypermethylated and hypomethylated promoters, but recruited DNMT3B to the former and TET2 to genes that become demethylated. TET2 converts 5-methylcytosine to 5-hydroxymethylcytosine, as part of the demethylation process [62]. Downregulation of *PU.1* mRNA by siRNAs lead to partially impaired DNA methylation, expression, and recruitment of TET2 and DNMT3B to PU.1 targets. DNMT3B plays a crucial role both in maintenance of DNA methylation patterns as well as in incorporating de novo hypermethylation of promoter CpG islands [63].

As shown by Nishikawa *et al.* [64] also DNMT3A has an important function in promoting osteoclastogenesis by increasing DNA methylation of anti-osteoclastogenic genes. The authors identified DNA-methylation regulated genes by comparing wild type bone marrow–derived monocyte-macrophage precursor cells (BMMs) with $Dnmt3a_{Rank}^{-/-}$ BMMs after RANKL treatment, applying three criteria: 1. Selection of genes downregulated during osteoclast differentiation of control cells. 2. Further selection of genes in which the reduced expression was clearly normalized by *Dnmt3a* deficiency. 3. Final selection of genes also having loci in which methylation was increased more in control cells than in $Dnmt3a_{Rank}^{-/-}$ BMMs after RANKL treatment. This strategy identified 19 genes with increased DNA methylation accompanied with reduced expression. These genes included interferon regulatory factor 8 (*Irf8)* which has a gene product that is a known negative regulator of osteoclast differentiation [65]. The results were corroborated by knockdown studies in mice and osteoclastic cells, e.g., *Dnmt3a* deficient osteoclast-precursors did not differentiate into mature osteoclasts, and mice with *Dntmt3a* deficiency developed osteopetrosis. Comparison of $Dnmt3a^{-/-}$ and $Dnmt3b^{-/-}$ osteoclast precursors as well as use of various cell specific targeted knockouts in mice confirmed that DNMT3A is of central importance in osteoclast differentiation.

De la Rica *et al.* [61] detected hypomethylation only in the gene body of *NFATC1* upon differentiation, while another study identified differential DNA methylation at the TSS of *NFATC1* between osteoarthritic and healthy cartilage isolated from the same person [66]. Although the cartilage probably did not contain osteoclasts, the study shows that DNA methylation also in the TSS of *NFATC1* has the potential also to play a part in its expression and activity of bone osteoclasts.

In summary, several studies show that DNA methylation in bone cells may have profound effects on gene expression and cell commitment and consequently to bone metabolism. The question regarding how the differential DNA methylation levels are regulated is largely unknown, but in some cases gene polymorphisms are important. Furthermore, most of the studies have been focused on promoter regions and function while regulation of methylations elsewhere is still inadequately covered. Although mother-daughter and twin studies have shown that 60 – 80% of the variation in BMD is heritable [67, 68], GWAS have so far only been able to explain around 6% of the variation [69]. Recent studies indicate that part of the missing heritability may be explained by DNA methylation.

Current studies are somewhat limited in mainly being performed *in vitro* and often focusing on few genes. However, recent studies involving human bone biopsies and methods for genome-wide methylation studies (reduced representation bisulfite sequencing (RRBS) and microchips covering a larger part of the methylome, like Illumina 450 k) is expected to provide better insight about genome DNA-methylation and its effect on bone tissue and gene-regulation. Furthermore, DNA methylation is not an isolated event, but also affects and is itself affected by other gene

regulatory mechanisms, such as gene silencing via histone modification. The Encyclopedia of DNA Elements (ENCODE) project (https://www.encodeproject.org/) seeks to identify functional elements in the human genome, and will be a valuable tool for identification of all aspects of genome organization, including DNA methylation. However, bone tissue and cells to date have paucity of relevant data and therefore are insufficiently represented.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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