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Research Article

In Silico Analysis of MicroRNA Regulation of Bone Development: Metal Response Element-Binding Transcription Factor 1 and Bone Signaling Pathways

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Abstract

Zinc (Zn) is an essential nutrient for bone growth and development. The Zn/metal response element-binding transcription factor 1 (MTF-1) regulates transcriptional activity by binding to the metal response elements (MRE) within the promoter regions of Zn-responsive genes. The effects of Zn on bone may be mediated through insulin-like growth factor 1, which intersects with pathways involved with bone metabolism, transforming growth factor β/bone morphogenic protein pathway (TGFβ), p38 mitogen-activated protein kinase pathway (MAPK), wingless-related integration site pathway (Wnt), and Hedghog pathways. The purpose of this *in silico* study was to determine whether microRNAs (miRNA) that are predicted to target MTF-1 also are predicted to target genes within the TGFβ, MAPK, Wnt, and Hedgehog signaling pathways. Ninety-five miRNAs were predicted to target MTF-1. Thirty-three (34.7%) of these miRNAs exhibited experimentally verified interactions with 68 genes among the TGFβ, MAPK, Wnt, and Hedgehog signaling pathways. hsa-miR-3613-3p is predicted to target 171 genes among the TGFβ, MAPK, Wnt, and Hedgehog signaling pathways; six of these miRNA/gene interactions have been experimentally verified. The signaling pathway genes for which miRNA interacts were experimentally verified are associated with molecular networks involved in binding (GO:0005488) and catalytic activity (GO:0003824);and cellular process (GO:0009987), biological regulation (GO:0065007), response to stimulus (GO:0050896), developmental process (GO:0032502), and metabolic process (GO:0008152) within biological networks. These results provide evidence for links between MTF-1 and bone development signaling pathways, and suggest that factors regulating cellular Zn homesostasis through miRNA regulation of MTF-1 may also affect expression of bone development genes.

Keywords: Metal-responsive transcription factor 1; Transforming growth factor β/Bone morphogenic protein; Wingless-related integration site; p38 mitogen-activated protein kinase; Hedgehog; microRNA; hsa-miR-3613-3p

Abbreviations

IGF-1: Insulin-Like Growth Factor 1; IGFBP: Insulin-Like Growth Factor Binding Protein; MAPK: P38 Mitogen-Activated Protein Kinase; MiRNA: MicroRNA; MRE: Metal Response Element; MTF-1: Metal-Responsive Transcription Factor 1; TGFβ: Transforming Growth Factor B/Bone Morphogenic Protein Pathway; Wnt: Wingless-Related Integration Site; Zn: Zinc

Introduction

Adequate zinc (Zn) nutriture is important for normal growth and development. Weanling rats fed Zn-adequate or Zn-deficient diets exhibit significant differences in their growth rates [1,2]. Impaired growth in humans, as determined by 20% prevalence in the population exhibiting low length- or height-for-age in children less than five years of age, is considered a useful indicator of low Zn intakes within that population [3]. Zn supplementation is effective in increasing growth rates in children exhibiting impaired growth due to Zn deficiency [4]. Zn is also known to play a key role in bone metabolism, as data in young mice and cell culture models clearly indicate that Zn deficiency leads to impaired skeletal growth [5-9]. Our research team has recently demonstrated that Zn supplementation significantly increases bone formation activity in humans [10].

The mechanisms involved with bone development are complex and involve numerous regulatory elements [11]. Bone mass in humans increases during fetal growth, is steadily maintained throughout childhood, peaking at ~25-30 years of age, and decreases as one ages [12-15]. Bone modeling during growth proceeds through the organized action of bone deposition and resorption to allow bones to expand and lengthen (i.e., periosteal apposition and endochondral ossification, respectively) into their mature form [16]. The amount of bone mineral acquired from birth to adulthood follows age- and sex-specific patterns, and the rates of bone mass accrual differ throughout the pubertal phases of adolescence. Approximately 90% of adult bone mass is achieved during puberty, with 40% occurring during the four years surrounding the peak in bone mass gain [17]. Changes in the structure and composition of bone (i.e., cartilage and cortical and trabecular bone) also occur during puberty, as do increases in the inner and outer dimensions of long bones, which provide greater

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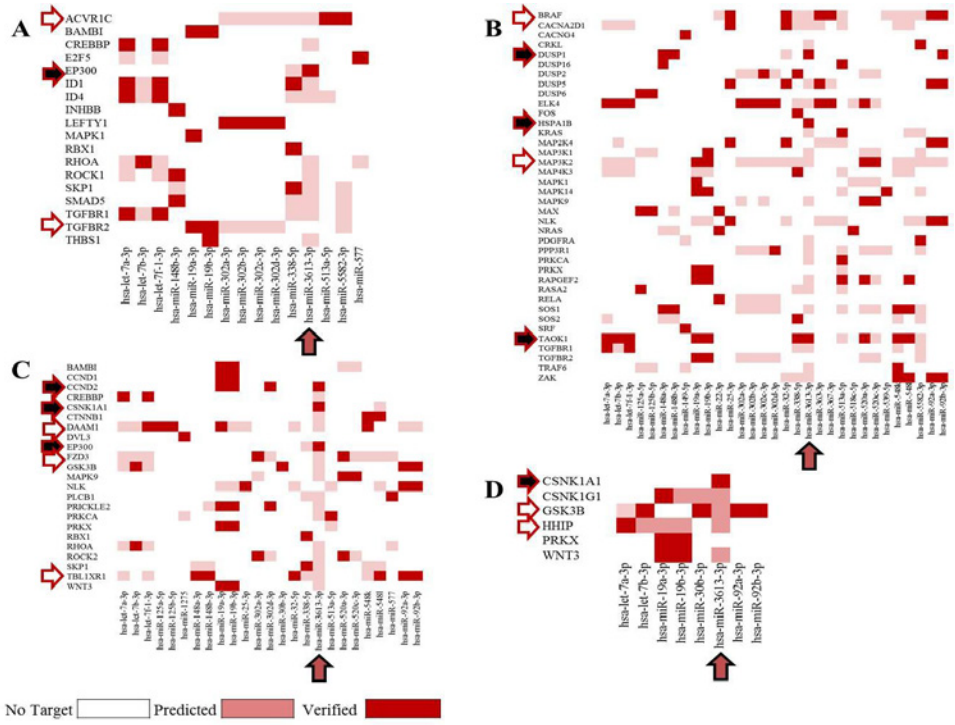

Figure 1: Heatmaps of experimentally verified and predicted miRNA/gene interactions.
Note: The miRNAs shown are those that have been experimentally verified to interact with at least one of the genes within the TGFβ (A), MAPK (B), Wnt (C), or Hedgehog (D) signaling pathways. The color gradation indicates whether there is no miRNA/gene interaction (white), a predicted miRNA/gene interaction (light color), or and experimentally verified miRNA/gene interaction (dark color). The red arrows highlight hsa-miR-3613-3p, the miRNA that exhibited the largest number of predicted and verified miRNA/gene interactions. The black arrows indicate the genes that have been experimentally verified to interact with hsa-miR-3613-3p. The white arrows indicate the genes within each pathway that are predicted and/or verified to interact with the largest number of miRNAs.

structural strength to the skeleton. Increases in trabecular bone occur between sexual maturity stages 3 and 4 [18], and the density of cortical bone is lower among children *vs.* adults and increases more rapidly with epiphyseal fusion [19-21].This period of rapid bone mass accretion, due to heightened modeling and remodeling processes, is considered an opportunity for diet to have an effect on optimizing peak bone mass and favorably altering bone size and shape.

The effects of Zn on bone growth may be mediated through insulin-like growth factor 1 (IGF-1), as levels increased following Zn supplementation of Zn-deficient children [4]. The combination of Zn and insulin-like growth factor 1increased the amounts of protein and DNA, and cellular proliferation, in cell culture models [7]. Zn is thought to partition insulin-like growth factor binding protein/ insulin-like growth factor 1 complex to the cell surface, increasing its proximity to the receptor [22].

There are other cellular mechanisms involved in bone growth and development. Important signaling pathways involved with bone development include the transforming growth factor β/ bone morphogenic protein (TGFβ), p38 mitogen-activated protein kinase (MAPK), wingless-related integration site (Wnt), Hedgehog, Notch, and fibroblast growth factor (FGF) pathways [23]. The IGF-1 pathway intersects with the MAPK, Wnt, and Hedgehog signaling pathways [24,25], and functions synergistically with TGFβ during mesenchymal stem cell chondrogenesis [26]. Hence, part of the Zn-effect on bone growth and development may be mediated through its interaction with the IGFBP/IGF-1 complex, thereby affecting these four signaling pathways.

Metal response element-binding transcription factor-1 (MTF-1) is a Zn-finger DNA binding protein of the Cys_2His_2 family of transcription factors that functions as an intracellular Zn sensor and transcriptional regulator of metallothionein, and SLC30A1 (ZNT1) [27-30]. Of the six Zn fingers, the finger at position one functions as the Zn sensor [31]. Computational analysis of cis-regulatory motifs predicted the presence of transcription factor binding sites within the promoter regions of numerous miRNA genes [32]. It is postulated that MTF-1serves as a master regulator of miRNA gene expression, along with c-Myb, NF-Y, Sp-1, and AP-2α [32]. The identification of other genes, such as those that are stress-related, secretory liver proteins, liver transcription factors, signal pathway factors, and tissue-specific proteins, as targets for MTF-1 [33], adds further support for Zn and MTF-1 as playing an essential role in regulation of intracellular biochemical pathways.

Genes whose transcription is regulated by Zn through MTF-1 contain within their promoters metal response elements (MRE). Several genes within signaling pathways involved in bone growth and development contain MREs, including TGFβ receptor 1, inhibin/activin BC subunit, activin receptor IIB, fibroblast growth factor receptor, and Notch-1 [33]. Cellular Zn homeostasis and physiological pathways are influenced through Zn-bound MTF-1 which then binds to the MRE [31,34], though other Zn-regulatory

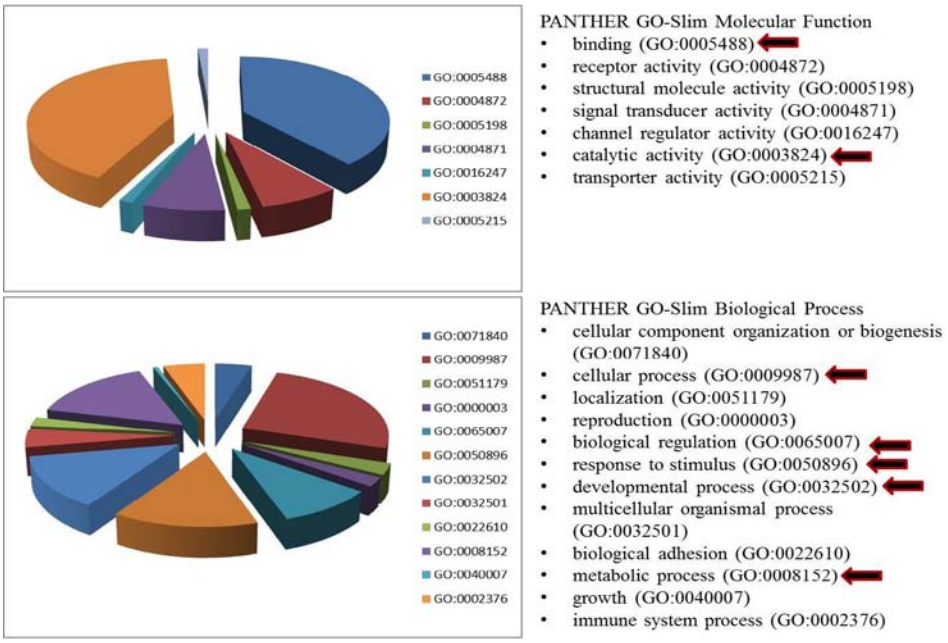

Figure 2: PANTHER Gene Ontology overrepresentation.
Note: Analysis of the genes predicted to be targets for the miRNAs that were verified experimentally to interact with TGFβ, MAPK, Wnt, or Hedgehog signaling pathway genes. The arrows indicate the GO categories containing the highest number of genes analyzed.

factors may be involved [35]. Little is known about the regulation of MTF-1 synthesis, nevertheless it is a putative target for small non-coding RNAs such as microRNAs. The purpose of this *in silico* investigation is to determine whether the miRNAs that are predicted to target MTF-1 also target genes in TGFβ, MAPK, Wnt, and Hedgehog signaling pathways, pathways that are associated with bone development. The results suggest that significant regulation of bone development is mediated through miRNAs that also regulate expression of the intracellular Zn sensor, MTF-1.

Materials and Methods

The miRNAs that are predicted to target MTF-1 were identified using the miRDB.og database, which uses the Mir Target algorithm to predict miRNA-target interactions [36,37]. Target scores ≥ 81 were considered likely miRNA/gene interactions; there were 95 miRNAs that fit this threshold score. These miRNAs were then used to query the mirPath v.3 (DIANA Tools) database [38]; the output being a list of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways containing predicted gene targets. The KEGG signaling pathways that are involved in osteoblast differentiation and bone development were chosen for further analysis [23]. Contained within the output is a list of each of the miRNAs predicted to target each of the genes within a particular KEGG pathway. DIANA-Tar Base v7.0 was used to identify which of the miRNA/gene interactions had been experimentally verified [39]. Therefore, for each KEGG pathway the miRNAs for which there was at least one experimentally verified miRNA/gene interaction were analyzed further.

The Panther Overrepresentation Test (release 20160715) (PANTHER version 11.1 Released 2016-12-28; [40-42]) was performed using the genes predicted to be targets for those miRNAs for which there was at least one experimentally verified miRNA/gene interaction. Each miRNA was used to query the miRDB.org database for their respective predicted targets, using a Target Prediction Score of ≥81 as the cutoff. The gene list was tested against the PANTHER GO-Slim Molecular Function and –Slim Biological Process data sets. The Bonferroni correction for multiple testing is used by the program to identify significantly overrepresented annotated gene ontologies. Only those results with $p < 0.05$ are reported.

Results and Discussion

The mirPath database query yielded 75 KEGG pathways in which their genes were predicted to be targets for the 95 miRNAs chosen in this study (Supplement Table 1). The number of predicted miRNA/gene interactions within the osteoblast/bone development signaling pathways, TGFβ, MAPK, Wnt, and Hedgehog, were determined (Supplement Tables 2-4). Of the miRNAs predicted to interact with MTF-1, 100% putatively targeted the genes within the MAPK signaling pathway; 98% putatively targeted the genes within the Wnt signaling pathway; 94% putatively targeted the genes within the TGFβ signaling pathway; and 81% putatively targeted the genes within the Hedgehog signaling pathway. However, only a small proportion of the miRNA/gene interactions have been experimentally verified (MAPK, 32.6%; Wnt, 26.9%; TGFβ, 16.8%; Hedgehog, 10.4%; Supplement Table 5).

Hsa-miR-3613-3p exhibited the most verified and predicted interactions among all of the miRNAs analyzed across the four signaling pathway (Figure 1A-D; Supplement Table 2). The altered expression of hsa-miR-3613-3p is observed in tissue and plasma patients afflicted with non-small cell lung cancer [43], and is expressed in neuroblastoma and colon cancer cell lines [44,45]. There are six genes within the four signaling pathways in which their interaction with hsa-miR-3613-3p has been verified: CCND2, CSNK1A1, DUSP1, EP300, HSPA1B, and TAOK1. CCND2 is a member of cyclin

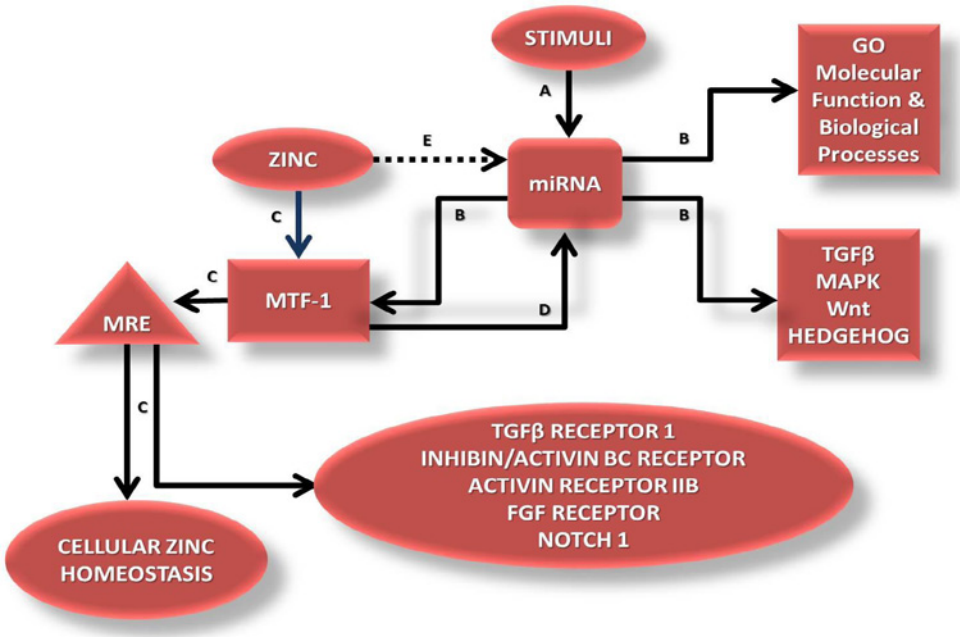

Figure 3: A model for the Zn-dependent, and miRNA, regulation of bone growth and development signaling pathways.
Note: (A) – Environmental, physiological stimuli induce miRNA synthesis. (B) – miRNA influence gene expression by binding to the 3´ UTR (untranslated region) of target mRNA, thereby inhibiting translation. There are 95 miRNAs that are predicted to target MTF-1. These miRNAs also target genes overrepresented in the PANTHER Gene Ontology Molecular Function and Biological Processes networks and in signaling pathways involved with osteogenesis and bone development. (C)– MTF-1 is a intracellular zinc sensor. Zinc/MTF-1 binds to the MRE-containing genes (metal response element), resulting in their transcription and in the maintenance of cellular Zn homeostasis. The MRE was also found in promoters of genes associated with osteogenic and bone development pathways [27-33]. (D)– Lee, et al. [32] postulates that MTF-1 is one of several master regulators of transcription of miRNAs, thereby adding another control through which Zn regulates gene expression. (E)– Zn may regulate miRNA expression through other transcriptional regulators such as the transcription factor KLF4 and/or miRNA genes containing the Zn transcriptional regulatory element in their promoters [35].

family and is experimentally verified to be involved in the regulation of protein phosphorylation (GO:0001934) and cyclin-dependent protein serine/threonine kinase activity (GO:0045737) [45,46]. CSNK1A1 is casein kinase 1 α1, and is shown to participate in protein phosphorylation (GO:0006468) and Golgi organization(GO:0007030) [47,48]. DUSP1 is dual specificity phosphatase 1, responds to oxidative stress (GO:0006979) and exhibits negative regulation of MAPK kinase activity (GO:0043407) [48-50]. EP300 is E1A binding protein P300, a histone acetyltransferase involved with chromatin remodeling. It is involved in the cellular response to hypoxia (GO:0001666) and histone H2B acetylation (GO:0043969) [51,52]. HSPA1B is a member of the heat shock protein 70 family, regulates protein ubiqutination (GO:0031396), and the cellular response to oxidative stress (GO:0034599) [53,54]. TAOK1 is a serine/theronine protein kinase. It is involved with the cellular response to DNA damage (GO:0006974) and the positive regulation of the JNK cascade (GO:0046330) [55,56]. The many predicted and verified hsa-miR-3613-3p/gene interactions suggests this miRNAmay play a key regulatory role in]cellular physiology, as well as in bone growth and development.

Eighty-nine of the 95 miRNAs targeting MTF-1 putatively target at least one of the 64 TGFβ signaling pathway genes (Supplement Tables 2,3). The miRNA/gene pairs for which interactions have been experimentally verified are shown in Figure 1A and Supplement Table 5. Fifteen miRNAs have been verified to interact with 18 genes in the pathway. The genes verified and predicted to interact with the largest number of miRNAs are ACVR1C (8 miRNAs) and TGFBR2 (7 miRNAs).

All of the miRNAs that target MTF-1 also putatively target at least one of the 193 genes in the MAPK signaling pathway (Supplement Table 2,3). The miRNA/gene pairs for which interactions have been experimentally verified are shown in Figure 1B and Supplement Table 5. Thirty-one miRNAs have been verified to interact with 38 genes in the pathway. The genes verified and predicted to interact with the largest number of miRNAs are BRAF (14 miRNAs) and MAP3K2 (19 miRNAs).

There are 113 genes within the Wnt signaling pathway. These genes are predicted to be targets for 93 of the miRNAs that target MTF-1 (Supplement Tables 2,4). The miRNA/gene pairs for which interactions have been experimentally verified are shown in Figure 1C and Supplement Table 5. Twenty-five miRNAs have been verified to interact with 23 genes in the pathway. The genes which are predicted to interact with the largest number of miRNAs are DAAM1 (13 miRNAs), FZD3 and TBL1XR1 (11 miRNAs each).

The Hedgehog signaling pathway contains 43 genes. Compared to the other signaling pathways, only 77 miRNAs that target MTF-1 are predicted to also target the Hedgehog signaling pathway genes (Supplement Tables 2,4).The miRNA/gene pairs for which interactions have been experimentally verified are shown in Figure 1D and Supplement Table 5. The interactions of only eight miRNAs and six genes have been experimentally verified within the Hedgehog signaling pathway. The genes which are predicted to interact with the largest number of miRNAs are GSK3B (6 miRNAs) and HHIP

(5 miRNAs).

The genes with reported experimental verification of their interaction with the miRNAs that are predicted to target MTF-1 were analyzed for their overrepresentation among annotated gene ontology molecular function and biological processes networks (Figure 2). The genes associated with these miRNAs were particularly involved with binding (GO: 0005488) and catalytic activity (GO: 0003824) within the molecular function category. The biological processes that were overrepresented by these genes include cellular process (GO: 0009987), biological regulation (GO: 0065007), response to stimulus (GO: 0050896), developmental process (GO: 0032502), and metabolic process (GO: 0008152) (Figure 2).

A model for the interactions between Zn, miRNAs, and signaling pathways involved with bone growth and development is proposed (Figure 3). The synthesis of miRNA is influenced by environmental and physiological stimuli. The miRNA inhibiting translation of their target genes by binding to the 3′ untranslated region of target mRNA.There are 95 miRNAs that are predicted to target MTF-1. These miRNAs also target genes within signaling pathways involved with osteogenesis and bone development. MTF-1 is an intracellular zinc sensor, and upon binding Zn will bind to the MRE-containing gene promoters. The result is transcription of genes involved in the maintenance of cellular Zn homeostasis. Certain genes within osteogenic and bone development pathways also contain MREs in their promoters [27-33]. MTF-1 is predicted to regulate miRNA expression as well [32], thereby adding another control through which Zn regulates gene expression. Other Zn-dependent regulators of miRNA transcription may be discovered, such as the Zn-finger transcription factor KLF4 and/or miRNA genes containing the Zn transcriptional regulatory element in their promoters [35].

Conclusion

The results from this *in silico* analysis suggest that miRNAs may play a significant role in regulating cellular Zn homeostasis by targeting the cellular Zn sensor, MTF-1. The miRNAs that are predicted to target MTF-1 also target the genes in TGFβ/BPM, MAPK, Wnt, and Hedgehog signaling pathways, and are likely to play an important role in regulating bone development. This analysis identified the genes most targeted by the MTF-1 targeting miRNAs, as well as the miRNAs that target the most genes within the signaling pathways. Among these genes, the MAPK and Wnt signaling pathways share the most genes. The miRNA that is predicted to target the most genes among these signaling pathways is hsa-miR-3613-3p. The genes that are targets for the miRNAs identified in this study are also involved in numerous molecular functions and biological processes within the cell, particularly regulation, development, and metabolism.

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