

Research Article

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

Grider A^{1*}, Bakre AA², Laing EM¹ and Lewis RD¹¹Department of Foods and Nutrition, University of Georgia, USA²Department of Infectious Diseases, University of Georgia, USA***Corresponding author:** Grider A, Department of Foods and Nutrition, University of Georgia, 280 Dawson Hall, Athens, GA 30602, USA**Received:** January 25, 2017; **Accepted:** February 28, 2017; **Published:** March 06, 2017**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 (TGF β), p38 mitogen-activated protein kinase pathway (MAPK), wntless-related integration site pathway (Wnt), and Hedgehog 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 TGF β , 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 TGF β , MAPK, Wnt, and Hedgehog signaling pathways. hsa-miR-3613-3p is predicted to target 171 genes among the TGF β , 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 homeostasis 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; Wntless-related integration site; p38 mitogen-activated protein kinase; Hedgehog; microRNA; hsa-miR-3613-3p

Abbreviations

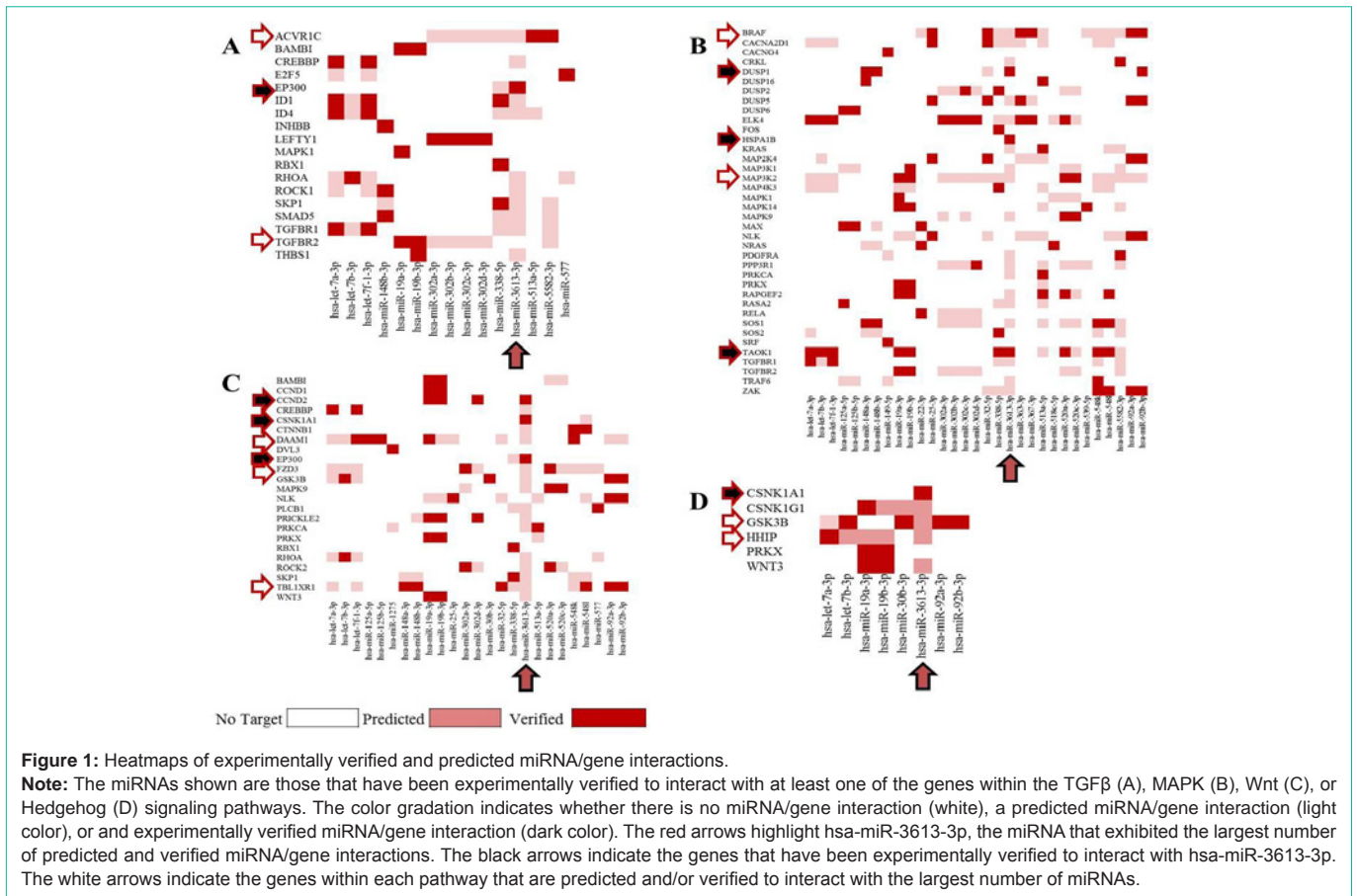
IGF-1: Insulin-Like Growth Factor 1; IGFBP: Insulin-Like Growth Factor Binding Protein; MAPK: P38 Mitogen-Activated Protein Kinase; MiRNA: MicroRNA; MRE: Metal Response Element; MTF-1: Metal-Responsive Transcription Factor 1; TGF β : Transforming Growth Factor B/Bone Morphogenic Protein Pathway; Wnt: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

Introduction

Adequate zinc (Zn) nutrition 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



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.

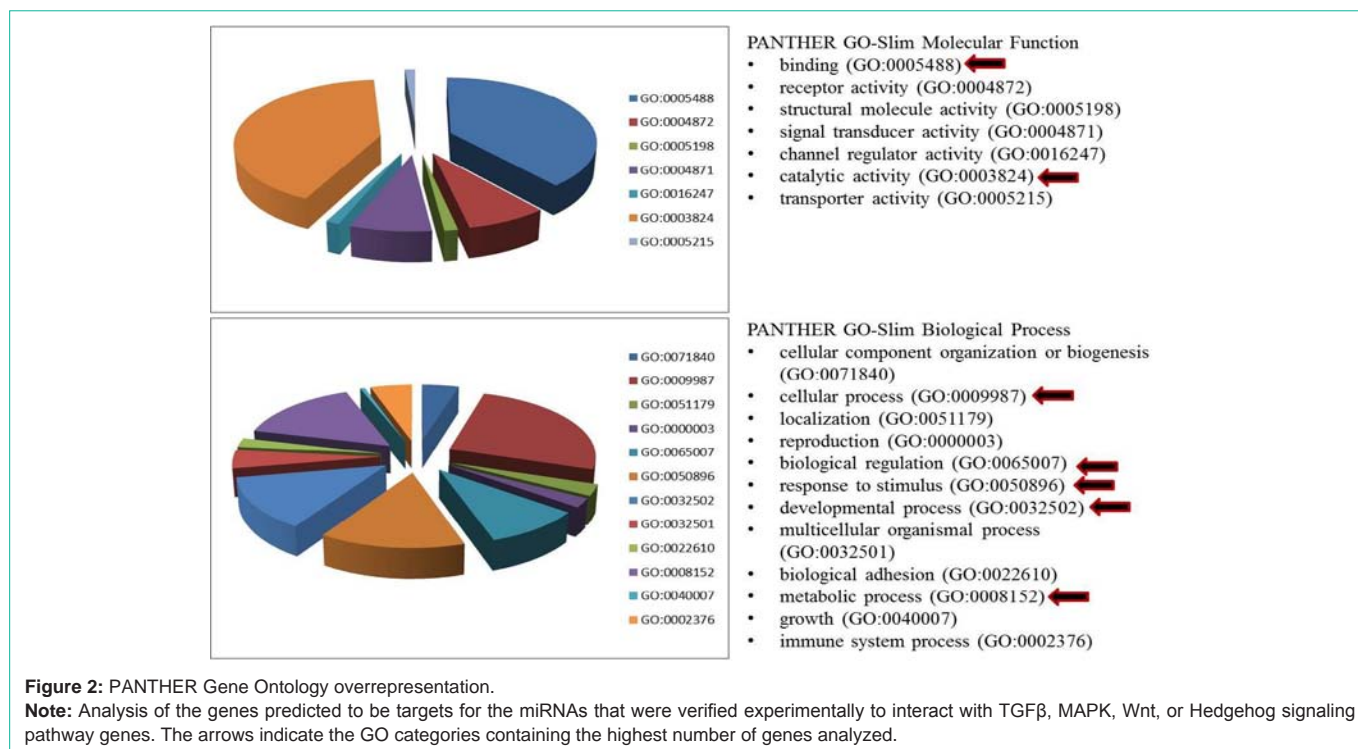
The effects of Zn on bone growth may be mediated through insulin-like growth factor 1 (IGF-1), as levels increased following Zn supplementation of Zn-deficient children [4]. The combination of Zn and insulin-like growth factor 1 increased the amounts of protein and DNA, and cellular proliferation, in 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 Cys₂His₂ 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-1 serves 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



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.org 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 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

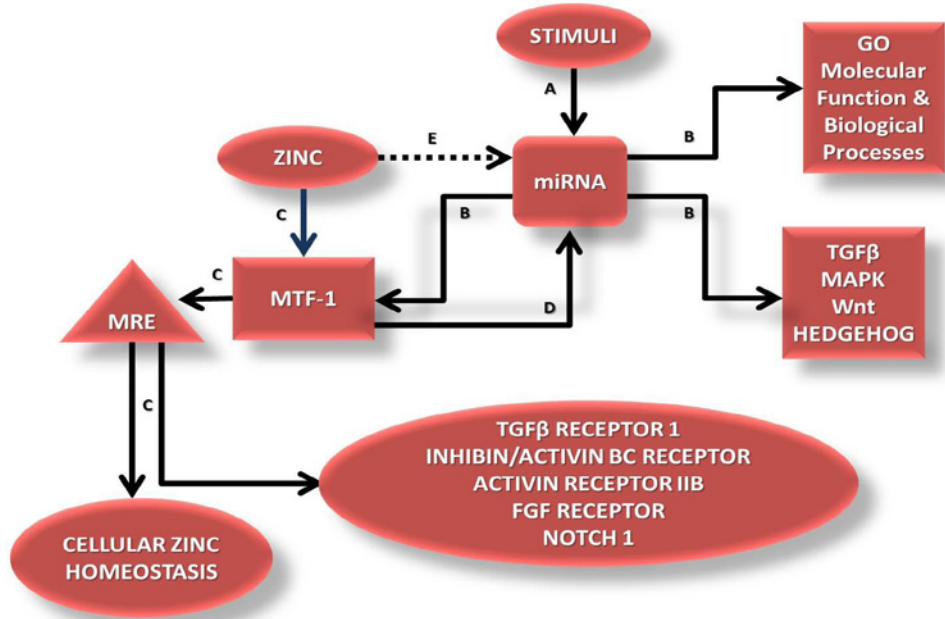


Figure 3: A model for the Zn-dependent, and miRNA, regulation of bone growth and development signaling pathways. **Note:** (A) – Environmental, physiological stimuli induce miRNA synthesis. (B) – miRNA influence gene expression by binding to the 3’ UTR (untranslated region) of target mRNA, thereby inhibiting 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 α 1, 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 ubiquitination (GO:0031396), and the cellular response to oxidative stress (GO:0034599) [53,54]. TAOK1 is a serine/threonine 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 miRNA may 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 TGF β 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 TGF β /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.

References

- Chu Y, Mouat MF, Harris RB, Coffield JA, Grider A. Water maze performance and changes in serum corticosterone levels in zinc-deprived and pair-fed rats. *Physiol Behav.* 2003; 78: 569-578.
- Williams RB, Mills CF. The experimental production of zinc deficiency in the rat. *Br J Nutr.* 1970; 24: 989-1003.
- Gibson RS, Hess SY, Hotz C, Brown KH. Indicators of zinc status at the population level: a review of the evidence. *Brit J Nutr.* 2008; 99: S14-S23.
- Hamza RT, Hamed AI, Sallam MT. Effect of zinc supplementation on growth hormone-insulin growth factor axis in short Egyptian children with zinc deficiency. *Ital J Pediatr.* 2012; 38: 21.
- Hambidge K, Casey C, Krebs N. Trace Elements in Human and Animal Nutrition. 5th edn. Orlando: Academic Press; 1986; 1-138.
- MacDonald RS. The role of zinc in growth and cell proliferation. *J Nutr.* 2000; 130: 1500S-1508S.
- Matsui T, Yamaguchi M. Zinc Modulation of insulin-like growth-factors effect in osteoblastic MC3T3-E1 cells. *Peptides.* 1995; 16: 1063-1068.
- Moonga BS, Dempster DW. Zinc is a potent inhibitor of osteoclastic bone-resorption *in vitro*. *J Bone Miner Res.* 1995; 10: 453-457.
- Yamaguchi M. β -Alanyl-L-histidinato zinc and bone resorption. *GenPharmacol.* 1995; 26: 1179-1183.
- Berger PK, Pollock NK, Laing EM, Chertin V, Bernard PJ, Grider A, et al. Zinc supplementation increases procollagen type 1 amino-terminal propeptide in premenarcheal girls: a randomized controlled trial. *J Nutr.* 2015; 145: 2699-2704.
- Kozhemyakina E, Lassar AB, Zelzer E. A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development.* 2015; 142: 817-831.
- Loro ML, Sayre J, Roe TF, Goran MI, Kaufman FR, Gilsanz V. Early identification of children predisposed to low peak bone mass and osteoporosis later in life. *J Clin Endocrinol Metab.* 2000; 85: 3908-3918.
- Bonjour JP, Theintz G, Buchs B, Slosman D, Rizzoli R. Critical years and stages of puberty for spinal and femoral bone mass accumulation during adolescence. *J Clin Endocr Metab.* 1991; 73: 555-563.
- Kroger H, Kotaniemi A, Kroger L, Alhava E. Development of bone mass and bone density of the spine and femoral neck - a prospective study of 65 children and adolescents. *Bone Miner.* 1993; 23: 171-182.
- Magarey AM, Boulton TJC, Chatterton BE, Schultz C, Nordin BEC, Cockington RA. Bone growth from 11 to 17 years: relationship to growth, gender and changes with pubertal status including timing of menarche. *Acta Paediatr.* 1999; 88: 139-146.
- Parfitt AM. The two faces of growth: benefits and risks to bone integrity. *Osteoporos Int.* 1994; 4: 382-398.
- Baxter-Jones AD, Faulkner RA, Forwood MR, Mirwald RL, Bailey DA. Bone mineral accrual from 8 to 30 years of age: an estimation of peak bone mass. *J Bone Miner Res.* 2011; 26: 1729-1739.
- Gilsanz V, Roe TF, Mora S, Costin G, Goodman WG. Changes in vertebral bone density in black girls and white girls during childhood and puberty. *New Eng J Med.* 1991; 325: 1597-1600.
- Weaver CM, Gordon CM, Janz KF, Kalkwarf HJ, Lappe JM, Lewis R, et al. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. *Osteoporosis Int.* 2016; 27: 1281-1386.
- Kirmani S, Christen D, van Lenthe GH, Fischer PR, Bouxsein ML, McCready LK, et al. Bone structure at the distal radius during adolescent growth. *J Bone Miner Res.* 2009; 24: 1033-1042.
- Wang QJ, Wang XF, Iuliano-Burns S, Ghasem-Zadeh A, Zebaze R, Seeman E. Rapid Growth produces transient cortical weakness: a risk factor for metaphyseal fractures during puberty. *J Bone Miner Res.* 2010; 25: 1521-1526.
- McCusker RH. Controlling insulin-like growth factor activity and the modulation of insulin-like growth factor binding protein and receptor binding. *J Dairy Sci.* 1998; 81: 1790-1800.
- Chen G, Deng C, Li YP. TGF-beta and BMP signaling in osteoblast differentiation and bone formation. *Int J Biol Sci.* 2012; 8: 272-288.
- Guntur AR, Rosen CJ. IGF-1 regulation of key signaling pathways in bone. *Bonekey Rep.* 2013; 2: 437.
- Tahimic CG, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. *Front Endocrinol.* 2013; 4: 6.

26. Longobardi L, O'Rear L, Aakula S, Johnstone B, Shimer K, Chytil A, et al. Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF- β signaling. *J Bone Miner Res*. 2006; 21: 626-636.
27. Saydam N, Adams TK, Steiner F, Schaffner W, Freedman JH. Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1 - identification of signal transduction cascades that control metal-inducible transcription. *J Biol Chem*. 2002; 277: 20438-20445.
28. Langmade SJ, Ravindra R, Daniels PJ, Andrews GK. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. *J Biol Chem*. 2000; 275: 34803-34809.
29. Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochemical pharmacology*. 2000; 59: 95-104.
30. Daniels PJ, Bittel D, Smirnova IV, Winge DR, Andrews GK. Mammalian metal response element-binding transcription factor-1 functions as a zinc sensor in yeast, but not as a sensor of cadmium or oxidative stress. *Nucleic acids research*. 2002; 30: 3130-3140.
31. Bittel DC, Smirnova IV, Andrews GK. Functional heterogeneity in the zinc fingers of metalloreulatory protein metal response element-binding transcription factor-1. *J Biol Chem*. 2000; 275: 37194-37201.
32. Lee J, Li Z, Brower-Sinning R, John B. Regulatory circuit of human microRNA biogenesis. *PLoS Comp Biol*. 2007; 3: e67.
33. Lichtlen P, Wang Y, Belser T, Georgiev O, Certa U, Sack R, et al. Target gene search for the metal-responsive transcription factor MTF-1. *Nucleic Acids Res*. 2001; 29: 1514-1523.
34. Koizumi S, Suzuki K, Ogra Y, Gong P, Otuska F. Roles of zinc fingers and other regions of the transcription factor human MTF-1 in zinc-regulated DNA binding. *J Cell Physiol*. 2000; 185: 464-472.
35. Hardyman JE, Tyson J, Jackson KA, Aldridge C, Cockell SJ, Wakeling LA, et al. Zinc sensing by metal-responsive transcription factor 1 (MTF1) controls metallothionein and ZnT1 expression to buffer the sensitivity of the transcriptome response to zinc. *Metallomics*. 2016; 8: 337-343.
36. Wang X. Improving microRNA target prediction by modeling with unambiguously identified microRNA-target pairs from CLIP-ligation studies. *Bioinformatics*. 2016; 32: 1316-1322.
37. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res*. 2015; 43: D146-D152.
38. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res*. 2015; 43: W460-W466.
39. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucl Acids Res*. 2015; 43: D153-D159.
40. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 2000; 25: 25-29.
41. Gene Ontology Consortium. Gene Ontology Consortium: going forward. *Nucleic Acids Res*. 2015; 43: D1049-D1056.
42. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc*. 2013; 8: 1551-1566.
43. Pu Q, Huang Y, Lu Y, Peng Y, Zhang J, Feng G, et al. Tissue-specific and plasma microRNA profiles could be promising biomarkers of histological classification and TNM stage in non-small cell lung cancer. *Thorac Cancer*. 2016; 7: 348-354.
44. Boratyn E, Nowak I, Horwacik I, Durbas M, Mistrz A, Kukla M, et al. Monocyte chemoattractant protein-induced protein 1 overexpression modulates transcriptome, including microRNA, in human neuroblastoma cells. *J Cell Biochem*. 2016; 117: 694-707.
45. Ji H, Chen M, Greening DW, He W, Rai A, Zhang W, et al. Deep sequencing of RNA from three different extracellular vesicle (EV) subtypes released from the human LIM1863 colon cancer cell line uncovers distinct miRNA-enrichment signatures. *PLoS One*. 2014; 9: e110314.
46. Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Molecular and cellular biology*. 1994; 14: 2077-2086.
47. Gonzales ML, Mellman DL, Anderson RA. CK1 α is associated with and phosphorylates star-PAP and is also required for expression of select star-PAP target messenger RNAs. *J Biol Chem*. 2008; 283: 12665-12673.
48. Chia R, Haddock S, Beilina A, Rudenko IN, Mamais A, Kaganovich A, et al. Phosphorylation of LRRK2 by casein kinase 1 α regulates trans-Golgi clustering via differential interaction with ARHGEF7. *Nat Comm*. 2014; 5: 5827.
49. Keyse SM, Emslie EA. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature*. 1992; 359: 644-647.
50. Lewis T, Groom LA, Sneddon AA, Smythe C, Keyse SM. XCL100, an inducible nuclear MAP kinase phosphatase from *Xenopus laevis*: its role in MAP kinase inactivation in differentiated cells and its expression during early development. *J Cell Sci*. 1995; 108: 2885-2896.
51. Tropberger P, Pott S, Keller C, Kamieniarz-Gdula K, Caron M, Richter F, et al. Regulation of transcription through acetylation of H3K122 on the lateral surface of the histone octamer. *Cell*. 2013; 152: 859-872.
52. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, Livingston DM. Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Gene Dev*. 1999; 13: 64-75.
53. Lee H, Sengupta N, Villagra A, Rezaei-Zadeh N, Seto E. Histone deacetylase 8 safeguards the human ever-shorter telomeres 1B (hEST1B) protein from ubiquitin-mediated degradation. *Mol Cell Biol*. 2006; 26: 5259-5269.
54. Dias V, Junn E, Mouradian MM. The role of oxidative stress in Parkinson's disease. *J Parkinson Dis*. 2013; 3: 461-91.
55. Raman M, Earnest S, Zhang K, Zhao Y, Cobb MH. TAO kinases mediate activation of p38 in response to DNA damage. *Embo J*. 2007; 26: 2005-2014.
56. Zihni C, Mitsopoulos C, Tavares IA, Ridley AJ, Morris JD. Prostate-derived sterile 20-like kinase 2 (PSK2) regulates apoptotic morphology via C-Jun N-terminal kinase and Rho kinase-1. *J Biol Chem*. 2006; 281: 7317-7323.
57. Merkerova M, Vasikova A, Belickova M, Bruchova H. MicroRNA expression profiles in umbilical cord blood cell lineages. *Stem Cell Dev*. 2010; 19: 17-26.
58. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*. 2010; 141: 129-141.
59. Dews M, Fox JL, Hultine S, Sundaram P, Wang W, Liu YY, et al. The myc-miR-17~92 axis blunts TGF β signaling and production of multiple TGF β -dependent antiangiogenic factors. *Cancer Res*. 2010; 70: 8233-8246.
60. Kishore S, Jaskiewicz L, Burger L, Hausser J, Khorshid M, Zavolan M. A quantitative analysis of CLIP methods for identifying binding sites of RNA-binding proteins. *Nat Methods*. 2011; 8: 559-564.
61. Lipchina I, Elkabetz Y, Hafner M, Sheridan R, Mihailovic A, Tuschl T, et al. Genome-wide identification of microRNA targets in human ES cells reveals a role for miR-302 in modulating BMP response. *Genes Dev*. 2011; 25: 2173-2186.
62. Gottwein E, Corcoran DL, Mukherjee N, Skalsky RL, Hafner M, Nusbaum JD, et al. Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. *Cell Host Microbe*. 2011; 10: 515-526.
63. Xue Y, Ouyang K, Huang J, Zhou Y, Ouyang H, Li H, et al. Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated microRNA circuits. *Cell*. 2013; 152: 82-96.
64. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature*. 2013; 495: 333-338.
65. Karginov FV, Hannon GJ. Remodeling of Ago2-mRNA interactions upon cellular stress reflects miRNA complementarity and correlates with altered

- translation rates. *Genes Dev.* 2013; 27: 1624-1632.
66. Balakrishnan I, Yang X, Brown J, Ramakrishnan A, Torok-Storb B, Kabos P, et al. Genome-wide analysis of miRNA-mRNA interactions in marrow stromal cells. *Stem Cells.* 2014; 32: 662-673.
67. Kameswaran V, Bramswig NC, McKenna LB, Penn M, Schug J, Hand NJ, et al. Epigenetic regulation of the DLK1-MEG3 microRNA cluster in human type 2 diabetic islets. *Cell Metab.* 2014; 19:135-145.
68. Pillai MM, Gillen AE, Yamamoto TM, Kline E, Brown J, Flory K, et al. HITS-CLIP reveals key regulators of nuclear receptor signaling in breast cancer. *Breast Cancer Res Treat.* 2014; 146: 85-97.
69. Skalsky RL, Corcoran DL, Gottwein E, Frank CL, Kang D, Hafner M, et al. The viral and cellular microRNA targetome in lymphoblastoid cell lines. *PLoS Pathog.* 2012; 8: e1002484.
70. Whisnant AW, Bogerd HP, Flores O, Ho P, Powers JG, Sharova N, et al. In-depth analysis of the interaction of HIV-1 with cellular microRNA biogenesis and effector mechanisms. *MBio.* 2013; 4:e000193.
71. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell.* 2007; 27: 91-105.
72. Qin X, Wang X, Wang Y, Tang Z, Cui Q, Xi J, et al. MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells. *Proc Natl Acad Sci USA.* 2010; 107: 3240-3244.
73. Lin SL, Chang DC, Ying SY, Leu D, Wu DT. MicroRNA miR-302 inhibits the tumorigenicity of human pluripotent stem cells by coordinate suppression of the CDK2 and CDK4/6 cell cycle pathways. *Cancer Res.* 2010; 70: 9473-9482.
74. Haecker I, Gay LA, Yang Y, Hu J, Morse AM, McIntyre LM, et al. Ago HITS-CLIP expands understanding of Kaposi's sarcoma-associated herpesvirus miRNA function in primary effusion lymphomas. *PLoS Pathog.* 2012; 8: e1002884.
75. Wang K, Wang X, Zou J, Zhang A, Wan Y, Pu P, et al. miR-92b controls glioma proliferation and invasion through regulating Wnt/beta-catenin signaling via Nemo-like kinase. *Neuro Oncol.* 2013; 15: 578-588.
76. Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell.* 2013; 153: 654-665.
77. Farazi TA, Ten Hoeve JJ, Brown M, Mihailovic A, Horlings HM, van de Vijver MJ, et al. Identification of distinct miRNA target regulation between breast cancer molecular subtypes using AGO2-PAR-CLIP and patient datasets. *Genome Biol.* 2014; 15: R9.