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MiRNA–target interactions in osteogenic signaling pathways involving zinc via the metal regulatory element

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Abstract Adequate zinc nutriture is necessary for normal bone growth and development, though the precise mechanisms for zinc-mediated bone growth remain poorly defined. A key transcription factor activated by zinc is metal response element-binding transcription factor 1 (MTF-1), which binds to the metal regulatory element (MRE). We hypothesize that MREs will be found upstream of miRNA genes as well as miRNA target genes in the following bone growth and development signaling pathways: TGF-β, MAPK, and Wnt. A Bioconductor-based workflow in R was designed to identify interactions between MREs, miRNAs, and target genes. MRE sequences were found upstream from 64 mature miRNAs that interact with 213 genes which have MRE sequences in their own promoter regions. *MAPK1* exhibited the most miRNA–target interactions (MTIs) in the TGF-β and MAPK signaling pathways; *CCND2* exhibited the most interactions in the Wnt signaling pathway. Hsa-miR-124-3p exhibited the most MTIs in the TGF-β and MAPK signaling pathways; hsa-miR-20b-5p exhibited the most MTIs in the Wnt signaling pathway. *MYC* and hsa-miR-34a-5p were shared between all three signaling pathways, also forming an MTI unit. JUN exhibited the most protein–protein interactions, followed by MAPK8. These in silico data support the hypothesis that intracellular zinc status plays a role in osteogenesis through the transcriptional regulation of miRNA genes via the zinc/MTF-1/MRE complex.

Keywords Metal regulatory element-binding transcription factor 1 · MicroRNA · Metal regulatory element · MIR34A · Zinc · Osteogenesis

Introduction

Zinc (Zn) is an essential trace element involved in fundamental biochemical and physiological pathways. It is necessary for enzyme catalysis, protein structure, and various regulatory functions as approximately 2800 enzymes and proteins bind Zn (10% of the human genome) (Andreini et al. 2006), and approximately 3% of our genome encodes zinc finger transcription factors (Klug 2010). Approximately 4% of the worldwide population is Zn deficient (Wuehler et al. 2005), and children are the most affected (Hambidge and Krebs 2007; Tuerk and Fazel 2009). Proper Zn nutriture is necessary for normal growth and development in animals and humans. Consumption of

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a Zn-depleted diet resulted in reduced bone Zn and growth in rodent animal models (Chu et al. 2003; Grider et al. 2007; Keller et al. 2000). Manipulation of the Zn nutritional environment affects osteogenic biomarkers (Berger et al. 2015; Cho et al. 2007; Seo et al. 2010). Current recommendations for assessing the risk of Zn deficiency in populations includes the functional assessment of length- or height-for-age measurements (Hess et al. 2009; International Zinc Nutrition Consultative Group et al. 2004).

The molecular mechanisms associated with the effect of Zn on bone growth and metabolism are the focus of ongoing research. Circulating levels of insulin-like growth factor-1 (IGF-1) correlate with dietary Zn levels and Zn status in children in some studies (Bougle et al. 2004; Cesur et al. 2009; Hamza et al. 2012; Imamoglu et al. 2005) but not others (Park et al. 2017). Zn supplementation increases the synthesis of IGF-1 in bone (Igarashi and Yamaguchi 2001) and the binding of IGF-1 to its receptor (McCusker 1998; McCusker et al. 1998; Sackett and McCusker 1998a; Sackett and McCusker 1998b). Signaling pathways that are also involved with bone formation include transforming growth factor β/bone morphogenic protein (TGF-β/BMP), wingless-type (Wnt), p38 mitogen-activated protein kinase (MAPK), Hedgehog, Notch, and fibroblast growth factor (FGF). Significant cross-talk occurs between these pathways (Chen et al. 2012). The TGF-β/BMP, Wnt, MAPK, and Hedgehog pathways also intersect with the IGF-1 pathway (Guntur and Rosen 2013; Longobardi et al. 2006; Tahimic et al. 2013).

We hypothesize that the Zn-dependent regulation of bone growth and development involves microRNAs (miRNAs) and metal regulatory elements (MREs), ultimately forming complex and precise regulatory cascades of gene expression (Arora et al. 2013). MiRNAs are small, noncoding RNAs involved in the posttranslational regulation of gene expression through binding to seed sequences in the 3′ untranslated regions (UTR) of mRNA, resulting in transcriptional repression (Lee et al. 1993), or in the 5′ terminal oligopyrimidine tracts of ribosomal protein mRNA, resulting in translational activation (Orom et al. 2008). MiRNAs have also been shown to form complexes with certain regulatory proteins resulting in translational activation (Eiring et al. 2010; Vasudevan et al. 2007). The MRE is a conserved 7-base motif (5′-TGCRCNC-3′; R = A or G, N = any nucleotide) that is bound by metal transcription factor-1 (MTF-1), a 72.5 kDa Zn-finger (ZF) protein in the Cys_2His_2 family of transcription factors (Stuart et al. 1984; Langmade et al. 2000; O'Halloran 1993; Saydam et al. 2002). MTF-1 contains six ZFs and exhibits Zn-dependent binding to the MRE, with ZF1 and possibly ZF3 and ZF6 responsible for its Zn-sensing and MRE binding properties (Bittel et al. 2000; Chen et al. 1999; Chen et al. 1998; Guerrerio and Berg 2004).

Results from in vivo and in vitro studies indicate Zn-dependent differential miRNA expression (Grider et al. 2015; Liuzzi 2014; Ryu et al. 2011). MTF-1 has been proposed as a master regulator for miRNA expression (Lee et al. 2007). The results of in silico studies from this laboratory support the hypothesis that genes within osteogenic signaling pathways are regulated by Zn mediated by MTF-1/MRE/miRNA interactions (Grider et al. 2017). The purpose of this investigation is to identify the locations of MREs upstream genes of miRNAs which then target genes within the TGF-β, MAPK, and Wnt signaling pathways. It must be noted, however, that previous results indicate that the presence of an MRE upstream from the transcriptional start site (TSS) of a gene does not necessitate its Zn-dependent or MTF-1 dependent regulation. The results presented here show that both miRNAs and their target genes contain MREs upstream from their TSSs, suggesting that the regulation of osteogenesis by Zn involves complex interactions between Zn, MRE/gene, and MRE/miRNA/gene associations. A model of feed-forward loops is proposed.

Materials and methods

All analyses were performed using the human hg38 genome assembly (Dec. 2017, Genome Reference Consortium GRCh38.p12). Bioconductor packages were run using R Studio (Huber et al. 2015; Team 2016). A summary of the workflow is shown in Fig. 1.

Positions of the MRE consensus sequence 5′-TGCRCNC-3′ in GRCh38.p12 were recorded using Biostrings (v2.48.0) (Pagès et al. 2018). BiomaRt (v2.36.0) was used to find the genomic coordinates of all HUGO Gene Nomenclature Committee (HGNC)-named genes (Durinck et al. 2005, 2009). Pre-miRNAs with TSSs predicted in human embryonic stem cells (hESC) by the microTSS algorithm

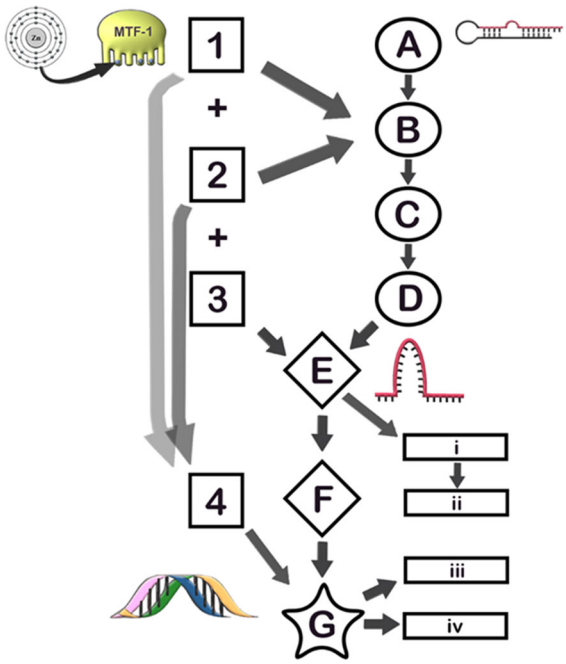

Fig. 1 In silico experimental pipeline. (*1*) Biostrings: identified all metal regulatory element (MRE) motifs (5′-TGCRCNC-3′) throughout the human genome. (*2*) BiomaRt: mapped the location of all HUGO Gene Nomenclature Committee (HGNC) named genes in the genome. (*3*) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways used to categorize genes from osteogenic development pathways TGF-β (hsa04350), MAPK (hsa04010), and Wnt (hsa04310). (*4*) Output—460 genes with an upstream MRE within − 7Kbp were found in these three KEGG pathways; 413 genes with MREs not interrupted by another gene (Supplementary Table 2) continued through the pipeline. (*A*) 86 pre-miRNAs whose transcriptional start sites (TSSs) were predicted by the microTSS algorithm. (*B*) GenomicRanges: identified the 85/86 pre-miRNAs with one or more MREs in the range − 7 to + 1 Kbp from their predicted TSSs; 44 pre-miRNA genes with MREs not interrupted by another gene continued through the pipeline. (*C*) miRBase: converted the 44 pre-miRNAs into 73 mature miRNAs. (*D*) multiMiR: find TarBase-verified miRNA–target gene interactions (MTIs) of the 73 mature miRNAs. (*E*) 64/73 mature miRNAs have TarBase-verified MTIs with genes from the KEGG pathways. (*F*) 241 gene targets from the miRNAs. (*G*) Of the 241 gene targets, 213 also have an MRE. (*i*) mirdb via multiMiR: finds all predicted gene targets of the miRNAs. (*ii*) Panther GO-Slim biological process for the predicted targets. (*iii*) Venn diagram identifies shared genes and miRNA between signaling pathways. (*iv*) Protein–protein interactions mapped using STRING

(Georgakilas et al. 2014) were queried in the range of -7Kbp upstream and +1Kbp downstream, based on previous analysis, (Francis and Grider 2018) using GenomicRanges (v1.32.3) (Lawrence et al. 2013). microTSS genomic coordinates were batch converted from hg19 to hg38 using liftOver (Kent et al. 2002). Genes that were interrupted between their TSSs and nearest MRE were excluded from our analysis. The pre-miRNAs with proximal MREs were converted to their mature miRNA sequences using miRBase (Griffiths-Jones 2004; Griffiths-Jones et al. 2006, 2008; Kozomara and Griffiths-Jones 2011; Kozomara and Griffiths-Jones 2014). TarBase (experimentally verified) miRNA–target interactions (MTIs) between these mature miRNAs and genes were identified using miRTarBase via multiMiR (Database Version: 2.2.0 Updated: 2017-08-08) (Ru et al. 2014). These MTIs were sorted into their KEGG signaling pathways (Kanehisa et al. 2017); interactions in the TGF-β, MAPK, and Wnt pathways were chosen for further analysis. GenomicRanges was used to find genes in these pathways that have MRE sequences without interruption by other genes in their upstream -7Kbp region (Supplementary Table 1).

Venn diagrams (Oliveros 2007–2015) were produced from the lists of MTIs from each signaling pathway to identify those genes and miRNAs that are shared between pathways (Supplementary Table 2). The gene cohort with experimentally verified MTIs was analyzed by the Panther GO-Slim Biological Process (Panther Overrepresentation Test release 20171205; PANTHER version 13.1 Released 2018-02-02; $\alpha = 0.05$) (Ashburner et al. 2000; Gene Ontology Consortium 2015; Tahimic et al. 2013; Zhao et al. 2017). In addition, genes from osteogenic KEGG pathways for which there was at least one experimentally verified MTI were subsequently used to query miRDB via multiMiR for predicted gene interactions (target scores ≥ 81) (Wang 2016; Wong and Wang 2015). The Panther Overrepresentation Test was performed on this cohort of predicted gene targets as well. Fisher's exact testing with false discovery rate multiple test correction was used to identify significantly overrepresented annotated gene ontologies.

The experimentally verified MTI genes that are shared between the signaling pathways were analyzed for their protein–protein interactions using the STRING database (v10.5) (Szklarczyk et al. 2015, 2017). The interactions were visualized using the Network viewer. The analysis was performed using the highest minimum required interaction score (0.900). Only experimentally derived and database

interaction sources are reported (Supplementary Table 3).

Results

The TSSs for 86 pre-miRNA genes were predicted by the microTSS algorithm; 44 of these pre-miRNAs contain an MRE within the range specified, without interruption between the MRE motif and the MIR gene TSS by the presence of another gene. These 44 pre-miRNAs equate to 73 mature miRNAs. Within the TGF-β, MAPK, and Wnt signaling pathways, 65 of the 73 mature miRNAs have Tarbase-verified MTIs (hereafter called "verified MTIs") with 241 genes. Of the 460 genes in these three signaling pathways, 413 have an MRE in the range specified. Of those 413 genes, 213 have verified MTIs with 64 mature miRNAs which also have MREs. The TGF-β pathway contained MTIs consisting of 39 miRNAs/44 genes, the MAPK pathway contained MTIs consisting of 57 miRNAs/130 genes, and the Wnt pathway contained MTIs consisting of 43 miRNAs/61 genes. *MAPK1* exhibited 10 MTIs, the most verified MTIs in the TGF-β and MAPK signaling pathways. *CCND2* exhibited 9 MTIs, the most verified MTIs in the Wnt signaling pathway. Hsa-miR-124-3p targeted the most genes in the TGF-β (12 genes) and MAPK (32 genes) signaling pathways. Hsa-miR-20b-5p targeted the most genes, 12, in the Wnt signaling pathway (Table 1; Supplementary Table 1).

Genes with MREs and verified MTIs that are shared between two or more signaling pathways are organized in Venn diagrams (Fig. 2). *MYC* is the only gene that is shared among TGF-β, MAPK, and Wnt signaling pathways. MAPK and Wnt pathways share 11 other genes; TGF-β and MAPK pathways share five genes; and TGFβ and Wnt pathways share four genes (Fig. 2a; Supplementary Table 2). There are 28 miRNAs that are shared among the three pathways (Fig. 2b; Supplementary Table 2). There are seven miRNAs that are experimentally verified to target *MYC* (Table 2); hsa-miR-34a-5p exhibits the most MTIs within each signaling pathway. The seven miRNAs targeting *MYC* are within the cohort of 28 miRNAs that interact with all three pathways (Fig. 2b, c).

The protein–protein interactions of the genes shared between the three signaling pathways that are targets for miRNAs that contain MREs upstream from their TSSs, and that also contain MREs within their own promoter regions, were identified using the String database (v10.5) (Fig. 3; Supplementary Table 3). *JUN* exhibits the most protein–protein interactions (10), and *MAPK8* exhibits the second most interactions (9). *MYC*, though involved with all three signaling pathways, interacts with just five other proteins.

There are 21 genes with verified MTIs that are common to at least two signaling pathways; these were used to query the Panther GO-Slim Biological Process database to identify overrepresented gene ontologies (Fig. 4). Intracellular signal transduction exhibited the highest enrichment score. This cohort is also significantly enriched in genes involved with cellular process, cell proliferation, and transmembrane receptor protein serine/threonine kinase signaling pathway. The 64 miRNAs with MREs that target genes in the osteogenic pathways exhibited 6886 predicted MTIs with 3613 genes (only target scores ≥ 81 were analyzed). This larger cohort of genes was enriched most in cellular process, followed by localization, metabolic process, and cell communication (data not shown).

Discussion

Zn is crucial for healthy bone growth and development, but the mechanism for its effects is complex and not fully elucidated. There is continued interest in understanding the translational control of osteogenesis through the nexus of miRNAs and osteogenic signaling pathway genes (Fushimi et al. 2018; Lian et al. 2012; Liu et al. 2018; Vimalraj and Selvamurugan 2013). Previous in silico analyses support the associations between Zn, MTF-1/MRE, and miRNAs in regulating osteogenic signaling pathways (Grider et al. 2017). This investigation serves to expand on the previous results, further supporting the role Zn plays in osteogenesis through its interaction with the MTF-1/MRE complex and miRNAs.

TGF-β, MAPK, and Wnt signaling pathways play key roles in osteogenesis (Baron and Kneissel 2013; Chen et al. 2012; Wu et al. 2016b). The data indicate that 213 genes within these three pathways have experimentally verified MTIs with 64 miRNAs. The MTIs in these pathways share a single gene, *MYC*, a

Table 1 The five highest miRNA/target gene interactions (MTIs) within each signaling pathway

(A) Signaling pathway genes with the highest number of MTIs

TGF-β		MAPK		Wnt	
GENE	MTI	GENE	MTI	GENE	MTI
MAPK1	10	MAPK1	10	CCND2	9
SMAD4	8	IGFR1	9	SMAD4	8
TGFBR2	8	TAOK1	9	CCND1	7
ACVR1	7	RPS6KA5	8	MYC	7
ACVR1B	7	TGFBR2	8	TP53	7

(B) miRNAs that target most genes in each signaling pathway

TGF-β		MAPK		Wnt	
Mature miRNA	MTI	Mature miRNA	MTI	Mature miRNA	MTI
hsa-miR-124-3p	12	hsa-miR-124-3p	32	hsa-miR-20b-5p	12
hsa-miR-20b-5p	11	hsa-miR-19b-3p	19	hsa-miR-106a-3p	11
hsa-miR-19b-3p	9	hsa-miR-34a-5p	19	hsa-miR-34a-5p	11
hsa-miR-106a-3p	8	hsa-miR-106a-3p	17	hsa-miR-124-3p	10
hsa-miR-34a-5p	7	hsa-miR-20b-5p	9	hsa-miR-19b-3p	9

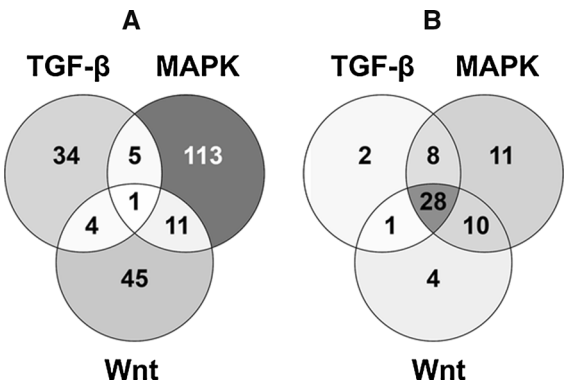

Fig. 2 Venn diagrams of shared signaling pathway genes and miRNAs. **a** Genes with MREs in their promoter regions that are targets for miRNAs that contain MREs within their own promoter regions. **b** MiRNAs that target the genes in 2A

Table 2 MYC miRNA/target gene interaction

Mature miRNA	TGF-β MTI	MAPK MTI	Wnt MTI
hsa-miR-34a-5p	7	19	11
hsa-miR-148a-3p	5	6	4
hsa-miR-222-3p	4	9	5
hsa-miR-940	3	12	9
hsa-miR-148a-5p	2	2	2
hsa-miR-92a-2-5p	2	7	3
hsa-miR-19b-2-5p	1	4	1

MYC is shared by the TGF-β, MAPK, and Wnt signaling pathways. The list of mature miRNAs that target *MYC*. Each of these miRNAs also target other genes within the signaling pathways (MTI). Hsa-miR-34a-5p exhibits the most MTIs within each signaling pathway

proto-oncogene involved in cell cycle progression and transformation, and apoptosis. Others have shown that *MYC* expression is increased with activation of Wnt/β-catenin, and correlated with reduced terminal differentiation of osteoblasts, increased bone mass through osteoblast proliferation, and reduced bone strength (Chen et al. 2015; Li et al. 2017). The addition of EDTA to cultured HL-60 cells, a model of Zn deficiency, decreased the expression of *MYC* and cellular proliferation; Zn addition reversed these effects (Morimoto et al. 1992). Our data indicate that *MYC* contains several MREs in its promoter, supporting the hypothesis that Zn regulates *MYC* expression via MTF-1.

We also observed that seven miRNAs formed MTIs with *MYC*; these miRNAs also contain MREs upstream from their TSSs. Hsa-miR-34a-5p targets *MYC* and additionally exhibits the largest number of experimentally verified MTIs with this gene in each signaling pathway. The expression of this miRNA is

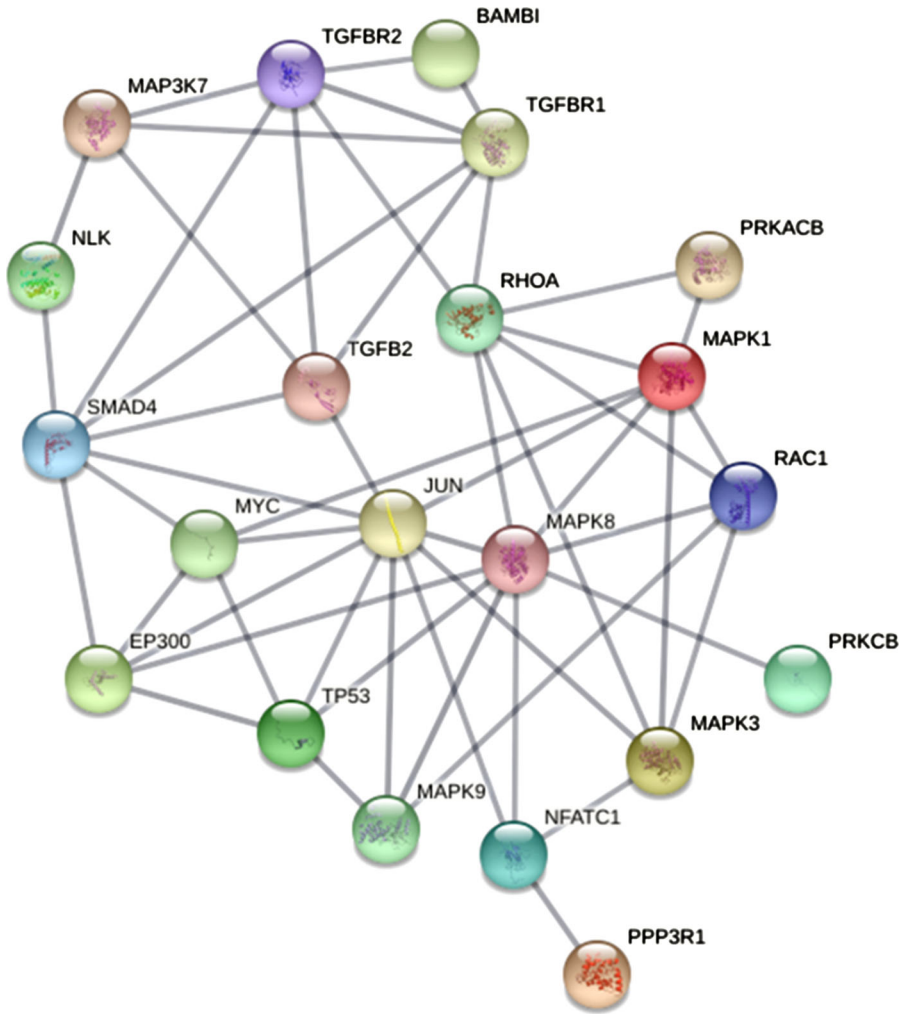

Fig. 3 Protein–protein interactions between miRNA–target gene interactions. The sources for determining active interactions were from experiments and databases. The minimum interaction score used to generate the interactions web was 0.900

induced in mice by dietary Zn (Liuzzi 2014). This miRNA has been studied extensively and inhibits osteoblast differentiation and bone formation. In models of human stromal (skeletal, mesenchymal) stem cell (hMSC) differentiation, overexpression of hsa-miR-34a in hMSCs reduced heterotopic bone formation by 60%. Conversely, in miR-34a-deficient hMSC, in vivo bone formation was increased by 200% (Chen et al. 2014). The available data indicate that osteoblast differentiation is inhibited by both *MIR34A* and *MYC* gene products. Others have found that the deletion of *MYC* in osteoclasts increases bone mass in mice which have undergone ovariectomy (Bae et al. 2017). The results from our in silico analysis suggests a complex relationship between the MRE, hsa-miR-34a-5p, and *MYC* effects on osteogenesis. MTF-$1_{(Zn)}$ activates gene expression via the MRE in both *MYC* and *MIR34A*; hsa-miR-34a-5p is expected to inhibit *MYC* but may act synergistically with the MRE in the *MYC* promoter to stimulate *MYC* transcription; the result is increased osteoblast proliferation, and decreased osteoblast differentiation and bone strength. These data suggest that the role of Zn in osteogenesis is a complex series of reactions between MTF-1/MRE, and genes in the signaling pathways that remain to be elucidated.

Genes that exhibit the most verified MTIs include *MAPK1* and *CCND2*. *MAPK1* exhibited the most MTIs within the TGF-β and MAPK signaling pathways. *MAPK1* is a member of a serine/threonine kinase family of extracellular signal-regulated kinases that are involved in numerous phosphorylation reactions associated with development and cellular differentiation (Kyosseva 2004; Martin-Blanco 2000). It has

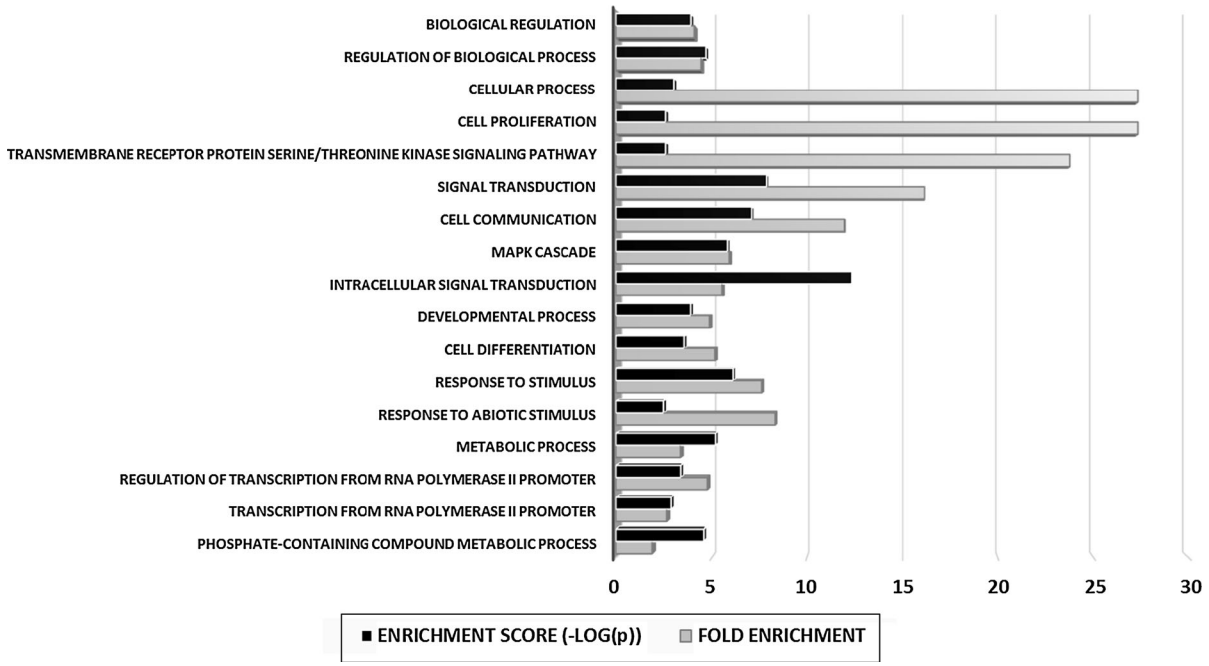

Fig. 4 Gene ontology enrichment of the MTI genes. The MTI genes were used to query the PANTHER GO-Slim Biological Process database. Significance was determined using the Fisher's Exact test with the false discovery rate multiple test correction. All values reported in the graph exhibited a false discovery rate < 0.05

also been reported to function as a transcriptional repressor (Hu et al. 2009). The interaction that *MAPK1* and *MYC* exhibit may involve phosphorylation or transcriptional repression of *MYC* by *MAPK1*. *CCND2* encodes cyclin D2, which complexes with cyclin-dependent kinases at the beginning of the G_1 phase of the cell cycle (Sherr 1994; Vermeulen et al. 2003). *CCND2* is not shared between Wnt and the other signaling pathways. Nevertheless, it is transcriptionally upregulated by the *MYC* gene product (Bouchard et al. 1999, 2001; Mai et al. 1999). *CCND2* and *MYC* are also targeted by different sets of miRNAs.

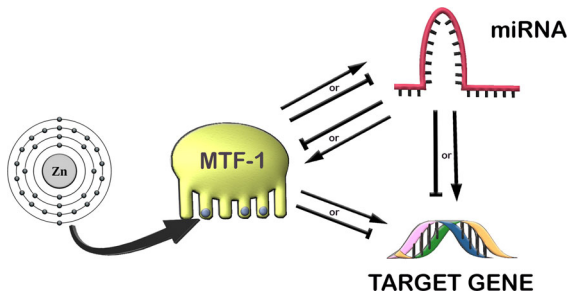

Fig. 5 Zn activates MTF-1 and MTF-1 will bind to the MRE in promoters of genes. These genes include miRNAs, which are themselves repressors of gene activity. A feed–forward loop can arise in which MTF-1 and miRNAs impact the expression of each other and of target genes

The top five MTI miRNAs are the same among the three signaling pathways, though in different orders. Hsa-miR-124-3p is involved in the most MTIs in the TGF-β and MAPK signaling pathways. The down-regulation of this miRNA is correlated with increased tumorigenesis and poor clinical outcomes, whereas upregulation results in decreased cell proliferation and tumor suppression (Feng et al. 2016; Yang et al. 2017; Zhang et al. 2015; Zhou et al. 2017). Hsa-miR-20b-5p is involved in the most MTIs in the Wnt signaling pathway. This miRNA also functions as a tumor suppressor (Xin et al. 2016) and is GO-linked to apoptosis and autophagy (Wu et al. 2016a). Our data support a role for Zn, through its binding to MTF-1 and subsequent MRE activation, in the transcriptional regulation of these miRNAs. Their specific effects on osteogenesis, though, remain to be determined.

MAPK8 and *JUN* exhibit the most protein–protein interactions among the MTI gene products shared between the TGF-β, MAPK, and Wnt signaling pathways. The *JUN* gene product, c-Jun, is activated by phosphorylation and is a necessary component of

the AP-1 DNA-binding complex (Miller et al. 2010; Vasilevskaya and O'Dwyer 2003). c-Jun also forms a complex with β-catenin and TCF4 to upregulate *MYC* transcription at the 3′ enhancer region (Yochum et al. 2008). Our analysis indicates that several mitogen-activated protein kinases, also called c-Jun N-terminal kinases, are responsible for phosphorylating c-Jun, including the gene product of *MAPK8*, *JNK1* (Bubici and Papa 2014). We report that *JUN*, *MAPK8*, and *MYC* are involved in MTIs, and each MTI component contains one or more MREs upstream from its TSS.

These observations support the hypothesis that the transcriptional regulation of osteogenesis by Zn occurs at multiple nodes within osteogenic signaling pathways. MREs are located upstream from the TSSs of both components of the MTI. We propose that feed-forward loops are involved with the Zn-dependent transcriptional regulation of osteogenesis through the MTF-1/MRE/MTI interactions (Fig. 5). The results from this study support the supposition that Zn regulation of bone growth and development occurs at the most fundamental levels. Future studies are planned to validate these complex Zn-dependent regulatory interactions as they relate to bone growth and development.

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