A Computational Survey of the intronic microRNAs in the X chromosomes of Human and Chimpanzee, their target gene interactions and its impact on gene regulation

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Abstract

The knowledge of what separates us genetically from our less-evolved relatives is crucial for gaining new biomedical insights about the human-chimpanzee relatedness for the use of appropriate stand-in towards the development of new treatments and diagnostic aids for various ailments. Although the genomes of humans and chimpanzees share 99% similarity, significant differences exist between the two species in their non-coding intronic regions. However, no work has been carried out in the aspects of target prediction concerning the 'predicted homology' in their microRNA sequences. Non-coding miRNAs which are post-transcriptional regulators of development, differentiation, growth, and metabolism, harboring the intronic regions may be crucial for expanding the horizons of our understanding. In this study, we proposed to perform the target prediction for the human-chimp miRNA homologs in the PHEX gene of the human X chromosome using various computational tools and databases. We identified a total of 1296 human miRNAs, 46, 957 gene targets, and 30, 563 targets of human and homologous chimp miRNAs respectively. Furthermore, we analysed gene interacting networks to identify the top interacting targets in both the species. Finally, we interpreted the biological importance of top-interacting miRNAs and their targets. The results demonstrated varying levels of multiplicity and cooperativity between the predicted miRNAs and target genes in the two genera. Such miRNAs may be responsible for the dysregulation of gene expression in several signaling pathways.

Keywords: miRNA, gene targets, intronic miRNA, miRNA prediction, human miRNAs, PHEX miRNAs, chimpanzee homologues, experimentally-validated miRNA targets, miRNA computational survey, miRNA target multiplicity, miRNA target cooperativity

1. Introduction

MicroRNAs (miRNAs) are small endogenous ~21–22 nucleotide non-coding RNAs present in the genomes of all multicellular eukaryotes, which are negative regulators of gene expression [1, 2]. It is believed that a gene may have several target sites for the same or different miRNAs, while a single miRNA can interact with more than a hundred genes during the miRNA biogenesis process [3-5]. This serves as the basis for the emergence of highly interconnected

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and complex miRNA-based regulatory networks [6, 7]. This discovery has unfolded new facets of gene regulation in multicellular eukaryotes, which could positively influence our understanding of the molecular basis of diseases, identification of potential biomarkers, and design of novel therapeutic rationale. Experimental studies in the past have revealed that miRNAs play crucial roles in a wide range of biological processes like development, signaling, apoptosis, cell fate, and differentiation, and the deregulation of which could trigger tumor initiation and progression by switching inappropriate molecular programs conducting uncontrolled proliferation [8, 9]. Recent studies have provided a piece of strong evidence for miRNA regulation of many essential oncogenes, including BCL2, RAS, MYC, p53 [9-12]. Previous studies have discovered that some miRNAs present in the genome as clusters, which constitute two or more miRNAs occupying neighboring positions within a few kilobases of each other and are transcribed as a polycistronic structure, may have a cooperative function [13, 14]. Following this finding, it has also been ascertained that at a 10 kb distance threshold, up to 48% of known human miRNA genes form clusters in the human genome, which are highly conserved in many vertebrates [15]. Additionally, it is believed that 50 to 80% of the miRNAs are encoded within introns of host mRNA genes [16].

While human miRNAs are relatively well investigated, it seems that studies on these small RNA molecules in the closest human-animal relative, the chimpanzee, are far from complete [17]. Investigation of chimpanzee miRNAs will help us strengthen our understanding of their biological functions and correlate them for studying complex human disease conditions at microRNA level. To date, only 600 miRNAs from the chimpanzee genome (*Pan troglodytes*) have been deposited in mirBase database, most of which originating from the studies of Berezikov et al. and Dannemann et al. Even though the chimpanzee genome complete sequence was first released in 2005, data about the expression and function of chimp miRNAs is yet to be exploited completely [14, 17, 18].

In this study, we computationally searched for the miRNA homologs in the introns of ‘phosphate-regulating gene with Homologies to Endopeptidases on the X-chromosome’ (PHEX) gene on the human X chromosome at Xp22.2. This computational survey was carried out with special reference to the identification of their homologs on the orthologous loci in *Pan troglodytes*. It was hypothesized that humans and chimps share numerous identical miRNAs at the sequence level though, they may not be necessarily networked to the same target genes. This, in turn, may result in entirely new regulatory events giving rise to novel gene products and metabolomes, which are the basis of their genus specificity.

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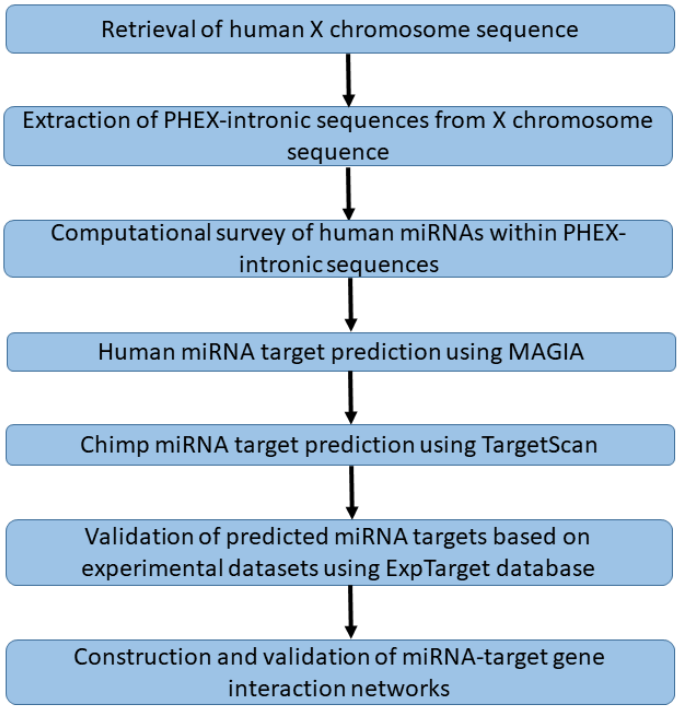
2. Materials and Methods

In this comparative genomic study, we designed an effective strategy to identify the microRNAs within the introns of the PHEX gene on the human X chromosomes and their homologous miRNAs within the corresponding genomic elements of the chimpanzee X chromosome. Schema 1 illustrates the schema provides a guided example of the strategy employed to conduct the computational screening of miRNAs.

2.1 Retrieval of human X chromosome and PHEX gene sequences

To begin with, the complete sequence of the human X chromosome was retrieved from the NCBI Nucleotide database at http://www.ncbi.nlm.nih.gov/. It contains about 2000 genes out of the approximately estimated 20,000 to 25,000 total genes in the human genome. From a set of 2000 genes, only the first gene, Phosphate-regulating gene with Homologies to Endopeptidases of the X chromosome was focused in this study. With an aim of analysing only the intronic regions of the PHEX gene, all the intronic stretches encapsulated in the gene were manually searched for and identified in the NCBI-Nucleotide database record. Subsequently, a master table was created containing the information on all the identified introns and their positions in the gene. DNA sequences of all the individual human PHEX-introns were downloaded from the UCSC Genome Browser using the genomic coordinates obtained from the NCBI Nucleotide database file. The PHEX-intronic sequences were converted to the FastA format using the alignment editor, BIOEDIT for further computational analyses.



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Schema 1. depicts the pipeline utilized in this study. This genome-wide strategy was proposed to computationally identify the conserved miRNA homologs in both the species, their potential target genes, and possible co-regulatory gene interaction networks, using the publicly available bioinformatics tools and database resources.

2.2 Computational screening of miRNAs in human PHEX-intronic sequences

The miRNAs along with their genomic co-ordinates of all the 21 obtained Intron sequences of the PHEX gene were predicted and catalogued from Welcome Trust Sanger Institute's miRBase, Release 16 at http://www.mirbase.org/. miRBase is a searchable database of published miRNA sequences, and miRBase-BlastN was used to search for all the miRNAs residing in the intronic sequences of the Phex gene on the human X chromosome. The sequences and their genomic positions of the miRNAs predicted were stored in the master table, in FastA format for further analysis.

2.3 Human miRNA target prediction

For each miRNA in the PHEX-intron sequences on the human X chromosome, one or more target genes specific to human miRNA dataset were predicated using MiRNA and Genes Integrated Analysis (MAGIA), a web-based tool that allows to retrieve and browse updated miRNA target prediction. This tool allowed to access a target prediction database and carry out a miRNA/gene expression profiles integrated analysis, by adopting different statistical measures of profiles relatedness, algorithms for expression profile combination and target prediction methods. It allowed querying the miRNA target prediction database on each individual miRNA entry of the catalogue made in the previous step. The output files were generated with different algorithms of three different target prediction tools namely miRanda, PITA and TargetScan, or Boolean combinations thereof applied to user-defined selections of up to twenty miRNA and/or targets in a single hit list. Thus, multiple targets for each miRNA in the catalogue were predicted.

2.4 Target prediction of homologous chimp miRNAs

Similarly, targets specific to all the homologous chimp miRNAs were predicted using TargetScan at http://www.targetscan.org/, having the human miRNA catalogue as the reference.

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2.5 Validation of the predicted miRNA targets using experimental sets

A vast number of gene targets were predicted for the intronic miRNAs on the PHEX gene of human and chimp genomes. Since the network elaborated from them was too complicated, ExprTargetDB was used at http://www.scandb.org/apps/microrna/, in order to refine the miRNAs and their targets across the human genome, to only those which have been experimentally cross validated. The p-value was set at 0.10 and the prediction algorithm used was Tarbase.

2.6 Construction and visualization of miRNA-target gene interaction network

After being validated using ExprTargetDB, the predicted top scoring conserved miRNA targets in the PHEX gene of both human and chimp genomes were imported as a two-column excel file input. It contained one column containing all the candidate miRNAs and the next adjacent column containing their respective gene targets, into Cytoscape, an integrated bio-molecular interaction networks software for the construction of probable miRNA-gene interaction networks**.** Eventually, the constructed gene networks were visualized and analyzed using cyto-Hubba, a facilitated platform for the analysis and visualization of molecular interaction networks supported by a Java plugin.

3. Results

3.1 Extraction of PHEX-intronic sequences from the genome of human X chromosome

The human X chromosome constitutes about 1200 genes, of which the first gene, namely PHEX, was analyzed. When viewed in the UCSC Genome Browser, the exact locus and the size of the PHEX gene was visualized between the nucleotide bases 22,050,921 - 22,266,476 on the X chromosome, and it is 215,556 bp long (Fig 1). The total length of the entire intronic stretches in this gene was calculated to be 212676 basepairs, which accounts for approximately 98.66% of the entire gene. The exons within this gene are 2880 base pairs long, which comprises 1.34 % of the total genomic content. From the entire contigs assembly, 21 introns identified on the PHEX gene of the human X chromosome were manually catalogued (Suppl. Table S1). We downloaded all the 21 DNA sequences of the respective PHEX-introns from the UCSC genome browser for further analysis.

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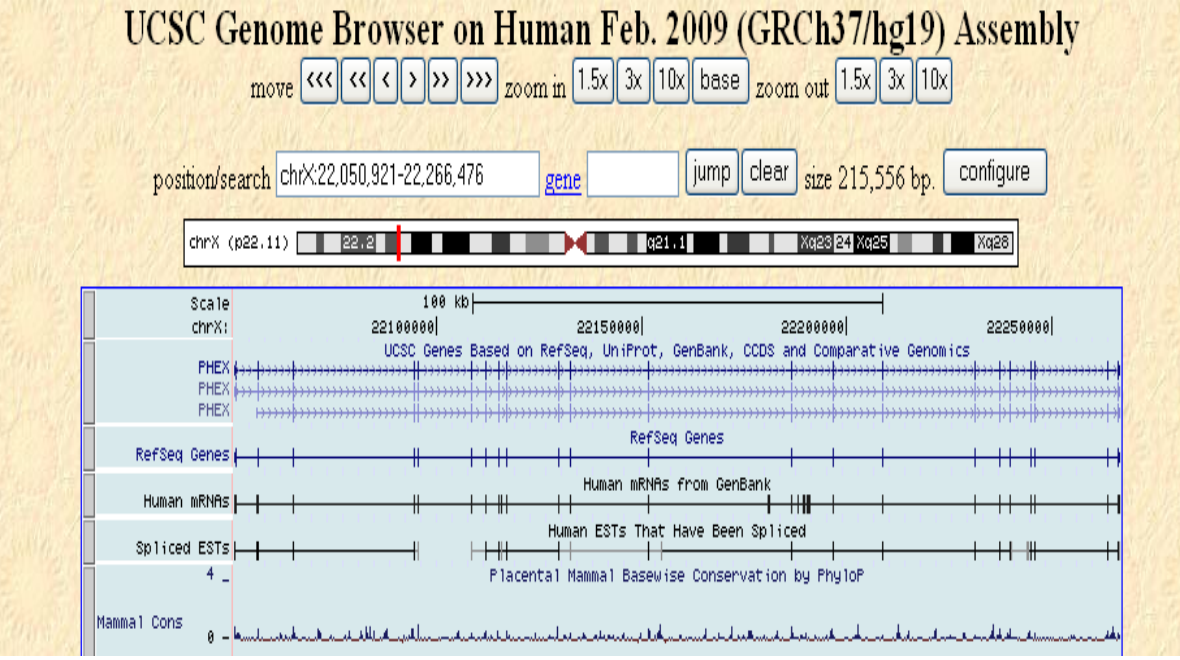

Figure 1: Locus and length of the Phex gene on the human X chromosome viewed in UCSC genome browser.

3.2 Prediction of miRNAs within human PHEX-introns

By means of the microRNA database miRBase16.0, we conducted a detailed analysis to determine the miRNAs harboring the PHEX-introns on the human X chromosome. Subsequently, we predicted a total of 1296 miRNAs in all the 21 PHEX-intronic regions alone. It was noteworthy that the number of miRNA candidates found within each of the 21 PHEX-introns was variable. It was noteworthy that the number of miRNA candidates found within each of the 21 PHEX-introns was variable. With the highest number of miRNAs, the twelfth intron encompassing184 miRNAs, introns 18, 3, and 15 were found to contain 173, 168, and 131 miRNAs respectively. Introns 8, 4, 6, 10, 7, 16, and 21 were predicted to consist of 11, 11, 14, 17, 18, 29, and 32 miRNAs respectively. Added to this, the size of the introns in the PHEX gene also varied as expected. The existence of the number of miRNAs in each intron was directly proportional to the size of their corresponding intron. For instance, the twelfth intron being the largest in the PHEX gene consisted of 184 miRNAs, while the thirteenth intron, which is of moderate size, displayed 48 miRNAs, and 11 miRNAs corresponded to the eighth intron. Notably, no miRNAs were present within the nineteenth intron.

As far as the predicted miRNAs were concerned, we noticed that more than half of them were repeatedly present throughout the intronic sequences. For example, the miRNA hsa-miR-

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1273 was discovered to occur twenty-two times in the introns 2, 3, 6, 12, 13, 18, and 20, and hsa-let-7f was present seven times in certain introns. Out of the 1296 miRNAs predicted overall, we found that 527 miRNAs were unique to the introns of the PHEX gene. The rest of the 769 microRNAs were found interspersed across the genome in various chromosomes ranging from 1 to 22. Although 1296 miRNAs were predicted across all the introns, only 136 miRNAs were uniquely distinguished within the human PHEX gene. This was because the predicted miRNAs overlapped between the introns of the PHEX gene. Similarly, we observed that a few of them possessed multiple genomic coordinates. An example of this is hsa-mir-3670, which was found in PHEX-introns 2, 11, and 12, also appeared on chromosome 16 at multiple loci (Suppl. Table S1).

3.3 Human miRNA target prediction

After analyzing the results obtained from miRBase miRNA screening, we predicted 136 unique human miRNAs on the PHEX gene of the human X chromosome. They were subjected to further analysis via MAGIA to identify their potential target genes. For each miRNA located on the intron sequences of the PHEX genes on the human X chromosome, 46, 957 specific targets were predicted using the MAGIA tool, which predicts the targets with the algorithms Miranda, PITA, and TargetScan. The Boolean logical operator intersection was used for stringent prediction so that only when a miRNA was predicted by all the algorithms, it would report an miRNA. The results obtained using optimal prediction threshold parameters for each algorithm were meticulously cataloged and analyzed. According to the predictions, miRNAs such as hsa-mir-4313, hsa-mir-761, hsa-mir-3148, hsa-mir-3672, hsa-mir-3188, hsa-mir-2054, hsa-mir-3135, hsa-mir-3927, hsa-mir-1273c, and hsa-mir-1273e and their associated gene targets were not available in the TargetScan database (Suppl. Sheet S2 & S3).

3.4 Predicted chimp miRNA target genes

In the same way, 121 homologous chimp miRNAs were predicted after analyzing the results obtained from TargetScan. 30, 563 conserved miRNA targets were predicted in the light of the human miRNA catalog. The output file listed out all the miRNA gene targets, a short description of the genes predicted, their representative miRNAs, and the total number of conserved and poorly conserved sites in the seed region of the mRNA in terms of 8mer and 7mer, and their context scores (Suppl. Sheet S4 & S5).

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3.5 Validation of the Predicted miRNA Targets using Experimental Sets

The miRNAs and their targets predicted in the previous steps generated an exhaustive list of target genes in both the human and the chimp genomes. Those predicted human gene targets were refined to only those, which have been experimentally cross-validated. Expression-corroborated Catalogue of MicroRNA Targets (ExprTargetDB) generated output files cataloging the gene transcripts and their differential expression in both the species. A total of 37, 508 targets were predicted for the 136 human miRNAs.

According to the experimentally validated human miRNA target catalog, a couple of its target genes, cyclinD1 (CCND1) and cyclinA (CCNA), which are implicated in primary melanomas, were reported as downregulated in the mRNA expression of epithelial, macrophage, and muscle cells. Similarly, DHFR, a target gene of hsa-mir-24 that was implicated in the tumor cell proliferation, was determined to be upregulated during the mRNA cleavage of lymphocytes. Cyclin-dependent kinase inhibitor 2A (CDKN2A/ P16) controlled by has-mir-24 in the mRNA expression of epithelial and lymphocyte cells was reported to be downregulated in cervical carcinomas. Similar observations were made on has-mir-34a to its gene targets, cyclin-dependent kinase (CDK6) and CCND1 that play a crucial role in the mRNA cleavage of epithelial, lymphocyte, and muscle cells were differentially expressed, in other words, downregulated and upregulated, in the development of non-small-cell lung cancer and the tumor proliferation respectively. Likewise, CDKN1A, tumor suppressor candidate 2 (TUSC2), and suppressor of fused homolog (SuFu) genes controlled by hsa-mir-106a and hsa-mir-378 respectively were dysregulated in the breast, colorectal, and papillary thyroid carcinomas (Suppl. Sheet S6). Surprisingly, hsa-let-7b was discovered to be associated with more than 80 gene targets (Fig 2).

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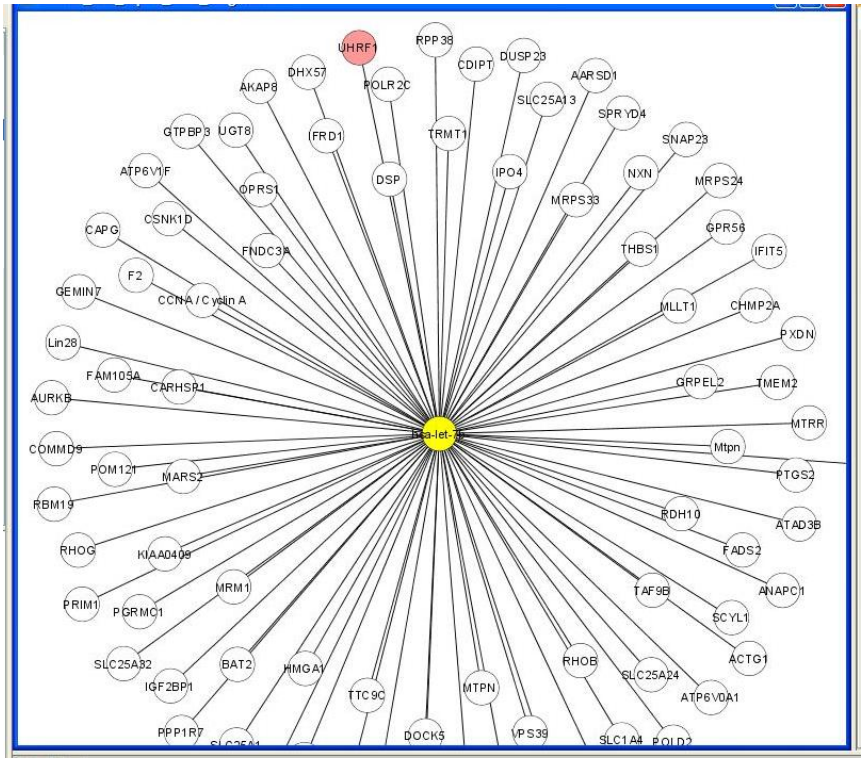

Figure 2: The molecular interaction networks of hsa-let-7b and its gene targets: this gene network depicts the interactions between the human miRNA, hsa-let-7b which regulates 80 different target genes that participates in various biological processes.

Likewise, we cataloged the differential gene expression of several target genes associated with chimp homologous miRNAs. According to the ExprTargetDB chimp catalog, Beta Secretase1 (BACE1) gene target of hsa-mir-107 was downregulated in epithelial, lymphocyte, and muscle cells. Our study discovered another target, RbI2 of hsa-mir-17-5p to be upregulated in the adipocytes during the early development of the lungs. It was interesting to observe that certain gene expression patterns of the experimentally-verified target genes among the conserved miRNAs predicted in both species were in agreement with each other. For instance, CDKN1A which is generally expressed in lymphocyte, macrophage, and muscle cells, was identified to be downregulated in the mesenchymal-epithelial transition (MET) of primary ovarian cancers in chimpanzee; however, the same CDKN1A was observed to be upregulated in human breast carcinomas (Suppl. Sheet S6).

3.5 Analysis of Human and Chimp miRNA-Target Gene Interaction Networks

The construction of probable miRNA-gene interaction networks for all the identified intronic miRNAs between both the species deciphered significant regulatory miRNAs from the miRNA

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pool considered for this study. Their gene specificity between both the species was examined in the complex interaction networks constructed (Fig 3 & 4). The constructed molecular interaction networks rendered top ten miRNAs within human PHEX-introns, such as hsa-let-7b, hsa-miR-24, hsa-miR-106a, hsa-miR-145, hsa-miR-34a, hsa-miR-27b, hsa-miR-378, hsa-mir-103 and hsa-mir-214, as interacting with their specific targets (Fig 5). In the same way, the gene interaction networks constructed on the conserved chimp miRNAs delineated the top ten miRNAs, namely hsa-miR-17-5p, hsa-miR-129, hsa-miR-127, hsa-miR-130, and hsa-miR-107 that were ranked in it (Fig 6). In addition to this, genes that significantly interacted with the top ten miRNAs like ALK4, CKDN2A, NOTCH1, DHFR, KIAA0152, MAPK14, CCND1/CYCLIN D1, etc., were also catalogued (Table 1).

Rank	Top interacting human miRNAs	Top interacting miRNAs in Chimp
1	hsa-let-7b	hsa-miR-24
2	hsa-miR-24	hsa-miR-17-5p
3	hsa-miR-106a	hsa-miR-145
4	hsa-miR-145	hsa-miR-34a
5	hsa-miR-34a	hsa-miR-129
6	hsa-miR-27b	hsa-miR-127
7	hsa-miR-378	hsa-miR-130
8	hsa-mir-103	hsa-miR-378
9	hsa-mir-214	hsa-miR-107

Table 1: Top Interacting miRNAs in Humans and Chimps

When we analyzed the above-mentioned gene interaction networks, it was revealed that certain gene targets were unique to only their top-interacting human miRNAs. Analogously, there were unique targets that were interacting with some of the top-ranked chimp miRNAs (Table 2).

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miRNA	Gene targets common to human and chimp	Gene targets unique to human	Gene targets unique to chimp
hsa-miR-24	ALK4, CKDN2A, NOTCH1, DHFR, KIAA0152 MAPK14	None	None
hsa-miR-145	TMOD3, FLJ21308, IRS1,	None	None
hsa-miR-34a	E2F3, CDK6, CCND1/CYCLIN D1	None	None
hsa-miR-378	"SuFu, TUSC2/Fus1, Fusion"	None	None
hsa-let-7b	CCND1/CYCLIN D1		
hsa-miR-27b		CYPIBI, NOTCH1	
hsa-miR-106a		RB1, ARID4B, MYLIP, HIPK3, CDKN1A,	
hsa-mir-103		GPD-1	
hsa-mir-214		PTEN	
hsa-miR-17-5p			RB12, NCOA3, E2F1, Rb12/p130, CDKN1A/p21,
hsa-miR-129			CAMTA1, EIF2C3
hsa-miR-127			Rt1/Peg11, BCL6
hsa-miR-130			MAFB, CSF1/MCSF
hsa-miR-107			NF1A, BASE1

Table 2: miRNAs having unique gene targets in both the species

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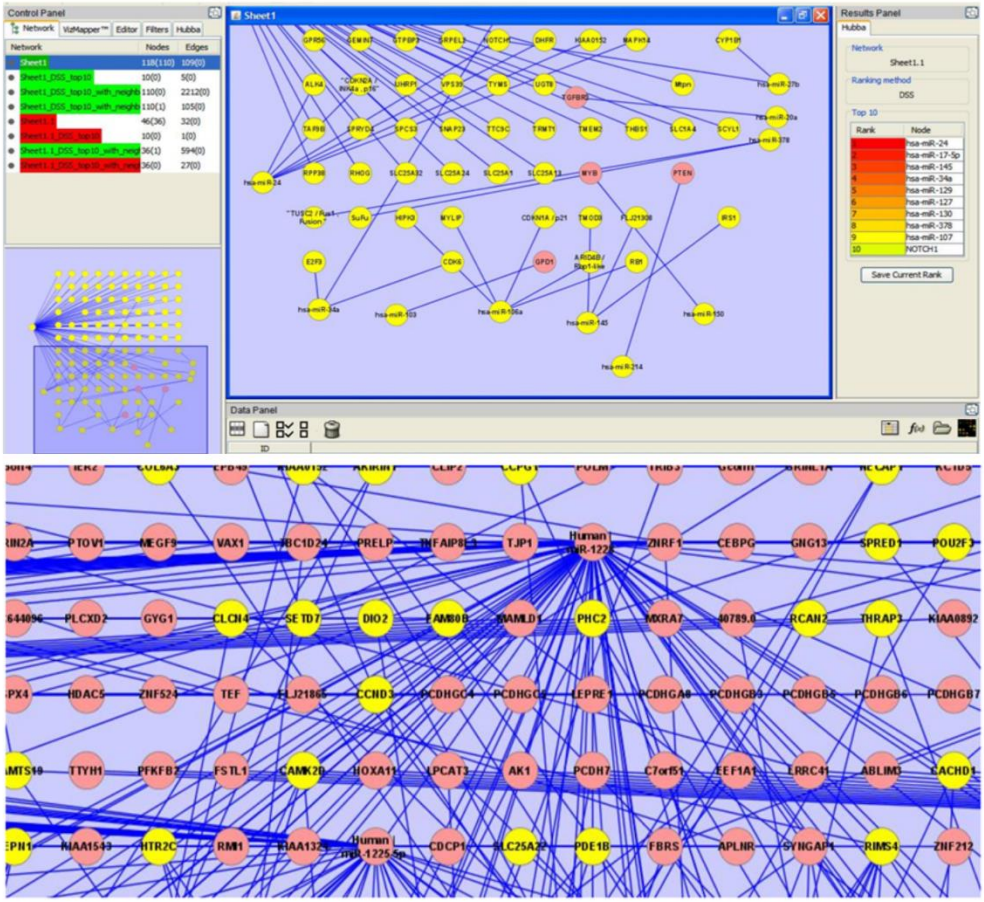

Figure 3: Complex molecular interaction networks of human miRNAs and their experimentally-validated target genes

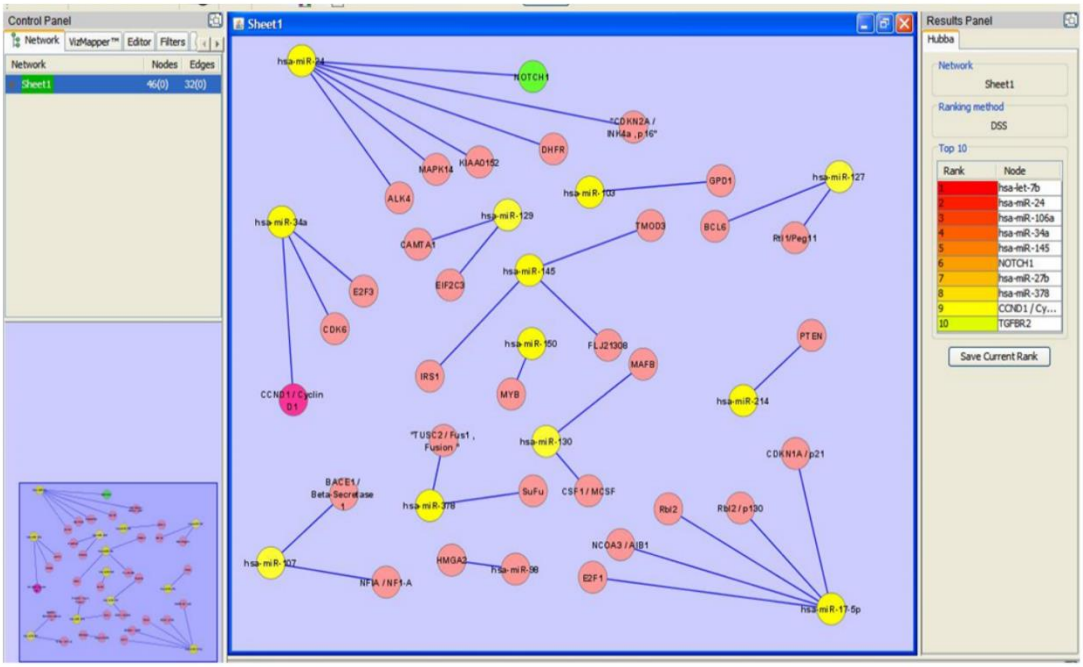

Figure 4: Complex gene interaction networks of chimp miRNAs and their target

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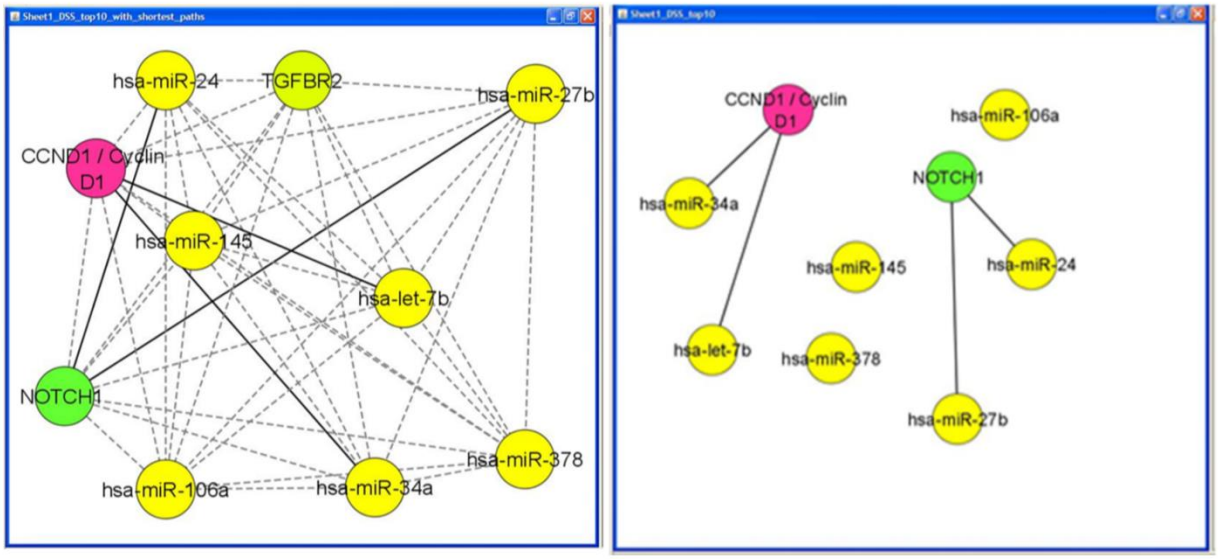

Figure 5: Top-ten-ranked human miRNAs and their target genes in the interaction network

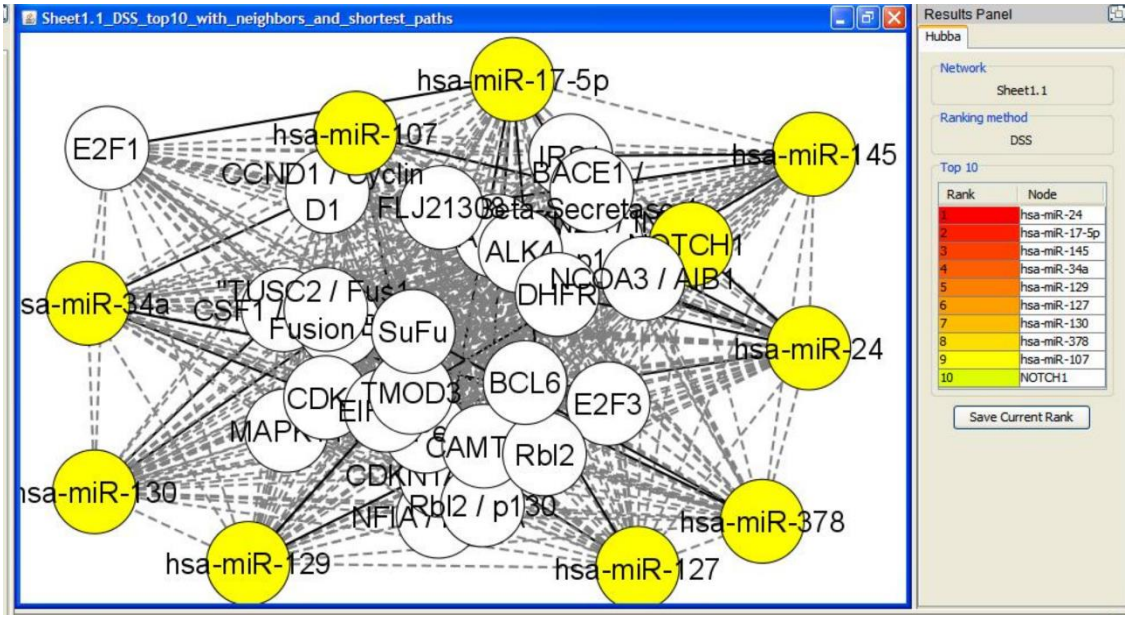

Fig 6: Top-ten-ranked chimp miRNAs and their target genes in the interaction network

4. Discussion

4.1 Importance of miRNA study on human and chimp X chromosome

It is well known that the X chromosome is one of the two sex chromosomes that is shared by males and females in humans and many other organisms. Diverse studies in the past have confirmed that the X chromosome is highly conserved among other mammalian species. [22, 23]. Experimental evidence suggests that the X chromosome is fairly a large chromosome that accounts for about 5% of the total human genome and carries substantially more active genes than the Y chromosome [24, 25]. It contains many essential genes that are not only

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responsible for sex determination but also for early embryonic development [26, 27]. Numerous X-linked Mendelian disorders and women-related cancers have been associated with the abnormalities of the X chromosome so far [28, 29]. Moreover, X chromosome-located miRNAs have been reported to be involved in crucial functions related to immunity and cancers [30].

MicroRNAs (miRNAs) are 21–23 nucleotides long genetic elements and they are members of a growing family of non-coding transcripts, participating in the regulation of a diverse collection of biological processes and various diseases by RNA-mediated gene-silencing mechanisms. They comprise well-equipped molecular machinery to regulate the expression of protein-coding genes through a complex network of pathways [31]. A recent research group has suggested miRNAs as key regulators of diverse vital programs implicating in processes such as developmental timing control, hematopoietic cell differentiation, apoptosis, cell proliferation, and organ development. According to their study, it was speculated that more than one-third of all protein-coding genes were known to be regulated by these miRNAs [31]. Because of their involvement in various developmental and physiological processes, understanding the distribution patterns and the regulatory mechanisms of microRNAs on the X chromosome of humans and chimp is advantageous for inventing new therapies against many diseases.

4.2 Screening of miRNAs in sequences with predicted homology in human and chimp

Vesselin Baev *et al.* in 2009, reportedly searched and analyzed the chimp homologs of the human pre-miRNA and mature miRNA sequences and identified hundreds of 100% and near 100% identical miRNAs. They further hypothesized that the 100% and near 100% identity determined in this study allowed them to predict that the newly discovered chimp orthologs were functional meaning, they have the same proposed function in both the genera [32]. Nevertheless, the study was incomplete, as the gene targets of human and chimp homologs were not analyzed. The sequence identity of the human and chimp miRNAs, their conservation patterns, and the specificity of their respective gene targets in both the species, with an emphasis on the differences in their targets and their interaction networks, were some of the areas that we proposed to be studied.

Genetic screening approaches for studying miRNAs in genomes are limited by their low efficiency, time-consuming procedures, and high cost involved. Hence, computational approaches have been developed as an alternative to complement the genetic screening efforts for identifying the miRNA genes. [33]. However, the prediction of miRNAs based on the informatics approach is a strenuous task because of the variability in the precursors and the

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enzymes involved in their complex biological processes. Successful prediction algorithms achieve a full-function recognition of mature miRNAs directly from the candidate hairpin secondary structures, high conservation of miRNAs at the sequence and target levels, and high minimal folding free energy index level across species, based on comparative genomics and phylogenetic analysis approaches with high accuracy [34, 35]. miRBase database provides comprehensive data with all the miRNAs mapped to their genomic coordinates, to facilitate the microRNA genomics study. Clusters of miRNA sequences in the genome are highlighted, and the overlap of miRNA sequences with annotated scripts are described in the miRBase repository. The genomic miRNAs of a wide spectrum of model organisms could be easily retrieved from miRBase [36]. In agreement with the previous research evidence, we strategized to survey the miRNAs concealed within the genomic sequences that were easily retrieved from the public domain genomic database.

In the present study, based on their sequence homology, we computationally examined for the candidate miRNAs, segment by segment on all the intronic sequences of the PHEX gene of the human X chromosome using miRBase-Blast tool. Out of the overall 1900 miRNAs encoded in the human genome [37], we identified about 7.16% of the miRNAs within the PHEX gene of the X chromosome through this study. Precisely, of the total number of the predicted PHEX-miRNAs predicted in this study, over 10% of them were unique miRNAs with the remaining repeats in the miRNA catalogue generated. The observation of miRNAs encoded within the introns of host mRNA genes suggests that there is co-regulation between the miRNA biogenesis and pre-mRNA splicing process. Hence, it is believed that the expression levels of intronic miRNAs and their host genes often are highly correlated, presumably because they are co-transcribed [38]. It has been reported earlier that miRNAs are transcribed from intergenic regions, introns, more infrequently from within exons of known protein-coding genes [19]. For this reason, intronic miRNAs may be co-transcribed with the ‘parent’ mRNAs, or may be transcribed independently [20]. A major bottleneck to studying miRNA across primates is the lack of experimentally verified data in non-human primates [38]. Literature says that only 12 of the 300 approximately known primate species have any entries in miRNAs [39]. For this reason, researchers attempt to predict non-human primate miRNAs based on their conserved patterns in the genomic sequences [40]. In our investigation, we noticed that more than 80% of the unique miRNAs of the human X chromosome to be conserved in the chimp genome at their sequence level.

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4.3 Validation of the predicted genes targets improves the quality of the target prediction algorithm

The knowledge of miRNA transcript targets regulated by miRNAs is crucial to correlate the associated abnormal or altered gene expression with the genetic and epigenetic events that drive a wide spectrum of human diseases [41, 42]. Therefore, with the goal of studying the miRNA target genes in both the species based on the complementarity between the miRNAs and targeted mRNAs, conserved miRNA targets were predicted for both human and chimp miRNAs tabulated in the previous step. This prediction was done with the state-of-the-art miRNA target prediction tools namely, MAGIA and TargetScan. Several studies in the past have employed these tools for the refined prediction of miRNA gene targets in various species [43, 44]. The use of Boolean operator intersection of all the three algorithms in MAGIA for identifying only the miRNAs substantially reduced the total number of predicted human PHEX-intronic miRNAs and placed much weight on the prediction accuracy as well. However, MAGIA did not support the target prediction of the Chimpanzee genus yet. Thus, the chimp miRNA target prediction was performed using TargetScan. Moreover, only the homologous miRNAs had to be selected to make a meaningful comparison of the gene targets. Most of the miRNA target prediction algorithms currently available to date take advantage of different biochemical/thermodynamic properties of the sequences of miRNAs and their gene targets. Although they have been of value to researchers, the prediction results of these methods are largely uncorrelated, and their degree of overlap is poor. Moreover, those miRNA targets that have been identified across diverse species are yet to be validated mainly due to the unavailability of large-scale experimental detection of the targets. Since ExprTarget, integrates some of the most frequently used miRNA target prediction methods as well as the expression datasets on HapMap cell lines generated in a laboratory [45], we conducted an analysis using the database of experimentally supported miRNA targets as gold standard.

This rationale of filtering out unlikely miRNA-target interactions and thus, eliminating the false-positives among the predicted targets greatly improved the miRNA target prediction when compared to the existing prediction algorithms [46, 47]. This analysis further reduced the total number of miRNAs whose target and interactions had to be studied. The final number of the human miRNAs and targets studied were 11 which included hsa-let-7b, hsa-miR-20a, hsa-miR-24, hsa-miR-27b, hsa-miR-34a, hsa-miR-103, hsa-miR-106a, hsa-miR-145, hsa-miR-150, hsa-miR-214 and hsa-miR-378. The total number of interacting gene targets of all these miRNAs was 117. This analysis further reduced the total number of miRNA whose target and

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interactions had to be studied. The final number of the human miRNAs and targets studied were 13 which included hsa-miR-103, hsa-miR-107, hsa-miR-17-5p, hsa-miR-98, hsa-miR-127, hsa-miR-129, hsa-miR-130, hsa-miR-145, hsa-miR-150, hsa-miR-214, hsa-miR-24, hsa-miR-34a and hsa-miR-378. The total number of interacting genes was found to be 37. The specificity and the number of gene targets corresponding to each miRNA differed between the species as anticipated.

4.4 Shared/common top interacting miRNAs among the human and chimp genera

The computation of the gene interaction networks of the experimentally validated miRNAs and their gene targets were summarized using the Double Screening Scheme (DSS) to get the top interacting nodes, which in turn were ranked using the DSS. As hypothesized, we obtained a different set of top interacting miRNA nodes in the human and the chimp networks. The top interacting miRNAs in the human were a subset of hsa-let-7b, hsa-miR-24, hsa-miR-106a, hsa-miR-145, hsa-miR-34a, hsa-miR-27b, hsa-miR-378, hsa-mir-103 and hsa-mir-214 (Top – has-let-7b and bottom – has-mir-214). The top interacting miRNAs in the chimp comprised a subset that included the miRNAs - hsa-miR-24 with a maximum number of interactions, hsa-miR-17-5p, hsa-miR-145, hsa-miR-34a, hsa-miR-129, hsa-miR-127, hsa-miR-130, hsa-miR-378, and hsa-miR-107 with the least number of interactions.

There were four top interacting miRNAs which were common to the two genera but their ranks varied with respect to one another. The shared miRNAs were - hsa-miR-24, hsa-miR-145, hsa-miR-34a and hsa-miR-378. It was interesting to observe that though the total number of validated miRNAs totaled to 11, the total number of interactions of those miRNAs numbered to 117 whereas, the total number of validated miRNAs of the chimp were 13 but their target gene interactions totaled to only 37. One of the reasons for this was the presence of has-let7b as the top interacting node in the human subset. This miRNA had a multiplicity value of 89 which dominated the picture of the interactions. Apart from this observation, there were varying levels of multiplicity and cooperativity between the miRNAs and their targets in the two genera. Of all the observations, some of the more observable cases of multiplicity was the case of has-let7b in the human miRNA – target gene interaction network. We observed cooperativity in the human network where has-34a and has-let7b were found to regulate expression of CCND1/Cyclin D1. The other case of cooperativity observed in the human network was when the NOTCH1 gene was found to be regulated by has-mir-24 and has-mir-27b. It was worth an observation that in the case of the chimp miRNA – gene network, this

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case of cooperativity was replaced by multiplicity. has-mir-34a was found to be involved in regulating the expression of three genes, namely, CCND1/Cyclin D1, CDK-6 and E2F3. Similarly, the observed cooperativity involving has-mir-24 and has-mir-27b was replaced by a case of multiplicity when the single miRNA the hsa-mir-24 responsible for the regulation of NOTCH1 was also found on a multiplicity network involving 4 other genes too, namely, ALK4, CKDN2A, DHFR, KIAA0152 and MAPK14.

Let-7 miRNAs are believed to be downregulated in various types of cancers such as hepatocellular carcinoma, gastric adenocarcinoma, melanoma, renal cell carcinoma, Burkitt lymphoma, pancreatic, ovarian, prostrate, and breast cancers [48]. Accumulating evidence has proved let-7 to have therapeutic efficacy in animal models of cancers [49]. Early studies have revealed that Let-7b is implicated in osteoporosis as it downgrades CCND1 to repress osteogenic proliferation and differentiation [50]. Validated targets of hsa-mir-24 including ALK-4, DHFR, and Notch1 have been discovered to be a target of methotrexate, and participating in intracellular folate metabolism, erythroid and neuronal differentiation [51-53]. IRS1, a target of hsa-mir-145 is implicated in granulosa cell proliferation, which in turn contributes to abnormal folliculogenesis in patients with polycystic ovary syndrome [55].

4.5 Multiplicity and cooperativity in human and chimp genera

As it has been reported in literature, regulation by miRNAs is obviously not as simple as one miRNA–one target gene, as perhaps the early examples *(lin-4* and *let-7)* seemed to indicate. The distribution of predicted targets reflects more complicated combinatorics, both in terms of target multiplicity (more than one target per miRNA) and signal integration (more than one miRNA per target gene).

The distribution of the number of target genes (and target sites) per miRNA is highly non-uniform, ranging from zero for seven most miRNAs in this study. It is difficult to describe this in detail, beyond the candidate miRNAs discussed in this study and beyond the identification and annotation of target genes. Specific processes in an organism appear to be regulated by each miRNA or each set of co-expressed miRNAs. Groups of targets may reflect a reaction, a pathway, or a functional class. Although all miRNA–target pairs are subject to the condition of synchrony of expression, it is likely that typically one miRNA regulates the translation of a number of target messages and that, in some cases, the target genes as a group are involved in a particular cellular process as is already known for the case of *lin-4* (Ambros et al, 2003).

5. Conclusion

We have herein furthered the work carried out by Vesselin Baev *et al.* in 2009, with the inclusion of the target prediction of the human miRNA homologs in the chimp. The current status of cataloging chimpanzee miRNAs and predicting novel precursors and mature miRNAs is incomplete. The target prediction of the chimp miRNAs too is not widely available. Only when these discrepancies are removed, there would be any meaningful study with regard to the miRNA mediated gene expression regulation between human and the other primate genera.

We conclude that multiplicity of targets and cooperative signal integration on target genes are key features of the control of translation by miRNAs. Neither multiplicity nor cooperativity is a novel feature in the regulation of gene expression. Indeed, regulation by transcription factors appears to be characterized, at least in eukaryotes, by analogous one-to-many and many-to-one relations between regulating factors and regulated genes. We are, of course, aware that the control cycles and feedback loops involving miRNAs cannot be adequately described without more detailed knowledge of the control of transcription of miRNA genes, about which little is known at present.

Supplementary Materials: Table S1: Documented PHEX gene with the intronic miRNAs sequences, their positions and genomic loci, Sheet S2: MAGIA scores of the gene targets predicted for human miRNAs and the target count for each miRNA, Sheet S3: Catalog of conserved targets, conserved and poorly conserved target sites predicted for human miRNAs, Sheet S4: Catalog of conserved targets, conserved and poorly conserved target sites predicted for chimp miRNAs, Sheet S5: Consolidated report of conserved targets, conserved and poorly conserved target sites predicted for human and chimp miRNAs, Sheet S6: ExprTargetDB results of experimentally validated gene targets of human and chimp.

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Conflicts of interest

The authors declare no conflict of interest.

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