Published in final edited form as:

Exp Gerontol. 2020 February ; 130: 110800. doi:10.1016/j.exger.2019.110800.

Accumulation of Kynurenine elevates oxidative stress and alters microRNA Profile in Human Bone Marrow Stromal Cells

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Abstract

Kynurenine, a metabolite of tryptophan breakdown, has been shown to increase with age, and plays a vital role in a number of age-related pathophysiological changes, including bone loss. Accumulation of kynurenine in bone marrow stromal cells (BMSCs) has been associated with a decrease in cell proliferation and differentiation, though the exact mechanism by which kynurenine mediates these changes is poorly understood. MiRNAs have been shown to regulate BMSC function, and accumulation of kynurenine may alter the miRNA expression profile of BMSCs. The aim of this study was to identify differentially expressed miRNAs in human BMSCs in response to treatment with kynurenine, and correlate miRNAs function in BMSCs biology through bioinformatics analysis. Human BMSCs were cultured and treated with and without kynurenine, and subsequent miRNA isolation was performed. MiRNA array was performed to

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Conceptualization; Data curation; Formal analysis; SF, SD, KS, AKM, AK

Investigation; Methodology; SD, KS, KS, HK, RK, AKM, AK

Funding acquisition, Resources; SF, CMI, MWH, WDH, RK.

Software; SD, SF

Validation; SD, KS, SF,

Roles/Writing – original draft; SF,SD

Writing – review & editing. SF, CMI, MWH, WDH

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Conflict of interest: The authors also declare that there is no other conflict of interest regarding the publication of this manuscript.

identify differentially expressed miRNA. Microarray analysis identified 50 up-regulated, and 36 down-regulated miRNAs in kynurenine-treated BMSC cultures. Differentially expressed miRNA included miR-1281, miR-330-3p, let-7f-5p, and miR-493-5p, which are important for BMSC proliferation and differentiation. KEGG analysis found up-regulated miRNA targeting glutathione metabolism, a pathway critical for removing oxidative species. Our data support that the kynurenine dependent degenerative effect is partially due to changes in the miRNA profile of BMSCs.

Keywords

Kynurenine; oxidative stress; microRNAs; Human Bone Marrow Stromal Cells

1. Introduction

In the United States, the projected population over the age of 65 is expected to rise from 40 million in 2010, to over 80 million in 2050 (Howden and Meyer, 2011; Ortman, et al., 2014). The oldest age category of 85 and older is expected to rise to 21% of the population, increased from 14% in 2010 (Ortman, et al., 2014). This significant rise in the elderly population has made understanding the pathophysiology of aging increasingly important to the future of healthcare. Osteoporosis and loss of bone mineral density significantly affects aging populations, and is a major cause for morbidity and mortality in the elderly in the form of fractures. At the age of 50, women have an estimated 53.2% and men an estimated 20.7% lifetime risk of a fracture at any site (Van Staa, et al., 2001). One of the most devastating injuries in the elderly, a femur/hip fracture, has a lifetime risk of 11.4% in women, and 3.1% in men at the age of 50 (Van Staa, et al., 2001). Hip fractures are associated with an increase in all-cause mortality, with a 1-year mortality up to 25% (Katsoulis, et al., 2017; Braithwaite, et al., 2003). Osteoporosis is a multifactorial disease and little is known about it.

Tryptophan, as an essential amino acid, plays an important role in protein synthesis and bone biology (Young, 2013; Badawy, 2017; van der Goot and Nollen, 2013; Refaey, et al., 2017). Recent studies from our group and others showed that the tryptophan metabolite kynurenine plays an important role in the pathophysiology of multiple age-related diseases (Young, 2013; Badawy, 2017; van der Goot and Nollen, 2013; Refaey, et al., 2017). Tryptophan is metabolized to a number of bioactive molecules including serotonin and melatonin, with 95% of tryptophan entering the kynurenine pathway (Young, 2013; Badawy, 2017; Pandi-Perumal, et al., 2013). The first and rate-limiting step of tryptophan catabolism is controlled by indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), forming N-Formylkynurenine prior to conversion to kynurenine (KYN) (van der Goot and Nollen, 2013). TDO is primarily located in the liver and accounts for most KYN production, while IDO is more broadly expressed in the liver, brain, kidney, and immune cells such as macrophages and T cells (Refaey, et al., 2017; Metz, et al., 2007; Metz, et al., 2014; Palego, et al., 2016). Kynurenine can then be converted to multiple different active metabolites, including kynurenic acid (KA), xanthurenic acid (XA), quinolinic acid (QA) and others (Song, et al., 2017).

The age-associated accumulation of kynurenine is well documented, and due in part to increases in inflammation and circulating cytokines in older populations (Bruunsgaard, et al., 2001; Oxenkrug, 2011; Pertovaara, et al., 2006). This increase in circulating cytokines, specifically interferon-gamma, causes an increase in IDO1 and IDO2 mediated tryptophan degradation (Oxenkrug, 2011; Brooks, et al., 2016). Increased IDO activity shunts tryptophan away from serotonin and melatonin formation, a metabolite associated with protective effects against oxidative stress, towards kynurenine metabolite production (Oxenkrug, 2011; Mehrzadi, et al., 2017; Wang, et al., 2013). This age associated increase in kynurenine pathway activity has been associated with a number of diseases more prevalent in older populations including cardiovascular disease, neurodegenerative disorders, diabetes, and malignancy (Metz, et al., 2007; Song, et al., 2017; Breda, et al., 2016; Munn and Mellor, 2016; Oxenkrug, 2015). Our group and others previously reported that elevated levels of kynurenine were associated with bone loss. Furthermore, we also reported that kynurenine treatment decreases osteogenic differentiation of bone marrow stromal cells (Refaey, et al., 2017). Recent studies suggested that KYN induces oxidative stress and exerts negative effects on cellular health (Reyes Ocampo, et al., 2014; Sas, et al., 2018; Varma and Hegde, 2010). Oxidative damage has previously been shown to be an important mediator of decreases in osteogenic differentiation and age-related bone loss (El Refaey, et al., 2014; Manolagas, 2010; Sendur, et al., 2009). In addition, melatonin has been shown to play a role in decreasing reactive oxygen species formation in bone marrow mesenchymal stem cells when exposed to hypoxic conditions and inflammatory cytokines such as TNF- α , and shown to promote cell viability when directly exposed to hydrogen peroxide (Mehrzadi, et al., 2017; Wang, et al., 2013; Wang, et al., 2015; Qiu, et al., 2019; Maria, et al., 2018). However, the mechanism by which kynurenine and kynurenine pathway metabolites, and the associated increases in oxidative stress, induces these changes in osteogenic differentiation is not yet fully understood.

It has previously been seen that miRNAs, which are single-stranded non-coding oligonucleotides typically around 20 nucleotides in length, are capable of regulating BMSC proliferation and differentiation, and therefore contribute to musculoskeletal development (Baglio, et al., 2015; Kim, et al., 2012; Lian, et al., 2012; Schoolmeesters, et al., 2009). MiRNA has been found to suppress osteogenic differentiation in BMSCs in response to aging and oxidative stress, and have been associated with osteoporotic fractures (Davis, et al., 2017; Seeliger, et al., 2014). This indicates a role of miRNAs in the regulation of bone homeostasis in aging populations. Limited research has also shown miRNA influence of the kynurenine pathway, supporting the potential relationship between miRNA and tryptophan breakdown in BMSCs (Duan, et al., 2017; Huang, et al., 2018; Moloney, et al., 2017). Though roles of kynurenine and miRNA have been independently established as contributing to BMSC differentiation and bone biology, no studies have investigated the importance of kynurenine-mediated miRNA regulation. The purpose of this study is to determine the differentially expressed miRNAs in BMSCs in response to an increase in kynurenine levels. The aim of this study was to identify differentially expressed miRNA in cultured BMSCs in response to treatment with kynurenine and to identify their potential role in altering BMSC proliferation and differentiation through bioinformatics analysis. Understanding the mechanism by which BMSCs lose their ability to differentiate with age

may allow for new targeted therapies which could help prevent the progression of age-related musculoskeletal diseases, such as osteoporosis.

2. Materials and Methods:

2.1. Culture and Kynurenine (KYN) Treatment of Human BMSCs (hBMSCs):

Human BMSCs were purchased from LaCell (New Orleans, LA, 70112, USA) and cells were treated with KYN as mentioned below. Human BMSCs were cultured on 24 well plates and treated with or without KYN at low (10 μ M) and high (100 μ M) concentrations for 24 hours in DMEM with 2% FBS for hydrogen peroxide estimation. For miRNAs array and RT-PCR analysis, cells were treated with KYN (50 μ M) for 6hrs.

2.2. Total RNA isolation, Gene Expression Analysis and hydrogen peroxide Estimation:

Total RNA was isolated using QIAGEN total RNA isolation kit (Cat No: 217004) followed by microRNA Array, mRNA and miRNA analysis. For hydrogen peroxide estimation, cells were cultured on 6 well plates and treated with KYN (10 μ M) for 24hrs. Hydrogen peroxide estimation was performed using Amplex red assay (Catalog No. A22188, Fisher Scientific). After treatment, media was removed and cells were suspended in sodium phosphate buffer (0.05 M, pH7.4, 100 mL) and plated in triplicate in a flat-bottom 96-well plate. The reaction was started by adding AmplexTM Red reagent, horse-radish peroxidase and p-tyramine. After 30 min incubation in the dark, the production of H₂O₂ was quantified at 37°C in multi-detection fluorescence reader (Syner-gy H1, BioTek Instruments) at an emission wavelength of 590 nm upon excitation at 545 nm. The specific final fluorescence emission was calculated against a standard curve of H₂O₂ incubated simultaneously.

2.3. Microrna Array and Bioinformatics Analysis:

The quality of RNA samples was characterized on an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) using an RNA6000 Nano Chip (Agilent). Microarrays were performed on miRNA using an Affymetrix GeneChip[®] miRNA 4.0 array at the Integrated Genomics Core, Augusta University, GA, USA. Details of the procedure can be found online at <http://www.augusta.edu/cancer/research/shared/genomics>. The miRNA profile was analyzed for hierarchical clustering of miRNA to generate heat maps. The results were normalized using robust multichip averages. T-tests were used to calculate the p-value (0.05) to determine whether there is a significant difference (1.5 fold) for miRNA expression between the control and the treatment groups. Principal component analysis (PCA) was performed between KYN treatment and control samples. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analyses were performed using DIANA-miRPath v. 3.0 (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>) on differentially expressed microRNAs target genes. Bioinformatics software (http://www.targetscan.org/vert_71/) was used to predict targets genes of differentially regulated miRNAs of musculoskeletal importance.

2.4. Validation of miRNA using real time-PCR:

Two hundred nanograms of enriched small RNA were converted into cDNA using RT2 miRNA First Strand Kit (SABiosciences Corporation, Frederick, MD, USA). Fifty

picograms of cDNA were amplified in each qRT-PCR using syber green dye and miRNA specific primers. The real-time qRT-PCR was performed on a Bio-rad q-pcr machine with following cycling parameters: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. SYBR Green fluorescence was recorded during the annealing step of each cycle. The average of RNU6 (RNA, U6 small nuclear 2) and SNORD (small nucleolar RNA, C/D box) was used as normalization reference genes for miRNAs.

2.5. Statistical analysis:

Data are presented as fold-changes or percentages with mean $\pm$ SEM as indicated in the figure legends. GraphPad Prism 5 (La Jolla, CA) was utilized to perform unpaired t-tests as appropriate. A p-value of 0.05 was considered significant.

3. Results

3.1. Kynurenine induces oxidative stress in hBMSCs:

The age-associated accumulation of kynurenine is well documented in different organ and tissues (Bruunsgaard, et al., 2001; Oxenkrug, 2011; Pertovaara, et al., 2006). To mimic similar conditions, hBMSCs were treated with KYN (10 μ M and 100 μ M) for 24hrs to estimate hydrogen peroxide, which is marker for oxidative stress marker. We found that both low and high doses of KYN showed significant (p Value=0.0121) elevation of hydrogen peroxide generation in hBMSCs (Figure 1(a)). For gene expression analysis, cells were treated with KYN (50 μ M) for 6hrs. Real time PCR showed significant up-regulation of CYP1A1 (p Value=0.0029) and CYP1B1 (p Value=0.008) (Figure 1(b&c)). This shows that KYN induces oxidative stress through activation of AhR signaling. Previously, similar results were reported in a number of different *in vitro* and *in vivo* studies (Hanieh, 2014; Wang, et al., 2018).

3.2. MicroRNA Expression Profile Following KYN:

To identify miRNAs that were differentially expressed following KYN treatment, we conducted a comprehensive miRNA microarray analysis on hBMSCs that were treated with or without KYN. The miRNAs that exhibited a significant ($p < 0.05$) 1.5-fold difference in expression after KYN treatment were selected for analysis. Our analysis identified 86 miRNAs that were differentially expressed ($p < 0.04$) in response to KYN. Out of 86 miRNAs, 50 up-regulated and 36 were down-regulated in the presence of KYN. The list of up-regulated and down-regulated miRNAs after KYN treatment is shown in Table 1. Hierarchical cluster analysis using the normalized miRNA expression data confirmed that the expression of miRNAs in KYN treated BMSCs can be clearly distinguished from the controls (Figure 2a).

3.3. Principal Component Analysis (PCA):

We performed a PCA to explore the relationships between the control and KYN treated samples. The PCA graph (Figure 2b) shows the presence of the cluster of KYN treatment samples that is clearly distinct from the control (non-treated) samples.

3.4. Validation of Differentially expressed microRNAs:

To further verify the results obtained from miRNA microarrays, we performed real-time PCR on randomly selected miRNAs (miR-24-2-5p, miR-128-3p, miR-493-5p, miR-5006-5p and 362-5p) to validate our findings. MiRNA real-time PCR showed similar changes as noted in the miRNA array (Figure 3). In KYN (50 μ M) treated samples, we found that miR-24-2-5p ($p = 0.0407$), miR-128-3p ($p = 0.011$), miR-493-5p ($p = 0.0208$) and miR-5006-5p ($p = 0.0138$) were significantly up-regulated and miR-362-5p ($p = 0.0207$) was significantly down-regulated.

3.5. Signaling Pathway Predictions:

After KYN treatment, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation and GO analysis to determine functions of the miRNAs found to be differentially expressed. The KEGG annotation analysis showed that a number of molecules were affected by these miRNAs. The important KEGG signaling for up-regulated miRNAs are ECM-receptor interaction, Glutathione metabolism, and Cyanoamino acid metabolism (Table.2a) and down-regulated are Biotin metabolism and Glycosphingolipid biosynthesis (Table.2b). Details of KEGG annotation analysis are shown in Table.2 (a&b). The gene ontology analysis showed that more than 54 and 43 biological processes were associated with the up-regulated and down-regulated miRNAs (Supplementary Table. S1). The most common GO pathways regulated by both up-regulated and down-regulated miRNAs are organelles, ion binding, cellular nitrogen compound metabolic process, small molecule metabolic process and gene expression. Details of the GO analyses are shown in Supplementary Table. S1.

3.6. Bioinformatics miRNAs Target Prediction:

We can derive some functional predictions of the differentially regulated miRNAs based on miRNA targets predicted from the in-silico analysis. We analyzed the potential targets of miRNAs that are differentially expressed following KYN, with the criteria that the miRNAs must bind the 3' UTR of the mRNA with its seed sequence. We used Targets.org target prediction tools to identify miRNA targets and their signaling pathways. We identified a number of miRNA targets of musculoskeletal and stem cell differentiation related genes (Figure 4). The lists of miRNA targets are shown in Table 3.

4. Discussion

Oxidative stress and inflammatory factors are both known to contribute to age-related diseases, and have been proposed as major contributing factors in dysregulation of bone marrow stromal cell physiology (Almeida and O'Brien, 2013; Hohn, et al., 2017). Kynurenine, a metabolite of tryptophan degradation, has been well documented to increase with age, in part due to increased IDO activity by circulating cytokines (Bruunsgaard, et al., 2001; Oxenkrug, 2011; Pertovaara, et al., 2006; Brooks, et al., 2016). Our group previously showed that mice given kynurenine supplementation had accelerated bone loss, and BMSCs treated with kynurenine had decreased osteogenic differentiation (Refaey, et al., 2017). It is well known that kynurenine and its oxidative metabolites induce oxidative stress in a number of different cell types and organs (Reyes Ocampo, et al., 2014; Sas, et al., 2018; Varma and

Hegde, 2010). However, the mechanism by which kynurenine pathway metabolites and oxidative stress induce changes in bone marrow stromal cell physiology is poorly understood. Our study aimed to identify differentially expressed miRNA in BMSCs in response to treatment with kynurenine, and to identify downstream target genes of these differentially expressed miRNA that may contribute to resulting changes in BMSC proliferation and differentiation.

Our data showed that kynurenine treatment elevated oxidative stress in human BMSCs. We found significantly elevated levels of hydrogen peroxide generation after 24hrs of treatment. Furthermore, we also found induction of cytochromes P4501A1 (Cyp1A1) and P450B1 (Cyp1B1) gene expression, which is an indication of elevated oxidative (Delescluse, et al., 2001; Zhou, et al., 2017). Our study found 50 up-regulated and 36 down-regulated miRNAs in BMSCs treated with kynurenine compared to control cultures (Table.1). We utilized published literature to identify miRNAs in our data set that had previously been associated with changes in response to oxidative stress or inflammation, as well as associated regulation of BMSC differentiation and senescence (Table.4).

The miRNA-210-3p has been known for its role in regulation of oxidative stress and the cell cycle in multiple tissues (Chan and Loscalzo, 2010; Dai, et al., 2019; Kim, et al., 2009). MiR-210-3p up-regulation has also been shown to protect against reactive oxygen species (ROS) formation in hypoxic states, and protect against apoptosis (Chan and Loscalzo, 2010; Chan, et al., 2009). Kim *et al*/previously reported that miR-210-3p has cytoprotective and antiapoptotic effects after ischemic preconditioning through inhibition of FLASH/*Casp8ap2* in bone marrow-derived mesenchymal stem cells (Kim, et al., 2009). Our study found that kynurenine treatment down-regulated miR-210-3p expression in hBMSCs. We speculate that this down-regulation increases BMSCs susceptibility to ROS, impairing cellular defense mechanisms and further supporting the important role of miR-210-3p in protecting cells against oxidative damage. Another miRNA, miR-493-5p, has been shown to be differentially regulated in aging tissue, as well as in osteosarcoma cells (Chen, et al., 2018; Zhang, et al., 2019). Zhang *et al* illustrated the role of miR-493-5p in inhibiting KLF5, a gene important for cell proliferation, differentiation, and apoptosis (Zhang, et al., 2019). Our study found miR-493-5p up-regulated in response to kynurenine in BMSCs, indicating a possible role of miR-493-5p in decreasing cell proliferation, differentiation and increase apoptosis of BMSCs in elevated kynurenine levels conditions such as aging.

In order to further characterize targets of differentially expressed miRNAs in our data set, bioinformatic analysis was utilized. From our data set only some of the miRNAs have been studied before at functional level (Table.3). As illustrated above, much is still unknown about the breadth of specific miRNA influence. *In silico* bioinformatic analysis using TargetScan was performed to predict genes targeted by select miRNA that are critical for stem cell proliferation, osteogenesis, and musculoskeletal development (Table.3) (Agarwal, et al., 2015). Our data showed that some of the selected miRNA target multiple genes critical for early progenitor cell function, including multiple Hox genes important for differentiation and proliferation (Bhatlekar, et al., 2018; De Kumar, et al., 2017; Gouti and Gavalas, 2008). WNT signaling genes were also targeted, which are associated with osteogenic

differentiation, cell proliferation, and chondrocyte maturation (Boyan, et al., 2018; Chen, et al., 2014; Teufel, et al., 2018; Zmuda, et al., 2009).

To further identify the biological role of differentially expressed miRNA found in kynurenine-treated BMSCs, KEGG pathway annotation and GO analysis were performed (Table.2 and Supplementary Table. S1) (Vlachos, et al., 2015). KEGG pathway annotation of the 20 most upregulated miRNAs of our data set showed regulation of multiple pathways, most importantly ECM-receptor interaction and glutathione metabolism (Table.2). The extracellular matrix is critical in bone remodeling, and influences the pathophysiology of osteoporosis (Alford, et al., 2015; Sroga and Vashishth, 2012; Sroga and Vashishth, 2018). Glutathione is well known to remove reactive oxygen species to prevent oxidative damage in cells throughout the body (Back, et al., 1998; Chandra, et al., 2000; Hayes and McLellan, 1999; Michiels, et al., 1994). Mentioned previously, oxidative stress is a contributing factor to osteoporosis, and has been proposed to mediate kynurenine-induced bone loss (El Refaey, et al., 2014; Manolagas, 2010; Sendur, et al., 2009; El Refaey, et al., 2015). These results further support a role of increased oxidative stress in BMSCs with increased kynurenine. Down-regulated miRNA in kynurenine-treated BMSCs were most relevantly found to regulate glycosphingolipid synthesis. Glycosphingolipids are important components of the cell membrane, and have been found to influence osteoclast activity and differentiation (Ersek, et al., 2015; Fukumoto, et al., 2006). Changes in glycosphingolipid expression are also associated with different stages of BMSC differentiation (Rosu-Myles, et al., 2013). GO analysis of upregulated miRNA showed involvement in multiple processes necessary for BMSC differentiation and proliferation, such as the mitotic cell cycle, phospholipid metabolic processes, cytoskeletal protein binding, and enzyme binding (Supplementary Table. S1). In addition, GO analyses of down-regulated miRNA showed involvement in TLR signaling, further supporting the role of kynurenine in the elevation of inflammation (Bruunsgaard, et al., 2001; Oxenkrug, 2011; Pertovaara, et al., 2006; Brooks, et al., 2016).

Our study is the first to identify changes in miRNA expression in human BMSCs in response to kynurenine. We speculate that these changes in miRNA expression pattern are in response to kynurenine-mediated increases in oxidative stress, which alters cellular processes important in normal BMSCs metabolism (Figure.4). Future studies will be needed to validate how miRNAs identified in our study influence BMSC differentiation and proliferation. The effect of these differentially expressed miRNAs on bone loss in animal models should also be examined. In addition, increased kynurenine metabolite production and the associated decrease in melatonin production may exert cumulative effects on BMSC metabolism and increased susceptibility to oxidative damage, and should be investigated. Circadian influence on bone metabolism has previously been illustrated through changes in melatonin production, as well as melatonin-independent circadian changes (Guntur, et al., 2011; Song, et al., 2018). Changes in AhR expression have also been shown to alter circadian rhythmicity (Jaeger, et al., 2017; Tischkau, 2019).

Overall, this study contributes to the understanding that increases in kynurenine pathway activity alters miRNA profiling. These changes in miRNA profiling partially affect BMSCs biology, and may play a vital role in aging-related musculoskeletal homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

This publication is based upon work supported in part by the National Institutes of Health (National Institute on Aging-AG036675 S.F, W.D.H, M.H, C.S.). The above-mentioned funding did not lead to any conflict of interests regarding the publication of this manuscript.

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Highlights

1. Kynurenine induces oxidative stress in hBMSCs
2. Microarray analysis identified 50 up-regulated, and 36 down-regulated miRNAs in kynurenine-treated hBMSC cultures.
3. These differentially expressed miRNA are important for BMSC proliferation, and differentiation.
4. KEGG pathway analysis showed these miRNA target glutathione metabolism, a pathway critical for removing reactive oxidative species.

Figure. 1. Kynurenine induces hydrogen peroxide generation in hBMSCs.

(a) hBMSCs cells were treated with KYN (10 and 100μM) and intracellular accumulation of Hydrogen peroxide was measured in the control and KYN treated cells after 24hrs (n=6, #p= 0.001). Real-time PCR showing changes in gene expression in hBMSCs after 6hrs of KYN treatment (b) CYP1A1 and (c) CYP1A1. Data (n= 6) are represented as the fold change in expression compared with control (*p= 0.04, #p= 0.001).

Figure 2.

Differential miRNA expression in human bone marrow stromal cells after Kynurenine treatment ((50 μ M), n=3 each group). The heat-map shows the differential expression pattern of miRNAs compared to control group. (b) Principle component analysis (PCA) mapping of Kynurenine treatment and control samples. Control group (indicated by golden color) was clustered distinctly from the Kynurenine treated group (indicated by blue color). The results were normalized using robust multichip averages. T-tests were used to calculate the p-value (0.05) to determine whether there is a significant difference (1.5 fold) for miRNA expression between the control and the treatment groups.

Figure 3.

Validation of miRNA array data on randomly picked miRNAs. Real-time PCR showing change in miRNA expression in kynurenine (50 μM) treated samples compared to control (a) miR-24-2-5p; (b) miR-128-3p; (c) miR-493-5p; (d) miR-5006-5p and (e) miR-362-5p (n = 4-6, * p = 0.05, # p = 0.01)

Figure 4. Kynurenine dependent miRNAs mediated signaling in human bone marrow stromal cells. Schematic diagram showing the contribution of key miRNAs in cell proliferation and differentiation of human bone marrow stromal cell.

Table.1.

Table showing miRNAs differentially regulated in presence of KYN in human bone marrow stromal cells.

Species Scientific Name	Probeset ID	Transcript ID(Array Design)	p-value(Kyn vs. control)	Fold-Change(Kyn vs. control)
Homo sapiens	MIMAT0000705_st	hsa-miR-362-5p	0.00060	-3.28528
Homo sapiens	MIMAT0005939_st	hsa-miR-1281	0.00362	-2.79347
Homo sapiens	MIMAT0000751_st	hsa-miR-330-3p	0.00184	-2.73427
Homo sapiens	MIMAT0000267_st	hsa-miR-210-3p	0.01158	-2.25788
Homo sapiens	MIMAT0018968_st	hsa-miR-4449	0.01893	-2.10157
Homo sapiens	MIMAT0019723_st	hsa-miR-4656	0.02707	-2.09407
Homo sapiens	MIMAT0004592_st	hsa-miR-125b-1-3p	0.01633	-2.01039
Homo sapiens	MIMAT0026636_st	hsa-miR-668-5p	0.01033	-1.95357
Homo sapiens	MIMAT0004804_st	hsa-miR-615-5p	0.00642	-1.86682
Homo sapiens	MIMAT0027496_st	hsa-miR-6798-5p	0.00412	-1.82968
Homo sapiens	MIMAT0027357_st	hsa-miR-6728-5p	0.02293	-1.81138
Homo sapiens	MIMAT0015036_st	hsa-miR-3162-5p	0.05499	-1.7836
Homo sapiens	MIMAT0004494_st	hsa-miR-21-3p	0.03644	-1.75477
Homo sapiens	MIMAT0020600_st	hsa-miR-5095	0.00001	-1.73149
Homo sapiens	MI0022631_st	hsa-mir-6786	0.00005	-1.68325
Homo sapiens	MIMAT0003336_st	hsa-miR-658	0.01897	-1.67349
Homo sapiens	MIMAT0004748_st	hsa-miR-423-5p	0.00185	-1.67135
Homo sapiens	MIMAT0001627_st	hsa-miR-433-3p	0.04030	-1.66801
Homo sapiens	MIMAT0004679_st	hsa-miR-296-3p	0.00954	-1.66451
Homo sapiens	MIMAT0002175_st	hsa-miR-485-5p	0.02897	-1.65444
Homo sapiens	MIMAT0004602_st	hsa-miR-125a-3p	0.02432	-1.65179
Homo sapiens	MIMAT0004982_st	hsa-miR-939-5p	0.04923	-1.64675
Homo sapiens	MI0017304_st	hsa-mir-4673	0.04697	-1.64315
Homo sapiens	MIMAT0019767_st	hsa-miR-4682	0.03980	-1.62301
Homo sapiens	MIMAT0000430_st	hsa-miR-138-5p	0.00000	-1.6156
Homo sapiens	MI0008331_st	hsa-mir-1910	0.00398	-1.61398
Homo sapiens	MIMAT0003881_st	hsa-miR-668-3p	0.01049	-1.61078
Homo sapiens	MIMAT0019855_st	hsa-miR-4732-5p	0.00415	-1.60154
Homo sapiens	MIMAT0004803_st	hsa-miR-548a-5p	0.00024	-1.59918
Homo sapiens	MIMAT0005936_st	hsa-miR-1278	0.01049	-1.58805
Homo sapiens	MIMAT0005929_st	hsa-miR-1275	0.02075	-1.56677
Homo sapiens	MIMAT0020601_st	hsa-miR-1273f	0.02648	-1.55552
Homo sapiens	MIMAT0002811_st	hsa-miR-202-3p	0.03384	-1.5554
Homo sapiens	MIMAT0003161_st	hsa-miR-493-3p	0.04921	-1.54454
Homo sapiens	MI0016428_st	hsa-mir-3921	0.03672	-1.54347
Homo sapiens	MIMAT0000722_st	hsa-miR-370-3p	0.02453	-1.53817

Species Scientific Name	Probeset ID	Transcript ID(Array Design)	p-value(Kyn vs. control)	Fold-Change(Kyn vs. control)
Homo sapiens	MIMAT0027511_st	hsa-miR-6805-3p	0.04503	1.51356
Homo sapiens	MIMAT0019862_st	hsa-miR-4736	0.00131	1.52144
Homo sapiens	MI0016890_st	hsa-mir-4523	0.00979	1.52344
Homo sapiens	MIMAT0027398_st	hsa-miR-6749-5p	0.01044	1.52935
Homo sapiens	MI0000803_st	hsa-mir-330	0.01195	1.53713
Homo sapiens	MIMAT0002805_st	hsa-miR-489-3p	0.02087	1.53787
Homo sapiens	MIMAT0017983_st	hsa-miR-3606-5p	0.00345	1.54464
Homo sapiens	MIMAT0005572_st	hsa-miR-1225-5p	0.00736	1.55646
Homo sapiens	MIMAT0005878_st	hsa-miR-1287-5p	0.00138	1.56307
Homo sapiens	MIMAT0000232_st	hsa-miR-199a-3p	0.00058	1.5649
Homo sapiens	MIMAT0004563_st	hsa-miR-199b-3p	0.00058	1.5649
Homo sapiens	MI0022558_st	hsa-mir-6723	0.03760	1.57969
Homo sapiens	MIMAT0015089_st	hsa-miR-3202	0.00399	1.58496
Homo sapiens	MIMAT0000417_st	hsa-miR-15b-5p	0.00099	1.59133
Homo sapiens	MIMAT0000062_st	hsa-let-7a-5p	0.00057	1.5914
Homo sapiens	MI0000298_st	hsa-mir-221	0.03408	1.59836
Homo sapiens	MIMAT0025857_st	hsa-miR-892c-5p	0.00023	1.60193
Homo sapiens	MIMAT0025848_st	hsa-miR-6511b-3p	0.00178	1.60328
Homo sapiens	MIMAT0022279_st	hsa-miR-5582-5p	0.01092	1.60639
Homo sapiens	MI0022661_st	hsa-mir-6816	0.00003	1.62863
Homo sapiens	MI0022635_st	hsa-mir-6790	0.00149	1.63096
Homo sapiens	MI0025898_st	hsa-mir-8062	0.00015	1.63733
Homo sapiens	MI0005534_st	hsa-mir-891b	0.00399	1.68558
Homo sapiens	MIMAT0021086_st	hsa-miR-5094	0.03907	1.68635
Homo sapiens	MIMAT00055/7_st	hsa-miR-1226-3p	0.00105	1.71573
Homo sapiens	MIMAT0019871_st	hsa-miR-4741	0.04835	1.72331
Homo sapiens	MI0015858_st	hsa-mir-4259	0.00233	1.77213
Homo sapiens	MIMAT0022693_st	hsa-miR-204-3p	0.01413	1.77695
Homo sapiens	MIMAT0005948_st	hsa-miR-664a-5p	0.01008	1.77733
Homo sapiens	MI0022682_st	hsa-mir-6836	0.04317	1.78137
Homo sapiens	MIMAT0019798_st	hsa-miR-4701-5p	0.00457	1.78877
Homo sapiens	MIMAT0027497_st	hsa-miR-6798-3p	0.02523	1.78962
Homo sapiens	MIMAT0004959_st	hsa-miR-216b-5p	0.01548	1.79158
Homo sapiens	MIMAT0004588_st	hsa-miR-27b-5p	0.04084	1.79382
Homo sapiens	MIMAT0015070_st	hsa-miR-3188	0.00259	1.84481
Homo sapiens	MIMAT0027504_st	hsa-miR-6802-5p	0.05218	1.88947
Homo sapiens	MIMAT0019210_st	hsa-miR-3157-3p	0.01476	1.9804
Homo sapiens	MIMAT0000414_st	hsa-let-7g-5p	0.02230	1.98574
Homo sapiens	MIMAT0014983_st	hsa-miR-3121-3p	0.01324	1.99195

Species Scientific Name	Probeset ID	Transcript ID(Array Design)	p-value(Kyn vs. control)	Fold-Change(Kyn vs. control)
Homo sapiens	MIMAT0000067_st	hsa-let-7f-5p	0.00015	2.03909
Homo sapiens	MIMAT0030420_st	hsa-miR-7845-5p	0.03259	2.09834
Homo sapiens	MIMAT0004568_st	hsa-miR-221-5p	0.00633	2.1047
Homo sapiens	MIMAT0002813_st	hsa-miR-493-5p	0.00324	2.19161
Homo sapiens	MIMAT0023693_st	hsa-miR-6068	0.00239	2.34777
Homo sapiens	MIMAT0003251_st	hsa-miR-548a-3p	0.01027	2.5563
Homo sapiens	MIMAT0000424_st	hsa-miR-128-3p	0.00231	2.60041
Homo sapiens	MIMAT0004497_st	hsa-miR-24-2-5p	0.01329	2.69645
Homo sapiens	MIMAT0021033_st	hsa-miR-5006-5p	0.00810	2.85836
Homo sapiens	MIMAT0019020_st	hsa-miR-4486	0.00195	2.87605
Homo sapiens	MIMAT0000226_st	hsa-miR-196a-5p	0.01334	3.17675

Table.2.

Selected KEGG biological pathways potentially affected by (a) miRNAs up-regulated and (b) miRNAs down-regulated in the presence of KYN in human bone marrow stromal cells.

Table.2 (a).			
KEGG pathway	p-value	#genes	#miRNAs
ECM-receptor interaction	6.28E-07	19	9
Axon guidance	0.00256757	32	14
Base excision repair	0.01479837	8	6
Cocaine addiction	0.01479837	14	10
Glutathione metabolism	0.01517146	15	8
Cyanoamino acid metabolism	0.0366651	5	3
Table.2 (b).			
KEGG pathway	p-value	#genes	#miRNAs
Prion diseases	2.23E-19	4	4
Biotin metabolism	0.00022473	1	1
Glycosphingolipid biosynthesis - lacto and neolacto series	0.00022473	4	3

Table.3.

Predicated targets of differentially regulated miRNAs of stem cell biology

miRNA	Stem cell	Osteogenic	Chondrogenic
miR-330-3p	GDF6	SMAD7, TGFB3, FGFR1	HoxC8, RUNX1, FGFR1, SOX6, HDAC5
miR-210-3p	--	FGFRL1	FGFRL1
miR-615-5p	HOXA11, SOX7	WNT10B, WNT7B, WNT11, GDF11, FGF11, FGFR3, MAP2K7, FKBP5, FKBP10, TGFB2, MSX2	HOXD4, WNT9A, FGFR3, TGFB2, TNIK, EGR2, CHRDL1
miR-5095	WNT7A, SOX7, SOX17	HOXD12, WNT7B, GDF11, FGF2, SMAD5, ROCK2, FKBP5, CSF3, CAMSAP1, TGFB1	SOX5, FGFR1OP, FGF2, COL9A3, COL2A1, HS2ST1, DSEL, CHRDL1, TGFB1
miR-1226-3p	HOXA4, HOXA11, HOXB9, HOXA13, WNT3, WNT7A, SOX11, SOX7	HOXD12, WNT11, GDF11, FGF2, CALCRL, STAT5A, ATF2	HOXD11, WNT9A, SOX5, SOX6, GDF5, FGF2, RUNX1, FGFR3, SMAD3, TNIK, HDAC4, HDAC5, DSEL
let-7f-5p	HOXA1, HOXB1, HOXA9, GDF6	FGF11, COL1A2, COL1A1, TGFB1	WNT9A, SOX6, SMAD2, TGFB1, TNIK, HS2ST1
miR-493-5p	WNT5A, SOX1	FGF2	SOX5, TNIK, PAPLN
miR-128-3p	HOXA13, HOXA9, SOX7, SOX11, GDF6,	GDF11, CBF-B, SMAD5, MAP2K7, TGFB1	RUNX1, SMAD2, FKBP14, TNIK, HDAC4
miR-5006-5p	HOXB1, HOXC13, WNT3, WNT7A, GDF6, SYNPO2	HOXD12, WNT7B, WNT4, GDF11, RUNX2, CBF-B, FGF2, SPOCK2, ROCK2, ZDHHC7, FKBP5, MSX2	SOX9, FGFR3, FGFR1OP, SMAD3, SMAD2, FKBP14, TNIK, HDAC4, DSEL, CHDRL1

Genes Searched (for reference)

Stem cell target miRNA: HOX, WNT, SOX, GDF, OCT, NANOG, SYNPO2, HoxC8

Osteogenic: RUNX2, Cbf-beta, FGF, COL1, HOXC8, SMAD, SPOCK2, CALCRL, FAK, ROCK2, MAP2K7, STAT5A, ZDHHC7, ATF2, FKBP5, CSF3, CAMSAP1, TGF-beta, RSK2, MSX2

Chondrogenic: RUNX1, FGFR1OP, TNIK, EGR2, SERPINC1, HDAC4, HS2ST1, DSEL, PAPLN, CHRDL1, L-Sox5, Sox6, Sox9, COL2, HDAC5, TGF-beta

Table.4.

List of published function of identify miRNAs that had previously been associated with changes in response to oxidative stress, inflammation, BMSC differentiation and senescence

miRNA	Biological Function
miR-362-5p	Up-regulated in non-small cell lung carcinoma, and targets Sema2A (downregulated), increasing cell invasion and migration (Luo, et al., 2018). Targets (PI3K)-C2P in neuroblastoma cells, decreasing proliferation (Wu, et al., 2015).
miR-1281	Upregulated in response to ER stress through interactions with p53, and enhances ER-induced apoptosis in osteosarcoma cells. Inhibits USP39 expression in osteosarcoma cells (Jiang, et al., 2018).
miR-330-3p	Targets SOD2 and MMP9 and decreases expression, which act on T-cell differentiation and NK cell cytotoxicity, respectively (Petty, et al., 2016). Overexpression inhibits SPHK1 and S1PR1 in gastric cancer, decreasing cancer cell proliferation (Wang, et al., 2018). Expression decreased in osteosarcoma cell lines, and decreased proliferation through Bmi -1 regulation (Zheng, et al., 2018).
miR-210-3p	Responds to HIF, and upregulated in hypoxia (termed hypoxamir), controlling multiple downstream targets in states of hypoxia. Downstream targets include E2F3, CASP8AP2, HOXA1, APC, and others involved in proliferation (Chan and Loscalzo, 2010). Important in maintaining cell cycle progression under hypoxic conditions through targeting BARD1 (Dai, et al., 2019).
miR-615-5p	Tumor suppressor role, and able to target directly to certain oncogenes, including IGF2, AKT2, and SHMT2 (Dong, et al., 2017; Gao, et al., 2015).
miR-5095	Inhibition involved in the progression of multiple cancers (NSCLC, cholangiocarcinoma, glioblastoma, hepatocellular carcinoma), leading to cell proliferation and migration due to downstream interactions with beta-catenin, MBD2, and additional pathways (Hu, et al., 2019; Ren, et al., 2018; Zhang, et al., 2018). Inhibited by long-noncoding RNA LINC01296 in NSCLC and cholangiocarcinoma. Targets MYCN downstream (Hu, et al., 2019; Zhang, et al., 2018).
miR-1226-3p	Up-regulation increases reactive oxygen species, and induces cell death, acting as a tumor suppressor through interactions with MUC1 (Jin, et al., 2010).
let-7f-5p	Important role in MSC differentiation. Decreases apoptosis in MSCs, and targets caspase-3 downstream (Han, et al., 2018). Upregulation inhibits pro-apoptotic proteins including TP53 and caspase-3 (Tie, et al., 2018).
miR-493-5p	Decreased expression in aging skeletal muscle, with an increase in target FGB (component of fibrinogen) expression (Chen, et al., 2018). Targets ITGB1 expression, which when increased improves prognosis in NSCLC (Liang, et al., 2017). Downregulation associated with prostate cancer progression and targets c-Met, CREB1, and EGFR (Wang, et al., 2017). Exerts tumor suppressive role in osteosarcoma by targeting KLF5 (Zhang, et al., 2019).
miR-128-3p	Up-regulation suppresses inhibitors of β -catenin and TGF- β signaling pathways, increasing tumorigenicity (Cai, et al., 2017). Down-regulation decreases NF- κ B signaling activity through upregulation of TNFAIP3, and decreases the inflammatory response in RA (Xia, et al., 2018). Involved in TGF β signaling in breast cancer and NSCLC (Cai, et al., 2017; Breunig, et al., 2018).