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The miR-181 Family: Wide-Ranging Pathophysiological Effects on Cell Fate and Function

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Abstract

MicroRNAs (miRNAs) are epigenetic regulators that can target and inhibit translation of multiple mRNAs within a given cell type. As such, a number of different pathways and networks may be modulated as a result. In fact, miRNAs are known to regulate many cellular processes including differentiation, proliferation, inflammation and metabolism. This review focuses on the miR-181 family and provides information from the published literature on the role of miR-181 homologs in regulating a range of activities in different cell types and tissues. Of note, we have not included details on miR-181 expression and function in the context of cancer since this is a broad topic area requiring independent review. Instead, we have focused on describing the function and mechanism of miR-181 family members on differentiation toward a number of cell lineages in various non-neoplastic conditions (e.g. immune/hematopoietic cells, osteoblasts, osteoclasts, chondrocytes, adipocytes). We have also provided information on how modulation of miR-181 homologs can have positive effects on disease states such as cardiac abnormalities, pulmonary arterial hypertension, thrombosis, osteoarthritis and vascular inflammation. In this context, we have used some examples of FDA-approved drugs that modulate miR-181 expression. We conclude by discussing some common mechanisms by which miR-181 homologs appear to regulate a number of different cellular processes and how targeting specific miR-181 family members may lead to attractive therapeutic approaches to treat a number of human disease or repair conditions, including those associated with the aging process.

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1. MicroRNAs

Non-coding microRNAs (miRNAs) are epigenetic regulators that function at the post-transcriptional level to suppress protein production. One of the earliest reports on the existence of miRNAs was the identification of let-7 in the nematode *Caenorhabditis elegans* (Lee, 1993). Since then, a PubMed search for "miRNA" shows over 140,000 publications to date, thus highlighting how much the field has grown exponentially over the years. An excellent tool for the miRNA community is miRBase, a public repository and online resource for miRNA sequences and annotations (http://mirbase.org). The latest release of miRBase (v22.1) contains miRNA sequences from 271 organisms: 48,860 mature miRNA sequences and 38,589 precursor miRNA hairpin sequences (Kozomara, 2019). Unlike long non-coding RNAs, many miRNAs are phylogenetically conserved (Ibáñez-Ventoso, 2008), thus permitting studies in a number of different organisms as a means to understand the role of human miRNAs in tissue development, homeostasis and disease.

Many miRNA genes are transcribed as independent transcriptional units (i.e. intergenic localization), having their own promoter and other regulatory elements. Alternatively, genes encoding miRNAs can also be found within introns of protein coding genes (i.e. intragenic). In this case, miRNA expression may be regulated by the host gene promoter or via its own independent promoter within the transcriptional unit (Pepe, 2017; Veronese, 2011). In addition, genes encoding miRNAs can also exist in clusters, meaning a set of two or more miRNAs that are transcribed from physically adjacent miRNA genes. Three additional criteria are required to properly define a miRNA cluster: miRNAs should be transcribed in the same orientation, and are not separated by a transcriptional unit or a miRNA in the opposite direction. With respect to distance limits within a miRNA cluster, miRBase has set 10 kilobases (kb) as the default parameter. As will be described further, miR-181 family homologs exist in clusters.

With respect to biosynthesis and function (Figure 1), miRNA genes are generally transcribed by RNA polymerase II as large primary miRNA (pri-miRNAs) transcripts ranging in length from several hundred to thousands of nucleotides (nt) in length. In the case of clustered miRNAs, a large primary polycistronic transcript is formed. These pri-miRNA transcripts are then processed in the nucleus by a Drosha-containing complex to form precursor (pre) miRNAs (~60–150 nt in length) which are then shuttled to the cytoplasm via the RNA binding protein, Exportin 5. Further processing of these hairpin pre-miRNAs takes place in the cytosol by the endonuclease, Dicer, forming mature miRNA duplexes ranging from ~19–24 nt. Following separation of the duplex by helicases, one of the RNA strands (the functional 5p or 3p strand) will commonly enter the RNA induced silencing complex (RISC) and interact with an Ago protein as well as bind, via its seed sequence, to a complementary sequence within the 3'-untranslated sequence (UTR) of a target mRNA. As a result, degradation of the target mRNA, or inhibition of translation commonly occurs. In

rare cases, miRNAs have been shown to enhance target gene expression via non-canonical mechanisms (Zhang, 2014). A recent study involving metabolic labelling of hundreds of functional miRNAs in murine cells revealed a median half-life ranging from 11 h to 34 h, which is longer than the typical half-life of mRNAs (Kingston, 2019). Half-lives for individual miRNAs were also found to vary between cell types, suggesting that cell-specific factors play a role in regulating miRNA stability. Although miRNAs account for only 1–5% of the human genome (Berezikov, 2005), up to 60% of protein-coding genes can be regulated by miRNAs (Friedman, 2009).

Levels of protein suppression induced by miRNAs are generally modest compared to siRNAs. However, unlike siRNAs, miRNAs have the potential to target tens to hundreds of mRNAs within a given cell type resulting in modulation of many cellular pathways and networks. This feature, in addition to their small size and high conservation between species, renders miRNAs as attractive therapeutic targets (Diener, 2022). A Phase 2 clinical trial was recently completed to test the effects of a miR-29 mimic drug (MRG-201; remlarsen) keloid prevention (Gallant-Behm, 2019). In addition, Phase 1 and 1b clinical trials have been carried out to test the effects of a miR-92a antagomir (MRG-110) in wound healing (Abplanalp, 2020) and a miR-21 antagomir (CDR132L) to treat the effects of heart failure (Täubel, 2021). Ongoing trials feature anti-miR-21 (RG-012; lademirsen) for Alport syndrome (Phase II) (Rubel, 2022) and an artificial engineered miR (AMT-130) for Huntington's Disease (Phase I/II) (Rodrigues, 2020). In addition, a vast number of active and recruiting studies on http://clinicaltrials.gov/ include outcome measures for a range of miRNAs in various human diseases. Clinical studies for additional miRNAs, such as anti-miR-122 drug miravirsen and miR-34 mimic drug MRX34, have recently been halted due to limited efficacy and immune-related patient deaths, respectively. Findings from these clinical studies may lead to identification of new miRNA therapeutic targets and disease biomarkers. As will be discussed more in the following sections, members of the miR-181 family have been shown to regulate many cellular processes including cell differentiation, metabolism and growth factor signaling. As such, targeting miR-181 homologs could be an attractive therapeutic strategy to treat specific disease states. Of note, this review will not cover miR-181 expression and function in cancer since this is a broad area of research and there have been some recent review articles on this topic (Braicu, 2019; Feng, 2018; Gu, 2018; Indrieri, 2020; Yang, 2022).

2. The miR-181 family

Advances in understanding miRNA biology in addition to the increasing availability of diverse sequenced genomes have revealed that miRNAs were present at the dawn of metazoan evolution (Grimson, 2008; Wheeler, 2009). A number of reports have proposed that the expansion of miRNA genes in higher organisms is an important causal factor regulating increased vertebrate complexity (Berezikov, 2005; Simkin, 2020; Wheeler, 2009). A miRNA family is a group of miRNAs that are derived from a common ancestor. Normally, members of a miRNA family are functionally similar but are not necessarily conserved in primary sequence of secondary structure.

The miR-181 gene family is an ancient one that originated in the invertebrate urochordata species (Yang, 2014). Phylogenetic analysis of the vertebrate miR-181 family suggests that expansion of this gene family formed through gene duplications, including whole genome duplications and segmental duplications of the common ancestor gene (Yang, 2014). There are six mature miRNAs expressed in the vertebrate miR-181 family – miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c and miR-181d (Figure 2). They are encoded for by three independent paralog sequences located on three separate chromosomes (Ji, 2009). All four sets of mature miRNAs (miR-181a, miR-181b, miR-181c and miR-181d) share the same "seed" sequence "ACAUUCA". In humans, three paralog precursor transcripts are found on Chromosome (Chr)-1 (miR-181a-1 and miR-181b-1), on Chr-9 (miR-181a-2 and miR-181b-2), and on Chr-19 (miR-181c and miR-181d). However, in mouse, the three paralogs are on Chr-1 (miR-181a-1 and miR-181b-1), on Chr-2 (miR-181a-2 and miR-181b-2), and Chr-8 (miR-181c and miR-181d). The human and mouse miR-181-a/b-1 cluster is found in an intron of a non-coding RNA host gene (MIR181A1-HG), while the miR-181-a/b-2 cluster is found in an intron of the NR6A1 gene, and the miR-181-c/d cluster is found in an uncharacterized sequence on either Chr-19 (human/rat/monkey) or Chr-8 (mouse) (Das, 2017a).

Mice lacking genes encoding each of the miR-181 clusters have been generated (Fragoso, 2012; Henao-Mejia, 2013). Interestingly, a striking dose-dependent reduction in size, body weight and viability was observed in mice lacking multiple miR-181 alleles (Williams, 2013). Triple knockout mice lacking all miR-181 clusters have never been obtained due to embryonic lethality. These mouse genetic studies points to a critical role for miR-181 family members in regulating processes controlling growth and/or development. The following sections will focus on reported roles of miR-181 homologs in regulating cell fate and function in a range of different biological systems.

3. Modulation of hematopoiesis by miR-181 homologs

One of the earliest published reports on miR-181 was by Bartel's group who investigated miRNA expression in murine hematopoietic cells, an approach that likely detected all miR-181 homologs (Chen, 2004). This study showed very high expression of miR-181 in the thymus that contains mainly T lymphocytes. High expression was also found in the brain and lung with lower levels detected in bone marrow, muscle and spleen. Within mouse bone marrow, miR-181 was detectable in undifferentiated progenitor (Lin^{-}) cells and specifically upregulated in differentiated B lymphocytes. Ectopic over-expression of miR-181 in progenitor murine bone marrow cells in vivo supported in vitro findings whereby a doubling of B lymphoid lineage cells (CD19+) was found in addition to a substantial decrease in CD8+ T cells. Overall, these studies highlighted that miR-181 homologs play a role in regulating normal hematopoietic lineage differentiation.

The study by Liu *et al* reported that the activities of pre-mir-181a-1 and pre-mir-181c differ in the regulation of early T cell development (Liu, 2008). They showed that ectopic expression of pre-mir-181a-1 in thymic progenitor cells promoted CD4+ and CD8+ double positive T cell development. However, over-expression of pre-mir-181c did not have such enhancing effects. Of note, the previously mentioned study by Chen *et al* (Chen, 2004)

found that over-expression of the mir-181 gene (amplified from chromosome 1, hence the mir-181a-1 homolog) promoted B cell production and decreased CD8+ T cell numbers in murine bone marrow. The differences in functionality reported for miR-181a-1 are likely due to the different cell types / tissue location where the miRNA was ectopically over-expressed (i.e. thymic progenitors versus bone marrow cells). Different subsets of target mRNAs will undoubtedly be expressed in thymic progenitors when compared to bone marrow cells, thereby resulting in varying functional outcomes. Also of interest in this study by Liu *et al* was the finding that nucleotides within the stem loop of pre-mir-181a-1 and pre-mir-181c appeared to explain why only the former could enhance T cell differentiation in vitro while the latter could not. One would expect similar functions given that both homologs share the same seed sequence. It was subsequently proposed in this paper that differences in the stem loop nucleotide sequence may influence a number of factors such as pri-and pre-miRNA processing as well as transport/subcellular localization of pre-miRNAs. Reports in other systems also suggest that the sequence of pre-miRNAs can influence loading of mature miRNAs in specific RISCs which would subsequently result in different efficiencies of gene silencing as well as different subsets of target mRNAs prone to miRNA-mediated suppression (Förstemann, 2007; Steiner, 2007).

An additional role for the miR-181a/b cluster in regulating human natural killer (NK) cell development was reported by Cichocki *et al* (Cichocki, 2011). It is not clear if pre-miRNAs from the 181a/b-1 or 181a/b-2 cluster were over-expressed in CD34+ hematopoietic stem cells or if only the mature miR-181a/b miRNAs were ectopically expressed. Regardless, they reported that miR-181a/b promoted NK cell development *in vitro*, in part, by targeting nemo-like kinase (NLK), thereby enhancing Notch signaling. Further evidence suggesting a role for the miR-181 family in regulating hematopoietic cell differentiation was reported by Su *et al* who showed that miR-181a inhibited granulocyte and macrophage-like differentiation of CD34+ hematopoietic progenitor cells by targeting and downregulating expression of PRKCD, CTDSPL and CAMKK1 (Su, 2015). Another study reported that miR-181a over-expression in CD34+ hematopoietic progenitors, promoted megakaryocyte (MK) differentiation (Li, 2012b). The major mechanism by which miR-181a induced these effects was due to targeting and suppressing the RNA binding protein, Lin28, thereby disturbing the Lin28 / let-7 reciprocal regulatory loop. As discussed by the authors, this regulatory loop may likely be important in the context of controlling cell differentiation in general.

Further evidence to support a role for miR-181a/b in immune cell fate was demonstrated by Henao-Mejia *et al* showing a complete absence of NKT cells in the thymus and periphery of mice lacking the miR-181a/b-1 cluster (Henao-Mejia, 2013). This is a rare example of how the absence of a single miRNA cluster can result in the loss of an entire cell lineage. In addition, these mice also displayed defects in T cell development and early B cell development. The major mechanism driving these immune cell defects was found to be due to increased levels of the miR-181a/b target gene, phosphatase and tensin homolog (PTEN), that primarily functions to inhibit the PI3K/AKT pathway. Subsequently, miR-181a/b-1 null mice have reduced PI3K/AKT signaling which led to dysregulation of thousands of genes most likely because the PI3K/AKT pathway regulates many important processes in the cell including metabolism, proliferation, differentiation and autophagy. Specifically, it was found

that significant metabolic reprogramming due to loss of miR-181a/b-1 was a major factor causing the defects in immune cell function and differentiation. It is also interesting that these immune cell defects were not found in mice devoid of miR-181a/b-2 or miR-181c/d, even though the mature miRNAs from each family member contain the same seed sequence. This suggests that distinct processes may regulate transcriptional activation of each of the miR-181 gene clusters and/or that other non-seed sequence nucleotides are important in mRNA targeting. For more detailed information on miR-181-mediated effects on T cell function and differentiation, the reader is referred to recent review articles (Grewers, 2020; Kim, 2021).

4. Expression and function in skeletal cell differentiation

As mentioned in Section 2, transgenic mice devoid of each miR-181 cluster have been generated and a dose-dependent reduction in body size was shown following deletion of multiple miR-181 alleles (Henao-Mejia, 2013). This suggests a potential role for miR-181 family members in regulating skeletal development and homeostasis. Cartilage and bone tissue are the principal elements of the skeleton. Chondrocytes are the cells present in growth plate and articular cartilage that generate a type II collagen and proteoglycan-rich extracellular matrix (ECM). Osteoblasts are bone-forming cells that synthesize an ECM rich in mineral and type I collagen while osteoclasts function to resorb bone. The activity of the bone anabolic osteoblasts and bone resorbing osteoclasts regulate bone turnover and maintain a healthy homeostasis.

Two studies by Bakhshandeh *et al* reported miRNA signatures associated with *in vitro* chondrogenesis (Bakhshandeh, 2012b) or osteogenesis (Bakhshandeh, 2012a) of unrestricted somatic stem cells (USSCs) from human placental cord blood. In both studies, miR-181a (the a1 homolog derived from chromosome 1) expression increased at day 21 of chondrocyte and osteoblast differentiation. An increase in miR-181 paralog expression was also found during osteogenic differentiation of a murine cell line as well during development of murine tibia and calvaria (Bhushan, 2013). Studies from the McAlinden group reported miRNA expression patterns in chondrocytes from distinct regions of human embryonic growth plate cartilage at a time point prior to endochondral bone formation (McAlinden, 2013). Of the miRNAs that were found to be differentially-expressed between regions containing progenitor, differentiated or hypertrophic chondrocytes, levels of miR-181a-1 and miR-181a-2 showed higher expression in the hypertrophic chondrocyte zone. Gabler *et al* (Gabler, 2015) and Lv *et al* (Lv, 2020b) described increases in miR-181 expression at days 14, 21, and 42 of bone marrow-derived MSC chondrogenesis and an association with enhanced hypertrophic cartilage formation, supporting findings from the McAlinden group. Two studies reported increased levels of miR-181a during bone marrow MSC chondrogenesis although a correlation with enhancing hypertrophy was not discussed (Barter, 2015; Vail, 2022). More recently, Melnik *et al* identified *R-Spondin 2* (*RSPO2*) as a key target for miR-181a in regulating MSC hypertrophy (Melnik, 2021).

The study by Bhushan *et al* showed that over-expression of miR-181a via mimic transfection of a murine osteo-progenitor cell line or primary calvarial osteoblasts promoted osteogenic differentiation (Bhushan, 2013). They showed that one of the mechanisms by which

miR-181a increased osteogenesis was by inhibiting TGF-β signaling via targeting *Tgfβi* (Tgf-beta induced) and *TβR-I/Alk5* (TGFβ-type I receptor). More recently, studies in the McAlinden group showed that both miR-181a and miR-181b expression increased during osteogenic induction of human skeletal progenitor cells and that over-expression of the miR-181a/b-1 cluster enhanced osteogenic differentiation (Zheng, 2019). Mechanistically, this study revealed that miR-181a/b-1 over-expression resulted in suppression of the target mRNA, PTEN, a subsequent increase in the PI3K/AKT signaling cascade and enhanced mitochondrial respiration. A similar mechanism was reported in studies from an independent group (Lv, 2020a). More recently, another study reported a pro-osteoblast function for miR-181a/b-1 (Qi, 2021) while over-expression of miR-181a alone was found to promote osteogenesis by targeting *PBX1* (Liu, 2020). Additionally, it was reported that miR-181c can enhance murine osteoblast differentiation and its expression was inversely correlated with human postmenopausal bone disease (Ma, 2020). There are limited and contradictory findings on the function of miR-181d on osteogenesis: one study demonstrated an inhibitory effect on osteogenic induction of human bone marrow-derived MSCs (BMSCs), in part, by targeting SMAD3 (Xie, 2018), while another group showed osteogenic enhancing function in BMSCs (Liu, 2021).

With regards to osteoclasts, miR-181a mimic treatment was found to inhibit osteoclastogenesis in vitro (Fu, 2021). However, a contradictory result was published whereby miR-181a over-expression resulted in enhancement of osteoclastogenesis in vitro, in part, by targeting DUSP6 (Zhang, 2021). In other studies, anti-osteoclastogenic function was reported following over-expression of miR-181b (Han, 2020) and miR-181c (Yu, 2021). For miR-181d, one report described mimic-induced enhancement of osteoclast differentiation via targeting and suppression of OPG (Sun, 2019).

Taken together, while the majority of reports point to a role for miR-181 homologs in enhancing chondrocyte and osteoblast differentiation, further studies are needed to resolve some of the contradictory functions reported on the role of specific family members on osteogenesis and osteoclast development.

5. Regulation of skeletal tissue homeostasis

With respect to post-natal cartilage tissue, published reports point to an anti-anabolic role for miR-181 in mature articular chondrocytes by either suppression of pro-anabolic factors (Sumiyoshi, 2013) or promotion of apoptosis and pro-catabolic factors, in part, by targeting PTEN (Wu, 2017). Song *et al* reported higher levels of miR-181b in mouse articular cartilage following induction of osteoarthritis (OA) by joint destabilization surgery (Song, 2013). They subsequently showed that intra-articular injection of antagomir-181b attenuated the effects of OA. Similarly, Nakamura *et al* found higher levels of miR-181a in OA facet joint cartilage compared to control specimens and showed that injection of miR-181a mimics into the facet joints of rats induced cartilage degradation (Nakamura, 2016). Follow up studies by this group revealed that antagomir-181a attenuated OA symptoms induced within the facet joints of rats or knee joints of mice (Nakamura, 2019). More recently, antagomir-181a antisense oligonucleotides were shown to dampen cartilage breakdown in a

mouse model of temporomandibular joint OA due, in part, to increased levels of its target gene *Sirt1* (Qi, 2022).

In human chondrocytes, de Palma *et al* identified that miR-181a is upregulated in osteoarthritic cartilage chondrocytes but decreased when loaded with hydrostatic pressure (De Palma, 2018). Min *et al* identified the *SBP2* (Selenocysteine Insertion Sequence Binding Protein 2) as a key target of miR-181a in the regulation of cartilage homeostasis (Min, 2018). Xue *et al* showed that miR-181a upregulation in osteoarthritic cartilage was localized to chondrocytes in damaged cartilage when compared to chondrocytes from smooth regions of the same tissue (Xue, 2018). They also showed that miR-181a can alter the chondrocyte response to oxidative stress, rendering these cells more susceptible to the damaging effects of reactive oxygen species (Xue, 2018). In addition, Cheleschi *et al* identified miR-181a as a mediator of apoptosis and oxidative stress in OA chondrocytes via regulating the NF-kB pathway (Cheleschi, 2019). Zeng *et al* showed that miR-181a over-expression enhanced apoptosis in human OA chondrocytes and that it could directly target *OPN (Zeng, 2022)* while another study found that miR-181c could also target *OPN* and reduce synoviocyte proliferation and catabolic gene expression, which would be advantageous in the context of OA (Wang, 2017). Interestingly, a recent study revealed that miR-181c, via targeting *SMAD7*, could enhance chondrogenesis as well as ectopic cartilage production in mice. The authors conclude that miR-181c enhanced cartilage repair, yet the ex vivo mouse model used in their study does not represent a cartilage injury model (Zhang, 2022). It remains to be determined how miR-181c functions in the context of regulating cartilage repair or OA.

On the subject of bone repair / homeostasis, levels of miR-181a were found to be significantly upregulated in fracture callus repair tissue 14 days post-fracture in rats (Waki, 2016) while serum levels of miR-181a were found to be decreased in patients with delayed tibial fracture healing compared to control patients with normal healing (Guo, 2022). Circulating levels of miR-181c were reported to be downregulated in the serum of postmenopausal women with osteopenia or osteoporosis, yet increased in patients treated with bisphosphonates (Ma, 2020). This study also showed that miR-181c could promote differentiation and mineralization of osteoblasts in vitro. Another study suggested that miR-181a may induce bone loss, although this was not tested directly in an *in vivo* model (Zhang, 2021). Other studies have demonstrated that one mechanism by which estrogen preserves bone mass is by decreasing miR-181 in BMMSCs to control osteoclast differentiation (Shao, 2018; Shao, 2015). While in vitro analyses of miR-181 function in regulating bone cell differentiation are informative, the effects of these miRNA homologs on regulating bone turnover and repair is more complex given the multiple cell types involved in these processes

6. Regulation of myogenesis and adipogenesis

The published literature informs us that miR-181 family members can also regulate differentiation of other cell types as well. There have been many reports on effects of miRNAs on skeletal muscle cell differentiation and, among these, miR-181 homologs have been shown to increase during myogenesis and promote differentiation (Wang, 2018). Specifically, one study showed that miR-181, by targeting the homeobox protein

(Hox-A11; a repressor of the differentiation process), functions in establishing the muscle cell phenotype (Naguibneva, 2006). It has also been reported that miR-181a has positive synergistic effects on myogenic differentiation, in part, by targeting a glucose-regulated protein (GRP78/BIP) to activate ER stress-mediated apoptosis (Wei, 2016). A pro-myogenic function for miR-181a was reported in chicken myoblasts (Yuan, 2022) as well as sheep skeletal muscle satellite cells, in part by targeting YAP1 (He, 2022). Even in fish, miR-181b was demonstrated to enhance muscle growth partly due to potential targeting of myostatin b (Zhao, 2019). To add another level of complexity, Wang et al reported that the long non-coding RNA (lncRNA), lncDLEU2, functions as a miR-181a sponge to inhibit muscle differentiation. By inhibiting miR-181a activity, levels of the miR-181a target, SEPP1, were found to increase which further explained how myogenic differentiation was suppressed by this lncRNA (Wang, 2020).

With respect to adipogenesis, transcriptomic analysis revealed that expression levels of miR-181a and miR-181c functional strands significantly increased during in vitro adipogenic differentiation (Hou, 2018). In other studies, miR-181a over-expression has been reported to enhance adipogenesis, in part, via targeting TNF-α (Li, 2013) or by targeting and suppressing Smad7 and Tcf7l2 (Ouyang, 2016). Porcine pre-adipocyte differentiation was found to be enhanced by miR-181a over-expression and TGFBR1 was identified as a target gene in this study (Zhang, 2019). A more recent study demonstrated that miR-181a enhanced adipogenesis of immortalized bone marrow-derived stromal cells (iBMSCs) via directly targeting period circadian regulator 3 (PER3) which subsequently increased baseline expression of the pro-adipogenic transcription factor, PPARγ (Knarr, 2019). Another study identified the matrix associated metalloproteinase (Adamts1) as a negative regulator of the adipocyte lineage as well as a target of miR-181d, thereby suggesting a pro-adipogenic function for this miR-181 family member as well (Chen, 2016a). However, contrary to these findings, one report has shown that over-expression of miR-181b suppressed adipogenesis and that IRS2 was a potential target gene regulating this function (Chen, 2016b).

7. Functional roles in the context of obesity

While not directly related to adipocyte differentiation, multiple miRNAs have been validated to regulate fatty acid metabolism, insulin sensitivity and dysregulate metabolic homeostasis that contribute to the development of obesity (Dávalos, 2011; Trajkovski, 2011). High-fat fed mice shows an activation of miR-181 in adipocytes, mainly in white adipose tissue (WAT), that ultimately leads to adipocyte proliferation (Virtue, 2019). The authors further investigate that in response to the high-fat diet, the gut microbiota produces tryptophan-derived metabolites that activate miR-181 transcription in adipocytes. In fact, in obese human children the miR-181 expression in WAT and the elevated circulating tryptophan-derived metabolites confirmed the role of miR-181 for the development of obesity, insulin resistance and WAT inflammation (Virtue, 2019). Another study reported that miR-181a, via targeting the TCA cycle metabolic enzyme, isocitrate dehydrogenase I (IDH1), inhibited lipid accumulation while transgenic mice engineered to over-express miR-181a exhibited decreased body weight under high fat diet conditions compared to control littermates (Chu, 2015). However, this study did not reveal if the primary, precursor or mature miR-181a was cloned into the targeting vector for generation of these miR-181a transgenic mice.

Also, data demonstrating successful over-expression of miR-181a in various tissues of these animals was not shown. In hepatocytes, miR-181c regulates lipid biosynthesis by targeting IDH1. In vivo, using an adeno-associated virus (serotype 8; AAV-8) over-expression of miR-181c shown to offer beneficial effect against diet-induced obesity by inhibiting WAT synthesis (Akiyoshi, 2021). In another study, lipid accumulation was found to stimulate miR-181c transcription in cardiomyocytes (Roman, 2020). The authors demonstrated that over-expression of miR-181c due to high-fat diet can lead to cardiac dysfunction during obesity by translocating into the mitochondrial compartment. Implementing knock down approach using antagomir-181c or using miR-181c knock-out mice, the cardioprotective effect during obesity has been documented (Roman, 2020). Hence, miR-181 may consider a potential future therapeutic option against obesity and obesity related human health condition.

8. Modulation of cardiac function

Apart from miR-181d (Belkaya, 2014; Das, 2012), all other miR-181 family members are expressed in the heart. RNA sequencing data from human cardiac cells shows that, of the miR-181 family, miR-181a is the most abundant in heart tissue. Therefore, most of the literature on cardiac function is focused on miR-181a and/or its clustered miRNA, miR-181b. It has been estimated that in the mouse heart, 60% of miR-181a/b is from Chr 1 (miR-181a/b-1 cluster), while the rest is derived from Chr 2 (miR-181a/b-2 cluster) (Das, 2017a). In studying both miR-181a/b-1 and miR-181c/d knock-out mouse models, it has been documented that over-expression of miR-181a/b plays a cardioprotective role; whereas, over-expression of miR-181c can cause cardiac damage (Das, 2017a). Several studies have elucidated that the downregulation of mRNA targets, such as Akt3 (Yuan, 2019), PI3KR3 (Yuan, 2019), GLP-1 (Sassoon, 2016), Hox-A11 (Li, 2009), PTEN (Das, 2017a), acts as a possible mechanism by which miR-181a/b can protect the myocardium from ischemia/reperfusion (I/R) injury. Another important role of the miR-181 family has been studied in chronic heart failure (CHF) patients. It has been shown that there is significant downregulation of miR-181 in the peripheral blood with aging. This study also correlated the number of B-cells with miR-181 expression. This study concluded that miR-181 may play an important role in immunosenescence in aged CHF patients (Seeger, 2013).

In contrast, it has been demonstrated that miR-181c is encoded for in the nucleus, matures in the cytoplasm and finally translocates into the mitochondrial fraction of cardiomyocytes (Das, 2014; Das, 2012; Das, 2017a). This unique functional mechanism categorized miR-181c as a “mitomiR” (i.e. a miRNA found localized to the mitochondria). Some mitomiRs have been found to translocate into the mitochondrial fraction and bind to the 3’-end of a mitochondrial gene or 3’-UTR of a nuclear mRNA present in the mitochondria (Baradan, 2017; Das, 2017b). There are several unanswered questions regarding why and how miR-181c translocates into the mitochondria in cardiomyocytes. Why does miR-181c translocate to the mitochondria only in cardiomyocytes? The “seed” sequence of miR-181c is homologous to miR-181a/b, and so why does miR-181c not bind to the same target mRNAs as miR-181a/b in the cytoplasm? Is there a hexanucleotide element at the 3’-end (Hwang, 2007) of miR-181c which directs it towards the mitochondrial compartment in cardiomyocytes? Even though there are several theories of how mitochondrial translocation

of a miRNA occurs (Macgregor-Das, 2018), the mechanism by which miR-181c translocates into the mitochondrial compartment in cardiomyocytes remains unknown. A recent study showed that miR-181c acts as an anterograde signal and alters mitochondrial function in the heart, which further regulates mitochondrial retrograde signals through inactivation of a transcription factor, Sp1. Sp1 inactivation transcriptionally inhibits the MICU1 gene that is responsible for mitochondrial Ca^{2+} import. In summary, miR-181c over-expression can cause cardiac damage through mitochondrial Ca^{2+} overload (Banavath, 2019).

9. Preserving the vascular function

Large artery stiffness, which occurs with aging and pathophysiological conditions, is a major and independent risk factor for a wide range of cardiovascular diseases. This includes isolated systolic hypertension, “an endemic condition responsible for a large proportion of the global burden of cardiovascular morbidity and mortality” (Chirinos, 2019). MicroRNAs have emerged as important modifiers of vascular structure and function (Nanoudis, 2017). Hori et. al. (Hori, 2017) was the first to describe the significant down-regulation of miR-181b in the aorta of aging mice. Furthermore, deletion of the miR-181a/b-1 locus results in increased vascular stiffness and associated extracellular matrix remodeling by collagen deposition. Several studies have utilized bioinformatic approaches to identify mRNA targets of miR-181b in the aorta. These include caspase recruitment domain family member 10 (Card10) (Lin, 2016) and importin-α3 (Sun, 2014; Sun, 2012). Endothelial cell activation and dysfunction have been linked to the pathophysiology of several arterial inflammatory diseases. The circulating miR-181b level is significantly lower in patients with sepsis compared with control intensive care unit patients (Sun, 2012). It has been shown that both *in vitro* and *in vivo* over-expression of miR-181b can protect against aortic inflammation by targeting importin-α3 and thereby modulating NF-κB-responsive genes such as adhesion molecules VCAM-1, E-selectin and tissue factor (Sun, 2014; Sun, 2012).

Tissue Factor (TF) is the primary initiator of the extrinsic clotting cascade which, if dysregulated, can lead to cardiovascular complications. It has been shown that miR-181 over-expression can cause lower levels of TF mRNA levels in THP1 cells. *In vivo*, using miR-181 knock-out mice, it has been observed that there is higher TF expression in the heart and aortic tissue. Therefore, it has been proposed that downregulation of miR-181 may contribute to increased thrombogenicity in diabetic patients, and may identify patients at risk for thrombosis (Witkowski, 2020). In another study, the anti-inflammatory role of miR-181b has been demonstrated by its ability to target Card10 mRNA in arterial endothelial cells. Card10 downregulation could alter TNF-α-induced NF-κB activation. Furthermore, systemic delivery of miR-181b has been shown to offer protection against arterial thrombosis (Lin, 2016).

Downregulation of miR-181a has been observed in patients with coronary artery disease (Su, 2019). In animal studies using apoE knock-out mice, it has been found that both strands of miR-181a-5p and miR-181a-3p are downregulated in aortic plaques as well as in the circulation. Moreover, the authors have demonstrated that loss of miR-181a-5p and miR-181a-3p decreases pro-inflammatory gene expression and the infiltration of macrophages and T cells into the mouse aortic lesions. In this study, it has been proposed

that miR-181a-5p and miR-181a-3p prevent endothelial cell activation through blockade of NF-κB signaling by binding to the 3'-UTR of TAB2 and NEMO, respectively (Su, 2019). Endothelial-mesenchymal transition is a very common symptom of endothelial dysfunction, which is involved in the pathogenesis of pulmonary arterial hypertension. In monocrotaline-induced pulmonary arterial hypertension, miR-181b plays an inhibitory role in endothelial-mesenchymal transition by targeting endocan and TGF-βR1 mRNAs (Zhao, 2020).

Vascular smooth muscle cells (VSMCs) play a pivotal role in the pathophysiology of multiple vascular diseases, such as vascular stiffness. Small RNA sequencing study revealed that miR-181a/b-1 cluster expression is one of the top five highly abundant miRNAs in VSMCs (Tuday, 2019). It has been shown that downregulation of miR-181b can cause severe vascular stiffness by activating TGF-β signaling (Hori, 2017). Recently, it has been shown that a novel miRNA-degrading enzyme, the translin/trax (TN/TX) complex, targets a small subpopulation of miRNAs and can degrade pre-miR-181b in VSMCs. Blocking degradation of miR-181b might be a fruitful approach to prevent or reverse vascular stiffness, and Tuday et al. (Tuday, 2019) found that TN knock-out mice, which lack this miRNA-degrading enzyme, exhibit selective elevation of miR-181b levels in the aorta. Strikingly, these mice do not develop increased aortic stiffness following chronic exposure to high-salt water (HSW; 4% NaCl in drinking water for three weeks) (Tuday, 2019). Based on the functional role of miR-181b both in endothelial cells and VSMCs in the aorta, miR-181b over-expression or inhibition of miR-181b degradation is now considered a potential treatment for preserving vascular function.

10. Conclusions and Perspectives

This review has highlighted the wide-ranging effects of miR-181 family members on regulating both differentiation and function of a number of distinct cell types. For example, many studies that over-expressed or inhibited miR-181 homologs have revealed, with a few exceptions, a pro-differentiation role toward a number of cell lineages including osteoblasts, hypertrophic chondrocytes, adipocytes, myoblasts, B cells, NK/NKT cells and megakaryocytes (Figure 3; Table 1). However, there are some examples of miR-181 members having an inhibitory function on differentiation of other cell types such as osteoclasts (Figure 3). This is most likely due to specific cell types expressing distinct populations of mRNAs that can be targeted by miR-181 homologs, thereby inducing different effects.

Many miR-181 target mRNAs have been identified in the context of regulating cell differentiation (Table 1). Of these, PTEN appears to be a common target of miR-181a/b in promoting differentiation of B cells, T cells, NKT cells and osteoblasts. By suppressing PTEN, PI3K/AKT signaling will be enhanced which can lead to altered cellular metabolism thus likely driving differentiation. In the context of miR-181a/b induction of osteoblast as well as adipocyte differentiation, suppression of TGF-β signaling has been identified as a common mechanism (Table 1). It will be interesting to manipulate (over-express) levels of miR-181a/b in vivo as a means to promote bone formation in the context of bone fracture healing. In fact, a positive role for miR-181a has been implicated in fracture healing from a recent study on the effects of a long non-coding RNA that can regulate this miRNA (Guo,

2022). On the other hand, inhibiting miR-181a/b could be a strategy to suppress formation of abnormal heterotopic bone. Local delivery of miR-181a/b in pre-clinical mouse models of endochondral bone fracture or heterotopic ossification is currently being investigated in the McAlinden group. Given the pro-adipogenesis function of miR-181a/b reported in vitro, the levels of adipocytes should be monitored in the context of bone repair as it would not be desirable to induce increased levels of these cells in this scenario.

With respect to other disease states, the therapeutic benefit of over-expressing specific miR-181 homologs has been reported in the context of suppressing arterial thrombosis, atherosclerosis, heart failure, pulmonary arterial hypertension as well as vascular inflammation and stiffness (Table 2). In some of these studies, suppression of PTEN, TGF-β signaling and inflammation are common mechanisms induced by miR-181 homologs, similar to their mechanism in controlling cell differentiation (Table 1). In addition, a positive therapeutic effect of cardiosphere-derived cell (CDC) injections as a treatment for Duchenne muscular dystrophy (DMD) was recently reported in Phase 2 of the HOPE-2 clinical trial (McDonald, 2022). It was suggested that the therapeutic action of CDCs may be due to the contents of their exosomes, including the anti-inflammatory actions of miR-181b and miR-146a. Future studies will be required to examine the potential therapeutic effects of miR-181b over-expression as a treatment for DMD. Interestingly, miR-181c appears to have opposing roles to miR-181a/b homologs in the context of chronic heart failure due to the observation that miR-181c can translocate into the mitochondria and regulate mitochondrial function (Table 2) (Roman, 2020). Another interesting pathological feature about miR-181c is that, in hepatocytes, over-expression of miR-181c prevents diet-induced obesity (Akiyoshi, 2021). However, over-expression of miR-181c leads to cardiac dysfunction through the mitochondrial pathway (Roman, 2020). Therefore, it is imperative to consider off-target effect of a miRNA delivery strategy. Organ/cell-specific delivery of miRNA will be ideal to perform miRNA therapeutics, such as AAV8-miR-181c complex that shows promising results as a liver-specific delivery approach (Akiyoshi, 2021). Even though miR-181 family members share the same seed sequence, it is clear that they may induce different functions depending on the cell type where they are expressed, baseline expression levels as well as their location within the cell.

Finally, there are a number of studies reporting decreased levels of miR-181 family members in the context of aging. For example, levels of specific miR-181 homologs were found to be lower in serum from older individuals (Noren Hooten, 2013), human naïve CD4+ T cells (Li, 2012a), murine NK cells (Lu, 2021), aged muscle (Mercken, 2013) and aged, calorie-restricted brain tissue (Khanna, 2011). It will be interesting in future studies to explore the therapeutic potential of targeting miR-181 homologs as a means to regulate immune cell responses, muscle function, chronic inflammation, bone repair, or other cellular processes that are known to be suppressed with increased age.

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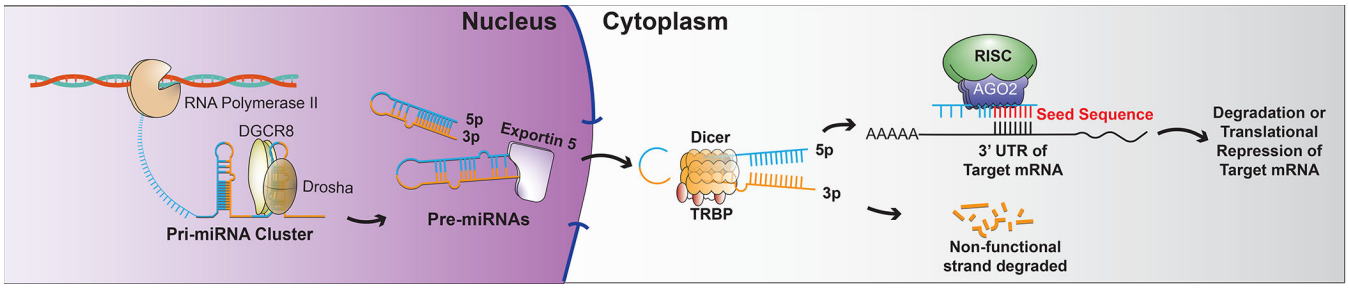

Figure 1. Synthesis and function of microRNAs.

Left panel shows RNA polymerase II-mediated transcription of a clustered microRNA gene in the nucleus resulting in formation of primary (pri) miRNAs which are then processed to precursor (pre) miRNAs and transported to the cytoplasm. Right panel shows processing of pre-miRNAs in the cytoplasm by a Dicer-containing enzyme complex and interaction of the functional (5p) mature miRNA strand, via its seed sequence, with a complementary sequence on the 3'UTR of a target mRNA. This interaction is mediated by the RNA-induced silencing complex (RISC) containing Ago2 protein. The result of this interaction is either mRNA degradation or suppression of mRNA translation.

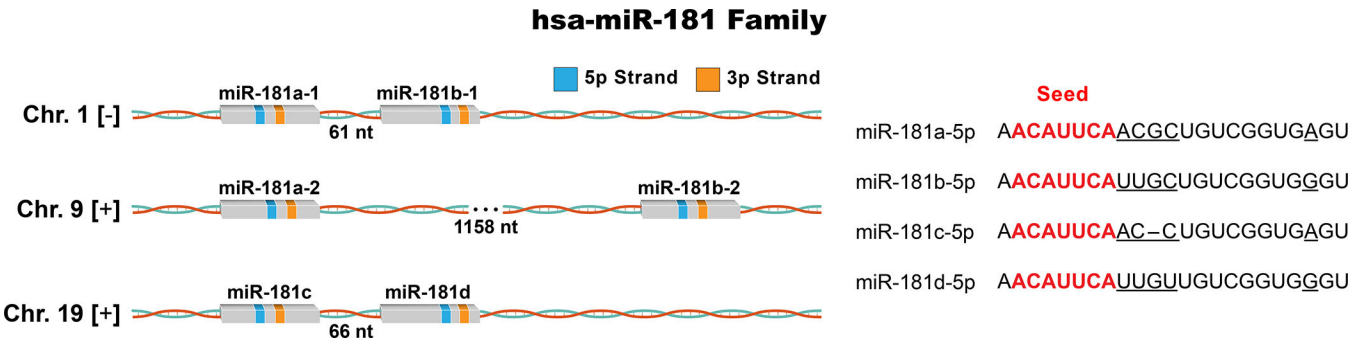

Figure 2. The human miR-181 family.

The human (homo sapiens; hsa) miR-181 family is encoded for by 6 genes shown by the grey boxes located on chromosome (chr) 1, 9 and 19. The blue and orange-boxed areas represent the mature 5p and 3p strand, respectively, within each miR-181 gene. Each chromosome contains two clustered miRNA genes: miR-181a/b-1 (Chr 1), miR-181a/b-2 (Chr 9) and miR-181c/d (Chr 19). The number of nucleotides (nt) separating these clustered miRNA genes is shown. Right panel show the sequence of each mature miR-181 strand. The seed sequence (red font) is conserved between each family member. Underlined regions indicate regions that are not conserved between the family members.

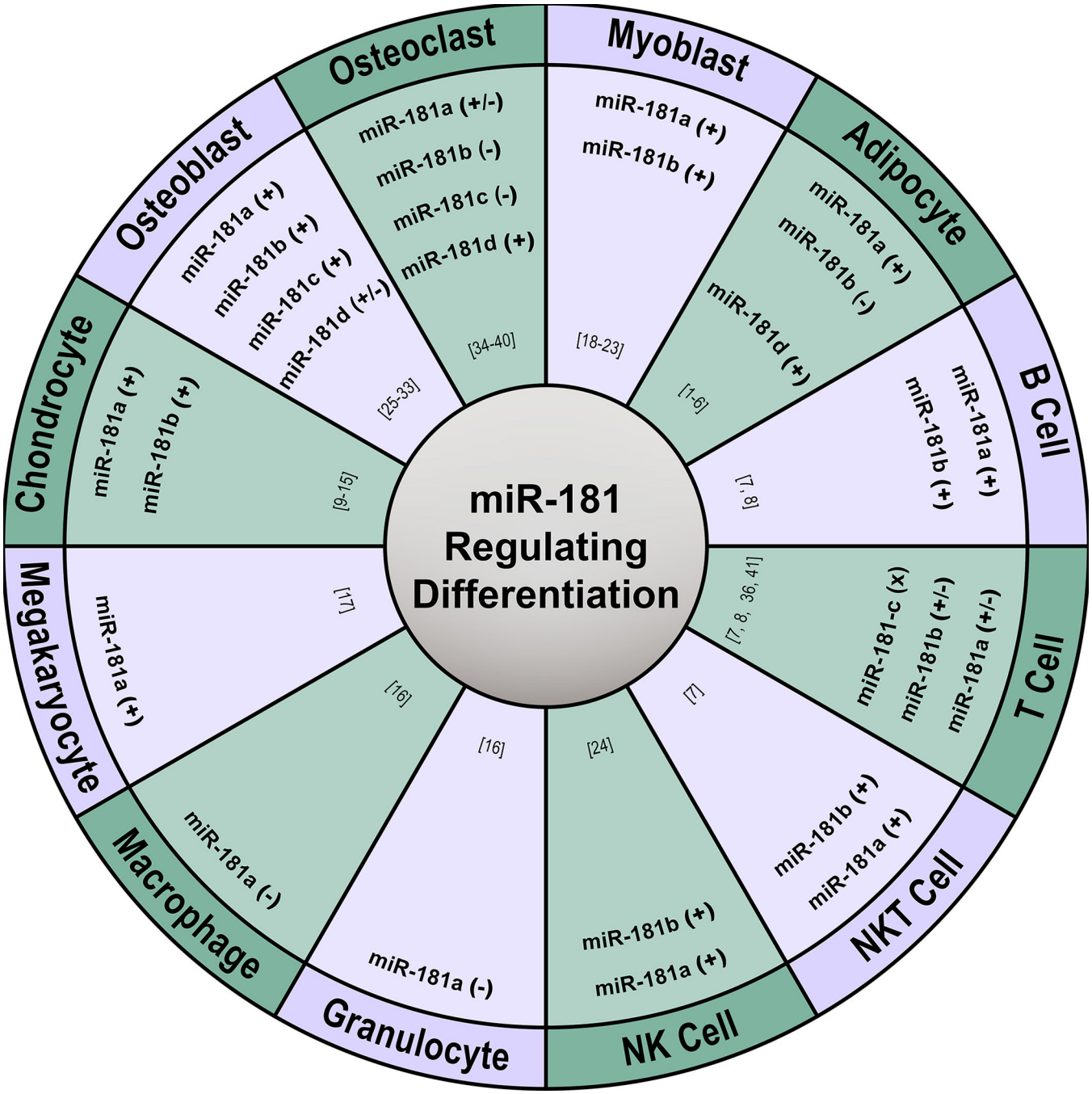

Figure 3. miR-181 regulates differentiation of many cell types.

The published literature informs us that miR-181 homologs can either enhance (+) or suppress (−) differentiation toward various cell lineages. There are also mixed reports (+/−) on whether a specific miR-181 homolog has positive or negative effects on differentiation. Some studies also report no effect (x) of a miR-181 family member on cell differentiation. See Table 1 for information about miR-181 targets in each differentiation lineage. References: [1] (Li, 2013) [2] (Ouyang, 2016) [3] (Zhang, 2019) [4] (Knarr, 2019) [5] (Chen, 2016b) [6] (Chen, 2016a) [7] (Henao-Mejia, 2013) [8] (Chen, 2004)

[9] (Gabler, 2015) [10] (Lv, 2020b) [11] (Barter, 2015) [12] (Vail, 2022) [13] (Melnik, 2021) [14] (Bakhshandeh, 2012b) [15] (Zhang, 2022) [16] (Su, 2015) [17] (Li, 2012b) [18] (Naguibneva, 2006) [19] (Wei, 2016) [20] (Yuan, 2022) [21] (He, 2022) [22] (Wang, 2020) [23] (Zhao, 2019) [24] (Cichocki, 2011) [25] (Bhushan, 2013) [26] (Liu, 2020) [27] (Bakhshandeh, 2012a) [28] (Zheng, 2019) [29] (Qi, 2021) [30] (Lv, 2020a) [31] (Ma, 2020) [32] (Liu, 2021) [33] (Xie, 2018) [34] (Zhang, 2021) [35] (Shao, 2015) [36] (Shao, 2018) [37] (Fu, 2021) [38] (Han, 2020) [39] (Yu, 2021) [40] (Sun, 2019) [41] (Liu, 2008)

Table 1.

Regulation of cell differentiation by miR-181 family members

Cell Type	Homolog	Differentiation	Target(s)	Ref
Adipocyte	a	Enhance	TNF-α	(Li, 2013)
			Smad7, Tcf7l2	(Ouyang, 2016)
			TGFβR1	(Zhang, 2019)
			PER3	(Knarr, 2019)
	b	Suppress	IRS2	(Chen, 2016b)
	d	Enhance	Adamts1	(Chen, 2016a)
B Lymphocyte	a1	Enhance	PTEN	(Henao-Mejia, 2013)
			N/A	(Chen, 2004)
	b1	Enhance	PTEN	(Henao-Mejia, 2013)
			N/A	(Chen, 2004)
Chondrocyte	a	Enhance	N/A	(Gabler, 2015)
			N/A	(Lv, 2020b)
			N/A	(Barter, 2015)
			N/A	(Vail, 2022)
			RSPO2	(Melnik, 2021)
	a1	Enhance	N/A	(Bakhshandeh, 2012b)
	b	Enhance	N/A	(Gabler, 2015)
	c	Enhance	SMAD7	(Zhang, 2022)
Granulocyte	a	Suppress	PRKCD, CTDSPL, CAMKK1	(Su, 2015)
Macrophage	a	Suppress	PRKCD, CTDSPL, CAMKK1	(Su, 2015)
Megakaryocyte	a	Enhance	Lin28	(Li, 2012b)
Myoblast	a	Enhance	Hox-A11	(Naguibneva, 2006)

Cell Type	Homolog	Differentiation	Target(s)	Ref
			GRP78/BIP	(Wei, 2016)
			ASNS, SMYD1, FOS	(Yuan, 2022)
			YAP1	(He, 2022)
			SEPP1	(Wang, 2020)
	b	Enhance	Mstnb	(Zhao, 2019)
NK Cell	a	Enhance	NLK	(Cichocki, 2011)
	b	Enhance	NLK	(Cichocki, 2011)
NKT Cell	a1	Enhance	PTEN	(Henao-Mejia, 2013)
	b1	Enhance	PTEN	(Henao-Mejia, 2013)
Osteoblast	a	Enhance	Tgfβi, TβR-I/Alk5	(Bhushan, 2013)
			PBX1	(Liu, 2020)
	a1	Enhance	N/A	(Bakhshandeh, 2012a)
			PTEN	(Zheng, 2019)
			N/A	(Qi, 2021)
	b	Enhance	PTEN	(Lv, 2020a)
	b1	Enhance	PTEN	(Zheng, 2019)
			N/A	(Qi, 2021)
	c	Enhance	N/A	(Ma, 2020)
	d	Enhance	MAPK1	(Liu, 2021)
		Suppress	SMAD3	(Xie, 2018)
Osteoclast	a	Enhance	DUSP6	(Zhang, 2021)
			FasL	(Shao, 2015)
			FasL	(Shao, 2018)
		Suppress	CREB1	(Fu, 2021)

Cell Type	Homolog	Differentiation	Target(s)	Ref
	b	Suppress	OSM	(Han, 2020)
	c	Suppress	SFRP1	(Yu, 2021)
	d	Enhance	OPG	(Sun, 2019)
T Lymphocyte	a	Enhance	FasL	(Shao, 2018)
	a1	Enhance	PTEN	(Henao-Mejia, 2013)
			N/A	(Liu, 2008)
		Suppress	N/A	(Chen, 2004)
	b1	Enhance	PTEN	(Henao-Mejia, 2013)
		Suppress	N/A	(Chen, 2004)
	c	No Effect	N/A	(Liu, 2008)

Table 2.

Functional roles of miR-181 family members in disease

Condition	Homolog	Function	Targets	Ref
Arterial Thrombosis	b	Suppress	Card10	(Lin, 2016)
Atherosclerosis	b	Suppress	IPOA3	(Sun, 2014)
Myocardial Ischemia/ Reperfusion Injury	a	Suppress	PTEN	(Das, 2017a)
			Hox-A11	(Li, 2009)
	b	Suppress	PTEN	(Das, 2017a)
			Akt3, PI3KR3	(Yuan, 2019)
	c	Enhance	mt-COX1	(Das, 2017a)
			mt-COX1, Sp1	(Banavath, 2019)
Obesity-induced Cardiomyopathy	c	Enhance	mt-COX1	(Roman, 2020)
Osteoarthritis	a	Enhance	N/A	(Nakamura, 2016)
			N/A	(Nakamura, 2019)
			Sirt1	(Qi, 2022)
	b	Enhance	N/A	(Song, 2013)
Vascular Inflammation	a	Suppress	TAB2, NEMO	(Su, 2019)
	b	Suppress	importin-α3	(Sun, 2012)
Vascular Stiffness	b	Suppress	TGF-βi	(Hori, 2017)