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MicroRNAs: Modulators of Cell Identity, and their Applications in Tissue Engineering

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Abstract: MicroRNAs post-transcriptionally regulate the expression of approximately 60% of the mammalian genes, and have an important role in maintaining the differentiated state of somatic cells through the expression of unique tissue-specific microRNA sets. Likewise, the stemness of pluripotent cells is also sustained by embryonic stem cell-enriched microRNAs, which regulate genes involved in cell cycle, cell signaling and epigenetics, among others. Thus, microRNAs work as modulator molecules that ensure the appropriate expression profile of each cell type. Manipulation of microRNA expression might determine the cell fate. Indeed, microRNA-mediated reprogramming can change the differentiated status of somatic cells towards stemness or, conversely, microRNAs can also transform stem- into differentiated-cells both *in vitro* and *in vivo*. In this Review, we outline what is currently known in this field, focusing on the applications of microRNA in tissue engineering.

Keywords: Cell fate, ESC, iPSC, microRNA, stemness, tissue engineering.

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) of approximately 22 nucleotides responsible for specific regulation of gene expression in a post-transcriptional manner, and, thereby, have an important role in several biological processes, such as development, cell proliferation, and apoptosis, among others [1-3]. Their genes correspond to 1-3% of all genes of the genome [4], and may be responsible for the regulation of approximately 60% of the coding genes [5]. The specificity of miRNAs is given by the seed region (nucleotides 2 to 8) that requires full complementarity to the mRNA-target [6-8]. The RNase III members, Drosha and Dicer, together with their specific partners, Dgcr-8 and TRBP, are crucial for miRNAs biogenesis, since they slice, respectively, primary and precursor miRNAs to yield the mature ones [9-12].

In vertebrates, approximately 70% of the miRNA genes are intragenic, usually being expressed in synergy with the host gene [13]. Forty percent of all miRNAs are organized in clusters and transcribed in a polycistronic fashion. Usually, these clustered miRNAs are members of a family, showing overlapping functions [13, 14]. Each cell type has a combination of either isolated or clustered expressing miRNAs, which regulate coding genes in a tissue-specific manner and, therefore, they are essential to the maintenance of cell identity and functional phenotype [15]. In this article, we review miRNAs involved in pluripotency maintenance, cell fate decision, differentiation-state safeguarding, focusing on how this knowledge has been used in tissue engineering.

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MiRNAs IN STEM CELLS

Embryonic stem cells (ESCs) are derived from pre-implantation blastocysts, and have the ability of self-renewal and the latent capacity to differentiate in cells of all three embryonic germ layers, therefore being pluripotent [16, reviewed in 17]. Because of these remarkable characteristics, ESCs have been extensively used as model in developmental and therapeutic studies, including tissue regeneration, transplantation, and drug screenings [18-21]. To sustain the undifferentiated state, they have a unique gene expression profile [22-26] regulated by a highly expressed set of transcriptional factors, including Oct4, Nanog, and Sox2 that, in this scenario, have overlapping functions [27-29]. In addition to OCT4 and NANOG, the most commonly used markers of ESCs are the cell surface antigens (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81) and tissue-nonspecific alkaline phosphatase [30]. Mouse pluripotent stem cells can also be derived from the epiblast of peri-implantation blastocysts, and are called EpiSC [31, 32]. These are epigenetically more similar to human ESCs (hESCs), being dependent on bFGF for self maintenance [33]. The human equivalent of mouse ESCs (mESCs), which is dependent on LIF, small molecule inhibitors of ERK1/ERK2 and GSK3b signaling, and express SSEA-1, has only recently been derived from human blastocysts and appear to be in an earlier epigenetic developmental state – therefore, they are called naïve hESCs, whereas the FGF-dependent hESCs are called primed hESCs [34, 35].

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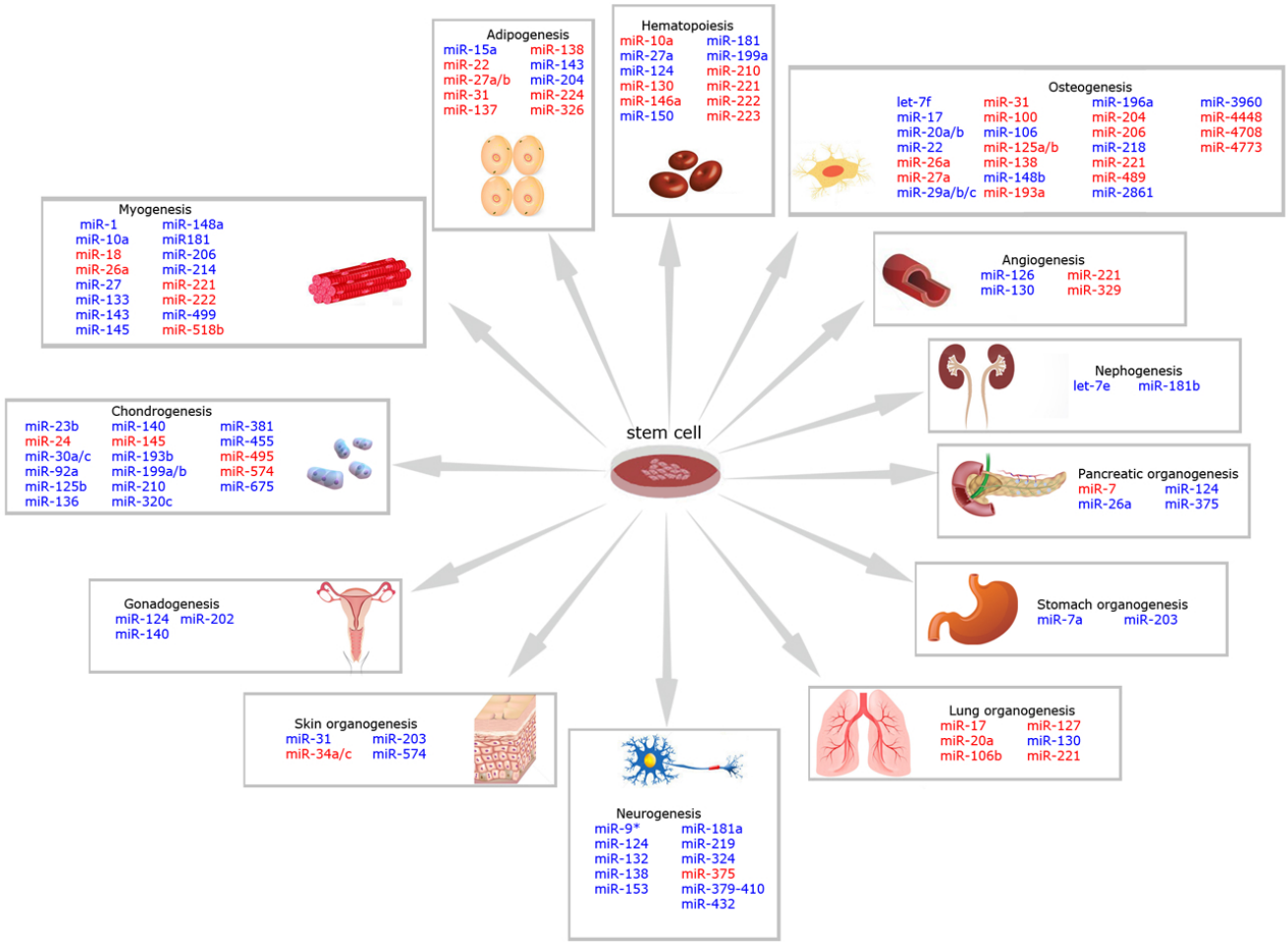

Fig. (1). MiRNAs involved in cell fate during genesis and development of different tissues and organs in mammals. In blue, miRNAs that positively regulate differentiation; in red, miRNAs that negatively regulate differentiation. Images were obtained from www.shutterstock.com website. This figure graphically represents Supplementary Table **S1**. For further information about each miRNA, please see referred Table.

Epigenetic modifications are also essential for pluripotency maintenance. The early steps of embryonic development are marked by global DNA demethylation, and this "permissive" epigenetic state at pre-implantational phase is pivotal for the expression of the above mentioned transcription factors [36]. By the time of implantation, methylation of CpG dinucleotides is reestablished throughout the genome as result of the *de novo* activation of highly expressed DNA methyltransferases Dnmt3a and Dnmt3b, which triggers the differentiation process [37, 38]. When most of the DNA methylation marks have already been established, the expression of many genes belonging to the epigenetic machinery is decreased, concomitantly with the differentiation process [39, reviewed in 40].

More recently, the maintenance of pluripotency has also been associated to miRNA expression. Since the first discoveries of the regulatory effect of small ncRNAs *lin-4* and *let-7* in *Caenorhabditis elegans*, it is well established that miRNAs are involved in development [1, 41]. Indeed, disruption of *Dicer* gene in mice compromises the entire miRNA biogenesis and is lethal early in development [42]. Although mouse Dicer-null ESCs are viable, they have a slow proliferation rate and fail to differentiate [43, 44]. Mouse Dgcr8-deficient ESCs also exhibit delayed proliferation rates with prolonged G0 and G1 phases [45], and when induced to differentiation, show aberrant expression of specific differentiation markers, such as a delayed expression of primitive ectoderm marker (*FgF5*), a weak or absent expression of endoderm (*Hnf4a* and *Afp*), mesoderm (*Brachyury*, *Bmp4*, *Gata*) and ectoderm (*Sox1*) markers, and incomplete repression of pluripotency [43, 46]. Additionally, mouse Dicer-null ESCs showed significant hypomethylation of the genome, including the promoter of *Oct4* gene (also known as *Pou5f1* gene) [47, 48], which impairs differentiation.

Three miRNAs clusters *hsa-miR-371-373* (ortholog of the mouse cluster *mmu-miR-290-295*), *hsa/mmu-miR-302-367* and *hsa/mmu-miR-17-92* are highly expressed in ESCs [49-54], therefore known as ESC-enriched miRNAs. They share similar seed sequences, suggesting an overlapping regulation of their targets [45, 55].

The *hsa-miR-371-373* cluster transcribes four miRNAs [50], whereas its murine counterpart, *mmu-miR-290-295* transcribes seven [49], and accounts for up to 70% of the total miRNAs expressed in mESCs [56]. At the stage of four to eight cells, mouse embryos already show miR-290-295 expression, which decreases after embryonic day 6.5 [57],

and during *in vitro* differentiation of ESCs to embryoid bodies [52]. These miRNAs are involved in (a) the regulation of transition from G1 to S phase of the cell cycle through targeting of cell-cycle inhibitors [45]; (b) repression of mesoderm and primordial germ cell differentiation pathways [58]; (c) repression of epithelial-mesenchymal transition [59]. Transfection of miRNAs belonging to the miR-290-295 cluster restores many of the defects exhibited by mouse Dicer-null ESCs. This phenomenon was seen by two independent studies showing that miR-290-295 members inhibit the expression of the retinoblastoma-like 2 protein (Rbl2), which is a transcriptional repressor of DNA methyltransferases – Dnmt3a and Dnmt3b [47, 48]. Thus, the authors of these two studies [47, 48] proposed that this is the major mechanism by which cells regulate DNA *de novo* methylation during early development. Accordingly, introduction of miR-290, miR-302 and miR-17-92 mimics was able to re-establish the proliferation rates of Dgcr8-deficient ESCs [45]. However, miR-290-295 function is still controversial, since recently it was shown that, although Rbl2 mRNA levels are increased in mouse Dicer-null ESCs, its protein remains at low levels [60].

The other highly expressed miRNA cluster in ESCs, *miR-302-367*, transcribes eight and five miRNAs, in human and mice, respectively [49, 50]. This cluster confers stemness proprieties to hESCs by controlling LEFTY1 and LEFTY2 expression, two inhibitors of TGFβ/ Nodal pathway that have an essential role in signaling early cell fate determination [61]. This cluster is also important to control cell cycle, since its inhibition leads to arrest of hESCs in G1 phase by targeting CYCLIN D1 [62].

Finally, *miR-17-92* cluster comprises six miRNAs in human and mice, is overexpressed in mESC and hESC [50, 51], and has an important role in cell cycle regulation [63, 64]. Despite being associated to maintenance of ESC pluripotency, this cluster is also widely expressed in many cell types [65-67]. In humans, its overexpression may lead to several malignancies, since it is located at the genome region 13q31-q32 that is frequently found amplified in lymphomas and other cancer types [68, 69].

Interestingly, expression profiles for mESCs and EpiSCs reveal that they have differences in the expression of several miRNAs, including these important ESC-enriched miRNA clusters, *miR-17-92, miR-290-295* and *miR-302-267*. The former two are more highly expressed in mESC, whereas the latter is in EpiSC [51]. Additionally, although barely expressed, members of Let-7 miRNA family, which are differentiation markers, are enriched in EpiSC in comparison to mESC, and that may reflect a degree of commitment to differentiation. Thus, miRNAs may have redundant and specific roles in regulation of pluripotency [51]. In humans, *hsa-miR-302b* expression is indicative of pluripotency in naïve and primed hESC, while expression of *hsa-miR-371-373,* the human ortholog of *mmu-miR-290-295*, is increased in naïve when compared to primed hESCs [33, 55].

Nevertheless, Oct4, Sox2 and Nanog regulate *miR-290-295* and *miR-302-367* gene clusters in mice and humans, reinforcing miRNAs role in pluripotency control and in the early steps of differentiation [56, 62, 70].

Adult stem cells, such as mesenchymal, bone marrow and hematopoietic stem cells are also capable of self-renewal and have the plasticity to differentiate into one or multiple cell types, functioning as a quiescent reservatory for tissue maintenance and repair throughout the life span [reviewed in 71]. As its embryonic counterparts, they have miRNAs that may participate in the maintenance of cell identity [72, 73], such as miR-489 that is highly expressed in mouse muscle stem cells (satellite cells), but is downregulated during cell activation [74].

MiRNAs IN INDUCED PLURIPOTENT STEM CELLS

Since the reversion of differentiated fibroblasts into pluripontent cells by the introduction of the defined reprogramming factors OCT4, SOX2, KLF4, and c-MYC (OSKM) in 2006, induced pluripotent stem cells (iPSCs) have been the center of many studies in cell therapy [75, 76]. IPSCs raise great interest in regenerative medicine because of their potential to overcome the issue of histocompatibility between cells and patient. Different methods have been used to deliver the reprogramming factors, including *(a)* integrative retrovirus vectors; *(b)* non-integrative vectors, such as adenovirus, Sendai virus, and plasmids; *(c)* DNA-free transfections, such as mRNAs and fusion proteins; and *(d)* excision after integration, such as *piggyBac* transposon and Retrovirus with loxP construction followed by Cre recombinase mRNA transfection [reviewed in 77]. Accordingly, new approaches to promote a better understanding of the mechanisms involved in maintenance of pluripotency, to achieve higher reprogramming efficiency, and to guarantee the safe use of iPSCs to therapy are of great importance. In this context, the introduction of ESC-enriched miRNAs has been used to improve cell reprogramming.

Transient transfection of miRNAs miR-291-3p, miR-294, and miR-295 enhances the efficiency of the reprogramming of mouse embryonic fibroblasts achieved by retroviral deliver of *Oct4*, *Sox2* and *Klf4*. Among them, miR-294 showed the best results, increasing tenfold the efficiency rates [78]. Similarly, the expression of miRNAs from the *miR-302-367* cluster also enhances retroviral reprogramming of human fibroblasts with *OCT4*, *SOX2* and *KLF4*, either with or without *c-MYC* [79]. Conversely, inhibition of Let-7 family members, which are known to be robust maintainers of the differentiated state in mouse embryonic fibroblasts, enhanced in over fourfold the efficiency of reprogramming carried out by *Oct4*, *Sox2* and *Klf4* [80]. Other examples of miRNAs expression manipulation to improve the efficiency of reprogramming are the transfection of miR-93 and miR-106b [81], the knockout of *miR-34* [82] and *miR-199a-3p* [83], among others [84-86].

Strikingly, the reprogramming of human skin cancer cells [87] and human fibroblasts [88] into a pluripotent state by the introduction of *miR-302-367* cluster *per se* was reported. However, few studies have used this methodology recently, and reprogramming of somatic cells through transfection of members of the cluster miR-302-367 alone [89] or combined with miR-200c or miR-369 [90] yield no clones or resulted in a low efficient rate. Nevertheless, the use of miRNAs as OSKM adjuvant to produce iPSC might be a good strategy to improve the efficiency of somatic cells reprogramming [89].

As any other cell-therapy approach, miRNA use must be in consonance with the applications intended for iPSCs, since some differentiated cells retain more plasticity than others, and some cell types present a more robust epigenetic memory after reprogramming [reviewed in 91].

MiRNAs IN CELL FATE DECISION

Cell differentiation is a complex pathway that depends on both activation of lineage-specific genes and repression of pluripotency-related ones. However, a coordinated modulation of *Oct4, Sox2* and *Nanog* expression in early steps of differentiation process contributes to specific germ layer induction of mESCs, despite combined expression of *Oct4* and *Sox2* suppresses germ layer differentiation [92]. Therefore, high Oct4 or Sox2 levels promote mesendodermal or neural ectoderm differentiation, respectively, while Nanog downregulation is decisive for lineage commitment [92]. Similar phenomenon was seen in a study using hESCs, whereas each factor is *per se* involved in a specific cell fate [93]. Likewise, *miR-302-367* cluster that, as seen before, has an important role in the maintenance of pluripotent cells, is also expressed in the human endodermal lineage [94], providing evidence that this cluster has a role in organogenesis. Indeed, hsa-*miR-302-367* cluster promoter is targeted by GATA6 transcription factor in early stages of lung epithelial development, promoting the proliferation of lung endoderm progenitor cells, proper apical-basal polarity and preventing its complete differentiation. Therefore, this cluster seems to be essential for the correct development of a single-layered lung epithelium [95]. Additionally, miR-17-92 that is also enriched in hESC [50], has an important role in the early stages of lung morphogenesis, regulating the proliferation-differentiation balance of lung epithelial progenitor cells [65]. As the cells commit to differentiation, Oct4, Sox2 and Nanog are downregulated, and consequently the clusters regulated by them, *miR-290-295* and *miR-302-367* in mice and human, respectively, are also silenced [56, 62]. Prior to being silenced, Oct4, Sox2 and Nanog also upregulate the expression of some miRNAs specifically associated with differentiation in mESCs, such as miR-9, miR124a, miR-155 and miR-708, which at least are in part responsible for proper cell fate determination [56]. Indeed, miRNAs are essential for ESCs specific-differentiation and maintenance of the differentiated status. Accordingly, miRNAs expression is frequently globally downregulated in tumors, which are less differentiated cells [15, 96].

Similarly, adult stem cells also have miRNAs involved in the commitment of their differentiation. For instance, expression of miR-590 and miR-199a in adult cardiomyocyte promotes re-entrance in cell cycle, resulting in cardiac repair in an *ex-vivo* mouse model [97].

One of the first miRNAs recognized by its role in differentiated tissues was *let-7* [41]. With its orthologs organized in large families along the vertebrate genomes, *Let-7* is upregulated in differentiating and differentiated mouse cells [98-100]. Although mature *Let-7* is poorly expressed in mESCs, its primary transcript is abundant [56]. The processing from pri-Let-7 to Let-7 mature duplex is inhibited by the RNA binding protein Lin28, which prevents differentiation and stabilizes mESC status [101].

Since then, many other miRNAs have been reported as having an important role in early steps of differentiation and maintenance of the differentiated status. Examples are miR-21 and miR-22 which were also reported as overexpressed in differentiated cells [49]. Indeed, Nanog and Sox2 are direct targets of miR-21 [102], and this miRNA may have an important role promoting adipocyte differentiation [103] as well as in bone formation, since it is overexpressed during the initial steps of osteogenic differentiation [104]. MiR-22, by its turn, has been reported as a maintainer of progenitor cells in murine mammary epithelium [99], promoter of osteogenic differentiation and inhibitor of adypogenic differentiation [105]. Moreover, a set of miRNAs was found to be up-regulated (miR-297, miR-96, miR-214, miR-125a, miR-424, miR-21, miR-29c, miR-7) or down-regulated (miR-376a) in mouse blastocysts when compared to the morula stage, indicating that they are involved in trophectoderm determination [106]. Furthermore, the different miRNA profiles characterizing the three germ layers in gastrulating embryo implicate the involvement of miRNAs in the differentiation of mesoderm, endoderm, and ectoderm [107-109]. In the Supplementary Table **S1** we show a comprehensive list of miRNAs involved in tissue regulation, organogenesis and development.

Once differentiation is established, each cell type will express its own set of miRNAs. Accordingly, tissues with the same ontogenetic origin have similar expression profiles, which are different from those of tissues originating from different embryonic layers [15]. Since miRNAs have been widely implicated in the control of stem cells fate, a better understanding of the relationships among miRNAs, transcription factors, signaling pathways, chromatin remodeling factors, and extracellular clues have a pivotal importance in developing new strategies to tissue engineering.

MiRNAs AS PROMISING TOOLS FOR TISSUE ENGINEERING

Tissue engineering (TE) is an interdisciplinary field that combines cells, engineered materials, and biomedical technology towards the development of bio-artificial tissue-like structures to restore, replace, maintain or improve the function of tissues or organs [110].

Currently, several tissues and organs are being engineered [111-114]. The obstacles in TE are to attain specific cell types, to develop appropriated scaffolds, and to promote the release of growth factors and other molecules from scaffolds in order to resemble organogenesis [115, 116]. The success of implant engineered tissues is also challenged by the difficult formation of blood vessel network, tissue innervation, and by inflammatory and immunological responses [reviewed in 117], making the transition from research stage to clinical trials limited to avascular or thin tissues, such as cartilage and skin [118, 119]. Given the role of miRNAs in many biological processes, including cell differentiation and maintenance of cell identity, modulation of these small molecules in combination with stem cells and/or bioartificial scaffolds has been providing encouraging results. Indeed, strategies for vascularization of bioartificial tissues, which are insofar mainly based on the delivery of angiogenic growth factors [120-122], have recently advanced with the

Table 1. MicroRNAs employed for tissue engineering approaches.

MicroRNA	MicroRNA delivery	Result of microRNA manipulation	Ref.
miR-1 and miR-206	Myogenic progenitor cells were transfected with each microRNA separately and cultured in a 3-D culture system.	Improvement of myogenic progenitor cells differentiation.	[129]
miR-21	Chondrocytes were cultured on an atelocollagen gel complexed with the miRNA.	Improvement of proliferation and matrix synthesis of the chondrocytes.	[127]
miR-26a	Bone marrow mesenchymal cells were cultured in an hydrogel system that releases a chemically modified miR-26a, which is a miRNA enhancer (agomiR-26a), and then this construction was implanted into calvarial bone defect in mice.	Improvement of bone regeneration and modulation of angiogenesis- osteogenesis coupling.	[125]
miR-29a	Human vascular smooth muscle cells were seeded with polyglycolic acid scaffolds in the presence of miRNA-29a inhibitor.	Improvement of elastin levels in bioengineered human vessels.	[126]
miR-29b	Fibroblasts were cultured in collagen-based scaffolds doped with miR-29b and these were applied to rat wound model.	Improvement of the wound healing response through reduced wound contraction and reduction of collagen type I production after the injury.	[116]
miR-30c	Treatment of osteoblastic cells with nano-bioglass ceramic particules (nBGC) was able to induce miR-30c expression.	The indirect upregulation of this miRNA may lead to the osteoblastic differentiation.	[131]
miR-31	Osteo-inductive bone marrow stromal stem cells transduced with anti-miR-31 lentiviral vectors were seeded on polyglycerol sebacate scaffolds and used to repair critical-sized calvarial defects in rats.	Improvement of ostogenic differentiation, biocompatibility and regeneration rate in the repair of *in vivo* large bone defects.	[132]
miR-132	The microRNA was encapsulated in a targeted biodegradable polymer nanoparticle and delivered to endothelial cells before transplantation.	Improvement of endothelial cells transplantation through vascularization enhancing.	[124]
miR-133	Myoblasts were transfected with anti-miR-133 and cultured in a collagen/matrigel construct.	Improvement of myogenic differentiation and increased peak forces after electrical stimulation.	[128]
miR-221, miR-222, miR-140, miR-143, and miR-145	In 3D agarose cultures, chondrocytes treated with TGF-β1 showed downregulation of miR-221 and miR-222 expression and inscreased expression of miR-140, miR-143 and miR-145.	Alterations in microRNAs expression due to treatment of cells with TGF-β1, which is known to enhance chondrocytic differentiation, may represent a promising role in the tissue engineering of the articular cartilage superficial zone.	[133]
miR-148b	Rat mesenchymal stem-cells were transfected with the microRNA lyophilized on a microporous titanium implant.	Improvement of osteogenic differentiation of the stem cells.	[135]
miR-148b and miR-489	Human mesenchymal stem-cells were transfected with miR-148b mimic and miR-489 inhibitor and cultured in a 2-D surface or capsuled in a 3-D scaffold.	Improvement of osteogenesis through the sensibilization of the cells to osteogenic signals.	[130]

use of miR-132 that indirectly induces *Ras* overexpression, enhancing neovascularization rate [123]. When this miRNA is encapsulated in biodegradable polymer nanoparticles, it improves vessel formation in human endothelial cells transplanted in immunodefficient mice. This approach allows the release of these small RNAs for weeks, longer than conventional lipid-based transfection [124]. Similarly, localized and sustained expression of miR-26a *in vivo* positively regulates osteogenesis-angiogenesis coupling, therefore, providing an enhanced efficiency in bone regeneration [125]. Zhang and colleagues have also reported that the inhibition of miR-29 in bioengineered vessels may increase the expression of its target gene, *ELN*, which has a major function in maintaining the integrity of the extracellular matrix of arteries [126].

Other studies also showed that TE could be potentially improved by the transfection of cells with miR-21, since it promoted high proliferation rates and high matrix synthesis of rat chondrocytes cultured in atelocollagen gel [127], and by implanting scaffold embedded with miR-29b in cutaneous injury, which was able to improve extracellular matrix remodeling of treated excisional wounds [116].

However, the most studied miRNAs in TE are related to directing cell fate. Examples of this are *(a)* the inhibition of miR-133, which enhances skeletal murine myoblast differentiation and the response to electrical stimulation in three-dimensional (3D) bioartificial muscle [128]; *(b)* the transfection of miR-206 in satellite cells, which increases their dif-

ferentiation in a bioartificial muscle construct [129]; *(c)* the introduction of miR-148b mimic and miR-489 inhibitor, which improves osteogenesis from human mesenchymal stem cells and the expression of osteogenic markers, also in a 3D scaffold [130], *(d)* the usage of nano-bioglass ceramic particles (nBGC) that stimulates miR-30 expression in osteoblastic cells, inducing their differentiation [131], *(e)* the transfection of miR-31 inhibitor in bone marrow stromal cells, which increases osteogenic differentiation, bone mineral density, biocompatibility and regeneration rate [132]. Finally, miRNAs seem to have an important role in the development of engineered tissues with a refined architecture. For instance, articular cartilage is subdivided in specific zones that seem to be frequently lost in monolayer expanding cultures of chondrocytes. Superficial zone-specific miRNAs expression is also lost in this process, and the TGF-β-directed differentiation of chondrocytes in a 3D agarose culture was able to reestablish their expression. Therefore, manipulation of miRNA expression might be useful to the correct assembly of a complex engineered tissue [133]. A complete list containing all miRNAs used so far in TE is reported in Table **1**. Thus, we expect that miRNAs will become an increasingly important tool for controlling cell fate for TE, and the prominent candidates to this purpose are listed in Supplementary Table **S1**.

Finally, miRNAs might potentially be used to monitor the graft status, since in a mouse model of heart transplantation, allograft rejection seems to be associated with specific miRNA signatures. Moreover, miR-182 was found overexpressed in peripheral blood mononuclear cells and plasma in mice with allograft rejection [134].

CONCLUSION

MicroRNAs have an essential role in maintenance of cell pluripotent and differentiated states, as well as in cell fate decisions, working as modulators of cell identity. Accordingly, these small regulators might *(a)* assist the reprogramming of iPSC, an important source of cells for TE; *(b)* direct and maintain tissue-specific differentiation; *(c)* guarantee proper vascularization of engineered tissue. A better understanding of miRNAs involvement in tissue formation, regeneration and function will provide more efficient engineered tissues. Thus, on the whole, despite few studies have been performed so far, the results are very promising and warrant remarkable advances in the next future.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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