Chapter

MicroRNAs in the Functional Defects of Skin Aging

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Abstract

Humankind has always been intrigued by death, as illustrated by the eternal quest for the fountain of youth. Aging is a relentless biological process slowly progressing as life cycle proceeds. Indeed, aging traduces an accumulation of physiological changes over time that render organisms more likely to die. Thus, despite our mastery of advanced technologies and robust medical knowledge, defining the molecular basis of aging to control lifespan is still currently one of the greatest challenges in biology. In mammals, the skin is the ultimate multitasker vital organ, protecting organisms from the world they live in. As a preferential interface with the environment, the skin is reflecting the internal physiological balances. The maintenance of these balances, called homeostasis, depends on the concurrent assimilation of diversified signals at the cellular level. MicroRNAs (miRNAs) are noncoding RNAs that regulate gene expression by mRNAs degradation or translational repression. Their relatively recent discovery in 2000 provided new insights into the understanding of the gene regulatory networks. In this chapter, we focused on the role of three miRNA families, namely miR-30, miR-200, and miR-181, playing a key role in the progression of the skin aging process, with particular input in mechanistic considerations related to autophagy, oxidative stress, and mitochondrial homeostasis.

Keywords: skin, microRNA, epidermis, keratinocyte, fibroblast, aging, autophagy, oxidative stress, mitochondria, miR-30, miR-200, miR-181

1. Introduction

Skin, the largest vital organ in the body, is made of three distinct layers from the top to the depth: the epidermis, which is a fine layer of epithelial keratinized cells called keratinocytes; the dermis consisting of fibroblasts in an intracellular matrix with various additional structures such as hear follicles, sweat glands, nerve endings, and capillaries; and the profound subcutaneous tissue called hypodermis. As a physical barrier between the body and the environment, the skin is affected by both intrinsic and extrinsic aging. Intrinsic or chronological aging is a natural continuous dynamic process that normally begins in the mid-1920s. During this inexorable process, the skin undergoes a physiological deterioration characterized by skin atrophy, increased physical and immunological vulnerability, with a reduced capacity of tissue repair in case of wounding. More precisely, intrinsic aging is leading to a 10–50% thinning of the epidermis, the flattening of the dermal-epidermal junction, an atrophy of the dermis with disorganization of the collagen and elastic fibers, a reduction of the microvasculature, and a loose of adipose tissue [1]. The

thinning of the epidermis and the reduction of the skin regeneration capacities are mainly linked to the dysfunctions of the epidermal stem cell compartment, which progressively lose its capacity to generate progenitor cells that are able to ensure the physiological renewal of the epidermis or to sustain wound repair [2].

Skin is also constantly exposed to environmental insults such as ionizing or UV radiations, chemicals, or climatic variations [3]. UV exposure is the main player in extrinsic skin aging and leads to phenotypic changes named photoaging. The photo-aged skin is characterized by a thickening of the epidermis with abnormal keratinocytes differentiation, an accumulation of abnormal elastic tissues (called solar elastosis) with a disorganization and degradation of the collagen fibers in the dermis, an abnormal pigmentation, and an activation of the immune response [4]. Skin is affected by both UV-A and UV-B radiations. UV-B rays are mainly limited to the superficial epidermal part of the skin and directly induce DNA lesions such as cyclobutane dimers and 6–4 photoproducts in exposed cells, leading to keratinocytes senescence, apoptosis, or carcinogenesis [5]. UV-A rays penetrate deeper into the dermis and induce DNA, protein, and lipid damages through the generation of reactive oxygen species (ROS), which in turn activate MAP-kinase p38, JNK, and ERK pathways with induction of the AP1 transcription factor resulting in the expression of the MMP1, -3, and -9 responsible for extracellular matrix degradation [6]. ROS also oxidate cellular components including proteins, lipids, DNA, and RNA, with altered metabolism and further damages.

ROS are also produced during the chronological aging process mainly through mitochondrial activity of the electron transport chain and this is the basis of the free radical theory of aging [7]. It states that mutations acquired in mitochondrial DNA (mtDNA) during life can disrupt metabolisms in the mitochondria and increase ROS. In the skin, mtDNA mutations accumulate with age and UV stress can accelerate this damage. Mitochondrial dysfunctions specifically contribute to skin aging phenotype especially through abnormal pigmentation and hair graying and loss [8]. More precisely, mitochondria play a pleiotropic role in pigmentation by modulating the melanin production through interacting with melanosomes [9]. Moreover, increased oxidative stress linked to mitochondrial dysfunction is observed in aged melanocytes and hair follicle epithelium [10].

Elimination of damaged mitochondria seems then to be a safety mechanism preserving cellular function, tissue homeostasis and organismal soundness. Selective mitochondrial autophagy, named mitophagy, has been described to ensure this function. Autophagy is a cellular quality control mechanism preliminary nonselective playing an essential role in cells for bulk proteins and organelle recycling. Mitophagy modulates the turnover of mitochondria under equilibrium conditions and adjusts the number of mitochondria according to the cellular needs. Increasing evidence suggest that impairment of mitophagy is involved in aging and age-related diseases [11] but the genetics and epigenetics mechanisms modulating mitophagy during aging remain to be better understood.

Multiple epigenetic changes are considered as reliable hallmarks of tissue aging such as modification of DNA methylation motifs, histone post-translational modifications, and modulation of noncoding RNA expression [12]. The latter is an emerging scientific domain in which an incredibly expanding number of studies have been published over the last decade, highlighting day after day the key roles of long noncoding RNAs, circular noncoding RNAs, and microRNAs (miRNAs) in the control of physiologic balances. For example, the stem cell function is governed by numerous factors such as growth factors, cellular metabolism, mediators of inflammation, extracellular matrix, interaction with niche cells, and so on. It has been clearly described that the imbalance between stem cells renewal and commitment can give rise to deleterious effects leading to pathologies or accelerated aging [13, 14]. Recent single-cell analyses

from multiple tissues, including epidermis, revealed a clonal heterogeneity of gene expression level among a defined cell population [15–17], thus reflecting a distinct fluctuating transcriptome in individual cells which governs cell fate. Thus, cell fate decisions rely on the integration of dynamic regulatory networks of gene expression and these fluctuating transcriptomes are likely to be under the control of noncoding RNAs, which cooperate with each other and are co-regulated.

Isolated human primary keratinocytes are a valuable model for studying epidermal aging as they retain features of the tissue they are extracted from. Recently, we took advantage of this model to identify miRNAs modulated with chronological aging through a genome-wide expression analysis of cell extracted from skin biopsies of healthy infants (3–6 years old), young adults (20–40 years old), and aged adults (60–71 years old). This microarray screening allowed us to identify 60 miRNAs significantly modulated ($P < 0.05$, fold change >1.5) between at least two of the three sample groups analyzed [18]. Most of them were differentially expressed between the youngest group and the two adult groups. Considering that physiological aging starts as early as 20 years old and that our cell samples were prepared from photo-protected skin areas, one can speculate that constant modulation of miRNAs expression as soon as 20 years could constitute an epigenetic signature of intrinsic chronological aging. Thus, according to our miRNome analysis during aging, this signature would be constituted by the overexpression of miR-181d-5p, miR-1972, miR-200c-5p, miR-30a-3p, miR-30a-5p, miR-30c-2-3p, miR-30c-5p, miR-365a-5p, miR-4298, miR-6812-5p, and miR-6831-5p and the underexpression of miR-4443. Among these miRNAs of the epidermis aging signature, no mechanistic studies are referenced in the literature for miR-1972, miR-4298, miR-6812-5p, and miR-6831-5p, thus limiting any biological interpretation. Furthermore, if few studies are published to date for miR-365 and miR-4443, they mostly come from cancerology studies, as it is traditionally the case for miRNA studies. Considering the debate on the relevance of the cancer cell lines as reliable mechanistic models [19–21], we decided here to focus on data obtained from pathological and physiological models excluding the cancer field, as much as we can. Consequently, we will focus here our attention on miRNA members from three different families. Interestingly enough, the miR-30 family is highly represented in this signature, and a recurrent biological pathway targeted by miR-30 is autophagy. In addition, many members of the miR-200 family have been associated to oxidative damage. Finally, the miR-181 family is progressively enriched with overexpression of additional members (miR-181a-2-3p and miR-181b-5p) with elderly. Several published studies converge toward a control of mitochondrial homeostasis by miR-181. As described earlier, the autophagic flux, the response to oxidative stress and the maintenance of functional mitochondria are all affected with skin aging and thus constitute cellular processes of particular interest regulated by miRNAs.

2. The miR-30 family in the control of the autophagic flux

The miR-30 family is composed of six members (miR-30a, miR-30b, miR-30c-1, miR-30c-2, miR-30d, and miR-30e) transcribed from three clusters of two genes located on human chromosome 1 (miR-30c and miR-30e), chromosome 6 (miR-30a and miR-30c-2), and chromosome 8 (miR-30b and miR-30d) [22]. Each gene is able to produce two mature miRNA sequences, the 3p and 5p strands (**Table 1**), with various abundances. We specifically observed an induction of miR-30a-3p, miR-30a-5p, miR-30c-2-3p, and miR-30c-5p in aged human skin [18]. In our miRNome analysis, the miR-30a is the most overexpressed miRNA with aging, with a 4- to 6-fold increase depending on the mature strand. For a long time, mir-30a-5p has been associated with the regulation of autophagy in various cancer cells [24, 25]

microRNA family	Mature sequences (*homo sapiens*)	Sequence alignments (ClustalW)	Lentgh (nt)	Identity (%)
miR-30	miR-30a-5p	UGUAAACAUCCUCGACUGGAAG--	22	100.00
	miR-30b-5p	UGUAAACAUCCUACACU--CAGCU	22	85.00
	miR-30c-1-5p	UGUAAACAUCCUACACUCUCAGC-	23	77.27
	miR-30c-2-5p	UGUAAACAUCCUACACUCUCAGC-	23	77.27
	miR-30d-5p	UGUAAACAUCCCCGACUGGAAG--	22	95.45
	miR-30e-5p	UGUAAACAUCCUUGACUGGAAG--	22	95.45
	miR-30-5p consensus	********** *** **		
	miR-30a-3p*	CUUUCAGUCGGAUGUUUGCAGC	22	100.00
	miR-30b-3p*	CUGGGAGGUGGAUGUUUACUUC	22	63.64
	miR-30c-1-3p*	CUGGGAGAGGGUUGUUUACUCC	22	59.09
	miR-30c-2-3p*	CUGGGAGAAGGCUGUUUACUCU	22	54.55
	miR-30d-3p*	CUUUCAGUCAGAUGUUUGCUGC	22	90.91
	miR-30e-3p*	CUUUCAGUCGGAUGUUUACAGC	22	95.45
	miR-30-3p consensus	** ** * ***** *		
miR-200	miR-200a-5p*	CAUCUUACCGGACAGUGCUGGA	22	100.00
	miR-200b-5p*	CAUCUUACUGGGCAGCAUUGGA	22	77.27
	miR-200c-5p*	CGUCUUACCCAGCAGUGUUUGG	22	68.18
	miR-141-5p*	CAUCUUCCAGUACAGUGUUGGA	22	81.82
	miR-429-5p*	N/A	-	-
	miR-200-5p consensus	* **** * *** * *		
	miR-200a-3p	UAACACUGUCUGGUAACGAUGU-	22	100.00
	miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA-	22	81.82
	miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA	23	77.27
	miR-141-3p	UAACACUGUCUGGUAAAGAUGG-	22	90.91
	miR-429-3p	UAAUACUGUCUGGUAAAACCGU-	22	77.27
	miR-200-3p consensus	*** **** * ***** *		
miR-181	miR-181a-1-5p	AACAUUCAACGCUGUCGGUGAGU	23	100.00
	miR-181a-2-5p	AACAUUCAACGCUGUCGGUGAGU	23	100.00
	miR-181b-1-5p	AACAUUCAUUGCUGUCGGUGGGU	23	86.96
	miR-181b-2-5p	AACAUUCAUUGCUGUCGGUGGGU	23	86.96
	miR-181c-5p	AACAUUCAAC-CUGUCGGUGAGU	22	100.00
	miR-181d-5p	AACAUUCAUUGUUGUCGGUGGGU	23	82.61
	miR-181-5p consensus	******** ******** **		
	miR-181a-1-3p*	-ACCAUCGACCGUUGAUUGUACC	22	100.00
	miR-181a-2-3p*	-ACCACUGACCGUUGACUGUACC	22	86.36
	miR-181b-1-3p*	-CUCACUGAACAAUGAAUGCAA-	21	52.38
	miR-181b-2-3p*	-CUCACUGAUCAAUGAAUGCA--	20	55.00
	miR-181c-3p*	AACCAUCGACCGUUGAGUGGAC-	22	90.48
	miR-181d-3p*	--CCACCGGGGGAUGAAUGUCAC	21	61.90
	miR-181-3p consensus	** * *** **		

Multiple sequence alignment of miRNA mature 5p or 3p strands was done for each family using Clustal Omega program [23]. Mature sequences with an asterisk (*) correspond to the passenger strand, whereas the seed sequences in the guide strand are indicated in bold. The identity between multiple miRNA strands is expressed as relative to the first miRNA for each guide or passenger strand within a family. Stars (*) are aligned with conserved nucleotides among the different members of either guide or passenger strands for each family.

Table 1.
Mature sequences of miRNAs from miR-30, miR-200, and miR-181 families.

and, more recently, in other several types of normal cells such as cardiomyocytes [26, 27], endothelial cells [28–30], thymocytes [31], lens epithelial cells [32], or hepatic stellate cells [33].

Autophagy is well-conserved catabolic process across phyla that directs the degradation of either bulk or selective cellular components. Molecular

mechanisms governing autophagy in mammals has been extensively reviewed [34]. Briefly, the process is regulated by a core machinery involving a step-by-step interaction of multiple molecular partners called autophagy-related (ATG) proteins. The initiation step is under the control of the protein kinase ULK1, which phosphorylates Beclin-1 (BECN1) on S14, thus boosting the activity of the VPS34-P150 complexes that induce the nucleation of the autophagophore. Subsequent phagophore extension requires first the intervention of ATG5-ATG12-ATG16L complexes. The closure of the autophagosome relies then on the activity of the ATG4-ATG3-ATG7 complexes that convert the inactive microtubule-associated protein LC3-I into the active LC3-II form by conjugation with phosphatidylethanolamine. Finally, LC3-II allows the autophagosomes to fuse with lysosomes to form autolysosomes where all contents are enzymatically digested (**Figure 1**).

Plethora of microRNAs have been shown to modulate the different proteins involved at each step of the autophagic process [35–37]. Although the six members of the miR-30 family have distinct mature sequences, the seed sequence is perfectly

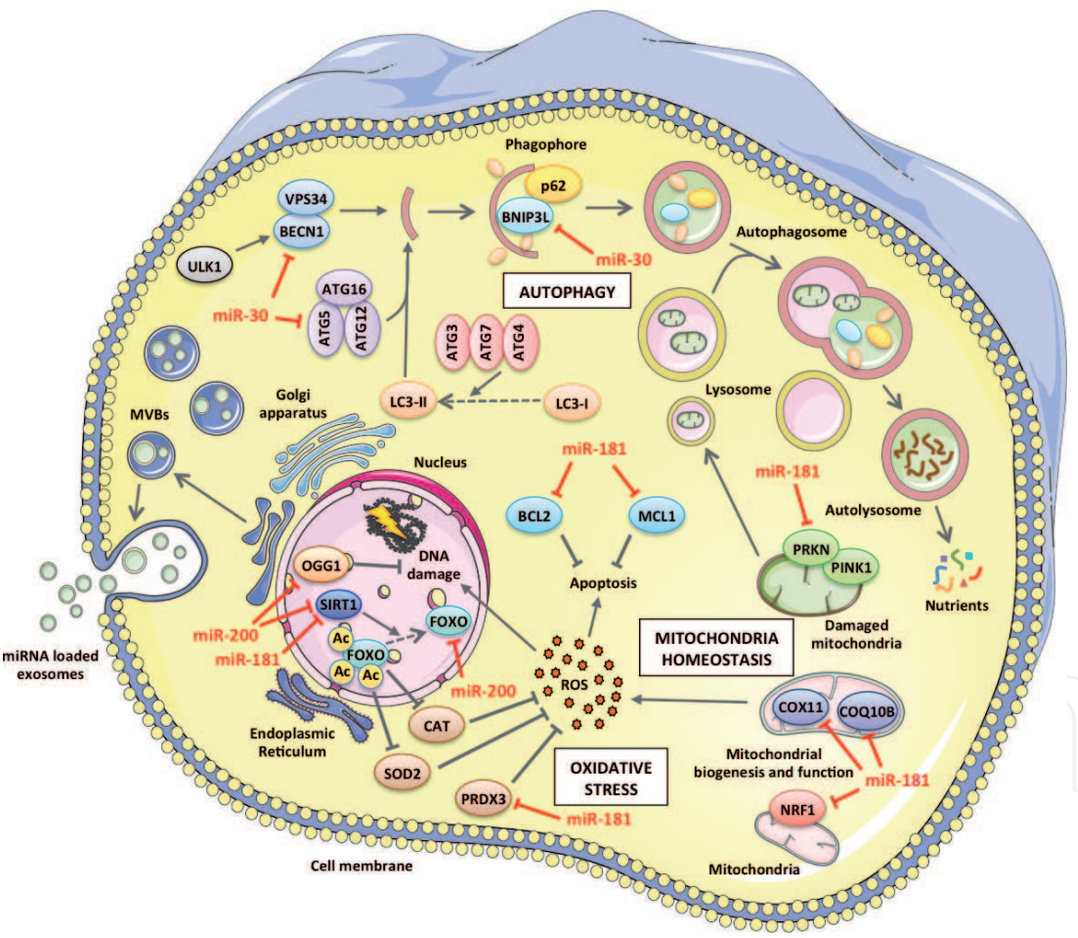

Figure 1.
MiRNAs regulate multiple gene regulatory networks implicated in skin aging. Members of the miR-30, miR-181, and miR-200 families are overexpressed with skin aging and regulate critical cellular processes such as autophagy, oxidative stress, and mitochondria homeostasis. Autophagy is regulated by the interaction of multiple molecular partners called autophagy-related (ATG) proteins and leads to the enzymatic digestion of autophagosome content. Oxidative stress is characterized by an accumulation of reactive oxygen species (ROS), which disturbs cellular homeostasis and leads to DNA damage and apoptosis. Mitochondria homeostasis relies on an effective biogenesis and a proper elimination of compromised mitochondria. MiR-30, miR-181, and miR-200 directly or indirectly target multiple key proteins implicated in these different processes. Fusion between multivesicular bodies (MVBs) and cell membrane allows the liberation of exosomes and their miRNA cargo in the extracellular compartment. Red inhibitory arcs symbolize miRNA inhibitory effect by inducing mRNA decay or translation inhibition. Gray inhibition arcs indicate inhibition effect on proteins or processes. Continuous plain gray arrows represent activation effect on protein or process. Continuous straight gray arrows represent intracellular dynamics. Dotted straight gray arrows represent protein post-translational modifications.

conserved (**Table 1**). Thus, it is not surprising that other members of the miR-30 family have been involved as well in the negative control of autophagy, such as miR-30b in vascular smooth muscle cell [38], miR-30c in neurons [39], miR-30d in astrocytes [40], and miR-30e in cardiomyocytes [41]. The different members of the miR-30 family have been primarily associated to the targeting of *BECN1* [24–33]. Importantly, this regulation of *BECN1* by miR-30a has been established *in vivo* as well [30, 39, 41, 42]. *ATG5* is another recurrent downstream target of all the members of the miR-30 family [43–52]. Finally, *BNIP3L* (*aka NIX*) is an additional factors of the autophagy pathway negatively controlled by both miR-30c [53] and miR-30d [50, 51], whereas miR-30d also decreases the luciferase activity of reporter plasmids carrying the 3′UTR of *ATG2B* or *ATG12* [50, 51].

The functional link between these miRNAs and the mechanisms of skin aging is not clarified yet; however, the decline in effectiveness of autophagy is clearly one of the hallmarks of aging [54]. This has been observed in diverse organisms from nematodes to rats, including human cells, and this is accompanied in part by a downregulation of *ATG5* and *BECN1* [55], which are notorious targets of the miR-30 family members. Accordingly, members of the miR-30 family have been shown to be induced in senescent cells, including aged keratinocytes [18, 56]. Apparent conflicting data exist in the literature as a recent study comparing gene expression from young (9–18 years old, average 12.7) and aged dermal fibroblasts (50–94 years old, average 67) revealed by RNA-seq analysis that the major autophagy-modulating genes (*BECN1*, *MAP1LC3B*, *ATG5*, *ATG7*, *ULK1*, *PIK3C3*, *mTOR*) were not differentially expressed [57]. However, the downregulation of mRNA target expression by miRNA binding in the 3′UTR occurs in two manners, either through mRNA decay or translation inhibition [58, 59]. Thus, the measure of mRNA levels does not simply reflect the final activity of the protein, and for example, miR-30a has been preferentially associated to *BECN1* translation inhibition rather than mRNA decay in endothelial cells [29, 30]. Indeed, in the same study, even though the mRNA levels of multiple effectors of autophagy were not downregulated with aging in dermal fibroblasts, excessive residual autophagic bodies were found in these cells, thus exposing an impaired autophagic flux in aged skin [57]. This is consistent with a previous report showing a nearly 80% reduction in the autophagic flux, as determined by RT-qPCR and immunocytofluorescence analysis of LC3B expression in synchronized aged normal human skin fibroblasts and compared to young fibroblasts [60]. Likewise, accumulation of autophagic vacuoles containing debris and deformed mitochondria was found in both senescent human keratinocytes and aged dermal fibroblasts by transmission electron microscopy analysis [61, 62].

In fibroblasts, the defect in the autophagic flux was identified at the final degradation step of the autophagolysosome and was correlated with weakened turnover of dermal extracellular proteins, possibly leading to a collapse of the dermis structure and skin fragility [62]. In keratinocytes, the autophagic process, especially the nucleophagy, plays a key role in the control of the terminal differentiation [63, 64]. In addition, it has been demonstrated that *Becn1* also plays a crucial role for skin development in mice [65]. Indeed, conditional knockout of *Becn1* in the epidermis layer results in mice having stiff and shiny skin with extensive water loss and death within a day after birth. The silencing of BECN1 in human keratinocytes is associated with a considerable drop in expression of the keratins 1 and 10 (KRT1 and KRT10) together with puncta formation of the integrin alpha 6 (ITGA6), suggesting a failure in the normal endosomal trafficking. Since the skin phenotype is not observed in *ATG5* or *ATG14* KO mice, the authors suggested that BECN1 is required for normal mouse skin development through the regulation of the endocytic pathway but autonomously from the autophagy pathway. However, an ATG5/ATG7-independent alternative autophagy has been described earlier [66–68], and

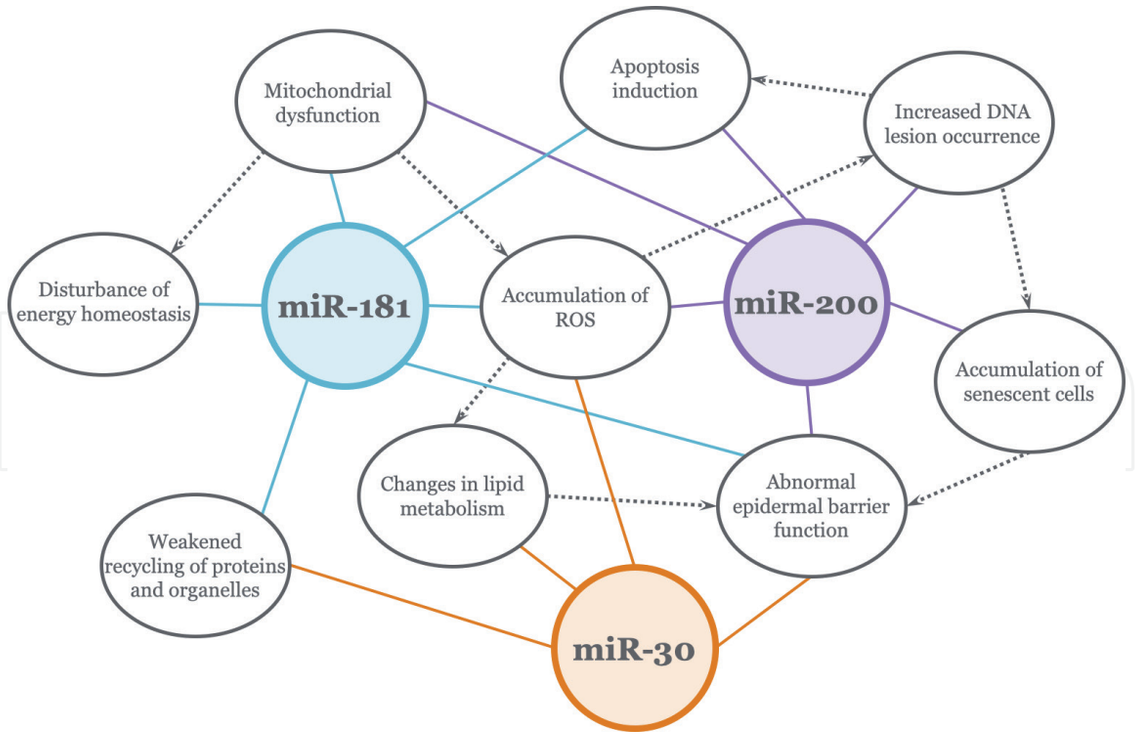

Figure 2.
Features of skin aging resulting of combined miRNA action. Overexpression of miR-30, miR-181, and miR-200 family members recapitulates many of the skin aging features. Each trait is directly regulated by one, two, or three of the families presented here. Some of the alterations related to miRNA regulation will also indirectly contribute to the exacerbation of the other traits. Colored lines represent direct effect of the corresponding miRNA. Dotted gray arrows symbolize indirect action of one feature on another.

thus, we cannot totally exclude the direct contribution of the autophagy pathway in normal skin development.

The importance of the multiple autophagy pathways in skin function has been recently reviewed elsewhere [69]. The causative link between miR-30 members' induction, autophagy reduction, and tissue aging still needs to be demonstrated especially in skin for miR-30a. Toward this aim, we already showed that a reduced epidermal differentiation is correlated with an abnormal barrier function in an organotypic skin models prepared with keratinocytes artificially overexpressing miR-30a [18]. Finally, the impairment of the various modes of autophagy (aggrephagy, lipophagy and mitophagy) leads to the accumulation of protein aggregates, to the aberrant handling of lipid droplets causing changes in lipid metabolism, and to the accumulation of dysfunctional mitochondria responsible for ROS production, which are all some features of aged tissue (**Figure 2**), including skin [70]. Interestingly, the upregulation of miR-30b and miR-30d (clustered at the same locus) significantly increases by about twofold in retinal epithelial cells treated with a sublethal dose of H_2O_2, a potent inducer of ROS [71]. All of these converging data from the literature strongly suggest that the negative regulation of the autophagic pathway by miR-30 could be an important mechanism contributing to tissue aging.

3. The miR-200 family in the control of the oxidative balance

Oxidative stress results from an imbalance between the production of free radicals and their molecular scavengers aiming at the restoration of the redox equilibrium in the cell. At moderate levels, ROS induce biochemical modifications of lipids, proteins, and DNA and thus take part in the signaling cascades controlling cellular processes such as differentiation or trafficking of intracellular vesicles. However,

a sustained excessive concentration of ROS will disturb the cellular homeostasis and eventually induces cell death or senescence. Every day, parts of the skin are directly exposed to solar radiations that induce the production of ROS. Since keratinocytes are exposed to both UV-A and UV-B, they display a powerful antioxidant system and an efficient DNA repair machinery compared to dermal fibroblasts.

Since the epidermis turnover takes about a month, even a little decline in this ROS scavenging mechanism will contrariwise strongly affect keratinocytes homeostasis and leads to a progressive accumulation of senescent cells that no longer participate in the regenerative process. Indeed, the steady-state ROS levels were found to be 2.6-fold higher in primary human keratinocytes from old donors (60–82 years) compared to young ones (2–45 years) [72]. This was correlated with a particular increase in 8-hydroxy-2′-deoxyguanosine (8-OH-dG) residues, a highly mutagenic DNA lesion leading to the transversion of GC to TA upon replication by a DNA polymerase [72, 73].

Consistently, a previous report has shown that about 3% of proliferating human keratinocytes contain these oxidized guanines versus 19% in senescent human keratinocytes [74]. The 8-oxoguanine DNA glycosylase (OGG1) is a key enzyme coordinating the removal of 8-OH-dG lesions by catalyzing the first step of the repair process (**Figure 1**), and its expression level is significantly decreased with aging in keratinocytes [72]. *In silico* analysis indicated that *OGG1* was a potential target of miR-33a and miR-200a, with respectively two putative seed sequences and one putative seed sequence in its 3′UTR. In primary keratinocytes from human elderly donors, only miR-200a was strongly upregulated, whereas miR-33a was downregulated, suggesting that only miR-200a was responsible for *OGG1* lower expression [72]. This prediction was confirmed as overexpression of miR-200a-3p mimics by transient transfection reduced both endogenous *OGG1* expression and luciferase activity derived from a reporter plasmid exhibiting the *OGG1* 3′UTR sequence. Concomitantly with OGG1 downregulation, a significant increase of the senescence marker CDKN2A (*aka* P16INK4) was observed, thus showing a direct link between the redox balance and senescence in aged keratinocytes (**Figure 2**).

To note, we have not observed a differential expression of miR-200a-3p in our own miRNA sequencing between keratinocytes from young (3–6 years), adult (20–40 years), and elderly (60–71 years) human skin samples [18]. This difference may come from the differential segregation of young and old keratinocytes in the two studies and/or from the anatomical location of the biopsies as sun-exposed or photo-protected areas would present a different pattern of expression. Nevertheless, the miR-200 family is composed of five members, including miR-141, miR-200a, miR-200b, miR-200c, and miR-429 (**Table 1**), and we effectively detected a significant twofold increase in miR-200c-5p levels in both adult and aged keratinocytes as compared to the young cells from photo-protected skin biopsies [18]. The functional significance of the elevated level of expression of the so-called passenger strand still needs to be depicted. Recently, a study on miR-122 has opened up a new perspective in the miRNA field as it showed that the passenger strand is not always an innocent bystander but could also cooperates with the guide strand to achieve the same function through different mechanisms [75]. In agreement with our miRNome analysis in aged keratinocytes, miR-200c-3p was increased by about threefold in aged skin fibroblasts (65–80 years) as compared to young ones (4–6 years), isolated from skin biopsies protected from sun exposition [76]. Thus, overexpression of miR-200 family members cannot be solely interrelated to photo-aging but also to intrinsic chronological aging.

Interestingly, the existence of a crosstalk between the miR-200 family and oxidative stress has been investigated in other physiological contexts. Oxidative stress induced by H_2O_2 treatment in normal liver cells or normal endothelial cells

triggers progressive overexpression of all of the five members, with discrepancy among them [77, 78]. Even if the five members of the miR-200 family are divided into two clusters, namely miR-200a/miR-200b/miR-429 on chromosome 1 and miR-200c/miR-141 on chromosome 12, it has been reported that the promoters of both miR-200 clusters comprise TP53-binding sites and that all of the three transcription factors TP53/TP63/TP73 are able to activate the transcription of the miR-200 family members [77–80]. MiR-200c-3p has been shown to target *SIRT1*, *FOXO1,* and *ZEB1*, all of them being downmodulated in aged skin cells [76, 78, 81]. A complex regulatory loop exists between *SIRT1* and *FOXO1* as the latter is a direct target of the deacetylase SIRT1, and at the same time, FOXO transcription factors are regulating *SIRT1* expression. MiR-200c-3p directly targets both *SIRT1* and *FOXO1* (**Figure 1**) [76]. Thus, by decreasing SIRT1 level, FOXO transcription factors become hyperacetylated, which in turn provokes their detachment from *SIRT1* promoter, thus further decreasing *SIRT1* expression [82]. FOXO1 hyperacetylation similarly decreases the expression of the ROS scavenger catalase (CAT) and superoxide dismutase 2 (SOD2) [83, 84].

Another consequence of miR-200c-induced *SIRT1* downregulation is the increased acetylation of TP53, a post-translational modification associated to apoptosis induction via a *TP53* transcription-independent pathway [85]. The overexpression of miR-200c-3p effectively enhances apoptotic DNA fragmentation and increases the percentage of senescent cells together with overexpression of CDKN1A (*aka* p21 WAF1/CIP1) [78]. Finally, miR-200c is also targeting the transcription factor ZEB1, which has been recently associated to ROS-induced senescence in human dermal fibroblast [86]. In physiological conditions, ZEB1 positively regulates the expression of the DNA methyltransferase *DNMT1* that methylates CpG islands in the *TP53* promoter, thus decreasing the transcription rate. In this study, the authors found that *ZEB1* expression is strongly repressed by elevated ROS levels but they are still interrogating the mechanistic relationship between ROS and *ZEB1* expression. One can speculate that miR-200c-3p overexpression during oxidative stress is one part of the answer.

Moreover, miR-200a-3p is regulating the ROS-stress response signaling by targeting the MAP kinase p38 alpha (*aka* MAPK14), which normally activates the expression of *NRF2* (*aka NFE2L2*). NRF2 is a well-known master regulator of adaptive protection against oxidative stress in cells and especially in keratinocytes [87, 88]. Indeed, a gradient of *Nrf2* expression was spotted in the murine epidermis, with higher levels of Nrf2 in the suprabasal differentiated cells and lower levels in the proliferating basal cells [89]. The gradient of *Nrf2* expression and activity is crucial for long-term epidermis homeostasis. In one hand, high concentrations of Nrf2 will establish a safeguard for suprabasal keratinocytes daily assaulted by pollutants and radiations, thus maintaining the skin functional integrity. On the other hand, low concentrations of Nrf2 will preferentially orient basal transit amplifying keratinocytes toward apoptosis under stress conditions, which is imperative for the elimination of mutated stem/progenitor cells and potential malignant transformation. Furthermore, it has been demonstrated that NRF2 also improves human keratinocyte differentiation *in vitro* by increasing the expression of Keratin-10 and Loricrin, even if the underlying mechanism has not been addressed yet [90]. Finally, the inhibition of MAPK14 signaling targeted by miR-200a-3p triggers a lack of NRF2, which will directly affect keratinocyte differentiation together with an accumulation of ROS and the generation of mitochondrial injury resulting in cell death [77, 90]. A defective keratinocyte differentiation program and an increased keratinocyte apoptosis are two hallmarks of epidermis aging that may fit with the consequences of miR-200 overexpression (**Figure 2**).

4. The miR-181 family in the control of mitochondria homeostasis

In our microarray approach aiming at identifying modulated miRNA with epidermis aging, we found out that three members of the miR-181 family were significantly upregulated, namely miR-181-a (fold change 1.61), miR-181-b (fold change 1.54), and miR-181-d (fold change 2.40). Two of which were previously associated with keratinocytes replicative senescence: miR-181a (fold change 1.30) and miR-181b (fold change 1.38) and with human skin aging as well, although this latter result was not statistically significant in this particular study [81]. Additionally, miR-181a was also found to be tightly related to human dermal fibroblasts senescence [91], making it a consistent miRNA associated with skin aging. The miR-181a and miR-181b are two intronic clustered miRNAs existing in double copies on chromosome 1 (miR-181a-1 and miR-181b-1) and on chromosome 9 (miR-181a-2 and miR-181b-2), whereas the miR-181c and miR-181d constitute a third cluster on chromosome 19 (**Table 1**).

Multiple targets have been identified for the miR-181, including SIRT1, a key regulator of cell survival in the context of oxidative stress. The essential crosstalk between oxidative stress and SIRT1 has been fully reviewed elsewhere [92]. As discussed previously, SIRT1 deacetylates the FOXO transcription factors and subsequently stimulates the expression of antioxidants. In addition, the SIRT1-FOXO axis is also involved in autophagy induction. SIRT1 promotes the activation of FOXO transcription factors that positively regulate the expression of several autophagy-related genes such as *ULK1*, *MAP1LC3A/B*, *GABARAPL1*, *ATG12*, and *BNIP3* [93–97]. Moreover, SIRT1 directly deacetylates the proteins ATG5, ATG7, and ATG8, thus controlling the dynamic of protein interaction and assembly requisite in the progression of the autophagic flux [98]. The importance of SIRT1 in regulating the autophagic flux was also demonstrated *in vivo* with a knockout mouse model. Indeed, the $Sirt1^{-/-}$ mice partially resemble the $Atg5^{-/-}$ mice, including the accumulation of damaged organelles and notably atypically shaped mitochondria, disturbance in energy homeostasis, and early perinatal mortality [98].

The disruption of the mitochondrial function is also retrieved when miR-181 are overexpressed, independently of *Sirt1* expression. Indeed, miR-181 additionally targets several members of the BCL2 family: BCL2 and MCL1, two major antiapoptotic effectors, and to a lesser extent the proapoptotic effector BIM (**Figure 1**) [99]. BCL2 is the most famous member of the family, and it has a role in almost all the main pathways governing cell aging. First, BCL2 promotes longevity by favoring the antiapoptotic signaling [100]. Second, BCL2 has an antioxidant function as it relocates glutathione to the mitochondrial membrane [101]. Third, BCL2 inhibits starvation-induced autophagy both *in vitro* and *in vivo* by binding to BECN1. Importantly, only BCL2 proteins localized at the endoplasmic reticulum present an inhibitory effect on starvation-induced autophagy, whereas BCL2 proteins localized at the mitochondrial membrane do not play a role in this process [102]. Likewise, MCL1 has been shown to regulate the balance between apoptosis and autophagy under stress conditions [103]. Thus, the miR-181 family seems to finely control the cell fate by favoring the cell death via apoptosis over the cell survival through autophagy within oxidative environment.

Remarkably, consistent enriched expression of miR-181 is found in mitochondria across different cell models even though these miRNAs are not encoded in the mitochondrial genome but come from the nucleus [104, 105]. The precise subcellular localization of particular miRNAs at the mitochondria led to the classification of miRNAs such as "mitomiRs," a group of approximately 60 members [106]. The miR-181a is one of the most consistent—if not the most consistent—mitomiR. Since mitochondria play a key role in the aging process, it is reasonable

to assume that mitomiRs disrupt gene regulatory networks eventually contributing to tissue decline with aging. Very recently, a seminal study has demonstrated that miR-181a/b is controlling a group of elemental genes for mitochondrial biogenesis and function [107]. NRF1, a master regulator of mitochondrial biogenesis; the cytochrome *c* oxidase assembly protein COX11 and the coenzyme Q-binding protein COQ10B, two actors of the mitochondrial respiratory chain assembly; and the thioredoxin-dependent peroxide reductase PRDX3, another potent ROS scavenger, are newly validated direct targets of miR-181a/b (**Figure 1**). In accordance, the inactivation of miR-181a/b stimulates both the mitochondrial biogenesis and activity in a knock out mouse model.

In order to keep up with redox equilibrium, the cell has to maintain a perfectly tuned balance between mitochondrial biogenesis and its recycling. The elimination of defective mitochondria is mediated by the BCL2-related outer membrane protein BNIP3L/NIX, which contains a conserved LC3-binding motif and acts as a receptor for addressing damaged mitochondria to autophagosomes, which then deliver the organelle to lysosomes for degradation and recycling. A recent *in vitro* study has shown that transient transfection of miR-181a decreases the colocalization of mitochondria with lysosomes after drug-induced mitochondria depolarization [108]. Under stress conditions, the depolarization of the mitochondria switches the localization of the PTEN-induced serine/threonine kinase 1 (PINK1) from the inner membrane to the outer membrane where it quickly accumulates, flagging the damaged organelle for elimination. From the outer mitochondrial membrane, PINK1 phosphorylates the cytosolic Parkin (PRKN) ubiquitin ligase, which in turn is recruited to the mitochondria (**Figure 1**). The addition of ubiquitin chains on several outer membrane mitochondrial proteins establishes a signal for the selective autophagic removal of the mitochondria, a process called mitophagy [109]. Interestingly enough, the TargetScan prediction algorithm shows no conserved putative binding site in the 3′UTR sequence of *PINK1* mRNA, whereas only one putative miR-181 binding site is present in the 3′UTR sequence of *PRKN*. The direct targeting of *PRKN* by miR-181a was demonstrated *in vitro* both at the mRNA level and the protein level [108]. This inhibition of *PRKN* by miR-181a was further associated to a substantial inhibition of the entire mitophagy process.

Accumulating compromised mitochondria will ultimately lead to the failure of the respiratory chain to produce ATP and will alongside generate even more ROS affecting in cascade the global cell homeostasis. A positive feedback loop may exist between miR-181 and oxidative stress since a recent study has shown that H_2O_2 was able to boost the expression of miR-181a, probably through the activation of the NF-κB signaling [110]. Altogether, these data show that miR-181 overexpression observed with skin aging would exert a deleterious effect by simultaneously preventing mitochondrial turnover and overactivation of cell death through apoptosis (**Figure 2**). However, it is to be noted that *miR-181a/b*$^{-/-}$ mouse model shows normal lifespan, with no apparent skin defects and thus cannot solely recapitulate the aging process [111].

5. Circulating miRNAs in the spreading of the aging message

MiRNAs have been found in all biological fluids such as blood, saliva, urine, or breast milk. These circulating miRNAs are very stable as they are protected from RNAse degradation thanks to a packaging into extracellular vesicles (EVs) made of a lipid bilayer [112, 113] or through complexing with carrier proteins [114, 115]. Indeed, an important part of circulating miRNAs is bound to the argonaute (AGO) proteins and is released in the extracellular compartment after cell death [116].

As AGO proteins are very stable in the presence of both RNAses and proteases, conjugated miRNAs are secured [117]. EVs are classically divided into three different groups: (1) apoptotic bodies, with a diameter comprised between 1 and 5 μm, (2) microvesicles, formed by direct budding of the plasma membrane and ranging in size from 0.1 to 1 μm, and (3) exosomes, the smaller EVs derived from an endosomal origin with a diameter ranging between 30 and 150 nm (**Figure 1**). They represent a new class of paracrine factors mediating cell-to-cell communication [118]. They transfer a complex signal to more or less distant recipient cells through their composite cargo, including proteins, mRNAs, lipids, noncoding RNAs and particularly miRNAs, thus modulating their behavior [112, 118, 119]. Interestingly, miRNAs enclosed in exosomes do not necessarily reflect their relative abundance in the parent cell, indicating that the exosomal miRNA loading occurs through a selective sorting [120, 121]. This allows a controlled released of particular messages to the recipient cells depending on the biological context. The intercellular communication via circulating miRNAs is likely to be involved in aging and age-related diseases. It was recently observed that human senescent dermal fibroblasts, which progressively accumulate in aging tissues [122], release more exosomes than proliferating cells [123]. In accordance, unpublished data from our group suggest that aged keratinocytes also secrete more exosomes as compared to young keratinocytes.

It is now well admitted that exosomes from senescent cells and their miRNA cargo are part of the senescent-associated secretory phenotype (SASP) [123]. Indeed, most of the miRNAs contained in exosomes are predicted to silence proapoptotic pathways and so could be involved in the propagation of senescent cells in tissue, thus greatly contributing to the aging process [122]. In parallel with an increased secretion of exosomes, two recent studies have demonstrated that immune cells are more capable of exosome uptake by internalization in older people [124, 125]. Even if the molecular mechanisms behind these observations are not understood yet, the decreased clearance of senescent cells by the innate immune system is clearly another factor enforcing tissue aging [126].

Specific circulating miRNAs have already been associated with different age-related diseases. For example, a concordance has been noticed between miR-29-3p increase in exosomes released by bone marrow mesenchymal stem cells and aging [127]. This miR-29-3p increase leads to insulin resistance in adipocytes, myocytes, and hepatocytes by downregulation of SIRT1 protein level. Insulin resistance is often developing in elderly type 2 diabetes patients. This suggests a significant role of exosomal miRNAs in aging-associated insulin resistance and represents a new therapeutic target. Additionally, circulating miR-34a in plasma is also increased during aging. This increase is even more important in age-related hearing loss patients and has been correlated with a decrease of diverse miR-34a target expression (*SIRT1*, *BCL2* and *E2F3*) in both plasma and hearing-related tissues [128]. Besides, circulating level of miR-130b was found raised with obesity, another metabolic disorder that accelerates the rate of aging by contributing to the accumulation of the pro-oxidative advanced glycation end-products and therefore shortening life span [129, 130]. The miR-130b directly regulates the expression of the master epidermis transcription factor ΔNp63, a predominant isoform of TP63, which controls the skin stem cell maintenance and longevity and which expression is decreased with skin aging [81, 131]. Interestingly, the miR-181 clustered genes, which expressions are increased with aging, are all negatively regulated by ΔNp63. If miR-130b is slightly increased with keratinocyte replicative senescence, its expression does not change in aged skin biopsies [18, 81], suggesting that the negative modulation of ΔNp63 with aging may come from exosomal release of miR-130b. Thus, the dissemination of local high concentration of miR-130b from other altered skin or body compartments with aging could lead to the increased expression of miR-181 family

members in the epidermis through miR-130b-dependent inhibition of the ΔNp63 action at the genomic loci.

In skin, few things are known concerning implication of circulating miRNAs in aging. However, several studies have demonstrated age-related changes of circulating miRNAs expression level in biological fluids [128, 132], including a decrease of miR-181a, miR-200c, and miR-30b in serum of older individuals [133, 134], one representative from each miRNA family that we focused on here. The observation that these miRNAs are increased in aged human primary keratinocytes compared to young cells [18] but decreased in the serum of elderly people well illustrates the fact that the proportion of miRNAs released by cells does not necessarily reflect the variations in parent cells. In another pathological context, the acute myocardial infarction, miR-30a, is enriched in exosome from patient serum [135]. *In vitro*, miR-30a enrichment in exosomes released by cardiomyocytes is repeated during hypoxia and leads to a reduction of autophagy in cardiomyocytes. To date, very few studies have focused on modulation of exosomes content in miRNA in aged skin cells. A recent study demonstrates that miR-23a-3p, which is enriched in exosomes released by senescent fibroblasts, has an impact on skin homeostasis. Indeed, miR-23a-3p seems to improve the migration of keratinocytes on a scratch closure assay *in vitro* and impairs keratinocytes differentiation [136]. In addition, another study has demonstrated that exosomes, and more specifically miRNA cargo, released by keratinocytes after UV-B exposure influence the activity of melanocytes [119]. The UV-B irradiation changes the exosome composition and lead to the modulation of skin pigmentation by multiple pathways.

Taken together, all of these emerging data exemplify the theory that circulating miRNAs and particularly miRNA exosome cargo play a crucial role in cell-to-cell communication. They are implicated in multiple physiological and pathological mechanisms, including aging and age-related diseases. As they are present in all the biological fluids, and more interestingly in blood, they could be used as biomarkers for various human diseases that limit lifespan. Moreover, the capability of exosomes to transfer information and affect the behavior of distant cells is very interesting for the development of new therapies. In skin context, the exosomes of the different cell types and their roles in skin homeostasis are not really described for the moment. It could be also interesting to consider if some miRNAs implicated in the complementary aging processes, as the ones we described here, are present in exosomes derived from skin cells and how they could affect some gene regulatory networks in recipient cells.

6. Conclusion

Many miRNAs have been already described in the skin to be involved in either keratinocytes or fibroblasts senescence [137]. However, cellular senescence is only one parameter contributing to tissue aging. In this chapter, we described how miRNAs could drive tissue decline with aging, by regulating complex gene regulatory networks with a special focus on autophagy, oxidative stress, and mitochondria homeostasis. Indeed, since one miRNA is targeting multiple effectors at the same time, a dynamic buffering of inter-related pathways will ultimately tip the balance toward a cell fate or another. Here, we tend to demonstrate that some miRNAs are consistently found to be indirectly or directly associated to diverse mechanisms of aging, namely the members of the miR-30, miR-200 and miR-181 family. Interestingly, the three cellular processes detailed in here are closely nested. Autophagy is crucial to remove havocs due to oxidative stress, including damaged mitochondria. Mitochondria are also a direct source of ROS, so maintaining the

homeostasis of this particular organelle ensures a good oxidative equilibrium. One particular finding when we gather different studies from the literature is the fact that the three miRNA families described here, miR-30, miR-200, and miR-181, are all upregulated following an oxidative stress. Since all of them could also worsen this oxidative stress by acting on different pathways, it is not clear what is the cause and what is the consequence so far. Finally, miRNAs are clearly exported out of the parent cell and could possibly diffuse into the whole body. According to all of the emerging data exposed in this chapter, it appears quite clear that circulating miRNAs have a central role in the propagation of functional defects associated to tissue aging. A central fundamental question still remains to date: What is driving in a first place the modulation of miRNAs expression with aging?

Conflict of interest

The authors declare no conflict of interest.

Notes

All of the artworks used in **Figure 1** were adapted from the illustration bank of Servier Medical Art (https://smart.servier.com) provided by Les Laboratoires Servier under a Creative Commons Attribution 3.0 Unported License.

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