MicroRNAs and their roles in wound repair and regeneration

Stuart J Mills & Allison J Cowin

ABSTRACT

Areas of non-coding DNA, originally termed "junk DNA", contain sequences for small non-coding RNAs, which when processed into smaller products called microRNAs (miRNAs), facilitate a high level of control on gene transcription and protein production. MiRNAs have been identified in the skin controlling processes such as cell proliferation, differentiation and apoptosis, and as such they have been shown to have important roles in skin morphogenesis, wound healing and regeneration. They can exert control by up-regulating as well as down-regulating specific processes. Additionally, there is a high degree of complexity with miRNAs with one miRNA capable of controlling many genes and more than one miRNA per gene so they can potentially offer a broad treatment therapy rather than a single gene targeting approach. Thus, miRNAs provide an interesting and exciting opportunity to develop new therapeutics and for furthering our understanding of the regulatory control in disease. Here we review the current knowledge of miRNA expression and function in the skin and how miRNAs may modulate wound repair and tissue regeneration.

INTRODUCTION

It was originally estimated that there were between 35,000 and 120,000 encoding genes within the DNA; however, studies revised this number down to 20,000–25,000 and it was suggested that a high level of control exists to regulate gene expression in this lower than expected number of genes[1]. Non-coding RNAs, in particular microRNAs (miRNAs), have been proposed as one mechanism for regulating gene expression[1-3]. Approximately 1000 miRNAs exist in human cells, which regulate around 30% of the genes[2]. MiRNAs are a novel class of endogenous, single-stranded RNAs, 19–24 nucleotides in length, which regulate development (stem cell proliferation, cardiac and skeletal muscle development, neurogenesis and haematopoiesis)[1-4], cell differentiation, proliferation, apoptosis and are involved in several other processes including insulin secretion, cholesterol metabolism, the immune response and heart disease[4]. MiRNAs control gene expression on a variety of levels and through several mechanisms including altering chromatin structure, epigenetic memory, transcription, RNA splicing, editing, translation and turnover[3]. Various skin defects including altered skin organisation have been observed when miRNA function has been perturbed[3]. This has led to the identification of a number of miRNAs that play a significant role in wound healing and regeneration[3,5].

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THE DISCOVERY OF miRNAs

The first hints of the influence of RNA on gene transcription were seen in experiments carried out by two groups looking to increase the purple pigment in petunias[6,7]. They discovered that over-expression of the pigment synthesis enzyme in the flower, instead of increasing the purple pigmentation, actually introduced "co-suppression" (gene silencing/RNA interference) and predominantly white flowers were produced[6,7]. Subsequent studies in *Caenorhabditis elegans* (*C. elegans*) and *Drosophila Melanogaster* showed that this post-transcriptional gene silencing also occurred in other organisms in a similar manner[8].

It was later found that, although sense and antisense single-stranded RNA (ssRNA) could silence gene expression, double-stranded RNA (dsRNA) actually triggered mRNA destruction with its sequence determining which mRNA was targeted[8]. Fire and Mello are credited with discovering miRNAs and were awarded the Nobel Prize for this work. The first miRNA found was lin-4 (Figure 1), discovered by Lee *et al.* (1993)[9] in *C. elegans* and the second was let-7[10].

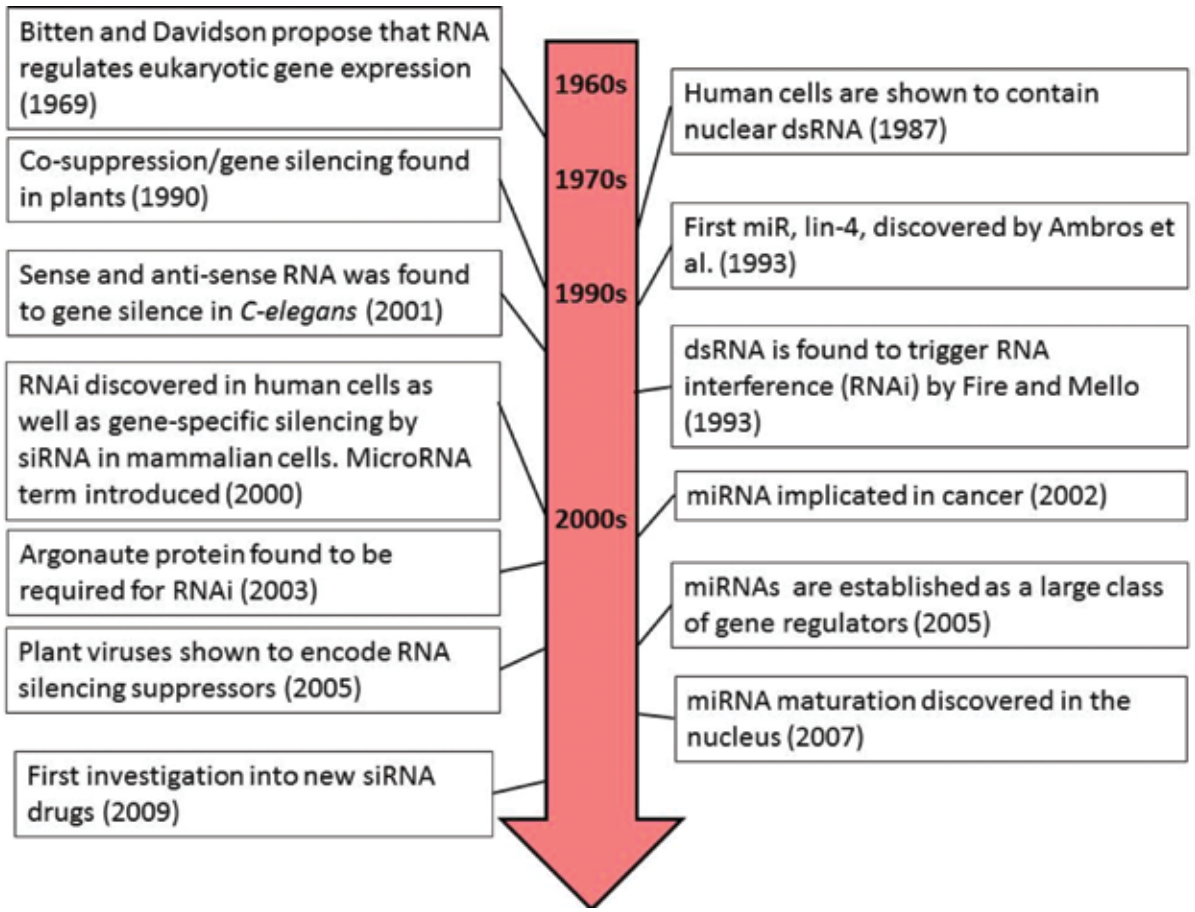

Figure 1: Time line of miRNA discoveries.

FORMATION OF miRNAs

Primary miRNAs (pri-miRs) are formed from RNA polymerase II transcription and are often hundreds to thousands of nucleotides long[1]. They contain a 5' 7-methyl guanoside cap and a 3' poly-A-tail. The first cleavage of the pri-miRs is initiated by Drosha, a dsRNA nuclear RNase III, which is a specific endonuclease that introduces staggered cuts on each strand of the RNA helix. This results in the formation of a 70-nucleotide stem-loop (hairpin) called precursor miRNA (pre-miRs). Drosha also requires a cofactor, DiGeorge Syndrome Critical Region Gene 8 (DGCR8), as alone it has no RNase activity[1]. The primary and secondary structures of the pre-miR are conserved to allow enzymatic processing leading to the formation of mature miRNAs. It is thought that RanGTP is required for this process (Figure 2). The pre-miR is then cleaved by Dicer, a multi-domain ribonuclease, to produce the 19-24 nucleotide mature small dsRNA – microRNA. This works in conjunction with a dsRNA tar-binding protein (TRBP) in humans[11,12]. Interestingly, inhibition of Dicer does not lead to a build up of all pri-miRs suggesting further levels of regulation exist[12]. The miRNA is then incorporated into the RNA-induced silencing complex (RISC) and is catalysed by the Argonaute protein. The interaction occurs at the 3'UTR of the mRNA and a matching 5'UTR of the miRNA called a seed element. The RISC and Argonaute protein are essential to this process and are located in P-bodies in the cytoplasm[1].

ACTION OF miRNAs

One miRNA can potentially regulate hundreds of genes and one gene can be regulated by more than one miRNA[4]. MiRNAs can be found in the introns of non-coding protein genes or introns and exons of coding RNA genes, thus miRNAs are under the transcriptional regulation of the host gene. This suggests that there are three transcriptional regulatory mechanisms for miRNAs; (i) intronic where they are encoded in the gene transcript precursors and share the same promoter, (ii) intergenic are located in the non-coding regions transcribed unidentified promoters and (iii) polycistronic which are derived from primary transcripts containing multiple hairpins with different hairpins giving rise to different miRNAs[1]. Clearly miRNA expression is itself tightly controlled and adds an additional level of complexity to the regulation of gene transcription.

MiRNAs bind to a complementary 'seed sequence', which tends to be in the 3'UTR of the target RNA. If there is a high degree of complementarity the miRNA can cleave the target RNA or if there is limited complementarity translation, is inhibited by mRNA decapping and/or deadenylation[13]. In this respect miRNAs are different to small interfering RNAs (siRNA) as there does not have to be exact sequence complementation to the binding RNA and they can bind within exons unlike siRNA.

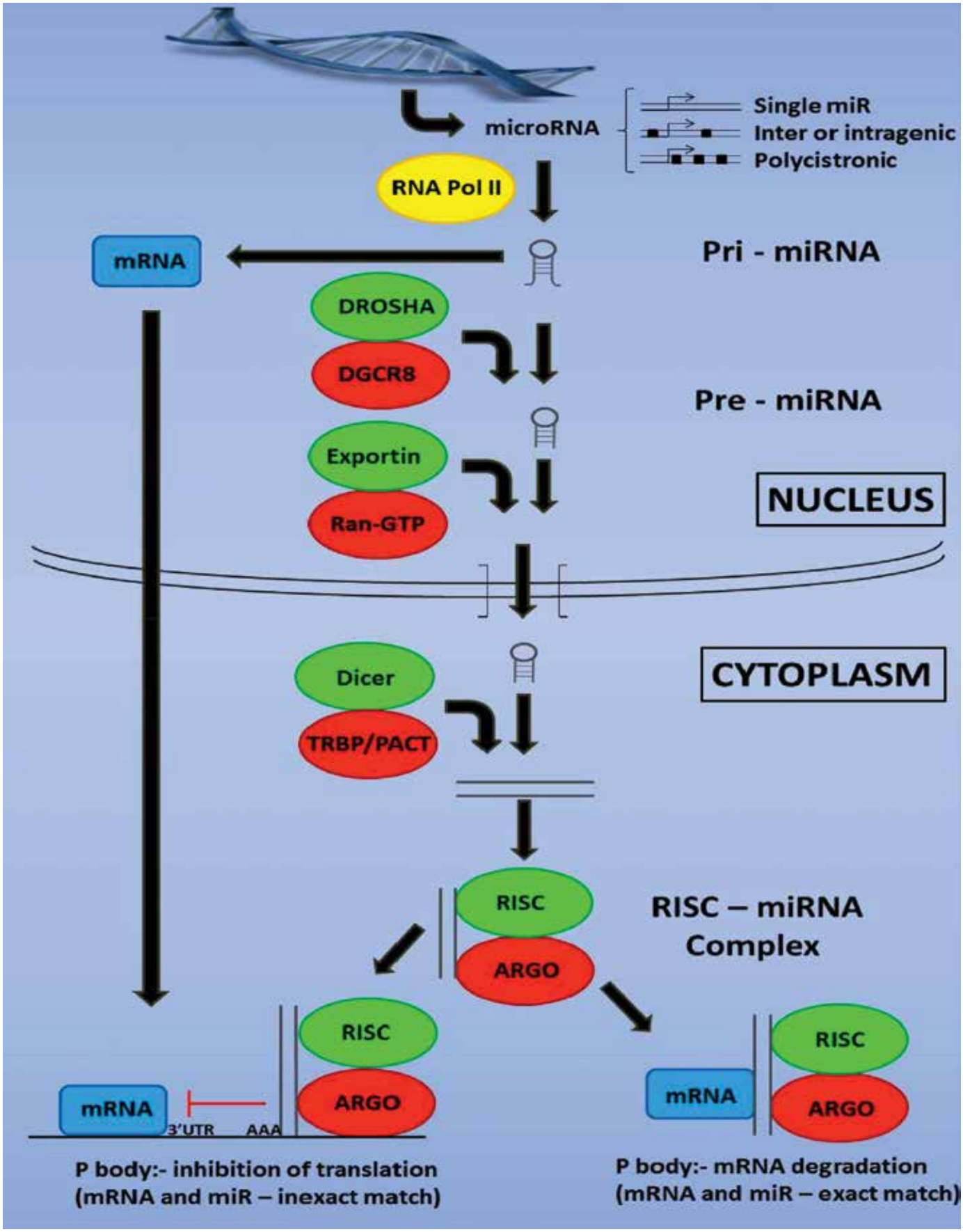

Figure 2: Overview of miRNA formation.

There are several potential ways that miRNAs can contribute to disease; (i) a miRNA can obtain a mutation resulting in a loss of function or copy number, (ii) a miRNA may obtain a mutation resulting in a gain of function or an increase in copy number, (iii) a target site may acquire a mutation and the miRNA no longer be able to bind, and (iv) a gene may acquire a new and undesired miRNA target sequence that will result in splicing. MiRNAs can also arise synthetically caused by virus insertions into the DNA.

MiRNAs AND SKIN MORPHOGENESIS

The formation and maintenance of the skin is a tightly regulated process involving the proliferation and differentiation of skin stem cells. Once the skin is formed, cells, such as keratinocytes, fibroblasts, melanocytes and dendritic cells, continually proliferate and act to maintain the appropriate barrier function and regulatory mechanisms of the skin. Any perturbation of this process can lead to a range of skin conditions or disruptions in processes such as wound healing.

In 2006 Yi *et al.*[12] created a mouse line that contained a conditional knockout of the Dicer gene to discover how its ablation affected embryonic skin progenitors. Initially they investigated the expression of miRNAs in wild-type mice, in particular the differences in expression between the interfollicular epidermis and the hair follicle. They discovered that miR-122 and miR-199 were up-regulated in hair follicles but were not found in the interfollicular epidermis whereas miR-19, miR-20, miR-93, miR-129, miR-141 and miR-200s were all preferentially expressed in the interfollicular epidermis[12]. MiR-16 was most abundant in the skin and hair follicle[12]. In the Dicer knockout mice there was an inhibition of the formation of mature miRNAs in the mice. Interestingly, rRNA and tRNA expression were unaltered, suggesting that RNA metabolism was unaffected by Dicer ablation. There was also a lack of build up of certain pri-miRs suggesting another level of control on miRNA expression[12].

In the Dicer knockout mice, within the first week of birth, cell fate and differentiation were not markedly impaired and, in the interfollicular epidermis, apoptosis was not significantly up-regulated. Overall there was a slight increase in the rate of apoptosis in the Dicer knockout mice skin but this was compensated for by an increase in proliferation. Despite a complete basement membrane being formed, these mice began to lose weight 1-2 days after birth and did not survive after 4-6 days as a result of dehydration. Hair follicles in the skin evaginated into the epidermis instead of invaginating downwards into the dermis. There was also a continual proliferation of hair

Table 1: A list of miRNAs identified in skin.

MicroRNA	Cell	Action	Author
Lin-4		First miR found	10
Let-7 **Let-7b** **Let-7f**		May be involved in melanomas via regulation of N-Ras, Raf, c-myc, cyclins D1/D3, Cdk4 and cyclin A. Show some signs of metatstatic potential	58
miR-k12-11		Viral miR which targets BACH-1, FOS and LDOC-1	59, 60
miR-15		Induces apoptosis by targeting Bcl-2	51
miR-16	Mice skin	Most abundant in the skin and hair follicle; induces apoptosis by targeting Bcl-2	13, 51
miR-18		Down-regulates CTGF and up-regulates angiogenesis	52
miR-19	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle; down-regulates Tsp-1 and this up-regulates angiogenesis	13
miR-20 **miR-20a** **miR-20b**	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle; impairs angiogenesis (20a and 20b)	13, 53
miR-21	Mice; all structural and inflammatory cells	Increases fibroblast proliferation; up-regulated in psoriasis	26, 31
miR-24	Mice	Down-regulated by TGF-β1 and a modulator of fibroblastic mitosis during wound healing	54
miR-29 **miR-29b** **miR-29c**	Mice embryo	Increases in scarless healing and targeted – Smads, β-catenin and Ras (29b & 29c). Shown to regulate TGF-β3; can regulate ECM proteins (29b & 29c); up-regulated in "adult" 29b & 29c and regulate Smads, β-catenin and Ras	5, 41
miR-34a		Regulated by p53 which promoted apoptosis	61
miR-93	Skin	Preferentially expressed in interfollicular epidermis but not the hair follicle	13
miR-96		Down-regulated in melanoma cell lines	55
miR-99		May be involved in melanomas via regulation of N-Ras, Raf, c-myc, cyclins D1/D3, Cdk4 and cyclin A. Show some signs of metatstatic potential	4
miR-101	Zebrafish; salamander	Down-regulated during fin regeneration in zebrafish and salamander regeneration	40
miR-122	Skin	Expressed in hair follicle but not the interfollicular epidermis; miR-122 knock down disrupts cholesterol synthesis	1, 3, 13
miR-125 **miR-125b**	Zebrafish; mice; in all structural cells	Regulate TNF-α (125b); increases fibroblast proliferation (125b); down-regulated in psoriasis and represses TNF-α. Expressed in ectodermal origin with its highest expression in bladder, lung, prostate and colon; down-regulated in psoriasis (125b). In low levels in inflammatory cells when compared to fibroblasts, keratinocytes and melanocytes	1, 26, 31
miR-126		Up-regulated angiogenesis	53
miR-129	Skin	Preferentially expressed in interfollicular epidermis but not the hair follicle	13
miR-132	THP-1 cells	Up-regulated in response to LPS in THP-1	21
miR-133a **miR-133b**	Zebrafish; salamander	Down-regulated during fin regeneration in zebrafish and salamander regeneration	40
miR-137		Modulates microphthalmia-associated transcription factor a regulator of melanocyte cell growth, maturation, apoptosis and pigmentation	62
miR-141	Skin; colorectal cancer cells Zebrafish; salamander	Preferentially expressed in interfollicular epidermis but not the hair follicle; levels negatively correlate to SIP1 (Zeb2) and regulate cell migration and invasion; highly expressed in ovarian cancer with poor prognosis. Associated with EMT and E-Cadherin. miR-141 inhibits SIP and inhibits migration and invasion of colorectal cells; down-regulated during fin regeneration in zebrafish and salamander regeneration	13, 41

Table 1 (continued): A list of miRNAs identified in skin.

MicroRNA	Cell	Action	Author
miR-143	Mice	Increases fibroblast proliferation;	31
miR-145	THP-1 cells	Inhibition of miR-146a leads to an increase in TRAF6 and an increase in IL-6	21
miR-146 **miR-146a** **miR-146b**	Zebrafish; THP-1 cells All immune cells	Regulated TNF-α (146a); up-regulated in response to LPS (and less so to TNF-α and IL-1β) in THP-1. There are also complementary sites in the TRAF6 and IRAK1. Flagellin treatment (TLR5) also up-regulated miR-146. No effect on TLR3, 7 and 9. Inhibition of miR-146a leads to an increase in TRAF6 and an increase in IL-6; overexpressed in psoriasis and organs with a high leukocyte infiltration. Targets TRAF and IRAK1 in the TNF-pathway and is NF-kB dependent (146a). NF-kB inhibits TNF-α induced apoptosis and may regulate overexpression of 146a; up-regulated in psoriasis. 146a absent on keratinocytes and dermal fibroblasts but is in immune cells; up-regulated in melanoma cell lines	1, 4, 21, 26, 31, 55
miR-155	THP-1 cells	Up-regulated in response to LPS in THP-1 cells	56
miR-182		Down-regulated in melanoma cell lines	55
miR-183		Down-regulated in melanoma cell lines	55
miR-192	Mice embryo; mesangial cells	Increases in scarless healing and targeted – Smads, β-catenin and Ras. Inhibited by TGF-β1 and leads to fibrosis possibly via inhibition of E-cadherin; induced by TGF-β1, which enhanced collagen Iα2 by targeting SIP1 (ZEB2); down-regulated in melanoma cell lines	5, 55, 57
miR-194		Down-regulated in melanoma cell lines	55
miR-196	Salamander; zebrafish	Up-regulated after tail removal in salamanders and is implicated in the regulation of Hoxb18. Its inhibition leads to stunted tail regrowth. Msx1, BMP4 and Pax7 are up-regulated in miR196 inhibited cells; interestingly miR-196 down-regulated in zebrafish regeneration	40
miR-199	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle;	13
miR-200 **miR-200a** **miR-200b** **miR-200c**	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle (200); involved in EMT by inhibiting cell migration by targeting E-Cadherin, ZEB1, ZEB2 (SIP1) and PDGF-D; down-regulated in melanoma cell lines	13, 41, 55
miR-203	Mice epidermis; keratinocytes	Up-regulated in suprabasal cells when compared to basal cells. Represses p63 enabling differentiation; may have a role in squamous cell formation. Its upregulation in psoriasis leads to a down-regulation of SOCS-3 which leads to an increase in STAT3. Represses p63 which inhibits the proliferation of stem cell, up-regulated in psoriasis; down-regulated in melanoma cell lines	1, 4, 24, 55
miR-204		Up-regulated in melanoma cell lines	55
miR-206	HeLa cells	Has a complementary sequence to the 3'UTR of notch3 and has been shown to be down-regulated notch3 expression in HeLa cells which blocks the anti-apoptotic action of notch3	63
miR-210		Involved in hypoxia and keratinocyte proliferation. Hypoxia stabilises HIF-1α which up-regulates miR-210 this silences E2F3 gene and inhibits keratinocyte proliferation;	27
miR-211		Targets c-KIT which binds SCF this inhibits its action and leads to a reduction in VEGF, this decreases angiogenesis; influences the expression of eNOS; up-regulated in melanoma cell lines	1, 53, 55
miR-212		Targets c-KIT which binds SCF, this inhibits its action and leads to a reduction in VEGF, this decreases angiogenesis; influences the expression of eNOS	1, 53
miR-218		Is a tumour suppressor and is inactivated in colon, lung and breast cancers. Located in the SLIT2 gene	64

Table 1 (continued): A list of miRNAs identified in skin.

MicroRNA	Cell	Action	Author
miR-221		Involved in hypoxia and keratinocyte proliferation. Involved in melanocyte regulation by downregulating p27Kip1/CDKN1B and c-KIT receptor; up-regulated in melanoma cell lines	27
miR-222		Involved in hypoxia and keratinocyte proliferation.	27
miR-224		Down-regulated in melanomas	55
miR-296		Associated with an up-regulation in angiogenesis	53
miR-335		Down-regulated in melanomas	55
miR-376		Up-regulated in melanomas	55
miR-LAT	HeLa cells	Down-regulated by TGF-β and Smad3 which reduces apoptosis in HeLa cells (Bcl-2)	65

follicle cells that led to the formation of cysts, which appeared to be linked to the lack of expression of keratin 15, a specific marker for hair follicle stem cells[12]. There was also a loss of the expression of sonic hedgehog (shh) and notch1, which are key proteins to controlling cell differentiation and tissue formation. Their loss together with the loss of keratin 15, suggested that miRNAs are involved in the formation of the epidermis and also in the regulation and maintenance of hair follicle stem cells.

MiR-203 has also been linked to skin morphogenesis as it is up-regulated 25-fold at E13.5-15.5 in the suprabasal cells of mouse skin[14]. One of the targets of miR-203 is p63, which initiates epithelial stratification and maintains the proliferative potential of mature keratinocytes in the basal layers of the epidermis[14]. When stem cells in the epidermis are proliferating and differentiating into stratified epithelium miR-203 is expressed to suppress p63 activity enabling differentiation to take place[14]. These studies clearly showed that miRNAs and the proteins involved in their formation are intimately linked with the formation and maintenance of the skin (a more detailed list is shown in Table 1).

MiRNAs AND WOUND HEALING

The process of wound healing in skin is a complex process consisting of overlapping events including inflammation, re-epithelialisation, matrix deposition and remodelling, angiogenesis and often the formation of a scar. It is a tightly controlled process with the main cells involved being neutrophils, monocytes/macrophages, keratinocytes, fibroblasts and endothelial cells. Any disruption in this process, which may cause an inhibition in proliferation, differentiation or migration of any of these cells, is likely to result in problematic wound healing, such as chronic wounds and/or excessive scarring[15]. Several miRNAs have been identified that influence the wound healing process (Table 2) and these could possibly be targeted to formulate treatments to help improve healing.

MiRNAs and inflammation

Inflammation is a key phase during the wound healing process[15]. Its main functions are to remove infection by initiating an immune response and to provide an array of cytokines and growth factors to allow subsequent phases of the wound healing process to occur[15]. Inflammation has to be tightly controlled as any perturbation can result in excessive inflammatory cell recruitment resulting in delayed healing and/or the formation of chronic wounds which fail to heal[15,16]. In a study by Harris *et al.* (2008)[17] miR-126 was identified by microarray as being highly expressed in human umbilical vein endothelial cells (HUVEC). Knocking down miR-126 expression increased leukocyte adhesion to endothelial cells and allowed tumour necrosis factor alpha (TNF-α) induced expression of vascular cell adhesion molecule-1 (VCAM-1). *In vivo* this could facilitate the leukocytes to leave the blood stream and in a wound site would lead to promotion of the inflammatory response[17]. LPS induced knockdown of miR-125b has also shown to increase TNF-α expression[18], which can lead to an induction of miR-31 and miR-17-3p expression[19]. This results in an inhibition of E-selectin and intracellular adhesion molecule-1 (ICAM-1) respectively[19] (Figure 3) and reduces adhesion of neutrophils to endothelial cells. This demonstrated that microRNAs might also function in a negative feedback mechanism to regulate vascular inflammation[19].

Transforming growth factor beta (TGF-β) plays a central role in several phases of wound healing, including inflammation[15]. It is released early on in the inflammatory response from the platelets and acts as a chemoattractant for inflammatory cells such as neutrophils, monocytes and macrophages[15]. This process may be mediated by miR-128a which has been found to bind to the 3'UTR of TGFβR1. This could in turn affect the signalling pathway of TGF-β, controlled by the Smad proteins, which themselves have been show to play a role in the formation of miRNAs in the nucleus[18]. Upon reaching the wound site immune cell differentiation has also been shown to

be under the regulation of miRNAs. PU.1, a transcription factor, was found to up-regulate miR-424, which resulted in differentiation of monocytes into macrophages[18]. MiR-223 has also been shown to play a role in myeloid cell differentiation[18].

Another mediator involved in the early stages of inflammation is platelet-derived growth factor (PDGF), which is expressed by resident inflammatory cells and acts to recruit monocytes to the wound site to prevent infection. Eberhart *et al.* (2008)[20] found in zebrafish that Mirn140 inhibited the expression of PDGF-α and although this has not been confirmed in humans, it is further evidence that some miRNAs may have a negative influence on inflammation. A similar mechanism has been shown by Nakamachi *et al.* (2009)[21] who found that miR-124a could inhibit the pro-inflammatory macrophage chemoattratant protein (MCP-1), which again could dampen the inflammatory response.

MiR-21, miR-146a and miR-155 all regulate Toll-like receptors, which are crucial in recognising some types of pathogens and signalling through these receptor results in an immune response[22-24]. Nuclear factor-kappa B (NF-kB) is a product of the MyD88 dependant TLR4 signalling pathway and increases the expression of miR-146a, which then targets both IRAK1 and TRAF6. Both of these are involved in TLR4/MyD88/NF-kB signalling, which up-regulates the inflammatory response. Although it is still to be confirmed, this suggest that miR-146a could be acting as a negative regulator of the TLR4/MyD88 pathway to prevent excessive inflammation[22]. MiR-146a has also been found to target IRAK2, which is involved in TLR signalling to up-regulate interferon-gamma (IFN-γ)[18]. MiR-21 also appears to act as a negative regulator of TLR signalling by inhibiting the pro-inflammatory cytokine PDCD4, which results in reduced expression of NF-kB and an up-regulation of IL-10[18,23]. PTEN and NFkB have also been shown to be a targets of miR-21. MiR-155 expression is also increased during the macrophage inflammatory response and is under the control of the JNK pathway[25]. Interestingly, miR-155 has been shown to act in both a pro- and anti-inflammatory manner and is known to be important in lymphocyte differentiation[24,25]. When targeted by interleukin 10 (IL-10) its expression is inhibited in a STAT-3 dependant manner. This then prevents miR-155 inhibition of SHIP1 and the activation of PIP3 helping to resolve the inflammatory response[18].

IL-10 also helps to control the influx of immune cells by down-regulating pro-inflammatory cytokines allowing a resolution of the

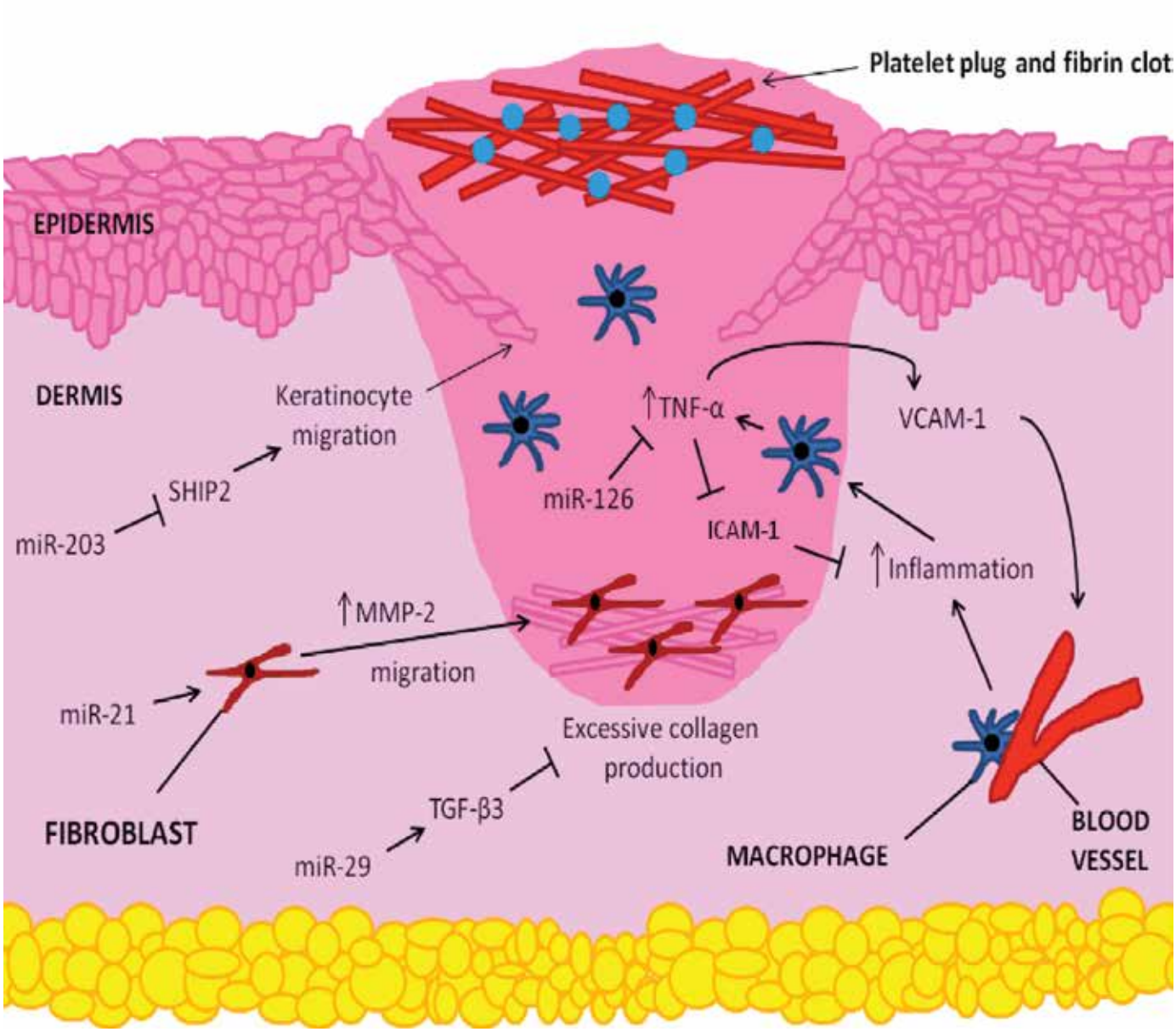

Figure 3: Schematic diagram illustrating the potential regulatory effects of miRNAs during the wound healing process.

inflammatory phase. Ma *et al.* (2010)[26] have found that miR-4661 can competitively bind to the 3'UTR of IL-10. This prevents tristetraprolin binding and degrading IL-10. This means that miR-4661 can stabilise the IL-10, giving a prolonged effect in resolving inflammation[26]. Together these studies suggest that miRNAs play an important role in several aspects of inflammation and can either act to promote or dampen the response. The negative regulation of inflammation is an important facet of the wound healing process, as unchecked inflammation can result in chronic wounds, such as diabetic ulcers[15].

MiRNAs and re-epithelialisation

Re-epithelialisation is an essential stage of wound healing as it re-establishes the epidermal integrity, limiting the risk of infection and reducing water loss through the skin. As mentioned above, miR-203 has been identified as having an important function in keratinocyte regulation[14]. Its expression is dependent on calcium levels, which when elevated led to an activation of protein kinase C/activator protein-1 (PKC/AP-1) pathway involving c-Jun and JunB. Activation of the PKC/AP-1 pathway leads to an increase in expression of miR-203 and results in the suppression of p63, allowing keratinocyte differentiation (Figure 3)[14,27]. MiR-203 is also present in psoriatic plaques and an increase in expression of miR-203 leads to a reduction in the levels of suppressor of cytokine signalling (SOCS-3), suggesting a potential role for this miRNA both in inflammation and re-epithlialisation[28].

Another miRNA involved in re-epithelialisation is miR-210[29]. Biswas *et al.*[29] using a bipedicle flap wounding model in mice showed that

induction of hypoxia led to the stabilisation of hypoxia inducible factor 1α (HIF-1α), which resulted in the up-regulation of miR-210[29]. This in turn silenced the E2F3 gene and inhibited keratinocyte proliferation at the wound edge, preventing re-epithelialisation. Another critical component of re-epithelialisation is the migration of keratinocytes, which has been found to be up-regulated by miR-205[30,31]. MiR-205 acts by inhibiting the expression of SH2-containing phosphoinositide 5-phosphatase 2 (SHIP2), which in turn up-regulates Akt signalling and leads to faster actin remodelling and migration of the keratinocytes[30,31].

Together these studies show that miRNAs can act to promote the re-epithelialisation process at various stages, including proliferation and migration and contribute to the re-formation of a mature epidermis.

MiRNAs, matrix formation and remodelling

Matrix formation and remodelling is primarily carried out by skin fibroblasts[15]. Under the influence of specific growth factors and cytokines, fibroblasts deposit collagen and remodel the matrix with the help of specific matrix metalloproteinases[32]. For this to occur resident fibroblasts proliferate and surrounding fibroblasts migrate into the wound site. Gu and Iyer[33] carried out a study to determine if miRNAs were involved in this process. They found that fibroblasts stimulated with either growth factors or serum had an increased rate of proliferation with an up-regulation of a group of miRNAs, including let-7, miR-21, miR-125b and miR-143, previously shown to be involved in cell differentiation, proliferation and apoptosis[33]. The expression of these miRNAs correlated to a regulation of the transcription factors; c-Myc, SRF, JunB, EGR2 and EGR3[33].

TGF-β is also a known regulator of matrix formation and remodelling and stimulates fibroblast migration into the wound site[15]. Once activated, the fibroblasts deposit collagen I and III throughout the wound[32]. Chen *et al.*[34] carried out an investigation to see if TGF-β1 action could be altered using miRNAs. These authors engineered four miRNAs to the chick TGF-β1 sequence and injected them into injured chick tendons and analysed expression of TGF-β1 after 1 and 6 weeks. TGF-β1 was down-regulated as was collagen III expression but collagen I synthesis was unaffected in the injured tendons that received the miRNA injections. Interestingly, Zhang *et al.*[35] using neutralising antibodies to TGF-β1, found that collagen I production was inhibited when TGF-β1 was down-regulated, suggesting that the miRNAs created by Chen *et al.* may influence other TGF-β isoforms and that different miRNAs may control collagen I and collagen III expression[34,35]. Connective tissue growth factor (CTGF) was also down-regulated in this study and this may influence scarring, as it is a downstream mediator of TGF-β induced fibrosis[34].

TGF-β isoforms have been shown to be involved in scar formation with TGF-β1 and TGF-β2 promoting scarring while TGF-β3 is anti-scarring[36]. Embryonic wounds up to late gestation heal without the formation of a scar, unlike adult healing and TGF-β is intimately involved in this process[36,37]. Cheng *et al.*[38] carried out a genome wide array on miRNAs in E16 mice (scar free healing) and E19 mice (scar forming) to investigate the scarring phenotypes. MiR-29b, miR-29c and miR-192 had the highest fold changes in Balb/C mice, with all being up-regulated at E19. These miRNAs had targets of; Smads, β-catenin and Ras[38]. Another investigation which linked miR-29 and scar forming/fibrosis was carried out by Maurer *et al.* [39] who studied fibroblasts from systemic sclerosis (SSc) patients. They discovered

Table 2: MiRNAs involved in the wound healing process.

MicroRNA	Site	Function	Studies
miR-16	Mice skin	Most abundant in the skin and hair follicle; Induces apoptosis by targeting Bcl-2	13, 51
miR-18		Down-regulates CTGF and may up-regulates angiogenesis	52
miR-19	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle; down-regulates Tsp-1 and this up-regulates angiogenesis	13
miR-20 **miR-20a** **miR-20b**	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle; impairs angiogenesis (20a and 20b)	13, 53
miR-21	Mice; all structural and inflammatory cells;	Increases fibroblast proliferation; up-regulated in psoriasis	26, 31
miR-24	Mice	Down-regulated by TGF-β1 and a modulator of matrix formation in wound healing	54
miR-29 **miR-29b** **miR-29c**	Mice embryo;	Increases in scarless healing and targeted – Smads, β-catenin and Ras (29b & 29c). Shown to regulate TGF-β3; can regulate ECM proteins (29b & 29c); up-regulated in "adult" 29b & 29c and regulate Smads, β-catenin and Ras	5, 41
miR-93	Skin	Preferentially expressed in interfollicular epidermis but not the hair follicle	13
miR-99		Maybe involved in melanomas via regulation of N-Ras, Raf, c-myc, cyclins D1/D3, Cdk4 and cyclin A. Show some signs of metatstatic potential	4
miR-101	Zebrafish; salamander	Down-regulated during fin regeneration in zebrafish and salamander regeneration	40
miR-122	Skin	Expressed in hair follicle but not the interfollicular epidermis; miR-122 knock down disrupts cholesterol synthesis	1, 3, 13
miR-125 **miR-125b**	Zebrafish; mice; in all structural cells;	Regulate TNF-α (125b); increases fibroblast proliferation (125b); down-regulated in psoriasis and represses TNF-α. Expressed in ectodermal origin with its highest expression in bladder, lung, prostate and colon; down-regulated in psoriasis (125b). In low levels in inflammatory cells when compared to fibroblasts, keratinocytes and melanocytes	1, 26, 31
miR-126		Up-regulated angiogenesis	53
miR-129	Skin	Preferentially expressed in interfollicular epidermis but not the hair follicle	13
miR-132	THP-1 cells	Up-regulated in response to LPS in THP-1	21
miR-133a **miR-133b**	Zebrafish; salamander	Down-regulated during fin regeneration in zebrafish and salamander regeneration	40
miR-141	Skin; colorectal cancer cells; Zebrafish; salamander	Preferentially expressed in interfollicular epidermis but not the hair follicle; levels negatively correlate to SIP1 (ZEB2) and regulate cell migration and invasion; highly expressed in ovarian cancer with poor prognosis. Associated with EMT and E-Cadherin. miR-141 inhibits SIP and inhibits migration and invasion of colorectal cells; down-regulated during fin regeneration in zebrafish and salamander regeneration	13, 41
miR-143	Mice	Increases fibroblast proliferation	31
miR-145	THP-1 cells	Inhibition of miR-146a leads to an increase in TRAF6 and an increase in IL-6	21
miR-146 **miR-146a** **miR-146b**	Zebrafish; THP-1 cells; All immune cells;	Regulated TNF-α (146a); up-regulated in response to LPS (and less so to TNF-α and IL-1β) in THP-1. There are also complementary sites in the TRAF6 and IRAK1. Flagellin treatment (TLR5) also up-regulated miR-146. No effect on TLR3, 7 and 9. Inhibition of miR-146a leads to an increase in TRAF6 and an increase in IL-6; overexpressed in psoriasis and organs with a high leukocyte infiltration. Targets TRAF and IRAK1 in the TNF-pathway and is NF-kB dependant (146a). NF-kB inhibits TNF-α induced apoptosis and may regulate overexpression of 146a; up-regulated in psoriasis. miR-146a absent on keratinocytes and dermal fibroblasts but is in immune cells; up-regulated in melanoma cell lines	1, 4, 21, 26, 55

Table 2 (continued): MiRNAs involved in the wound healing process.

MicroRNA	Site	Function	Studies
miR-155	THP-1 cells	Up-regulated in response to LPS in THP-1 cells	56
miR-192	Mice embryo; mesangial cells	Increases in scarless healing and targeted – Smads, β-catenin and Ras. Inhibited by TGF-β1 and leads to fibrosis possibly via inhibition of E-cadherin; induced by TGF-β1, which enhanced collagen Iα2 by targeting SIP1 (ZEB2); down-regulated in melanoma cell lines	5, 55, 57
miR-196	Salamander; zebrafish	Up-regulated after tail removal in salamanders and is implicated in the regulation of Hoxb18. Its inhibition leads to stunted tail regrowth. Msx1, BMP4 and Pax7 are up-regulated in miR-196 inhibited cells; interestingly miR-196 down-regulated in zebrafish regeneration	40
miR-199	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle	13
miR-200 **miR-200a** **miR-200b** **miR-200c**	Mice skin	Preferentially expressed in interfollicular epidermis but not the hair follicle; involved in EMT by inhibiting cell migration by targeting E-Cadherin, ZEB1, ZEB2 (SIP1) and PDGF-D; down-regulated in melanoma cell lines	13, 41, 55

that miR-29 expression was significantly reduced in SSc fibroblasts when compared to healthy controls, which led to the over-expression of collagen I and III. Other miRNAs involved in this process are miR-24, which is down-regulated by TGF-β1, and miR-29 which was further shown to regulate TGF-β3[40]. A study by Madhyastha *et al.* [41] investigated miRNA expression in the fibroblasts of diabetic mice. Madhyastha *et al.* discovered that miR-21 in particular had decreased expression in the diabetic wounds, which led to a delay in the migration of fibroblasts into the wound site. MiR-21 has also been investigated in myocardial tissue and it has been shown that it can regulate matrix metalloproteinase-2 via the pTEN pathway[42]. When miR-21 function was inhibited a reduction in MMP-2 expression was observed which limited the capacity of fibroblasts to degrade the wound matrix.

All of these studies show that miRNAs are intimately involved in matrix deposition and the remodelling phase via the control of growth factors, collagen expression and matrix remodelling enzymes.

MiRNAs AND REGENERATION

Regeneration is another important aspect of skin function. It differs from wound healing in that the newly formed skin is indistinguishable from surrounding undamaged skin. This is often seen in embryonic healing and in some higher vertebrates, such as salamanders and fish. Salamanders (for example, newts) and fish (for example, zebrafish) have a rapid initial healing of the epithelium and the establishment of an underlying area of rapid cell division within a group of progenitor cells called a blastema. Dorsal/ventral patterns are of particular importance to obtain perfect regeneration. Proteins that appear to be involved in this process are Pax6, Pax7 and Msx1/2, which are expressed during embryogenesis and regeneration. In mammals, during embryogenesis, these are expressed in the embryo once the neural tube has matured. The central nervous system is patterned along the dorsal/ventral axis by the Hox-a and Hox-b cluster genes[43]. Wnt signalling has also been shown to be important in regeneration.

Salamanders are one of the more widely studied models of regeneration due to the reformation of their tails after removal. MiR-196 is up-regulated after tail removal in salamanders and has been implicated in the regulation of Hoxb8 gene expression. MiR-196 is also up-regulated 24 hours post amputation and is expressed up to 14 days post-regeneration. Inhibition of this miRNA caused severe defects in tail outgrowth with the tails formed being consistently shorter[43]. The tails also had an aberrant terminal vesicle and spinal cord structure. Cell proliferation and the rate of apoptosis were not affected, although there was a suggestion that reduced cell proliferation was observed in the blastema[43]. Slight reductions in HoxA9 and HoxC10 expression were observed, as was markedly higher expression of Pax7, though this was restricted to the dorsal most cells. Msx1 and BMP4, all downstream signals from Pax7, were up-regulated in animals treated with the anti-miR/antagomiR for miR-196. Similarities in gene array expression in zebrafish dorsal fin regeneration and tail reformation in the salamander suggests that miR-101, miR-133 and miR-141 play important roles in tissue regeneration[43,44].

In fetal skin, wounds regenerate up to the E16 stage of gestation in mice without scar formation[45]. As mentioned earlier, expression of miR-29b and 29c is increased in E19 mice (scar forming) when compared to the E16 mice (non-scar forming). MiR-29b and 29c regulate genes such as Smads and β-catenin, both of which are involved in TGF-β signalling[44]. As both of these miRNAs have reduced expression at the non-scarring stage in mice it suggests that alternate mechanisms may be in action. Reports in the literature suggest that TGF-β is involved in wound healing in the E16 mice[45] but to what extent, which isoforms are more prominent and whether there are other mechanisms involved has yet to be completely elucidated.

MiRNAs as markers of disease

It has been suggested that miRNAs can be used as biomarkers for diseases and that simple blood screens could be used to determine if patients are at risk of different disease pathologies[5,13]. Leidinger *et al.*[46] profiled the miRNA signature of 900 melanoma patients and compared them to healthy controls. They found that there were 51 differentially regulated miRNAs between the two groups with 16 significantly deregulated miRNAs in the melanoma patients. This supports the hypothesis that the miRNA profile could be used to determine the success of any administered treatments[47]. This type of profiling does not need to be limited to the identification of different cancers, but could also be adaptable to identifying conditions such as cardiovascular disease[48], or potentially patients susceptible to chronic non healing wounds or hypertrophic scarring.

MiRNA therapies and delivery methods

Due to their mechanism of action, miRNAs can either up-regulate or down-regulate protein expression. They can also target multiple genes involved in the area of interest and therefore have much wider ranging effects and even possibly overcome existing compensatory mechanisms currently in place, which a single gene target therapy cannot always accomplish. In simple terms miRNA manipulation can be achieved in two ways; by either increasing the expression of a particular miRNA or, by blocking its action, that is, up-regulation or down-regulation of a miRNA expression. MiRNAs therefore have several advantages as therapeutic targets, unlike the traditional manipulation of a single gene of interest, which has met with limited success in the field of wound healing.

Increasing the expression of a miRNA can be achieved using mimics, which are small, double-stranded oligonucleotides. The double-stranded nature allows a ”guide” strand to be processed by RNA-induced silencing complex (RISC) to form a mature miRNA which incorporates the ”passenger” strand of the mimic[13]. These have been used with some success *in vitro* but as yet the effects are still to be replicated *in vivo*[13]. Stable expression of miRNAs can also be attained using insertion into a viral vector in a pre-miR or pri-miR form and this has been shown to have some potential by up-regulating miR-155 expression in the treatment of leukaemia and breast cancer[13].

To inhibit the action of miRNAs competitive oligonucleotides can be used to either prevent the maturation of the target miRNA or by inhibiting the mature miRNA. There are several methods available that have had some success. One of the simplest ways is to create an anti-miR or antagomiR that directly targets the mRNA of interest and is chemically modified to prevent premature breakdown. This was done by Krutzfeldt *et al.*[49] who created an antagomiR for miR-122 in the liver using a 2’-O-methyl modified, cholesterol-conjugated, single-stranded RNA oligo. This was directly injected into the mice and was shown to reduce miR-122 levels. Most of this action occurred in the liver, where most substrates are metabolised, but some miR-122 inhibition was observed in other tissues[49,50]. Other modified oligonucleotides exist including 2’-O-methoxymethyl modified oligonucleotides and locked nucleic acid (LNA) oligonucleotides[51]. LNA oligonucleotides have also been used to reduce the expression of miR-122 in non-human primates, which resulted in a decrease in plasma cholesterol levels[52]. An antagomiR to miR-126 has also been used to disrupt angiogenesis in mice[53].

MiRNA “sponges” have been used as competitive inhibitors that bind miRNA of interest near a bulge region preventing its cleavage by the RISC to form mature miRNA[13]. One of the advantages of sponges is that they utilise a heptameric seed to not only block a single miRNA but a whole family of miRNAs that may be involved in a complex process such as wound healing[13]. “Erasers” are similar to sponges but only have two copies of the complementary antisense sequence to the target miRNA. Although this can reduce their effectiveness compared to the sponges, it does limit unanticipated side effects of inhibiting large numbers of miRNAs, which may control more that the process being studied. To prevent any side effects “masks” can be used, which instead of targeting miRNAs, directly target the mature mRNA and thus prevent the miRNA/mRNA interaction.

To date only limited success has been obtained in the delivery of the miRNA to target tissues due to the difficulty in getting the miRNA across the lipid membrane. One method of doing this is to package the miRNA in liposomes or nanoparticles. Both of these methods have been used successfully in transferring miRNA into the target tissue[13]. Viruses are another alternative method of delivery either via adenovirus or lentivirus and again these methods have been used with some success[13].

CONCLUSION

MiRNA research is relatively new and as such there is still much to understand about their functions and potential usefulness as therapeutic tools. The discovery of miRNAs has opened the door to many new directions in the treatment of various conditions as wide-ranging as cardiovascular disease, cancer and chronic wound repair. As miRNAs appear to exert control in up-regulating as well as down-regulating multiple processes, they potentially offer a far greater treatment opportunity than the kind of single gene targeting approach that has been trialled to date. A disadvantage is that the miRNA of interest may play an important regulatory role in other processes in other tissues, where an alteration in its expression could be deleterious. MiRNAs also have the ability to be excellent biomarkers for the identification of conditions as well as for determining the efficacy of selected treatments. Therefore, the field of miRNAs and how it impacts on wound healing and tissue regeneration provides an interesting and exciting opportunity for the development of new therapeutic strategies and for furthering our understanding of the regulatory control that exists in the skin.

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