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Review

Restoring the Final Frontier: Exosomal MicroRNA and Cutaneous Wound Repair

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Non-healing wounds present a major healthcare challenge associated with the ageing population, the rising incidence of diabetes and the obesity epidermic. Driven by the need to expand therapeutic options for the treatment of such wounds, a large body of evidence has emerged in recent years demonstrating that microRNAs (miRNAs) modulate various aspects of cutaneous wound healing through effects on diverse cell types, including keratinocytes, fibroblasts, endothelial cells and macrophages. However, clear translational pathways for non-invasive cutaneous delivery of miRNAs to facilitate wound repair have not yet been established. The recognition that miRNAs can be actively partitioned into extracellular vesicles (EVs)—exosomes, microvesicles and apoptotic bodies—has stimulated research into the regulation, function and translational exploitation of EV-derived miRNAs both as a novel mode of intercellular signalling and as a tool for miRNA transfer to cells for therapeutic purposes. In particular, because mesenchymal stem cells (MSCs) were found to support wound healing, there is much interest in the therapeutic potential of EVs, especially exosomes, derived from these cells. In this review, we survey some of the main mesenchymal stem cells (MSCs) for which exosomal miRNAs have been evaluated in the context of skin repair, including exosomes from adipose-derived MSCs, bone MSCs, amniotic MSCs and umbilical cord MSCs. Epithelial stem cell (EPSC)-derived exosomes are also considered, from keratinocytes and epidermal stem cells. The picture that emerges from studies on exosomes from various cell types reveal they share a limited set of exosomal miRNAs enhancing wound repair. We suggest a need for direct comparison of exosomal miRNA profiles from a range of MSCs and EPSCs. The ability of exogenous exosomal miRNAs to promote healing of chronic diabetic wounds also warrants further attention in order to more fully establish their therapeutic potential.

Key words: microRNA; Extracellular Vesicles; Exosomes; Wound Healing; Skin; Stem Cells.

INTRODUCTION

Chronic wounds have emerged as a major public health challenge associated with enormous negative implications on quality of life and healthcare budgets [1, 2]. Much of the increased prevalence of chronic wounds including foot ulcers, pressure ulcers, venous ulcers and other non-healing wounds stems from the ageing population, the elevated incidence of diabetes and the obesity epidemic [1, 3]. Early estimates put the costs of managing chronic wounds in the UK at £5.3 billion annually [4] and $25 billion per year in the USA [5]. However, a recent assessment based on Medicare expenditures related to wound care spanning various ulcers, surgical wounds, traumatic wounds and, respectively, associated infections, estimated the total cost ranged from $28.1 billion at the lower end to $96.8 billion at the upper limit [6].

Cutaneous wound healing is a complex physiological process that helps preserve the integrity of the organism by restoring the skin barrier. This process is mediated by extensive cross-talk between diverse cell types including keratinocytes, fibroblasts, endothelial cells and immune cells, especially macrophages, through overlapping stages comprising inflammation, proliferation, angiogenesis, epithelialisation and remodelling [7-10]. Despite healing rates of only 30 to 56% there are only four approved therapies for chronic cutaneous wounds to date [11]. Hence, there is a clear need to develop additional therapies with improved efficacy.

A large body of evidence has emerged showing small non-coding RNAs of the microRNA (miRNA) family enhance diverse aspects of wound healing [12-22] while other miRNAs exert an inhibitory role [23-34]. However, these knowledge gains have not translated into patient benefit and only 3 trials relevant to cutaneous wound repair were returned in a search of the ClinicalTrial.gov database (condition or disease: microRNA; other terms: wound healing; searched 30th April 2021. NCT02024243; NCT03601052 NCT03603431). Further, the most efficient mechanisms for delivery of miRNA mimics or inhibitors to target cells to promote wound repair have not been established.

Extracellular vesicles (EVs), comprising exosomes (EXOs), microvesicles (MVs) and apoptotic bodies (ABs), have emerged as novel mediators of intercellular communication [35-37]. In particular, because EVs carry

diverse molecular cargoes, including miRNA, RNA, DNA, proteins and metabolites and given that miRNAs regulate wound repair, there is great interest in understanding and exploiting exosomal miRNA to enhance wound repair. In this review, we summarise the mechanisms of miRNA biogenesis and introduce EVs in some detail, with a focus on EXOs. We then review recent insights in relation to the deployment of EXOs and other EVs from various mesenchymal and epithelial stem cells to promote wound repair in the skin. Circulating blood-based EXOs are not considered within the scope of the present work.

MICRORNA BIOGENESIS AND FUNCTION: A BRIEF OVERVIEW

MicroRNAs (miRNA) are small regulatory RNA molecules that silence gene expression by post-transcriptional mechanisms that culminate in destabilisation and degradation of their messenger RNA targets [38, 39]. Canonical miRNAs are generated through a well-established mechanism that involves transcription of diverse genomic loci to yield a primary transcript that can be thousands of base pairs long [40-42]. These pri-miRNAs fold into a hairpin structure that is cleaved by a complex of DROSHA and DGCR8, also known as the Microprocessor [43], for which structural mechanisms have recently been established [44]. The resulting precursor miRNAs (pre-miRNAs) are exported from the nucleus predominantly but not exclusively by the exportin 5 (XPO5) complex [45, 46]. In the cytoplasm, the loop region of the pre-miRNA hairpin is cleaved by DICER complex leaving the duplex of the 5 prime and

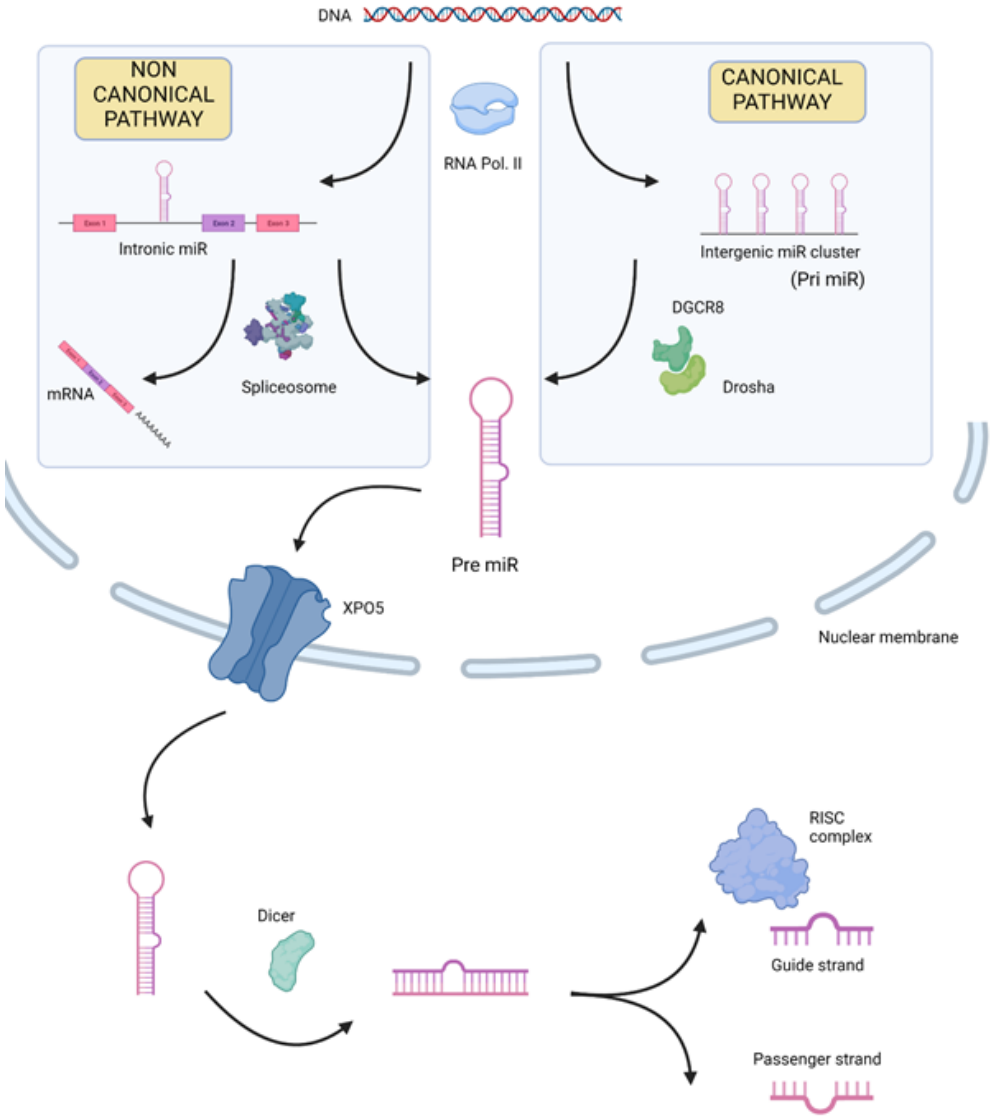

Figure 1: miRNA Biogenesis

miRNA may be produced through a canonical or a non-canonical pathway. In the canonical pathway miRNA genes, which often contain clusters of miRNAs, are transcribed by RNA Pol II to produce a primary miRNA transcript (pri-miR). This is then processed by a complex containing DROSHA and DGCR8 to yield a pre-miR consisting of a single 60-70nt stem-loop structure. In the non-canonical pathway, miRNA are encoded within the introns of mRNA and are processed by the spliceosome to produce a pre-miR. The pre-miR is exported to the cytoplasm by XPO5. Here, it is processed by DICER to produce a 21-nt double stranded RNA. One or both of these strands will then associate with AGO2 to form the mRNA-targeting RISC complex.

3 prime (-5p) and (-3p) arms. Non-canonical miRNA biogenesis occurs through multiple mechanisms [47], including a DROSHA-independent approach in which “mirtrons” are spliced out of introns and de-branched to yield a pre-miRNA [48] and a mechanism for miR-451 which depends on DROSHA but not DICER [49].

One strand of the mature duplex is loaded into the argonaute (AGO) protein to form the RNA-induced silencing complex (RISC). This “guide” strand directs the AGO to the 3’UTR of target mRNA transcripts, while the “passenger” strand is unwound and largely degraded (Figure 1). The guide and passenger strands were initially distinguished as miR and miR*, respectively. However, evidence soon emerged indicating the so-called passenger strands can persist, bind AGO and mediate gene silencing [50-53]. Therefore miRNAs are increasingly designated with -5p or -3p to indicate which arm of the duplex they arise from, although this approach fails to distinguish the dominant major strand from the less dominant or minor strand, as others have observed [54].

The choice of guide or passenger strand depends on the identity of the 5′ nucleotide of each strand and relative thermodynamic stability of the ends of the duplex [55-57], reviewed in [54]. The strand with its 5′ terminus at the end of the duplex with lower thermodynamic stability is preferentially loaded into AGO as the guide strand, with a preference for 5′ terminal UMP>AMP>CMP>GMP [56]. However, careful analysis across *C. elegans, D. melanogaster* and *H. sapiens* suggests that only half of miRNAs follow one or other of these rules, and ~20% do not follow either rule, indicating that there is complexity in the control of strand selection that has not yet been uncovered [54].

EXTRACELLULAR VESICLES: A BRIEF PRIMER

The classification of EVs into three classes EXOs, MVs and ABs reflects differences in their biogenesis (Figure 2). Exosomes are derived from the endosomal pathway and are the smallest of the three, with a size range of 40-150 nm [58-60]. Microvesicles overlap with EXOs at the lower end of their size range, ~100 nm in diameter [61], but can also be much larger, with an average reported upper end of 350 nm [61] with some reports up to approximately 1 μm [62]. Apoptotic bodies (ABs) are produced by cellular breakdown during apoptosis and are the largest class, being 1-5 μm [62].

The first step in EXO biogenesis is endocytosis of the plasma membrane to form an endosome. Invaginations of the endosome membrane result in the formation of intraluminal vesicles (ILVs) within a structure called the mutlivesicular body (MVB). The MVB can either be targeted to the lysosome for degradation of vesicle contents or can fuse with the plasma membrane to release EXOs. The proteins which regulate these processes are shared with other intracellular trafficking pathways, and as a result, very few unique markers for exosomes exist. The endosomal sorting complex required for transport (ESCRT) regulates MVB formation, recognising ubiquitinated membrane proteins and sorting

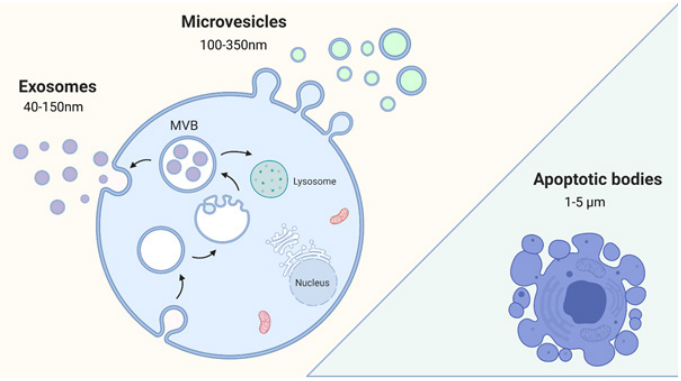

Figure 2: Biogenesis of exosomes, microvesicles and apoptotic bodies

Although overlapping in size and sharing expression of many miR and protein, microvesicles and exosomes are derived by different mechanisms. Microvesicles are derived by budding directly from the plasma membrane. Exosomes are derived from the endosomal pathway. Endocytosis results in formation of an endosome. Invagination of the endosome membrane results in formation of intraluminal vesicles within a multivesicular body (MVB). Fusion of the MVB with the plasma membrane releases exosomes. Apoptotic bodies, which are much larger than the other classes, are derived by blebbing from dying cells.

these into ILVs [63]. ESCRT-independent pathways also exist, such as those involving the tetraspanin CD63 [64], which is also the most reliable membrane epitope used to identify EXOs [62], CD81 and CD9 being also present in subsets of EXOs.

Microvesicles (MVs) were first described in platelets [65] and they are best characterised as products of various blood cell types. Other names used for MVs include shedding vesicles, microparticles and ectosomes. Microvesicles are produced by budding directly from the plasma membrane and do not arise from an intracellular precursor, but much less is known about their synthesis compared to EXOs. Annexin A1 is a unique marker for microvesicles [62]. A role in cell signalling was demonstrated when it was shown that MVs mediate horizontal transfer of a truncated, oncogenic form of the epidermal growth factor receptor (EGFR) between neighbouring cells [66].

Purification of EVs is normally achieved based on size. In a standard protocol a series of centrifugations of increasing speed is used to remove larger particles, before ultracentrifugation at 100,000g is used to pellet the smallest EVs [67]. Size exclusion chromatography is also increasingly being used. In practise it is very hard to obtain a pure population of one class or the other due to overlapping size and epitope expression and most published studies are probably describing a heterogeneous population of EVs. Furthermore, this classification may be over-simplistic and additional classes have since been described. For these reasons, current recommendations from the International Society for Extracellular Vesicles (ISEV) [68] suggest providing quantitative details on isolation methods such as

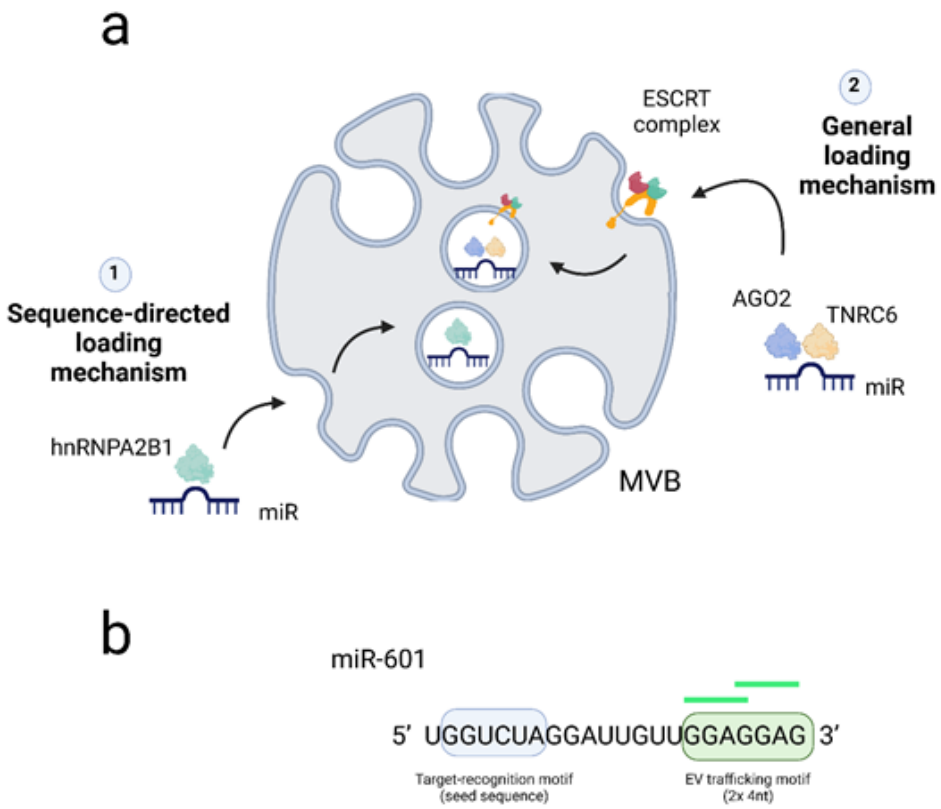

Figure 3: Potential mechanisms of miRNA trafficking into exosomes
a. *Two of the best described mechanism involve a sequence-directed mechanism by which the ribonucleoprotein hnRNPA2B1 recognises an EV trafficking motif within the miR and a general mechanism by which proteins such as TNRC6 interact with both the RISC complex (represented here by AGO2) and the ESCRT complex within the MVB membrane.* ***b.*** *miR-601 is an example of a miRNA containing a cis-acting trafficking motif located towards the 3' end. The mRNA target recognition motif is located at the 5' end.*

centrifugal force used. ISEV recommend using the general term extracellular vesicle (EV) for all particles and describing them by physical characteristics such as size (the terms small EV, medium EV and large EV may be appropriate) or epitope expression [68].

All three classes of EVs have been shown to carry RNA and to deliver this to target cells. The miRNA cargo of EXOs appears to be distinct from that of ABs and MVs, which are more similar [69]. The presence or otherwise of other nucleic acids within EVs is more controversial. For example, DNA fragments greater than 3 kb and ribosomal RNA can be eluted with EVs from size exclusion columns or co-precipitated by ultracentrifugation making it difficult to confirm the source of these molecules [70].

Interestingly, cells undergoing apoptosis are able to use ABs in order to signal to their neighbours. These vesicles can be taken up by target cells [71]. For example, in atherosclerosis vascular endothelial cells release ABs which carry miR-126 [72]. miR-126was delivered to neighbouring cells where it inhibits the negative regulator RGS16, leading to production the chemokine CXCL12, recruiting progenitor cells for repair [72]. Next generation sequencing of primary human keratinocytes identified 608 miRNAs present in ABs (for this study, defined as particles pelleting at 3000g), of which about two thirds appear to be shared with other classes of EV [69].

Larger MVs (defined in this study as particles pelleting at 25,000g but not at 3000g) from keratinocytes have been shown to transfer miR-21-5p to fibroblasts [73], as discussed below.

MECHANISMS of microRNA LOADING

A selective mechanism exists to load miRNA into EVs (Figure 3). This can be seen, for example, in analysis of activation-induced changes in miRNA expression in T lymphocytes and their EVs which demonstrated that miRNA expression changes in secreted EVs are not the same as changes in the cells themselves [74]. Therefore, the EV miRNA population is not simply a snapshot of miRNA present in the parental cell, but rather is specifically trafficked to these structures.

Several mechanisms underpinning selective miRNA loading into EVs have been demonstrated, and it seems that there may be more than one way to achieve this. It is also possible that passive loading occurs in addition to active mechanisms. Naked miRNA is quickly degraded by RNases present within the cell, and therefore all stable miRNA must be associated with protein [70]. It is these RNA-binding proteins, or proteins that interact with them, that regulate trafficking.

TABLE 1: Selection of exosomal microRNAs associated with wound healing

Source of exosomes	MicroRNA	Target cells examined	Supporting animal work	Reference
ADSC	miR-21-5p	HaCaT keratinocytes	Wounded mouse skin	[91]
ADSC electroporated with miR-21-5p	miR-21-5p	HaCaT keratinocytes	Streptozotocin-induced diabetic rat wounds	[93]
BMSC	miR-126-3p	HUVEC	Streptozotocin-induced diabetic rat wounds	[96]
hAMSCs	miR-135a-5p	BJ human fibroblast cell line	Wounded rat skin	[106]
hAECs	Small RNAs implicated but not defined	HDF	Wounded mouse skin	[108]
ucMSC	miR-21-5p	HDF	Wounded mouse skin	[112]
UCB	miR-21-3p	HDF HMEC	Wounded mouse skin	[116]
Human keratinocytes expressing a miR-21 mimic*	miR-21-5p	HDF HUVEC	Streptozotocin-induced diabetic rat wounds	[115]
Human epidermal keratinocytes	miR-21-5p	Macrophages	Various transgenic/ knockout mice	[119, 120]
Human epidermal stem cells	miR-425-5p miR-142-3p	HDF	Wounded rat skin	[127]

ADSC, adipose-derived stem cells; hAMSC, human amniotic mesenchymal stem cells; hAECs, human amniotic epithelial cells; BMSC, bone mesenchymal stem cells; HDF, human dermal fibroblasts; HMEC, human microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ucMSC, umbilical cord-derived mesenchymal stem cells; UCB, umbilical cord blood.
**Protocols used were aimed at capturing microvesicles rather than exosomes*

Specific trafficking of miRNA subsets may be regulated by a cis-acting sequence motif, recognised by RNA-binding proteins somewhat analogous to the Zipcode binding proteins of mRNA [75]. Short motifs have been identified within populations of EV enriched mRNA [76]. The heterogeneous nuclear ribonucleoproteins (hnRNPs) appear to be involved in trafficking of both mRNA and miRNA [74]. hnRNPA2B1 recognises a 21-nt sequence called RNA trafficking sequence (RTS) in neuronal trafficked mRNA [77], whereas an element of this same sequence, a shorter 4-nt motif, is present in the 3′ half of miRNA trafficked into T-cell EXOs [74]. Thus, miRNA have a cis-acting sequence motif located adjacent to the 5′ target-binding seed sequence. It is worth noting that trafficking of mRNA may be regulated through differential splicing to alter the 3′ UTR [78] whereas miRNA are not spliced, therefore any targeting motif present within a miRNA will always direct trafficking if the relevant RNA-binding protein is present.

Interestingly, TNRC6 (also known as GW182), which interacts with Argonautes in processing (P) bodies, also appears to interact with the ESCRT complex on MVBs to load miRNA into EXOs [79, 80]. Another P-body protein, YBX1, has also been shown to play a role in miRNA loading into EVs [81]. Interestingly, Argonaute proteins have been reported to be absent in EXOs by many studies [62, 79, 81], indicating that the RISC must be disassembled as miRNA are trafficked into EXOs.

RNA editing is another potential regulatory mechanism. There is evidence that miRNAs sometimes contain non-template-encoded nucleotides and this may be a mechanism for sorting. In one study, 3′ adenylation was associated with cellular miRNA while 3′ uridylation seemed to label miRNAs enriched in EVs [82].

MESENCHYMAL STEM CELL-DERIVED EXOSOMES

Exosomes from various sources have proved to be a rich source of miRNAs that can facilitate wound closure but work on mesenchymal stem cells (MSC)-derived exosomes (MSC-EXO) has outpaced work on epithelial stem cell exosomes (EPSC-EXO). Major sources of MSCs include adipose tissue, bone marrow, synovium, placenta and umbilical cord [83], and their translational utility in wound repair stems from studies showing MSC differentiation into various cell types required for tissue regeneration in wounded skin. For instance,

adipose-derived stem cells (ADSCs) differentiate into keratinocytes, fibroblasts and endothelial cells [84], while bone-marrow derived MSC (BMSC) differentiated into keratinocytes, endothelial cells and monocytes [85]. However, the limited persistence of MSCs in damaged tissue, with less than 1% of such cells remaining in the damaged tissue after 1 week, combined with the observation that conditioned media from MSC cultures have regenerative capacities that match or even exceed those of their parent cells has led to the notion that EVs rather than MSCs themselves are the main drivers of MSC-mediated wound repair [83]. We assess the use of EVs from these tissues to promote wound repair in a miRNA-dependent manner as summarised in Table 1.

Adipose-derived Mesenchymal Stem Cell Exosomes: fat cell functions beyond fat

Adipose-derived stem cells (ADSCs) are multipotent stem cells derived from the stromal vascular fraction of subcutaneous adipose tissue [86]. These cells are attractive for regenerative medicine due to their relative ease of isolation. The ability of ADSCs and EXOs thereof to promote wound healing has gained substantial support in recent years [84, 87-89]. In excisional mouse wounds, the ADSC-EXOs boosted wound closure, whether delivered by local injection or intravenous administration [90, 91]. Elevated miR-21-5p was detected at the wound edge after application, though the precise cells in which miR-21-5p was raised compared to controls were not defined [91]. Conceivably both fibroblasts and keratinocytes are target cells for ADSC-EXO miRNA applied to wounded skin, but the relative accumulation of ADSC-EXOs (or any other exosomes) and their miRNA cargoes into fibroblasts and keratinocytes when applied to (wounded) skin is unclear. Studies on cultured HDFs (human dermal fibroblasts) showed the ADSCs-EXOs were internalised by the cells, promoting migration, proliferation and collagen synthesis in a dose-dependent manner [90]. However, the miRNAs involved in these effects of ADSCs-EXOs on fibroblasts were not defined. There appears to be only limited evidence of essential roles for ADSC-EXOs miRNAs in fibroblasts at present. A recent screen identified 292 differentially expressed miRNAs in ADSC-EXOs compared to ADSC themselves and although important targets were predicted no experimental support was provided to confirm the functional significance of ADSC-EXO-derived miRNA on fibroblasts [92].

In contrast, ADSC and ADSC-EXOs promoted the migration and proliferation of HaCaT keratinocytes at least partly through miR-21-5p, and may involve miR-21-5p-dependent silencing of transforming growth factor beta (TGF-β1) expression, elevation of matrix metalloproteinase-9 (MMP-9) and repression of tissue inhibitor of metalloproteinase-2 TIMP-2 [91]. Further studies also show enhancement of wound healing by ADSC-EXOs loaded with miR-21-5p mimic. These "engineered" ADSC-EXOs promoted HaCaT keratinocyte proliferation and migration compared to native ADSC-EXOs and accelerated re-epithelialisation, angiogenesis and blood vessel maturation when applied topically to a rat diabetic wound model, thus enhancing wound closure [93]. Mechanistically, the effects of the engineered ADSC-Exos were associated with elevated expression of β-catenin, Wnt4 and MMP-7 in HaCaT keratinocytes [93]. However, the targets directly repressed by miR-21-5p to thus modulate the β-catenin, Wnt4 and MMP-7 axis were not defined nor were the mechanisms by which ADSC-Exos-miR-21-5p promotes angiogenesis explored. More importantly, head-to-head comparisons of ADSC-Exos-miR-21-5p loaded with other pro-healing miRNAs such as miR-31-5p, miR-132-3p, miR-129-5p and miR-335-5p, which have recently been reviewed [94], would help establish the relative potencies of ADSC-EXOs loaded with each of these miRNAs.

Bone Mesenchymal Stem Cells: hypoxic enhancement of exosome performance

Bone Mesenchymal Stem Cells (BMSCs) are non-haematopoietic multipotent stem cells derived from the bone marrow, and can differentiate into osteoblasts, chondrocytes, and adipocytes. Perhaps surprisingly, there is evidence that EXOs from BMSCs can stimulate proliferation of HaCaT keratinocytes and dermal fibroblasts, though a role for miRNA was not established in that work [95].

Recent studies have also shown that BMSC-derived EXOs can enhance the angiogenic behaviour of human umbilical vein endothelial cells (HUVECs), and accelerate cutaneous wound closure in diabetic rats [96]. Further, the authors examined the impact of hypoxia on BMSC-EXOs function, given that hypoxia plays keys roles in wound healing and can boost the ability of ADSC-EXOs to promote angiogenesis [97, 98]. Hypoxic BMSC-EXOs out-performed normoxic counterparts in relation to both angiogenesis by HUVECs and wound healing in diabetic rats [96]. Crucially, the pro-angiogenic miRNA miR-126-3p was elevated ~5-fold in EXOs from hypoxic BMSCs, and inhibition of miR-126-3p in HUVECs abrogated the proangiogenic impact of hypoxic BMSC-EXOs, consistent with the known roles of miR-126, [96, 99]. Elevation of exosomal miR-126-3p by direct transfection of BMSCs has also very recently been shown to enhance angiogenesis, and wound closure in mouse skin was thus accelerated [100].

Together, the above studies suggest that BMSC-derived EXOs can be exploited to accelerate cutaneous wound healing and elevation of miR-126-3p in such EXOs drives an angiogenic program to support wound repair. Questions remain, however, concerning the full spectrum of exosomal miRNAs from hypoxic BMSCs and whether the same cohort of exosomal miRNAs are consistently elevated by hypoxia treatment of diverse MSCs. The impact of hypoxia on the generation and function of exosomal miRNA for cutaneous wound healing has received limited attention, despite the prominence hypoxia-induced EVs have gained over the last decade [101].

Human Amniotic Mesenchymal Stem Cell Exosomes: exploiting placental cells for wound repair

The amniotic membrane of the placenta has long been recognised as a source of multipotent stem cells that can be accessed with limited ethical constraints [102, 103]. Amniotic epithelial cells and amniotic mesenchymal cells have gained traction as multipotent cells that can be deployed in a wide range of applications [104]. Amniotic membrane and composites thereof have been deployed in a wide range of applications associated with the repair of cutaneous, ocular, oral, gastric and urinary defects [105]. Recent studies have implicated exosomal miRNAs from both human amniotic mesenchymal stem cells (hAMSC) and human amniotic epithelial cells (hAECs), in relation to wound healing. For instance, hAMSC and exosomes thereof promoted closure of rat wounds, the hAMSC exosomes boosted migration of a human fibroblast cell line [106]. Furthermore, miR-135a-5p was found to contribute to the effects of hAMSC exosomes on wound closure and fibroblast migration, particularly when overexpressed in the hAMSC prior to isolation of exosomes [106]. The pro-healing effects of miR-135a-5p were linked to its ability to repress large tumour suppressor 2 (LATS2), a Ser/Thr kinase associated with the Hippo pathway [107]. It was not clear, however, whether miR-135a-3p, the partner strand from the pre-miR-135a duplex, was also loaded into hASMC exosomes. Feinberg and colleagues have shown that miR-135a-3p regulates wound closure, and inhibition of miR-135a-3p increased angiogenesis, granulation tissue formation wound repair in diabetic mouse wounds [32]. Therefore, it is important to determine whether the pro-healing potential of miR-135a-5p in hAMSCs exosomes is counterbalanced by putative miR-135a-3p contributions.

Interestingly, human amniotic epithelial cells (hAECs) have also been found to possess small RNAs that can support fibroblast migration and accelerate wound closure in mice [108]. However, the specific miRNAs underpinning such pro-healing activity were not defined. We also do not know at present the relationships between hAEC and hAMSC exosomes in terms of the total amount of miRNAs per exosome and the specific miRNA profiles associated with each source.

Human Umbilical Cord Mesenchymal Stem Cell Exosomes: towards scar-free wound healing

The umbilical cord and umbilical cord blood provide MSCs with distinct miRNA profiles [109],and EXOs from both have been exploited to promote wound healing. Studies have shown that umbilical cord-derived MSCs (ucMSCs) EXOs can impair the TGF-β signalling pathway and this appears to dampen fibroblast differentiation into myofibroblasts [110]. This is important because myofibroblasts are scar-forming cells that drive the overproduction of extracellular matrix (ECM) proteins associated with scarring [111]. Consistent with this, Xing and colleagues found that ucMSC-EXO appeared to promote repair of mouse wounds, with a reduction in scarring [112]. This was attributed to the ability of uMSC-EXO to prevent TGF-β-dependent transdifferentiation of fibroblasts into myofibroblasts. Crucially, using RNAseq, the authors identified 10 miRNAs that were highly expressed in ucMSC-EXO. These miRNAs appeared to be actively partitioned into EXOs because, with the exception of miR-21-5p, their levels were relatively high in the ucMSC-EXOs compared to ucMSCs [112]. Moreover, none of the 10 miRNAs identified by Xing and colleagues were enriched in an independent microarray-based assessment of miRNAs in EVs from ucMSC [113]. It is conceivable that the specific accumulation in EXOs is no longer detectable in such bulk EV analysis, lending credence to the notion of selective miRNA secretion into ucMSC-EXOs, though technical differences (RNAseq versus microarray) may be a confounding factor.

Notably, miR-21-5p was the most abundant miRNA in both ucMSC and ucMSC-EXO [112]. More importantly, miR-21-5p, miR-23a-3p, miR-125b-5p, and miR-145-5p, were all predicted and shown to target genes associated with the TGF-β/SMAD2 pathway, such as TGF-β2, TGF-βR2, and SMAD2. When transfected into HDFs, these miRNAs individually impaired TGFβ-induced activation of SMAD2 and expression of alpha smooth muscle actin (α-SMA), a hallmark of myofibroblast differentiation, [8]. On the other hand, early work had indicated that a miR-21-5p mimic boosted differentiation of (MRC-5) lung fibroblasts into myofibroblasts [114], and similar observations were recently reported in skin fibroblasts [115]. Hence it appears that while miR-21-5p can dampen TGFβ signalling to α-SMA induction, the main outcome in unstimulated fibroblasts transfected with miR-21-5p is the promotion of fibroblast:myofibroblast differentiation.

Exosomes from umbilical cord blood (UCB) have also been evaluated [116]. Injection of UCB-EXOs accelerated re-epithelialisation, angiogenesis and wound closure, with reduced scarring [116]. Of a selection of miRNAs (miR-21, miR-214, miR-126, miR-27b, miR-125b and miR-19b, both 5p and 3p strands, respectively) miR-21-3p was the most abundant in UCB-EXOs. The UCB-EXOs were internalised by endothelial cells (ECs) and HDFs, promoting migration and proliferation of these cells in a miR-21-3p dependent manner [116]. The effects of miR-21-3p on HDF and ECs were attributed to silencing of PTEN and SPRY1 as treatment with UCB-EXOs lead to depletion of these negative regulators of signalling and this was accompanied with enhanced activation of the Akt pro-survival and ERK1/2 mitogenic pathways [116]. Interestingly, very recent studies observed miR-21-3p was depleted in the serum of diabetes patients, in HDFs stimulated with high glucose and in the skin of db/db mice, where wounding further reduced miR-21-3p expression [117]. A miR-21-3p mimic enhanced HDF proliferation as well as collagen and growth factor expression and accelerated wound closure in mouse skin. Mechanistically, the effects of miR-21-3p were linked to direct repression of SPRY1 by miR-21-3p [117]. Together, these findings on miR-21-3p function in exosomal and non-exosomal contexts suggest that earlier studies of miR-21 in wound repair using pre-miR-21 or miR-21 mimic [12, 19] may reflect activity of both miR-21-5p and miR-21-3p.

EPITHELIAL STEM CELL-DERIVED EXOSOMES

Epidermal Keratinocyte Microvesicles and Exosomes: miR-21-5p

Comparative analysis of miRNA profiles from keratinocyte EXOs, MVs and ABs defined a cohort of 437 miRNAs that were shared by all three EVs [118]. Few of these have been studied specifically in relation to intercellular communication during wound healing. However, recent work by Bi and co-workers showed that, upon lentiviral overexpression in primary human keratinocytes, miR-21-5p partitions into MVs that promoted wound closure in diabetic rats [115]. Studies on cultured cells indicated the pro-healing effects of the miR-21-5p-laden MVs was associated with enhanced angiogenesis, fibroblast migration and, importantly, fibroblast differentiation into myofibroblasts [115].

Furthermore, work by Roy and colleagues showed that miR-21-5p from keratinocyte EXOs promotes the differentiation of inflammatory macrophages to fibroblast-like cells during wound repair [119]. This transdifferentiation of macrophages into fibroblast-like cells is thought to play an important role in the resolution of inflammation during wound repair and blockade of miRNA loading into EXOs impaired the resolution of wound inflammation by promoting the persistence of wound macrophages [119, 120]. Interestingly, macrophages have recently been shown to undergo a process of transdifferentiation into myofibroblasts in kidney and macular fibrosis [121-124]. Although macrophage-myofibroblast transition (MMT) has not been established in skin wounds, it is plausible that miR-21-5p functions first to support the transition of macrophages to fibroblast-like cells and then to promote differentiation of the fibroblast-like cells into myofibroblasts. This model is consistent with the observations demonstrating a role for miR-21-5p in fibroblast differentiation into myofibroblasts [114, 115]. Since, however, myofibroblasts drive the ECM production associated with scarring, it seems miR-21-5p has a complex pathophysiological role in wound healing, promoting wound repair on the one and driving myofibroblast activation on the other. This calls for careful comparison of miR-21-5p concentrations and spatiotemporal dynamics of miR-21-5p in scar versus scar-free wound healing, particularly in terms of how miR-21-5p levels affect the fine balancing act that myofibroblasts perform in mediating wound contraction on the one hand without excessive scarring on the other [125, 126].

Epidermal Stem Cell Exosomes: miR-425-5p and miR-142-3p

Beyond miR-21-5p, there is evidence for other keratinocyte-derived EXOs in the promotion of wound repair. Zhang and co-workers observed very recently that wound closure in rat skin was accelerated by EXOs reportedly from epidermal stem cells [127]. Levels of scarring were apparently reduced in the EXO-treated wound compared to EGF or PBS-treated controls, and wound repair was associated with increased angiogenesis and innervation in the wound bed [127]. Importantly, the reduced the expression of TGF-β1, SMAD2, α-SMA and collagen 1 in the healing tissue, with TGF-β1 expression also blunted in cultured HDFs [127]. These effects were tentatively attributed to miR-425-5p and miR-142-3p because these miRNAs were elevated in EXOs and repressed TGF-β1 expression HDFs [127]. However, further studies are required to determine whether these miRNAs abrogate TGF-β1-dependent differentiation of fibroblasts to myofibroblasts. Nonetheless, when combined with earlier observations on the ability of miR-21-5p to abrogate TGF-β1 signalling (see ucMSC-EXO section above), it seems that any miRNA that dampens the TGF-β1 pathway in HDFs may prevent fibroblast differentiation to myofibroblast to thus limit scarring. However, comparisons of the relative efficacy with which unrelated miRNAs can block myofibroblast activation during wound repair have not been reported to our knowledge.

CONCLUSION

With such a plethora of EXO sources, which also include endothelial progenitor cells [128, 129], it is clear that there is a need for standardisation of methods for purification and physico-chemical characterisation of EXOs [130]. Furthermore, studies that characterise and compare the exosomal miRNA profiles of the different cell types whose EXOs can repair wounded skin are required in order to establish which miRNA functions are most consistently associated with wound healing EXOs. It is not known at present if EXOs from adipose, bone marrow, amniotic, umbilical and epithelial sources are equally effective at targeting keratinocytes, fibroblasts, macrophages and endothelial cells, respectively, to modulate wound healing. Hence, there is also a need to determine the relative levels of EXOs uptake by different recipient cells in the skin, and define the underlying mechanisms associated with each recipient cell type.

In addition, the stability of EXOs and their cargoes should be studied under real-world conditions that would be associated with their translational deployment in clinical settings. Some authors point out that stem cells may contribute to tumour formation, thrombosis, and adverse immune responses, using such arguments to justify the therapeutic potential of EXOs [113, 125]. However, there is a long history of safe stem cell transplantation in the context of haematological malignancies [126] and adult MSCs appear to be safe [127]. Hence the use of MSC-derived exosomes rather than MSCs in wound healing may ultimately depend on pragmatic considerations such as the fact that they can be freeze-dried or spray-dried for deployment in cold supply chains at 4°C, which is not feasible for cells [131, 132].

Finally, although some studies have examined the wound healing potential of exosomal miRNA in diabetic rodent models, translational exploitation of these approaches will benefit from further studies using human wounded skin models. Human skin organoids used by Keswani, Feinberg and co-workers [32, 133] and *ex vivo* human skin deployed by Landén and colleagues [134] will enable deeper insight into the translational potential

of exosomal miRNAs for diabetic wound healing, especially when combined with high glucose, reactive oxygen species (ROS) generators and advanced glycation end products [135, 136]. Studies on rodents can in turn be designed mainly for evaluation of safety and biodistribution.

In summary, the evidence from the last decade has revealed that exosomal miRNAs from diverse cell types can target diverse pathways to promote cutaneous wound healing. However, our understanding of the exosomal miRNA signatures across various cells from which EXO have been obtained using standardised conditions is limited and the relative levels of EXO uptake by skin, immune and endothelial cells warrant further investigation, along with the mechanisms of uptake under conditions that mimic the diabetic microenvironment.

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