The Role of CAF derived Exosomal MicroRNAs in the Tumour Microenvironment of Melanoma

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Abstract

Exosomes play a crucial role in the crosstalk between cancer associated fibroblasts (CAFs) and cancer cells, contributing to carcinogenesis and the tumour microenvironment. Recent studies have revealed that CAFs, normal fibroblasts and cancer cells all secrete exosomes that contain miRNA, establishing a cell-cell communication network within the tumour microenvironment. For example, miRNA dysregulation in melanoma has been shown to promote CAF activation via induction of epithelial-mesenchymal transition (EMT), which in turn alters the secretory phenotype of CAFs in the stroma. This review assesses the roles of melanoma exosomal miRNAs in CAF formation and how CAF exosome-mediated feedback signalling to melanoma lead to tumour progression and metastasis. Moreover, efforts to exploit exosomal miRNA-mediated network communication between tumour cells and their microenvironment, and their potential as prognostic biomarkers or novel therapeutic targets in melanoma will also be considered.

Key words: Melanoma; Cancer Associated Fibroblasts; Exosomes; micro-RNA; Metastasis.

1.1 Melanoma

Melanoma is an aggressive type of skin cancer that manifests when melanin pigment producing melanocytes accumulate mutations most frequently due to UV-induced DNA damage¹. Whilst melanoma represents less than 5% of all skin cancers, it is responsible for the majority of deaths, mainly due to metastasis to secondary distal organs, such as the brain². Survival from American Joint Committee on Cancer (AJCC) stage IV melanoma was abysmal until the relatively recent advent of targeted (BRAF and MEK inhibitors) and checkpoint blockade. However, although early detection has enabled most people to be treated promptly for melanoma and survive, primary and secondary resistance remains a significant problem in management of disease. This is due to a diverse range of resistance mechanisms and tumour heterogeneity, highlighting the urgency to develop novel therapeutics to combat the issue of melanoma metastasis³.

1.2 Seed-soil theory for metastasis in melanoma

Melanoma cells originate in the epidermis from melanocytes but can become metastatic when tumour cells develop the ability to invade the dermis⁴. To successfully metastasise, tumour cells must be able to manipulate a local or distal environment to optimise growth and survival conditions. Paget's 'seed-soil' theory hypothesises that an environment any distance from the primary tumour can be primed to support the metastasis of the cancer cells⁵. With regard to melanoma, the secondary site whose cell population undergoes phenotypic changes, termed the tumour microenvironment (TME), is controlled by factors released by melanoma cells before they even start to invade the dermis and metastasise⁴. Once melanoma cells acquire invasive properties, they can circulate in the blood as 'seeds', which can reach the TME 'soil' prepared ahead that possesses enhanced properties to allow the melanoma to thrive⁵⁻⁶.

1.3 The tumour microenvironment in melanoma

The process of metastasis in melanoma is extremely inefficient, with a minute percentage of cancer cells disseminating from the original tumour able to successfully metastasise into a secondary tumour⁷. The likelihood of circulating tumour cells (CTCs) successfully seeding at a remote site is highly dependent on how hospitable the environment is to allow cell survival, termed the pre-metastatic niche,

in addition to the tumour cell's biological variations⁸. Although the underlying mechanisms of pre-metastatic niche formation remain poorly understood, what is known is that before cancer cells leave their primary site and enter into circulation, they prime the TME by causing non-cancerous cells within the secondary site to undergo various biochemical and physiological changes^{6,9}. This alters the phenotypes of various cell types within the stroma, such as fibroblasts and immune cells, to become pro-inflammatory (e.g. tumour-associated macrophages (TAMs)) and immunosuppressive (e.g. cancer associated fibroblasts (CAFs)), conditioning the TME for the arrival of the CTCs¹⁰.

The TME is a complex biosystem that relies on crosstalk between cancer and non-malignant cells, where the cancer's trajectory depends on the spatiotemporal communication between many different cell types⁷. The populations of non-malignant cells between melanoma patients can vary greatly, but will usually include CAFs, endothelial cells, macrophages, natural killer cells, and various T cells and B cells¹¹. Recent evidence has highlighted the contribution of CAFs to melanoma progression, metastasis and drug resistance, via both direct cell-cell interaction and secretion of transforming agents to distal cells¹².

2.1 Cancer associated fibroblasts in melanoma

One of the most abundant cell types in typical TME stroma is the fibroblast, a spindle shaped cell that secretes a variety of extracellular matrix (ECM) proteins that function to maintain connective tissue framework and aid in wound healing¹³. Normal fibroblasts (NFs) are important inhibitors of early stage melanoma development, by preventing the epithelial-mesenchymal transition (EMT) and inducing G1/S cell cycle arrest in melanoma cells¹⁴. However, over time these quiescent NFs become activated by constant signalling cues released from melanoma cells causing them to differentiate into CAFs, acquiring tumour promoting properties and physiological characteristics of myofibroblasts¹⁵. These include enhanced proliferation, and increased expression of α -smooth muscle actin (α -SMA), various pro-inflammatory cytokines and proteolytic enzymes like matrix metalloproteinases (MMPs) to promote ECM remodelling and desmoplasia to prime the pre-metastatic niche¹⁶. Although CAFs are more genetically stable than the corresponding tumour cells, they also have increased genetic and

epigenetic alterations that drive the irreversible transformation from NFs, although the specifics of this transdifferentiation process have yet to be fully elucidated¹⁷.

Studies in melanoma have found that culturing metastatic cells with NFs results in pro-inflammatory gene expression and recruitment of NFs to CAFs to meet the structural (ECM proteins) and chemical (CAF's secretome) requirements for melanoma growth¹⁸⁻¹⁹.

2.2 CAF exosomes

One of the most important mediators of pre-metastatic niche formation is the secretion of extracellular vesicles (EVs), such as exosomes from both the primary tumour cells and transformed stromal cells at the secondary site²⁰. Exosomes are a subtype of EVs, characterised by a lipid bilayer membrane extracellular structure and a small size of approximately 30-150nm, released by almost all cell types and present in all biological fluids²¹. They originate from endosomal packaging into multi-vesicular bodies (MVBs) within the cell, which can then fuse with the outer cell membrane to release the intraluminal vesicles into the extracellular space²². Exosomes carry a wide range of biologically active cargo inside and within their membranes, including proteins, lipids, metabolites and nucleic acids, which can be trafficked in circulation to be internalised by recipient cells to exert their effects²³. Therefore, exosomes have a significant role in intercellular communication between neoplastic and distal non-malignant cells, where their higher specificity to certain cell types and resistance against circulating RNAses compared to small molecules make them an ideal vehicle for bi-directional crosstalk²⁴. Tumour cells have been found to secrete larger amounts of exosomes than non-malignant cells, with altered cargo and markers, meaning exosomes isolated from cancer patient's biological fluids have the potential to act as prognostic or diagnostic markers to predict the cancer's metastatic ability and response to certain therapeutics²⁵⁻²⁶.

In addition to direct interaction with carcinoma cells and secretion of biologically active molecules, CAFs themselves secrete their own subsets of exosomes containing functional molecules that can exert pro-metastatic and angiogenic effects²⁷. This results in a crosstalk between both CAFs and cancer cells (Fig. 1), reciprocally releasing and internalising each other's exosomes to promote and maintain a

favourable TME²⁸. Whilst these exosomes contain an assortment of potentially transforming proteins, lipids and DNA, recent studies have also highlighted the importance of dysregulated micro-RNAs (miRNAs) in both the activation of CAFs and functional modulation of cancer cells²⁹⁻³⁰. In fact, it has been shown that CAFs fail to undergo significant genetic changes, raising the possibility that CAF gene expression may be controlled epigenetically, or post-transcriptionally via miRNAs³¹.

2.3 Exosomal miRNAs

MiRNAs belong to the non-coding RNA (ncRNA) family, and are short 18-24 nucleotide transcripts that generally post-transcriptionally regulate the translation of target mRNAs³². Recent studies indicate that humans transcribe ~2300 different miRNAs that act to silence gene expression by typically binding to the 3' untranslated region (UTR) of specific mRNAs via complementary base pairing³³. Target genes of miRNAs have been found to control a plethora of cellular processes, including cell cycle progression, apoptosis, migration and differentiation³⁴.

MiRNA genes are transcribed in the nucleus by RNA polymerase II or III (Fig. 2), where the primary miRNA transcripts (pri-miRNAs) undergo cleavage via a microprocessor complex composed of endonuclease Drosha and RNA binding protein DGCR8³⁵. Hairpin pre-miRNAs are then exported to the cytoplasm, where they undergo further cleavage via the endonuclease Dicer to form dsRNA miRNA duplexes. The complementary strand of the mature miRNA sequence is degraded, facilitating formation of the miRNA-induced silencing complex (RISC) with proteins such as Dicer and Argonaute 2 protein (Ago2)³⁶ that targets complementary sequences in the 3' UTR of target mRNAs to repress translation.

An intriguing aspect of miRNA packaging into EVs is how exactly this process is regulated. Several elegant reports have identified a range of accessory proteins that are required for the packaging of specific miRNA in EVs and includes proteins such as Ago2 and Y-box protein 1, which drives packaging of miR-223 in CD63-positive exosomes³⁷⁻³⁸. Moreover, bioinformatic analysis of miRNAs present in primary T lymphoblast-derived exosomes uncovered a short consensus sequence (GGAG) within a subset of packaged miRNA, termed an EXOmotif³⁹. MiRNAs harbouring EXOmotifs are recognised and bound by heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), which

regulates their loading into exosomes. Indeed, mutagenesis of exosome enriched miRNA EXOmotifs or knockdown of hnRNPA2B1 expression significantly reduced packaging of these miRNA in exosomes³⁹. Due to the abundance of RNAses in blood, naked miRNAs are rapidly degraded, and therefore require being bound to other proteins (such as RISC) or transported within EVs, such as exosomes, to exert their effects⁴⁰. It is unclear if all EV-packaged miRNAs also associate with RISC or if some mature miRNAs are packaged directly into MVBs and ultimately EVs. However, these pathways are likely not mutually exclusive, and evidence exists to support both models⁴⁰.

MiRNAs have also been found to be involved in processes typically dysregulated in cancers, such as uncontrolled proliferation, metastasis and drug resistance⁴¹⁻⁴², where stress signals have been found to regulate the production of the pri-miRNAs by controlling specific transcription factors⁴³. Tumour cells can therefore prime the pre-metastatic niche by altering their vesicular miRNA content to favour oncogenic processes, such as invasion and proliferation, within cells present at the secondary site. Moreover, activated CAFs have also been shown to possess an altered miRNA secretome that serves to maintain ideal TME conditions and fosters drug resistance and metastatic progression in the primary tumour²⁹.

3.1 Melanoma miRNA and CAF development

MiRNAs from melanoma-derived exosomes have been implicated in the activation of NFs to transform towards a CAF phenotype, where the significant changes in CAF gene expression may be due to the fact that individual miRNAs can regulate hundreds of genes¹¹. A combination of melanoma patient miRNA profiling and *in vitro* CAF studies have given great insight into some of the miRNAs differentially expressed in melanoma that influence CAF formation.

Oncogenic miRNA miR-21 has been found to be upregulated in a variety of cancers, including melanoma, where primary melanoma tissues show increased miR-21 expression when compared to benign nevi⁴⁴. Moreover, miR-21 has been shown to be implicated in metastasis and invasion in multiple cancer types due to its influence on gene expression of ECM genes, such as PTEN, a tumour suppressor gene involved in melanoma progression⁴⁵. A study by Li *et al.* demonstrated that depletion of miR-21

in NFs prevented the activation into CAFs when the fibroblasts were stimulated with TGF- β 1, a well-established CAF activator⁴⁶. Additional studies using miR-21 from malignant oesophageal and colorectal cancer cells (CRC) found that miR-21 can activate NFs to produce CAF phenotypic markers S100A4 and α -SMA, respectively⁴⁷⁻⁴⁸. Moreover, it has been shown that miR-21 levels in EVs are more abundant compared to free floating miR-21 in human liver cancer serum and plasma samples, highlighting its prevalence in EV mediated crosstalk within the TME⁴⁹.

Another established miRNA implicated in CAF activation in the melanoma TME is miRNA-211. A seminal study by Dror *et al.* found that miR-211 was upregulated in mature melanosomes released by melanoma cells, which when internalised by NFs induced CAF formation. The increased levels of miR-211 in CAFs were confirmed to be due to the melanosome delivery, and depletion of miR-211 expression levels before melanosome treatment in NFs prevented CAF formation. MiR-211 was discovered to specifically target tumour suppressor IGF2R, leading to hyper-activation of IGF1R/MAPK signalling. This resulted in an increased rate of collagen production by CAFs to produce a more contractile phenotype, as well as increased motility and proliferation⁵⁰. However, other studies have found that miR-211 is downregulated in melanoma tissue compared to healthy controls, highlighting the complex relationship this miRNA may have in melanoma progression that will require further investigation⁵¹⁻⁵². These data taken together have led to a model hypothesis, where the melanoma cells themselves have low miR-211 levels but produce melanosomes containing upregulated miRNAs such as miR-211, that have the ability to reprogram NFs within the TME³⁰. The study by Dror *et al.* report that melanosomes and exosomes share ~70% of their miRNAs, where exosomes contained unique enriched miRNAs involved in Wnt signalling, and the melanosomes contained miRNAs involved in ERB/MAPK signalling⁵⁰. Genes relating to the Wnt signalling pathway have been found to be highly upregulated in CAFs isolated from CRC patients, such as *Wnt2* expression⁵³. Interestingly, our analysis of public data (GSE72229), which details transcriptomic changes in human fibroblasts treated with melanoma-derived EVs revealed differential expression of several genes involved in the Wnt signalling pathway, including *WNT5B* and *DKK3*. This suggests that either melanosomes are also modulating Wnt signalling or perhaps that melanoma-derived exosomes are delivering miRNAs that

regulate the Wnt-pathway, raising the intriguing possibility that melanosomes and exosomes may deliver their own subsets of miRNAs targeting different pathways to achieve the same outcome of CAF activation and pre-metastatic niche establishment.

MiR-155 is another important miRNA that has been found to be preferentially upregulated in melanoma when compared to normal tissue⁵⁴. A recent study by Shu *et al.* discovered that human melanoma-derived exosomes (HMEXs) can be taken up by adult dermal fibroblasts and are responsible for acidification of the stroma to favour pre-metastatic niche formation⁵⁵. When NFs were exposed to the melanoma exosomes, they underwent a phenotypic change to favour aerobic glycolysis and suppress oxidative phosphorylation, promoting extracellular acidification. MiRNAs miR-155 and miR-210 were discovered by immune-biochip to be present within the HMEXs, and were also found to be upregulated in six melanoma cell lines. Inhibition of miR-155 and miR-210 activity within the HMEXs resulted in reversal of the of the exosome-dependant metabolic reprogramming in dermal fibroblasts⁵⁵. Another study discovered that melanoma secreted exosomal miR-155 can induce a proangiogenic switch in normal fibroblasts to transform into CAFs, causing higher expression of proangiogenic factors, such as VEGFa and MMP9¹⁶. MiR-155 does this by directly targeting suppressor of cytokine signalling 1 (SOC1) to downregulate it, resulting in increased activation of the JAK2/STAT3 signalling pathway. Reduction of miR-155 in the exosomes was shown to alleviate angiogenesis *in vivo* and *in vitro*¹⁶, highlighting with other evidence the importance that miR-155 plays in the activation of CAFs and the crosstalk between CAFs and melanoma.

In addition to these verified CAF transforming miRNAs, there are other dysregulated miRNAs in melanoma that need to be experimentally assessed in terms of EV levels and CAF promoting properties. A miRNA that may have a potential role in melanoma CAF formation is miR-222, found to be enriched in melanoma exosomes that can drive tumorigenesis by inhibiting expression of p27, c-Fos and CDKN1B⁵⁶. In fact, it was discovered that both CAF transformer miR-211 and miR-222 are upregulated in melanoma, due to silencing of repressor transcription factor promyelocytic leukaemia zinc finger (PLZF)⁵⁷. MiR-222's role in melanoma CAF formation has yet to be elucidated, however a recent study has shown that miR-222 has the ability to transform NFs into CAFs to allow growth and metastasis of

breast cancer. Enhanced expression of miR-222, or downregulation of Lamin B receptor, a direct target of miR-222, resulted in increased expression of characteristic CAF markers, such as increased migration and senescence⁵⁸. Therefore, it would be interesting to see if this miRNA had the same or similar role for melanoma derived CAFs.

Many miRNAs have this potential role in melanoma derived CAF formation, as they are found to be upregulated in melanoma cells and the miRNA has been found to drive CAF formation in cancers other than melanoma. Another example of this is upregulation of miR-214 in malignant melanoma, that drives tumorigenesis by directly targeting Transcription Factor AP-2 Alpha (TFAP2) to downregulate it⁵⁹. However, in gastric cancer, it was found that miR-214 was downregulated in CAFs compared to NFs, and upregulation of miR-214 in CAFs resulted in inhibition of migration and invasion of cancer cells. This implies that miR-214 may have different cell-specific outcomes and that more work is needed to understand better its role in melanoma and CAFs⁶⁰. In addition to these established CAF-transforming melanoma miRNAs, there are a plethora of miRNAs that are found to be upregulated in melanoma but have yet to have their CAF changing properties investigated. For example, in addition to miR-21 and miR-155, Volinia *et al.* found that miR-17-5p, miR-107, miR-130, miR-181b and miR-221 were upregulated in melanoma solid tumours compared to normal tissue⁶¹. Dror *et al.* found that in addition to miR-211, melanoma cells had upregulated levels of miR-149, miR-23, miR-let7a and -miR-let7b⁵⁰. Knowing the roles miR-21, miR-155 and miR-211 have in CAF formation in melanoma, it would be interesting to investigate whether these other miRNAs have functions in these activation processes.

3.2 CAF miRNA and melanoma development

The study of CAF miRNA in cancer progression is an emerging field and while it is known that miRNAs are deregulated in both malignant cells and CAFs, we still have a long way to go to fully understand the complex interactions of miRNA cross-talk between both cell types in the TME. Multiple studies have revealed that miRNAs released from CAFs can change cancer cell phenotypes to increase their aggressiveness, and that crosstalk between CAFs and cancer cells exist⁶². From existing data, we can predict potential CAF miRNA prospects for melanoma research.

In addition to miR-21 being upregulated in melanoma, it is also increased in melanoma-associated CAFs treated with the known CAF activator, TGF- β 1⁴⁶. TGF- β has been shown in multiple studies to be upregulated in melanoma, where expression levels increase as the disease advances⁶³. MiR-21 is one of the most well-known oncogenic miRNAs in melanoma progression, being implicated in invasion, metastasis, proliferation and genetic instability⁶⁴. Therefore, it is possible that miR-21 could act as a bidirectional mediator of TME development in melanoma, that if secreted by both CAFs and malignant cells drive a pro-oncogenic phenotype between both cell types. This has been found to be the case in oesophageal squamous-cell carcinoma (SCC), where miR-21 was overexpressed in both patient SCC and SCC-associated stromal fibroblasts, acting to increase migration and invasion capability in the tumour while also activating CAFs⁴⁷.

Melanoma progression is influenced not only by increased levels of pro-oncogenic miRNAs, but also the decreased levels of miRNAs that possess tumour suppressive properties. One miRNA that could be potentially important in melanoma TME development is low expression of miR-148a, although further study is required to determine if it is downregulated in CAFs. Tian *et al.* reported that *miR-148a* expression was lower in patient-derived tumour compared to normal controls and increased *miR-148a* expression *in vitro* inhibited metastasis⁶⁵. Moreover, methylation-dependant silencing of the *miR-148a* gene has been associated with lymph node metastasis of melanoma⁶⁶. Multiple studies have found that levels of miR-148a are significantly downregulated in CAFs from endometrial cancer and oral squamous cell carcinoma (OSCC) tissue, and that overexpression of miR-148a impaired migration and invasion in both cancers⁶⁷⁻⁶⁸. One of the direct targets of miR-148a is WNT-1, a proto-oncogene protein of Wnt/ β -catenin signalling pathway, where significantly higher WNT-1 expression was found in breast cancer tissue compared to normal tissue⁶⁹. The Wnt/ β -catenin has been implicated in melanoma derived CAF activation, where it was found that Yes-associated protein (YAP) is an important β -catenin-interacting partner in stromal fibroblasts. Human and murine melanoma studies discovered that YAP is highly expressed in CAF nuclei, where its nuclear translocation is modulated by the Wnt/ β -catenin pathway⁷⁰. Together, these data advocate a new avenue of research into the role of miR-148a downregulation in melanoma development and CAF activation. If found to be under-expressed in

melanoma CAFs, upregulation of miR-148a could become a potential therapeutic target in the future of melanoma treatments.

A recent study by Jorge *et al.* analysed potential miRNA target genes that could impact melanoma-TME crosstalk and found that miR-342 was downregulated in a subset of melanoma patients with worse clinical prognosis, where lower levels were associated with worse overall survival¹¹. Strikingly, they were even able to detect an increase in hsa-miR-342-3p expression in the plasma of melanoma survivors compared to metastatic melanoma patients¹¹. MiR-342 targets *PPM1F* transcripts and leads to decreased PPM1F levels, which have been shown to promote invasion and migration in breast cancer cells⁷¹. Moreover, a recent study by Shi *et al.* found that miR-342 prohibits cell proliferation and invasion in melanoma by targeting the Zinc-finger Ebox binding homeobox 1 (ZEB1) gene⁷². ZEB1 is a known transcription factor that has been found to cause MAPK inhibitor resistance in melanoma⁷³. Furthermore, miR-342 has been found to be downregulated in breast cancer CAFs, causing increased expression of α -SMA, migration and invasion⁷⁴. At present, the expression of miR-342 by CAFs in melanoma has yet to be explored, however, our analysis of existing public data (GSE100508) reveals that miR-342-3p is significantly decreased in circulating EVs isolated from metastatic melanoma patients, suggesting that this target might have some utility as a liquid biomarker in melanoma.

Another potential tumour suppressor miRNA that may play an important role in the suppression of CAF activation is miR-145. Melling *et al.* discovered that fibroblast cells treated with TGF- β acquired a CAF myofibroblast-like phenotype, which was also associated with an increased expression of miR-145. In fact, CAFs appeared to revert to a fibroblast-like state when miR-145 was overexpressed, due to a miR-145/TGF- β negative feedback loop preventing TGF- β -induced CAF differentiation⁷⁵. MiR-145 has been found to be under expressed in melanoma, and induced overexpression of miR-145 in metastatic melanoma has been shown to suppress invasion and migration⁷⁶. In addition, two separate studies have reported that miR-145-5p was able to inhibit proliferation, invasion and metastasis in melanoma cell lines and tissues⁷⁷⁻⁷⁸. Therefore, this suggests that therapeutically exploiting the expression of miR-145 in CAFs may have a dual benefit and inhibit both melanoma metastasis and pro-oncogenic transformation of stromal fibroblasts, thus preventing optimal TME formation.

3.3 CAF exosome crosstalk in melanoma metastasis

Collectively, current evidence suggests that bi-directional crosstalk occurs between melanoma cells releasing miRNAs within EVs that are internalised by distal NFs, leading to their re-programming as CAFs. Subsequently, CAFs then secrete their own unique population of miRNAs within EVs that can support or sometimes hinder metastasis (Fig. 3). Such crosstalk has been well documented to increase cancer aggressiveness, promoting increased melanoma proliferation, metastasis and even drug resistance^{12,28}. In fact, it was found in one early study that 9 out of 11 advanced metastatic melanoma cell lines derived from primary lesions or distal metastases could be constantly stimulated to grow when co-cultured with dermal fibroblasts that produced stimulatory growth factors⁷⁹. However, what is relatively unknown is the role that miRNAs play in melanoma-CAF signalling and whether CAF-related miRNA expression profiles could have clinical relevance. More detailed understanding of the complex dynamics involved in the non-autonomous mechanisms of metastasis and how miRNAs contribute to this will allow us to exploit them to create more targeted therapeutics for melanoma patients.

4.1 Therapeutic benefits of targeting CAFs

A great deal of progress has been made in recent years to improve treatment options for melanoma. Over 50% of metastatic melanoma patients harbour a v-raf murine sarcoma viral oncogene homolog B (*BRAF*) oncogenic mutation, mainly a V600E residue change, resulting in hyperactivation of the MAPK/ERK signalling pathway⁸⁰. Targeted therapy using BRAF protein inhibitors such as Vemurafenib/PLX4032 (Zelboraf®) and Dabrafenib (Tafinlar®) have shown clinical efficacy and have been approved by the FDA for melanoma treatment⁸⁰. Immune checkpoint inhibitors within the past decade have proven seismic for melanoma treatment, with the FDA approving the use of ipilimumab in 2011 after it was shown to improve overall survival in metastatic melanoma patients⁸¹. However, despite these advancements, melanoma still proves to be challenging to treat, with poor prognosis, high recurrence, and limited response to treatments in some patients¹⁸. This therefore highlights the urgency to develop alternative treatments to prevent melanoma metastasis, which may be used as an adjunctive therapy targeting non-malignant cells alongside primary treatment.

Targeting stromal fibroblasts that contribute towards the TME has many advantages over targeting malignant cells, making it an exciting treatment prospect in melanoma. One of the most evident advantages involves the issue of melanoma cells acquiring drug resistance due to increased levels of genetic instability and aneuploidy leading to oncogenic genetic alterations¹. Stromal fibroblasts and CAFs are more genetically stable in comparison to malignant cells, so they are less likely to mutate and become resistant to treatments¹². In addition, tumour cells within the same tumour are likely to evolve into heterogeneous groups due to the continuous accumulation of genetic mutations as the disease progresses, meaning all malignant cells may not respond to the same treatment. However, a characteristic these malignant cells all share is the requirement for pro-angiogenic growth conditions in the TME, which will most likely involve CAFs²⁷. Therefore, targeted therapies exploiting CAFs may provide a tumour-resistant microenvironment that could inhibit the growth and invasion of primary melanoma cells harbouring different combinations of mutations.

CAFs have the potential to influence cancer cells in various ways to increase tumour aggressiveness, which may be important in treating late stage melanoma resistant to current therapies, where survival rates are extremely poor. They have been shown to change their ECM and protease expression profile in proteins such as fibronectin and MMPs, to promote ECM remodelling for TME development and may even prevent therapeutic drugs reaching melanoma cells¹⁸, indeed, enhanced tumorigenicity and decreased sensitivity to anti-cancer drugs are induced by malignant cells interacting with ECM components²⁷. In addition, CAFs secrete a plethora of cancer phenotype transforming factors, including growth factors, cytokines and miRNAs¹². Indeed, it appears that CAFs serve as a critical signalling centre and TME remodeller to aid in creation of the pre-metastatic niche. Their multiple mechanisms for increasing tumorigenicity in melanoma have highlighted promising avenues for combination treatment targeting both malignant cells and CAFs. However, further unravelling of the complexities of CAF-melanoma crosstalk will be necessary before these novel therapeutic strategies can be used clinically. If it becomes possible to discriminate between CAF and NF populations, for example their miRNA expression profile, then it could be possible to selectively target CAFs so only fibroblasts that promote cancer get affected by treatment, reducing side effects. What has become clear in recent years

is the exciting potential of miRNAs to be used as a prognostic marker or a target for therapeutics in melanoma, as discussed below.

4.2. MiRNAs as a prognostic biomarker

Recent strides in melanoma biomarker research have accelerated the efficacy of early diagnosis and predicted prognosis, a key determinant in achieving higher survival rates in patients. Traditional melanoma biomarkers are often proteins and can have shortcomings that make them unsuitable for early and accurate diagnosis or predictive treatment response. For example, lactate hydrogenase (LDH) levels have been found to be upregulated in melanoma, with levels appearing to increase as the disease progresses, recognised in the melanoma AJCC staging system as the only blood-based biomarker in widespread use³. However, the sensitivity of the LDH marker appears to reduce as the disease progresses⁸², highlighting the importance of addressing the current limitations in melanoma testing by looking for better informative and non-invasive biomarkers. As large amounts of miRNAs produced by melanoma or stromal cells can be transported within EVs via circulatory body fluids, they have the potential to be used as predictive, prognostic and diagnostic markers for melanoma. These miRNAs could be collected using a non-invasive method such as blood collection, reducing the need for invasive pathological diagnosis requiring a tumour tissue biopsy from the patient³.

A study by Stark *et al.* measured the expression of a panel of 17 miRNAs (MELmiR-17) in tissue and sera of melanoma patients compared with healthy controls, including miR-211 and miR-145, which were all found to be upregulated in melanoma. They found within this panel a subset of 7 melanoma-specific miRNAs (MELmiR-7) that were expressed in a stage specific manner, with highest expression in stage III melanoma, and was shown to have high sensitivity (93%) and specificity (82%). In fact, it was shown that measurement of MELmiR-7 expression levels outperformed measuring LDH levels in prediction of patient overall survival⁸². Therefore, this unique subset of miRNAs may be able to be used as a primary screening tool for previously undetectable melanoma, and could act as a prognostic marker for overall survival or reoccurrence. This signal may have even greater powerful prognostic capability if melanoma- or CAF-derived EVs are first purified from blood prior to miRNA analysis.

One of the most common characteristics of a TME is hypoxia, where malignant cells transform their metabolism to favour anaerobic glycolysis over oxidative phosphorylation to overcome oxygen deficiency⁸³. MiR-210 has been shown in multiple studies to be upregulated in a hypoxic state by multiple cancers, and is reported as an oncogenic miRNA⁸⁴. A study by Ono *et al.* used a direct miRNA assay on plasma from melanoma patients to determine if miR-210 expression levels could predict early metastatic recurrence. They discovered that the levels of cell-free miR-210 were significantly higher in melanoma patients compared to controls, and the levels were higher in metastatic melanoma compared to primary tissues. When comparing measuring levels of miR-210 with LDH before melanoma reoccurrence, they found that miR-210 gave a more accurate indicator of disease reoccurrence⁸⁵. A seminal study by Shu *et al.* reported that exosomes derived from six different melanoma lines were found to contain miR-210, and addition of these exosomes to dermal fibroblasts caused them to reprogram to create a more acidic TME, highlighting the importance of EVs in melanoma-CAF crosstalk⁵⁵.

MiR-221 is known to be abnormally expressed in melanoma, and is thought to act by downregulating c-KIT receptor and p27Kip to drive a more malignant phenotype⁸⁶. Kanemaru *et al.* sought to find out whether circulating miR-221 in malignant melanoma patients could be used as a novel tumour biomarker. Using sera samples from 94 malignant melanoma patients, they discovered that not only was miR-221 upregulated in melanoma compared to healthy control samples, but also that miR-221 expression was higher in patients with stages I-IV compared to those with melanoma *in situ*. In addition, a longitudinal study showed that levels of miR-221 were correlated with tumour thickness, indicating that miR-221 may have uses as a prognostic marker in melanoma⁸⁷.

Biopsies of melanoma tumours can sometimes be histopathologically ambiguous, leading to the possibility of incorrect diagnosis, unjustified overtreatment of benign lesions producing side effects in patients, or undertreatment of metastasising melanomas³. Using next-generation sequencing of the melanoma miRNA transcriptome, Kozubek *et al.* defined a set of 40 miRNAs that were either over or under-expressed in melanoma compared to benign nevi. One of the miRNAs that was found to be significantly decreased in expression in malignant melanoma was miR-211, which was shown to

accurately discriminate between malignant and benign tumours⁸⁸. This discovery led to the development of a potential accompanying test using miRNA *in situ* hybridisation to detect miR-211 levels to predict benign or malignant outcomes in patients to a high accuracy (92%)⁸⁹. The test was shown to effectively categorise melanomas based on their metastatic ability effectively in histologically ambiguous melanoma lesions, suggesting that this test could be useful for melanoma patients with hard to diagnose tumours.

In addition to these exciting likely prognostic miRNAs, there exist many more miRNAs that could potentially be used for melanoma prognosis, but are understudied, opening exciting avenues of research for the future. For example, one study found that miR-17, miR-19a, miR-21, miR-126 and miR-149 were expressed in EVs at increased levels in metastatic sporadic melanoma compared to familial melanoma patients⁹⁰. Thus, it would be interesting to determine whether these miRNAs could be used to discriminate between melanoma types for a more accurate diagnosis and predictor of targeted treatment outcome. Whilst Dror *et al.* discovered the upregulated miR-211 causes hyperactivation of the MAPK/ERK, they also discovered four other miRNAs previously associated with melanoma to be upregulated in mature melanosomes; miR-149, miR-23, miR-let7a and miR-let7b⁵⁰. It would therefore be fascinating to discern whether these miRNAs can also be significantly detected in melanoma patient body fluids and if they are able to predict the outcome of the patient. Overall, whilst significant advances have been made in the use of miRNAs for prognostic biomarkers in melanoma, further characterisation of the melanoma/TME subpopulation miRNA transcriptome is necessary to develop more sensitive and accurate methods to diagnose melanoma and predict outcome in the future. Multiple methods to detect miRNAs within EVs, such as *in situ* probes, biosensors and DNA enzyme probes, have allowed the acceleration of the research into how miRNAs influence the TME⁹¹.

4.3 Emerging therapeutics targeting miRNAs in melanoma

Due to the abnormal expression of miRNAs being closely related with malignant cancer progression, growing interest in miRNA-based targeted therapies has developed in recent years. MiRNA targeted therapies fall into three main categories: (1) Inhibition of the import, expression or function of oncogenic miRNAs that are found to be upregulated in tumour tissue (2) Import of EVs into

cancer/activated stromal cells containing tumour suppressing miRNAs that are downregulated in cancer and the TME; (3) Design of artificial miRNAs that can target specific oncogenic genes to silence them⁹². Emerging therapeutic treatment options for melanoma using miRNAs remains relatively understudied. However, recent evidence has provided promising insight into the potential of exploiting miRNAs as an adjunctive therapy in melanoma.

Heparan sulphate proteoglycans (HSPGs) are proteins found in the ECM and basement membrane and have been found to be cleaved by an endo- β -glucuronidase (HPSE), which allows metastasis to occur as it is easier for cancer cells to migrate and invade tissues⁴. In one study, synthesised miR-155 mimics were shown to cause a decrease in both the mRNA and the protein expression of HPSE in transfected melanoma cells⁹³. The down regulation of HPSE via miR-155 was shown to abolish migration, invasion and adhesion properties of melanoma cells *in vitro*. It was also observed that this artificial miRNA was also capable of inhibiting expression of chemokines interleukin-8 (IL8) and chemokine ligand 1 (CXCL1) at the transcription and translational levels. The levels of p38 MAPK, JNK and ERK phosphorylation were found to be reduced in miRNA transfected cells, suggesting that the expression of *IL8* and *CXCL1* may be mediated by HPSE-induced phosphorylation of the MAPK/JNK/ERK pathway⁹³. The fact that both HPSE and the cytokines IL8 and CXCL1 are implicated in melanoma suggests that using these artificial miRNAs may provide an applicable treatment for melanomas with high invasion and migration abilities⁴. However, a recent study by Zhou *et al.* reported that melanoma cells secrete exosomes containing miR-155-5p, which can trigger the proangiogenic switch to reprogram CAFs, highlighting this miRNA's complicated role in EV mediated TME crosstalk¹⁶. Further work is required to discover whether this miRNA can be made to be expressed in a tissue or cell-type specific manner and whether there are suitable delivery systems, such as EVs or viral vectors, to deliver the miRNA effectively *in vivo* systems.

Genistein is a natural isoflavone isolated from soybeans that has been shown to reduce proliferation, migration and adhesion and induce apoptosis in melanoma cells by downregulating the FAK/paxillin and MAPK pathways as a dual functional effect⁹⁴. A study by Sun *et al.* found that genistein was able to inhibit *in vitro* and *in vivo* human uveal melanoma cell growth, but additionally altered expression

of miR-27a in a dose dependant manner. Therefore, it is likely that genistein acts to influence miR-27a levels, which may affect expression of miR-27a target gene zinc finger and BTB domain containing 10 (ZBTB10), to exert growth inhibitory mechanisms in melanoma⁹⁵. Therefore, the use of genistein to target miR-27a upregulation to prevent melanoma growth and metastasis may be an attractive therapeutic option in future melanoma research. In addition, a study on gastric cancer cell lines found that the cancer cells release miR-27a within exosomes that can regulate the transformation of fibroblasts into CAFs, suggesting that if this is also the case in melanoma, this treatment may be effective in preventing the formation of the TME⁹⁶.

One other exciting insight into miRNAs being involved in melanoma therapy utility is from a study into miR-26a by Reuland *et al.* Via a microarray screening of melanoma-associated miRNAs, they identified miR-26a as being strongly downregulated in melanomas when compared to healthy melanocytes. Further experiments showed that miR-26a possessed a role in promoting apoptosis in melanoma, where transfection of miR-26a resulted in significant programmed cell death induction in most of the melanoma cell lines tested⁹⁷. Screening of potential downstream effectors of miR-26a identified silencer of death domains (SODDs), an important mediator to protect melanoma cells from apoptotic mechanisms by binding to tumour necrosis factor receptor-1 (TNFR-1) to inhibit multimerization⁹⁷⁻⁹⁸. Therefore, SODD deregulation may be sufficient to inhibit apoptosis in melanoma, and therapies using miR-26a may prove useful to directly target SODD and bypass the mechanisms involved in avoiding cell death. Alternatively, miR-26a could be used as an adjunctive therapy alongside traditional treatments targeting cell death to possibly enhance their effects. Additionally, a recent study reported that miR-26a regulates the secretion of EVs from prostate cancer cells by targeting genes regulating EV release⁹⁹. This would be interesting to research in melanoma, as if miR-26a is found to regulate melanoma EV release too, it can be a potential target to prevent melanoma-TME crosstalk occurring.

Therefore, it can be concluded that miRNAs hold a key role in potential additional therapeutic targets in melanoma. However, one of the biggest challenges when trying to scale up miRNA treatments from *in vitro* to *in vivo* is protection of miRNAs from enzymatic degradation in circulation, plus trying to ensure only target cells receive the miRNAs. One way to circumvent these problems is the potential use

of EV shuttles such as exosomes to deliver miRNAs, so they remain stable in circulation and have the potential to be delivered to certain recipient cells types due to differing receptors on the EV surface¹⁰⁰. Melanoma primary tumours are easily accessible, so could have exosomes delivered to the malignant melanocyte cells via local injection, limiting any negative off-target effects. However distal melanoma metastases may require systemic injection, complicating treatment¹⁰⁰. Further understanding of specific miRNA and EVs roles in melanoma progression will be necessary to determine the safety and therapeutic efficacy of exosomal miRNAs in a clinical context.

Conclusions

In conclusion, exosomes and other EVs are a newly established category of intercellular communicatory mediators, that contain a wide range of various biological signalling molecules to exert a variety of effects within the TME, such as increased invasiveness, in melanoma cells. MiRNAs have long been implicated in melanoma progression, and as a result, the function of miRNAs within exosomes in cancer research has become an increasingly important matter of contention in research. Increasing evidence in recent years has highlighted a critical role of circulating miRNAs on development of the melanoma TME, where studies have found miRNAs to be expressed in both malignant and surround stromal cell populations. It has become clear that a bi-directional crosstalk is paramount for the constant evolution of the TME to retain optimal conditions for the CTCs, with miRNAs from malignant cells and CAFs found to modify the phenotypes of cells within the TME to favour oncogenesis. Although substantial progress has been made in the last decade within this field, gaps remain in terms of the more complicated details of the melanoma-TME crosstalk and the melanoma miRNA transcriptome that will need to be filled before miRNAs can be fully exploited in diagnosis, prognosis and clinical therapies for melanoma patients.

Authors' contributions

Mikayla Shelton, Wayne Roberts, Julia Newton-Bishop and James R. Boyne: Writing-Original Draft, Investigation, Writing-Review & Editing. Chinedu Anthony Anene, Jeremie Nsengimana: Investigation, Writing-Review & Editing.

Declaration of Competing Interests

The authors declare no competing interests.

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Figure Legends

Fig 1: Extracellular vesicles (EVs) mediate a crosstalk between cancer associated fibroblasts (CAFs) and melanoma cells. Melanoma cells at the primary site can release exosomes that contain a variety of signalling factors, such as various nucleic acids and proteins. These exosomes can enter circulation and travel to a secondary site within the body to transform normal fibroblasts (NFs) into CAFs. CAFs then secrete their own EVs that can transform the stromal environment to favour tumour growth, such as acidic extracellular pH.

Fig 2: MiRNA processing for exosomal release into the extracellular space. MiRNA precursors are cleaved in the nucleus then exported to the cytoplasm via Ran-GTP dependant protein Exportin 5. Pre-miRNA is then further cleaved to form mature miRNA, which can either repress translation of its complementary mRNA within the cell or leave the cell via EVs to generate changes in other cells.

Fig 3: miRNAs that have a potential role in bi-directional crosstalk between melanoma cells and dermal fibroblasts/CAFs. miRNAs overexpressed are represented by black arrows, and under expressed by red dashes. These miRNAs inhibit (red lines) or activate (black arrows) their target downstream pathways to cause the phenotypic change within the cell. miRNAs overexpressed in melanoma with unknown effects on CAFs are shown within the blue box.

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