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

Shelton, M.[1], Anene, A.[2], Nsengimana, J.[3], Roberts, W.[4], Newton-Bishop, J.[5], Boyne, J. R[1].

1. School of Applied Sciences, University of Huddersfield, Huddersfield, HD1 3DH. 2. Centre for Cancer Genomics and Computational Biology, Barts Cancer Institute, Queen Mary University of London, London, EC1M 6BQ. 3. Population Health Sciences, Institute Faculty of Medical Sciences, Newcastle University, Newcastle, NE1 7RU. 4. School of Clinical and Applied Science, Leeds Beckett University, Leeds, LS1 3HE. 5. University of Leeds School of Medicine, Leeds, United Kingdom, UK.

Address correspondence to: J. R. Boyne

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 damage[1]. 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 brain[2]. 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 metastasis[3].

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 dermis[4]. 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 cells[5]. 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 metastasise[4]. 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 thrive[5-6].

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 tumour[7]. 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 variations[8]. 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 CTCs[10].

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 types[7]. 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 cells[11]. 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 cells[12].

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 healing[13]. 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 cells[14]. 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 myofibroblasts[15]. 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 niche[16]. Although CAFs are more genetically stable than the corresponding tumour cells, they also have increased genetic and

77 epigenetic alterations that drive the irreversible transformation from NFs, although the specifics of this
78 transdifferentiation process have yet to be fully elucidated[17].

79 Studies in melanoma have found that culturing metastatic cells with NFs results in pro-inflammatory
80 gene expression and recruitment of NFs to CAFs to meet the structural (ECM proteins) and chemical
81 (CAF's secretome) requirements for melanoma growth[18-19].

82 **2.2 CAF exosomes**

83 One of the most important mediators of pre-metastatic niche formation is the secretion of extracellular
84 vesicles (EVs), such as exosomes from both the primary tumour cells and transformed stromal cells at
85 the secondary site[20]. Exosomes are a subtype of EVs, characterised by a lipid bilayer membrane
86 extracellular structure and a small size of approximately 30-150nm, released by almost all cell types
87 and present in all biological fluids[21]. They originate from endosomal packaging into multi-vesicular
88 bodies (MVBs) within the cell, which can then fuse with the outer cell membrane to release the
89 intraluminal vesicles into the extracellular space[22]. Exosomes carry a wide range of biologically active
90 cargo inside and within their membranes, including proteins, lipids, metabolites and nucleic acids,
91 which can be trafficked in circulation to be internalised by recipient cells to exert their effects[23].
92 Therefore, exosomes have a significant role in intercellular communication between neoplastic and
93 distal non-malignant cells, where their higher specificity to certain cell types and resistance against
94 circulating RNAses compared to small molecules make them an ideal vehicle for bi-directional
95 crosstalk[24]. Tumour cells have been found to secrete larger amounts of exosomes than non-malignant
96 cells, with altered cargo and markers, meaning exosomes isolated from cancer patient's biological fluids
97 have the potential to act as prognostic or diagnostic markers to predict the cancer's metastatic ability
98 and response to certain therapeutics[25-26].

99 In addition to direct interaction with carcinoma cells and secretion of biologically active molecules,
100 CAFs themselves secrete their own subsets of exosomes containing functional molecules that can exert
101 pro-metastatic and angiogenic effects[27]. This results in a crosstalk between both CAFs and cancer cells
102 (Fig. 1), reciprocally releasing and internalising each other's exosomes to promote and maintain a

favourable TME[28]. 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 cells[29-30]. 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 miRNAs[31].

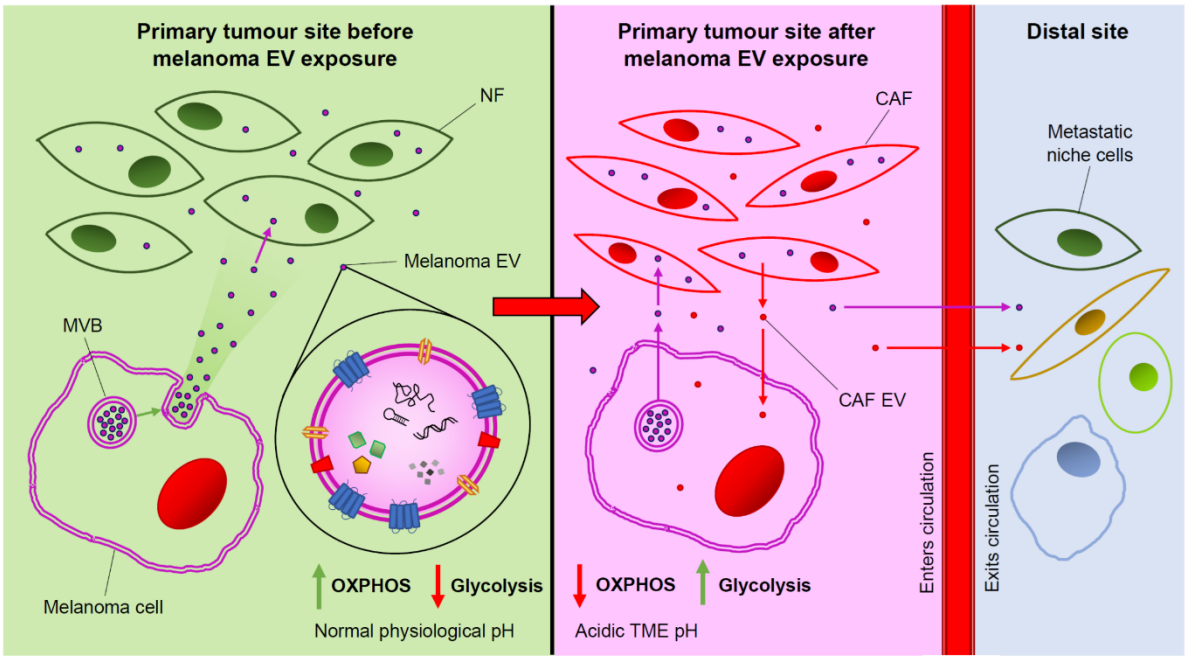

Fig 1: Extracellular vesicles (EVs) mediate a crosstalk between cancer associated fibroblasts (CAFs) and melanoma cells. Melanoma cells at the primary site can release exosomes that contain a variety of signalling factors, such as various nucleic acids and proteins. These exosomes are taken up by local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

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 mRNAs[32]. 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 pairing[33]. Target genes of miRNAs have been found to control a plethora of cellular processes, including cell cycle progression, apoptosis, migration and differentiation[34].

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 DGCR8[35]. 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 exosomes[37-38]. 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 EXOmotif[39]. 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 exosomes[39]. 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 effects[40]. 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 models[40].

MiRNAs have also been found to be involved in processes typically dysregulated in cancers, such as uncontrolled proliferation, metastasis and drug resistance[41-42], where stress signals have been found to regulate the production of the pri-miRNAs by controlling specific transcription factors[43]. 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.

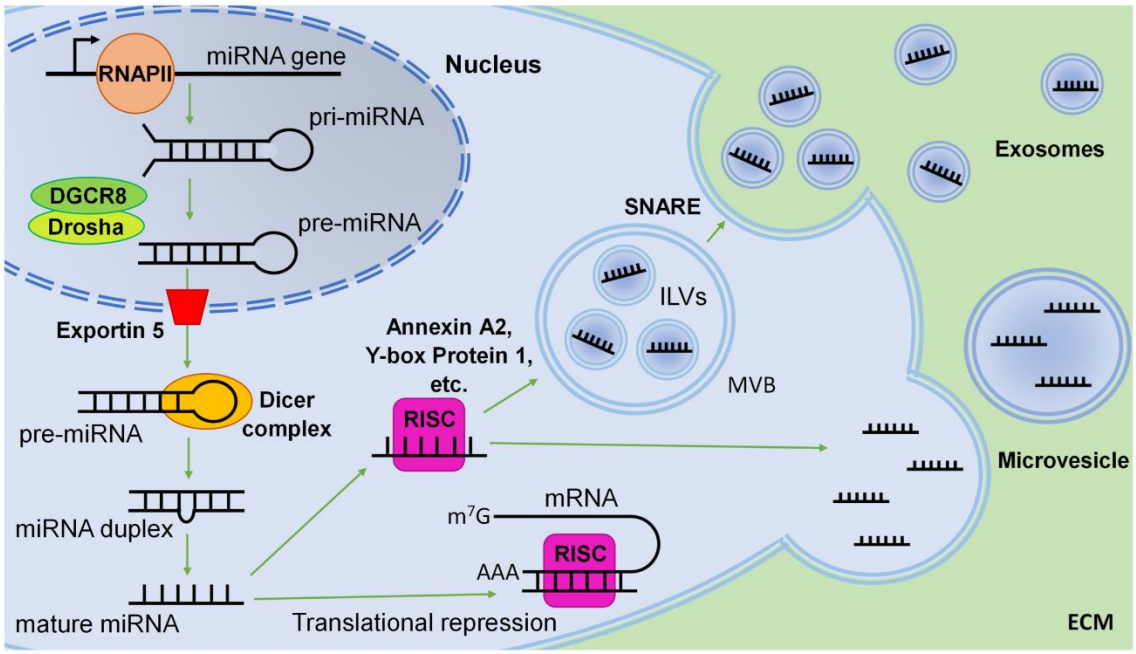

Fig 2: miRNA processing for exosomal release into the extracellular space. Primary miRNA (pri-miRNA) transcripts are cleaved in the nucleus via the microprocessor complex and exported to the cytoplasm via Ran-GTP-dependant protein Exportin-5. Precursor miRNA (pre-miRNA) is then further cleaved by Dicer to form mature miRNA, which can either repress translation of its complementary mRNA within the cell via association with RISC, or exit the cell via packaging within EVs. The precise mechanisms of miRNA sorting into EVs has yet to be elucidated, but numerous proteins have been implicated, including Argonaut 2 (Ago2), Y-box Binding Protein 1, Heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1, hnRNPA1, hnRNPC, hnRNPQ/SYNCRIP, Caveolin-1 (Cav-1), neutral sphingomyelinase-2 (nSMase-2), Alix and Annexin A2, depending on the specific miRNA being packaged. MVB (Multivesicular body), SNARE (SNAP Receptor), ECM (Extracellular matrix), RISC (RNA-induced silencing complex).

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 tumour[29].

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 genes[11]. 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 nevi[44]. 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 progression[45]. 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-β1, a well-established CAF activator[46]. 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, respectively[47-48]. 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 TME[49].

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 proliferation[50]. 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 investigation[51-52]. 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 TME[30]. 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 signalling[50]. Genes relating to the Wnt signalling pathway have been found to be highly upregulated in CAFs isolated from CRC patients, such as *Wnt2* expression[53]. 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 tissue[54]. 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 formation[55]. When NFs were exposed to the melanoma exosomes, they underwent a phenotypic change to favour aerobic glycolysis and supress 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 fibroblasts[55]. 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 MMP9[16]. 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 CDKN1B[56]. 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 senescence[58]. 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 it[59]. 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 CAFs[60]. In addition to these established CAF-transforming melanoma miRNAs, there are a plethora or 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 tissue[61]. Dror *et al.* found that in addition to miR-211, melanoma cells had upregulated levels of miR-149, miR-23, miR-let7a and -miR-let7b[50]. 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 exist[62]. 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-β1[46]. TGF-β has been shown in multiple studies to be upregulated in melanoma, where expression levels increase as the disease advances[63]. MiR-21 is one of the most well-known oncogenic miRNAs in melanoma progression, being implicated in invasion, metastasis, proliferation and genetic instability[64]. Therefore, it is possible that miR-21 could act as a bi-directional 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 CAFs[47].

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 metastasis[65]. Moreover, methylation-dependant silencing of the *miR-148a* gene has been associated with lymph node metastasis of melanoma[66]. 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 cancers[67-68]. 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 tissue[69]. 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

pathway[70]. 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 survival[11]. 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 patients[11]. MiR-342 targets *PPM1F* transcripts and leads to decreased PPM1F levels, which have been shown to promote invasion and migration in breast cancer cells[71]. 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) gene[72]. ZEB1 is a known transcription factor that has been found to cause MAPK inhibitor resistance in melanoma[73]. Furthermore, miR-342 has been found to be downregulated in breast cancer CAFs, causing increased expression of α-SMA, migration and invasion[74]. 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 differentiation[75]. MiR-145 has been found to be under expressed in melanoma, and induced overexpression of miR-145 in metastatic melanoma has been shown to supress invasion and migration[76]. In addition, two separate studies have reported that miR-145-5p was able to inhibit proliferation, invasion and metastasis in melanoma cell lines and tissues[77-78]. 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 factors[79]. 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.

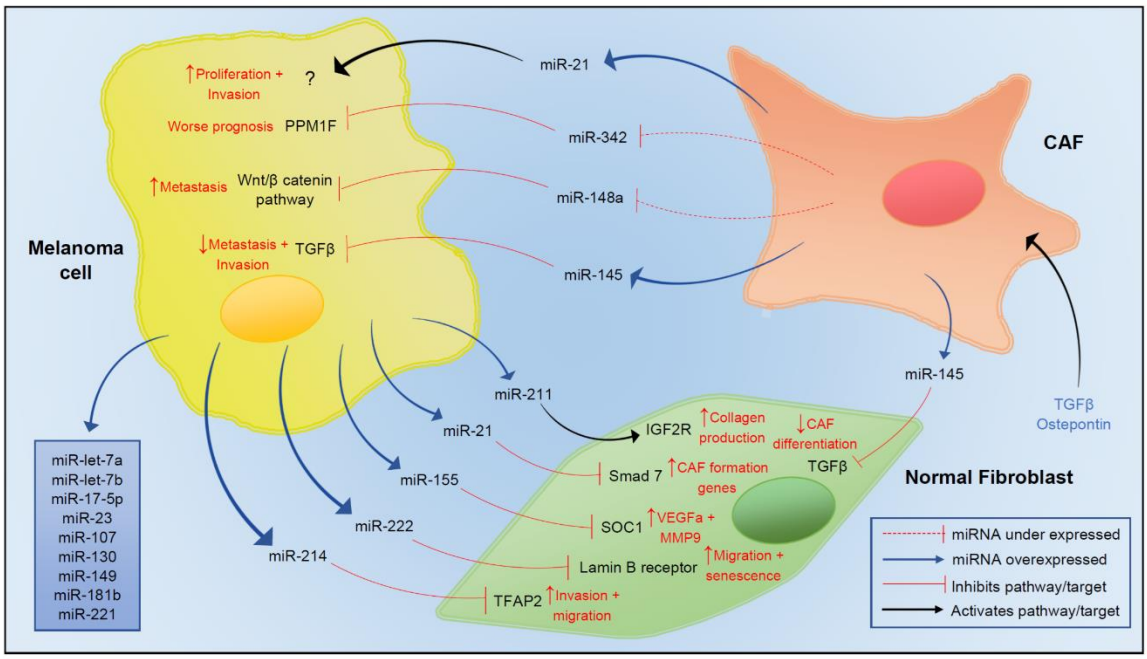

Fig 3: miRNAs that have a potential role in bi-directional crosstalk between melanoma cells and dermal fibroblasts/CAFs. miRNAs overexpressed are represented by blue arrows, and under expressed by red dashes. These miRNAs inhibit (red lines) or activate (black arrows) their target downstream pathways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

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 pathway[80]. 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 treatment[80]. 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 patients[81]. However, despite these advancements, melanoma still proves to be challenging to treat, with poor prognosis, high recurrence, and limited response to treatments in some patients[18]. 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 alterations[1]. Stromal fibroblasts and CAFs are more genetically stable in comparison to malignant cells, so they are less likely to mutate and become resistant to treatments[12]. 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 CAFs[27]. 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 cells[18], indeed, enhanced tumorgenicity and decreased sensitivity to anti-cancer drugs are induced by malignant cells interacting with ECM components[27]. In addition, CAFs secrete a plethora of cancer phenotype transforming factors, including growth factors, cytokines and miRNAs[12]. 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 tumorgenicity 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 use[3]. However, the sensitivity of the LDH marker appears to reduce as the disease progresses[82], 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 patient[3].

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 survival[82]. 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 deficiency[83]. MiR-210 has been shown in multiple studies to be upregulated in a hypoxic state by multiple cancers, and is reported as an oncogenic miRNA[84]. 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 reoccurrence[85]. 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 crosstalk[55].

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 phenotype[86]. 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 melanoma[87].

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 melanomas[3]. 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 tumours[88]. 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 patients[90]. 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-let7b[50]. 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 TME[91].

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 supressing miRNAs that are downregulated in cancer and the TME; (3) Design of artificial miRNAs that can target specific oncogenic genes to silence them[92]. 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 tissues[4]. 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 cells[93]. 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 pathway[93]. 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 abilities[4]. 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 crosstalk[16]. 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 effect[94]. 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 melanoma[95]. 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 TME[96].

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 tested[97]. 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 multimerization[97-98]. 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 release[99]. 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 surface[100]. 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 treatment[100]. 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, Julia Newton-Bishop and James R. Boyne: Writing-Original Draft, Investigation, Writing-Review & Editing. Anthony Anene, Jeremie Nsengimana: Investigation, Writing-Review & Editing.

Declaration of Competing Interests

The authors declare no competing interests.

Acknowledgements

This work was supported by a British Skin Foundation studentship awarded to MS.

References

1. Gray-Schopfer, V., Wellbrock, C. & Marais, R. 2007, "**Melanoma biology and new targeted therapy**", *Nature*, vol. 445, no. 7130, pp. 851-857.

2. Matthews, N.H., Li, W.Q., Qureshi, A.A. *et al.* **"Epidemiology of Melanoma"**. In: Ward, W.H., Farma, J.M., editors. *Cutaneous Melanoma: Etiology and Therapy* [Internet]. Brisbane (AU): Codon Publications; 2017 Dec 21. Chapter 1. Available from: https://www.ncbi.nlm.nih.gov/books/NBK481862/ doi:10.15586/codon.cutaneousmelanoma.2017.ch1

3. Davis, L.E., Shalin, S.C., Tackett, A.J. *et al.* 2019, "**Current state of melanoma diagnosis and treatment**", *Cancer Biology and Therapy*, vol. 20, no. 11, 1366-1379.

4. Zbytek, B., Carlson, J.A., Granese, J. *et al.* 2009, "**Current concepts of metastasis in melanoma**", *Expert Review of Dermatology*, vol. 3, no. 5, pp. 569-585.

5. Langley, R.R. & Filder, I.J. 2007, "**The seed and soil hypothesis revisited - the role of tumor-stroma interactions in metastasis to different organs**", *International Journal of Cancer*, vol. 128, no. 11, pp. 2527-2535.

6. Olmeda, D., Cerezo-Wallis, D., Riveiro-Falkenbach, E. *et al.* 2017, "**Whole body imaging of lymphovascular niches identifies premetastatic roles of MIDKINE**", *Nature*, vol. 546, no. 7660, pp. 676-680.

7. Hanahan, D. & Weinberg, R.A 2011, "**Hallmarks of Cancer: The Next Generation**", *Cell*, vol. 144, no. 5, pp. 646-674.

8. Gener Lahav, T., Adler, O., Zait, Y. *et al.* 2019, "**Melanoma-derived extracellular vesicles instigate proinflammatory signaling in the metastatic microenvironment**", *International Journal of Cancer*, vol. 145, no. 9, pp. 2521-2534.

9. Lui, Y. & Cao, X. 2016, "**Characteristics and Significance of the Pre-metastatic Niche**", *Cancer Cell*, vol. 30, no. 5, pp. 668-681.

10. Monteran, L. & Erez, N. 2019, "**The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor Microenvironment"**, *Frontiers in Immunology*, vol. 10, pp. 1835.

11. Jorge, N.A.N., Cruz, J.G.V., Pretti, M.A.M. *et al.* 2020, "**Poor clinical outcome in metastatic melanoma is associated with a microRNA-modulated immunosuppressive tumor microenvironment**", *Journal of Translational Medicine.* 18:56. doi: 10.1186/s12967-020-02235-w

12. Zhou, L., Yang, K., Andl, T. *et al.* 2015, "**Perspective of Targeting Cancer-Associated Fibroblasts in Melanoma**", *Journal of Cancer*, vol. 6, no. 8, pp. 717-726.

13. Sorrell, J.M. & Caplan, A.I. 2004, "**Fibroblast heterogeneity: more than skin deep**", *Journal of Cell Science.* 117:667-675. doi: 10.1242/jcs.01005

14. Zhou, L., Yang, K., Wickett, R.R. *et al.* 2016, "**Dermal fibroblasts induce cell cycle arrest and block epithelial–mesenchymal transition to inhibit the early stage melanoma development**", *Cancer Medicine*, vol. 5, no. 7, pp. 1566-1579.

15. Kalluri, R. 2016, "**The biology and function of fibroblasts in cancer**", *Nature Reviews Cancer*. 16:582–598. doi: 10.1038/nrc.2016.73

16. Zhou, X., Yan, T., Huang, C. *et al.* 2019, "**Melanoma cell-secreted exosomal miR-155-5p induce proangiogenic switch of cancer-associated fibroblasts via SOCS1/JAK2/STAT3 signaling pathway**", *Journal of Experimental and Clinical Cancer Research.* 37:242. doi: 10.1186/s13046-018-0911-3

17. Liu, T., Han, C., Wang, S. *et al.* 2019, "**Cancer-associated fibroblasts: an emerging target of anti-cancer immunotherapy**", *Journal of Hematology and Oncology*. 12:86. doi: 10.1186/s13045-019-0770-1

18. Flach, E.H., Rebecca, V.W., Herlyn, M. *et al.* 2011, "**Fibroblasts contribute to melanoma tumor growth and drug resistance**", *Molecular Pharmaceutics,* vol. 8, no. 6, pp. 2039-2049.

19. Gallagher, P.G., Bao, Y., Prorock, A. *et al.* 2005, "**Gene Expression Profiling Reveals Cross-talk between Melanoma and Fibroblasts: Implications for Host-Tumor Interactions in Metastasis**", *Cancer Research,* vol. 65, no. 10, pp. 4134-4146.

20. Guo, Y., Ji, X., Liu, J. *et al.* 2019, "**Effects of exosomes on pre-metastatic niche formation in tumors**", *Molecular Cancer*. 18:39. doi: 10.1186/s12943-019-0995-1

21. Harding, C.V., Heuser, J.E. & Stahl, P.D. 2013, "**Exosomes: Looking back three decades and into the future**", *The Journal of Cell Biology*, vol. 200, no. 4, pp. 367-371.

22. Hessvik, N.P. & Llorente, A. 2018, "**Current knowledge on exosome biogenesis and release**", *Cellular and Molecular Life Sciences*, vol. 75, no. 2, pp. 193-208.

23. Maia, J., Caja, S., Strano Moraes, M.C. *et al.* 2018, "**Exosome-Based Cell-Cell Communication in the Tumor Microenvironment**", *Frontiers in Cell and Developmental Biology*. 6:18. doi: 10.3389/fcell.2018.00018

24. Zhao, H., Achreja, A., Iessi, E. *et al.* 2018, "**The key role of extracellular vesicles in the metastatic process**", *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1869, no. 1, pp. 64-77.

25. André, M.R., Pedro, A. & Lyden, D. 2016, "**Cancer Exosomes as Mediators of Drug Resistance".** In: Rueff J., Rodrigues A., editors. *Cancer Drug Resistance* [internet]. Humana Press, New York, NY; 2016 Feb 25. Methods in Molecular Biology, vol 1395. Available from: https://doi.org/10.1007/978-1-4939-3347-1_13

26. Lener, T., Gimona, M., Aigner, L. *et al.* 2015, "**Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper**", *Journal of Extracellular Vesicles.* 31:4:30087. doi: 10.3402/jev.v4.30087.

27. Yamaguchi, H. & Sakai, R. 2015, "**Direct Interaction between Carcinoma Cells and Cancer Associated Fibroblasts for the Regulation of Cancer Invasion**", *Cancer (Basal),* vol. 7, no. 4, pp. 2054–2062.

28. Yang, X., Li, Y., Zou, L. *et al.* 2019, "**Role of Exosomes in Crosstalk Between Cancer-Associated Fibroblasts and Cancer Cells**", *Frontiers in Oncology.* 9:356. doi: 10.3389/fonc.2019.00356

29. Yang, F., Ning, Z., Ma, L. *et al.* 2017, "**Exosomal miRNAs and miRNA dysregulation in cancer-associated fibroblasts**", *Molecular Cancer.* 16:148. doi: 10.1186/s12943-017-0718-4

30. García-Silva, S. & Peinado, H. 2016, "**Melanosomes foster a tumour niche by activating CAFs**", *Nature Cell Biology*. 18: 911-913. doi: 10.1038/ncb3404

31. Aprelikova, O., Palla, J., Hibler, B. *et al.* 2012, "**Silencing of miR-148a in cancer-associated fibroblasts results in WNT10B-mediated stimulation of tumor cell motility**", *Oncogene*. 32:3246-3253. doi: 10.1038/onc.2012.351

32. Mendell, J.T. 2005, "**MicroRNAs: Critical Regulators of Development**, **Cellular Physiology and Malignancy"**, *Cell Cycle*. 4:9:1179-1184. doi: 10.4161/cc.4.9.2032

33. Alles, J., Fehlmann, T., Fischer, U. *et al.* 2019, "**An estimate of the total number of true human miRNAs**", *Nucleic Acids Research*, vol. 47, no. 7, pp. 3353-3364

34. Wahid, F., Shehzad, A., Khan, T. *et al.* 2010, "**MicroRNAs: Synthesis, mechanism, function, and recent clinical trials**", *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1803, no. 11, pp. 1231-1243.

35. MacFarlane, L.A. & Murphy, P.R. 2010, "**MicroRNA: Biogenesis, Function and Role in Cancer**", *Current Genomics,* vol. 11, no. 7, pp. 537-561.

36. Kogure, A., Kosaka, N. & Ochiya, T. 2019, "**Cross-talk between cancer cells and their neighbors via miRNA in extracellular vesicles: an emerging player in cancer metastasis**", *Journal of Biomedical Science.* 26:7. doi: 10.1186/s12929-019-0500-6

37. Shurtleff, M.J., Temoche-Diaz, M.M., Karfilis, K.V. *et al.* 2016, "**Y-box protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction**", *eLife.* 5:e19276. doi: 10.7554/eLife.19276

38. McKenzie, A.J., Hoshino, D., Hong, N.H. *et al.*, 2016, "**KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes**", *Cell Reports,* vol. 5, no. 15, pp. 978-987.

39. Villarroya-Beltri, C., Gutiérrez-Vázquez, C., Sánchez-Cabo, F. *et al.* 2013, "**Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs**", *Nature Communications.* 4:2980. doi: 10.1038/ncomms3980

40. Matsuzki, J. & Ochiya, T. 2017, "**Circulating microRNAs and extracellular vesicles as potential cancer biomarkers: a systematic review**", *International Journal of Clinical Oncology.* 22:413-420. doi: 10.1007/s10147-017-1104-3

41. Wei, L., Wang, X., Lv, L. *et al.* 2019, "**The emerging role of microRNAs and long noncoding RNAs in drug resistance of hepatocellular carcinoma**", *Molecular Cancer*. 18:147. doi: 10.1186/s12943-019-1086-z

42. Wang, H., Tan, Z., Hu, H. *et al.* 2019, "**microRNA-21 promotes breast cancer proliferation and metastasis by targeting LZTFL1**", *BMC Cancer*. 19:738. doi: 10.1186/s12885-019-5951-3

43. Dews, M., Homayouni, A., Yu, D. *et al.* 2006, "**Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster**", *Nature Genetics*, vol. 38, no. 9, pp. 1060-1065.

44. Satzger, I., Mattern, A., Kuettler, U. *et al.* 2012, "**microRNA-21 is upregulated in malignant melanoma and influences apoptosis of melanocytic cells**", *Experimental Dermatology,* vol. 21, no. 7, pp. 509-514.

45. Nicoloso, M.S., Spizzo, R., Shimizu, M. *et al.* 2009, "**MicroRNAs--the micro steering wheel of tumour metastases**", *Nature Reviews Cancer,* vol. 9, no. 4, pp. 293-302.

46. Li, Q., Zhang, D., Wang, Y. *et al.* 2013, "**MiR-21/Smad 7 signaling determines TGF-β1-induced CAF formation**", *Scientific Reports.* 3:2038. doi: 10.1038/srep02038

47. Nouraee, N., Van Roosbroeck, K., Vasei, M. *et al.* 2013, "**Expression, tissue distribution and function of miR-21 in esophageal squamous cell carcinoma**", *PLOS One,* vol. 8 no. 9. doi: 10.1371/journal.pone.0073009

48. Bullock, M.D., Pickard, K.M., Nielsen, B.S. *et al.* 2013, "**Pleiotropic actions of miR-21 highlight the critical role of deregulated stromal microRNAs during colorectal cancer progression**", *Cell Death & Disease.* 4:e684. doi: 10.1038/cddis.2013.213

49. Ramshani, Z., Zhang, C., Richards, K. *et al.* 2019, "**Extracellular vesicle microRNA quantification from plasma using an integrated microfluidic device**", *Communications Biology.* 2:189. doi: 10.1038/s42003-019-0435-1

50. Dror, S., Sander, L., Schwartz, H. *et al*. 2016, "**Melanoma miRNA trafficking controls tumour primary niche formation**", *Nature Cell Biology*. 18:1006–1017. doi: 10.1038/ncb3399

51. Mazar, J., DeYoung, K., Khaitan, D. *et al.* 2010, "**The regulation of miRNA-211 expression and its role in melanoma cell invasiveness**", *PLOS One.* 5:11:e13779. doi: 10.1371/journal.pone.0013779

52. Li, N., Liu, Y., Pang, H. *et al.* 2019, "**Methylation-Mediated Silencing of MicroRNA-211 Decreases the Sensitivity of Melanoma Cells to Cisplatin**", *Medical Science Monitor,* vol. 25, pp. 1590-1599.

53. Aizawa, T., Karasawa, H., Funayama, R. *et al.* 2019, "**Cancer-associated fibroblasts secrete Wnt2 to promote cancer progression in colorectal cancer**", *Cancer Medicine,* vol. 8, no. 14, pp. 6370-6382.

54. Grignol, V., Fairchild, E.T., Zimmerer, J.M. *et al.* 2011, "**miR-21 and miR-155 are associated with mitotic activity and lesion depth of borderline melanocytic lesions**", *British Journal of Cancer,* vol. 105, no. 7, pp. 1023-1029.

55. Shu, S.L., Yang, Y., Allen, C.L. *et al.* 2018, "**Metabolic reprogramming of stromal fibroblasts by melanoma exosome microRNA favours a pre-metastatic microenvironment**", *Scientific Reports.* 8:12905. doi: 10.1038/s41598-018-31323-7

56. Felicetti, F., De Feo, A., Coscia, C. *et al.* 2016, "**Exosome-mediated transfer of miR-222 is sufficient to increase tumor malignancy in melanoma**", *Journal of Translational Medicine.* 14:56. doi: 10.1186/s12967-016-0811-2

57. Felicetti, F., Errico, M.C., Bottero, L. *et al.* 2008, "**The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms**", *Cancer Research,* vol. 68, no. 8, pp. 2745-2754.

58. Chatterjee, A., Jana, S., Chatterjee, S. *et al.* 2019, "**MicroRNA-222 reprogrammed cancer-associated fibroblasts enhance growth and metastasis of breast cancer**", *British Journal of Cancer,* vol. 121, no. 8, pp. 679-689.

59. Penna, E., Orso, F., Cimino, D., *et al.* 2013, "**miR-214 coordinates melanoma progression by upregulating ALCAM through TFAP2 and miR-148b downmodulation**", *Cancer Research,* vol. 73, no. 13, pp. 4098-4111.

60. Wang, R., Sun, Y., Yu, W. *et al.* 2019, "**Downregulation of miRNA-214 in cancer-associated fibroblasts contributes to migration and invasion of gastric cancer cells through targeting FGF9 and inducing EMT**", *Journal of Experimental & Clinical Cancer Research*. 38:20. doi: 10.1186/s13046-018-0995-9

61. Volinia, S., Galasso, M., Costinean, S. *et al.* 2010, "**Reprogramming of miRNA networks in cancer and leukemia**", *Genome Research,* vol. 20, no. 5, pp. 589-599.

62. Schoepp, M., Ströse, A.J. & Haier, J. 2017, "**Dysregulation of miRNA Expression in Cancer Associated Fibroblasts (CAFs) and Its Consequences on the Tumor Microenvironment**", *Cancers,* vol. 9, no. 6. doi: 10.3390/cancers9060054

63. Busse, A. & Keilholz, U. 2011 "**Role of TGF-β in melanoma**", *Current Pharmaceutical Biotechnology,* vol. 12, no. 12, pp. 2165-2175.

64. Melnik, B.C. 2015, "**MiR-21: an environmental driver of malignant melanoma?**", *Journal of Translational Medicine.* 13:202. doi: 10.1186/s12967-015-0570-5

65. Tian, Y., Wei, W., Li, L. *et al.* 2015, "**Down-Regulation of miR-148a Promotes Metastasis by DNA Methylation and is Associated with Prognosis of Skin Cancer by Targeting TGIF2**", *Medical Science Monitor.* 21:3798-3805. doi: 10.12659/MSM.894826

66. Lujambio, A., Calin, G.A., Villanueva, A. *et al.* 2008, "**A microRNA DNA methylation signature for human cancer metastasis**", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 36, pp. 13556-13561.

67. Min, A., Zhu, C., Peng, S. *et al.* 2015, "**Downregulation of Microrna-148a in Cancer-Associated Fibroblasts from Oral Cancer Promotes Cancer Cell Migration and Invasion by Targeting Wnt10b**", *Journal of Biochemical and Molecular Toxicology,* vol. 30, no. 4, pp. 186-191.

68. Aprelikova, O., Palla, J., Hibler, B. *et al.* 2013, "**Silencing of miR-148a in cancer-associated fibroblasts results in WNT10B-mediated stimulation of tumor cell motility**", *Oncogene,* vol. 32, no. 27, pp. 3246-3253.

69. Jiang, Q., He, M., Ma, M.T. *et al.* 2016, "**MicroRNA-148a inhibits breast cancer migration and invasion by directly targeting WNT-1**", *Oncology Reports,* vol. 35, no. 3, pp. 1425-1432.

70. Liu, T., Zhou, L., Yang, K. *et al.* 2019, "**The β-catenin/YAP signaling axis is a key regulator of melanoma-associated fibroblasts**", *Signal Transduction and Targeted Therapy*. 4:63. doi: 10.1038/s41392-019-0100-7

71. Jurmeister, S., Baumann, M., Balwierz, A. *et al.* 2012, "**MicroRNA-200c Represses Migration and Invasion of Breast Cancer Cells by Targeting Actin-Regulatory Proteins FHOD1 and PPM1F**", *Molecular and Cellular Biology,* vol. 32, no. 3, pp. 633-651.

72. Shi, Q., He, Q. & Wei, J. 2018, "**MicroRNA-342 Prohibits Proliferation and Invasion of Melanoma Cells by Directly Targeting Zinc-finger E-box Binding Homeobox 1**", *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics*. 26:9. doi: 10.3727/096504018X15193823766141

73. Richard, G., Dalle, S., Monet, M.A. *et al.* 2016, "**ZEB1-mediated melanoma cell plasticity enhances resistance to MAPK inhibitors**", *EMBO Molecular Medicine,* vol. 8, no. 10, pp. 1143-1161.

74. Zhao, L., Sun, Y., Hou, X. *et al.* 2012, "**MiRNA expression analysis of cancer-associated fibroblasts and normal fibroblasts in breast cancer**", *The International Journal of Biochemistry & Cell Biology*, vol. 44, no. 11, pp. 2051-2059.

75. Melling, G.E., Flannery, S.E., Abidin, S.A. *et al.* 2018, "**A miRNA-145/TGF-β1 negative feedback loop regulates the cancer-associated fibroblast phenotype**", *Carcinogenesis,* vol. 39, no. 6, pp. 798-807.

76. Dynoodt, P., Speeckaert, R., De Wever, O. *et al.* 2013, "**miR-145 overexpression suppresses the migration and invasion of metastatic melanoma cells**", *International Journal of Oncology,* vol. 42, no. 4, pp. 1443-1451.

77. Jin, C., Wang, A., Liu, L. *et al.* 2019, "**miR-145-5p inhibits tumor occurrence and metastasis through the NF-κB signaling pathway by targeting TLR4 in malignant melanoma**", *Journal of Cellular Biochemistry,* vol. 120, no. 7, pp. 11115-11126.

78. Liu, S., Gao, G., Yan, D. *et al.* 2017, "**Effects of miR-145-5p through NRAS on the cell proliferation, apoptosis, migration, and invasion in melanoma by inhibiting MAPK and PI3K/AKT pathways",** *Cancer Medicine,* vol. 6, no. 4, pp. 819-833.

79. Cornil, I., Theodorescu, D., Man, S. *et al.* 1991, "**Fibroblast cell interactions with human melanoma cells affect tumor cell growth as a function of tumor progression**", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 88, no. 14, pp. 6028-6032.

80. Cheng, L., Lopez-Beltran, A., Massari, F. *et al.* 2018, "**Molecular testing for BRAF mutations to inform melanoma treatment decisions: a move toward precision medicine**", *Modern Pathology,* vol. 31, no. 1, pp. 24-38.

81. Smylie, M.G. 2020, "**Use of immuno-oncology in melanoma**", *Current Oncology,* vol. 7, no. S2, pp. S52-S58.

82. Stark, M.S., Klein, K., Weide, B. *et al.* 2015, "**The Prognostic and Predictive Value of Melanoma-related MicroRNAs Using Tissue and Serum: A MicroRNA Expression Analysis**", *EBioMedicine,* vol. 2, no. 7, pp. 671-680.

83. Laitala, A. & Erler, J.T. 2018, "**Hypoxic Signalling in Tumour Stroma",** *Frontiers in Oncology,* vol. 8, no. 189. doi: 10.3389/fonc.2018.00189

84. Wang, J., Zhao, J., Shi, M. *et al.* 2014, "**Elevated expression of miR-210 predicts poor survival of cancer patients: a systematic review and meta-analysis**", *PLOS One.* 9:2:e89223. doi: 10.1371/journal.pone.0089223

85. Ono, S., Oyama, T., Lam, S. *et al.* 2015, "**A direct plasma assay of circulating microRNA-210 of hypoxia can identify early systemic metastasis recurrence in melanoma patients**", *Oncotarget,* vol. 6, no. 9, pp. 7053-7064.

86. Felicetti, F., Errico, M.C., Segnalini, P. *et al.* 2008, "**MicroRNA-221 and -222 pathway controls melanoma progression",** *Expert Review of Anticancer Therapy,* vol. 8, no. 11, pp. 1759-1765.

87. Kanemaru, H., Fukushima, S., Yamashita, J. *et al.* 2011, "**The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker**", *Journal of Dermatological Science,* vol. 61, no. 3, pp. 187-193.

88. Kozubek, J., Ma, Z., Fleming, E. *et al.* 2013, "**In-Depth Characterization of microRNA Transcriptome in Melanoma**", *PLOS One,* vol. 8, no. 9. doi: 10.1371/journal.pone.0072699

89. Babapoor, S., Horwich, M., Wu, R. *et al.* 2016, "**microRNA in situ hybridization for miR-211 detection as an ancillary test in melanoma diagnosis**", *Modern Pathology.* 29:461-475. doi: 10.1038/modpathol.2016.44

90. Pfeffer, S.R., Grossmann, K.F., Cassidy, P.B. *et al.* 2015, "**Detection of Exosomal miRNAs in the Plasma of Melanoma Patients**", *Journal of Clinical Medicine,* vol. 4, no. 12, pp. 2012-2027.

91. Liu, Q., Peng, F. & Chen, J. 2019, "**The Role of Exosomal MicroRNAs in the Tumor Microenvironment of Breast Cancer**", *Europe PMC.* 20:16. doi: 10.3390/ijms20163884

92. Ji, W., Sun, B. & Changqing, S. 2017, "**Targeting MicroRNAs in Cancer Gene Therapy",** *Genes,* vol. 8, no. 1, pp. 21

93. Liu, X., Fang, H., Chen, H. *et al.* 2012, "**An Artificial miRNA against HPSE Suppresses Melanoma Invasion Properties, Correlating with a Down-Regulation of Chemokines and MAPK Phosphorylation**", *PLOS One,* vol. 7, no. 6. doi: 10.1371/journal.pone.0038659

94. Cui, S., Wang, J., Wu, Q. *et al.* 2017, "**Genistein inhibits the growth and regulates the migration and invasion abilities of melanoma cells via the FAK/paxillin and MAPK pathways**", *Oncotarget,* vol. 8, no. 13, pp. 21674-21691.

95. Sun, Q., Cong, R., Yan, H. *et al.* 2009, "**Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression**", *Oncology Reports,* vol. 22, no. 3, pp. 563-567.

96. Wang, J., Guan, X., Zhang, Y. *et al.* 2018, "**Exosomal miR-27a Derived From Gastric Cancer Cells Regulates the Transformation of Fibroblasts Into Cancer-Associated Fibroblasts**", *Cellular Physiology and Biochemistry,* vol. 49, no. 3, pp. 869-883.

97. Reuland, S.N., Smith, S.M., Bernis, L.T. *et al.* 2013, "**MicroRNA-26a Is Strongly Downregulated in Melanoma and Induces Cell Death through Repression of Silencer of Death Domains (SODD)**", *Journal of Investigative Dermatology,* vol. 133, no. 5, pp. 1286-1293.

98. Antoku, K., Maser, R.S., Scully, W.J. *et al.* 2001, "**Isolation of Bcl-2 binding proteins that exhibit homology with BAG-1 and suppressor of death domains protein**", *Biochemical and Biophysical Research Communications,* vol. 286, no. 5, pp. 1003-1010.

99. Urabe, F., Kosaka, N., Sawa, Y. *et al.* 2019, "**miR-26a regulates extracellular vesicle secretion from prostate cancer cells via targeting SHC4, PFDN4 and CHORDC1**", *bioRxiv.* 6:18. doi: 10.1101/646380

100. Wang, M., Yu, F., Ding, H., *et al.* 2019, "**Emerging Function and Clinical Values of Exosomal MicroRNAs in Cancer**", *Molecular Therapy Nucleic Acids,* vol. 16, pp. 791-804.