

MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION IN CUTANEOUS MELANOMA

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1 **ABSTRACT**

2 The Warburg effect in tumour cells is associated with the upregulation of glycolysis to
3 generate ATP, even under normoxic conditions and the presence of fully functioning
4 mitochondria. However, scientific advances made over the past 15 years have reformed this
5 perspective, demonstrating the importance of oxidative phosphorylation (OXPHOS) as well as
6 glycolysis in malignant cells. The metabolic phenotypes in melanoma display heterogeneic
7 dynamism (metabolic plasticity) between glycolysis and OXPHOS, conferring a survival
8 advantage to adapt to harsh conditions and pathways of chemoresistance. Furthermore, the
9 simultaneous upregulation of both OXPHOS and glycolysis (metabolic symbiosis) has been
10 shown to be vital for melanoma progression. The tumour microenvironment (TME) has an
11 essential supporting role in promoting progression, invasion and metastasis of melanoma.
12 Mesenchymal stromal cells (MSCs) in the TME show a symbiotic relationship with melanoma,
13 protecting tumour cells from apoptosis and conferring chemoresistance. With the significant
14 role of OXPHOS in metabolic plasticity and symbiosis, our review outlines how mitochondrial
15 transfer from MSCs to melanoma tumour cells plays a key role in melanoma progression and
16 is the mechanism by which melanoma cells regain OXPHOS capacity even in the presence of
17 mitochondrial mutations. The studies outlined in this review indicate that targeting
18 mitochondrial trafficking is a potential novel therapeutic approach for this highly refractory
19 disease.

20

21 **KEY WORDS**

22 Melanoma, Skin Cancer, Metastasis, Tumour Microenvironment, Oxidative Phosphorylation,
23 Mitochondrial transfer

24 **INTRODUCTION**

25 Melanoma is the most aggressive, deadly form of skin cancer (1) — despite accounting for
26 only 5% of cases, it constitutes the main cause of deaths from skin cancer (2). It is also one of
27 the fastest growing cancers worldwide (2), with the UK reporting 16,000 new cases every year
28 (3). Along with the long-standing global trend of incidence rise (4), worldwide mortality rates
29 are expected to increase from 61,850 in 2016 to 108,630 by 2040 (5).

30

31 Melanoma is highly curable when limited to the primary site (6); metastatic melanoma,
32 however, confers a poor prognosis of a median survival of six months (7). Current systemic
33 therapies in patients with metastatic melanoma have a varied response rate, and tumour
34 resistance develops rapidly in the majority of patients (6, 8-10). Further research is therefore
35 required to understand the pathophysiology of this highly refractory disease, in the context
36 of the role of metabolism (oxidative phosphorylation and/or glycolysis) in melanoma, and the
37 interaction of melanoma with the tumour microenvironment (TME), which supports its
38 survival and proliferation, and contributes to drug resistance.

39

40 Primary cutaneous melanoma comprises a distinctly heterogeneous population of both
41 cancerous and non-cancerous cells (11, 12), including fibroblasts, adipocytes and other niche
42 cells such as mesenchymal stromal cells (MSCs), which make up the extracellular matrix,
43 endothelial cells of the microvasculature, and immune cells (11-13). In addition to the cellular
44 component of the tumour microenvironment (TME), the non-cellular component consists of
45 several growth factors, chemokines and cytokines (14). Melanoma cells can manipulate the
46 close association between themselves and the TME to facilitate tumour progression, invasion
47 and metastasis (15, 16). Currently, immune cells in the TME have been the focus of much

48 interest in an attempt to understand how an immunosuppressive microenvironment that
49 allows for proliferation, growth and invasion of melanoma is created (10), while, by contrast,
50 relative little research has been carried out on the role of MSCs in the TME in melanoma
51 growth.

52

53 In this review, we explore the symbiotic relationship between melanoma and MSCs and the
54 ensuing metabolic advantage conferred on melanoma. We begin by describing the
55 metabolism of melanoma and metabolic plasticity in melanoma cells before introducing
56 metabolic symbiosis with MSCs and outlining potential mechanisms of transfer of
57 mitochondrial DNA from MSCs to melanoma to facilitate oxidative phosphorylation.

58

59 **MSCs IN THE TME**

60 MSCs — spindle-shaped cells that are present in bone marrow, adipose, skin, umbilical cord,
61 blood and various other tissues (17-20) — are highly proliferative and can differentiate into
62 various cells such as osteoblasts, chondrocytes and adipocytes (17-19). These properties, in
63 addition to their ability to home towards injured tissue, can be exploited by melanoma, which,
64 like many other solid cancers, behaves like tissues that do not heal (21, 22): increasing
65 evidence has shown that, like a chronic, non-healing wound, melanoma secretes
66 chemoattractants (23), similar to those used in inflammatory signalling pathways (24), to
67 attract and direct MSCs towards the tumour sites and form part of the TME to contribute
68 towards tumour progression, invasion and metastasis (23, 25-28).

69

70

71

72 ***MSCs and melanoma growth***

73 A positive effect for MSCs on tumour incidence was first demonstrated by co-injecting
74 allogeneic mice with B16 melanoma cells and MSCs: not only was the incidence of tumour
75 formation 100% when the melanoma cells and MSCs were injected together versus 0% in the
76 control group (29, 30), but the onset of tumour formation was faster when soluble MSC-
77 derived factors were added (31). Kucerova *et al.* demonstrated this increased tumour
78 incidence and growth using the human melanoma cell line A375 and human MSCs, as well as
79 showing that this increase was dependent on the dose of MSCs (32). MSCs also abrogated
80 tumour latency *in vivo* for low numbers of cells that would otherwise not normally produce
81 tumours if injected alone (32). Furthermore, MSCs were shown to protect melanoma cells
82 from sustaining cellular stress in response to systemic treatment, such as doxorubicin, and
83 cytotoxicity by inhibiting apoptosis. Notably, the effect of MSCs on tumour initiation was
84 reported in experiments using low volumes of A375 melanoma cells, mimicking minimal
85 residual disease that is common following radiotherapy treatment. Together, these data
86 demonstrate the pro-oncogenic role of MSCs on melanoma growth.

87

88 ***Additional pro-oncogenic roles of MSCs***

89 MSCs also display various other pro-oncogenic behaviours, which are outlined here but not
90 covered in detail as they are not the focus of this review. MSCs have been reported to increase
91 the motility and invasiveness of melanoma by communicating with melanoma-derived
92 exosomes, to manipulate melanoma cells towards a more metastatic phenotype via the
93 process of epithelial–mesenchymal transition (EMT) (33) and by increasing the porosity of
94 blood vessels, thereby facilitating tumour migration (33). Current reports have demonstrated
95 the ability of MSCs to support neovascularisation in a mouse model of melanoma through the

96 secretion of pro-angiogenic factors (27, 28, 34). Kucerova *et al.* demonstrated enhanced
97 melanoma growth as a result of this proangiogenic cellular milieu created by the mutual
98 crosstalk between melanoma and MSCs (32). In addition to the secretion of various factors,
99 Vartanian *et al.* provided direct evidence that melanoma can educate MSCs to engage in
100 vasculogenic mimicry, a process in which MSCs adopt certain endothelial-cell-like properties
101 to directly contribute to the formation of the tumour vasculature (28). Several studies have
102 also demonstrated the ability of MSCs to differentiate into carcinoma-associated fibroblasts
103 (CAFs) (28, 34), a key cellular component of the growth-supporting TME, aiding the formation
104 of the stem-cell niche and promoting stemness in the tumour (23, 35). Not only do these CAFs
105 and MSCs promote tumour growth, but they have also been shown to have
106 immunomodulatory functions — for example, reducing T-cell proliferation and the number
107 of tumour-infiltrating T and B cells *in vivo*, and producing cytokines — thereby creating a
108 highly effective immunosuppressive TME for melanoma proliferation (23, 36).

109

110 ***MSC–melanoma symbiosis confers metabolic advantage***

111 The processes of MSC-mediated tissue repair, which involves activation, migration and
112 homing to TME, and MSC differentiation and subsequent secretion of factors (by both
113 melanoma cells and MSCs) produce a strong pro-oncogenic symbiotic relationship between
114 MSCs and melanoma cells (21). This symbiotic relationship provides a metabolic advantage
115 that is effective for melanoma proliferation and metastasis (37-39). Given the significance of
116 metabolism in melanoma, supported by the growing evidence of its impact on the efficacy of
117 current systemic therapies for this highly refractory disease (38), below we explore the
118 symbiotic relationship between MSCs and melanoma, and how it might arise.

119

120 **METABOLISM OF MELANOMA**

121 Due to its significant influence on all aspects of tumorigenesis, metabolic reprogramming has
122 been widely accepted as one of the hallmarks of cancer (40). Determining the biochemical
123 pathway that melanoma cells use for energy production allows researchers to understand the
124 influence of metabolism on the symbiotic relationship between melanoma and MSCs and its
125 corresponding pro-oncogenic role (41).

126

127 ***Glycolysis in melanoma***

128 In the 1920s, Warburg reported that, even in presence of oxygen, cancer cells take up glucose
129 for glycolysis (42). This preferential method for energy production adopted by cancers was
130 termed 'aerobic glycolysis' (also known as the Warburg effect) and was demonstrated to
131 provide ATP necessary for survival and proliferation of the tumour (43).

132

133 Melanoma has been demonstrated to be associated with a glycolytic phenotype (44, 45).
134 Aerobic glycolysis in melanoma cells is driven by a multitude of factors, including activation
135 of oncogenes, the presence of a hypoxic TME and an absence of tumour suppressors (46).
136 Approximately 50–60% of melanomas contain a *BRAF* gene mutation (47), the most frequent
137 of which (*BRAF*V600E, accounting for 90% of *BRAF* mutations (48) and rendering the gene
138 product B-Raf constitutively active (49)) has been shown to be associated with higher glucose
139 uptake and subsequent glycolysis (50). B-Raf activates the mitogen-activated protein kinase
140 (MAPK) pathway, which promotes hypoxia-inducible factor 1 α [HIF1 α (master regulator of
141 glycolysis)], resulting in an increase in glycolysis (50). Furthermore, B-Raf inhibits
142 microphthalmia-associated transcription factor (MITF) and peroxisome proliferator-activated
143 receptor-gamma coactivator 1 α (PGC-1 α), thereby inhibiting oxidative phosphorylation

144 (OXPHOS) (43, 51, 52). OXPHOS is the main pathway for energy production in mitochondria
145 via aerobic respiration. Providing direct evidence for this B-Raf mediated upregulation of
146 aerobic glycolysis, Hall *et al.* (44) demonstrated a 14–16-fold higher extracellular acidification
147 rate (ECAR, resulting from respiratory and glycolytic acidification) in melanoma cells
148 compared to melanocytes. Furthermore, treatment with the glycolysis inhibitor 2-Deoxy-D-
149 glucose (2-DG) induced a significant drop in ATP production by melanoma cells, causing them
150 instead to revert to OXPHOS for energy production. Analysis of the ECAR (a surrogate marker
151 for glycolysis) and oxygen consumption rate (OCR; a surrogate marker for OXPHOS) in these
152 cells uncovered a lower OCR/ECAR ratio, indicating the upregulation of glycolysis rather than
153 low oxygen consumption or lower OXPHOS capacity. In fact, the absolute OCR values were
154 higher in melanoma cell lines compared to melanocytes, with corresponding high OXPHOS
155 enzyme activity. Therefore, although glycolysis is upregulated in melanoma, OXPHOS also
156 plays a role.

157

158 ***Oxidative phosphorylation in melanoma***

159 Whether a specific metastatic lesion relies on either glycolysis or OXPHOS depends upon the
160 heterogeneity of individual tumour types (53). Tumours behave individually, with each cancer
161 demonstrating its own metabolic properties (46, 53). To add further complexity, even within
162 an individual tumour, the constituent cells can be heterogeneous, displaying different energy
163 metabolic phenotypes (46). For example, large B cell lymphomas can be split into a low
164 OXPHOS subset and a high OXPHOS subset; the latter subset show an upregulation of
165 mitochondrial electron transport chain components (54). Whilst many melanomas have an
166 aerobic glycolytic phenotype, a subset has been shown to present with higher OXPHOS
167 phenotype (38, 52). Fischer *et al.* have identified that 35–50% of BRAF-mutant and wild-type

168 cell lines and patient samples can be categorised into this subset (38), indicating that a
169 significant proportion of melanoma cells present with a higher OXPHOS phenotype. PGC-1 α
170 is a member of a family of transcriptional coactivators that play a central role in the regulation
171 of cellular energy metabolism and mitochondrial biogenesis (55). Regulatory mechanisms to
172 suppress OXPHOS mediated via the PGC-1 α pathway fail to occur in high OXPHOS melanomas
173 (38). Higher PGC-1 α levels are correlated with poorer survival in melanoma patients (52). The
174 PGC-1 α -driven high OXPHOS subset demonstrated an improved tolerance to the damaging
175 effects of reactive oxygen species (ROS), indicating their increased ability to survive under
176 conditions of oxidative stress (52). *In vivo* experiments in mice demonstrated that PGC-1 α
177 knockdown resulted in reduced metastasis of melanoma (56), highlighting the pro-oncogenic
178 role of OXPHOS in melanoma progression and metastasis.

179

180 ***OXPHOS and glycolysis in melanoma***

181 Ho *et al.*(57) suggested that both OXPHOS and glycolysis play a significant role in the
182 progression of melanoma and generation of ATP. They discovered two patient populations
183 within their melanoma cohorts: one with high serum levels of lactate dehydrogenase (LDH)
184 and one with normal serum LDH levels. The high serum LDH population had a corresponding
185 high ECAR, suggesting that glycolysis was the predominant metabolic pathway. By contrast,
186 in the normal serum LDH population, the tumours demonstrated elevation of several OXPHOS
187 enzymes and higher OCR, indicating that OXPHOS was the predominant metabolic pathway.
188 However, it is important to note that, although high serum LDH levels are associated with
189 poor prognosis in metastatic melanoma patients (58), serum LDH levels might not necessarily
190 always be a marker of tumour-associated increased cell turnover, as patients can have high
191 LDH levels and perform poor clinically due to other factors such as tissue damage, severe

192 infections and renal failure (59). The OCR rates were higher in both populations of melanoma
193 patients, as well as in melanoma samples from patient tumour biopsy samples and cell lines
194 in culture, compared to normal melanocytes. Thus, OXPHOS and glycolysis both play a
195 significant role in melanoma metabolism (60, 61).

196

197 **METABOLIC PLASTICITY**

198 Although it is simpler to categorise melanoma into a glycolytic or OXPHOS phenotype, an
199 increasing body of evidence suggests that the nature of metabolic phenotypes in melanoma
200 is dynamic — this is termed ‘metabolic plasticity’ (38, 40). Jose *et al.*(40) demonstrated that
201 the metabolic phenotype of melanoma is not fixed during tumorigenesis and, in fact,
202 melanoma has a ‘hybrid’ glycolysis/OXPHOS metabolic phenotype, intuitively conferring
203 selective advantages on tumour cells (45). Importantly, this hybrid phenotype provides
204 tumour cells with the flexibility to use different energy sources to meet their bioenergetic
205 needs in the different and changing TME (62). In a glucose-deprived environment, tumour
206 cells are metabolically reprogrammed towards elevated levels of OXPHOS with decreased
207 glycolysis, whereas in hypoxic conditions, the melanoma cells preferentially use glycolysis,
208 uncoupling from the TCA cycle and attenuating mitochondrial respiration (45).

209

210 Metabolic plasticity confers on melanoma cells not only the ability to adapt to harsh TME
211 conditions but also a degree of chemoresistance, thereby providing a survival advantage in
212 treatment-induced conditions (38). The use of targeted systemic therapy such as BRAF
213 inhibitors (e.g. vemurafenib) to target BRAFV600E is associated with a switch from glycolysis
214 to OXPHOS (63). Similarly, Haq *et al.* demonstrated that treatment with MAPK inhibitors
215 resulted in increased PGC-1 α -driven OXPHOS (51). Notably, an increase in PGC-1 α -driven

216 OXPHOS is observed in 30–50% of *BRAF*-mutant melanomas with *de novo* and acquired
217 resistance to MAPK inhibitors (64) and, in these circumstances, PGC-1 α knockdown resulted
218 in reduced tumour growth (64). This metabolic switch from glycolysis to OXPHOS and the
219 corresponding adaptive resistance was observed in patients treated with inhibitors of BRAF
220 or MEK (MAPK and ERK kinase; an upstream activator of MAPK), alongside increased
221 mitochondrial content, mitochondrial activity and mitochondrial oxidative capacity (37, 65-
222 68). These observations highlight the ability of melanoma to redirect the metabolic
223 phenotypes to confer multiple pathways of chemoresistance. Collectively, it is clear that
224 metabolic plasticity confers a significant survival advantage on cancer cells.

225

226 **METABOLIC SYMBIOSIS**

227 Within melanoma tumours, the extent of tissue perfusion and oxygenation depends on the
228 location and physical distance of the tumour cells from the local vasculature (57). Accordingly,
229 melanoma cells located in the poorly perfused centre of tumour masses are more likely to be
230 predominantly dependent on glycolysis, whereas tumour cells closer to the vasculature at the
231 periphery are more likely to be dependent on OXPHOS (57). It has, however, been proposed
232 that these two spatially distinct cell populations might be linked, such that the end products
233 from glycolysis (e.g. lactate) are utilised to feed into the TCA cycle for OXPHOS (57).

234

235 Indeed, Ho *et al.* (57) reported that, although melanoma patients showed high levels of serum
236 LDH, monocarboxylate transporters MCT4, (the principal transporter for lactate efflux (69)
237 and a downstream effector of hypoxia-inducible factor (HIF)-1 α (70)), indicating that the
238 melanoma cells predominantly used glycolysis for energy production, the serum lactate levels
239 remained constant. Although it is plausible that the lactate levels might not be affected by

240 the tumour, the above data demonstrating upregulation of glycolysis make it more likely that
241 the lactate levels remain unchanged due to other processes. Ho *et al.* suggested that
242 increased levels of lactate resulting from glycolysis are taken up by the metabolically
243 symbiotic melanoma cells that use OXPHOS as their primary mechanism of energy
244 production. When enzymes associated with OXPHOS and glycolysis were both expressed at
245 higher levels, it was evident that OXPHOS and glycolysis were both upregulated in melanoma,
246 compared to normal tissues, demonstrating a further stage to metabolic plasticity (57). This
247 co-operation of both OXPHOS and glycolysis occurring at the same time has been coined
248 'metabolic symbiosis' (Figure 1A). Several papers (71) have reported this phenomenon and
249 demonstrated its importance in melanoma initiation, growth and metastasis.

250

251 **THE REVERSE WARBURG EFFECT**

252 *In vivo* work carried out over the past decade has demonstrated that metabolic
253 reprogramming involves not only cancer cells but also the MSCs and CAFs in the TME (62, 72).
254 Whereas the Warburg effect refers to glycolysis being the preferential method of energy
255 production in tumour cells, according to the 'Reverse Warburg' effect, tumour cells, by
256 secreting ROS (by-product of OXPHOS melanoma cells), stimulate cells in the surrounding
257 TME to undergo aerobic glycolysis, resulting in the secretion of metabolites, such as lactate,
258 into the TME via MCT4 (73). These metabolic intermediates can be taken up by tumour cells,
259 via MCT1, to feed into the TCA cycle for OXPHOS-mediated energy production (74), similar to
260 the situation in metabolic symbiosis outlined above. Loss of Cav-1, a protein involved in
261 endocytosis and vesicular transport, in TME cells results in a positive-feedback loop of
262 oxidative stress in these cells, consequently increasing OXPHOS in tumour cells (62). This
263 Reverse Warburg effect was initially reported in a variety of cancers (38) but is as yet to be

264 fully elucidated in melanoma. However, taking together the use of OXPHOS in the periphery
265 of melanomas, the metabolic symbiosis reported earlier, and Ho *et al.*'s findings of increased
266 expression of MTC1 and MTC4 in melanoma (57), it is likely that the Reverse Warburg effect
267 occurs in the TME of melanoma (Figure 1B).

268

269 The above research has demonstrated that the oncological hallmark of altered metabolism is
270 not only due to the regulation for growth, but can be primary cause for tumour initiation,
271 progression, metastasis and chemoresistance. Due to the heterogeneous dynamism (metabolic
272 plasticity) between glycolysis and OXPHOS of melanoma, the effective blockade of OXPHOS
273 (e.g. using inhibitors of mTORC1) as well as glycolysis (e.g. MAPK pathway inhibitors) has been
274 shown to resensitise melanomas that are resistant to inhibitors of BRAF and other MAPK
275 pathway components **[AU: OK?]**, and thus to be a promising form of treatment (64, 75).
276 Previous work has shown that upregulation of aerobic glycolysis in tumour cells is due to the
277 presence of mitochondrial DNA (mtDNA) mutations, which were assumed to impair OXPHOS
278 capacity. However, several papers have demonstrated that these mtDNA mutations do not
279 necessarily equate to a compromise in OXPHOS capacity. Conversely, although cancer cells
280 retain OXPHOS capacity, they can also possess mtDNA mutations due to damaging effects of
281 higher ROS secretion in mitochondria from inefficient repair mechanisms, close proximity and
282 vulnerability of mtDNA (43, 46, 50, 76). Consequently, further research was carried out to
283 discover why melanoma cells with mtDNA mutations still possessed the capacity to use
284 OXPHOS for energy production, as well as to develop more effective OXPHOS therapies
285 against melanoma.

286

287

288 **MITOCHONDRIAL TRANSFER**

289 In 2010, Berridge and Tan (77) designed a model of B16 melanoma cell lines with severe
290 mtDNA damage caused by the mitochondrial gene deletion p0 to investigate mitochondrial
291 OXPHOS. The authors observed that not only did the p0 cells grow at half the rate of their
292 parental cells *in vitro*, but they also underwent delayed primary subcutaneous melanoma
293 growth and reduced lung metastasis formation in mouse models *in vivo*, compared to B16
294 parental cells (77). At the time these studies were carried out, this delay was suggested to be
295 due to the time taken to adapt to auxotrophic requirements and local microenvironmental
296 conditions. However, a series of *in vitro* experiments demonstrating mitochondrial trafficking
297 in other cancers (Table 1) as a prerequisite for aerobic respiration, tumour growth, metastasis
298 and chemoresistance (78-88) implied that the delay might be the result of mitochondrial
299 trafficking from MSCs in the TME to tumours. Additional investigations in other cancers into
300 the mechanisms and stimuli behind mitochondrial trafficking, such as NADPH-oxidase-2-
301 driven and CD38-driven in acute myeloid leukaemia and multiple myeloma, respectively, have
302 led to the development of effective therapeutic agents targeting mitochondrial trafficking,
303 with demonstrated effective tumour regression (83, 84, 89).

304

305 ***Mitochondrial transfer in melanoma***

306 Consistent with the results obtained in other tumours, Tan *et al.* (88) subsequently
307 demonstrated, in 2015, that the delay in melanoma tumour growth when B16p0 cells were
308 injected in NOD/SCID mice was due to the time taken for these cells to acquire mtDNA from
309 the TME *in vivo*. In 2017, Dong *et al.* (90) demonstrated that the tumours that grew from
310 injected B16p0 cells *in vivo*, after a delay, contained host TME mtDNA (confirmed via single-
311 cell droplet PCR methods), and that the B16p0 cells had acquired mitochondria from host

312 MSCs by the presence of double-positive cells when B16p0 cells with nuclear-targeted blue
313 fluorescent protein were injected into C57BL/GN mice with red fluorescent mitochondria in
314 mouse MSCs.

315

316 The primary role of mitochondria is to produce energy via OXPHOS (91), and mtDNA encodes
317 peptides that are essential for this task (92). Accordingly, Dong *et al.* demonstrated that the
318 injected B16p0 cells that gained mtDNA subsequently contained mtDNA-encoded proteins
319 and fully assembled respirasomes, with a higher OCR and increased ATP production than
320 injected B16p0 cells that failed to gain mtDNA. These results demonstrated that the mtDNA
321 transferred to the B16p0 cells was functional and conferred similar OXPHOS respiration rates
322 and respiration recovery to those of their parental B16 cells.

323

324 Finally, Dong *et al.* (90) provided direct evidence for the requirement of OXPHOS respiration
325 mediated by mtDNA transfer from MSCs to melanoma cells in tumour formation. B16p0 cells
326 with OXPHOS respiration suppressed (via inhibition of the catalytic subunits of CI and CII)
327 formed tumours with an even longer lag period of 15–40 days compared with B16p0 cells
328 without OXPHOS suppressed, *in vivo*. A similar pattern was observed for parental B16 cells
329 with OXPHOS knocked down (90). This indicates a shift in viewpoint regarding cancer
330 metabolism, with mitochondrial DNA mutations not compromising OXPHOS capacity. These
331 results collectively demonstrate that melanoma cells gain mtDNA from the MSCs and their
332 subsequent rapid OXPHOS recovery is a prerequisite for driving efficient tumour formation.
333 Although mitochondrial trafficking in melanoma has only been shown in the murine B16 cell
334 line, the extensive experiments mentioned above, coupled with the importance of
335 mitochondrial trafficking demonstrated in several other human cancers, make it very

336 plausible that mitochondrial trafficking plays a vital role in human melanoma progression. A
337 major gap in the literature therefore exists, and further experiments are required to
338 demonstrate role of mitochondrial trafficking in other cell lines and human melanoma.

339

340 Despite intracellular transfer of mtDNA being the most likely transfer mechanism, other
341 possible explanations for mtDNA acquisition and respiration recovery have been suggested.
342 First, it is plausible that a few tumour cells with mitochondria and mtDNA replicate their
343 mtDNA and proliferate at much faster rate than tumour cells without mtDNA, and that the
344 tumour cells without mtDNA might then be progressively removed by autophagy. However,
345 markers for autophagy, such as LC3A11 protein, were lower in B16p0 cells compared with B16
346 cell lines, suggesting that this is not the case (90). Another possible explanation is the
347 presence of B16p0 cells that contained residual undetectable mtDNA. However, this theory
348 was rejected by Dong *et al.*, who used assays that were able to detect heteroplasmy down to
349 0.5%. The absence of mtDNA in p0 cells was further reinforced by confocal microscopy
350 analysis, and the absence of any latent respirasomes/supercomplexes prior to mtDNA
351 acquisition was shown via native blue gel electrophoresis. Thus, the only plausible mechanism
352 of mtDNA acquisition in tumour cells is thought to be transfer from host TME.

353

354

355 **MECHANISM OF MTDNA TRANSFER**

356 Studies carried out over the past 15 years have demonstrated that mitochondria can cross
357 cell boundaries and be transferred horizontally between cells (93). The main mechanisms of
358 mtDNA transfer from MSCs to tumour cells are tunnelling nanotubules (TNTs), microvesicles
359 and gap junctions, although other plausible mechanisms exist that require further research,
360 such as cell fusion and direct mtDNA secretion into extracellular media (93-95) (Figure 2).

361

362 **Tunnelling nanotubules**

363 Tunnelling nanotubes (TNTs) are filopodial extensions (bundles of rod-like shaped parallel
364 actin filaments) of cell cytoplasm that connect two cells via open-ended channels (96, 97)
365 (Figure 2A). TNTs have been shown to facilitate the transfer of biomaterial such as cellular
366 organelles, cytoplasmic molecules and membrane molecules between cells (97). Koyangi *et*
367 *al.* were the first to document (in 2005) whole mitochondrial transfer through TNTs from
368 cardiomyocytes to endothelial progenitor cells (98); mitochondrial transfer into melanoma
369 via TNTs was not demonstrated until 2017 (90).

370

371 **Extracellular vesicles (EVs)**

372 mtDNA can also be horizontally transferred through the movement of mitochondrial-derived
373 products or intact mitochondria in EVs — exosomes or microvesicles, respectively (93) (Figure
374 2B). Exosomes are small (30-100nm in diameter) membrane-encapsulated vesicles formed in
375 the endosomal pathway (94, 99, 100). During the endosomal pathway, segments of
376 endosomal membrane bud inside the endosome as a collection of intraluminal vesicles (ILVs)
377 to form multivesicular bodies (MVBs) (94). These MVBs move to the cell's surface plasma
378 membrane release ILVs (exosomes) externally into the extracellular media (94, 101). In

379 contrast, microvesicles, largest EVs (50-1000nm in diameter) (99), are formed directly from
380 external budding and fission of the plasma membrane of the cell into the extracellular media
381 (94, 102, 103). Guescini *et al.* demonstrated the potential of exosomes as vectors for
382 mitochondrial transfer in glioblastoma and astrocyte cells, which routinely secrete EVs into
383 the intercellular space (104). Isolation of these purified EVs demonstrated the presence of
384 mtDNA and absence of nuclear DNA. Furthermore, high mtDNA levels and mitochondrial
385 proteins were shown to be present in exosomes released into the intercellular media by
386 skeletal muscle cells (105). Although these papers implied that EVs could function as mtDNA
387 carriers [AU: OK?], Islam *et al.* (106) were the first to demonstrate mitochondrial transfer
388 from MSCs to lung alveolar epithelial cells via EVs *in vivo*. Furthermore, Sinclair *et al.* (95)
389 demonstrated that mitochondrial trafficking was reduced by 34.7% after inhibition of
390 endocytosis of EVs into lung epithelial cells. These results provide *in vivo* evidence for the
391 transfer of mitochondria from MSCs to tumour cells via EVs, although transfer from MSCs to
392 melanoma by this means has not yet been reported.

393

394 **Gap junctions**

395 Gap junctions are intercellular channels composed of two connexons, joined together in the
396 intercellular space, that directly connect the cytoplasm of two different cells (107) (Figure 2C).
397 Whereas TNTs facilitate long-distance communication, gap junctions promote close cell-to-
398 cell communication (107). Islam *et al.* (106) demonstrated gap-junction-mediated
399 mitochondrial transfer from MSCs and a subsequent increase in ATP production for tissue
400 repair in alveolar epithelial cells in an *in vivo* mouse model of acute lung injury. These results
401 were reproducible in other models comprising MSCs with haematopoietic stem cells (108) or
402 epithelial cells (95), with a increase in mtDNA transfer and mitochondrial content in recipient

403 cells. Most current literature agrees that gap junctions are one of the main mechanisms for
404 mitochondrial transfer from MSCs to the target cell (93), although this method of
405 mitochondrial transfer has so far not been demonstrated in melanoma.

406

407 **Alternative mechanisms**

408 In the three main transfer mechanisms outlined above, mitochondrial transfer is quick and
409 unidirectional. However, Sinclair *et al.* (95) demonstrated that, although inhibition of all these
410 mechanisms reduced mitochondrial transfer, it did not completely prevent it, indicating the
411 possibility that additional mechanisms exist.

412

413 Cell fusion, whereby the plasma membranes of two cells fuse and merge together whilst
414 retaining their nuclei (94), is a contentious form of intercellular communication (Figure 2D).
415 Evidence for mitochondrial transfer via cell fusion remains scarce, as it is difficult to ascertain
416 whether the host cell remains as a host cell after fusion (93). Wada *et al.* modelled cell fusion
417 *in vitro* by developing microfluidic devices that fused paired single cells together through a
418 microslit to form a cytoplasmic connection (109). They demonstrated that this cell fusion
419 system enabled whole mitochondria to be transferred from parental osteosarcoma cells to
420 p0 osteosarcoma cells and that, after transfer, the fused cells would spontaneously
421 disconnect and recover in normal culture. Further data are required to substantiate cell fusion
422 as a method of transfer from MSCs to cancer.

423

424 Although Guescini *et al.* demonstrated the transfer of mtDNA via EVs, they also showed that
425 a significant proportion of mtDNA was free in the intercellular media (104). Other studies
426 have identified the release of endogenous mtDNA, as 'damage'-associated molecular

427 patterns (DAMPs), into the intercellular media in response to injury and inflammation (110).
428 As carcinogenesis mimics a chronic inflammatory state (111), it is likely that tumours secrete
429 mtDNA into the media, identifying mtDNA secretion into the media as another potential
430 method of mitochondrial transfer (Figure 2E).

431

432 **CONCLUSIONS AND DISCUSSION**

433 In melanomas, the TME is known to be important for conferring treatment resistance to the
434 tumour cells. The bulk of the TME is formed by MSCs and the cells they give rise to. In contrast
435 with melanoma cells, MSCs have stable genomes, and so offer themselves as a better
436 potential for therapeutic targeting. OXPHOS plays a significant role in metabolic plasticity,
437 metabolic symbiosis and the homeostasis of the high OXPHOS subset in melanoma, allowing
438 the development of treatment resistance. MSCs ensure that melanoma cells can retain an
439 independent OXPHOS capacity via mitochondrial trafficking to melanoma cells. Mitochondrial
440 trafficking has been shown to be a prerequisite for continued aerobic respiration, subsequent
441 tumour growth, metastasis and the development of chemoresistance and, consequently,
442 inhibition of this process has been integrated into the treatment pathway for other cancers
443 (112, 113).

444

445 In this review, we have highlighted mitochondrial trafficking as a potential target to combat
446 the prevalent resistance to current therapies in melanoma. We also outline the need for
447 further research into the different potential mechanisms of mitochondrial trafficking. As
448 mentioned above, only TNT-mediated transfer has definitively been demonstrated as a
449 means for mitochondrial transfer to melanomas; the fact that EVs and gap junctions are
450 commonly employed for mtDNA transfer by most cancers highlights the need for further
451 research to elucidate if these important mechanisms occur in melanoma as well, to facilitate
452 the development of targeted therapeutics against this highly refractory disease.

453

454

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2. Study concepts and design: Prakrit Kumar, Stuart Rushworth, Marc Moncrieff
3. Literature research: Prakrit Kumar, Stuart Rushworth
4. Figure preparation: Prakrit Kumar, Jamie Moore, Stuart Rushworth
5. Manuscript preparation: Prakrit Kumar, Jamie Moore, Marc Moncrieff, Kristian Bowles and Stuart Rushworth
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746 **TABLES**

747 **TABLE 1:** Mitochondrial transfer from the TME to cancer cells

748 **FIGURE LEGENDS**

749 **Figure 1: Metabolism in melanoma. A)** The smooth co-operation of OXPHOS and glycolysis in
750 the two spatially distinct melanoma cell populations (melanoma cells in the centre that use
751 glycolysis versus melanoma cells in periphery that use OXPHOS mainly for energy production)
752 promotes melanoma initiation, growth and metastasis of melanoma through metabolic
753 symbiosis, whereby the waste products from glycolysis are used to feed into the TCA cycle for
754 OXPHOS in melanoma cells in the periphery. **B)** Mesenchymal stromal cells (MSCs) migrate
755 from the bone marrow and liver towards the melanoma, where they are then manipulated
756 by tumour cells to produce lactate and other macromolecules via glycolysis, for use by
757 melanoma cells that mainly use OXPHOS in the peripheral part of the tumour (Reverse
758 Warburg).

759

760 **Figure 2: Mechanisms of mtDNA transfer A)** tunnelling nanotubules (TNTs), **B)** microvesicles
761 and **C)** gap junctions, as well as other plausible mechanisms that require further research,
762 such as **D)** cell fusion and **E)** direct mtDNA secretion into extracellular media.