

Obesity and Melanoma: the role of FGF-21

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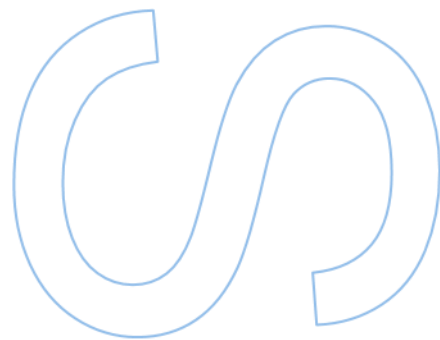
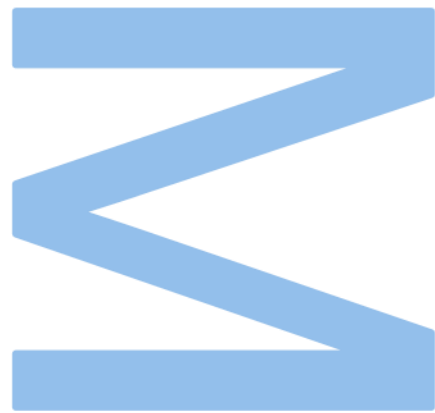
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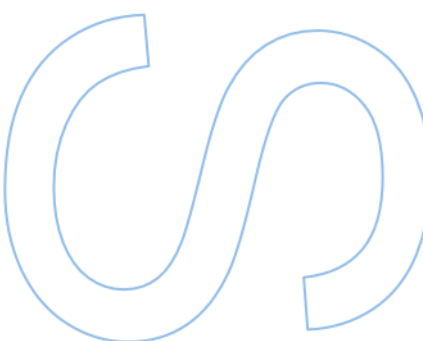
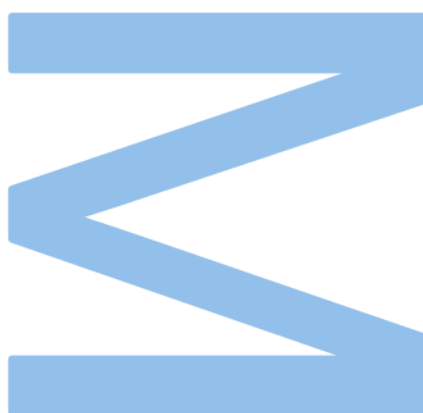
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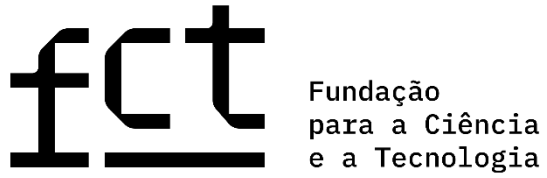
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Resumo

O melanoma é a forma mais agressiva de cancro da pele, apresentando um elevado risco de metástase. Recentemente, a obesidade foi identificada como fator de risco para numerosas formas de cancro, aumentando a incidência e mortalidade. No entanto, a associação entre a obesidade e o melanoma é complexa e ainda não é completamente compreendida.

O fator de crescimento de fibroblastos (FGF)-21 é reconhecido primariamente pelo seu envolvimento na regulação metabólica. No entanto, o FGF-21 tem ganho atenção, recentemente, devido ao seu papel emergente em vários processos fisiológicos, incluindo processos relacionados com a obesidade e o cancro. De facto, relatórios anteriores do nosso grupo revelaram níveis circulantes notavelmente elevados de FGF-21 em ratinhos obesos portadores de melanomas.

Aqui, levantamos a hipótese de que o FGF-21 desempenha um papel na relação entre a obesidade e o melanoma. Assim, o presente trabalho foi dividido em dois objetivos principais: o primeiro objetivo foi criar uma linha celular estável de melanoma B16F10 com duplo repórter, expressando tanto a luciferase de pirilampo (Luc) como a proteína verde fluorescente (GFP), que posteriormente será enxertada em ratinhos com obesidade induzida por dieta com alto teor de gordura (HFD) para futuros estudos *in vivo*, e o segundo objetivo envolveu a investigação *in vitro* dos efeitos do FGF-21 e do secretoma de adipócitos na progressão de melanoma B16F10.

Após a transdução de lentivírus com um construto policistrónico de Luc e GFP, mediado por um péptido de autoclivagem F2A, sob um promotor de citomegalovírus, foram estabelecidas com sucesso duas linhas celulares policlonais B16F10 de duplo repórter que expressam estavelmente Luc e GFP. Estas linhas celulares foram posteriormente caracterizadas, e comportam-se de forma semelhante às células parentais B16F10, ao analisar parâmetros como morfologia, migração, proliferação, viabilidade e progressão do ciclo celular. Em seguida, o impacto de meio condicionado (CM) de adipócitos 3T3-L1, FGF-21 recombinante e um inibidor de FGFR-1, PD173074, individualmente e em combinação, foi explorado em células de melanoma. Embora o tratamento com FGF-21 tenha aumentado a adesão de células B16F10 em 34% e as taxas de proliferação de melanoma tenham sido aumentadas pela ação do CM de adipócitos, nenhuma outra observação significativa foi encontrada. De facto, a estimulação e/ou inibição do FGF-21 mostraram efeitos negligenciáveis, sugerindo que o FGF-21 pode não ser um elemento-chave direto nesta relação.

No total, o futuro enxerto de B16F10-Luc-GFP em ratinhos obesos-HFD melhorará o nosso modelo de pesquisa, permitindo a visualização *in vivo* e *ex vivo* de melanomas primários e metástases, proporcionando uma melhor compreensão dos mecanismos moleculares subjacentes, e revelando as evidências necessárias para confirmar ou excluir o FGF-21 como um mediador-chave na relação obesidade-melanoma.

Palavras-chave: Melanoma, B16F10, Obesidade, 3T3-L1, FGF-21, GFP, Luciferase.

Abstract

Melanoma is the most aggressive form of skin cancer, presenting a high risk of metastasis. Recently, obesity has been identified as a risk factor for numerous forms of cancer, raising both incidence and mortality. Nevertheless, the association between obesity and melanoma is complex and not fully understood.

Fibroblast growth factor (FGF)-21 is primarily known for its involvement in metabolic regulation. However, FGF-21 has recently gained significant attention due to its emerging role in various physiological processes, including those related to obesity and cancer. In fact, previous reports from our research group unveiled remarkably high circulating levels of FGF-21 in obese mice bearing melanomas.

Herein, we hypothesize that FGF-21 plays a role in the relationship between obesity and melanoma. Hence, the present work was divided into two major objectives: the first aimed to create a dual-reporter stable murine B16F10 melanoma cell line expressing both firefly luciferase (Luc) and green fluorescent protein (GFP), that will later be engrafted into high fat diet (HFD) obesity-induced mice for future *in vivo* studies, and the second aim involved the *in vitro* investigation of the effects of FGF-21 and adipocyte secretome on B16F10 melanoma progression.

Upon lentivirus transduction with a polycistronic construct of Luc and GFP, mediated by a F2A self-cleaving peptide, under a cytomegalovirus promoter, two dual-reporter B16F10 polyclonal cell lines that stably expresses Luc and GFP were successfully established. These cell lines were later characterized and behave similarly to parental B16F10 cells, when analyzing parameters such as morphology, migration, proliferation, viability, and cell cycle progression. Next, the impact of 3T3-L1 adipocyte conditioned medium (CM), recombinant FGF-21 and a FGFR-1 inhibitor, PD173074, individually and in combination, on melanoma cells was explored. Although treatment with FGF-21 increased B16F10 cell adhesion by 34% and adipocyte CM enhanced melanoma proliferation rates, no other significant observations were found. In fact, FGF-21 stimulation and/or signaling inhibition showed negligible effects, suggesting that FGF-21 may not be a direct key player in this relationship.

Altogether, the future engraftment of B16F10-Luc-GFP in HFD-obese mice, will improve our research model, enabling the *in vivo* and *ex vivo* visualization of primary melanomas and metastasis, providing better understanding of the underlying molecular mechanisms,

and unveiling the evidence needed to confirm or exclude FGF-21 as a key mediator in the obesity-melanoma relationship.

Keywords: Melanoma, B16F10, Obesity, 3T3-L1, FGF-21, GFP, Luciferase.

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List of Abbreviations

7-AAD	7-AMINOACTINOMYCIN D
ANOVA	ANALYSIS OF VARIANCE
AT	ADIPOSE TISSUE
ATCC	AMERICAN TYPE CULTURE COLLECTION
BCC	BASAL CELL CARCINOMA
BRDU	BROMODEOXYURIDINE
CM	CONDITIONED MEDIUM
CSD	CUMULATIVE SOLAR DAMAGE
CTLA	CYTOTOXIC T LYMPHOCYTE-ASSOCIATED ANTIGEN
DAPI	4',6-DIAMIDINO-2-PHENYLINDOLE
DMEM	DULBECO'S MODIFIED EAGLE'S MEDIUM
ERK	EXTRACELLULAR-SIGNAL-REGULATED KINASE
FACS	FLUORESCENCE ACTIVATED CELL SORTING
FBS	FETAL BOVINE SERUM
FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
FGF	FIBROBLAST GROWTH FACTOR
FGFR	FGF RECEPTOR
GFP	GREEN FLUORESCENT PROTEIN
HFD	HIGH-FAT DIET
HGF	HEPATOCYTE GROWTH FACTOR
IBMX	3-ISOBUTYL-1-METHYLBXANTHINE
IL	INTERLEUKIN
JNK	C-JUN N-TERMINAL KINASE
LUC	FIREFLY LUCIFERASE
MAPK	MITOGEN ACTIVATED PROTEIN KINASE
MCC	MERKEL CELL CARCINOMA
NCS	NEWBORN CALF SERUM
NMSC	NON-MELANOMA SKIN CANCER
NRAS	NEUROBLASTOMA RAS VIRAL (V-RAS) ONCOGENE HOMOLOG
ORO	OIL-RED-O
PBS	PHOSPHATE BUFFERED SALINE

PD	PROGRAMMED DEATH
PI	PROPIDIUM IODIDE
PTEN	PHOSPHATASE AND TENSIN HOMOLOG
ROS	REACTIVE OXYGEN SPECIES
RSV	ROUS SARCOMA VIRUS
RT-QPCR	QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION
SAT	SUBCUTANEOUS ADIPOSE TISSUE
SCC	SQUAMOUS CELL CARCINOMA
SEM	STANDARD ERROR OF THE MEAN
TDT	TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE
TNF	TUMOUR NECROSIS FACTOR
TUNEL	TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE- MEDIATED DEOXYURIDINE TRIPHOSPHATE NICK-END LABELLING
UP	UNIVERSITY OF PORTO
UV	ULTRAVIOLET
VAT	VISCERAL ADIPOSE TISSUE
VEGF	VASCULAR ENDOTHELIAL GROWTH FACTOR

1. Introduction

1.1 Skin cancer

The skin is the largest human organ and primarily functions as a barrier against the environment (Zaidi & Lanigan, 2010). It consists of several types of cells, organized in two layers: epidermis and dermis (Figure 1). The dermis is composed of connective tissue and is responsible for the elasticity and strength of the skin. It presents fibroblasts, mast cells, dermal dendrocytes, nerves and blood vessels. In the dermis, we can also find epidermal appendages, such as sweat glands, hair follicles and sebaceous glands. The outermost layer, the epidermis, is a stratified epithelium mostly composed of keratin-producing cells, keratinocytes (McGrath et al., 2004). It also presents Langerhans cells, Merckels cells and melanocytes, which are cells that produce melanin, a photoprotective pigment. The dermoepidermal junction connects the dermis and epidermis, and provides nourishment to the last one. Beneath the skin there is a subcutaneous tissue layer comprised of fat tissue, connective tissue, nerves and blood vessels (Zaidi & Lanigan, 2010).

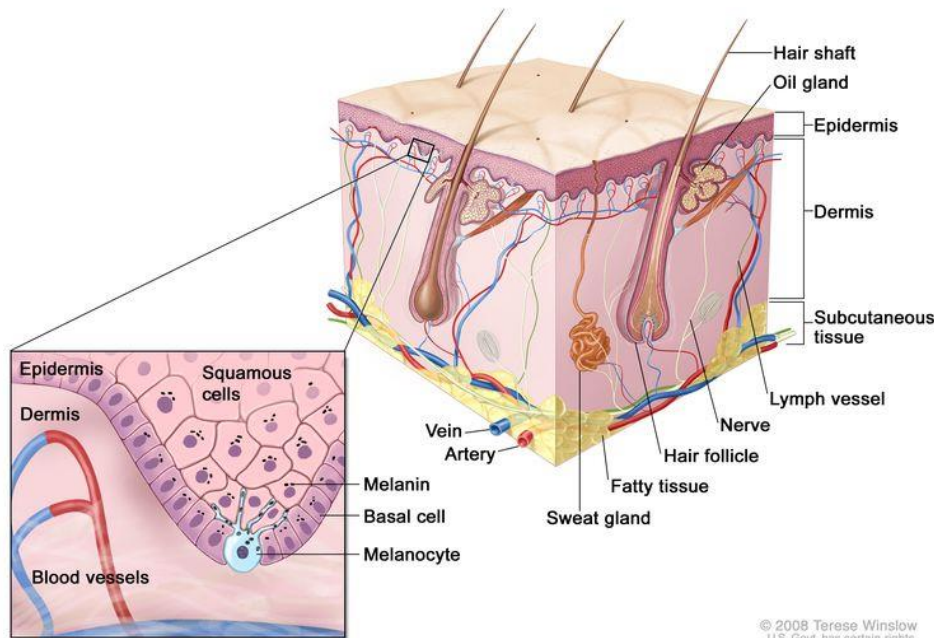


Figure 1: Skin structure. The skin is composed by the dermis and epidermis. The dermis is comprised of connective tissue, fibroblasts, mast cells, dermal dendrocytes, nerves and blood vessels. In addition, sweat glands, hair follicles and sebaceous glands are also located in the dermis. The epidermis is composed of keratinocytes, Langerhans cells, Merckels cells and melanocytes (located in the basal layer of the epidermis). Beneath the skin there is a layer of subcutaneous tissue made up of fat tissue, connective tissue, nerves and blood vessels. Adapted from (Board, 2023).

Skin cancer is the most common malignancy in the world, affecting mostly fair-skin and Caucasian populations, and its incidence has been rising in the last decades (Lomas et al., 2012). In 2020, over 1.5 million new skin cancer cases were reported with over 120 thousand associated deaths (Lopes et al., 2022).

Skin cancer can be mainly categorized into two categories: melanoma or non-melanoma skin cancer (NMSC). The majority of NMSC develops from keratinocytes and can be further classified into other subtypes, the most common being the Basal Cell Carcinoma (BCC) and Squamous Cell Carcinoma (SCC). NMSC also includes Merkel Cell Carcinoma (MCC) together with a host of rare tumors. BCC generally present very low metastasis rates and its cases represent about 70-80% of total skin cancer diagnoses. However, most cases are not recorded in cancer registries, due to their simpler treatment (Dacosta Byfield et al., 2013; Nikolouzakis et al., 2020). On the other hand, SCC presents a higher metastatic risk and corresponds to about 20% of NMSC cases.(Nikolouzakis et al., 2020). MCC is a fast-growing rare type of skin cancer and develops from the malignant transformation of Merckel cells. Non-melanoma skin cancer commonly arises in sun-exposed areas, including the head, face and arms (Cives et al., 2020). In fact, the development of BCC, SCC, and MCC involves a complex interplay of various factors, but the predominant risk factor is exposure of the skin to physical carcinogens. Specifically, ultraviolet (UV) radiation possesses the capacity to directly initiate the malignant transformation of precursor cells (Cives et al., 2020; Lear et al., 2007). Surgery is the standard treatment for NMSC and is curative in approximately 90% of cases. In certain cases, non-surgical option, including cryotherapy, photodynamic therapy and radiotherapy may be utilized (Craythorne & Al-Niami, 2017).

1.2. Melanoma

Cutaneous melanoma is a malignancy that arises from the melanin pigment-producing cells known as melanocytes, primarily found in the skin. Despite representing a low percentage, approximately 1%, of total skin cancer diagnoses cutaneous melanoma is undoubtedly the most aggressive type of skin cancer (Domingues et al., 2018).

In 2020, over 300,000 new cases of melanoma were diagnosed globally, with over 57,000 associated deaths, representing about 50% of total skin cancer deaths (Sung et al., 2021). In Portugal, about 1000 new cases of melanoma and 300 associated deaths were registered, according to GLOBOCAN 2020 data (IARC, 2021). Cutaneous melanoma presents a 5-year relative survival rate of 98% when diagnosed at a localized

initial stage. Contrarily, survival rates drop drastically to 23% for patients with metastatic disease and distant lesions (Carr et al., 2020). Therefore, early melanoma detection is essential for patient survival, although melanoma mortality rates have been declining in the last decade, as a result of novel treatment options and regimens, such as targeted pharmacological agents and immunotherapies (Mason et al., 2019).

1.2.1. Etiology and classification of melanomas

Cutaneous melanoma is a form of cancer that encompasses several subtypes with distinct characteristics such as cell origin, clinical presentation, and treatment response.

Traditionally, melanomas have been classified several categories, including: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and acral lentiginous melanoma (Scolyer et al., 2011). More recently, the World Health Organization presented a multidimensional classification of melanoma based on a progression model in which intermediate tumors, or melanocytomas, are precursors to melanoma. This model integrates knowledge about genetic drivers and histopathological presentation (Ferrara & Argenziano, 2021).

Melanomas can be mainly classified as being or not derived from exposure to the sun. Further categorization takes into account the degree of cumulative solar damage (CSD) of the skin surrounding the primary melanoma. The “UV signatures”, or point mutations, characterized by transitions of cytosine to thymidine at dipyrimidine sites, are associated with the degree of CSD of melanomas: therefore, high CSD melanomas present a heightened tumor mutation burden. High CSD tumors include lentigo maligna and nodular melanomas; on the other hand, low CSD melanomas comprise low superficial spreading melanomas. Other melanomas such as acral and mucosal melanoma are not etiologically related to UV exposure, but can still be categorized on the basis of their driving mutations (Ferrara & Argenziano, 2021).

Benign neoplasms, or nevi, progress to melanoma as they accumulate genetic alterations, that incrementally disrupt biological pathways. Melanocytic nevi are stable and partially transformed masses of proliferated melanocytes that, despite retaining mechanisms that suppress tumor growth, have attained mutations that promote cell proliferation (Yeh & Bastian, 2021). Usually these naevus-initiating mutations affect the mitogen-activated protein kinase pathway (MAPK), the most common being *BRAFV600E*, found in approximately 50% of melanomas. (Pollock et al., 2003). These

benign neoplasms may progress to malignancies once cells additionally acquire mutations that corrupt tumor suppressive mechanisms, such as TERT promoter mutations or an inactivation of CDKN2A (Yeh & Bastian, 2021). Other commonly observed mutations in melanoma include *NRAS* (neuroblastoma RAS viral (v-ras) oncogene homolog), accounting for 20% of cases, proto-oncogene *c-KIT*, *p53* and *PTEN* (phosphatase and tensin homolog) (Tímár & Ladányi, 2022). In fact, studies have demonstrated that the phosphatidylinositol-3 kinase (PI3K)-AKT pathway complements the activation of the MAPK pathway in promoting melanocyte transformation. PTEN mutations or loss have been reported in a significant proportion of melanoma cases (10-30%). Still, a large percentage of melanomas, 30–40%, do not harbor known driver mutations and are considered to be ‘pan-negative’ (Jamme et al., 2022).

Altogether, many known mutations drive the transformation of melanocytes into melanoma and contribute to the metastatic phenotype. Nevertheless, the discovery of previously unknown key genetic mutations and pathways presents promising opportunities for the development of new targeted therapies to treat melanoma.

1.2.2. Risk Factors

The main risk factor for melanoma is UV exposure, which is modulated by its wavelengths, individual melanin profiles and genetic factors (Saginala et al., 2021).

UV radiation has a direct effect on DNA, inducing photoproducts such as thymidine-dimers which may lead to replication errors, mutated molecules and ultimately cancer, if left unrepaired (Bowden et al., 2010). Members of the MAPK family, which are in charge of cell proliferation and differentiation, such *NRAS* and *BRAF*, are among the proteins that are frequently mutated (Edlundh-Rose et al., 2006). Stage-IV melanoma patients often present *p53* gene mutations, which are linked to a poorer prognosis (Kappes et al., 2006; Saginala et al., 2021).

UVA is characterized by being the main source of UV environmental exposure and longer wavelengths (315 – 400 nm) that pierce deeply into the skin. On the contrary, UVB shorter wavelengths (280 – 315 nm) penetrate more superficially into the dermis yet are drastically more genotoxic than UVA, because of their ability to promptly interfere with DNA (Rashid et al., 2023).

Melanoma rates are increased in individuals whose skin is occasionally exposed to intense solar radiation, as opposed to continuous low-intensity sun exposure. People

with skin that is dark, or darkens easily in reaction to sun exposure without burning, demonstrate lower melanoma incidence (Conforti & Zalaudek, 2021). Pheomelanin, which is produced in greater quantities in people with light skin, is less effective at blocking UV radiation than eumelanin, which is produced in greater quantities by those with dark skin (Saginala et al., 2021). While UV exposure constitutes the major risk factor for melanoma development, there are other, mainly non-modifiable, risk factors. Familial melanomas account for up to 10 % of cases, 30 % of which arise from CDKN2A mutations (Rossi et al., 2019; Saginala et al., 2021). The number of nevi or moles is also a factor - individuals with over 100 moles, or moles exhibiting a dysplastic appearance are especially prone to the development of melanoma (Conforti & Zalaudek, 2021; Zalaudek et al., 2020). Recently, obesity has been postulated as a risk factor for several types of cancer, including melanoma (Karimi et al., 2016).

1.2.3. Melanoma treatment

There are several treatment options for melanoma, depending on the genetic profile of the tumor, its stage and location. Surgery is typically the primary form of treatment, for individuals with stage I to IIIB melanoma. For patients presenting a single metastasis, metastasectomy is a standard care practice, and in certain metastases cases, chemotherapy may also be advised. Although radiotherapy is not commonly used in primary melanoma treatment, it can be beneficial for addressing brain and bone metastases (Domingues et al., 2018). Previously, individuals presenting melanoma with inoperable metastases displayed average survival times ranging from 8 to 12 months (Ugurel et al., 2017).

However, recent advancements in understanding the molecular and genetic factors implicated in the malignant transformation of melanocytes have led to the discovery of novel therapeutic targets (Domingues et al., 2018). The recent introduction of immune checkpoint inhibitors and selective kinase inhibitors, as treatment options for advanced metastatic disease, has played a crucial role in the increase of the median survival of patients (Ugurel et al., 2017). Immune checkpoint blockers, such as selective inhibitors of programmed death (PD)-1, nivolumab and pembrolizumab, and cytotoxic T lymphocyte-associated antigen (CTLA)-4 receptors ipilimumab, target the tumor immunosuppressive system (Domingues et al., 2018).

The discovery of many driving mutations and signaling pathways in melanoma gave rise to the introduction of targeted molecular inhibitors in melanoma treatment (Amann et al., 2017). Most selective kinase inhibitors target the frequently altered MAPK pathway and

include BRAF and MEK inhibitors. BRAF protein inhibitors such as vemurafenib, dabrafenib and encorafenib or MEK inhibitors cobimetinib, binimetinib and trametinib are approved drugs, alone or in combination, for treatment of advanced melanoma (Amann et al., 2017; Domingues et al., 2018).

Despite the major recent advancements made in the field of melanoma treatment, there are still several obstacles, such as disease recurrence and the commonly detected resistance to immunotherapy observed in patients. (Domingues et al., 2018). Additional research is imperative as melanoma continues to present a significant challenge without a universally effective treatment.

1.3. Obesity

The prevalence of obesity has risen exponentially in industrialized countries, as a consequence of an increasingly sedentary lifestyle (Bhupathiraju & Hu, 2016). When energy consumption exceeds energy expenditure, the surplus energy is stored as fatty acids in the adipocytes. Consequently, adipose tissue will expand by increasing the size and number of adipocytes to accommodate excess calories. In obesity, the uncontrolled accumulation of fat leads to adipose tissue (AT) structural changes and dysfunction, constituting an increased risk for several diseases (Longo et al., 2019; Zhang et al., 2023).

AT primarily consists of adipocytes, and various stromal cells such as endothelial and immune cells. It is the major component of the hypodermis, which is located subcutaneously (Olszanska et al., 2021). AT's function was traditionally thought to be limited to insulation against cold temperatures and energy storage (Booth et al., 2015). AT can be categorized into white adipose tissue or brown adipose tissue, and can be located subcutaneously (SAT) or viscerally (VAT) (Olszanska et al., 2021).

Obesity is a prominent cause in the development and is associated with the incidence of multiple co-morbidities including metabolic syndrome, type 2 diabetes, cardiovascular and musculoskeletal disease (Scherer & Hill, 2016). In addition, epidemiological studies identify obesity as a risk factor for numerous forms of cancer, raising both incidence and mortality (Renehan et al., 2008). Nevertheless, existing epidemiological data show that the association between obesity and increased melanoma incidence is not as strong (Smith et al., 2020).

Adipose and tumoral tissues display several common features along their fast expansion, such as tissue neovascularization, immune alterations and abnormalities in genetic expression (van Kruijsdijk et al., 2009). Although not yet fully elucidated, the progression of melanoma in obese individuals involves a complex interplay of numerous factors: angiogenesis, inflammation, oxidative stress, insulin-resistance and adipokines are some of the mechanisms postulated to be involved in the complex interplay between obesity and melanoma (Figure 2).

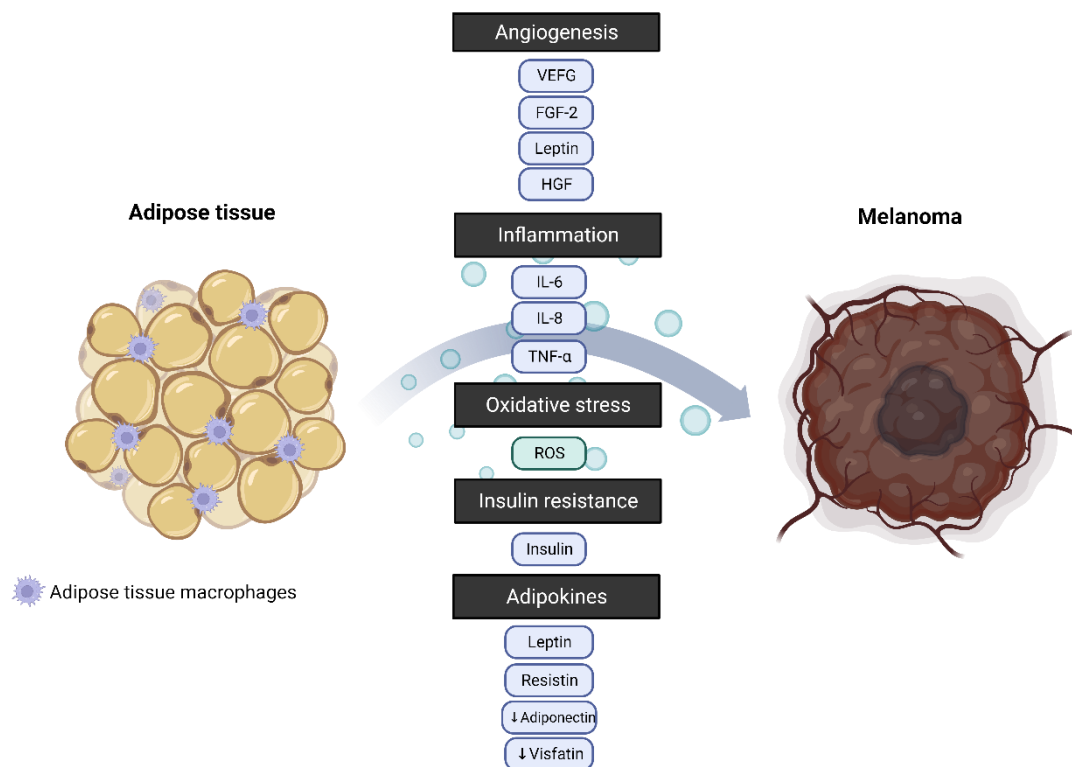


Figure 2: The interconnections between obesity and melanoma involve various processes, including adipokine secretion, insulin-resistance, inflammation, oxidative stress and angiogenesis.

1.3.1. Angiogenesis

Adipose tissue has the capacity to expand almost indefinitely in adulthood, a property that is not observed in most tissues. This expansion has to be accompanied by changes in the vascular network, including the generation of new blood vessels – a process known as angiogenesis (Nishida et al., 2006). Adipocytes play a critical role in the regulation of this process, by secreting several molecules known to modulate angiogenesis, such as: vascular-endothelial growth factor (VEFG), hepatocyte growth factor (HGF), leptin,

fibroblast growth factor (FGF)-2, tumor necrosis factor (TNF)-alfa and beta, among others (Cao, 2010).

Angiogenesis is also critical to tumor progression and spread, and may be dependent or independent of hypoxic conditions. Some of the angiogenic molecules secreted by adipocytes play a significant role in the early trigger of angiogenesis in tumors, independently of hypoxia, leading to a faster tumor growth and metastasis in obese individuals (Meierjohann, 2015).

1.3.2. Inflammation

Obesity leads to the accumulation of macrophages in the adipose tissue and a state of chronic low-grade inflammation (Weisberg et al., 2003). These macrophages not only have immunological functions, but also play a role in angiogenesis, by producing angiogenic factors and cytokines. Interleukin (IL)-6, IL-8 and TNF-alpha are some of the inflammatory cytokines produced by macrophages associated to the AT and associated to the tumor (Mantovani & Sica, 2010). The released factors are known to contribute to melanoma growth, angiogenesis, and metastasis (Karimi et al., 2016).

1.3.3. Oxidative Stress

Obesity is associated with systemic oxidative stress, as a result of reduced antioxidant capacity, exacerbated reactive oxygen species (ROS) production and inflammatory processes observed in obese individuals (Karimi et al., 2016).

The excessive production of ROS can lead to irreversible modifications in cellular components such as proteins and DNA, resulting in altered cell behavior and genetic mutations (Ziech et al., 2011). Additionally, ROS and oxidative stress can modulate various signal transduction pathways involved in cell proliferation, ultimately, enhancing tumor progression (Matés et al., 2008).

1.3.4. Insulin-resistance

Chronic inflammation caused by adipose tissue-derived inflammatory cytokines has been linked to the development of insulin resistance and type 2 diabetes (Lee, 2013). This leads to an increase in the secretion of insulin by the pancreas, as a compensatory mechanism for the impaired uptake of insulin by cells (Lackey & Olefsky, 2016). Malignant cells respond to high concentrations of extracellular insulin through the activation of pathways such as extracellular-signal-regulated kinase (ERK) and PI3K (De Pergola & Silvestris, 2013). Additionally, tumor cells exhibit an overexpression of insulin

receptors, which further promotes cellular proliferation induced by insulin stimulation (De Pergola & Silvestris, 2013; Karimi et al., 2016).

1.3.5. Adipokines

Adipose tissue is mainly made up of adipocytes, cells that not only store fat, but also are responsible for the release of various substances, such as lipids, exosomes and adipokines. Adipokines are bioactive molecules that can regulate metabolic responses locally and systemically (Olszanska et al., 2021). They include hormones, metabolic, angiogenic and inflammatory factors, and may be secreted by adipocytes or other cell types present in the adipose tissue (Fain et al., 2004). In normal circumstances, these adipokines play a role in regulating energy balance related processes (Olszanska et al., 2021). However, obesity leads to significant changes in the structure and composition of the AT, disrupting its microenvironment and physiological processes (Fasshauer & Blüher, 2015). The altered secretion of factors by dysfunctional adipocytes is linked to various diseases, including cancer (Blüher, 2013).

Hypersecretion of leptin and resistin, and hyposecretion of adiponectin and visfatin, commonly observed in obese individuals, have been linked to an unfavorable prognosis in melanoma (Oba et al., 2016). Leptin plays a significant part in promoting carcinogenesis due to its pro-inflammatory, angiogenic and anti-apoptotic properties (Somasundar et al., 2004). Adiponectin is known to improve insulin sensitivity and suppress cell proliferation (Kelesidis et al., 2006). Studies point to an inverse correlation between cancer incidence and stage, and adiponectin levels (Cust et al., 2007; Kelesidis et al., 2006; Wei et al., 2005).

1.3.5.1. FGF-21

The FGF family consists of 22 factors, subcategorized as normal or hormone-like FGFs. These ligands are crucial for several processes, such as: metabolic regulation, cellular growth, angiogenesis, wound healing, among others (Beenken & Mohammadi, 2009).

FGF-21 plays a key role in the maintenance of metabolic balance and its secretion is greatly influenced by changes in the nutritional state (Potthoff et al., 2012). The liver is mainly responsible for the release of circulating FGF-21, although, under stressful conditions, an expression of this protein may be also observed in the pancreas, kidney, muscle or adipose tissue (Dai et al., 2021).

To exert its effects, FGF-21 requires binding to a complex formed by a FGF receptor (FGFR) and a beta-Klotho co-receptor. The FGFRs are transmembrane proteins that possess tyrosine kinase activity. In particular, the major receptor subtype, FGFR-1, shows wide-spread expression among normal and tumoral cells, such as melanoma (Czyz, 2019; Ye et al., 2016).

In adipocytes, FGF-21 was shown to stimulate the secretion of adiponectin, suggesting that it may be one of the key mediators for the beneficial effects of FGF-21 (Lin et al., 2013). The upregulation of adiponectin expression was achieved by FGF-21 autocrine signaling, intermediated by the c-Jun N-terminal kinase (JNK) pathway (Han et al., 2021).

Treatment with FGF-21 over an extended period has shown to decrease body mass, enhance energy consumption and improve the regulation of glucose and lipid levels, in mice fed with a high fat diet (HFD) (Xu et al., 2009). In studies involving obesity and type 2 *diabetes mellitus*, FGF-21 was reported as responsible for the reduction of blood glucose and insulin levels, and insulin sensitivity enhancement (Berglund et al., 2009; Kharitononkov et al., 2005; Woo et al., 2013).

New research has suggested a correlation between FGF-21 and the development of various cancers, including colon, thyroid and liver cancers (Kang et al., 2019; Qian et al., 2018; Singhal et al., 2018). Studies conducted *in vivo* have observed increased expression and serum levels of FGF-21 in breast, renal and papillary thyroid tumors (Akyol et al., 2017; Kang et al., 2019; Knott et al., 2016).

In an *in vitro* study involving prostate cancer cells, it was reported that FGF-21 inhibited cell proliferation, migration and invasiveness, while promoting cell apoptosis. Cancer cells display high metabolic activity and are susceptible to nutrient deprivation: the authors theorize that FGF-21 alters the prostate cancer cells glucose metabolism, resulting in cell proliferation inhibition (Dai et al., 2021).

In mice inoculated with B16F10 melanoma cells, HFD-induced obesity led to a significant increase in FGF-21 serum levels and FGFR-1 expression. FGF-21 was pointed out as the probable mediator of the decreased metastatic potential of melanoma observed in obese mice, due to its metabolism regulation and anti-inflammatory properties (Fonseca et al., 2021).

1.4. Rationale and Objectives

The incidence of melanoma has been progressively rising in recent years in many western countries and obesity has been nominated as one of the causes for the heightened incidence of this form of skin cancer (Karimi et al., 2016). Literature on this subject highlights numerous possible factors and biological processes and mechanisms subsidizing the obesity-related melanoma development. Indeed, previous data from our research group showed that diet-induced obesity led to a significant increase in FGF-21 serum levels and FGFR-1 expression (Fonseca et al., 2021).

Therefore, we aim to uncover the biological role of adipocyte-released factors, focusing on FGF-21, in our attempt to identify obesity-associated mechanisms of melanoma carcinogenesis, by using cell culture models of 3T3-L1 preadipocytes and genetically engineered dual-reporter murine malignant melanoma B16F10 cells.

In this study, our research endeavors were divided into two distinct aims, each encompassing specific and defined objectives as outlined below:

- investigate the *in vitro* effects of adipocyte secretome, FGF-21 and FGFR-1 blockage on B16F10 melanoma progression.
- create a dual-reporter stable murine B16F10 melanoma cell line expressing both firefly luciferase (Luc) and green fluorescent protein (GFP), that will later be engrafted into high fat diet obesity-induced mice for future *in vivo* studies.

2. Materials and Methods

2.1. Cells and cell culture

B16F10 (ATCC - American Type Culture Collection #CRL-6475), a murine melanoma cell line with high lung tropism, and 3T3-L1 pre-adipocytes (ATCC #CL-173) were purchased from the ATCC. Cells were grown in Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/L) medium with L-Glutamine (DMEM-HA-P10, Capricorn Scientific, Germany), supplemented with sodium bicarbonate (1.5 g/L), 1% (v/v) penicillin-streptomycin solution (Corning, United States of America – USA) and 10% (v/v) heat inactivated fetal bovine serum (FBS) (Corning, USA) or newborn calf serum (NCS) (PAN-Biotech, Germany), respectively. Cultures were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in a cell culture incubator (SCA-80DRS, ASTEC bio, Japan). B16F10 cells were split every 2 to 3 days, in a 1:12 ratio. 3T3-L1 cell culture medium was changed every 2 to 3 days and cells were split every 4 days, in a 1:10 ratio. Both cell lines were split using 0.05% trypsin–EDTA (AppliChem, Germany) to remove cells enzymatically (1 min, 37°C), and were subcultured in plastic culture dishes (nØ 60 mm; Orange Scientific, Belgium).

2.2. Creation of a Dual-Reporter Melanoma Cell Line

B16F10 cells expressing firefly luciferase (Luc) and green fluorescent protein (GFP) (B16F10-Luc-GFP) were generated by transfection with premade lentiviral particles (LVP020, Amsbio, United Kingdom). The lentivirus features a construct with Luc and GFP under a cytomegalovirus (TetCMV) promoter and mediated by a F2A element; the antibiotic selection marker (puromycin) is expressed under a Rous sarcoma virus (RSV) promoter. B16F10 cells were seeded in a 96 well plate with a density of 2.5×10^3 cells per well and incubated overnight. Afterwards, cell culture medium was refreshed and 10, 20, 30 or 40 µL (multiplicity of infection: 10, 20, 30, 40, respectively) of lentiviral particles was added to each well. After 48 h of incubation, the medium containing the lentivirus was removed and the transfected cells were selected by adding complete medium supplemented with 1 µg/ml of puromycin (Santa Cruz Biotechnology). Cells were regularly observed under a fluorescence microscope (EVOS M7000, Thermo Fisher Scientific, USA) and the two cultures that exhibited the highest levels of GFP-positive cells and GFP fluorescence intensity (L20 and L40) were chosen for cell sorting.

2.3. Fluorescence Activated Cell Sorting of B16F10-Luc-GFP

B16F10 cells transfected with the lentivirus were cell sorted to ensure the stable expression of Luc and GFP. B16F10-Luc-GFP cultures were harvested and centrifuged twice at 75 g for 5 m. The remaining cell pellet was resuspended in PBS supplemented with 10% FBS. GFP-positive cells were sorted in a BD FACS Aria II model cell sorter (BD Biosciences) and cultured in complete medium supplemented with 1 µg/mL of puromycin. A week after, cells were subjected to another round of cell sorting, to ensure the final cell line presents a stable expression of GFP and luciferase. The resulting cell lines were used for further assays.

2.4. Luciferase and GFP detection

To detect luciferase activity in transfected cells, the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher Scientific, USA) was used. B16F10 and B16F10-Luc-GFP cells were plated in a 96 well plate with a density of 1, 5 and 10×10^3 cells per well. Following overnight incubation, cells were washed with PBS and 50 µL of 1X Cell Lysis Buffer was added. The plate was shaken for 15 minutes and 20 µL of cell lysate was added to an opaque 96 well plate. Finally, 50 µL of Working Solution containing D-luciferin was pipetted into each well and left to stabilize for 10 minutes, before reading luminescence in a multimode microplate reader (Varioskan LUX, Thermo Fisher Scientific, USA). The expression of GFP in B16F10-Luc-GFP cells was evaluated by fluorimetry using the Varioskan LUX microplate reader, set at wavelengths 487 nm for excitation and 512 nm for emission.

2.5. Adipocyte differentiation and conditioned medium harvesting

3T3-L1 pre-adipocytes were seeded in a concentration of 3×10^3 cells/cm² in culture dishes and grown till 100% confluence was reached (day 0). At day 2, an induction medium consisting of 10 µg/mL recombinant insulin (Lantus, Sanofi, USA), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, I5879, Sigma-Aldrich, Germany), 1 µM dexamethasone (D4902, Sigma-Aldrich, Germany) in DMEM, supplemented with 10% FBS and 1% of penicillin-streptomycin solution, was added to the cells, to initiate 3T3-L1 pre-adipocyte differentiation into adipocytes. In day 4, this medium was replaced with a maintenance medium, consisting of 10 µg/mL insulin in complete medium, and changed every 2 to 3 days. By day 11, 3T3-L1 are expected to be differentiated; cell cultures were carefully washed with PBS and incubated in serum free medium for 24 h. In day 12, the

adipocyte conditioned medium (CM) was collected and centrifuged at 300 g for 5 m, and the resulting supernatant was stored at -80°C for future treatments.

An Oil-Red-O (ORO) staining was performed on differentiated 3T3-L1 cells, in order to visualize and confirm lipid accumulation of adipocytes. Cells were washed with PBS and fixed, for 30 minutes, with 10% formalin (v/v) (20909.290, VWR, France). After washing with deionized water, cells were treated with 60% (v/v) isopropanol (216022500, Biochem Chemopharma, France) for 5 minutes. Then, cells were incubated for 30 minutes with a working solution comprised of ORO (O0625, Sigma-Aldrich, Germany) stock solution (0.5% (w/v) in isopropanol) diluted in water (3:2). Afterward, cells were washed with deionized water and stained with hematoxylin (GHS3-50ML, Sigma-Aldrich, Germany). After washing excess dye with tap water, representative pictures of the cells were taken, with an inverted microscope (EVOS XL Core, Thermo Fisher Scientific), at 200X magnification.

2.6. Cell Treatment

In order to evaluate the effects of adipocyte CM on melanoma cells *in vitro*, recombinant mouse FGF-21 (110 ng/mL, 230-30069-10, RayBiotech, USA), a FGFR inhibitor - PD173074 (25 nM, 331-11686-3, RayBiotech, USA) and 3T3-L1 adipocyte conditioned media was tested on B16F10 cells. The tested concentrations were chosen based on literature and our previous works (Fonseca et al., 2021). Cells were incubated with the respective treatments for 24 h, in serum-free conditions.

2.7. Spread and Adhesion Evaluation

B16F10 and B16F10-Luc-GFP cells were seeded in 24 well plates, at a concentration of 2.5×10^4 cells per well, in medium without serum supplemented with the respective treatments, and incubated for 40 min or 3 h, for adhesion or spread tests, respectively. Next, cells were washed 3 times with PBS and fixated with 200 µL methanol (Chem-Lab, Belgium) for 30 min at 4°C. Cells were then dyed with a 0.5% crystal violet (Sigma-Aldrich, Germany) solution in 25% methanol for 20 min at room temperature. Excess dye was washed off with tap water and cells were left to dry overnight. To complete the spread assay, each cell well was photographed 5 times at 200X magnification, under an inverted microscope (EVOS M7000). 1000 cells from 5 fields for each treatment were analyzed by the CellProfiler software (Broad Institute, USA) for several morphologic parameters, such as area, sphericity, size and perimeter. For the adhesion assay, 200 µL of 10% (v/v) acetic acid (Scientific Laboratory Supplies, United Kingdom) was added

to the cultures to dissolve for 30 minutes. Cell adhesion was quantified as a measure of absorbance (590 and 690nm, Varioskan LUX).

2.8. Wound healing assay

B16F10 and B16F10-Luc-GFP cells were seeded at a concentration of 9×10^4 cells per well, in a 24 well plate. After 2 days, when cultures had reached confluence, the cell monolayer was scratched using a sterile yellow pipette tip. Cells were then washed with PBS and incubated with the respective treatments. Images were taken at 0 h and 12 h after injury, using an inverted microscope (EVOS XL Core, Thermo Fisher Scientific) at 200X magnification. The areas of the lesions were analyzed using the ImageJ software (National Institutes of Health, USA) and cell migration was quantified by subtracting areas observed at 12h by 0h.

2.9. Viability and cell cycle analysis

7-Aminoactinomycin D (7-AAD) is a fluorescent dye that binds to double stranded DNA and only penetrates dead or dying cells with damaged membranes, thus allowing for cell viability analysis. Propidium iodide (PI) is a dye that stains DNA and RNA. A solution of PI and RNase allows for the determination of cell DNA content and G0/G1, S and G2/M cell cycle analysis.

Briefly, 24 h after treatment cells were trypsinized and harvested and centrifuged for 5 min at 75 *g*. The supernatant was discarded, and the pellet was resuspended in PBS and centrifuged at 300 *g* for 5 min. For the 7-AAD viability analysis, the supernatant was removed, and the cells were incubated for 30 minutes with 100 μ L of PBS and 5 μ L of 7-AAD (BD Biosciences, USA). Another 300 μ L of PBS was added and samples were analyzed with a flow cytometer. For the cell cycle evaluation, cells were fixated with 3 mL of 70% ethanol for 30 min on ice. Afterwards, samples were centrifuged for 10 min at 500 *g*, the supernatant discarded, and centrifuged twice with PBS at 400 *g* for 5 min. The final cell pellet was incubated for 30 minutes in the dark with 500 μ L of PI staining solution: RNase A (Qiagen, Germany) 0.2 mg/mL and PI (Abcam, United Kingdom) 20 μ g/mL in Triton-X100 0.1% (Merck, Darmstadt, Germany), and samples were examined through flow cytometry (NovoCyte® 3000 Flow Cytometer; Acea Biosciences). 7-AAD-staining was assessed in the BL4 channel, which is equipped with a band pass filter 675/30 nm, and DNA content was assessed in the BL3 channel, which is equipped with a band pass filter 615/20 nm, by employing blue laser excitation at 488 nm. Results were analyzed with the FlowJo 10.9.0 software (Becton, Dickinson and Company, USA).

2.10. BrdU incorporation assay

A BrdU Cell Proliferation ELISA kit (ab126556, Abcam, United Kingdom) was used in order to assess melanoma cell proliferation. This kit relies on the detection and quantification of bromodeoxyuridine (BrdU), a thymidine analog, incorporated in DNA synthesized by proliferating cells.

B16F10 and B16F10-Luc-GFP cells were seeded in a 96-well plate with a density of 5×10^3 cells per well and left to grow for 24 h. Subsequently, the cells were incubated with the respective treatments for another 24 h. At the 20h mark, 20 μ L of BrdU label was added to each well and cells were incubated for the final 4 h. Next, after fixating cells for 30 min with 200 μ L of fixing solution, the plate was washed three times with 1X wash buffer and cells were incubated for 1 h with 100 μ L anti-BrdU monoclonal detector antibody. The plate was washed again three times with 1X wash buffer, and 100 μ L of 1X peroxidase goat anti-mouse IgG conjugate was added to each well and left to incubate for 30 minutes. Cells were washed again with 1X wash buffer. Following the incubation of cultures with 100 μ L of TMB peroxidase substrate for 30 minutes, 100 μ L of stop solution was pipetted into each well. Absorbances at 450/550 nm were read using a plate reader (Varioskan LUX, Thermo Fisher Scientific, USA).

2.11. Apoptosis Evaluation

An *In situ* Cell Death Detection Kit (11684795910, Roche, Switzerland) was used to detect apoptosis of B16F10 cells by labelling DNA strand breaks by the enzyme terminal deoxynucleotidyl transferase (TdT) with fluorescein dUTPs.

Briefly, B16F10 were seeded in a 96 well plate with a density of 3×10^3 cells per well and, 24 h later, were incubated with respective treatments. After 24 h, cell medium was removed and a fixation solution consisting of 200 μ L of 10% (v/v) formalin in PBS was added to cells for 1 h. After washing the wells with PBS, the cells were incubated for 5 min at 4° C with 100 μ L of permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). Next, cells were washed twice with PBS; between washes, the positive control well was incubated with 25 μ L DNase I for 5 minutes. Cultures were then incubated for 1 h, at 37° C in the dark, with 25 μ L of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) reaction mixture. Cells were washed once with PBS, treated with 25 μ L 4',6-diamidino-2-phenylindole (DAPI) (1 ng/mL), to counter-stain nuclei, for 10 min and washed again with PBS. TUNEL-positive cells were counted by visualization under a fluorescence microscope (EVOS M7000) at

200X magnification on 3 random fields for each well/treatment. The percentage of apoptotic cells was obtained by dividing TUNEL-positive cells by the number of nuclei stained with DAPI.

2.12. Statistical analysis

Results are shown as arithmetic means $\pm$ standard error of the mean (SEM). The value of n indicates the number of replicates of at least 2 independent experiments. Results were analyzed with GraphPad Prism 9.4.1 (GraphPad Software, USA). One-Way Analysis of variance (ANOVA) was used for comparison between groups. Comparisons with $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Development of a stable B16F10-Luc-GFP dual reporter cell line

Reporter cell lines are valuable tools in cancer research. Engineered cell lines that express different reporter genes, typically fluorescent proteins or luminescent enzymes, can be used to study cancer biology, namely the cellular processes at the tumor microenvironment, understand the metastatic process while allowing live *in vivo* imaging in animal models. Herein, a dual reporter melanoma cell line, expressing both GFP and Luc, for fluorescence and bioluminescence activity detection was established.

B16F10 cells were transduced with 10, 20, 30 or 40 μ L of lentiviral particles with a construct encoding firefly Luciferase and GFP. After monitoring via fluorescence microscopy, we selected cells treated with 20 (hereby named L20) or 40 μ L (L40) of lentivirus, due to their high GFP expression. These cultures underwent two rounds of FACS (Figures 3A and 3B) to ensure the stable expression of GFP. On both rounds, L40 cells (right images A and B) displayed a higher proportion of GFP-expressing cells than L20 (left images A and B), and more intensely fluorescent cells. The resulting polyclonal cell lines were used for further characterization and analysis.

The stable B16F10-Luc-GFP L20 and L40 cell lines were analyzed to ensure the transduced genes expression. A quantitative reverse transcription polymerase chain reaction (RT-qPCR) kit was employed to analyze the mRNA expression of Luc, GFP and GAPDH (housekeeping gene); however, we were not able to amplify the desired sequences, due to unknown errors (data not shown). Therefore, we proceeded to quantify the fluorescence and bioluminescence of transduced cells (Figure 4A, B and C). As shown in Figure 4B, GFP expression is present on both L20 and L40 cell lines, and is proportional to cell density. Consistent with the analysis obtained in FACS, L40 cells express higher levels of GFP fluorescence. Similarly, luciferase activity was confirmed on both lines (Figure 4A). After D-luciferin exposure, L40 cells displayed higher levels of relative luminescence units, when compared to L20 cells. As expected, the parental cell line did not display GFP or luciferase activities.

Next, to ensure no changes on B16F10 cellular physiology ensued after lentiviral transduction, we compared several cell parameters of B16F10-Luc-GFP to the parental cell line.

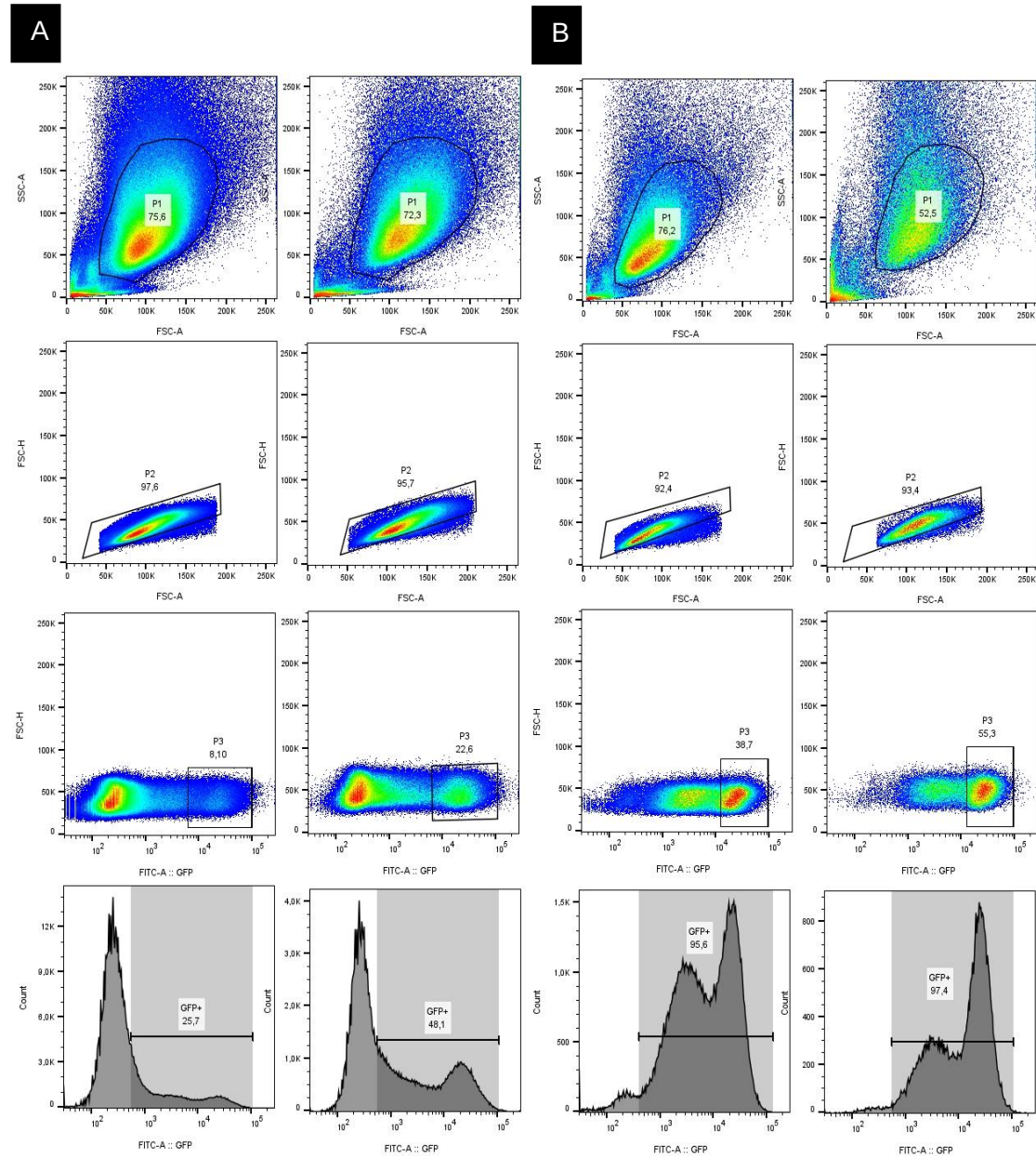


Figure 3: Gating strategy for first round (A) and second round (B) of FACS of B16F10-Luc-GFP L20 (left) and L40 (right). Cells were gated by FSC-A versus SSC-A (P1), a singlet population was gated from FSC-A versus FSC-H (P2), GFP-positive cells were then plotted versus FSC-H (P3), and finally, the percentage of GFP-positive cells was plotted in a histogram.

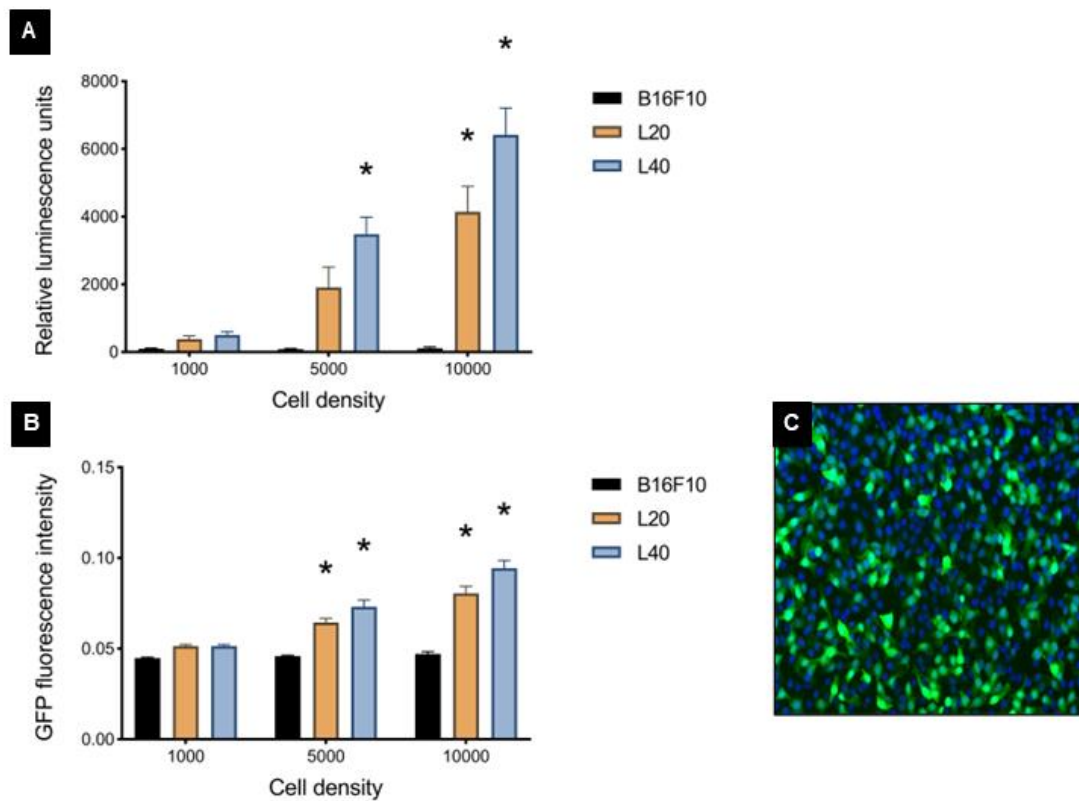


Figure 4: GFP and luciferase activity analysis of the stable B16F10-Luc-GFP cell line. A) 1000, 5000 and 10000 L20 and L40 cells were seeded and GFP fluorescence intensity was measured, with a Varioskan LUX, and compared to the parental cell line (control) (n=6). B) L20 and L40 cells were seeded in indicated cell densities and luciferase bioluminescence intensity was measured, after D-luciferin incubation, with a Varioskan LUX, and compared to the parental cell line (control) (n=6). C) Representative image of the B16F10-Luc-GFP cell line, displaying GFP-positive cells, with nuclei counter-stained with DAPI (200X). Data shown as arithmetic means $\pm$ standard error of the mean (SEM). *($P < 0.05$) denotes significant difference from control, by Two-Way ANOVA.

Regarding cell migration (Figure 5A) and proliferation, no statistically significant differences were observed between cell lines (Figures 5B and 5C, respectively).

However, some differences were found when analyzing cell adhesion and spread between transduced and non-transduced B16F10 cells. L20 cells adhered 41.7% more than the parental cell line, to the assay plate (Figure 5D). Furthermore, L20 cells exhibited smaller areas than the control (Figure 5E and F). On the other hand, L40 cells had larger areas and perimeters, and were longer, when compared to the control.

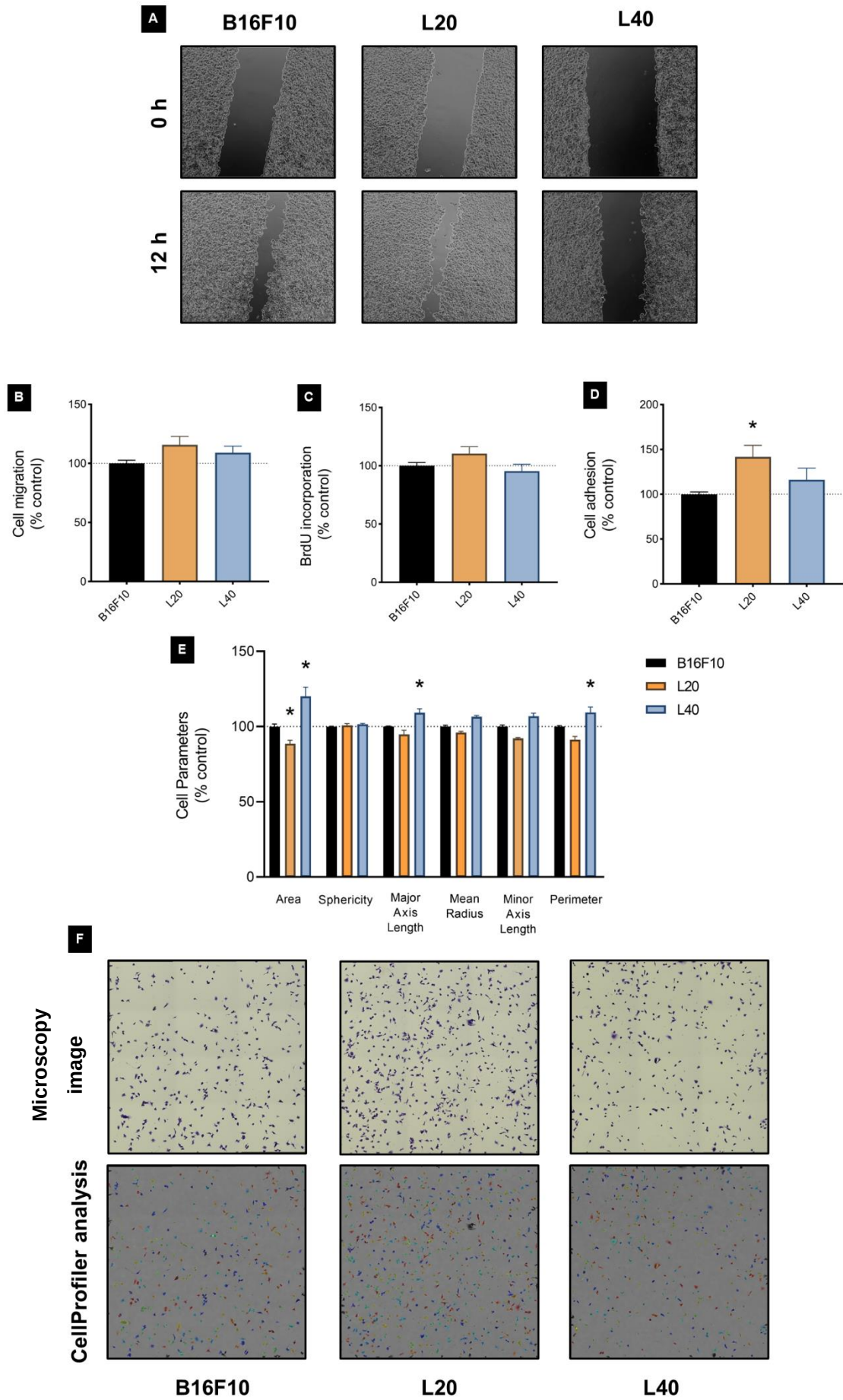


Figure 5: *In vitro* analysis of B16F10-Luc-GFP L20 and L40 cell migration, proliferation, adhesion and spreading, and comparison to parental cell line (control). **A)** Cell monolayers were scratched using a sterile pipette tip (0h) and incubated with fresh serum-free medium for 12 h. Images were taken at 0 and 12 h after the scratch. The images shown are representative of the migration exhibited by the cells (100X magnification). **B)** Cell migration was quantified using the ImageJ software (n=8). Results are presented as migrated area (% of control). **C)** Cells were serum deprived for 24 h. The cell proliferation rate was assessed by BrdU incorporation quantification (n=9). Results are presented as absorbance (% of control). **D)** Cells were seeded, left to adhere for 40 min and stained with crystal-violet. Results are presented as absorbance (% of control). **E)** Cells were seeded, left to spread for 3 h and stained with crystal-violet. Images were taken on 5 random fields of each well and analyzed with the CellProfiler Software for several morphologic parameters (n=6). Results are presented as % of control. **D)** The images shown are representative of B16F10, L20 and L40 cells fixated and stained with crystal violet (200X) (top) and its respective analysis on the software (bottom). Data shown as arithmetic means $\pm$ standard error of the mean (SEM). *($P < 0.05$) denotes significant difference from control, by One-Way ANOVA.

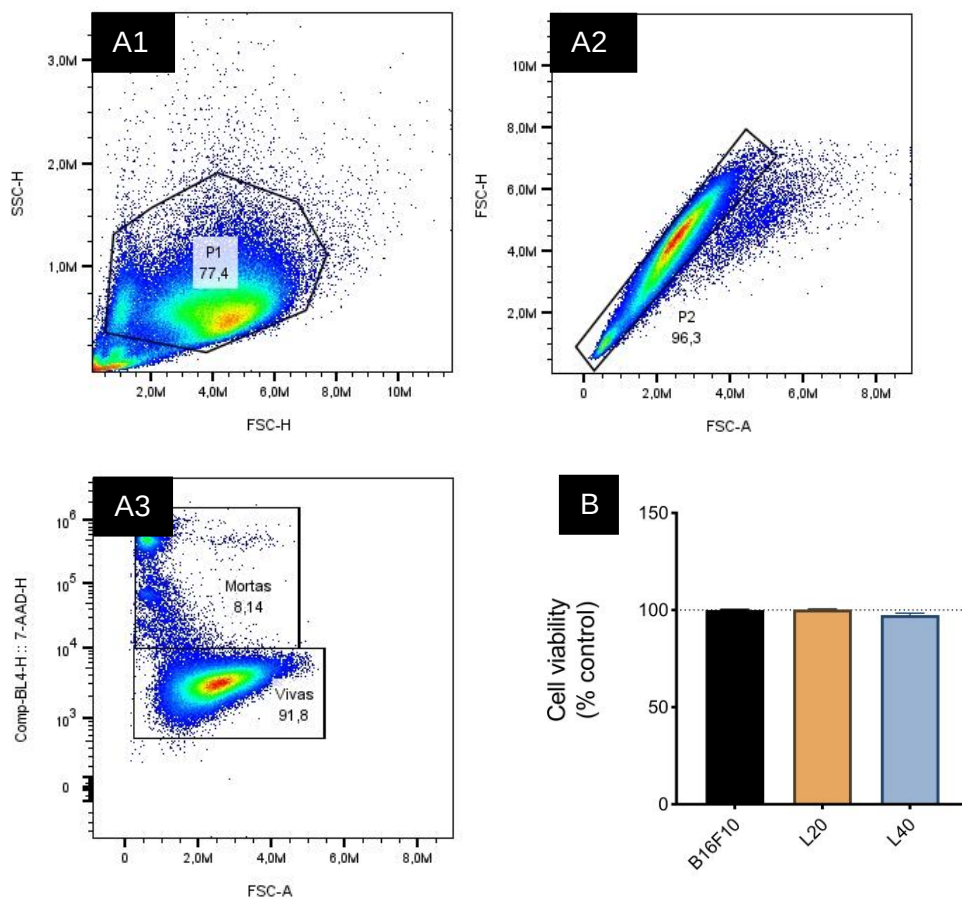


Figure 6: A - Gating strategy for 7-AAD cell viability analysis. **A1)** Cells were gated by FSC-A versus SSC-A (P1). **A2)** A singlet population was gated from FSC-A versus FSC-H (P2). **A3)** Cells were plotted by 7-AAD-H versus FSC-A and live and dead cell populations were gated. **B)** Cells were serum deprived for 24 h. Cell viability was assessed by 7-AAD incorporation analysis (n=3). Results represent non-stained (viable) cells and are presented as % of control. Data shown as arithmetic means $\pm$ standard error of the mean (SEM). *($P < 0.05$) denotes significant difference from control, by One-Way ANOVA.

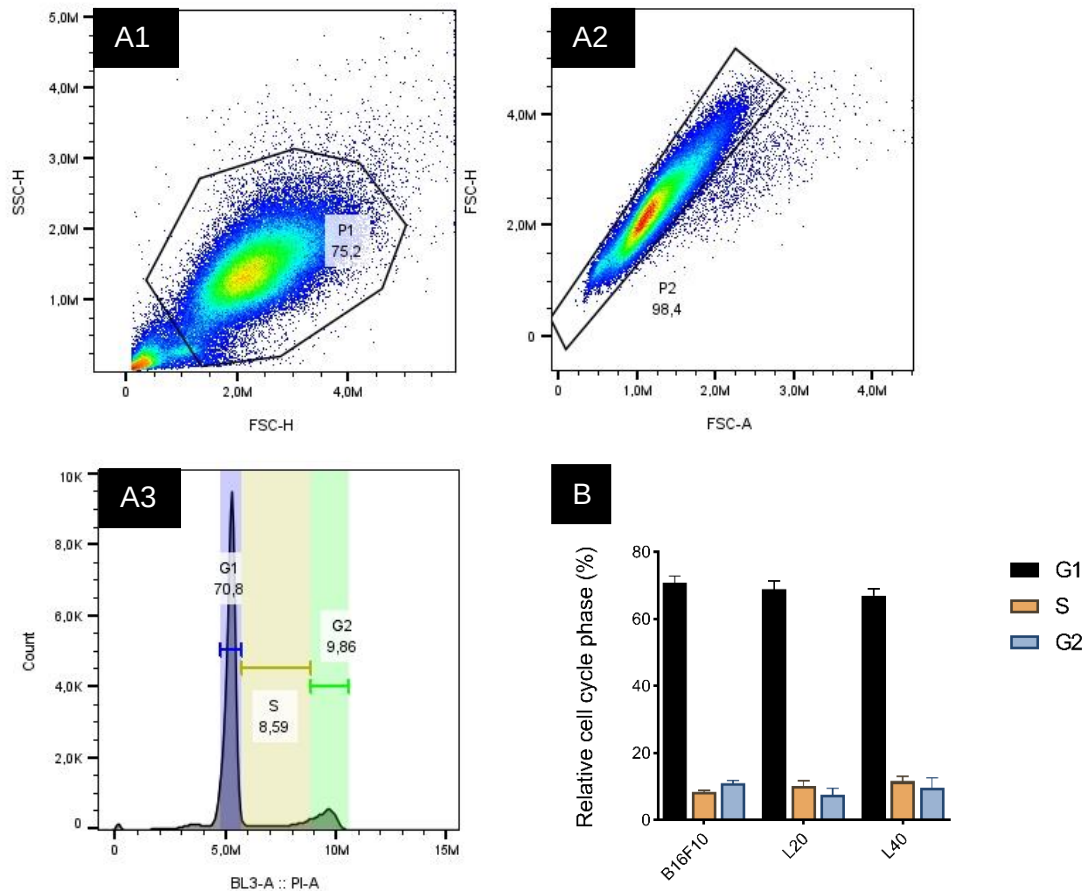


Figure 7: A - Gating strategy for PI cell cycle analysis. A1) Cells were gated by FSC-H versus SSC-H (P1). A2) A singlet population was gated from FSC-A versus FSC-H (P2). A3) Cells were plotted by PI-A versus cell count and the different cell cycle phases were gated based on the Watson (pragmatic) model. B) Cells were serum deprived for 24 h. Cell viability was assessed by PI cell cycle analysis (n=3). Results are presented as % of relative cell cycle phase. Data shown as arithmetic means.

Afterwards, cell viability and cell cycle progression were assessed using the 7-AAD live/dead exclusion and PI-staining flow cytometry analysis, respectively. No statistically significant differences were observed for the number of cells 7-AAD positive (Figure 6A and B) nor the G0/G1, S, and G2/M populations (Figures 7A and B) between L20, L40 Luc-GFP and non-transduced B16F10 cells. Thus, there was no evidence that the lentiviral transduction had an influence on the viability and cell cycle progression of B16F10-Luc-GFP cells (Figures 6 and 7, respectively).

Altogether, Luc-GFP L20 and L40 cells display comparable characteristics to the parental B16F10 cells, with minor to no differences when comparing features such as morphology, migration, proliferation, viability, and cell cycle progression. The future

engraftment of the B16F10-Luc-GFP cell line generated on a diet-induced obesity murine model will enable the *in vivo* and *ex vivo* visualization of melanoma cells, and further investigation of the effects of obesity on melanoma.

3.2. *In vitro* evaluation of the effect of adipocyte conditioned medium and FGF-21 on melanoma progression

In an attempt to deepen our understanding of the association between obesity and melanoma, an *in vitro* cell culture model of obesity was employed. 3T3-L1 preadipocytes represent a readily accessible cell line commonly utilized in the study of adipogenesis (Bahmad et al., 2020). The differentiation of 3T3-L1 preadipocytes into mature adipocytes within a culture setting is a widely adopted procedure with established protocols (Zhao et al., 2019). In our research, we employed conditioned media (CM) obtained from fully differentiated 3T3-L1 cells (Figure 8A and B) to investigate the potential impact of adipocyte-secreted factors, particularly FGF-21, a physiologically relevant adipokine, on malignant melanoma B16-F10 cells. Furthermore, we also assessed the impact of recombinant FGF-21 and a FGFR-1 inhibitor, PD173074, individually and in combination, on melanoma cells to enhance our experimental model.

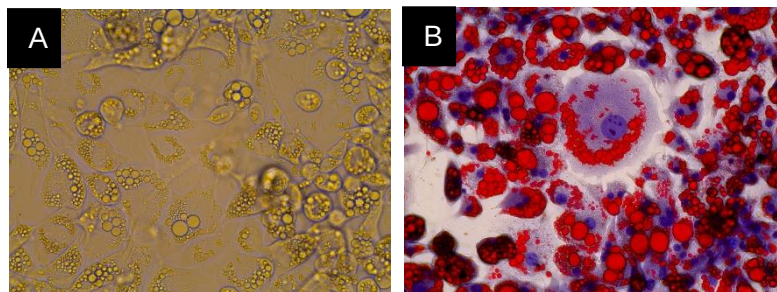


Figure 8: Representative images of differentiated 3T3-L1 cells unstained (A) or stained with Oil-Red-O (B) captured under an inverted microscope (200X). 3T3-L1 pre-adipocytes were differentiated into adipocytes after a 12 day treatment with an induction mixture. An ORO-hematoxylin staining was performed on differentiated 3T3-L1 cells, to inspect lipid accumulation in adipocytes. The adipocyte conditioned medium was collected and used as an *in vitro* model of obesity.

Cancer cells exhibit acquired traits that allow them to survive, proliferate and disseminate even under adverse conditions. These hallmarks include: cell proliferation signaling maintenance, the evasion of growth suppression and of programmed cell death, angiogenesis induction, replicative immortality, metabolic reprogramming, immune system evasion, and metastasis and invasion activation (Hanahan, 2022).

Migration is an altered cancer feature that plays a critical role in the process of metastasis (Paul et al., 2017). Therefore, we first evaluated the melanoma cells migration when treated with adipocyte CM, FGF-21 and/or FGFR-1 inhibitor, by the injury assay after an injury was made to the cell monolayer (Figure 9A). The results showed no significant differences between treatment groups and/or the controls regarding B16F10 motility, although a tendency was observed in cell treated with CM to enhance melanoma cell migration (Figure 9B).

Then we stained melanoma cells with crystal violet, after a 40 m incubation with standard treatments, to assess the adhesive properties of cells. Only treatment with FGF-21 had an effect on the adhesion of B16F10 cells, showing an increase by 34%, when compared with nontreated cells (Figure 9C).

Afterwards, we evaluated the proliferation of cells melanoma cells, after a 24 h treatment with our previously mentioned compounds (figure 10A). Our results reveal that melanoma cells treated with adipocyte CM display enhanced BrdU incorporation rates, when compared to untreated B16F10 cells. Contrastingly, treatment with FGF-21 and PD173074, or just the inhibitor, appears to lower the proliferative capacity of melanoma cells.

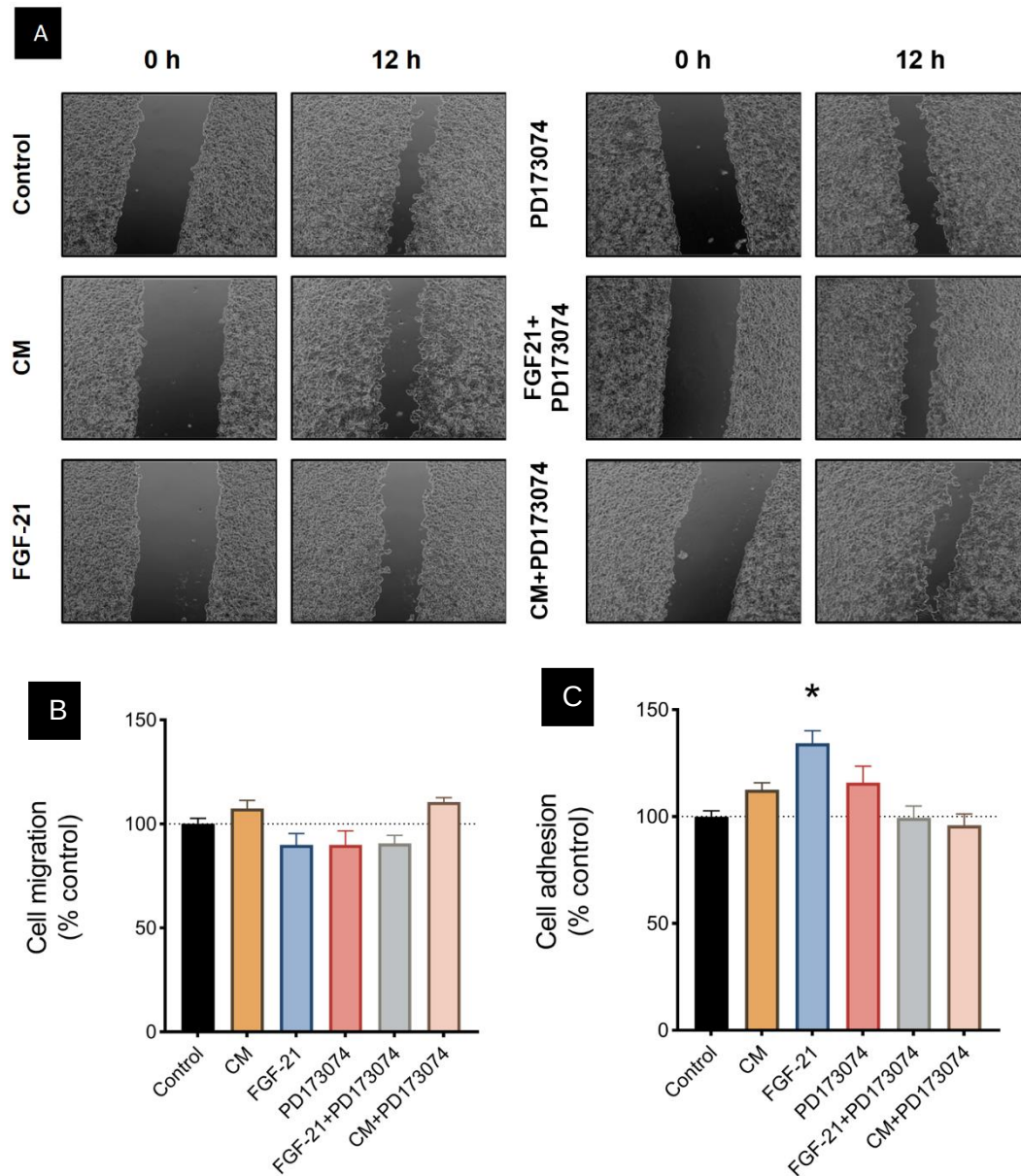


Figure 9: *In vitro* analysis of the effect of obesity on melanoma cell migration and adhesion. **A)** B16F10 cell monolayers were scratched using a sterile pipette tip (0h) and incubated with respective treatments for 12 h. Images were taken at 0 and 12 h after the scratch. The images shown are representative of the migration exhibited by the cells (100X magnification). **B)** Cell migration was quantified using the ImageJ software (n=8). Results are presented as migrated area (% of control). **C)** Cells were seeded with the standard treatments, left to adhere for 40 min and stained with crystal-violet. Results are presented as absorbance (% of control) (n= 4-6). Data shown as arithmetic means $\pm$ standard error of the mean (SEM). *(P < 0.05) denotes significant difference from control, by One-Way ANOVA.

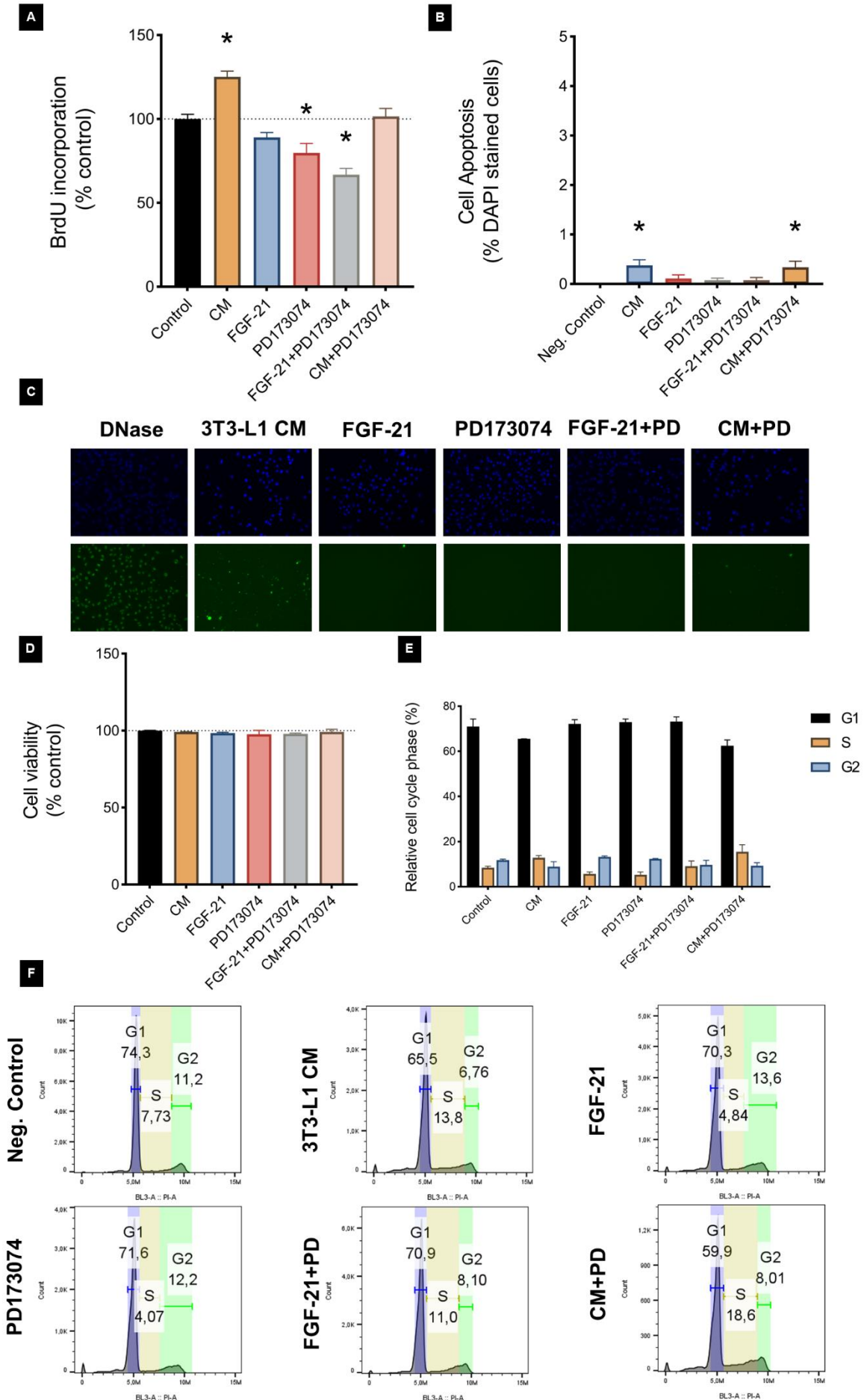


Figure 10: *In vitro* analysis of the effect of obesity on melanoma cell proliferation, apoptosis, viability and cell cycle progression. B16F10 cells were incubated with 3T3-L1 CM, FGF-21, PD173074 or a combination of these, for 24 h. **A)** The cell proliferation rate was assessed by BrdU incorporation quantification (n=9). **B)** The cell apoptotic rate was assessed by dividing TUNEL-positive cells by the number of nuclei stained with DAPI. (n=9-18). Results are presented as % of DAPI-stained nuclei. **C)** Representative images of DAPI (top) and TUNEL-stained (bottom) cells, taken with an inverted microscope (200X). **D)** Cell viability was assessed by 7-AAD incorporation analysis (n=3). Results represent non-stained (viable) cells and are presented as % of control. Gating strategy shown previously was used. **E)** Cell cycle progression was assessed by PI cell cycle analysis (n=3). Results are presented as % of relative cell cycle phase. Gating strategy shown previously was used. **F)** Representative images of the different cell cycle phases, gated based on the Watson (pragmatic) model. Data shown as arithmetic means $\pm$ standard error of the mean (SEM). *(P < 0.05) denotes significant difference from control, by One-Way ANOVA.

Our findings imply that treatment with adipocyte CM is able to stimulate melanoma cell proliferation. Thus, we then evaluated the cell cycle progression of treated cells, after the relative determination of cell DNA content, dyed with PI stain. Surprisingly, there was no evidence that our treatments had an influence on the cell cycle progression of melanoma cells (Figure 10E).

Additionally, no statistically significant effects were observed on melanoma cell viability (Figure 10D). Nevertheless, after TUNEL analysis, we observed that CM treatment, with or without PD173074, enhances, very slightly, the number of B16F10 apoptotic cells (figure 10B and C). The 3T3-L1 CM contains proteins and other molecules that stain fluorescently in the presence of the TUNEL reagent; thus, the low number of observed apoptotic cells may be, in fact, artifacts.

4. Discussion

Melanoma is the most aggressive form of skin cancer, presenting a high risk of metastasis. Melanoma accounts for a high number of deaths and its incidence has been rising worldwide (Saginala et al., 2021). Recently, obesity has been postulated as a risk factor for several types of cancer (Dobbins et al., 2013). However, the association between obesity and an increased incidence of melanoma remains inconclusive (Smith et al., 2020).

The present work was divided into two major objectives and tasks: the first task was to create a dual-reporter stable B16F10 cell line expressing both Luc and GFP, that after engraftment in HFD-obese mice, will enable the *in vivo* and *ex vivo* visualization of melanoma cells; the second task involved the *in vitro* investigation of the effects of FGF-21 and adipocyte CM on melanoma.

The application of the B16F10 cell line in the research of melanoma progression has been widely documented in the academic literature (Culp et al., 2006). B16F10 cells are originated from a subclone of the B16 tumor line, obtained from pulmonary melanoma metastasis in C57BL/6 mice of the B16-F0 parent cell line 10th passage (Nicolson et al., 1978). B16F10 cells exhibit epithelial-like morphology, are highly proliferative, metastatic and adherent, and will rapidly form tumors and metastases after being implanted into syngenic C57BL/6 animals (Nicolson et al., 1978). Luc and GFP are two widely utilized reporters in various scientific and experimental contexts that enable the visualization of cells *in vivo* and *ex vivo* (Welsh & Kay, 1997). Herein, we have successfully established two dual-reporter B16F10 polyclonal cell lines that stably expresses Luc and GFP: L20 and L40. Importantly, these cell lines behave similarly to parental B16F10 cells, when analyzing cell parameters such as morphology, migration, proliferation, viability and cell cycle progression. L20 cells were smaller than control cells and its adhesive properties were enhanced; L40 cells, on the other hand, displayed larger areas and perimeters than parental B16F10 cells. Nevertheless, we believe these effects are not significant from a biological point of view.

Other studies have established dual reporter Luc-GFP cell lines with success, including melanoma cells (Day et al., 2009).

Furthermore, reporter gene transfection enhances our ability to unravel underlying mechanisms involved in disease progression. Utilizing B16F10 cells expressing luciferase facilitates the *in vivo* monitoring of the primary and metastatic melanoma tumor

development in a non-invasive manner, employing the sensitive and accurate bioluminescence imaging technique (Tiffen et al., 2010). Moreover, GFP expression provides a straightforward means of visualizing and analyzing tumoral tissues *ex vivo* using methods such as fluorescence microscopy.

Additionally, the C57Bl/6 mice model is an excellent model that can mimic the complications, observable in humans, of diet-induced overweight and obesity, the long-term high-fat diet consumption, such as dyslipidemia, insulin resistance, hyperinsulinemia, hypertension, among others. (Preguiça et al., 2020). Accordingly, an *in vivo* model using diet-induced obese C57Bl/6 mice injected with B16F10-Luc-GFP cells will be employed to research the impact, and underlying molecular mechanisms, of obesity and fat-rich diets on the primary growth and metastasis of melanoma.

Obesity has been recognized as a contributing factor to increased incidence and mortality in various malignancies (Renehan et al., 2008). However, the link between obesity and melanoma remains ambiguous (Smith et al., 2020). Previous studies from our team showed that diet-induced obesity led to a significant increase in FGF-21 circulating levels (Fonseca et al., 2021). Therefore, the second objective of the present work concerned the exploration of the effects of FGF-21 and 3T3-L1 adipocyte CM on melanoma cells using an *in vitro* cell culture model.

In an attempt uncover the effects of obesity on melanoma, we have employed 3T3-L1 adipocyte conditioned medium as an *in vitro* model of obesity. 3T3-L1 preadipocytes are a widely used murine cell line in adipogenesis research (Bahmad et al., 2020). The preadipocyte differentiation into adipocytes is attained by well-established protocols, that include the addition of an induction mixture of dexamethasone, IBMX and insulin (Zhao et al., 2019). Mature 3T3-L1 secretome analysis reveals similar patterns to those observed in the serum obese individuals, such as increased levels of adipokines including leptin, resistin, VEGF and endocan, and decreased levels of adiponectin (Coelho et al., 2016; Coelho, 2017). Therefore, the conditioned medium of these cells was used to study the *in vitro* effects of adipocyte-released factor on B16F10 cells.

Our results show that treatment with adipocyte CM enhanced B16F10 cell proliferation, whereas the addition of the FGFR-1 inhibitor nullified this effect. Coelho et al. (2016) previously studied the effects of adipocyte CM on melanoma cells. In line with our results, B16F10 cells treated with 3T3-L1 CM exhibited increased BrdU incorporation rates. However, in contrast to our data, adipocyte CM also increased melanoma cell migration and adhesion, and reduced cell apoptosis, while we did not observe any significant effects in regard to those parameters. We believe this discrepancy is due to variability in

the secretome composition of the mature 3T3-L1 CM. There are several factors that may affect the process of 3T3-L1 preadipocyte differentiation into mature adipocytes, including the origin of the cell line, its passaging number or even the type or provider of the culture dishes (Zhao et al., 2019). Therefore, the adipokines present 3T3-L1 CM in this study may be vary from the profile of the adipocyte CM used in the referenced study, thus leading to different effects when applied to melanoma cells.

Treatment with a FGFR-1 inhibitor, significantly lowered cell proliferation. Moreover, the addition of the inhibitor to the adipocyte CM nullified the CMs proliferation enhancing properties. Hence, we can deduce another member of the FGF family with affinity to this receptor may be present in the adipocyte CM and be responsible for the increased proliferation of melanoma cells. Inhibition of FGFR-1 has been correlated to smaller prostate, breast and lung cancer tumors (Turner et al., 2010; Weiss et al., 2010; Yang et al., 2013). In melanoma, the inhibition of FGFR-1 gene leads to cell proliferation decline (Becker et al., 1992). FGFR-1 mediates the uptake of FGF-21 and others FGFs, including FGF-2. The overexpression of this ligand has been observed in melanoma cells, where it acts as autocrine growth factor (Metzner et al., 2011).

Metzner et al. (2011) reported that the inhibition of FGF2/FGFR-1 leads to a decrease in the proliferation and colony formation of melanoma cells. In fact, the study by Fonseca et al. (2021) also reported elevated FGF-2 serum levels in HFD-obese mice, although not as preeminent as FGF-21 levels. Therefore, we can hypothesize that FGF-2 is one of the factors present in the 3T3-L1 CM responsible for the cell proliferation enhancement, and that its effect is blocked upon FGFR-1 inhibition.

FGF-21 is regarded as a crucial factor in the maintenance of the lipid and glucose metabolism, and is linked with blood glucose and insulin level reduction, and insulin sensitivity enhancement in obese individuals (Berglund et al., 2009; Kharitononkov et al., 2005; Woo et al., 2013). A recent study by our group prompted us to investigate the relation between FGF-21, obesity and melanoma (Fonseca et al., 2021). In this study, obese mice engrafted with melanoma cells displayed a reduced metastatic potential, when compared to control animals. In mice inoculated with B16F10 cells, HFD-induced obesity led to an increase in FGF-21 serum levels and FGFR-1 expression. Thus, FGF-21 was suggested as the probable cause of the decreased metastatic potential of melanoma observed in obese mice, due to its metabolism regulation and anti-inflammatory properties (Fonseca et al., 2021).

In the present work, treatment with FGF-21 alone enhanced melanoma cell adhesion, but did not affect other cell parameters. Nevertheless, when added to PD173074, the

proliferation exhibited by melanoma cells was further decreased. We deduce FGF-21 is not significantly expressed by 3T3-L1 adipocytes. FGF-21 is mainly produced in the liver, although it may be produced by adipocytes under certain conditions. Therefore, in order to reproduce and further investigate the protective effects of FGF-21 on obesity and melanoma, *in vivo* studies have to be carried out. These findings suggest that FGF-21s action on melanoma does not occur directly, on the melanoma cells, but rather via a systemic indirect mechanism. Thus, simple *in vitro* cell culture models with adipocyte CM, FGF-21 and B16F10 melanoma cells may be too simplistic, and unable to represent the complexity of obesity-associated alterations.

Contradictory findings have been reported regarding FGF-21 and its effect on cancer. Recent reports have suggested a correlation between FGF-21 and colon, thyroid and liver cancer development (Kang et al., 2019; Qian et al., 2018; Singhal et al., 2018).

Moreover, *in vitro*, the administration of FGF-21 resulted in the enhancement of thyroid cancer cells migrative and invasive properties (Kang et al., 2019). Similar results were reported by Yu et al. (2021), who observed increased migration and proliferation of lung cancer cells mediated by FGF-21. In contrast, in a study involving prostate cancer cells, FGF-21 treatment inhibited cell proliferation, migration and invasiveness, while promoting cell apoptosis (Dai et al., 2021). In fact, Fisher et al. (2010) reported that the elevated serum FGF-21 levels observed in obese mice, and further exogenous administration of FGF-21, did not exert any of the favorable effects that are expected of FGF-21, such as increased glucose tolerance. The author described obesity as a FGF-21 resistant state. In obese individuals, the state of chronic inflammation may lead to beta-klotho expression repression, thus impairing the action of FGF-21 on adipocytes (Díaz-Delfín et al., 2012).

In fact, these FGF-21 antagonistic effects raise a question: are the high levels of FGF-21 observed in obese and cancer patients in fact involved in the obesity-cancer relationship, or are they a simple consequence of obesity and cancer metabolic deregulation?

Altogether, to determine the role of FGF-21 in the obesity-melanoma association, future studies will involve the engraftment of B16F10-Luc-GFP cells into HFD-obese mice. This research model will enable the *in vivo* and *ex vivo* visualization of primary melanomas and metastasis, facilitating the understanding of underlying molecular mechanisms involved, and providing valuable evidence towards confirming or excluding FGF-21 as a key mediator in this relationship.

5. Conclusion

In conclusion, this study delves into the intricate relationship between obesity and melanoma, shedding light on the potential effects of FGF-21 in this complex association. While obesity has been widely recognized as a risk factor for various types of cancer, the precise impact of obesity on melanoma remains elusive. Herein, we encompassed the creation of a novel dual-reporter B16F10 cell line, enabling to visualize melanoma cells both *in vivo* and *ex vivo* within an obese murine model.

Unraveling the relation obesity-melanoma, and its underlying factors, will lead to the discovery of new therapeutic targets and agents. Moreover, identifying and integrating obesity as a risk factor in the development, diagnosis and prognosis of melanoma will facilitate the therapeutic monitoring of patients and the development of personalized treatment strategies, resulting in improved outcomes for obese melanoma patients.

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