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BIOQUÍMICA EM SAÚDE

Umbilical Cord Mesenchymal Stem Cells Effect on Melanoma Progression

Pablo Rende Fernandes

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ESCOLA SUPERIOR DE SAÚDE

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List of Publications

Over the course of this dissertation, two abstracts were created and subsequently accepted for presentation at a conference, as outlined below.

Fernandes, P., Gomes, A., & Coelho, P. (2023). Secretome of Umbilical Cord Mesenchymal Stem Cells: Potential Effects on Melanoma. *Stem Cells Translational Medicine*, *12*(Suppl 1), S29. Abstract 27 at Cord Blood Connect 2023 conference. (September 8, 2023 – September 10, 2023). https://doi.org/10.1093/stcltm/szad047.028

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Resumo

O melanoma, um tipo de cancro da pele que tem origem nos melanócitos, caracteriza-se pela sua natureza agressiva e propensão para metastizar, o que torna o seu tratamento um desafio. Os tratamentos existentes para o melanoma metastático têm uma eficácia limitada, evidenciando a necessidade de novas abordagens terapêuticas. Em modelos pré-clínicos de cancro, as células estaminais mesenquimais do cordão umbilical humano (CEMCUhs) têm demonstrado efeitos anti-tumorais promissores, impedindo a proliferação e a metastização das células malignas. Neste trabalho, investigou-se o efeito do meio condicionado (MC) obtido a partir de CEMCUhs na viabilidade, proliferação, ciclo celular, migração e adesão de melanócitos malignos utilizando modelos de cultura de células *in vitro*. As CEMCUhs, foram isoladas do cordão umbilical de sete recém-nascidos saudáveis por digestão enzimática, cultivadas e o seu MC coletado e armazenado para ensaios subsequentes. Em seguida, células de melanoma maligno B16F10 foram tratadas com uma concentração final de 100% de MC ou com meio padrão sem soro (controlo) durante 24 horas. A viabilidade celular avaliada pelo ensaio MTT e pelo ensaio de coloração com 7-aminoactinomicina D (7-AAD), demonstrou que as células B16F10 diminuíram a sua viabilidade em 32% e 11%, respetivamente ($p<0,05$, n=7). Em seguida, a proliferação e a progressão do ciclo celular foram avaliadas por citometria de fluxo com as marcações Ki-67 e iodeto de propídeo (PI), respetivamente. A expressão do marcador de proliferação Ki-67 foi reduzida em 14% ($p<0,05$, n=7), com um aumento concomitante do número de células na fase G0/1 (6%). Ao avaliar a capacidade migratória, o CEMCUh-CM reduziu a mesma em 24% ($p<0,05$ vs. controlo, n=7), enquanto que aumentou a adesão em 16% ($p<0,05$ vs. controlo, n=7). Os nossos resultados mostraram que o secretoma das CEMCUhs reduziu a viabilidade e a capacidade de proliferação das células do melanoma, levando a uma acumulação das células na fase celular G0/1. Este estudo sugere que o efeito parácrino das CEMCUhs inibe o crescimento das células do melanoma, com uma diminuição significativa da sobrevivência global das células. São necessárias mais investigações para caracterizar as vias moleculares destes promissores efeitos do secretoma das CEMCUhs na inibição da proliferação das células do melanoma e para estabelecer a sua segurança e eficácia. Em suma, os nossos resultados suportam uso de terapias baseadas em secretoma das CEMCUhs no melanoma.

Palavras-chave: Melanoma, CEMCUhs, Secretoma, Células B16F10; Terapia Acelular

Abstract

Melanoma, a skin cancer originating from malignant transformation of melanocytes, is characterized by its aggressive nature and high tendency to metastasize, rendering melanoma management challenging. The few existing treatments for metastatic melanoma have limited effectiveness, underlying a demand for novel therapeutic strategies. In preclinical cancer models, human umbilical cord mesenchymal stem cells (hUCMSCs) have shown promising anti-cancer effects, hampering both the proliferation and metastasis of malignant cells. In this work, we investigated the impact of conditioned media (CM) obtained from hUCMSCs on viability, proliferation, cell cycle, migration, and adhesion of melanoma cells using *in vitro* cell culture models. hUCMSCs, isolated from the umbilical cord of seven healthy neonates by enzymatic digestion followed by direct plastic adherence method, were cultivated and their CM stored for subsequent assays. Then, malignant melanoma B16F10 cells were treated with final concentration of 100% of CM or standard serum-free media (control) for 24 hours. Cell viability was assessed using MTT assay and 7-aminoactinomycin D (7-AAD) staining assay. When treated with hUCMSCs secretome, B16F10 cells decreased their viability by 32% and 11% as shown by the MTT assay and 7-AAD exclusion stain, respectively ($p<0.05$, n=7). Next, proliferation and cell cycle progression were evaluated by flow cytometry analysis with Ki-67 and propidium iodide (PI) stains, respectively. The expression of Ki-67 proliferation marker was reduced by 14% ($p<0.05$, n=7) with a concomitant increase in the number of cells in G0/G1 phase arrest (6%). hUCMSC-CM reduced cell migration, assessed by scratch assay, by 24% ($p<0.05$ vs control, n=7) while enhancing cell adhesion capacity, evaluated by the crystal violet assay, by 16% ($p<0.05$ vs control, n=7). Our results showed that hUCMSCs secretome reduced the viability and proliferation capacity of malignant melanoma cells with a concomitant arrest of cells in G0/G1 cell cycle stage. This study suggests hUCMSCs paracrine signaling induces melanoma cells into a quiescent growth inhibition state with a concomitant decrease in overall cell viability and survival. Further research is required to characterize the molecular pathways of these promising effects of hUCMSCs secretome in inhibiting melanoma cells proliferation and establish their safety and efficacy. Altogether, our findings support hUCMSCs paracrine component therapies for melanoma.

Keywords: Melanoma, hUCMSCs, CM, Secretome, B16F10 cells; Accelular Therapy

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List of Acronyms

ANOVA	Analysis of Variance
ASCs	Adult Stem Cells
ATMSC	Adipose Tissue Mesenchymal Stem Cell
BCC	Basal Cell Carcinoma
BM	Bone Marrow
BRAF	B-Raf proto-oncogene
CDK	Cyclin-Dependent Kinase
CKI	Cyclin-Dependent Kinase Inhibitors
CM	Conditioned Media
cSCC	Cutaneous Squamous Cell Carcinoma
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
ESCs	Embryonic Stem Cells
EVs	Extracellular Vesicles
FDA	Food and Drug Administration
FDMSC	Fetal Dermal Mesenchymal Stem Cell
FSCs	Fetal Stem Cells
HOVEC	Human Ovarian Microvascular Endothelial Cells
HSCs	Hematopoietic Stem Cells
hUC	Human Umbilical Cord
hUCMSCs	Human Umbilical Cord Mesenchymal Stem Cells
IFN	Interferon
IL	Interleukin
iPSCs	Induced Pluripotent Stem Cells
MAPK	MAP kinase
MITF	Microphthalmia-Associated Transcription Factors
MMPs	Metalloproteinases
MSH	Melanocyte-Stimulating Hormone

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NF1	Neurofibromin 1
NK	Natural Killer
PBS	Phosphate-Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PD-1	Programmed Cell Death 1
PI	Propidium Iodide
POI	Primary Ovarian Insufficiency
POT1	Protection of Telomere-1
PTEN	Phosphatase and tensin homolog
RT	Room Temperature
SD	Standard Deviation
TERT	Telomerase Reverse Transcriptase
TME	Tumor Microenvironment
UCB	Umbilical Cord Blood
UCT	Umbilical Cord Tissue
UV	Ultraviolet
7-AAD	7-aminoactinomycin D

1. Introduction

1.1. The Hallmarks of Cancer

Cancer is an abnormal condition in which a group of cells disobey the physiological rules of cell division and grow uncontrollably. Cancer cells do not respond to the numerous signals and mechanisms that normally control cell cycle, resulting in uncontrolled growth and proliferation of malignant cells (Hanahan and Weinberg, 2000). Cancer is one of the most severe diseases that affect the global population (Zhang et al., 2007), accounting for nearly 10 million deaths worldwide every year (Ferlay et al., 2021).

The process by which healthy cells develop into malignant cells is complex and multifactorial, with several endogenous and exogenous factors contributing to cancer development (Brennan et al., 2010). Cancer cells exhibit a series of genetic alterations that activate oncogenes and/or disable tumor suppressor genes and stability genes, which often amplify mutations in other genes (Costello and Franklin, 2012).

According to the extended hallmarks of cancer (Hanahan and Weinberg, 2000, 2011), ten different features are acquired and shared along the multistep development and progression of cancer (Figure 1): (i) tissue invasion and metastasis, (ii) tumor-promoting inflammation, (iii) limitless replicative potential, (iv) sustained angiogenesis, (v) self-sufficiency in growth signals, (vi) evasion of immune system destruction, (vii) deregulating cellular energetics, (viii) evasion of programmed cell death (apoptosis), (ix) insensitivity to growth-inhibitory (antigrowth) signals, and (x) genome instability and mutation.

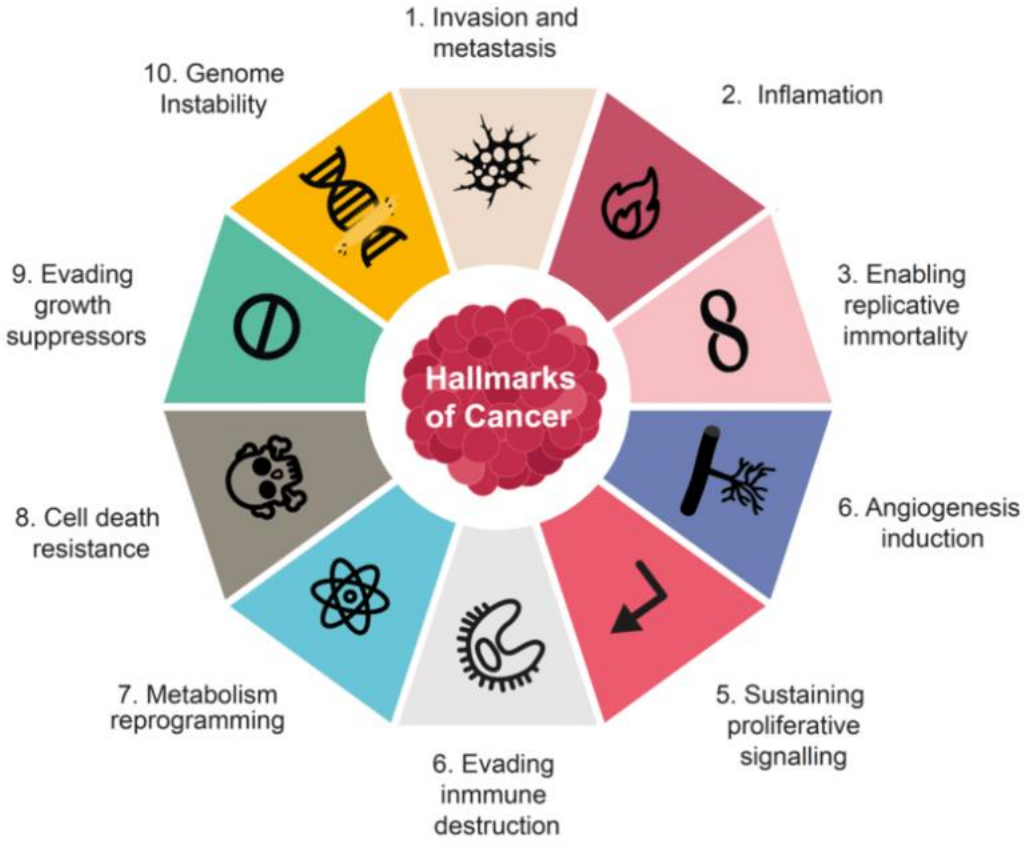


Figure 1: The Hallmarks of Cancer. Figure from (Hanahan and Weinberg, 2000, 2011).

Each of these capabilities represents the successful disruption of a control or defence mechanism against cancer that is hardwired into cells and tissues (Hanahan and Weinberg, 2000, 2011).

As cancer progresses, tumors become highly heterogeneous, creating a mixed population of cells characterized by different molecular features and diverse responsivity to therapies (Dagogo-Jack and Shaw, 2018). Thus, a thorough knowledge of these intricate events is essential to establish accurate and effective treatments. Since the discovery of malignancy, significant research has been directed toward finding novel and high-quality treatment approaches for cancer (Zaigham and Sakina, 2018). The choice of therapy and its likelihood of success depends on the cancer type, anatomical location, and progression stage. Surgery, radiation-based surgical knives, chemotherapy, and radiation therapy are some of the traditional and most commonly used treatment modalities (Attama et al., 2022), while modern approaches also include hormone therapy, small molecule inhibitors, anti-angiogenic treatment regimens, stem cell therapies, immunotherapy, and dendritic cell-based immunotherapy (Charmsaz et al., 2019; Zaigham and Sakina, 2018). While a complete cure for cancer remains a

goal, it is important to recognize that current therapies, while not always curative, still play a crucial role in extending survival, managing the disease, and improving patients' quality of life (Khan et al., 2005; Pucci et al., 2019). Although cancer therapies are effective to varying degrees, they all have their own set of limitations and potential side effects. Thus, the pursuit of effective cancer treatment strategies involves a delicate balance between maximizing therapeutic benefits while minimizing the adverse effects that can impact patients' overall well-being (Palumbo et al., 2013).

1.2. Skin Cancer: The Most Common Neoplasm

Skin is the biggest organ in the human body, covering about 2 m^2 of surface and weighing approximately 3.6 kg in adulthood. It performs crucial roles in controlling body temperature, producing antimicrobial substances to fight infections, and ensuring protection against physical, chemical, and biological threats (Gilaberte et al., 2016; Park, 2022). Histologically, the skin is composed of three distinct layers, from the outermost to the innermost: the epidermis, which contains pilosebaceous units that include a hair follicle and sebaceous glands and are connected with the interfollicular epidermis through the infundibulum (Blanpain and Fuchs, 2006). The skin epidermis also contains other appendages, such as sweat glands, which regulate body temperature through perspiration (Blanpain and Fuchs, 2006). The majority of cells in the epidermis are keratinocytes, constituting around 90% of its cellular composition, but the epidermis is also comprised of melanocytes, Langerhans cells, Merkel cells, and inflammatory cells (Jiang et al., 2020); the dermis, composed of an upper (papillary) and a lower (reticular) layer of fibroblasts, blood vessels, immune cells, and extracellular matrix (ECM) (Hsu et al., 2014; Lynch and Watt, 2018); underneath the dermis, the hypodermis (or subcutaneous adipose tissue) is composed of adipocytes, blood vessels, and inflammatory cells (Driskell et al., 2014). The hypodermis is important for thermoregulation and mechanical protection (Driskell et al., 2014; Hsu et al., 2014).

Skin diseases affect millions of people worldwide and cover a wide range of problems, from chronic diseases such as atopic dermatitis and psoriasis to neoplasms. Although potentially treatable, skin tumors are associated with a high mortality rate (Basra and Shahrukh, 2009; Lim et al., 2017; Richard et al., 2022). In fact, skin cancer is the most common malignancy in the world, with rising incidence rates and a growing global burden (Global Burden of Disease Cancer, 2017; Lomas et al., 2012). Skin cancer can be categorized into two major types: cutaneous

melanoma, which originates from the skin's melanocytes, and nonmelanoma skin cancer, also known as keratinocyte cancer, which includes cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC) (Khayyati Kohnehshahri et al., 2023).

1.2.1. Melanoma: Epidemiology, Etiology, and Pathophysiology

Cutaneous melanoma is a cancer that arises from the malignant transformation of the melanin-producing melanocytes. Melanocytes are cells derived from the neural crest found typically in the basal epidermis, hair follicles, gastric mucosal surfaces, meninges, and uveal and choroidal layers of the eye (Jean, 2002). As ultraviolet (UV) radiation causes damage to melanocytes, keratocytes in the skin produce a melanocyte-stimulating hormone (MSH) that binds the melanocortin-1 receptor on the surface of the local melanocytes, which induces the production and release of melanin (Riley, 1997). Melanin has a complex set of antioxidant and pro-oxidant properties (Obrador et al., 2019; Sarangarajan and Apte, 2006). Its conversion from an antioxidant to a pro-oxidant agent under the influence of various etiological factors such as UV radiation, heavy metals, herbicides, etc., is the critical and earliest pathogenetic event that initiates carcinogenesis. The pro-oxidant action of the melanin results in an increase in the levels of intracellular oxygen radicals, which in turn causes damage to the DNA molecule of the melanocyte. These mutations promote excessive activation of various cell signalling pathways and result in the uncontrolled proliferation, dedifferentiation, and immortalization of cells (Meyskens et al., 2004).

Cutaneous melanoma comprises only 1% of all skin cancers and is the third most prevalent skin cancer after BCC and cSCC, respectively. Nonmelanoma skin cancers, especially BCC, are more common and make up the majority of skin cancer cases. (Miller and Mihm, 2006; Sboner et al., 2003). Despite this, melanoma is responsible for approximately 90% of deaths associated with cutaneous tumors (Garbe et al., 2022; Hogue and Harvey, 2019). Survival rates of advanced melanoma are low; the 5-year survival rate in patients with stage IV melanoma is only about 6% (Spencer et al., 2016). Over the past few decades, the incidence rate of cutaneous melanoma has been rising dramatically throughout the world (Apalla et al., 2017). Melanoma incidence has steadily increased by 4–6% per year in populations with light skin, such as those in North America, Northern Europe, Australia, and New Zealand (Kosary et al., 2014).

The incidence and prevalence rates of cutaneous melanoma continue to rise year after year, in 2020, an estimated 325 000 persons (174 000 males, 151 000 females) worldwide were

diagnosed as having melanoma, and approximately 57 000 persons (32 000 males, 25 000 females) died of the disease (Arnold et al., 2022). The number of newly diagnosed cases of melanoma was estimated to increase by more than 50% by 2040, to 510 000. Similarly, melanoma deaths were estimated to increase by approximately 68%, from 57 000 in 2020 to 96 000 in 2040, assuming rates in 2020 remained stable. Melanoma, which accounts for approximately 1.7% of all cancer diagnoses (Sung et al., 2021), is one of the most prevalent cancers in the world, with 57,000 probable fatalities in the same time frame.

It is estimated that skin melanoma accounted for 4% of all new cancer diagnoses in EU-27 countries in 2020 (all cancers, excluding non-melanoma skin cancers) and for 1.3% of all deaths due to cancer (Manola et al., 2021). This made it the sixth most frequently occurring cancer (after breast, colorectal, prostate, lung, and bladder cancers) and one of the 20 most frequent causes of cancer death.

Today, melanoma is considered a multifactorial disease brought on by genetic predisposition and environmental exposure (Rashid et al., 2022). Increased exposure to UV radiation is unquestionably acknowledged as the main environmental risk factor for the development of cutaneous melanoma (Garland et al., 1993; Tucker and Goldstein, 2003). Upon exposure to excessive UV light, the skin's cellular biology undergoes dysplastic change (Uong and Zon, 2010), leading to melanocyte deregulation and ultimately to neoplastic changes within the melanocyte (Bandarchi et al., 2010; Shain and Bastian, 2016). According to experimental research, UV radiation exposure frequently causes DNA mutations including the formation of pyrimidine dimers or C-T transitions (Agar and Young, 2005). Melanoma that occurs in chronically sun-exposed areas such as the face or forearm, and melanomas occurring in older-aged individuals, exhibit a high load of UV radiation signature mutations such as B-Raf proto-oncogene (BRAF), neurofibromin 1 (NF1) and NRAS (Bastian, 2014; Candido et al., 2014). Melanoma that develops in less sun-exposed locations, such as the proximal extremities or trunk, or that appears in younger-aged people is frequently linked to intermittent sun exposure and typically has lower levels of BRAF mutations (Bastian, 2014; Curtin et al., 2005).

The most important host risk factors are the number of melanocytic nevi, a family history of melanoma, and genetic susceptibility. Melanocytic nevi are benign accumulations of melanocytes or nevus cells and may be congenital or acquired. Approximately 25% of melanoma cases occur in conjunction with a pre-existing nevus (Bevona et al., 2003).

Moreover, the total nevus count is positively correlated with melanoma risk and varies based on the number, size, and type of nevi (Grob et al., 1990; Halpern et al., 1991; Holly et al., 1987).

A family history of melanoma also constitutes a strong risk factor for the disease. In a study of families with inherited melanoma, Tsao *et al.* found that there was a definite pattern of autosomal-dominant inheritance, with numerous family members affected in more than the first generation (Tsao and Niendorf, 2004). The most frequent genetic anomalies identified in these families were changes in cyclin-dependent kinase inhibitor 2A (p16). Other high-penetrant genetic mutations associated with hereditary melanoma include c-KIT/KIT, cyclin-dependent kinase-4 (CDK4), telomerase reverse transcriptase (TERT), and the protection of telomere-1 (POT1), whereas the melanocortin-1 receptor and microphthalmia-associated transcription factors (MITF) are considered to have intermediate penetrance (Pham et al., 2020; Potrony et al., 2015; Zocchi et al., 2021)

The chance of developing melanoma is also increased in people with family cancer syndromes such as familial retinoblastoma, Li-Fraumeni cancer syndrome, and Lynch syndrome type II (Markovic et al., 2007). Moreover, phenotypic traits like red hair, fair skin, numerous freckles, light eyes, UV sensitivity, and an inability to tan increase the risk of developing melanoma by about 50% (Titus-Ernstoff et al., 2005).

Although oncogenic driver mutations in BRAF and NRAS are present in 40–50% and 15%–20% of melanomas, respectively, 30–40% of melanomas do not harbour identifiable driver mutations by conventional clinical assays and are considered to be 'pan-negative' (Palmieri et al., 2015).

1.2.2. Management of Melanoma

The majority of melanoma patients have localized primary disease that may be effectively treated surgically (Lee et al., 2017). However, surgical therapy for advanced melanoma is not curative (Jenkins and Fisher, 2021; Lee et al., 2017; Nguyễn et al., 2020). Treatment of advanced melanoma remains a challenge since melanoma is a solid tumor with a high number of driver mutations and malignant melanocytes exhibit high aggressiveness and have the ability to evade the immune system (Sung and Chang, 2022).

In the past decades, a dramatic evolution in the treatment of patients with unresectable or metastatic melanoma has been observed, with the development of immune checkpoint blockade strategies targeting the PD-1 (programmed cell death 1) and CTLA-4 (cytotoxic T

lymphocyte antigen 4) co-inhibitory receptors, as well as MAP kinase (MAPK) molecular targeted therapy directed at oncogenic BRAF and MEK signalling pathways (Curti and Faries, 2021; Villani et al., 2021). Both approaches have proven effective in the treatment of advanced melanoma. Before 2010, the only primary FDA (Food and Drug Administration) approved treatments for advanced melanoma were high-dose interleukin-2 (IL2) (Atkins et al., 1999) and dacarbazine (Luke and Schwartz, 2013). In 2010, the results of the first phase I trial of the BRAF inhibitor PLX4032 (vemurafenib) were published (Flaherty et al., 2010). That same year, the results of the first phase III trial of the anti-CTLA-4 monoclonal antibody, ipilimumab, were published demonstrating improved overall survival (Hodi et al., 2010). Since then, nearly a dozen new treatments/treatment regimens for melanoma, have been approved by the FDA, including 4 systemic immunotherapy treatments/combinations (ipilimumab, nivolumab, pembrolizumab, combination ipilimumab-nivolumab) (Daud et al., 2016; Larkin et al., 2019; Larkin et al., 2015), single-agent BRAF inhibitors (vemurafenib, dabrafenib) (Falchook et al., 2012; Flaherty et al., 2010), combination BRAF-MEK inhibitor regimens (dabrafenib-trametinib, vemurafenib-cobimetinib, encorafenib-binimetinib) (Flaherty et al., 2012), and 1 intra-lesional immunotherapy involving a modified oncolytic herpes virus (talimogene laherparepvec, T-VEC) (Kaufman et al., 2014). These treatments gained their initial indications in advanced, unresectable melanoma, and several have since gained approval in the adjuvant setting.

While there are several molecular targeted therapies and treatment regimens currently accessible for addressing metastatic melanoma, patients frequently exhibit rapid resistance to these interventions (Patel et al., 2021). The emergence of resistance poses a substantial clinical hurdle, emphasizing the ongoing imperative for research and the creation of novel approaches and treatments to overcome drug resistance and fight advanced melanoma.

1.3. Stem Cells

Stem cells are a unique population of cells present in all stages of life that have the ability to self-renew and differentiate into multiple cell lineages, giving rise to mature cell types with specialized functions (Choumerianou et al., 2008; Jensen et al., 2009; Thomson et al., 1998). The ability of stem cells to differentiate into multiple cell types is called potency (Poliwoda et al., 2022). Self-renewal, on the other hand, implies that a cell can undergo either symmetric divisions into daughter cells which retain full stem cell characteristics, as such maintaining the stem cell population, or asymmetric divisions where only one of the two daughter cells remains

a stem cell while the other starts to differentiate (Ulloa-Montoya et al., 2005) (Figure 2). Therefore, these cells are key mediators in the development of neonates and restorative processes after injury or disease as they are the source from which specific cell types within differentiated tissues and organs are derived (Kolios and Moodley, 2013).

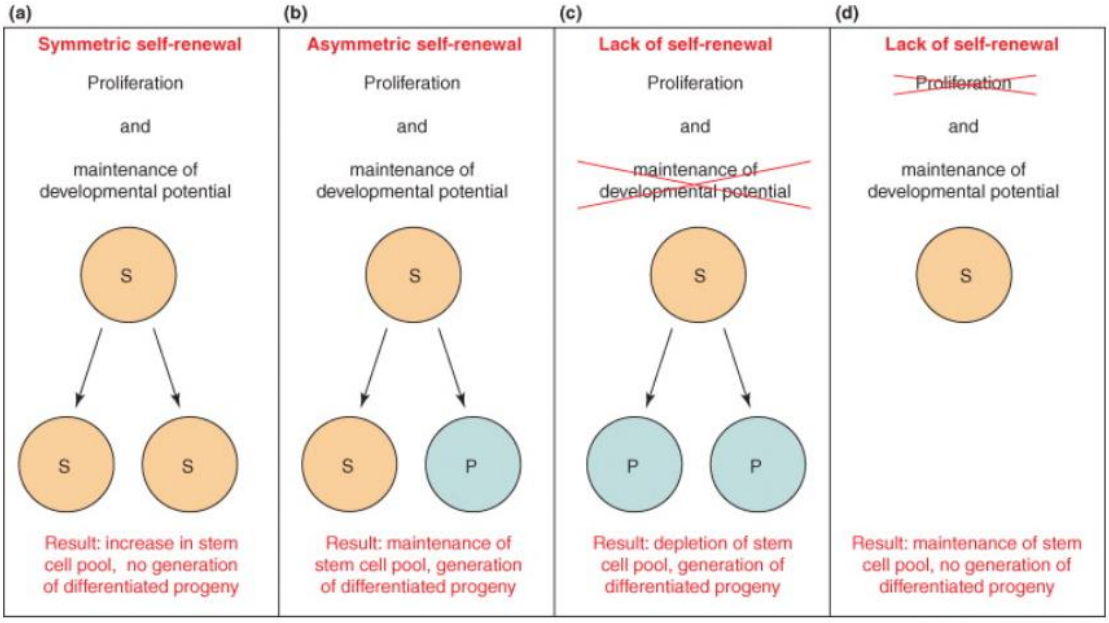


Figure 2: Stem cell self-renew. A stem cell can self-renew by dividing symmetrically to generate two stem cells **(a)**, or asymmetrically to generate a stem cell and a restricted progenitor **(b)**. A loss of either developmental potential **(c)** or the ability to proliferate **(d)** results in a failure of stem cells to self-renew. Figure from (Molofsky et al., 2004).

Based on their differentiation potential, stem cells can be classified into:

i. Totipotent stem cells: the most undifferentiated cells found in early development. They can differentiate into embryonic and extraembryonic tissues, forming the embryo and the placenta (Rossant, 2001).

ii. Pluripotent stem cells: differentiate into 3 germ layers – ectoderm, endoderm, and mesoderm – from which all tissues and organs develop (P. De Miguel et al., 2010). Recently, Takahashi and Yamanaka generated pluripotent cells by reprogramming somatic cells (Takahashi and Yamanaka, 2006). These cells are called induced pluripotent stem cells (iPSCs).

iii. Multipotent stem cells: found in most tissues and differentiate into cells from a single germ layer (Ratajczak et al., 2012). Mesenchymal stem cells (MSCs) are the most recognized multipotent cells.

iv. Oligopotent stem cells: can self-renew and form 2 or more lineages within a specific tissue. Hematopoietic stem cells (HSCs) are a typical example of oligopotent stem cells, as they can differentiate into both myeloid and lymphoid lineages (Marone et al., 2002).

v. Unipotent stem cells: can self-renew and differentiate into only one specific cell type and form a single lineage such as muscle stem cells, giving rise to mature muscle cells and not any other cells (Beck and Blanpain, 2012; Bentzinger et al., 2013).

According to their origin, stem cells can be classified as embryonic stem cells (ESCs), fetal stem cells (FSCs), and adult stem cells (ASCs) (Nawab et al., 2019).

ESCs lines are derived from the inner cell mass of the blastocyst that originates five days after the fertilization. ESCs are pluripotent and give rise, during development, to all of the three primary germ layers (Odorico et al., 2001). ESCs can differentiate into any cell type *in vitro* through gene transcription, either naturally from the body or artificially (through chemical inducement) (Reubinoff et al., 2000).

FSCs are multipotent, meaning they exist only in specific fetal tissues and give rise to differentiated cells of that tissue (O'Donoghue and Fisk, 2004). These cells are a relative newcomer in the field, with unique and fascinating characteristics, and recent studies have provided an impressive wealth of information and new insights into our understanding of the biology of stem cells in general, as well as putative therapeutic strategies (Pappa and Anagnou, 2009).

ASCs are undifferentiated cells that reside among differentiated cells in a tissue or organ. They can renew themselves and differentiate to yield the major cell types of a specialized tissue or organ. The primary role of ASCs in a living organism is to maintain and repair the tissue in which they reside. ASCs can be obtained from several adult tissues or, in some cases, from neonatal tissues (Barrilleaux et al., 2006).

1.3.1. Human Mesenchymal Stem Cells: Sources and Clinical Applications

MSCs are multipotent adult stem cells, that can self-renew and stay "undifferentiated" due to some intrinsic or extrinsic suppressed factors until activated (Bianco et al., 2008; Jiang et al., 2002; Wagner and Ho, 2007). MSCs have the potential to generate mesodermal cell lineages

such as adipocytes, osteoblasts, and chondrocytes (Phinney and Prockop, 2007; Pittenger et al., 1999). On the other hand, MSCs can also trans-differentiate into neural cells from the ectodermal layer, and hepatic cells and pancreatic cells from the endodermal layer (Y. Zhang et al., 2012). The differentiation capacity of MSCs coupled with their release of trophic factors and immunomodulatory properties holds great promise for cell-based therapies and tissue engineering (Murray and Péault, 2015).

MSCs can be isolated from multiple human tissues and display a variety of in vitro characteristics, including their proliferation capacity and differentiation potential, which influence their applicability (Ahani-Nahayati et al., 2018; Marofi et al., 2019). Therefore, selection of an adequate cell source for their clinical use should ideally be based on their logistical, practical, and functional behavior (Heo et al., 2016). Today, the major and most well-known sources of MSCs are bone marrow (BM) (Pittenger et al., 1999) and umbilical cord (UC) (Erices et al., 2000). However, MSCs can also be isolated from adipose tissue (Zhang et al., 2006), dental pulp (Seifrtová et al., 2012), endometrium (Schüring et al., 2011), peripheral blood (Ab Kadir et al., 2012), skin (Riekstina et al., 2008), placenta (Raynaud et al., 2012), synovial fluid (Morito et al., 2008), salivary gland (Rotter et al., 2008), Wharton's jelly (Hou et al., 2009), among others.

1.3.1.1 Bone Marrow

BM is a spongy tissue present within the central cavities of the axial and long bones of the body that has been shown to perform many important regenerative functions. There are two types of BM: red marrow and yellow marrow (Cahn, 1940). All mature blood cell types, except for lymphocytes, are found in this fraction of the red marrow, where hematopoiesis, the process of producing blood cells, takes place. The stroma of the BM contains the yellow marrow and consists of all tissues that are not related to hematopoiesis (blood vessels, adipocytes, osteoblasts, and connective fibroblasts).

BM has two distinct stem cell types: HSCs and MSCs. MSCs in BM are a rare population of cells (~0.001% to 0.01% of the nucleated cells), located perivascularly, directly on blood vessel surfaces along with sympathetic nerves (Pittenger et al., 1999), and play a prominent role in HSCs support (Greenbaum et al., 2013; Méndez-Ferrer et al., 2010; Pinho et al., 2013). Although present as a rare cell population in the BM, MSCs are expandable in culture and multipotent, capable of differentiating into several cell types (Pittenger et al., 1999; Prockop, 1997).

Many beneficial qualities of BM-MSCs for regenerative treatment have been widely characterized, including their multipotency, anti-inflammatory, and immune-modulatory activities (Nguyen et al., 2013; Wada et al., 2013). This stem cell population can promote angiogenesis and assist hematopoiesis. Additionally, the release of paracrine factors by BM-MSCs alters the surrounding microenvironment, a trait very relevant to organ and tissue repair (Pittenger et al., 2019). While BM-MSCs do not engraft easily after transplantation, they can promote the surrounding tissue survival by releasing a panoply of paracrine factors (Leong et al., 2012; Saeedi et al., 2021). Due to their therapeutically advantageous characteristics, BM-MSCs have been used and researched as an encouraging option in cellular treatment and reconstruction of human tissues (Margiana et al., 2022).

The application of BM-MSCs has demonstrated promising therapeutic results in the treatment of neurological diseases including amyotrophic lateral sclerosis (Oh et al., 2015; Syková et al., 2017), spinal cord injury (Mendonça et al., 2014; Vaquero et al., 2017), Parkinson's disease (Giordano et al., 2014), and stroke (Jaillard et al., 2020; Law et al., 2021). For the treatment of liver diseases such as cirrhosis (Suk et al., 2016), and acute-on-chronic liver failure (Schacher et al., 2021), the ability of BM-MSCs to develop into the endodermal lineage (hepatocyte-like cells) makes them an appealing option. Numerous studies focusing on heart failure have used BM-MSCs to develop cutting-edge treatments to improved cardiac function (Chan et al., 2020; Hare et al., 2017; Mathiasen et al., 2020; Xu et al., 2019; Yagyu et al., 2019). These reports demonstrate that BM-MSCs therapy can be an effective, achievable, and safe process that remarkably improves cardiac function and promotes patients' quality of life. BM-MSCs have also been shown to be a promising, safe, and effective alternative for bone regeneration (Chahal et al., 2019; Hernigou et al., 2021; Jayankura et al., 2021; Lamo-Espinosa et al., 2020; Lamo-Espinosa et al., 2018) and wound regeneration (Falanga et al., 2007; Hertegård et al., 2020).

1.3.1.2 Umbilical Cord

Human umbilical cord (hUC) is the link between the mother and the fetus during pregnancy, connecting the growing embryo or fetus to the placenta (Stefańska et al., 2020). This structure allows oxygen and nutrients exchanges between the maternal and fetal circulatory systems, as well as the removal of waste products from the fetal circulation through maternal processes. The hUC comprises three unbranched vessels, two arteries, and one vein, surrounded by a stromal connective tissue called Wharton's jelly, which is covered by the amniotic epithelium

(Gomes et al., 2021; Nagamura-Inoue and He, 2014). Several groups have classified the hUC into different compartments, including (i) the amniotic epithelial membrane, (ii) the cord lining, (iii) the intervascular Wharton's jelly, and (iv) the perivascular zone around the umbilical blood vessels (Bongso and Fong, 2013; Troyer and Weiss, 2008).

Despite being traditionally regarded as medical waste, both the UC blood (UCB) and the UC tissue (UCT) are excellent sources of MSCs (Kim et al., 2004; Lee et al., 2004; Mareschi et al., 2001; Romanov et al., 2003). Comparatively, to other depots of MSCs in the adult organism, human umbilical cord mesenchymal stem cells (hUCMSCs) are preferred candidates for cell-based therapies and regenerative medicine due to the non-invasive isolation method, lack of ethical concerns, lower immunogenicity, faster self-renewal ability, more stable doubling time, and higher proliferation potency (Baksh et al., 2007; Li et al., 2015). The use of hUCMSCs in regenerative medicine and organ grafts has the potential to significantly improve the quality of life for millions of patients while simultaneously address the enormous unmet need for organ transplants (Alatyyat et al., 2020).

In fact, hUCMSCs are being used to treat a variety of diseases, in both preclinical and clinical trials, and significant therapeutic advances have been made using these cells due to their unique modes of action (differentiation, immune regulation, paracrine effects, anti-inflammatory action, anti-fibrotic activity, and non-coding RNA regulation) that are crucial for their therapeutic applications (Figure 3) (Xie et al., 2020).

UCMSCs have been employed in preclinical studies to treat myocardial infarction (Nascimento et al., 2014), traumatic brain damage (Peng et al., 2015), and improve hematopoiesis following cord blood transplantation (Wu et al., 2013a; Wu et al., 2013b). Under clinical trials, hUCMSCs have been used to treat refractory systemic lupus erythematosus (D. Shi et al., 2012; Sun et al., 2010; Wang et al., 2014; Woodworth and Furst, 2014), type 2 diabetes mellitus (Kong et al., 2014), sequelae of thoracolumbar spinal cord injury (Cheng et al., 2014), autism (Lv et al., 2013), hereditary spinocerebellar ataxia (Jin et al., 2013), spinocerebellar ataxia and multiple system atrophy-cerebellar type (Dongmei et al., 2011), spinal cord injury (Liu et al., 2013), primary biliary cirrhosis (L. Wang et al., 2013), sequelae of traumatic brain injury (S. Wang et al., 2013), acute-on-chronic liver failure (M. Shi et al., 2012), decompensated liver cirrhosis (Z. Zhang et al., 2012), stroke (Jiang et al., 2013), and steroid-resistant severe acute graft-versus-host disease (Chen et al., 2014).

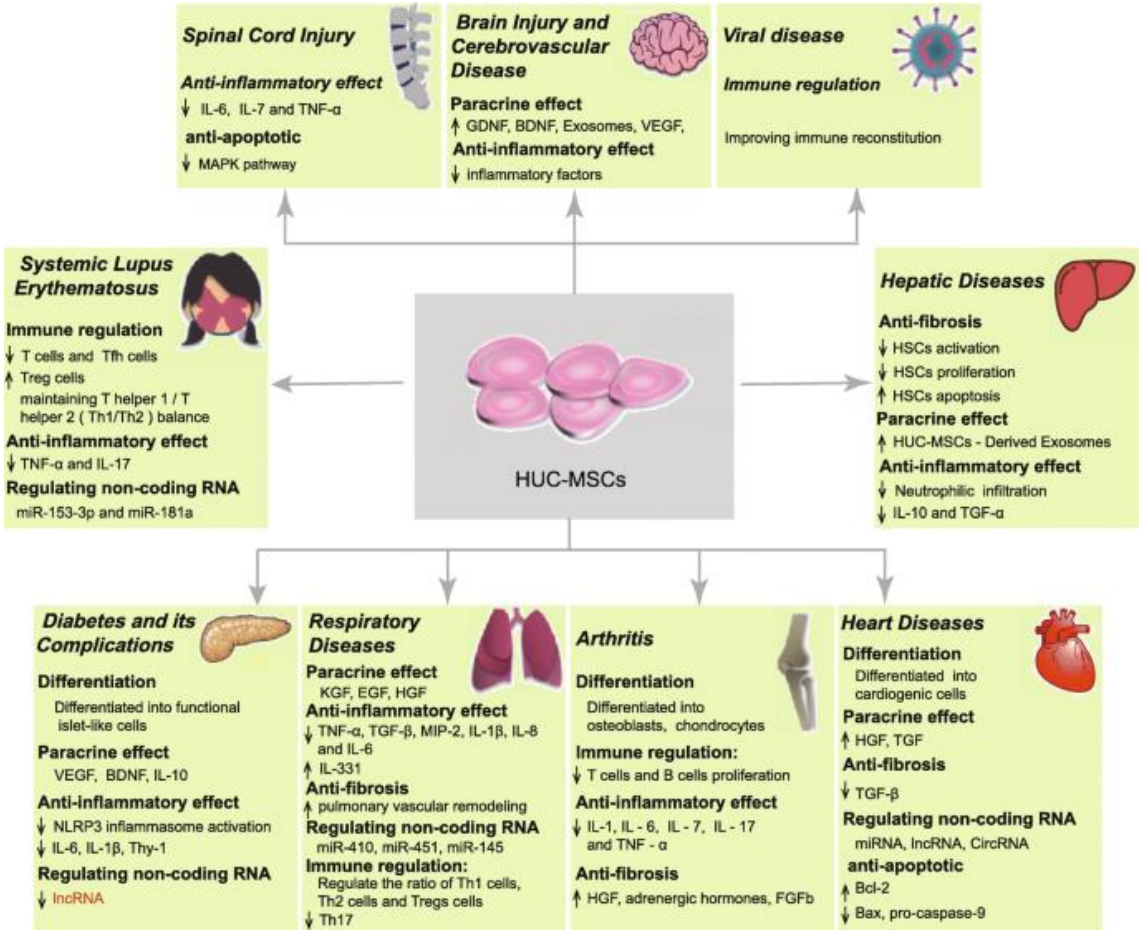


Figure 3: Human UCMSCs clinical applications and mechanisms of action. This figure illustrates how human UCMSCs are used to treat a variety of diseases, including those that affect the liver, the brain, the spine, the heart, and the respiratory system as well as viral infections, systemic lupus erythematosus, arthritis, and diabetes and its complications. Figure from (Xie et al., 2020).

1.4. Effects of Human Mesenchymal Stem Cells on Melanoma

Recent progress in regenerative medicine highlights the intense research in the field of stem cell differentiation. The identification and characterization of adult stem cells involved in tissue homeostasis offer new perspectives for cellular therapies that circumvent biological and ethical problems concerning the use of embryonic stem cells (Fritz and Jorgensen, 2008). Nowadays, MSCs are recognized as promising tools for gene therapy to treat different diseases, including cancer (Hmadcha et al., 2020). This is attributed to the mechanism of action of MSCs, which includes their ability to migrate to the injury site (Caplan et al., 2019; Nitzsche et al., 2017; Ullah et al., 2019), the paracrine effect of their secretome (Fiore et al., 2018; Li et al., 2018), and their immunomodulatory actions (Weiss and Dahlke, 2019; Zheng et al., 2018).

MSCs therapy on cancer is a controversial subject. Several studies have unveiled the crosstalk between MSCs and tumor cells resulting in tumor progression and metastasis through modulation of signal transduction pathways (Wang et al., 2015; Zhong et al., 2017), while other studies suggest that MSCs affect the pathways that can suppress both proliferation and apoptosis (Lin et al., 2016; Yulyana et al., 2015), raising safety concerns for clinical application of MSCs in oncology (Barkholt et al., 2013).

According to the literature, MSCs are attracted to tumor sites where they stimulate angiogenesis and tumor growth by differentiating into myofibroblasts associated with cancer and secreting angiogenic cytokines (Tsai et al., 2011; Walter et al., 2009; Zhang et al., 2013). Meanwhile, the recruited MSCs also enhanced tumor metastasis via increasing lysyl oxidase (El-Haibi et al., 2012). Additionally, it has been shown that MSCs create a cancer stem cell niche where tumor cells can maintain their capacity to proliferate and sustain the malignant process (Ramasamy et al., 2007). On the other hand, it has been shown that unmodified MSCs, through the paracrine factors they secrete, have antitumor effects both *in vitro* and in various *in vivo* animal models of cancer (glioma, melanoma, hepatoma, prostatic and breast cancer cells) (Nakamura et al., 2004; Qiao et al., 2008; Sousa et al., 2023). Additionally, it has been demonstrated that MSCs derived from hUCMSCs slow the spread of breast cancer by inducing tumor cells death and suppressing angiogenesis (Leng et al., 2014). Moreover, studies conducted in both *in vitro* and *in vivo* environments have demonstrated that MSCs derived from fetal skin can inhibit the development of human hepatocellular carcinoma (HCC) cells and can reduce cell division, colony formation, and oncogene expression (Qiao et al., 2008).

The discrepancies in the ability of MSCs to promote or suppress tumor development may be attributable to differences in experimental tumor models, MSCs tissue source, dose or timing of the MSCs treatment, cell delivery method, control group chosen, and other experimental conditions (Bajetto et al., 2017; Bortolotti et al., 2015).

Regarding the use of MSCs in melanoma, a study demonstrated that MSCs stably transduced with a retroviral vector expressing the cytokine IL-12 strongly reduced the formation of lung metastases of B16F10 melanoma cells (Elzaouk et al., 2006). The activity of the MSCs (IL-12) cells was dependent on the presence of natural killer (NK) cells in this experimental setting. Petrov *et al.* also showed that a single intravenous injection of xenogeneic BM-MSCs significantly increased the survival rate of tumor-bearing animals (Petrov et al., 2020). This

phenomenon can be related to *in vivo* participation of BM-MSCs in reprogramming of resident tissue macrophages, including within the tumor microenvironment.

Other studies demonstrated the effectiveness of MSCs in reducing the growth of melanoma cells, significantly increasing the survival rate in a mouse melanoma model. It was shown that systemic administration of MSCs producing both interferon (IFN) α and IFN-β reduced the growth of melanoma cells and significantly prolonged survival (Ren et al., 2008; Seo et al., 2011). Additionally, MSCs expressing fusion yeast cytosine deaminase:uracil phosphoribosyltransferase in combination with 5-fluorocytosine mediated a long-term tumor-free survival in tumor-bearing animals, evidencing that MSCs can be efficiently used in targeted immunotherapeutic approaches towards melanoma (Kucerova et al., 2014).

2. Objectives

This master dissertation intends to elucidate the yet much controversial role of MSCs in cancer therapy. The MSCs herein used, were isolated from the umbilical vein of healthy neonates using direct plastic adherence method. These cells, being isolated from a neo-natal tissue, besides dodging the epigenetic alterations inherent to adult cells, also present an easier, inexpensive, less invasive, noncontroversial source of MSCs (Weiss and Troyer, 2006).

Although MSCs therapy has contradictory effects on cancer cells, it has recently become evident that MSCs secretome have antitumor effects both *in vitro* and in various *in vivo* cancer models, with promising therapeutic effects. Notably, the MSCs secretome is beginning to be considered an active pharmaceutical component, in which its vesicular portion has revealed promising characteristics for use as a drug delivery system, mainly due to its homing capacity, thus opening an opportune window for compounds specific and targeted (drugs, proteins, etc.) released into damaged lesions (Bari et al., 2018).

In this context, this work aimed to unveil the role of the secretome derived from hUCMSCs in melanoma cancer cells, as a potential therapeutic candidate. To achieve this, hUCMSCs were cultured in standard culture conditions, from which the MSCs secretome was collected. Subsequently, the secretome effect on melanoma cells dynamics was evaluated, namely cell viability, cell proliferation, cell cycle as well as the adhesion and motility capacity of melanoma cells.

3. Methods and Materials

3.1. Cell Culture

The B16F10 cell line was purchased from American Type Culture Collection (ATCC CRL-6475). The B16F10 cell line was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Corning®) supplemented with 10% fetal bovine serum (FBS, Corning®), 2 mM L-glutamine (Corning®), and 1% penicillin/streptomycin (Corning®) in a 5% CO_2 incubator at 37 °C (MCO-170AC incubator; PHC Corpotation, Japan) (standard culture conditions).

Subculture was routinely performed when cells reached approximately 75–80% confluence and media change was conducted every 2-3 days. Unless stated otherwise, all treatments were conducted in serum-free conditions using DMEM supplemented only with 1% penicillin/streptomycin as a control.

3.2. Culture of hUCMSC and Collection of Conditioned Media

hUCMSCs were isolated from the UCT of seven healthy neonates (n=7) using the direct plastic adherence method. Briefly, UC samples were washed with PBS (Phosphate-buffered saline) to remove residual cord blood. Then, blood vessels and clots were scraped and washed away, and UCT was mechanically and enzymatic digested. After obtaining the hUCMSCs, cells were plated and incubated in a 5% CO_2 atmosphere at 37°C in MesenCult™ Basal Medium (STEMCELL Technologies, France) supplemented with 10% (v/v) MesenCult™ Stimulatory Supplement (STEMCELL Technologies, France) and 1% antibiotic-antifungal (v/v, Sigma-Aldrich, Germany). UCMSCs was propagated to the 3rd-7th passages and characterized using accepted MSC-positive markers anti-CD73, CD90, and CD105 (BD Pharmingen, BD Biosciences, CA, USA), negative hematopoietic markers anti-CD45, CD34, CD11b, CD19, and HLA-DR (BD Stemflow PE hMSCs Negative Cocktail, BD Biosciences, CA, USA) and cellular viability (BD 7-AAD, BD Biosciences, CA, USA) by flow cytometry (BD FACSCalibur™ Flow Cytometer; BD Biosciences, CA, USA).

Afterwards, hUCMSCs were propagated and allowed to reach confluence. Then, cultures were washed with HBSS (Hanks' Balanced Salt solution) and incubated in serum-free DMEM. After 24h, CM (conditioned media) was collected from the hUCMSCS cultures, spin for 5 minutes at 300g and the supernatant was stored at -20ºC for the subsequent treatments.

Treatments were performed for 24 hours, using a final concentration of 100 % of hUCMSC-CM.

3.3. Cell Viability assay

3.3.1. MTT assay

Effects of CM on cellular viability of B16F10 cells were determined using the MTT assay. MTT assay is based on the conversion of MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Alfa Aesar™) to insoluble formazan crystals by mitochondrial NAD(P)H dependent oxidoreductase enzymes released in living cells. First, B16F10 cells were seeded into 96-well plates in 100 µL of complete DMEM at a concentration of 2.5×10^4 cells/well and then incubated for 24 h. The next day, the culture medium was removed, the cells were rinsed with PBS (PanReac AppliChem), and then treated with CM. After being treated with CM and incubated for 24 h, 10 µL/well MTT (5 mg/mL) was added to each well. The medium was then removed after 2 h incubation and 150 µL/well DMSO (Dimethyl sulfoxide; Honeywell®) was added to dissolve the reduced formazan product. Finally, the absorbance was determined spectrophotometrically at a dual wavelength of 570/690 nm using a Multiskan SkyHigh microplate reader (Thermo Scientific™)

3.3.2. Aminoactinomycin D staining assay

7-AAD (7-aminoactinomycin D) is a vital dye. The reactivity of 7-AAD is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable and the ones with permeable membranes that take up the dye are dead. When 7-AAD-bound cells pass through the laser beam of the cytometer, they emit red fluorescence. This fluorescence is detected, and the data are collected and analyzed to determine the cell viability and DNA content of each cell.

Briefly, 5×10^4 cells/well were seeded in a 1 mL medium in a 12-well plate and allowed to adhere for 24 h. Afterwards, cells were treated with an 800 µL CM for 24 h. At the end of incubation period, cells were harvested with 0.25% Trypsin-EDTA and washed in PBS. Cells were resuspended in PBS and stained with 5 µL of BD 7-AAD (BD Biosciences, CA, USA). Stained cells were analyzed using a flow cytometer (NovoCyte® 3000 Flow Cytometer; Acea Biosciences) and FlowJo version 7.6.1 (Ashland, Oregon, USA) software was used to analyze the flow citometry data. Acquisition rate was kept below 10.000 events/sec for all analysis. 7-AAD-staining was assessed in the BL4 channel, which is equipped with a band pass filter 675/30 nm, by employing blue laser excitation at 488 nm. Cells were gated based on their forward scatter and sideward scatter and 7-AAD negative cells were considered viable.

3.4. Cell Proliferation assay

To determine how CM affects the proliferation of the B16F10 cells, Ki-67 expression was evaluated. Antigen Ki-67 is a nuclear protein that is expressed in actively dividing cells during all phases of the cell cycle, except for the resting phase (G0) (Gerdes et al., 1984). Because the Ki-67 antigen is present in all proliferating cells (normal and tumor cells), it soon became evident that the presence of this structure is an excellent operational marker to determine the growth fraction of a given cell population (Scholzen and Gerdes, 2000). For this reason, antibodies against the Ki-67 protein were increasingly used as diagnostic tools in different types of neoplasms. The percentage of cells staining positive for the Ki-67 antigen is called the Ki-67 index and it is directly related to cell proliferation. The Ki-67 index provides information about the growth characteristics of a tumor and the effects of certain drugs on it (Adan et al., 2016).

Briefly, cells (5×10^4 cells/well) were seeded in a 1 mL medium in 12-well plates. After 24 h incubation, the culture medium was replaced with an 800 µL CM, and cells were incubated for an additional 24 h. At the end of treatment, the Ki-67 proliferation assay was conducted by harvesting and washing the cells with PBS. Afterward, cells were fixed/permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, USA) and stained with an antibody solution containing 5 µL anti-Ki67-fluorescein isothiocyanate (FITC) kit (Invitrogen, USA), incubated for 30 min, and subjected to flow cytometry analysis (NovoCyte® 3000 Flow Cytometer; Acea Biosciences). Ki-67 positive cells were assessed in the BL1 channel, which is equipped with a band pass filter 530/30 nm, by employing blue laser excitation at 488 nm. FlowJo version 7.6.1 (Ashland, Oregon, USA) software was used to analyze the flow citometry data.

3.5. Cell Cycle assay

Flow cytometry was employed to analyze the cell cycle by quantifying cellular DNA content using the DNA intercalating propidium iodide (PI) dye. This method facilitates the segregation of cells into three distinct populations corresponding to the different phases of the cell cycle: G0/G1 phase, S phase, and G2/M phase.

Briefly, B16F10 cells were seeded in 12-well plates containing complete medium at a concentration of 5×10^4 cells/well. After overnight incubation, cells were treated with 800 µL of CM for 24 hours. Cells were harvested with 0.25% Trypsin-EDTA and fixed/permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, USA).

Afterwards, cells were stained with 20 µg/mL PI (Abcam) and 1% RNase A (QIAGEN, Valencia, CA, USA) for 30 minutes. Stained cells were suspended in PBS and analyzed using a flow cytometer (NovoCyte® 3000 Flow Cytometer; Acea Biosciences). 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. FlowJo version 7.6.1 software was used for citometry data analysis.

3.6. Adhesion assay

The adhesion capacity of B16F10 was determined by crystal violet (Sigma-Aldrich) staining of adherent cells. Briefly, the B16F10 cells (5×10^4 cells/well) were seeded in 500 µL of CM, in 24-well plates and allowed to adhere for 40 min in a 5% CO_2 incubator at 37 °C. After removal of CM, cells were extensively washed 3 times in PBS to remove any non-adherent cells, then fixed in absolute methanol for 30 min at 4°C and stained with 10% crystal violet (200 µL/well) for 20 min at room temperature (RT). After 8 washing cycles in deionized H_2O to remove crystal violet excess, the dye bound to adherent cells was solubilized using a 10% (v/v) acetic acid solution (200 µL/well; Sigma-Aldrich) on an orbital shaker at 150 rpm for 30 min at RT. The absorbance of the eluate was measured at 590 nm using a microplate read (Multiskan SkyHigh microplate reader; Thermo Scientific™). The background absorbance was measured at 690 nm.

3.7. Scratch Wound Healing assay

B16F10 cells (6×10^4 cells/well) were seeded into 24-well plates and allowed to adhere overnight. After reaching 100% confluence, a 'reference line' was scratched at the bottom of the plate using a sterile 200 µL pipette tip. Subsequently, cells were washed three times with PBS to remove the cell debris and were further incubated with 400 µL/well in hUCMSCS-CM to observe the migration of melanoma cells into the cell-free area. Two photomicrographs of each scratch were obtained at the initial time of wound creation and the same location was photographed at 3, 6, 12, and 24 h with an inverted microscope (Evos XL Core; Invitrogen). CellProfiler (www.cellprofiler.org,(Carpenter et al., 2006)) was used to quantify the area of the remaining wound.

3.8. Statistical analysis

Each experiment was performed with 3 or more replicates for each CM, and all values were expressed as the mean ± SD (standard deviation). All statistical calculations and generation of

graphs were performed using GraphPad Prism® version 9.4.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). For comparison between two groups Student t-test was used. Two-way analysis of variance (ANOVA) test with repeated measures, followed by multiple comparisons with Bonferroni's *post-hoc* test was used to determine statistical significance of cell cycle and scratch wound healing assays. Normality distribution of data and the homogeneity of variance were assessed by the Shapiro–Wilk and Levene test, respectively. p-value < 0.05 was considered statistically significant.

4. Results

It has recently become evident that MSCs have potential antitumor effects unveiling a novel and promising therapeutic tool in cancer. Notably, the MSCs factor-rich secretome is now regarded as an active pharmaceutical component, with promising characteristics and numerous bioactive molecules, factors and vesicles that can play a role in many biological processes and have beneficial therapeutic effects (Bari et al., 2018; Crivelli et al., 2017). Accordingly, in this work, CM from expanded hUCMSCs, isolated from the UCT of seven healthy neonates (n=7), was used to explore the potential effects of hUCMSCs' secretome on the viability, proliferation, cell cycle, motility, and adhesion of malignant melanoma B16F10 cells.

4.1. Effect of CM on Cell Viability

In order to evaluate the efficacy of potential new cancer treatments or to investigate the behavior of cancer cells under diverse conditions, cell viability assays for cancer cells are crucial in both cancer research and drug development (Adan et al., 2016). Viability assays help determine whether cancer cells are alive, dying, or dead and provide valuable information about treatment responses and overall cytotoxicity. Therefore, this work first aimed to evaluate the effect of hUCMSC-CM on B16F10 cells viability. For this, cell viability was evaluated according to the metabolic activity of the cells (MTT) and according to the integrity of the cell membrane (7-AAD).

4.1.1. MTT assay

The MTT assay is based on the principle that mitochondrial dehydrogenase enzymes, in viable cells, cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal

which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells (Kamiloglu et al., 2020).

Then, B16F10 malignant melanoma cells were treated with CM at a concentration of 100% for 24 h. As is shown in Figure 4, a significant decrease in the number of viable cells was observed as compared to controls (32%; $p < 0.0001$ *vs* control, n=7). As a result, it was shown that CM has a cytotoxic effect on treated cells as opposed to the control.

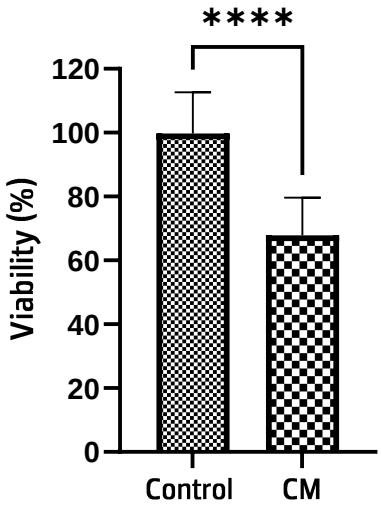


Figure 4: Evaluation of the cytotoxic effect of the hUCMSCs conditioned medium (CM) on the viability of B16F10 cells through MTT assay. In comparison to untreated cells, CM-treated melanoma cells showed a statistically significant decrease in viability after 24 hours (32%; ****$p < 0.0001$ *vs* Control). Bars represent the means ± SD from three independent experiments, normalized as percentage of Control (untreated B16F10).

4.1.2. Aminoactinomycin D staining assay

To assess the live and dead fractions of cells after treatment with hUCMSC-CM, B16F10 cells were treated for 24 h, then stained with the fluorescent DNA-binding agent 7-AAD, which is not cell membrane permeable in intact cells but permeable in damaged cells. After B16F10 cells exposure to hUCMSC-released factors, approximately 11% more cells had lost membrane integrity and were in a necrotic or apoptotic stage (stained with 7-AAD) compared to the control (10.84%; $p < 0.05$ *vs* control, n=7; Figure 5A/B), indicating that over 89% of cells remained healthy.

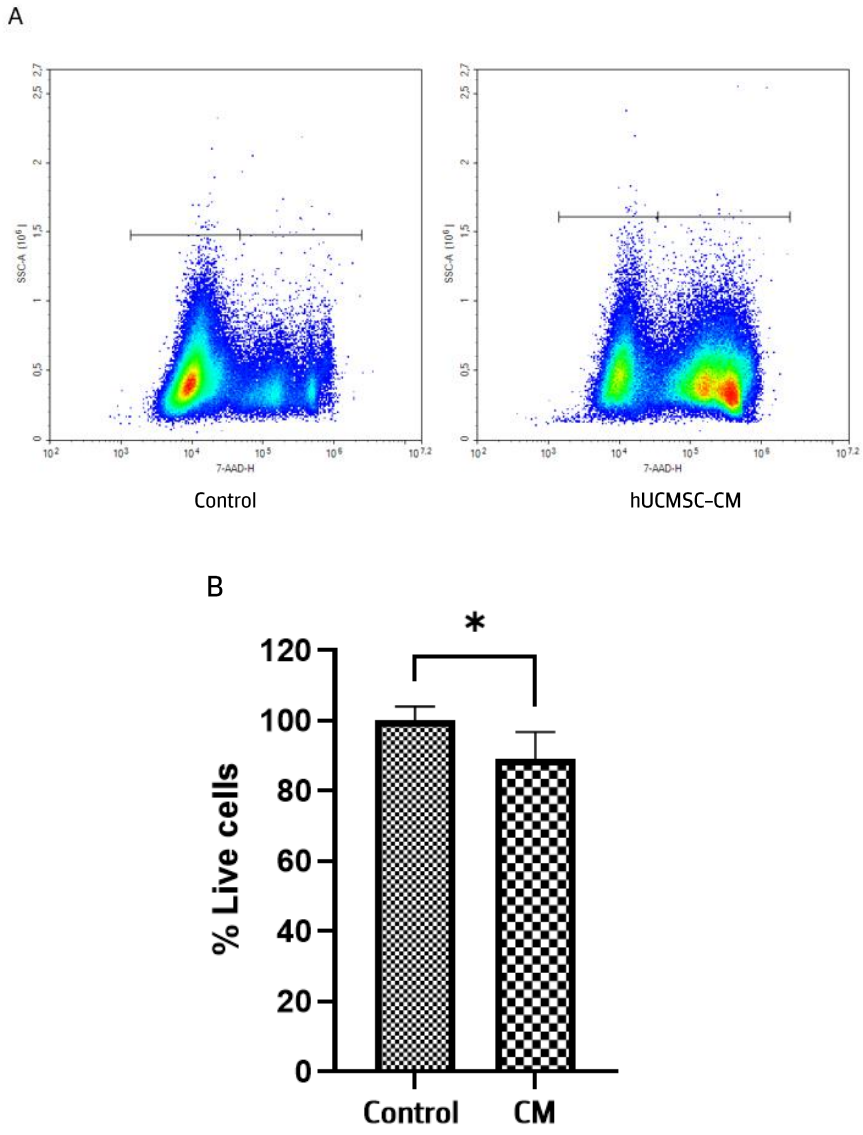


Figure 5: Cell viability according to 7-aminoactinomycin D (7-AAD) staining, a fluorescent DNA-binding agent. A – Representative cytometry plots of viable cells (7-AAD negative) and dead cells (7-AAD positive). B – hUCMSCs conditioned medium (CM) promoted a statistically significant decrease in the percentage of live B16F10 cells compared to Control (10.84%; *p <0.05 *vs* Control, n=7). The results present the means ± SD from three independent experiments, normalized as percentage of Control (untreated B16F10).

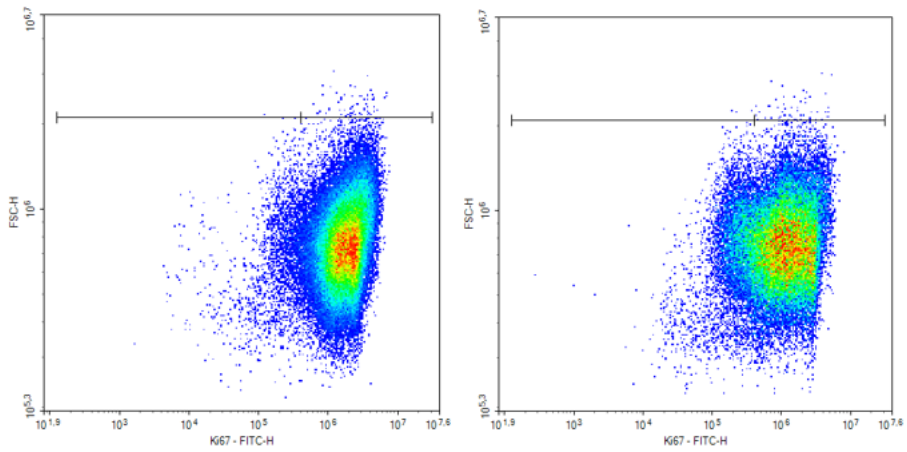
4.2.Effect of CM on B16F10 Proliferation

The development and continued growth of cancers involve significantly increased rates of cell proliferation. Proliferation rates can provide useful information on prognosis and aggressiveness of individual cancers and can be used to guide treatment protocols in clinical practice (Beresford et al., 2006). Information on proliferation rates during or after systemic therapy may be utilized as predictors of response and allow further tailoring of therapy for the

development of therapeutic agents, some of which may be targeted directly at specific points in the cell division pathway.

Therefore, to determine how hUCMSC-CM affects the proliferation of the B16F10 cells, expression analysis of the proliferation marker Ki-67 was investigated. For this purpose, the B16F10 cell line was treated with CM for 24 h. At the end of treatment, the Ki-67 proliferation assay was conducted by harvesting, staining, and subjected the cells to flow cytometric analysis. After 24 h of B16F10 treatment with hUCMSCS-CM, the percentage of Ki-67-positive B16F10 cells decreased to 85.95% (p <0.01) when compared to control (100%) (Figure 6). This result indicates that the factors present in the CM have an overall inhibitory effect on the proliferation of B16F10 cells.

A



Control hUCMSC-CM

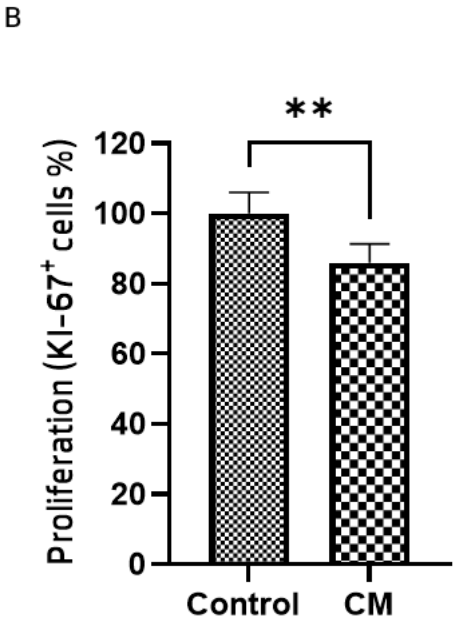


Figure 6: Effect of conditioned medium (CM) on the proliferation of B16F10 cells. A – Representative images of the quantification by flow cytometry of Ki-67 expression in B16F10 cells. B – Ki-67 expression significantly decreased in CM treated cells compared to untreated cells (14.5%; **p <0.1 *vs* control, n=7). The results present the means ± SD from three independent experiments, normalized as percentage of Control (untreated B16F10).

4.3.Effect of CM on Cell Cycle

Cancer is frequently considered to be a disease of the cell cycle (Park and Lee, 2003). As such, it is not surprising that the deregulation of the cell cycle is one of the most frequent alterations during tumor development. Cell cycle progression is a highly ordered and tightly regulated process that involves multiple checkpoints responsible for ensuring the order of events in the cell cycle, and that integrate DNA repair with cell cycle progression. Measuring the cell cycle of cancer cells is crucial for understanding their behavior, proliferation rate, and response to various treatments (Sun et al., 2021).

To investigate the effect of hUCMSC-CM on melanoma cell proliferation involved the regulation of the cell cycle, the cell cycle phase of B16F10 cells was examined by PI staining. B10F16 cells were treated with CM for 24 h before PI staining. The results revealed that hUCMSCs paracrinally influenced the cell cycle phase of melanoma cells. B16F10 melanoma cells cultured with hUCMSC-CM showed an increase in the G0/G1 phase of the cell cycle compared to the controls, which suggests that more melanoma cells are arrested at the G0/G1 phase in the presence of CM. The proportion of cells in the G0/G1 phase was 62.47% and 56.72% for B16F10 cells treated with and without hUCMSC-CM, respectively (p <0.01) (Figure 6A). This increase

was coupled with a decreased percentage of tumor cells in the S phase. The percentage of B16F10 cells cultured with hUCMSC-CM in the S phase was 27.05%, whereas it was 31.87%, for B16F10 cells cultured in control medium (p <0.05).

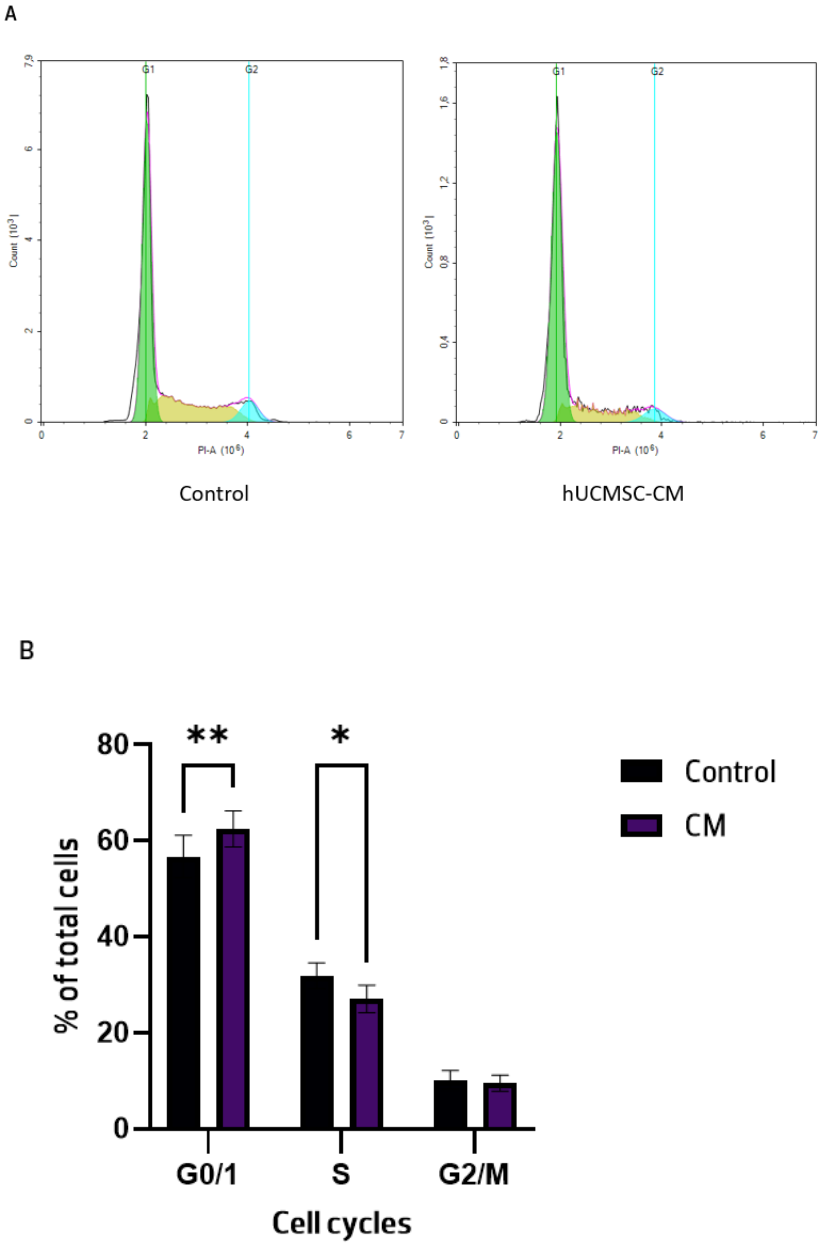


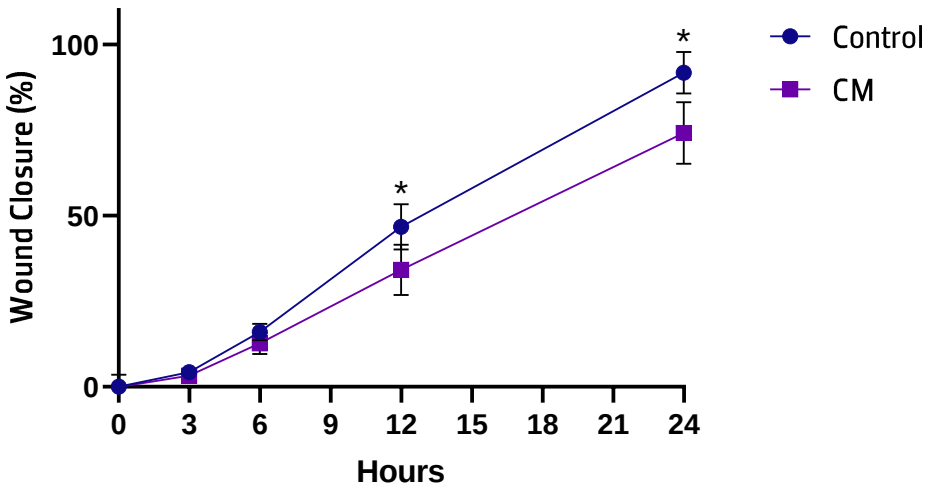
Figure 7: Cell-cycle arrest of B16F10 cells treated with hUCMSC conditioned media (CM). The cell-cycle phase distribution of B16F10 was analyzed after harvesting by flow cytometry. A – Representative cytometry plots of events by DNA content show cell cycle distribution of B16F10 cells. B – Bar graph of % of cell cycle phase distribution indicated that treated-B16F10 with CM showed an increase in the G0/G1 phase of the cell cycle compared to the controls (5.75%; **p <0.01 *vs* Control, n=7). G1 arrest occurred concomitantly with a reduction in the percentage of S phase cells (*p <0.05). All experiments were independently conducted in triplicate and values were expressed as the mean±SD. The *p-value* was obtained using two-way ANOVA with post-hoc Bonferroni's multiple comparison (*p <0.05, **p <0.01).

4.4. Effect of CM on Cell Migration

Cell migration plays a crucial role in both physiological and pathologic processes, such as morphogenesis, immune cell trafficking, inflammation, and cancer metastasis (Justus et al., 2014; Pijuan et al., 2019). Along cancer progression, metastasis is the final cause of death in 90% of patients, and there are significant gaps in our understanding of the metastatic genesis (Chaffer and Weinberg, 2011). The wound healing assay is a simple, inexpensive, and highly reproducible approach for studying cell migration *in vitro* (Rodriguez et al., 2005). Wound healing assays can replicate some aspects of cell migration that occur *in vivo*. The foundation of this assay is the fact that the creation of an artificial wound in cells growing in a monolayer initiate cell migration.

To further evaluate the safety of the possible use of hUCMSC-CM in cancer therapy, the next step of this study was to evaluate to what extent the latter would affect the migratory capacity of the melanoma cancer cell line B16F10 *in vitro*. Thus, melanoma monolayer cultures were subjected to a mechanical injury and treated with CM. The closure of the wound site was then monitored for 24 h (Figure 8A). After 24 h, a statistically significant reduction in cellular migration was observed for cells exposed to the CM when compared to the control (18%; $p<0.05$ vs control, n=7; Figure 8B). Three other time points were also analysed, at 3 h, 6 h and 12 h post injury (Figure 8B). 3 h and 6 h after performing the scratch, it was not possible to observe a significant difference in motility in the case of treated-B16F10 when compared to controls (untreated). On the other hand, after 12 h it was observed a significant reduction in cellular motility of treated cells compared to untreated cells (13%; $p < 0.05$ *vs* control, n=7). Overall, a decrease in wound closure was observed as early as 12 h after exposure to time points, being less pronounced between 0 and 6 h time points.

A



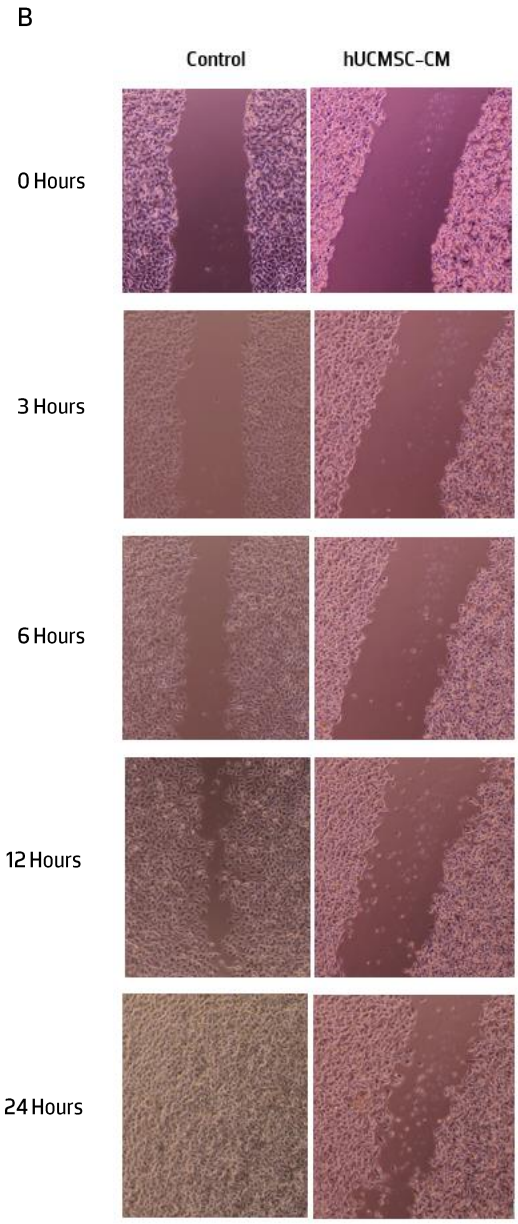


Figure 8: hUCMSCs-CM inhibited B16F10 cell line migration: B16F10 cell lines were cultured with CM, and the effect on cell migration was assessed by scratch assay. A - Results are presented as the mean value (±SD) percent of wound closure of three independent experiments for each time point/condition. *p-value* was obtained using two-way ANOVA with post-hoc Bonferroni's multiple comparison (p <0.05). B - Representative images of wound closure photographed immediately (0 h) and after 3 h, 6 h, 12 h and 24 h of cultured cells' area.

4.5.Effect of CM on Cell Adhesion

Adhesion is essential for cell communication and regulation, as well as tissue development and maintenance (Khalili and Ahmad, 2015). Cell adhesion has a role in stimulating signals that regulate cell differentiation, cell cycle, cell migration, and cell survival (Huang and Ingber, 1999). Changes in cell adhesion provide cancer cells the ability to defy the social order, which results in

the destruction of histological structure, the morphological hallmark of malignant tumors (Hirohashi and Kanai, 2003). Tumor cells are characterized by changes in adhesivity to ECM, which may be related to the invasive and metastatic potential.

The crystal violet assay was chosen to evaluate cell adhesion, as it indicates the relative density of cells adhered to the surface of the plate, which were evaluated after 40 min of incubation. After the incubation period, it was found that the number of adhered treated-B16F10 cells was increased by 15.4% about the no-treatment control (p <0.01 vs control, n=7) (Figure 9), indicating that the factor present in the CM contributed positively to cell adhesion.

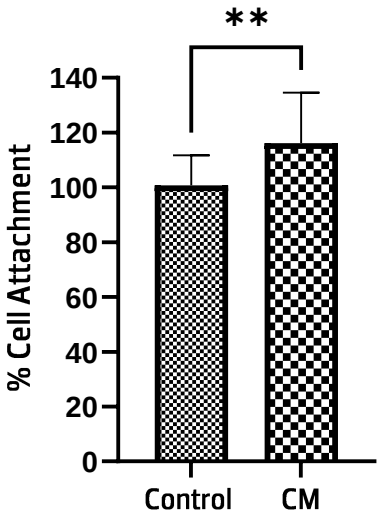


Figure 9: Effect of CM on the adhesion capacity of the B16F10 cell line. Percentage of adhered cells, after 40 min of treatment, increased significantly in relation to the Control (15.45%; **p <0.01 *vs* Control, n=7). The results present the means ± SD from three independent experiments, normalized as percentage of Control (untreated B16F10).

5. Discussion

MSCs are adult multipotent stem cells that have a high proliferative capacity and the ability to differentiate into a variety of cell lines (Chamberlain et al., 2007). These cells are easily isolated from many types of tissue, being considered excellent candidates for use in stem cell therapy due to their unique set of properties such as low immunogenicity, homing, suppress the inflammatory response and assist in wound sites and tumors (Ullah et al., 2019). However, despite major insights obtained in recent years, the exact mechanisms through which MSCs interact with tumor cells still remain to be clarified. The tumor-promoting or -suppressing effects of MSCs have been subject of controversy over the past decade (Galland and Stamenkovic, 2020; Klopp et al., 2011; Waterman et al., 2012). While some reports suggest that MSCs promote carcinogenesis (Galiè et al., 2008; Karnoub et al., 2007; Yan et al., 2012), other

research indicates that MSCs may inhibit tumor growth (Attar-Schneider et al., 2016; Cousin et al., 2009; Mirabdollahi et al., 2019). This duality might be explained by i) the use of various tumor models, ii) the functional heterogeneity of MSCs preparations, iii) various MSCs sources (such as BM, adipose tissue, peripheral blood and UC), iv) the dose or timing of MSCs injection, and v) the potential involvement of numerous mechanisms, including chemokine signalling, vascular support, and immune modulation (Klopp et al., 2011).

Nowadays, instead of cell-based therapies many studies propose the use of MSCs secretome, which includes the secreted paracrine factors, exosomes and/or microvesicles (Kusuma et al., 2017), to treat various diseases, including cancer (Grisendi et al., 2010; Kim et al., 2002). Herein, we evaluated the potential efficacy of the complete secretome derived from hUCMSCs in the progression of melanoma, at the cellular level, to determine whether the factors release by hUCMSCs have beneficial and anti-tumor effects and can therefore be used as an alternative therapy, or whether the hUCMSCs CM has tumor growth-promoting effects that exacerbate carcinogenesis.

To study the interaction between the secretome of hUCMSCs and melanoma, we used the B16F10 cell line – a murine metastatic melanoma cell line. The B16F10 melanoma cells were treated with a final concentration of 100% hUCMSC-CM in an effort to evaluate some major tumor cell hallmarks and characteristics, such as viability, proliferation, cell cycle, adhesion, and cell motility.

Initially, the effect of CM on the cell viability of the B16F10 cell line was assessed using the MTT and the 7-AAD staining assays. Although the values from the two viability assays differed, the findings of both experiments showed that, after 24 hours of treatment, the CM caused a significant decrease in the number of viable B16F10 cells, indicating that the hUCMSCs have a paracrine cytotoxic effect on melanoma viability.

In fact, MSC-CM have an intrinsic ability to attenuate the growth of several types of cancer (Hussein et al., 2019; Maj et al., 2017; Sousa et al., 2023). Ahn *et al.* demonstrated that MSC-CM inhibits A375SM and A375P human melanoma cell lines growth when treated with adipose tissue MSC-CM (ATMSC-CM), showing that ATMSC-CM has *in vitro* anti-proliferative effects against human melanoma cells (Ahn et al., 2015). Another study showed a reduction of A375 cell line treated with fetal dermal MSC-CM (FDMSC-CM) in a dose-dependent manner (Sun et al., 2019), demonstrating that FDMSC-CM is able to significantly decrease cell viability. However, Viera de Castro *et al.* showed that SNB-19 human glioblastoma cell line exposed to the

hUCMSC-CM exhibit increased viability *in vitro*, showing that CM may contribute to tumor growth/proliferation, influencing positively of in critical hallmark features of melanoma (Vieira de Castro et al., 2017).

In fact, the several bioactive factors secreted by MSCs are known to have a variety of biological effects and the molecular composition of the secretome is, in part, dependent on the MSCs source (Costa et al., 2021; González-González et al., 2020). Therefore, MSCs can differentially express and secrete regulatory factors, some of which are only released in specific micro-environments (Eleuteri and Fierabracci, 2019). Thus, we hypothesized that hUCMSC-CM appears to have a cytotoxic effect on the melanoma cell line, further supporting the current studies that show that MSC-CM inhibits the bioactivity of cancer cells and has antitumor effects *in vitro* (Ahn et al., 2015; Sun et al., 2019; Wan et al., 2023).

Previous research has reported that MSCs can be used to restrain tumor cells proliferation by two mechanisms (Zhang et al., 2017): 1) MSCs-derived exosomes and cytokines directly reprogram the signaling pathways and regulate the expression of growth factors; 2) genetically engineered MSCs can specifically migrate to various tumors and locally secrete therapeutic proteins, such as IFN-β and IFN-γ, IL-12 and 24, tumor necrosis factor-related apoptosis inducing ligand or suicide gene/enzyme prodrug. Herein, the effect of hUCMSC-CM on B16F10 cell proliferation was evaluated by quantifying the expression of the nuclear proliferation marker Ki-67. The results obtained indicated a decrease in the percentage of Ki-67-positive B16F10 cells after a 24 h exposure to hUCMSC-CM. Li *et al.* showed that MSCs inhibited the proliferation and induced the apoptosis of adenocarcinoma human alveolar basal epithelial A549 cells, probably through down-regulating the expression of proliferating cell nuclear antigen (PCNA) and inhibiting the formation of CDK2 complexes (Li et al., 2011). On the contrary, another study demonstrated an increase in the proliferation of endothelial cells, stimulating ovarian angiogenesis in Primary Ovarian Insufficiency (POI) (Al-Hendy, 2019). According to their results, human ovarian microvascular endothelial cells (HOVEC) treated with MSC-CM showed a greater population of Ki-67 positive cells, inducing an increase in HOVEC cell proliferation. Anh *et al.* (Ahn et al., 2015) found that proliferation of melanoma cells was inhibited after treatment with ATMSC-CM, which indicates that certain soluble factors secreted by ATMSCs may paracrinally inhibit melanoma cell proliferation without cell-to-cell contact, while Khakoo *et al.* reported that MSCs inhibit the proliferation rate of Kaposi's sarcoma by direct cell-to-cell

interactions (Khakoo et al., 2006). Our findings add up to the evidence supporting the paracrine inhibitory effects of MSCs on malignant melanoma proliferation.

Tumorigenesis is the result of cell cycle deregulation, which leads to uncontrolled cellular proliferation, a hallmark of cancer cells (Golias et al., 2004). Most, if not all, human cancers present altered cell cycle regulators, especially in the G1 phase and the G1/S phase transition progression, a period in which cell proliferation and differentiation are strictly regulated (Malumbres and Carnero, 2003). In our study, the effect of hUCMSC-CM on the cell cycle of B16F10 cells was evaluated by PI staining. After 24 h of treatment with MSCs secretome, a higher amount of B16F10 cells were found in the G0/G1 phase. Therefore, we can hypothesize that hUCMSC-CM represses cell growth via cell cycle arrest in the G0/G1 phase.

In accordance, *Anh et al.* observed an increase in melanoma cells cultured with ATMSC-CM in the G0/G1 phase of the cell cycle, which was associated with a decrease in the percentage of tumor cells in the S phase (Ahn et al., 2015). To confirm what could be causing the cells to arrest the cell cycle, they assessed cyclin D1 levels. The main families of regulatory proteins that play major roles in controlling cell-cycle progression are the CDKs, cyclins, CDK inhibitors (CKI), and the tumor-suppressor gene products p53 and pRb (Gali-Muhtasib and Bakkar, 2002). They showed that cyclin D1 levels decreased in A375SM and A375P cells treated with ATMSC-CM, which probably indicates that ATMSC-CM can negatively regulate the level of the cyclin D1 protein, leading to cell cycle arrest in melanoma cells. Zhang *et al.* also demonstrated that MSCs had an inhibitory effect on A375 cells by arresting the cell cycle, verifying that the number of cells in the G1 phase increased, while the number of cells in the S phase decreased (Zhang et al., 2017). They concluded that MSCs had an inhibitory effect on the proliferation of A375 cells through the secretion of cytokines without direct simulation of tumor cells and caused cycle arrest of A375 cells in the G1 phase through downregulation of NF-κB signaling.

Epithelial-mesenchymal transition (EMT) is a well-known phenomenon frequently connected to development and cancer progression (Shirkoohi, 2013). This process is only possible because the absence of cell-cell adhesion molecules facilitates the separation of tumor cells from the primary tumor (Jiang et al., 2015). MSCs have few contact sites on their surface that serve to mediate their interaction with neighboring cells, which lowers their ability for adhesion (Kokkinos et al., 2007). In contrast, epithelial cells exhibit numerous points of cell-cell contact that keep cells arranged in a layer. In this sense, we demonstrate that B16F10 cells, after treatment with hUCMSC-CM, have their adhesion capacity significantly increased. This fact is

not compatible with the EMT phenotype, in which cell adhesion molecules, such as cadherins, have their expression or function altered, promoting carcinoma progression and metastasis (Lamouille et al., 2013). Although our results are not compatible with the EMT phenotype, we cannot conclude whether CM really had an inhibitory effect on the promotion of melanoma, since to ensure that the increase in adhesion capacity after CM treatment is associated with an EMT-like process, several other parameters, in addition to adherence, needed to be evaluated. Furthermore, it is important to note that the effects of the MSCs secretome on cancer cells are context-dependent and may vary depending on the type of cancer, the specific MSCs population, and the microenvironment in which they interact.

Tumor cells reproduce normal cell migration, driving cancer progression and compromising anti-cancer therapeutic approaches due to tumor expansion and metastasis (Krakhmal et al., 2015; Limia et al., 2019). Therefore, we evaluated the effect of hUCMSC-CM on the migratory capacity of B16F10 cells through the scratch wound healing assay. Migration was assessed by measuring the area occupied by cells in the scratch after being treated with CM.

During the first 6 h evaluated, no significant change was observed in the migration of treated cells in relation to control (untreated). In the time range of 12 h to 24 h, migration of B16F10 melanoma cells was markedly reduced. Our results suggested that hUCMSC-CM significantly inhibited the migratory capacity of the B16F10 melanoma cells as evidenced by the reduction in the migratory capacity of cells present on the scratch compared to control. Although recent reports determine the exact mechanism by which MSCs inhibit the migration and invasion of cancer cells (Clarke et al., 2015; Dasari et al., 2010; Li et al., 2010), our findings are in agreement with other reports (Ahn et al., 2015; Sun et al., 2019) showing that MSCs inhibit the cellular migration of melanoma cells. TIMP-1, TIMP-2 and inhibitors of matrix metalloproteinases (MMPs) were identified as candidates for this inhibition (Clarke et al., 2015). Another study demonstrated that the inhibitory effect on tumor migration was mediated by up-regulation of phosphatase and tensin homolog (PTEN) in glioma cells by UCBMSCs (Dasari et al., 2010).

Overall, the results of the present study support that hUCMSC-CM inhibits the bioactivity of malignant melanocytes aggressiveness and has antitumor effects *in vitro.* Despite the increase in cell adhesion, the reduction in melanocyte survival, proliferation, and motility showed inhibitory effects that MSC-CM had directly on melanoma progression. Therefore, there is strong evidence in favor of the enormous potential of CM produced from hUCMSCs to be employed in acellular therapy for the treatment of melanoma and potentially other cancers.

6. Conclusion

At present, the primary approach for addressing localized melanoma involves the surgical extraction of both the tumor and adjacent healthy tissue (Lee et al., 2017). Nevertheless, when it comes to advanced melanoma, surgical interventions are not curative. (Jenkins and Fisher, 2021; Lee et al., 2017; Nguyễn et al., 2020). Consequently, given the rising incidence and mortality rates associated with melanoma, it becomes imperative to explore novel strategies aimed at enhancing the efficacy of melanoma treatments (Davis et al., 2019; Rebecca et al., 2012).

Extensive studies explore the use of MSCs as therapeutics agents; however, given the controversies attributed to MSCs-based cellular therapy, secretome-, EV (extracellular vesicles)- and exosome-based strategies are an appealing alternative to explore the therapeutic potential of MSCs, strategy that can benefit from the immunomodulatory, anti-inflammatory, and regenerative properties of MSCs without inducing side effects such as immunogenicity or tumoral transformation (Jammes et al., 2023).

Thus, this dissertation aimed to evaluate the effects of CM derived from hUCMSCs on melanoma tumor progression *in vitro*. To this end, a murine metastatic melanoma cell line (B16F10) was treated with CM and its effect on the dynamics of melanoma cells was then evaluated. In this study, it was demonstrated that hUCMSCs secrete paracrine factors that exert cytotoxic and anti-proliferative effects, leading to cell cycle arrest, as well as anti-migratory effects. Therefore, our data supports the potential of the MSCs secretome to be used in acellular therapy to combat melanoma.

However, additional investigations would be necessary to further characterize the effects of CM in other melanoma cellular parameters, such as, apoptosis (caspase activation or Annexin V staining); invasion potential (ECM transwells); the formation of new blood vessels (angiogenesis); anchorage-independent proliferation (colony formation assay); actin cytoskeleton staining to provide insights into alterations in cell spreading and cytoskeleton organization, which can be indicative of changes associated with cell migration and invasion; immunofluorescence analysis and western blot to detect and quantify the expression of proteins involved in signaling pathways relevant to cancer.

In fact, the molecular characterization of the specific set of factors and mechanisms involved in these therapeutic effects would provide valuable tools to better understand the underlying

biological processes and circuits that, ultimately could contribute to reinforce the translational potential of hUCMSCs-derived pharmacological agents for the treatment of melanoma.

Tumors develop in complex and dynamic microenvironments that influence their growth, invasion, and metastasis. Tumor microenvironment (TME) is a complex environment with a panoply of cell types: tumor cells, tumor stromal cells (stromal fibroblasts, endothelial cells and immune cells) and the non-cellular components of ECM (Jahanban-Esfahlan et al., 2018; Jahanban-Esfahlan et al., 2017). Therefore, understanding the interaction between cells in the tumor milieu can be used to develop therapeutic strategies to predict and neutralize the tactics used by cancer cells to survive and resist anticancer modalities. One of the key players in this complex environment are stromal cells who have often been considered as 'accessories in crime,' as they can actively support and subsidize tumor growth.

In the ever-evolving landscape of cancer research and therapeutic development, CM derived from various cellular sources, including MSCs, has emerged as a promising avenue. Future studies will unveil the profound impact of CM on the behavior of stromal cells within the intricate TME. The MSCs potential to reprogram stromal cells within the TME, shifting them from a pro-tumor phenotype to an anti-tumor phenotype through the action of secreted immunomodulatory factors (D'souza et al., 2015; Dalmizrak and Dalmizrak, 2022). Therefore, understanding the interaction between MSCs CM and cells in the tumor milieu can be used to develop therapeutic strategies to predict and neutralize the tactics used by cancer cells to survive and resist anticancer modalities.

Furthermore, the future will see ongoing exploration into other MSCs paracrine properties, the secretion of EVs, including exosomes containing microRNAs. The understanding of the content of these EVs and how they influence neighboring cells and actively participate in intricate cell-to-cell communication networks, transmitting messages that effectively reshape tumor and stromal cell behavior could have implications for the development of new therapies for cancer, including melanoma.

As the fields of stem cell and cancer research advance, the application of MSCs paracrine component, from diverse cellular sources, offers the potential to revolutionize cancer therapy. Such strategies are poised to provide more effective treatments, leveraging the dynamic interactions within the TME to combat cancer with greater precision and efficacy. In hindsight, the utilization of hUCMSC-CM unveiled its remarkable potential in exerting antitumor effects on

melanoma cells, hinting at a transformative stride forward in the battle against melanoma and other cancer types.

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Appendix

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Preclinical Studies

OXFORD

Abstract 27

Secretome of Umbilical Cord Mesenchymal Stem Cells: Potential Effects on Melanoma

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Introduction: Melanoma, a tumor resulting from the malignant transformation of melanocytes, is characterized by its aggressive nature and propensity to metastasize. Current treatment options for advanced melanoma are limited and mostly ineffective, highlighting the need for novel therapeutic approaches. Mesenchymal stem cells (MSCs) have increased considerable attention due to their anti-cancer and immunomodulatory properties. In particular, human umbilical cord MSCs (hUCMSCs) have shown promise in various therapeutic applications.

Objectives: The objective of this research was to assess and understand the potential therapeutic effects of conditioned media (CM) derived from hUCMSCs on melanoma cells in vitro.

Methods: hUCMSC, isolated from the umbilical cord of seven healthy neonates by enzymatic digestion followed by direct plastic adherence method, were cultivated and their CM stored for subsequent assays. Then, malignant melanoma B16F10 cells were treated using a final concentration of 100% of hUCMSC-CM for 24 hours. Afterward, their viability, adhesion, and motility were accessed by MTT, crystal violet, and scratch wound healing assays, respectively.

Results: The MTT assay showed the inhibition of B16F10 cell viability in all treated groups compared to the control group (32%; $p<0,05$ vs control, $n=7$). Moreover, crystal violet staining revealed that hUCMSC-CM enhanced the adhesion capacity of melanoma cells by 16% ($p<0,05$ vs control, $n=7$). In terms of motility, the hUCMSC-CM reduced migration by 24% ($p<0,05$ vs control, $n=7$) after 24 hours.

Discussion: Our results suggest that hUCMSCs may exhibit dual effects on melanoma cells. On one hand, certain factors produced by hUCMSCs have shown potential in reducing the viability and motility of B16F10 melanoma cells, indicating a potential therapeutic benefit. On the other hand, it has also been observed that CM of hUCMSCs can increase the adhesion capacity of melanoma cells.

While hUCMSC-released factors may exhibit beneficial effects on melanoma cells, further research and clinical trials are needed to establish their safety, efficacy, and optimal therapeutic protocols for malignant melanoma.

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Human Umbilical Cord Mesenchymal Stem Cells Secretome Affects Melanocytes Proliferation and Cell Cycle Progression

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Abstract:
Melanoma, a skin cancer originating from malignant transformation of melanocytes, is characterized by its aggressive nature and high tendency to metastasize, rendering melanoma management challenging. The few existing treatments for metastatic melanoma have limited effectiveness, underlying a demand for novel therapeutic strategies. In preclinical cancer models, human umbilical cord mesenchymal stem cells (HUC-MSCs) have shown promising anti-cancer effects, hampering both the proliferation and metastasis of malignant cells. In this line, we investigated the impact of conditioned media (CM) obtained from HUC-MSCs on viability, proliferation, and cell cycle of melanoma cells using *in vitro* cell culture models. HUC-MSCs, isolated from the umbilical cord of seven healthy neonates by enzymatic digestion followed by direct plastic adherence method, were cultivated and their CM stored for subsequent assays. Then, malignant melanoma B16F10 cells were treated with final concentration of 100% of CM or standard serum free media (control) for 24 hours. Cellular viability, proliferation and cell cycle progression were evaluated by flow cytometry analysis with 7-aminoactinomycin D (7-AAD), Ki-67 and propidium iodide (PI) stains, respectively. When treated with HUC-MSCs secretome, B16F10 cells decreased their viability by 18% when compared to the control group as shown by the 7-AAD exclusion stain ($p<0.05$, $n=7$). Moreover, the expression of Ki-67 proliferation marker was reduced by 14% when B16F10 cells were exposed to HUC-MSCs released factors ($p<0.05$, $n=7$). Concomitantly, PI cell cycle analysis revealed that HUC-MSCs CM significantly increased the number of cells in G0/G1 phase (10%) and decreased the S and G2/M populations (15%) ($p<0.05$, $n=7$). Our results showed that HUC-MSCs secretome reduced the viability and proliferation capacity of malignant melanoma cells with a concomitant arrest of cells in G0/G1 cell cycle stage. This study suggests HUC-MSCs paracrine signaling inducing melanoma cells into a quiescent state and growth inhibition with a concomitant decrease in overall cell viability and survival. Further research is required to characterize the molecular pathways of these promising effects of HUC-MSCs secretome in inhibiting melanoma cells proliferation and establish their safety and efficacy. Our findings support HUC-MSCs paracrine component therapies for melanoma.

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