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Investigation of the expression of pro-angiogenic markers in cutaneous tumours *in vivo* and *in vitro*

By

Masibulele Nonxuba

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South Africa

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Supervisor: Prof P. Mabeta

Co-supervisor: Prof N.M. Bida

UNIVERSITY OF PRETORIA

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Executive summary

Cutaneous tumours are common types of cancers that develop from the skin and account for 40% of the cases worldwide. In particular, melanomas and cutaneous haemangiomas are commonly occurring skin tumours that are characterised by aggressive growth. Angiogenesis, the development of cutaneous tumours such as haemangioma and melanoma, has been associated with angiogenesis the formation of new blood vessels from existing vasculature - promoting tumour proliferation, survival, and metastasis in the case of melanoma. As a result, there is a need to identify angiogenic markers as a tool that could systematically disrupt the vessel formation process.

This study aimed to investigate markers of angiogenesis in cutaneous tumour cells and biopsies. Endothelial cells cultured in different medium conditions (normal, melanoma-conditioned, and serum-free) were analysed for growth and cell morphology. Vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor and platelet-derived growth factor-BB (PDGF-BB) were quantified in an ELISA kit to determine their expression level as angiogenic biomolecular markers, employing melanoma B16-F10 cell lines. Formalin-fixed haemangioma biopsy tissues were investigated for the presence of EC using IHC and a light microscope. The expression of Bcl-2 and VEGF-R were also analysed following immunoreactivity against appropriate antibodies.

In this study, a time-dependent cell growth assay was employed, using crystal violet as the investigative tool. The results of this study demonstrated that the cells grown in a melanoma-conditioned environment induced a proliferative effect while those incubated with serum-free medium exhibited cell growth inhibition. When cells were treated with a dose-dependent Nocodazole, cell growth inhibition was also initiated. The cells showed a healthy spindle-like shape with well-defined nucleoli in both conditions. No morphological difference existed between the cell populations cultured in a different medium. High expression levels of VEGF-A and bFGF in melanoma B16-F10 cells were identified and significantly ($p < 0.05$) associated with the progression of the disease. Although no scientific significance was found for PDGF in

the cutaneous tumour cells, their expression was depicted. Dilated vessels with red blood cells were observed in tumour tissues of haemangioma. While the expression of Bcl-2 in cutaneous tumour tissues was barely expressed, the antibodies against VEGF-R strongly depicted the molecular expression.

In conclusion, the collectively identified molecular markers *in vivo* and *in vitro* serve as potential prognostic markers that could enable clinicians to diagnose the disease early and lower its burden through revolutionised anti-angiogenic therapy.

Keywords: Cutaneous tumour, melanoma, angiogenesis, haemangioma, and growth factors.

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
Akt	Protein kinase B
BCC	Basal cell carcinoma
Bcl-2	B-cell lymphoma-2
bFGF	Basic fibroblast growth factor
bFGFR	Basic fibroblast growth factor receptor
BRAF/RAF	v-Raf murine sarcoma viral oncogene homolog B1
cm	Centimetres
CO_2	Carbon dioxide
cSCC	Cutaneous squamous cell carcinoma
CSL	CBF1, Suppressor of Hairless, Lag-1
CTLA	Cytotoxic T-lymphocyte antigen
CV	Crystal violet
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
EC	Endothelial cell
ECM	Extracellular matrix

ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular-signal-regulated kinase
FCS	Foetal calf serum
FGF	Fibroblast Growth Factor
H&E	Haematoxylin and Eosin
HIF	Hypoxia-inducible factor
h	Hour(s)
IA	Intussusceptive angiogenesis
IHC	Immunohistochemistry
IL-8	Interleukin-8
MAPK	Mitogenic-activated protein kinase
MCM	Melanoma-conditioned medium
MEK	MAPK/ERK kinase
min	Minutes
ml	Millilitres
mm	Millimetres
MMP	Matrix metalloprotease
mTOR	Mechanistic target of rapamycin
NICD	Notch intracellular domain
nm	Nanometre
NRAS	Neuroblastoma threonine-protein kinase

PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK	Phosphoinositide-dependent kinase
pH	Potential of hydrogen
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PlasDIC	Polarization optical differential interference contrast
rpm	Revolutions per minute
RT	Room temperature
SA	Sprouting angiogenesis
SFM	Serum-free medium
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

SYMBOLS

%	Percentage
/	Per
<	Lesser-than
=	Equals to
±	Plus-minus
µl	Microlitre
µm	Micrometre
ºC	Degrees Celsius
x	Multiplication
µg	Microgram

CHAPTER ONE

LITERATURE REVIEW

1.1. Cutaneous tumours

Cutaneous tumours are the most common cancer types with high incidence rates globally (1). Their development results from genotypic, phenotypic, and environmental factors that pose challenging conditions ranging from benign lesions to premalignant lesions and aggressive tumours (2). Melanoma and non-melanoma skin cancer, which comprise basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC), are the most common types of skin cancer presented (1).

1.1.1. Basal cell carcinoma

Basal cell carcinoma is a keratin skin tumour type that often causes considerable morbidity due to tumour growth, local invasion, and destruction of surrounding normal tissues, even though it rarely metastasizes (3). While BCCs are considered disruptive when treated inadequately, they generally have a good outcome when diagnosed early (4). On clinical presentation, it is predominantly found on the head and trunk, exhibiting pinkish pearly papules with underlining ulceration. The onset of this pathophysiological disease is highly associated with chronic sun exposure as a critical risk factor (5).

Unlike the cSCC, as detailed below, BCC generally develops without a precursor lesion and due to their different histological presentation, it consists of three subtypes including nodular, micronodular, superficial and morpheaform BCC. While nodular and superficial BCCs are

considered the least aggressive subtypes, the aggressive micronodule and morpheaform BCC are associated with a high risk of recurrence (6, 7).

1.1.2. Squamous cell carcinoma

Squamous cell carcinoma is a non-melanoma cutaneous tumour that develops primarily on sun-exposed skin, including the face, scalp, and hands (8). However, a small proportion of cSCC develops on skin-covered areas, including the genital area, buttocks, and feet (9, 10). Empirical evidence suggests that the identified skin-covered areas in which cSCC develops are consistent with the role of non-actinic factors associated with the development of cSCC (8). The development of this keratinocyte carcinoma is a gradual process that either arises de novo or has evolved from a progressive actinic keratosis to an invasive cSCC (11).

Clinically, the progressive development of cSCC is initially presented with plaques, papules or nodules and ulcerative or hyperkeratotic lesions (12). Even though cutaneous cSCC is usually not fatal and is easy to treat in the early stages, it can be very complicated once it infiltrates nerves and deeper structures and can re-occur locally or metastasize, leading to significant morbidity and mortality (8).

1.1.3. Cutaneous haemangioendothelioma

Cutaneous haemangioendothelioma is an avascular tumour that presents a biological behaviour that is intermediate between haemangioma and conventional angiosarcoma (13). This soft tissue tumour type originates from the vascular endothelium with clinical features that include solitary erythematous mass, multiple dome-shaped masses, or dermal nodules over the extremities. Moreover, it may also present as a non-healing ulcer (14, 15). This low-graded malignancy is highly associated with benign tumours that rarely metastasize and locally aggressive tumours (16).

Among the subtypes of haemangioendothelioma, epithelioid haemangioendothelioma is a skin-limited and an infrequent cancer type, with low cases reported worldwide (15, 17). It is

often related to multi-systemic localizations. This neoplasm is often misdiagnosed, as it accounts for less than 1% of all vascular tumours, and the primary cutaneous lesions are challenging to diagnose because of the non-specific clinical presentation of a red nodule, which may or may not be tender (18).

1.1.4. Cutaneous haemangioma

Cutaneous haemangioma is a common benign vascular tumour that mainly develops in paediatrics, displaying aggressive complications (19). It is often presented with a typical evolution profile that consists of rapid endothelial proliferation during the first year of life and a slow involution that is usually completed by 5 to 10 years of age, developing within and under the skin (20). Clinically, cutaneous haemangioma causes the skin to bulge and become bright red or bluish if they are profound. Most deep haemangiomas grow between 0.5 and 5 cm across, although sometimes they grow much more significantly in the head and neck regions (21). Similarly, it is characterised by numerous abnormal blood vessel structures. However, an immediate and adequate treatment approach is required when a cutaneous haemangioma tumour is in areas at risk for functional complications that include a considerable size, consistent bleeding, ulceration, or superinfection (22).

1.1.5. Cutaneous melanoma

Cutaneous melanoma is an aggressive skin cancer type with high lethality (23). While there are several types of melanoma (mucosal and ocular melanoma), cutaneous is the most prevalent (24, 25). It arises from pigment-producing melanocytes owing to a genetic mutation and tumour microenvironment alterations that alter the cell cycle, causing uncontrollable cell division (26). The onset of melanoma development can be visually detected in all types of cutaneous presenting malignancies. Although this poses a unique prognostic factor for curative treatment, the surface-level appearance of melanoma is often presented in various types of lesions. Thus, it is challenging to identify whilst maintaining a reasonable, and valid negative rate accurately (27).

1.2. Epidemiology of cutaneous melanoma

While melanoma accounts for 5% of all known cutaneous tumours, it is responsible for 75% of skin-related cancer deaths (28). These incidences have been steadily increasing, with 232 100 cases reported in 2018 worldwide (29). In South Africa, melanoma-related incidences are estimated yearly at 4.76 per 100 000 persons (30). The tendency of melanoma to increase has been associated with different geographic areas, populations and gender, as illustrated in figure 1.1 (31).

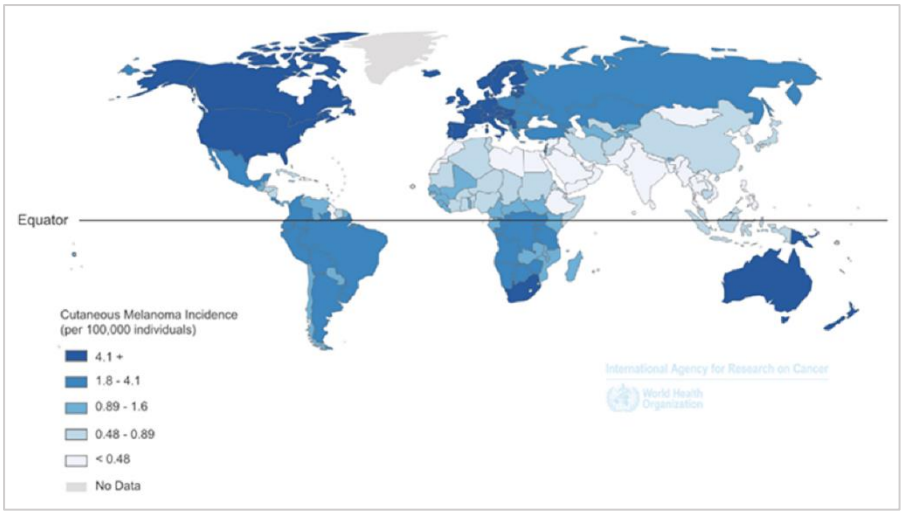

Figure 1.1: Schematic presentation of demographic regions of cutaneous melanoma incidence rate. *Reprint with permission from Horrell et al. (32).*

Non-melanoma skin cancer is a global health concern, with 20 times higher incidences when compared with those reported in melanoma, causing significant morbidity. Despite being a common disease, incident reports are often excluded. This is because non-melanoma skin cancer is often under-diagnosed or treated within primary care (33).

1.2.1. Aetiology and risk factors

Epidemiological studies have documented skin cancer as a multi-factorial disease arising from an interaction between environmental exposure and genetic susceptibility (34, 35). Its

association with squamous carcinogenesis is due to its ability to induce immune-related mechanisms and indirectly damage the deoxyribonucleic acid (DNA) by generating high reactive free radicals and oxidation injury. As a result, empirical evidence from studies investigating the pattern and timing of sun exposure has recognised ultraviolet (UV) radiation as the main environmental factor that risks the development of cutaneous melanoma and keratinocyte skin cancer (36, 37).

1.2.1.1. Environmental Factors

It is well established that long-term skin exposure to UV radiation increases the risks of skin cancer. According to Sample and He (38), about 60-70% of cutaneous melanoma-related incidences are caused by UV radiation exposure: UV-A (315-400 nm) and UV-B (280-315 nm), respectively. Through animal studies and clinical observations, UV-A is a crucial factor that plays a significant role in tumorigenesis by indirectly altering the DNA molecule by producing oxidative stress (39).

When the skin is exposed to UV-A, oxidative DNA-based modification occurs, following an indirect reaction through the excitation of a cellular photosensitizer and the generation of reactive oxygen species, mainly singlet oxygen (38). However, Ultraviolet-B causes direct DNA damage through which photoproducts such as cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone are induced. As a result, DNA mutation occurs, and the stability of the epidermal cells is compromised (36, 40).

1.2.1.2. Genetic Factors

The transformation of cutaneous cells is thought to occur by the sequential accumulation of genetic alterations that can activate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (Akt) signalling pathways (41). In 2005, Curtin *et al*. (42) reported high levels of v-Raf murine sarcoma viral oncogene homolog B1 *(BRAF)* mutation in melanoma patients who showed no evidence of chronic sun-induced damage, suggesting gene alteration as a potential factor in melanoma pathology. Also, a recent study

attested to that of Curtin *et al*. (42), advocating that a *BRAF* mutation — a gene related to the MAPK family — is expressed in 40-50% of cutaneous melanoma-related cases (41).

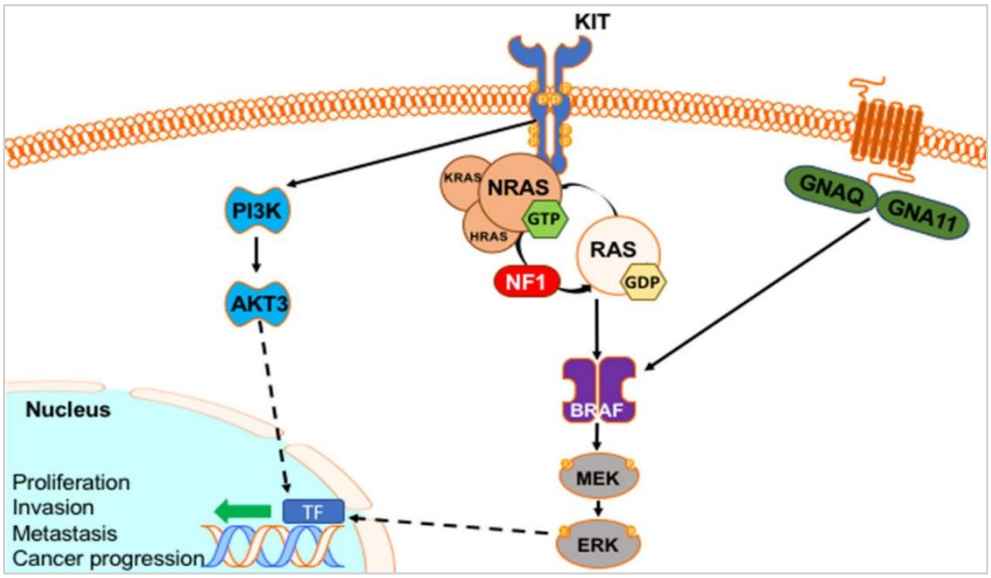

Figure 1.2: Schematic illustration of the steps involved in the process of carcinogenesis through NRAS gene mutation. Upon NRAS activation by tyrosine kinase receptor, *BRAF* is activated through a cascade of biochemical events, leading to the activation of a dual-specificity serine/threonine kinase MEK1 and MEK2. The phosphorylation of ERK1/2 by MEK1 results in cell proliferation and migration. In the event of PI3K/Akt signalling pathway, NRAS activates PI3K through a cascade of biochemical processes, causing the activation of Akt by binding to the pleckstrin homology domain of Akt. Following the conformational change of Akt, mTOR phosphorylates Akt leading to signalling that impacts various cell functions, including cell growth, cell cycle entry, proliferation and migration (43). *Reprinted with permission from Shaughnessy et al. (44)*

Cell growth and inhibition of pro-apoptotic signalling are achieved through the activation of MAPK/Akt either by mutated BRAF V600E or NRAS (Figure 1.2). Similarly, the activation of Akt stimulates angiogenesis by increasing hypoxia-inducible factor (HIF) translation and activating endothelial nitric oxide synthase, which increases nitric oxide production. However,

the pathological mechanisms underlining melanoma genetics are incompletely understood (45, 46).

1.3. Angiogenesis in physiology and pathology

De novo blood vessel formation primarily occurs during embryogenesis through a physiological process termed vasculogenesis. These primitive vessels are derived from an aggregate of precursor cells, angioblasts, located in the splanchnic mesoderm. The differentiation of angioblast cells proliferates and coalesces into the primary capillary plexus, which serves as a scaffold of angiogenesis (47).

Angiogenesis is a physiological process that is facilitated through sequential, multi-step biochemical processes involving an endothelial cell (EC) losing its state of quiescence and emerging from the already existing vasculature (48). Consecutively, the EC proliferates and migrates, resulting in vascular remodelling and tube formation. This highly regulated, complex mechanism is known for its crucial role as a prerequisite for embryonic development, the endometrium cycle and wound healing (49, 50).

In pathology, angiogenesis is a process of uncontrollable and disordered vessel formation fundamentally facilitated by the imbalance of pro-angiogenic and anti-angiogenic factors, as illustrated in figure 1.3 (51). This process plays a primary role in clinical cases such as cancer, atherosclerosis, and chronic inflammation. Empirical evidence from cancer studies demonstrated that the inability of the nearest blood capillary to supply oxygen and nutrients to the growing solid tumour cells and to facilitate the removal of waste materials beyond a diameter of 1 - 2 mm necessitates the development of angiogenesis. With a subsequent number of experiments confirming the phenomenon mentioned above, scholars also revealed that the initiation of angiogenesis in the absence of adequate blood supply prevents tumour cells from becoming necrotic and apoptotic. As a result, solid tumour cells continue to grow, initiating angiogenic switch and metastases (49).

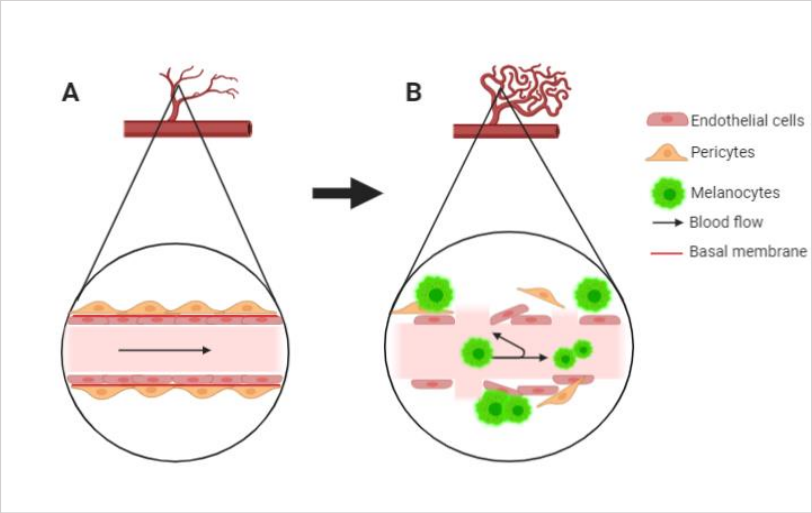

Figure 1.3: Schematic diagram depicting the morphology of angiogenic vessels in a physiological and pathological setting. a) In normal physiology, vessels are presented with endothelial cells, basal membrane and pericytes that are in place, allowing one-way directed blood flow. b) However, in the event of pathology, angiogenesis is characterised by aggressive growth, resulting in irregular patterns and tortuous vessels. In this regard, blood flow is compromised because of the detached pericytes and ECs (53). *Figure designed by Nonxuba M. using BioRender.*

In an event where tumour cells have grown beyond a few millimetres in diameter, their ability to grow and survive is threatened mainly by the inadequate supply of their metabolic needs. As a result, tumour cells exploit their microenvironment by releasing cytokines and growth factors in response to a hypoxic surrounding and inflammatory infiltrate (49). Consequently, quiescent cells are activated and initiated into a cascade of events that quickly becomes dysregulated. This means that even though the induction of angiogenesis may provide the essential needs required by the tumour at first, the ultimate response is poor. This is because of the faulty regulation involved in downstream signals and the lack of cross-regulatory control with the host tissue, causing tortuous vessels (52).

1.3.1. Angiogenic switch: The initiation of tumour angiogenesis

As previously mentioned, the progression of tumour cells is often accompanied by the ingrowth of blood vessels, ensuring that the malignant cells have complete access to the capillary network for rapid expansion. However, at an early stage of cancer progression, the lack of active blood vessel formation in small dormant tumours is frequently observed in the human tissue microenvironment and transgenic mouse cancer models. These observations suggested that tumour angiogenesis is linked to a switch in the equilibrium between positive and negative regulators. During standard physiological conditions, vascular quiescence is generally regulated by the dominant influence of anti-angiogenic inhibitors over angiogenic stimuli. However, in tumour angiogenesis, fast-growing vessels are influenced by the upregulation of pro-angiogenic factors and the downregulation of such inhibitors (53-55).

Apart from the increased expression of pro-angiogenic factors, the angiogenic switch may also be triggered either by the recruitment of immune cells, activation of endogenous stimuli that include the upregulation of hypoxia and inflammation or by additional genetic alterations of tumour cells. This, in turn, triggers a defined cascade that involves the production of proteolytic enzymes and the degradation of the perivascular extracellular matrix (ECM) and basement membrane through coordinated chemical signals (56). Subsequently, the formation of primary sprouts leads to blood vessels characterised by irregular patterns and a tortuous morphology.

The result of this occurrence causes abnormal vessel growth. In this case, pericytes – usually found around the intervals of healthy blood vessels, maintaining vascular stability – are often detached from the ECs of tumour vasculature. Also, tumour vessels are thin-walled and destabilised due to an adequate basement membrane and impaired endothelial junctions. Consequently, the impaired endothelial integrity causes an increase in vascular leakage, alternately altering the pressure of the interstitial fluid, causing it to increase and promote a slow, irregular blood flow. Due to the impaired blood vessels, a hypoxic environment is generated, upregulating angiogenic molecules to promote malignant growth (53). Also, other abnormalities that result in this pathophysiological tumour microenvironment include low

glucose concentrations and acidic extracellular pH (57, 58). However, the morphogenetic events that lead to angiogenesis remain poorly understood and are being debated (53).

1.3.2. Mechanisms of angiogenesis in cutaneous tumours

The increasing evidence suggesting that angiogenesis exists in two properties – sprouting and intussusceptive angiogenesis (IA) - that co-exist in pathophysiological settings has been braced by the literature search. During sprouting, the formation of new blood vessels is governed by the release of angiogenic factors in response to a hypoxic environment. In contrast, IA results from high-sheared stress (59).

In 1977, Ausprunk and Folkman observed and described for the first time the process whereby the newly formed vessels budged out from the pre-existing vessel and migrated parallel to the capillary bed, calling it sprouting angiogenesis (SA) (60). Years later, the work of Gerhardt *et al*. (61) refined our understanding of SA by identifying “tip cells” and “stalk cells” as two EC types essential for the process, even though filopodia were previously described by Kurz *et al*. (62) and Ruhrberg *et al*. (63) in 1996 and 2002, respectively. Gerhardt suggested that the cells at the sprout's tip exhibited a morphology characterised by numerous filopodial extensions resembling axonal growth cones (61). As a result, it was recently noted that only a fraction of ECs exhibited a tip cell behaviour that enabled them to initiate sprouting, whilst others served as a stalk, maintaining the structural and functional integrity of the vessel (64).

These findings supported the primary studies that described the functional purpose of tip cells using the mouse's retina, encouraging that these cells play a crucial role in guiding the route at which the vessels extend and look into the signalling environment (65). Research also found that the stalk cells that trailed from behind participated highly in vessel proliferation and were later identified as the nascent sprout’s building block (66).

Intussusceptive angiogenesis is another process in which angiogenesis exists and was formally recognised in the 1800s. In contrast to sprouting, the pre-existing blood vessel, as demonstrated in figure 1.4, splits into two new vessels through the formation of intraluminal

pillars. As such, the ECs would be remodelled by increasing in volume and becoming thinner. Based on this, IA is regarded as a dynamic, fast, and metabolically undemanding process because it does not undergo endothelial proliferation but progresses through intraluminal EC rearrangements (67).

The event of IA is believed to be stimulated by the enhanced blood flow dynamics in arterial branches. In this particularity, IA restores the increase in blood flow by splitting vessels into two lumens to shear stress (68). Similarly, it has been postulated that the postneovascular of IA may be used to evade anti-neovascular therapies. As such, vessels use IA as their alternate growth mechanism in a context where vascular endothelial growth factor (VEGF) signalling is inhibited, for example (52).

The succession of IA in pathology was described in phases by Burri *et al*. (69), although the exact mechanism remains poorly understood. During the first phase, the vessel's walls protrude into the lumen, resulting in EC junctions being altered so that a bilayer of vessels may be formed. The formation of a pillar between an endothelial protrusion in phase 2 follows this. In phase 3, the pillar core is formed at the point of contact between the walls and filled with myofibroblasts, pericytes and interstitial fibres. During the fourth phase, the new cells begin forming the collagen fibres in the core and help provide an ECM for the growth of the vessel lumen. After that, the core splits the existing blood vessel lumen into two (69, 70).

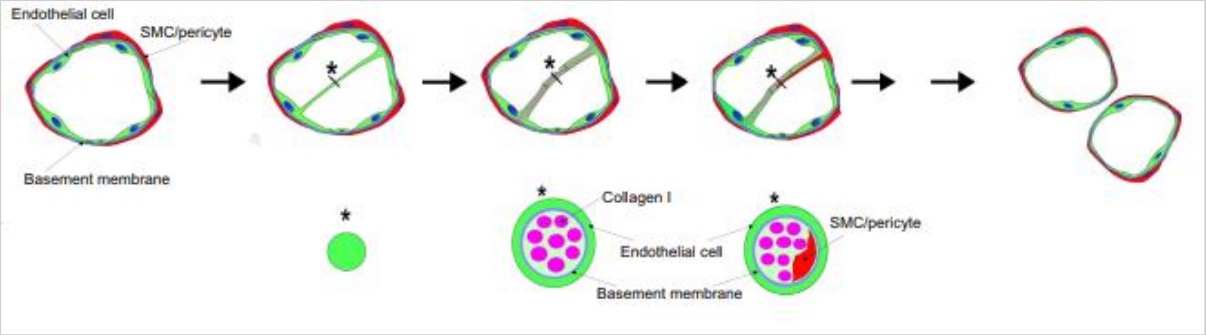

Figure 1.4: Cross-section of angiogenesis by intussuscepted growth. Initially, the ECs from opposite capillary walls protrude into the vessel's lumen, resulting in the endothelial bilayer and basal membrane perforation. Pillar formation occurs through the development of collagen I and IV and smooth muscle cells or pericytes. After a downstream formation of a pillar, vascular splitting follows. *Reprinted with permission from Pandita A* (70).

Amongst the above-mentioned mechanical processes that stimulate angiogenesis, several voltage-gated channels have been identified as part of the mechanical stimuli that respond to blood flow and hydrostatic pressure in ECs (71). Their role in tumour progression has mainly been described in sodium, calcium, and potassium channels, playing a pivotal role in angiogenic proliferation. However, little is known regarding their functional pathway in pathological settings (72, 73).

1.4. The role of angiogenesis in melanoma tumours

The development of angiogenesis in melanoma was first described by Warren and Shubik, who transplanted melanoma cells into a hamster pouch (74). Since then, numerous studies have been conducted to elucidate the relationship between angiogenesis and cutaneous tumour progression. Through immunohistochemical analysis, a strong linkage between microvascular density and the progression of the disease was brought to light, acknowledging its crucial role in the vertical growth phase - a stage for metastasis (23, 49).

Among the primary studies, Straume *et al*. (75) justified the phenomenon of microvascular density by demonstrating the onset of angiogenesis during the radial growth phase of

melanoma, resulting in disease progression. Similarly, Pastushenko *et al*. (76) reassured the significant correlation between intratumoral blood vasculature and distal organ metastasis in a retrospective multicentre study. As a result, different pan-endothelial cell markers have been used to estimate microvascular density, with cluster of differentiation 31 (CD31) being one of the widely accepted markers (77).

Platelet-endothelial cell adhesion molecule-1, also known as CD31, is a single-chain type-1 transmembrane protein that is expressed in human granulocytes, monocytes, and platelets. As a molecule belonging to the immunoglobulin gene superfamily, CD31 plays a meaningful role as an adhesion receptor molecule in adjacent ECs and between leukocytes and platelets (78). In ECs, CD31 is known for its involvement in cell-cell contact formation and stabilisation, modulation of cell migration and maintaining the barrier of vascular permeability. It also involves other cellular mechanisms, including cell migration and signalling, angiogenesis, and leukocyte transmigration (79, 80).

1.5. Factors that promote angiogenesis in melanoma

The secretion of angiogenic biomarkers by melanoma cells, promoting a rich vascular network, has been correlated to the transition from the radial to the vertical growth phase, where several cells express the laminar receptors, which enable their adhesion to the vascular wall, favouring tumour cell extravasation and metastases (81).

A plethora of growth factors in most cancer types, as illustrated in figure 1.5, have been identified as progressive role players of angiogenic tumour growth, either direct or indirect. However, in melanoma, the heterogenic factors of angiogenesis explored during histological studies to understand the biology underlining the progression of the disease remains controversial. This circumstance is based upon melanoma being a multifactorial aetiology, and its genetic and immunological background remains unclear. *In vivo* studies have indicated high growth factors in melanoma cell lines that initiate the breakdown of proteolysis or basement membrane and stimulate ECs for tumour growth and survival (82). These factors

indirectly stimulate the ECs through ligand binding by releasing direct-acting factors from microphages or pro-inflammatory cytokines (50).

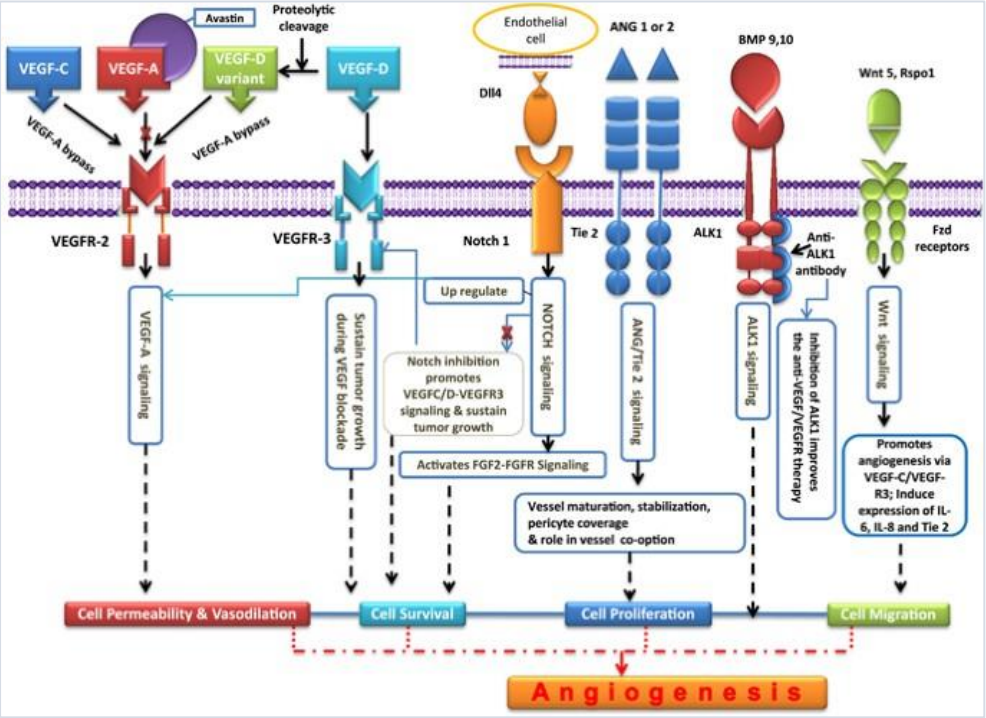

Figure 1.5: Schematic illustration of angiogenic growth factors involved in promoting angiogenesis. Various downstream signalling pathways are activated upon ligand binding, resulting in SA (83). *Reprinted with permission from Gacche* (84).

Various types of pathways facilitate the stimulation of angiogenic melanoma by growth factors. While MAPK is the primary route involved in the melanoma gene, the Notch pathway is another significant pathway, acknowledging its regulatory role in cell fate, differentiation, proliferation, and homeostasis. It is also associated with apoptosis, tumour growth and suppression (85). This fundamental pathway is subjected to a wide array of regulatory influences and protein-protein interactions and is correlated with other signalling pathways like MAPK and Akt pathways (86).

The Notch pathway is a highly conserved intercellular signalling whose cascades are illustrated by multiple ligands of the Jagged (Jagged1, Jagged2) and Delta (Delta-likes 1, 3, and 4) families. Of these ligands, vesicular ECs that compose “stalk” components of a growing capillary sprout adjacent to the tip cells express notch-1 and -4 receptors. Even though Dll1, Dll4 and jagged 1 are also depicted in ECs, Dll4 is exclusively expressed, playing a pivotal role in angiogenesis. Its expression is highly observed across the “tip” cells of sprouting vessels and is commonly held to be induced by VEGF-A (86).

Upon ligand binding, the transmembrane Notch (Notch1 - 4) receptors changes in confirmation and activates by proteolytic cleavage. As illustrated in figure 1.6, a protease known as ADAM on the extracellular surface cleaves the extracellular domain off the notch receptor and effectively dislodges it from the plasma membrane. On the other hand, the Notch intracellular domain (NICD) is cleaved off by the gamma-secretase, allowing the NICD to migrate to the nucleus. However, the NICD binds to other complex proteins (Mastermind-like and CSL) before translocating to the nucleus. As a complex, these proteins act as a transcriptional factor and regulate the expression of various target genes like vesicular endothelial growth factor receptor (VEGFR) in ECs (87).

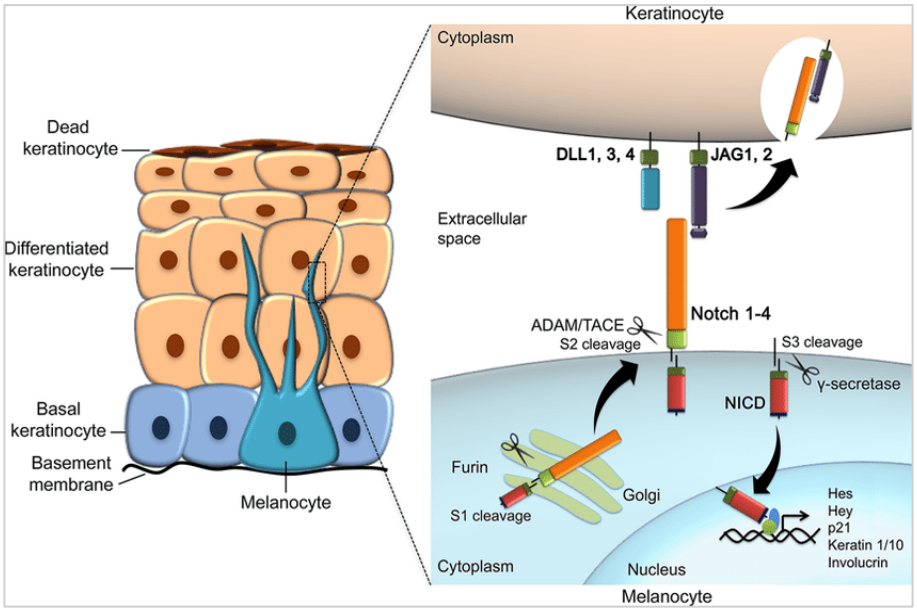

Figure 1.6: Schematic illustration of the DII4/Notch pathway in cutaneous melanoma. The stimulation of VEGFR in the endothelial tip cell by VEGF up-regulates the expression of DII4. The expressed DII4 across the surface area of the presenting endothelial tip cell interact in trans with the Notch transmembrane receptor of the endothelial stock cell. Upon Notch ligand binding, a two-way proteolytic cleavage is catalysed at the juxtamembrane region where ADAM cleaves the extracellular Notch domain whilst the gamma-secretase cleaves the Notch intracellular domain (NICD) (88). As a result, the NICD is translocated into the nucleus, where it forms a transcriptional complex with CSL and mastermind-like 1 to induce the transcription of the target gene. *Reprinted with permission from Dantonio et al. (89)*

Research has publicized that VEGF-A induces the up-regulation of Dll4 in ECs for angiogenic functionality. The elevated Dll4, in turn, acts as a negative feedback mechanism, preventing the accumulation of pathological angiogenesis. However, when the Dll4-notch signalling is blocked, this negative feedback mechanism is interrupted. As a result, there will be an increase in microvascular density, and because these fast-growing vessels are dysfunctional and unable to perfuse adequate blood supply, tumour hypoxia will also increase. Considering this event, studies have postulated that anti-Dll4 drugs could be of considerable interest as a potential therapeutic measure (88).

1.5.1. Vascular endothelial growth factor

It was not until 1989 that the VEGF was cloned for the first time, recognising it as a prototypic pro-angiogenic factor and a major regulatory factor in both standard settings and metastatic diseases (90). The VEGF family is known for constituting five subclasses of glycoprotein isoforms: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and the placental growth factor. The secretion of these proteins is primarily regulated by a hypoxic tumour microenvironment in which the HIF-1∝ is transcribed (91).

Three extracellular tyrosine kinase receptors commonly referred to as VEGFR-1, VEGFR-2, and VEGFR-3, serve as the binding receptors for the VEGF ligands (92). Having frequently been expressed in monocytes/macrophages, endothelial and smooth muscle cells, VEGF receptors cause dimerisation and activation by transfer relation (93). In human ECs, the signalling pathway of VEGF-A in response to angiogenesis is carried out through the binding of VEGFR-2. However, the ligand-binding effect of VEGF-A on VEGFR-1 remains a mystery, even though VEGF-A has a high affinity for VEGFR-1 compared to VEGFR-2. In contrast, VEGFR-3 is mainly found in the lymphatic ECs, playing a significant role in lymphangiogenesis (92, 94).

Although recent studies have described VEGF-A as an autocrine regulator for angiogenesis and vascular homeostasis, it is wildly believed that the actions of VEGF-A are attributed to a paracrine mechanism by tumour cells. This means that the secretion of VEGF-A is primarily produced by tumour cells even though they cannot respond to them directly since they lack cell-surface VEGF receptors. However, active tumour-associated ECs express numerous receptors of VEGF-A but produce an insufficient amount of VEGF-A. Based on this, enough VEGF-A that can drive tumour angiogenesis originates from various host cells, including tumour-associated stromal cells, to mention a few. In bits and pieces, these findings explain why some patients fail to benefit from a good prediction of drugs targeted for the VEGFR-2 pathway when expressing high levels of VEGF-A in blood or tumour tissues (95).

The vesicular permeability factor, popularly known as VEGF-A, and its receptors, have been acknowledged by countless histological studies concerning the prognosis of malignant melanoma. The underlined relation of VEGF-A with the progression of angiogenesis was highlighted owing to being overly expressed in most parts of cancer, reflecting the aggressiveness with which tumour cells spread (96, 97). Since then, the highest observed factor has helped predict the value in identifying high-risk patients with poor prognoses. Accordingly, the expression of angiogenic factors has been chiefly used as biomarkers, providing advanced evidence of angiogenetic activity (98).

Despite VEGF-A being the most influential factor that drives angiogenesis in melanoma, it is incapable of cascading every pathway involved in forming an anastomosing network of capillaries required for perfusion on its own. Thus, factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) come into play, stimulating angiogenic cascades later in the process, as described below (99).

1.5.2. Platelet-derived growth factor

Platelet-derived growth factors are a heparin-binding family of polypeptide growth factors denoted PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF–DD. These ligands exert their physiological effects by binding on the two structurally related cell surface tyrosine kinase receptors, platelet-derived growth factor receptor-∝ and -β (PDGF-R∝/β). Although sharing evolutionary relationships, PDGF-R∝ and PDGF-Rβ exhibit different functional roles and are ligand-specific. As a result, PDGF-AA and PDGF-CC exclusively bind to the ∝-receptor, and the β-receptor responds solely to PDGF-DD, whilst PDGF-BB binds to both the ∝- and the β-receptor (100).

Several scholars have extensively studied the role of PDGF as a regulator for mesenchymal cell proliferation, survival, and migration in melanoma. This homodimeric growth factor has also been acknowledged for its meaningful functionality as a molecule that regulates the interactions between the ECs and the pericytes. As a result, *in vivo* and *in vitro* studies have

associated its expression in the tumour microenvironment with the induction of tumour angiogenesis, recruitment of tumour stroma fibroblast, stimulation of tumour cells through an autocrine mechanism, and control of tumour cell pressure (101, 102).

During the development of vasculature in both physiological and pathological settings, PDGF-BB and PDGF-Rβ are highly expressed, acknowledging their role in promoting the recruitment of perivascular cells and enhancing the proliferation of tumours and ECs (101, 102). These findings are based on the primary knockout studies that exhibited pericyte deficiency in the microvasculature as the prominent phenotype that causes lethal haemorrhage and oedema at late gestation (103, 104).

The molecular mechanisms that are inducted by the expression of PDGFs are influenced by PI3K/MAPK intracellular signalling. In this regard, a cascade of auto-phosphorylation is initiated, resulting in the proliferation, migration and apoptosis of ECs (100, 105).

1.5.3. Basic fibroblast growth factor

The basic fibroblast growth factor, also known as fibroblast growth factor-2 (FGF-2), is an essential pro-angiogenic factor that serves versatile functions, which include the migration of macrophages for wound healing in standard physiological settings. Similarly, FGF-2 is known for its pathological involvement, where it promotes EC proliferation and migration, ECM degradation, as well asmodulating cell adhesion molecules (106). The basic fibroblast growth factor is a family of 22 cell signalling members with which FGF-1 and -2 are extensively studied, along with their cell membrane receptor tyrosine kinases (94).

In human melanoma, the overexpression of bFGF has been associated with matrix metalloproteinases (MMPs), which are enzymes secreted by tumour cells for tissue remodelling. The secretion of MMPs results in ECM degradation by which the inactive matrix-bounded bFGF is released, resulting in VEGF-A upregulation. This phenomenon is thus the angiogenic stimulator in melanoma cancer (94, 107).

Upon encountering a specific ligand, the activation of integrins triggers a signal transduction pathway through which a wide range of intracellular kinase molecules, including integrin-linked kinase and focal adhesion kinase, are recruited. As a result, these kinases undergo autophosphorylation that cascades into c-Src activation (108). Following this, scaffolding proteins such as CRK-associated substrates and growth factor receptor-bound protein 2 lead to the activation of MAPK or PI3K/Akt transduction pathway. Through this pathway, the survival and malignancy of a tumour are assured. Although the integrin signalling pathway has been extensively characterised, new specificities and signalling components are still being discovered (109).

1.6. Molecule activated by angiogenic factors: Bcl-2

The B-cell lymphoma-2 (Bcl-2) has been intensely studied over the years, owing to its essential functional role in regulating mitochondrial apoptosis, tumorigenesis, and cellular responses to cancer therapy (110, 111). While pro-apoptotic members govern the Bcl-2 family to ensure organismal health, other members exhibit anti-apoptotic characteristics, facilitating carcinogenesis and angiogenic formation (111).

The ability of Bcl-2 to influence capillary network remodelling in the tumour microenvironment has been discussed in various histotypes (112-114). Most recently, the expression of Bcl-2 in haemangioma has been linked with tumour angiogenesis through a mechanism that is associated with the expression of VEGF-A (115).

Briefly, through VEGFR and VEGF-A interaction, PI3K is recruited and activated by phosphorylation. The activated PI3K phosphorylates PIP2 to form PIP3. After that, PIP3 phosphorylates PDK1/2 which in turn activates PKB/Akt. This leads to the up-regulation of Bcl-2, promoting EC survival and proliferation (115, 116). This mechanical pathway is suggested to enhance EC sprouting in a hypoxic tumour microenvironment, ensuring the continuous, uninterrupted flow of nutrients to the tumour cells (117-119).

1.7. Current treatments of common cutaneous tumours and their limitations

Over the past years, the rapid prevalence of skin cancer has emerged as a global health concern despite the breakthroughs made to advance disease therapy (120). While skin cancer can be managed effectively or even be cured through surgical excision when detected early, treatment options become limited and ineffective when the tumour is metastatically advanced (121). As a result, numerous studies have emerged, aiming to identify critical regulatory elements and signalling pathways that can advance the development of novel therapeutic strategies (49, 107).

1.7.1. Chemical therapy

Chemotherapy remains the mainstay of treatment out of conventional therapies such as surgery and radiotherapy, which play a limited role in advanced melanoma cancer (122). In South Africa, melanoma patients from public health care sectors are widely administered chemotherapy even though it has been reported with a poor overall survival rate (123). In non-melanoma cancer, chemotherapy is rarely used because most tumours are often cured at an early stage. However, when metastatic, particularly cSCC, epidermal growth factor receptor antagonists are employed (124).

1.7.1.1. Dacarbazine treatment

Dacarbazine (DTIC) is a United States Food and Drug Administration-approved cytotoxic agent used to treat melanoma cancer. Since 1975, it has remained the standard golden treatment despite the absence of clear evidence regarding survival benefits (125, 126). Dacarbazine is intravenously administered as a pro-drug, requiring conversion in the liver for activity (127). When DTIC is bio-activated into a 5-(3-methyl-triazen-1-yl)-imidazole-4-carboxamide compound, DTIC is extensively metabolized into diazomethane, an alkylating agent causing DNA methylation, which prevents defective cells from replicating (128).

Several studies have looked at DTIC as an anti-melanoma drug, associating it with poor overall survival benefits, mainly when administered as a single agent (129, 130). A response

rate ranging from 10 - 25% with median response durability of 7 - 8 months has been reported in various clinical trials. However, tumour resistance and high-grade toxicity (including lung injury) remain huge threats to overall survival (131, 132).

1.7.2. Anti-angiogenic therapy

In his 1971 treatise, Judah Folkman considered the correlation between tumour microvessels and the progression of tumour cell growth, postulating that the success of a malignant tumour is angiogenic dependent. His theory became a breakthrough for what is now known as an antiangiogenic therapy, implicating that the cancerous cells could be disrupted using anti-angiogenetic targets as an effective therapeutic measure (23). Since then, chemical signals that systematically disturb the vessel formation process in pathological settings have been described (56). Based on this process, numerous preclinical and clinical trials have been established to eradicate angiogenesis by concentrating the microenvironment with anti-angiogenetic factors, as listed in Tables 1.1 and 1.2. However, little progress has been made despite advanced research over the past decades (133).

Since melanoma is a highly vascularized cancer type, theoretically, it should be amenable to treatment by angiogenic inhibition. Even though there is an excellent scientific rationale to support this argument, clinical evidence still shows insignificant benefits (134). Consequently, none of the trialled angiogenic drugs has been approved for melanoma treatment. The reason for this discrepancy is the emergence of acquired resistance that often results from alternate pathway activation or gene mutation within the pathway (107). Moreover, it was noted that the anti-angiogenic therapy response was different across tumour types or the lesion site within the same tumour (135).

Table 1.1: Molecular targets of angiogenic drugs. *Adapted with permission from Ria et al.* (81)

Categories	Molecular targets	Name
Angiogenic growth factors and the receptors	VEGF	Bevacizumab
	Tyrosine kinase receptors	Sorafenib
	VEGF receptors	PTK/ZK DC101
Receptors for ECM and integrins	Integrin αvβ3	Vitaxin (MEDI-52)
	Integrin αvβ3/ αvβ5	Cilengitide (EMD 121974)
Components of ECM and proteases	MMPs	Batimastat Marimastat
	ECM	Edostain
Complex mechanism of action	Angiogenesis inhibitors and immunomodulators	Thalidomide Lenalidomide
	Cyclooxygenase-2	Celecoxib

VEGF - Vascular Endothelial Growth Factor; ECM – Extracellular Matrix, MMPs - Matrix Metalloprotease

Table 1.2: Angiogenesis inhibitors used to treat melanoma and their success.

Angiogenic biomarkers	Angiogenic inhibitors	Success	Reference
VEGF-A	Bevacizumab	Phase 2 clinical trial	(136, 137)
VEGFR and PDGFR	Axitinib	Phase 2	(138)
bFGF	Human Anti-FGFR4 antibody	Phase 1 clinical trial	(139, 140)
IL-8 (CXCL8)	Human anti-IL-8 antibody (ABX-IL8)	Phase 1b/2 clinical trial	(141, 142)
PDGFR-$\propto/-\beta$	Imatinib mesylate	Pre-clinical trial	(143)
Angiopoietin-2	MEDI-3617	Phase 1 clinical trial	(144)
MMP-14	DX-2400	Pre-clinical study *in vivo* mouse models	(145)
Integrin- αvβ3	Etaracizumab (MEDI-522)	Phase 2	(146)
Tyrosine kinase inhibitor	Sorafenib	Phase 2	(147)

VEGF-A – Vascular Endothelial Growth Factor-A, bFGF – Basic Fibroblast Growth Factor, IL-8 – Interleukin-8, PDGFR-∝/-β – Platelet-Derived Growth Factor Receptor-∝/-β, MMP – Matrix Metalloprotease.

1.7.2.1. Bevacizumab treatment

Bevacizumab, a licenced anti-VEGF-A antibody for various solid tumours, is popularly known for its role in neutralising expressed VEGF-A selectively, even though it has shown limited benefits in melanoma and other types of cancerous cells (94, 148, 149). It is being clinically trialled for malignant melanoma as a humanised monoclonal antibody and in combination with other therapies, namely cytotoxic chemotherapy (136). Findings from the preclinical study suggested that the ectopic expression of αIIβ3 in melanoma cells could be another novel target, even though further exploitation of antibody therapy is needed (150). Similarly, the 2009 preclinical study data suggested that bevacizumab, in combination with erlotinib, a tyrosine kinase receptor inhibitor, is effective (151). However, even though these studies showed encouraging results, combination therapies still show graded 3 or 4 adverse events

in administered patients with insignificant benefits (136, 137). As such, further evaluation of therapeutic strategies that target multiple angiogenic pathways may be warranted for patients with advanced malignant melanoma.

1.7.2.2. Propranolol treatment

Propranolol is a non-selective β-adrenergic receptor blocker, and its antiproliferative effects on haemangioma were serendipitously discovered in 2008 (152). Clinically, it has been shown to promote vasoconstriction, inhibit angiogenesis and induce apoptosis (153). Similarly, propranolol is suggested to inhibit angiogenic-promoting factors that are implicated in proliferating haemangioma, VEGF-A and MMP-2 and 9 (154). Although the definite mechanism of action in haemangioma regress is unclear, its favourable resolution rate and minimum adverse events favoured propranolol as an ideal standard care treatment compared to other therapeutic options (155).

1.8. Motivation for the research study

Cutaneous cancer is a skin-associated malignancy that is caused by long-term skin exposure to UV radiation or underlining gene mutations that alter the cell DNA, causing uncontrollable cell division. Its association with significant resistance and high metastatic ability in the case of melanoma is a global health concern whose prevalence is increasingly getting linked to a high mortality rate (23).

Since 1971, anti-angiogenic therapy has received considerable attention in a world with growing interest in cancer treatment. Factors that induce intratumoral angiogenesis have been described and targeted as angiogenic biomarkers for angiogenic disruption (94, 156). However, despite the critical knowledge obtained over the years concerning angiogenesis in promoting tumour cell egress, little has been achieved thus far due to the complex nature of the disease (133). As a result, there is an emerging need to identify novel molecular markers

for cutaneous tumours that would be useful for early diagnosis and predicting the progression of the disease.

1.9. Contribution of the research study

The present study, investigated the progression of angiogenesis in pathophysiological settings, along with the factors involved, in such a way that it advances the understanding of the aggressive nature of this disease and assists in the development of new anti-angiogenic therapies. Furthermore, it will advance the identification of the diagnostic biomarkers that could be most effective at a particular stage of disease progression.

1.10. Research aims and objectives

The present study aimed to investigate markers of angiogenesis in cutaneous tumour cells and biopsies.

The objectives were:

- To determine growth patterns of skin tumour cells *in vitro* as an indicator of angiogenic marker effectiveness using crystal violet and light microscopy
- To measure the levels of angiogenic markers, VEGF-A, PDGF-B, and bFGF *in vitro* using an Enzyme-Linked Immunosorbent Assay (ELISA)
- To study the histology of cutaneous tumour tissue using H&E and light microscopy
- To determine the presence of Bcl-2 and VEGFR in tumour tissue using immunohistochemistry (IHC)

CHAPTER TWO

METHODS AND MATERIALS

2.1. Study design

This cross-sectional study was conducted at the Angiogenesis Laboratory, Basic Medical Science, at the University of Pretoria, as summarised in figure 2.1.

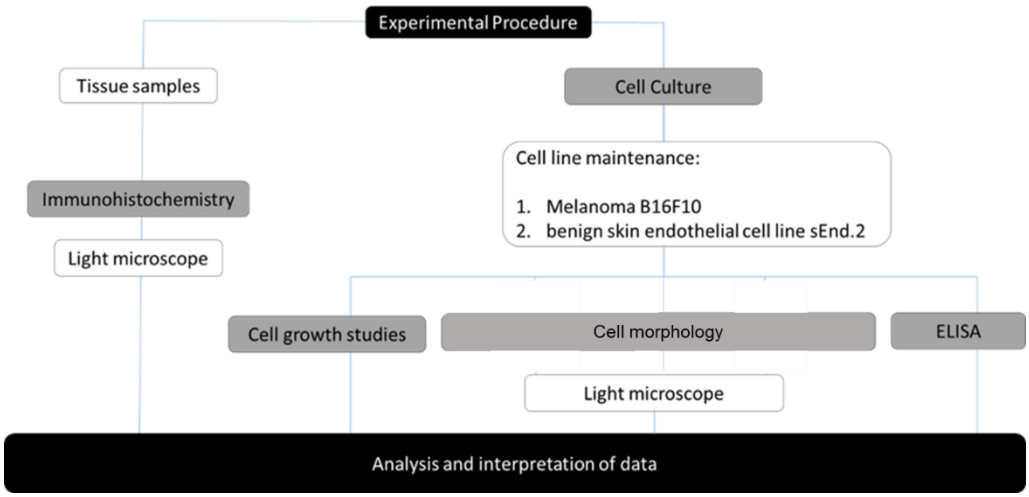

Figure 2.1: Flow diagram illustrating the study design. *Figure designed by Nonxuba M. using Microsoft PowerPoint Presentation (2016)*.

2.2. Study methods

2.2.1. Cell culture maintenance

In the present study, the human melanoma cell line B16-F10 was obtained from the American Type Culture Collection (Manassas, Virginia, United States of America) and the benign skin endothelial cell line sEnd.2, provided by Prof M.S. Pepper (University of Pretoria, Pretoria, South Africa) were grown in sterile 25 cm² cell culture flasks as indicated in figure 2.1. Both the melanoma and endothelioma cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, United States of America), supplemented with 10% foetal calf serum (FCS) (Scientific Group, Midrand, South Africa) and 1% penicillin-streptomycin (Whitehead Scientific, Johannesburg, South Africa). The cell lines were maintained in a humidified incubator at 37˚C and 5% carbon dioxide (CO_2).

2.2.2. Sub-culturing adherent cells

Cell passage and splitting were performed when cells had reached their 80 – 100% confluence. Cell confluence was assessed by a Zeiss Axiovert CLT 40 light microscope (Zeiss, Oberkochen, Germeny). The cells were grown in a 25 cm² cell culture flask. Before cell passaging, the growth medium in the flask was discarded, and the cells were rinsed once with 2 ml of pre-warmed phosphate-buffered saline (PBS) to remove any FCS particles. Afterwards, the flask was incubated for 20 min at 37˚C and 5% CO_2. Following incubation, the PBS solution was discarded, and the cells were exposed to 2 ml of 1:1 diluted trypsin-Ethylene Diamine Tetra Acetic Acid solution (Sigma-Aldrich, Cambridge, United Kingdom) for cell splitting.

After gently rocking the flask to get complete coverage of the cell layer, the cell culture flask was incubated at 37˚C and 5% CO_2 for 30 min until the monolayer was dislodged from the flask. After that, 3 ml of pre-warmed growth medium containing 10% FCS was dispersed across the cell layer surface by pipetting several times to stop the trypsin enzymatic reaction.

A single-cell suspension was generated, transferred into a 15 ml centrifuge tube and span for 10 min at 2000 rpm. After centrifuging at room temperature (RT), the supernatant was discarded, and a pellet containing the cells was kept for cell counting, as detailed in section 2.2.3.

2.2.3. Hemacytometer cell counting

Principle

Cell enumeration is an essential procedure that is used by clinical and research laboratories to determine the concentration of cells in a given sample — an analysis that assesses the kinetics of growth (157). This technique was employed using a hemacytometer of (4 x 4) gridlines to determine viable cells from dead cells using a hemacytometer, as illustrated in figure 2.2.

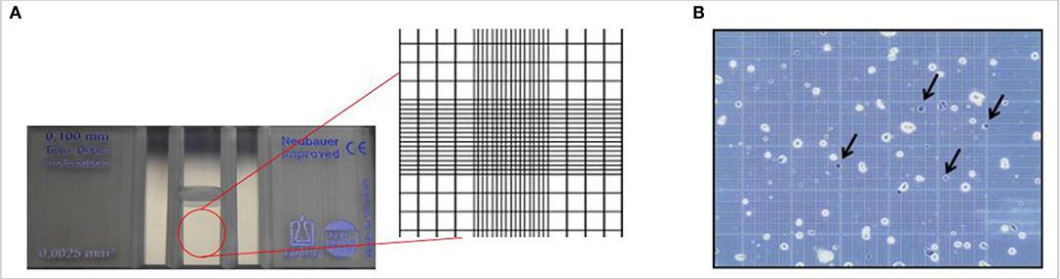

Figure 2.2: Overview illustration of a hemacytometer for cell counting. **(A)** Zoomed inset of a hemacytometer showing the Neubauer chamber with 4 counting grids. **(B)** Cells stained with trypan blue dye with dead cells indicated by an arrow (157). *Reprinted with permission from Creative Commons Attribution 4.0 International Licence*.

Procedure

The cell pellet was gently re-suspended in pre-warmed 1 ml of the growth medium, and 2 µl of cell suspension was transferred into a 2 ml Eppendorf tube. Of the same tube, 18 µl of PBS

and 20 µl of trypan blue were added, giving a concentration of cells with a twenty times dilution factor. Dead cells took up the blue dye and were left uncounted. Depending on the pellet size, the number of viable cells was counted by gently filing the two chambers of the bright-line hemacytometer (Hausser Scientific, Horsham, United States of America) with trypan blue-treated cell suspension. Before the mixture was transferred, a coverslip was put in place to allow the cell suspension to be drawn out by capillary action. In a case where cell suspension was too concentrated, PBS was used for dilution. For visual acquisition, the Neubauer chamber of haemocytometer was placed directly under the Zeiss Axiovert CLT 40 light microscope (Zeiss, Oberkochen, Germeny), and the cells were counted as previously described by Freshney (1995) (158) through a 10x objective lens. The average of three counts was calculated to calculate the number of cells in suspension. To calculate the density of cells, the following equation was employed:

$$\text{Cell density (cells/ml)} = \frac{Total\ number\ of\ viable\ cells}{Number\ of\ squares\ (4)} \text{ x } \textit{Dilution factor x } 10^4$$

After cell counting, the cell suspension was seeded into a multi-well cell culture plate for subsequent experiments or split into a fresh 25 cm² cell culture flask to grow more cells. Cell incubation was always kept at 37˚C humidified atmosphere and 5% CO_2.

2.2.4. Cell growth assay

Principle

Crystal violet (CV), a monochromatic indicator that binds protein and deoxyribonucleic acid (DNA) of live cells, was employed to study cell viability under diverse stimulus conditions using a previously described protocol (159). In theory, CV assumes that the live cells will remain

attached to the plate because they have an intact cell membrane, allowing them to adhere, while dead cells will detach because of a compromised or no cell membrane. Subsequently, these cells get lost from their cell population. The dye acts as an intercalating dye that enables DNA quantification, which is always held directly proportional to the cell biomass attached to the plate. Therefore, the dye easily penetrates dead cells' cytoplasm and stains the nucleus dark blue and the cytoplasm light blue (160).

Procedure

Following the determination of cell density, a time-depended study was conducted with 24 h and 48 h intervals. Exponentially growing endothelial cells (ECs) (sEnd.2 cell line) were seeded in sterile 96-well culture plates (Nest Biotech, China) at a density of 5 000 cells per well. To allow cell attachment, cells were incubated at humidified 37°C and 5% CO_2. Each well contained 100 µl of DMEM-cell suspension mixture. Cells were grown to 60% confluence.

The quantification of monolayer cells was determined using spectrophotometry, employing CV as a DNA stain. After 24 h in the endothelial culture medium, 100 µl of serum-free medium (SFM) (control), B16-F10 derived melanoma-conditioned medium (MCM) and 10% serum-containing medium were added to cultured cells, and incubation continued for 24 - 48 h. After 24 – 48 h, cells were fixed with 30 µl of 1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, United States of America) in PBS and left at RT for 15 min after shaking the plate gently. The glutaraldehyde was then discarded, and 100 µl of 0.1% CV solution (Sigma-Aldrich, DE) in PBS was used to stain the cells. This was to allow rapid, accurate and reproducible quantification of cell numbers in a culture grown in 96-well plates (161).

After incubating the plate for 30 min at RT, the CV was discarded, and the microtiter plate was immersed under running tap water for 10 min and left to dry overnight at RT. When the plate was completely dry the following day, the wells were solubilised with 100 µl of 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and left overnight at RT. The absorbance of the dye was read at 570 nm with an EL_X800 Universal Microplate Reader from Bio-Tek Instruments

Inc (Vermont, United States of America). Three wells were analysed for each time interval. Studies were undertaken in triplicate. The data from the obtained CV results were used to calculate the concentration of cell growth.

2.2.5. Cell morphological studies

Principle

Cell morphology is a typical histological image processing and pattern recognition for abnormality identification and quantitative characterization of cell structures and inner components. The critical issue of this analysis is to advance the understanding of malignancy and behaviour in pathogenesis, providing early detection and a better prognosis (162). As such, the Polarization optical differential interference contrast (PlasDIC), functionally known for its ability to contrast with cells in plastic dishes or plates, was employed to observe the different architectural arrangements of endothelial cells.

Procedure

At 60% cell confluence, a density of 2.5 x 10^6 ECs per well was seeded in 100mm x 20mm heat-sterilized coverslips Petri-dishes (Nest Biotech, China) and cultured overnight. After 24 h in an endothelial culture medium, B16-F10 derived MCM was added to the ECs, and intubations continued for 24 – 48 h under normoxic or hypoxic conditions. After 24 - 48 h, cells were visualised and evaluated under the Zeiss Axiovert CFL 40 light microscope (Zeiss, Oberkochen, Germany) at 40 × magnification. The PlasDIC contrast images were captured using a Zeiss Axiovert MRm digital camera (Zeiss, Oberkochen, Germany) using AxioVision 4.5 software.

2.2.6. Enzyme-linked immunosorbent assay

Principle

An Enzyme-Linked Immunosorbent Assay (ELISA) is a colourimetric analysis that is technically designed to detect and quantify antigens in biological samples using antibodies and colour changes (163). This technique is often done in multi-well microtiter plates to determine the presence of an antibody related to certain pathological conditions (164).

Procedure

A Quantikine® ELISA (R&D Systems, Inc. United States of America) kit was used to quantify the expression of VEGF-A, bFGF or PDGF-BB proteins in the cells. A sandwich ELISA was performed to quantify VEGF-A, bFGF or PDGF-BB in cells according to the manufacturer's instructions. As such, 5 000 cells grown in DMEM (Sigma-Aldrich, St. Louis, Missouri, USA), supplemented with 10% foetal calf serum (FCS) (Scientific Group, Midrand, ZA) and 1% penicillin-streptomycin (Whitehead Scientific, Johannesburg, ZA) were seeded in each well at approximately 80% of cell confluence.

The well plate strips were pre-coated with 200 µl primary antibodies that were VEGF-A, bFGF or PDGF-BB specific (ThermoFisher Scientific, Massachusetts, United States of America). Cells were pipetted directly into the well, and the proteins present in the sample were allowed to bind to the wells through the immobilized antibody. The plate was subsequently covered with the adhesive strip and incubated for 2 h at RT. Following the 2 h incubation, each well was aspirated and washed in three changes of 400 µl wash buffer using a squirt bottle. After the last wash, the wash buffer was removed by inverting the plate and gently blotting it against a clean paper towel to remove any remaining wash buffer.

Afterwards, each well was incubated with 200 µl of anti-mouse VEGF-A, bFGF, and PDGF-BB conjugates (ThermoFisher Scientific, Massachusetts, United States of America). The microplate was covered with a new adhesive strip and incubated for 2 h at RT. After 2 hours, each well was aspirated and washed in three changes of 400 µl wash buffer using a squirt

bottle. After the last wash, the wash buffer was removed by inverting the plate and gently blotting it against a clean paper towel to remove any remaining wash buffer. Following this, 200 µl of prepared horseradish peroxidase - Streptavidin solution was added to each well and incubated for 30 min at RT. Subsequently, 50 µl of stop solution was added to each well, followed by the colour changes in the wells (from blue to yellow). The optical density of each well was determined within 30 min. The spectrophotometry was set to 570 nm with an EL_X800 Universal Microplate Reader from Bio-Tek Instruments Inc (Vermont, United States of America). Experimental samples were taken in triplicates and repeated three times.

2.2.7. Histological processing

Principle

Histological processing is a procedure that involves various stages as illustrated in figure 2.3, designed to get fixed tissues into paraffin to remove all extractable water from the tissue and replace it with a supporting medium that provides sufficient rigidity to enable sectioning of the tissue without damage or distortion (165).

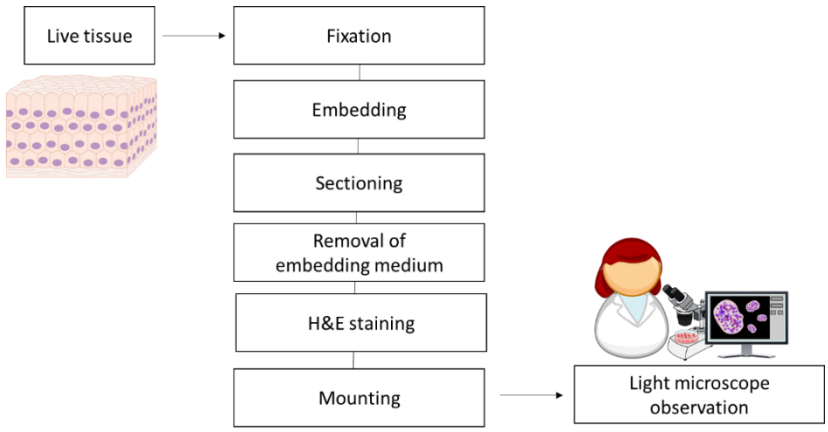

Figure 2.3: Flow diagram illustrating the primary stages involved in histological processing. *Figure designed by Nonxuba M. using Microsoft PowerPoint Presentation (2016)*.

Procedure

The Elivision Plus detection kit (Maixin-Bio, Fuzhou, China) was used as a guideline to analyse immunohistochemistry where tissue biopsies that were formalin-fixed and embedded in paraffin were continually cut into 5 µm sections using a microtome. The sections were then floated on a 40˚C water bath containing distilled water (ddH_2O), transferred onto a poly-1-lysine-coated slide, and dried overnight. Before deparaffinisation, slides were kept in an incubator at 55˚C for 10 min for proper fixation of tissues to the slides, limiting the probability of sections floating during antigen retrieval. Subsequently, slides were repeatedly deparaffinised in xylene twice for 5 min each and rehydrated with two changes of graded ethanol (80%, 100%) for 3 min each. To complete the rehydration process, the sections were immersed twice in ddH_2O for 5 min each. Before the antigen retrieval, the slides were immersed in 600 ml of 10 mM sodium citrate buffer (pH 6.0) to remove the cross-link formed by formalin fixation. Afterwards, antigen retrieval was performed with a microwave treatment for 10 min at 98˚C, and the slides were cooled down for 30 min.

In preparation for chromogenic staining, slides were rinsed in ddH_2O three times for 5 min each and once in PBS for 5 min. The activity of endogenous peroxidase that may lead to background staining was blocked by placing the sections into 0.3% hydrogen peroxide in ethanol for 10 min at RT. After that, the sections were washed in ddH_2O twice for 5 min before washing with PBS for 5 min. Subsequently, 5% bovine serum albumin in PBS was used to block non-specific binding antibodies to the tissues for 1 hr in the humidified chamber. After the sections were incubated, the blocking solution was removed.

Following the removal of the blocking solution, primary mouse monoclonal antibodies, VEGFR (1:100; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.) and Bcl-2 (1:100; Santa Cruz Biotechnology, Inc., Dallas, United States of America) were incubated with overnight at 4˚C. After rinsing the slides in three changes of PBS for 2 min each, a FITC-conjugated anti-rabbit secondary antibody (ThermoFisher Scientific, Massachusetts, United States of America) was incubated for 30 min using a dilution of 1:5000 at RT. After that, slides were rinsed twice with PBS for 2 min each. Thereafter, slides were

counterstained with haematoxylin, as detailed in section 2.2.8 and mounted in a fluorescent mounting medium (Dako Cytomation). Olympus Cell® system and Olympus 1X-81 inverted fluorescence microscope was used for visualisation and image acquisition (Olympus Inc. Japan).

2.2.8. Light microscope: Haematoxylin and eosin staining

Principle

The H&E stain uses two combinations of dye that involve the chemical attraction between tissue samples and dye. It is used to demonstrate a broad range of cytoplasmic, nuclear, and extracellular matrix features of the tissue. Haematoxylin stain is a basic blue dye that contrasts the nucleic acid moieties. However, the acidic eosin stain dyes the cytoplasm and extracellular matrix (ECM) components red/pink in colour (166).

Procedure

Following the paraffin-embedded sections, the cells were stained for morphological observations using a Zeiss Axiovert CFL 40 light microscope (Zeiss, Oberkochen, Germany). The H&E stains were used to provide detailed visualization of morphological changes exhibited by the cells.

Slides were immersed in Bouin's fluid for 30 min and rinsed in 70% ethanol for 2 min. After rinsing the slides with ddH_2O, Mayer's haematoxylin solution was stained for 20 min at RT. The slides were then rinsed from the reverse sides with ddH_2O and 70% ethanol for 2 min each, respectively. After that, 1% of the eosin Y dye solution was stained for 2 min. Dehydration was executed through two changes of 70% ethanol, 95% ethanol and 100% ethanol immersion for 2 min each. This was followed by clearing the slides in two changes of xylol for 2 min each and mounting the slides with a xylene-based mounting medium. The slides were then left to dry overnight.

2.3. Ethical and legal clearance

Ethical clearance (660/2020) (see Annexure II) was obtained from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria. The study's author complied with the Human Tissue Act (167) and the HPCSA Ethical Guidelines (168) for using human sample-related research.

2.4. Data analysis

The data were analysed using SPSS software. Column analysis was used to determine normality. The three proportions of interest were reported along with 95% confidence intervals. A one-way analysis of variance (ANOVA) and Fisher-LSD exact test were employed to compare the association between channel presence and protein expression, VEGF-A, bFGF and PDGF-B, respectively, and the odds ratio and its 95% confidence interval were determined. The level of significance was designated at 0.05.

CHAPTER THREE

RESULTS

3.1. Growth characterisation: Crystal violet

A time-dependent cell growth study was conducted to determine the effect of melanoma-conditioned medium (MCM) and medium with 10% of serum on endothelial cell (EC) growth. The ability of the B16-F10-conditioned medium and medium+serum to promote sEnd.2 cell growth was explored, and fixed monolayer cells were quantified under a spectrophotometer, employing crystal violet (CV) as a deoxyribonucleic acid (DNA) stain.

As illustrated in Figure 3.1, the ECs treated with MCM and medium+serum showed a significant increase ($p < 0.05$) in cell viability by 63% and 63%, respectively, after 24 h when compared to the control (serum-free medium - SFM). The increase in cell viability was further enhanced as the treatment time increased to 48 h (figure 3.2), showing a significant difference ($P < 0.05$) between the control and MCM and medium+serum. It was also noted that after 24 h of cell growth, there was a significant difference between the cell viability of the conditioned melanoma and medium+serum ($p < 0.05$). When the control average was compared at 24 h (figure 3.1A), to that of 48 h (figure 3.2B) a significant decrease of $P < 0.05$ was noted.

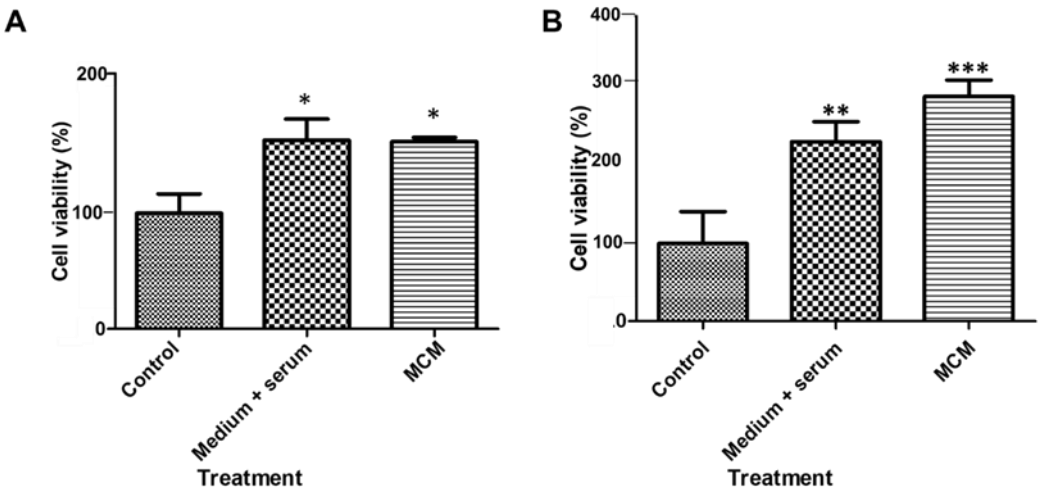

Figure 3.1: Cell growth effect of MCM, medium+serum and control on endothelial cells *in vitro*. **(A)** Cells at 24 h incubation and **(B)** at 48 h incubation. Each point represents the mean ± standard deviation of at least three independent experiments done in triplicates. The significant difference was found at *P < 0.05; **P < 0.01; ***P < 0.001. Melanoma conditioned medium – MCM.

Based on the results obtained above where the induction of cell growth inhibition by, the control (SFM) on EC was observed, a dose-dependent study was further conducted to determine whether the culturing of cells in SFM led to cell damage compared to medium with 10% serum since it is universally known that cells cultured without serum can undergo apoptosis. In this regard, Nocodazole, a known apoptotic-inducing drug was incubated in cultured growing cells at different dosages (0.01 µg/mL; 0.1 µg/mL; 0.25 µg/mL) so to compare.

After 48 h of incubation, a significant difference (P<0.05) in cell growth inhibition was noted across all concentrations of Nocodazole when compared to the control (figure 3.2). As the concentration of Nocodazole increased, more cells were inhibited from growth. This observation was found to be significant (P<0.05); having a concentration of 0.25 µg/mL to have a significant decrease of viable cells compared to the 0.01 µg/ml and 0.1 µg/mL concentration (see annexure V). When compared to SFM however, Nocodazole exhibited

similar behaviour to which cell growth inhibition was induced. Even though there was a slight difference between the 0.25 µg/mL dose of Nocodazole and SFM after 48 h, a significant difference of P <0.05 was found across all doses of Nocodazole.

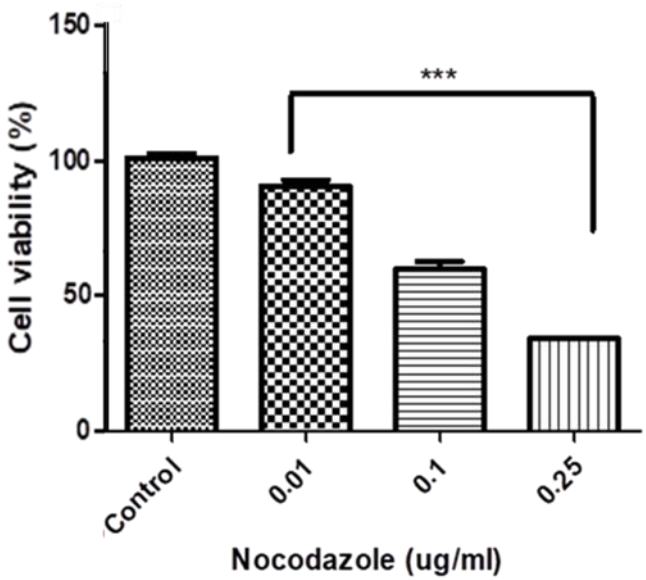

Figure 3.2: Effects of Nocodazole on sEnd.2 cell viability. After 48 h of exposure, a significant difference (P<0.05) was found compared to the control. Each point represents the mean ± standard deviation of at least three independent experiments done in triplicates.

3.2. Growth characterisation: Cell morphology

To ascertain the morphological changes that were characterised by sEnd.2 cell line growth in different adherent conditions, the PlasDIC light microscope was employed to focus on the visual appearance of cells in culture and how quickly they adapted or re-adapted. As illustrated in Figure 3.3, the cells were exposed to the conditioned melanoma medium following 24 and 48 h.

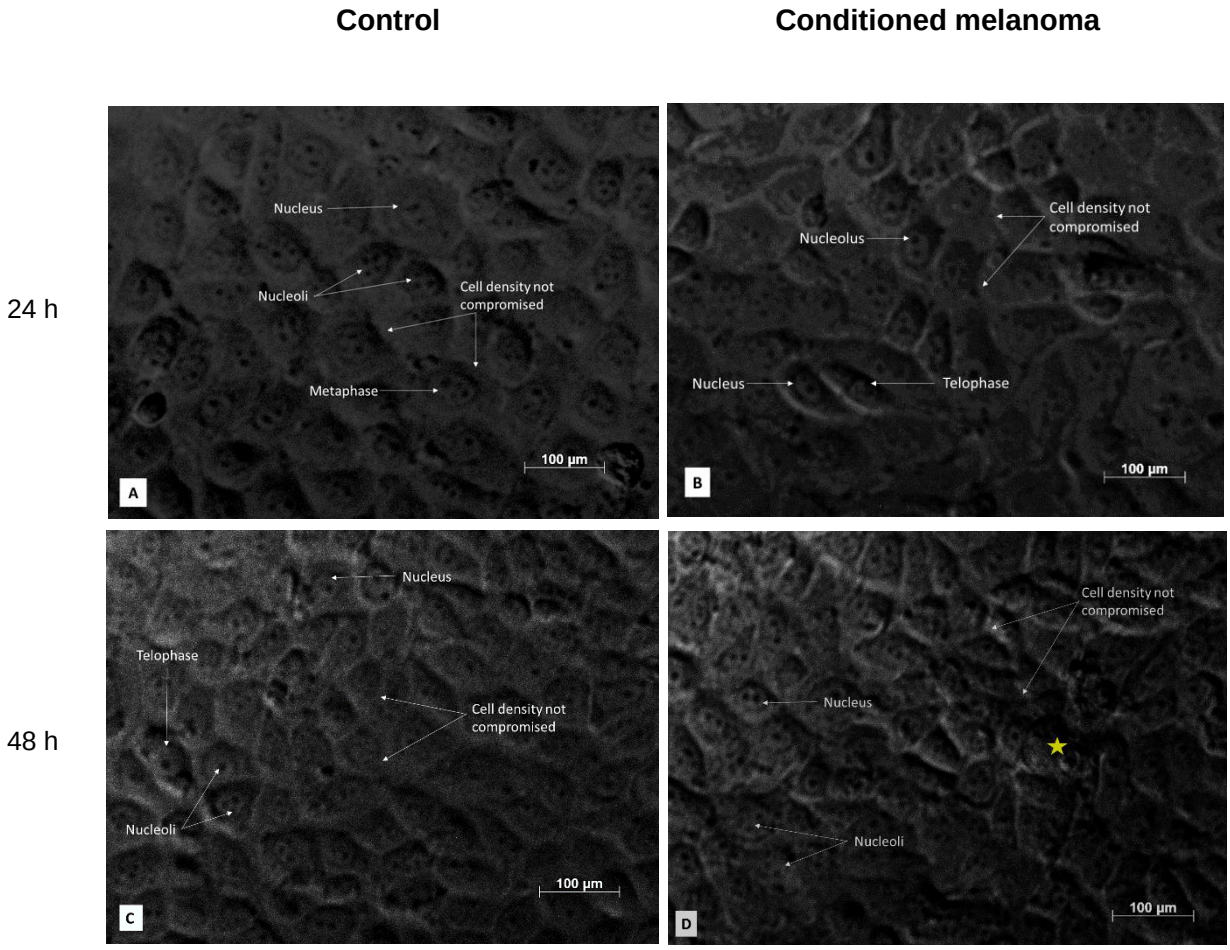

Figure 3.3: Morphological appearances of sEnd.2 cell established in different adherent conditions. **(A)** Cell arrangement at 24 h with a well-defined nucleus and prominent nucleoli but **(B)** with the high confluence in melanoma-conditioned cells. **(C)** Endothelial cells after 48 h with an increased confluence and **(D)** cell compaction (yellow star). Light microscopic images viewed at x 40 magnification.

Over a 24-h period in culture, the control cells exhibited a typical morphology. The spindle-shaped cells adhered firmly, demonstrating mitotic growth as illustrated in Figure 3.3A. Following 48 h, the cell density remained uncompromised (figure 3.3C) with increased cell confluence. Even though the nucleocytoplasmic ratio of the cells had slightly increased after 48 h, mitotic division (telophase) was still observed.

Although melanoma-conditioned cells at 24 h showed no morphological differences from the 24-h control, the plated epithelioid-shaped cells were more confluent (figure 3.3B). As the treatment time increased to 48 h, no morphological change was observed in cultured cells, although an atypical cell arrangement was seen, as illustrated in Figure 3.3D. While the melanoma-conditioned cells displayed a typical spindle-like cell structure as seen in control cells, they also displayed an increase in cell confluence and nuclear-cytoplasmic ratio.

As seen in control cells, the cells grown in a conditioned medium exhibited a non-tormented cell density in both time intervals. Unlike the control cells, the melanoma-conditioned cells showed a reduction in cell size, indicating shrinkage or a physiological disturbance in the nucleocytoplasmic ratio.

3.3. ELISA – Protein expression

Following morphological growth studies in different cell lines, a time-dependent study utilising ELISA as a technical tool was carried out to identify and quantify the presence of angiogenic proteins that promote tumour cell growth. Antibodies that respond positively to VEGF-A, bFGF and PDGF-BB were added to determine their expression levels in control ECs and melanoma B16-F10 cell lines cultured in DMEM.

A multivariable statistical model was constructed, as illustrated in Figure 3.4-6, correlating the concentration of the expressed candidate angiogenic promoting factor in treated and untreated cell samples with the time to which the disease progressed. Compared to the control sample, as the time went from 24 – 48 h, the intensity of expression by VEGF-A was

significantly increased ($p < 0.05$), as shown in figure 3.4, indicating strong participation in promoting tumour growth.

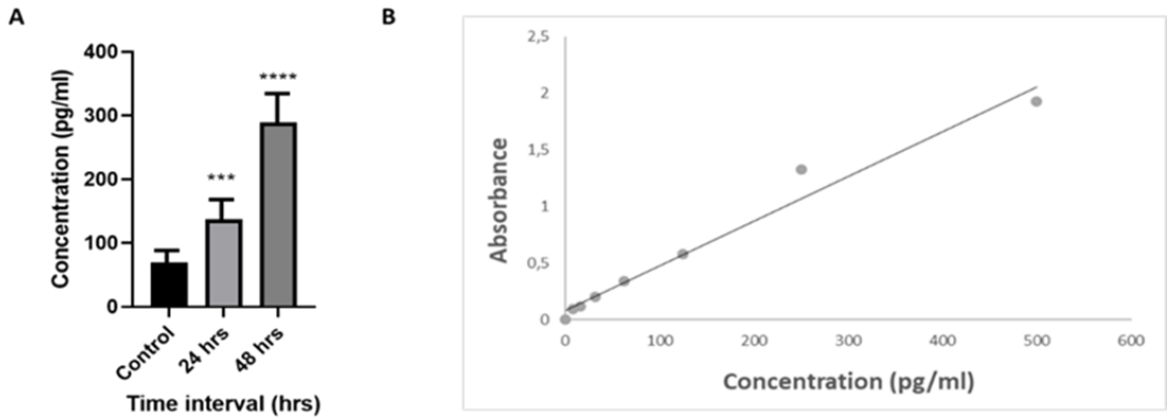

Figure 3.4: The expression levels of VEGF-A in melanoma B16-F10 cell line and the control endothelioma cells. **(A)** The concentration levels of VEGF-A protein at different periods. **(B)** Standard curve graph comparing the concentration of PDGF-BB against the absorbance reading. Each point represents the mean ± standard deviation of at least three independent experiments done in triplicates. A significant difference was found at $P < 0.05$.

When a similar comparison of variants was crossed over to the identified bFGF growth factor, as shown in figure 3.5, a significant difference was observed ($p < 0.05$) between the study samples at control, 24 and 48 h. Protein levels of bFGF exhibited exponential growth at 48 h, associating them with cell growth characterisation. Even though it might have seem to have been a slight change, a significant difference ($p < 0.05$) between the samples of the control and that of 24 h was found .

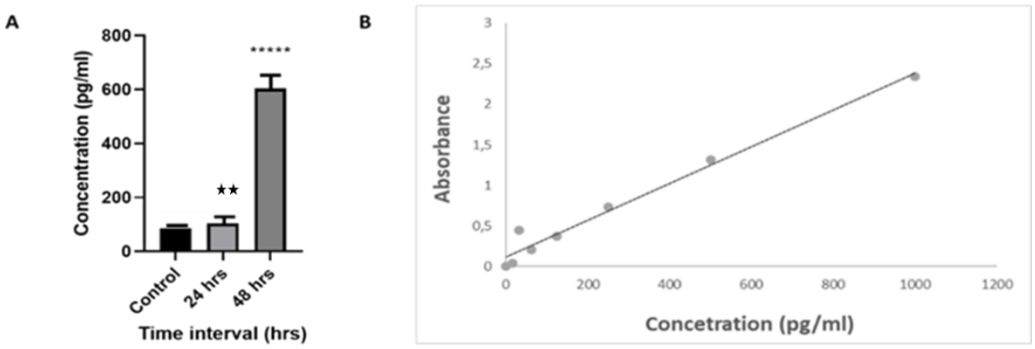

Figure 3.5: The expression levels of bFGF in melanoma B16-F10 cell line and the control endothelioma cells. **(A)** The concentration levels of bFGF protein at different times. **(B)** Standard curve graph comparing the concentration of bFGF against the absorbance reading. Each point represents the mean ± standard deviation of at least three independent experiments done in triplicates. Significance indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

Surprisingly, PDGF-BB proteins were not highly expressed in the control endothelial and the melanoma cells at 24 h, as illustrated in figure 3.6. Although a significant increase in protein expression was noted at 48 h compared to the cell samples at the control and 24 h, there was no significant difference found.

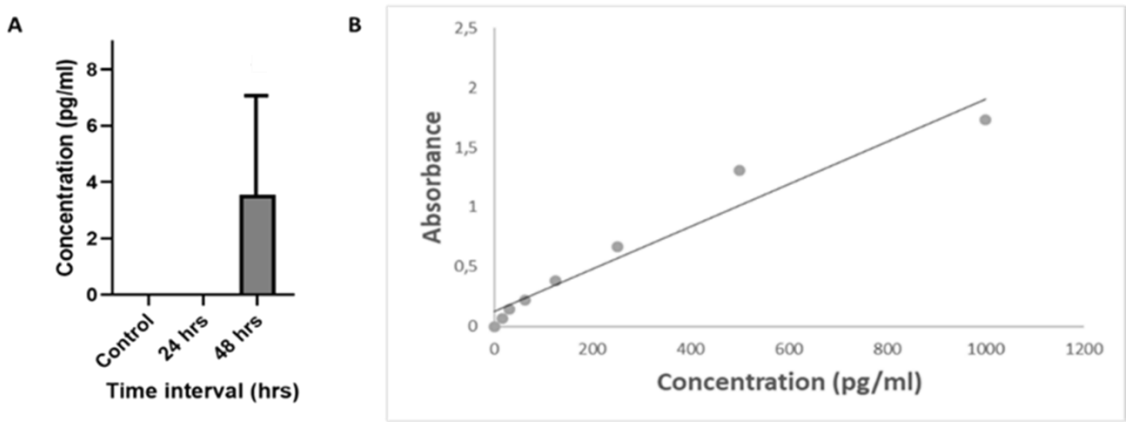

Figure 3.6: The expression levels of PDGF-BB in the melanoma B16-F10 cell line and the control endothelioma cells. **(A)** The concentration levels of PDGF-BB protein at different periods. **(B)** Standard curve graph comparing the concentration of PDGF-BB against the absorbance reading. Each point represents the mean ± standard deviation of at least three independent experiments done in triplicates. There was no significant difference found.

3.4. Histopathology of tumour tissue

To confirm the presence of ECs in cutaneous tissues, paraffin-embedded slides were prepared from a sample of haemangioma tissue biopsies for histology and IHC. As illustrated in Figure 3.7, the histological examination revealed dilated vascular lumens infiltrated with red blood cells. Figure 3.7.A. shows the fibrous stroma intervening with the dilated vascular channel. Furthermore, a monolayer of normal-appearing and well-differentiated ECs characterised the tumour vessels. As indicated by a blue arrow in Figure 3.7.B, small vascular channels with intervening fat lining were observed. No atypia was observed.

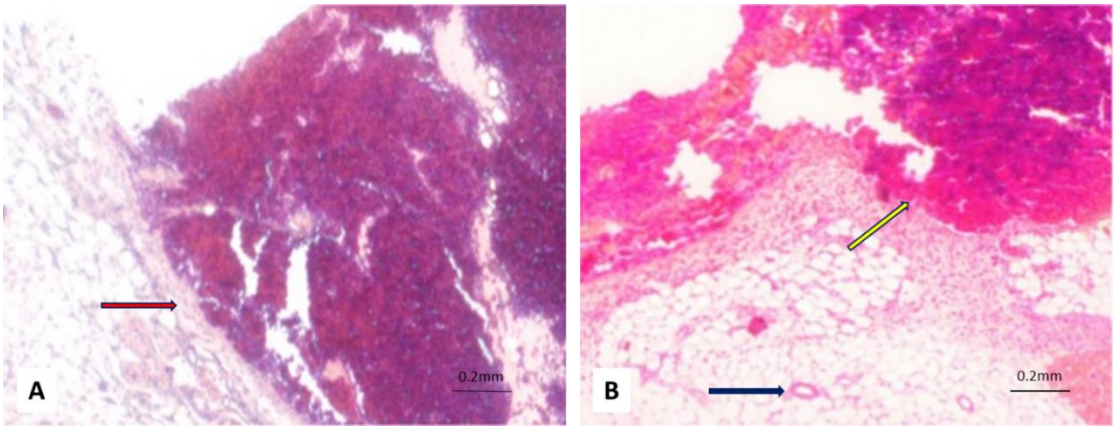

Figure 3.7: Histology and immunohistochemistry of cutaneous haemangioma. **(A)** A well-dilated vascular lumen with intervening fibrous stoma (red arrow). **(B)** Small vascular channels lined by adipose (blue arrow) and large vascular channels filled with red blood cells (yellow arrow).

3.5. Immunoexpressing Bcl-2 and VEGF-R proteins

In a cutaneous tumour, several different biomolecules that comprise the microenvironment play a significant role in tumour progression. Many of these molecules are optimally concentrated, and the expression of Bcl-2 and VEGF-R have been shown to have a role in

the cutaneous tumour environment. As such, cultured tumour tissues of haemangioma were evaluated for expressing Bcl-2 and VEGF-R using an immunoreactive assay. The sections were labelled with antibodies against Bcl-2 and VEGF-R, and the images were captured at multiple fields of view at 20x magnification.

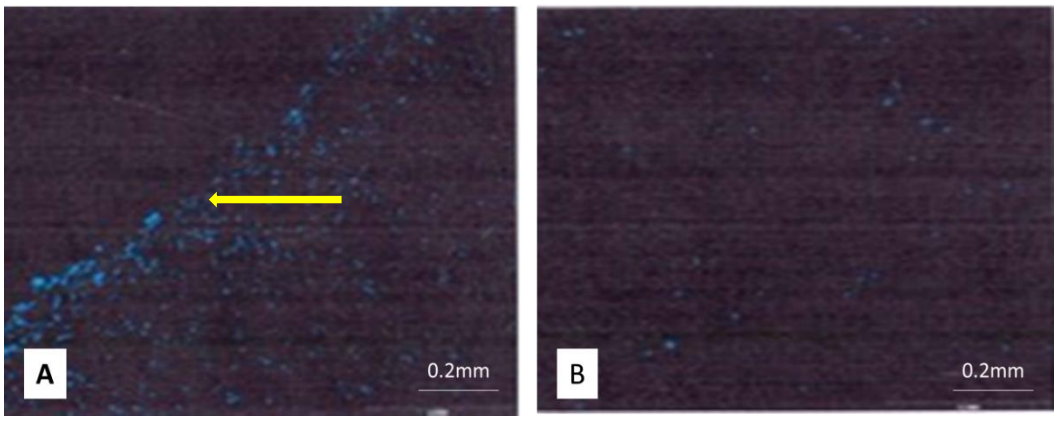

Figure 3.8: Representative images showing the expression of Bcl-2. The expression of Bcl-2 in **(A)** the control and **(B)** in the tumour.

The immunostaining of cells that responded positively to the antibodies against Bcl-2 in cultured specimens is shown in figure 3.9. The results showed very faint staining of Bcl-2 in a tumour section, suggesting a markedly reduced expression compared to the control. Intense staining of Bcl-2 was observed in EC lining the tumour vasculature, as indicated by an arrow in figure 3.9.B. As illustrated in figure 3.9, VEGF-R was highly expressed in endothelial cells of haemangioma, whereas low immunoreactivity of VEGF-R was exhibited in the control specimen.

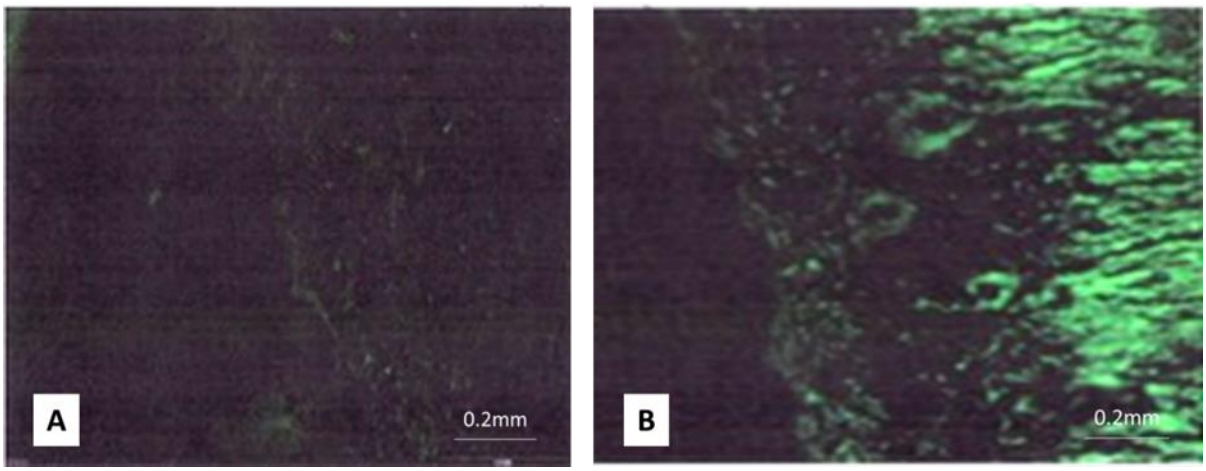

Figure 3.9: Representative images showing the expression of VEGF-R. The expression of VEGF-R in **(A)** the control and **(B)** in the tumour.

CHAPTER FOUR

DISCUSSION

4.1. Cell growth and differentiation

Endothelial cells (EC) are dynamic structures that form a building block of vessels that play a significant role in physiological and pathological angiogenesis. While angiogenesis is a highly regulated process controlled by a balanced, complex multifactorial system in physiological processes, in cancer, the induction of vessel formation is a crucial process that serves as an egress route, obliging tumour growth, invasion and metastasis (51). The activation of ECs in cutaneous tumours is believed to be activated along the gradient of tumour-promoting factors, initiating cells to proliferate, aggregate into disordered blood vessels and present with an aggressive phenotype. As a result, understanding the tumour microenvironment stress factors' influence on ECs to support their growth and survival requires in-depth knowledge (169).

In this current study, a time-dependent analysis was employed to evaluate the kinetic potential of ECs surviving under different medium conditions. Because various factors affect the growth pattern of ECs *in vitro*, the cell viability of ECs was tested in two different conditions, employing crystal violet (CV) as a deoxyribonucleic acid (DNA) stain. Cells were seeded and grown in melanoma-conditioned medium (MCM) and medium+serum. While the growth of cells was induced in EC grown in medium with serum at different time intervals, the control (serum-free medium: SFM) showed a reduction in cell density at both time intervals, indicating an antiproliferative effect. These results aligned with those reported by Danciu *et al.* (170), exhibiting a drop in growth fraction in all four cell lines of melanoma cultured in SFM while those cultured in medium with serum indicated growth activity. Most recently, Wu and Naar

(171) showed a plateaued growth in melanoma cells cultured in 0% serum medium, arguing that the cells might have survived due to *de novo* fatty acid and *de novo* cholesterol synthesis-activated gene expression. While the growth of cells in a serum-containing medium can be supported by the nutrients and growth factors that enable their survival, the apoptotic activity reported in cells cultured in SFM may be explained by the reduction in basal cellular activity, causing the proliferating cell to be more homogenous due to their sudden withdrawal from the cell cycle to enter the quiescent G0/G1 phase (172).

When the cells were cultured in MCM, a significant increase in cell viability was only noted after 48 h. These findings were consistent with Danciu *et al.* (170), postulating that the B16-F10 is a highly metastatic cell line after displaying a high absorbance value. Similarly, Liu *et al*. (173) showed an exponential growth curve in untreated B16-F10 cell lines. As a result, the high cell density observed in B16-F10 reflects the high proliferative activity in melanoma cells and rapid augmentation of the tumour (174).

The effectiveness of Nocodazole, a mitotic-blocking agent, which in part has been used in malignantly altered cells to suppress tumour cell growth by acting on and damaging the mitotic microtubules (mitotic spindles) was assessed for EC viability (175). From the present findings, the ECs appeared to have been susceptible to the growth-inhibitory effects of the drug. These results are coherent with those obtained in a previous study, demonstrating the permeability of EC in a dose-dependent way. In that study, Nocodazole was found to have compromised cell viability by disrupting the peripheral microtubules, triggering a cascade of reactions that resulted in vascular EC dysfunction (176). A similar observation was noted in *in vitro* studies where Nocodazole induced microtubule disassembly in EC, compromising cell viability (177) and inhibiting cell migration and growth in BME cell lines (178). The decreased cell viability in Nocodazole treated cells as seen in the present study can be accounted for by the fact that Nocodazole contains vascular-disruptive properties that directly inhibit proliferation, migration, and tube formation in endothelial cells *in vitro* by inducing cell arrest in G2/M (176,179-181). Also, while no apoptotic bodies might have been seen in morphological studies due to

inconclusive results from Nocodazole, CV however, showed a significant reduction in cell numbers following Nocodazole treatment (see annexure V).

After reaching confluence, the morphological appearance of cells adhered in different medium compositions was viewed under a PlasDIC light microscope. The cells revealed no morphological changes even after the incubation time had increased. However, there was an increase in cell confluence from both medium conditions, with melanoma B16-F10 conditioned media more susceptible to growth. These findings were coherent with the observations made by Das *et al*. (182) in an *in vitro* study where the behaviour of melanoma cell lines was investigated under hypoxic conditions. They suggested that the MCM prevented EC apoptosis, indicating viability. Sanooghi *et al*. (183) debated these findings, showing that after 48 h in culture, melanoma cells exhibited alterations in morphological characteristics. The use of different melanoma cell lines may be the cause of this discrepancy.

To enhance contrast and discerning between nuclei and cytoplasm in ECs, the H&E staining was employed to confirm the morphological characterisation of melanoma B16-F10 and that of sEnd.2 cell line. After leaving the slides to dry overnight, a large area of compromised cell density was observed, with an increased nucleocytoplasmic growth. Both the ECs of the control and conditioned medium showed morphological alterations. This heterogenic differentiation in cells can be explained by the heterogeneity influenced by the tumour cell secretions (184). Lasfar *et al*. (185) stated that B16-F10 is a poorly immunogenic tumour, characterised by inefficient major histocompatibility complex-restricted antigen-presenting. As such, B16-F10 cells expressing IFN-alpha may exhibit a decrease in tumorigenic, causing them not to proliferate.

Conversely, Chen (186) conflicted with the results of the current study, stating that the cells of B16-F10 displayed progressive, and aggressive growth. The cells featured clear and regulated cytoplasm, as well as nuclei with prominent nucleoli. According to Zhang *et al*. (187), the degradation in morphological characteristics may be evidence of G-protein coupled receptor-4 (GPR4), intriguing structural distraction and genetic engineering. In tumour cells, GPR4 is

believed to establish advantageous growth or inhibit growth, depending on which tumour type of common skin cancer (188). If the cell line of the current study significantly expressed GPR4, it may have modified their cell morphology and inhibited their ability to proliferate and migrate.

4.2. The molecular expression of pro-angiogenic markers

In 1939, Ide suggested that tumour cells secreted specific molecular factors capable of initiating the growth of blood vessels (189). This discovery formed the foundation of Folkman's intuition, suggesting that tumour growth and metastasis are angiogenic dependents (23). Based on these findings, anti-angiogenic therapy was introduced. Systematically, the treatment allows growing tumour cells to be starved of nutrients and oxygen by eliminating the density of microvessels in the microenvironment using angiogenic inhibitors. Even though several factors that influence the development of distorted vessels in the cancer microenvironment have been identified and studied, the dynamic nature of pro-angiogenic factors and their mechanisms require in-depth knowledge (94).

During the growth of cutaneous tumour cells, various growth factors and cytokines are secreted into the tumour microenvironment. Their receptors in different stages of tumour progression respond to autocrine and paracrine effects that support tumour growth by acquiring the ability to develop vessel streams for metastasis (50, 82, 190). However, the expressed angiogenic factors have yielded conflicting results. For this reason, it was assumed that the variability in immunochemistry protocol differences in the tumour location, the choice of antibodies, and cut-offs might be the cause (191).

Due to complex interactions of a high order between molecules constituting different regulatory networks, our understanding of how a particular factor function still do not entirely clarify the emerging new features, although the functional roles of the growth factors in angiogenesis have been previously studied. Subsequently, the present study investigated vesicular endothelial growth factor (VEGF-A), basic fibroblast growth factor (bFGF) and platelet-derived

growth factor-BB (PDGF-BB) expression using ELISA to study their potential in malignant B16-F10 cells.

Vesicular endothelial growth factor-A is an essential angiogenic-promoting factor. Its participation in EC growth and proliferation has been extensively studied (90). In support of this, the levels of VEGF-A in melanoma B16-F10 cells exhibited high expression compared to the control ECs, leading us to believe that the growth of tumour cells is mediated by VEGF autocrine loop signalling pathway, stimulating tumour growth metastasis (192). Mehnert *et al.* (193) braced these findings by observing intense VEGF expression in melanoma cells compared to benign ones, suggesting that VEGF is highly associated with tumour progression. Coherently, Ni *et al.* (194) found a correlation between the overexpressed VEGF-A and melanoma growth, proliferation and invasion *in vitro*. Using biopsy samples, Rajabi *et al.* (58) also showed a significant relationship between the intensity of VEGF and tumour progression. Their findings postulated that the elevated levels of VEGF in growing tumour cells are influenced by hypoxia and are associated with the transformation of melanocytes. While several studies support the effect of VEGF-A on disease progression, some previous studies showed conflicting results (195, 196). The fault behind this discrepancy lies in using different techniques to identify or quantify the expression of VEGF-A and the sensitivity of conjugative antibodies.

The bFGF mediates the development of tumour cells by stimulating angiogenesis and induces mitogenic effects on ECs and fibroblasts. Its expression in melanoma cells is functionally known for its role in cell differentiation *in vitro* (106). In 2017, Ghassemi *et al.* (197) provided evidence that the expression of FGF5 in melanoma cells exhibited pro-tumorigenic function. Similarly, the proliferative effect of melanoma B16-F10 by bFGF secretion was investigated, employing anti-bFGF. While the control cells proliferated, the anti-bFGF-treated cells showed growth inhibition. As a result, the induction of apoptosis in melanoma cells by bFGF antibodies was assumed to have been caused by the blockage of its survival pathway, proving bFGF to be a pro-tumorigenesis (195). These results are in line with the observations of the present

study, exhibiting overexpression of bFGF in the studied melanoma cells, indicating tumour growth and survival. Interestingly, the findings of the current report demonstrated that the increased levels of bFGF in a time-dependent manner led to an increase in VEGF-A expression. Subsequently, this raises an argument that the bFGF may promote angiogenic and tumour growth by directly stimulating ECs or indirectly by upregulating VEGF-A (198).

In vitro studies have shown that PDGF-BB can directly induce EC proliferation, migration, and tube formation in melanoma cancer. Despite its role in fibroblast proliferation, the production of PDGF-BB by the tumour endothelium is essential for adequate recruitment and proper integration of pericytes into the vascular wall (101, 102). While primary studies correlated a positive expression of PDGF-BB with tumour progression (199, 200), literature speculated that B16-F10 cells lack the presence of PDGF-BB or contain low expression (101), as observed in the present study. A reasonable explanation for these results may be a reflection of interdependent ECs and pericytes to which the disruption of normal EC-pericyte homeostasis by tumours occurs, releasing factors that participate in tumour expansion and metastasis (201-203). In this context, VEGF-A often plays a significant role. An over production of this factor may cause a downregulation of pericyte coverage leading to low PDGF-BB expression and leaky vessels (204, 205).

4.3. Histopathology of cutaneous tumours

While angiogenesis is an essential aspect of tumour growth, histological images were developed, following H&E staining, to characterize the presents of the blood vessels and their surrounding tissues. As observed in the present study, Kita and Long (206) demonstrated that the histological features of haemangioma comprised vascular channels of different sizes and a single layer of normal-appearing ECs around the vessel's lumen. These observations were also described by Luca *et al*. (207) in haemangioma sections, stating that the vessel lining had one or two layers of spindle-shaped nucleated ECs. Aboutalebi *et al*. (208), argued that the haemangioma capillaries are often surrounded by fibrofatty stroma. Collectively, these

features indicate proliferative ECs, supporting their role in the angiogenic formation and tumour growth (206, 208).

4.4. The expression of Bcl-2 and VEGF-R

Over the past years, understanding the molecular bases of a tumour microenvironment has been the centre of attention in various tumour types. Molecular studies have identified numerous biological factors that regulate the development and maintenance of cutaneous tumours to ensure tumour growth and survival (50). Given this, the expression of Bcl-2 and VEGF-R in various cutaneous microenvironments has been correlated with tumour growth and angiogenesis, despite the reported conflicting results (117, 209, 210). As such, the present study investigated the expression of Bcl-2 and VEGF-R, employing IHC analysis.

The expression of Bcl-2, a protein addressed in various cutaneous tumour studies for its role as a critical regulator for apoptosis resistance in malignant melanoma and non-melanoma biopsy samples, was correlated with its role in tumour progression (211). Among these studies, the expression of Bcl-2 in melanoma and BCC patients was associated with poor prognostic significance through multivariate IHC analysis (212, 213). Most recently, Yang *et al*. (214) and Tang and Zhang (117) detected Bcl-2 in proliferative haemangioma tissues, suggesting that its expression promotes tumour growth by inhibiting endothelial apoptosis.

While the control section expressed high levels of Bcl-2 in the present study, the tumour had a reasonably low expression, suggesting that the presence of Bcl-2 in haemangioma is not always the case. These results aligned with those observed in SCC or haemangioma tissue samples where the Bcl-2 proteins could not be identified (209). For this reason, Morales-Ducret *et al*. (211) argued that the histogenetic differences that are presented by a pool of different cell populations in the cutaneous tumour microenvironment might account for this discrepancy.

The expression of VEGF-R in haemangioma tissue culture was observed in a previous study, suggesting its involvement in a broad spectrum of cutaneous neoplasm (215). These results

were supported by the present study, and such consistency was also found in a study by Xu (216), where high levels of VEGF-R expression in proliferative haemangioma were detected compared to involuting haemangioma and normal tissue. In addition to these studies, Ou *et al*. (217) demonstrated that the presence of VEGF-R2 played a proliferative role in haemangioma in a knock-out study that leads to the viability of tumour cells decreasing. As seen in the present study, Nye *et al*. (218) described a strong expression of VEGF-R around the ECs lining the vasculature of non-melanoma skin cancer. Thus the expression of VEGF-R in cutaneous tumours, haemangioma, in particular, supports the concept that VEGF-A is an essential molecular factor that promotes tumour progression by stimulating angiogenesis (210, 218).

CHAPTER FIVE

CONCLUSION

A wide variety of factors that can initiate a cascade of events leading to endothelial cell (EC) sprouting have been studied to better understand this process, particularly in a cutaneous tumour microenvironment. Cell growth and morphological studies for endothelial sEnd.2 and melanoma B16-F10 cell lines, employing crystal violet and light microscope, respectively, were used. The data obtained from the assays employed in the present study allowed us to conclude that a cutaneous tumour is the most proliferative and aggressive type and the induction of cell proliferation reflects DNA damage caused by impaired DNA replication.

Secondly, the investigation sought to understand the crucial pathophysiological determinants of cutaneous tumour cells by quantifying angiogenic proteins providing insights that may further guide the discovery of novel regulators of skin cancer biology and result in the implementation of new treatment rationales for therapeutic benefit. We evaluated the corresponding levels at which vesicular endothelial growth factor (VEGF-A), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF-BB) were expressed. Unlike PDGF-BB, the VEGF-A and bFGF database results suggested their pattern of involvement in proliferating ECs of melanoma. At the same time, VEGF-A is perhaps the most critical factor in promoting neovasculature in most tumour cells of cutaneous and providing the most potent treatments. Nevertheless, the role of supporting elements such as bFGF and PDGF-BB still requires more understanding.

Finally, while the histological features of haemangioma may present with dilated blood vessels that feed into the development of the tumour, the involvement of Bcl-2 in endothelial

haemangioma was not demonstrated, despite the contradicting results obtained from previous studies, suggesting the heterogeneity of this tissue allows the growth of the tumour despite the anti-apoptotic functionality of Bcl-2. The expression of VEGF-R, however, was increased, suggesting its involvement with VEGF for tumour growth.

Although numerous *in vivo* and *in vitro* studies have provided practical tools for angiogenic promoting factors, we are still far from a complete understanding of their mechanisms that can lead to better therapeutic initiations for effective inhibition of a single critical step in the cascade, completely suppressing angiogenesis and therefore tumour growth. However, the collectively identified molecular markers in the present paper serve as potential prognostic markers, enabling clinicians to diagnose the disease early and lower its burden through revolutionised anti-angiogenic therapy.

CHAPTER SIX

RECOMMENDATIONS

6.1. Recommended prospects

The process of pathological angiogenesis in melanoma and non-melanoma tumours remains the major clinical challenge for tumour development and metastasis. Undoubtedly, the underlining mechanisms of this pathology still require further detailed investigation. Because angiogenesis involves several molecular markers and signalling pathways that act synergistically, studying these two objectives on tissue organoids or 3-D cultured cell lines could provide an in-debt understanding of the disease. Secondly, Due to time constraints, the angiogenic molecular factors discussed in this paper were by no means exhaustive. As such, factors such as Ang-2, PlGF, IL-8, MMP-2 and MMP-14 in benign and metastatic melanoma tumour cell lines and tissues using protein analytic assays.

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ANNEXURE I: Declaration

DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

Full names of student **:** Masibulele Nonxuba

Student number **:**20799978

Topic of work **:**Investigation of the expression of pro-angiogenic markers in cutaneous tumours *in vivo* and *in vitro*

DECLARATION

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this protocol (e.g., essay, report, project, assignment, dissertation, thesis, etc.) is my own original work. Where other people's work has been used (either from a printed source, Internet, or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow anyone to copy my work with the intention of passing it off as his or her own work.

.....

SIGNATURE

ANNEXURE II: Ethical clearance letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

11 November 2022

Approval Certificate
Annual Renewal

Dear Mr M Nonxuba,

Ethics Reference No.: 660/2020 – Line 6
Title: Investigation of the expression of proangiogenic markers in melanoma in vivo and in vitro

The **Annual Renewal** as supported by documents received between 2022-10-26 and 2022-11-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-11-09 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-11-11.
- Please remember to use your protocol number (660/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Professor Werdie (CW) Van Staden
MBChB, MMed(Psych), MD, FCPsych(SA), FTCL, UPLM
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

ANNEXURE III: MSc committee letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

6 October 2020

Dr P Mabeta
Department of Physiology
Faculty of Health Sciences

Dear Dr,

Mr M Nonxuba, Student no 20799978

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

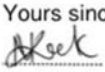
Student name	Mr Masibulele Nonxuba	**Student number**	20799978
Name of study leader	Dr Peace Mabeta		
Department	Human Physiology		
Title of MSc	Investigation of the expression of proangiogenic markers in melanoma *in vivo* and *in vitro*		
Date of first submission	August 2020		

MSc Committee, School of Medicine
Faculty of Health Sciences
University of Pretoria,
Private Bag X323
Pretoria 0001, South Africa
Tel +27 (0)12 319 2325
Fax +27 (0)12 323 0732

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

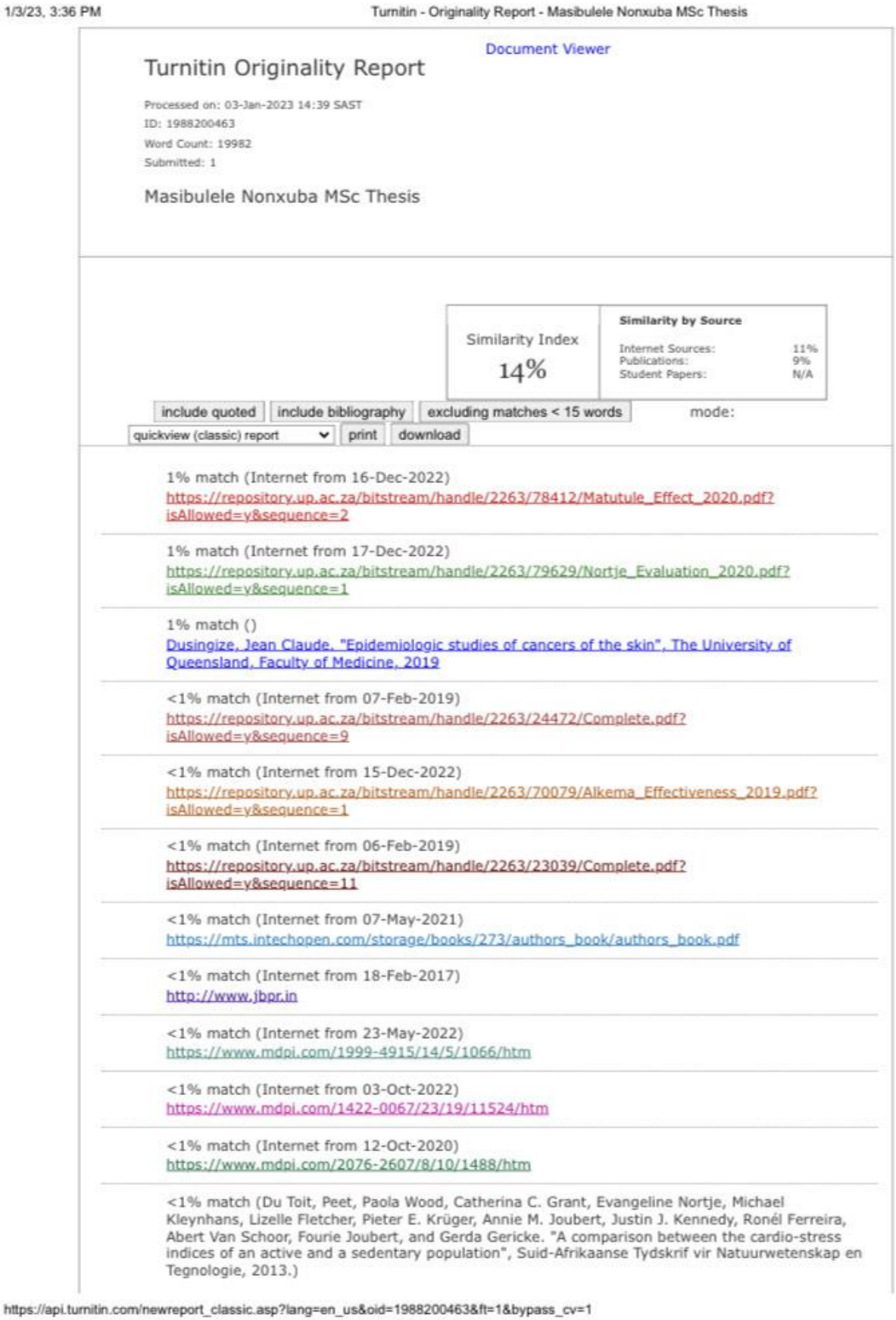
Comments to study leader August 2020	• Please update the MoA –supervisor's initials on each page. • Include the two cell lines in the objectives. • The methodology needs to be clear and sufficiently detailed. The principle of a method should not be included in the materials and methods. • Clarify and confirm the biopsy samples that you will receive. Include a statement that Mr Matlala and you will be working on a larger study to characterise melanoma. • Different stages of melanoma will be included – please explain how this will be analysed further. • How will protein concentration be determined? Different contradicting statements make it difficult to determine how it will be done. • Western blot method should be expanded on – more detail is required. Explain how expression will be measured – compared to what? • Secondary antibodies are not stated in your protocol. Please expand and provide more detail. • Correct all typographical errors and terms used incorrectly, e. g. antigen repair versus correct term antigen recovery; wrong buffers indicated in methodology (MOPS vs TRIS glycine buffer). Capitalize the "w" in Western blot. • Please include for each method the expected results, how the results will be interpreted, and how the results will be captured and stored. • Revise the reference list and correct inconsistencies. • Include a detailed description of the microscopy to be done. • Please include a description in the data management section how the candidate will comply with the new UP research data management policy. All metadata and final data should be uploaded on the UP research data management system.
October 2020	• Thank you for submitting the revised protocol and requested documents.
Decision	This protocol has been provisionally approved. Please submit the revised protocol to ethics, and supply the MSc committee with proof of acceptance. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.

Yours sincerely



Prof Marleen Kock
Chair: MSc Committee

ANNEXURE IV: Turnitin report



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ANNEXURE V: Nocodazole-treated morphological cells

The sEnd.2 cell lines were treated with Nocodazole at 0.01 µg/mL; 0.1 µg/mL; 0.25 µg/mL for 48 h in a 96-well plate. After 48 h of incubation, the plate was placed under the PlasDIC microscope to determine the drug effects on the cell morphology. As such, server cell detachment was observed in cells treated with Nocodazole at 0.1 µg/mL and 0.25 µg/mL drug concentration as indicated in Figure 1C and 1D. The cells appeared to be round with an undefined morphology. In contrast, the cells treated at 0.01 µg/mL showed not much change compared to the control as seen in figure 1A and 1B. The cells maintained their well-defined morphology, however, fewer than those of the control.

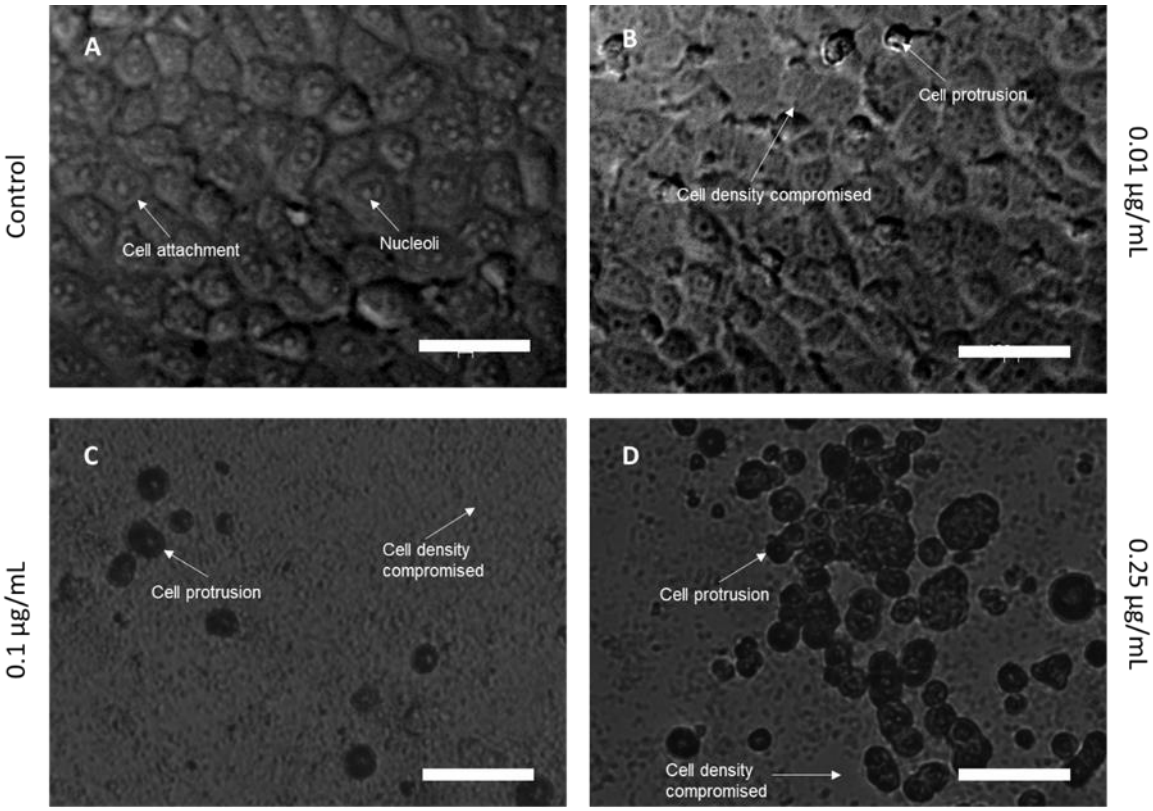

Figure 1: Morphological observation of sEnd.2 cells treated with Nocodazole after 48 h. Cells were either grown in (A) normal medium, or treated with Nocodazole at (B) 0.01 µg/mL, (C) 0.1 µg/mL, and (D) 0.25 µg/mL. Images were captured at 40X magnification with a scale bar of 100µm.

The findings of the present paper are coherent with a previous study, where the adherence to Nocodazole-treated ECs was found to have significantly decreased compared to the untreated cells (1). This repetitive influence of Nocodazole on the ECs as seen in the present paper and those of the past is thought to be caused by its ability to readily induce microtubule disassembly, causing the disruption of the actin cytoskeleton, a key role player in cell structure maintenance (2).

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