Effect of tumor microenvironment-derived factors on melanoma cell growth and drug-response: an *in vitro* study in three-dimensional cultures

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Abstract

The incidence rate of malignant melanoma is increasing in Norwegian population as well as worldwide. Malignant melanoma is one of the most aggressive human cancers showing exceptional abilities to metastasize and develop resistance to therapy. Currently there are no effective treatments against metastatic melanoma. Traditionally, melanoma aggressiveness was linked to intrinsic properties of the malignant cells themselves. However, it is becoming apparent now that the tumor microenvironment (TME) – non-malignant (stroma) cells, soluble molecules and extracellular matrix components – can play an important role in modulating metastatic properties and sensitivity to drugs in cancer cells.

In the present work we investigated how various TME factors like extracellular matrix components (fibronectin and laminin) and soluble factors released from mice organs – common sites of melanoma metastasis - affected melanoma cells, specifically their metastasis-associated properties like growth/proliferation and sensitivity to the experimental dug Elesclomol. The study was based on three-dimensional (3D) *in vitro* cultures, where melanoma cells were grown in a Collagen or Matrigel matrix in the presence of investigated factors of TME. Two melanoma cells lines, Melmet 1 and Melmet 5 derived from the metastatic melanoma patents with different clinical indications were employed.

It was observed that fibronectin and laminin did not have a notable effect on cell growth or viability. However, the soluble factors from the organs showed a slight stimulating effect on cell growth and a notable effect on cell morphology and growth pattern. The latter was especially pronounced for the bone marrow-derived factors. Comparison of the sensitivity of Melmet cells to Elesclomol in 3D versus 2D revealed that 3D cultures were less sensitive to the drug, and that Melmet 5 was less sensitive compared to Melmet 1. The sensitivity was not modulated by the soluble factors derived from the healthy or metastatic brain.

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Content

Abstract……..3
Acknowledgment……..4
Content……5
Abbreviations……7
1. Introduction……9
1.1 Melanoma: clinical aspects and biology……9
1.2 Tumor microenvironment (TME) and its role in metastasis……..12
1.3 Melanoma therapy……16
1.4 Three-dimensional *in vitro* cultures – an in vitro model to study cancer properties in the setting of TME……19
Aim of the study……..21
2. Materials and methods……..23
2.1 Cell lines: Melmet 1 and Melmet 5 ……23
2.2 General cell work: cell culturing in 2D……24
2.3 Cells adhesion to extracellular matrix (ECM) proteins……26
2.4 Cell culturing in 3D……27
2.4.1 Collagen cultures……27
2.4.2 Matrigel cultures……29
2.4.3 Treatment with Elesclomol in 3D……30
2.4.4 3D cultures supplemented with fibronectin and laminin……30
2.4.5 3D cultures supplemented with organ-conditioned medium……31
2.4.6 3D cultures supplemented with organ-conditioned medium and treated with Elesclomol……33
2.5 Evaluation of cell viability and growth: measurement methods……..33
2.5.1 Cell viability measurement based on Calcein staining……33
2.5.2 Staining with Propidium Iodide for visualization of dead cells……34
2.5.3 Evaluation of cells metabolic activity by the MTS assay (“Cell Titer 96®Aqueous Cell Proliferation Assay”)……34
2.5.4 Evaluation of cell growth/proliferation by measuring intensity of the DsRed signal……34
3. Results……..37
3.1 Evaluation of melanoma cell growth and viability in 3D cultures……37

3.1.1 Optimization of culturing conditions for Melmet 5 in collagen matrixes: determination of suitable cell amount and serum concentration……..37
3.1.2 Evaluation of Melmet 1 growth in 3D matrixes……..40
3.2 Investigation of the effect of extracellular matrix (ECM) proteins……43
3.2.1 Evaluation of Melmet cell adhesion to various ECM proteins……43
3.2.2 Evaluation of the effect of the ECM proteins fibronectin and laminin: Melmet 5……..44
3.2.3 Evaluation of the effect of the ECM proteins fibronectin and laminin: Melmet 1 in collagen matrix……..48
3.3 Effect of organ-conditioned medium……49
3.4 Therapy-related studies……..55
3.4.1 Effect of Elesclomol on Melmet 5 in 2D and 3D cultures……55
3.4.2 Effect of organ-conditioned medium on Melmet 5 sensitivity to Elesclomol in 3D……57
3.4.3 Effect of Elesclomol on Melmet 1 in 2D and 3D cultures……58
3.4.4 Effect of organ-conditioned medium on Melmet 1 sensitivity to Elesclomol in 3D……62
4. Discussion……63
Conclusion……68
Future plans……...69
References……..70

Abbreviations	
Melmet 5	Melanoma 5 cell line
Melmet 1	Melanoma 1 cell line
ECM	Extracellular matrix
DMEM	Dulbecco's Modified Eagles medium
2D	Two-dimensional
3D	Three- dimensional
RPMI	Roswell Park Memorial Institute Medium
FBS	Fetal Bovine Serum
EDTA	Ethylenediaminetetraacetic acid
FN	Fibronectin
LN	Laminin
CM	Conditioned medium
TB	Trypan Blue
DTIC	Dacarbazine
BM	Bone Marrow
TME	Tumor Micro Environment
BRAF	V-raf murine sarcoma viral oncogene homolog B1
NRAS	Neuroblastoma RAS viral oncogene homolog
FDA	Food and Drug Administration
MAPK	Mitogen-activated protein kinase
CDKN2A	Cyclin dependent kinase inhibitor 2A
PTEN	Phosphatase and tensin homologue
PI	Propidium iodide
TRPMI1	Melastatin 1
MITF	Microphthalmia-assosiated transcription factor
αVß3	AlphaVbeta3 dimer
IL-2	Interleukin-2
ROS	Reactive oxygen species
TMZ	Temozolomide
TGFβ	Tumor Growth Factor β
MMP	Matrix Metalloproteinases
TAF	Tumor-assosiated fibroblasts
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor
BMDC	Bone marrow-derived cells
GIST	Gastrointestinal stromal tumor
Hsp	Heat shock protein
PDGFR	Platelet-derived growth factor receptor
VEGFR	Vascular endothelial growth factor receptor

1. Introduction

1.1 Melanoma: clinical aspects and biology

Melanoma is among the cancers that are rapidly increasing in Norway as well as worldwide. There are registered about 1300 new cases of malignant melanoma of the skin in Norway in 2008, and this is the second most common cancer (11%) after testicular cancer (20%) in men aged 25-49 years. In young women aged 15-24 years, melanoma is the second most common cancer (13%), after tumors of the central nervous system (21%) *(*Cancer in Norway 2008, Bray F.,Grimsrud T.,Haldorsen T., Cancer Registry of Norway 2009*)*. Malignant melanoma is the most dangerous form of skin cancer accounting for more than 70% of all skin cancer-related deaths (Villanueva and Herlyn 2008). According to the World Health Organization, the incidence of malignant melanoma is increasing faster than any other cancer in the world, especially in women younger than 40 years and in men older than 40 (Erickson and Driscoll). So it seems that melanoma mostly is affecting young and middle-aged people, unlike most other solid tumors, which mainly affect older adults. There exist several risk factors for the development of cutaneous malignant melanoma (see **Table 1**), but accumulating evidence suggests that exposure to sunlight and other sources of ultraviolet irradiation is the critical one (Gallagher, Spinelli et al. 2005).

Table1. Risk factors which increase the risk for development of cutaneous malignant melanoma.

Risk factors for cutaneous malignant melanoma
History of melanoma or nonmelanoma skin cancer
Family history of cutaneous malignant melanoma
Atypical nevi or numerous nevi
History of severe (blistering) sunburns or intense intermittent sun exposures
Light skin, blond hair
Giant melanocytic nevus

Patients with early stages of melanoma (when lesions are less than 1mm thick without metastases) have a good prognosis, because a lesion can be removed by surgery, which is currently the only curative treatment. However, melanoma can

progress rapidly from a surgically curable lesion to an aggressive metastatic disease (**Figure 1**), able to establish metastases in multiple organs and showing poor response to current therapies, resulting in high mortality. Thus, the prognosis for melanoma patients with distant metastases is very bad: a median survival is of only 6–9 months and a 5-year survival rate of less than 5% (Agarwala 2009).

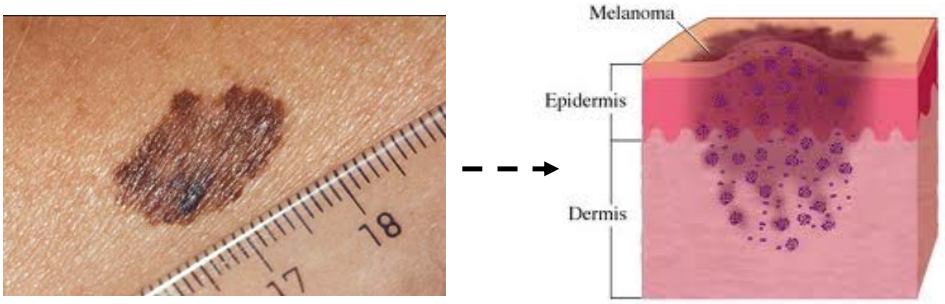


Figure 1. Melanoma lesion. Melanoma can progress rapidly from a surgically curable lesion in the epidermis to an aggressive metastatic disease invading the dermis.

Melanoma arises from malignant transformation of melanocytes which are pigmented cells found predominantly in the skin and eyes (Gray-Schopfer, Wellbrock et al. 2007). The progression from normal melanocytes to metastatic disease is a multistep process (see **Figure 2**). It starts with structurally normal melanocytes as benign nevi and develops to dysplastic nevi with structural atypia. These two phases can be identified as primary events in melanocytic abnormal proliferation. Further it is followed by a radial growth phase (a primary tumor), vertical growth phase which has a potential for metastasis and ends with metastatic melanoma. A primary tumor grows horizontally through the epidermis; over time, a vertical growth phase component intervenes and melanoma increases its thickness, invades the dermis and disseminate to regional lymph nodes and distant organs, like brain, lungs, liver (Miller and Mihm 2006; Gaggioli and Sahai 2007).

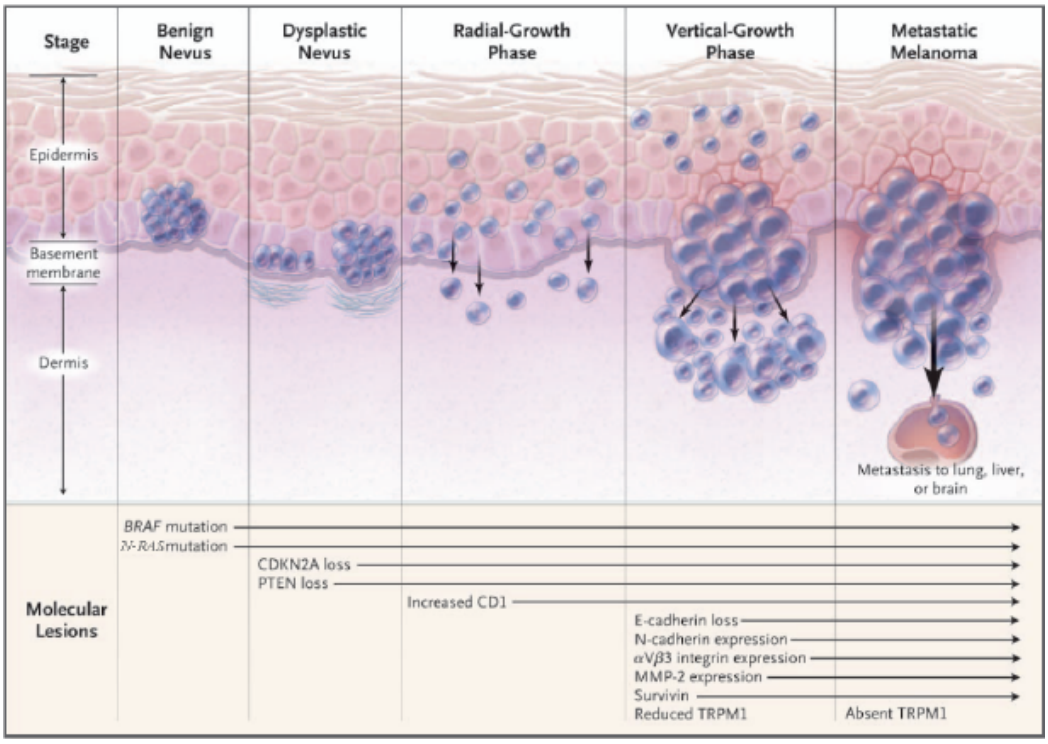


Figure 2. Melanoma progression. Mutations in BRAF and NRAS initiate the abnormal activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which disrupts growth control in normal melanocytes in benign nevi. Inactivation of cyclin dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathway, which encodes tumor-suppressor genes, triggers development of cytologic atypia in dysplastic nevi. Further progression of melanoma is associated with the overexpression of the CD1 oncogene and the decreased expression of melastatin 1(TRPMI1) and other genes underlying melanin synthesis, which is regulated by microphthalmia-assosiated transcription factor (MITF). The next step in development, i.e. progression from a vertical-growth phase to metastatic melanoma.This process is associated with alterations in control of cell adhesion: loss of E-cadherin and expression αVβ3 integrin, which induces expression of matrix metalloproteinase 2 - an upregulated enzyme in invasive melanoma that promotes melanoma cell dissemination via degradation of the collagen in basement membrane. In addition, αVβ3 integrin increases expression of the prosurvival genes and stimulates the motility of melanoma cells through the reorganization of melanoma cytoskeleton leading to formation of metastasis (adapted from Miller and Mihm, 2006).

To understand the onset of malignancy substantial advances have been made on genetic level. The genetic changes, associated with biology underlying the initiation and progression of melanoma, include: (a) mutation of BRAF and NRAS and consequently up-regulation of the RAS/RAF/MEK/ERK pathways; (b) mutation in PI(3)Ks pathway and loss of PTEN function; (c) down-regulation of the retinoblastoma protein (RB) by increased cyclin D1 or CDK4 activity; (d) inactivation of the CDKN2A p16 suppressor of CDK4; (e) intermediate levels of MITF, which regulate the melanogenesis and survival (see **Figure 2**) (Miller and Mihm 2006; Gray-Schopfer, Wellbrock et al. 2007).

Invasion and spread of melanoma are associated with alterations in cell adhesion. Integrins are main receptors for cell adhesion to extracellular matrix proteins, which regulates a variety of cellular processes, including proliferation and cell survival (Hynes 2002). The main ECM components that integrins bind to are collagen, laminin and fibronectin. The integrin alphaVbeta3 dimer appears to be particularly important for the invasive potential of melanoma cells (**Figure 2**) (Dang, Bamburg et al. 2006).

Other adhesion molecules, cadherins are part of larger protein complexes that link cell adhesion to control of cell morphology, motility, and intracellular signalling events. The biology of normal melanocytes is tightly controlled by keratinocytes that interact via E-cadherin. Loss of E-cadherin and gain of N-cadherin is a characteristic event associated with the disease progression from a vertical growth phase to metastasis (**Figure 2**). Increased levels of N-cadherin enable melanoma cells to interact instead with fibroblasts and vascular endothelial cells in the dermis (Smalley, Brafford et al. 2005). Thus, cells lacking E-cadherin and expressing N-cadherin are able to survive and as a result invade distant tissues effectively (Hsu, Meier et al. 2000).

Moreover, cell invasion is mediated by proteinases such as matrix metalloproteinases (MMPs). MMPs belong to a family of calcium- and zinc-dependent endopeptidase that can degrade and digest a wide range of interstitial and basement membrane proteins in the extracellular matrix. MMPs, particularly MMP-2 and MMP-9 are implicated in tumor cell invasion and represent an important stage of tumor progression by its expression in invasive melanoma (Simonetti, Lucarini et al. 2002; Schnaeker, Ossig et al. 2004).

1.2 Tumor microenvironment (TME) and its role in metastasis

One of the main hallmarks of malignant melanoma is its exceptionally high ability for metastasis. Metastasis is a very complex process. First, tumor cells have to leave the primary tumor by loosening cell-cell contacts and adhering to and degrading extracellular matrix (ECM). Then the tumor cells migrate through tumor endothelial basement membrane and through or between endothelial cells to enter the blood or lymphatics- intravasation process. Once in circulation, the tumor cells have to survive i.e. avoid immune recognition and resist the process of programmed cell death. Surviving cells can become arrested in the capillaries of distant organs,

where they must adhere to the endothelial basement membrane and extravasate into the tissue. Then, the metastasizing tumor cells have to survive the stresses of a new microenvironment characteristic for this organ, proliferate there, and successfully grow into a measurable metastatic lesion (see **Figure 3**) This distant site progresses can also develop through single cells, which might remain dormant for years (Steeg and Theodorescu 2008).

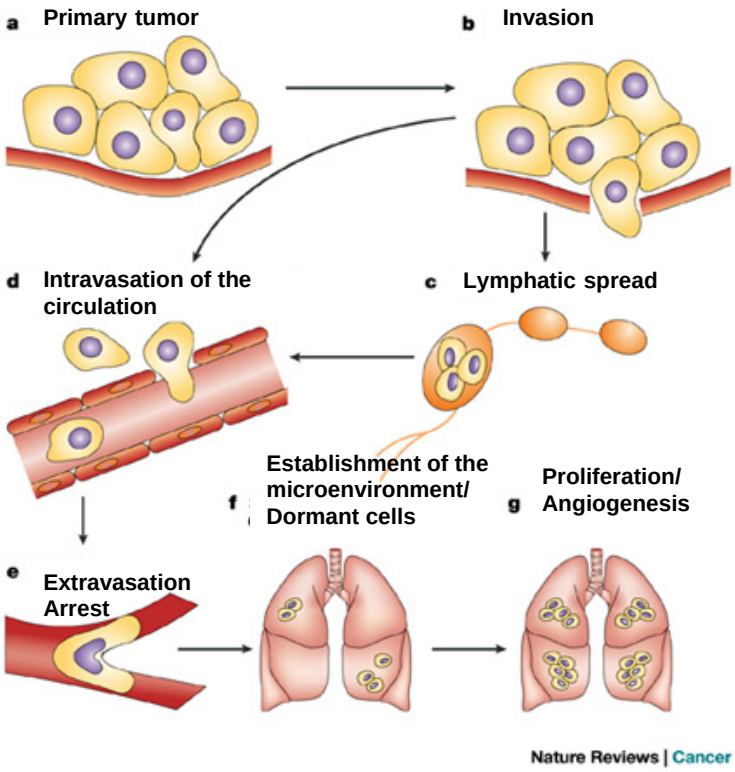


Figure 3. A schematic presentation of metastatic process. A- represents tumor growth, **b**- invasion, which requires reversible changes in cell–cell and cell–extracellular-matrix adherence, **c**- cells can metastasize via lymphatic or **d**- directly enter the circulation, **e**- survival and arrest of tumor cells and extravasation occurs next, **f**- establishment of the microenvironment through metastasizing cells or dormant cells, **g**- tumor are now progressively growing, development of a vascular network occurs (adapted from Steeg, 2003).

Earlier it was though that metastasizing cancer cells themselves independently could overcome all these obstacles along the metastatic cascade. However, now it is becoming more and more clear that tumor microenvironment (TME) plays a very important role in assisting tumor cells in metastasis. Interestingly, the association between TME and metastasis as a “Seed and soil” hypothesis was first time suggested by Stephen Paget over a century ago in 1889 to explain organ preferred metastasis. This hypothesis suggested that tumor cells (seed) can colonize

only these distant organs (soil) where there is a favorable environment for tumor growth. Stephen Paget concluded that metastases resulted only when the seed and soil were compatible (Paget 1989). However, only recently Pagets ideas received a lot of attention, and TME in cancer progression, metastasis and drug-response became a very actively investigated topic.

Tumor microenvironment generally consists of extracellular matrix (ECM) and different type of non-malignant cells like fibroblasts, endothelial cell and bone marrow-derived cells (BMDC), which include myeloid precursors, macrophages and other immune cells. All these cells can contribute to tumor progression and metastasis by releasing a variety of inflammatory-, growth- and survival-factors (chemokines, cytokines, growth factors (GFs)), extracellular proteases and proangiogenic factors, which altogether also belong to TME (Baglole, Ray et al. 2006; Mantovani, Allavena et al. 2008).

Bone-marrow derived cells (BMDC) are particularly interesting cell type lately discovered to be strongly involved in metastasis via the formation of a “pre-metastatic niche”. Thus, it has been suggested that primary tumor directs early changes within sites of future metastasis through the recruitment of a certain type of BMDCs to tumor-specific pre-metastatic sites. This leads to the changes preparing the tissue to receive the disseminating tumor cells (Kaplan, Riba et al. 2005; Kaplan, Psaila et al. 2006; Joyce and Pollard 2009). The changes reported to be associated with a ”pre-metastatic niche” include: 1) modulation of ECM like enhanced production of fibronectin (Kaplan, Riba et al. 2005); and 2) generation of an inflammatory state by inducing pro-inflammatory cytokines (Yan, Pickup et al.).

Recently inflammation has been suggested to represent one of the hallmarks of cancer (Hanahan and Weinberg). The association between inflammation and promotion of cancer was first observed in 1863 by Rudolf Virchow (Balkwill and Mantovani 2001). Recent studies support the notion that cancer-associated inflammation promotes tumor growth and progression (Coussens and Werb 2002; Balkwill, Charles et al. 2005). Thus, inflammation can supply bioactive molecules to the microenvironment, including growth factors that sustain proliferation, survival factors that limit cancer cell death, proangiogenic factors, ECM-modifying enzymes that facilitate angiogenesis, invasion and metastasis (Hanahan and Weinberg).

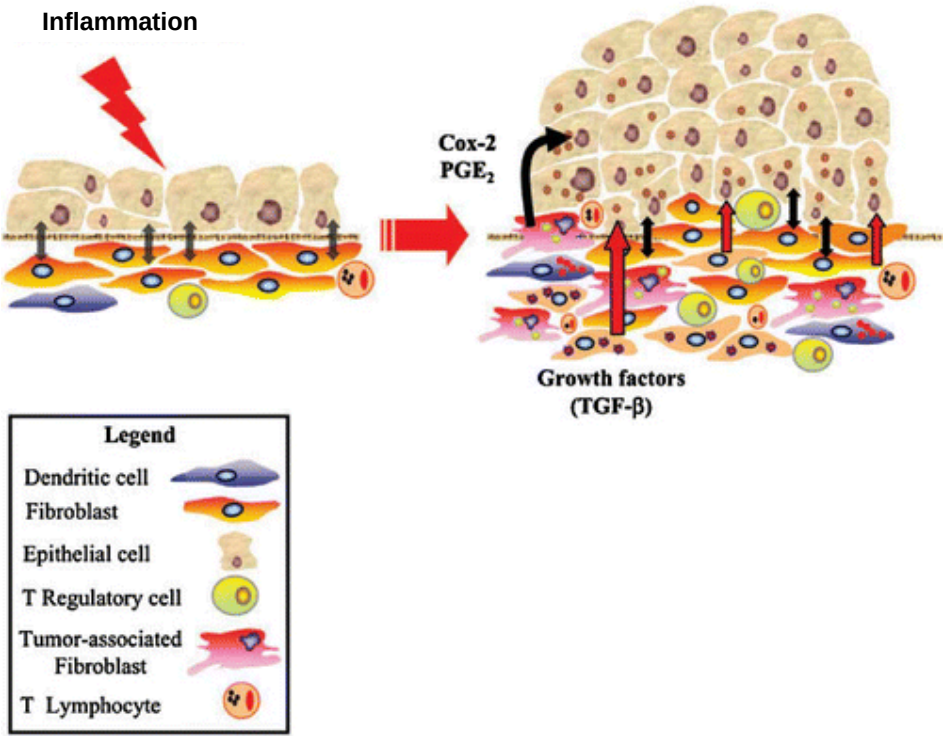


Figure 4. Impact of inflammation-related microenvironment factors on progression of epithelial cancers. Stroma cells, mainly fibroblasts and immune cells, are separated from the epithelium by the basement membrane. In association with inflammation, fibroblasts become activated, proliferate and differentiate into tumor-associated fibroblasts (TAFs). TAFs, in turn, secrete immunoregulatory molecules such as TGF-β, Cox-2 and PGE2. Increased Cox-2 expression is common in most cancers and PGE2 maintains tumorigenesis by immune suppression, angiogenesis and epithelial growth. Immune cells such as T lymphocytes (including T regulatory cells) and dendritic cells are recruited to the stromal compartment to continue the inflammatory state of the tumor microenvironment (adapted from Baglole et al., 2006).

Figure 4 illustrates the mechanism how the inflammation-related microenvironment factors could support cancer progression. Such mechanism has been suggested for epithelial cancers, which so far remains to be the best investigated cancer form with respect to the TME. However, other cancer forms can follow the similar mechanisms. Melanoma cells in generally express a variety of cytokines, chemokines and their receptors (Richmond, Yang et al. 2009). Growth factor TGFβ– is one of the most potent immunosuppressive cytokines. In cancer, together with IL-2, it targets and promotes differentiation of the regulatory T cells (Treg), a strongly immunosuppressive T cells found in melanoma and other tumors. This contributes to tumor escape from immune surveillance. In addition, TGFβ can promote tumor angiogenesis, invasion and metastasis by stimulating expression of various metastatic markers, including MMPs, VEGF and integrins (Li, Wan et al. 2006; Padua and Massague 2009).

COX 2 is inducible enzyme of inflammatory reactions (see **Figure 4**). It catalyzes the production of prostaglandins (PGE) in inflammatory processes and is frequently expressed in malignant melanomas and may have a correlation with cancer progression (Denkert, Kobel et al. 2001; Meyer, Vogt et al. 2009).

Besides various stroma cells and soluble factors facilitating metastasis, various components of the extracellular matrix - another constituent of TME - also can play a role in metastasis. Proteins such as collagen, laminin and fibronectin are the major structural components of ECM. Laminin is a main protein of basement membrane. Usually, melanoma cells have high affinity to exogenous laminin, which they have to degrade in order to cross basement membrane during metastatic dissemination (see **Figure 1**) (Terranova, Williams et al. 1984). Fibronectin has also been strongly linked to the metastasis process, since enhanced levels of fibronectin were observed in metastatic organs, particularly around the metastatic lesions. Interestingly, enhanced expression of fibronectin has been linked to the formation of a pre-metastatic niche, specifically in lungs, where fibronectin could further facilitate the development of a metastatic lesion as discussed above (Kaplan, Riba et al. 2005).

In summary, it appears that metastasis is governed on a systemic level i.e. involves various non-malignant (stroma) cells, soluble factors and ECM components. This research field is still at the very early phase, and the direct contribution of each element of TME in metastasis in various cancer forms, particularly in melanoma, remains to be elucidated. Eventually, this might uncover novel targets for therapy. It is proposed that targeting tumor cells together with the targeting of implicated TME factors can improve the efficiency of anticancer therapy.

1.3 Melanoma therapy

Melanoma is an extremely aggressive disease, and high resistance to therapy is one of the main hallmarks of metastatic melanoma. Generally, drug-resistance may be associated with communication between tumor cells and surrounding microenvironment (Meads, Gatenby et al. 2009). The pathways/oncogenes, microenvironmental factors and enzymes mentioned in the chapters above represents attractive potential targets and offer a novel approach for therapeutic strategies and treatment also in melanoma (see **Table 2**) (Gray-Schopfer, Wellbrock et al. 2007; Villanueva and Herlyn 2008).

Table 2. Single agents targeting different components of the melanoma tumor microenvironment in clinical trials (adapted from Villanueva and Herlyn, 2008).

Target	Compound	Mechanism	Phase	Comments
Vasculature	AG-013736	VEGFR and PDGFR inhibitor	2	Starting dose 5 mg twice daily ± 20% according to toxicity
	ABT-510	Synthetic analogue of thrombospondin-1/ angiogenesis inhibitor	2	100 mg twice daily in patients with metastatic melanoma did not demonstrate definite clinical efficacy
	AZD2171	ATP-competitive inhibitor of VEGFR family	2	May also inhibit Kit and (less potently) PDGFR-A and PDGFR-B
	VEGF Trap (Aflibercept)	Fusion protein that binds VEGF-A and placental growth factor	2	Effectively suppresses tumor growth and vascularization *in vivo*
	Bevacizumab	Anti-VEGF humanized monoclonal antibody binds to VEGF and inhibits VEGF receptor binding	1/2	Treatment with intravitreal bevacizumab for large uveal melanomas; efficacy of bevacizumab monotherapy being tested in trial in Norway
	Vorinostat (FR901228, Romidepsin)	Histone deacetylase inhibitor	2	Proapoptotic in preclinical studies; blocks hypoxia-induced angiogenesis and depletes Hsp90-dependent oncoproteins
Integrins	Volociximab (M200)	α5β1 integrin	2	Will also inhibit angiogenesis
	MEDI-522 (Abergrin)	αvβ3 integrin	2	Humanized monoclonal antibody potent *in vivo* inhibitor of tumor growth and metastasis
	EMD 121974 (Cilengitide)	αvβ3 and α5β1 integrins	2	Cyclic Arg-Gly-Asp (RGD) peptide; inhibiting endothelial cell–cell interactions, endothelial cell–ECM interactions, and angiogenesis
	CNTO 95	αv integrins	1/2	Human monoclonal antibody; inhibits melanoma cell adhesion, migration, and invasion
Matrix	COL-3 (NSC-683551)	MMP-2 and MMP-9	1	Completed
	Marimastat	MMP	2	Limited activity in melanoma; trials in Canada and Europe
	GC1008	Human anti-TGF- β monoclonal antibody	1	
Other kinase inhibitors	RAF-265	MAPK inactivation/ BRAF and VEGFR-2 inhibitor	1	Inhibits the RAF/MAPK pathway as well as VEGF and thus angiogenesis
	Sunitinib (SU11248)	Multikinase inhibitor including Kit, PDGFR, VEGFR	2	FDA approved for GIST and metastatic kidney cancer

However, despite big efforts during the last decades testing various treatment strategies, none significantly prolonged patient survival. An alkylating agent dacarbazine (DTIC) has been most commonly used single agent in metastatic melanoma treatment. It was FDA-approved for malignant melanoma in 1976 and still remains the sole approved chemotherapeutic drug for this cancer type. Unfortunately, DTIC alone or in combination with other chemotherapeutic agents elicit durable response in very few patients (i.e. less than 25%) (see **Table 2**) (Jilaveanu, Aziz et al. 2009).

Table 3. Respone rates of melanoma treatment with single chemotherapeutic agents (adapted from Jilaveanu, 2009).

Agent	Response rate, %
Dacarbazine	7-25
Temozolomide (TMZ)	21
Cisplatin (DDP)	16.3
Carboplatin	19
Zeniplatin	9.5
Vindesine	20
Vinflunine ditartrate	3
Vinorelbine tartrate	0
Carmustine	20
Semustine	15
Fotemustine	15.5
Cystemustine	17.9
Taxol	15.6
Taxotere	17

Immunotherapy has also been investigated in metastatic melanoma. High dose of Interleukin-2 (IL-2), which plays a central role in immunoregulatory process by cytotoxic activity and stimulation of natural killer cells, is another FDA-approved agent (in 1998) for advanced melanoma. Different regimes and schedules have been tried without association with better survival, but rather with even higher toxicity than with chemotherapeutics (Agarwala 2009).

Lack of efficient drugs against malignant melanoma stimulated an intensive research on new therapeutic options. Recently, a novel strategy for apoptosis stimulation, based on the induction of reactive oxygen species (ROS), has emerged. A small molecular drug named Elesclomol (Synta Pharmaceutical) is a ROS-inducing agent, which leads to apoptosis directly, or sensitizes cells to other agents. Generally, melanomas have high levels of ROS as compared to normal cells, and elevation of ROS beyond the “threshold” level induces cell death. In a recent Phase II

trial in metastatic melanoma Elesclomol demonstrated an improved patient survival (Kirshner, He et al. 2008; Tuma 2008).

Lately, a new promising drug PLX4032, a small molecular inhibitor of mutated BRAF ($BRAF^{V600E}$, a mutation observed in 40-60% melanomas) entered clinical trials. The first results were dramatic: the majority of the patients treated with PLX4032 exhibited complete or partial tumor regression. Unfortunately, the drug stopped working after ~7months, and at present a lot of research focuses on understanding the molecular mechanism of such resistance and the ways to overcome it (Ledford; Vultur, Villanueva et al.). Likely, PLX4032 has to be combined with other drugs to create a more potent cocktail against malignant melanoma.

1.4 Three-dimensional *in vitro* cultures – an in vitro model to study cancer properties in the setting of TME

As discussed above, TME components play an important role in cancer cell proliferation, invasion and survival. Therefore, there is a high need for models like cell culture systems in vitro, that would allow studying cancer cell behavior in the presence of TME components. One of such models is three-dimensional (3D) cell cultures, where the cells are embedded in a matrix of e.g. a collagen – the main component of ECM. Culturing cells within three-dimensional matrices is not a novel idea. Already in 1972, a model system was described for fibroblastic cells in the body using collagen I matrices polymerized *in vitro* to form a three-dimensional fibrous network. These three-dimensional collagen gels induced morphological changes in fibroblasts that partially mimicked connective tissue cells *in vivo* (Elsdale and Bard 1972).

Lately, it is becoming recognized that 3D matrix-based cell cultures are superior in vitro models compared to traditional 2D monolayer cultures grown on a plastic surface. There are some limitations to the 2D models that are apparent when compared to cells grown in a 3D matrix. For example, 3D models incorporate TME components like ECM and therefore, mimic the situation in vivo more closely. Important cell-cell or cell-ECM contacts, which are lost in ”flat” 2D monolayers, can be restored in matrix-based 3D systems. Having in mind that cell-cell or cell-matrix interactions are important in regulating cell survival and invasion, 3D models are highly relevant systems for *in vitro* studies of tumor cell growth, migration, invasion and drug response.

Interestingly, it has been shown previously that tumor cells responsiveness to drugs in 2D and 3D systems can differ tremendously, and that microenvironment components like ECM present in 3D cultures can influence drug-response (Smalley, Lioni et al. 2006; Serebriiskii, Castello-Cros et al. 2008). Furthermore, it has been shown that drug efficiency observed *in vivo* is closer to the efficiency seen in 3D than in 2D *in vitro* systems. Generally it has been observed that the sensitivity to drugs is lower *in vivo* and in 3D compared to 2D. This might explain an often seen situation: drugs that show high efficiency in 2D fail to show the same efficiency *in vivo* or in the clinic. Therefore, lately use of 3D systems for testing new therapeutic approaches gets in focus.

3D cell cultures are usually based on matrixes consisting of collagen or Matrigel that were also used in the present work. Collagen I is a matrix component highly relevant for melanomas: it is present in the skin where a primary tumor develops, while Matrigel is rich in laminin – the main component of the basement membrane that melanoma cells have to cross during dissemination from a primary tumor. Besides, 3D cultures can be supplemented with additional factors of TME, like various soluble factors or stroma cells. Such systems allow investigation of cancer cell behaviour in vitro in 3D and in the presence of relevant TME components (Studebaker, Storci et al. 2008).

Growing interest in the role of TME in cancer also boosted the interest in more relevant in vitro models like 3D systems. Therefore, the present project was built on 3D cell cultures to investigate how various components of TME affect melanoma cell behaviour.

The aim of the study

The present project focuses on metastatic melanoma and how its aggressive properties are influenced by the factors of tumor microenvironment (TME). The overall aim is to identify how various TME-associated factors affect melanoma cell growth and response to therapy in vitro. Importantly, the study is performed by employing three-dimensional (3D) matrix-based cell cultures that allow partial recapitulation of TME and therefore mimic the in vivo situation more closely.

The specific aims were:

1) To elucidate adhesion abilities of melanoma cell to various components of extracellular matrix (ECM) and to reveal how selected ECM proteins affect melanoma cell growth in 3D.
2) To evaluate the effect of soluble factors released from different organs – common sites of melanoma metastasis – on tumor cell growth and drug-response.
3) To compare melanoma cell sensitivity to the drug Elesclomol in 3D versus 2D cultures and to evaluate whether/how melanoma sensitivity to Elesclomol is modulated by the investigated TME factors.

2. Materials and methods

2.1 Cell lines: Melmet 1 and Melmet 5

Metastatic melanoma cell lines, Melmet 1 and Melmet 5, were established from the biopsies of metastatic melanoma patients (see **Table 4** for more information) at the department of Tumor Biology, The Norwegian Radiumhospital as described previously (Prasmickaite, Engesaeter et al.). Briefly, melanoma cells were isolated from the mechanically disintegrated biopsies by the help of immunomagnetic beads conjugated to the melanoma-specific antibody. The isolated tumor cells were cultured as two-dimensional (2D) monolayers in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS) and 5% L-Glutamin as described further in general cell work.

Table 4. Background information about Melanoma cell lines.

	Melmet 1	**Melmet 5**
Source	Female /36 years	Male /56 years
Tissue (sites of biopsies)	Subcutaneous	Lymph node
Distant metastasis	Brain, lung, breast, skin	Brain, lung, liver, abdomen
Tumorigenicity in mice (subcutaneous growth)	Yes	Yes
Metastatic ability in mice	Yes	Yes
***In vitro* response to the approved drug DTIC in 2D**	Poor	Poor

Both cell lines easily adhere to a bottom of a culture flask and form a monolayer, but Melmet 1 grow slower than Melmet 5. The morphology of Melmet cells grown in 2D is shown in **Figure 5**.

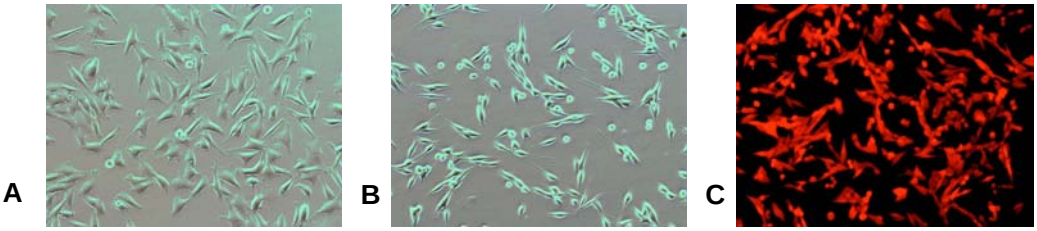


Figure 5. Morphology of Melmet cell lines. A: Melmet 1; **B:** Melmet 5; **C:** Melmet 5 DSRed fluorescence.

In addition, red colour-labelled Melmet cells lines, which stably express a fluorescent protein DsRed (further referred as DSRed Melmet 1 and DSRed Melmet 5) were used in some of the experiments described below. Due to the DsRed label, these cells can be tracked by a fluorescent microscopy or analysed by a plate reader measuring the fluorescence signal (see **Figure 5** C).

Both Melmet 1 and Melmet 5 cells show high abilities to metastasize and resist therapies and, therefore, are representative and useful models for the studies on malignant melanoma.

2.2 General cell work: cell culturing in 2D

Routinely, melanoma cells were grown in cell culture flasks in RPMI 1640 medium supplemented with FBS (10%) and L-Glutamine (5%) (further referred as "RPMI++") in an incubator with 5% CO_2 at 37ºC. The confluence of the cell monolayers was checked daily by an inverse microscope (Olympus CKX41). Cells were subcultured and the medium was replaced 2-3 times weekly. All procedures were performed under sterile conditions. Materials and instruments which were used for cell culturing are listed in **Table 2**.

For cell subculturing the growth medium was removed from the flask and the cell monolayer was first washed once with 2 ml EDTA. Then a new 1 ml of EDTA was added and incubated for about 2-3 minutes at room temperature (or in an incubator at 37ºC to speed up cell detachment), and the cell flasks were roughly shaked to facilitate cell detachment. When all the cells were detached, 2-3ml of new medium was added directly to the flask, and a homogenous cell suspension was prepared. The desired amount of this cell suspension was transferred to a new culture flask and diluted with fresh RPMI++ (the total volume of the medium culture flask was 10ml).

For applications where the cell concentration had to be determined, the detached cells in EDTA suspension were transferred to a new tube. The remaining cells were collected into 2-3ml new medium and transferred to the same tube. After centrifugation at 1200 rpm for 5 minutes, the supernatant was removed and the cell pellet was resuspended in fresh RPMI++ pippeting up and down to make a homogenous cell suspension. For cells counting, 10μl of the cell suspension was mixed with 10μl of trypan blue (which stains dead cells and, therefore, allows

exclusion of dead cells during counting), and 10μl of the mix was added directly to the Burker chamber for cell counting.

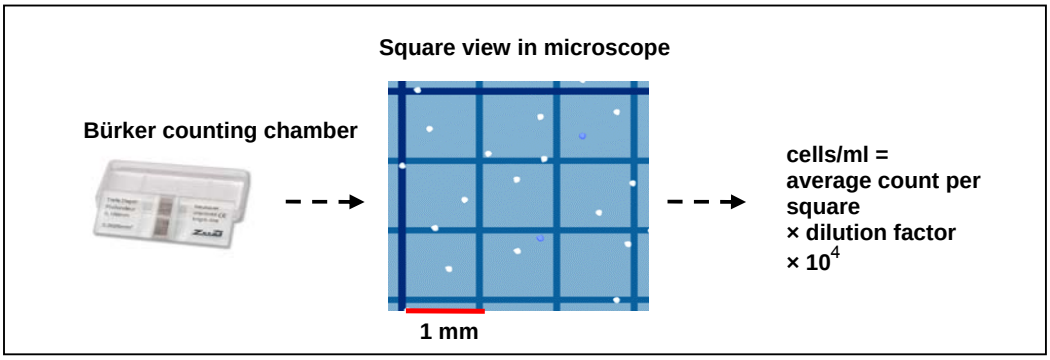


Figure 6. Cell counting method. Viable cells are counted in some squares of the Burker chamber. Then average count per square is multiplied by dilution factor and 10^4.

Further, viable cells were counted in some squares of the chamber (see **Figure 6**) so the average count per square could be determined. To get the finally cells number in 1 ml of cell suspension, average count per square was multiplied by the dilution factor and by factor 10^4.

All reagents, materials and instruments used in "General cell work: cell culturing in 2D" are listed in **Table 5**.

Table 5. Materials and methods used in general cell work.

Materials/instruments:	Company:
RPMI 1640	Lonza, Belgium
Glutamine	BibkoBRL, UK
Fetal Bovine Serum (FBS)	PAA, Austria
Cell growth flasks with filter-caps	NUNC™, Denmark
EDTA, Versene	Lonza, Belgium
2, 5, 10, 25 ml pipettes	Sarstedt, Numbrecht
15 and 50 ml tubes	Sarstedt, Numbrecht
Filter tips	Molecular Bio Produkts®
Burker chamber for cell counting	Marienfeld, Germany

2.3 Cells adhesion to extracellular matrix (ECM) proteins

Cell adhesion to various proteins of ECM was evaluated by employing ECM Cell Adhesion Array Kit (CHEMICON®) according to the manufacture's protocol. Briefly description of the procedure is as follows: A single cell suspension with a cell density of $2x10^6$ cells per ml was prepared in advance. The kit-strips consisting of wells coated with a specific protein of ECM were rehydrated with PBS for 10 minutes. PBS was removed by turning over the strips and gently tapping 100µL of the prepared cell suspension was added to each well (i.e. $200x10^3$ cells/well). Following the incubation for 2 hours at 37º C in a CO_2 incubator, the media was removed and the wells were gently washed (2-3 times) with the Assay buffer. After washing, the Cell Stain Solution was added to each well and incubated for 5 minutes at room temperature, removed and gently washed for 3-5 times with the deionized water. The wells were left to air dry for a few minutes. The Extraction Buffer was added to each well and absorbance was monitored at 540 nm on a Walac plate reader. "Cell Stain Solution" stains the adhered cells, therefore, the wells with many attached cells are strongly stained and lead to high absorbance. In this way, the absorbance intensity allows identification of ECM proteins that melanoma cells adhere to.

All reagents and materials used are listed in **Table 6**.

Table 6. Materials and instruments needed for performance of the ECM kit.

Materials/instruments:	**Company:**
ECM Cell Adhesion Array Kit (Colorimetric) Cat. No. ECM540 96 wells	CHEMICON® International
PBS	Lonza, Belgium
dH2O	-
Wallac plate reader	KSH-Productor Oy, Finland

2.4 Cell culturing in 3D

2.4.1 Collagen cultures

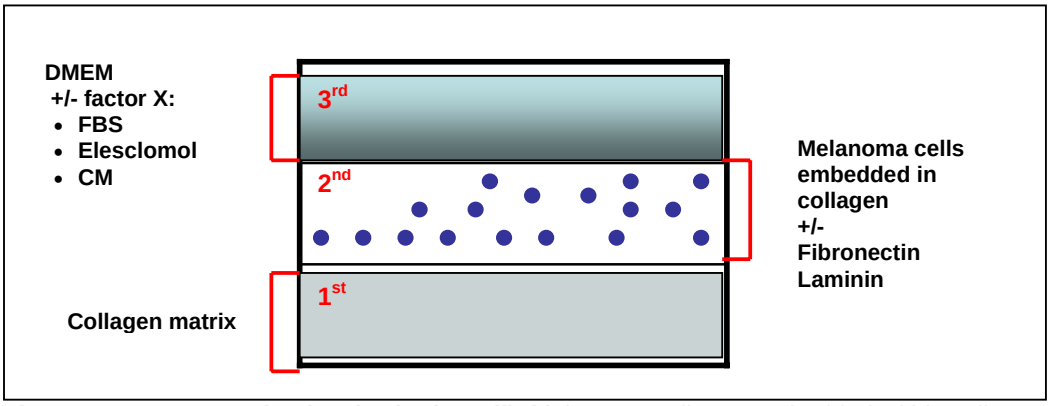


Figure 7. 3D systems *in vitro* (1 single well). Melanoma cells are embedded within collagen matrix. Such systems can be supplemented with investigating factors embedded with cells in 2nd layer or dissolved in the 3rd soluble layer.

3D cell cultures consist of three layers (see **Figure 7**): a 1st collagen layer, a 2nd collagen layer with embedded cells and a 3rd top layer - growth medium, which can be supplemented with various soluble factors or investigated drugs. The amounts of the components required for the preparation of each layer are listed in **Table 7**.

Table 7. Preparation of 3D collagen-based cell cultures in 96-well plates (the indicated amounts are needed for 1 well).

collagen final concentration 2.5 mg/ml			
	1st layer	2nd layer with cells (no cells in "blanc" samples)	3rd layer (growth medium)
4xDMEM++	10µl	10µl	100 µl 1xDMEM ++ (+/-factor X)
collagen I (stock 5mg/ml)	25µl	25µl	
NaOH 0.025N	1µl	1µl	
H2O	4µl	4µl	
1x DMEM++	10µl	-	
1x DMEM++ with cells	-	10µl containing desired amount of cells	
Total volume	50µl	50µl	100µl

Basic protocol for preparation of 3D collagen-based cell cultures:

Collagen I stock solution (5 mg/ml) was diluted with 4xDMEM supplemented with glutamine, antibiotics penicillin and streptomycin (further referred as "DMEM++"), H2O and NaOH (to adjust the final pH of the solution to 7, 4) as indicated in **Table 7**. All the ingredients and the tubes were kept on ice while mixing to avoid undesirable polymerization of the collagen. The culture plates were also pre-chilled on ice before application of the 1st collagen layer.

For the 1st layer, 50µl prepared as indicated in **Table 7** were applied into each well. Air-bobbles, if they were formed, were removed by the help of a needle. After application of the 1st layer, the plate was transferred to room temperature for at least 30 minute to allow polymerization of collagen. After polymerization, 50µl of the 2nd layer was added, which was prepared similarly to the 1st layer except that it was supplemented with desired amount of melanoma cells resuspended in 10µl 1x DMEM++ (see **Table 7**). Paralelly, "blank" wells lacking tumour cells in the 2nd layer were prepared by adding 50µl collagen solution on top of the polymerized 1st layer; "blanc" wells were used to measure a background signal (for the collagen and the investigating factors in 3rd top layer) which was substracted from a cell-mediated signal during final calculations (see chapter below). Subsequently, the plate was put into CO_2 incubator at 37°C for at least 30 minutes to allow collagen polymerization.

Finally, the polymerized gel was overlaid with 100µl of the 3rd top layer consisting of growth medium with or without investigated factor X. 1xDMEM medium supplemented with glutamine and penicillin/streptomycin was usually used as a "basic" growth medium, which could be further supplemented with FBS (further referred as "DMEM+2% FBS"), and/or a factor X e.g. investigated drugs or soluble molecules provided by conditioned media (see below). Eventually the plate was put into CO_2 incubator at 37°C and incubated for 1 to 5 days before the analysis. Samples were inspected visually every day by an inverse microscope (Olympus IX81). Besides, the plates were analyzed by a plate reader along the experiment or at the end of it.

All reagents, materials used in 3D collagen-based cell cultures are listed in **Table 8**.

Table 8. Materials and instruments used in preparation of 3D collagen models.

Materials/Instruments:	Company:
96 well black walled-clear bottom plate	Greiner bio-one, Germany
4× DMEM	Sigma Aldrich, Switzerland
1× DMEM	Sigma Aldrich, Switzerland
Phosphate Buffered Saline (PBS)	Lonza, Belgium
Sterile dH2O	-
15, 50 ml tubes	Sarstedt, Nümbrecht
Eppendorf tubes	Trefflab, Switzerland
Inverse microscope Olympus IX81	Olympus, Norway
Bovine Collagen I	Gibco ®
Synergy2 plate reader	BioTek, Norway
Walac plate reader	KSH-Productor Oy, Finland

2.4.2 Matrigel cultures

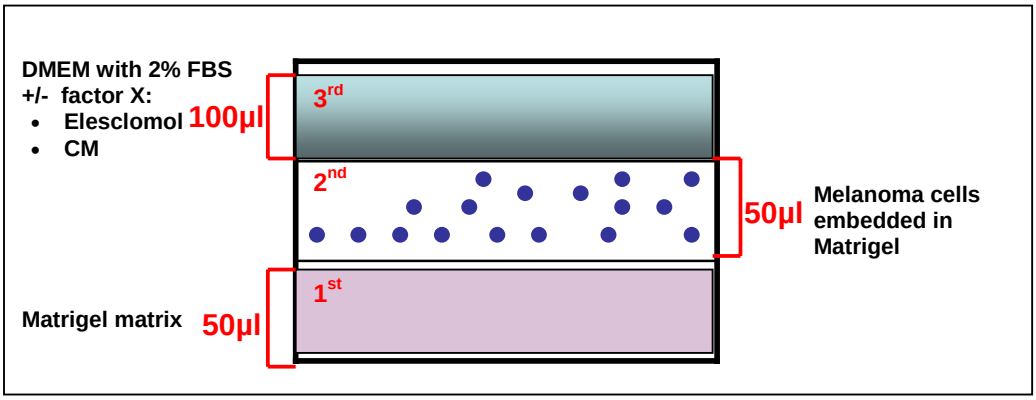


Figure 8. 3D systems *in vitro* (1 single well). Melanoma cells are embedded within matrigel matrix. Such systems were supplemented with investigating factors dissolved in the 3rd soluble layer.

Matrigel 3D cultures consisted of 3 layers as indicated in **Figure 8**.

Briefly, for the 1st layer, 50µl of Matrigel were applied directly into each well. Then, the plate, which was pre-chilled on ice before application of the 1st layer, was transferred to room temperature for at least 15min to allow polymerization of Matrigel. Bottle with Matrigel was also kept on ice during the application.

The desired amount of melanoma cells suspension was centrifuged at 1200 rpm for 5 minutes, the supernatant was removed and the cell pellet was gently resuspended in 50 µl Matrigel/well pippeting up and down to make a homogenous

cell suspension. Paralelly, “blank” wells lacking tumor cells in the 2nd layer were prepared by adding 50 µl Matrigel solution on top of the polymerized 1st layer. Bobbles, if they appeared, were removed with a needle. After application, the plate was put into CO_2 incubator at 37°C for at least 30 minutes to allow Matrigel polymerization.

Finally, the polymerized gel was overlaid with 100µl of the 3rd top layer consisting of DMEM+2% FBS with or without investigated factor X (see **Figure 8**).

All reagents and materials used in 3D Matrigel-based cell cultures are listed in **Table 9**.

Table 9. Materials and instruments used in preparation of 3D Matrigel based models.

Materials/Instruments:	Company:
96 well black walled-clear bottom plate	Greiner bio-one, Germany
1× DMEM	Sigma Aldrich, Switzerland
15, 50 ml tubes	Sarstedt, Nümbrecht
Eppendorf tubes	Trefflab, Switzerland
Inverse microscope Olympus IX81	Olympus, Norway
BD Matrigel™ matrix	BD Biosciences, USA
Walac plate reader	KSH-Productor Oy, Finland

2.4.3 Treatment with Elesclomol in 3D

To test cell sensitivity to therapy, Elesclomol was chosen as a drug. It is a new agent currently investigated for melanoma therapy at the Department of Tumor Biology. Melanoma cells were treated with various concentrations of Elesclomol. A serial dilution of Elesclomol stock of 10mM was made to obtain the desired final concentrations of the drug in DMEM+2% FBS. 100µl of each concentration Elesclomol solution was added in the 3rd top layer of the 3D cultures. After 3 days cell viability was evaluated by the MTS method as described below.

2.4.4 3D cultures supplemented with fibronectin and laminin

To modify the 3D models with some additional components of ECM, two well known noncollageneous proteins i.e. fibronectin (derived from human fibroblasts) and laminin (derived from human placenta) were incorporated into the 2nd layer while

making the 3D cultures. Briefly, 1µl of fibronectin or laminin stock solutions (0.5mg/ml) was mixed into the 50µl of the 2nd layer solution (consisting of collagen and cells) to obtain a final concentration of the proteins in the 2nd layer 10µg/ml. The layer was allowed to polymerized and overlaid with 3rd layer as described previously (see Cell culturing in 3D).

2.4.5 3D cultures supplemented with organ-conditioned medium

Growing cells both in vitro and in vivo in an organism are known to secrete various soluble mediator substances, like T-growth factors and cytokines that may promote the growth of new cells. To describe media in which cells have been cultivated for a period of time, a term “conditioned media” (CM) is often used. Such media is cell-free, but is enriched with various soluble factors produced by the cells while the media and the cells were in contact. The same term can be used to describe media, which has been in contact with an organ for some time.

In the present work, the conditioned media was prepared from brain, lungs, bone marrow and lymph nodes isolated from a healthy or a metastatic mouse (**Figure 9**). The mentioned organs represent commons sites of melanoma metastases, indicating that generally melanoma cells “like” to growth in the environment of these organs.

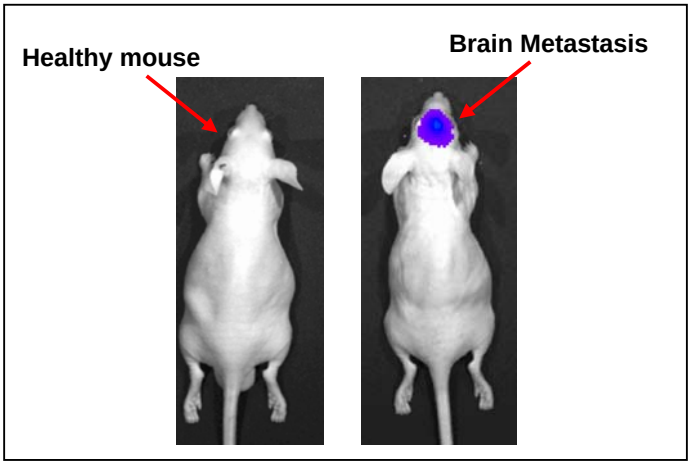


Figure 9. Imaged mice. Healthy mouse and mouse with established Mel 1 metastasis in the brain which were used in CM preparations.

To prepare a conditioned media, each organ was cut by a scalpel into small pieces (~ 2 mm in size) that were transferred into a flask containing DMEM+2% FBS

and incubated in 37ºC for 2 hours under constant agitation. For preparation of CM from brains and lungs, 2.5-3 ml of the medium was used for each organ. CMs from lymph nodes and bone marrow were prepared in the manner that organ-pieces were just covered with the medium (approximately 1.5 ml). After 2 hours of incubation, the conditioned medium was removed, filtrated through a 70µm filter for removal of the biggest tissue-pieces, and finally filtrated through 0, 22µm filter. The prepared CMs were stored at -80ºC in the freezer and thawed on ice when needed.

For use in the experiments where the effect of CMs in 3D cultures was investigated, the conditioned media was diluted with DMEM+2% FBS in a ratio 50:50. For one single well 50 µl of the CM was mixed with 50 µl of the growth media and added to the 3rd top layer of the collagen cultures.

After evaluation of the results from the first experiments using CMs and based on the previously published data in the literature (Valle, Zalka et al. 1992; Cruz-Munoz, Man et al. 2008), we decided to reduce the amount of CMs used. Further, 25 µl of the organ-conditioned media was mixed with 75 µl DMEM+2% FBS (i.e. ratio 25:75) and added to the 3rd top layer of the collagen matrixes.

The 3D cell cultures supplemented with CMs were incubated at 37ºC for 4-5 days before the effect was evaluated as described below (see Evaluation of growth/prolif and viability/metabolic activity: measurements methods).

All reagents and materials used in preparations of organ-conditioned medium are listed in **Table 10**.

Table 10. Materials and Instruments used in preparation of 3D cultures supplemented with organ-conditioned medium.

Materials/Instruments:	Company:
1× DMEM	Sigma Aldrich, Switzerland
Fetal Bovine Serum (FBS)	PAA, Austria
Cell growth flasks with caps	NUNC™, Denmark
Eppendorf tubes	Trefflab, Switzerland
Scalpels	Swann-Morton®, England
Petri dishes	NUNC™, Denmark
PBS	Lonza, Belgium
70µm filter	BD Falcon™, USA
20µm filter	CORNING®, Germany
Syringes and needles	Tamro, Finland
Pincette	Bastos Viegas, Portugal

2.4.6 3D cultures supplemented with organ-conditioned medium and treated with Elesclomol

For use in the experiments where the effect of Elesclomol treatment in the presence of CMs in 3D cultures was investigated, the conditioned media was diluted with DMEM+2% FBS in a ratio either 50:50 or 25:75. Further, a serial dilution of Elesclomol stock of 10mM was made till the desired final concentrations of the drug by suing the medium containing the conditioned medium as indicate above. For one single well 100µl of each desired concentration of Elesclomol solution was added in the 3rd top layer of the 3D collagen cultures and cells were incubated for the desired time before analysis.

2.5 Evaluation of cell viability and growth: measurement methods

Several methods based on different principles have been used to evaluate general cell viability and cell functions like metabolic activity and growth/proliferation.

To evaluate cell viability, we have used:

- Staining with the “live” dye Calcein
- Staining with the “dead” dye Propodium Iodide
- Measurement of cells metabolic activity by the MTS method

To evaluate cell growth/proliferation we have used:

- DsRed-labeled cells and analyzed the DsRed signal intensity

A short description of the mentioned methods is presented below.

2.5.1 Cell viability measurement based on Calcein staining

Calcein is a non-fluorescent compound which can be converted into the fluorescent polyanion calcein by cytosolic esterases in live cells. Therefore, Calcein fluorescence can be used as a measure of cell viability since viable cells fluoresce green. In the present work Calcein fluorescence was observed by microscopy and/or measured by a plate reader. Briefly, 50µl of PBS with 0, 25µl Calcein (stock 4 mg/ml) were added to a well with 3D cultures and incubated for 30 minutes at room temperature. Subsequently, the cells were observed by a fluorescence microscope

using a “FITC” filter (excitation at 450-490nm, emission at 515-565nm) or analyzed by a plate reader after excitation at 485nm and emission at 528nm.

2.5.2 Staining with Propidium Iodide for visualization of dead cells

Propidium Iodide (PI) is membrane impermeable and generally is excluded from viable cells. However, PI can enter dead cells where it binds to DNA or RNA so that the dead cells become stained. Therefore staining with PI can be used to visualize dead cells e.g. by microscopy. For cell staining with PI, 2µl of PI stock solution (1 mg/ml) was added to a well containing 3D cultures and immediately observed by a microscope using a “TRITC” filter (excitation at 550nm, emission at 570nm).

2.5.3 Evaluation of cells metabolic activity by the MTS assay (“Cell Titer 96®Aqueous Cell Proliferation Assay”)

The MTS method is based on the reduction (by dehydrogenase enzymes) of the tetrazolium salt, MTS, to a colored formazan compound by metabolically active mitochondria in viable cells in culture. Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce MTS into the formazan product, which is soluble in cell culture medium. Upon cell death, cells rapidly lose the ability to reduce MTS into formazan. The production of the colored formazan product, therefore, is proportional to the number of viable cells in culture.

MTS stock solution from Promega was added directly to a well at a ratio of 20µl MTS reagent to 100µl of culture medium in the top layer in 3D cultures or in 2D monolayers. Cells were incubated for 1–2 hours at 37°C, and then absorbance was measured at 490nm by a plate reader. Based on the absorption data the viability was calculated. The viability/metabolic activity data were presented either as % relative to controls, or as Absorbance at 490nm.

2.5.4 Evaluation of cell growth/proliferation by measuring intensity of the DsRed signal

In the experiments where the DsRed-labeled cells were used, cell growth/proliferation was evaluated by monitoring a DsRed fluorescence intensity by a

plate reader. This method is “noninvasive” i.e. cells stay unaffected and therefore can be analyzed repeatedly measuring the fluorescence in the same wells over time. Briefly, the DsRed-labeled Melmet cells were embedded in 3D matrix in black-walled clear-bottom 96 wells plates (see **Figure 10**) as described above in “General 3D culturing.” The black walls allowed to avoid fluoresce “leakage” to the neighboring wells during the measurements of the DsRed signal. The DsRed signal intensity was followed over time, usually up to 5 days after the initiation of the experiment. Measurements of the DsRed signal intensity was performed by a Synergy 2 plate reader (excitation at 530nm).

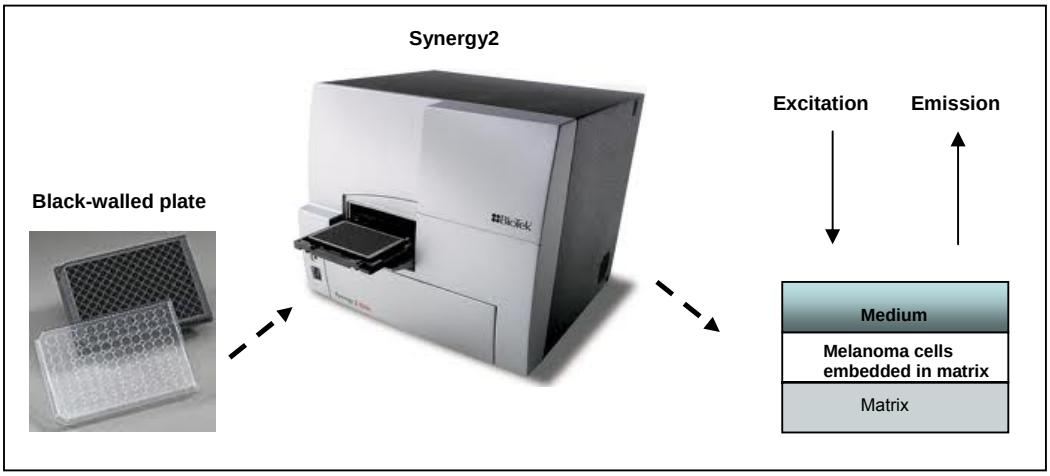


Figure 10. Schematic presentation of measurement of intensity of a DsRed signal. The DsRed labeled cells cultured in 3D (right) in black-well plates (left) were analyzed by a plate reader “Synergy2” (middle) measuring the DsRed fluorescence after excitation at 530nm.

3. Results

3.1 Evaluation of melanoma cell growth and viability in 3D cultures

To grow the cells in 3D in the presence of ECM components, Collagen is usually used as a matrix. Another often used matrix is Matrigel, which is generally richer in growth factors compared to collagen and therefore can be suitable for cells that do not grow well in collagen. However, Matrigel is also much more expensive and therefore, collagen is often the first choice in studies based on 3D cultures.

The aim of this part of the work was to find the conditions suitable for culturing of Melmet cells in 3D matrixes. For this, we have tested collagen I, Matrigel, the presence/absence of serum, different cell amounts, and followed Melmet cell growth and/or viability by various approaches. Specifically, we have measured: a) the DSRed signal intensity to reveal the increase in the number of DSRed-labeled Melmet cells (= cell growth); b) cells metabolic activity by the MTS method to reveal cell viability and/or grow; c) visualized live and dead cells by microscopy after staining of cell cultures with the “live and dead” dyes Calcein and Propidium Iodide. The choice of the method varied depending on the specific experiment.

We have started the study from Melmet 5 since this cell line is easy to work with, the cells grow fast and we knew from the previous studies that these cells can grow in collagen matrixes.

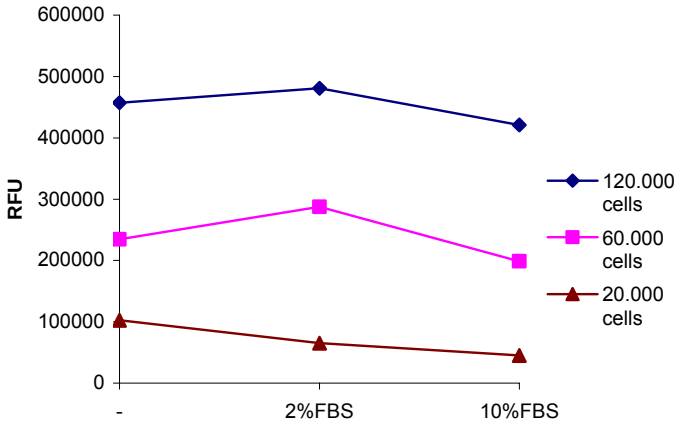
3.1.1 Optimization of culturing conditions for Melmet 5 in collagen matrixes: determination of suitable cell amount and serum concentration

The following parameters have been investigated first: 1) amount of Melmet cells seeded in a well; 2) presence/absence of serum (FBS) in a growth medium in a 3rd (top) layer of the 3D cultures. In these studies DSRed-labeled Melmet 5 cells were used, and the DSRed signal was measured.

First, a correlation between a cell number and DsRed fluorescence was evaluated. The DSRed signal for three different concentrations of cells (20.000/-, 60.000/- and 120.000/well) was measured after 24 hours of culturing. Cells were grown in 3D collagen matrix in the medium without serum, with 2% serum and with 10% serum.

As shown in the **Figure 11**, the DsRed signal intensity was higher for the higher amount of cells seeded, indicating that the signal intensity was proportional to

the cell number. This also indicated that measurements of the increase of the DsRed signal can be used to follow the increase in cell number, which indicates cell growth.



Figur 11. DSRed signal for three different concentrations of Mel5 DSRed cells in 3D cultures containing media of different compositions. "-"= wells containing RPMI++ medium without serum; "2%FBS" = wells containing the medium with 2 % serum; "10% FBS" = wells containing the medium with 10% serum. Relative Fluorescence Units (RFU) of the DSRed signal are shown on the Y axis, and the medium compositions are indicated on the X axis.

Under the conditions with 2% serum a slightly higher DsRed signal was registered for the two highest cell concentrations (see **Figure 11**). Although the difference was not significant, it suggested that the presence of 2% serum was beneficial for the cells. Serum promotes cell growth and/or survival, which might result in higher cell amounts and thus, higher DsRed fluorescence compared to the conditions without serum.

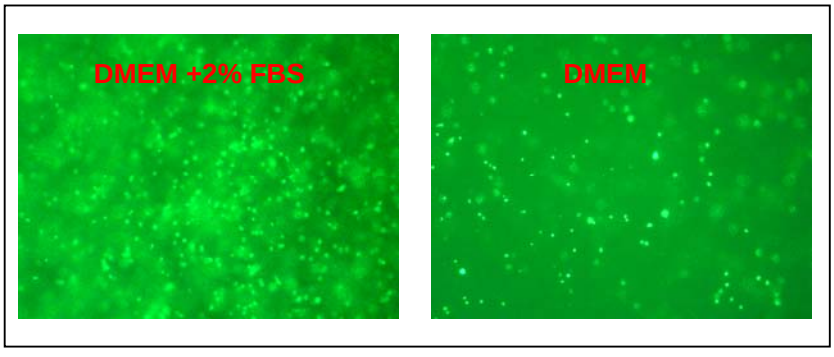


Figure 12. Visualization of the viable (i.e. green) Melmet 5 DSRed cells grown in DMEM medium with (left) or without (right) 2% serum in the 3rd layer of the 3D cultures. The cells were cultured for 4 days and stained with the "live dye" Calcein to identify the viable cells (pictures taken using 4x objective).

The later experiments supported this observation, and as can be seen in **Figure 12**, there was a higher amount of viable (i.e. green) cells in 3D cultures containing 2% serum compared to the serum-free cultures.

It should be noted that the intensity of the DsRed signal in cultures with 10% serum was reduced for all cell concentrations tested (**Figure 11**). Generally, 10% is a widely accepted serum concentration for cell culturing in vitro, which however might be not optimal for our experiments where the fluorescence has to be measured, since the presence of serum can interfere with the fluorescence measurements.

To investigate further cell growth in 3D over time in the presence versus absence of serum, we have followed the increase in DsRed signal for up to 5 days. Melmet 5 DSRed cells were cultured in 3D collagen using the medium with 2% serum or the serum-free medium in the 3rd layer. The DSRed signal was measured after 24, 96 and 120 hours of culturing.

As we can see in **Figure 13,** Melmet 5 cells were able to growth even under the conditions without serum where they doubled their number on day 5 compared to day 1. Under the conditions with 2% serum, the DsRed signal on day 5 was increased significantly more, approximately 5-fold compared to day 1. Likewise, on day 4 it was also observed a higher DsRed signal in serum-containing compared to serum-free medium. This confirms that the medium with 2% serum is beneficial for growing Melmet 5 cells for several days.

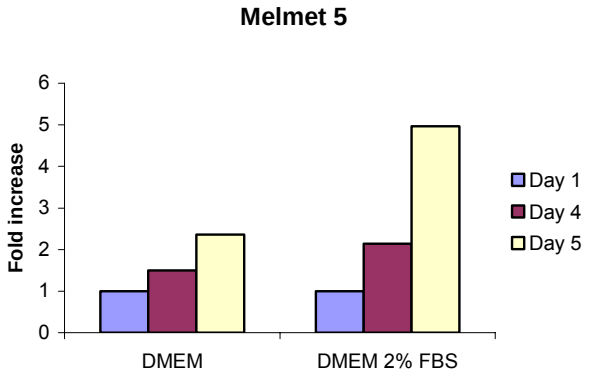


Figure 13. Melmet 5 cells growth during 5 days of culturing in 3D collagen matrixes. 60000 DSRed Melmet 5 cells were seeded in the 2nd layer of collagen matrixes as described in Material and Methods. The growth medium in the 3rd layer was either DMEM or DMEM+2 % FBS as indicated on the X axis; On days 1, 4 and 5 (indicating respectively 24, 96 and 120 hours after the initiation of the experiment) the DsRed signal was measured. Fold increase in the DsRed signal relative to the signal on day 1 is shown on the Y axis.

Summarizing, it seems that for Melmet 5 the collagen matrix and the medium with 2% serum might represent the suitable conditions for culturing in 3D. Also, 60000 cells/well seem to be a suitable cell concentration since this cell amount gave

easy-detectable (clearly above the background) fluorescence signals. Therefore, these conditions have been use for the majority of the subsequent experiments with Melmet 5.

3.1.2 Evaluation of Melmet 1 growth in 3D matrixes

Based on the results above, we have attempted to use similar culturing conditions also for Melmet 1 cells. However, we increased cell number to 100.000 cells/well, since Melmet 1 generally grows much slowed and gives lower DsRed signals compared to Melmet 5 (data not shown). Melmet 1 cells were seeded in 3D collagen matrix in the medium with 2 % serum in the 3rd layer. The visual inspection by the microscope of the similar cultures with Melmet 1 raised doubts to whether these cells were viable and growing suggesting that Melmet 1 cells might not like to be cultured under these conditions – the cells were round, dark and did not look viable (data not shown). Therefore, we decided to control if Melmet 1 cells are still viable and are proliferating during culturing for 4 days in a collagen matrix and instead of measuring the DsRed signal, we have measured cells metabolic activity by the MTS method, which is an accepted approach for evaluation of cell viability. The MTS assay was performed after 1, 3 and 4 days of culturing. Paralelly, the cells were also stained with live/dead dyes Calcein/PI at the same time points. It has to be noticed that cells were seeded on three different plates so that MTS-measurements and staining procedures at the early time points could not affect the growth of the cells that were analyzed at the later time points.

As we can see in **Figure 14 A,** the metabolic activity of Melmet 1 cells was reduced approximately 2-fold after 4 days of culturing in collagen, indicating that only ~ 50 % of the cells remained viable. **Figure 14 B** compares the MTS signal measured in the well with collagen without cells (=collagen background) and wells with cells in collagen, revealing that the signal “produced” by viable cells was only 1.4 fold above the background. This is not much having in mind that this system was planed to be used for therapy studies, where decrease in cell number (consequently absorbance) was expected.

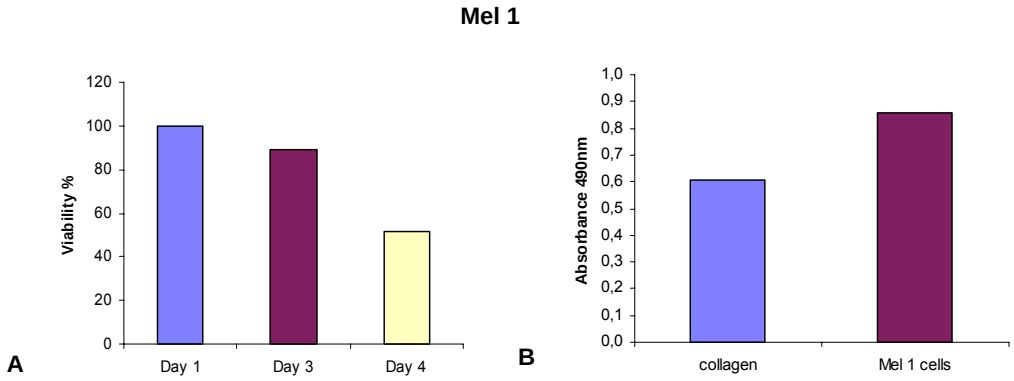


Figure 14. Metabolic activity (viability) of Melmet 1 during culturing in 3D collagen matrixes. Melmet 1 cells (100000 cells/well) were seeded in the 2nd layer of collagen. **A:** Cells metabolic activity was measured by the MTS method on days 1, 3 and 4 (indicating respectively 24, 72 and 96 hours after the initiation of the experiment) and are presented as % relative to day 1 and are indicated on the Y axis. **B:** Absorbance signal measured at 490nm (raw data) in cell-free collagen ("collagen" indicates a background signal) and collagen with Melmet 1 cells ("Mel 1 cells") on day 3. Absorbance signal is indicated on the Y axis.

Figure 15 confirms that the amount of viable (green) Melmet 1 cells in collagen on days 3 and 4 were reduced significantly compared to day 1; consequently, the amount of dead (red) cells was increased. Thus, pictures taken after 3 days of culturing shows us a lot of red cells, even though green cells are still in the majority, while after 4 days, the red cells are dominating. To note, some very few red cells are seen already after 24 hours of culturing in 3D collagen. It should be added that the ratios between green and red cells observable by the microscope varied depending on the area where the pictures were taken. Despite this variation, based on the results shown in **Figures 14 and 15** it can be concluded that on day 3, 10% and more of the cells are already dead and on day 4 this number reaches at least 50%, suggesting that the decline in cell viability of Melmet 1 started already after 2 days of incubation in collagen.

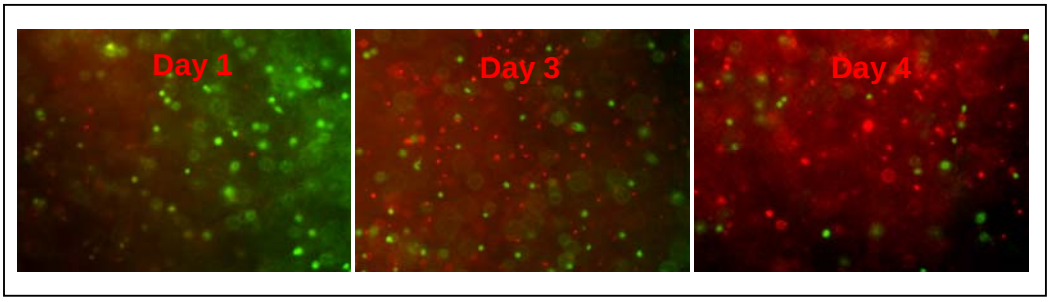


Figure 15. Evaluation of Melmet 1 viability in 3D collagen cultures. Visualization of cells stained with live/dead dyes Calcein/PI on days 1, 3 and 4 (pictures of the same area are taken using 10X objective and respective filters for Calcein (green) and PI (red) and subsequently combined).

To test whether Matrigel could be used instead of collagen for culturing of Melmet 1 cells, we performed a pilot experiment, where the cells were seeded in Matrigel in the medium with 2% FBS in the 3rd layer as described in Materials and Methods (see "Cell culturing in 3D: Matrigel cultures"). The cultures were inspected by microscopy, and cells metabolic activity was measured by the MTS method after 3 days of culturing.

Based on the microscopic evaluation we concluded that the cells looked "nicer" compared to the collagen cultures (data not shown). The MTS measurements revealed that the MTS signal detected in the wells with Matrigel+cells compared to the wells with only Matrigel was 1.7-fold higher (**Figure 16).** This indicates that the MTS signal produced by the viable cell was slightly higher in the Matrigel-based culture (**Figure 16**) compared to the collagen-based culture (**Fig. 14B**).

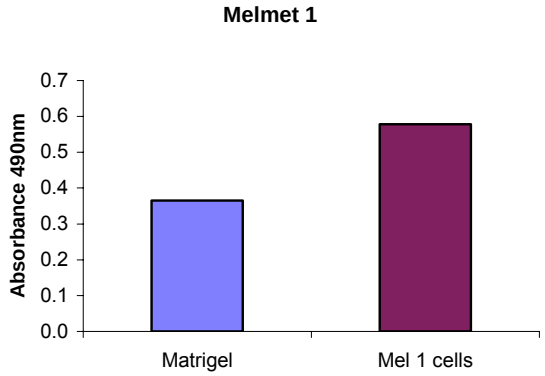


Figure 16. MTS signal intensity in Melmet 1 cultures in in Matrigel. Melmet 1 cells (100000 cells/well) were seeded in the 2nd layer of Matrigel matrix. Paralelly, a cell-free well containing only Matrigel in the 2nd layer was prepared for comparison. On day 3 (indicating 72 hours after the initiation of the experiment) the MTS signal was measured in the wells containing only Matrigel" or Matrigel with cells ("Mel1 cells") indicated on the X axis. The MTS absorbance data are presented on the Y axis.

Based on these experiments we concluded that Matrigel might be a more suitable matrix than collagen for culturing of Melmet 1 in 3D. Therefore, most of the subsequent studies with Melmet 1 were performed in Matrigel. However, it can be mentioned that one additional experiment using Melmet 1 and collagen supplemented with additional proteins form extracellular matrix was performed as described in the next section (see 3.2.3 Evaluation of the effect of the ECM proteins fibronectin and laminin: Melmet 1 in collagen matrix).

3.2 Investigation of the effect of extracellular matrix (ECM) proteins

Cell adhesion to the extracellular matrix (ECM) plays a major role in cellular communication and regulates a variety of cellular processes including proliferation, survival and motility. As discussed in the introduction, tumour cells can interact with various proteins of ECM, and these interactions might influence tumour cell growth and viability. Therefore, in the present part of the work we investigated how the selected proteins of ECM influence melanoma cell growth and viability. The focus was on Melmet 5, since Melmet 5 was more suitable for culturing in 3D collagen-based matrixes.

3.2.1 Evaluation of Melmet cell adhesion to various ECM proteins

To map the ECM proteins that the Melmet cells could adhere to, we have employed ECM arrays containing the most important proteins of ECM: collagens I, II, and IV, fibronectin, laminin, tenascin and vitronectin. **Figure 17** reveals the ability of Melmet cells to adhere to these proteins.

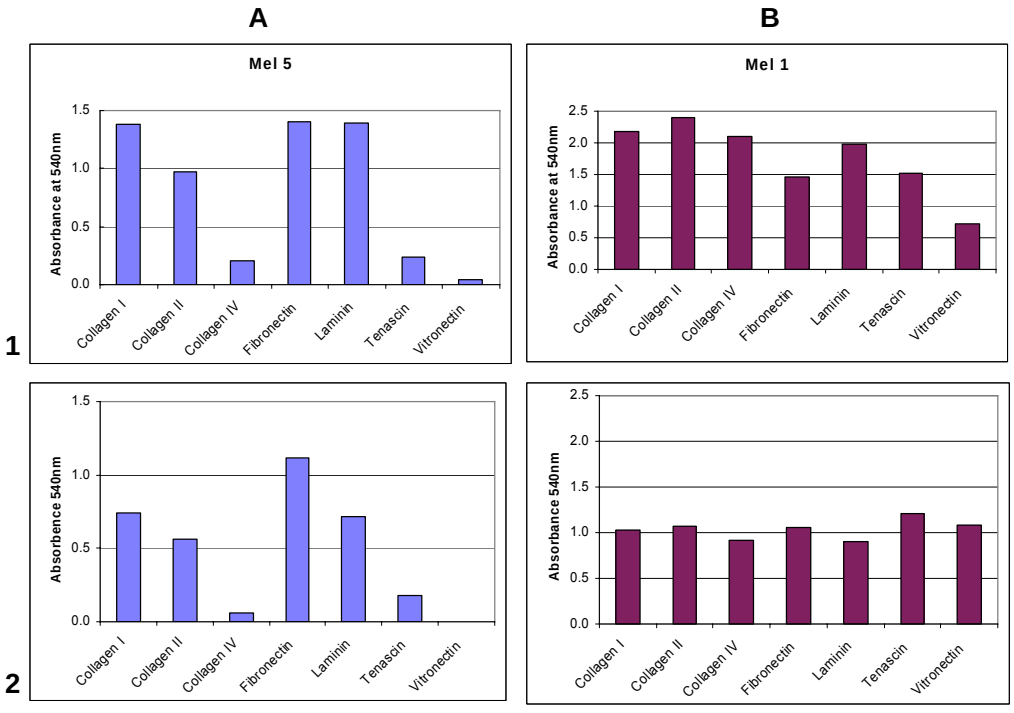


Figure 17. Cell adhesion to ECM components array profiles. A: Melmet 5. **B:** Melmet 1. 1 and 2 indicates two biologically different experiments performed for each cell line.

As can be seen Melmet 1 and Melmet 5 had different tendency regarding adhesion to selected ECM proteins (**Figure 17 A.** 1 and 2). Melmet 1 adhered almost equally efficiently to all investigated proteins, while Melmet 5 (see **Figure 17 B.** 1 and 2) was adhering very poorly to collagen IV and vitronectin, suggesting that Melmet 5 has low levels of these respective receptors.

It has to be noticed that it has been performed two independent biological experiments for each cell line using cells at different passage numbers. This explains some variations in the absolute numbers shown on axis Y in **Figure 17**. Despite this variation, it can be easily seen the same tendency regarding adhesion to selected proteins of ECM in both experiments for each cell line. To note, collagen I (the main component of collagen-matrix) and laminin (the main component of Matrigel-matrix) were among the proteins both Melmet cell lines adhered efficiently to.

3.2.2 Evaluation of the effect of the ECM proteins fibronectin and laminin: Melmet 5

Mapping of the adherence to various ECM proteins revealed that both Melmet 1 and Melmet 5 was efficiently binding to fibronectin (FN) and laminin (LN) and indicated that Melmet cells posses the receptors assuring binding to these two proteins. Therefore, it was of interest for us to explore whether the presence of these two proteins in 3D cultures affected growth of Melmet cells.

Fibronectin and laminin represent the ECM proteins that have been shown previously by others to be associated with metastasis (see Introduction section). First, we have focused on Melmet 5. 3D cultures were supplemented with these ECM proteins by adding Fibronectin or Laminin (at a final concentration 10µg/ml) to the 2nd collagen layer together with the DsRed-labeled Mel 5 cells as described in Materials and Methods (see section “3D cultures supplemented with fibronectin and laminin”). In the 3rd layer, the medium with or without 2% serum was used. Cell growth was followed for up to 4 days by measuring the DS Red signal. At the end of the experiment i.e. on day 4, all cultures were stained with the Calcein dye to quantify and visualize the viable cells. As can be seen in **Figure 18**, the DsRed signal on day 4 was increased approximately 1.5-2 fold compared to day 1, indicating an increase in cell number i.e. cellular proliferation during 4 days.

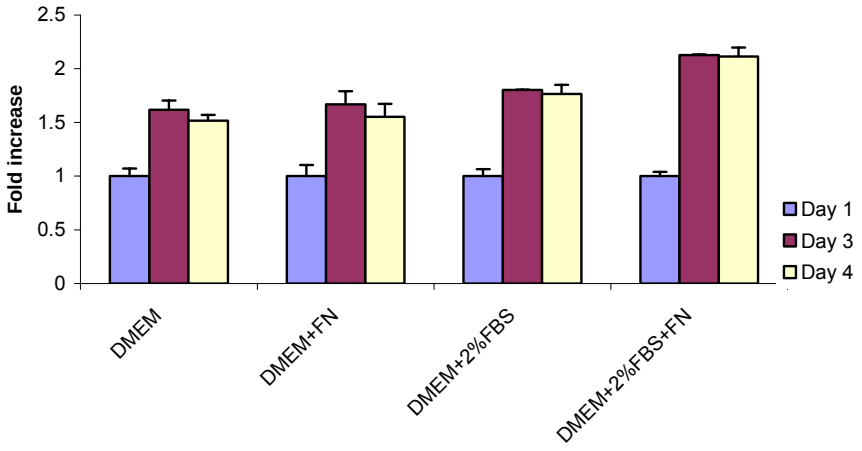


Figure 18. Melmet 5 cell growth in 3D collagen matrix in the presence or absence of fibronectin. 60000 DSRed Melmet cells were seeded in the 2nd collagen layer with or without 10µg/ml fibronectin (FN, indicated on the X axis) as described in Material and Methods. The growth mediums used in the 3rd layer where DMEM or DMEM+2%FBS. The DsRed signal was measured on days 1, 3 and 4 which corresponds to respectively 24, 72 and 96 hours after the initiation of the experiment. Fold increase relative to day 1 is shown on the Y axis. Errors bars indicate StDv from 2 parallels.

However, FN did not show a significant influence on cell proliferation or viability, since the intensity of both the DSRed and the Calcein signal were the same in the presence and absence of FN (**Figure 19 A**).

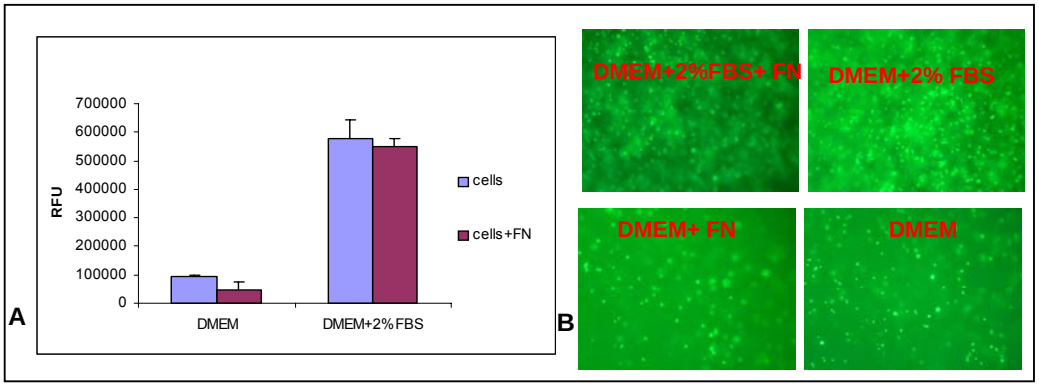


Figure 19. Evaluation of fibronectin effect on the viability of Melmet 5: staining with Calcein. Cells were cultured in 3D with or without 10µg/ml FN in the 2nd layer as described in the Figure 18 text. At the end of the experiment i.e. on day 4, the 3D cultures were stained by adding 0.25 µl Calcein as indicated in Materials and Methods. **A:** Green Calcein signal was measured by a plate reader Synergy 2 and Relative Fluorescence Units (RFU) are shown on the Y axis; media compositions in the 3rd layer are indicated on the X axis; error bars indicate StDv from 2 parallels. **B:** Viazualization of viable Calcein-stained cells by microscopy (pictures taken using 4x objective; the compositions in the 3rd layer and the presence of FN is indicated in the figure).

Furthermore, **Figure 19 B** illustrates that there was no visual difference in the amount of viable (green) cells in the presence versus absence of fibronectin.

However, **Figure 19 B** confirms that the amount of viable cells was higher when the medium with 2% serum was used compared to serum-free medium. This corresponds to the earlier observation (see **Figure 12**).

Similar experiments were performed to evaluate an effect of laminin on growth and viability of Melmet 5. As can be seen in **Figure 20**, laminin also did not stimulate cell growth significantly since the DsRED signal intensity in the presence and absence of LN were very similar despite big standard deviation.

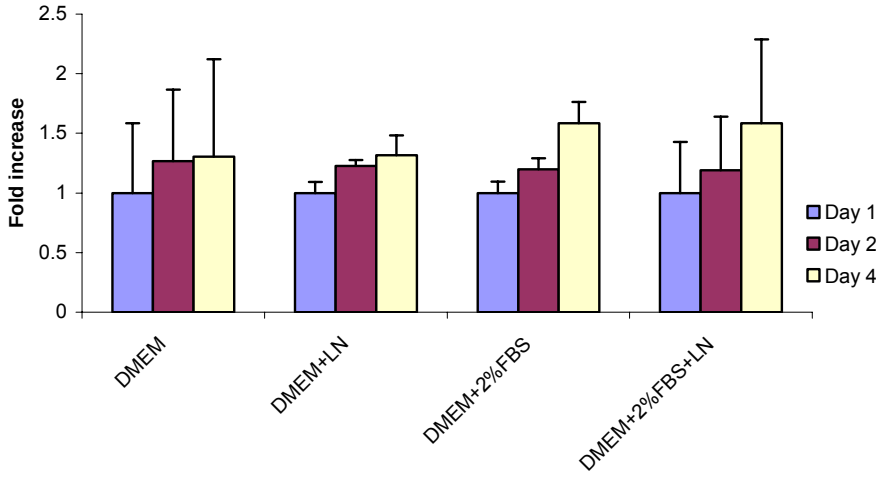


Figure 20. Melmet 5 cell growth in 3D collagen matrix in the presence or absence of laminin. 60000 DSRed Melmet cells were seeded in the 2nd collagen layer with or without 10µg/ml laminin (LN, indicated on the X axis) as described in Material and Methods. The growth mediums used in the 3rd layer were either DMEM or DMEM+2%FBS. The DsRed signal was measured on days 1, 3 and 4 which corresponds to respectively 24, 72 and 96 hours after the initiation of the experiment. Fold increase relative to day 1 is shown on the Y axis. Errors bars indicate StDv from 2 parallels.

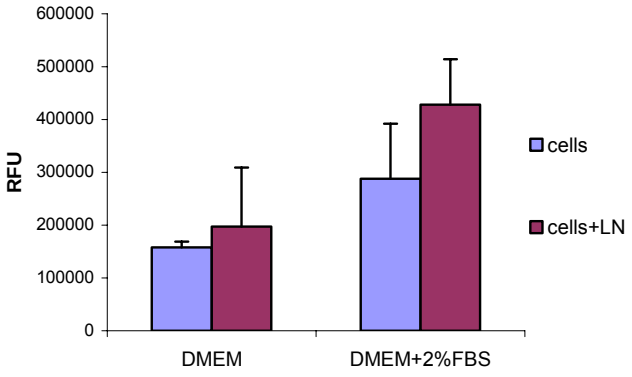


Figure 21. Measurement of Calcein signal on day 4. DMEM=wells containing DMEM in the top layer of 3D cultures; DMEM+2%FBS=DMEM supplemented with 2% serum in the top layer of 3D cultures; cells=wells containing cells; cells+LN=wells containing cells and 1µl Laminin embedded in the 2nd layer. Relative Fluorescence Units (RFU) is shown on the Y axis and media compositions on X axis. Error bars indicate StDv of 2 parallels.

However, measurements of the Calcein signal (**Figure 21**) suggested that cell viability could be slightly higher in cultures with LN compared to the cultures lacking LN, and this was more pronounced in cultures with 2% serum.

Therefore, we decided to investigate the potential effect of the ECM proteins on cell viability more closely by measuring cells metabolic activity. Thus, Melmet 5 cells were seeded in a collagen matrix supplemented with laminin or fibronectin as described above and after 4 days cells metabolic activity was measured by the MTS method. This time we could not see any difference between the cells grown in the presence of laminin or fibronectin versus the cells grown without these proteins (**Figure 22**).

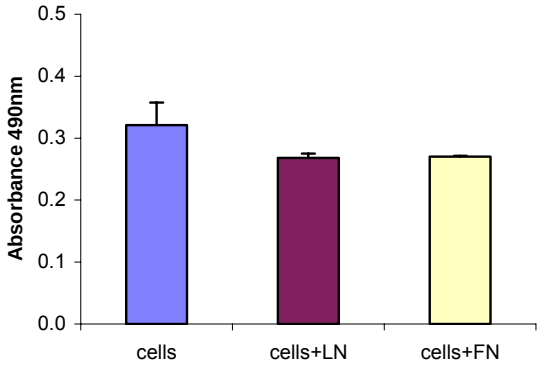


Figure 22. Melmet 5 cell metabolic activity in 3D collagen matrix in the presence or absence of laminin and fibronectin. 60000 Melmet 5 cells were seeded in the 2nd collagen layer with or without 10µg/ml FN and 10µg/ml LN (indicated on the X axis) as described in Material and Methods. The growth medium used in the 3rd layer was DMEM+2%FBS.The MTS absorbance signal (indicated on Y axis) was measured after 4 days which corresponds to 96 hours after the initiation of the experiment. Errors bars indicate StDv from 2 parallels.

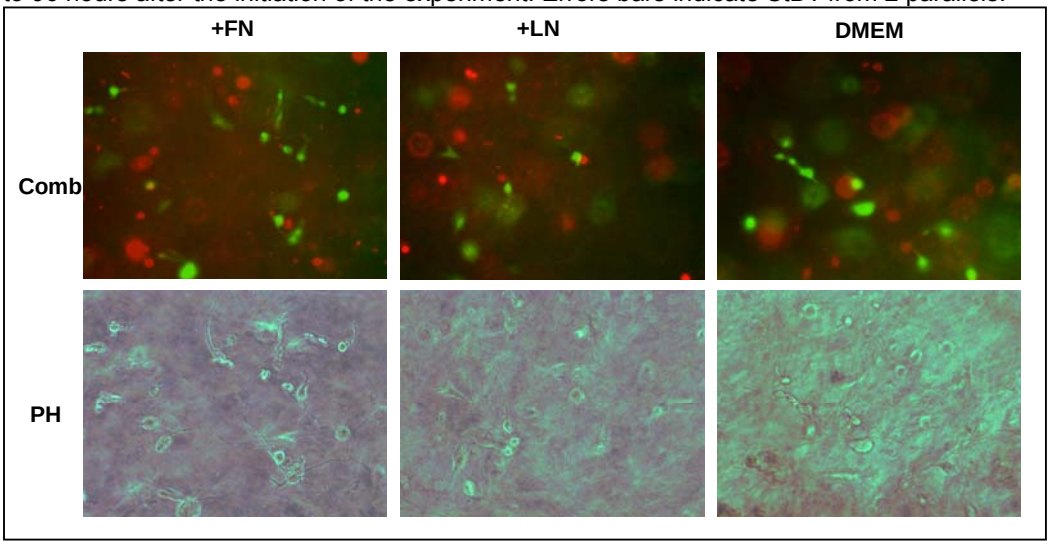


Figure 23. Evaluation of Melmet 5 viability in 3D collagen cultures supplemented with FN and LN. Combined picture (top) illustrating cells stained with live/dead dyes Calcein/ Propidium Iodide on day 4 and Phase contrast picture (bottom) of the same area. (Pictures are taken using 20X objective).

Also **Figure 23**, showing the viable and dead cells after staining of the cultures with Calcein/PI, indicates approximately equal amounts of green (viable) and red (dead) cells in the presence or absence of fibronectin and laminin.

Altogether, this indicates that we could not confirm by the MTS and the Calcein/PI staining methods that laminin or fibronectin has an effect on the viability of Melmet 5 grown in collagen.

3.2.3 Evaluation of the effect of the ECM proteins fibronectin and laminin: Melmet 1 in collagen matrix

Previously we have shown that Melmet 1 did not "like" to be cultured in collagen matrix using the conditions suitable for Melmet 5. However, assuming that fibronectin and laminin might have an effect on cell viability (see the data and the discussion above), we have performed a similar study on Melmet 1 and investigate whether addition of these proteins to collagen cultures could improve the growth/viability of Melmet 1. Thus we have cultured Melmet 1 cells in less dense collagen (1.5mg/ml instead of traditional 2.5mg/ml) supplemented with 10 µg/ml fibronectin or laminin. After 4 days cells metabolic activity and viability were evaluated by the MTS and Calcein/PI methods.

As can be seen in **Figures 24** and **25**, neither fibronectin nor laminin had a notable effect.

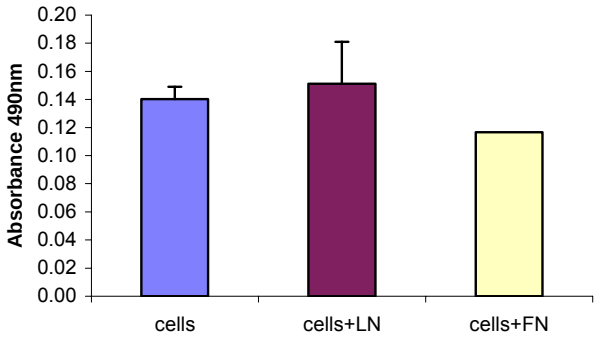


Figure 24. Melmet 1 cell metabolic activity in 3D less-dense collagen matrix (1, 5 mg/ml) in the presence or absence of laminin and fibronectin. 60000 Melmet 5 cells were seeded in the 2nd collagen layer with or without 10µg/ml FN and 10µg/ml LN (indicated on the X axis) as described in Material and Methods. The growth medium used in the 3rd layer was DMEM+2%FBS.The MTS absorbance signal (indicated on Y axis) was measured after 4 days which corresponds to 96 hours after the initiation of the experiment. Errors bars indicate StDv from 2 parallels.

However, it might seem from **Figure 25** that red (dead) cells are not dominating like it was the case in more-dense collagen cultures (see **Figure 23** above).

Summarizing, we conclude that fibronectin and laminin did not have a notable effect on cell growth or viability in collagen matrixes under the investigated conditions.

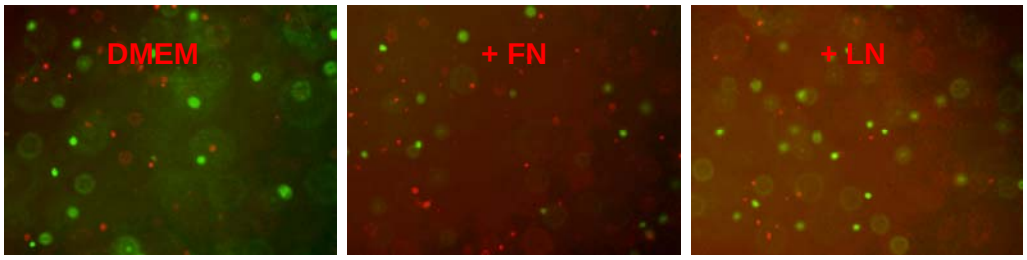


Figure 25. Evaluation of Melmet 1 viability in 3D collagen (1,5 mg/ml) cultures supplemented with FN and LN in the 2nd layer. Visualization of cells stained with live/dead dyes Calcein/ Propidium Iodide on day 4: combined pictures (pictures are taken using 10X objective).

3.3 Effect of organ-conditioned medium

To evaluate whether Melmet cells can be influenced by soluble factors released from the organs – common sites of melanoma metastasis – 3D cultures were supplemented with organ-conditioned medium (CM). Also here we focused on Melmet 5 cells. CMs were prepared from brain, lungs, bone marrow and lymph nodes of healthy mice as described in Materials and Methods, diluted 50:50 with fresh medium containing 2 % FBS and applied to the 3rd layer of the 3D cell cultures. In control wells, the medium with 2% serum without CM was applied to the 3D cell cultures. Cultures were grown for up to 5 days and the results were analyzed by measuring cells metabolic activity by the MTS method on days 3 and 5 (**Figures 26-27**). In addition, cells growth pattern was analyzed by microscopy (**Figures 28-29**).

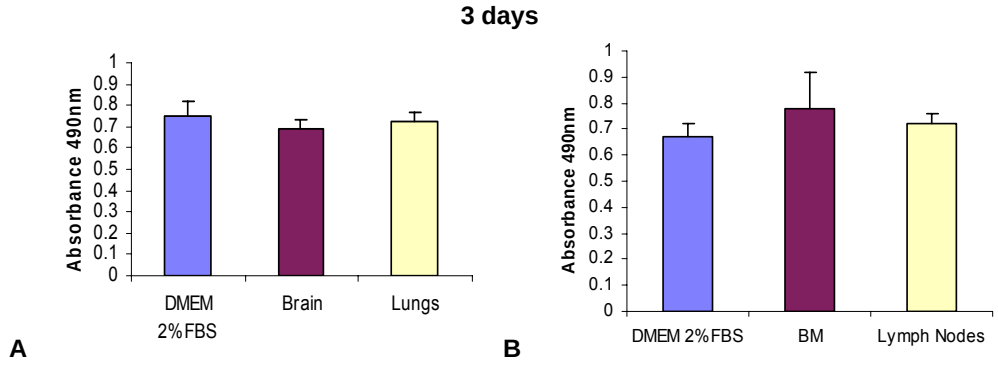


Figure 26. Metabolic activity of Melmet 5 cells incubated for 3 days in the presence of CM from A: Brain and Lungs; B: Bone Marrow (BM) and Lymph Nodes. CMs were diluted with DMEM+2% serum at a ratio 50:50 and added as a 3rd layer to the 3D cultures containing 60000 Melmet 5 cells as indicated in Materials and Methods. In control wells, DMEM+2% serum without CM was used. Cells metabolic activity was evaluated by the MTS method and is presented as absorbance on the Y axis. Error bars indicate StDv from 2 parallels.

As shown in **Figure 26**, none of the investigated CM affected cells metabolic activity during 3 days of culturing.

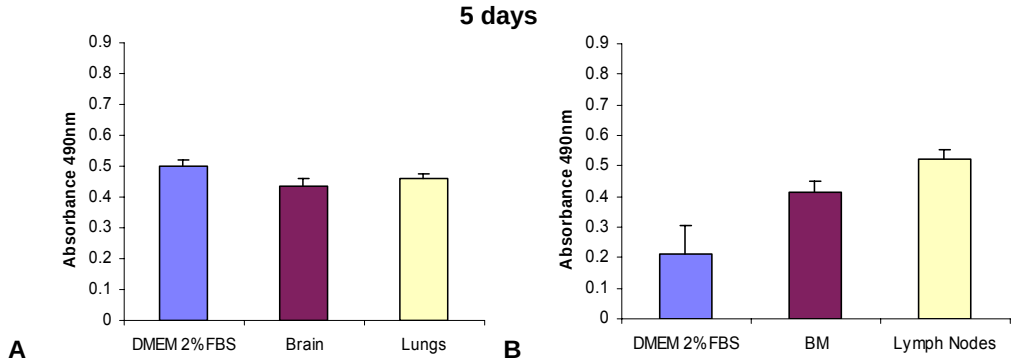


Figure 27. Metabolic activity of Melmet 5 cells incubated for 5 days in the presence of CM from A: Brain and Lungs; B: Bone Marrow (BM) and Lymph Nodes. CMs were diluted with [DMEM+2% serum] at a ratio 50:50 and added as a 3rd layer to the 3D cultures containing 60000 Melmet 5 cells as indicated in Materials and Methods. In control wells, DMEM+2% serum without CM was used. Cells metabolic activity was evaluated by the MTS method and is presented as absorbance on the Y axis. Error bars indicate StDv from 2 parallels.

However, it might seem that organ-derived cells released from bone marrow- and lymph node-conditioned media stimulate the survival and proliferation of Melmet 5 cells, particularly after 5 days in culture, suggesting that these organs may release

soluble factors stimulating growth of melanoma cells in collagen matrix (**Figure 27 B**).

Interestingly, microscopic observation of the cultures revealed that the conditioned media from brain and lungs had an effect on cells growth pattern and morphology inducing the formation of complex structures ("rings" and "chains" (**Figure 28**) compared to the cells incubated only with the medium +2% FBS, where mostly separated single cells were observed.

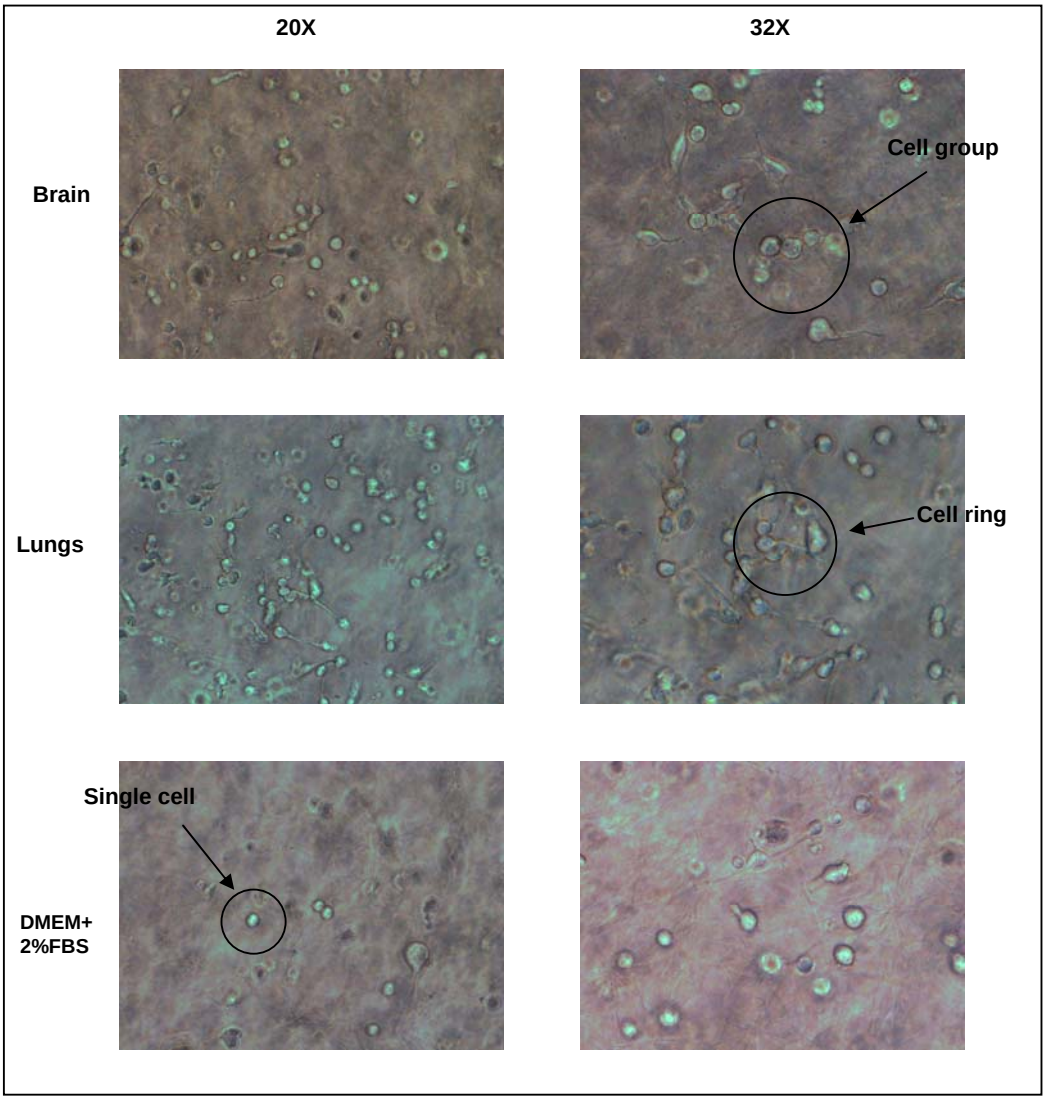


Figure 28. Growth pattern of Melmet 5 cells incubated with CM from lungs and brain. Phase contrast pictures were taken on day 5 using 20X (left) and 32x (right) objectives. CMs were diluted with the medium DMEM+2 % serum at a ratio 50:50. For comparison cells were cultured in the medium DMEM+2 % serum without CM.

Furthermore, the conditioned media from bone marrow induced even more complex cell structures, and these structures looked more like clumps of elongated cells (**Figure 29**)**.** Also the conditioned media from lymph nodes stimulated the formation of elongated complex cell structures as shown in (**Figure 29**).

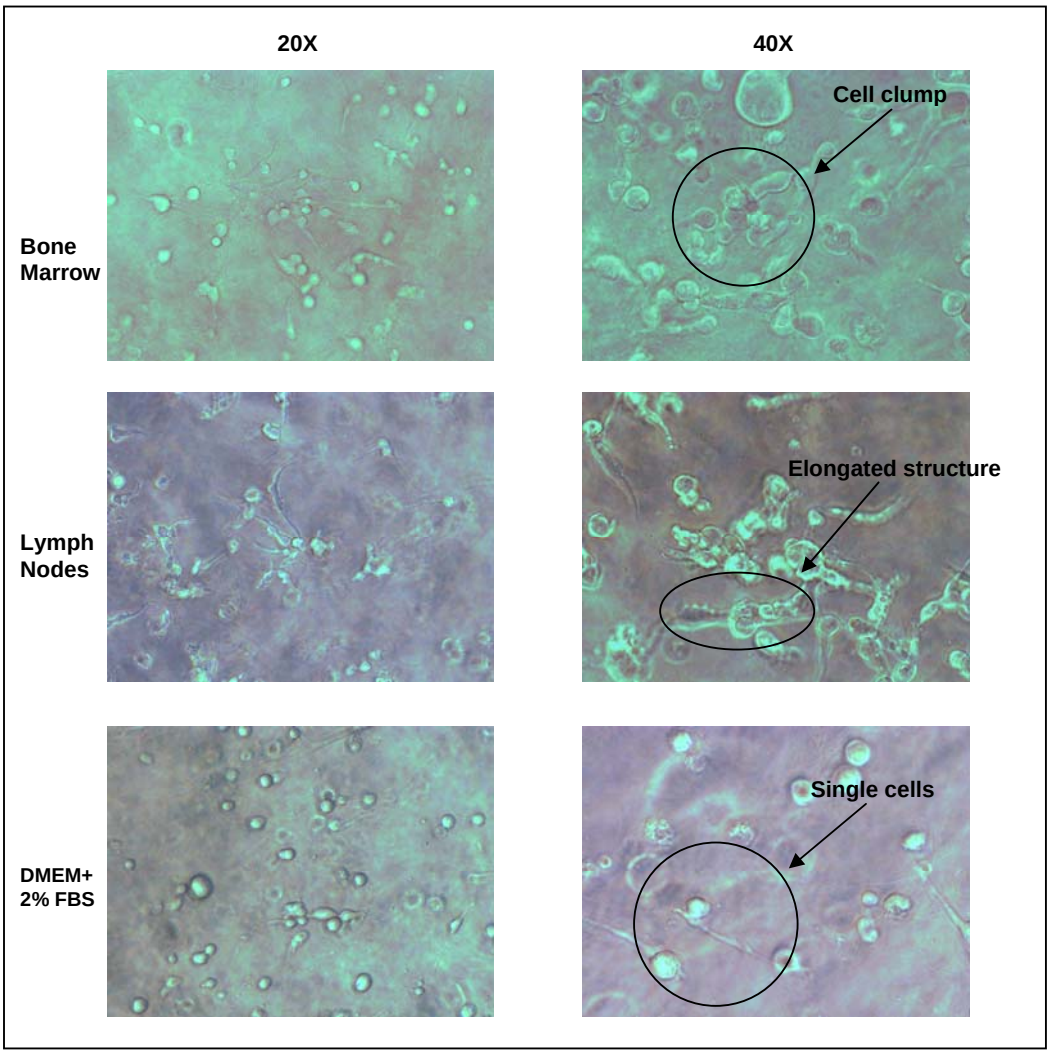


Figure 29. Growth pattern of Melmet 5 cells incubated with CM from bone marrow and lymph nodes. Phase contrast pictures were taken on day 3 using 20x (left); 40x (right) objectives. CMs were diluted with the medium DMEM+2 % serum at a ratio 50:50. For comparison cells were cultured in the medium DMEM+2 % serum without CM.

It has been published previously that too high amount of the CM might actually inhibit cell growth (Valle, Zalka et al. 1992). Therefore, we have performed a similar experiment but reduced the amount of CM added to 25 % instead of 50%. Thus, we diluted the CM from lungs, brain, lymph nodes and bone marrow with the

medium at ratio 25:75 and added to the 3rd layer of the 3D cell cultures. Cell metabolic activity was evaluated after 5 days by the MTS method.

In contrast to the previous experiment where 50% of CM was used (see **Figures 26 A and 27 A**), here the CM from the lungs and brain seem also to stimulate the cells (**Figure 30**) .As can be seen in **Figure 30**, a ~1, 5-fold increase in relation to DMEM+2%FBS was observed . CM from bone marrow and lymph nodes also showed a stimulating effect on the cells.

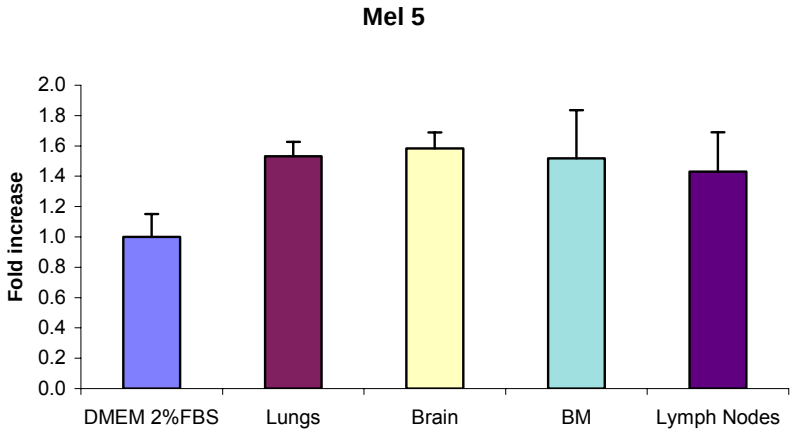


Figure 30. Effect of CM from brain, lungs bone marrow (BM) and lymph nodes on metabolic activity of Melmet 5. CMs were diluted with the medium [DMEM with 2% serum] at a ratio 25:75 and added as a 3rd layer to the 3D cultures containing 60000 Melmet 5 cells. In control wells, DMEM with 2% serum without CM was added to the cell cultures. Metabolic activity was evaluated by the MTS method and is presented on the Y axis as fold increase relative to DMEM+2%FBS. Error bars indicate StDv from 3 parallels.

To evaluate the effect of CM on Melmet 1, the cells were embedded in the 2nd Matrigel layer which was overlaid with CM diluted with the medium at a ration 25:75. 3D cultures were incubated for 4 days and cell metabolic activity was measured by the MTS method.

As we can see in **Figure 31**, all investigating CMs had more or less stimulating effect on the Melmet 1 cell, however, it should be noted that the standard deviations were very big.

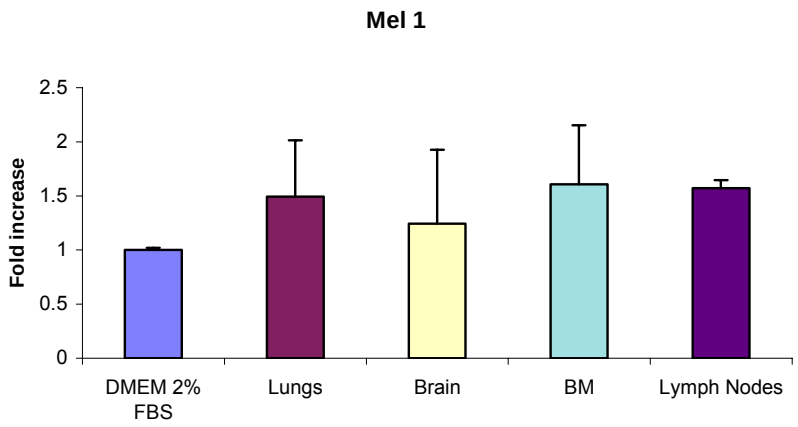


Figure 31. Incubation of Melmet 1 cells for 4 days in the presence of CM from brain, lungs bone marrow (BM) and lymph nodes. CMs were diluted with DMEM medium supplemented with 2% serum at a ratio 25:75 and added as a 3rd layer to the 3D Matrigel cultures containing 60000 Melmet 1 cells as indicated in Materials and Methods. In control wells, DMEM+2% FBS without CM was used. Cells metabolic activity was evaluated by the MTS method and is presented as fold-increase relative to DMEM+2% FBS on the Y axis. Error bars indicate StDv from 2 parallels.

Further we wanted to investigate whether CM prepared from a metastatic mouse will have a different effect on Melmet cells compared with CM from a healthy mouse. It is known that a growing tumour and its microenvironment might lead to secretion of various soluble factors (particularly those promoting tumour growth) different from normal conditions. Therefore, it was of interest to investigate how Melmet cells respond to soluble factors extracted from a mouse with metastases. We used only Melmet 5 cells for these studies. Thus, CM prepared from brain, lungs, lymph nodes and BM of a metastatic mouse was diluted with the medium DMEM+2 % FBS at a ratio 25:75 and added to the 3rd layer of 3D cell cultures. For comparison, we used CM prepared from a healthy mouse diluted at the same ratio 25:75. Cell metabolic activity was measured on the last day of incubation i.e. at day 5.

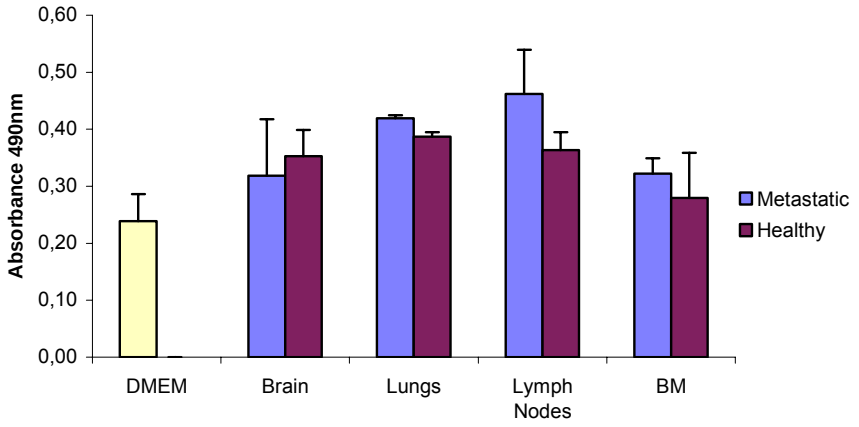


Figure 32. Incubation of Melmet 5 cells for 5 days in the presence of CM from brain, lungs, bone marrow and lymph nodes of metastatic and healthy mouse. CMs were diluted with DMEM+ 2% serum at a ratio 25:75 and added as a 3rd layer to the 3D collagen cultures containing 60000 Melmet 5 cells as indicated in Materials and Methods. In control wells, DMEM+ 2% FBS without CM was used. Cells metabolic activity was evaluated by the MTS method and is presented as absorbance on the Y axis. Error bars indicate StDv from 2 parallels.

As we can see in **Figure 32**, all CM seems to have a stimulating effect compared to the medium without CM. Interestingly, CM obtained from the lymph nodes, bone marrow and maybe lungs seems to stimulate cells to a greater extent than CM obtained from the same organs of a healthy mouse, however the difference was not big and not statistically significant.

3.4 Therapy-related studies

To investigate how the sensitivity of melanoma cells to drugs can be modulated by TME factors, we have measured cell viability after treatment with the drug Elesclomol. Elesclomol is a new ROS-inducing agent currently investigated on preclinical melanoma models at the Department of Tumour biology, where this project was performed. As previously, we have focused on Melmet 5

3.4.1 Effect of Elesclomol on Melmet 5 in 2D and 3D cultures

It is known that tumour cell sensitivity to drugs can differ when cells are treated in 2D versus 3D cultures (discussed in the Introduction). To compare Elesclomol effect on Melmet 5 cell viability in 3D and 2D cultures, we have tested

different concentrations of the drug: 2nM, 10nM, 50nM, 200nM, 1000nM and 2000nM (see Materials and Methods, “Elesclomol as factor X”).

For 3D experiments, 60.000 Melmet 5 cells/well were seeded in collagen matrix; Elesclomol at the mentioned concentrations was added to the 3rd (top) layer and the cultures were incubated for 3 days.

To evaluate Elesclomol effect on Melmet 5 in 2D cultures, 10000cells/well were seeded in the medium as described previously in Materials and Methods. The next day, the growth medium was replaced with the new medium containing Elesclomol at different dilutions and incubated for 3 days. Cell viability in both 3D and 2D cultures was measured after 3 days (72 hours) of the treatment by the MTS method.

As we can be seen in **Figure 33,** the viability of the cells was reduced with increasing concentration of the drug in both cultures.

In 2D cultures, we achieved complete response using 2000nM of Elesclomol. This is supported by microscopic visualization (see **Figure 34**), were we could not observe any viable cells after the treatment with 2000nM of the drug. Also when 1000nM of Elesclomol was used, the viability of the cells was significantly reduced reaching approximately only 35% (**Figure 33** “2D”).

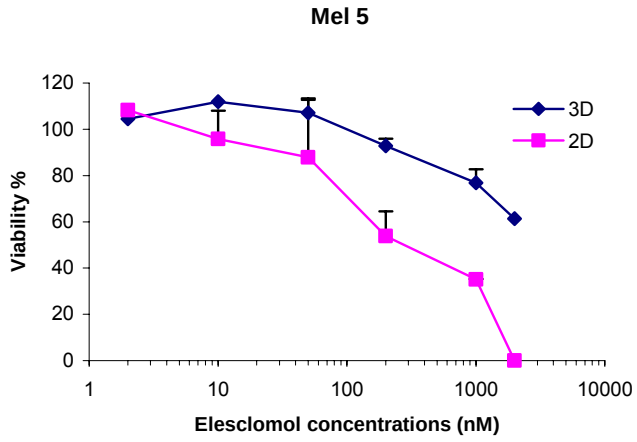


Figure 33. Viability of Melmet 5 cells in 2D and 3D following treatment with different consentrations of Elesklomol: 2nM, 10nM, 20nM, 50nM, 200nM, 1000nM and 2000nM. 3D: Cells (60000/well) were embedded in the collagen matrix. **2D:** Cells (10000/well) were seeded in RPMI++ on the day 0 and the next day the medium was replaced with the medium containing Elesclomol. Elesclomol was added at indicated concentrations in the 3rd (top) layer of the 3D cultures or to 2D monolayer’s on day 1 as described in Materials and methods. Cell viability was measured by the MTS method and is presented as percentage of untreated controls on the Y axis; used concentrations of the drug are indicated logarithmic on the X axis. Error bars indicate StDv from 2 independent experiments.

The microscopy pictures in **Figure 34** confirm that while most of the cells are dead/dying (rounded cells) we could still see some viable cells when 1000nM Elesclomol was used in 2D. The smallest concentrations of the drug used, 10nM hardly had an effect, while 50nM killed only ~10% of the cells in 2D.

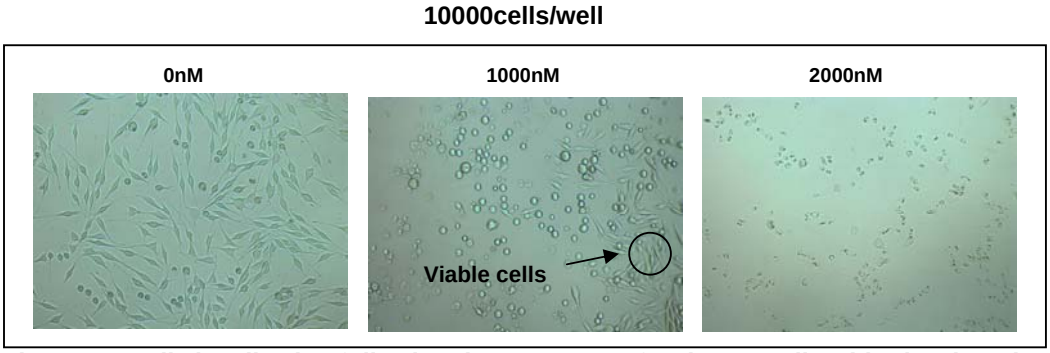


Figure 34. Cell visualization following the treatment of Melmet 5 cells with Elesclomol in 2D. Elesclomol concentrations used are indicated in the figure. Phase contrast pictures were taken using a 10X objective.

In 3D cultures, cell viability was reduced to approximately 60% after treatment with 2000nM Elesclomol, which induced complete toxicity in 2D (see **Figure 33 "3D"**). The low concentrations of the drug (2nM, 10nM, 50nM) did not have any inhibitory effect in 3D cultures.

These data support the previous observations that cells are more sensitive to the drug in 2D culture compared to cells grown in 3D matrixes supporting the theory that there is a difference between these two systems with respect to drug-response.

3.4.2 Effect of organ-conditioned medium on Melmet 5 sensitivity to Elesclomol in 3D

Further we wanted to investigate if organ-conditioned medium (CM) has an effect on the viability of Melmet 5 cells treated with Elesclomol. Besides, we wanted to evaluate whether CM prepared from a metastatic mouse and CM prepared from a healthy mouse can induce different effects. For these studies we have used CMs prepared from the brains.

Melmet 5 cells were embedded in the 2nd collagen layer. Elesclomol was diluted to 2000nM with the medium consisting of CM and DMEM +2% FBS at a ratio

25:75. The drug solution was added to the 3rd layer, and the MTS assay was performed after 3 days (i.e. 72 hours) of the treatment.

As can be seen in **Figure 35**, the treatment reduced cell viability to approximately 50-60% corresponding to the previous results obtained in 3D (see **Figure 33**). Unfortunately, we could not observe any significant effect of CM: although the cells treated in the presence of CM showed slightly higher sensitivity to the drug, the differences were not statistically significant. Furthermore, there was no significant difference between treatments in the presence of CM from the metastatic mouse compared to the CM from the healthy mouse.

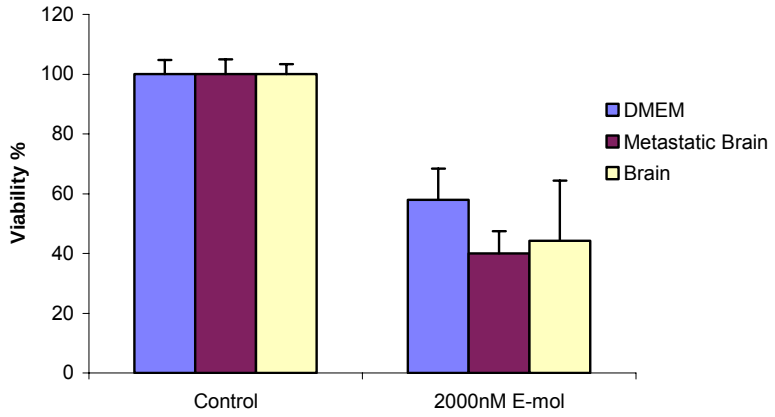


Figure 35. Effect of brain-CM from metastatic mouse versus healthy mouse on viability of Melmet 5 cells following treatment with Elesklomol. Melmet 5 cells (60000/well) were embedded in the 3D collagen matrix. Elesclomol (2000nM) was added to the growth medium in the 3rd (top) layer with or without CM from a metastatic or healthy brain; "control" = wells without Elesclomol; "2000nM E-mol" =concentration of Elesclomol is 2000nM per well; Cell viability was measured by the MTS method and is presented on the Y axis as percentage relative to untreated controls; used concentrations of the drug are indicated on the X axis. Error bars indicate StDv from 2 parallels.

3.4.3 Effect of Elesclomol on Melmet 1 in 2D and 3D cultures

To evaluate the effect of the therapy with Elesclomol on Melmet 1 cells, the studies in 2D were performed first. Since Melmet 1 are significantly bigger and grow significantly slower compared to Melmet 5, two cell densities were tested: 5000- and 10000 cells/well. Elesclomol serially diluted to different concentrations with the medium with 2 % FBS was added to the cells, and cell viability was measured on day 3 (i.e. after 72 hours) of treatment by the MTS method.

As can be seen in **Figure 36**, cells sensitivity to Elesclomol was dependent on cell density. For 5000cells/well, inhibitory effect of Elesclomol "starts" when the drug concentrations exceed 10nM, and the viability declines to ~40% when Elesclomol concentration reaches 20nM. At 50nM only ~10% of the cells are alive. This effect of Elesclomol is supported by the microscopy-studies shown in **Figure 37** (left panel), where we can see that very few cells stay viable at Elesclomol concentration of 50nM. When 200nM Elesclomol was used, there were no viable cells left (**Figure 36**) in the wells were 5000 cells were seeded.

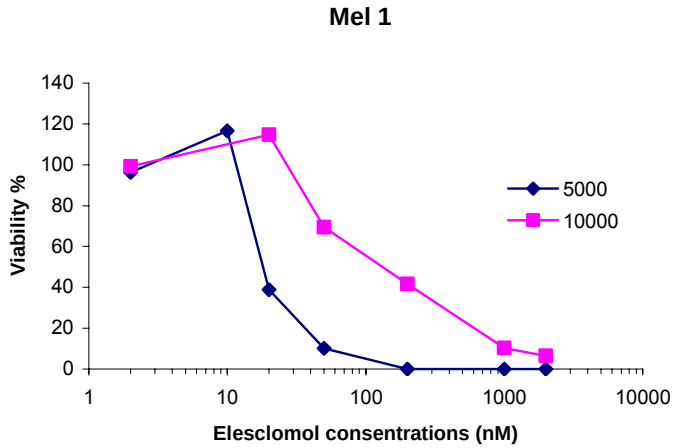


Figure 36. Viability of Melmet 1 cells in 2D following treatment with Elesklomol. Cells (5000/well and 10000/well) were seeded in 2D cultures as described in Materials and Methods. Elesclomol was diluted with the growth medium to 10nM, 20nM, 50nM, 200nM, 1000nM and 2000nM (as indicated on X axis, logarithmic scale) and added to the cells. Cell viability was measured by the MTS method and is presented as percentage of untreated controls on the Y axis.

On the contrary, for wells with more cells i.e. 10000 cells/well, the decline in viability starts at higher Elesclomol concentration i.e. exceeding 20nM, and cell viability goes down to ~70% at Elesclomol concentration of 50nM (see **Figure 36)**. Also **Figure 37** (right panel) indicates that there are left viable cells at Elesclomol concentration of 50nM. When cells were treated with 1000nM, the viability of the cells was reduced to approximately 10% (**Figure 36**), which was also confirmed by the microscopic inspection indicating the presence of some but very few viable cells (see **Figure 37** right panel).

Furthermore, this experiment has shown that 1000nM of Elesclomol is almost as effective as 2000nM for the treatment of 10000 cells/well (**Figure 36 "3D").**

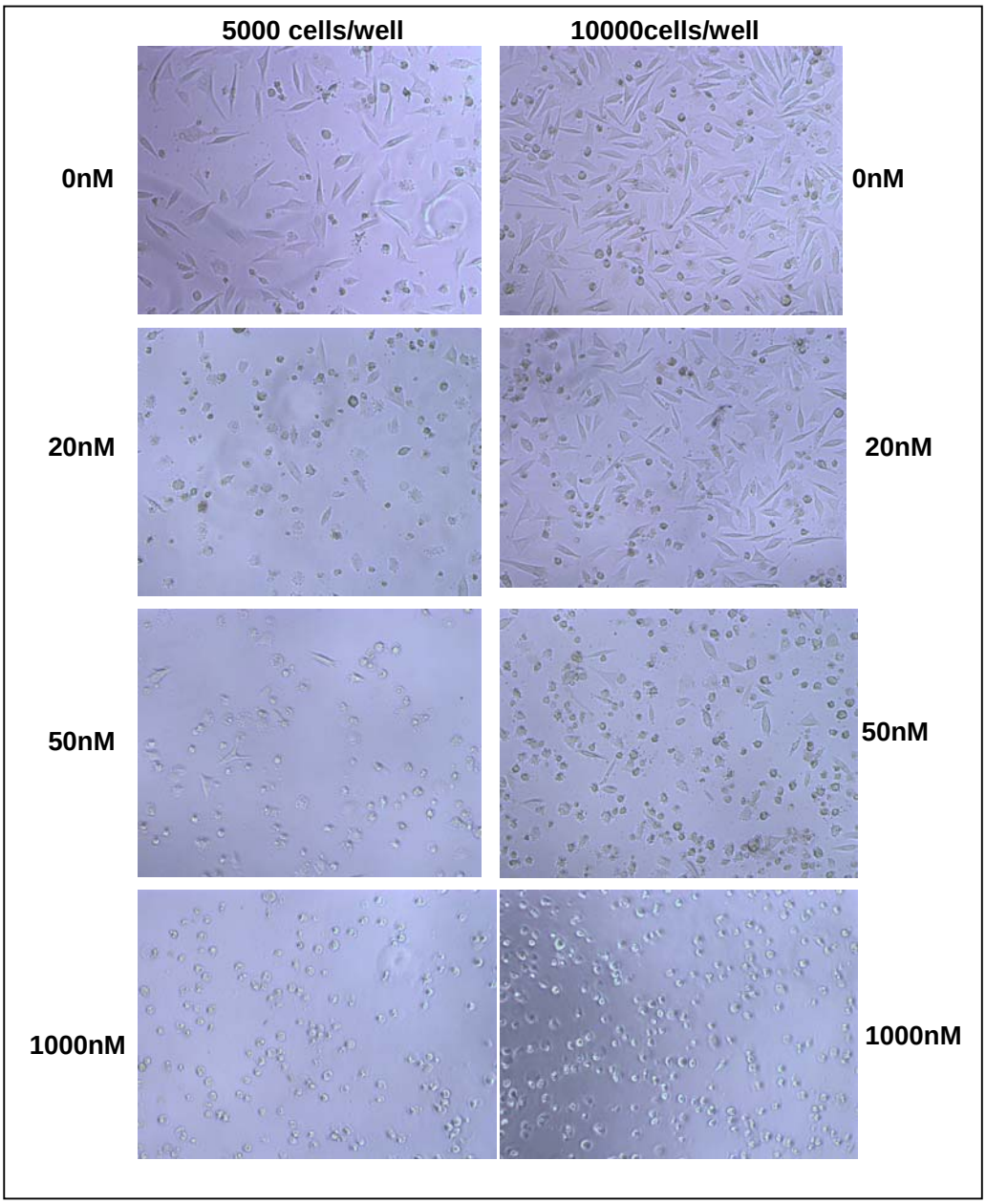


Figure 37. Cell visualization following the treatment of Melmet 1 cells with Elesclomol in 2D. 5000 cells (left panel) and 10000 (right panel) cells were treated with Elesclomol at the concentrations indicated on the figure. Phase contrast pictures taken using 10X objective.

To confirm that Melmet 1 is more sensitive to Elesclomol than Melmet 5 in 2D independently on cell density, we have seeded 10000 cells of Melmet 1 and 2x less (5000 cells) of Melmet 5 and treated them with 200nM Elesclomol for 3 days before microscopic inspection. As can be seen in **Figure 38**, 200nM Elesclomol had hardly any effect on Melmet 5 (the cells looked elongated and nice); while more than half of Melmet 1 cells seemed to be dead (small and round cells).

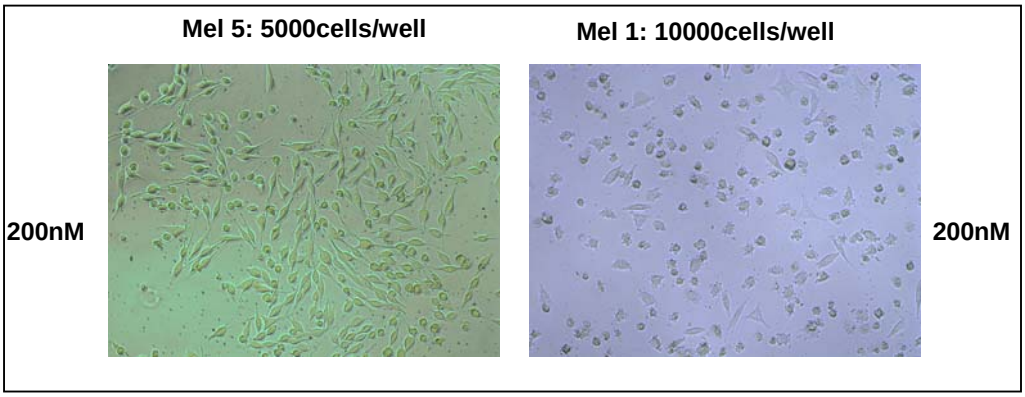


Figure 38. Cell visualization following the treatment (200nM Elesclomol) of 10000 Melmet 1 and 5000 Melmet 5 cells/well. Phase contrast pictures taken using 10X objective.

Further, the Elesclomol effect on Melmet 1 in 3D Matrigel-cultures was evaluated. The cells were embedded in the 2nd Matrigel layer and overplayed with Elesclomol serially diluted to concentrations: 50nM, 200nM, 1000nM and 2000nM. The MTS assay was performed after 3 days (i.e. 72 hours) of the treatment.

As can be seen in **Figure 39**, in 3D almost full effect of the treatment was achieved with the Elesclomol concentration of 2000nM, and ~ 40% of the cells were still viable following the treatment with 1000nM.

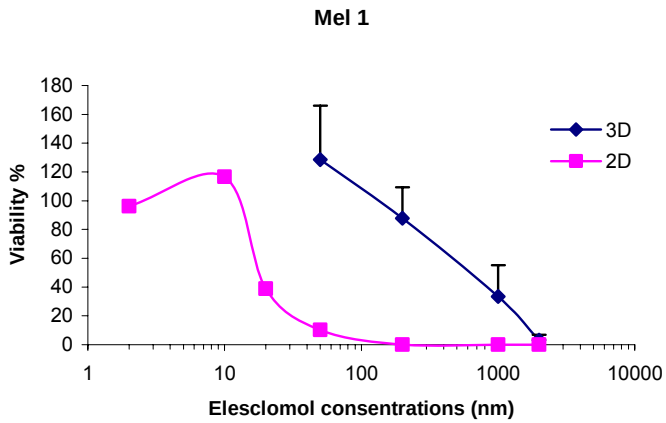


Figure 39. Comparison of Melmet 1 cell viability in 3D versus 2D following treatment with different concentrations of Elesclomol: 2nM, 10nM, 20nM, 50nM, 200nM, 1000nM and 2000nM. For 3D, cells (60000/well) were embedded in the 2nd layer of Matrigel matrix. Elesclomol was added at indicated concentrations in the 3rd layer. 2D cultures were prepared using 5000cells/well as described in Figure 33. Cell viability was measured by the MTS method and is presented as percentage of untreated controls (on the Y axis); used concentrations of the drug are indicated on the X axis (logaritmic scale). Error bars for 3D data indicate StDv from 2 parallels.

These results indicate that also Melmet 1 is less sensitive to the treatment in 3D than in 2D (compare blue versus pink line in **Figure 39**). Furthermore, comparison of the 3D data shown in **Figure 39** and **Figure 33** confirms that Melmet 1 is more sensitive to Elesclomol treatment compared to Melmet 5 also in 3D since we achieved the full response to the therapy using 2000nM Elesclomol on Melmet 1 in 3D.

3.4.4 Effect of organ-conditioned medium on Melmet 1 sensitivity to Elesclomol in 3D

Further, we investigated if brain-conditioned medium has an effect on viability of Melmet 1 cells treated with Elesclomol. The cells were embedded in the 2nd Matrigel layer. Elesclomol was diluted to 1000nM with the medium consisting of CM and DMEM+2% FBS at a ratio 25:75 and, added to the 3rd layer. The MTS assay was performed after 3 days (i.e. 72 hours) of the treatment.

As shown in **Figure 40**, 1000nM Elesclomol reduced cell viability to 20-40%, corresponding to the previous results shown in **Figure 39**. Interestingly, we could observe ~10% stimulating effect of CM on the viability of the treated Melmet 1 cells compared to the cells treated in the absence of CM. However, the difference was very small and needs further investigation before final conclusions.

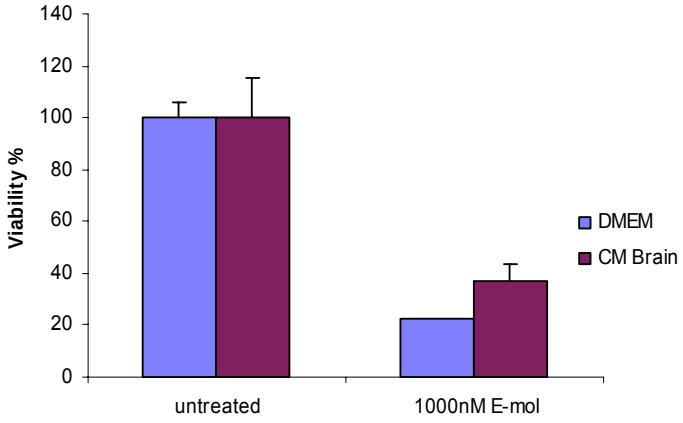


Figure 40. Effect of brain-CM from a healthy mouse on viability of Melmet 1 cells following treatment with 1000nM Elesklomol in 3D. Cells (60000/well) were embedded in the 3D Matrigel, and Elesclomol was added to the 3rd layer with or without CM from the brain; ”untreated” = wells without Elesclomol; “1000nM E-mol”=concentration of Elesclomol is 1000nM per well; Cell viability was measured by the MTS method and is presented in percentage relative to untreated controls (on the Y axis); used concentrations of the drug are indicated on the X axis. Error bars indicate StDv from 2 parallels.

4. Discussion

In the present work three-dimensional (3D) extracellular matrix-based cell cultures were employed to study in vitro whether and how various factors of tumor microenvironment modulate melanoma cell properties, specifically growth/proliferation and sensitivity to therapy.

Although 3D cultures mimic the body's conditions much better than traditional 2D cultures, generally 3D systems are NOT widely used models for in vitro studies, because they are much more difficult to make and to work with. Therefore, a big part of the present work was related to search of "optimal" conditions for culturing and analysis of Melmet cells in 3D. This explains why different analysis methods (i.e. measurement of DsRed fluorescence, Calcein signal, MTS signal and microscopic evaluation) have been used along the project to evaluate the behavior of melanoma cells in 3D. We were looking for culturing conditions and cell amounts that would lead to an easy measurable signal allowing to follow cell viability and growth. Furthermore, this signal had to be significantly above the background level, since this was necessary for therapy-studies where a decrease in cell-associated signal was expected as a result of cell killing by a drug. Thus, screening of several cell concentrations revealed that 60-100 thousand cells per well in a 96-well plate fulfill the above mentioned criteria. Initially we wanted to omit serum in our 3D cultures, since serum might interfere with fluorescence/absorbance measurements that our analysis methods were based on. However, comparison of cell growth and viability in serum-free *versus* serum-containing cultures revealed that the presence of serum was important for the cell state and therefore, we have chosen to supplemented the 3D cultures with low concentration (2%) of serum in the majority of the experiments.

Also we have observed that while Melmet 5 grew well in a matrix of collagen I, Melmet 1 did not do so. There could be several reasons for that. One of the possibilities could be lack of respective receptors for interaction with collagen I. However, our study where adhesion of Melmet cells to ECM proteins was evaluated revealed that Melmet 1 interacts efficiently well with all investigated proteins, including collagen I. Another possibility which was considered was related to the density of collagen matrix where the cells were embedded. It could be that the used concentration of collagen i.e. 2.5mg/ml formed too dense matrix for Melmet 1 cells, which are much larger in size compared to Melmet 5 growing well in 2.5mg/ml collagen. Indeed, we have observed that culturing of Melmet 1 in less dense collagen

(1.5mg/ml) seems to result in fewer dead cells than in dense collagen (see **Figure 15** "day 3" and **Figure 25** "DMEM"). This suggests that theoretically it might be possible to find more suitable conditions for culturing of Melmet 1 in collagen-based 3D. However, based on our observation that Melmet 1 cells looked "nicer" in Matrigel matrix than in collagen, we have chosen to focus on Matrigel instead of optimizing collagen conditions for Melmet 1.

Eventually, based on the said above, for Melmet 5, we have chosen to use collagen I matrix, 60000 cells/well and the medium with 2% serum in the 3rd (top) layer of 3D cultures. For Melmet 1 - Matrigel matrix, 100000 cells/well and the same medium with 2% serum were chosen as satisfactory conditions. Under these conditions the measured signal associated with the cells was significantly above the background level and thus: a) easy measurable; b) suitable for therapy-studies where a decrease in cell-associated signal was expected as a result of a drug effect.

Trying to bring the 3D models even closer to *in vivo* condition, we have supplemented them with additional components of ECM, fibronectin and laminin - two important proteins known to be linked to metastasis (discussed in the Introduction). The aim was to investigate whether the presence of these proteins modulate growth and viability of Melmet cells. It has been shown by Kaplan et al. that elevation of fibronectin level may be associated with the formation of a pre-metastatic niche, as described in the Introduction (Kaplan, Riba et al. 2005). Furthermore, it has been shown that transition from a dormant (i.e. non-proliferative) state to a proliferative state in breast cancer cells was dependent on fibronectin production (Barkan, Kleinman et al. 2008), and such transition must occur during metastasis formation in order to give macrometastases. Altogether this suggests that fibronectin might be a fundamental component in establishing a favorable microenvironment for tumor cell growth, proliferation and survival leading to metastases (Barkan, Green et al.). In our systems though, neither fibronectin nor laminin modulated notably growth and/or survival of Melmet cells. The lack of effect can not be explained by the lack of respective receptors on the surface of Melmet cells. Thus, our data on adhesion to ECM (**Figure 17**) indicated that both Melmet 1 and Melmet 5 could efficiently bind to these proteins. Generally, Melmet 1 demonstrated an efficient binding to all 7 ECM proteins that were investigated, while Melmet 5 lacked the ability to bind efficiently to collagen IV, vitronectin and tenescin. Altogether, our data does not exclude a possibility that fibronectin and laminin might have an effect under different conditions. However, here we conclude that under investigated conditions – when 10μg/ml of the

proteins was added to the 2nd layer of collagen containing cells – fibronectin and laminin did not affect significantly the cells properties that were analyzed.

To investigate how various soluble factors might affect melanoma cell growth and viability, we have supplemented 3D cultures with conditioned media (CM) prepared from various organs isolated from experimental animals. Mouse brain, lungs, bone/bone marrow and lymph nodes were used. These organs are known to represent common sites of melanoma metastases, indicating that melanoma cells like to grow in their microenvironments. When such CMs were added to the 3D cultures, we have observed a slight stimulatory effect on cell growth/viability. Although the stimulatory effect was not big i.e. did not exceed 2-fold, it was confirmed in several independent experiments in both Melmet lines. Interestingly, the effect was observed when the CMs were diluted with fresh medium at a ratio 25:75, and not at 50:50, indicating that the CM effect depends on the optimal (i.e. lower) concentration of CM. This corresponds to the earlier publications that also demonstrated that high concentration of CM might be not optimal and might even inhibit cell growth (Valle, Zalka et al. 1992; Cruz-Munoz, Man et al. 2008). The fact that CMs from all 4 organs induced quite similar effect (shown in **Figures 30** and **31**) might suggest that the effect was associated with the factors e.g. growth factors generally present in the organs i.e. in vivo. To note, Melmet 5 is known to be able to establish metastases in experimental animals in brain, lungs, bone marrow and lymph node (Prasmickaite et.al, manuscript in preparation), ant therefore the fact that Melmet 5 cells can respond to CM from these organs is not very surprising.

Further, we have observed that CM had a very clear effect on morphology and growth pattern in Melmet 5 – the cells formed more complex elongated structures. This effect was especially pronounced for CM from bone marrow and lymph nodes. The result that bone marrow factors affect tumor cells corresponds well to the recently proposed role of bone marrow-derived cells (BMDC) in the metastasis process. As discussed in the introduction, BMDC seem to be strongly involved in metastasis via the formation of a “pre-metastatic niche” (Kaplan, Riba et al. 2005) i.e. BMDC are recruited to metastatic sites to induce changes that stimulate growth of tumor cells. This suggests that BMDC might secrete factors required for stimulation of tumor growth, and therefore conditioned media from bone marrow might be rich in these factors. The significance of cell morphology changes induced by the CM from bone marrow (as well as from other organs) has not been investigated further in the present work. However, the observation that in the presence of CM tumour cells

“communicate” more forming complex structures/clumps instead of single separated cells is interesting and worth further investigation.

In addition, we have tested an effect of conditioned media prepared from a metastatic mouse. The idea behind this experiment was that the growing tumour i.e. metastases might induce the production of soluble factors that further stimulate tumor growth. Therefore, we speculated that CM from a metastatic mouse might have a more pronounced effect on tumor cell growth than the CM from a healthy mouse. However, our results indicated that although CM from a metastatic mouse had a small stimulatory effect on Melmet 5 growth, the difference was not significant compared to CM from a healthy mouse. It should be noted that this experiment was not optimally performed. First, the metastatic mouse had metastases only in the brain, and not in other investigated organs (**Figure 9** in Materials & Methods). Therefore it is probably not surprising that we could not detect a significant difference between the CM from “metastatic” compared to CM from healthy mouse lungs, bone marrow and lymph nodes. Secondly, the metastases formed in the brain were small i.e. contained relatively few tumor cells. Therefore, it can be that microenvironmental changes (e.g. secretion of soluble factors) induced by such small metastases are not strongly pronounced and are not very significant. Thus, this experiment should be repeated by preparing CM from a mouse with clearly detectable metastases not only in the brain, but also in other investigated organs.

A big part of the present project was devoted to therapy-related studies. The original idea was to use 3D cultures for evaluation of the effect of the new promising anti-melanoma drug PLX 4032 discussed in the Introduction. PLX targets mutated BRAF, and both Melmet 1 and Melmet 5 have this mutation (Prasmickaite, unpublished). Unfortunately, the delivery of this drug was delayed by the manufacturer, so that the drug was not available for the present project. Instead, we have used another drug, Elesclomol, which is proposed for melanoma therapy and is currently in clinical trials. Evaluation of Elesclomol efficiency in 2D in various melanoma cell lines (not Melmets) is ongoing in the Department of Tumour Biology, and therefore it was of great interest to evaluate Elesclomol in Melmet cell lines, particularly in 3D cultures. Evaluation of the Elesclomol effect in 2D versus 3D revealed that in both cell lines Elesclomol induced a stronger effect in 2D, indicating that cells growing in 2D were more sensitive to the drug. This correspond to the published data on other cell lines and other drugs (Smalley, Lioni et al. 2006; Serebriiskii, Castello-Cros et al. 2008) and supports the theory that there is a difference between 2D and 3D systems with respect to drug-response. This also

signifies the importance of performing preclinical tests of new drugs or drug combinations in 3D systems.

Although the sensitivity of Melmet 1 to Elesclomol in 2D was dependent on cell number/density, the general observation was that Melmet 1 was more sensitive than Melmet 5 both in 2D and 3D systems. Thus, in 3D cell viability was reduced by 50% when Elesclomol concentration exceeded 500nM for Melmet 1 and 2000nM for Melmet 5, indicating at least 4-fold difference in sensitivity between these cell lines. We have not investigated further the reasons for such difference. It might be that Melmet 1 and Melmet 5 have different levels of intracellular ROS. The action mechanism of Elesclomol is based on the elevation of the ROS levels above the "threshold", which is toxic for a cell. It might be that cells that are more sensitive to Elesclomol, like Melmet 1, also have higher levels of intracellular ROS.

Further, we have investigated, whether conditioned media from the brains might influence the response to Elesclomol. We hypothesized that soluble factors present in these organs might protect the tumor cells, increasing their viability and therefore reducing drug-effect. The idea was based on the fact that melanoma metastases are usually highly refractory to therapies. Unfortunately, our hypothesis was not supported by the experimental results. The only experiment that was performed indicated a slightly lower viability (i.e. better Elesclomol effect) when CM was used, and these data were not verified further.

Summarizing, this work was the initial evaluation on how various factors from tumor microenvironment affect malignant melanoma cells in 3D cultures in vitro. The established conditions and the obtained results put a basis for further studies on tumor-microenvironment interactions in malignant melanoma by using 3D matrix-based in vitro model systems.

Conclusion

- The conditions suitable for culturing of Melmet cells in 3D matrixes for up to 5 days have been established. These include: for Melmet 1- Matrigel matrix; for Melmet 5 - 2.5mg/ml collagen I matrix. The growth medium in the top layer of 3D cultures has to be supplemented with at least 2% serum.
- Cell adhesion to 7 proteins of ECM has been evaluated. Both Melmets adhered efficiently to laminin and fibronectin. Melmet 1 could efficiently bind to all other investigated proteins, while Melmet 5 showed low/no binding to collagen IV, tenascin and vitronectin.
- The presence of laminin and fibronectin in 3D cultures did not affect Melmet cell growth and/or viability.
- The conditioned media i.e. soluble factors extracted from brain, lungs, bone marrow and lymph node of experimental mice showed a slight stimulating effect on Melmet cell growth, and notable effect on cell morphology and growth pattern, particularly in the case of the media conditioned with bone marrow.
- Both Melmet cell lines demonstrated higher sensitivity to the drug Elesclomol in 2D than in 3D, and Melmet 1 cells were more sensitive than Melmet 5. The brain-conditioned media did not increase the resistance to Elesclomol.

Future plans

- More optimal culturing conditions for Melmet 1 in 3D remains to be found. Although we concluded here that Matrigel is an acceptable matrix for Melmet 1, generally the 3D culturing conditions for Melmet 1 were only satisfactory, but not very good. Further optimization, like changing concentration of the collagen (less dense matrixes) and varying cell number as well as supplementation with some additional TME factors may improve the quality of Melmet 1 3D cultures.
- An effect that organ-conditioned medium seem to have on morphology and growth pattern of Melmet 5 cells has not been investigated further in the present work, but is definitely an interesting observation which is worth further investigation, furthermore, in both cell lines.
- Further investigation of the effects of conditioned medium from metastatic organs compared to “healthy” organs remains to be performed. Preparation of CM from a more “optimal” mouse with notable metastases in multiple organs should be tested in the future.
- Therapy-related studies in 3D, testing e.g. the most promising anti-melanoma drug PLX4032 in the presence or absence of TME factors, like stroma cells or CMs, would be very interesting to perform.

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