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EFFECT OF SERUM STARVATION AND DOWN-REGULATION OF CD146 PROTEIN-EXPRESSION ON THE CELLULAR LIFE CYCLE OF ORAL CANINE MELANOMA CELLS

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Orientador: Prof. Dr. Andre Marcelo Conceição Meneses

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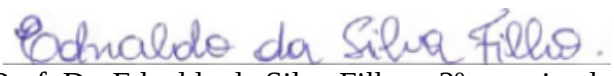

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LIST OF ABBREVIATIONS AND ACRONYMS

A32	Melanoma-associated antigen number 32
ATP	Adenosine triphosphate
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CD34	Cluster of differentiation number 34
CD106	Cluster of differentiation number 106
CD146	Cluster of differentiation number 146
CD146-shRNA1	Cluster of differentiation number 146 short hairpin ribonucleic acid 1
DMEN	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FCS-HI	Heat inactivated fetal calf serum
Mel-CAM or MCAM	Melanoma cell adhesion molecule
MUC18	Progression marker of melanoma number 18
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate-buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PI	Propidium iodide
S-Endo-1	Endothelial associated antigen number 1
siRNA	Small interfering ribonucleic acid
SK-MEL-28	Skin melanoma cell line number 28
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens protein number 1

ABSTRACT

AMORIM, B. S. **Effect of serum starvation and down-regulation of CD146 protein-expression on the cellular life cycle of oral canine melanoma cells.** [Efeito da ausência de soro e supressão da regulação da proteína de expressão CD146 no ciclo de vida de células de melanoma oral canino]. 2018. 37 f. Dissertation (Master in Health and Animal Production in Amazonia) – Postgraduate Program in Health and Animal Production in Amazonia, Federal Rural University of Amazonia, Belem, 2018.

The oral cavity is place of 6% of all tumors in dogs, being the fifth most important localization of neoplasms in this specie. Melanoma is a type of cancer that originates from melanocytes and its establishment involves several stages, until the formation of an invasive and metastatic tumor. The exact molecular mechanisms of the dysregulation in melanoma are yet unknown. Many studies have advanced our knowledge towards understanding the multiple roles of CD146 in biology and pathology and have pointed to the significance of CD146 as a potential diagnostic marker for certain lesions including melanomas. In the present study we examine the impact of different serum starved conditions and reduction of CD146 expression on the cellular life cycle of canine melanoma cell in order to obtain more information about the functional relationship of CD146 and nutritional undersupply on tumor cell proliferation and metastatic behavior. After cell culture, melanoma cells were serum starved and then proliferation assay was performed through ELISA technique. Then, FACS staining could analyze the CD146 down-regulation so the cell cycle could be done. Statistics was performed between the different cell densities, media times and concentrations, using the data obtained from proliferation assay, through procedures of SAS® University Edition. The 5,000 cells per well seeding density had a $R^2 = 0.70$, being the best density to perform the proliferation assay; specific siRNA number 34 proved a reduction in the percentage of cells expressing CD146; and compared to the siRNA scrambled control CD146 silenced cells showed an accumulation of cells within the G_0/G_1 phase demonstrating an inhibition in cell proliferation and cellular senescence. Further studies are needed to provide additional molecular information about the functional relationship of CD146 and the cell cycle analysis using melanoma cells.

Keywords: Media conditions; Cluster of differentiation 146; Melanoma cells life cycle.

RESUMO

AMORIM, B. S. **Efeito da ausência de soro e supressão da regulação da proteína de expressão CD146 no ciclo de vida de células de melanoma oral canino.** [Effect of serum starvation and down-regulation of CD146 protein-expression on the cellular life cycle of oral canine melanoma cells]. 2018. 37 f. Dissertação (Mestrado em Saúde e Produção Animal na Amazônia) – Programa de Pós Graduação em Saúde e Produção Animal na Amazônia, Universidade Federal Rural da Amazônia, Belém, 2018.

A cavidade oral é o local de 6% de todos os tumores em cães, sendo a quinta localização mais importante de neoplasias nesta espécie. O melanoma é um tipo de câncer que se origina em melanócitos e seu estabelecimento envolve vários estágios, até a formação de um tumor invasivo e metastático. Os mecanismos moleculares exatos da desregulação no melanoma ainda são desconhecidos. Muitos estudos tem avançado nosso conhecimento para a compreensão dos múltiplos papéis do CD146 na biologia e na patologia, e apontaram o significado do CD146 como um possível marcador de diagnóstico para certas lesões, incluindo melanomas. No presente estudo, examinamos o impacto de diferentes condições de inanição de soro e a redução da expressão de CD146 no ciclo de vida celular das células de melanoma canino, a fim de obter mais informações sobre a relação funcional de CD146 e a insuficiência nutricional na proliferação de células tumorais e comportamento metastático. Após o cultivo celular, células de melanoma foram submetidas à inanição de soro e, em seguida, o ensaio de proliferação foi realizado através da técnica de ELISA. Então, o teste em citômetro de fluxo pôde analisar a supressão da regulação de CD146 para que a análise do ciclo celular pudesse ser feita. A estatística foi realizada entre as diferentes densidades celulares, tempo de cultivo e diferentes concentrações utilizando os dados obtidos a partir do ensaio de proliferação, através de procedimentos do SAS® University Edition. A densidade de semeadura de 5.000 células por poço mostrou $R^2 = 0,70$, sendo a melhor densidade para realizar o ensaio de proliferação. O número específico de siRNA 34 provou uma redução na porcentagem de células que expressam CD146, e em comparação com as células de controle para CD146, estas mostraram um acúmulo maior dentro da fase G_0/G_1 demonstrando uma inibição na proliferação celular e na senescência celular. Fazem-se necessários mais estudos para fornecer informações moleculares adicionais sobre a relação funcional do CD146 e a análise do ciclo celular usando células de melanoma.

Palavras chave: Meios de cultivo; Cluster de diferenciação 146; Ciclo de vida das células de melanoma.

SUMÁRIO

1 CONTEXTUALIZAÇÃO

Mitocôndrias são organelas citoplasmáticas de dupla membrana, altamente dinâmicas, e que desempenham um papel importante no metabolismo energético através da fosforilação oxidativa, fase final da respiração celular. Manter a qualidade mitocondrial e a homeostase é indispensável para a realização das funções celulares normais. A disfunção mitocondrial é frequentemente associada a diversas condições patológicas, incluindo doenças neurodegenerativas, envelhecimento, doenças cardíacas, obesidade e câncer (FRANK *et al*., 2012).

A hipóxia e a inanição de nutrientes, juntamente com mitocôndrias disfuncionais, induzem a mitofagia, o que impede a produção de espécies reativas de oxigênio e conserva nutrientes valiosos de serem consumidos ineficientemente. Além disso, a incapacidade de regular adequadamente a rotatividade mitocondrial sob condições estressantes oncogênicas leva ao surgimento de efeitos positivos e negativos sob a carcinogênese (CHOURASIA *et al*., 2015).

O microambiente tumoral é geralmente hipóxico devido ao fornecimento limitado de oxigênio a partir de vascularização ineficiente ou insuficiente, o que também causa falta de componentes séricos (KOIZUME; MIYAGI, 2015). No entanto, estudos anteriores identificaram células tronco endoteliais e células precursoras endoteliais circulantes no adulto e demonstraram participar da formação de vasos sanguíneos novos em estados normal e patológico, incluindo neoangiogênese tumoral (MOORE, 2002; RAFII, 2000).

Em 1971, Folkman propôs que o crescimento tumoral e a metástase são dependentes da angiogênese e, portanto, o bloqueio da angiogênese pode ser uma estratégia para prevenir o crescimento tumoral. Diversos fatores, como fatores mecânicos, hormonais, imunológicos e de crescimento, estão envolvidos por sua estimulação ou inibição, na neoangiogênese, e um dos objetivos no campo biomédico é identificar e elucidar os mecanismos moleculares que são cruciais para a proliferação de células endoteliais e encontrar formas de supressão da formação de vasos sanguíneos durante processos patológicos, incluindo a tumorigênese (CARMELIET; JAIN, 2000). Um dos mecanismos para controlar o crescimento tumoral pode envolver a regulação direta do ciclo celular (MOTOKURA *et al*., 1991).

CD146 (cluster de diferenciação número 146) é uma molécula de superfície celular e membro da superfamília do gene Ig. Tem sido designado por nomes diferentes devido à sua identificação em diferentes tecidos por vários grupos de pesquisa independentes. Os sinônimos utilizados para CD146 são: marcador de progressão do melanoma (MUC18), antígeno associado ao melanoma (A32), molécula de adesão celular de melanoma (Mel-CAM ou MCAM) e antígeno associado endotelial (S-Endo-1) (JOHNSON *et al*., 1997; SHIH *et al*., 1997).

Sendo principalmente expresso em melanócitos malignos, CD146 também é detectável nos vasos sanguíneos, nas células foliculares do cabelo, nos trofoblastos intermediários, no microambiente tímico e nos linfócitos T periféricos ativados (como marcador de ativação) e linhagens celulares de leucemia T (SHIH *et* al., 1998; SEFTALIOGLU; KARAKOC, 2000).

No presente estudo, examinamos o impacto de diferentes condições de inanição de soro e a redução da expressão de CD146 no ciclo de vida celular das células de melanoma canino, a fim de obter mais informações sobre a relação funcional de CD146 e a insuficiência nutricional na proliferação de células tumorais e comportamento metastático.

1.1 Literature review

1.1.1 Basic effects of serum starvation on cells *in vitro*

Cell proliferation is essential for a number of biological processes, including immune response, maintenance of epithelial barrier functions and cellular differentiation. Cell synchronization offers a unique strategy to study the molecular and structural events taking place in a cell during the different stages of cell cycle. It is a procedure that aims to arrest cultured cells at different stages of the cell cycle, in order to obtain subpopulations of cells arrested at the same stage (LANGAN; CHOU, 2011). These subpopulations of cells are then used to study regulatory mechanisms of the cycle at the level of macromolecular biosynthesis (DNA synthesis, gene expression, and protein synthesis), protein phosphorylation and development of new drugs (PENG *et al*., 2014).

A number of methods have been established to synchronize mammalian cell cultures, which include counterflow centrifugal elutriation, mitotic shake off, chemically induced cell cycle arrest, and newer live cell methods, such as cell permeable dyes. One broadly applied method for cell synchronization is nutritional serum starvation, which can arrest cells at G_0/G_1 phase with high efficiency and no toxic effects. The effect of serum starvation on cell synchronization changes depending on the serum starvation time and serum concentration (TONG *et al*., 2016).

As close as the *in vitro* cell culture is to reality, it still causes problems for cell development. Its cell-cell and cell-matrix adhesion is reduced and not have the characteristics (heterogeneity and three-dimensional architecture) of a tissue *in vivo*, since its nutritional and hormonal environment is modified. The choice of an ideal medium is a path to follow in order to obtain cultures that expresses a specific function (ALVES; GUIMARÃES, 2013).

When studying the effect of bacteria and bacterial products on cells, serum starvation is often performed as a standard procedure (CHOWDHURY *et al*., 2006) to avoid unwanted

stimulation by the serum components. The full effect of serum starvation on cell behavior and inflammatory responses is unknown, though it has been suggested to induce various responses that can interfere with experimental results and conclusions (PIRKMAJER; CHIBALIN, 2011). Serum starvation has been shown to cause cells to undergo apoptosis and autophagy (GONZÁLEZ-POLO *et al*., 2005; VASUDEVAN; STEITZ, 2007), as well as superoxide production and increasing cell susceptible to inflammatory stimuli (RUSSELL; HAMILTON, 2014).

It is known that around the tumor microenvironment, the accumulation of stress factors including hypoxia, growth factor deprivation, acidosis, pH change, etc., can trigger even more active metastases. Alteration in the expression of many pro and anti-metastatic genes takes place at the molecular level (due to stress) that further aggravates the metastatic cascade and advancement of the disease. Neutralization of these built-up tumor-associated stresses has been proven efficacious to repeal tumor growth and metastasis (LUO *et al*., 2006).

1.1.1.1 Lack of growth factors and other serum components

Although serum provides optimal conditions for cell growth, its poorly defined complex and above all variable composition represents an important and undesirable confounding factor while performing bioassays (VAN DER VALK *et al*., 2010).

Programmed cell death serves as a natural barrier to cancer initiation and development. Because of their uncontrolled proliferation, cancer cells often find themselves in a microenvironment characterized by hypoxia and a minimal supply of serum components. Such severe serum starvation eventually induces the apoptosis of these tumor cells, restricting the expansion of the growing cell mass (OU *et al*., 2006).

There is still a lack of molecular understanding about why thick tumors are more prone to spread and metastasize compared to thin ones (REDPATH *et al*., 2014). Cancer cells overcome growth factor dependence by acquiring genetic mutations that functionally alter receptor-initiated signaling pathways (HSU; SABATINI, 2008). Oncogenic mutations can result in the uptake of nutrients, particularly glucose, that meet or exceed the bioenergetics demands of cell growth. Unlike most normal tissues, cancer cells tend to “ferment” glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation (WARBURG, 1956).

In vivo, oxidative stress can cause cell damage either directly or through altering signaling pathways. This kind of stress is a consolidating mechanism of injury in many types of diseased and pathological conditions, and it occurs when there is an imbalance between generation of reactive oxygen species and inadequate antioxidant defense systems, during cancer therapy, for example (DRYDEN *et al*., 2005). According to the Warburg effect (metabolic requirements of cell

proliferation), unlike normal cells, cancer cells gain energy primarily from glycolysis even under aerobic conditions, leading to increased reactive oxygen species levels (LIBERTI; LOCASALE, 2016).

The metabolism of glucose to lactate generates only two adenosine triphosphates (ATPs) per molecule of glucose, whereas oxidative phosphorylation generates up to 36 ATPs upon complete oxidation of one glucose molecule (LEHNINGER *et al*., 1993). One possible explanation for why a less efficient metabolism would be selected for proliferating cells is that inefficient ATP production is a problem only when resources are scarce. This is not the case for proliferating mammalian cells, which are exposed to a continual supply of glucose and other nutrients in circulating blood. Metabolic pathways and their regulation have only recently been studied in actively proliferating cells, and there is evidence that ATP may never be limiting in these cells (CHRISTOFK *et al*., 2008).

1.1.1.2 Effects on cellular life cycle

Many studies have shown that serum starvation and chemical inhibitors have caused cell cycle synchronization of the somatic cells (KHAMMANIT *et al*., 2008). Serum starved medium is widely used for synchronization donor cells by arresting them in the G_0/G_1 phase of the cell cycle, but it often reduces cell survival and increases the DNA fragmentation (BAGHDADCHI, 2013).

Serum starvation (supposedly) reduces basal cellular activity (CODELUPPI *et al*., 2011) and makes the population of proliferating cells more homogenous, since they withdraw from the cell cycle to enter the quiescent G_0/G_1 phase (PONTARIN *et al*., 2011). Moreover, its induced synchronization, followed by serum restimulation or preceded by serum shock (e.g., 50% serum), has been extensively used in cell cycle and circadian rhythm research ever since Arthur Pardee established the restriction point concept (BALSALOBRE *et al*., 1998).

In conceptualizing cell fate in higher organisms, it has been suggested that certain cells enter a state of terminal differentiation. Cells that can reenter the cycle are in contrast considered to be in the G_0 phase of the cell cycle, and this includes the several presumptively differentiated cells mentioned above (SALOMONI; CALLEGARI, 2010). It is absolutely necessary for this minority of cells to remain in G_0 since inhibition of cell cycle reentry (ALBERTS *et al*., 2002). On the other hand, un-regulated proliferation of cells in G_0 can result in malignant transformation (SALOMONI; CALLEGARI, 2010). This simple technique of serum depletion is used to establish a G_0 state, followed by the re-addition of serum to enable reentry into G_1 and S phases (LANGAN; CHOU, 2011). But the tumor microenvironment is characterized by cancer cell subpopulations with

heterogeneous cell cycle profiles. For example, hypoxic tumor zones contain clusters of cancer cells arrested in G_1 phase (BEAUMONT *et al.*, 2016).

1.1.1.3 CD146 expression and effects on cellular life cycle

Bardin *et al*. (2001) have previously shown that CD146 is a component of the endothelial junction localized outside the adherence junctions. The endothelial junctions play a fundamental role in endothelial integrity, vascular permeability and cellular traffic (BAZZONI; DEJANA, 2004). These junctions are tightly regulated structures composed of several adhesion molecules interacting with cytoskeletal proteins (DEJANA *et al*., 2000).

The adhesive function of CD146 was first demonstrated by the finding that melanoma cells bound to CD146 purified from melanoma cells in the solid phase (SHIH *et al*., 1994). CD146-mediated cell adhesion depends on the temperature and this adhesion can be partially inhibited by full-length CD146 or by a polyclonal antibody against CD146 (JOHNSON *et al*., 1997).

Previous studies have revealed a multi-functional role for CD146, not merely limited to cell adhesion but expanded to processes such as development, signaling, cell migration and motility, proliferation, differentiation, and immune response (WANG; YAN, 2013).

Although CD146 expression has been demonstrated in several different tumors, the functional role of CD146 in tumor progression has only been studied in melanoma and breast carcinoma. Most melanoma cells co-express CD146 and its receptor on the cell surface and the binding of CD146 to its receptor may trigger intracellular signals which are important for tumor invasion and metastasis (XIE *et al*., 1997), whereas the decreasing CD146 expression results in reduced tumorigenicity, indicating that CD146 may play an important role in promoting melanoma progression (SATYAMOORTHY *et al*., 2001).

According to Johnson *et al*. (1996), CD146 present on the endothelia of blood vessels penetrating primary and metastatic melanomas plays critical role in tumor angiogenesis and hematogenous spread. It is possible that the interaction between melanoma cells and endothelial cells mediated by CD146 is not only physical but may trigger a cascade of events leading to extravasation (SHIH, 1999).

It has so been proposed that CD146 mediates the metastasis of melanoma cells by impacting on only the later stages of metastasis, namely, extravasation and the establishment of new foci of growth (XIE *et al*., 1997). Cell populations showed the growth rate of CD146 cells was faster than those without it, and telomerase activities were also significantly higher (JIN *et al*., 2016). CD146 already demonstrated antiproliferative activity by modulating G_0/G_1 and S cell cycle regulators, may being a positive regulator of cell growth to promote mitogenic signal (CHEN *et al*., 2009).

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2 EFFECT OF SERUM STARVATION AND DOWN-REGULATION OF CD146 PROTEIN-EXPRESSION ON THE CELLULAR LIFE CYCLE OF ORAL CANINE MELANOMA CELLS

2.1 Introduction

The oral cavity is place of 6% of all tumors in dogs, being the fifth most important localization of neoplasms in this specie (SMITH, 2005). Melanoma is a malignant neoplasm that originates from melanocytes and it frequently affects the oral cavity, other common localization being skin and nail bed (SMITH *et al*., 2002).

In a retrospective study addressing this subject, melanomas represented 45,5% of the 57 malignant neoplasms founded in dogs (REQUICHA, 2010), almost like Schultheiss (2006) that presented a casuistic of 46 melanocytic neoplasms in dogs, finding a 69.5% prevalence of melanomas. While on the other hand, in a five years study performed by Manzan *et al*. (2005), just one case of oral melanoma was found via histological examination. In a period of six years, the Animal Pathology Service of the University of São Paulo School of Veterinary Medicine and Animal Science registered 2154 reports, of which 193 (8.9%) corresponded to melanocytic neoplasms in cats and dogs (TEIXEIRA *et al*., 2010).

According to Smith *et al*. (2002), melanoma is a relatively common disease in dogs, responsible for 7% of all malignant tumors. Nevertheless, cats rarely develop these neoplasms, having higher incidence in highly pigmented dog breeds, such as Boxer and Cocker Spaniel (MANZAN *et al.*, 2005). Generally, the difference between breed profiles can simply reflect changes in popularity of a breed in a certain moment or reflect local population and its cultural aspects (SCHULTHEISS, 2006), although the true incidence in individual breeds of dogs is poorly established (BERGMAN *et al*., 2013).

Melanocyte growth is controlled by the surrounding keratinocytes by extracellular communication through paracrine growth factors, by intracellular communication through second messengers and signal transduction and by intercellular communication through cell–cell adhesion molecules, cell–matrix adhesion, and gap junctional intercellular communication (HAASS *et al.*, 2004). Under normal conditions, homeostasis determines whether a cell remains quiescent, proliferates, differentiates, or undergoes apoptosis (BISSELL; RADISKY, 2001). Dysregulation of the homeostasis may disturb the balance of the epidermal melanin unit and trigger a continuous proliferation of the melanocytes, which may lead to the development of melanoma (HAASS *et al.*, 2005).

The exact molecular mechanisms of the dysregulation in melanoma are yet unknown. However, it is likely that melanoma cells escape from control through keratinocytes through down-regulation of receptors important for communication with adhesion to keratinocytes (e.g. E-cadherin); up-regulation of receptors and signaling molecules not found on melanocytes but important for melanoma–melanoma and melanoma–fibroblast interactions [e.g. N-cadherin, MCAM, zonula occludens protein-1 (ZO-1)]; and loss of anchorage to the basement membrane because of an altered expression of the extracellular-matrix binding integrin family (HAASS *et al.*, 2005).

It is possible that alterations in the composition of intercellular junctions of endothelial cells may be associated with diseases linked to altered endothelial permeability or vascular morphogenesis. The typical transmembrane protein is vascular endothelial (VE)-cadherin and occluding, but other adhesive molecules have been found to be concentrated at endothelial cell-cell contacts, including platelet endothelial cell adhesion molecule (PECAM), CD34, endoglin, and CD146 (LAMPUGANI; DEJANA, 1997).

CD146 is a cell adhesion molecule (CAM), which is located on the cell surface and it is involved in an extensive range of physiological processes, including cell-cell and cell-matrix interactions, cell migration, cell cycle, and signaling as well as morphogenesis during development and tissue regeneration (TRZPIS *et al.*, 2007). CD146 is a specific antigen in malignant melanoma, and it is also overly expressed on a variety of carcinomas, attracting plenty of attention becoming a potential marker for tumor diagnosis, prognosis, and treatment (WU, 2012).

Many studies have advanced our knowledge towards understanding the multiple roles of CD146 in biology and pathology and have pointed to the significance of CD146 as a potential diagnostic marker for certain lesions including melanomas (SHIH, 1999). Hence, the present study aimed at observing the CD146 protein-expression in oral canine melanoma cells during *in vitro* serum starved conditions and the effect of CD146 down-regulation on cell cycle, in order to potentially correlate changes in cell cycle metastasis initiation.

2.2 Material and methods

2.2.1 Cell culture

Canine oral melanoma cells originating from a primary tumor were established at the Research Group Oncology of the Veterinary Medicine University of Vienna. Melanoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEN 1x) High Glucose (4,5g/l) with Glutamax supplemented with 10% heat-inactivated (30min, 56°C) fetal calf serum (FCS-HI) and incubated at

37°C in a 5% CO_2 atmosphere. Maintenance of cells was carried out by passaging twice a week using 0,05% Trypsin-EDTA (ethylenediamine tetraacetic acid) and a splitting ratio between 1:4 to 1:8. Cell counts were obtained using a haemocytometer (Neubauer improved) by the trypan blue (0,4%) exclusion method.

2.2.2 Proliferation assay of serum starved canine melanoma cells

Canine melanoma cells were counted as described above and seeded in the appropriate medium at the appropriate density (1000, 5000 and 10,000 cells per well). Cell proliferation assay was performed in 96-well plates in a volume of 100µl per well for time points 0h, 24h, 48h and 72h (Chart 1). Analysis was conducted with CellTiter 96® AQ_{ueous} reagent (Promega) according to the recommendations of the manufacturer. In brief, 20µl of the reagent was applied into each well containing the samples in 100µl culture medium. The absorbance was measured after an incubation period of 1h at 37°C in a humidified 5% CO_2 atmosphere on an ELISA (Enzyme Linked ImmunonoSorbent Assay) plate reader (Molecular Devices "SpectraMax M3", SoftMax Pro6, Version 6.4). The reagent, a tetrazolium compound, is bioreduced by cellular NADPH or NADH of metabolically active cells into a soluble, colored formazan product recordable at 490nm.

Chart 1 – 96-well plate plan for observation and proliferation assay protocol.

	1	2	3	4	5
A	100µl Ms	100µl Ms	100µl Ms	100µl Ms	100µl Ms
B	100µl Ms	**100% Ms**	**100% Ms**	**100% Ms**	100µl Ms
C	100µl Ms	**75% Ms 25% Mn**	**75% Ms 25% Mn**	**75% Ms 25% Mn**	100µl Ms
D	100µl Ms	**50% Ms 50% Mn**	**50% Ms 50% Mn**	**50% Ms 50% Mn**	100µl Ms
E	100µl Ms	**25% Ms 75% Mn**	**25% Ms 75% Mn**	**25% Ms 75% Mn**	100µl Ms
F	100µl Ms	**10% Ms 90% Mn**	**10% Ms 90% Mn**	**10% Ms 90% Mn**	100µl Ms
G	100µl Ms	**100% Mn**	**100% Mn**	**100% Mn**	100µl Ms
H	100µl Ms	100µl Ms	100µl Ms	100µl Ms	100µl Ms

In the center (shades of blue), triplicates showing percentages of different culture media decreases, going from 100% Ms [DMEN + 10% FCS (HI); line B] to 100% Mn (DMEN without serum; line G). At the edges (gray color), only culture medium containing untreated cells.

2.2.3 CD146 down-regulation with siRNA, FACS staining protocol and analysis

Before transfection of the siRNA (small interfering RiboNucleic Acid), harvested cells were washed with PBS (Phosphate-Buffered Saline), ressuspended and counted to obtain at least 250,000 cells in 2.5ml medium (100,000 cells/ml).

In 1.5ml tubes, 10nM, 25nM and 50nM of negative (scrambled) control or CD146 specific siRNA were prediluted in 12µl of Opti-MEM® (reduced serum medium). Using a 6-well plate, 500µl of Opti-MEM® were put in each well to be transfected. Subsequently, 4.5µl of the prediluted negative control or specific siRNA was added to the prepared Opti-MEM® in the appropriate well. 5µl of Lipofectamine™ RNAiMAX was then added to each well containing the diluted siRNAs. This entire mixture was gently vortexed and incubated for 10-20 minutes for siRNA/Lipofectamine™complex formation.

2.5ml of the prepared cell-suspension was added to each well of siRNA/Lipofectamine™ complex to a final volume of 3ml per well and put in a humidified CO_2-incubator at 37°C. Flow cytometric analysis was performed after 48h as follows.

After removing the medium from the wells they were washed with 1ml of PBS, and the cells from each well were detached with 0.5ml of trypsin. As soon as the cells detached, 1ml of PBS was added, and the suspension was transferred to a 2ml tube. The tubes were centrifuged at 1,500 rpm at room temperature for 5 minutes, and the supernatant was discarded. The pellet was washed 2 times with 1ml of PBS containing 5% of FCS and centrifugation was performed after every wash.

The CD146 antibody (Merck/Millipore) or IgG1-isotype control antibody were prediluted 1:1000 in PBS 5% FCS and 100µl of this mixture were put in each tube. The tubes were incubated for 30 minutes at 4°C. The tubes were centrifuged, the supernatant was discarded, and the pellet was washed 2 times with 1ml of PBS 5% of FCS.

The Alexa Fluor 647 labeled secondary antibody (Abcam) was prediluted 1:2000 in PBS 5% FCS, and 100µl of this mixture were put in each tube. The tubes were incubated for 30 minutes at 4°C in the dark. The tubes were centrifuged, the supernatant was discarded, and the pellet was washed 2 times with 1ml of PBS. After each step, the cell pellet was vortexed to ensure that final solution would contain single cells.

Finally, the cell pellets were vortexed and resuspended in 0.5ml of FACS (fluorescence-activated cell sorting) flow solution, after being transferred to a 5ml FACS tube. The analysis was performed on a BD FACS Canto II™ (BD Biosciences, San Jose, CA, USA) flow cytometer using BD FACS DIVA™ Software 6.0.

2.2.4 Cell cycle analysis

For cell cycle analysis, harvested cells were washed with PBS, resuspended in 400µl ice cold PBS and fixed by drop wise addition of 800µl ice cold 100% ethanol. After an incubation period of a minimum of 30 minutes at 4°C fixed cells will be washed twice in PBS and stained with freshly prepared propidium iodide (PI) staining solution containing RNase for 30 minutes at 37°C. After staining, analysis was carried out using BD FACS Canto II™ (BD Biosciences, San Jose, CA, USA) flow cytometer and BD FACS DIVA™ Software 6.0.

2.2.5 Statistics

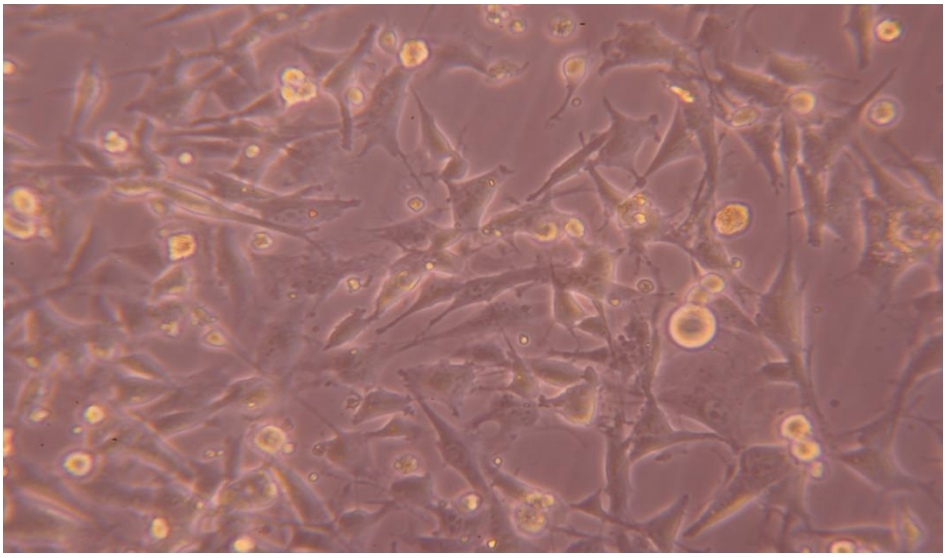
A factorial was performed between the different cell densities, media times and concentrations, using the data obtained from proliferation assay, through the PROC MIXED procedure of SAS® University Edition (SAS / STAT, SAS Institute Inc., Cary, NC). Due to the multicollinearity between measurements, an equation was also determined that could predict the absorbance value within each density. For this, a simple non-polynomial linear regression was calculated by the SAS MIXED PROC procedure. The determination coefficient was considered high when $R^2 \geq 0.70$, and P value was significant when less than 0.05 ($P < 0.05$).

2.3 Results

2.3.1 Cell culture

Canine oral melanoma cells originating from a primary tumor (Figure 1) were maintained in a T75 cell culture flask. All assays were done between passage number 55 and 60. One day before seeding for an experiment cells were splitted at a low ratio to guarantee that cells are in a proliferative growth phase.

Figure 1 – Canine melanoma cells derived from an oral primary tumor.

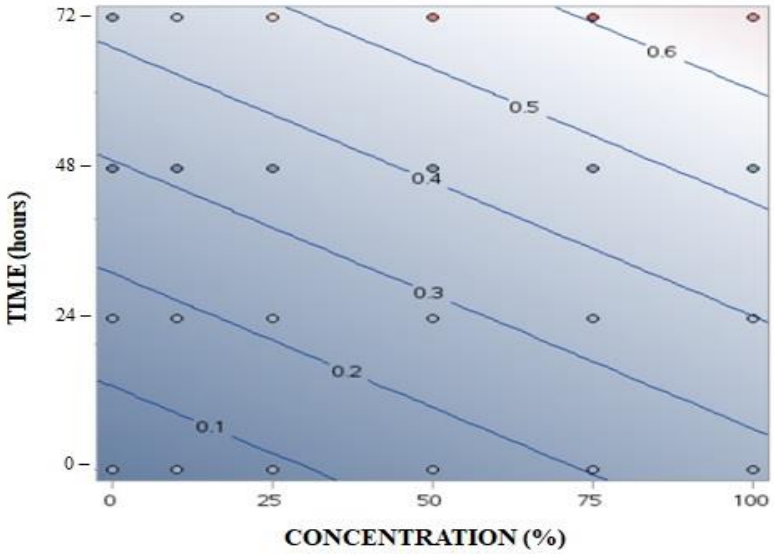


Melanoma cells attached to the flask, cultivated in DMEN + 10% FCS (HI) media, showing a good confluency in an 40x objective.
Source: Research Group Oncology of the Veterinary Medicine University of Vienna.

2.3.2 Proliferation of serum starved canine melanoma cells

The 96-well plates were seeded with a density of 1,000 (Chart 2), 5,000 (Chart 3) and 10,000 (Chart 4) cells per well and cell count was performed at 0h and after 24h, 48h and 72h for each type of medium. The mean values for each seeding density and for each different medium were calculated and results were represented in a chart.

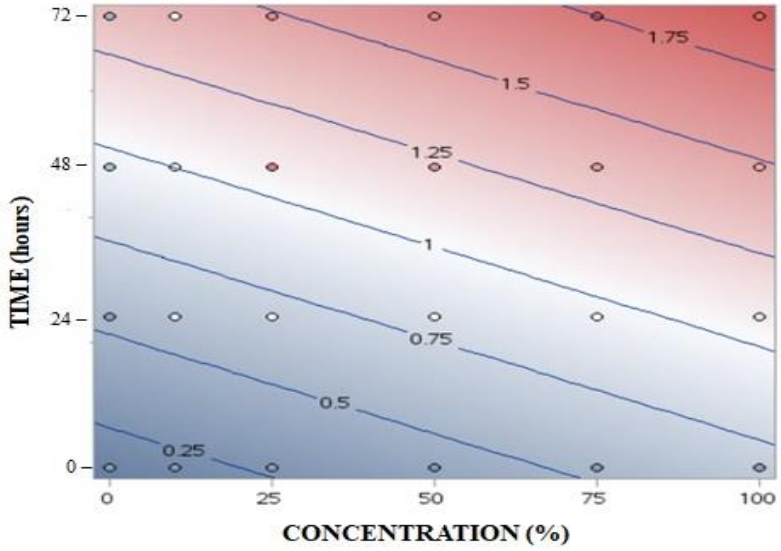
Chart 2 – Cell proliferation at different serum conditions, seeding density of 1,000 cells per well.



The concentration of the media was measure in percent and goes from 0 to 100% added with serum, and the time was measure in hours. The equation generated for the 1,000 cells density was Absorbance = 0.0055 x Time + 0.0024 x Concentration + 0.0260. The determination coefficient was $R^2 = 0.37$ and $P < 0.0001$.

Cell proliferation assay performed at a seeding density of 1,000 cells per well indicated no linear response between cell number and absorbance at 490nm. The linear growth phase of cells started just before 24h after initial seeding, but the determination coefficient was under 0.70 indicating that this seeding density of 1,000 cells per well is too low for the performance of the proliferation assay.

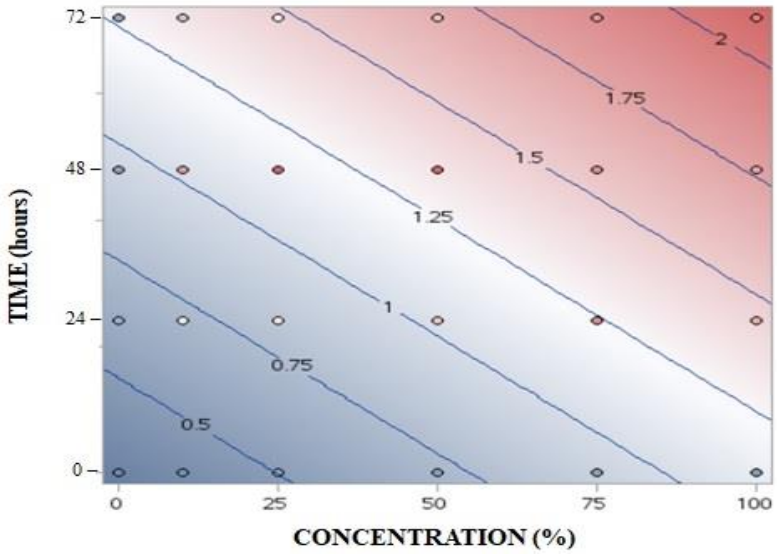
Chart 3 – Cell proliferation at different serum conditions, seeding density of 5,000 cells per well.



The concentration of the media was measure in percent and goes from 0 to 100% added with serum, and the time was measure in hours. The equation generated for the 5,000 cells density was Absorbance = 0.0167 x Time + 0.0053 x Concentration + 0.1433. The determination coefficient was $R^2 = 0.70$ and $P < 0.0001$.

Cell proliferation assay performed at a seeding of 5,000 cells per well showed linear growth phase of cells since time point 0h. After 24h, it was possible notice a higher consistence in absorbance for the 75 and 100% added serum media, and after 48h for the 25% added serum and 0% added serum media condition. This seeding density had a $R^2 = 0.70$, being the best density to perform the proliferation assay.

Chart 4 – Cell proliferation at different serum conditions, seeding density of 10,000 cells per well.



The concentration of the media was measure in percent and goes from 0 to 100% added with serum, and the time was measure in hours. The equation generated for the 10,000 cells density was Absorbance = 0.0134 x Time + 0.0082 x Concentration + 0.2981. The determination coefficient was $R^2 = 0.56$ and $P < 0.0001$.

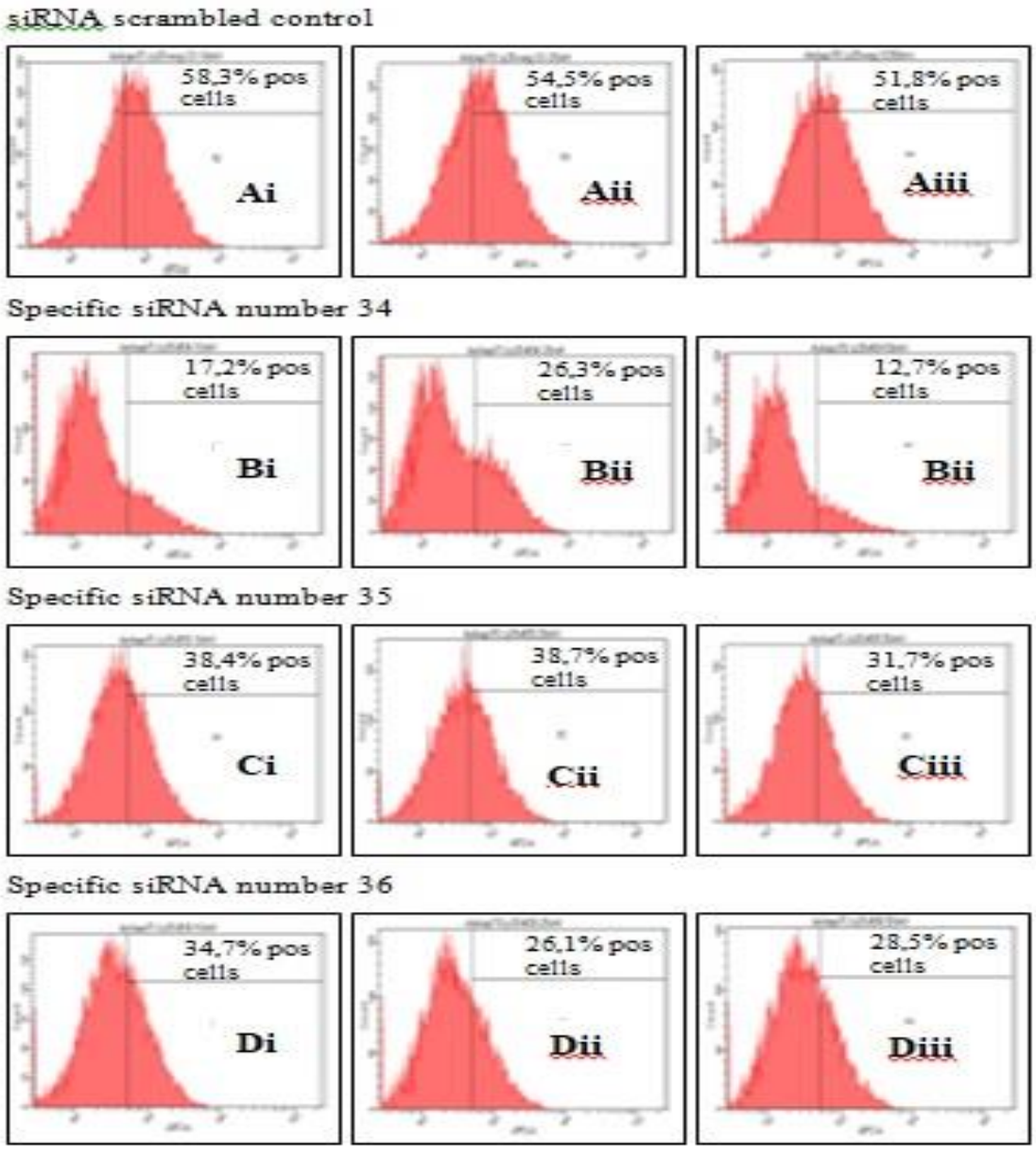
Cell proliferation assay performed at a seeding of 10,000 cells per well showed linear growth phase of cells starting at time point 0h. Non after 24h, it was possible notice a higher consistence in absorbance for the 100% added serum medium, after 24h for the 75% added serum medium and after 48h for the 50%, 25% and 0% added serum media condition. Even this density showing an earlier consistence of the data, the determination coefficient was under 0.70 so could not be good enough to perform the proliferation assay.

Condensing the data, cellular proliferation still happens over a time periode of 48h and still at serum reduced media condition of 10% Ms / 90% Mn. Hence, we established an incubation time of 48h for all further experiments.

2.3.3 Concentration-dependent downregulation of CD146

In order to measure the concentration-dependent downregulation of CD146 48h after siRNA transfection, FACS analysis was performed for siRNA scrambled control (negative) and for CD146-specific siRNAs number 34, 35 and 36. The siRNA scrambled control indicated the percentages of CD146 positive cells for each concentration applied (Figure 2 – A), while the specific siRNAs 34, 35 and 36 proved a reduction in the percentage of cells expressing CD146 (Figure 2 – B, C and D).

Figure 2 – FACS analysis showing the expression of CD146 for siRNA scrambled control and specific siRNAs at concentrations of 10nM, 25nM and 50nM.



Each chart represents a specific concentration for the analysis after a 48h incubation periode. (A) siRNA scrambled control. (Ai) 10nM. (Aii) 25nM. (Aiii) 50nM. (B) Specific siRNA #34. (Bi) 10nM. (Bii) 25nM. (Biii) 50nM. (C) Specific siRNA #35. (Ci) 10nM. (Cii) 25nM. (Ciii) 50nM. (D) Specific siRNA #36. (Di) 10nM. (Dii) 25nM. (Diii) 50nM.

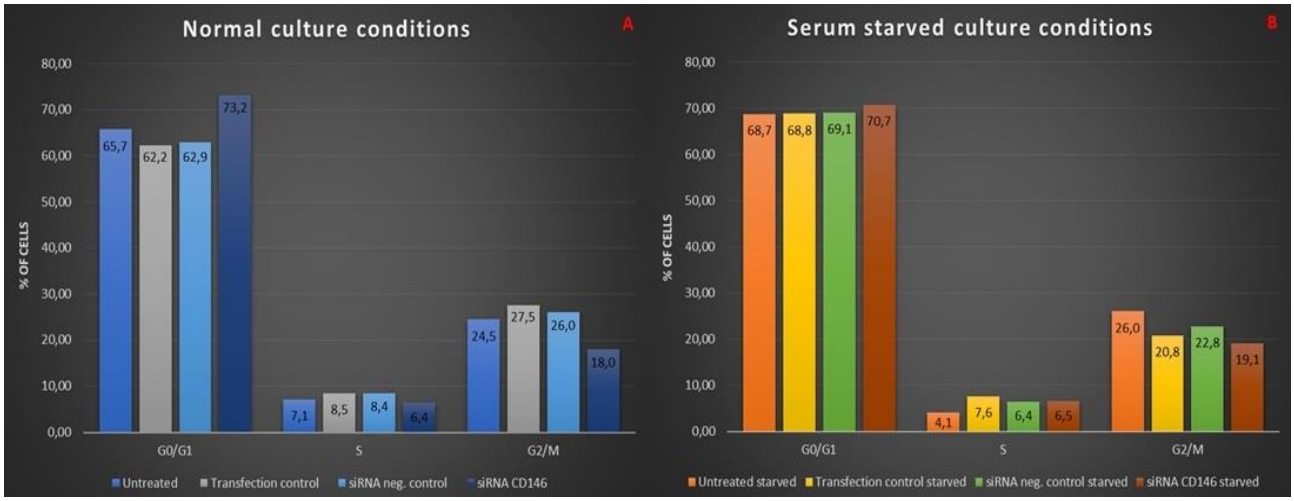
Concluding the results of the proliferation assay and CD146 RNA interference experiments cell cycle experiments were performed using CD146 specific siRNA number 34 at a concentration of 50nM with the media condition of 10% Ms / 90% Mn and after an incubation periode of 48h.

2.3.4 Cellular life cycle of CD146 silenced melanoma cells under serum starved conditions

Within the final experiment the inhibitory effect of CD146 specific RNAi in combination with serum starved cell culture conditions on cellular life cycle was performed. The different cell cycle phases, G_0/G_1, Sor G_2/M are characterized by varying DNA contents measurable by a strongly binding of PI to cellular DNA.

The effects of CD146 specific siRNA on cell cycle of canine melanoma cells investigated are shown in figure 3A. Compared to the siRNA scrambled control CD146 silenced cells showed an accumulation of cells within the G_0/G_1 phase demonstrating an inhibition in cell proliferation and cellular senescence. Additional analysis under serum starved conditions (Figure 3B) revealed only a minimal reduction in G_2/M phase and induction of G_0/G_1 arrest. These results may indicate that CD146 expression will be enhanced under serum starved conditions and partly reverse cellular senescence induced by CD146 silencing.

Figure 3 – Cell cycle analysis showing the expression of CD146 silenced melanoma cells



PI-staining and cell cycle analysis after 48h after transfection (A) under normal cell culture conditions (10% FCS) and (B) under serum starved (1% FCS) cell culture conditions.

2.4 Discussion

The choice of an ideal medium is a path to follow in order to obtain cultures expressing a specific function (ALVES; GUIMARÃES, 2013). Inoue *et al*. (2004) established and characterized four canine melanoma cell lines, and noticed there was a difference in proliferation patterns in the culture conditions between those lines. The authors suggested that intercellular adhesion activity or function and metabolism of the cells were different.

In this study, melanoma cells were first seeded in different densities and media conditions in order to find out if serum starvation would allow cell proliferation in all situations. Statistical data showed that 5,000 cells per well was the best density to perform the proliferation assay, but it also revealed that all types of densities and media conditions had a linear growth. The best time point to apply the assay occurred in 48h, yet once again it could be seen cell proliferation in all tested time points.

Under experimental cell culture conditions as well as in growing solid tumors, increased cell density imposes various challenges on cells: cadherin-mediated contact inhibition (NELSON; DANIEL, 2002), nutrient depletion, hypoxia, and mechanical stresses (POUYSSEGUR *et al*., 2006), and these facts may explain why the best density to be used is not 10,000 cells per well. In their study testing JAK inhibitor I, Kreis *et al*. (2007) could not detect any growth-inhibiting effects on a selection of melanoma cell lines even when using concentrations of up to 1µmol/L over a period of 24 or 48h. Haridas *et al*. (2017) realized experiments involving fibroblast cells and SK-MEL-28 melanoma cells separately while co-culture experiments used three ratios of both cell types in the same experiment, and all of them initialized by placing approximately 20,000 cells analyzed after a period of 48h, concluding that the spreading and growth patterns observed for primary fibroblast cells and SK-MEL-28 melanoma cells are not affected by growing them in monoculture or co-culture.

FACS analysis proved a reduction in the percentage of cells expressing CD146, especially in siRNA number 34. CD146 is well-known as an important marker for tumor invasion and metastasis (XIE *et al*., 1997), and its decreasing expression can result in reduced tumorigenicity (SATYAMOORTHY *et al*., 2001).

Osmotic pressure can induce CD146 expression. For example, high glucose (WANG *et al*., 2008), high Ca^{2+} concentration (SCHON *et al*., 2005) and increased cAMP (cyclic adenosine monophosphate) (RUMMEL *et al*., 1996) are able to up-regulate CD146 mRNA expression in a variety of cell types. This research analyzed serum starved conditions for oral canine melanoma cells and noticed that with a 10nM concentration of a specific siRNA the CD146 was best down-regulated.

It has been gradually clear that the manner of CD146 expression regulation is different in various tissues from embryo, tumor and adult (WANG; YAN, 2013). Since the first observation that CD146 was overly expressed in melanoma compared with normal melanocyte, a growing evidence has revealed that in the growth of primary and metastatic tumors, CD146 protein levels are significantly enhanced compared with normal control samples (MELNIKOVA; BAR-ELI, 2006). Although numerous proteins have been proved to be aberrantly up-regulated in tumors via genetic alterations, the genetic research results indicate that increased expression levels of CD146 in tumor

tissues is not due to translocation, amplification or mutation of the CD146 gene (LUCA *et al*., 1993).

On the one hand, knockdown of CD146 protein expression severely hinders vascular development, leads to poorly developed intersomitic vessels, and lacks of blood flow through the intersomitic vessel region (CHAN *et al*., 2005); on the other hand, the gain-of-function analysis of CD146 in zebrafish, in which enforcing expression of CD146 constructs, induces sprouting angiogenesis (SO *et al*., 2010). Angiogenesis is also required for the spread of a tumor, or metastasis. Tumors induce angiogenesis through secreting various growth factors (e.g. VEGF, vascular endothelial growth factor) (MURUKESH *et al*., 2010). Already in 1994, Johnson and colleagues reported that CD146 was overly expressed in tumor blood vessels, and CD146 up-regulation was closely associated with tumor angiogenesis.

Jin *et al*. (2016) demonstrated that CD146 suppression accelerated cellular senescence in mesenchymal stem cells from human umbilical cord, Similarly, results from a recent study showed that the expression of another vascular cell adhesion molecule, CD106, is markedly reduced in senescent bone marrow mesenchymal stem cells, which results in low homing activity (JUNG *et al*., 2011). Here, the analysis under serum starved conditions revealed only a minimal reduction in G_2/M phase and induction of G_0/G_1 arrest, and this may indicate that CD146 expression will be enhanced under serum starved conditions and partly reverse cellular senescence induced by CD146 silencing.

The proliferation activity of tumor cell is important in invasion/metastasis of tumor, but experiments involving melanoma cell cycle regulation using CD146 are yet little known. In the same cell culture condition, Chen *et* al. (2009) analyzed the proliferation of CD146-shRNA1 (CD146-short hairpin RNA1), transfected cells and control, and they all were almost similar on the first 24h. By time lapse, the cells of CD146 gene silencing grew more slowly than the cells of control. The cells of CD146-shRNA1 transfectant resulted in significant decrease in cell proliferation rate compared with control cells.

When studying the effect of CD146-specific shRNA on cell cycle in adenoid cystic carcino-melanoma, Chen *et* al. (2009) found that the control group resulted in cycling of about 48% of the cells in the S and G_2/M phases. In the shRNA1 group, the proliferation index value of cycling cells (combined total number of cells in the S and G_2/M phases) was decreased to about 36% with a concomitant increase in the number of cells in the G_0/G_1 phase, similar as the results founded in this test.

2.5 Conclusion

Increasing evidence demonstrates that CD146 is a multi-functional molecule implicated in a variety of biological and pathological processes. Better understanding the function of CD146 will not only benefit for investigation of CD146-related physiological processes, but also for CD146-associated pathological progressions, such as cancerous progression.

To summarize, our assay suggests that different serum starved conditions and reduction of CD146 expression can minimally affect the cellular life cycle of canine melanoma cells. Cell proliferation is better on the time point 48h in serum starved media, and siRNA number 34 is responsible for the best down-regulation of CD146 when compared with numbers 35 and 36. Further studies are needed to provide additional molecular information about the functional relationship of CD146 and the cell cycle analysis using melanoma cells.

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