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Diplomarbeit

Expression and Activity of Galectins in Human Melanoma Cell Lines

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Zusammenfassung

Hintergrund: Das Melanom ist einer der am schnellsten wachsenden Hauttumore. Obwohl das Melanom weniger als 5 % der bösartigen Tumore der Haut umfasst, ist es verantwortlich für fast 60 % der tödlichen Hautneoplasien. Galektine gehören zur Familie der Lektine, die kohlenhydratbindende Proteine sind und die an verschiedenen biologischen Vorgängen wie Zellwachstum, Zelladhäsion und Metastasierung beteiligt sind.

Ziele: Das Ziel der vorliegenden Diplomarbeit war es, die Expression und die biologische Aktivität einer Reihe von humanen galektinen in zwei humanen Melanom-Zelllinien (MCM1 und DLNxt Zellen) zu untersuchen, die sich in ihrer metastatischen Kapazität in vivo unterscheiden.

Methoden: Diese Arbeit untersuchte (1) die Gen-Expressionsmuster verschiedener Galektine mittels RT-qPCR, (2) die galektin-Expression und galektin-Bindungsstellen durch FACS-Analyse bzw. durch Fluoreszenz-Mikroskopie und (3) die Wirkung von Galektinen auf die Zellviabilität, Zelladhäsion und MMP3 und TNF-alpha mRNA-Spiegel.

Resultate: Die Gen-Expressionsanalysen zeigten unterschiedliche Galektin-Expressionsmuster in den Melanom-Zelllinien. Galektin-1 und -3 waren hoch in beiden Zelllinien exprimiert, während Galektin-7, -8 und -9 mittels RT-qPCR kaum nachweisbar waren. Das höchste Niveau von Galektin-3-Expression war in MCM1 Zellen zu sehen, während das höchste Expressionsniveau von Galektin-1 in DLNxt Zellen gefunden wurde. Die Unterschiede in der galektin-Expression sowie ihre Präsenz auf der Zelloberfläche, im Zytoplasma und in der Kernregion, wurden weiters mit Hilfe der Fluoreszenzmikroskopie gezeigt. Galektin-1, -3 und -9 waren

diffus im Zytoplasma verteilt, während Galektin-8 eine deutliche vesikuläre Akkumulation um die Kerne herum zeigte. Die Präsenz von galektin-Bindungsstellen an MCM1 und DLNxt Zellen wurde zudem mittels biotinylierten galektinen untersucht. Die Ergebnisse zeigten Unterschiede in der galektin bindung, nicht nur zwischen den Zellen, sondern auch zwischen den einzelnen Galektinen. Die Anwesenheit von galektin-Bindungsstellen wirft die Frage auf, ob Galektine die Zelladhäsion an Kulturflaschen beeinflussen können. Die Ergebnisse zeigten, dass die Anwesenheit von Galektin-1 die Zelladhäsion an Kulturflaschen nicht ändert. Im Gegensatz dazu erhöhte Galektin-8 die Adhäsion der DLNxt dosisabhängig, während die Adhäsion der MCM1-Zellen nicht betroffen war. Darüber hinaus dokumentieren RT-qPCR-Daten, dass Galektin-1 die Expression von MMP3 und TNF-alpha in DLNxt Zellen reguliert.

Schlussfolgerungen: Die in dieser Studie vorgestellten Ergebnisse zeigen, dass humane Melanomzellen mit unterschiedlichem metastatischem Potenzial, galektine auf mRNA- und Protein-Ebene exprimieren. Darüber hinaus wurde gezeigt, dass die Zelllinien die jeweiligen galektin-Bindungsstellen exprimieren und dass die Anwesenheit von galektinen einen Einfluss auf die mRNA-Expression von MMP3 und TNF-alpha und auf die Zelladhäsionseigenschaften hat. Zusammenfassend kann festgestellt werden, dass diese Studie einen Zusammenhang zwischen der Expression von galektinen und galektin-Bindungsstellen und ihrer Beteiligung an Zelladhäsion und Tumorverhalten zeigt.

Abstract

Background: Melanoma has been one of the fastest-rising malignancies over the past several decades. Although melanoma comprises less than 5 % of malignant skin tumors, it is responsible for almost 60 % of lethal skin neoplasia. Galectins belong to the family of lectins which are sugar binding proteins that are assumed to be involved in cell growth, adhesion and metastasis.

Aims: The aim of the present study was to investigate the expression and biological activity of a set of human galectins in two human melanoma cell lines (MCM1 and DLNxt cells), that differ in their metastatic capacity in vivo.

Methods: This thesis investigated (1) the gene expression pattern of different galectins using RT-qPCR, (2) the galectin expression levels and galectin binding-sites by FACS analysis and by fluorescence microscopy, respectively, and (3) the effect of galectins on cell viability, cell adhesion and MMP3 and TNF-alpha mRNA levels.

Results: Gene expression analyses showed a different galectin expression pattern among the melanoma cell lines. Galectin-1 and -3 were highly expressed in both cell lines, whereas Galectin-7, -8, and -9 were hardly detectable using RT-qPCR. The highest level of Galectin-3 expression was seen in MCM1 cells, whereas the highest level of Galectin-1 was found in DLNxt cells. The differences in galectin expression as well as their presence on the cell surface, in the cytoplasm and around nuclei, respectively, have been further demonstrated using fluorescence microscopy. Galectin-1, -3, and -9 were diffusely distributed in the cytoplasm, while Galectin-8 showed a clear vesicular accumulation around the nuclei. MCM1 and DLNxt cells were also monitored for accessible galectin-binding sites using biotinylated galectins.

Taking unlabeled cells as reference, both cell lines displayed different capacities to bind the labeled galectins. The results revealed differences in the galectin-binding, not only between the cells but also among the individual galectins. The presence of galectin-binding sites prompts the question if galectins could impact the cell adhesion to culture flasks. The results showed that the presence of galectin-1 did not alter cell adhesion to plastic dishes. In contrast, Galectin-8 increased the DLNxt adhesion in a dose-dependent manner, whereas the adhesion of MCM1 cells was not affected. Moreover, RT-qPCR data documented that Galectin-1 regulates the expression of MMP3 and TNF-alpha in DLNxt cells.

Conclusion: The results presented in this study demonstrate that human melanoma cells with different metastatic potential in vivo express galectins at the mRNA and protein level. In addition, we showed that the cell lines express the respective galectin binding sites and that the presence of galectins influences the mRNA expression levels of MMP3 and TNF-alpha and cell adhesion properties. Taken together, this study suggests a relationship between the expression of galectins and galectin binding sites and their involvement in cell adhesion and tumor behavior.

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1 Introduction

1.1 Skin

Skin is a vital organ that covers the entire outside of the body, forming a protective barrier against pathogens and injuries from the environment. The skin is the body's largest organ weights approximately 10 kg. It protects the organs inside the body against infection, heat and ultraviolet light from the sun. It helps to regulate the body temperature and to get rid of waste materials through the sweat glands. The skin also produces vitamin D and stores water and fat. The skin is composed of three primary layers. The layer at the surface is called the *Epidermis*. Below the epidermis are the inner layers: the *dermis* and the *hypodermis* [1, 2, 3]

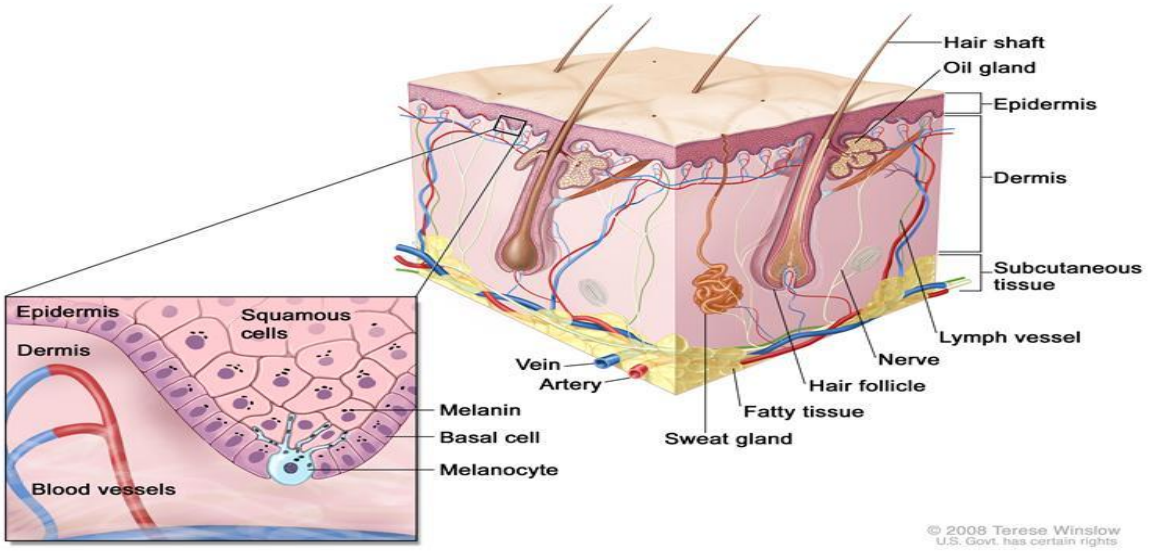


Figure 1: The Structure of the Skin[1]

1.2 The Epidermis

The epidermis contains the melanocytes, the cells in which melanoma develops, the Langerhans cells that are involved in the immune system, the Merkel cells that are considered as an adapting mechanoreceptor and sensory nerves.

[1] http://www.cancer.gov/cancertopics/factsheet/Sites-Types/metastatic

The epidermis layer itself is made up of five sublayers that work together to continually rebuild the surface of the skin.

The Basal Cell Layer (Stratum basale)

The basal cell layer contains cells called melanocytes. Through a process called melanogenesis, these cells produce melanin, which is a pigment found in the skin and eyes and which saves the skin from damaging ultraviolet rays, producing a suntan. Sometimes melanocytes cluster together and form moles also called *nevi*. Moles are common and are usually not cancerous.

The Squamos Cell Layer (Stratum spinosum)

The Squamos cell layer is located above the basal layer. It is the thickest layer of the epidermis and participates in the transfer of certain substances into and out from the body. The basal cells have been pushed upwards therefore these cells are now called squamos cells or keratinocytes. They produce keratin: a protective protein which is the main constituent of the structure of the skin, nails and hair. The layer also contains the Langerhans cells.

The Stratum Granulosum and the Stratum Lucidum

The Stratum Granulosum is superficial to the stratum spinosum, also called grainy layer. Stratum Granulosum consist of three to five keratinocytes derived from the Stratum Spinosum. Stratum Lucidum covers the Stratum Granulosum.

The Stratum Corneum

The Stratum Corneum is the outermost layer of the epidermis, composed of large, flat, polyhedral, plate-like envelopes filled with keratin, which is made up of dead cells that have migrated up from the Stratum Granulosum. [2, 3, 4]

1.3 Malignant Melanoma

Malignant melanoma is one of the most aggressive malignancies in human. This cancer can metastasize rapidly. It develops when melanocytes are damaged, which causes these cells to grow uncontrollably. The transformation of melanocytes into malignant melanoma involves the interplay between genetic factors, UV exposure and the tumor microenvironment [4]. Melanoma begins at the surface of the skin, but it can reach the blood and lymphatic vessels, and from there it can spread through the body, causing a life-threatening condition. With early detection and proper treatment, melanoma has a high cure rate [4, 10, 14].

1.3.1 Epidemiology of Melanoma

Melanoma has been one of the fastest-rising malignancies over the past several decades. Even though this cancer type comprises less than 5 % of malignant skin tumors, it is responsible for almost 60 % of lethal skin Neoplasia [8]. The annual increase in incidence rate of this malignancy varies between populations, but it is predominantly in the order of 3–7 % per year in white populations [8, 9] and the incidence is 5- to 20-fold lower in populations with darker skin color e.g. Africans, East Asians and Hispanics [8, 10]. The highest incidence rate appears to be in Queensland, Australia [8, 9, 10]. As reported in the period between 1980 and 1987 the annual incidence of melanoma was 55.8 per 100,000 inhabitants for men and 42.9 per 100,000 inhabitants for women. Similar incidence rate were reported also in New South Wales, Australia, where the annual incidence of melanoma in the period between 1986 and 1988 was 52.5 per 100 000 in men and 42.9 per 100,000 in women [10]. The mean annual incidence (age-standardized rate) of invasive cutaneous melanoma in Styria, a province of Austria, in the years 2001–2003, was 24.5 per 100,000 – lifetime risk 1 in 52 [51].

1.3.2 Risk Factors

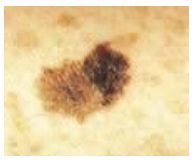
The list of risk factors that may have an importance in developing melanoma is very large and includes blond or red hair, fair sun-sensitive skin that has the tendency to tan poorly or to burn easily, presence of 50 to 100 or more atypical moles, Xerodema Pigmentosum, Immune system weakness due to disease or organ transplant or medication and genetic factors [2, 14]. The exposure of high penetrating UVB radiation leads to DNA lesion such as formation of cyclobutane pyrimidine dimer and pyrimidine pyridine photoproducts [4]. The incorrent repair of these products leads to a DNA mutation which could be responsible for the tumor progression [4, 10]. Increased UV-exposure is the main responsible for the three major types of skin cancer [4]:

- *Squamous cell skin cancer* starts in the squamous cells (thin flat cells found on the surface of the skin).
- *Basal cell skin cancer* starts in the basal cells (round cells that lie under the squamous cells).
- *Melanoma* starts in the melanocytes.

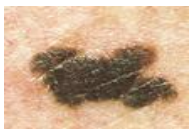
1.3.3 Diagnosis, Staging and Conventional Therapy

Early detection and treatment are possible because melanoma typically begins at the surface of the skin. The majority of persons (more than 85 %) diagnosed at the early stages are cured, whereas melanoma diagnosis at later stages of tumor progression is associated with a poor prognosis, because the melanoma can be highly malignant and metastasize to almost any organ of the body [10].

When looking for a melanoma on the skin, it is helpful to apply the ABCD rules [2, 10]:



A stands for **ASYMMETRY**; one half unlike the other half



B stands for **BORDER**; irregular, scalloped or poorly defined border

C stands for **COLOR**; varied from one area to another; shades of tan

D stands for **DIAMETER**; melanomas are usually greater than 6 mm (size of a pencil eraser) when diagnosed, but they can be smaller.

Figure 2: ABCD`s of Melanoma[2]

The primary melanoma progresses through two phases which are

(1) the radial growth phase characterized by horizontal spreading of transformed melanocytes within the epidermis and

(2) the vertical growth phase characterized by invasion of melanoma into the deeper dermis [2, 11].

Determining how far the cancer has spread is called staging. The staging of melanoma is based on the depth of the invasion into the dermis, determined by either Clark's method or Breslow's method [2, 10, 12].

[2]http://www.melanomafoundation.org/prevention/abcd.htm

a) In situ (*stage 0*) melanomas are noninvasive and have not broken the integrity of the epidermal basement membrane (these lesions are not included in melanoma statistics).

b) *Stage I* (1mm depth) and *stage II* (1–2.0 mm depth) melanoma is confined to the skin, shows an increased thickness and the skin may be intact or ulcerated and is treated by surgical excision.

c) *Stage III* (2.1–4 mm depth) melanoma has spread to a nearby lymph node and is found in increasing amounts within one or more lymph nodes and is treated by surgery with or without adjuvant therapy using interferon-α2b [10, 13].

d) In *stage IV* (4 mm depth) melanoma has spread to internal organs, beyond the closest lymph nodes to other lymph nodes, or areas of skin far from the original tumor. In addition to surgery and radiation therapy, systemic therapy (chemotherapy and immunotherapy) is used, but induces complete remissions in only a small proportion of patients [13]. The tumor can metastasize only if all stages of the cascade occur in the correct order. If only one step gets blocked, it can attenuate tumor cell metastases [14].

1.3.4 But why does Malignant Melanoma Get Dark?

The original sources of melanin are the melanoma cells. As already known, melanin is the dark pigment of the skin. Melanoma begins first in the melanoma cells and exhibits pigmented regions that are darker than the surrounding skin. Beside melanoma cells, they are two others melanin-containing cell types that also contribute to the dark color: keratinocytes and melanophages [5]. It is quite obvious that in the dark regions there is more melanin produced as compared to normal melanocytes in adjacent skin. Melanoma melanin appears brown-black consistent

with being eumelanin, compared to the gold-red colour of pheomelanin in normal skin [6]. Increased melanin synthesis and the production of eumelanin are both sings of a normal functioning of MC1-MC1R system – also known as melanocortin-1 [5, 6]. It plays a main role in melanoma progression. This system leads the activation of the proto-oncogene cMet, whose signaling pathway is a key regulator of metastasis in melanoma [7]. It has been speculated that the dark melanoma cells express an MC1-MC1R system, which is differently activated from that in healthy melanocytes, maybe through aberrant glycosylation or other post-translational modifications [6].

1.3.5 Metastatic Growth

The metastasis of the tumor is the important step for tumor spreading. Spreading is the potential of tumor cells to disperse from the primary site, movement away from the original site of disease, intravasation survival in circulation and finally their adhesion to the vessel wall and organ homing [14, 15, 16, 17]. Metastasis is a complex process and preventing tumor cells from migrating is of medical interest. For cells to spread and to be invasive they need to create space by degrading the cell-cell and the cell-matrix interaction [14, 17]. Melanocytic cells need the initial dissociation from the neighbouring keratinocytes followed by migration and dermis proliferation and eventually lymphovascular permeabilization [25].

1.4 The Genetics of Melanoma

Many genetic and molecular changes are involved in melanoma progression and metastasis, and their underlying mechanisms are largely undefined. Involved in melanoma invasion are tumor suppressor genes (p16 INK4a/p14ARF, PTEN and p53), transcription factors (CREB/ ATF-1, AP-2), oncogenes (BRAF, NRAS),

tyrosine kinases (c-kit, PDGF receptors), cell adhesion molecules (E-cadherin, β-integrin) and Matrix Metalloproteinases [18]. In 25–50 % of familial melanoma worldwide and in approximately 10 % of individuals with multiple primary melanoma, a CDKN2A **(**cyclin-dependent kinase [CDK] inhibitor 2a also p16) mutation has been identified. The CDKN2A locus encodes two proteins which are involved in growth regulation and apoptosis: namely the p16/INK4a and the p14/ARF. The first one inhibits the CDK 4/6 (cyclin-dependent kinase) which in cancer cells is constitutively active, leading to an inactivation of uncontrolled growth. The second one stabilizes p53, well known as a tumor suppressor and proapoptotic protein. Inactivation of p14/ARF leads to abrogation of the p53 pathway. Loss of both mechanisms allows the damaged cells to proliferate and leads to accumulation of further mutations [8, 10, 18, 19]. The most important molecular finding over the past decades was the high frequency of NRAS and BRAF mutations in melanoma and other cancers. BRAF mutation has been reported in 66 % of melanomas. BRAF proteins are serine-threonine kinases and mediators of RAS signaling, activates the ERK/MAPK pathway which mediates cell growth and proliferation downstream [10, 19, 20]. Mutations of BRAF in exon 15 as well as mutations of NRAS in exon 2 have been identified with high increase in nevi, cutaneous melanoma and metastasis and are preserved through tumor progression, indicating they are the key pathological events [20, 21].

1.4.1 Role of Adhesion Molecules in the Progression and Metastasis of Melanoma

Cell Adhesion Molecules (CAMs) are typically transmembrane receptors located on the cell surface. Cell surface adhesion molecules play vital roles in numerous cellular processes. Some of these including: cell growth, differentiation, embryogenesis,

immune cell transmigration and response, and cancer metastasis. Cell Adhesion molecules are composed of three domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain and an extracellular domain that interacts with other CAMs of the same kind homophilic binding or with other CAMs or the extracellular matrix-heterophilic binding. They can also mediate adhesion between two cells of the same type, a process called homotypic adhesion, or different cells type heterotypic adhesion. It is known that the homotypic interactions between tumor cells play a role in extravasation by supporting cell survival and helping organ homing, whereas the heterotypic interactions play a central role in tumor cell intravasation and extravasation. They were firstly described on the basis of the tissue in which they were initially identified. It is now clear that they are ubiquitous and not limited to a single tissue. CAMs have also been characterized as calcium-independent and calcium-dependent. This classification depends on whether calcium is needed for the function or not [14].

a) *Integrins – calcium-independent:* The Integrins are heterodimers, composed of an alpha and beta subunit, which are both fundamental for proper integrin function. The integrins contribute to cell growth by providing a link between cytoskeletal structures and the extra-cellular matrix proteins. They participate in both cell-matrix and cell-cell adhesion in a variety of physiologically important processes such as haemostasis and wound healing [22]. The adhesion to the extracellular matrix strongly depends on the extracellular pH. The cell-matrix interactions are promoted by acidic extracellular pH whereas they are impaired in an alkaline environment [23]. In some tumors, increased integrin expression is associated with increased malignancy and metastasis formation [24]. Cells that derived from a malignant melanoma tender to have an increased level of alpha 2 and 4 integrin

subunits. Over expression of the alpha4β1 receptor which mediates the interaction between melanoma cells and endothelial cells, may play an important role in melanoma metastasis. In this context, recent studies have documented an increased expression from alpha4β1 in 40 % of invasive and metastatic melanoma, whereas it is not expressed by normal melanocytic cells [22].

b) *Cadherins – calcium-dependent:* The most important members of this family are *E-cadherins* (epithelial), *P-cadherins* (placental) and *N-cadherins* (neural). Each member was found to regulate cell adhesion of particular cell types and thus was thought to be fundamental for the organization of the multicellular organism. The cadherins will preferentially bind to other receptors within their subclass-homotypic binding. However, they also possess a weaker binding affinity for members of a different subclass within the same superfamily-heterotypic binding. Melanocytic cells are found in normal and healthy skin, as a single cell**s** scatered around the keratinocytes. It has been shown that keratinocytes control proliferation, differentiation and expression of cell surface molecules on melanocytes. If this contact disappears, melanocytic cells proliferate rapidly [25]. E-cadherins mediates the adhesion between melanoma cells and keratinocytes [26]. A manifest loss of E-cadherin expression during melanoma development correlates with a loss of keratinocyte-mediated regulation of melanoma cells. In this case melanocytes become free from keratinocytes regulation, resulting in increased motility, proliferation and invasive potential of melanoma cells [27]. A loss of E-cadherin during progression of melanoma directly correlates with an increase expression of N-cadherin [26]. The last one mediates the interactions between melanoma cells and vascular endothelial cells as well as the communication between melanoma cells and fibroblast through gap-junction, and

also facilitates the migration of melanoma cells over dermal fibroblasts. This change on the repertoire of cadherins plays a key role in melanoma migration, survival and metastases [28].

1.5 Glycobiology of Melanoma Metastasis

1.5.1 Protein Glycosylation of Melanoma

Glycosylation is the enzymatic process that attaches glycans to proteins, lipids or other organic molecules. The carbohydrate side chains modulate the interaction of a protein with its environment, influencing its solubility, activity and biologic fate. This enzymatic process produces one of the fundamental biopolymers found in cells. Glycosylation types are classified according to the identity of the atom of the amino acid which binds the carbohydrate chain, namely [16, 29]**:**

a) *C-linked glycosylation* refers to the covalent attachment of a mannose residue to a tryptophan residue within an extracellular protein.

b) *N-linked glycosylation i*s based on the attachment of oligosaccharides to a nitrogen atom, usually the N4 of Asparagine side-chain, in the consensus sequence Asn-X-Ser/Thr, where X is any residue but a Proline. This process occurs on secreted or membrane bound proteins, mainly in eukaryotes and archaea – most bacteria do not carry this modification. N-glycosylation begins as a co-translational event in the endoplasmic reticulum, with additions of 14 sugars chain (including 2 N-acetylglucosamines, 9 mannoses and 3 glucoses) to an asparagine in the polypeptide chain of the target protein. After removing 3 glucose and 1 mannose residues, the protein is transferred to the Golgi apparatus where the glycans lose a variable number of mannose residues and acquire a more complex structure during a process called ‘terminal glycosylation’.

There are 3 types of mature N-glycans:

I. high mannose – those that have escaped terminal glycosylation,

II. hybrid,

III. complex – with different combinations of mannose, N-acetylglucosamine, N-acetylgalactosamine, fucose and sialic acid residues [29].

c) *O-linked glycans:* a post-translational event that takes place in the cis-Golgi compartment after N-glycosylation and folding of the protein. This is the addition of glycans to the hydroxyl group of serine or threonine residues by the enzyme UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosa-minyltransferase, followed by other carbohydrates such as galactose and sialic acid [16, 29]. O-linked glycans play important roles in protein localization and trafficking, protein solubility, antigenicity and cell-cell interactions.

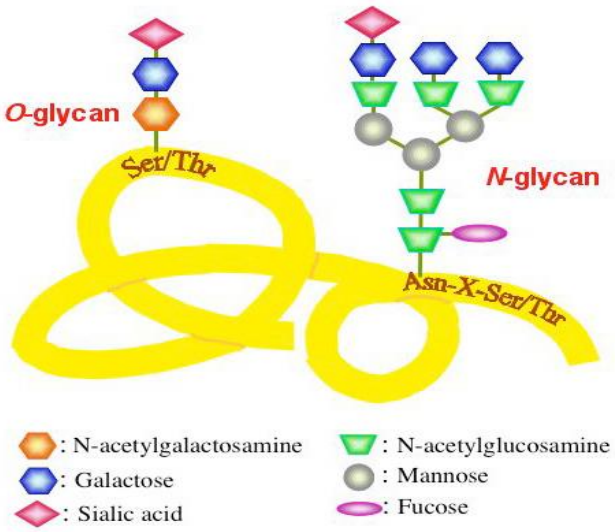


Figure 3: Examples of the attachment forms of glycans on a protein [16][3]

[3] Altered Glycosylation Contributes to the Tumor Behavior

1.5.2 Glycosylation Contributes to the Tumor Behavior

The interactions between metastatic tumor and extracellular matrix depend on modifications of cell surface glycoprotein's e.g. integrins and cadherins, respectively on the carbohydrate side chain at the cell surface. Both adhesion proteins are heavily glycosylated and alterations in their glycosylation may play a central role in giving rise to invasive and metastatic potential. For successful tumorigenesis there must be changes in cellular adhesivity which facilitate the disruption of normal tissue architecture [14].

1.5.3 Changes in Cell Surface Glycans Associated with the Progression of Human Melanoma?

The surface of mammalian cells is decorated with complex carbohydrates. Aberrant glycosylation accompanies various diseases including cancer [30, 31]. Many studies have reported that increased levels of sialylated or fucosylated complex type of β-1, 6-N-acetylglucosamine branching is associated with malignant transformation, including tumor invasion and metastasis [16, 29]. The enzyme that catalyzes this process is β-1, 6-N-acetylglucosaminyltransferase V, a Golgi complex enzyme. The appearance of increased enzymatic activity correlates with the metastatic potential and tumor progression [30]. This glycan structure is not produced by normal melanocytes but is characteristic of invasive and metastatic tumors [32]. The expression of β-1, 6-branched oligosaccharide correlates with increased melanin production, autophagy and high invasive potential in melanoma cells [33]. This was illustrated in experiments where inhibition of β-1, 6-branched oligosaccharide production virtually eliminated melanin synthesis and markedly decreased chemotactic motility in hybrids [33]. The hybrids were created by fusion in culture between mouse melanoma cells and human macrophages. It shows that the

metastatic and melanogenic phenotypes in hybrids were dependent on β-1, 6-branched oligosaccharide [33, 34, 35]. This study shows similarities to dark malignant melanoma and suggests the possibility that macrophage fusion with melanoma cells is the underlying mechanism [34, 35]. While fusion has been demonstrated in animal melanoma, analog proof of fusion in human melanoma still awaits analysis at the genetic level [5].

1.5.4 Galectins

Lectins are sugar binding proteins that are able to interpret the specific information carried by carbohydrate structures in animal cells. The animal lectins are divided into two main groups: C-type (calcium-dependent) and S-type (calcium-independent)

The most relevant group of **C-type** lectins is the Selectin family consisting of three members called E (endothelial), P (platelet) and L (lymphocytes). The Selectins are involved in lymphocyte homing to high endothelial venules and in the initial processes of attachment of leukocytes to endothelial cells at sites of inflammation [36, 37, 38].

S-type lectins, also known as Galectins, consist of beta-galactoside binding lectins containing homologous carbohydrate recognition domains (CRDs). Already have been 14 soluble galectins members identified, named by consecutive numbering. A common function of galectins is to cross-link structures containing N-acetyl-lactosamine located at the cell surface and within the extracellular matrix. They also possess hemagglutination activity, which is attributable to their bivalent carbohydrate binding properties [36, 37]. Galectins bind glycoconjugates of the extracellular matrix (ECM) via the CRDs. The galectin Carbohydrate Recognition Domain (CRD) consists of ~135 amino acids. Each of CRDs consists of three exons.

The amino acid sequence will be encoded by the middle one of these three exons. This exon represents four contiguous beta-strands and intervening loops, incorporating all amino acides that directly interact with the carbohydrate ligand [36, 37, 39]. Galectins-1, -2, -5, -7, -10, and -11 are prototypical galectins, consisting of a single CRD, Galectin-3 is also one CRD galectin, but it is unique in that it contains unusual tandem repeats of proline- and glycine-rich short stretch. Galectins-4, -6, -8, -9, and -12 are composed of two different CRDs joined by a linker peptide chain known also as tandem-repeat type. These molecules can cross-link glycoproteins due to the presence of more than one CRD, whereas the prototypical galectins must dimerize in order to cross-link target structures [36, 37]. The amino acids identity increases to 80–90 % when comparing only the CRDs. Within one mammalian species, the amino acids identity of the CRDs among different galectins ranges from approximately 20–40 % [37]. The expression levels of galectins are distinct but overlap in mammalian tissues. The localization as well as the expression levels are modulated by external stimuli, viral infections, inflammatory agents or tumor, whereas the secretion levels increases as a stress response which could be inflammation or heat shock. It is a fact that galectins play a key role in many pathological states, including autoimmune diseases, allergic reactions, inflammation, tumor cell metastasis, atherosclerosis and diabetic complications. Galectins can either allow or inhibit cellular adhesion by binding one of the interacting partners involved in the adhesion process [37, 40]. The effect on adhesion is dependent on the concentration and specificity of the galectin as well as the glycosylation state of the receptor to which it binds. They can bind to several adhesion molecules such as laminin or fibronectin e.g. Galectin-3 and -8. Diverse integrins serve as receptors for many galectins e.g. Galectin-1 and -3. Galectins have the ability to cross-link

glycoproteins of different cells or glycoproteins of the cell and the ECM. At high galectin concentrations, cell surface glycoproteins on individual cells may become cross-linked, which also can result in the loss of adhesive potential [37].

1.5.4.1 Galectin-1 Expression Correlates with Melanoma Progression

Galectin-1 is a homodimeric galectin composed of subunit of approximately 130 amino acids. Each subunit folds as one compact globular domain [37]. A Galectin-1 expression and over-expression in tumoric cells is a sign of malignant cells progression that could be related to tumor metastases. An interesting observation by Yoon et al. revealed that Galectin-1 is more expressed in malignant melanoma than in melanocytic nevus and dysplastic nevus. It suggests that Galectin-1 might contribute to the tumorigenesis and malignancy of melanocytes [41].

1.5.4.2 Galectin-3 Expression in Association with Melanoma Progression

Galectin-3 belongs to the chimera type of galectins, containing a short NH2- terminal domain of 12 amino acids, a collagen-like long sequence and a COOH-terminal domain. Galectin-3 has been found on the tumor cell surface and plays a role in tumor metastasis [42]. Several in vitro studies have demonstrated that the expression of the Galectin-3 correlates with the tumor invasion, metastatic cascade and inflammation [42, 43]. Very high serum concentrations have been reported in several carcinomas including Melanoma [43]. Vereecken and Heenen suggest two options that could explain the correlation of high serum levels of Galetin-3 with melanoma progression: The first option is that Galectin-3 could directly facilitate the interaction between metastatic cells with vascular endothelium cells and the adhesion of melanoma cells to elastin-rich tissues such as skin and lung tissues. The second option is that Galectin-3 releases advance inflammation which can be related to

tumor pressure in cancer patient. It can be produced by inflammatory cells and save them from apoptosis. This anti-apoptotic effect of Galectin-3 gives cells a selective survival advantage, which is very important in the vascular compartment, in which inflammatory and circulating tumor cells could be found in patients with metastatic melanoma. [43]

Galectin-1	Many cell types	Homodimer; Prototype; 14-15kDa (one CRD)
Galectin-2	Gastrointestinal tract	Homodimer; Prototype; 14kDa (one CRD)
Galectin-3	Many cell types	Monomer with one CRD; Chimera type; Pro-, Tyr-, Gly-rich repeats in N-terminal section. 27-36kDa
Galectin-4	Lung, Liver, Colon, Placenta	Monomer; Tandem-repeat type; 36kDa (two CRDs connected by a link peptide)
Galectin-5	Reticulocytes; Erythrocytes (rat)	Monomer; Prototype; 17kDa (one CRD)
Galectin-6	Small intestine; Colon	Tandem-repeat type with two CRDs
Galectin-7	Keratinocytes; Carcinoma cells	Homodimer; Prototype; 15kDa (one CRD)
Galectin-8	Several tissues; in tumor cell lines	Tandem-repeat type; 34kDa (two CRDs)
Galectin-9	Lung; Liver; Small intestine; B-cells	Tandem-repeat type; 36kDa (two CRDs)
Galectin-10	Lung	One CRD-like structure with specificity to D-Man;16.5kDa
Galectin-11	Sheep Gastrointestinal tract	One CRD resembling prototype galectins; 14kDa
Galectin-12	Several tissues	Tandem-repeat type with two CRDs; 35.3kDa
Galectin-13	Kidney; Bladder; Tumor cells	Homodimer; 16.1kDa; (one CRD)
Galectin-14	Ovine eosinophiles; Lung	One CRD resembling prototype galectins; 18.2kDa

Table 1: Members of the Galectin Family of Mammalian Lectins[4]

[4] The Emerging Funtionality of Endogenous Lectins:A Primer to the Concept and a Case Study on Galetins including Medical Implications

1.6 Aim of the Study

Recently, a set of functionally different melanoma cell lines was developed at the Department of Dermatology at the Medical University Vienna (unpublished data). Firstly, one melanoma cell line was established from a metastatic melanoma lesion of a single patient. The primer cell line was further cultured and 20 independent cell clones have been introduced. Only 8 of them (MCM-1; MCM-1a; MCM-1d; MCM-1g; MCM-1l; MCM-1k; MCM-1m and MCM-1q) were subcutaneously implanted in severe combined immune deficient mice (SCID) and have been tested for their tumorigenic potential.

It was shown that only the original cell pool and two clones (MCM1; MCM1-d; MCM-1g) have high transplant efficacy while the rest were not transplantable under standard conditions. Tumor associated tissue was stain with LYVE-antibody, and only one clone (MCM1-d) shows metastatic potential in one case to regional lymph nodes but not to lungs. No LYVE staining or lung metastases were observed with any of the clones other than MCM1-d. Cell line MCM1-DLNxt results from transplantation of clone MCM1-d, shows high transplant efficacy and complete staining with LYVE antibody. All animals had metastases in the regional lymph nodes and in the lungs [15].

As different galectin members are assumed to be involved in cell growth, adhesion and metastasis, the aim of the present study was to investigate the expression and biological activity of a set of human galectins in two different cell lines, the original cell pool MCM1 with tumorigenic potential but less metastatic spreading, and the malignant DLNxt cells. As such, we aimed to determine

(1) the gene expression pattern of different galectins using RT-qPCR,

(2) the galectin expression levels and galectin binding-sites by FACS Analysis and by fluorescence microscopy, respectively, and

(3) the effect of galectins on cell viability and cell adhesion.

The results of this work might contribute to clarify whether glycobiological aspects will affect or correlate with the metastatic cell behavior of the MCM-1 cell model.

2 Materials and Methods

2.1 Materials

All, the galectin antibodies used for the galectin expression and biotynilated galectins used to test the galectin-binding sites and the corresponding galectins used to determine the cell adhesion properties and the mRNA expression level of TNF-alpha such as MMP3, were kindly provided to us by Prof. Hans-Joachim Gabius, Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Germany. All other chemicals were of analytical grade and supplied by our laboratory, unless otherwise specified.

2.2 Cell-culture

Melanoma cell lines DLNxt and MCM1 were routinely grown as monolayer cultures in DMEM (**D**ulbecco's **M**odified **E**agle's **M**edium), supplemented with 10 % FCS (heat-inactivated **F**etal **C**alf **S**erum) and Penicillin/Streptomycin (10 ml/l), in a humidified atmosphere of 5 % CO_2 at 37 °C.

2.3 Splitting

The medium was aspirated and the cells were washed once with PBS (**P**hosphate-**B**uffer **S**aline). 0.25 % Trypsin/EDTA (**E**thylene**d**iamine**t**etra**a**cetic acid) was added onto the cell layer and incubated at 37 °C for about 5 minutes. The progress of detachment was monitored using the microscope. Cells were resuspended in complete medium and were transferred into a new culture flask, containing 10 ml fresh medium.

2.4 RNA Isolation and cDNA Synthesis

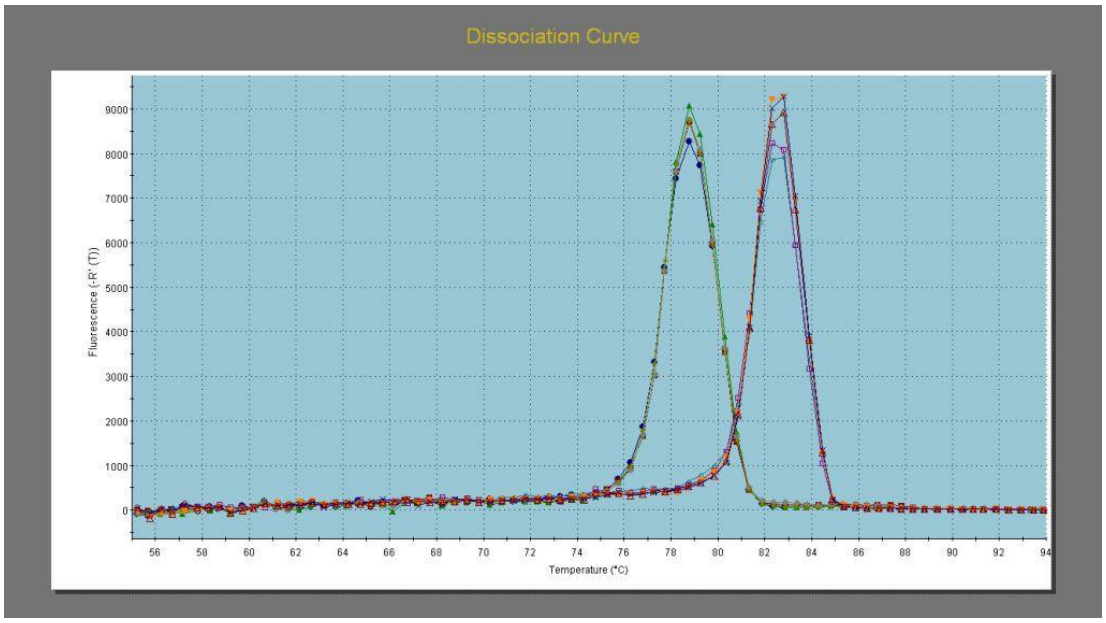
DLNxt cells were seeded at 6×10^4 cells/well into a 12 well plate and cultivated as described above. At 90 % confluency, cells were serum-starved by changing the

culture medium to 2 % ITS medium (**I**nsulin-**T**ransferrin-**S**elenium). After 6 hours incubation, fresh medium mixed with 10 µg/ml Galectin-1 was added to each well. Total RNA isolation and on-column DNase digestion (for DNA degradation) were performed using the NucleoSpin RNA II Kit following the manufacture supplied protocols. The quantity and integrity of isolated RNA samples were determined using the Agilent 2100 Bioanalyzer with Nano RNA chips (Agilent). For Real-time PCR experiments only those samples that gave RNA Integrity Numbers of 9.1–10 and showed no DNA contamination in their histogram were used. Cellular RNA was processed into cDNA using the High Capacity cDNA Reverse Transcription Kit. All cDNA preparations were diluted at ratio 1:5 with RNase-free water prior to Real-Time PCR.

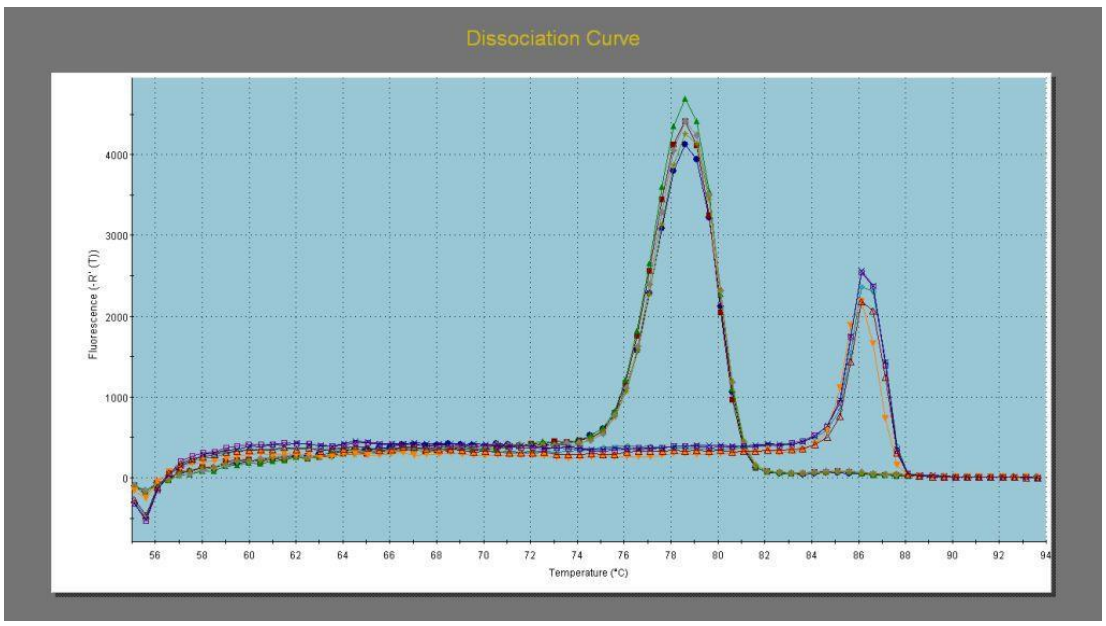
2.4.1 Real-time PCR

All RT-qPCR reactions were performed in 25 µl reaction mixtures containing 1 µl cDNA (**c**omplementary **D**eoxyribo**n**ucleic **a**cid), 12.5 µl SYBR Green Master Mix, primer pairs and nuclease-free water to 25 µl. Thermocycling conditions consisted of an initial polymerase activation step at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, at 55 °C for 1 min, and at 72 °C for 1 min. Afterwards, melting curves were generated to confirm a single gene-specific peak and to detect primer-dimer formation by heating the samples stepwise from 55 °C to 95 °C while continuously monitoring the fluorescence. NTC were included in each run to control for contaminations. The crossing point of the amplification curve with the threshold represented the cycle threshold (Ct). The reactions were run in duplicate and no-template as controls were included. Used target genes were MMP3 and TNF-alpha and as reference gene was used SDHA (**S**uccinate **de**h**y**drogenase complex, subunit

A). The results were analyzed using the MxPro real-time QPCR software (Stratagene).



A. MMP3



B. TNF alpha

Figure 4: Melting curves were generated to confirm a single gene-specific peak and to detect primer-dimer formation by heating the samples stepwise from 55 °C to 95 °C while continuously monitoring the fluorescence. MMP3 (figure 4 A. Second Peak) and TNF alpha (figure 4 B. Second Peak) with respect to SDHA (figure 4 A and 1 B. First Peak) used as reference gene.

2.5 MTT-Assay

The assay is based on the reducing of the yellow tetrazolium salt MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) to purple formazan crystals in mitochondria of metabolic active cells by dehydrogenase enzymes. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. Cells were seeded in 96 well tissue culture plates at a density of 3000 cells per well following incubation for 24 hours in a humidified atmosphere at 37 °C. 100 µl 2 % ITS medium containing 10 µg/ml, 5 µg/ml, and 0.5 µg/ml of Galectin-1, -1DTT, -3, -3T, -8, -9 were added to each well. The plate was then incubated at 37 °C for 48 hours. After incubation, the medium was removed and 20 µl MTT solution in 200 µl fresh ITS medium were added into each sample and melanoma DLNxt and MCM1 cells were incubated for 4 hours at 37 °C. After incubation, the plate was gently mixed by tipping at all four sides. The absorbance was measured by using a microplate-reader at 450 nm and 620 nm served as reference wavelength. The reference wavelength is used to correct for nonspecific background signals introduced by fingerprints or other factors.

2.6 FACS-Flow Cytometric Analysis

2.6.1 Galectin Expression in Melanoma Cells

For flow cytometry, 4×10^5 melanoma DLNxt and MCM1cell line were first washed with 500 µl DBPS (**D**ulbeco's **P**hosphate-**b**uffered **S**aline) and fixed by 4 % formalin. Membrane permeabilization was performed using 0.5 % Saponin. After 10 minutes and after centrifugation, the cells were incubated with 100 µg/ml and 50 µg/ml of Galectin-3 or Galectin-8-Antibodies for 30 min at 37 °C. Cells were

washed with FACS Buffer (DPBS with 1 % BSA, 0.01 % NaN3) and stained with anti-rabbit IgG-FITC (1:100, Sigma) for 60 min at room temperature.

2.6.2 Galectin Binding in Melanoma Cells

4×10^5 melanoma DLNxt and MCM1cell line were centrifuged for 3 minutes at 3000 rpm and the cell pellets were washed with 500 µl DPBS with 0.1 % BSA. Afterwards, cells were incubated with 50 µg/ml biotinylated Galectin-3 or Galectin-8 on ice for 30 minutes. The cells were again washed with washing buffer. Streptavidin-R-Phycoerythrin (1:40, Sigma) was added and the cells were incubated for 30 minutes on ice in the dark. Finally, the cells were fixed with 4 % formalin.

For checking purposes were used cells that were incubated with Streptavidin-R-**P**hyco**e**rythrin (PE) only or Anti-Rabbit-IgG-FITC as second antibodies.

Besides the protocol described above we used for fixation 2 % formalin. In addition to the use of Saponin, membrane permeabilisation was performed also with Triton X in concentrations of 0.1 %, 0.5 %, and 1 % and with methanol for 1 hour at –4 °C. We changed the incubation period for the first antibody to 1 hour. Incubated was also at 37 °C, on ice or at room temperature. The cells were also incubated with the second antibody at room temperature.

2.7 Fluorescence Microscopy

2.7.1 Galectin Expression in Melanoma Cell Lines DLNxt and MCM1

To test Galectin-1, -3, -8, and -9 expression in melanoma cells DLNxt and MCM1, 5×10^5 cells were cultured on glass cover slips for 2 days. At 90 % confluency, cells were washed three times with cold PBS and fixed with methanol at –20 °C for 20 min. After fixation they were washed again with PBS, and rehydration was performed using 1 ml 0.1 % BSA (**B**ovine-**s**erum **A**lbumin) per well for 60 min at

room temperature. Cells were then incubated with Galectin-1, -3, -8, and -9 antibodies (20 µg/ml) for 2 hours at 37 °C. Following additional washes in 0.1 % BSA cells were incubated for 1 hour at 37 °C with FITC (**f**luorescein **i**so**t**hio**c**yanate) and DAPI (4'-6-**d**i**a**midino-2-**p**heny**l**indole). After three washing steps with 0.1 % BSA, the cover slips were mounted on microscope slides using a drop of FluorSave (Fluorsave™ Reagent, 345789, Calbiochem®, Merck Biosciences) and the staining was observed under a fluorescence microscope.

2.7.2 Galectin Binding in Melanoma Cell Lines DLNxt and MCM1

To determine the Galectin-1, -3, -3T, -3P, -8, -8N, -9, and -9N binding sites, 5×10^5 cells were seeded onto glass cover slips and grown for 2 days. At 90 % confluence, cells were washed three times with cold PBS and fixed with methanol at –20 °C for 20 min. After fixation they were washed again with PBS, and rehydration was performed using 1 ml 0.1 % BSA per well for 60 min at room temperature. Cells were then incubated with labeled galectins (40 µg/ml) for 2 hours at 37 °C. Following additional washes in 0.1 % BSA cells were incubated for 1 hour at 37 °C with PE and DAPI. After three washing steps with 0.1 % BSA, the cover slips were mounted on microscope slides using a drop of FluorSave (Fluorsave™ Reagent, 345789, Calbiochem®, Merck Biosciences) and the staining was observed under a fluorescence microscope.

2.8 Cell Adhesion Assay

Adhesion assays not only analyze cell-cell interactions or cell-matrix interactions, but also provide information about other cellular functionalities such as receptor-ligand binding or changes in the intracellular signaling pathways [46]. 3×10^4 cells were suspended in 400 µl of complete medium and supplemented with Galectin-1 at

concentrations of 5 µg/ml, 20 µg/ml, 100 µg/ml, and Galectin-8 at concentrations of 200 µg/ml, 250 µg/ml, 300 µg/ml, and 350 µg/ml. Following incubation for 30 min at room temperature, the cell suspension was divided into 4 wells of a 96-well plate prior to incubation at 37 °C for 2 hours. Afterwards, unattached cells were removed by washing gently with 100 µl of PBS and attached cells were fixed by the addition of 100 µl of 2 % formalin for 20 min at room temperature. Cells that were not washed but directly fixed with 2 % formalin, served as 100 % value. Then, cells were washed three times with PBS and stained with 100 µl 0.1 % crystal violet in ethanol for 60 min at room temperature. Finally, the cells were washed three times with 100 µl PBS prior to solubilization of the dye with 100 µl acetic acid and incubation in an orbital shaker for 5 min at room temperature (150 rpm). Blanks without cells were included to test the background binding of crystal violet to the culture plate. The absorbance was measured at 570 nm using a microplate reader (TECAN, Austria). In addition, control samples lacking the addition of galectins were included.

3 Results

3.1 The Gene Expression Pattern of Galectin-1, -3, -7, -8, and -9 in Melanoma Cell Lines

The expression levels of Galectin-1, -3, -8, -9 in MCM1, MSM1D, MCM1G, and DLNxt, were quantified with respect to reference gene SDHA (Succinate dehydrogenase complex, subunit A) using Quantitative Real Time-PCR (RT-qPCR). Table 2 shows that Galectin-1 is highly expressed in MCM1D and MCM1G followed by DLNxt and MCM1. In comparison to Galectin-1, Galectin-3 is significantly less expressed in MCM1D, MCM1G and MCM1 and DLNxt cells. Galectin-8 and Galectin-9 are expressed only in a small extent, whereas Galectin-7 mRNA levels were hardly detectable using RT-qPCR.

	MCM1	MCM1D	MCM1G	DLNxt
LGALS1	7.27±0.82	11.06±1.53	10.57±1.03	8.26±1.20
LGALS3	1.08±0.12	3.34±0.55	1.25±0.20	0.61±0.10
LGALS7	0.00006±0.000	0.00006±0.000	0.00008±0.000	0.00014±0.00
LGALS8	0.16±0.02	0.19±0.02	0.20±0.03	0.19±0.03
LGALS9	0.01±0.00	0.018±0.003	0.016±0.005	0.003±0.001

Table 2: mRNA levels of Galectin-1, -3, -7, -8 and -9 in MCM1, MCM1D, MCM1G, and DLNxt cell lines. The mRNA expression was analyzed using RT-qPCR and quantified with respect to SDHA as reference gene (mean±SD)

3.2 FACS-Flow cytometric analysis

In order to determine the galectin expression in DLNxt cells, cells were incubated with Antigal-1, -3, and -8 and stained with Anti-Rabbit-IgG-FITC. In order to evaluate the presence of binding sites of galectins, cells were incubated with biotinylated Galectin-1 and -8 and stained with Streptavidin-R-Phycoerythrin. The data were analyzed using flow cytometry. Even though we evaluated all the alternatives as described in the methods section, we did not come to any results. For this reason we have changed to fluorescence microscope.

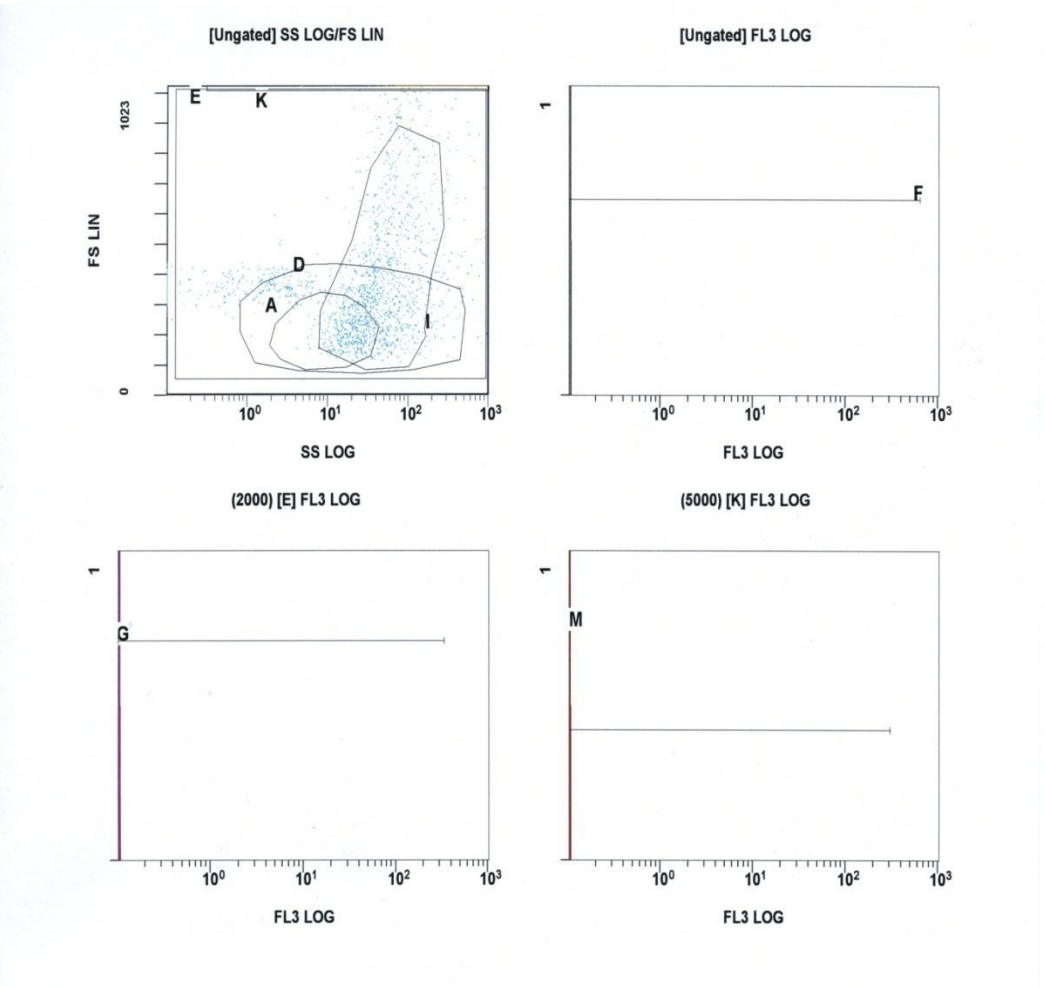


Figure 5: As negative controls, the cells were treated in parallel, under identical conditions as described in the methods section, except that the primary antibody or both the primary and the secondary antibodies were excluded from the diluting solutions.

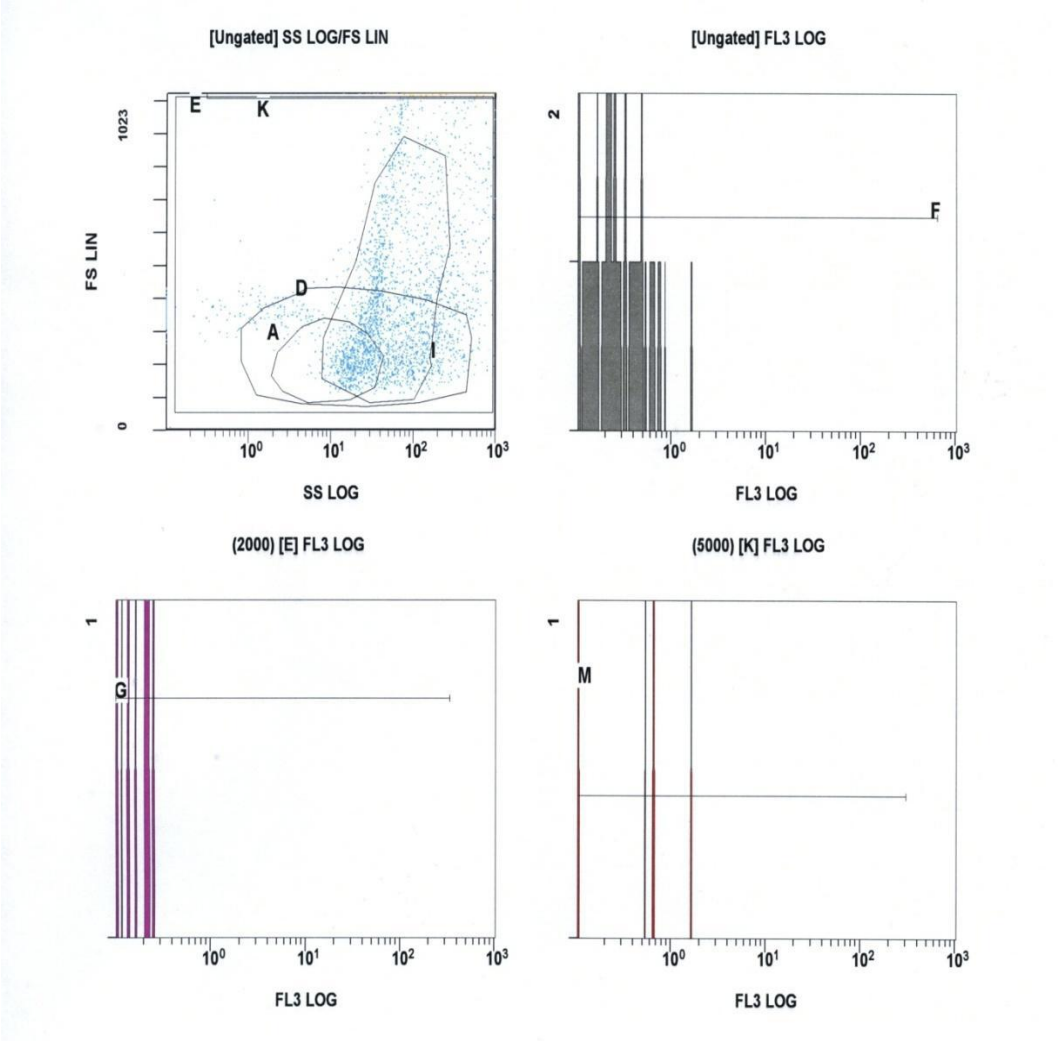


Figure 6: Analysis of galectin expression in DLNxt using Antigal-1, Antigal-3, and Antigal-8 at concentration of 50 µg/ml and 100 µg/ml. Fluorescence intensity was determined in the absence of the marker, which served as control (scan 1) and in the presence of Antigal-1, -3, and -8 (scans 2, 3, 4).

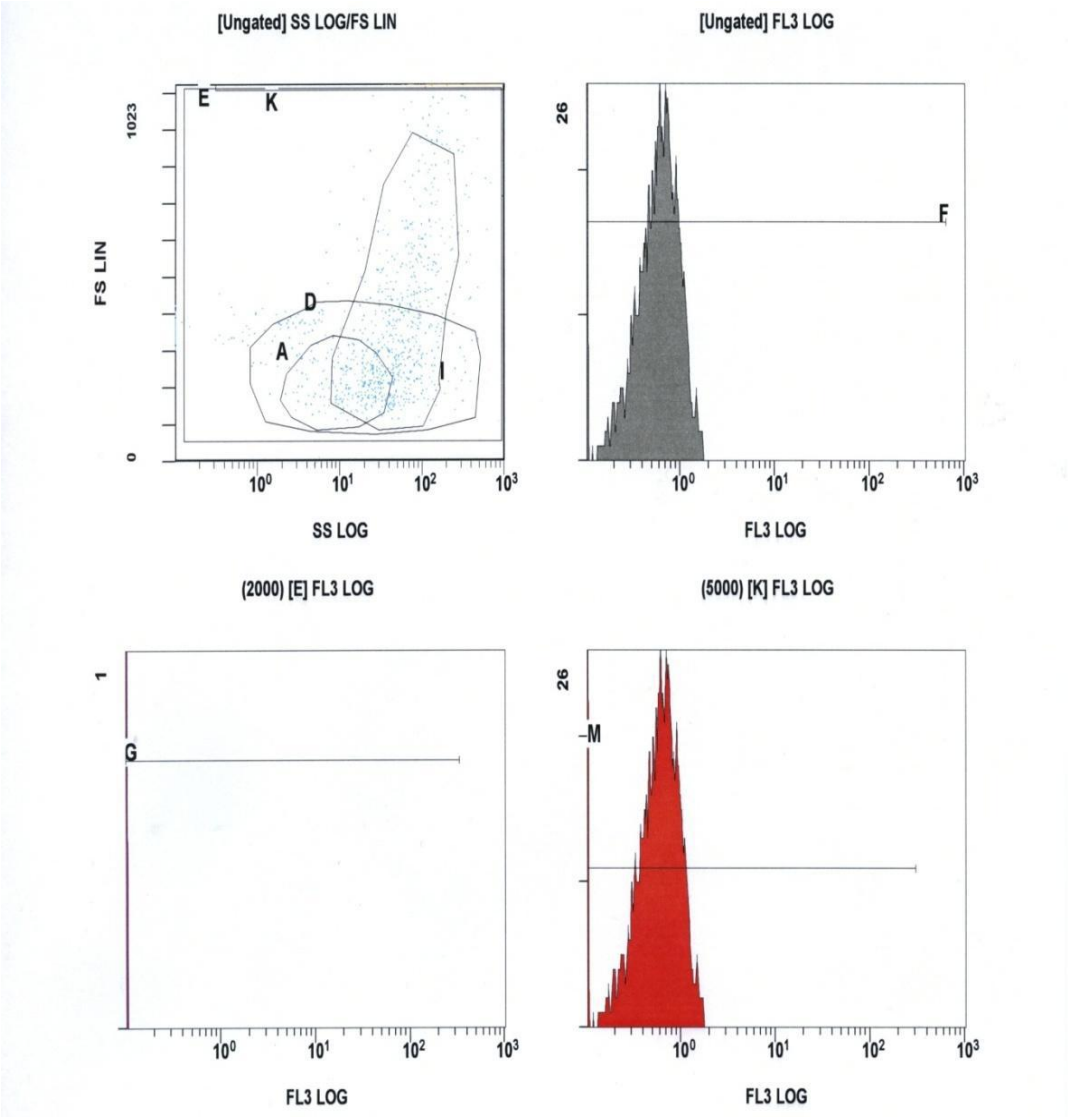


Figure 7: Analysis of galectin-binding on the cell surface of DLNxt using biotynilated Galectin-1 and -8 at concentration of 50 µg/ml. Fluorescence intensity was determined in the absence of the marker, which served as control (scan 1) and in the presence of biotynilated Galectin-1 and -8 (scans 2, 3, 4).

3.3 Fluorescence Microskopy

3.3.1 Galectin Expression in Melanoma cell Line MCM1

To confirm the expression of Galectin-1, -3, -8, and -9 by the MCM1 cell line the cells were stained with the corresponding galectin antibodies and observed under a fluorescence microscope (figure 8 A–B). From a comparison between panel B and panel A, it is obvious that Galectin-3 is markedly expressed than Galectin-1 followed by Galectin-8 expression (panel C). Galectin-1 is predominantly expressed in the cytoplasm, whereas Galectin-8 is accumulated in vesicular structures around the nuclei. Galectin-9 appears to be expressed to a very low extent (D).

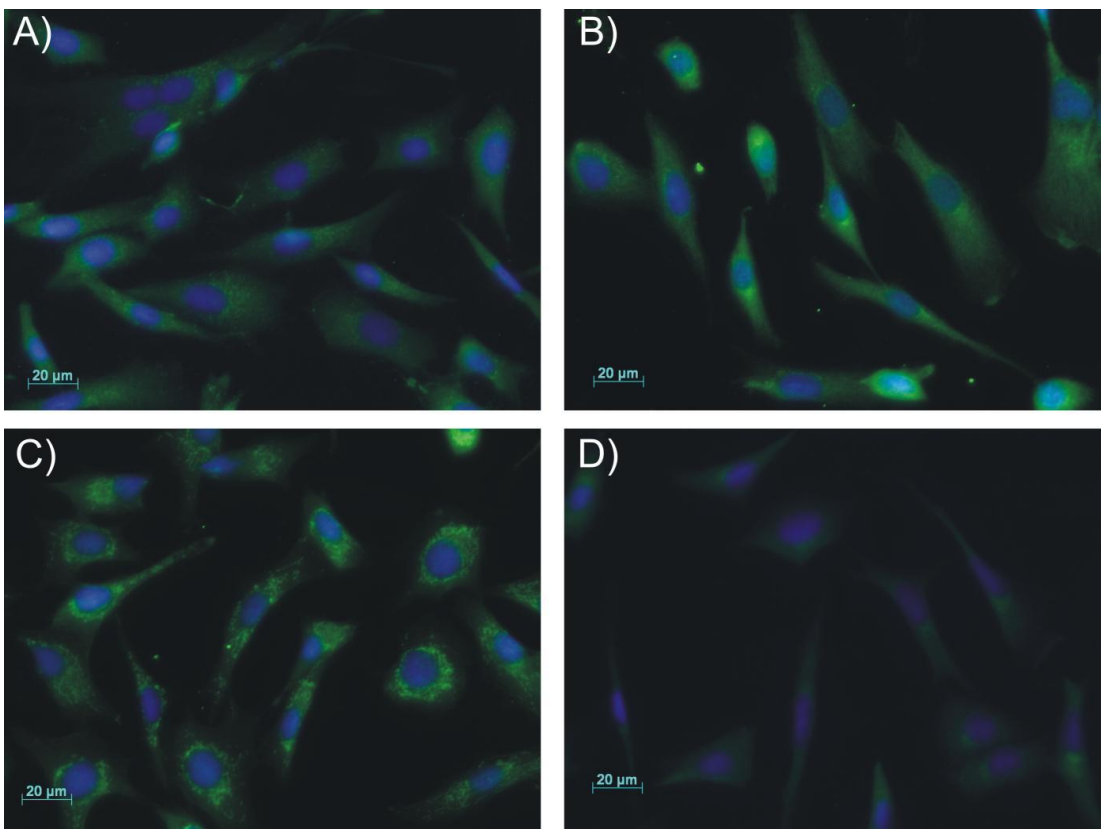


Figure 8: Analysis of galectin expression in MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with Antigal-1 (A), Antigal-3 (B), Antigal-8 (C), and Antigal-9 (D) and with FITC as second antibody. DAPI was used to stain the cell nuclei.

3.3.2 Galectin Binding to Melanoma Cell Line MCM1

In order to determine the binding intensities of Galectin-1, -3, -3T, -3P, -8, -8N, -9, and -9N in MCM1cells, permeabilized cells were stained with biotinylated galectins and monitored using fluorescence microscopy (figure 9–16). The findings show that all used biotinylated galectins specifically stained the MCM1 cells. The staining was in all of the cases predominantly cytoplasmic. Panel A of figures 9–16 shows the cell nuclei stained with DAPI, while panel B presents the fluorescence intensities corresponding to galectin binding. Panel C shows the DIC picture, and panel D the merged image. From a comparison between figure 9 with figure 10, it is obvious that both Galectin-1 and Galectin-3 weakly bind to MCM1 cells. The binding intensities of Galectin-3 and Galectin-3P (figure 10 and figure 12) are almost similar, whereas the binding of Galectin-3T (figure11) appears to be stronger. It is interestingly to see that the N-domain of Galectin-8N (figure 14), which seems to be important for the binding affinity, stained with lower intensity the MCM1 cells than the whole sequence of Galectin-8 (figure 13). In contrast, the N-domain of Galectin-9N stained intensely than Galectin-9 (figure 16 and figure 15). Of note, Galectin-9N shows a dot-like background staining which might be due to media components attached onto the cover slip during fixation.

3.3.2.1 Labeled Galectin-1

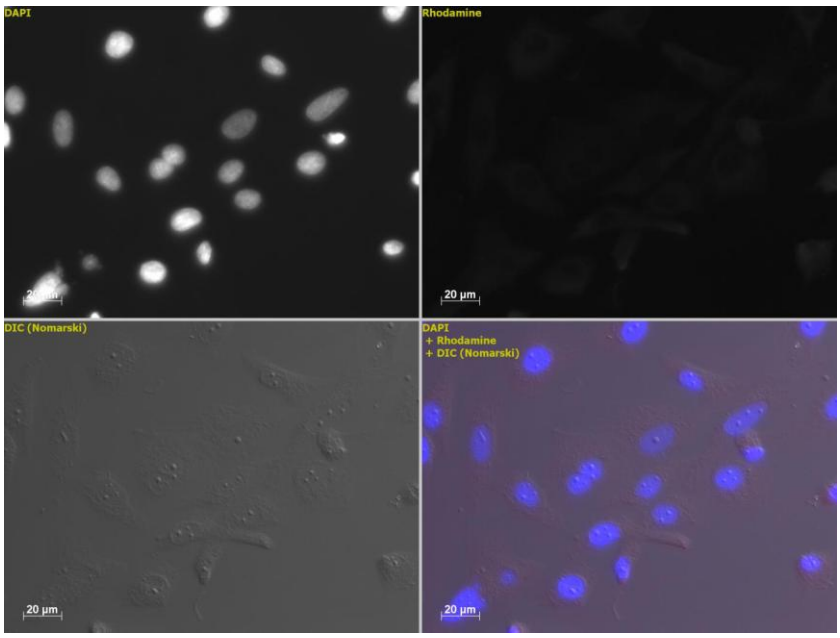


Figure 9: Analysis of Galectin-1 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-1 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-1. Panel C presents the DIC picture and panel D the merged image.

3.3.2.2 Labeled Galectin-3

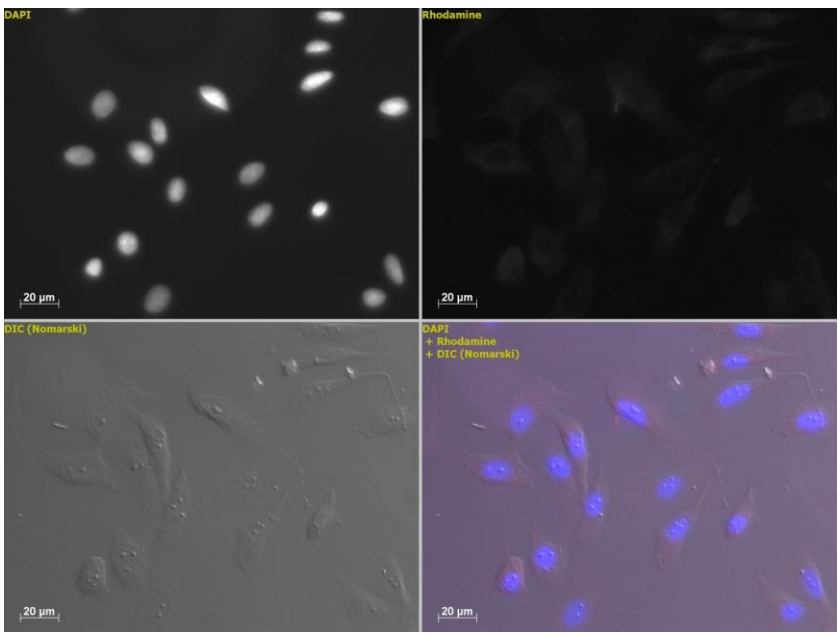


Figure 10: Analysis of Galectin-3 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-3 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-3. Panel C presents the DIC picture and panel D the merged image.

3.3.2.3 Labeled Galectin-3T

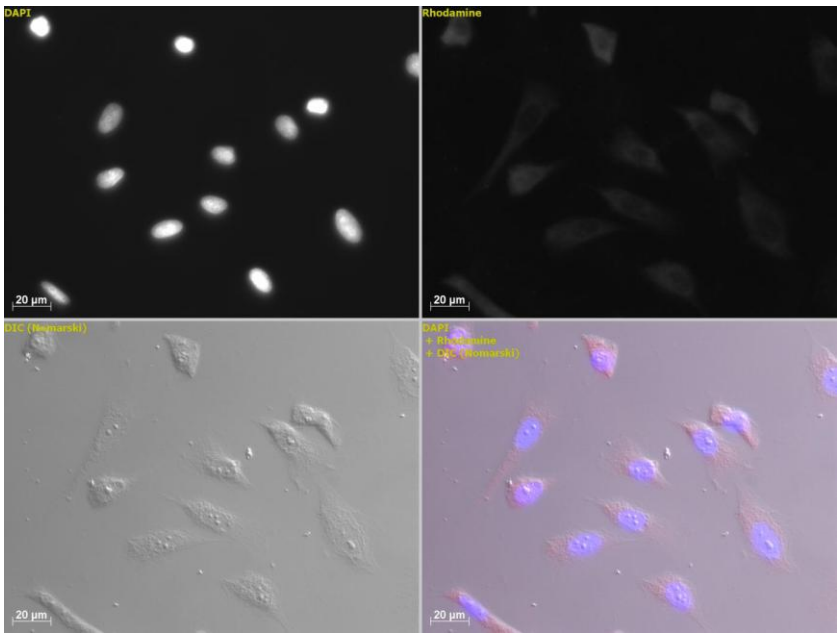


Figure 11: Analysis of Galectin-3T binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-3T and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-3T. Panel C presents the DIC picture and panel D the merged image

3.3.2.4 Labeled Galectin-3P

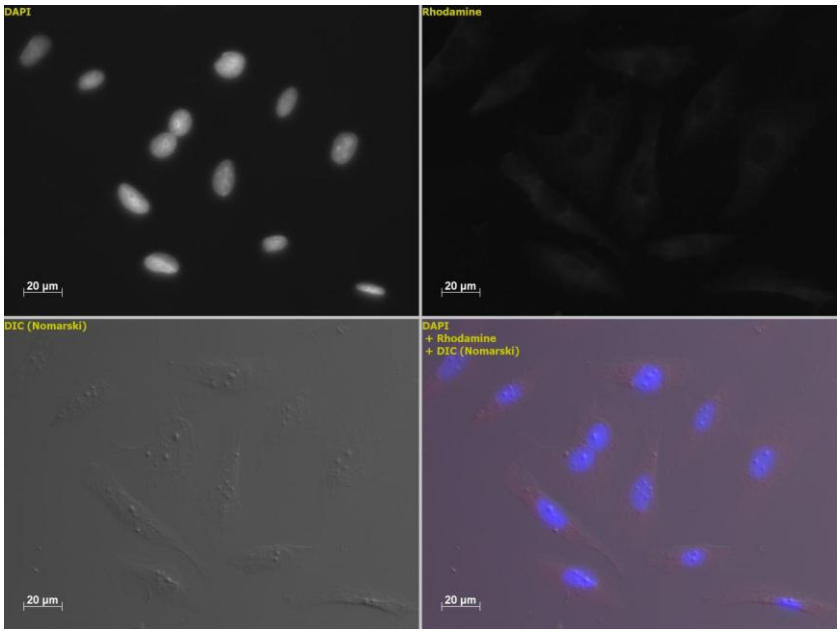


Figure 12: Analysis of Galectin-3P binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-3P and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-3P. Panel C presents the DIC picture and panel D the merged image.

3.3.2.5 Labeled Galectin-8

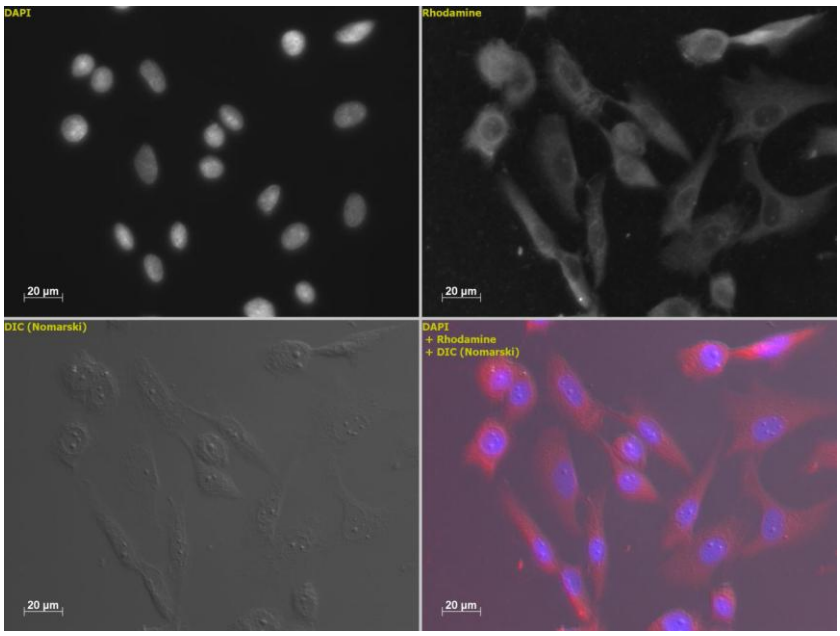


Figure 13: Analysis of Galectin-8 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-8 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-8. Panel C presents the DIC picture and panel D the merged image.

3.3.2.6 Labeled Galectin-8N

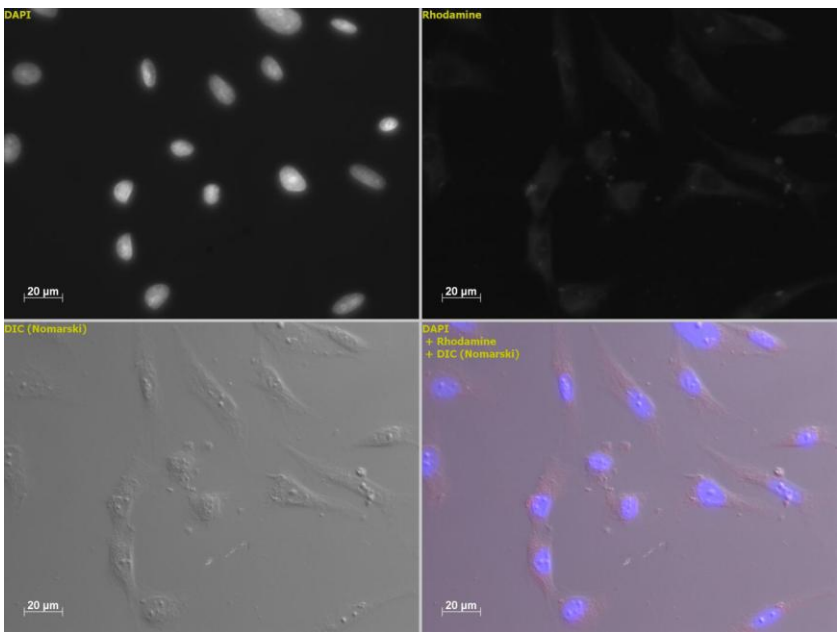


Figure 14: Analysis of Galectin-8N binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-8N and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-8N. Panel C presents the DIC picture and panel D the merged image.

3.3.2.7 Labeled Galectin-9

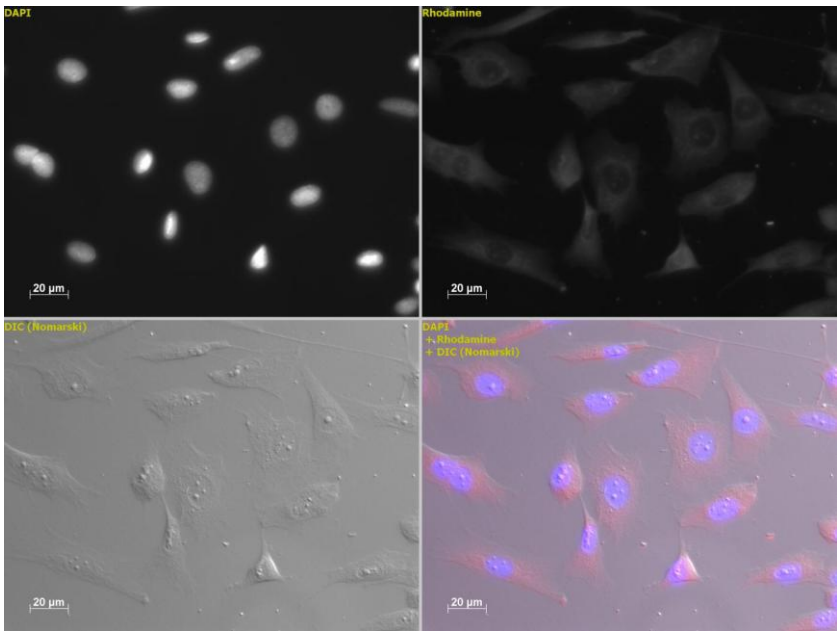


Figure 15: Analysis of Galectin-9 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-9 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-9. Panel C presents the DIC picture and panel D the merged image.

3.3.2.8 Labeled Galectin-9N

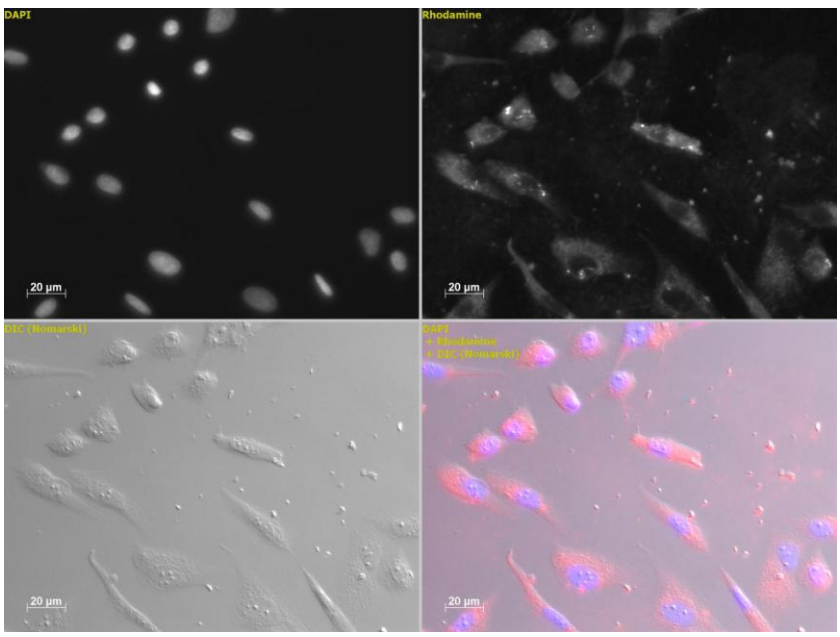


Figure 16: Analysis of Galectin-9N binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-9N and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-9N. Panel C presents the DIC picture and panel D the merged image.

3.3.3 Galectin Expression in Melanoma Cell Line DLNxt

To determine the expression of Galectin-1, -3, -8, and -9 by the melanoma cell line DLNxt, the cells were stained with galectin antibodies and observed under a fluorescence microscope (figure 17 A–D). All galectin antibodies that have been used stained specifically the DLNxt cells but with different intensity, as shown on figure 20 A–D. Panel A shows that Galectin-1 is markedly expressed in comparison to Galectin-3 (panel B), followed by Galectin-8 expression (panel C). DLNxt cells appear to express Galectin-9 in minor extent (panel D). Galectin-1 is diffusely distributed in the cytoplasm, Galectin-3 and -9 exhibited a similar but less distribution pattern in the cytoplasm (panel A, B, C), while Galectin-8 shows a clear vesicular accumulation around the nuclei.

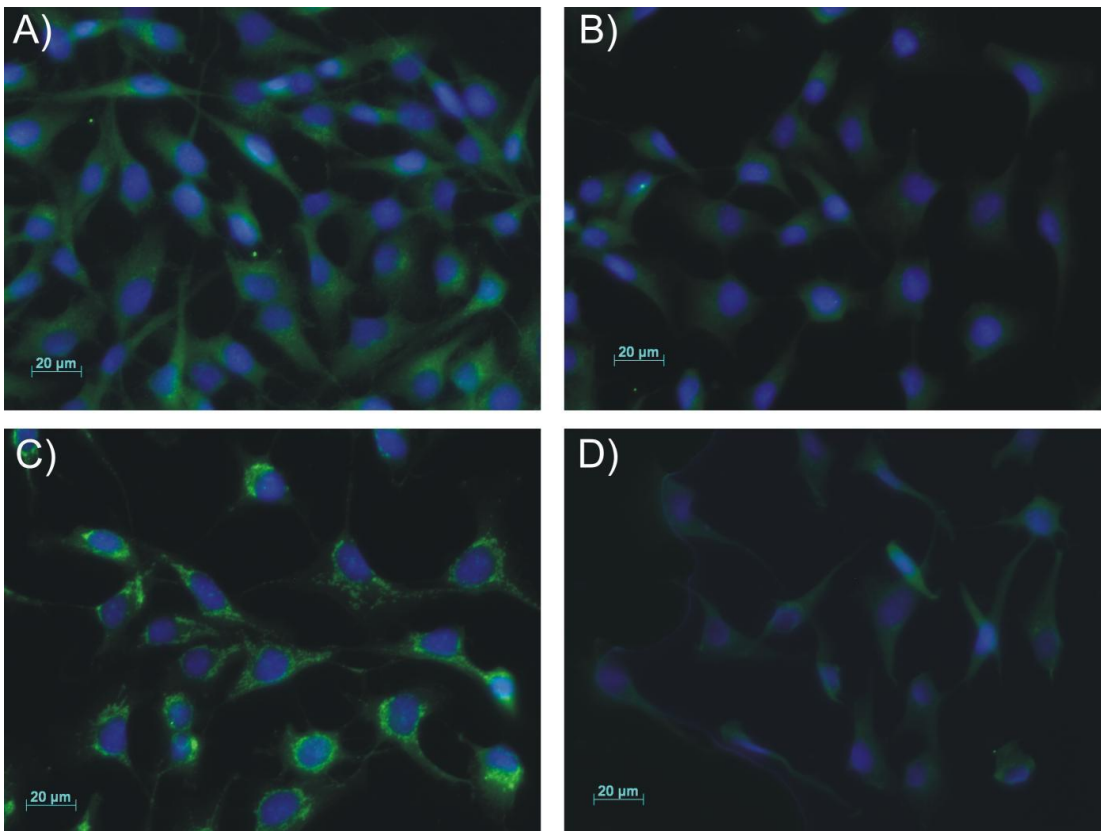


Figure 17: Analysis of Galectin expression on DLNxt cells by fluorescence microscopy. Fixed and permeabilized cells were stained with Anti-gal1 (A), Anti-gal3 (B), Anti-gal8 (C), Anti-gal9 (D), and with FITC as second antibody. DAPI was used to stain the cell nuclei.

3.3.4 Galectin Binding to Melanoma Cell Line DLNxt

In order to evaluate the presence of binding sites of galectin-1, -3, -3T, -3P, -8, -8N, -9, and -9N in DLNxt cells, the cells were stained with biotinylated galectins and observed under a fluorescence microscope (figures 18–26). Figure 18 shows that staining only with secondary antibody resulted in minor extent of unspecific binding. In contrast, figures 18–26 demonstrate that all used galectins specifically stained DLNxt cells. Panel A of figures 18–26 shows the cell nuclei stained with DAPI, whereas panel B presents the fluorescence intensities of galectin binding. Panel C shows the DIC picture, and panel D the merged image. From a comparison between figure 19 and 20, it is obvious that galectin-1 binds to a smaller extend to DLNxt cells than Galectin-3. The binding of Galectin-3 and -3T (figure 21) appears comparable whereas that of Galectin-3P (figure 22) appears less. Interestingly, Galectin-3, -3T, and -3P intensively stained the background in a dot-like manner, which might be due to media components attached onto the cover slip during fixation. Furthermore accumulates Galectin-3T around the nucleus, while Galectin-3 and -3P show occasionally cytoplasmic staining. Comparison between figure 23 and 24 makes clear that the whole sequence of Galectin-8 affects the binding, otherwise, the N-domain of Galectin-8, which is responsible for the binding on the cell surface, stained intensely in addition to a vesicular accumulation around the nuclei. The same is observed in case of Galectin-9 and -9N, where the N-domain shows an increased binding compared to the whole extent of Galectin-9 (figure 25 and 26).

3.3.4.1 Control

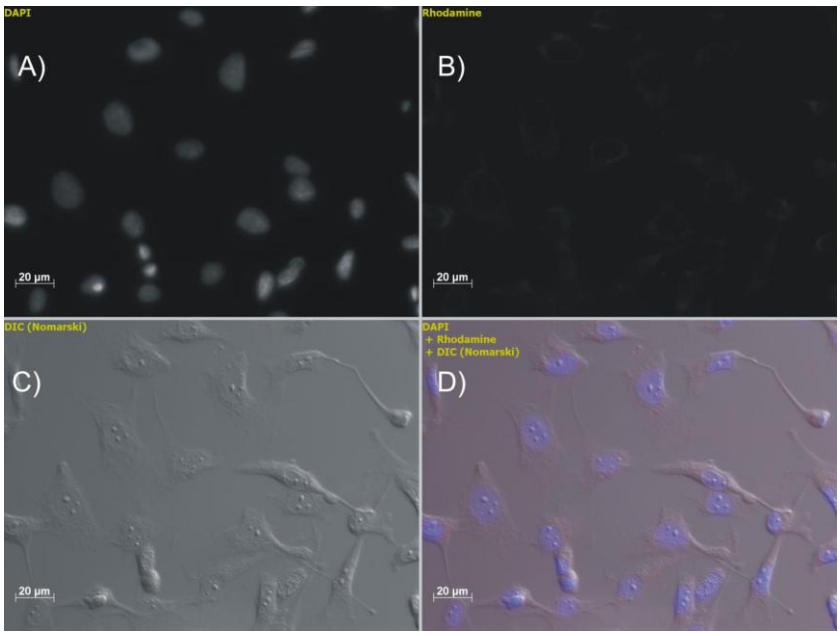


Figure 18: Untreated cells were used as control. The cells were stained only with FITC as second antibody.

3.3.4.2 Labeled Galectin-1

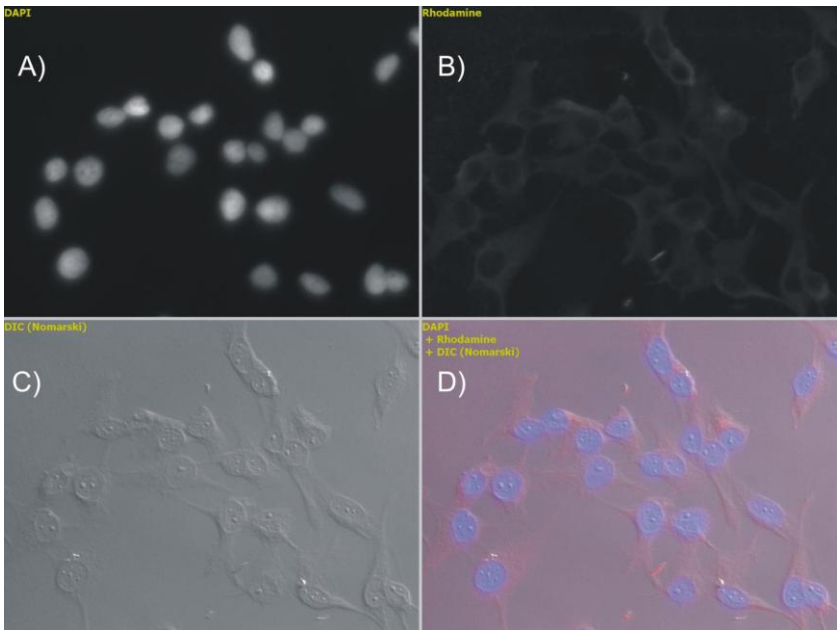


Figure 19: Analysis of Galectin-1 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-1 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-1. Panel C presents the DIC picture and panel D the merged image.

3.3.4.3 Labeled Galectin-3

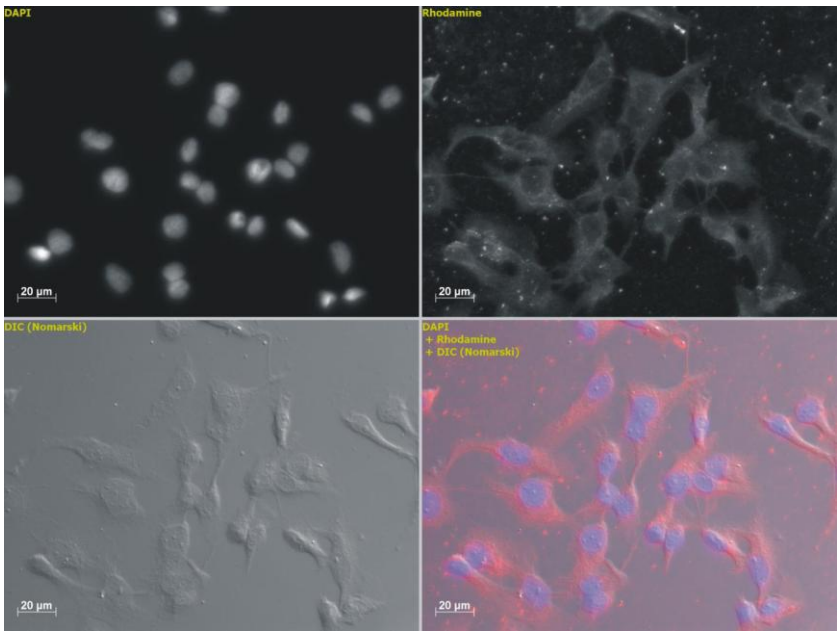


Figure 20: Analysis of Galectin-3 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-3 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-3. Panel C presents the DIC picture and panel D the merged image.

3.3.4.4 Labeled Galectin-3T

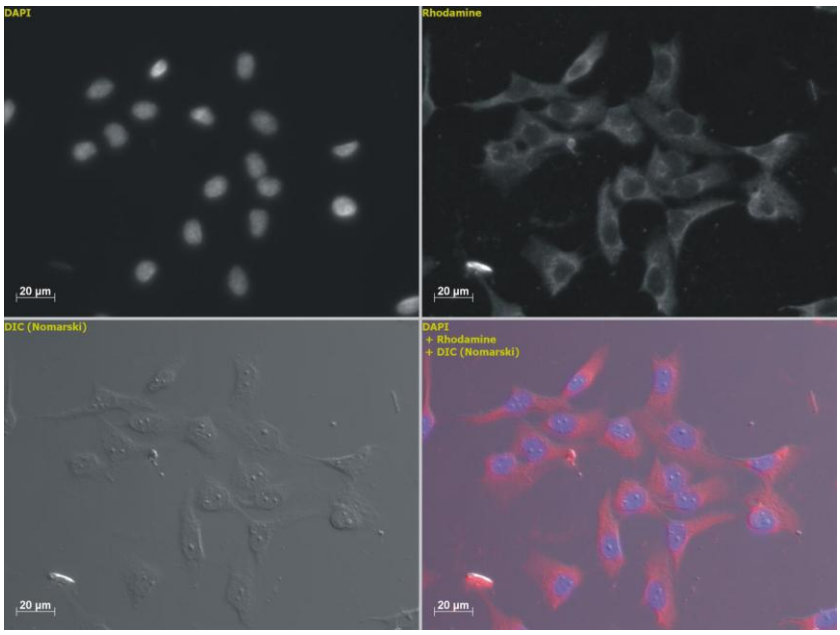


Figure 21: Analysis of Galectin-3T binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-3T and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-3T. Panel C presents the DIC picture and panel D the merged image.

3.3.4.5 Labeled Galectin-3P

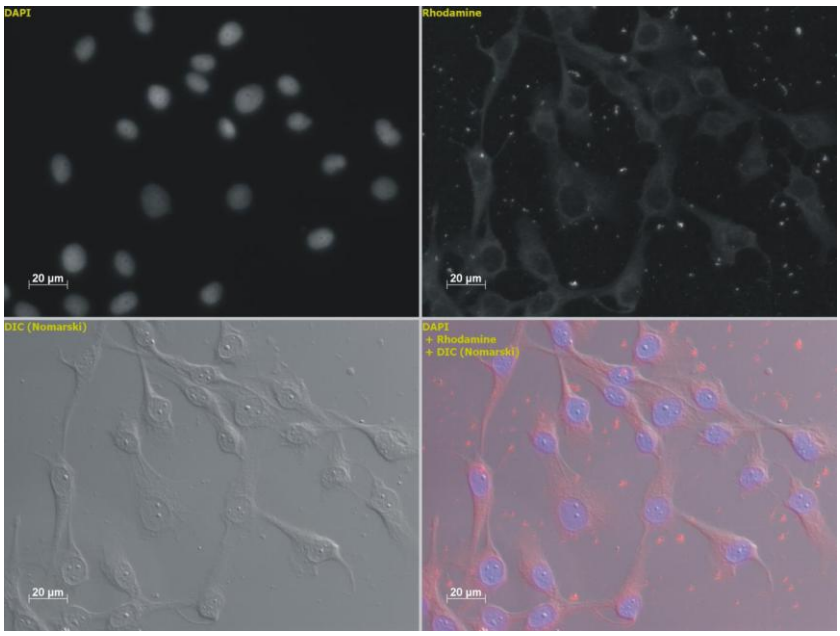


Figure 22: Analysis of Galectin-3P binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-3P and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-3P. Panel C presents the DIC picture and panel D the merged image.

3.3.4.6 Labeled Galectin-8

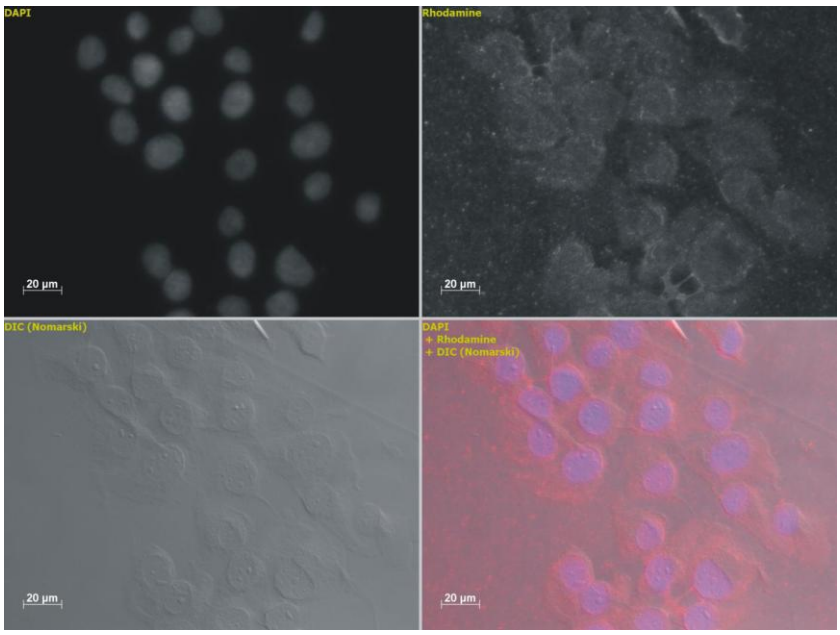


Figure 23: Analysis of Galectin-8 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-8 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-8. Panel C presents the DIC picture and panel D the merged image.

3.3.4.7 Labeled Galectin-8N

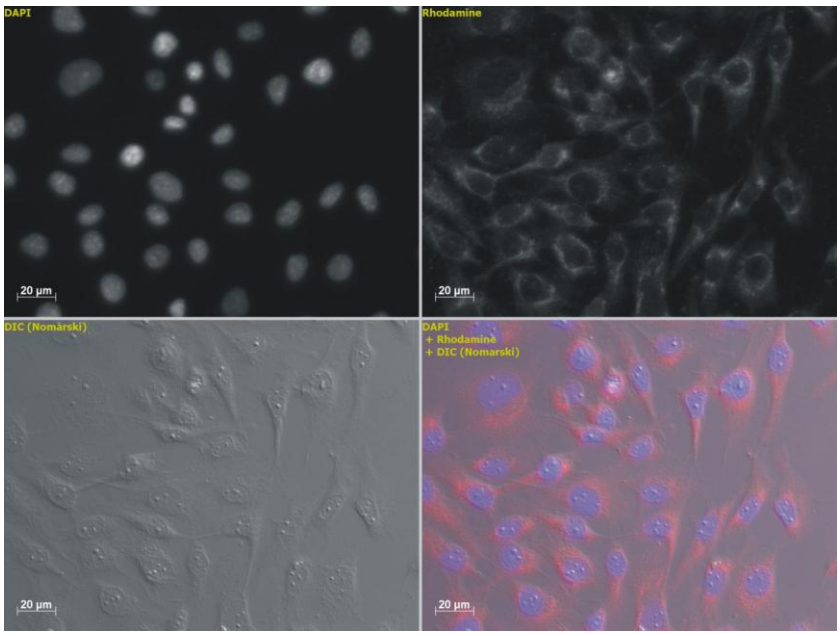


Figure 24: Analysis of Galectin-8N binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-8N and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-8N. Panel C presents the DIC picture and panel D the merged image.

3.3.4.8 Labeled Galectin-9

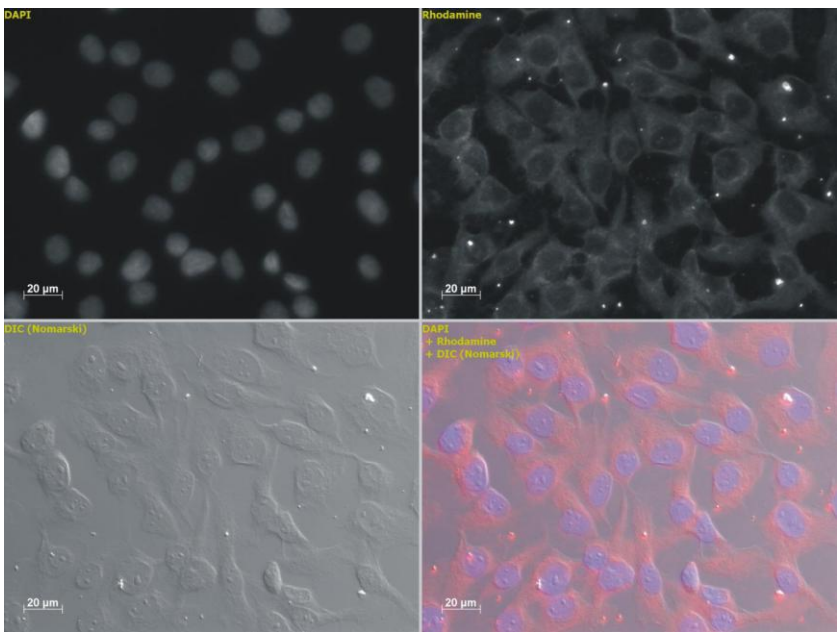


Figure 25: Analysis of Galectin-9 binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-9 and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-9. Panel C presents the DIC picture and panel D the merged image.

3.3.4.9 Labeled Galectin-9N

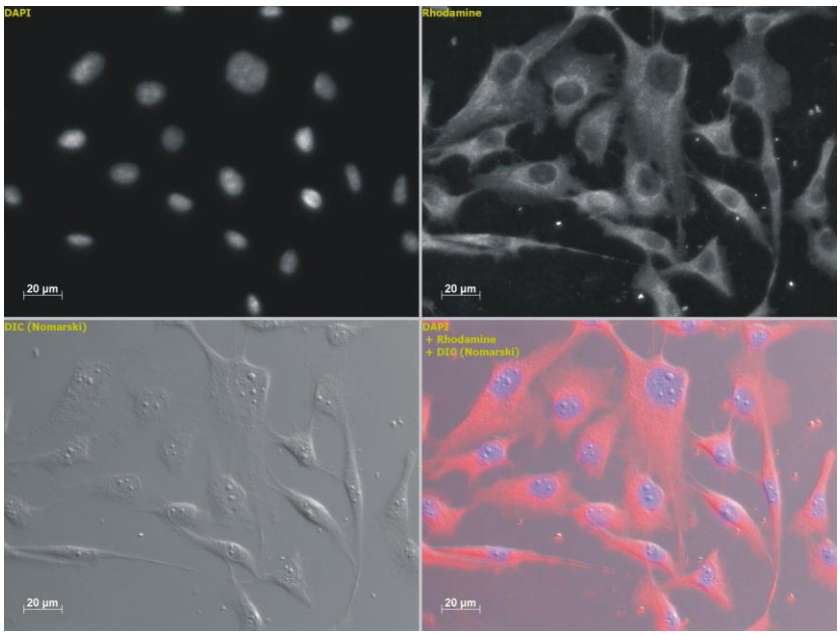
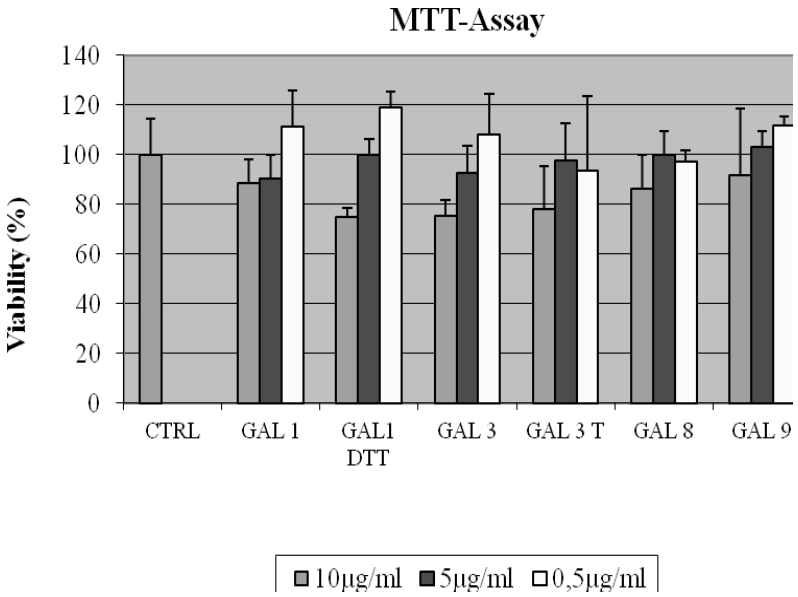


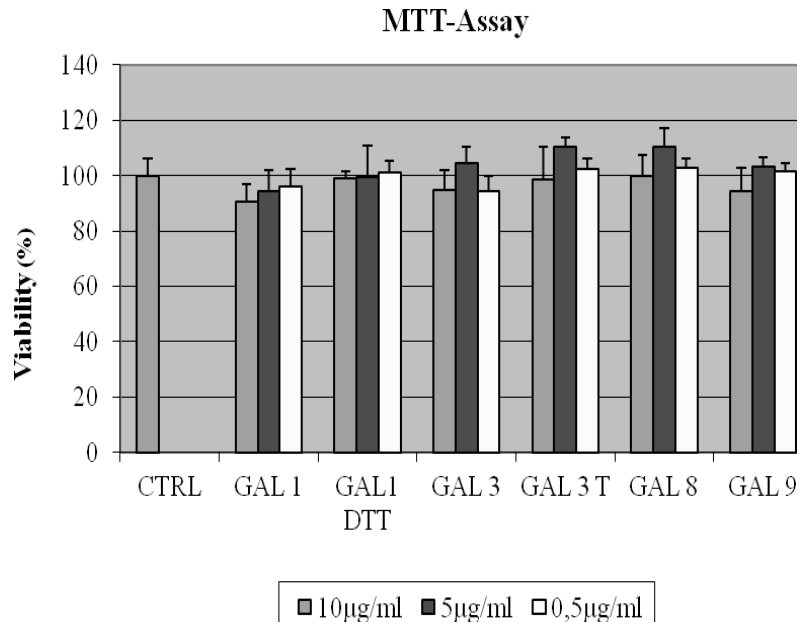
Figure 26: Analysis of Galectin-9N binding intensity on MCM1 cells by fluorescence microscopy. Fixed and permeabilized cells were stained with biotinylated Galectin-9N and with PE as second antibody. Panel A shows cell nuclei stained with DAPI, while panel B shows the fluorescence intensity of biotinylated Galectin-9N. Panel C presents the DIC picture and panel D the merged image.

3.4 Galectin Effect on DLNxt (A) and MCM1 (B) Cell Viability

In order to determine the cytotoxic effect of Galectin-1, -1DTT, -3, -3T, -8, -9 in DLNxt and MCM1 cell line, the MTT cell viability assay was used. Treatment of cells for 48 h with 10 µg/ml, 5 µg/ml, 0.5 µg/ml Galectin-1, -1DTT, -3, -3T, -8 and Galectin-9, did not show any significant cytotoxicity against the studied cell lines. These results (figure 1 A and 1 B) clearly demonstrate that survival of the cells was not affected by the presence of galectins.



A. DLNxt

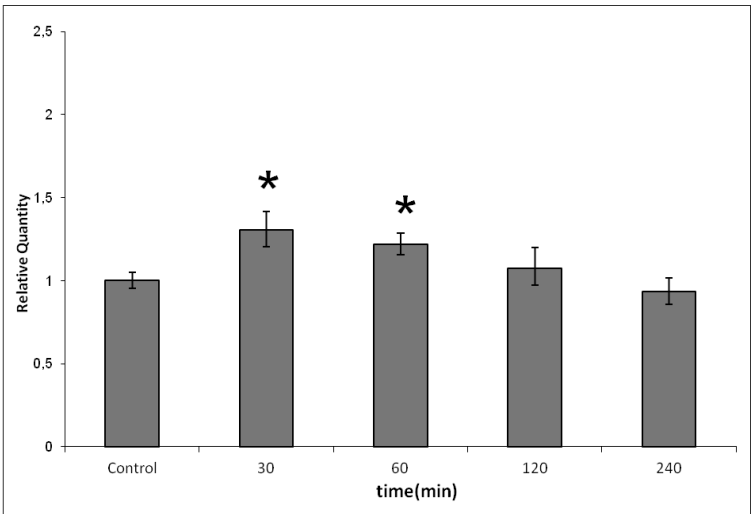


B. MCM1

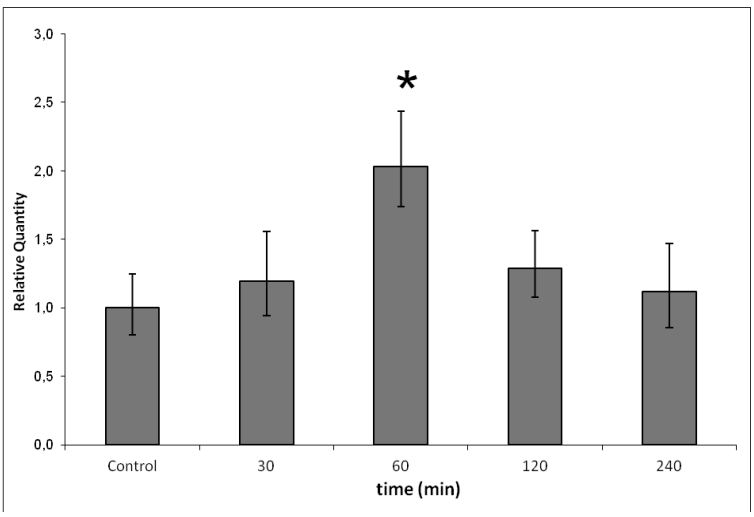
Figure 27: Galectin effect on DLNxt (A) and MCM1 (B) cell viability. The cells were seeded in 96-well plates at an initial density of 3×10^3 cells/well, and incubated with Galectin-1, -1DTT, -3, -3T, -8, and -9 for 48 h at 37 °C. Cell survival was assessed by MTT assay after 48 h. DLNxt and MCM1 cells incubated with medium alone served as controls. The values were normalized to untreated controls and represent the mean±SD.

3.5 Up-regulation of MMP3 and TNF alpha by Galectin-1

To evaluate whether Galectin-1 induces the mRNA expression level of MMP3 and TNF alpha, cells were treated with 10µg/ml Galectin-1 and analyzed by RT-q PCR. As shown in figure 2 A the expression level of MMP3 was only slightly affected by Galectin-1, showing a significant increase after 30 min and 60 min treatment. In contrast, TNF alpha (figure 2 B) was significantly up-regulated twofold by Galectin-1 after 60 min as compared to untreated cells.



A. MMP3



B. TNFalpha

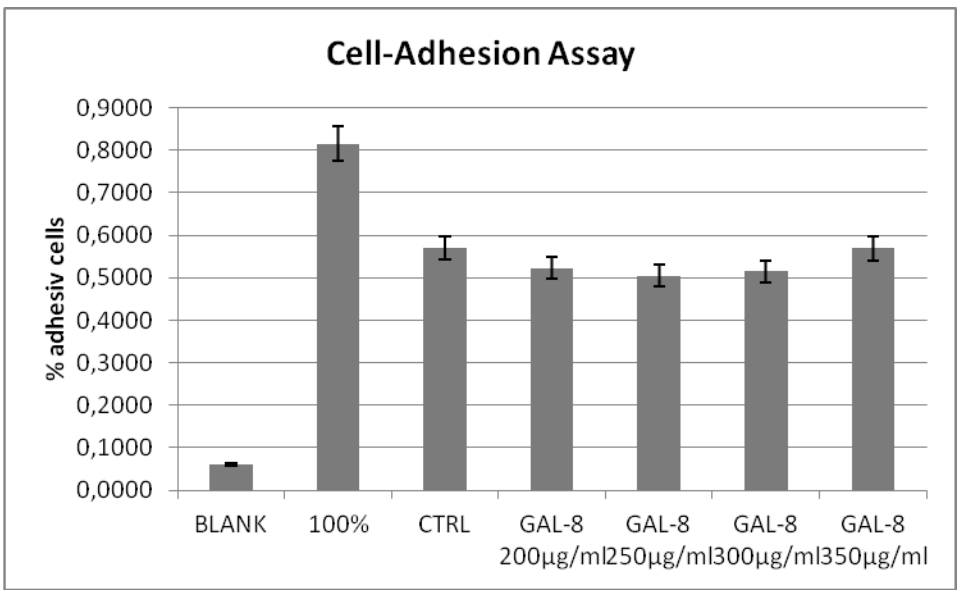
Figure 28: Time-dependent effects of Galectin-1 on MMP3 (figure 28 A) and TNF alpha (figure 28 B) mRNA expression in DLNxt cells as determined by RT-q PCR. Cells were incubated with Galectin-1 at a concentration of 10 µg/ml and RNA was isolated at different time points. Untreated cells served as control. Statistically significant differences are marked with asterisks ($p < 0.05$).

3.6 Cell Adhesion Assay

3.6.1 Effect of Galectin-1 and -8 on Adhesion

To investigate whether Galectin-1 or Galectin-8 have an influence on MCM1 and DLNxt cell adhesion, the cells were incubated with Galectin-1 and Galectin-8 for 2 hours and observed using a plate reader. When comparing the 100 % attachment value and the control, for both cell lines, it was observed a significant reduction in cell number detached through one wash step. As reported in figure 29 the adhesion of MCM1cells (figure 29 A) did not seem to be affected by Galectin-8, when compared to the untreated control cells. In contrast, the treatment with Galectin-8 significantly increased the attachment of DLNxt cells in a dose-dependent manner (figure 29 B) in relation to untreated control cells. The maximal effect was achieved at concentrations of 250 μg/ml and 300 μg/ml. Generally, the adhesion of DLNxt and MCM1 was not affected by the presence of Galectin-1 (data not shown).

A. MCM1



B. DLNxt

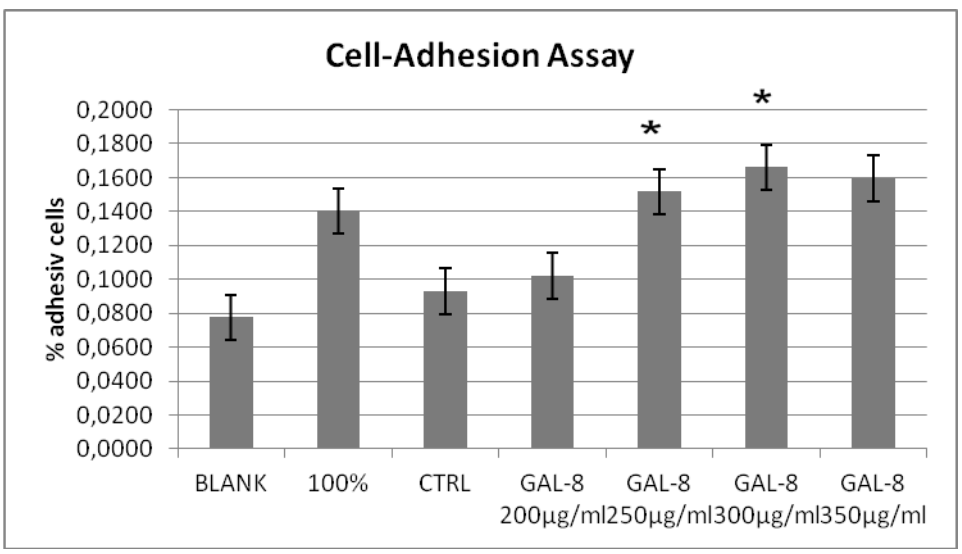


Figure 29: Dose-dependent Effect of Galectin-8 on DLNxt and MCM1 cell Adhesion. Cells were incubated with Galectin-8 at concentration of 200 µg/ml, 250 µg/ml, 300 µg/ml and 350 µg/ml for 2 h and observed using a plate reader. Untreated cells served as control. Statistically significant differences are marked with asterisks ($p < 0.05$).

4 Discussion

Malignant melanoma has developed as one of the fastest-growing and most aggressive malignancies over the past several decades. Malignant transformation of cells is the pathologic consequence of mutations in cellular genetic control mechanisms [4].

In this study we aimed to investigate the expression and biological activity of selected galectins in two functionally different melanoma cell lines. Members of galectins are supposed to mediate the cell adhesion, to regulate cell growth, cell proliferation, transformation, metastasis, and apoptosis. It has been demonstrated that the expression pattern of various galectins is altered in carcinomas [37, 38]. Studies have shown higher levels of Galectin-1 in malignant melanoma, which may contribute to tumorigenesis and metastasis [41]. Galectin-1 is a laminin binding protein that recognizes poly-N-acetyllactosamine chains. Castronovo et al. have investigated that Galectin-1 increases the melanoma cell attachment to laminin in dose-dependet manner and therefore could be a modulator of invasion and metastasis [44]. Bolander et al. in a study performed in patients with metastasizing malignant melanoma, have demonstrated a higher expression of Galectin-1 as compared to patients with normal tissues [45]. Galectin-3 is a B-galactoside binding protein that is expressed in a variety of neoplastic cell types. Overall, it is proposed that Galectin-3 may serve as a marker of progression in human melanoma. Vereecken and Heenen have already reported a high serum concentration of Galectin-3 in melanoma which might correlate with the melanoma progression. Furthermore they showed that Galectin-3 could be produced by the tumor cells themselves and save them from apoptosis [43]. Galectin-3 expression also correlated with the melanoma progression

from benign nevi to dysplastic nevi, followed by primary to metastatic melanoma [42].

In the present study we used the RT-qPCR to analyze the expression of Galectin-1, -3, -7, -8 and -9 in MCM1 and DLNxt cell lines. Among the cell lines as well as between the individual galectins, there were marked differences on the expression pattern. In general, the highest level of Galectin-3 expression was seen in MCM1 cell line, whereas the highest level of Galectin-1 expression was found in DLNxt cell line, followed by MCM1 cell line. The expression levels of galectin-7, -8, and -9 were hardly detectable using RT-qPCR. The differences in galectin expression as well as their presence on the cell surface, in the cytoplasm and around nuclei, respectively, have been further demonstrated using fluorescence microscopy. Furthermore MCM1 and DLNxt cells were monitored for accessible galectin-binding sites using biotinylated galectins. Taking unlabeled cells as reference, both cell lines displayed different capacities to bind the labeled galectins. There were differences in the galectin-binding, not only between the cells but also among the individual galectins. The presence of galectin-binding sites prompted the question if galectins could impact the cell adhesion to culture flasks.

Cell adhesion is a complex process involved in tissue development and diseases. Cells that are derived from multicellular organism are able to adhere to extracellular matrix proteins or other cells [46].

In the presented study we investigated how Galectin-1 and -8 may influence the cell adhesion of MCM1 and DLNxt cell lines to culture flask in vitro. The results showed that the presence of Galectin-1 did not alter cell adhesion to plastic dishes (data not shown). In contrast, Galectin-8 increased the DLNxt adhesion in a dose-dependent

manner, whereas the adhesion of MCM1 cells was not affected. These findings indicate that Galectin-8 over expression can be involved in cellular attachment, spreading and migration of human melanoma cells.

Galectin-8 is one B-galactoside-binding lectin, and it can modulate cell adhesion and migration, which is depending on the way how it interacted with the cells. On the one way the immobilized Galectin-8 functions as an ECM protein, whereas the soluble Galectin-8 blocks the cell adhesion [47].

Metastasis is a process that often needs close cooperation between cancer cells and inflammatory cells. The process of metastasis can be divided in four major steps [48]. One of these steps is cell spreading. For cells to spread, they need to leave their original growth site and to intravasate into blood vessels or lymph. This process could be promoted by inflammation, through mediator building that increase the vascular permeability. TNF alpha is a metastasis-promoting cytokine. It can induce up regulation of Integrin expression which is associated with increased malignancy of melanoma cells [49].

Matrix metalloproteinase's are involved in the degradation of ECM components in both physiological and pathological processes. It enables melanoma cell to progress and to metastasize [50].

In our study we investigated the effect of Galectin-1 on DLNxt cell line regarding the gene expression of TNF-alpha and MMP3, using the RT-PCR. The data showed that the presence of Galectin-1 increased the expression of TNF-alpha and MMP3. The expression of MMP-3 was only slightly affected, whereas the TNF-alpha expression was significantly up-regulated.

Galectin expression and their binding site in MCM1 and DLNxt are valuable tools for further examination between galetins and tumor biologically relevant parameters. Overall, our investigation suggests that galectins play a critical role in melanoma tumor growth and metastasis, at least in part by mediating melanoma plasticity and tumor behavior and could serve as marker for melanoma spreading.

5 Conclusion

The results presented in this study demonstrate that human melanoma cells with different metastatic potential in vivo express galectins at the mRNA and protein level. In addition, we showed that the cell lines express the respective galectin binding sites and that the presence of galectins influences the mRNA expression levels of MMP3 and TNF-alpha and cell adhesion properties. Taken together, this study suggests a relationship between the expression of galectins and galectin binding sites and their involvement in cell adhesion and tumor behavior.

List of References

[1] Wachtler F. (2000): *Lehrbuch der Zytologie, Histologie*. Wien: Facultas Verlag

[2] Böcker W./Denk H./Heitz PH. U.: *Lehrbuch der Pathologie* (3.Auflage). München: Elsevier (Urban und Fischer Verlag)

[3] Lippert H.: *Lehrbuch der Anatomie* (6. Auflage). München: Elsevier (Urban und Fischer Verlag)

[4] Pons M./Quintanilla M. (2006): *Molecular biology of malignant melanoma and other cutaneous tumors.* Clinical and Translational Oncology 8 (7), 466–474

[5] Lazova R./Pawelek JM. (2009): *Why do melanomas get so dark?* Experimental Dermatology 18 (11), 934–938

[6] Carlson JA./Linette GP./Aplin A./Ng B./Slominski A. (2007): *Melanocyte receptors: clinical implications and therapeutic relevance.* Dermatologic Clinics 25, 541–557

[7] McGill GG./Haq R./Nishimura EK./Fisher DE. (2006): *c-Met expression is regulated by Mitf in the melanocyte lineage*. The Journal of biological chemistry 281, 10365–10373

[8] Bandarchi B./Ma L./Navab R./Seth A./Rasty G. (2010): *From Melanocyte to Metastatic Malignant Melanoma.* Dermatology Research and Practice. Pii: 583748

[9] Lens MB./Dawes M. (2004): *Epidemiological Trends of Cutaneous Malignant Melanoma: Incidence of Cutaneous Malignant Melanoma.* The British Journal of Dermatology 150(2), 179–185

[10] Houghton AN./Polsky D. (2002): *Focus on melanoma.* Cancer Cell 2, 275–278

[11] Hsu MY./Meier F./Herlyn M (2002): *Melanoma development and progression: a conspiracy between tumor and host*. Differentiation 70, 522–536

[12] Clark WH Jr./From L./Bernardino EA./Mihm MC. (1969). *The Histogenesis and Biologic Behavior of Primary Human Malignant Melanomas of the Skin.* Cancer Research 29, 705–727

[13] Ko JM/Fisher DE. (2011): *A new era: melanoma genetics and therapeutics*. The Journal of Pathology 223, 241–250

[14]. McGary EC./Lev DC./Bar-Eli M. (2002): *Cellular Adhesion Pathways and Metastatic Potential of Human Melanoma*. Cancer Biology and Therapy 1(5), 459–465

[15] Hoeller Ch./Viernstein H. *Glycobiology of Melanoma Metastases*: Persönliche Mitteilungen

[16] Wang PH. (2005): *Altered Glycosylation in Cancer: Sialic Acids and Sialyltransferases.* Journal of Cancer Molecules 1(2), 73–81

[17] Srinivasan N./Bane SM./Ahire SD./Ingle AD./Kalraiya RD. (2009): *Poly N-acetyllactosamine substitutions on N- and not O-oligosaccharides or Thomsen-Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3.* Glycoconjugate Journal 26, 445–456

[18] Lin J./Hocker TL./Singh M./Tsao H. (2008): *Genetics of Melanoma Predisposition: Molecular Risk Assessment of Melanoma*. The British Journal of Dermatology 159(2), 286–291

[19] de Snoo FA/Hayward NK. (2005): *Cutaneous melanoma susceptibility and progression genes.* Cancer Letters 230, 153–186

[20] Davies H./Bignell GR./Cox C./Stephens P/Edkins S/Clegg S. et al. (2002): *Mutations of the BRAF gene in human cancer*. Nature 417, 949–954

[21] Omhol K./Platz A./Kanter L./Ringborg U./Hansson J. (2003): *NRAS and BRAF Mutations Arise Early during Melanoma Pathogenesis and Are Preserved throughout Tumor Progression*. Clinical Cancer Research 9(17), 6483–6488

[22] Epenetos AA/Pignatelli M. (1995): *Cell adhesion molecules in Cancer and Inflammation* (Volume 1). Chur, Switzerland: Harwood Academic

[23] Krähling H./Mally S./Eble JA./Noël J./Schwab A./Stock C. (2009): *The glycocalyx maintains a cell surface pH nanoenvironment crucial for integrin-mediated migration of human melanoma cells*. Pflügers Archiv European Journal of Physiology 458(6), 1069–1083

[24] Mizejewski GJ. **(**1999): *Role of integrins in cancer: survey of expression patterns.* Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine New York NY 222, 124–138

[25] Valyi-Nagy IT./Hirka G./Jensen PJ./Shih IM./Juhasz I./Herlyn M. (1993): *Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact.* Laboratory investigation a journal of technical methods and pathology 69(2), 152–159

[26] Tang A./Eller MS./Hara M./Yaar M./Hirohashi S./Gilchrest BA. (1994): *E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro*. Journal of Cell Science 107, 983–992

[27] Danen EH./de Vries TJ./Morandini R./Ghanem GG./Ruiter DJ./van Muijen GN. (1996): *E-cadherin expression in human melanoma.* Melanoma Research 6(2), 127–131

[28] Li G./Satyamoorthy K./Herlyn M. (2001): *N-Cadherin-mediated Intercellular Interactions Promote Survival and Migration of Melanoma Cells*. Cancer Research 61, 3819–3825

[29] Dall'Olio F. (1996): *Protein glycosylation in cancer biology: an overview*. Clinical Molecular Pathology 49(3), 126–135

[30] Laider P. et al. (2006): *Characterization of glycosylation and adherent properties of melanoma cell lines*. Cancer Immunology Immunotherapy 55, 112–118

[31] Yarema KJ./Bertozzi CR. (2001): *Characterizing glycosylation pathways* Genome Biology 2(5), 0004.1–0004.10

[32] Handerson T./Pawelek J. (2003): *β 1, 6-branched oligosaccharides and coarse vesicles: a common and pervasive phenotype in melanoma and other human cancers*. Cancer Research 63, 5363–5369

[33] Chakraborty AK./Pawelek JM. (2007): *β 1, 6-branched oligosaccharides regulate melanin content and motility in macrophage-melanoma fusion hybrids*. Melanoma Research 17, 9–16

[34] Pawelek JM./Chakraborty AK. (2008): *Fusion of tumour cells with bone marrow derived cells: a unifying explantation for metastases*. Nature Reviews Cancer 8, 377–386

[35] Pawelek JM./Chakraborty AK. (2008): *The cancer cell-leukocyte fusion theory of metastases*. Advances in Cancer Research 101, 397–444

[36] Leffler H./Carlsson S./Hedlund M./Qian Y./Poirier F. (2004): *Introduction to galectins*. Glycoconjugate. Journal 19(7-9), 433–440

[37] Barondes SH./Cooper DN./Gitt MA./Leffler H. (1994): *Structure and Function of a large Family of Animal Lectins*. The Journal of Biological Chemistry 269, 20807–20810

[38] Poirier F./Kimber S. (1997): *Cell surface carbohydrates and lectins in early development*. Molecular Human Reproduction 3(10), 907–918

[39] Gitt MA./Barondes SH. (1991): *Genomic sequence and organization of two members of a human lectin gene family*. Biochemistry 30(1), 82–89

[40] Barondes SH. (1984): *Soluble lectins: a new class of extracellular proteins*. Science 223(4642), 1259–1264

[41] Yoon J./Kim SM./Kim JG./Lee SH./Kim CY./Yoon TJ. (2010): *The expression of Galectin-1 in melanocytic nevus, dysplastic nevus and malignant melanoma*. Melanoma Research 20, 43–44

[42] Prieto VG./Mourad-Zeidan AA./Melnikova V. et al. (2006): *Galectin-3 Expression Is Associated with Tumor Progression and Pattern of Sun Exposure in Melanoma*. Clinical Cancer Research 12, 6709–6715

[43] Vereecken P/Heene M. (2006): *Serum Galectin-3 in Advanced Melanoma Patients: a Hypothesis on a Possible Role in Melanoma Progression and Inflammation***.** The Journal of International Medical Research 34, 119–120

[44] van den Brûle FA./Buicu C./Baldet M./Sobel ME./Cooper DN./Marschal P./Castronovo V. (1995): *Galectin-1 modulates human melanoma cell adhesion to laminin*. Metastasis Research Laboratory 209(2), 760–767

[45] Bolander A./Agnarsdottir M./Strömberg S./Ponten F./Hesselius P./Uhlen M./Bergqvist M. (2008): *The Protein Expression of TRP-1 and Galectin-1 in Cutaneous Malignant Melanomas.* Cancer Genomics and Proteomics 5, 293–300

[46] Humphries MJ. (2001): *Cell Adhesion Assays*. Molecular Biotechnology 18(1), 57–61

[47] Levy Y./Arbel-Goren R./Hadari YR./Eshhar Sh./Ronen D./Elhanany E./Geiger B./Zick Y. (2001): *Galectin-8 Functions as a Matricellular Modulator of Cell Adhesion*. The Journal of Biological Chemistry 276, 31285–31295

[48] Grivenikov SI./Greten FR./Karin M. (2010): *Immunity, Inflammation and Cancer*. Cell 140, 883–889

[49] Katerinaki E./Evans GS./Lorigan PC./MacNeil S. (2003): *TNF-alpha increases human melanoma cell invasion and migration in vitro: the role of proteolytic enzymes*. British Journal of Cancer 89(6), 1123–1129

[50] Nishi N./Shoji H./Seki M./Itoh A./Miyanaka H./Yuube K./Hirashima M./Nakamura T. (2003): *Galectin-8 modulates neutrophil function via interaction with integrin* alphaM. Glycobiology 13(11), 755–763

[51] Richtig E./Berghold A./Schwantzer G./Ott A./Wölfelmaier F./Karner B./Ludwig R./Denk H./Stering R./Leitner G./Lax S./Okcu M./Gerger A./Kerl H./Smolle J. (2007): *Clinical Epidemiology of Invasive Cutaneous Malignant Melanoma in the Austrian Province Styria in the Years 2001–2003 and Its Relationship with Local Geographical, Meteorological and Economic Data*. Dermatology 214, 246–252

Curriculum Vitae

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Education

2005	Study of Pharmacy at the University of Vienna
2004	Internship in the pharmacy of the city of Struga
2004 – 2000	Secondary School of Pharmacy in Skopje/Mazedonia
2000 – 1992	Primary School in Struga/Mazedonia

Work

Since 2007	McDonalds Restaurant
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Special Skills

Foreign Languages:	German in speech and writing
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