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THE ROLE OF GALECTIN-3 IN MELANOMA PROGRESSION

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DEDICATION

I dedicate this dissertation to my family for their unconditional love and support.

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ABSTRACT OF THE DISSERTATION

THE ROLE OF GALECTIN-3 IN MELANOMA PROGRESSION

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Melanoma is a highly aggressive type of skin cancer that accounts for the majority of all skin cancer-related deaths worldwide. Melanoma metastasis poses a poor prognosis with resistance to therapy and high mortality rate. Thus, it is imperative to explore molecular mechanisms governing melanoma metastasis to identify candidate diagnostic or prognostic markers and develop novel therapeutic opportunities. Galectin-3 (Gal-3) has emerged as a pleiotropic promoter of cancer initiation and progression, exerting varying activities depending on the cellular context and its interacting partner. Extracellular Gal-3 is involved in mediating crosstalk between melanoma cells and the tumor microenvironment (TME) through lattice formation, conferring survival advantages for tumor cells. However, whether intracellular Gal-3 promotes melanoma aggressive behavior remains unknown. Here, I explore extra-/intracellular expression of Gal-3 in melanoma patient sera, primary and metastatic melanoma samples, and several melanoma models and its causal role in metastatic behavior. In contrast to elevated Gal-3 in melanoma patient sera, Gal-3 expression was markedly downregulated in primary melanomas and

further depressed in metastatic melanomas. Enforced silencing of Gal-3 in melanoma cells promoted migration, invasion, colony formation, xenograft growth, and activated the canonical oncogenic PI3K/AKT, MAPK/ERK, and Wnt/β-catenin signaling pathways. My data also provide evidence of a negative regulatory role for Gal-3 on the expression of nuclear factor of activated T cells (NFAT1) and its downstream metastasis-associated effector proteins, namely matrix metalloproteinase-3 (MMP-3), interleukin-8 (IL-8), and glypican-6 (GPC6) in melanoma cells. Importantly, these results highlight the tumor-suppressive function of Gal-3 in melanoma cells, emphasizing the negative crosstalk between Gal-3 and NFAT1 and its role in dictating melanoma metastasis, and introducing GPC6 as a candidate target of the Gal-3-NFAT1 axis in melanoma. Overall, studies conducted in this dissertation implicate tumor-intrinsic Gal-3 as a candidate prognostic indicator of metastatic risk in melanoma patients.

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LIST OF ABBREVIATIONS

ACC	Adrenocortical carcinoma
AKT	Protein kinase B
ATCC	American Type Culture Collection
ATF	Activating transcription factor
AUC	Area under the curve
bFGF	Basic fibroblast growth factor
BLCA	Bladder urothelial carcinoma
BRCA	Breast invasive carcinoma
CBP	Carbohydrate-binding protein
CCK-8	Cell Counting Kit-8
cDNA	Complementary DNA
Ce6	Chlorine6
CESC	Cervical and endocervical cancers
CHO	Chinese hamster ovary
CHOL	Cholangiocarcinoma
CO2	Carbon dioxide
COAD	Colon adenocarcinoma
CRD	Carbohydrate recognition domain
CRE	cAMP-dependent response element
CREB	cAMP-response element binding factor
CRM1	Chromosomal region maintenance/exportin 1
CRP	C-reactive protein

CS	Chondroitin sulfate
CSPG4	Chondroitin sulfate proteoglycan 4
CTCs	Circulating tumor cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL8	C-X-C Motif Chemokine Ligand 8 (Aka interleukin-8)
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DSA	Datura stramonium agglutinin
EBP	Elastin-binding protein
EC	Endothelial cell
ECM	Extracellular matrix
EDPs	Elastin-derived peptides
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptors
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESCA	Esophageal carcinoma
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
G-CSF	Granulocyte colony-stimulating factor
Gal	Galectin

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnT	N-acetylglucosaminyltransferase
GPC6	Glypican-6
GSE	Genomic Spatial Event
GTEx	Genotype Tissue Expression
HEK	Human embryonic kidney
HIF	Hypoxia inducible factor
HIPK2	Homeodomain-interacting protein kinase-2
HNSC	Head and Neck squamous cell carcinoma
HTLV-I	Human T-lymphotropic virus type 1
ICAM-1	Intercellular adhesion molecule 1
ICI	Immune checkpoint inhibitors
IEG	Immediate early gene
IFN-γ	Interferon gamma
IGFIR	Insulin-like growth factor I receptor
IHC	Immunohistochemistry
IL	Interleukin
ILVs	Intraluminal vesicles
KICH	Kidney Chromophobe
KIRC	Kidney renal clear cell carcinoma

KIRP	Kidney renal papillary cell carcinoma
LacNAc	N-acetyllactosamine
LAML	Acute Myeloid Leukemia
LAMP	Lysosome-associated membrane protein
LDH	Lactate dehydrogenase
LDH	Lactate dehydrogenase
LEA	Lycopersicon esculentum agglutinin
LGALS3	Lectin, Galactoside-Binding, Soluble 3 (Gal-3 gene symbol)
LGG	Brain Lower Grade Glioma
LIHC	Liver hepatocellular carcinoma
LLPS	Liquid–liquid phase separation
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
M-MDSCs	Monocytic myeloid-derived suppressor cells
Mac-2BP	Mac-2-binding protein
MAPK	Mitogen-activated protein kinase
MCAM	Melanoma cell adhesion molecule
MCP	Modified citrus pectin
MEFs	Mouse embryonic fibroblasts
MESO	Mesothelioma
MIP	Molecular imprinted polymers
MMPs	Matrix metalloproteinases
MOI	Multiplicity of infection

MPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MT3-MMP	Membrane type 3 matrix metalloproteinase
MVB	Multivesicular body
NES	Nuclear export signal
NF-κB	Nuclear factor-κB
NFAT	Nuclear factor of activated T cells
nHEM	Normal human epidermal melanocytes
NK	Natural killer
NLS	Nuclear localization signal
nm	Nanometer
NPC	Nuclear pore complex
NSG	NOD-SCID IL-2Rγ-deficient
NTD	N-terminal domain
NTS	Acid N-terminal segment
OD	Optical density
OTSCC	Oral tongue squamous cell carcinoma
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCPG	Pheochromocytoma and Paraganglioma

PCR	Polymerase chain reaction
PD-1	Programmed death 1
PD-L1	Programmed death-ligand 1
PDT	photodynamic therapy
PGCCs	Polyploid/multinucleated giant cancer cells
PGIL	Photoactivable Gal-3-inhibitor nanoliposome
pH	Potential of hydrogen
PHA-L	Phaseolus vulgaris leukoagglutinin
PI3-K	Phosphateidylinositol-3-kinase
PI3K	Phosphoinositide-3-kinase
PRAD	Prostate adenocarcinoma
PVDF	Polyvinylidene fluoride
r	The correlation coefficient
READ	Rectum adenocarcinoma
RGP	Radial growth phase
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-seq	RNA Sequencing
RNAi	RNA interference
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
RPM	Rotation per minute
RSEM	RNA-Seq by Expectation-Maximization

RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
scRNA-seq	Single-cell RNA sequencing
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
shRNA	short hairpin RNA
SKCM	Skin cutaneous melanoma
STAD	Stomach adenocarcinoma
TCGA	The Cancer Genome Atlas
TGCT	Testicular Germ Cell Tumors
TGF-β1	Transforming growth factor β1
THCA	Thyroid carcinoma
THYM	Thymoma
TILs	Tumor-infiltrating T lymphocytes
TISCH2	Tumor Immune Single-cell Hub 2
TMA	Tissue microarray
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
UCEC	Uterine Corpus Endometrial Carcinoma
UCS	Uterine Carcinosarcoma
UVM	Uveal Melanoma
VEGF	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase

1. CHAPTER 1: INTRODUCTION

1.1. Preface: Galectin-3 (Gal-3) is one of the most extensively studied galectins in cancer research

Melanoma is an aggressive skin cancer that arises from melanocytes; the neural crest-derived melanin-producing cells of the skin [1, 2]. Although melanoma accounts for only 4% of skin cancers, it is the most lethal form of the disease worldwide with increased mortality rates over the past 30 years [3-5]. It is also one of the more common malignancies in adolescent populations [4]. Melanoma progression typically involves a stepwise cascade, characterized by an initial horizontal spread of malignant cells through the epidermis representing the radial growth phase (RGP), followed by a vertical growth phase (VGP) where cells invade the dermis, and eventually metastasize to distant organs [6, 7]. These changes are usually triggered by independent genetic alteration such as BRAF mutation and loss of the tumor suppressor protein PTEN, in addition to other epigenetic and environmental changes [8-10]. Early-diagnosed melanoma is the best scenario for patient survival, as it can be cured with surgical excision [11, 12]. In contrast, once melanoma metastasizes to visceral organs (Stage IV), it becomes a life-threatening disease with a poor outcome [12-14]. The emergence of several immune checkpoint inhibitors (ICI) and targeted therapies over the last decade has substantially improved patient survival. However, tumor cell resistance to these novel therapies is still a major concern [11, 15-17]. Understanding the molecular landscape driving melanoma progression paves the way to find out new diagnostic and therapeutic strategies more effective than

conventional modalities [8]. One of the most rapidly expanding areas of cancer research is the field of glycobiology, which influences various cancer cellular activities, including those functioning in melanoma cells [18].

Galectins are a subfamily of the carbohydrate-binding proteins (lectins) that bind specifically to β-galactosides of various glycoconjugates through their characteristic carbohydrate recognition domain (CRD) [19]. Although the first galectin was isolated and described in 1975, it was not until 1994 that the first crystal structure of the CRD was characterized and hence galectins were named and classified [20]. Since then, and with the huge advancement of biochemical techniques, galectins have gained attention as prominent effectors in cancer research. The fifteen members of the galectin family are classified, based on their molecular structures, into three main groups **(Figure 1.1):** (i) *Prototypical galectins* (Galectin-1, 2, 5, 7, 10, 11, 13, 14, and 15) which have a single CRD and can homodimerize; (ii) *Tandem-repeat galectins* (galectin-4, 6, 8, 9, and 12) which have two homologous CRDs per monomer connected by a linker protein; and (iii) *Chimeric galectin* (galectin-3) which has a single CRD linked to a N-terminal domain (NTD). The "multifunctionality" of galectins has been studied in different types of human cancer. However, the exact molecular mechanisms underlying these functions are still not fully understood [21]. The structurally distinct member of the galectin family, galectin-3 (Gal-3), has remarkably attracted the interests of cancer researchers.

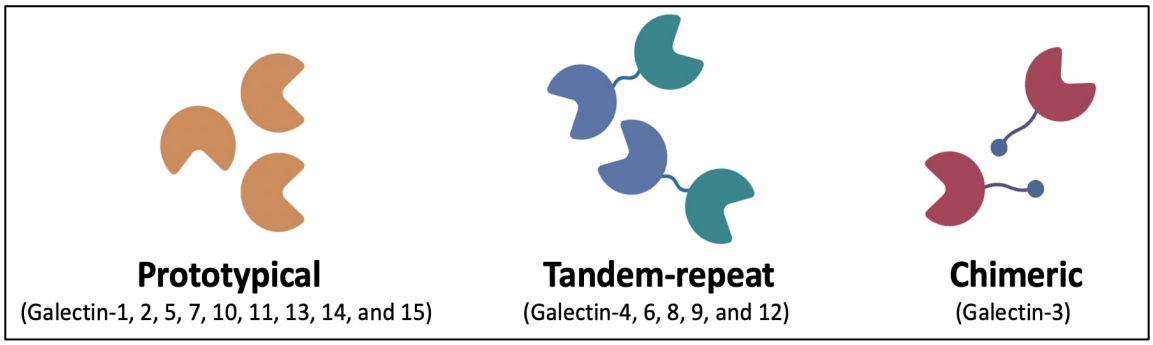


Figure 1.1. The three classes of the galectin family.
The fifteen members of the galectin family are classified according to their molecular structures into three major groups. The ***prototypical*** galectins have a single carbohydrate recognition domain (CRD) that can homodimerize. The ***tandem-repeat*** galectins have two homologous CRDs connected by a linker protein. The ***chimeric*** group has only one member (Galectin-3) which has a single CRD linked to a N-terminal domain.

In this Chapter, the established physical, biochemical, and biological characteristics of Gal-3 will be introduced. Then, the putative roles of Gal-3 in modulating cancer cell activities will be discussed, with particular emphasis on melanoma studies, recapitulating recent advances of Gal-3-based diagnostic and therapeutic applications.

1.2. Gal-3: A general overview

1.2.1. Gal-3 molecular structure

Gal-3 (previously known as Mac-2 antigen) is the only member in the chimeric galectin group. It is a 29–35kDa protein first identified as a murine macrophage surface antigen [22]. Later, it was found to be widely expressed by many human cell types [23-25]. Gal-3 is also known as CBP-35, hL-31, L-34, HL-29, RL-29, εBP, and LBP based on its varied molecular mass and the species from which it was isolated [24]. The Gal-3 structure has been broadly explored

aiming to provide a sound foundation for its structure-function relationship. Indeed, the unique chimeric structure of Gal-3 accounts for its distinguished biological roles among other galectin members [22]. Gal-3 has an unfolded N-terminal domain (NTD; residues 1–111) linked to a globular C-terminal carbohydrate-recognition domain (CRD; residues 112–250).

The *N-terminal domain* consists of a 21-amino-acid N-terminal segment (NTS), harboring two serine phosphorylation sites, and nine collagen-like repeats rich in proline and glycine [23]. The NTS sequence is essential for Gal-3 nucleocytoplasmic translocation and secretion [26, 27]. The *C-terminal domain* of Gal-3 has a 5-stranded β-sheet (F-face; F1-F5) in addition to the canonical 6-stranded β-sheet (S-face; S1–S6), both together form the CRD β-sandwich via intramolecular antiparallel folding [24]. The concave side of the β sandwich with its defined five subsites (A–E) accommodates the bound glycan. Of note, the conserved subsite C docks the β-galactose, while the adjacent residue occupies subsite D, forming the disaccharide-binding site. Other less conserved subsites are accountable for the variable specificity of individual galectins to longer glycans. Unlike other members of the galectin family, Gal-3 has a distinctive Asp-Trp-Gly-Arg (NWGR) motif within its C-terminal domain (residues 180–183). Interestingly, this motif is homologous to the anti-death motif of the Bcl-2 family [24], which could explain, in part, the anti-apoptotic activity of cytoplasmic Gal-3 (see below).

1.2.2. Gal-3 polymerization

Generally, Gal-3 exists as monomers in solution [28]. In the absence of glycosylated ligands, Gal-3 can homodimerize via its CRDs, while Gal-3 binding to its glycoprotein or glycolipid ligand triggers spontaneous oligomerization up to pentamers through NTD interactions in a dynamic process similar to liquid–liquid phase separation (LLPS) [29, 30] **(Figure 1.2)**. Gal-3 exhibits the pentameric structure exclusively outside of cells [31], where its formation is largely dependent on Gal-3 concentration as well as interacting glycoconjugate type and concentration [32]. Such extracellular arrangement forms a lattice-like structure by cross-linking between adjacent cells or between cells and extracellular matrix [33]. Gal-3 binding to cell surface glycosylated proteins such as integrins and epidermal growth factor receptors (EGFR), and extracellular matrix proteins like laminin and fibronectin has been reported to play vital roles in regulation of various extracellular biological processes [34-36].

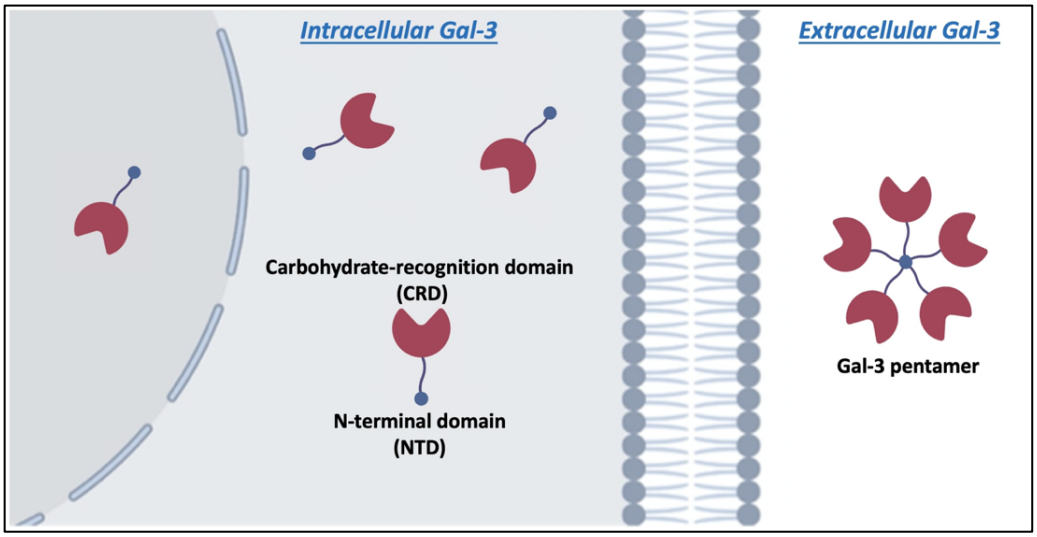


Figure 1.2. Gal-3 structure and polymerization.
Gal-3 is unique among other members of the galectin family by the ability of its monomeric molecules inside of the cell ***(Left Panel)*** to assemble extracellularly, upon binding to their glycosylated ligands, to form pentamers ***(Right Panel)***.

Mechanistically, a growing body of evidence suggests that both NTD and CRD are involved in Gal-3 polymerization [24]. Gal-3 aggregation is initiated by accumulating interactions between NTD's aromatic residues, namely tryptophan and tyrosine. These interactions are augmented by Gal-3-glycan binding and the evolving high local protein concentration, while they are blocked by lactose which interferes with Gal-3 binding to its ligand [30]. Recent mutagenesis data showed that intramolecular interactions between N-terminal proline residues and the F-face of the CRD are directly implicated in glycan-binding and subsequent Gal-3 polymerization [32].

1.2.3. Gal-3 gene and regulation of its expression

The gene expression profile of a given human protein is determined by a complex fine-tuned process that is regulated at various cellular levels, and varies depending on cell type, developmental stages, disease states and other environmental influences [37]. Several studies have been conducted to identify regulators of Gal-3 expression in cancer cells [24]. However, relevant data from melanoma studies are deficient. Understanding how Gal-3 is regulated may offer valuable insights into identifying novel molecular targets for future cancer therapies.

The human Gal-3 gene (LGALS3) is located on chromosome 14, locus q21–q22 [38]. The gene spans approximately 17 kilobases and consists of six exons separated by five introns. Multiple regulatory sequences are located within its promoter region including: two nuclear factor-κB (NF-κB)-like sites (between -229 and -105) and five cAMP-dependent response element (CRE) motifs

(between -836 and -513), suggesting that the transcription factors NF-κB and cAMP-response element binding factor (CREB) function as a potential regulators of Gal-3 gene expression [39]. This assumption is verified in subsequent studies. Dumic et al. were the first group to highlight the role of NF-κB as well as Jun protein in the induction of Gal-3 expression in cells exposed to UV stress [40]. Then, Liu et al. demonstrate that Gal-3 mRNA as well as protein levels are downregulated by the pro-apoptotic protein Nucling via interference with NF-κB activation through the nuclear translocation of NF-κB/p65 [41]. Data from another study show that vascular endothelial growth factor C (VEGF-C) induces cervical cancer invasiveness by upregulation of Gal-3 expression through NF-κB pathway [42]. Hsu et al. has demonstrated that HTLV-I Tax protein stimulates expression of Gal-3 through the CREB/ATF and the NF-KB/Rel transcription factor pathways, which further activates T cells and contributes to the enhanced cell growth seen in HTLV-1-induced adult T cell leukemia [43].

Additionally, five GC box motifs for binding of Sp1 transcription factor are present within the LGALS3 promoter, which is often associated with the promoters of housekeeping genes. However, the Gal-3 gene is known as an immediate early gene (IEG) that is rapidly activated in response to various stimuli, such as serum addition to serum-starved 3T3 mouse fibroblasts [39, 44]. Of note, Tat protein of HIV-1 can induce Gal-3 expression in various human cell lines through activation of Sp1 [45]. Homeodomain-interacting protein kinase-2 (HIPK2)-activated p53 is known to induce apoptosis via downregulation of Gal-3

expression at the promoter level, while loss of HIPK2 is associated with Gal-3 upregulation in well-differentiated thyroid carcinomas [46].

Zeng et al. report that Gal-3 expression is conspicuously dependent upon local oxygen tension. Using HeLa cells and mouse embryonic fibroblasts (MEFs) exposed to hypoxic conditions, their data show that hypoxia inducible factor (HIF)-1α interacts with hypoxia regulatory elements in the Gal-3 promoter region to upregulate Gal-3 expression [47]. Furthermore, several inflammatory cytokines and growth factors, such as interleukin (IL)-2, IL-4, IL-7, basic fibroblast growth factor (bFGF), Tumor necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been shown to enhance Gal-3 expression in different types of cancer cells, while transforming growth factor β1 (TGF-β1) and epidermal growth factor (EGF) are associated with reduced expression of Gal-3 [48]. Epigenetically, Ruebel et al. were the first group to hypothesize the negative correlation between CpG island methylation in the LGALS3 promoter region and Gal-3 protein expression levels in various types of human pituitary tumors, suggesting that Gal-3 gene expression is possibly regulated by DNA methylation of its promoter region [49].

1.2.4. Subcellular and extracellular distribution of Gal-3

Gal-3 is widely expressed in many human epithelial and immune cells, such as macrophages, activated B and T cells, NK cells, mast cells and dendritic cells [23, 50]. Gal-3 is expressed intracellularly; in cytoplasm, nucleus and mitochondria, where it participates in the regulation of cell proliferation, cell cycle, and apoptosis through modulation of many signaling pathways [24, 51]. It is also

secreted out of the cell where it largely modulates cell adhesion and immune surveillance [52]. Subcellular distribution of Gal-3 between the nucleus and the cytoplasm is suggested to be cell-type dependent, essentially governed by the balance between nuclear export and import of Gal-3, alongside the presence of nuclear/cytoplasmic retaining partners [53, 54]. However, it should be emphasized that intracellular Gal-3 localization is also impacted by experimental circumstances, including cell culture conditions, growth and age of cells, as well as disease or tumor stage [55].

Certain amino acid residues in the Gal-3 polypeptide chain control the directional movement of Gal-3 through the nuclear pore complex (NPC) via a receptor-mediated system. A nuclear localization signal (NLS), analogous to that of c-Myc and p53 NLSs, is considered essential for Gal-3 translocation into the nucleus mediated via the importin-α/β heterodimer [53, 56], while a leucine-rich nuclear export signal (NES) motif is thought to be crucial for Gal-3 translocation into the cytoplasm carried by the chromosomal region maintenance/exportin 1/Xpo1 (CRM1) [57]. Both NLS and NES were recognized near the C-terminus of the Gal-3 polypeptide chain with an overlapping sequence [57]. Gal-3, like other members of the galectin family, is characterized by lack of an amino-terminal signal peptide sequence, which is important to route cellular proteins via the classical secretion pathway. This implies that Gal-3 released into the extracellular space follows a non-classical secretion pathway, independent of the endoplasmic reticulum (ER)-Golgi complex [58]. Various mechanisms have been proposed to explain how Gal-3 is secreted out of the cell. Bänfer et al. have recently

suggested exosomal release of Gal-3 as a possible mechanism. The authors identified a tetrapeptide motif P(S/T) AP in the N-terminal domain of Gal-3 that can interact with Tsg101, a key component of the endosomal sorting complex required for transport (ESCRT), in a process similar to that of enveloped viruses release from infected cell [59]. Such interaction routes Gal-3 into intraluminal vesicles (ILVs) of the multivesicular body (MVB) to be delivered to lysosomes for degradation or released into extracellular media as exosomes [60]. However, the exact mechanism of Gal-3 release out of the enclosed exosome still needs to be elucidated in future studies.

1.3. Extracellular and cell-intrinsic Gal-3-binding partners

Asparagine (N)-linked-glycosylation is a post-translational protein modification that undergoes structural revision by cells undergoing adaption to various external stimuli, and accordingly facilitates regulation of cellular functions, such as cell proliferation, migration and differentiation [61]. Altered surface N-linked glycans, which are characteristic in cancer, often correlate with cancer progression and metastasis [62]. An example of this cancer-associated process is the upregulation of complex tri- and tetra-antennary N-linked glycans, mediated by the action of *N*-acetylglucosaminyltransferase-V (GnT-V, *Mgat5*), which has been shown to affect tumor growth and metastasis [63, 64]. Increased branching of N-linked glycans expressed on surface glycoproteins enhances Gal-3 – N-linked glycan interactions, which in turn, regulate diverse activities of cancer and immune cells [51, 65]. As such, in melanoma, upregulation of branched N-linked glycans, has been correlated with tumor growth and

metastasis [66]. Conversely, reduction of N-glycan branching either with the chemical inhibitor swainsonine [67, 68] or by the action of *N*-acetylglucosaminyltransferase-III (GnT-III, *Mgat3*) [69, 70], an enzyme responsible for the biosynthesis of bisecting N-linked glycans (GlcNAcβ1,4Man), suppresses metastasis. Gal-3 binds preferentially to internal linear N-acetyllactosamine (LacNAc) units within a linear poly-LacNAc chain which can be found on the branched N-linked glycans, while the presence of other modifications in these poly-LacNAcs can reduce or inhibit this binding [71, 72]. Therefore, identifying the level of N-glycan branching, the type of poly-LacNAc abundance, and the individual modified glycoproteins is an important step towards delineating the mechanisms that govern melanoma growth, survival, and metastasis from a Gal-3 – N-linked glycan-binding axis perspective.

In melanoma research, lectins have been used to purify and subsequently identify Gal-3 binding glycoproteins. To this end, lectin affinity chromatography has been employed using Gal-3 and either *Phaseolus vulgaris leukoagglutinin* (PHA-L) or *Datura stramonium agglutinin* (DSA) lectins on extracts from human [73, 74] and mouse melanoma cell lines [75, 76]. The glycoproteins identified by these methods include, but are not limited to, integrins, lysosome-associated membrane proteins (LAMPs), melanoma cell adhesion molecule (MCAM/MUC-18/CD146), basigin (CD147), chondroitin sulfate proteoglycan 4 (CSPG4), intercellular adhesion molecule 1 (ICAM-1/CD54), and Mac-2BP (Mac-2-binding protein/LGALS3BP) (**Table 1**).

Table 1. **Gal-3-binding Partners**

Protein identified	**Gal-3/ PHA-L**	**PHA-L**				**DSA**	**Refs**
	B16F10	A375	WM35	WM9	WM239	WM266-4	
Integrin subunit α2		•		•			**[73]**
Integrin subunit α3	•	•		•	•		**[73, 75, 76]**
Integrin subunit α5	•	•		•	•		**[73, 75, 76]**
Integrin subunit α6	•						**[75]**
Integrin subunit αv		•	•	•	•		**[73, 76]**
Integrin subunit β1	•	•	•	•	•		**[73, 75]**
Integrin subunit β3				•	•		**[76]**
LAMP-1 (CD107a)	•	•	•	•	•	•	**[73-76]**
LAMP-2 (CD107b)	•	•	•	•	•		**[73, 75, 76]**
MCAM (CD146)	•		•	•	•		**[75, 76]**

Basigin (CD147)	•					•	**[74, 75]**
Chondroitin sulfate proteoglycan 4 (CSPG4, MCSP)			•	•	•	•	**[74, 76]**
Intercellular adhesion molecule 1 (ICAM-1, CD54)	•		•	•	•		**[75, 76]**
Mac-2BP		•	•	•	•		**[73, 76]**
Tetraspanin-30, LAMP-3 (CD63)	•					•	**[74, 75]**
L1		•	•				**[73, 76]**
MEMD							**[73]**
Tumor rejection antigen p96					•		**[76]**
Melanoma associated antigen (p97)		•			•		**[73, 76]**
CD44	•						**[75]**

1.3.1. LAMPs

Affinity chromatography with recombinant Gal-3 of human melanoma A375 cell lysates identified LAMP-1 and LAMP-2 glycoproteins as Gal-3 ligands [77]. LAMPs are highly glycosylated proteins and are sensitive to endo-β-galactosidase digestion, indicating the presence of poly-LacNAc chains [78, 79]. They are found mainly in lysosomes forming a dense coat on the luminal side, serving as a barrier between the hydrolases and the membrane [80]. However, a small portion of these glycoproteins can also be found on the external surface of the cell membrane, probably acting as part of a defense mechanism of the cell against hydrolases, such as cathepsins, during lysosome-plasma membrane fusion [81]. Indeed, immunofluorescence on a permeabilized A2058 metastatic melanoma cell line shows that LAMPs accumulate at the edges of the cells intracellularly, while flow cytometry on non-permeabilized cells show that LAMPs are also expressed on the cell surface [82]. Increased LAMPs surface expression, by treatment with sodium butyrate, promotes Gal-3 binding to cell surfaces LAMP in a carbohydrate-dependent manner [82]. In another study, comparisons on low- and high-grade murine metastatic melanoma cell lines B16F1 and B16F10 respectively, show that LAMPs are expressed by both cell lines, though only LAMP-1 is expressed in higher amounts on the cell surface of the high metastatic cell line that may provide more abundant surface poly-LacNAcs for enhanced Gal-3-binding. In fact, downregulating LAMP-1 expression on B16F10 cells shows reduced Gal-3-binding [83].

1.3.2. Mac-2BP

Mac-2BP is a Gal-3-binding glycoprotein, which has been implicated in various types of cancers, including melanoma [84]. It is a secreted protein with seven potential N-linked glycosylation sites and can form homodimers and homomultimers of very high molecular mass [85]. Its expression has been shown to be elevated in melanoma patients [86]. An *in vitro* study shows that, when added exogenously, it binds to the surface of the melanoma A375 cells and forms cell-aggregates in a time- and dose-dependent manner, both inhibitable by lactose [87]. A more recent study shows that Mac-2BP is a ligand for various integrin subunits in a C8161 cell line and promotes cell migration that could be inhibited by an antibody against the β1 integrin subunit [88].

1.3.3. MCAM

MCAM, a member of the immunoglobulin superfamily, is a cell surface glycoprotein involved in numerous physiological and pathological processes of cells [89]. In melanoma, immunohistochemistry experiments have shown that MCAM glycoprotein is mainly detected in primary and metastatic melanoma tissues [90]. It was later shown that the expression of MCAM in primary melanomas is positively correlated with the vertical thickness of the melanoma, possibly reflecting its metastatic potential [91]. This is further supported using experimental metastasis studies, showing that MCAM abundance on human melanoma cell lines is positively correlated with metastatic potential [92-94]. In contrast, tumor growth and lung metastasis are inhibited when nude mice injected with $MCAM^{high}$ human metastatic cell lines (A375SM and WM2664) are

treated with the anti-human MCAM antibody ABX-MA1 [95]. Recent published results show that circulating Gal-3 interacts with endothelial MCAM to stimulate secretion of metastasis-promoting cytokines such, as granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6) [96].

1.3.4. Integrins

Integrins are cell adhesion receptors known to bind Gal-3 that regulate a diverse array of functions of solid tumors including the initiation of metastasis and providing the physical link between the extracellular matrix (ECM) and the actin cytoskeleton [97]. In melanoma, integrins contribute to the radial and vertical growth phases as well as metastasis through various mechanisms [98]. Their importance has been shown by *in vitro* data were cell migration was blocked by antibodies against α2, α3 and β1 integrin subunits [99, 100]. Furthermore, β1 integrin was found on the leading edge of melanoma cells during cluster migration, while a blocking antibody against β1 impaired the organized migration and path persistence [101]. *In vivo* data showed that increased expression of the α3/β1 heterodimer is associated with tumor progression in human melanoma patients, since its expression was positively correlated with tumor invasiveness [102]. In parallel to integrin expression, their function depends, among other factors [103], on the status of their N-glycosylation [104, 105]. Integrins glycosylated by GnT-V promote malignant progression, while glycosylation by GnT-III reverses the phenomenon [106].

1.3.5. CSPG4

Melanoma-associated chondroitin sulfate proteoglycan (CSPG4) is another protein identified by affinity chromatography as a Gal-3 binding partner. It is a protein originally discovered as a human melanoma plasma membrane antigen detected as two high molecular weight (~280 and >440kDa) polypeptides [107]. Later studies on the human melanoma M21 cell line reveal that the low molecular weight polypeptide (~280kDa) is the core N-linked glycan glycoprotein (biosynthetic precursor) upon which the chondroitin sulfate (CS) glycosaminoglycans are attached (>440kDa) [108, 109]. Research on human melanoma cell lines has indicated that CSPG4 influences positively the activity of the α4β1 integrin *in vitro* [110-112], the latter being confirmed to be important for melanoma progression and metastasis [113, 114]. In parallel, CSPG4 appears to be regulating the activity of matrix metalloproteinases (MMPs). Studies on human melanoma WM1341D cell line found that the membrane type 3 matrix metalloproteinase (MT3-MMP) and the CSPG4 are required for invasion, a process facilitated by the CS glycosaminoglycans [115], which subsequently contribute to the activation of Pro-MMP2 [116], a metalloproteinase expressed in melanomas [117]. Likewise, NG2, the rat homologue of CSPG4 [118], is associated with increased tumor growth and metastasis when both NG2-induced B16F1 or B16F10 cell lines are injected subcutaneously and intravenously respectively [119]. In an endothelial cell (EC) migration model system, soluble recombinant NG2 when exogenously added, can promote EC migration and *in vivo* angiogenesis. In this system, NG2, Gal-3 and α3β1 integrin form

multimolecular complexes in a carbohydrate dependent manner [120]. Furthermore, in parallel experiments, the same type of multimolecular complex forms on the A375 human melanoma cell line. The NG2 – Gal-3 interaction appears to be dependent on the N-linked glycans found proximal to the plasma D3 domain [121]. Interestingly, other studies report that Gal-3 can bind directly to CS glycosaminoglycans, further demonstrating the complexity of Gal-3's binding mechanism [122].

Importantly, the human enzyme I-branching β1,6-*N*-acetylglucosaminyl transferase 2, encoded by the *GCNT2* gene (76), forms I-branched (-3Galβ1,4GlcNAcβ1,3(Galβ1,4GlcNAcβ1,6)Galβ1-) poly-LacNAc chains, which decrease Gal-3-binding [72]. Human melanocytes express the GCNT2 enzyme and related N-linked glycans containing I-branched poly-LacNAc chains, whereas their abundance in human metastatic A375 and G361 melanoma cell lines is low, resulting in more linear (non-I-branched) poly-LacNAcs [123]. Increased abundance of linear poly-LacNAcs due to reduced expression of the *GCNT2* corresponds with human melanoma progression and human melanoma xenograft growth, colony formation and cell survival. These melanoma glycome traits have been suggested to increase Gal-3 binding to melanoma surface glycosylated proteins. Interestingly, the changes detected on the high molecular weight branched N-linked glycans are accompanied by changes in LEA lectin (poly-LacNAc) binding, but not PHA-L (branched N-glycan) lectin. To note, similarly, Gal-3 binding to N-linked glycans on CHO cells is not affected by silencing enzymes responsible for producing branched N-linked glycans [124].

Therefore, changes in PHA-L binding do not necessarily reflect changes occurring in poly-LacNAc chains that more closely impact Gal-3-binding.

1.4. Gal-3 involvement in cancer progression

Through its interactions with intracellular protein partners and extracellular glycosylated ligands, Gal-3 can either facilitate or impair diverse biological processes associated with tumor growth and metastasis [18] **(Figure 1.3)**. In this section, the impact of Gal-3 on various cancer hallmarks will be discussed, including the ability of tumor cells to maintain proliferative signaling, resist apoptotic stimuli, evade immune surveillance, promote angiogenesis, and migrate/invade ECM, highlighting relevant examples from melanoma studies.

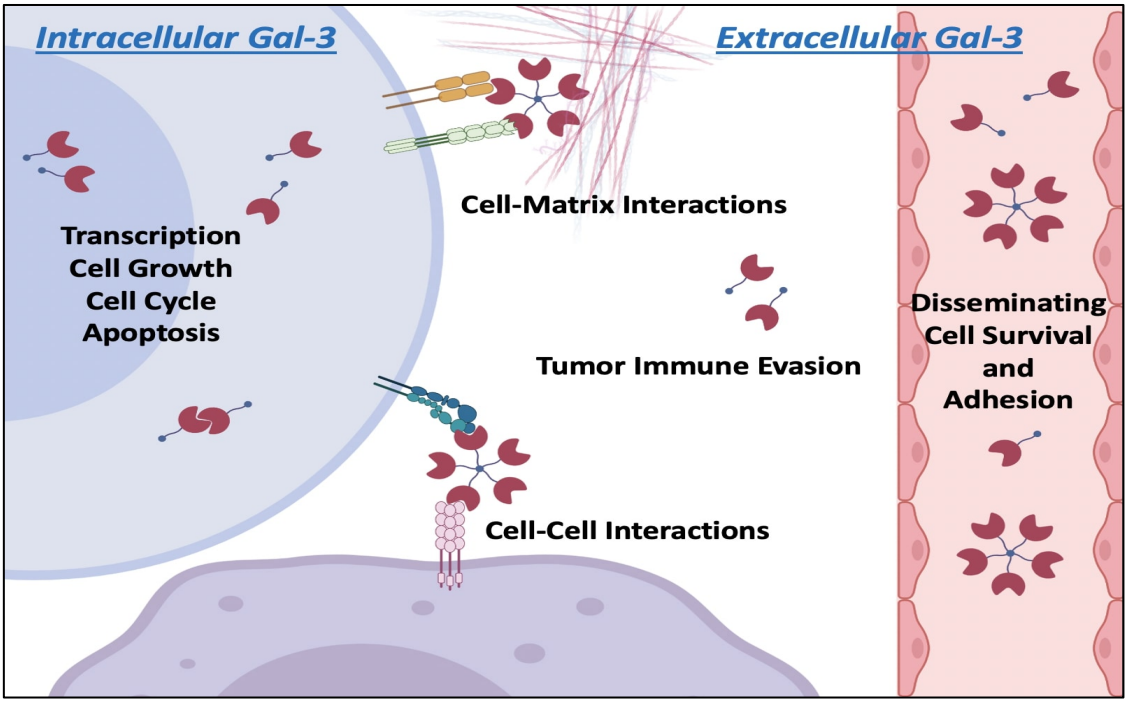


Figure 1.3. Schematic illustration of Gal-3 involvement in cancer progression. Gal-3 regulates diverse activities related to cancer progression, such as intracellular proliferative and apoptotic signaling, cell-cell and cell-matrix interactions, tumor immune surveillance, and metastasis to distal tissues.

1.4.1. Gal-3 and intracellular oncogenic signaling pathways

Uncontrolled proliferation is a distinctive feature of tumor cells, mediated by dysregulated signaling pathways and cell cycle machinery [125]. Through its carbohydrate-independent interaction with numerous intracellular oncogenic proteins, Gal-3 has been shown to affect downstream signaling cascades promoting uncontrolled growth of tumor cells [126]. Using HEK-293 cells co-transfected with K-Ras/Gal-3, Elad-Sfadia et al. demonstrated that Gal-3 can interact with activated K-Ras, maintaining its membrane anchorage and activation, and promoting phosphatidylinositol-3-kinase (PI3-K) and Raf-1 activation, while attenuating active ERK signaling through an unknown mechanism that is probably mediated by upregulation of ERK phosphatases [127]. Consistently, Song et al. report that Gal-3 silencing in pancreatic cancer cells can attenuate Ras activity and Ras downstream signaling cascades including both ERK and AKT phosphorylation, while cells overexpressed with Gal-3 show enhanced Ras activity and its downstream signaling [128]. Gal-3 was also found to affect tumor progression of oral tongue squamous cell carcinoma (OTSCC) through activation of the Wnt/β-catenin signaling pathway [129]. Moreover, Gal-3 is involved in cell cycle regulation where it mediates G1 arrest via downregulation of G1-S cyclin levels (cyclin E and cyclin A) and upregulation of cell cycle inhibitors ($p21^{WAF1/CIP1}$ and $p27^{KIP1}$) [130]. Thus, Gal-3 has a stimulatory effect on tumor cell proliferation by controlling proliferative signaling pathways and cell cycle progression.

Gal-3 studies on melanoma regarding this aspect are lacking. Indeed, the RAS/RAF/ERK signaling pathway is constitutively active in the majority of melanomas due to the dominance of the activating $BRAF^{V600E}$ mutation [131]. Therefore, future work should shed more light on how Gal-3 may modulate this oncogenic pathway in melanoma. Additionally, the potential role of Gal-3 in mediating melanoma resistance to the BRAF inhibitors should be considered and investigated.

1.4.2. Gal-3 and apoptotic signaling pathway

Disrupting the balance between pro-apoptotic and pro-survival factors is another hallmark of cancer [132]. Two major apoptotic pathways have been described: the intrinsic pathway that is initiated by signals generated within the cell and mediated by mitochondria, and the extrinsic pathway that is triggered by death molecules binding to surface death receptors [133]. The anti-apoptotic role of intracellular Gal-3 has been widely-studied in different types of cancer [134]. Its role in mediating tumor resistance via inhibition of chemotherapeutic-induced apoptosis has also been researched [135-138]. However, few studies have reported a pro-apoptotic activity of endogenous Gal-3 [139]. Accumulating evidence has shown that Gal-3 exerts its action through its interaction with components of the intrinsic and extrinsic apoptotic pathways [133]. Yu et al. reports that Gal-3 can translocate to the perinuclear mitochondrial membrane as a response to apoptotic stimuli, where it maintains mitochondrial integrity and inhibit cytochrome c release [140]. Ser^6 phosphorylation of Gal-3 is reported to be fundamental for its translocation from the nucleus to the cytoplasm, and hence

regulates Gal-3's antiapoptotic activity [137]. Bcl-2 is one of the first molecules identified as an intracellular Gal-3 ligand, raising the possibility that Gal-3/Bcl-2 interaction is involved in regulating mitochondrial integrity [140, 141]. On the other hand, the shared structural similarities between the NWGR motif of Gal-3 and the anti-death motif of Bcl-2 makes it tempting to speculate that this motif is crucial for the direct interaction of Gal-3 with the mitochondrial permeability transition pore (MPTP) to control its opening [141]. In fact, glycine to alanine amino acid substitution in the NWGR motif negatively impacts the antiapoptotic activity of Gal-3 [136]. Other molecules are involved in regulating the apoptotic signals through Gal-3-binding. Fukumori et al. suggest that intracellular Gal-3 modulates the CD95 apoptotic signaling pathway. Their data show that Gal-3 interacts with CD95 (APO-1/Fas), a member of the death receptor family, and subsequently promotes caspase-8 activation and caspase-3 cleavage, whereas it interferes with apoptotic signals from caspase-8 to mitochondria and represses C2-ceramide-induced apoptotic signals [142]. The yeast two-hybrid screen of a Jurkat T cell cDNA library using Gal-3 as a bait results in the recognition of AIP-1/Alix (ALG-2 linked protein or ALG-2 interacting protein-1) as another cytoplasmic Gal-3 binding partner [143]. AIP-1/Alix was first identified through its interaction with the apoptosis-linked-gene 2 (ALG-2) protein in a Ca^{2+}-dependent reaction [144, 145]. Later, AIP-1/Alix has been reported to inhibit insulin-like growth factor I receptor (IGFIR)-induced paraptosis, a non-apoptotic form of programmed cell death [146]. In addition, the pro-apoptotic molecule Nucling can modulate Gal-3 antiapoptotic activity, not only by transcriptional downregulation

of Gal-3 expression, as discussed above, but also through direct interaction with Gal-3 [41].

Data from melanoma studies are controversial with both pro- and anti-apoptotic activities documented. Borges et al. report that enhanced intracellular expression of Gal-3 in melanoma cells increases the susceptibility to reactive oxygen species (ROS) generated by isatin-Schiff base copper (II) complex, resulting in oxidative cellular damage and apoptosis [147]. In another study, Okra RG-I pectin, a Gal-3 inhibitor, has been shown to induce murine melanoma cell apoptosis and reduce melanoma cell growth *in vitro* [148]. The conflicting activity of Gal-3 was suggested to be subcellular localization-dependent, indicating that nuclear localization is usually associated with pro-apoptotic activity, whereas cytoplasmic localization is associated with anti-apoptotic effects [149]. Nonetheless, the precise mechanisms of how Gal-3 governs cell death pathways continue to be explored.

1.4.3. Gal-3 and cell adhesion, invasion, and metastasis

To successfully metastasize, cancer cells must pass through a series of complex events, which include separation from the primary tumor, invasion through surrounding tissues and basement membranes, entry and survival in the circulation and arrest in a distant target organ vasculature, extravasation and adaptation to the new organ growth environment [150, 151]. Early studies on colon cancer cells reveal that reduction of Gal-3 levels associate with marked decreases in spontaneous metastasis [152]. Similar results were obtained in melanoma models, where silencing of Gal-3 in metastatic melanoma cells

associates with significant reduction of lung metastasis after injection into athymic nude mice [153]. Nevertheless, the underlying molecular mechanisms are still under investigation.

Matrix metalloproteinases (MMPs) are a family of endopeptidases crucial for ECM remodeling, tumor cell invasion and metastasis. They are usually upregulated in response to external oncogenic stimuli [154]. In a study performed with mouse B16F10 melanoma cells, a direct interaction between Gal-3 and AP-1, a transcriptional factor regulating matrix metalloproteinase-1 (MMP-1) expression, was demonstrated, suggesting that Gal-3 can promote melanoma cell invasion and metastasis, at least in part, via induction of MMP-1 expression [155]. Additionally, data show that Gal-3 appears is an essential upstream regulator of MMP-2 [153] and MMP-9 [156] expression in melanoma cells. Interestingly, Gal-3 induced MMP-9 expression is mediated by LAMP1, one of the major carriers of poly-LacNAcs, through the p38 MAPK pathway [156].

Binding of Gal-3 lattices to glycosylated cell surface receptors have a dominant role in controlling cell aggregation and cell-matrix adhesion through modulating receptor trafficking and clustering [8, 48, 157, 158]. High elastin-derived peptides (EDPs) levels associated with advanced melanoma facilitate melanoma cell invasion predominantly through their interaction with Gal-3, αvβ3 integrin and elastin-binding protein (EBP) [159]. Furthermore, binding of Gal-3 lattices to cell surface receptors, such as EGF and TGF-β receptors, limits the internalization and degradation of these receptors, and thus boosts downstream cytokine signaling cascade [160]. On the other hand, Gal-3, which is highly-

expressed on the lung vascular ECs, can mediate organ specific metastasis by acting as an anchor for the circulating melanoma cells [83]. Hence, Gal-3 plays a dual role in enhancing distant melanoma metastasis, not only in the primary tumor site, but also in the metastatic site, and this is probably dictated by the glycan profile of the tumor cells. This hypothesis is supported by results obtained in a study performed on metastatic breast cancer showing that altered O-glycans on the tumor cell surface affects binding of Gal-3 to the tumor cells and regulates the metastatic events of the tumor [161].

1.4.4. Gal-3 and tumor angiogenesis

Tumor angiogenesis, formation of new blood vessels out of pre-existing vessels, is an essential requirement for tumor growth and metastasis to fulfill the increased demand of the growing tumor for oxygen and nutrients [8, 162, 163]. Gal-3 serves as a major player in promoting tumor angiogenesis. Previous studies on human umbilical vein endothelial cells demonstrated the possible role of Gal-3 in capillary tube formation *in vitro* and angiogenesis *in vivo* [164]. As discussed above, Gal-3 can form a complex with α3β1 integrin on the EC surface to potentiate EC function with pericyte-derived NG2 proteoglycan [120]. Furthermore, Gal-3 promotes angiogenesis through modulating vascular endothelial growth factor (VEGF)- and bFGF-mediated angiogenic response, where Gal-3 forms a complex with glycosylated αvβ3 integrin with subsequent activation of downstream signaling pathways that mediate new blood vessels growth [165, 166]. Moreover, microenvironmental Gal-3 potentiates VEGF

signaling and enhance macrophage migration to tumors, which in turn augments TGF-β1 signaling leading to more VEGF production [167].

1.4.5. Gal-3 and immunosuppressive tumor microenvironment

A distinguishing feature of a cancer cell is its ability to evade immune destruction by adapting different mechanisms to create an immunosuppressive microenvironment [18, 168]. Recently, there is a growing evidence that Gal-3 can alter the TME toward a more suppressive phenotype through divergent mechanisms, and thus facilitating melanoma progression [18]. In a study performed on 33 biopsies from 24 melanoma patients, a strong positive correlation was detected between Gal-3 expression and apoptosis of tumor-associated lymphocytes [169]. Another study demonstrated impaired IFN-γ secretion by human $CD8^+$ tumor-infiltrating T lymphocytes (TILs) as a response to extracellular Gal-3, probably as a consequence of the limited accessibility of T-cell receptors to CD8 molecules caused by Gal-3-lattice formation on top of these glycosylated receptors. Restoration of IFN-γ secretion occurred after treatment with GCS-100, a Gal-3 antagonist and also after treatment with anti-Gal-3 antibody [170]. Data from a recent study suggest that the reduced T-cell recruitment in various tumors and disruption of IFN-γ-induced chemokine gradient is caused by Gal-3 oligomerization on the surface of glycosylated IFN-γ and extracellular glycoproteins. This chemokine gradient is essential for proper tumor infiltration by T cells [171]. Moreover, Gal-3 secreted from tumor cells acts as a soluble inhibitory ligand of the NK cell receptor NKp30, highlighting another

mechanism through which Gal-3 compromises immune responses against tumors [172].

1.5. Diagnostic and prognostic values of Gal-3

Gal-3 is overexpressed in various human malignancies, such as breast cancer [173], cervical cancer [174], gastric cancer [175], colon cancer [176], bladder cancer [177], renal cell carcinoma [178], and melanoma [179]. Its upregulation, in many studies, correlates with tumor progression, metastasis and poor outcome [52]. On the other hand, a few cancer types exhibit decreased Gal-3 expression compared with their levels in corresponding normal tissues [179]. These findings raise the possibility of using Gal-3 as a biomarker to predict the progression of primary tumors [50, 179-182].

The diagnostic and/or prognostic significance of Gal-3 in melanoma was first investigated by Vereecken et al. who conducted an immunohistochemical study on various types of melanocytic lesions. Their data show that Gal-3 is considerably overexpressed in thin primary melanoma compared to its levels in benign melanocytic lesions, while metastatic melanomas display low Gal-3 levels compared to thin melanomas. Additionally, high Gal-3 levels are inversely correlated with melanoma aggressiveness as assessed by tumor thickness (Breslow index), the most important determinant of melanoma prognosis [183]. Brown et al. confirm the same observations using tissue microarray (TMA) and immunohistochemistry (IHC) on a larger sample size of benign, dysplastic, and malignant melanocytic lesions. Survival analysis data from this study reveal that high Gal-3 expression is associated with improved overall survival as well as

melanoma-specific survival [179]. Consistently, Abdou et al. demonstrate that malignant melanocytic lesions are associated with significantly higher levels of Gal-3 compared to benign naevi. They also report that localized cytoplasmic staining was a feature of benign melanocytic lesions, while malignant melanocytic lesions exhibit a nucleocytoplasmic staining pattern in approximately 43% of the cases. The nucleocytoplasmic pattern is linked to thick melanoma with poor prognosis, though this observation failed to attain statistical significance [182]. An earlier study conducted by Prieto et al., interestingly, also reports increased nuclear localization of Gal-3 in metastatic melanoma compared to the primary lesions. The authors also observe that lesions with nuclear to cytoplasmic Gal-3 ratio ≥ 1 are strongly correlated with poor survival, suggesting that Gal-3 translocation into the nucleus facilitates melanoma progression by mechanisms that remain poorly understood [184].

In addition to immunohistochemical studies, serum Gal-3 level has also been studied as a potential diagnostic/prognostic biomarker in patients with melanoma. Vereecken et al. report that Gal-3 levels are significantly higher in sera of metastatic melanoma patients compared with its levels in healthy individuals. Moreover, a significant positive correlation is observed between levels of serum Gal-3 and the two conventional melanoma markers: C-reactive protein (CRP) and lactate dehydrogenase (LDH) [185]. Another study from the same group clearly find that serum Gal-3 had a substantial independent prognostic value in stage III and stage IV melanoma patients, with a cut-off value of 10ng/ml [181]. Recently, an electrochemical biosensor based on molecular

imprinted polymers (MIP) detects Gal-3 in sera of melanoma patients, with a detection limit ranging from 0.5ng/mL to 5 µg/mL. Unlike other conventional methods, this device will provide a simple, low cost, fast, and sensitive approach to monitor Gal-3 levels for diagnosis as well as follow up of melanoma patients [5].

1.6. Gal-3-based therapeutic applications

Based on the various mechanisms through which Gal-3 contributes to tumor development and progression, it is hypothesized that blocking Gal-3 with specific inhibitors might impede tumor progression and metastasis. Several studies have validated the therapeutic efficacy of Gal-3 antagonists in different types of cancer e.g., prostate cancer [186], lung adenocarcinoma [187], multiple myeloma [188], either as a monotherapy or in combination with other conventional therapies. Studies on targeting Gal-3 in melanoma models are ongoing and will be highlighted in this section.

1.6.1. Modified citrus pectin (MCP)

Modified citrus pectin (MCP) is a complex polysaccharide of D-galacturonic acid residues linked together by alpha-1, 4 glycosidic bonds. MCP is obtained from the inner peel and pulp of citrus fruits and subjected to thermal, enzymatic and pH treatment to augment its intestinal absorption and enhance binding to its target molecules [189]. The anti-metastatic effect of MCP was first reported by Platt and Raz using the murine B16-F1 melanoma cells in an experimental metastasis model after incubation with various concentrations of MCP [190]. Since then, the antineoplastic properties of MCP have been studied

in many types of cancer [191]. By antagonizing Gal-3 action, MCP slows tumor growth, inhibits its metastasis and strengthens anti-tumor immunity [192]. Recently, Wang et al. has incorporated low molecular weight citrus pectin with the photosensitizer chlorin e6 (Ce6) into a liposome-based delivery system, named as photoactivable Gal-3-inhibitor nanoliposome (PGIL). They tested the therapeutic efficacy of PGIL, both *in vitro* and *in vivo*, using human A375 melanoma cells with the aim of reinforcing the tumor-suppressing effect of Gal-3 blockade with the anti-neoplastic properties of photodynamic therapy (PDT). The results of this study confirm the effectiveness of combining PDT and Gal-3-inhibitor as a novel approach for melanoma management [193].

1.6.2. Belapectin and immunotherapy

Despite great success of immune checkpoint inhibitors (e.g., monoclonal antibodies against programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T lymphocyte antigen 4 (CTLA-4)) in boosting survival rates of patients with advanced melanoma over the past decade, tumor-induced immunosuppression and drug resistance are still unresolved issues. The immunosuppressive properties of Gal-3 in melanoma provoke further thought about combining immunotherapy and Gal-3 inhibitors to enhance anti-tumor immunity to increase response rates to therapy and improving overall survival. In this regard, Belapectin (GR-MD-02), a novel apple pectin-derived Gal-3 inhibitor, has recently been investigated in a phase I clinical trial combined with pembrolizumab (anti-PD-1) for treatment of patients with metastatic melanoma. Data from this study has demonstrated favorable clinical response to the

belapectin + pembrolizumab therapy with enhanced T-cell activation and marked decline of circulating monocytic myeloid-derived suppressor cells (M-MDSCs) [194].

1.6.3. Gal-3-based cancer vaccination

Early studies report the detection of autoantibodies against Gal-3 in sera of melanoma patients [195]. Furthermore, $CD8^+$ and $CD4^+$ T-cell responses against Gal-3-derived peptides have been demonstrated in peripheral blood mononuclear cells (PBMCs) isolated from both healthy individuals and patients diagnosed with different types of cancer, including melanoma [196]. The antigenicity of Gal-3 has sparked the interest of researchers to propose novel immune-modulatory strategies for treating melanoma. Instead of targeting the extracellular Gal-3 protein, Gal-3-based vaccines were developed to augment immune response against Gal-3-expressing tumor cells. In a recent preclinical study on murine tumor model, Gal-3-derived peptide vaccine established a more immune permissive tumor microenvironment associated with prominent tumor growth delay [196]. Further studies are needed to validate these promising results and evaluate the anti-melanoma therapeutic efficacy of combined Gal-3-derived vaccine and immune checkpoint inhibitor in preclinical/clinical testing.

1.7. Overview

During the past two decades, there has been considerable research dedicated to elucidating the role of Gal-3 in tumor progression, with emphasis on its key roles in regulating different cancer hallmarks, such as intracellular oncogenic signaling, cell adhesion, invasion, metastasis, angiogenesis, and immunosuppressive microenvironments. The focus of this dissertation is to characterize the role of extra-/intracellular Gal-3 in melanoma progression. The expression patterns of Gal-3 in sera and tumor tissues from melanoma patients and its correlation with melanoma stage is covered in CHAPTER TWO. The functional influence of intracellular loss of Gal-3 on melanoma behavior is covered in CHAPTER THREE. The regulatory role of Gal-3 on the pro-oncogenic signaling pathways in melanoma is discussed in CHAPTER FOUR. Conclusions and future directions are discussed in CHAPTER FIVE.

In CHAPTER TWO, I report my analysis on the expression patterns of Gal-3 in the serum and tumor tissues of melanoma patients and its association with the stage of melanoma. My findings reveal that melanoma patients exhibit significantly elevated levels of circulating Gal-3 compared to healthy individuals, while there is no difference in Gal-3 levels between early and advanced stages of melanoma, suggesting its potential as a diagnostic marker for suspected melanoma cases but limited usefulness in monitoring melanoma progression. Moreover, I demonstrate a characteristic downregulation of tumor-intrinsic Gal-3 expression with melanoma progression, both in human melanoma samples and in murine melanoma models. This indicates a possible link between the loss of intracellular Gal-3 and the

metastatic capacity of melanoma cells, proposing a prognostic value of melanoma-intrinsic Gal-3 in predicting melanoma metastasis.

In CHAPTER THREE, I report my findings on the functional consequences of Gal-3 depletion in melanoma cells on their metastatic behavior. The results of my investigations demonstrate that Gal-3-silenced cells exhibit enhanced migration, invasion, and colony formation capacities compared with control cells. Moreover, loss of Gal-3 significantly promoted tumor growth in xenograft tumor models and resulted in higher tumor burden in in vivo metastatic studies compared to control cells, indicating that intracellular Gal-3 probably acts as a suppressor of melanoma metastasis.

In CHAPTER FOUR, I report my findings on the regulatory role of Gal-3 on the pro-oncogenic signaling pathways in melanoma. My data indicate that the downregulation of Gal-3 in melanoma cells resulted in continuous activation of the PI3K/AKT, MAPK/ERK, and Wnt/β-catenin signaling pathways. Additionally, my results support the existence of a negative regulatory function for Gal-3 on the expression of nuclear factor of activated T cells (NFAT1) and its downstream metastasis-associated targets: MMP-3, IL-8, and GPC6 in melanoma cells.

In conclusion, my data highlight the tumor-suppressive function of intracellular Gal-3 in melanoma cells, implicating tumor-intrinsic Gal-3 as a prognostic indicator of melanoma metastasis.

2. CHAPTER 2: EXPRESSION PATTERN OF GAL-3 IN THE SERUM AND TUMOR TISSUES OF MELANOMA PATIENTS AND ITS CORRELATION WITH MELANOMA STAGE

2.1. Abstract

Melanoma is a skin cancer characterized by a greater propensity for early metastasis with high mortality rates compared to many other cancer types. Identifying reliable biomarkers for melanoma diagnosis and prognosis is crucial for effective management. In this study, we explored the expression pattern of Gal-3 in the sera and tumor tissues of melanoma patients and its association with melanoma stage. Our results revealed that melanoma patients have significantly higher levels of circulating Gal-3 compared to healthy individuals with no difference in Gal-3 levels between early and advanced melanoma stages, suggesting a diagnostic role for Gal-3 in suspected melanoma cases and limited value in detecting advanced melanoma progression in patients with primary melanoma. On the other hand, we demonstrated that tumor-intrinsic Gal-3 expression is progressively downregulated with melanoma advancement to metastatic disease in human samples and in murine melanoma models, denoting a possible correlation between loss of intracellular Gal-3 and the metastatic capability of melanoma cells.

2.2. Introduction

Melanoma is potentially a life-threatening melanocyte-derived skin neoplasm known for its aggressive clinical behavior and poor outcomes in patients with late-stage, metastatic disease [197]. Melanoma incidence has been

increasing worldwide over the last few decades [198]. Despite recent breakthroughs in immunotherapy and targeted therapy which have noticeably increased the survival expectancy of melanoma patients [199], the prognosis of patients with late-stage disease remains dismal with a 5-year survival rate of only 32% [200]. Therefore, surveillance for distant metastasis in patients with primary melanoma is fundamental to provide an accurate prognosis, an optimal treatment choice, and a personized follow-up maintenance plan. The histopathological features of primary lesions (including Breslow thickness), sentinel lymph node biopsy, imaging studies, and routine blood testing (LDH, S100 protein) are collectively used to identify metastatic disease [201]. However, there is no absolute predictor of metastasis in patients with primary melanoma and lifelong follow-up is crucial for early detection of potential metastasis [201, 202]. In-depth understanding of the molecular mechanisms that govern melanoma metastasis could uncover new diagnostic markers and help identify targets to develop innovative therapeutic strategies [203].

Tumor metastasis is a complex, multi-step process, in which cancer cells with certain genotypic and phenotypic alterations dislodge from the primary tumor and migrate through the bloodstream or the lymphatic system as single cells or small clusters of cells, known as circulating tumor cells (CTCs) [204, 205]. A small fraction of CTCs can eventually settle in distant organs, grow, and form secondary tumors [206]. The intrinsic properties of CTCs, such as their ability to invade surrounding tissues, elude immune-mediated clearance, and adapt to local tissue microenvironmental factors, are among the major determinants of

their metastasis-initiating capacity [207]. Throughout this functional cascade, several factors, including adhesion molecules, proteases, and cytokines, are either upregulated or downregulated by cancer cells to facilitate migration, invasion, and metastasis formation [208-210].

To this end, galectins, a 15-member family of ß-galactoside-binding lectins, are known as crucial regulators of several cellular events [211]. The role of galectins in regulating tumor initiation, progression and drug resistance are also now gaining attention in the cancer research field [212, 213]. Gal-3 is the only chimera type galectin that has a single CRD linked to a matrix metalloproteinase cleavable N-terminal domain [24]. This characteristic structure allows oligomerization of Gal-3 monomers into pentamers through their N-terminal domains with formation of lattice-like structure [214]. While the carbohydrate-binding activity of pentameric Gal-3 predominates extracellularly to crosslink glycosylated proteins on cell surfaces and ECM [52], intracellular Gal-3 can engages in carbohydrate-independent interactions with other cytosolic proteins to impact many biological processes through the modulation of signaling pathways [24]. Gal-3 is among the most extensively studied galectins due to its aberrant expression and localization reported in many cancer types, including melanoma [23]. Membrane-bound and extracellular Gal-3 are theorized to bind cancer cell surface glycoconjugates to promote homotypic cell adhesion that facilitates the formation of apoptosis-resistant circulating emboli [215]. Furthermore, Gal-3 can also interact with CTCs and endothelial cells (ECs) to facilitate cancer cell extravasation and organ colonization [83]. Prior studies have

reported high serum Gal-3 levels in patients with melanoma, some of which suggested a positive correlation between Gal-3 serum levels and melanoma stage [181, 216, 217]. However, Gal-3 levels in melanoma cells and its relationship to clinical behavior is still unclear.

Here, we report that Gal-3 is upregulated in the sera of early as well as advanced-stage melanoma patients compared with healthy individuals, while tissue-intrinsic Gal-3 exhibit an apparently opposite trend where its expression is progressively downregulated from normal skin to primary melanomas, and further declined in metastatic melanomas. Moreover, our findings provide evidence supporting the role of various cells in the TME, mainly fibroblasts and monocytes/macrophages, in the production of Gal-3, which further highlights the intricate sources of extracellular and circulating Gal-3 in melanoma patients. Collectively, our data suggest that, unlike serum Gal-3, Gal-3 levels in melanoma cells inversely correlate with their metastatic abilities.

2.3. Materials & Methods

2.3.1. Gene expression data collection and processing.

Transcriptomic data of 33 cancers, including skin cutaneous melanoma (SKCM) samples (n = 481), were retrieved from The Cancer Genome Atlas (TCGA) database, while normal skin samples (n = 977) were collected from Genotype Tissue Expression (GTEx) database. Data were analyzed and visualized using UCSC Xena (http://xena.ucsc.edu) [218]. Exclusion criteria were as follows: (1) Samples with missing data regarding sample type or Gal-3 expression; (2) TCGA melanoma samples categorized as solid tissue normal or additional metastatic.

As a result, a total of 10,284 samples from 33 cancer types, including 471 SKCM samples (103 primary melanoma and 368 metastatic melanoma) and 812 normal skin samples were included in the study. The obtained gene expression profiles were generated using the Illumina HiSeq 2000 RNA Sequencing platform and presented as log2(value + 1) transformed RSEM normalized count. The Gene Expression Omnibus (GEO) browser (https://www.ncbi.nlm.nih.gov/geo/browse/) was searched for datasets that include primary and metastatic melanoma samples. The microarray datasets GSE8401 (included 31 primary melanoma samples and 52 metastatic melanoma samples) and GSE7553 (included 14 primary melanoma samples and 40 metastatic melanoma samples) were uploaded to the interactive web tool (GEO2R) (https://www.ncbi.nlm.nih.gov/geo/geo2r/) that uses 'limma' package of R programming language for gene expression analysis. The RNA-seq dataset GSE157740 (included 2 melanoma CTC lines and their patient-matched primary or metastatic specimens) was analyzed by the online GREIN platform (http://www.ilincs.org/apps/grein/?gse=) [219]. An adjusted p value < 0.05 was considered significant. The GEO browser was also searched for single-cell RNA sequencing (scRNA-seq) data of immune cells from human melanoma tumors. The scRNA-seq datasets GSE139249 (included 5 samples of melanoma metastases) and GSE123139 (included primary and metastatic melanoma samples from 25 patients) were analyzed and visualized using the Tumor Immune Single-cell Hub 2 (TISCH2) tool [220] to determine Gal-3 expression patterns across major cell lineages in each scRNA-seq cohort. The TISCH2

platform (http://tisch.comp-genomics.org/home/) provides the protocol of data collection, processing, and cell-type annotation.

2.3.2. Patients and sample processing.

Blood samples were collected from 44 melanoma patients admitted to the New York University Langone Health (NYULH) between July 2015 and October 2019. Blood samples from healthy control donors were obtained from the Biospecimen Repository Facility at Miami Cancer Institute Baptist Health-South Florida. Samples were centrifuged at 3000 rpm for 10 min, and serum was aliquoted and stored at −80 °C until further analysis.

2.3.3. ELISA assay.

Gal-3 was measured in the serum samples using a commercial enzyme-linked immunosorbent assay (ELISA) Kit (DGAL30, R & D systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The absorbance of samples was measured at 450 nm and a standard curve was generated using a Cytation 5 reader and Gen5 software (BioTek Instruments, Winooski, VT, USA). All samples were analyzed in triplicates. Gal-3 levels in melanoma patients were compared with those in age-matched healthy control donors. The results were expressed as ng/ml. Receiver Operating Characteristic (ROC) curve was plotted to determine the prognostic ability of serum Gal-3, and optimal cut-off value was determined by maximizing the sum of specificity and sensitivity.

2.3.4. Cell lines and cell culture

Human melanoma cell lines (SK-MEL-2, SK-MEL-5, A375 and A2058) and B16F0 murine melanoma cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). B16/BL6 murine melanoma cell line was purchased from Accegen Biotechnology (Fairfield, NJ, USA). Cells were grown in their respective culture media (Dulbecco's modified Eagle's medium (DMEM) for SK-MEL-5, A375, A2058 and B16F0 cell lines and RPMI 1640 medium for SK-MEL-2 and B16/BL6 cell lines) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% Antibiotic-Anti-mitotic (Gibco, Waltham, MA, USA), and maintained in a humidified 37 °C incubator with 5% CO2. Cell lines were passaged when they reached approximately 80% confluency and were regularly tested with PlasmoTest (InvivoGen, San Diego, CA, USA) to ensure the absence of mycoplasma contamination.

2.3.5. RT-qPCR Analysis

For gene expression analysis, RNA was extracted using RNeasy Plus kit (mini) (Qiagen, Ontario, Canada) and cDNA synthesized using SuperScriptTM VILOTM cDNA synthesis kit (Invitrogen; Thermo Fisher, CA, USA) per manufacturer's protocol. Real-time quantitative PCR was then performed with TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan® primers to amplify human LGALS3 (assay ID: Hs00173587_m1), human GAPDH (assay ID: Hs02786624_g1), mouse LGALS3 (assay ID: Mm00802901_m1), and mouse GAPDH (assay ID: Mm99999915_g1) per manufacturer's protocol.

2.3.6. Western blotting

The cells were lysed in Pierce™ RIPA buffer (Thermo Scientific™) with protease and phosphatase inhibitor cocktail (Thermo Scientific™). After a 30 min incubation on ice, cell lysates were centrifuged for 10 mins at 10,000 RPM in 4°C. Protein concentrations were calculated using Pierce™ BCA protein assay kit (Thermo Scientific™) per manufacturer protocol, and equal protein amounts from each sample were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). 30 µg of protein samples were boiled for 5 mins and subsequently loaded on a 4-12% gradient SDS PAGE gel (BioRad) for electrophoresis. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, Massachusetts), blocked for 1h at room temperature with Intercept® (TBS) blocking buffer (LI-COR, Lincoln, NE, USA), and incubated overnight at 4°C with primary antibodies against Gal-3 (1:1000, #125402, Biolegend, San Diego, CA, USA) and β-actin (1:1000, #8457, Cell Signaling, Danvers, MA, USA). The membranes were later washed and incubated with secondary antibodies (1:10,000, IRDye® 800CW Goat anti-Rat IgG and IRDye® 800CW Goat anti-Rabbit IgG, LI-COR, Lincoln, NE, USA) for 1hr at room temperature. The membranes were then visualized and analyzed with Li-Cor imager.

2.3.7. Flow cytometry

To analyze surface expression of Gal-3, cells were harvested using Accutase (Fisher), washed with PBS and resuspended in Alexa Fluor 647 anti-mouse/human Mac-2 (Galectin-3) antibody (Biolegend, #125408, San Diego, CA,

USA) and Aqua Live/Dead stain (Invitrogen Life Technologies, Carlsbad, CA, USA) for 30 min on ice. Single-color, isotype, and unstained controls were also prepared for validation. Cells were washed, resuspended in 200µl of PBS. Flow cytometric were acquired using FACSCelesta (BD Biosciences, San Jose, CA, USA) and analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

2.3.8. Statistical Analysis

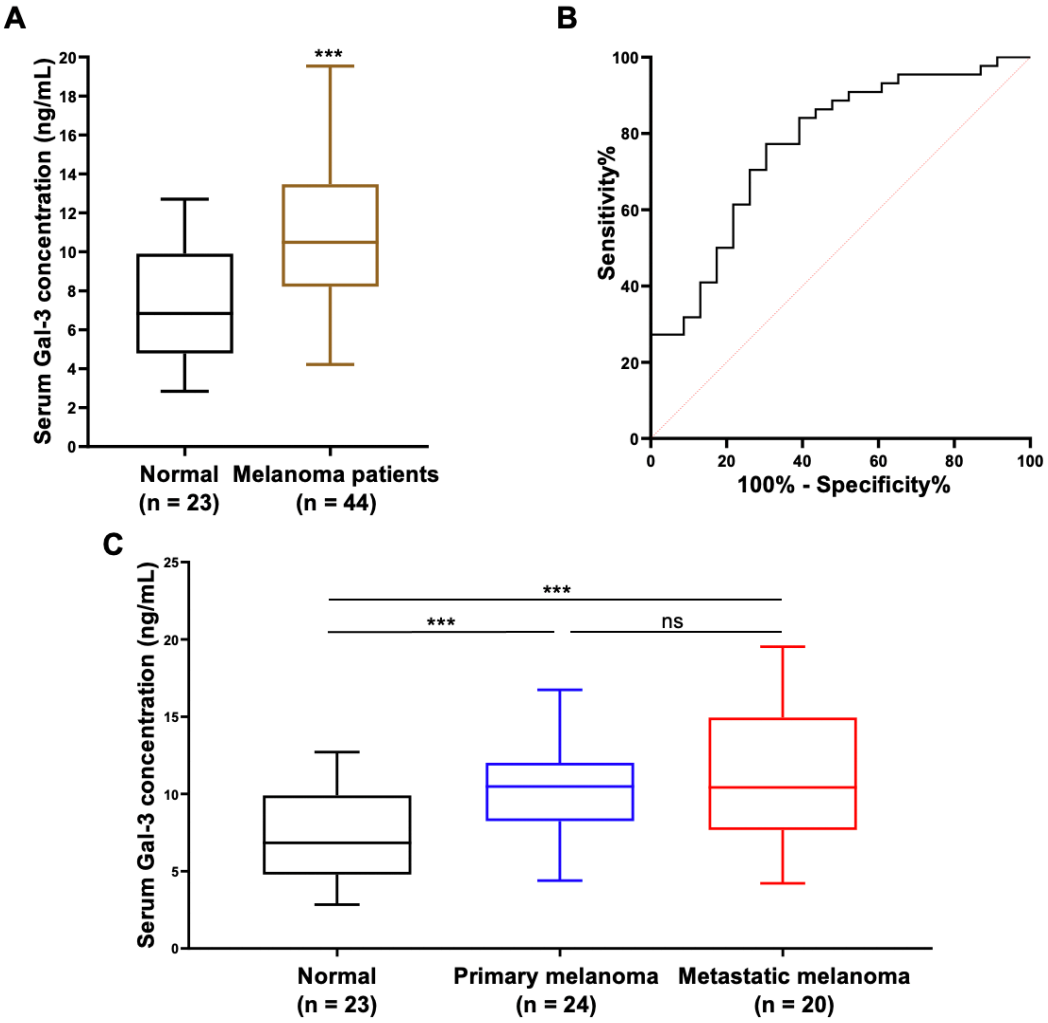
Prism 8.0 software (GraphPad) was used for statistical analysis. For normally distributed data involving two groups, unpaired two-tailed Student's *t*-test was used. For non-normally distributed data, analysis was performed using a Mann–Whitney test; normality was assessed using a Shapiro–Wilk test. Throughout, data are presented as the means ± SEM, unless otherwise noted. *P* value of <0.05 was considered statistically significant.

2.4. Results

2.4.1. Gal-3 is elevated in the sera of melanoma patients with no significant difference between early and advanced melanoma stages.

To explore the possible role of serum Gal-3 as a biomarker of melanoma progression, circulating Gal-3 levels were measured in sera from forty-four patients with melanoma and twenty-three age-matched healthy controls. Twenty-four patients presented with primary melanoma (stage 0, I or II), while twenty patients presented with metastatic melanoma (stage III or IV). We found that melanoma patients had significantly elevated circulating levels of Gal-3 compared to age-matched healthy subjects (10.76 (±3.45) ng/mL vs. 7.24 (±2.95) ng/mL, p<0.001) (**Figure 2.1 A**). The area under receiver operating characteristic

(ROC) curve for Gal-3 to predict melanoma was determined at >7.74 ng/ml. This cut-off value had a sensitivity of 77.3%, a specificity of 69.6%, positive and negative predictive values of 82.9 and 61.5%, respectively. The area under the curve (AUC) was 0.771 [95% CI, 0.651–0.891] ($p<0.001$) (**Figure 2.1 B**). No significant difference of circulating Gal-3 levels was detected between patients who developed metastases, including regional lymph node metastases, and those with non-metastatic melanoma (11.22 (±4.14) ng/mL vs. 10.38 (±2.78) ng/mL, $p=0.43$) (**Figure 2.1 C**). These data suggest that serum Gal-3 can be a useful diagnostic biomarker for melanoma, in combination with conventional melanoma markers. However, its utility as a prognostic indicator of melanoma progression and metastasis is limited.



<u>Figure 2.1.</u> Gal-3 is elevated in the sera of melanoma patients.
ELISA-measured serum Gal-3 levels depicted significantly higher circulating levels of Gal-3 in melanoma patients, compared to age-matched healthy subjects **(A)**. The area under receiver operating characteristic (ROC) curve for Gal-3 to predict melanoma was determined at a cut-off value of 7.74 ng/ml with a sensitivity of 77.3% and a specificity of 69.6% **(B)**. No significant expression difference was detected between patients with metastatic melanoma and those with non-metastatic melanoma **(C)**. (***p<0.001)

2.4.2. Gal-3 is progressively downregulated from normal skin to primary melanomas, and further depressed in metastatic melanomas.

First, we applied a pan-cancer analysis of Gal-3 expression in samples from 33 different types of cancer (n = 10,284) collected in the TCGA database. We found that melanoma is among the top Gal-3-expressing tumors (**Figure 2.2**).

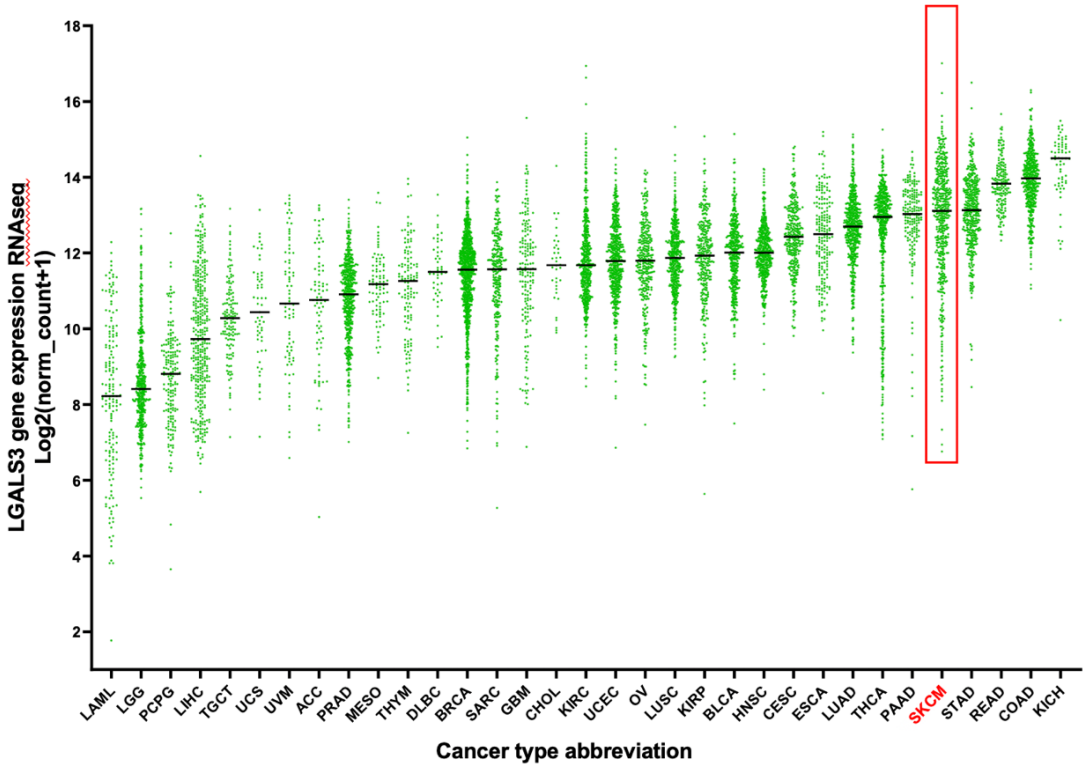


Figure 2.2. Melanoma is among the top Gal-3-expressing tumors in TCGA pan-cancer data.
Transcriptional expression analysis of Gal-3 in 10,284 samples from 33 different types of cancer collected in the TCGA database. Each green dot represents one patient. Medians are depicted as horizontal black lines. SKCM=Skin cutaneous melanoma

However, Gal-3 expression was significantly downregulated in the TCGA melanoma samples (n = 471) compared to its expression in the GTEx normal skin tissues (n = 812) (p<0.001) (**Figure 2.3**).

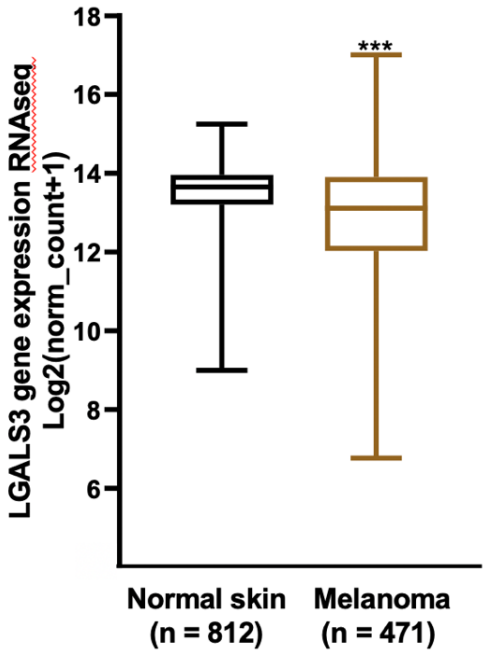
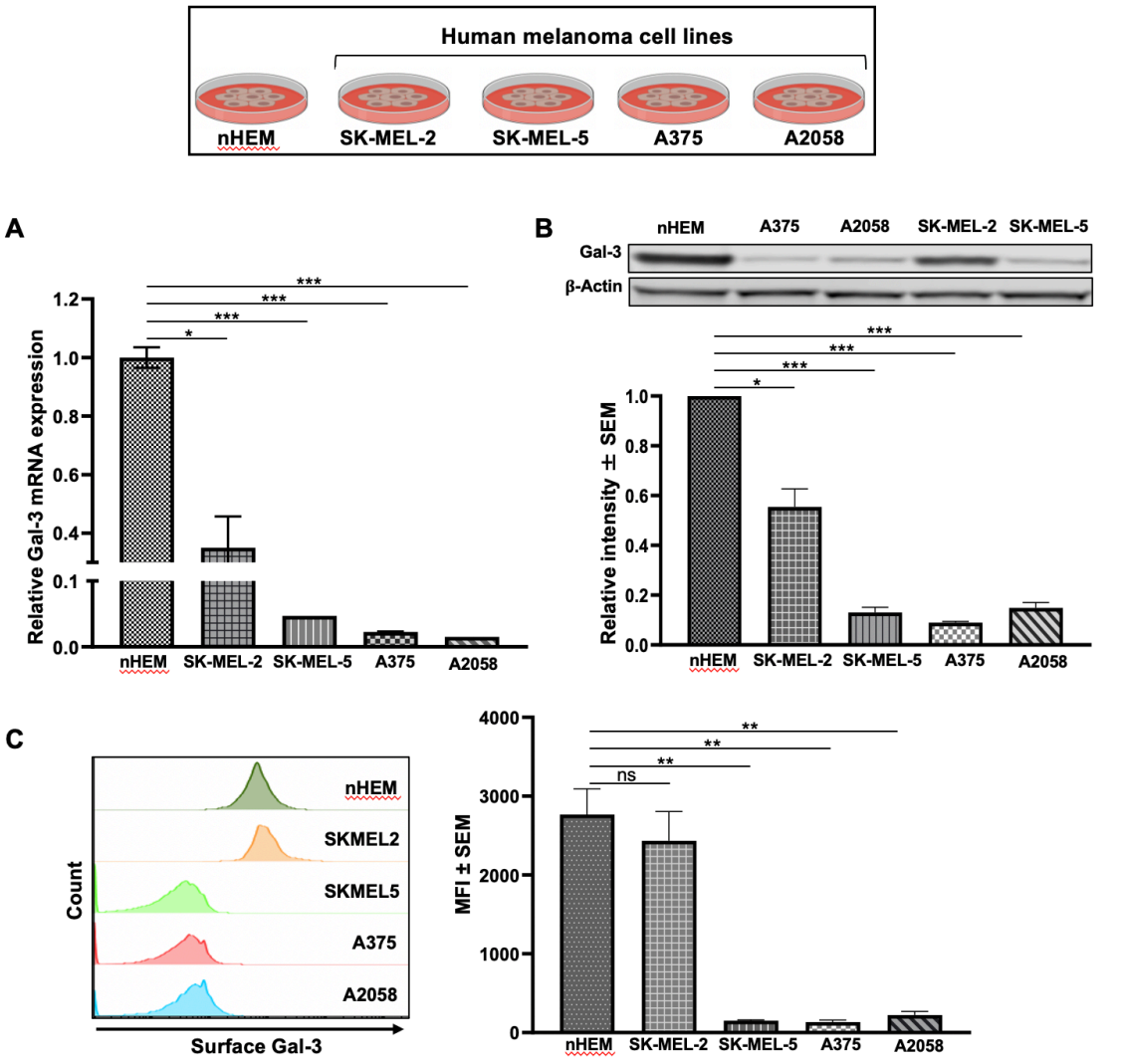


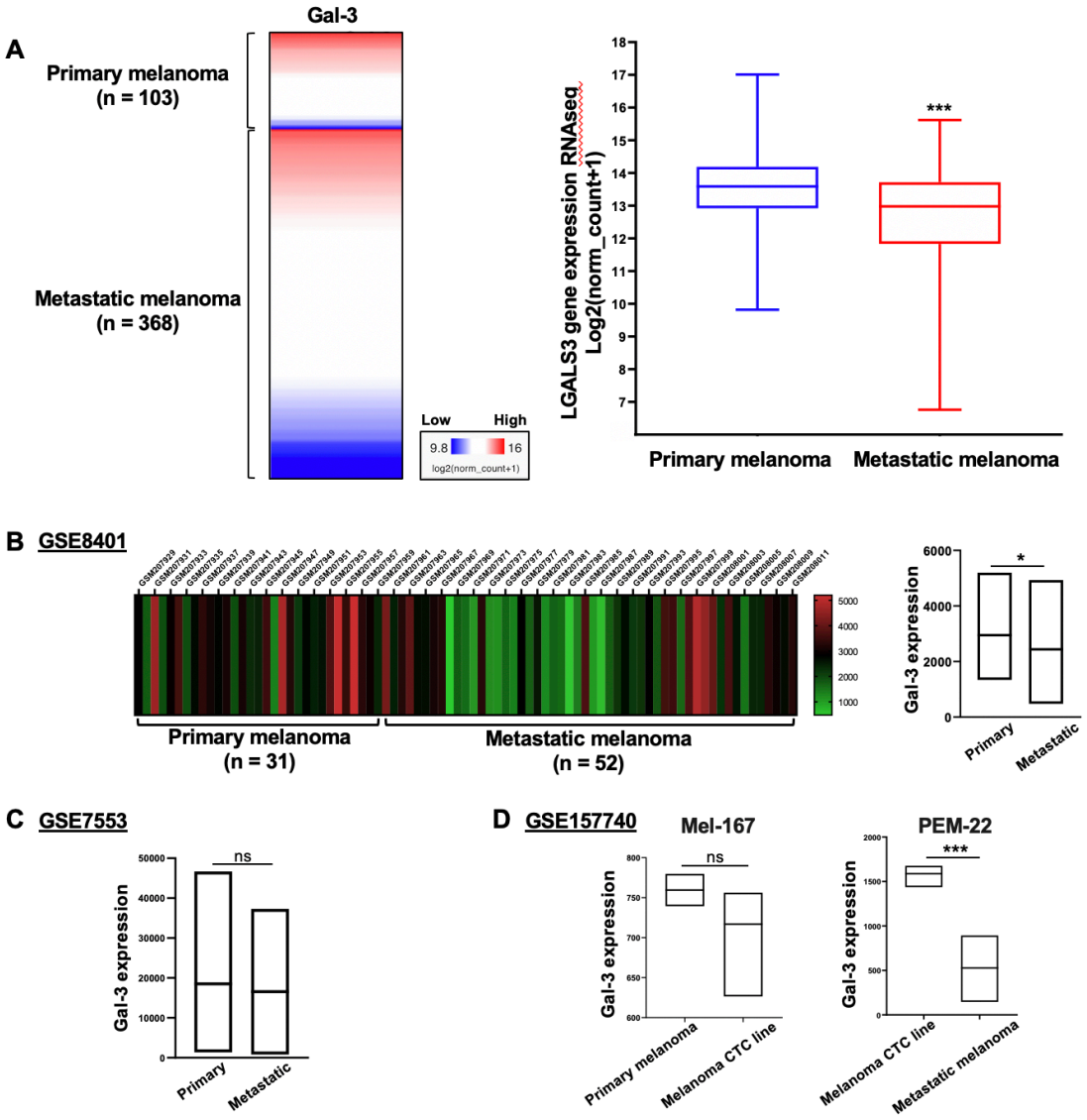
Figure 2.3. Gal-3 is downregulated in melanoma samples compared with normal skin tissues.
Transcriptional expression analysis of Gal-3 in 471 TCGA melanoma samples versus 812 GTEx normal skin tissues. (***p<0.001)

Considering that melanocytes are underrepresented in the normal skin epidermal layer with a keratinocyte–melanocyte ratio of approximately 35:1 [221], we sought assessing Gal-3 expression levels in normal human epidermal melanocytes (nHEM) and in a panel of human melanoma cell lines (A375, A2058, SK-MEL-2 and SK-Mel-5) using RT-qPCR (**Figure 2.4 A**), and immunoblotting (**Figure 2.4 B**). Our results showed that nHEM exhibited significantly higher Gal-3 expression compared with the four human melanoma cell lines included in this study: SK-MEL-2 ($p<0.05$), SK-MEL-5 ($p<0.001$), A375 ($p<0.001$), and A2058 ($p<0.001$). We also analyzed the expression of membrane bound Gal-3 by flow cytometry (**Figure 2.4 C**), which showed a significantly higher expression of Gal-3 on nHEM surface compared to SK-MEL-5 ($p<0.01$), A375 ($p<0.01$), and A2058 ($p<0.01$), with no significant difference of surface expression of Gal-3 between nHEM and SK-MEL-2. These data indicate that melanoma cells express variable levels of Gal-3, both intracellularly and extracellularly.



<u>Figure 2.4.</u> Normal melanocytes express higher levels of Gal-3 compared with melanoma cell lines.
The expression levels of Gal-3 in melanoma cell lines of variable metastatic potential versus nHEM were examined by RT-PCR **(A)**, Western blot **(B)**, and flow cytometry **(C)**. (***p<0.001, **p<0.01, *p<0.05)

We further explored the TCGA database to compare the Gal-3 expression profiles of primary melanoma samples (n = 103) versus metastatic melanoma samples (n = 368). Data analysis revealed a significantly higher expression of Gal-3 in primary melanomas compared with metastatic melanomas ($p<0.001$) (**Figure 2.5 A**). Consistently, data analysis of the microarray dataset GSE8401 demonstrated lower expression of Gal-3 in metastatic melanoma samples (n = 52) compared to primary melanomas (n = 31) ($p<0.05$) (**Figure 2.5 B**), while no significant difference between metastatic (n = 40) and primary melanomas (n = 14) was observed in the dataset GSE7553 (**Figure 2.5 C**). Furthermore, RNA-seq dataset GSE157740 was analyzed to compare Gal-3 transcriptional profiles of PEM-22 melanoma CTC lines versus six patient-matched–independent archival metastatic lesions, while transcriptional profiles of Mel-167 CTCs were compared versus patient-matched primary tumor. Data analysis demonstrated that Gal-3 expression in Mel-167 CTCs was lower than its levels in patient-matched primary tumors. However, it failed to achieve a statistically significant difference (**Figure 2.5 D, left panel)**. On the other hand, Gal-3 expression is downregulated in metastatic lesions compared to patient-matched PEM-22 CTCs ($p<0.001$), suggesting a predilection of Gal-3–deficient cells in metastatic foci (**Figure 2.5 D, right panel)**.



<u>Figure 2.5.</u> Gal-3 is downregulated in metastatic melanomas compared to primary melanomas.
Transcriptional expression analysis of Gal-3 in metastatic melanomas versus primary melanomas retrieved from TCGA SKCM database **(A)**, the GEO microarray datasets GSE8401 **(B)** and GSE7553 **(C)**, and the GEO RNA-seq dataset GSE157740 **(D)**. (***p<0.001, **p<0.01, *p<0.05)

To further confirm the negative correlation between the metastatic potential of melanoma cell and intrinsic Gal-3 expression levels, we investigated the expression levels of Gal-3 in a murine melanoma cell line with low metastatic potential (B16 cells) and in its highly metastatic subline (B16/BL6). A marked decline of Gal-3 expression was observed in B16/BL6 cells versus B16 cells using RT-qPCR (**Figure 2.6 A)**, immunoblotting (**Figure 2.6 B)**, and flow cytometry analysis (**Figure 2.6 C)** ($p<0.001$, $p<0.001$, $p<0.001$, respectively). Together, these results led us to hypothesize that loss of intracellular Gal-3 in melanoma cells may promote its metastatic dissemination.

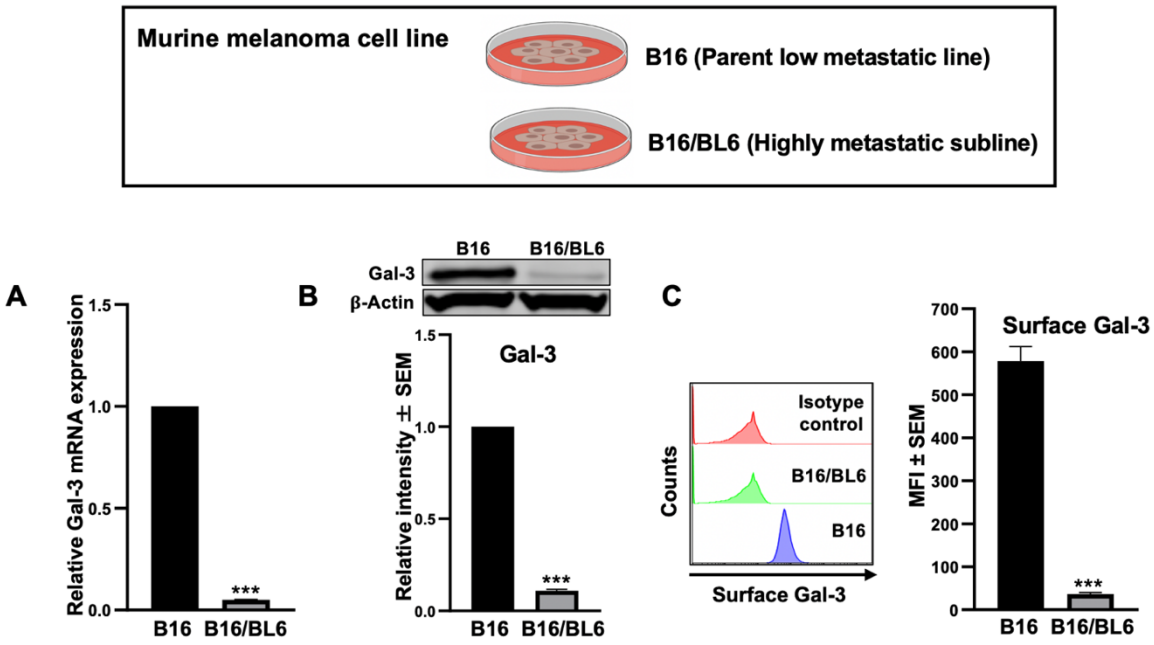


Figure 2.6. Loss of Gal-3 in the highly metastatic murine melanoma subline B16/BL6 versus the parent B16 line.
Gal-3 expression analysis in the highly metastatic murine melanoma cell subline (B16/BL6) compared with its parent line (B16) by RT-PCR **(A)**, Western blot **(B)** and flow cytometry **(C)**. (***$p<0.001$)

2.4.3. Gal-3 is expressed by other cells in the TME.

To determine possible sources of microenvironmental Gal-3 rather distinct from cancer cells, we analyzed scRNA-seq data of immune and stromal cells within human melanoma samples. Data analysis of two GEO datasets: GSE139249 (**Figure 2.7 A**) and GSE123139 (**Figure 2.7 B**), by the TISCH2 webtool revealed variability in Gal-3 expression among immune and stromal cells in the TME, with fibroblasts and monocytes/macrophages constitute the major contributors to the Gal-3 pool in the melanoma microenvironment. These findings indicate that, besides cancer cells, extracellular Gal-3 can be derived from different cells within the TME, which probably represent fundamental sources of the metastasis-promoting extracellular and circulating Gal-3.

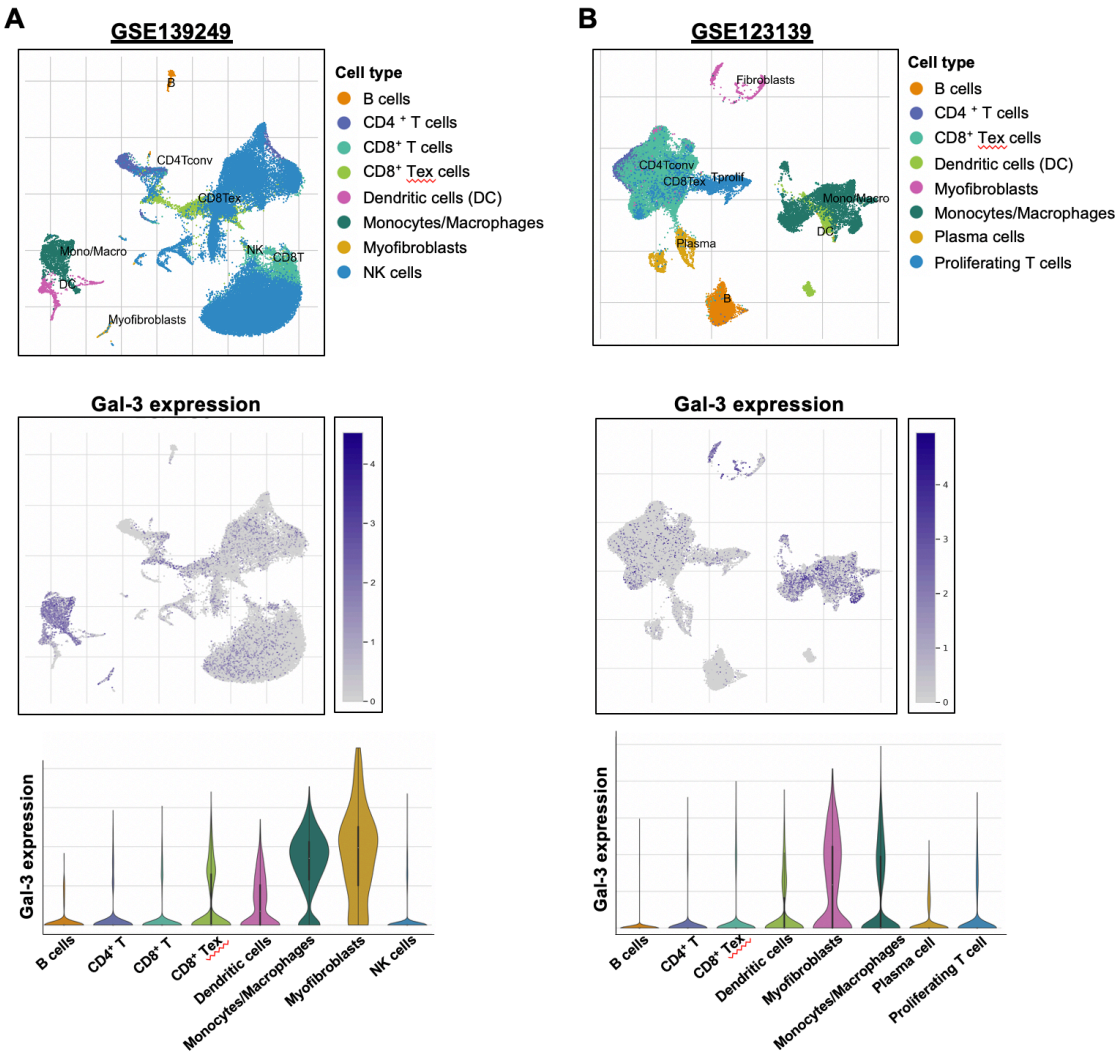


Figure 2.7. Gal-3 is expressed by immune and stromal cells in the TME. scRNA-seq data analysis of immune cells within human melanoma samples retrieved from the GEO datasets GSE139249 **(A)** and GSE123139 **(B)**. The distribution of various cells in melanoma TME within each dataset is depicted in the upper panels. The middle panels represent heatmaps of Gal-3 expression levels in the TME cells. The lower panels represent quantification of Gal-3 expression data, categorized based on cell type.

2.5. Discussion

Gal-3 has gained considerable attention over the past years owing to its diverse activities in cancer investigations [222]. The dysregulated expression of Gal-3 in melanoma has been extensively studied with a view to potential applications in melanoma diagnosis and therapeutics [214]. In this study, we have provided evidence supporting the negative correlation between Gal-3 expression in melanoma cells and their metastatic potential. The expression of Gal-3 was found to be lower in TCGA melanoma samples compared with GTEx normal skin tissues. Additionally, a significant trend of Gal-3 loss was observed in metastatic melanoma samples versus primary melanomas in patient cohorts retrieved from both TCGA and GEO databases as well as murine melanoma models.

Earlier studies sought to evaluate the validity of serum Gal-3 levels as a potential biomarker for predicting advanced melanoma [181, 216, 217]. Vereecken et al. report that Gal-3 levels are significantly higher in sera of metastatic melanoma patients compared with its levels in healthy individuals. Moreover, a significant positive correlation is observed between levels of serum Gal-3 and the two conventional melanoma markers: C-reactive protein (CRP) and lactate dehydrogenase (LDH) [185]. Another study from the same group clearly find that serum Gal-3 has a substantial independent prognostic value in stage III and stage IV melanoma patients, with a cut-off value of 10ng/ml [181]. Interestingly, an electrochemical biosensor based on molecular imprinted polymers (MIP) has been recently developed to detect Gal-3 in sera of

melanoma patients, with a detection limit ranging from 0.5ng/mL to 5 μg/mL, providing a simple, low cost, fast, and sensitive approach to monitor Gal-3 levels for diagnosis as well as follow up of melanoma patients [5]. In consistent with these data, we have found that Gal-3 levels are significantly higher in serum samples from melanoma patients compared to healthy individuals. However, no difference in serum Gal-3 level was detected between patients with and without metastatic dissemination. Serum Gal-3 in cancer patients can be derived from cancer cells as well as immune and stromal cells in the TME as a host response to tumor antigen [223]. In this regard, we revealed that fibroblasts and monocytes/macrophages are major sources of extracellular Gal-3 in melanoma TME, likely contributing to the overall levels of Gal-3 in the bloodstream of melanoma patients. Besides, serum Gal-3 has been considered as an inflammatory marker with reported high levels in a plethora of other diseases [224-226], making the reliability of serum Gal-3 as a predictive marker of melanoma metastasis is questionable.

On the other hand, tumor-intrinsic levels of Gal-3 and its relationship to melanoma stage and clinical outcome is currently lacking and not well-understood. The diagnostic and/or prognostic significance of Gal-3 in melanoma was first investigated by Vereecken et al. who conducted an immunohistochemical study on various types of melanocytic lesions. In alignment with our notion, the authors show that Gal-3 is considerably overexpressed in thin primary melanoma compared to its levels in thick and metastatic melanomas. Additionally, high Gal-3 levels are inversely correlated with

melanoma aggressiveness as assessed by tumor thickness (Breslow index), the most important determinant of melanoma prognosis [183]. Brown et al. confirm the same observations using tissue microarray (TMA) and immunohistochemistry (IHC) of various melanocytic lesions. Survival analysis data from this study reveal that high Gal-3 expression is associated with improved overall survival as well as melanoma-specific survival [179]. On the contrary, an immunohistochemical study, conducted by Prieto et al. using TMA blocks of melanocytic lesions, reports a statistically significant acquisition of Gal-3 expression with melanoma progression [184]. The authors reveal an increased nuclear localization of Gal-3 in metastatic melanoma compared to the primary lesions. They also observe that lesions with nuclear to cytoplasmic Gal-3 ratio ≥ 1 are strongly correlated with poor survival, suggesting that Gal-3 translocation into the nucleus facilitates melanoma progression by mechanisms that remain poorly understood [184]. It is noteworthy that an under- or over-estimation of Gal-3 expression levels in clinical melanoma samples may be a consequence of the well-established phenomenon of melanoma heterogeneity, suggesting that a single tumor biopsy might occasionally be non-representative of the entire tumor [227, 228].

In conclusion, our data suggest a negative association between the expression levels of Gal-3 in melanoma cells and their metastatic capability. Thus, we propose that tumor-intrinsic Gal-3 levels could be added to the conventionally used prognostic indictor “Breslow index” for a stronger prediction of metastasis in melanoma patients. However, further investigations are required on a larger sample size and multiple biopsies from melanocytic lesions at

different stages of melanoma to validate the observed decline in Gal-3 expression during melanoma progression. Additionally, in vitro studies are necessary to investigate the functional impact of intracellular Gal-3 on cancer cell behavior and elucidate its potential mechanistic role in governing melanoma metastasis, which will be highlighted in Chapters Three and Four.

3. CHAPTER 3: TUMOR-INTRINSIC GAL-3 NEGATIVELY REGULATES MELANOMA METASTASIS

3.1. Abstract

Intracellular Gal-3 exerts diverse functions within cancer cells that influence metastatic behavior. Nevertheless, the precise role of intracellular Gal-3 in regulating melanoma metastasis remains poorly elucidated. We have reported a significant trend of Gal-3 loss in metastatic melanoma samples versus primary melanomas. Hence, in this Chapter, we aim to investigate the functional consequences of Gal-3 downregulation on melanoma cell behavior. Using the SK-MEL-2 cell line, which is known for its low metastatic phenotype and high Gal-3 expression, we established Gal-3 knockdown lines through lentiviral-mediated transduction. We report that Gal-3-silenced cells display increased migration and invasion capacities, and enhanced colony formation compared to control cells. Furthermore, loss of Gal-3 significantly promotes tumor growth in a xenograft tumor model and induces higher tumor burden in the in vivo metastatic studies compared to control cells. These findings illuminate the apparent opposing roles of Gal-3 in melanoma progression, where intracellular Gal-3 serves as a metastasis-suppressive protein, while extracellular Gal-3 acts as a metastasis-promoting molecule.

3.2. Introduction

Extracellular Gal-3 role in cancer progression and metastasis is mainly attributed to its function as an adhesion molecule, crosslinking Gal-3 ligands expressed on tumor cell surface to ECM proteins and vascular ECs which is a

fundamental step in early stages of tumor invasion and metastasis [83, 229, 230]. Intracellular Gal-3 expression, in the nucleus and the cytoplasm, participates in the regulation of various intracellular processes, including cell proliferation, apoptosis, angiogenesis, immune response modulation, and tumor progression through modulation of diverse signaling pathways inside the cells [24, 51].

Early studies reveal that loss of Gal-3 in cancer cells associate with marked decreases in spontaneous metastasis [152]. Similar results were obtained in melanoma models, where silencing of Gal-3 in metastatic melanoma cells associates with significant reduction of lung metastasis after injection into athymic nude mice [153]. Nevertheless, the underlying molecular mechanisms are still poorly understood. In a study performed with mouse B16F10 melanoma cells, a direct interaction between Gal-3 and AP-1, a transcriptional factor regulating matrix metalloproteinase-1 (MMP-1) expression, was demonstrated, suggesting that Gal-3 can promote melanoma cell invasion and metastasis, at least in part, via induction of MMP-1 expression [155]. Additionally, data show that Gal-3 appears is an essential upstream regulator of MMP-2 [153] and MMP-9 [156] expression in melanoma cells. The anti-apoptotic role of intracellular Gal-3 has been widely studied in different types of cancer [134]. However, few studies have reported a pro-apoptotic activity of endogenous Gal-3 [139]. Accumulating evidence has shown that Gal-3 exerts its action through its interaction with components of the intrinsic and extrinsic apoptotic pathways [133]. Yu et al. reports that Gal-3 can translocate to the perinuclear mitochondrial membrane as a response to apoptotic stimuli, where it maintains mitochondrial

integrity and inhibit cytochrome c release [140]. Bcl-2 is one of the first molecules identified as an intracellular Gal-3 ligand, raising the possibility that Gal-3/Bcl-2 interaction is involved in regulating mitochondrial integrity [140, 141].

Data from melanoma studies are controversial with both pro- and anti-apoptotic activities documented. Borges et al. report that enhanced intracellular expression of Gal-3 in melanoma cells increased the susceptibility to reactive oxygen species (ROS) generated by isatin-Schiff base copper (II) complex, resulting in oxidative cellular damage and apoptosis [147]. In another study, Okra RG-I pectin, a Gal-3 inhibitor, has been shown to induce murine melanoma cell apoptosis and reduce melanoma cell growth *in vitro* [148]. The conflicting activity of Gal-3 was suggested to be subcellular localization-dependent, indicating that nuclear localization is usually associated with pro-apoptotic activity, whereas cytoplasmic localization is associated with anti-apoptotic effects [149]. Nonetheless, the precise mechanisms of how Gal-3 governs cell death pathways continue to be explored.

Here we report that Gal-3-depleted cells exhibit augmented migration, invasion, and colony formation abilities when compared with control cells. Moreover, the absence of Gal-3 significantly accelerated tumor growth in a xenograft tumor model and resulted in enhanced metastatic colonization in vivo. These observations shed light on the dual extra-/intracellular roles played by Gal-3 in driving melanoma progression.

3.3. Materials & Methods

3.3.1. Cell lines and cell culture

The human melanoma cell line SK-MEL-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% Antibiotic-Anti-mitotic (Gibco, Waltham, MA, USA), and maintained in a humidified 37 °C incubator with 5% CO2. Cell lines were passaged when they reached approximately 80% confluency and were regularly tested with PlasmoTest (InvivoGen, San Diego, CA, USA) to ensure the absence of mycoplasma contamination.

3.3.2. Generation of stable Gal-3 silencing

SK-MEL-2 cells were infected with three different shRNA-carrying lentiviral particles directed against Gal-3 or with scrambled control shRNA-carrying lentiviral particles (GeneCopoeia, Rockville, MD, USA) according to the manufacturer's protocol (Refer to **Table 2** for oligonucleotide details). Briefly, 2×10^4 cells/well were plated in 96-well plate and incubated overnight with the lentiviral particles at a multiplicity of infection (MOI) of 5 in serum-free medium. On the next day, cells were replaced with fresh complete medium, and two days later, cells were selected in 1mg/ml Hygromycin (Corning, Corning, NY, USA) containing medium for 1 week to generate stable lines (SK-MEL-2 Scr and SK-MEL-2 Gal-3KD).

Table 2. Oligonucleotide targets for each shRNA lentiviral particle

	Gene	Oligonucleotide sequences	Comments
1	Human LGALS3 shRNA #1	CCTCGCATGCTGATAACAATT	shRNA target sequence
2	Human LGALS3 shRNA #2	CCACGCTTCAATGAGAACAAC	shRNA target sequence
3	Human LGALS3 shRNA #3	GGAATTTCTGGTGACATAGAC	shRNA target sequence
4	Human LGALS3 scrambled control	GCTTCGCGCCGTAGTCTTA	Non-target sequence

3.3.3. RT-qPCR Analysis

For gene expression analysis, RNA was extracted using RNeasy Plus kit (mini) (Qiagen, Ontario, Canada) and cDNA synthesized using SuperScriptTM VILOTM cDNA synthesis kit (Invitrogen; Thermo Fisher, CA, USA) per manufacturer's protocol. Real-time quantitative PCR was then performed with TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan® primers to amplify human LGALS3 (assay ID: Hs00173587_m1) and human GAPDH (assay ID: Hs02786624_g1) as an internal control per manufacturer's protocol.

3.3.4. Western blotting

The cells were lysed in Pierce™ RIPA buffer (Thermo Scientific™) with protease and phosphatase inhibitor cocktail (Thermo Scientific™). After a 30 min incubation on ice, cell lysates were centrifuged for 10 mins at 10,000 RPM in 4°C. Protein concentrations were calculated using Pierce™ BCA protein assay kit (Thermo Scientific™) per manufacturer protocol, and equal protein amounts from each sample were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). 30 µg of protein samples were boiled for 5 mins and subsequently loaded on a 4-12% gradient SDS PAGE gel (BioRad) for electrophoresis. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, Massachusetts), blocked for 1h at room temperature with Intercept® (TBS) blocking buffer (LI-COR, Lincoln, NE, USA), and incubated overnight at 4°C with primary antibodies against Gal-3 (1:1000, #125402, Biolegend, San Diego, CA, USA) and β-actin (1:1000, #8457, Cell Signaling,

Danvers, MA, USA). The membranes were later washed and incubated with secondary antibodies (1:10,000, IRDye® 800CW Goat anti-Rat IgG and IRDye® 800CW Goat anti-Rabbit IgG, LI-COR, Lincoln, NE, USA) for 1hr at room temperature. The membranes were then visualized and analyzed with Li-Cor imager.

3.3.5. Flow cytometry

To analyze surface expression of Gal-3, cells were harvested using Accutase (Fisher), washed with PBS and resuspended in Alexa Fluor 647 anti-mouse/human Mac-2 (Galectin-3) antibody (Biolegend, #125408, San Diego, CA, USA) and Aqua Live/Dead stain (Invitrogen Life Technologies, Carlsbad, CA, USA) for 30 min on ice. Single-color, isotype, and unstained controls were also prepared for validation. Cells were washed, resuspended in 200µl of PBS. Flow cytometric were acquired using FACSCelesta (BD Biosciences, San Jose, CA, USA) and analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

3.3.6. Transwell migration and invasion assays.

Cells (2×10^5) were seeded on top of the filter membrane in an 8.0- µm-pore transwell insert (Corning Incorporated, Corning, NY, USA) with serum-free medium. The inserts were placed into a 24-well plate with medium supplemented with 30 % FBS as a chemo-attractant. For invasion assay, the top filter membrane was coated with a layer of Matrigel before loading the cells. After incubation for 48 h, cells in the upper chamber were removed by a cotton swab gently. The migrated/invaded cells attached to the lower surface of the filters

were fixed in 4 % paraformaldehyde and stained with 0.5% crystal violet. For quantification, the migrated/invaded cells were counted at 5 randomly selected areas in each well under 40× magnification using EVOS ® FL Imaging System (Life Technologies, Grand Island, NY, USA). Data were expressed as mean ± SD from three independent experiments.

3.3.7. Clonogenic assay

Cells (10^3) were seeded in a 6-well culture plate and incubated at 37 °C. Fresh medium was replaced every after 24-48 h until the 10 days of incubation. Colony formation was evaluated by fixing the cells with 100% methanol, staining with 0.5% crystal violet, and imaging using EVOS ® FL Imaging System (Life Technologies, Grand Island, NY, USA). Cell survival was calculated (treated count/untreated count).

3.3.8. Tumor xenograft mouse model

A total of 5×10^6 SK-MEL-2 Scr or Gal-3KD cells were injected subcutaneously into the right flank of 6- to 8-week-old NOD-SCID IL-2Rγ-deficient (NSG) mice (bred in-house, strain from the Jackson Laboratory). Both male and female mice were equally included in the studies. Tumor growth was assessed every 2-3 days by a vernier caliper. Tumor volume was calculated using the formula: $V = LW^2/2$, where L is the length (longest dimension), and W is the width (shortest dimension). At the endpoint (21 weeks post injection), the mice were sacrificed, and tumors were excised and weighed. All experimental procedures were conducted as per FIU IACUC protocol.

3.3.9. Generation of luciferase-expressing SK-MEL-2 cells

SK-MEL-2 Scr and Gal-3KD cells were transduced with firefly luciferase lentivirus or negative control lentivirus (BPS Bioscience, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, 2×10^4 SK-MEL-2 Scrambled control or Gal-3KD cells were plated in 96-well plate and incubated overnight with the lentiviral particles at a multiplicity of infection (MOI) of 5 in serum-free medium. On the next day, lentivirus-containing medium was replaced with fresh complete medium, and two days later, cells were selected for 10 days in selection medium containing 4 µg/mL puromycin (Gibco, Waltham, MA, USA) to generate stable lines expressing luciferase cells (SK-MEL-2 Scr.Luc and SK-MEL-2 Gal-3KD.Luc) and negative control cells (SK-MEL-2 Scr.NegCtrl and SK-MEL-2 Gal-3KD.NegCtrl) respectively. An in vitro luciferase activity assay was performed to verify successful expression of firefly luciferase using the Bright-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) per manufacturer protocol. Briefly, luciferase-expressing and negative control SK-MEL-2 Scr and Gal-3KD cells were seeded in a 96-well white bottomed plate in a serial dilution of 1,000,000, 500,000, 250,000, 125,000, 62,500, and 31,250 cells/well. On the next day, the Bright-Glo Luciferase Assay reagent was added to each well and incubated for 5 min. The number of photons emitted per second over a 30 sec exposure period was measured using the AMI HT imager (Spectral Instruments Imaging, Tucson, AZ), and quantified using the Aura Imaging Software (https://spectralinvivo.com/software/).

3.3.10. In vivo metastasis assay

1×10^6 cells of either SK-MEL-2 Scr.Luc or Gal-3KD.Luc cells were injected intravenously into the tail veins of 6- to 8-week-old NOD-SCID IL-2Rγ-deficient (NSG) mice (bred in-house, strain from the Jackson Laboratory). Metastatic tumor formation and colonization were monitored bi-weekly using an AMI HT imager. Briefly, mice were anesthetized with 2% isoflurane and maintained under anesthesia by continuous inhalation of isoflurane until imaging was complete. Mice were injected intraperitoneally with 200 µl of 15 mg/ml D-luciferin solution (VivoGlo™Luciferin, In Vivo Grade, Promega, Madison, WI, USA). Ventral and lateral images of the mice were taken 10 min later using AMI HT imaging system and quantified using the Aura Imaging Software. The conditions for bioluminescence acquisition were as follows: open emission filter, exposure time 30 seconds, binning medium for 8, field of view 12.9 cm, and f/stop as 1. Rainbow images show the relative levels of luminescence ranging from low (blue), to medium (green), to high (yellow/red).

3.3.11. Statistical Analysis

Prism 8.0 software (GraphPad) was used for statistical analysis. For normally distributed data involving two groups, unpaired two-tailed Student's *t*-test was used. For non-normally distributed data, analysis was performed using a Mann–Whitney test; normality was assessed using a Shapiro–Wilk test. Throughout, data are presented as the means ± SEM, unless otherwise noted. *P* value of <0.05 was considered statistically significant.

3.4. Results

3.4.1. Validation of shRNA-mediated knockdown of Gal-3 in SK-MEL-2 melanoma cells

To determine the functional consequences of Gal-3 downregulation in melanoma cells, SK-MEL-2 cell line was selected for experimental Gal-3 silencing and further studies due to its high inherent Gal-3 expression (**Figure 2.4 A, B, and C**) and its low metastatic phenotype [231, 232], making it ideal for testing our hypothesis. First, we established Gal-3 knockdown lines of SK-MEL-2 cells by lentiviral-mediated transduction of cells with three different LGALS3 shRNAs. Stable knockdown of Gal-3 was verified by RT-qPCR (**Figure 3.1 A**), immunoblotting (**Figure 3.1 B**), and flow cytometry analysis (**Figure 3.1 C**). LGALS3 shRNA#1 had the greatest knockdown efficiency on Gal-3 expression among the three used constructs ($p<0.001$). Hence, this line was used in the entire downstream analyses (hereafter referred to as Gal-3KD).

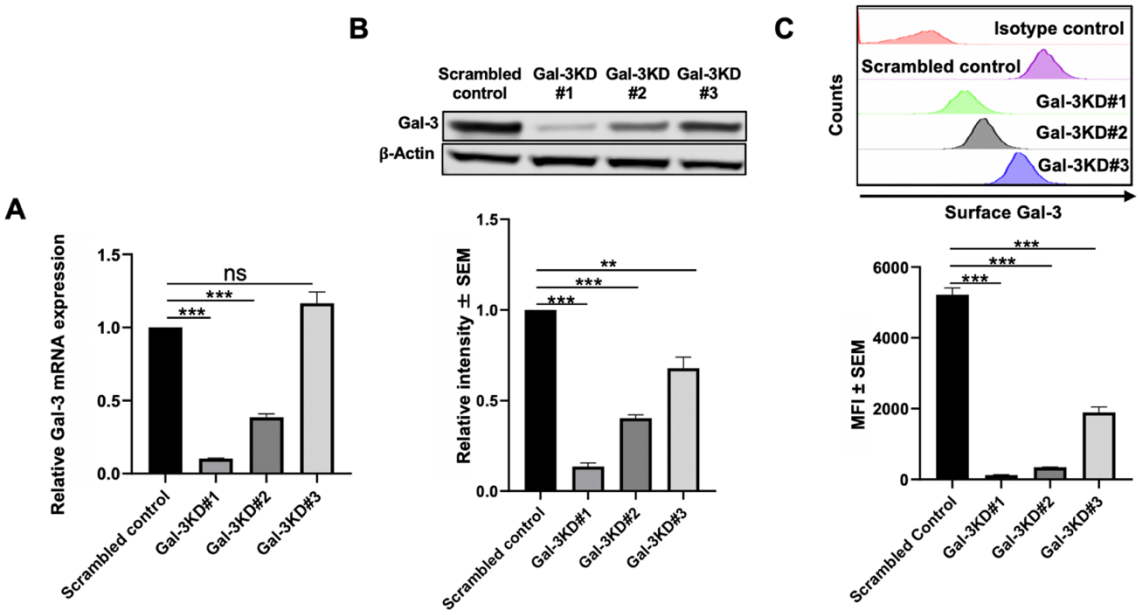


Figure 3.1. Knockdown of Gal-3 within SK-MEL-2 melanoma cell line using small hairpin RNAs (shRNAs).
Gal-3 silencing in SK-MEL-2 melanoma cells was performed using three different shRNA constructs, then validated by RT-PCR **(A)**, Western blot **(B)**, and flow cytometry **(C)**. (***p<0.001, **p<0.01, *p<0.05)

3.4.2. Enforced knockdown of Gal-3 enhances the metastatic abilities of melanoma cells in vitro

First, we observed a change in the morphology of Gal-3-silenced cells grown in regular media, which appeared bigger in size with frequent giant multinucleated cells compared to parental cells (**Figure 3.2 A, arrows)**. Metastatic abilities of Gal-3-silenced cells were assessed in vitro using the transwell migration as well as invasion assays. Our results showed that Gal-3 knockdown significantly enhanced the migration and invasion capacities of SK-MEL-2 cells ($p<0.01$ and $p<0.01$, respectively) (**Figure 3.2 B and C**). Lack of Gal-3 resulted also in significantly increased number and size of colonies compared to the mock control cells ($p<0.05$) as evidenced by the colony formation assay (**Figure 3.2 D**). Next, we established a xenograft tumor model in NSG mice to assess the influence of Gal-3 silencing on tumor growth in vivo. By following tumor volume over time, we found that loss of Gal-3 significantly promoted tumor growth in vivo ($p<0.05$) (**Figure 3.2 E**).

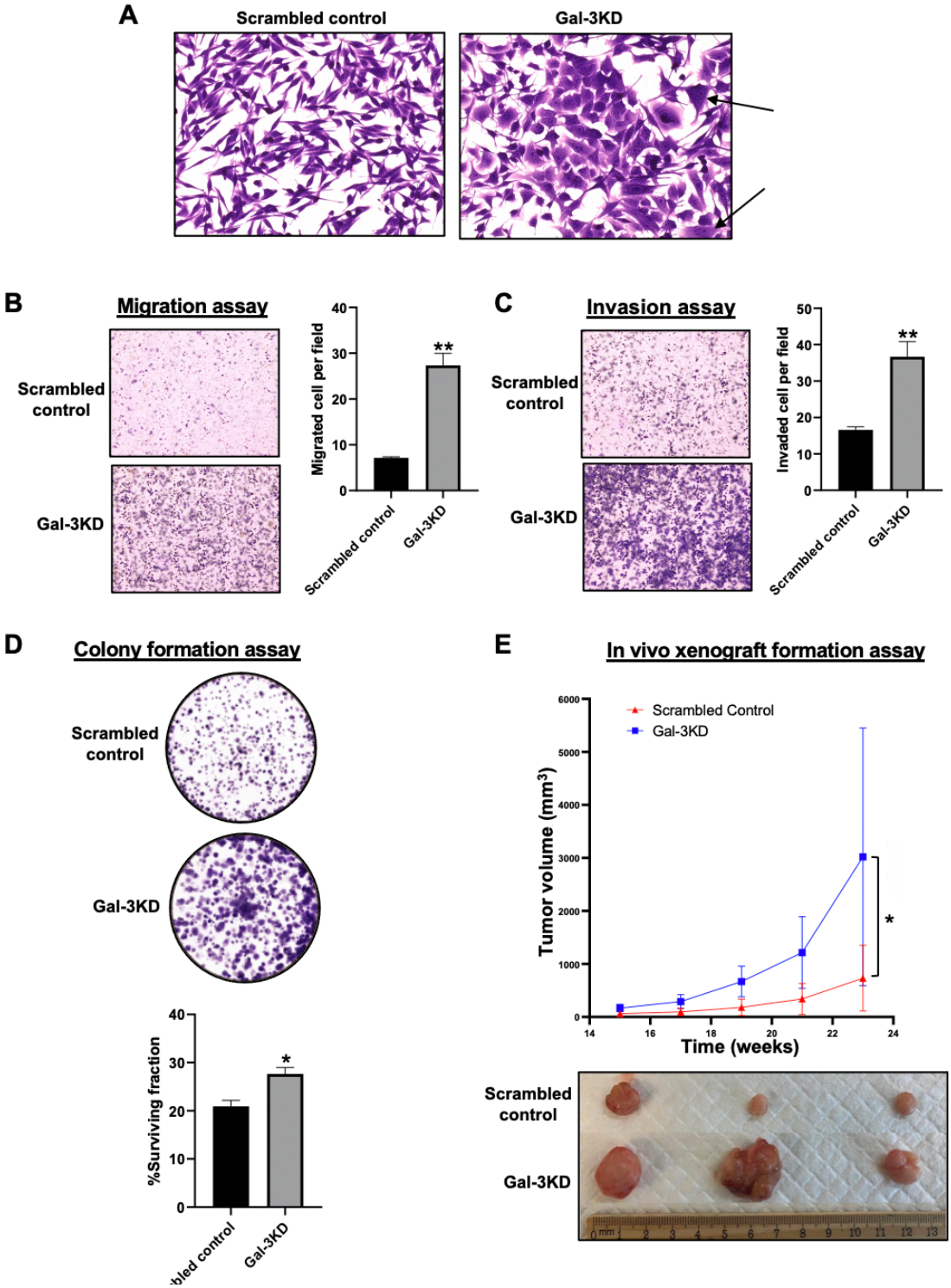


Figure 3.2. Loss of Gal-3 enhances the metastatic behavior of melanoma cells. Enforced silencing of Gal-3 within SKMEL2 melanoma cells resulted in change of cell morphology with frequent giant multinucleated cells **(A)** and enhanced aggressive behavior as indicated by data from migration assay **(B)**, invasion assay **(C)**, colony formation assay **(D)**, and in vivo xenograft formation assay **(E)**. (**p<0.01, *p<0.05)

3.4.3. Gal-3 depletion promotes metastatic colonization of melanoma cells in vivo

Prior *in vivo* metastatic studies showed that SK-MEL-2 cells have a low metastatic phenotype [232]. To explore whether Gal-3-depleted SK-MEL-2 cells can initiate metastatic colonization in vivo, we first generated SKMEL-2 cell lines expressing luciferase by lentiviral transduction. Successful luciferase expression by SK-MEL-2 Gal-3KD and scrambled control cells was verified using an in vitro luciferase activity assay (**Figure 3.3 A**). Then, we injected the luciferase-labeled cells into NSG mice via the tail vein. Bioluminescence imaging was performed bi-weekly using IVIS Spectrum Imaging System until termination of the study 12 weeks post-injection. SK-MEL-2 Gal-3KD.Luc cells exhibited higher tumor burden compared with Scr.Luc control cells by 8 weeks post-injection ($p<0.001$) (**Figure 3.3 B**). Altogether, these data indicate that Gal-3 expression is inversely correlated with melanoma progression and highlights its potential use as a prognostic biomarker in patients with primary melanoma.

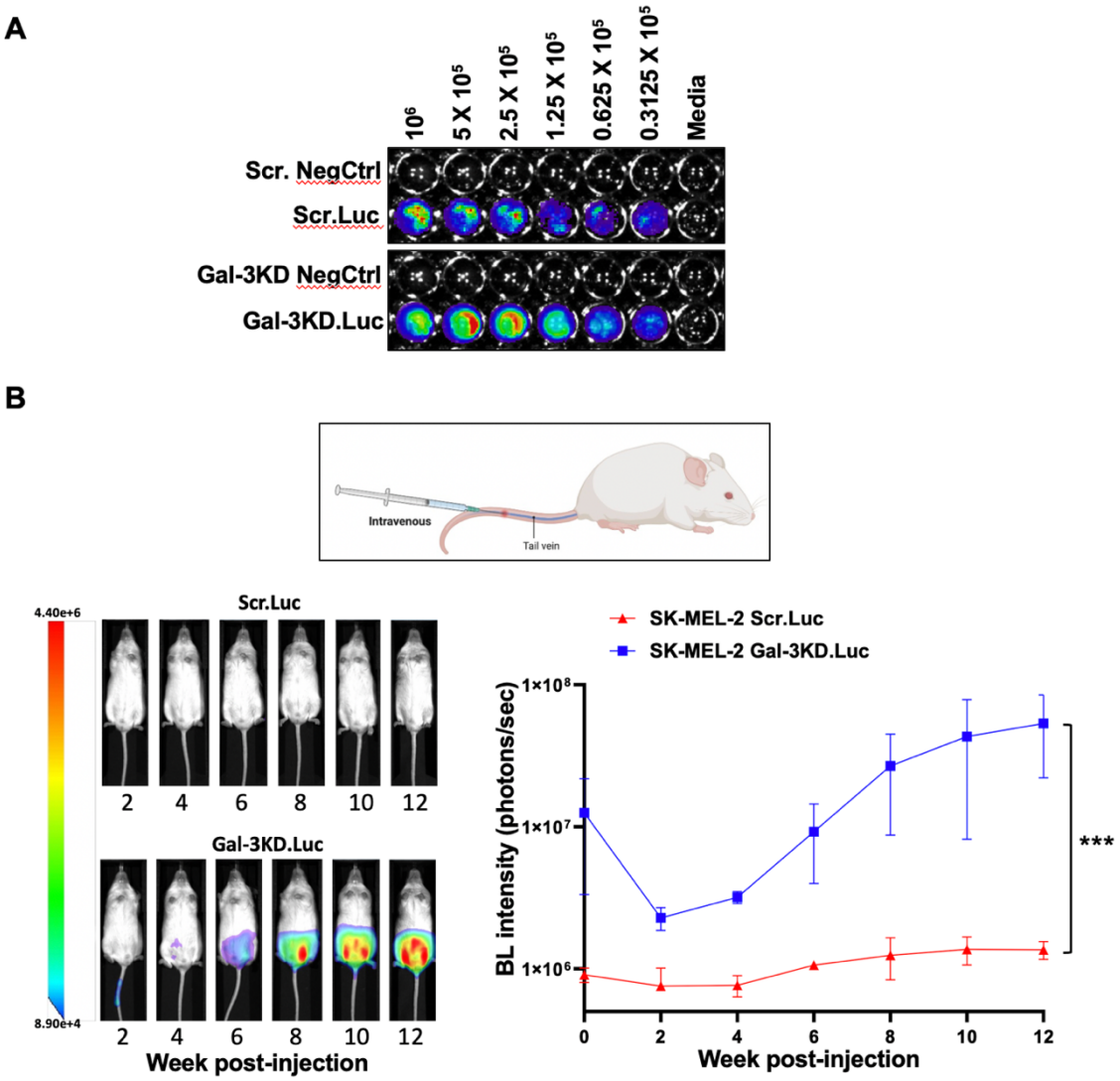


Figure 3.3. Intracellular Gal-3 depletion promotes metastatic colonization of melanoma cells *in vivo*.
In vitro assay of luciferase activity in serially diluted SK-MEL-2 Scr.Luc and Gal-3KD.Luc cells following puromycin selection **(A)**. Monitoring intravenously injected luciferase-labelled SK-MEL-2 cells into NSG mice by the in vivo imaging system. Ventral images of SK-MEL-2 Scr.Luc **(top)** and Gal-3KD.Luc **(bottom)** cells were obtained 2, 4, 6, 8, 10, and 12 weeks after the intravenous injection **(B, left panel)**. Average tumor growth rates as measured by photon flux in SK-MEL-2 Scr.Luc and Gal-3KD.Luc injected mice **(B, right panel)**. The photon counts are indicated by the color scale. (***p<0.001)

3.5. Discussion

The role of Gal-3 in driving cancer metastasis has been debated topic among researchers. Even though there are several studies supporting the pro-metastatic role of intracellular Gal-3 in many cancer types including melanoma [50, 233, 234], a growing body of evidence has shown the opposite [235-237]. Here, our in vitro and in vivo studies revealed enhanced melanoma migration, invasion and metastatic colonization abilities induced by depletion of cellular Gal-3, confirming a metastasis-suppressive function for intracellular Gal-3 in melanoma.

In accordance with our findings, Hayashi et al. demonstrate that Gal-3 knockout in the low metastatic B16 melanoma cells greatly enhances its in vivo metastatic abilities [238]. It has been proposed that the release of matrix metalloproteinases (MMPs) from growing melanomas eventually brings about the consumption of intracellular Gal-3 stores as a substrate which in turn facilitates melanoma cell shedding from the primary tumor into the circulation [179, 183]. Another mechanism has been recently revealed by Herrera-Reinoza et al. who show that loss of Gal-3 is associated with marked decline in the elastic modulus of melanoma cells, and hence higher capability to metastasize [239].

On the contrary, Mourad-Zeidan et al. report that loss of Gal-3 in melanoma cells resulted in reduced tumorigenic and metastatic potential and impaired angiogenesis [153]. Likewise, Braeuer et al. show that Gal-3 silencing in melanoma cells inhibits tumor growth and metastasis [240]. We speculate that this contradiction highlights a potentially complex role for Gal-3 in melanoma progression and it can be attributed to many factors. First, the diverse and

sometimes opposing roles played by Gal-3 in cancer progression ultimately depend on its cellular localization, which dictates its structure and in turn its biological functions [214]. Importantly, in scenarios where Gal-3 is experimentally overexpressed in cancer cells and implicated in enhancing their metastatic behavior, we cannot exclude the possibility that Gal-3 exerts such pro-metastatic activities upon its secretion to the extracellular milieu. Accordingly, interplay between intracellular and microenvironmental Gal-3 likely defines the clinical outcome of melanoma. Second, dysregulated subcellular distribution of Gal-3 has been associated with altered tumor behavior in melanoma [182] and prostate cancer [241, 242]. Taking into consideration that subcellular localization of Gal-3 is affected by experimental circumstances such as tumor stage, aging state of cells, and cell culturing conditions [55], the possibility of altered tumor behavior by virtue of variable experimental cell lines and growth conditions cannot be omitted.

Interestingly, we have shown that loss of Gal-3 is associated with increased number of polyploid/multinucleated giant cancer cells (PGCCs). This phenomenon is described in cancer cells as a response to stressful conditions triggered by either endogenous stimuli, such as hypoxic microenvironment [243] or exogenous insults, such as chemotherapeutic agents [244, 245] and ionizing radiation [246]. Although PGCCs have been associated with suppressed proliferative capacity, it is becoming increasingly evident that their presence is associated with a more metastatic phenotype with poorer clinical outcomes [247-

249]. Further studies are still necessary to elucidate the mechanistic details of how Gal-3 depletion promotes PGCCs abundance.

Taken together, lack of Gal-3 in melanoma cells enhances their metastatic behavior, highlighting the potential use of intracellular Gal-3 level as a prognostic marker in melanoma patients.

4. CHAPTER 4: GAL-3 MODULATES THE ACTIVITIES OF INTRACELLULAR ONCOGENIC SIGNALLING PATHWAYS

4.1. Abstract

The intricate molecular mechanisms underlying the development and progression of melanoma encompass the dysregulation of multiple signaling pathways. Gaining a comprehensive understanding of the interactions within these pathways is crucial for identifying novel therapeutic targets for melanoma. Gal-3 has been recognized as a regulator of pro-oncogenic signaling pathways in different cancer types. In this Chapter, we aim to examine the potential regulatory role of Gal-3 on the intracellular canonical signaling pathways in melanoma cells. We also investigate the possible relationship between Gal-3 and NFAT1 in melanoma. Our results reveal that Gal-3 silencing results in the activation of MAPK/ERK, PI3K/AKT, and Wnt/β-catenin pathways. Interestingly, the metastasis-promoting transcription factor NFAT1 is found to be upregulated in metastatic melanoma samples and correlated negatively with Gal-3 expression. Furthermore, Gal-3 knockdown leads to a significant increase in NFAT1 expression as well as its downstream pro-metastatic effectors MMP-3, IL-8, and GPC6, compared with control cells. Collectively, these findings suggest a negative regulatory role for intracellular Gal-3 on pro-oncogenic signaling pathways and NFAT1 expression, hindering their pro-metastatic functions.

4.2. Introduction

The development and progression of melanoma involve complex molecular mechanisms, including dysregulation of several signaling pathways

[250]. Understanding the interplay and aberrations within these pathways and identifying effector molecules that modulate their activities are fundamental for developing targeted therapies and improving clinical outcomes of melanoma patients. Gal-3 serves as a key regulator of diverse pro-oncogenic signaling pathways in different types of cancer [222]. However, there is a lack of research studies regarding this aspect in the context of melanoma [214].

The mitogen-activated protein kinase (MAPK) pathway is one of the most frequently dysregulated signaling pathways in melanoma [251]. It plays a crucial role in regulating cell growth, differentiation, survival, and migration [252]. This pathway is activated by the Ras-Raf-MEK-ERK cascade, wherein Ras activates Raf, which subsequently phosphorylates and activates MEK, leading to ERK activation [253]. In melanoma, activating mutations in the BRAF gene, particularly the V600E mutation, occur in 40–50% of cases of melanoma, resulting in constitutive activation of the MAPK/ERK pathway [254]. This aberrant signaling leads to uncontrolled cell proliferation, survival, and cell cycle dysfunction [255]. Targeted therapies against BRAF and MEK have shown significant clinical benefits in patients with melanoma harboring BRAF mutations [256].

The Phosphatidylinositol 3-kinase (PI3K)/AKT is another major pathway that is activated by growth factors and other extracellular signals [257]. When activated, PI3Ks phosphorylate a lipid called phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 then recruits AKT, which is a serine/threonine kinase. AKT phosphorylates several downstream targets, including proteins that regulate

cell growth, proliferation, and survival [258]. The PI3K/AKT pathway is frequently dysregulated in melanoma and contributes to tumor initiation, growth, migration, invasion, and resistance to therapy [259]. Various genetic alterations, such as activating mutations in PI3K, loss of the tumor suppressor PTEN, or activation of receptor tyrosine kinases (RTKs), result in hyperactivation of this pathway [260].

The Wnt/β-catenin pathway plays a critical role in embryonic development, tissue homeostasis, and cell fate determination [261-263]. Dysregulation of this pathway has been implicated in various cancers, including melanoma [264]. In the absence of Wnt signaling, β-catenin is targeted for degradation, preventing its translocation into the nucleus [265, 266]. Aberrant activation of the Wnt pathway, caused by mutations in β-catenin or APC genes, leads to the accumulation of β-catenin and its translocation into the nucleus. Nuclear β-catenin forms complexes with transcription factors, activating target genes involved in cell proliferation, survival, and metastasis [265]. Cyclin D1 and c-Myc stand out as the most prominent target genes that play significant roles in mediating oncogenicity [267].

Nuclear factor of activated T cells 1 (NFAT1), a transcription factor belongs to the NFAT family, is well-known for its role in T cell development and activation [268]. Intriguingly, NFAT1 has recently emerged as a crucial player in various cellular processes related to tumor initiation, proliferation, metastasis, and drug resistance [269-271]. In melanoma, NFAT1 has been reported as a metastasis-promoting molecule by activating downstream targets such as MMP-3, IL-8, and MMP-9 [240, 272].

Here, we report that Gal-3 depletion in melanoma cells results in a sustained activation of the PI3K/AKT, MAPK/ERK, and Wnt/β-catenin signaling pathways. We also observed a negative regulatory role for Gal-3 on the expression of NFAT1 and its downstream metastasis-associated targets: MMP-3, IL-8, and GPC6 in melanoma cells. As such, our results provide evidence for a negative regulatory loop between Gal-3 and NFAT1 that may dictate the metastatic phenotype of melanoma cells.

4.3. Materials & Methods

4.3.1. Gene expression data collection and processing

Transcriptomic data of cutaneous melanoma (SKCM) samples (n = 481) were retrieved from The Cancer Genome Atlas (TCGA) database. Data were analyzed and visualized using UCSC Xena (http://xena.ucsc.edu) [218]. Exclusion criteria were as follows: (1) Samples with missing data regarding sample type or Gal-3 expression; (2) Samples categorized as solid tissue normal or additional metastatic. As a result, a total of 471 SKCM samples (103 primary melanoma and 368 metastatic melanoma) were included in the study. The obtained gene expression profiles were generated using the Illumina HiSeq 2000 RNA Sequencing platform and presented as log2(value + 1) transformed RSEM normalized count.

4.3.2. Cell lines and cell culture.

The human melanoma cell line SK-MEL-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI

1640 medium supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% Antibiotic-Anti-mitotic (Gibco, Waltham, MA, USA), and maintained in a humidified 37°C incubator with 5% CO2. Cell lines were passaged when they reached approximately 80% confluency and were regularly tested with PlasmoTest (InvivoGen, San Diego, CA, USA) to ensure the absence of mycoplasma contamination.

4.3.3. RT-qPCR Analysis

For gene expression analysis, RNA was extracted using RNeasy Plus kit (mini) (Qiagen, Ontario, Canada) and cDNA synthesized using SuperScriptTM VILOTM cDNA synthesis kit (Invitrogen; Thermo Fisher, CA, USA) per manufacturer's protocol. Real-time quantitative PCR was then performed with TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan® primers to amplify human NFAT1 (assay ID: Hs00905451_m1), mouse NFAT1 (assay ID: Mm01240677_m1), human MMP-3 (assay ID: Hs00968305_m1), human IL-8 (assay ID: Hs00174103_m1), human GPC6 (assay ID: Hs00170677_m1), human GAPDH (assay ID: Hs02786624_g1), and mouse GAPDH (assay ID: Mm99999915_g1) per manufacturer's protocol.

4.3.4. Western blotting

The cells were lysed in Pierce™ RIPA buffer (Thermo Scientific™) with protease and phosphatase inhibitor cocktail (Thermo Scientific™). After a 30 min incubation on ice, cell lysates were centrifuged for 10 mins at 10,000 RPM in 4°C. Protein concentrations were calculated using Pierce™ BCA protein assay kit

(Thermo Scientific™) per manufacturer protocol, and equal protein amounts from each sample were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). 30 µg of protein samples were boiled for 5 mins and subsequently loaded on a 4-12% gradient SDS PAGE gel (BioRad) for electrophoresis. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, Massachusetts), blocked for 1h at room temperature with Intercept® (TBS) blocking buffer (LI-COR, Lincoln, NE, USA), and incubated overnight at 4°C with primary antibodies against p-ERK1/2 (1:1000, #5863, Cell Signaling, Danvers, MA, USA), total ERK1/2 (1:1000, # MAB1576, R&D Systems, Minneapolis, MN, USA), p-AKT (1:1000, #4060, Cell Signaling, Danvers, MA, USA), total AKT (1:1000, #2920, Cell Signaling, Danvers, MA, USA), β-catenin (1:1000, #8480, Cell Signaling, Danvers, MA, USA), Cyclin D1 (1:1000, #2978, Cell Signaling, Danvers, MA, USA), c-Myc (1:1000, #5605, Cell Signaling, Danvers, MA, USA), Gal-3 (1:1000, #125402, Biolegend, San Diego, CA, USA), NFAT1 (1:1000, #5861, Cell Signaling, Danvers, MA, USA) and β-actin (1:1000, #8457, Cell Signaling, Danvers, MA, USA). The membranes were later washed and incubated with secondary antibodies (1:10,000, IRDye® 800CW Goat anti-Rat IgG and IRDye® 800CW Goat anti-Rabbit IgG, LI-COR, Lincoln, NE, USA) for 1hr at room temperature. The membranes were then visualized and analyzed with Li-Cor imager.

4.3.5. Statistical Analysis

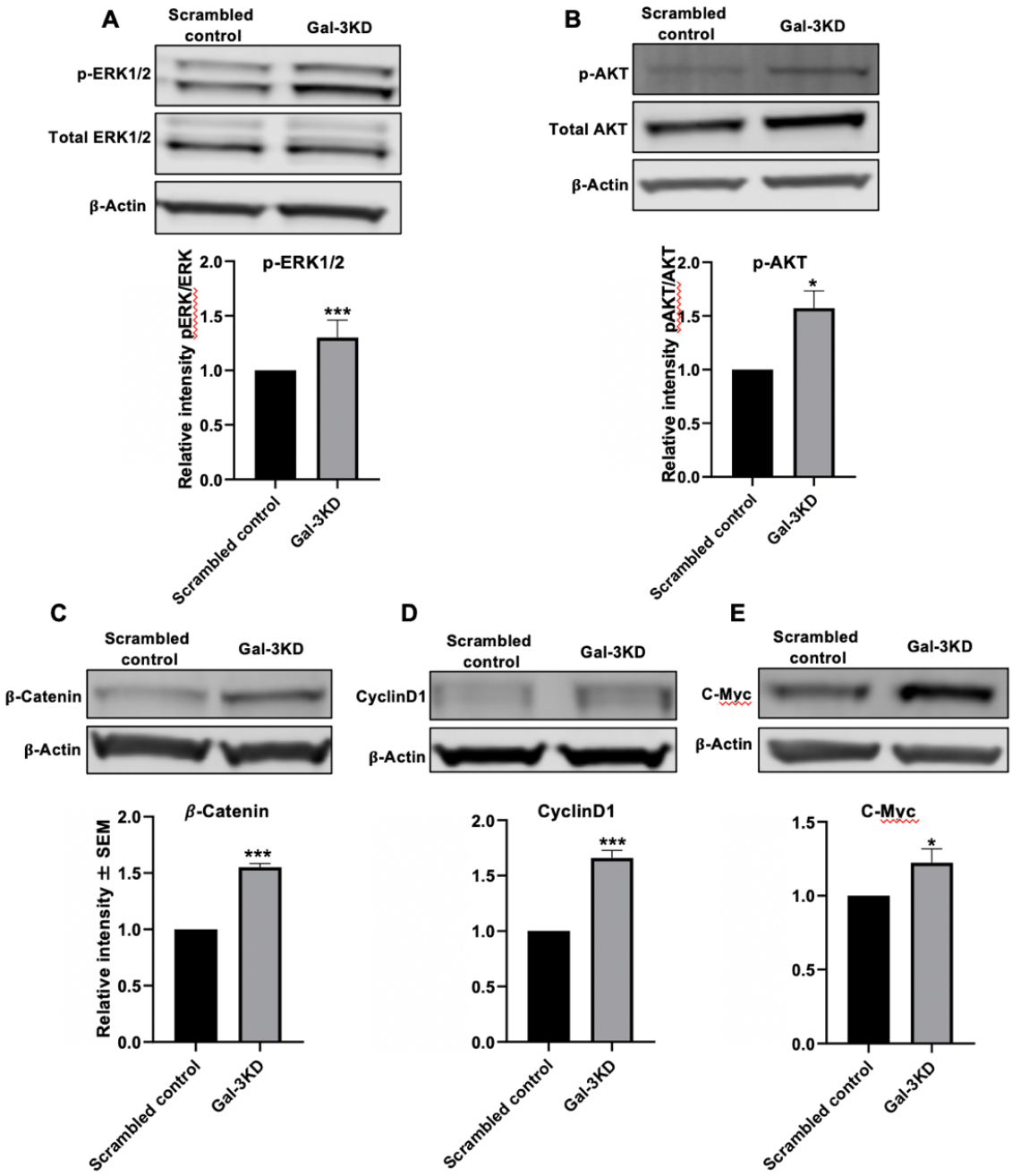
Prism 8.0 software (GraphPad) was used for statistical analysis. For normally distributed data involving two groups, unpaired two-tailed Student's *t*-test was

used. For non-normally distributed data, analysis was performed using a Mann–Whitney test; normality was assessed using a Shapiro–Wilk test. Spearman's correlation test was used to investigate the correlation between the expression levels of Gal-3 and NFAT1 or GPC6 in TCGA melanoma data. Throughout, data are presented as the means ± SEM, unless otherwise noted. *P* value of <0.05 was considered statistically significant.

4.4. Results

4.4.1. Gal-3 regulates the activation of pro-oncogenic signal pathways in melanoma

To assess whether Gal-3 knockdown triggered canonical intracellular signaling, we analyzed activation of the pro-survival molecules AKT and ERK1/2. Immunoblotting confirmed the activation of MAPK/ERK and PI3K/AKT signaling pathways in Gal-3KD cells versus control cells, as evidenced by increased phosphorylation of ERK1/2 ($p<0.001$) (**Figure 4.1 A**) and AKT ($p<0.05$) (**Figure 4.1 B**), respectively. Dysregulation of the Wnt/β-catenin signaling pathway was also implicated in melanoma progression [273]. To test whether Gal-3 downregulation affects Wnt/β-catenin signaling, we measured the protein expression levels of β-catenin, c-Myc, and cyclin D1, which are important components of the Wnt/β-catenin pathway. We found that loss of Gal-3 resulted in increased β-catenin ($p<0.001$) (**Figure 4.1 C**), cyclin D1 ($p<0.001$) (**Figure 4.1 D**), and c-Myc expression ($p<0.05$) (**Figure 4.1 E**). Together, these data indicate that intracellular Gal-3 depletion promotes the activation of MAPK/ERK, PI3K/AKT, and Wnt/β-catenin signaling pathways.



<u>Figure 4.1.</u> Intracellular Gal-3 regulates the activities of intracellular signaling pathways in melanoma cells.
Effect of Gal-3 silencing within SKMEL2 melanoma cells on the activity of the Ras/MAPK **(A)**, PI3K/Akt **(B)**, and Wnt/β-catenin **(C-E)** signaling pathways as examined by Western blot. (***p<0.001, **p<0.01, *p<0.05)

4.4.2. Intrinsic Gal-3 suppresses the pro-metastatic activity of NFAT1 in melanoma.

A growing body of evidence suggests that the NFAT1 is involved in driving melanoma progression [272, 274, 275]. Herein, NFAT1 expression was explored in melanoma samples from the TCGA database, and we found that NFAT1 is upregulated in metastatic melanoma samples compared with primary melanomas ($p<0.001$) (**Figure 4.2 A**). NFAT1 was also upregulated in the highly metastatic B16/BL6 cell line compared with the parental B16 cell line ($p<0.001$) as evidenced by RT-qPCR analysis (**Figure 4.2 B**).

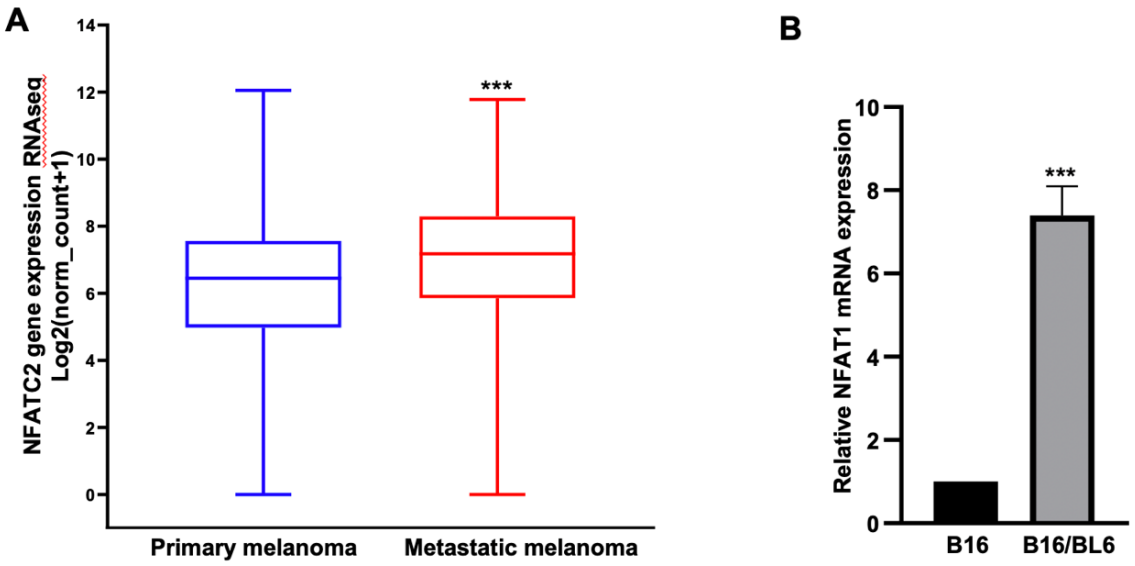


Figure 4.2. NFAT1 is upregulated in metastatic melanomas versus primary melanomas.
Transcriptional expression analysis of NFAT1 in human metastatic melanomas versus primary melanomas retrieved from TCGA SKCM database **(A)**. NFAT1 expression analysis in the highly metastatic murine melanoma cell subline (B16/BL6) compared with its parent line (B16) by RT-PCR **(B)**. (***$p<0.001$)

To test the possible relationship between Gal-3 and NFAT1 expression in melanoma, Spearman's correlation analysis was applied to calculate the correlation coefficient between the expression levels of Gal-3 and NFAT1 in the TCGA melanoma database. Data analysis showed that the Gal-3 expression is significantly negatively correlated with NFAT1 expression in melanoma samples (r=-0.3079, P<0.001) (**Figure 4.3 A**). To further investigate the possible negative regulatory role of Gal-3 on NFAT1 expression, NFAT1 expression was assessed in the three established Gal-3-silenced SK-MEL-2 lines (Gal-3KD#1, Gal-3KD#2, and Gal-3KD#3) using immunoblotting. Data analysis demonstrated a significant upregulation of NFAT1 in the three lines (p<0.001, p<0.001, and p<0.05, respectively), with an obvious negative correlation with Gal-3 expression levels inside these cells (**Figure 4.3 B**).

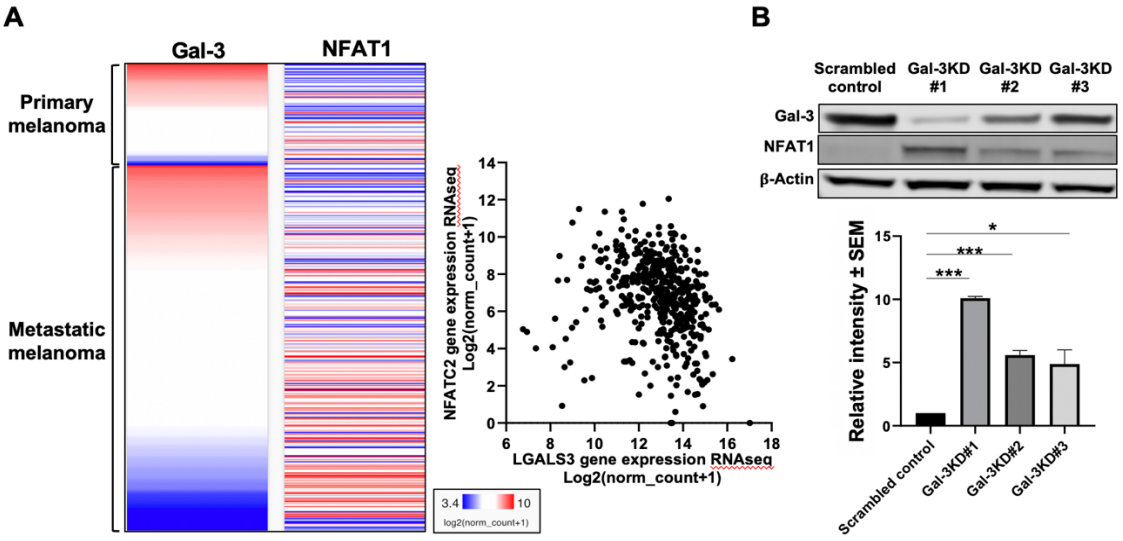


Figure 4.3. NFAT1 expression levels are negatively correlated with Gal-3 expression in melanoma.
Spearman's correlation analysis between the expression levels of Gal-3 and NFAT1 in TCGA SKCM database (r=-0.3079, P<0.001), represented as a heat map **(A, left panel)** and scatter plot **(A, right panel)**. NFAT1 expression analysis in the three established Gal-3-silenced SK-MEL-2 lines (Gal-3KD#1, Gal-3KD#2, and Gal-3KD#3) compared with the scrambled control as examined by Western blot **(B)**. (***p<0.001, **p<0.01, *p<0.05)

NFAT1 was shown to exert its pro-metastatic function in melanoma cells by regulating other effector proteins, such as matrix metalloproteinase (MMP)-3 and interleukin (IL)-8 [274]. Therefore, we sought to characterize the expression levels of NFAT1 and its target proteins in Gal-3-depleted melanoma cells using RT-qPCR. Interestingly, we found a significant upregulation of NFAT1 ($p<0.001$) (**Figure 4.4 A**), MMP-3 ($p<0.001$) (**Figure 4.4 B**), and IL-8 ($p<0.01$) (**Figure 4.4 C**) in SK-MEL-2 Gal-3KD cells compared with control cells. Collectively, these results suggest an inhibitory role for Gal-3 on the expression of NFAT1, and hence its downstream effector proteins.

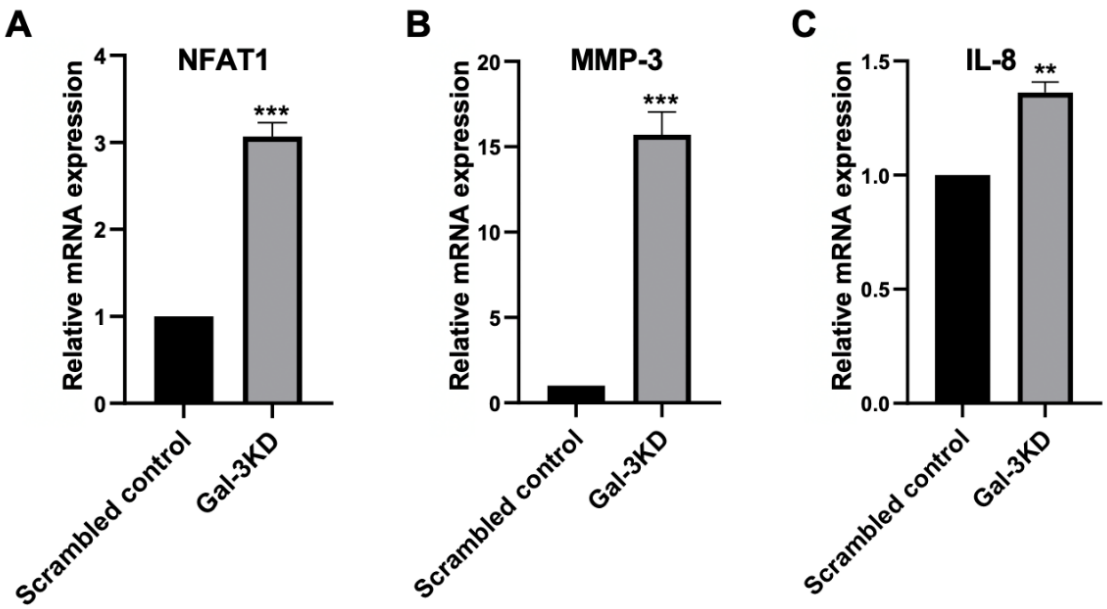
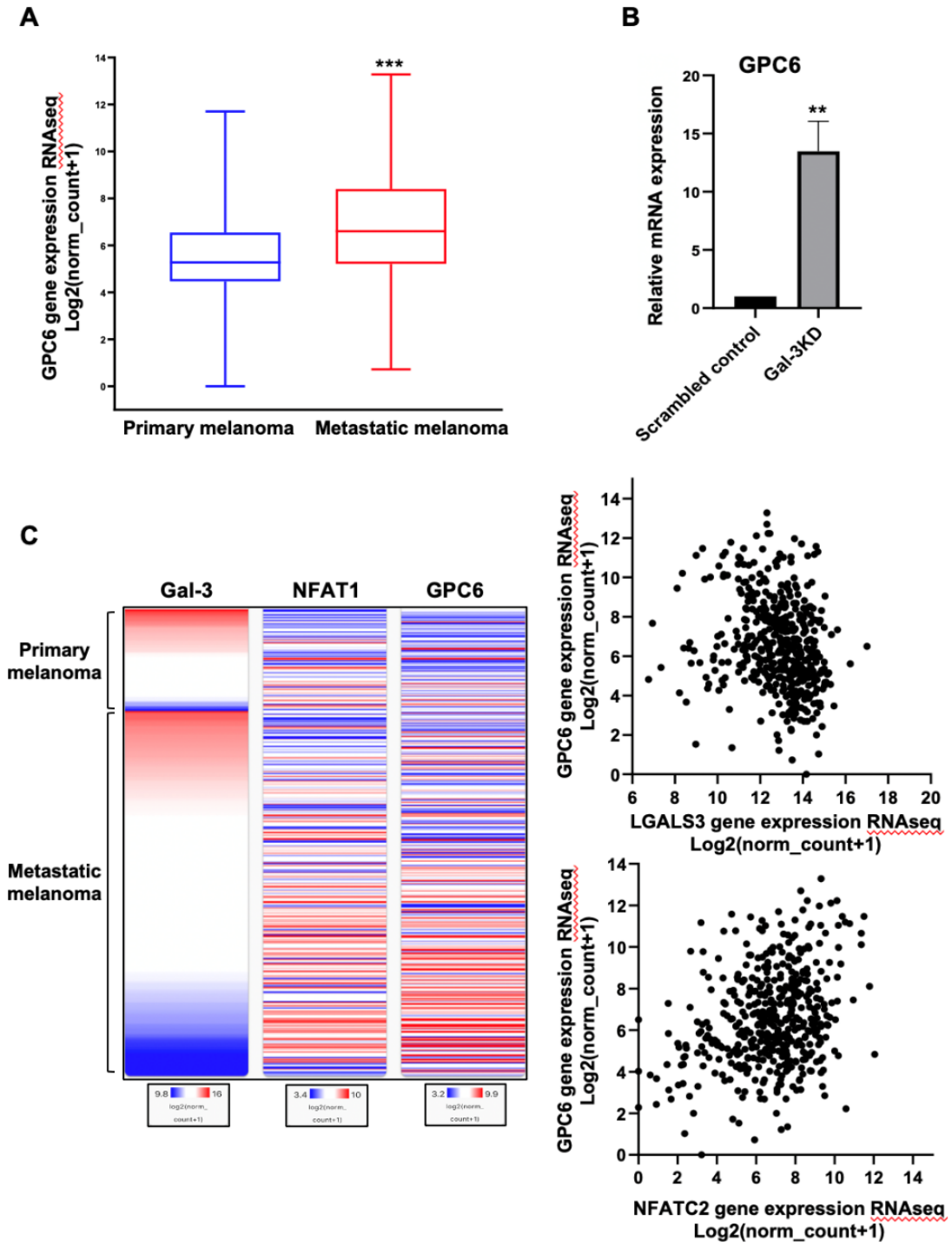


Figure 4.4. Gal-3 regulates the expression of NFAT1 and its downstream targets MMP-3 and IL-8 in melanoma cells.
Effect of Gal-3 silencing within SKMEL2 melanoma cells on the expression levels of NFAT1 **(A)**, MMP-3 **(B)**, and IL-8 **(C)** by RT-PCR **(B)**. (***$p<0.001$)

4.4.3. Glypican-6 is a potential target of Gal-3-NFAT1 axis in melanoma.

Glypican-6 (GPC6) is also shown to be regulated by NFAT1 to mediate tumor cell invasion in breast cancer [276]. Although GPC6 was identified as a potential marker of melanoma metastasis [277], its regulation by NFAT1 has not been established in melanoma. Here, we revealed a significant upregulation of GPC6 expression in Gal-3-silenced cells compared to scrambled control cells using RT-qPCR ($p<0.01$) (**Figure 4.5 A**). By analyzing TCGA melanoma data, GPC6 was shown to be markedly upregulated in metastatic melanomas compared to primary melanomas ($p<0.001$) (**Figure 4.5 B**). Moreover, Spearman's correlation analysis was carried out to evaluate the relationships between GPC6 and Gal-3 or NFAT1 in the TCGA melanoma data. Interestingly, we observed a significant negative correlation between GPC6 and Gal-3 expression levels ($r=-0.2838$, $P<0.001$) (**Figure 4.5 C, heatmap and right upper panel**) and a significant positive correlation between GPC6 and NFAT1 expression levels ($r=0.2557$, $P<0.001$) (**Figure 4.5 C, heatmap and right lower panel**). These data propose a negative regulatory role for Gal-3 on GPC6 expression in melanoma cells, probably mediated via NFAT1.



<u>Figure 4.5.</u> GPC6 is a potential target of Gal-3-NFAT1 regulatory axis in melanoma.
Transcriptional expression analysis of GPC6 in human metastatic melanomas versus primary melanomas retrieved from TCGA SKCM database **(A)**. Effect of Gal-3 silencing within SKMEL2 melanoma cells on the expression levels of GPC6 by RT-PCR **(B)**. Spearman's correlation analysis between GPC6 and Gal-3 (r=-0.2838, P<0.001) **(C, heatmap and right upper panel)** or between GPC6 and NFAT1 (r=0.2557, P<0.001) **(C, heatmap and right lower panel)** in the TCGA melanoma data. (***p<0.001)

4.5. Discussion

Dysregulated intracellular signaling pathways is one of the major hallmarks of cancer [212, 278]. Gal-3 has been shown to modulate the activities of the diverse intracellular signaling pathways in various types of cancer [222]. In this study, we demonstrate that depletion of Gal-3 in melanoma cells leads to persistent activation of the PI3K/AKT, MAPK/ERK, and Wnt/β-catenin signaling pathways. Furthermore, we detect consistent negative correlation between Gal-3 and NFAT1 by analyzing TCGA melanoma data, besides data from murine B16 melanoma model as well as in vitro studies on Gal-3-silenced cells, raising the possibility that NFAT1 expression is negatively regulated, either directly or indirectly, by Gal-3. Our findings are further supported by the demonstration of upregulated expression of NFAT1 pro-metastatic targets; MMP-3, GPC6, and IL-8 in Gal-3-silenced cells.

Uncontrolled proliferation is a distinctive feature of tumor cells, mediated by dysregulated signaling pathways [125]. Through its carbohydrate-independent interaction with numerous intracellular oncogenic proteins, Gal-3 has been shown to affect downstream signaling cascades promoting uncontrolled growth of tumor cells [126]. Using HEK-293 cells co-transfected with K-Ras/Gal-3, Elad-Sfadia et al. demonstrated that Gal-3 can interact with activated K-Ras, maintaining its membrane anchorage and activation, and promoting PI3-K and Raf-1 activation, while attenuating active ERK signaling through an unknown mechanism that is probably mediated by upregulation of ERK phosphatases [127]. Consistently, Song et al. report that Gal-3 silencing in pancreatic cancer

cells can attenuate Ras activity and Ras downstream signaling cascades including both ERK and AKT phosphorylation, while cells overexpressed with Gal-3 show enhanced Ras activity and its downstream signaling [128]. Gal-3 was also found to affect tumor progression of oral tongue squamous cell carcinoma (OTSCC) through activation of the Wnt/β-catenin signaling pathway [129]. Thus, Gal-3 has a stimulatory effect on tumor cell proliferation by controlling proliferative signaling pathways. In contrast to these findings, our data suggest an inhibitory role for intracellular Gal-3 on the activities of intracellular signaling pathways in melanoma. We report that Gal-3 depletion leads to the activation of MAPK/ERK and PI3K/AKT signaling pathways, as evidenced by increased phosphorylation of ERK1/2 and AKT, respectively. Furthermore, loss of intracellular Gal-3 resulted in the upregulation of β-catenin, cyclin D1, and c-Myc, suggesting an enhanced activity of the Wnt/β-catenin pathway. It is worth noting that Gal-3 studies on melanoma regarding this aspect are lacking. Indeed, the RAS/RAF/ERK signaling pathway is constitutively active in the majority of melanomas due to the dominance of the activating $BRAF^{V600E}$ mutation [131]. Therefore, comprehensive studies are necessary to establish a more definitive understanding of Gal-3's role in modulating the activities of intercellular signaling pathways.

For a few decades, NFAT1 has been known for its principal roles in both innate and adaptive immune responses [268]. However, its involvement in cancer progression is currently the focus of many studies [270, 271]. NFAT1 has recently been reported as a metastasis-promoting molecule in melanoma [240,

272]. Mechanistically, MMP-3 has been identified as downstream targets of NFAT1 in melanoma [274], where their expression promotes melanoma cell invasive abilities [279, 280]. IL-8, a neutrophil chemotactic factor, is also regulated in melanoma cells by NFAT1 [274], where it is secreted to attract neutrophils that facilitate CTCs extravasation [205, 281]. GPC6 is another novel target gene of NFAT1 that, upon expression, promotes invasion of cancer cells [276]. Li et al. have proposed GPC6 as a new marker of melanoma metastasis [277]. In this study, we detected consistent negative correlation between Gal-3 and NFAT1 by analyzing TCGA melanoma data besides data from murine B16 melanoma model and in vitro studies on Gal-3-silenced cells, raising the possibility that NFAT1 is negatively regulated, either directly or indirectly, by Gal-3. Our findings were further supported by the demonstration of upregulated expression of NFAT1 targets; MMP-3, GPC6, and IL-8 in Gal-3-silenced cells.

In conclusion, our data suggest that decreased expression of Gal-3 is associated with the advancement of melanoma probably via activating oncogenic signaling pathways and unleashing NFAT1-dependent expression of metastasis-associated effector proteins such as MMP-3, GPC6, and IL-8. To the best of our knowledge, this study represents the initial investigation emphasizing the negative crosstalk between Gal-3 and NFAT1 and its role in dictating melanoma metastasis and introducing GPC6 as a candidate promising target of the Gal-3-NFAT1 axis in melanoma.

5. CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Although progress has been made in characterizing the role of the beta-galactoside-binding lectin, galectin-3 (Gal-3) in cancer development and progression, its precise function in melanoma remains unclear. The general purpose of this dissertation was to investigate the role of Gal-3 in melanoma progression and metastasis.

First, I investigated the expression patterns of Gal-3 in serum and tumor tissues from melanoma patients and examined its association with the stage of melanoma. The results showed significantly higher levels of circulating Gal-3 in the serum of melanoma patients compared to healthy individuals with no differences between early and advanced stages of melanoma, proposing its potential as a diagnostic marker for suspected melanoma cases but limited utility in tracking melanoma progression.

Additionally, I observed a consistent downregulation of Gal-3 expression within the tumor cells as melanoma advances from localized to metastatic stages, both in human melanoma samples and murine melanoma models. Based on these findings, I hypothesized that, unlike extracellular and circulating Gal-3, intracellular Gal-3 may hinder primary melanoma progression to a metastatic disease, suggesting a prognostic value of tumor-intrinsic Gal-3 in predicting melanoma metastasis.

Next, I sought to determine the functional consequences of Gal-3 depletion in melanoma cells using RNA interference (RNAi) to selectively silence Gal-3 in melanoma cells. A series of experiments and analyses were conducted

on the Gal-3-silenced cells to unravel the functional significance of Gal-3 in shaping melanoma metastatic behavior. My findings demonstrated that Gal-3-silenced cells exhibited enhanced migration, invasion, and colony formation abilities compared to control cells. The loss of Gal-3 also significantly accelerated tumor growth and enhanced tumor cell metastasis in the *in vivo* studies.

Accumulating evidence points to the crucial role of dysregulated intracellular signaling pathways in the development and progression of melanoma [250]. Therefore, I investigated the involvement of Gal-3 in regulating pro-oncogenic signaling pathways in melanoma. The results indicated that the downregulation of Gal-3 resulted in sustained activation of the PI3K/AKT, MAPK/ERK, and Wnt/β-catenin signaling pathways in melanoma cells. Mechanistically, I observed a negative association between Gal-3 and the transcription factor nuclear factor of activated T cells (NFAT1) in melanoma samples and murine melanoma models. This observation was subsequently investigated using Gal-3-silenced cells which confirmed the negative regulatory role of Gal-3 on NFAT1 expression in melanoma cells. These findings were further supported by the demonstration of upregulated expression of the NFAT1 targets, namely metalloproteinase-3 (MMP-3), interleukin-8 (IL-8), and glypican-6 (GPC6), in Gal-3-silenced cells.

MMP-3 is a member of the matrix metalloproteinase family, responsible for the cleavage of various ECM substances, such as type IV collagen, laminin, and E-cadherin, making it a significant player in driving tumor invasion [279]. IL-8 is a pro-inflammatory cytokine that mediates tumor metastasis via facilitating

invasion, migration, and angiogenesis [274, 282]. GPC6 is a cell-surface proteoglycan that enhances proliferation and invasion of tumor cells in many cancer types [276, 283]. Thus, the metastasis-suppressive function of Gal-3 in melanoma cells is probably mediated via Gal-3-dependant downregulation of NFAT1 and its metastasis-associated downstream targets MMP-3, IL-8, and GPC6.

Overall, this dissertation contributes valuable knowledge to the field of melanoma research by highlighting the tumor-suppressive function of intracellular Gal-3 and its potential as a prognostic indicator for melanoma metastasis (**Figure 5.1**).

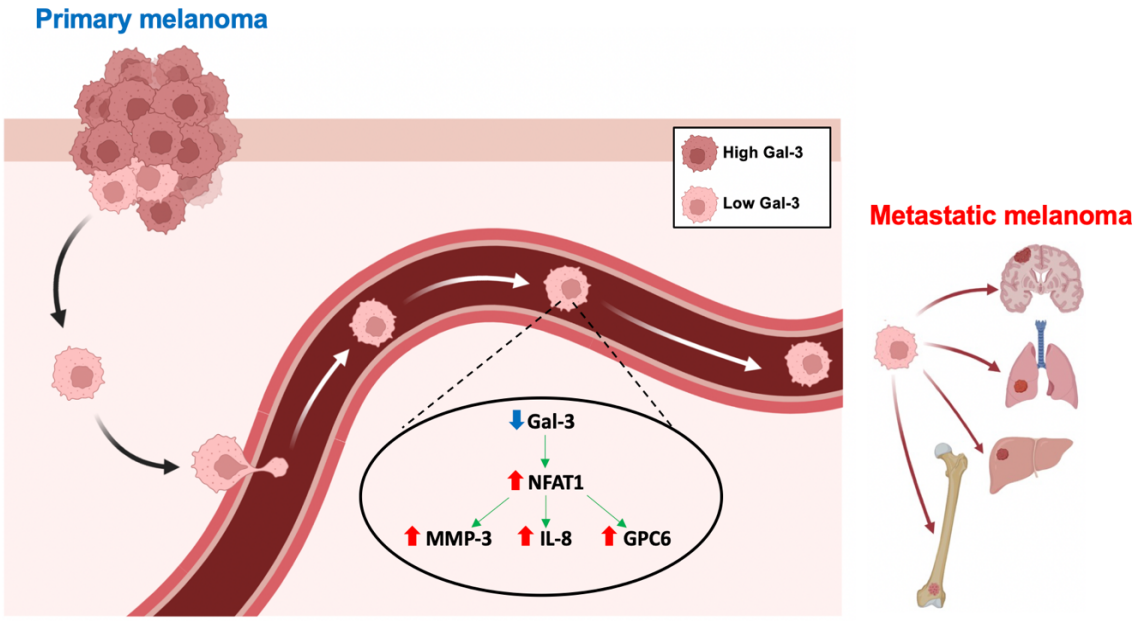


Figure 5.1. Proposed role of tumor-intrinsic Gal-3 in regulating melanoma metastasis.
Gal-3 is expressed at high levels in primary melanoma (Left). Diminished intracellular Gal-3 levels results in unleashing of NFAT1-dependent expression of MMP-3, GPC6, and IL-8, which collaborate to facilitate dislodging of melanoma cells from the primary tumor and migration through the bloodstream to initiate metastatic colonization in distal organs (Right).

This dissertation has fulfilled the aim of investigating Gal-3's role in melanoma metastasis through comprehensive analysis of Gal-3 expression patterns in serum and tumor tissues of melanoma patients in addition to *in vitro* as well as *in vivo* studies performed on Gal-3-silenced melanoma cell lines. However, there were some limitations and/or pitfalls in this project which are addressed below.

The first limitation was the lack of tumor tissue samples from melanoma patients for immunohistochemical Gal-3 staining studies. We have obtained a few melanoma samples from our collaborators at Baptist Health-South Florida/Miami Cancer Institute. However, the sample size was insufficient to achieve statistical significance. Immunohistochemical studies on human primary and metastatic melanoma samples will serve as compelling evidence to support the reliability of tumor-intrinsic Gal-3 as a prognostic marker. Alternatively, we are planning to perform immunohistochemical studies on melanoma tissue microarrays (TMAs). TMAs are constructed by extracting small tissue cores from individual tissue specimens and then embedding them into a new paraffin block. This will allow us to analyze large cohorts of patient samples in a time-effective manner.

The second limitation was the use of a single melanoma cell line, SK-MEL-2, to perform the functional assays proposed in this study. We selected SK-MEL-2 cell line for experimental Gal-3 silencing and further studies due to its high inherent Gal-3 expression compared with other melanoma cell lines included in the study (**Figure 2.4 A, B, and C**) and also due to its characteristic low

metastatic phenotype [231, 232] which makes it ideal for testing our hypothesis. However, using multiple cell lines is essential to strengthen the reliability and validity of our observations and helps differentiate between general cancer cell behavior and cell line-specific effects. Therefore, we are planning to include more melanoma cell lines in this study, assess their Gal-3 expression levels to identify another suitable candidate for Gal-3 silencing, and then repeat the same experiments performed on SK-MEL-2 cell line.

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PUBLICATIONS AND PRESENTATIONS

1. Mohammed NB, Lau LS, Souchak J, Ahluwalia MS, Osman I, Dimitroff CJ. Tumor-intrinsic galectin-3 suppresses melanoma metastasis. *Cancer Research* in preparation.
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11. Mohammed NB and Dimitroff CJ. The metastasis-suppressive function of intracellular galectin-3 in melanoma. Poster presented at the AACR Annual Meeting, April 2023, Orlando, FL, USA. Published Cancer Res 2023;83(7_Suppl): Abstract nr 3602.
12. Mohammed NB and Dimitroff CJ. Intracellular galectin-3 dictates the metastatic potential of melanoma. Poster presented at the Graduate Student Appreciation Week (GSAW) Scholarly Forum, April 2023, Miami, FL, USA.
13. Mohammed NB and Dimitroff CJ. Tissue galectin-3: A novel prognostic index for patients with melanoma. Poster presented at the FIU CTS/Cleveland Clinic Florida Research and Innovation Center, Translational Research Symposium, March 2023, Port St. Lucie, FL, USA.
14. Mohammed NB and Dimitroff CJ. "How is the "Sugar coat" of a melanoma cell adapted to enhance tumor progression?" Oral presentation at the HWCOM 8th Annual Research Symposium, April 2022, Miami, FL, USA.
15. Mohammed NB, Carroll J, Chakraborty A, Ortega L, and Dimitroff CJ. "Galectin-3: Opposing roles in melanoma progression". Poster presented at the NIH/NCI Alliance of Glycobiologists for Cancer Research U01 Steering Committee Meeting, April 2022, Miami, FL, USA.
16. Mohammed NB, Carroll J, Chakraborty A, Ortega L, and Dimitroff CJ. "Metastatic melanoma glycome controls the pro-metastatic properties of Galectin-3". Poster presented at the Graduate Student Appreciation Week (GSAW) Scholarly Forum, April 2022, Miami, FL, USA.
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