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BSc in Biology

IDENTIFYING MOLECULAR MECHANISMS AND THERAPEUTIC TARGETS IN MEDULLOBLASTOMA

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

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Every great scientific truth goes through three stages. First, people say it conflicts with the Bible. Next, they say it had been discovered before. Lastly, they say they always believed it.

Louis Agassiz 1807-1873

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ABSTRACT

Medulloblastoma (MB) is a malignant pediatric brain tumor originating in the cerebellum, exhibiting significant heterogeneity across its subgroups. Sonic Hedgehog (SHH) MB, a particularly well characterized subgroup, arises from cerebellar granule neuron precursors, with aberrations in the SHH signalling pathway. Galectin 3 (GAL3) plays a critical role in tumor progression by modulating immune responses and cellular processes and is notably upregulated in SHH-MB. To elucidate the mechanisms underlying GAL3 overexpression in SHH-MB, a GAL3 Knockdown (KD) was performed in DAOY and tNES cell lines. Our results suggests that GAL3 reduction leads to the decreased in tumor homing capacity and growth, *in vivo*. Additionally, the study emphasises the correlation between GAL3 and the arginine metabolism, revealing that the GAL3 KD disrupts the expression of arginine transporters and the enzyme Argininosuccinate Synthase 1 (ASS1) in DAOY cell lines. The metabolic vulnerabilities of tumor cells, particularly in amino acid pathways, are a promising avenue for new therapies. One potential strategy is targeting GAL3 which, when combined with arginine depletion strategies, could significantly enhance treatment efficacy. For SHH-MB, this combination therapy offers hope for more effective outcomes, especially in pediatric patients, potentially minimizing harmful side effects.

Keywords: Medulloblastoma, Cancer metabolism, Galectin-3, Arginine synthesis, Arginine-deprivation

RESUMO

Meduloblastoma (MB) é um tumor cerebral pediátrico maligno, que tem origem no cerebelo, sendo caracterizado por uma significativa heterogeneidade entre os seus subgrupos. O subgrupo Sonic Hedgehog (SHH) MB, notavelmente bem caracterizado, origina-se a partir de progenitores neurais granulares, que exibem alterações na via de sinalização de SHH. A Galactina-3 (GAL3) apresenta um papel essencial na progressão tumoral, uma vez que modula respostas imunes e influencia processos celulares, estando sobre expressa em SHH-MB. Com o propósito de elucidar os mecanismos subjacentes à sobre expressão de GAL3 em SHH-MB, foi realizado um “Knockdown” (KD) no gene da GAL3 nas linhas celulares DAOY e tNES. Os resultados obtidos indicam que a redução dos níveis da GAL3 provocam uma diminuição na capacidade de retorno do tumor ao seu sítio de origem e crescimento tumoral, *in vivo*. Adicionalmente, este estudo revela uma correlação entre a GAL3 e o metabolismo da arginina, demonstrando que a redução nos níveis de GAL3 perturba os níveis de expressão dos principais transportadores de arginina e da enzima Argininossuccinato Sintase 1 (ASS1) na linha celular DAOY. As numerosas vulnerabilidades metabólicas das células tumorais, incluindo nas vias de aminoácidos, oferecem um vasto leque de oportunidades para o desenvolvimento de novas terapias. Abordagens terapêuticas combinadas, envolvendo a inibição de GAL3 e estratégias de depleção de arginina, podem representar uma via bastante promissora para o tratamento de SHH-MB, potencialmente resultando em tratamentos mais eficazes e menos prejudiciais para os pacientes pediátricos.

Palavras-chave: Meduloblastoma, Metabolismo do cancro, Galactina-3, Síntese da arginina, Depleção de arginina.

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Symbols and Abbreviations

%	Percentage
>	Higher then
<	Lower then
Δ	Delta
°C	Celsius degrees
+	Plus
ADC	Arginine Decarboxylase
AGM	Arginine Agmatinase
ARG	Arginase
ASL	Argininosuccinate Lyase
ASS1	Argininosuccinate Synthase 1
ATPF51	ATP synthase inhibitor factor 1
BCA	Bicinchoninic Acid
BCL2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
CATs	Cationic Amino acid Transporters
CCND1	Cyclin D1
cDNA	complementary Deoxyribonucleic acid
CNS	Central Nervous System
CK1	Casein Kinase 1
CRD	Carbohydrate Recognition Domain
Ct	Cycle Threshold
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
EGFR	Epidermal Growth Factor Receptor
EGFP	Enhanced Green Fluorescent Protein
EGL	External Granule cell Layer
FBS	Fetal Bovine Serum
FGF2	Fibroblast Growth Factor 2
Exp	Experiment

g	grams
h	hours
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GAL	Galectin
GBP	Glycan Binding Protein
GLI	Glioma associated oncogene
GNP	Granule Neural Precursor
GCP	Granule Cell Precursor
GS	Gorlin Syndrome
GSK3	Glycogen Synthase Kinase 3
HEPES	Hydroxyethyl Piperazinyl Ethanesulfonic Acid
HH	Hedgehog
IGL	Internal Granule cell Layer
Il7	Interleukin-7
iPS cells	Induced Pluripotent Stem cells
KD	Knockdown
KDa	kilodaltons
KO	Knockout
KRAS	Kirsten Rat Sarcoma
L	Liters
LATs	L-type Amino acid Transporters
LFS	Li-Fraumeni Syndrome
LGALS1	Galectin-1
LGALS3	Galectin-3
LGALS9	Galectin-9
LOF	Loss of Function
MB	Medulloblastoma
mg	milligrams
mL	millilitres
mM	millimolar
MOI	Multiplicity of Infection
mRNA	messenger Ribonucleic Acid

mTOR	mammalian Target Of Rapamycin
NES cells	Neuroepithelial Stem cells
ng	nanograms
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NGS	NOD/SCID/IL2Ry
PKA	Protein Kinase A
OAT	Ornithine AminoTransferase
OCT	Ornithine Carbamoyl Transferase
ODC	Ornithine Decarboxylase
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDG	Phosphate Dependent Glutaminase
ProDH	Proline Dehydrogenase
PTCH1	Patched1
P5CS	Pyrroline5 Carboxylate Synthase
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RNA-seq	Ribonucleic Acid Sequencing
RPMI	Roswell Park Memorial Institute
RT-pPCR	Real Time- quantitative Polymerase Chain Reaction
SCG2	Secretogranin II
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SHH	Sonic Hedgehog
SHH-MB	Sonic Hedgehog Medulloblastoma
sh-RNA	Short Hairpin Ribonucleic Acid
SLC	Solute Carrier
SMO	Smoothened
SUFU	Suppressor of Fused
TBST	Tris- Buffer Saline with 0.1% Tween-20

TCA	Tricarboxylic Acid
tNES cells	tertiary Neuroepithelial Stem cells
TP53	Tumor Protein 53
µg	Microgram
µm	Micromolar
µl	Microliter
WB	Western Blot
WNT	Wingless
WNT-MB	Wingless Medulloblastoma

1. Introduction

1.1. Medulloblastoma

The central nervous system (CNS) is composed of the brain and the spinal cord. The brain, which is embedded within the skull, is divided into three major components: the brainstem, the cerebrum, and the cerebellum ¹. The cerebellar cortex is a remarkably simple laminar structure, with two principal groups of neurons, the granule cells and the Purkinje cells. The most prevalent neuronal subclass within the cerebellum, is the cerebellar granule neurons. Granule neurons serve an essential role in coordinating afferent input to and motor output from the cerebellum through their excitatory connections with Purkinje neurons. Although the cerebellum represents only 10% of the brain volume, it bears more than half of a total amount of neurons. The cerebellum is involved in the control of movement by forming an internal model through repeated performance and feedback, allowing the movement to be executed skilfully. It is also involved in cognitive functions, including sensory-motor learning, speech, and spatial memory. Cerebellar development is tightly regulated, and dysfunction in this process could lead to cancer, as well as, neurological disorders ¹⁻³. Cancer is among the leading causes of death worldwide and it is characterized by the proliferation of cells that have managed to evade central endogenous control mechanisms. Cancer develops due to genetic abnormalities caused by somatic or germline mutations, as well as a plethora of progressive events inducing genomic instability, some of them also related to environmental factors, such as ultraviolet radiation or chemicals in the breathing air. The dysregulation of oncogenic pathways may activate hallmark processes necessary for the initiation and progression of tumorigenesis ^{4,5}. Medulloblastoma (MB) is a brain cancer, developed in the cerebellum, as represented by **Figure 1.1**.

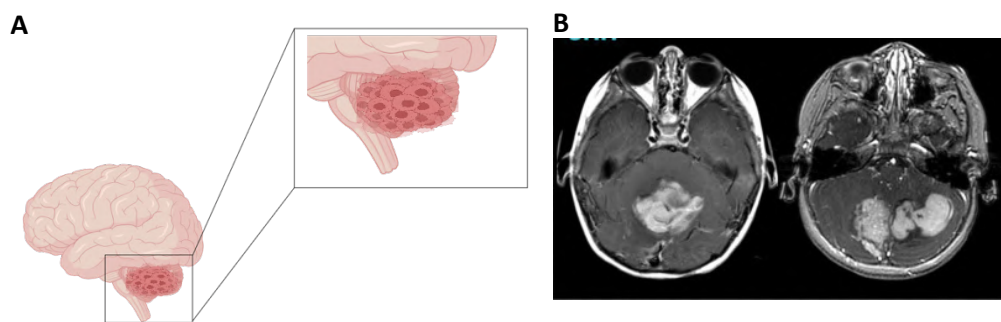


Figure 1.1 Medulloblastoma Localization. **A-** Schematic representation of the Sonic Hedgehog MB localization in the cerebellum. **B-** Axial contrast-enhanced T1 MRI scans of Sonic Hedgehog MB. Image adapted from: Zhang *et al.*, 2022 ⁶.

The name “medulloblastoma” was first introduced by Harvey Cushing and Percival Bailey in 1925. Drs. Cushing and Bailey had initially used the term “spongioblastoma cerebelli” to describe

the posterior fossa tumor seen in pre-adolescents. They changed the name to medulloblastoma to distinguish it from a distinct glial tumor. The two recognized that MB cells resembled undifferentiated cells present in embryonal stages, and speculated that, MB arose from multipotent stem cells thought to be present in the neural tube ^{7,8}. Nowadays, the World Health Organization (WHO) defines MB as a “malignant invasive embryonal tumor of the cerebellum with preferential manifestation in children, predominantly neuronal differentiation and an inherent tendency to metastasize via cerebrospinal pathway” ⁹. Most MB are sporadic and arise in the posterior fossa due to the deregulation of cerebellar development, however, in rare cases, MB can be associated with inherited disorders such as Li-Fraumeni syndrome (LFS), Turcot or Gorlin syndrome (GS) ⁶.

There has been a revolution in cancer genomics in the last two decades, and neuro-oncology is not an exception. While many different pediatric brain tumors were initially aggregated together under the denomination of primitive neuroectodermal tumors, new research methods enable the classification and grouping of brain tumors and a comprehensive molecular characterization of new rare diseases ¹⁰. MB represents a heterogeneous tumor with multiple subtypes, sharing a primitive embryonal phenotype, composed of malignant tumor cells which are dominated by neural antigen expression ¹¹. Recent discoveries have provided valuable insight into substantial clinical and biological heterogeneity within this disease-encompassing at least four molecularly distinct subgroups (Wingless [WNT], Sonic Hedgehog [SHH], Group 3 and Group 4), as the name indicates, WNT-MB and SHH-MB display deregulation in WNT signalling and SHH signalling, respectively. Group 3 tumors have the poorest prognosis of all MB subgroups and are frequently associated with genomic instability and *MYC* amplification, and present large cell/anaplastic histology. Group 4 are the most frequent group, and exhibit an intermediate prognosis, however, the underlying biology of Group 4 is less understood than of the other subgroups ¹². Each group is characterized by discrete genomic landscapes, patient demographics, and clinical phenotypes. The association between genotypes, transcriptional profiles, and patient age at diagnosis suggests that MB subgroups arise from the transformation of different cell types in precise spatial-temporal patterns. WNT- and SHH-MB are thought to originate from cells in the extracerebellar lower rhombic lip, and from cerebellar granule neuron precursors (GNPs), respectively. In contrast, cellular origins of Group 3 and Group 4 MB remain unconfirmed ¹³. Each subgroup with prognostically relevant genomic, epigenomics, and transcriptomic signatures. Histologically defined categories include classic, desmoplastic/nodular, anaplastic/large cell, and MB with extensive nodularity ^{10,14}. **Figure 1.2.** stratifies the 4 molecular subgroups of MB. The current idea is that each different MB group is derived from a specific cell of origin, which therefore determinates the clinical and molecular behaviour of the disease. Additionally, the four MB

groups have recently been subdivided into 12 subtypes which display specific molecular and clinical characteristics ¹⁵.

	WNT	SHH	Group 3	Group 4
Age Group	Child	Infant and Adult	Infant	Child
Gender ratio (♂:♀)	1:1	1:1	2:1	3:1
Outcome	Very good	Infants good, others intermediate	Poor	Intermediate
Anatomic diagnosis	Brainstem, 4 th ventricular	Cerebellar hemisphere	Midline, 4 th ventricle	Midline, 4 th ventricle
Metastasis at Diagnosis	5-10%	15-20%	40-45%	35-40%
Pattern of recurrence	Rare. Can be local or metastatic	Local	Metastatic	Metastatic

Figure 1.2. Molecular subgroups of MB. WNT- Wingless, SHH- Sonic Hedgehog. Figure adapted from: Kuzan-Fisher *et al.*, 2018¹⁶.

MB is the most malignant childhood brain tumor, accounting for 30% of pediatric brain tumors and 7% to 8% of all brain malignancies. Leptomeningeal dissemination occurs in up to 40% of patients at time of diagnosis and almost all patients present with metastases at time of recurrence ^{10,16–18}. The incidence of MB peaks occurs during the first decade of life, with a higher incidence noted in children between 3 and 4 years of age and between 8 and 10 years of age. Although MB is mainly a pediatric disease, it can occur in adults. Fewer than 5% of cases are associated with the hereditary cancer predisposition syndromes ¹⁷.

Since, MB generally occurs in the posterior fossa, its diagnosis and treatment are much more challenging and often delayed. The 5-year overall survival of MB patient is 60-80% under the current standard multimodal treatment consisting of maximal safe tumor resection, chemotherapy and, for non-infant (>3-5 years) patients, craniospinal irradiation ¹⁶. However, the long-term use of these therapeutic regimes has shown to induce substantial toxic damages, most notably in developing young patients. The inability of the therapy to recognize both the inter- and intra-tumoral heterogeneity of MB subgroups and the lack of clinical biomarkers to stratify patients, are the major drawbacks underlying the adverse effects ⁹. Therefore, even though the long-term survival of MB patients is relatively high (60-80%), the impact of treatment-related morbidities on the quality of life in survivors remains high. Still, more than one-third of patients die within 5 years after diagnosis, and the median survive of relapse/refractory MB is less than 1 year ^{11,19}. To further improve the survival rate of MB patients and identify novel treatment options, new disease-specific models are required.

1.2. Sonic Hedgehog (SHH) medulloblastoma

Hedgehog (hh) proteins constitute a family of secreted signalling proteins that together regulate multiple aspects of animal development, tissue homeostasis and regeneration²⁰. The Hedgehog signalling pathway, is an evolutionary conserved pathway of signal transmissions from the cell membrane to the nucleus. The hh signalling pathway plays a significant role in the normal embryonic development of invertebrates and vertebrates²¹.

Cerebellar granule neurons (CGN) are the largest neuronal population of the nervous system, and these cells arise from granule cell precursors (GCPs). GCPs populate the surface of the cerebellum and forms the external granule cell layer (EGL). After one week of proliferation, GCPs start to differentiate and migrate towards the internal granule cell layer (IGL). Although different molecules promote the proliferation of neuronal precursors, Shh is the most important mitogen for GCPs. SHH-MB arise during the post-natal period where GCPs undergo a rapid and massive proliferation. This massive proliferation is due principally to the SHH mitogen, that is released from the Purkinje cells²². Recent single-cell RNA-seq experiments show that among all the cerebellar cell types, GCP gene expression most closely matches SHH-MB gene expression, supporting the conclusion that GCPs are also the cell of origin of SHH-MB¹⁰. **Figure 1.3**, is a schematic representation of the cerebellum development.

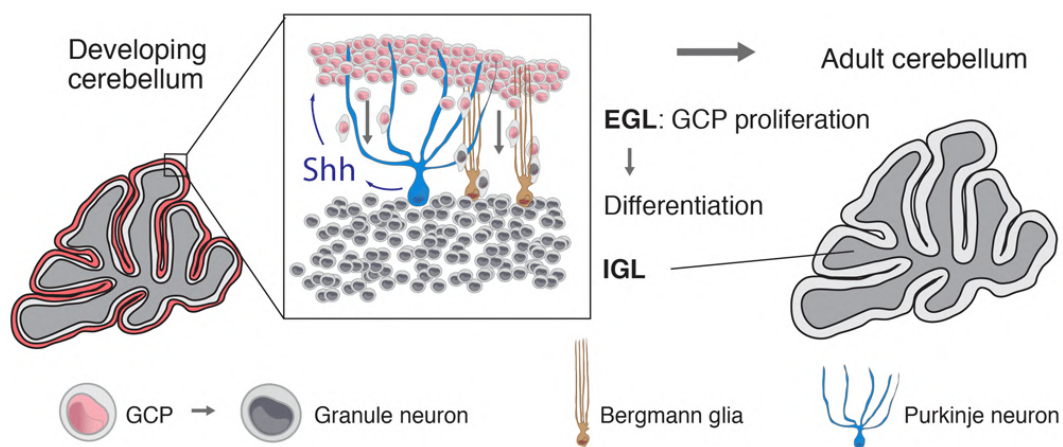


Figure 1.3. Cerebellum development. GCPs, the progenitors that give origin to Granule neurons, proliferate in the EGL of the cerebellum in response to Purkinje neuron-derived SHH. Granule neurons populate the IGL after GCPs differentiate and migrate through the Purkinje cell layer using the radial process of Bergmann glia. Image from: Tamayo-Orrergo *et al.*, 2019¹⁰. GCP- granule cell precursors; EGL- external granule layer; IGL- internal granule layer; Shh- Sonic hedgehog.

The SHH-activated subgroup has a characterized molecular and genetic profile offering a wide array of appealing targets. In the canonical signalling pathway, the SHH cascade is initiated with the binding of the SHH ligand to Patched1 (PTCH1) transmembrane receptor, thus removing the blockade of the latter on Smoothened (SMO), which is a G protein coupled receptor²³. In this turn, SMO translocate to the primary cilium where it causes the activation of proteins belonging

to the glioma-associated oncogene (GLI). The active form of GLIs dissociate from suppressor of fused (SUFU), a protein encoded by a tumor suppression gene. The activate form of GLIs, the final effectors of the pathway, are able to move to the nucleus and positively influence the transcription of specific target genes. These target genes include *CCND1*, *IL7* and *BCL2* being involved in cell proliferation²⁴. In the absence of SHH ligand, PTCH1 gets localized in the plasma membrane in the primary cilium and inhibits SMO. GLIs are phosphorylated through PKA/CK1/GSK3(cAMP-dependent protein kinase/casein kinase 1/ glycogen synthase kinase3) complex and undergo a proteolytic cleavage that allows the detachment from SUFU. As a consequence, GLIs are converted in their repressor form and subsequently transfer to the nucleus to repress the expression of particular target genes (*CCND1*, *IL7* and *BCL2*)^{2,23}. The canonical SHH pathway is represented by **Figure 1.4**. In addition to this canonical pathway, multiple alternative non-canonical pathways have been shown to activate SHH signalling downstream of SMO. The SHH-MB group compromises 30% of all MBs.

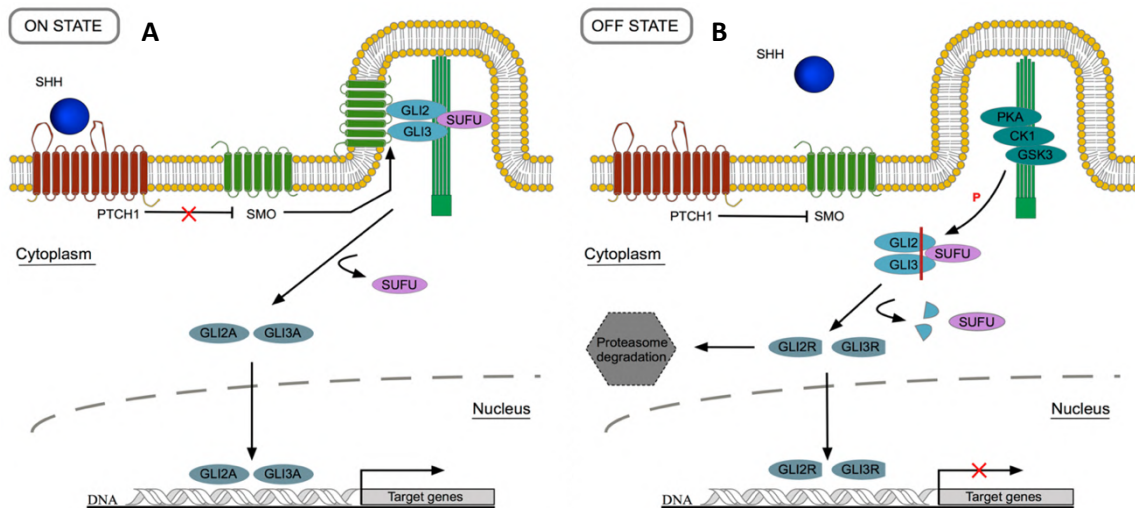


Figure 1.4. Overview of canonical SHH signalling pathway. **A-** Representative signalling cascade when the SHH signalling is switch on. In the presence of SHH ligand, PTCH1 gets inhibited by the SHH ligand-dependent interaction. This activates SMO, allowing its translocation to the cilia. Subsequently, GLI proteins dissociate from SUFU to enter the cell nucleus and activate the target genes. **B-** Representative signalling cascade when the SHH signalling is shut off. In the absence of the SHH ligand, PTCH1 gets localized to the plasma membrane and inhibits SMO. This causes the GLI family proteins to stay restrained by SUFU to facilitate their phosphorylation. Consequently, the GLI proteins undergo proteolysis to generate GLI-R that supresses the transcription of target genes. SHH- sonic hedgehog; PTCH1- Patched; SMO- Smoothened; SUFU- suppressor of fused. Figure adapted from: Fernandes-Silva *et al.*, 2017²³.

Consequently, aberrant expression of hh-target genes leads to excessive cell proliferation and tumorigenesis. SHH signalling, which normally stimulates the proliferation of lineage-restricted cerebellar GNPs during cerebellar development, can lead to MB formation when aberrantly activated^{25,26}.

SHH-MB are genetically characterized by either genetic or somatic mutations and aberrant expression in one of the SHH pathway genes. GS, also known as basal cell carcinoma syndrome, is an inherited autosomal dominant disorder caused by germline inactivation of *PTCH1* and *SUFU*, that is associated with an increased risk of development of skin cancer and MB. LSF is also an inherited autosomal dominant condition caused by germline mutations in *TP53* that is associated with an increased risk of a spectrum of various cancers, including MB. Somatic mutations affecting genes involved in SHH signalling have been extensively described in SHH-MB and occur across all molecular subtypes. Large-scale human sequencing studies have revealed frequent loss-of-function (LOF) mutations and focal deletions in *PTCH1* (40%) and *SUFU* (13%), activating mutations in *SMO* (9%), and amplifications of *MYCN* (13%) and *GLI2* (6%)^{27,28}.

SHH-MB is an heterogeneous disease, where multiple events and mechanisms can be involved in tumor formation. The complexity of SHH-MB can reshape how new therapies for SHH-MB are imagined and oriented research towards novel approaches.

1.3. Galectins

Canonical pathways of immune regulation involve ligand-receptor interactions that transmit signalling outcomes. The discrete nature of such ligand-receptor pairs, including the specific signalling pathways they engage, lends well to genetic and pharmacologic approaches to define their biological functions²⁹.

Mammalian cells express various glycan binding proteins (GBP) that recognize glycosylated proteins and glycosylated lipids. Galectins (GALs) were the first family of GBPs for which immune regulatory activity was described and they represent the most ancient arm of mammalian GBP evolution. GALs represent a family of evolutionary lectins that preferentially bind β -galactose-containing glycoconjugates via their carbohydrate recognition domains (CRDs), which consists of approximately 130 amino acids. The CRD mainly detects glycoproteins sites on other proteins for binding. Different galectins have been identified to bind with immune cells, such as cytotoxic T cells, dendritic cells, and macrophages, to regulate cancer cell immunosurveillance³⁰. There are 11 galectins in humans (GAL1, GAL2, GAL3, GAL4, GAL7, GAL8, GAL9, GAL10, GAL12, GAL13, GAL14). The interaction between galectins and their specific ligands may lead to a broad range of biological activities that are dependent on the cell type and tissue context, the expression levels of individual galectins, and the potential receptors involved³¹. GALs can be detected in the cytoplasm and exhibit a secretory form, the secretory proteins are then embedded inside lipid bilayer vesicles and transported into the Golgi apparatus, after which the vesicles and can be further fused with the cell membrane to export the protein outside the cell³². GALs are soluble proteins that can mediate their functions both intracellularly and

extracellularly. Once, in the extracellular medium, galectins can transduce intracellular signals, leading to modulation of a wide range of cellular processes including proliferation, differentiation, and survival. Furthermore, GALs can also control intracellular processes, including mRNA splicing, cell cycle progression, apoptosis, among others ³³. **Figure 1.5** summarizes the immunomodulatory roles of galectins in inflammation.

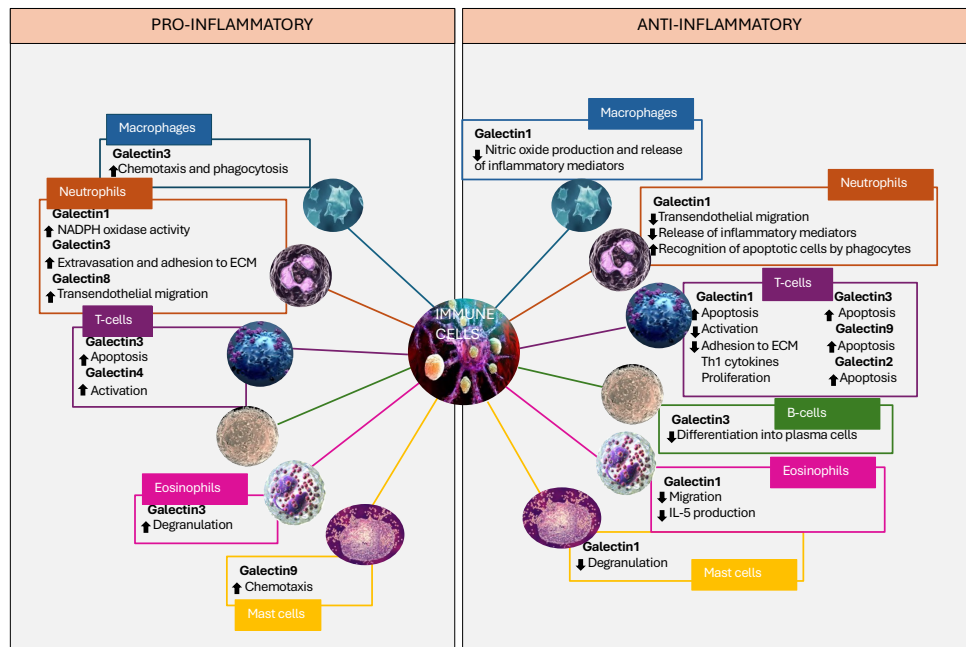


Figure 1.5. Galectin Immunomodulatory role in inflammation. The effect of galectins on immune system cells promotes different processes that directly the outcome of the disease. ↓, ↑ Means decrease or increase. Figure adapted from: Sergio Martin-Saldaña *et al.*, 2022 ³⁴.

The interactions in the tumor microenvironment are considered another critical mechanism for cancer progression that assimilates peripheral cells through secretion of various molecules. Furthermore, there are various cells surrounding the microenvironment, such as stromal cells and immune cells, which are reportedly regulated by GALs ³⁵. For example, in tumor cells, intracellular galectins can enhance oncogenic signals and reduce apoptosis that promote tumor transformation and proliferation. GALs have pivotal roles in the innate and adaptive immune response, also the effect of GALs on immune system cells promotes different processes that directly affect the outcome of the disease. Additionally, what makes them appealing targets for immunomodulatory therapies, is that galectins are notably regulated by activated T-cells and B-cells, among other immune cells ^{34,36}.

Galectin-3 (GAL3) is one of the most studied members of the galectin family that mediates various biological processes, such as growth regulation, immune function, cancer metastasis and apoptosis. As a multifunctional protein involved in multiple pathways of many diseases, GAL3 has generated significant interest ³⁷. Human GAL3 is a protein coded by a single gene, *LGALS3*, located on chromosome 14, is widely expressed in human tissues, including all types of immune

cells, epithelial cells, endothelial cells and sensory neurons³⁸. GAL3 is distributed in the nucleus, cytoplasm, and cell surface. It can also be secreted into the extracellular matrix. Nuclear and cytoplasmic GAL3 expression is closely engaged in processes that are pivotal for basic cellular functions, such as pre-mRNA splicing, regulation of cell growth, cell cycle progression, apoptosis and tumor-suppressor or -promoter function³³. Extracellular GAL3 acts as an adhesion molecule by cross-linking adjacent cells, and cells and extracellular matrix and is also associated with the occurrence, growth, embolization, and metastasis of cancer cells³⁹. Because of the expression of GAL3 in various of tissues and cell types, and its involvement in various human diseases, this molecule is particular interest due to its remarkable role in controlling cancer progression. GAL3 is often highly expressed in various solid and malignant tumors, which is generally correlated with the progression of cancer³⁸, suggesting that this molecule plays an important role in disease outcome. There are some indications that GAL3 has an important role in the initiation of mammary carcinomas, possibly through its interactions with oncogenic RAS (Rat Sarcoma virus) proteins. GAL3 preferentially binds to K-RAS, modulating gene expression at the transcriptional level^{40,41}. There is also substantial evidence that indicating that GAL3 is associated with mediating important tumor-related functions, such as tumorigenesis, neoplastic transformation, tumor cell survival, angiogenesis, tumor metastasis and regulation of apoptosis^{42,43}. GAL1 and GAL9, expression has also been described to be altered in some tumors, including lung cancer and breast cancer, contributing to tumor cell proliferation, differentiation and metastases^{44 43,45}. **Figure 1.6** highlights the contribution of GAL3, GAL1 and GAL9 in the tumor microenvironment. However, the role of galectins and their expression levels in MB are still unknown.

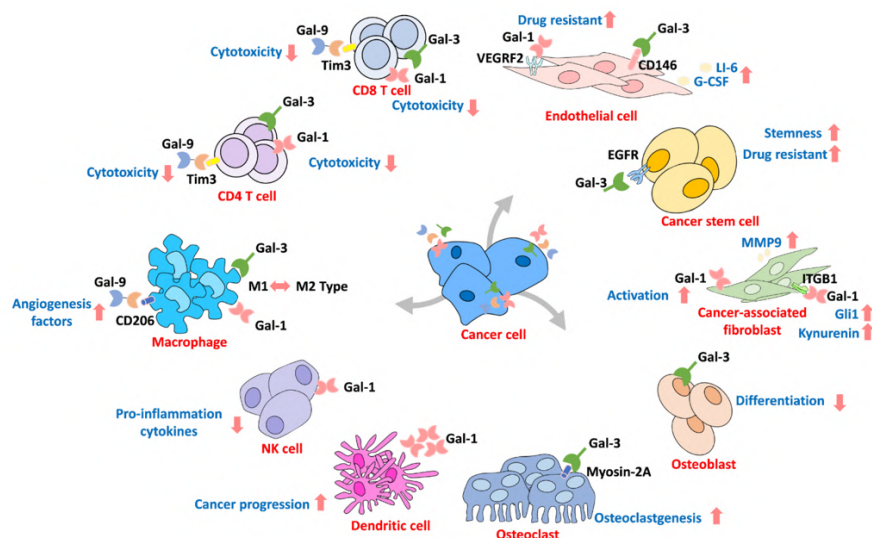


Figure 1.6. The Galectin family contributes to tumor microenvironment. GALs can act as a reprogramming messenger to connect tumor cells and other cells in the microenvironment. ↓, ↑ Means decrease or increase. Figure from: Chien-Hsiu Li *et al.*, 2021³³.

1.4. Galectin-3 is upregulated in SHH-MB and its downregulation affects biological pathways involved in amino acid metabolism

Although current treatments for MB have significantly improved survival of affected children, they often result in devastating side effects. With this in mind, our lab identified and tested different therapeutic targets against MB, developing new models that mimic the initiation and progression of the disease. We have established, through cellular reprogramming, healthy neural stem cells carrying a germline mutation known to activate the SHH signalling pathway. Our lab induced pluripotent stem (iPS) cells by the expression of reprogramming factors in skin fibroblasts. The fact that iPS cells and their derivatives mimic early stages of human development, makes this model more attractive to study MB ⁴⁶.

It is also well established that the CNS develops from a small number of highly plastic progenitors called neuroepithelial cells, these cells have been shown to be competent in generating granule cells, a major cell population in the cerebellum. Long-term self-renewing neuroepithelial-like stem cells (NES cells) are able to maintain their stem cell properties even after long-term propagation, *in vitro*. So, we have generated these Gorlin NES cells to form tumors when exposed to permissive environment ⁴⁶. However, not all NES cells are able to form tumors, only Gorlin NES cells are able to do it, healthy NES cells do not form tumors. To test if the Gorlin NES cells could give rise to tumors, *in vivo*, the cells were orthotopically transplanted into the cerebellum of immunodeficient mice, after some weeks we collected the tumor and kept the Primary tNES cells in culture in neural stem cell media (NES media). This procedure was performed two more times, generating Secondary and Tertiary tNES cells, as it is represented by **Figure 1.7**.

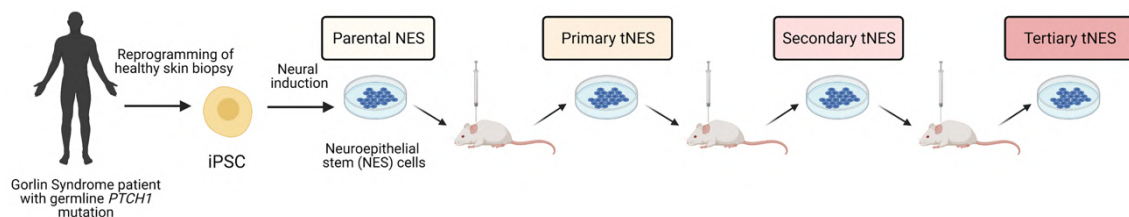


Figure 1.7. Schematic representation of the model created previously in our lab, to study SHH-MB. iPS cell technology was used to derive a MB model by creating NES cells from a Gorlin Syndrome patient. Orthotopically transplantations were performed in mice to form tumors. Three generations of NES cells were created (primary tNES, secondary tNES and tertiary tNES). Figure created using BioRender.

Unpublished data from our lab show that GALs, including GAL3, are upregulated in SHH-MB. Further analysis reveals that GAL3 was more upregulated in Gorlin tertiary tNES, when compared with primary and secondary tNES. With the purpose of determine if GAL3 is associated with an increase in malignancy in SHH-MB, our lab members performed a Knockout (KO) of the GAL3 gene on tertiary tNES cell lines. Upon the KO of *LGALS3*, migration, proliferation, 3D spheres formation and tumor formation, *in vivo*, are all decrease, compared with the control tNES cell

lines. These interesting findings, suggests that the upregulation of GAL3 in tertiary tNES is correlated with an increase in malignancy and a tumorigenic behaviour, in SHH-MB.

Also, upon the KO of *LGALS3*, a metabolite profile and a metabolite set enrichment analysis were performed. Interestingly, these results show that the loss of GAL3 impairs amino acid uptake and downstream amino acid metabolism, especially the arginine metabolism (Van Bree *et al.*, 2024/unpublished data).

1.5. The Arginine Metabolism

Arginine, the substrate of arginase, is a semi-essential amino acid, because under certain conditions, such as growth during infancy, pregnancy, severe immune challenge, or burn injuries, humans require a supplemental intake of dietary arginine⁴⁷. Arginine is involved in numerous biological functions including cell proliferation, cell signaling, muscle contraction, immunity, neurotransmission, vasodilation, among others⁴⁸.

Interestingly, minor arginine disturbances might prompt cellular and organ dysfunctions, so its metabolism is highly intricate and tightly regulated. Three major resources of arginine include: 1) arginine-enriched nutrition supplement from dietary intake, such as chicken, pork loin and peanuts, 2) endogenous synthesis from citrulline (15% of total arginine production) and, 3) protein catabolism (approximately 80% of the circulating arginine). In the endogenous synthesis, glutamine and glutamate are taken up by the small intestine and finally converted to citrulline in a series of reactions taking place at the small intestine. Citrulline is subsequently released by the intestines into the blood stream, with an efficient bypass of the first-pass metabolism. Once in the systemic circulation, all fluxes of citrulline in the plasma feed into de novo arginine synthesis by the kidneys. In the kidney, argininosuccinate synthase 1 (ASS1) converts citrulline to argininosuccinate, and subsequently, argininosuccinate lyase (ASL) converts it to arginine, which ultimately reenters circulation in the bloodstream^{48–50}. The inter-organ arginine metabolism is represented in **Figure 1.8**.

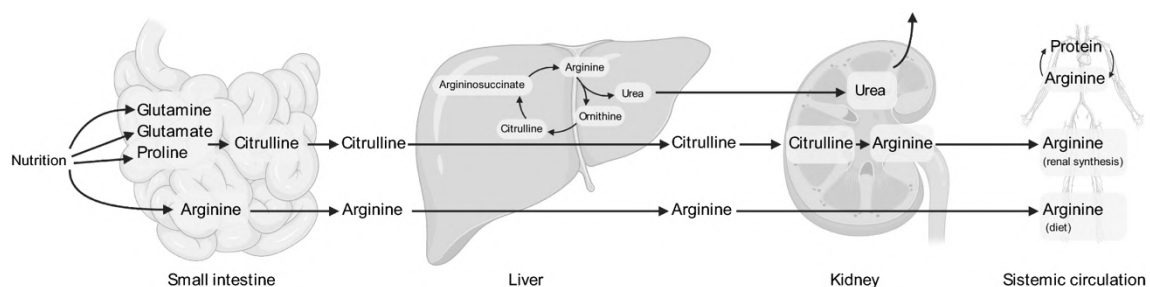


Figure 1.8. Inter-organ arginine metabolism and the intestinal renal arginine synthesis axis. Figure from: L ndez *et al.*, 2021.⁴⁷

Arginine, endogenously, is synthesized in the cytoplasm from citrulline through consecutive reactions catalyzed by ASS1 and ASL. It can be converted into nitric oxide (NO), urea and agmatine. NO participates in a variety of signaling processes and in the tissues defense against pathogens but is also a toxic agent when produced in excess⁵¹. Thus, the regulation of supply of arginine for NO generation in neural cells may be of pivotal importance for a delicate balance between normal and pathophysiological events in the periphery and CNS. Arginine is converted in downstream metabolites such as putrescine via two different pathways. Depending on the reaction, either agmatine or ornithine is the intermediate metabolite. Putrescine is an important polyamine. Polyamines, play an important role in several physiological and biochemical processes of living organisms. They control and regulate various important cellular and genetic functions, such as proliferation, transcription, translation, and post-translational modifications^{49–51}. The endogenous synthesis of arginine is represented in **Figure 1.9**.

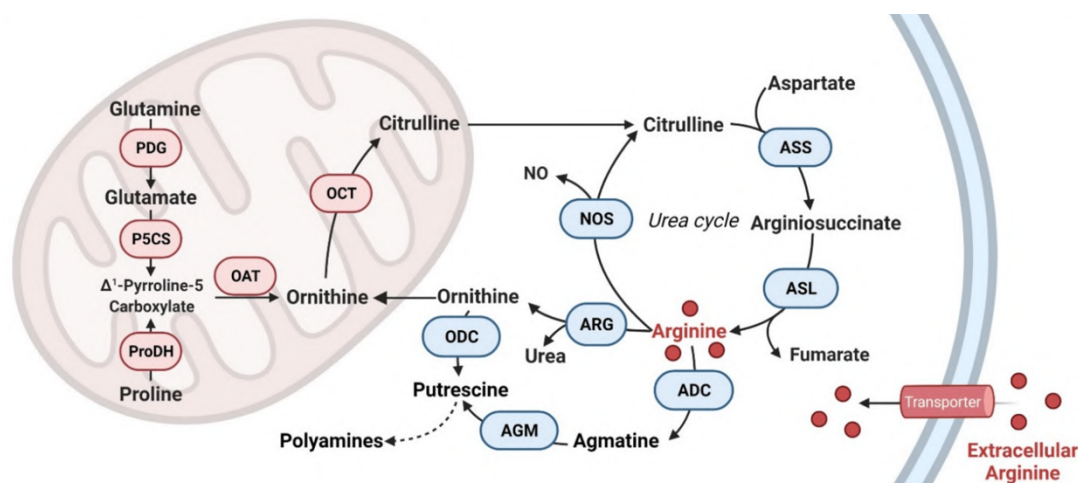


Figure 1.9. Arginine-related Metabolic pathways. In normal cells, arginine can be directly transported into the cell or synthesized from citrulline and aspartate in the urea cycle. Arginine can be converted into nitric oxide, urea and agmatine. ASL: argininosuccinate lyase, ASS: argininosuccinate synthase, ADC: arginine decarboxylase, AGM: agmatinase, ODC: ornithine decarboxylase, ARG: arginase, NOS: nitric oxide synthase, OCT: ornithine carbamoyl transferase, OAT: ornithine aminotransferase, PDG: phosphate-dependent glutaminase, P5CS: pyrroline-5-carboxylate synthase, ProDH: proline dehydrogenase. Figure from: Cheng *et al.*, 2021⁴⁸.

The availability of arginine for metabolic functions is also determined by activities of its transporters in the plasma and mitochondrial membranes. Consequently, basically every cell needs to transport arginine across the plasma membrane, however, the lipid bilayer of biological membranes is impermeable for hydrophobic solutes such as amino acids. Specialized carrier proteins are therefore necessary to provide adequate import and export routes.⁵² Multiple transport systems mediate the influx of amino acids across plasma membrane into mammalian cells⁵³. Arginine transport is mostly mediated by members of the solute carrier 7 family (SLC7), which is divided into two subgroups: cationic amino acids transporters (CATs SLC7A1-4) and L-

type amino acid transporters (LATs). CATs ensure cellular arginine uptake extracellularly, where SLC7A1, SLC7A2 and SLC7A3 are main arginine transporters ^{52,54}.

Progress in the last decade has revealed that cancer is a metabolic disorder in which several, if not most, major metabolic pathways are rewired to enhance cell proliferation ⁵⁵. An important step in tumor development is a metabolic adaptation to cope with the demand of rapid cell division as well as hypoxia, and nutritionally deprived microenvironment ⁴⁸. Different tumors utilize different strategies to reprogram their metabolic pathways, which can be exploited therapeutically. The altered metabolic pathways include carbohydrate, amino acid, nucleotide, fatty acid, and lipid metabolism ⁵⁰. For instance, tumor cells are “addicted” to certain external nutrients, including arginine, to cope with its high metabolic demands. Interestingly, one of the most common metabolic defects of tumor cells is the impaired intrinsic ability to synthesize arginine ⁵⁵. The suppressed or decreased expression of ASS1 in some tumors, including SHH-MB, is making it the most prevalent metabolic deficiency in this cancer and rendering cancer cells “addicted” to external arginine ^{54,56}. Given the importance of arginine in cellular processes and being the most consumed amino acids in the inner mass of tumor, it is counterintuitive that ASS1 is epigenetically suppressed in cancer cells ^{48,55}. One possible explanation is that ASS1 diverts aspartate to arginine synthesis from pyrimidine/purine synthesis, which is needed for tumor cells. Several studies have also reported that the ASS1 has been found to support the proliferation of human colon cancer cells, *in vitro*, as well as the migration and metastatic potential of human gastric cell lines, both *in vivo* and *in vitro*. It is possible that the high levels of ASS1 support tumor proliferation and aggressiveness by increasing the supply of arginine and NO, meaning that ASS1 deficiency in tumors, including SHH-MB, can be a selective trait of the tumor cells ^{57,58}.

Importantly, the downregulation of ASS1 in tumors results in arginine auxotrophic, an intrinsic dependence of the cells on exogenous arginine due to their inability to synthesize it. In these circumstances, arginine becomes an essential amino acid, generating a vulnerability that can be used to treat cancer using arginine-depriving agents. Therefore, amino acid starvation therapy has gained significant momentum in recent years ⁵⁸.

1.6. Aim of the study

With the purpose of defining the underlying mechanisms of how GAL3 affects the malignancy in SHH-MB, a Knockout of the *LGALS3* gene was performed in our lab. The generated cellular model allowed us to draw important conclusions and foresee that the continuation of these studies could lead to significant discoveries in the therapy of MB. Important data generated by our lab members showed a connection between GAL3 and the arginine metabolism and we came across promising conclusions. This project arises as a continuation of that study.

Here, we aim to elucidate the importance of GAL3 in SHH-MB progression as well as other metabolites, using *in vivo* models. Specifically, we aim to understand the role of GAL3 on the molecular mechanisms important for tumor growth and homing capacity by performing a Knockdown of the *LGALS3* gene encoding for this protein. We also aim to identify other potential tumor specific targets that can be exploited for target therapies in SHH-MB.

2. Materials and Methods

2.1. Animal Models

Mouse experiments were approved by Stockholm's North Ethical Committee of Animal Research (ethical permit 6548/18).

All zebrafish experiments were conducted in accordance with the national ethical guidelines and regulations (N207/14).

Female NOD/SCID/IL2Ry (NSG) 1 to 4 months old mice were injected subcutaneously into the right flanks with 5×10^6 DAOY cells. Tumor growth was monitored by caliper measurement of three dimensions (width, length and height). Mice were housed in specific pathogen free conditions where light, temperature, and relative humidity were controlled. Food and water were available *ad libitum*.

Zebrafish Tg (fili1:EGFP) embryos aged about 4 hours old (1k-cell stage) were used for transplantation. Fertilized eggs were obtained from natural spawning and staged according to standard procedures.²¹ Transplanted zebrafish embryos were kept for a maximum of 3 days after transplantation. Experiments on embryos younger than 5 days do not require an ethical permit.

2.2. Cell culture and cell lines

tNES cell lines (G1A#14.40.86 p8) were grown in high density monolayers, passaged using TrypLE Select 1X (ThermoFisher, A1217701) and plated onto plates coated with 0,1 mg/ml Poly-L-ornithine (Sigma, P3655) and 1 µg/ml Lamin (Sigma, L2020), in complete neural stem cell medium (DMEM/F-12, GlutaMAX™ (Thermo Fisher, 31331093) supplemented with 1% N2 supplement (ThermoFisher, 17502001), 1% Penicillin-Streptomycin (Sigma, P4333), FGF2 (10 ng/ml) (QKine via Bionordika, Qk053), EGF (10 ng/ml) (PeproTech, AF100-15), 0.1% B27 supplement (ThermoFisher, 17504044), in a humidified environment at +37°C and 5% of CO₂. The same media was used for DAOY and UW228-3 cell lines grown in NES media, plus 1% Fetal Bovine Serum (FBS) (Fisher Scientific, SV30160.03HI).

DAOY and UW228-3 cell lines were grown in high density monolayers, passaged using TrypLE Select 1X, counted and plated onto flasks. DAOY cell lines were cultured in DMEM media (Thermo Fisher, 41966-029) supplemented with 10 % of FBS, 1% Penicillin-Streptomycin, 1% of non-essential amino acids (Thermo Fisher Scientific 11140050), 1% HEPES Solution (Hyclone, SH30237.01) and 1% of GlutaMax (Thermo Fisher Scientific, 35050061). UW228-3 cell lines were cultured in RPMI media (Thermo Fisher, 61870044) supplemented with 10% FBS,

1% Penicillin-Streptomycin, both cell lines were grown in a humidified environment at 37°C and 5% of CO₂.

2.3. Real-time quantitative PCR

For RNA extraction, cells were lysed using TRIzol® Reagent. Total RNA was isolated using Direct-zol RNA MiniPrep (Zymo Research) kit according to manufacturer's instructions. The complementary DNA was synthesized using iScript™ cDNA (BioRad) and real-time PCR (iTaQ universal SYBR Green Supermix, BioRad) was performed using ABI StepOne machine. Each PCR experiment was carried out in triplicates, for each gene. Fold changes in the genes of interest were calculated after normalization with endogenous controls (*ATPF51* and *GADPH*) and using the comparative threshold ($\Delta\Delta C_t$) method. The primer sequences are listed in the supplementary material, Table 1.

2.4. Western blot analysis

Approximately 0.5×10^6 cell lysates from each cell line (DAOY, UW228-3 and tNES) were prepared using Radioimmunoprecipitation Assay (RIPA) lysis buffer (Sigma) with 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Final protein concentration of the cell lysates was determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA), as the standard. 2x Laemmli Sample Buffer (Bio-Rad) was added to the samples and they were boiled at 95°C for 5 minutes, and equal amounts of total protein extracts were separated by Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Bolt™ 3-8% Tris-Acetate gel or 12% Tris-Bis gel). Following electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Bio-Rad). Membranes were stained with Ponceau to verify equal transfer. After blocking in 5% non-fat milk during 1 hour at room temperature, membranes were probed at 4°C overnight with the primary antibodies that were diluted in Tris- Buffer Saline with 0.1% Tween-20 (TBST) (TBST/tween; 5% (w/v) non-fat milk powder or 5% (w/v) BSA). The primary antibodies used are listed in Table 2, in the supplementary material. Then, the appropriate secondary antibody conjugated to horseradish peroxidase was applied at room temperature for 1 hour. Immunoreactive proteins were developed using ECL SuperSignal West Dura Extend Duration Substrate Kit (Thermo Fisher Scientific). The images were detected in a ChemiDoc XRS+ (BioRad). The secondary antibodies list is on Table 3, in the supplementary material.

2.5. *Lentiviral shRNA-mediated KnockDown of LGALS3*

Lentiviral particles were previously generated in the lab, pLko1- shLGALS3 (Sigma, LGALS3#1 TRCN0000029304, LGALS3#2 TRCN0000029305). For transduction, DAOY and tNES cell lines were seeded into 6 well plates and transduced with the virus at 70% confluency, 24h after the transduction, cells were selected with 1 µg/mL and 0.5 µg/mL puromycin, respectively. Knockdown efficiency was verified by assessing GAL3 protein levels.

2.6. *ZebraFish embryo transplantation of cell cultures and treatments*

Confluent cell cultures were labelled with Vybrant™ Dil cell-labeling solution (ThermoFisher, V22885) in the corresponding culture media, with a dilution of 1:200, for 15 minutes according to manufacturer's instructions. Cells were washed with PBS for 5 minutes, followed by trypsinization and centrifugation at 300g for 3 minutes. Cells were resuspended and centrifuged twice to assure complete removal of the dye. Fluorescent labelled tumor cells were transplanted in the blastula-stage of zebrafish embryos. (Van Bree *et al*, 2024/unpublished data)

2.7. *Hight-throughput imaging of zebrafish embryos*

For high-throughput imaging of the transplanted zebrafish embryos, imaging 96-well plates (Ibidi, 89621) with agarose molds were prepared²⁰. Single embryos were distributed into wells together with 150 µl of exposure medium (160 µg/ml tricaine, 30 mg/L phenylthiourea in E3 medium). Embryos were manually oriented into position and imaging using ImageXpress Nano (Molecular Devices) every 24 hours. After imaging, the plate was moved into a wet chamber inside an incubator at 33°C. Zebrafish embryo images were analysed for tumor location (hindbrain, mid/forebrain, pericardium, yolk sac, and body/tail area) using Image J 1.53. Unfocussed images or dead embryos were excluded from the analysis. All data was analysed by two people individually of which researcher 2 was blinded. To compare those two analysed datasets, each image was quantitatively classified from area with the highest percentage of tumor cells, to area with the lowest percentage of cells.

2.8. *Luciferase activity measurement*

To measure luciferase activity, treated embryos were transferred into a white-bottom 96-well plate (Greiner, 655074). Exposure medium was removed before adding 100 µl of freshly prepared lysis buffer containing 10% glycerol, 1% Triton-X 100, 25 mM Tris-phosphate pH 7.8 adjusted with phosphoric acid, 8 mM magnesium chloride, and 1 Mm DL-dithiothreitol (Sigma, cat#G012,

T8787, T1503, 438081, M8266, 43815, respectively) in dH₂O. The plate was placed on a rocking platform and embryos were lysed for 45 minutes at room temperature. 100 µl of freshly prepared substrate buffer, 25mM Tris-phosphate pH 7.8, 1 mM DL-dithiothreitol, 0.25 mg/ml D-luciferin (BioThema, BT11-1000K), and 1 mM ATP (ThermoFisher, R0441) in dH₂O was added and incubated for 5 minutes in the dark at room temperature. Luminescence was measured using the INFINITE M PLEX (Tecan, 1901001687).

2.9. Effects of Arginine Depletion by Live Cell Imaging

Five replicates of each cell line and condition were seeded on 96 well plate and left overnight, in a humidified environment at 37°C and 5% of CO₂. On the next day, the cells were washed with PBS, and NES culture media without Arginine (DMEM: F12 without L-Arginine and L-Lysine, ThermoFisher, 88370), supplemented with L-Lysine (L-Lysine monohydrochloride, Sigma Aldrich, L8662-25G) was added. L-arginine (L-Arginine monohydrochloride, Sigma Aldrich, A6969-25G), was also added to the control conditions. The cells were directly placed into the IncuCyte.

The IncuCyte S3 (ESSEN Bioscience) was used to assess the proliferation of transduced DAOY and tNES cell lines. Cells were seeded in 96-well plates and images of the cells were captured every four hours for up to three days, cell confluency was analysed using IncuCyte software and cell death through apoptosis SYTOXTM Green nucleic acid stain (Invitrogen, S7020). Data shows the results from independent experiments.

2.10. Statistical analysis

Data is presented as means ± standard deviation (SD) for at least three independent experiments. Significance is highlighted with *, **, ***, and **** indicating $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. Statistical significance was analyzed with the Student t test, and all statistical analyses were performed using GraphPad Prism9.5.0.

3. Results

3.1. Changing to NES media does not impair Galectin-3 expression

UW228-3 and DAOY are SHH-derived MB cell lines, derived from different patients. We started by culturing the cells in their respective media, **Figure 3.1A** and **3.1C**. After a few passages the cells were concurrently changed to NES media. The switch to NES media is important to standardize all the cell lines to the same conditions (once, tertiary tNES cell lines that will be used further in the experiments, are cultured in NES media). In **Figure 3.1B** and **3.1D** we can visualize that upon the changing to NES media, both cell lines change their morphology, due to a possible adaptation to NES media.

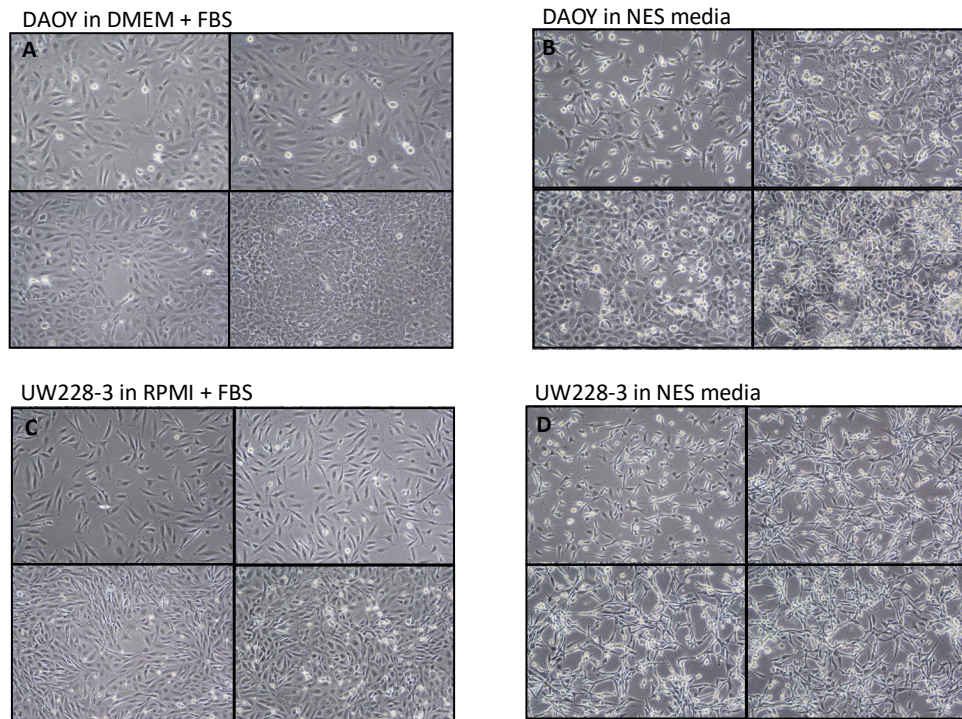


Figure 3.1. Microscopic images of DAOY and UW228-3 cell lines at different confluences. A- DAOY cell line in DMEM +10% FBS media. B- DAOY cell line in NES media. C- UW228-3 cell line in RPMI +10% FBS media. D- UW228-3 cell line in NES media.

It is important to confirm that the levels of GAL3 are not downregulated when changing the media, on both cell lines. With this purpose, RT-qPCR and Western Blot (WB) analyses were conducted. We started by the RT-qPCR analysis, and **Figure 3.2A** and **3.2B**, show that the levels of GAL3 are not downregulated in NES media. We proceeded with the WB analysis (**Figure 3.2C** and **3.2D**), that also confirms that the levels of the GAL3 protein are not downregulated upon the media change. These results allowed us to continue into further experiments.

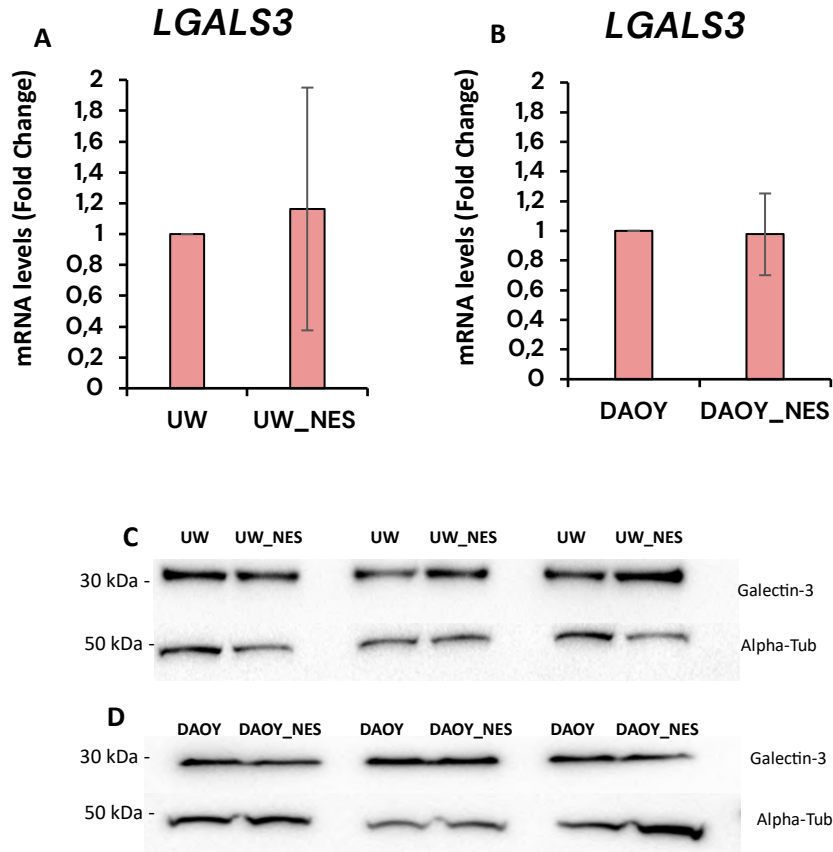


Figure 3.2. Comparison of galectin-3 levels between different medias. **A-** RT-qPCR analysis representing the LGALS3 levels between 3 biological replicates of UW and UW_NES. **B-** RT-qPCR analysis representing the LGALS3 levels between 3 biological replicates of DAOY and DAOY_NES. **C-** Western Blot analysis of Galectin-3 protein levels in 3 biological replicates of UW and UW_NES cell lines. **D-** Western Blot analysis of Galectin-3 protein levels in 3 biological replicates of DAOY and DAOY_NES cell lines. Alpha-tub were used as loading control in Western blot as indicated. RT-qPCR data is presented as a mean $\pm$ SD of three independent experiments. UW- UW228-3 cell lines cultured in RPMI + FBS media; UW228-3_NES- UW228-3 cell line cultured in NES media; DAOY- DAOY cell lines cultured in DMEM + FBS media; DAOY_NES- DAOY cell line cultured in NES media.

It has been previously reported by the lab, through RNA sequencing, that the genes *LGALS1*, *LGALS9*, *NES*, *SCG2* and *CCND1* were upregulated in UW228-3 when cultured in NES media (Van Bree *et al.*, 2014/unpublish data). These genes are involved in stemness, cell proliferation and cell differentiation. So, based on these results, we performed confirmatory RT-qPCR analysis. Results presented on **Figure 3.3** show that these genes are indeed upregulated in NES media.

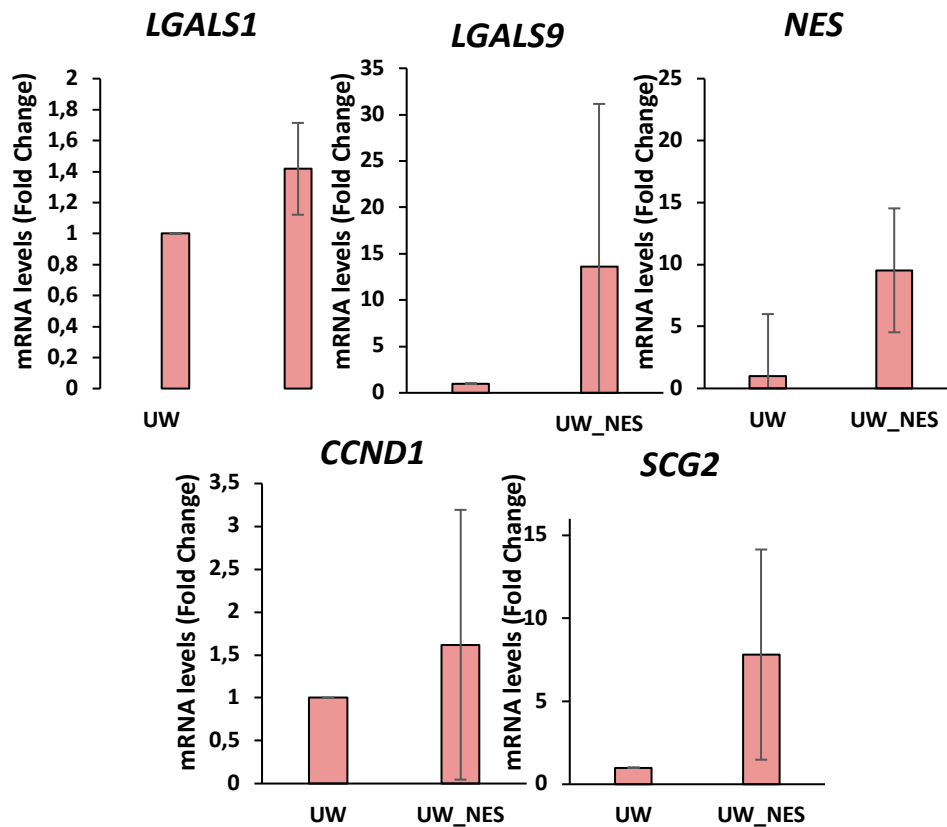


Figure 3.3. RT-qPCR analysis for genes that were proven to be upregulated in NES media, in UW228-3 cell lines. A RT-qPCR analysis confirm that the genes *LGALS1*, *LGALS9*, *NES*, *SCG2* and *CCND1*, in UW228-3 cell lines are upregulated upon the change to NES media. RT-qPCR data is presented as a mean $\pm$ SD of three independent experiments. UW- UW228-3 cell lines cultured in RPMI + FBS media; UW228-3_NES- UW228-3 cell line cultured in NES media.

3.2. Efficient Knockdown of Galectin-3 in medulloblastoma cell lines

- Lentivirus Transduction

As presented on **Figure 3.2**, both DAOY and UW228-3 cell lines, showed no downregulation on GAL3 levels in NES media, suggesting that we could choose both cell lines to perform the lentivirus Knockdown (KD). The remaining experiments were performed with the DAOY cell line.

The lentivirus transduction was performed in tertiary tNES and DAOY cell lines (**Figure 3.4**). DAOY cells were transduced with a higher viral dose, since preliminary data indicated that these cells are less sensitive for the virus particles, compared to the tertiary tNES. First, we optimized the concentration of puromycin on non-transduced cell lines, showed in **Figure 3.5**. Once the lentivirus construct contains the puromycin resistance gene we performed a puromycin selection, to select only the transduced cells (**Figure 3.6**).

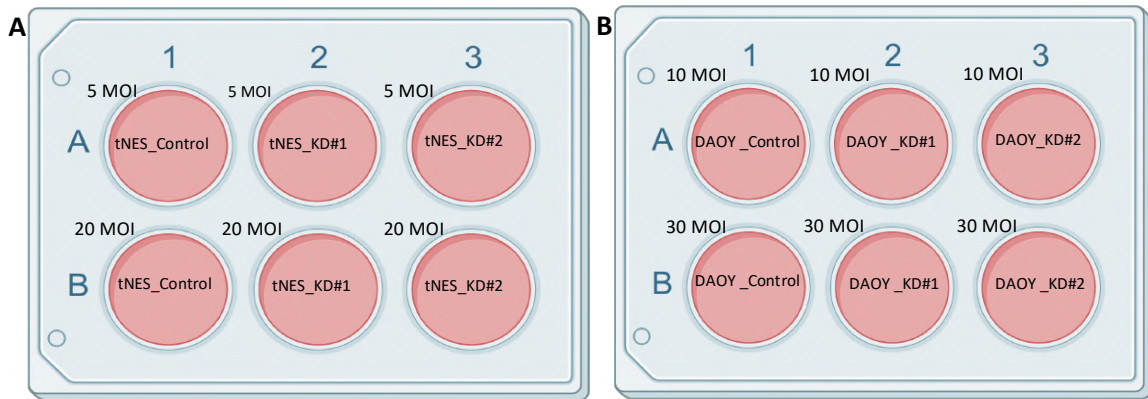


Figure 3.4. Schematic representation of the 6 well plate set up for the lentivirus transduction. A- Representative image of the plate set up with the tNES cell line for lentivirus transduction. The 5 and 20 multiplicities of infection (MOI) were chosen. One well per cell line and condition. **B-** Representative image of the plate set up with the DAOY cell line for lentivirus transduction. The 10 and 30 MOI were chosen. One well per cell line and condition.

- Optimization of the puromycin concentration:

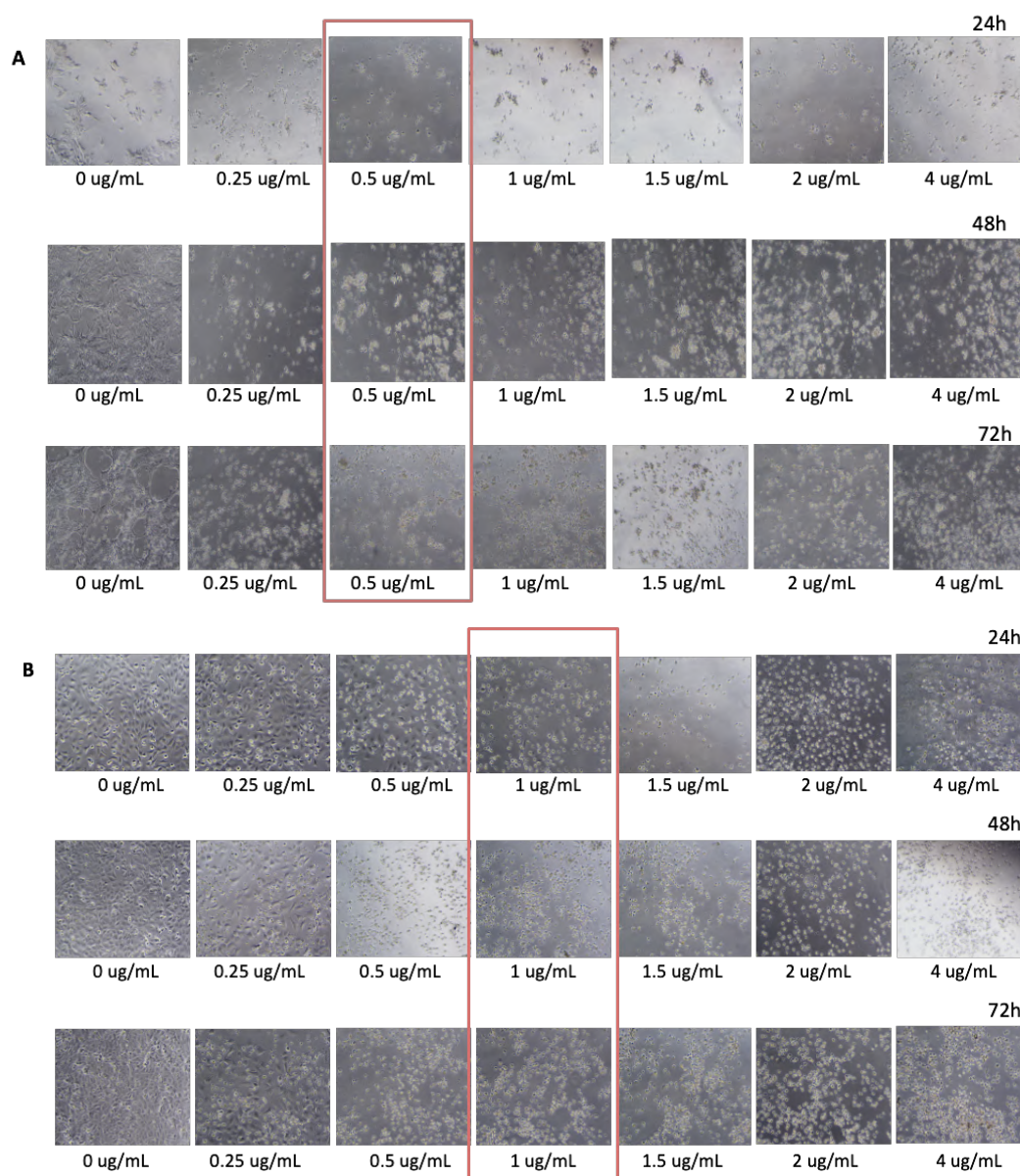


Figure 3.5. Puromycin Optimization on non-transduced tNES and DAOY cell lines. **A-** Puromycin optimization on non-transduced tNES cell line. Different puromycin concentrations (ug/mL) were tested. A timepoint of 72h was used for the optimization and photos of the cells were taken every 24 hours. The pink box shows the concentration of puromycin selected. **B-** Puromycin optimization on non-transduced DAOY cell line. Different puromycin concentrations (ug/mL) were tested. A timepoint of 72h was used for the optimization and photos of the cells were taken every 24 hours. The pink box shows the chosen concentration of puromycin, for each cell line.

Figure 3.5 shows that different concentrations of puromycin affect the cells viability, in different timepoints. Results from **Figure 3.5A**, show that 24h in the presence of 0.5 ug/mL of puromycin there are less tNES cells in the plate surface, suggesting that the cells are detaching and dying. On the other hand, the number of DAOY cells in the plate surface seems to start to decrease when the cells were treated with 1ug/mL of puromycin for 24h longer (**Figure 3.5B**), indicating that at that timepoint and antibiotic concentration the cells start to die. Taking these results in

consideration, the chosen concentrations of puromycin to perform the puromycin selection after the lentivirus transduction were 0.5 ug/mL and 1 ug/mL, for tNES and DAOY cell lines, respectively.

- Puromycin Selection

Unexpectedly, tNES cells started dying during the puromycin selection (**Figures 3.6A and 3.6B**). This cell death may be explained by the fact that the cells were not able to integrate the lentiviral construct properly, so we needed to repeat the lentivirus transduction, but with a much lower concentration of the virus. On the other hand, as showed in **Figure 3.6C**, the DAOY cells seemed to survive the puromycin selection, so the cells were kept in culture. However, the DAOY cells under the condition of 30 MOI (**Figure 3.6D**), stopped proliferating and started to die after the puromycin selection, again possibly due to the fact that the cells weren't able to properly integrate the lentivirus construct. So, we discarded this condition, and we just kept in culture the DAOY under the condition of 10 MOI.

Lentivirus transduction was repeated with 0.5 MOI and 3 MOI for tNES and 5 MOI for DAOY (**Figure 3.7**).

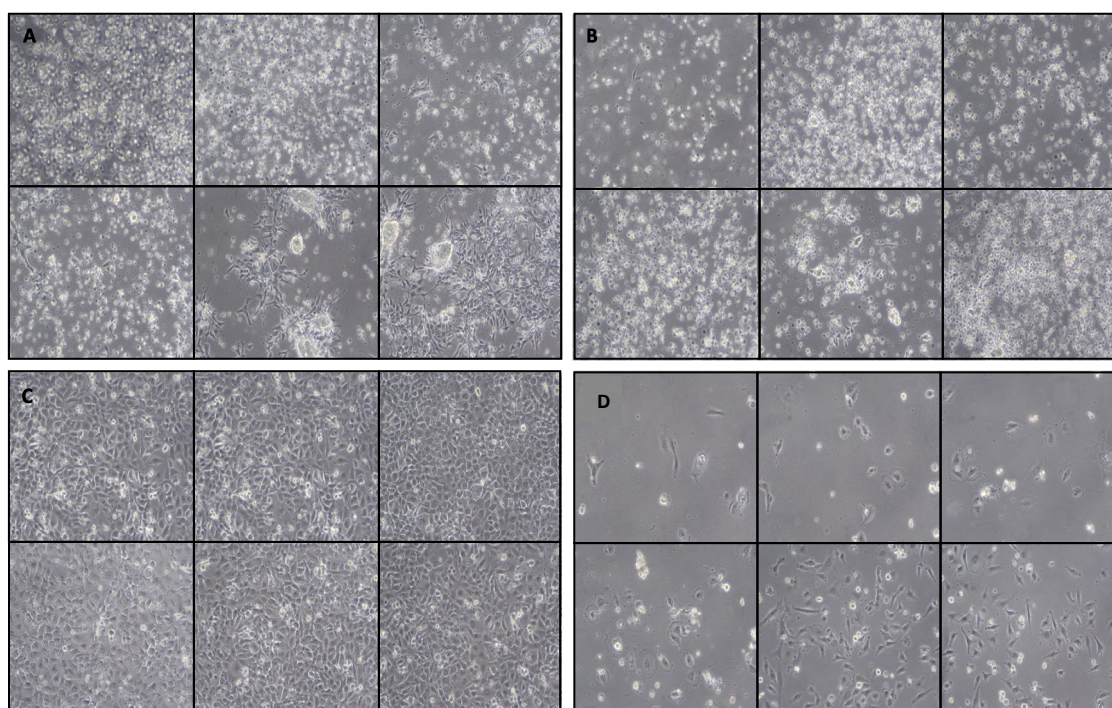


Figure 3.6. Puromycin selection on tNES and DAOY cell lines. A- Microscopic images of Puromycin Selection on tNES cell lines, using 5 MOI. B- Microscopic images of Puromycin Selection on tNES cell lines, using 20 MOI. C- Microscopic images of Puromycin Selection on DAOY cell lines, using 10 MOI. D- Microscopic images of Puromycin Selection on DAOY cell lines, using 30 MOI.

- Second Lentivirus Transduction

Lentivirus transductions were performed in tertiary tNES and DAOY cell lines for a second time, using lower MOI concentrations for both cell lines, as represented in **Figure 3.7**.

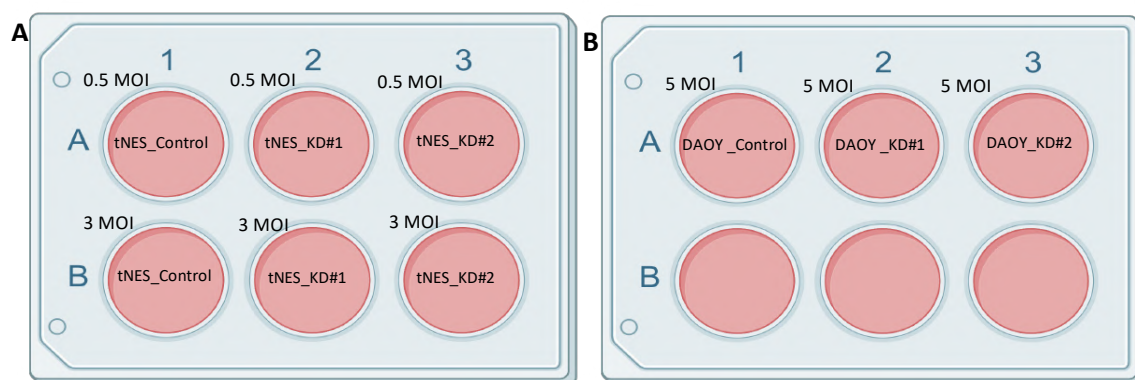


Figure 3.7. Schematic representation of the 6 well plate set up for the second lentivirus transduction. **A-** Representative image of the plate set up with the tNES cell line for lentivirus transduction. The 0.5 and 3 MOI were chosen. One well per cell line and condition. **B-** Representative image of the plate set up with the DAOY cell line for lentivirus transduction. The 5 MOI was chosen. One well per cell line and condition.

As before, tNES cells also started dying during the puromycin selection, due to the low concentration of the viral construct that was not enough to provide puromycin resistance in enough cells, so the cells died when the puromycin was introduced on the media (**Figure 3.8A**). On the other hand, in **Figure 3.8B**, the tNES cells survived after the puromycin selection, so we kept them in culture. Results from **Figure 3.8C**, show that the DAOY_Control cells stopped proliferating and started dying, so we needed to discard these cell line. Taking in consideration these results we selected the best concentrations of MOI as 3 MOI for tNES and 10 MOI for DAOY cell line.

- Puromycin Selection

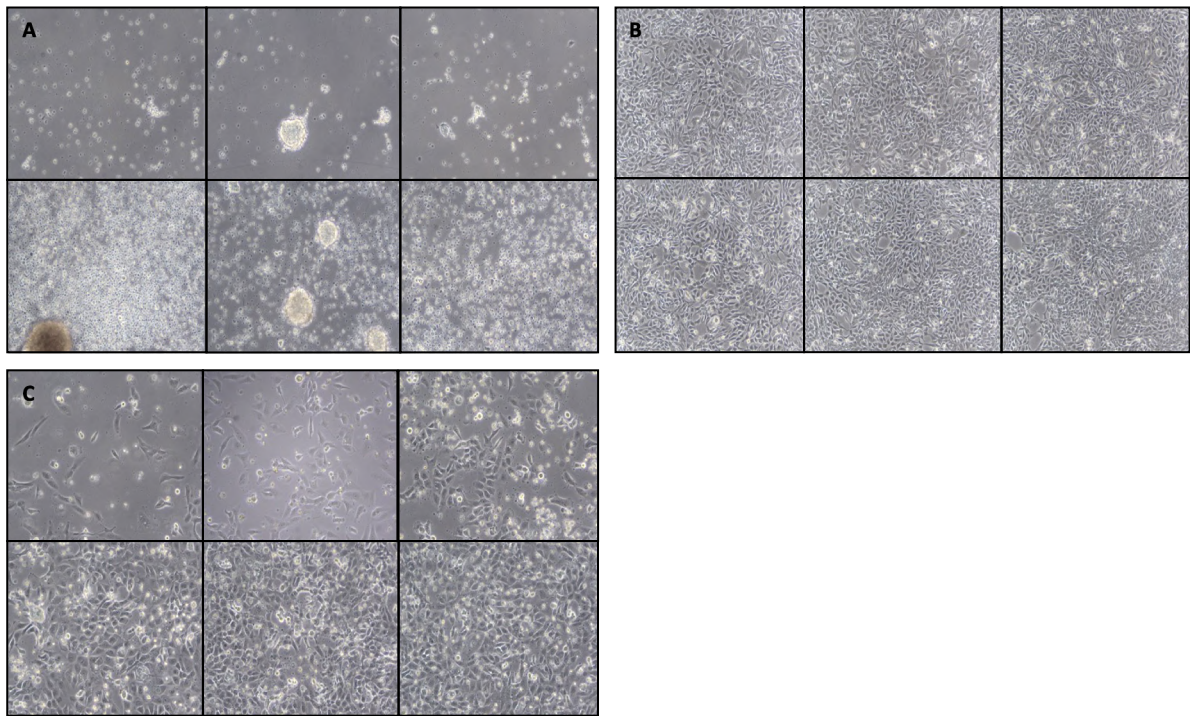


Figure 3.8. Second puromycin selection on tNES and DAOY cell lines. **A-** Microscopic images of tNES cells after puromycin selection, using 0.5 MOI. **B-** Microscopic images of tNES cells after puromycin selection, using 3 MOI. **C-** Microscopic images of DAOY cell after puromycin selection, using 5 MOI.

After having transduced both cell lines, the efficiency of the transduction was confirmed by WB and RT-qPCR analysis, to evaluate the levels of the GAL3 protein and *LGALS3*, respectively.

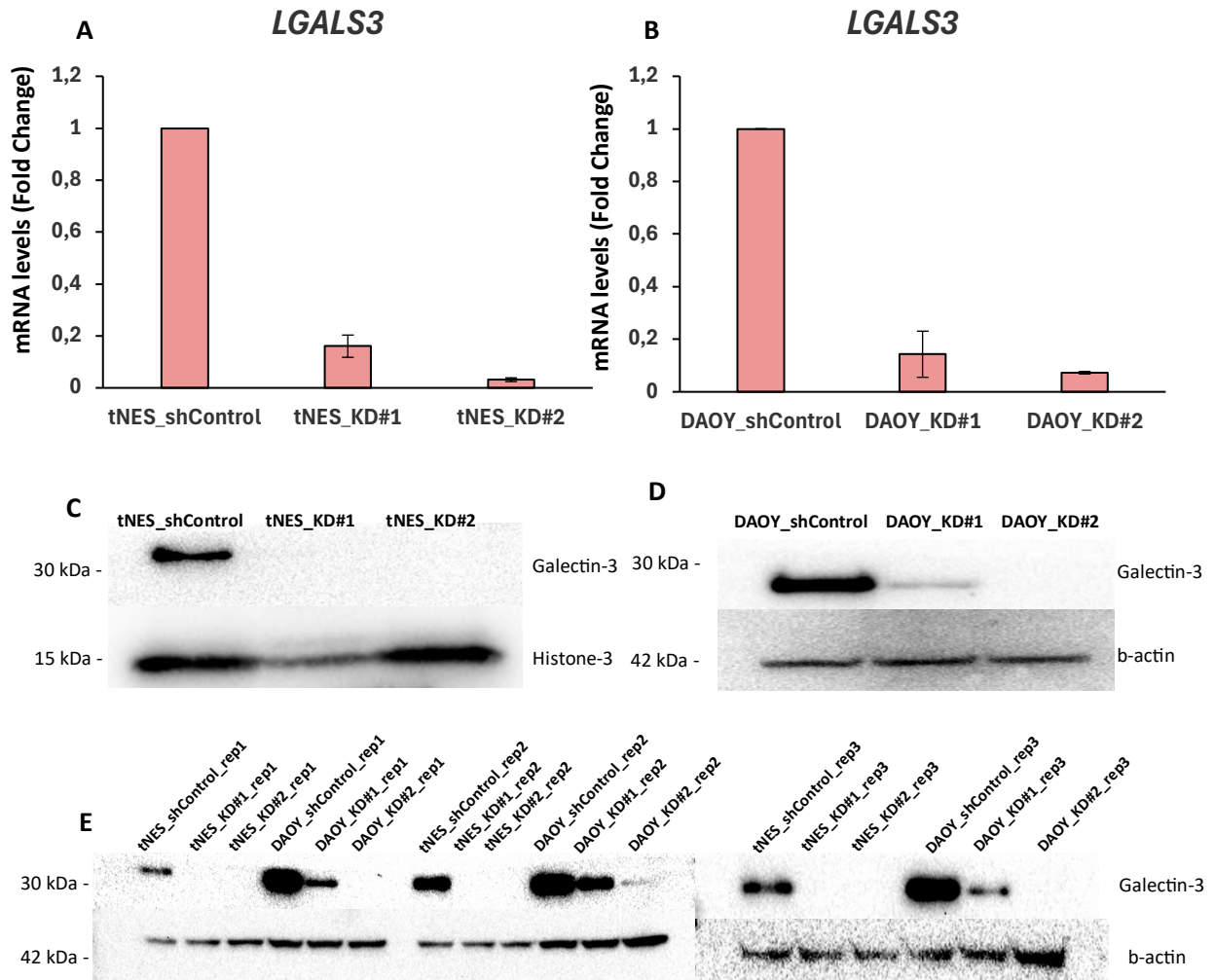


Figure 3.9. Comparison of GAL3 levels in different cell lines. **A-** RT-qPCR analysis showing the *LGALS3* levels between the control and the KDs, in tNES cell lines. **B-** RT-qPCR analysis showing the *LGALS3* levels between the control and the KDs, in DAOY cell lines. **C-** Western Blot analysis of Galectin-3 protein levels between the control and the KDs, in tNES cell lines. **D-** Western Blot analysis of Galectin-3 protein levels between the control and the KDs, in DAOY cell lines. **E-** Western Blot comparison analysis of the Galectin-3 protein levels among all three replicants in tNES and DAOY cell lines. Histone 3 and b-actin were used as loading control in Western blot as indicated. RT-qPCR data is presented as a mean $\pm$ SD of three independent experiments.

The levels of the *LGALS3*, as presented in figures **Figure 3.9A** and **3.9B**, and the levels of GAL3 protein, depicted in **Figure 3.9C** and **3.9D**, are downregulated on both cell lines due to the effective lentivirus transduction. **Figure 3.9E**, shows the comparison between the GAL3 protein levels in tNES and DAOY cell lines, where the KD is once again confirmed. These results allowed us to move into further experiments.

3.3. *In vivo* experiments

3.3.1. Tumor cell transplantations into Zebrafish embryos as an *in vivo* experiment for tumor localization - GAL3 Knockdown, impacts on the tumor localization and homing capacity.

An unpublished study performed by our lab members, showed that various types of brain cancer are able to home to their environment of origin, by intrinsic morphogenetic cues in the early developing zebrafish embryos. So, in this study we transplanted zebrafish embryos and with fluorescent imaging and further image analysis we are able to follow the tumor location, as described in **Figure 3.10A**. Since it is known that GAL3 plays an important role in the tumor microenvironment, we hypothesized that GAL3 might also influence the homing capacity of our MB cells. To test this hypothesis, a total of 3 cell lines were transplanted (tNES_shControl, tNES_KD#1, tNES_KD#2). Each embryo was divided into five areas, namely hindbrain, mid/forebrain, pericardium, yolk sac, and body/tail area, **Image 3.10B**, to quantify tumor location. All data was analysed by two people individually of which researcher 2 was blinded, Supplementary Figure 1.

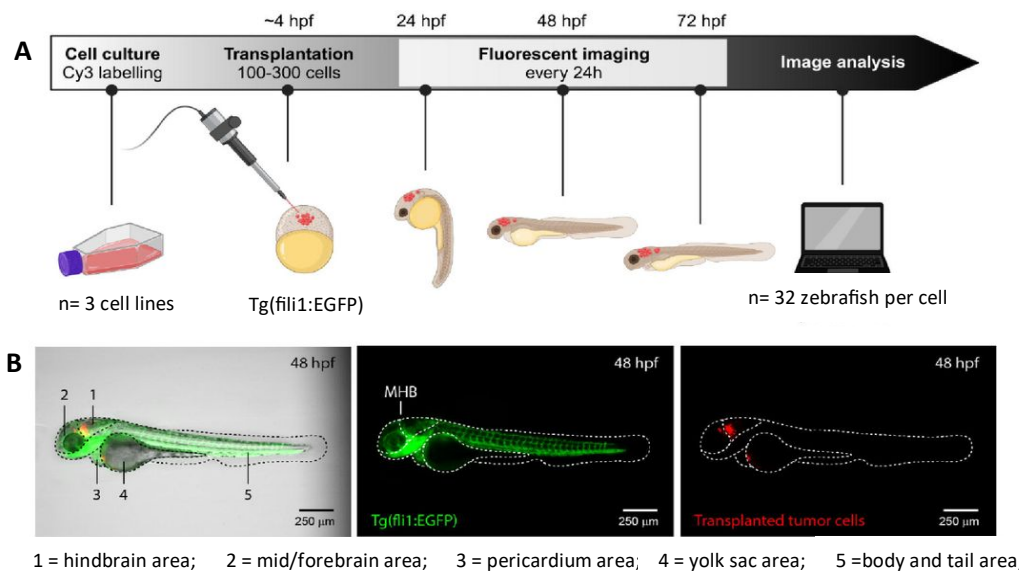


Figure 3.10. Homing of fluorescently labelled tumor cells transplanted in zebrafish embryos can be examined with fluorescent imaging and image analysis. **A-** Schematic overview of the experimental workflow for zebrafish embryo transplantations followed by tumor location analysis. **B-** Representative image of a tNES cell transplanted zebrafish embryo, displaying the five defined locations for location analysis. Left panel: bright field with GFP and Cy3 overlay. Middle panel: expression of enhanced GFP in the vasculature of the fli1: EGFP zebrafish embryo (GFP, green). MHB= mid-hindbrain boundary. Right panel: labelled tumor cells (CY3, red). Figure adapted from: Niek van Bree *et al.*, 2024/unpublished data.

After analysing all the images per cell line, **Figure 3.11A**, we quantified the tumor location. Upon the quantification, we observed a higher degree of brain localization when tNES_shControl was transplanted, compared to the tNES_KD#1 and tNES_KD#2, that showed a higher degree of localization on the body regions, **Figure 3.11B**. Further analysis revealed that the tNES_shControl homed 39,1% into the hindbrain, 34,6% into the mid/forebrain, 11% into the pericardium, 7,8 into the yolk sac, 7,5% into the body/tail area. The tNES_KD#1 homed 34,3% into the hindbrain, 20,5% into the mid/forebrain, 16,8% into the pericardium, 22,1% into the yolk sac and 6,3% into the body/tail area. The tNES_KD#2 homed 35,3% into the hindbrain, 28,7% into the mid/forebrain, 23,4% into the pericardium, 5,4% into the yolk sac and 7,2% into the body/tail area. With a significant statistical difference in the homing cells between the shControl and the tNES_KD#1 and tNES_KD#2. **Figure 3.11C**. Taken together, these results suggest that the GAL3 KD, impacts on the tumor localization and homing capacity, upon transplantation in developing zebrafish embryos.

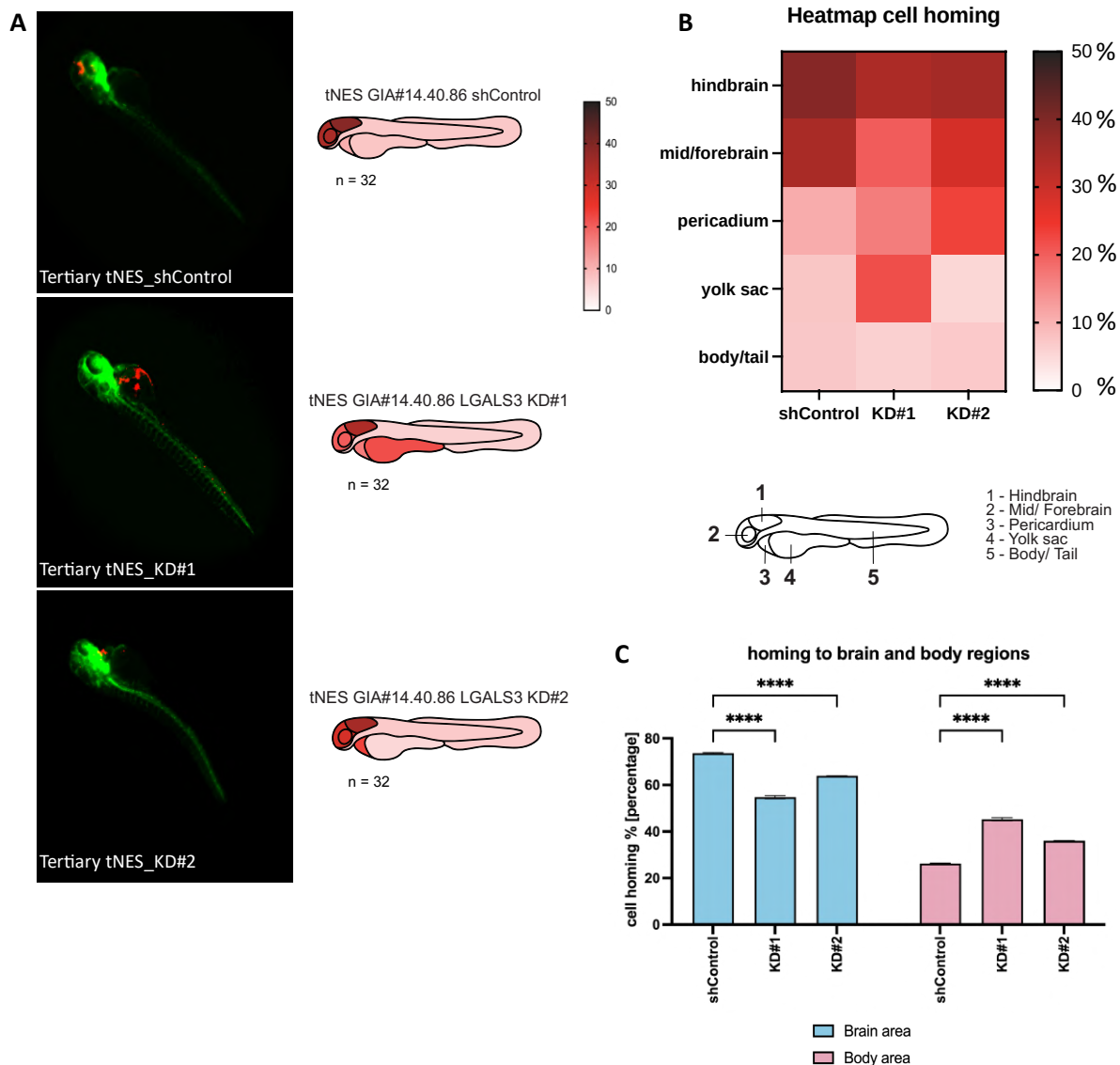


Figure 3.11. tNES_Control cell line home more towards the brain region after transplantation into the blastula-stage of zebrafish, whereas tNES_KD1 and tNES_KD2 cell lines home more towards the body region A- Representative images of fli1: EGFP zebrafish embryos 48 hpf transplanted with tNES_Control, tNES_KD1 and tNES_KD2. B- Brain/Body area distribution of transplanted tNES_Control, tNES_KD1 and tNES_KD2 cell lines. Heatmap representing the brain/body distribution of tumor cell lines. C- Percentages of the tumor cell homing, per cell line in the brain/body regions. Data is presented as a mean $\pm$ SD of thirty-two independent experiments; Statistical analysis: t test with **** indicating $p < 0.0001$.

Our analysis using Pearson correlation revealed that all five anatomical areas showed a strong positive correlation when comparing the two analysed data (Supplementary Figure 1). As both analyses were very similar, average values were used for further presentation of the data.

- Luciferase Activity

Transduced tertiary tNES cell lines have a luciferase gene, so to enhance the quantitative analysis of tumor cell growth and localization, we measured bioluminescence. As shown in **Figure 3.12**, the bioluminescence is too low, to detect any variations among the shControl and the tNES_KD#1 and tNES_KD#2. The low bioluminescence levels are correlated with the cell line, and this data does not provide consistent information.

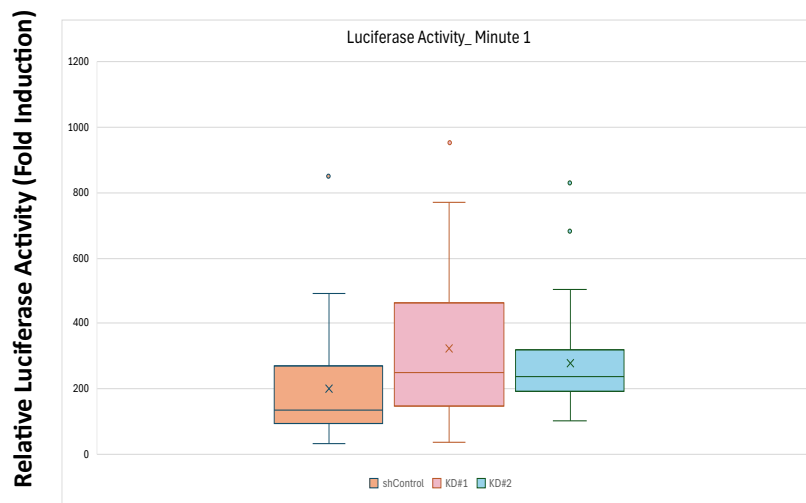


Figure 3.12. Luciferase activity of transplanted tNES. Data is presents as a mean $\pm$ SD of thirty-two independent experiments; Statistical analysis: t test with * indicating $p < 0.05$.

3.3.2. DAOY transduced cells subcutaneously transplanted into mice as an *in vivo* experiment for tumor growth and proliferation - DAOY *LGALS3* KD1 cells seems to originate a smaller and lighter tumor, compared with the DAOY *LGALS3* KD2 cells, and with the shControl.

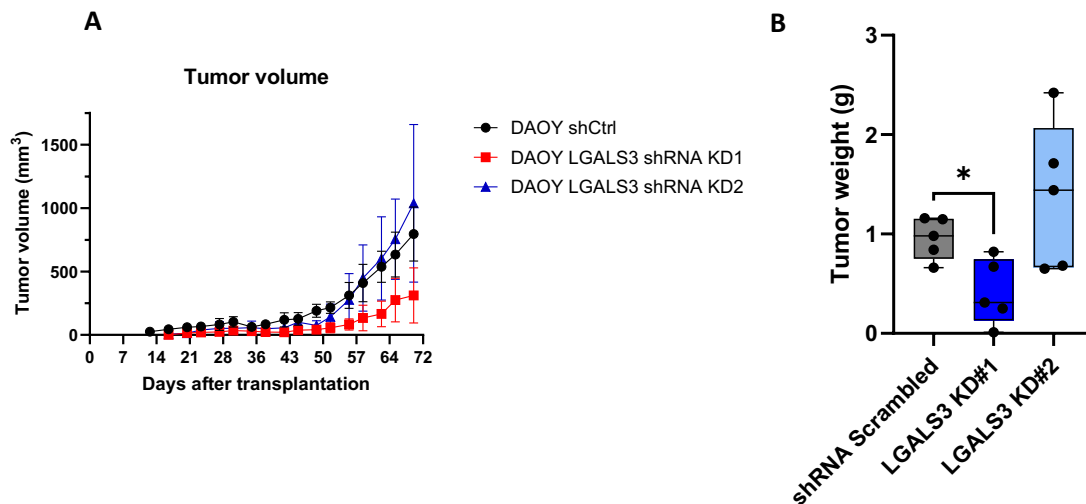


Figure 3.13. Mice subcutaneous transplantations DAOY *LGALS3* KD. **A-** Evolution of the tumor volume (mm³) during a timepoint of 72 days after transplantation with DAOY_shControl, DAOY_KD1 and DAOY_KD2. **B-** Evolution of tumor weight (g) during a timepoint of 72 days after transplantation with DAOY_shControl, DAOY_KD1 and DAOY_KD2. shRNA Scrambled: sh_control. Data is presented as a mean $\pm$ SD of five independent experiments; Statistical analysis: t test with * indicating $p < 0.05$.

A difference in tumor growth rate, between the sh_Control and the KDs, can be observed in **Figure 3.13A**. KD1 cells seem to have a slower growth, leading to a less voluminous tumor, when compared with the other cell lines. In **figure 3.13B**, there is also a significant statistically difference on the tumor weight among the cell lines, where, once again the KD1 cells originate a lighter tumor compared with the KD2 and even to the sh_control. The fact that, DAOY *LGALS3* KD1 cells, originates smaller and lighter tumors compared with the sh_control, suggests that these cell line have a tendency to decrease the tumor malignancy.

3.4. Changing to NES media does not downregulate arginine transporters expression

The arginine transporters (SLC7A1, SLC7A2, SLC7A3) are very important for the uptake of arginine from the media and the enzyme Argininosuccinate Synthase 1 (ASS1) is essential for the endogenous production of arginine. So, it is highly important to ensure that NES media does not impact cell arginine content by downregulating the levels of these transporters and enzyme. To confirm this, a RT-qPCR analysis was conducted. Results from **Figure 3.14**, show that the levels of the genes encoding for SLC7A1, SLC7A2, SLC7A3 and ASS1 are not downregulated by NES media. These results were important and allowed us to move on with the experiments.

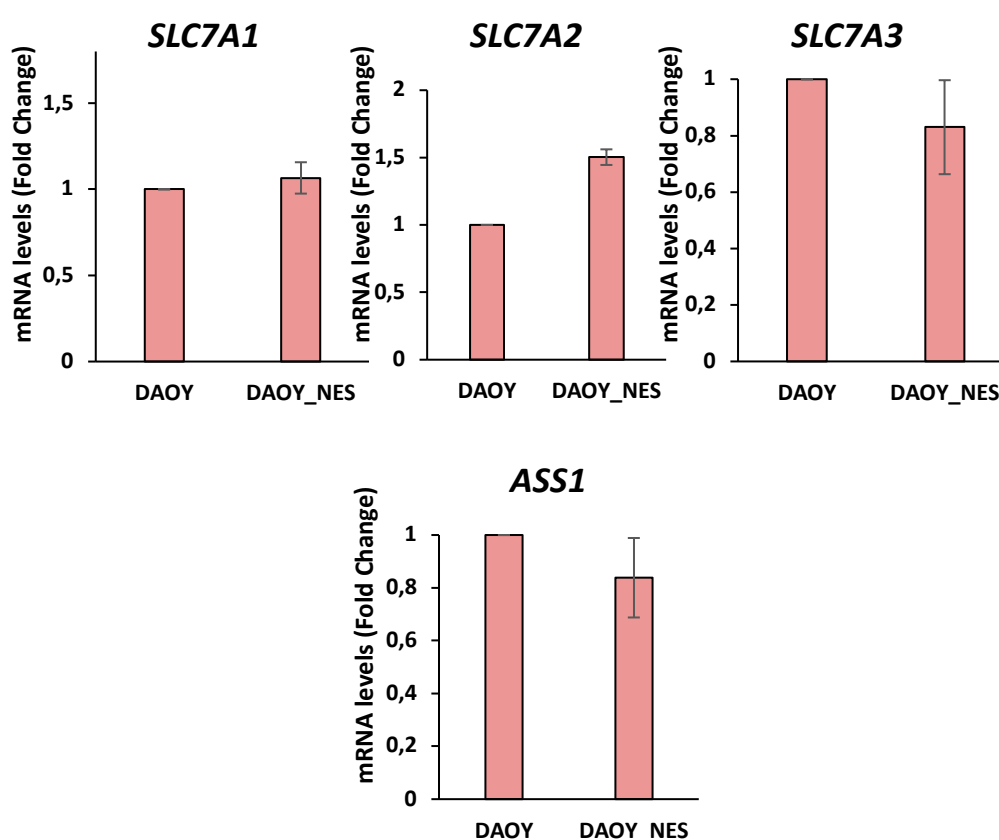


Figure 3.14. Comparison of SLC7A1, SLC7A2, SLC7A3 arginine transporters and the enzyme ASS1 levels in the presence of different culture media. RT-qPCR analysis representing the *SLC7A1*, *SLC7A2*, *SLC7A3*, *ASS1* levels from 3 biological replicates of DAOY- DAOY cell lines cultured in DMEM + FBS media and DAOY_NES- cell lines cultured in NES media. RT-qPCR data is presents as a mean $\pm$ SD of three independent experiments.

3.5. Knockdown of Galectin-3 seems to downregulate arginine transporters in DAOY cell lines, but not in tNES cell lines

With the purpose of verifying if there are any changes on the expression of the arginine transporters SLC7A1, SLC7A2, SLC7A3 and the enzyme ASS1 levels in the transduced cell lines after the GAL3 gene was KD, a RT-qPCR analysis was performed. In **Figure 3.15A**, the KD of GAL3 does not seem to provoke changes on the levels of SLC7A1, SLC7A2, SLC7A3 and ASS1, in tNES cell lines. However, in DAOY cell lines shRNA lentivirus KD of the GAL3 gene seems to downregulate SLC7A1, SLC7A2, SLC7A3 and ASS1 (**Figure 3.15B**). Both KD1 and KD2, show a downregulation among all arginine transporters and on the enzyme, in DAOY cell lines.

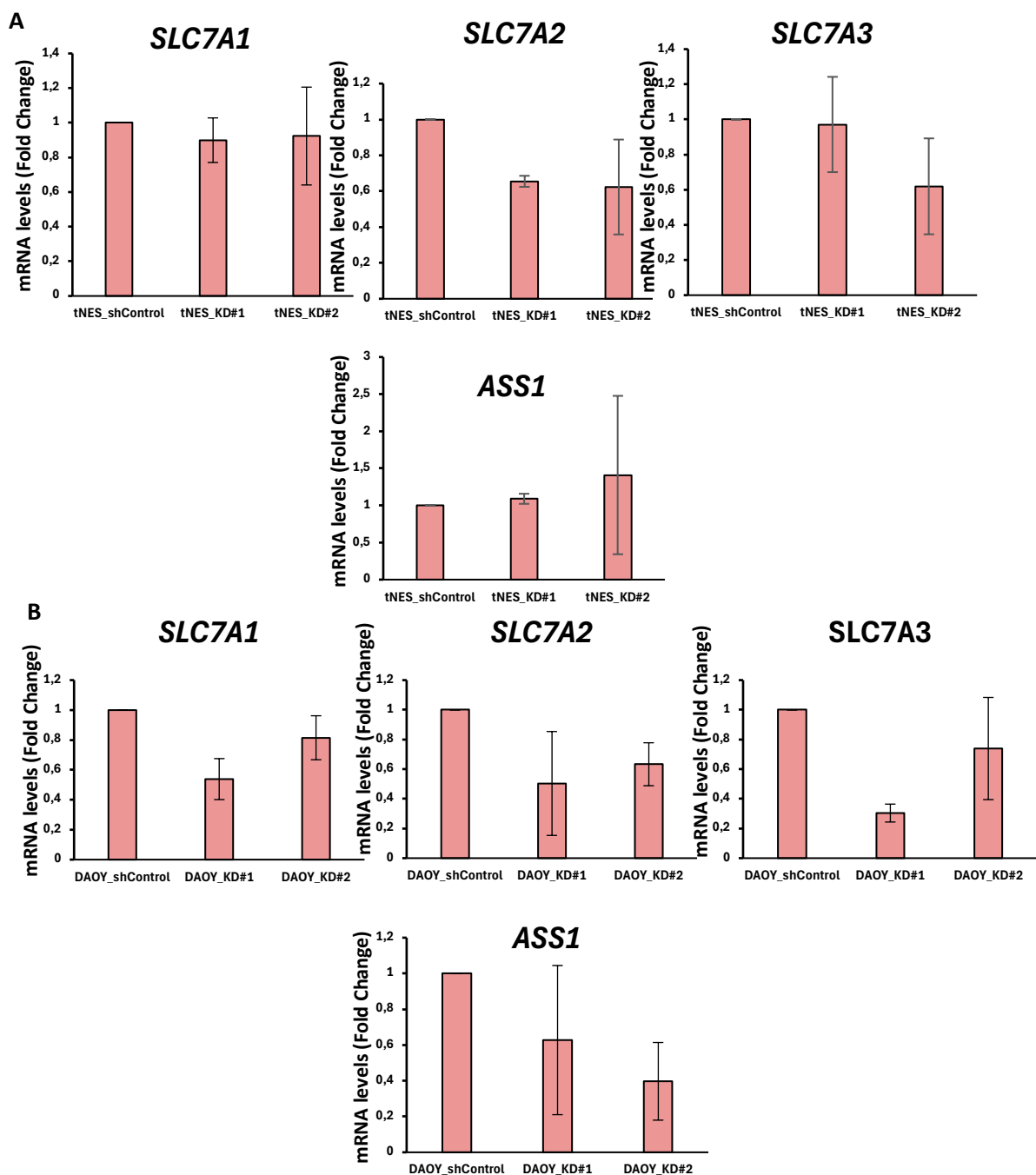


Figure 3.15. Comparison between SLC7A1, SLC7A2, SLC7A3 arginine transporters and the enzyme ASS1 levels on transduced tNES and DAOY cell lines. **A-** RT-qPCR analysis representing the *SLC7A1*, *SLC7A2*, *SLC7A3*, *ASS1* levels between 3 biological replicates of tNES_shControl, tNES_KD#1 and tNES_KD#2. **B-** RT-qPCR analysis representing the *SLC7A1*, *SLC7A2*, *SLC7A3*, *ASS1* levels between 3 biological replicates of DAOY_shControl, DAOY_KD#1 and DAOY_KD#2. RT-qPCR data is presents as a mean $\pm$ SD of three independent experiments.

3.6. Arginine Depletion Media

In order, to study the proliferation rate in DAOY transduced cell lines, we performed an IncuCyte experiment, where the cells were cultured in rich media (NES media), and in arginine depletion media (NES media, without arginine). In **Figure 3.16A**, **3.16B** and **3.16C** we can observe that there is a considerable difference in confluency between the cells cultured with or without arginine. The slope of the proliferation curve of cells cultured in arginine depletion media, after a timepoint of 14 to 28 hours, start to decrease, resembling a flat line. Thus, a decrease in confluency can be observe, meaning that the cells are unable to proliferate due to the depletion of arginine from the media. However, the cells cultured in rich media, show higher levels of confluency, meaning that they can proliferate faster when there is arginine in the media, despite the downregulation on the arginine transporters in the KD cells.

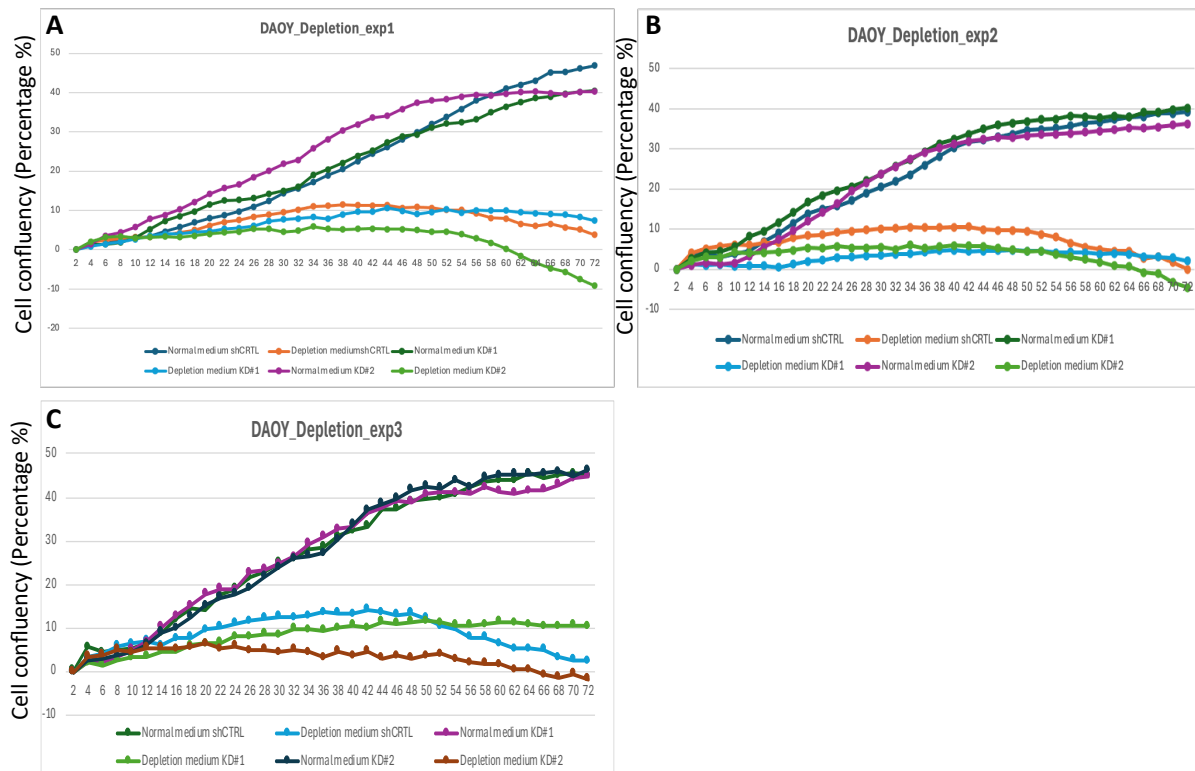


Figure 3.16. Arginine Depletion Media seems to have an effect in cell confluency. **A-**DAOY cell lines (DAOY_shControl, DAOY_KD#1 and DAOY_KD#2), cultured in rich media and in Arginine depletion media. Cell confluency was measure during a timepoint of 72h, for replicant one. **B-** DAOY cell lines (DAOY_shControl, DAOY_KD#1 and DAOY_KD#2), cultured in rich media and in Arginine depletion media. Cell confluency was measure during a timepoint of 72h, for replicant two. **C-** DAOY cell lines (DAOY, DAOY_shControl, DAOY_KD#1 and DAOY_KD#2), cultured in rich media and in Arginine depletion media. Cell confluency was measure during a timepoint of 72h, for replicant three.

Figure 3.17 shows the behaviour of each cell line in rich media and arginine depletion media. In **Figure 3.17A, 3.17D** and **3.17G** it can be observed that the DAOY_shCtrl has the same behaviour in all three experiments. In all of the experiments, a higher confluency is noted when the cells are grown in the rich media compared with the depletion media. Also, in **Figure 3.17B, 3.17E** and **3.17H**, we can visualize that all the DAOY_KD#1, maintains the same behaviour, where the proliferation curve of the cells in depletion media adopt a flat line at after early timepoints. As depicted in **Figure. 3.17C, 3.17F** and **3.17I**, the DAOY_KD#2 shows a similar tendency as the one observed for DAOY_KD#1. Based on these results, we conclude that the arginine depletion media clearly has an impact in the cells proliferation, regarding the GAL3KD.

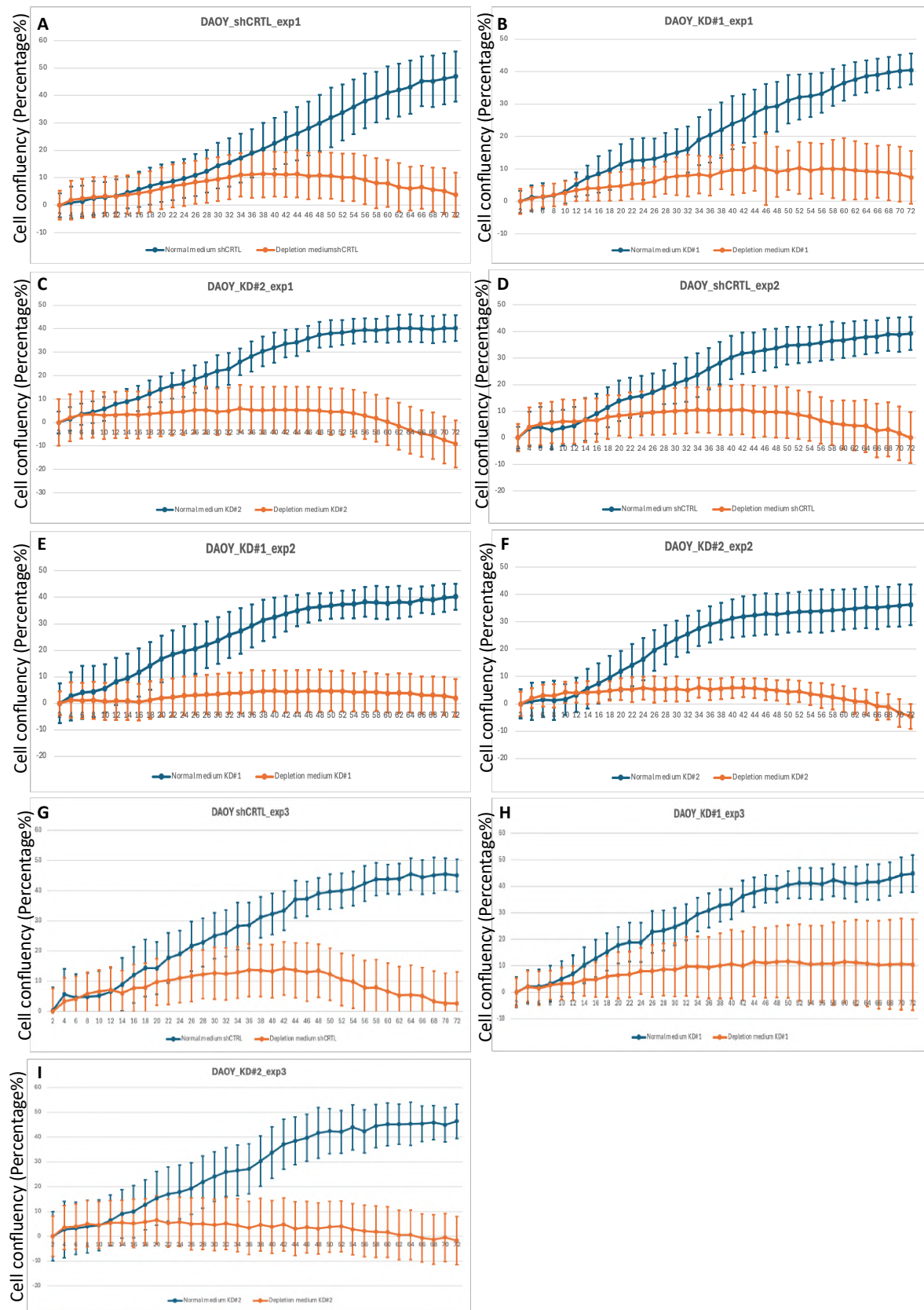


Figure 3.17. Differences in the confluency between transduced DAOY cell lines in arginine depletion media and rich media. A-DAOY_shControl, cultured in rich media and in Arginine depletion media, for replicant one. **B-** DAOY_KD#1, cultured in rich media and in Arginine depletion media, for replicant one. **C-** DAOY_KD#2, cultured in rich media and in Arginine depletion media, for replicant one. **D-** DAOY_shControl, cultured in rich media and in Arginine depletion media, for replicant two. **E-** DAOY_KD#1, cultured in rich media and in Arginine depletion media, for replicant two. **F-** DAOY_KD#2, cultured in rich media and in Arginine depletion media, for replicant two. **G-** DAOY_shControl, cultured in rich media and in Arginine depletion media, for replicant three. **H-** DAOY_KD#1, cultured in rich media and in Arginine depletion media, for replicant three. **I-** DAOY_KD#2, cultured in rich media and in Arginine depletion media, for replicant three.

Taken together, these results suggest, that the transduced DAOY cell lines in rich media are proliferating at a much higher rate compared with the cells grown in arginine depleted media. **Figure 3.18** shows representative images of the data presented in Figure 3.17 exp 3. The images in **Figure 3.18**, shows the confluency of each transduced DAOY cell line *per* condition, where a higher confluency in the rich media compared with a lower confluency in the depletion media, in all cell lines and conditions, is noticeable. These results combined, provide enough evidence that the arginine depletion media impairs on the confluency and proliferation of DAOY cell lines, regarding the GAL3 KD.

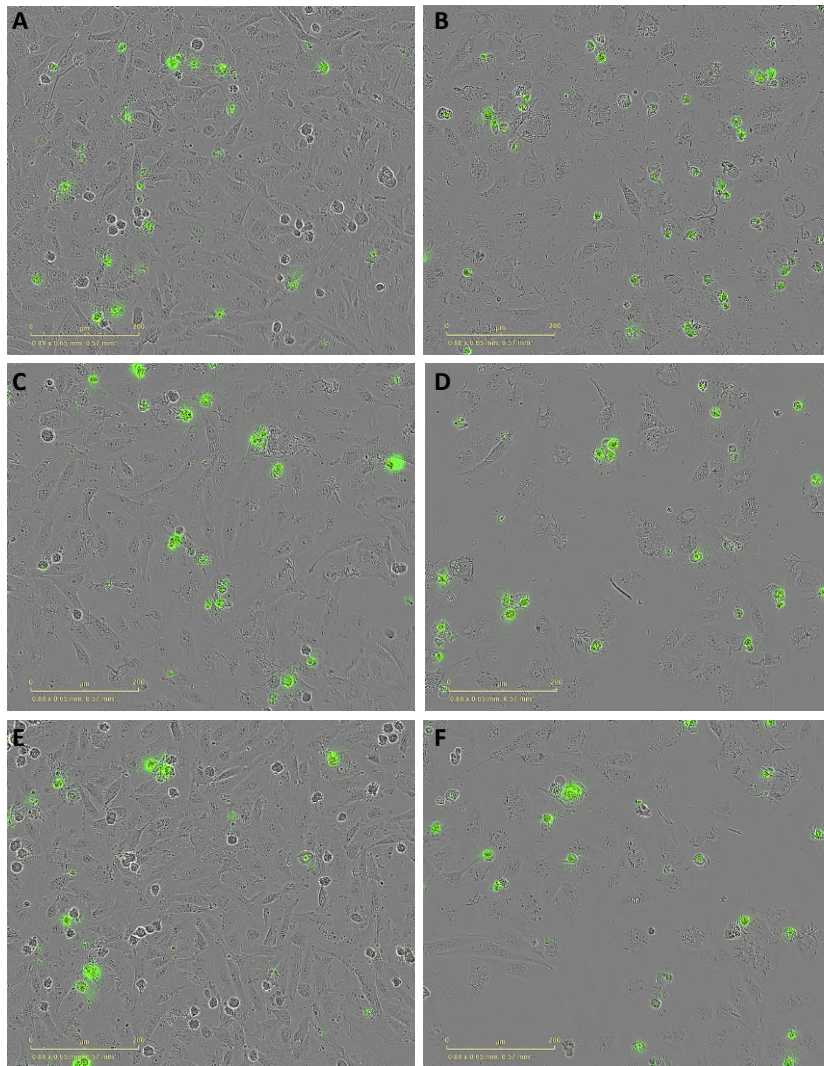


Figure 3.18. Transduced DAOY cell lines are more confluent in rich media compared to arginine depletion media. A- DAOY_shControl_exp3 in rich media. **B-** DAOY_shControl_exp3 in depletion media. **C-** DAOY_KD#1_exp3 in rich media. **D-** DAOY_KD#1_exp3 in depletion media. **E-** DAOY_KD#2_exp3 in rich media. **F-** DAOY_KD#2_exp3 in depletion media. Images from IncuCyte. SYTOX™ Green nucleic acid stains the dead cells by apoptosis.

4. Discussion

This study consists of using SHH-MB derived cell lines from different patients to better understand the underlying mechanism of this pediatric malignancy. The overexpression of GAL3 is associated with an increase in SHH-MB malignancy. Therefore, we studied the involvement of this protein in tumor proliferation and homing capacity using *in vivo* models. Additionally, in this study, we also emphasise the correlation between GAL3 and the arginine metabolism, unrevealing new promising therapeutic approaches.

Cancer research requires *in vitro* models capable of producing reliable information through mimicking the cells phenotype as it exists in the target tissue. Thus, the use of *in vitro* models, continues to be crucial in cancer research. The performance of cancer cells depends on many aspects, a critical one being the media in which the cell resides. Several researchers have reported that gene expression patterns of cancer cells can vary significantly according to the conditions under they are cultured. So it's highly important to have a controlled and standardized media on the different cell types used in this study ⁵⁹. Both DAOY and UW228-3 are established SHH-MB cell lines, used frequently in research, and they were our starting point in this study. We standardized all external conditions and progressively changed the media of both cell lines to NES media (Figure 3.1). Upon changing the media, both DAOY and UW228-3 cells changed their morphology, presenting a more elongated cytoplasm (Figure 3.1B and Figure 3.1D, respectively), suggesting that these cells were able to adapt to the new media culture conditions. Upon the change to the new growth conditions, the metabolic characterization of these tumor cells can change, due to several adaptations of the cell ⁶⁰. With this in mind, it was important to be sure, that the protein GAL3 was not downregulated in DAOY, and UW228-3 cell lines, cultured in NES media (Figure 3.2).

As mentioned above cancer cells have the ability to change gene expression patterns according to the to the conditions, they are cultured in ⁵⁹. In NES media is expected that the SHH signalling is more active. The genes *LGALS1*, *LGALS9*, *NES*, *CCND1* and *SCG2* are involved in enhancing SHH signalling, tumor proliferation and differentiation, in stemness and in angiogenesis, therefore, we expect their upregulation in NES media. Similar to GAL3, GAL1 and GAL9 are the most extensively studied galectins and are also implicated in alterations in oncogenic pathways, apoptosis and metastasis ⁶¹. GAL1 contributes to tumor growth and progression by promoting tumor migration, metastasis and immunomodulation. GAL9 has been also reported to induce tumor growth in melanoma ^{61,62}. Nestin (*NES*), an intermediate filament protein, was originally identified in neural stem cells, which has been used as a marker for neural stem cells. Nestin expression is recently found in many tumor tissues including brain tumors. Nestin-expressing progenitors are found to serve as a tumor initiating cells in medulloblastoma and glioblastoma.

Nestin also plays important functions during tumor progression, where it has been previously reported that it mediates medulloblastoma tumorigenesis by augmenting the SHH signalling.⁶³ When the cells are switched to growth in NES media we expect a more active SHH signalling, implicating an upregulation of the *NES* gene. Secretogranin II (*SCG2*) is a secretory protein that mediates neural differentiation involved in angiogenesis and in promoting tumor growth⁶⁴. Also, Cyclins D1 (*CCND1*) and D2, are upregulated in response to SHH signalling in cerebellar cells. *CCND1* are expressed in proliferating GNPs, and their joint removal results in an exceptionally hypoplastic cerebellum, indicating that *CCND1* play a crucial role in the rapid postnatal expansion of the GNP population⁶⁵. These genes *LGALS1*, *LGALS9*, *NES*, *CCND1* and *SCG2* contribute to the formation of the neurogenic niche, enhancing SHH signalling in NES media, as showed in Figure 3.3.

To study the role of *LGALS3* gene in DAOY and tNES cell lines, a KD was performed. Interference RNA (RNAi) has emerged as a powerful tool for the study of gene function. A form of RNAi involves the use of short hairpin RNAs (shRNAs) synthesized within the cell by DNA vector-mediated production. Lentiviral-mediated transduction provides a convenient method of introducing shRNA into dividing and non-dividing cells and, in general, is less toxic to the cells than adenoviral-mediated transduction⁶⁶. shRNA is used to generate stable KD cell lines, eliminating the need for multiple rounds of transfection, increasing the reproducibility of the results. Additionally, two successful KDs can also provide a useful control for off-target KDs since it is statistically unlikely that different sequences will produce the same off-target KDs. For this reason, we used KD#1 and KD#2, to target *LGALS3* gene, as illustrated in Figure 3.4 and 3.7. To successfully select the transduced cells, a puromycin optimization (Figure 3.5) and selection was performed. Puromycin is an antibiotic, that can be used as a selection marker. It was introduced on the successfully transduced cells, a lentiviral construct that contains the resistance gene against puromycin, meaning that when the transduction is not efficiently done, the cells will not be able to survive the selection. If the concentration of the viral construct is too high, it can result in an inefficient transduction, leading to cell death (Figures 3.6A, 3.6B and 3.6D), and the same, if the concentration of the viral construct is too low, where the cells are not able to achieve 100% infection (Figure 3.8A and 3.8C). On the other hand, when the concentration of the virus is correctly aligned with the cells, they survived the puromycin selection, showing an efficient lentivirus transduction, as demonstrated in figures 3.6C and 3.8B. Therefore, the efficiency of the KD can be rapidly and easily determined by RT-qPCR and Western Blot techniques. As showed Figure 3.9, both KDs of *GAL3* in DAOY and tNES cell lines were successful.

The results from *in vitro* studies, provide insight information about the molecular mechanisms that can influence cancerous cells under defined conditions. However, these results are extremely difficult to extrapolate to a clinical effect since they were not performed in an intact, living

organism, where far more biological variables are in play. This emphasizes the importance of using animal models. The process of selection an animal model for biomedical research is a very intricate part, as all models have various limitations. Many factors should be taken into consideration during the selection of an ideal animal model for biomedicine trials. The most important criteria are the proper selection of models in terms of resemblance between animal species and humans in terms of physiological and/or pathophysiological aspects ⁶⁷. Zebrafish embryos have emerged as a promising, *in vivo*, model for many human cancers, and have been proven to be a good model system for high-throughput transplantation studies. Figure 3.10. Also, human xenografts in growing mice are a well-established and useful model for studying human tumor biology in a system that better mimics the primary tumor as compared to cells grown *in vitro* ⁶⁸. Therefore, the transduced tertiary tNES MB cell lines, were transplanted in zebrafish embryos, and the home capacity of the of these cell lines were analysed. Cell homing is the property by which circulating cells can enter their corresponding organ of origin. Homing to the natural microenvironment of human cells transplanted into blastula embryos has been observed before, and it was postulated that the early zebrafish embryo contains homing cues that correctly guide transplanted melanoma and glioma cells ⁶⁹. There are several important cues in the regulation of cell homing, including the interactions of adhesion molecules (selectins and integrins). Cell homing requires the upregulation of cell adhesion molecules, and previous studies have reported that GAL3 increases cancer cell heterotypic adhesion to vascular endothelium and promotes endothelial secretion of cytokines, these cytokines enhance tumor cell adhesion to endothelial cells through upregulation of E-selectin and integrin in endothelial cells ⁷⁰⁻⁷³. The KDs (tNES_KD#1 and tNES_KD#2) have a significantly reduction in the GAL3 expression, and the fact that these cells are more spread on the body region instead of being more localized on the brain region, means that the reduced levels of GAL3, influenced negatively the cells homing capacity, due to the reduction on the adhesion interactions needed for the homing of the cells ⁷⁴. On the other hand, the higher degree of brain localization when tNES_shControl was transplanted can be explained by the functional expression of GAL3. In summary, we demonstrate that the reduction of GAL3 expression affect the home capacity of the cells, in the zebrafish model, as showed in Figure 3.11. The lack of home capacity reduces the tumorigenesis, once we don't believe that these cells will be able to develop a tumor outside their origin site (brain region), due to the lack of a neural microenvironment.

There are pre-clinical studies describing the capacity of GAL3 in promoting tumor growth and metastasis, predominantly in tumors outside the CNS. However, the role of GAL3 in brain tumors has been less studied ⁷⁵. We previously showed that the cells homing capacity was influenced by the decreased levels of GAL3 in zebrafish, so we wondered if the decreased levels of GAL3 can also influence the tumor growth and proliferation, in mice. EGFR is the epidermal growth factor

receptor and induces cell proliferation upon activation, DAOY cell lines express EGFR. EGFR-mediated pathways are important in a variety of cellular processes, including cancer cell migration, invasion and growth. Thus, researchers have revealed intracellular and extracellular functions of the GAL3 protein in the regulation of EGFR expression. The cross-link between GAL3 and growth factors receptors, such as EGFR, have been implicated in modulating tumor cell behaviour, such as tumor growth, migration, and metastasis. As a result, reduced levels of GAL3 can negatively impair this interaction contributing to the decrease of tumor volume and growth ⁷⁶. Moreover, several studies have showed that the GAL3 KD results in a perturbed cell-cycle progression, including cell-cycle arrest at the G1 phase, and hypo-phosphorylation of the several tumor suppressor proteins ⁷⁶. These data align with our observations, as we can see in Figure 3.13A and 3.13B the loss of GAL3 in KD#1 cell line strongly reduced subcutaneous, *in vivo*, tumor volume and weight, compared with the sh_Control, suggesting that the decreased levels of GAL3 also impact on tumor growth. However, the KD#2 in *LGALS3* gene didn't cause the same tumor volume and weight reduction, as KD#1, as it was expected. This different behaviour on the KDs cannot be justified by a non-effective GAL3 KD in KD#2, as we can confirm by figure 3.9. So, we hypothesized that the KD#2, may have re-establish the GAL3 levels, reverting the effect of the KD. To verify this hypothesis, further studies on the tumor tissue, need to be performed to quantify the levels of GAL3.

Notably, for the first time, GAL3 inhibitors are being explored for the treatment of CNS disorders, including cancers. Therefore, Galectin inhibition has emerged as a promising avenue in cancer research ⁴³.

Arginine is a semi-essential amino acid which is key in any neurological, immunological, and proliferative cellular functions. As a precursor for a plethora of proteins, this amino acid also has critical roles in cellular metabolism ⁵⁷. When arginine is sourced extracellularly, it crosses the cell membrane via specific cell membrane amino acid transporters ⁷⁷. Tumor cells frequently overexpress specific types of SLCs transporters, to meet their high arginine demand, therefore cancer cells have the ability to quickly and effectively regulate the functions of some vital proteins and transporters ⁷⁸. This, emphasises the importance to confirm that the main arginine transporters (SLC7A1, SLC7A2 and SLC7A3) and the enzyme ASS1, responsible for arginine synthesis, are not downregulated after the change to the standardized NES media, Figure 3.14. Functional regulation of cell surface proteins, including the arginine transporters, is an essential process for maintaining cell homeostasis. GALs are important for the regulation of cellular functions, and the dysregulation of galectin-lattice can result in cellular dysfunctions that are frequently associated with pathogenesis of various diseases ⁷⁹. It has been previously reported that galectins form a complex with cationic amino acid transporters in cancer cells. Cationic amino acid transporters, for example SLC7A3, bound to galectins using beta-galactosidase epitope. Moreover, the

disruption of the GAL3-SLC7A3 bindings, can significantly reduce cell surface expression of arginine transporters. The reduced cell surface residency of arginine transporters attenuates the cellular arginine uptake activities⁷⁷. SLC7s contribute to the basal arginine uptake and the reduced cell surface expression of these transporters presumably decrease the constitutive cellular arginine uptake function. Therefore, the binding of SLC7s with the galectin-lattice is important for sustaining the cellular arginine uptake that is a crucial process to properly regulate the whole-body metabolism⁸⁰. In concordance with this, the downregulation of GAL3 in the KD cell lines disrupts the galectin-SLC7 binding leading to a significantly reduced SLC7A1, SLC7A2 and SLC7A3 transporter levels, in DAOY cell lines (Figure 3.15B). These results indicate that the galectin-lattice stabilizes arginine transporter and consequently sustains its expression levels on the cell surface. As it was reported previously GAL3 is upregulated in several cancers, including SHH-MB, where it is involved in the modulation of the cellular stress responses, survival and metabolism, and the downregulation of GAL3, can disrupt these processes, potentially making cells more susceptible to metabolic stress that might exacerbate the metabolic vulnerabilities of the cell. These metabolic vulnerabilities can be associated with ASS1 deficiency, particularly in cancer cells. For instance, a more hostile environment might push cancer cells to adapt by altering their metabolic enzyme expression, including decrease the expression levels of ASS1 (Figure 3.15B). On the other hand, the GAL3 KD in tNES cells seems not downregulate the arginine transporters and the ASS1 enzyme (Figure 3.15A). In this cell line, the KD is not sufficient to reduce the arginine transporters and the ASS1 enzyme expression levels. To overcome this issue in this specific cell line, a more effective technique should be used, a Knockout of the *LGALS3* gene, for example.

As mentioned previously, cancer cells need extracellular arginine due to their high metabolic demands, this results in cancer cells becoming wholly dependent on extracellular arginine for survival. When these cells don't have access to external arginine, they starve. Arginine starvation causes global transcriptional suppression of metabolic genes including those involved in mitochondrial functions, glycolysis, and DNA repair genes. Removal of arginine causes fragmentation of mitochondria and impairment of mitochondrial functions. Dysfunctional mitochondria could potentially affect the conversion of ornithine to citrulline since that process happens in the mitochondria. Furthermore, the urea cycle is also closely linked to the TCA cycle as the TCA cycle produces oxalacetate, which gets converted to aspartate that gets utilised in the urea cycle for conversion to argininosuccinate. The perturbation of both mitochondria and TCA cycle can affect arginine biosynthesis.⁴⁸ Thus, mitochondria is targeted by arginine deprivation and arginine represents a major regulator of mitochondrial activities in cancer metabolism. Therefore, just the removal of external arginine will impair on the endogenous production of arginine by the mitochondria, in cancer cells. Additionally, many cancers, including SHH-MB,

lose the ability to produce endogenous arginine, due to the reduced levels of ASS1, becoming wholly dependent on extracellular arginine for survival.⁵⁷

SHH-MB DAOY cell line already has reduced levels of ASS1, and upon the GAL3 KD the expression levels of ASS1 were even lower, so these cells need to rely on extracellular arginine for a continued growth and survival. If the cells are growing on arginine depleted media, they don't have access to external arginine, so we expect that the cells will not be able to proliferate. However, if the cells are growing on a rich media with arginine, they should be able to uptake the extracellular arginine from the media and proliferate. The KD#1 and KD#2 cells, have a significant downregulation of the arginine transporters, but they are still able to proliferate at the same rate than the sh_Control, meaning that the lower expression of the transporters is not showing an impact on the cells capacity to proliferate and survive, Figure 3.16 and 3.17. However, with very low proliferation rates in the cells cultured in arginine depleted media, can be very difficult to observe a difference on the proliferation between the cells with the reduced levels of arginine transporters and enzyme (KD#1 and KD#2), compared with the sh_Control. This data emphasizes, how essential arginine is for the ability of SHH-MB cells to proliferate, and its deprivation cause a decrease in these cancer cells proliferation and growth. Furthermore, there are still numerous therapeutically highly relevant questions, for example, how arginine deprivation affects tumor cells motility and invasiveness, and this is of particularly interest in MB, due to its highly motility and invasive nature⁸¹.

We showed that arginine deprivation affects the proliferation in SHH-MB. The low level of proliferation is correlated with a decrease in cell confluency and consequently with cell death. Caspase-dependent apoptosis is the mechanism associated with arginine-deprivation induced cell death, however this is probably not the cell death mechanism occurring when transduced DAOY cells die on the arginine depleted media, as showed in Figure 3.18. It has been proven that arginine deprivation inhibits mTOR (mammalian Target Of Rapamycin), which is a negative regulator of autophagy. Autophagy, often triggered by starvation protect cells from nutrient stress, however prolonged arginine deprivation leads to excessive and aberrant autophagy⁷⁹. More studies need to be done, to know exactly the cell death mechanism associated with arginine deprivation in SHH-MB.

Our results highlight the critical role of GAL3 in SHH-MB progression and how its overexpression can contribute to tumorigenesis. Our findings suggest that an effective KD of GAL3 can effectively reduce SHH-MB homing capacity and growth, *in vivo*. Moreover, the decrease expression of GAL3, upon the KD is also able to downregulate important arginine transporters and the enzyme ASS1. Therefore, the downregulation of ASS1, incapacitates the cell to produce endogenous arginine, relying only on external arginine. Our results showed that SHH-

MB is heavily dependent on extracellular arginine due to their high metabolic demands and reduced capacity for endogenous arginine synthesis. Furthermore, more studies need to be done to fully understand how the GAL3 downregulation affects the endogenous arginine cycle. Nevertheless, our data highlights GAL3 and arginine as critical regulators in SHH-MB cell metabolism and provide promising insights for novel therapeutic strategies, such as combining GAL3 inhibitors with arginine-depleting therapies.

5. Concluding Remarks

Galectin-3 is upregulated in many solid tumors, including SHH-MB, and its overexpression is closely associated with an increase in tumorigenesis. Through the use of patient derived SHH-MB cell lines and *in vivo* models, our findings suggests that an effective Knockdown of Galectin-3 not only significantly reduces tumor homing capacity and growth, *in vivo*, but also disrupts the expression of key arginine transporters and enzyme, in DAOY cell lines. This disruption is particularly significant, as arginine is essential for tumor cell survival, especially in SHH-MB, which already exhibits reduced levels of ASS1, a key enzyme in the endogenous arginine synthesis. This metabolic disruption, force cancer cells to depend only on extracellular arginine for survival.

Given the challenges of current treatment for SHH-MB, which are often associated with severe side effects, this study offers promising insights for the development of more targeted and less harmful treatments, such as the combination of GAL3 inhibitors with arginine-depleting therapies. Furthermore, the reduction in tumor size through such strategies could facilitate surgical intervention and improve survival rates in affected pediatric patients. However, further research is required to fully understand the implications of GAL3 downregulation on the endogenous arginine cycle and to optimize therapeutic interventions for this aggressive malignancy.

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7. Supplementary Material

Table 7.1. Primers and their sequences used in RT-qPCR.

Primer	Primer Sequence
GADPH foward	TGCACCACCAACTGCTTAGC
GADPH reverse	GGCATGGACTGTGGTCATGAG
ATP5F1 foward	GTATTGCCCCGCGTACATGG
ATP5F1 reverse	AGGACATACCCTTTAAGCCTGA
LGALS3 foward	GTGAAGCCCAATGCAAACAGA
LGALS3 reverse	AGCGTGGGTAAAGTGGAAGG
LGALS1 foward	TCGCCAGCAACCTGAATCTC
LGALS1 reverse	GCACGAAGCTCTTAGCGTCA
LGALS9 foward	GGACGGACTTCAGATCACTGT
LGALS9 reverse	CCATCTTCAAACCGAGGGTTG
CCND1 foward	CAATGACCCCGCACGATTTC
CCND1 reverse	CATGGAGGGCGGATTGGAA
SCG2 foward	GAAGCCGAATGGATCAGTGGA
SCG2 reverse	TGGTCTAAGTCAGCCTCTGAG
NES foward	CTGCTACCCTTGAGACACCTG
NES reverse	GGGCTCTGATCTCTGCATCTAC
SLC7A1 foward	ATCATCGGTACTTCAAGCGTAGC
SLC7A1 reverse	GGCGTTCAGAGTCATGTGTGT
SLC7A2 foward	CTCTGCTATGCCGAATTTGGG
SLC7A2 reverse	TCCGACAGTCACGTAGGTGT
SLC7A3 foward	GCAACAGCTCTCTGATCATCTT
SLC7A3 reverse	CGTCTGCGTACCAGCTTTTG
SLC7A11 foward	GGTCCATTACCAGCTTTTGTACG
SLC7A11 reverse	AATGTAGCGTCCAAATGCCAG
ASS1 foward	CTTGGGGCCAAAAAGGTGTTC
ASS1 reverse	GAGGTAGCGGTCCTCATACAG

Table 7.2. Primary Antibodies used in Western Blot.

Antibody Target	Source	Identifier	Dilution
α -tubulin	Santa Cruz	sc-32293	1:10000
Histone 3	Abcam	ab1791	1:1000
β -actin	Santa Cruz	sc-47778	1:10000
Galectin-1	Cell Signaling	40103S	1:500
Galectin-3	Cell Signaling	87985S	1:500

Table 7.3. Secondary Antibodies used in Western Blot.

Antibody	Source	Identifier	Dilution
Anti-mouse	Invitrogen	61-6520	1:5000
Anti-rabbit	Invitrogen	65-6120	1:5000

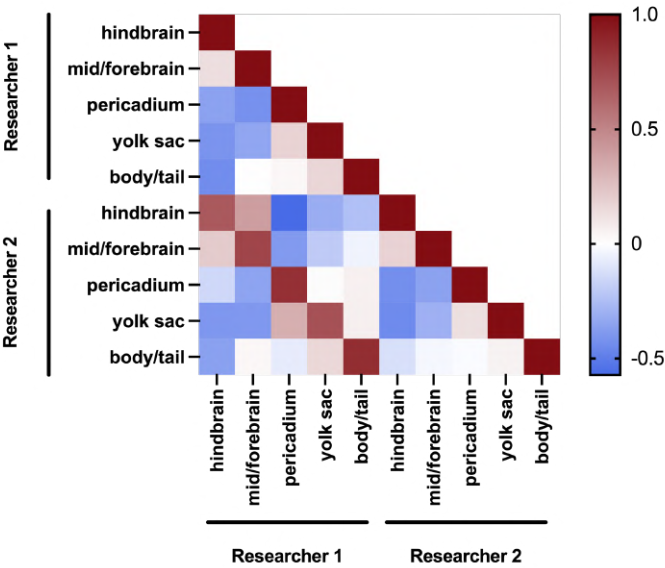


Figure 7.1. Pearson Correlation matrix between the analyzed datasets of researcher 1 and researcher 2 revealing high correlation between individually analyzed datasets.



