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GDF1 AS A REGULATOR OF CERAMIDE METABOLISM AND HEMATOPOIESIS IN ACUTE MYELOID LEUKEMIA

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BY

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LIST OF ABBREVIATIONS

ACTB.....β-actin

ALL.....acute lymphocytic leukemia

AML-MRC.....AML with MDS-related changes

AML.....acute myeloid leukemia

APL.....acute promyelocytic leukemia

ApoM.....Apolipoprotein M

AraC.....cytarabine

ATRA.....all-trans retinoic acid

C1P.....ceramide-1-phosphate

C1PP.....ceramide-1-phosphate phosphatase

CAR.....chimeric antigen receptor

CDase.....ceramidase

CERK.....ceramide kinase

CERS.....ceramide synthase

CERS1.....ceramide synthase 1

cHL.....classical Hodgkin lymphoma

circRNA.....circular RNAs

CLL.....chronic lymphocytic leukemia

CML.....chronic myeloid leukemia

COPD.....chronic obstructive pulmonary disease

CXCL12.....C-X-C motif chemokine 12

CXCR4.....C-X-C chemokine receptor type 4

DMT.....demethyltransferase

DN-AML.....*de novo* AML

DNMT.....DNA methyltransferase

EPR.....enhanced permeation retention

FB1.....Fumonisin B1

FLT3……..... FMS-like tyrosine kinase-3

FTY720.....Fingolimod

G-CSF.....granulocyte colony stimulating factor

GBA.....cerebrosidase

GCS……glucosylceramide synthase

GDF.....growth/differentiation factor

GDF1.....growth/differentiation factor 1

GDF3.....growth/differentiation factor 3

GM3.....monosialodihexosylganglioside 3

GPCR.....G-protein coupled receptor

HAT.....histone acetylase

HDAC.....histone deacetylase

HDMT.....histone demethyltransferase

HIF-1α.....hypoxia-inducible transcription factor-1α

HL.....Hodgkin lymphoma

HMT.....histone methyl transferase

HPC.....hematopoietic progenitor cell

HSC.....hematopoietic stem cell

I2PP2A.....inhibitor 2 of protein phosphatase 2A

iPSC.....induced pluripotent stem cell

Lag1.....longevity-assurance gene 1

Lep.....leptin

Lip-C6.....nanoliposomal C6-ceramide

Lip-Ghost.....nanoliposomal Ghost

lncRNA.....long noncoding RNA

MDS.....myelodysplastic syndrome

miRNA.....microRNA

MM.....multiple myeloma

MSC.....mesenchymal stem cell

NG2.....neuron-glial antigen 2

NHL.....non-Hodgkin lymphoma

NHVDL.....New Hampshire Veterinary Diagnostic Laboratory

NLPHL.....nodular lymphocyte predominant Hodgkin lymphoma

nSMase2.....neutral sphingomyelinase 2

PcG.....polycomb-group protein

piRNA.....piwi-interacting RNA

RISC.....RNA-induced silencing complex

RNA POL II.....RNA polymerase II

RT-qPCR.....real-time quantitative PCR

S1P.....sphingosine-1-phosphate

S1PP.....sphingosine-1-phosphate phosphatase

S1PR……..sphingosine-1-phosphate receptor

SCF.....stem cell factor

SMPD1.....acid sphingomyelinase

SGMS.....sphingomyelin synthase

SPHK.....sphingosine kinase

STAT3.....signal transducer and activator of transcription 3

TBP.....TATA-Box binding protein

TF.....transcription factor

TGFβ.....transforming growth factor beta

TGFβR1.....transforming growth factor beta receptor 1

THPO.....thrombopoietin

VCAM-1.....vascular cell adhesion molecule 1

VPA.....valproic acid

ABSTRACT

GDF1 AS A REGULATOR OF CERAMIDE METABOLISM AND HEMATOPOIESIS IN ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is cancer of the myeloid lineage of blood cells. In AML, hematopoietic precursors acquire mutations or chromosomal changes that cause differentiation arrest. This results in an uncontrolled proliferation of these malignant cells in the bone marrow that can interfere with normal blood cell production. AML progresses rapidly and can be fatal within weeks if left untreated. The only current curative treatment is bone marrow transplant, which has potentially life-threatening side effects. Thus, a better understanding of the underlying biology of AML is needed to enable the development of better therapeutic modalities.

Ceramides are a family of wax-like lipids in the broader category of sphingolipids. Ceramide is comprised of a sphingoid base linked to a fatty acid through an amide group. Recently, ceramides have been appreciated for their bioactive functions including as regulators of apoptosis. This is significant because ceramide metabolic routes persist in AML that may be exploited for therapeutic development. Recently, it was shown that nanoliposomal C6-ceramide (Lip-C6) exerts unique therapeutic efficacy towards AML with myelodysplastic syndrome-related changes (AML-MRC). In contrast, other forms of AML were resistant to Lip-C6 due to enhanced ceramide metabolism. Interestingly, in these Lip-C6-resistant AMLs the gene encoding for growth/differentiation factor 1 (GDF1) is downregulated. GDF1 is encoded from a rare

bicistronic gene that also encodes for ceramide synthase 1. GDF1 is presently only appreciated to have roles in embryonic and cardiac development. However, its genetic link to a ceramide biosynthesizing enzyme as well as its inverse relation to Lip-C6-resistant AML suggest that it may also exert a role in ceramide metabolism.

This dissertation research first explored a hypothesis that GDF1 regulates ceramide detoxification in AML. *GDF1* expression was variably expressed across AML subtypes but was mostly downregulated in Lip-C6-resistant AML. Moreover, there was an inverse relationship between *GDF1* expression and genes encoding for ceramide neutralizing enzymes. This inverse relationship was validated as treatment of AML cell lines with recombinant GDF1 downregulated the expression of these same genes. Interestingly, recombinant GDF1 was also able to uniquely promote SMAD2/3 phosphorylation while concurrently downregulating STAT2 tyrosine phosphorylation in a transforming growth factor beta receptor 1 (TGFβR1)-dependent manner. Next, this dissertation research evaluated the ability of GDF1 to regulate hematopoiesis and exert an anti-AML therapeutic effect. Recombinant GDF1 restored hematopoiesis and promote erythropoiesis (red blood cell development) both *in vitro* and *in vivo*. GDF1 was unable to impact hematopoiesis in normal bone marrow, suggesting that its hematopoietic-regulatory therapeutic effect was specific to abnormal and malignant situations. Furthermore, GDF1 treatment exerted combinatorial anti-AML efficacy with cytarabine and extended the overall survival of mice engrafted with a highly aggressive AML. The final aspect of this dissertation studied a novel transgenic obese AML mouse model to evaluate links between obesity and AML. Obesity provokes profound changes in lipid homeostasis including by upregulating sphingolipid biosynthesis. Transgenic obese AML mice developed a robust leukemia burden compared with non-obese counterparts. More so, genes responsible for regulation of the ceramide-mediated

NADPH oxidase 2 were upregulated in these transgenic obese AML mice. This demonstrated a further sphingolipid-mediated dysfunction that can contribute to the development and progression of AML.

Overall, this dissertation research has uncovered important sphingolipid metabolic biology that underlies the development and progression of AML. This has revealed pathways associated with resistance to ceramide-elevating therapeutics such as Lip-C6. Most noteworthy, this research has identified for the first time an ability for GDF1 to regulate ceramide metabolism, hematopoiesis, and erythropoiesis. It has also shown that GDF1 can exert effects through a unique TGFβR1-dependent mechanism regulating both SMAD2/3 and STAT3 signaling. Collectively, this has revealed an anti-AML therapeutic ability for GDF1 linked to the regulation of ceramide metabolism.

CHAPTER 1

INTRODUCTION

1.1. Influences on the Hematopoietic Stem Cell Niche[1]

Hematopoietic stem cells (HSCs) are rare, self-renewing and multipotent progenitors that sustain hematopoiesis throughout the lifespan (Crane et al., 2017). This process involves a complex variety of interactions with supporting cells and ultimately generates all types of blood cells, including erythrocytes, platelets, and leukocytes. In this process, HSCs first give rise to an array of hematopoietic stem progenitors which have restricted self-renewing capacity before terminal differentiation into mature cells, such as erythrocytes, megakaryocytes that produce platelets, and leukocytes. In homeostasis, HSCs are mostly in a dormant state, striking a remarkable balance between quiescence and self-renewal. But in cases of hematopoietic stresses such as severe infection or blood loss, HSCs activate to regenerate hematopoietic cells.

HSCs normally reside in the bone marrow within the medullary cavities of long bones and axial bones, although they can transiently expand into facultative niches in extramedullary tissues such as the liver and the spleen under severe hematopoietic stresses (Crane et al., 2017). The bone marrow microenvironment provides signals for the maintenance and regulation of HSCs, directing proliferation, apoptosis, extra-medullary mobilization, and quiescence (Geiger et al., 2013). In the past decade, advancements in imaging, HSC biomarker discovery, and improvement in functional genomic techniques have allowed for a deeper understanding of the

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mechanisms within the bone marrow microenvironment that regulate HSCs. This review will summarize the current understanding of the adult hematopoietic bone marrow microenvironment, focusing on cellular and molecular participants and their contributions to normal HSC regulation, as well as the crosstalk between the niche and HSCs in malignant transformation.

The HSC niches

Human bone marrow is located within the trabecular region of the large bones, which is lined by the endosteum, formed by osteoblasts (Calvi and Link, 2014; Wang et al., 2013a). This tissue is highly vascularized and rich in micro vessels and capillaries (Nombela-Arrieta et al., 2013; Wang et al., 2013a). In the center of the bone marrow, longitudinal large arteries give rise to smaller radical arteries, which then branch into arterioles. Arterial circulation transitions to venous circulation near the endosteal surface. The network of arterial vessels progressively transforms into wider, irregularly shaped sinusoids which eventually coalesce into a big collecting central sinus and drain into the venous circulation (Nombela-Arrieta et al., 2013; Wang et al., 2013a).

Within the bone marrow, local tissue microenvironments with a distinct cellular and molecular makeup, termed “niches”, maintain and regulate HSCs (Morrison and Spradling, 2008). The bone marrow is a complex cellular environment with HSCs residing in close contact with multiple cell types, including perivascular mesenchymal stromal cells and endothelial cells (Ehninger and Trumpp, 2011; Morrison and Spradling, 2008; Nombela-Arrieta et al., 2013; Wang et al., 2013a). These cells secrete growth factors and chemokines that participate in normal hematopoiesis and support progenitor cells. Confocal imaging and spatial modelling studies have

confirmed that HSCs are widely distributed throughout the bone marrow. In addition, they are more common at the periphery of the marrow near the bone surfaces compared to the medullary regions of the bone, and in the metaphysis of the bone (Acar et al., 2015) (Figure 1.1). In these niches, approximately 80% of HSCs reside adjacent to sinusoidal blood vessels in the bone marrow; the remainder are mainly associated specifically with arterioles or adjacent to transition zone vessels in the endosteal region (Crane et al., 2017; Nombela-Arrieta et al., 2013; Spencer et al., 2014; Wang et al., 2013a).

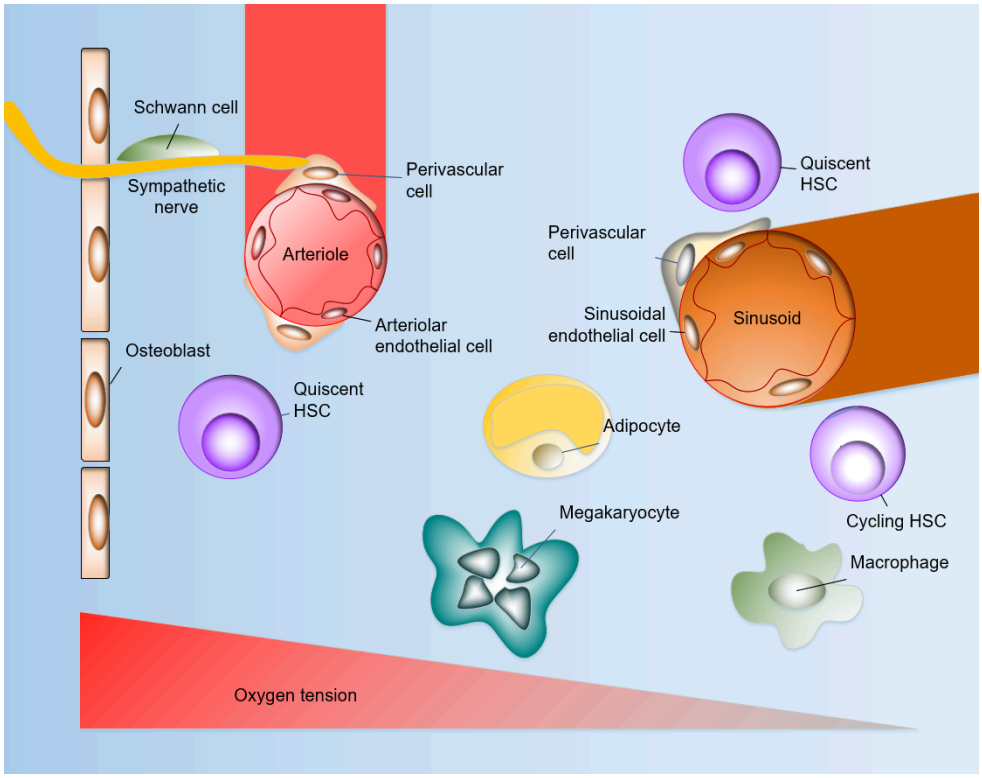

Figure 1.1. Schematic representation of the HSC niche. The bone marrow microenvironment is composed of different cell populations that coordinately contribute to the regulation of HSCs. These include perivascular stromal cells, endothelial cells, megakaryocytes, sympathetic neurons and non-myelinating Schwann cells, adipocytes, monocytes and macrophages. The arteriolar niche has a higher ratio of quiescent HSCs, whereas majorities of both quiescent and cycling HSCs localize to the sinusoidal niche. The absolute pO_2 of the bone marrow is quite low despite very high vascular density, with the lowest pO_2 in perisinusoidal regions. The endosteal region, by contrast, is less hypoxic as it is perfused with small arteries.

Growth factors that modulate HSC function

The normal function of HSCs is supported by cells that reside within the bone marrow niche. These supporting cell types that are responsible for the production of growth factors are known to be required for HSC maintenance, including C-X-C motif chemokine 12 (CXCL12), stem cell factor (SCF) and thrombopoietin (THPO) (Crane et al., 2017). Other factors in the bone marrow may also modulate HSC function in a non-cell-autonomous way by promoting hematopoietic regeneration after injury, although they are not necessarily required for HSC maintenance or hematopoiesis.

C-X-C motif chemokine 12 (CXCL12)

CXCL12, also known as stromal cell-derived factor 1, is a chemokine protein ubiquitously expressed in many tissues and cell types that functions as an HSC homing signal to the marrow (Calvi and Link, 2014). In the bone marrow, CXCL12 is constitutively expressed at high levels by perivascular stromal cells. When deleted from mice, absence of CXCL12 is universally fatal shortly after birth (Hoffman and Calvi, 2014). Its receptor, CXCR4, is mainly expressed on immature and mature hematopoietic cell types. This CXCL12/CXCR4 signaling closely regulates HSC retention and maintenance in bone marrow niches (Katayama et al., 2006; Lai et al., 2014; Walenkamp et al., 2017). Disruption of this connection leads to release of HSCs into the peripheral blood with limited effect on the function of HSC (Calvi and Link, 2014; Spiegel et al., 2008). Additionally, depletion of bone marrow macrophages reduces CXCL12 expression in the bone marrow and promotes HSC mobilization and extramedullary hematopoiesis (Chow et al., 2011). Similarly, deletion of *Cxcl12* from arteriolar Neuron-glial

antigen 2 (NG2)-expressing cells results in HSC reductions and altered HSC localization within the bone marrow (Asada et al., 2017).

This signaling is also vital for subsequent repopulation of HSCs in the bone marrow (Lai et al., 2014). Deletion of *Cxcr4* in adult mice resulted in severe reduction of HSC numbers and increased sensitivity to myelotoxic injury (Sugiyama et al., 2006). Deletion of *Cxcl12* from perivascular stromal cells or endothelial cells depletes HSCs from the bone marrow. In contrast, conditional deletion of *Cxcl12* from osteoblasts in murine lines leads to loss of lymphoid progenitors but had little or no effect on the number of HSCs (Birbrair and Frenette, 2016; Ding and Morrison, 2013). Despite this, mice with deletion of *Cxcl12* maintain normal levels of B-cell and T-cell progenitors (Hoffman and Calvi, 2014). However, ablation of osteoblasts eventually results in pancytopenia, likely implicating an indirect regulation of HSCs by osteoblasts (Ding et al., 2012; Morrison and Scadden, 2014; Visnjic et al., 2004). Indeed, proximal osteolineage cells upregulate genes encoding cell surface and immune response proteins, such as CXCL12 and vascular cell adhesion molecule 1 (VCAM-1), providing a possible mechanism by which osteoblasts can regulate HSCs. However, their exact function in hematopoiesis is incompletely understood (Birbrair and Frenette, 2016; Silberstein et al., 2016).

Stem cell factor

SCF, also known as KIT ligand, is a growth factor that is required for HSC maintenance. In the bone marrow, SCF is primarily expressed by perivascular and endothelial cells in proximity to cells that also express CXCL12 (Birbrair and Frenette, 2016; Ding et al., 2012; Morrison and Scadden, 2014). It activates signaling by the c-KIT receptor tyrosine kinase, which is widely expressed on HSCs and mast cells (Ho et al., 2017; Ogawa et al., 1991). SCF is present

in both membrane-bound and soluble forms (Crane et al., 2017). HSCs are depleted in *Sl*/*Sld* mutant mice, which lack membrane-bound SCF but express the soluble form (Barker, 1994). This indicates that the membrane bound SCF is more important for HSC maintenance. Endothelial and leptin receptor-expressing perivascular stromal cells are the major sources of SCF for HSC maintenance in normal adult bone marrow (Ding et al., 2012). Deletion of *Scf* from each cell population has additive effects on HSC depletion. HSCs are depleted from bone marrow when *Scf* is deleted from either endothelial cells or leptin receptor-expressing perivascular stromal cells (Ding et al., 2012). In this scenario, adipocytes have the capacity to synthesize SCF to maintain the HSC population (Zhou et al., 2017). In fact, adipocytes in the long bones promote hematopoietic recovery through production of SCF after irradiation (Zhou et al., 2017). In contrast, HSC frequency or function was not affected by conditional deletion of *Scf* from osteoblasts, hematopoietic cells, or perivascular mesenchymal stem cells (MSCs) (Ding et al., 2012).

Thrombopoietin

THPO is mostly produced by the liver and kidneys, in addition to the bone marrow stroma, and it to a much lesser extent is critically involved in the maintenance of adult quiescent HSCs (de Graaf and Metcalf, 2011; Morrison and Scadden, 2014; Qian et al., 2007; Yoshihara et al., 2007). Unlike other hormones, THPO is not negatively regulated; rather it is directly regulated by platelet quantities (de Graaf and Metcalf, 2011; Morrison and Scadden, 2014; Qian et al., 2007; Yoshihara et al., 2007). In its normal state, THPO produced by the liver promotes megakaryocyte growth, migration and platelet production (Khodadi et al., 2016). Secreted THPO binds to the THPO receptor, which is also known as the myeloproliferative leukemia protein

(MPL), and activates a signaling cascade that results in megakaryocyte maturation within the marrow (Khodadi et al., 2016). Maturing megakaryocytes within the marrow interact with numerous cells, including osteoblasts, osteoclasts, HSCs, and plasma cells (Khodadi et al., 2016). Loss of THPO or THPO signaling results in amegakaryocytic thrombocytopenia and eventually progresses to bone marrow failure via loss of HSC quiescence (Decker et al., 2018; Qian et al., 2007; Yoshihara et al., 2007). Murine THPO-knockout models have demonstrated that absence of THPO results in failure to reconstitute marrow cellularity following irradiation (Decker et al., 2018). Further, liver-specific deletion of murine *Thpo* reduces both marrow megakaryocytes and HSCs without decreasing the overall marrow cellularity, implicating THPO as a critical factor in the maintenance of HSCs (Decker et al., 2018). THPO ensures HSC chromosomal integrity and function in response to irradiation by regulating the DNA-damage response (de Laval et al., 2014). THPO receptor agonists drive HSCs into self-renewing divisions, leading to quantitative expansion of functional HSC (Geiger et al., 2013). Further, depletion of megakaryocytes results in a loss of HSC quiescence and increases the expansion of HSCs, likely by a concomitant increase in THPO-signaling (Bruns et al., 2014, 2014). In *ex vivo* models, supplementation of HSC cultures with THPO results in accelerated expansion of HSCs (de Graaf and Metcalf, 2011).

Indeed, during times of hematopoietic distress in which THPO signaling may be enhanced, such as during periods of thrombocytopenia or during treatment with THPO-mimetics such as eltrombopag and romiplostim, HSCs exit their quiescent phase and begin to divide through an myeloproliferative leukemia-mediated mechanism (Kovtonyuk et al., 2016). However, long term increases in THPO or treatment with THPO-mimetics results in bone marrow fibrosis through transforming growth factor-beta (TGFβ), platelet-derived growth factor,

and mechanisms resulting in increased bone marrow reticulin production (Khodadi et al., 2016; Kuter, 2009). THPO-mediated marrow fibrosis may be reversible upon normalization of THPO levels or cessation of therapy with THPO-mimetics (Kuter, 2009).

Angiogenin

Angiogenin is a secreted ribonuclease with a broad range of effects, including angiogenesis, neurogenesis, and immune regulation (Yoshihara et al., 2007). In HSCs, angiogenin promotes quiescence, but it promotes proliferation in lineage-committed myeloid progenitors; the exact mechanism is not yet understood (Yoshihara et al., 2007). Angiogenin is expressed at a higher level in mesenchymal progenitors, osteolineage-committed progenitors, and periarteriolar sheath cells. It restricts the proliferation of early lymphoid progenitors and HSCs, thereby promoting HSC self-renewal and repopulating potential (Qian et al., 2007). Supplemental therapy with angiogenin promotes hematopoietic regeneration following bone marrow failure in stem cell transplantation, while deletion of angiogenin from those cells resulted in an increased number of long-term HSCs and more active cycling of HSCs (Yoshihara et al., 2007).

Granulocyte colony stimulating factor (G-CSF)

G-CSF is a cytokine that is mainly produced by the endothelium and macrophages. It is clinically used to promote HSC mobilization into the bloodstream and to increase the production of granulocytes, either to reconstitute a depleted immune system or to prepare a patient for a bone marrow donation. To promote mobilization, G-CSF suppresses the expression of CXCL12 from marrow stromal cells, downregulating the homing signal that attracts HSCs to the marrow

niche while upregulating CXCR4 (Calvi and Link, 2014; Hoffman and Calvi, 2014). Interestingly, activating osteoclasts via administration of receptor activator of nuclear factor kappa-B-ligand results in mobilization of HSCs, but inhibition of osteoclasts with calcitonin mitigates the G-CSF-induced mobilization of HSCs (Calvi and Link, 2014). It has been hypothesized that activated osteoclasts secrete cathepsin K which may cleave CXCL12 and result in mobilization of HSCs (Calvi and Link, 2014). Further, neutrophils are believed to play a role in G-CSF-mediated HSC mobilization, although this too is incompletely understood (Calvi and Link, 2014). Recently, it was found that these two functions induced by G-CSF work independently. While cells with limited regenerative potential are induced to repopulate by G-CSF, dormant HSCs are only mobilized into the blood without any proliferation (de Graaf and Metcalf, 2011).

Bone marrow niche cells that regulate HSCs

The HSC niche can be regulated by various types of cells, including perivascular stromal cells and endothelial cells, which can produce factors including CXCL12 and SCF. Several other cell types also contribute to maintenance of the HSC niche and can leverage additional factors including CXCL4, G-CSF, and TGFβ (Ehninger and Trumpp, 2011).

Bone marrow stromal cells

CXCL12-abundant reticular cells reside in the marrow at sites closely associated with HSCs (Hoffman and Calvi, 2014). They have been found to largely overlap with nestin-GFP+ stromal cells and leptin receptor-expressing cells in studies where both are defined by transgenic expression using defined stromal-specific promoters (Anthony and Link, 2014; Ding et al., 2012;

Nagasawa et al., 2011; Omatsu et al., 2010; Sugiyama et al., 2006). These stromal cells are all MSCs that have both adipogenic and osteogenic potential. They differentiate into adipocytic and osteoblastic mesenchymal lineages in the adult bone marrow, producing large amounts of proteins for the storage of nutritional energy or bone formation (Nagasawa et al., 2011; Omatsu et al., 2010; Zhou et al., 2014). HSCs are depleted from the bone marrow when *Scf* or *Cxcl12* are conditionally deleted from leptin receptor-expressing CXCL12-abundant reticular cells. This implicates them in a maintenance role for the HSC niche (Asada et al., 2017; Ding et al., 2012; Zhou et al., 2014).

Osteoblasts

It was previously believed that osteoblasts do not directly promote HSC maintenance as they do not express the crucial niche factors (Ding et al., 2012; Morrison and Scadden, 2014). However, evidence that osteoblast ablation eventually causes pancytopenia has led to the hypothesis that osteoblasts indirectly regulate HSCs, likely via crosstalk (Visnjic et al., 2004). Recently, stable genome-wide transcriptional differences have been identified by single-cell RNA-seq and transcriptional comparison between HSC-proximal stromal cells and osteolineage cells either in proximity to transplanted HSCs or at a distance. Proximal osteolineage cells displayed a significant upregulation of genes encoding cell-surface proteins and those involved in immune response, such as CXCL12, VCAM-1, and angiogenin, lending further support for osteoblastic regulation of HSCs (Silberstein et al., 2016).

Adipocytes

Bone marrow-resident adipocytes are abundant, and their population size increases with age (Anthony and Link, 2014). Adipocytes have been shown to negatively affect HSC maintenance, and are increasingly present after chemotherapy and radiation (Naveiras et al., 2009). Recently, bone marrow adipocytes were found to also synthesize SCF after depletion of endothelial cells and leptin receptor-expressing stromal cells (Zhou et al., 2017). Despite being an important source of SCF in both locations, adipocytes in long bones promote hematopoietic recovery after irradiation, while in caudal vertebrae they inhibit hematopoietic regeneration. Importantly though, adipogenesis can promote initial hematopoietic recovery following irradiation (Zhou et al., 2017). This is in accordance with the finding that adipocyte-rich bone marrow has decreased numbers of HSCs compared with adipocyte-poor bone marrow (Naveiras et al., 2009).

Endothelial cells

Endothelial cells lining the blood vessels of the bone marrow are also indispensable for HSC maintenance through producing niche factors CXCL12 and SCF and are a central component in the maintenance of these cells (Crane et al., 2017; Ding and Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013). Despite the relatively small amount of CXCL12 and SCF expressed, conditional deletion of *Cxcl12* or *Scf* from endothelial cells has been shown to lead to a decrease in the number of HSC in bone marrow (Ding et al., 2012, 2012). HSC frequency and function were not affected when *Scf* was conditionally deleted from other cells types such as osteoblasts, megakaryocytes or other hematopoietic cells (Ding et al., 2012). The HSC niche thus

depends on SCF or CXCL12 that is produced by endothelial cells and leptin receptor-expressing stromal cells.

Neuronal cells

Bone marrow is highly innervated (Katayama et al., 2006). Sympathetic nervous fibers generally run alongside arterioles, yet subsets are also found in the sinus wall and the hematopoietic parenchymal tissue, indicating existence of an interconnected network (Giles et al., 2016). The nervous system is not required for the maintenance of HSCs in the bone marrow but is critical for bone marrow regeneration after chemotherapy (Lucas et al., 2013). Signals from the sympathetic nervous system coordinate the circadian egress of HSCs into circulation by regulating local production of CXCL12 in the synapsed perivascular cells (Giles et al., 2016; Méndez-Ferrer et al., 2008). Genetic or pharmacological ablation of adrenergic signaling inhibits G-CSF-induced HSC mobilization (Méndez-Ferrer et al., 2008). Pharmacological or genetic ablation of adrenergic neurotransmission inhibits G-CSF-induced HSC mobilization (Katayama et al., 2006). Lastly, non-myelinating Schwann cells are autonomic nerve-ensheathing glial cells. They are a producer of TGFβ, which is a quiescence signal for HSC (Yamazaki et al., 2011).

Megakaryocytes

Megakaryocytes are terminally-differentiated hematopoietic cells that play an inhibitory role in HSC expansion via the secretion of CXCL4 and TGFβ (Bruns et al., 2014; Zhao et al., 2014). Studies showed that depletion of megakaryocytes resulted in specific loss of HSC quiescence and led to a marked expansion of functional HSCs. In contrast, conditional deletion of *Cxcl4* or *Tgfβ1* in megakaryocytes increased HSC activation and proliferation (Bruns et al.,

2014; Zhao et al., 2014). While in response to stress, fibroblast growth factor 1 signaling from megakaryocytes transiently dominates over TGFβ inhibitory signaling to stimulate HSC expansion to facilitate the recovery of the hematopoietic system (Zhao et al., 2014).

Monocytes and macrophages

Higher levels of G-CSF are expressed in monocytes and macrophages as part of the response to sepsis and other inflammatory conditions (Burberry et al., 2014). Depletion of bone marrow macrophages reduces CXCL12 expression in the bone marrow and promotes HSC mobilization and extramedullary hematopoiesis (Chow et al., 2011). Monocytes and macrophages with high expression of α-smooth muscle actin and cyclooxygenase-2 maintain HSCs and protect them from exhaustion during stress situations by producing prostaglandin E2. Moreover, macrophages maintain the quiescence of HSCs through the DARC/CD82 ligand interaction and downstream TGFβ signaling (Hur et al., 2016).

Sinusoidal and arteriolar niches

Functionally distinct perivascular niches are created by sinusoids and arterioles (Acar et al., 2015; Asada et al., 2017; Chen et al., 2016; Itkin et al., 2016; Kunisaki et al., 2013). There is a higher ratio of quiescent to cycling HSCs associated with arterioles (Asada et al., 2017; Kunisaki et al., 2013). However, However, the majority of quiescent and cycling HSCs are localized to the sinusoids (Acar et al., 2015; Asada et al., 2017; Itkin et al., 2016; Kunisaki et al., 2013). The sinusoidal and arteriolar microenvironments differ with respect to vessel wall permeability, oxygen tension and niche factors produced by the residing hematopoietic cells (Asada et al., 2017; Crane et al., 2017; Itkin et al., 2016; Kunisaki et al., 2013; Spencer et al.,

2014). First, sinusoids are very leaky as they have a fenestrated basal lamina. They promote HSC activation and are the exclusive site allowing for cell migration to and from the bone marrow. In contrast, less permeable arterial blood vessels maintain HSCs in a low oxidative state, keeping them in a quiescent state (Itkin et al., 2016). HSCs are also more resilient after irradiation thus they may become more dependent on periarteriolar niches during the regeneration of hematopoiesis (Ito and Suda, 2014). Second, direct measurement of local oxygen concentration in the bone marrow showed that the lowest oxygen tension can be found in deeper perisinusoidal regions. The endosteal region, by contrast, is less hypoxic as it is perfused with small arteries (Spencer et al., 2014). Thirdly, cytokines produced in distinct vascular niches contribute to HSC maintenance differently. Selective *Cxcl12* deletion from arteriolar NG2-expressing cells caused HSC reductions and altered HSC localization in bone marrow. In comparison, deletion of *Scf* in sinusoidal leptin receptor-expressing cells led to reductions in bone marrow HSC numbers (Asada et al., 2017).

Differentiation hierarchy and metabolic features of HSCs

HSCs rely heavily on anaerobic glycolysis and have relatively inactive mitochondria irrespective of oxygen tension, in a manner similar with the Warburg effect in cancer cells (Ito and Suda, 2014). The activity of specific metabolic pathways and the differentiation state of stem cells are clearly related (Figure 1.2). Cell differentiation is accompanied by a shift from anaerobic glycolysis to mitochondrial respiration; oxygen tension increases from the endosteum to the sinusoids (Gaspar et al., 2014; Wang and Zhong, 2018). The level of oxygen in the various locations within the bone marrow niche are directly responsible for CXCR4 function and expression, with higher CXCR4 function and expression in relatively hypoxic environments

(Wang and Zhong, 2018). Moreover, stem cell fate is dependent on the degree of activation of mitochondrial metabolism, in addition to other factors (Bigarella et al., 2014). Initially, human embryonic stem cells rely entirely on glycolysis for their source of energy regardless of oxygen availability (Panopoulos et al., 2012). After birth, adult stem cells such as HSCs and their progenitor cells reveal a preference for aerobic glycolysis and a repression of oxidative phosphorylation (Yeo et al., 2013). Later, terminally differentiated hematopoietic cells lose their colony-forming capacity and shift from glycolysis to mitochondrial oxidative phosphorylation to produce ATP. In addition, reprogramming somatic cells to a primitive stage results in a metabolic switch from oxidative to glycolytic phenotype (Prigione et al., 2014). These results indicate that the differentiation hierarchy is in close relationship with metabolism, and loss of primitive stem cell potential is accompanied by a remodeling of the mitochondrial compartment. Differentiation of HSCs is accompanied by a biogenic shift from glycolysis to mitochondrial oxidative phosphorylation (Zhang and Sadek, 2014) (Figure 1.2).

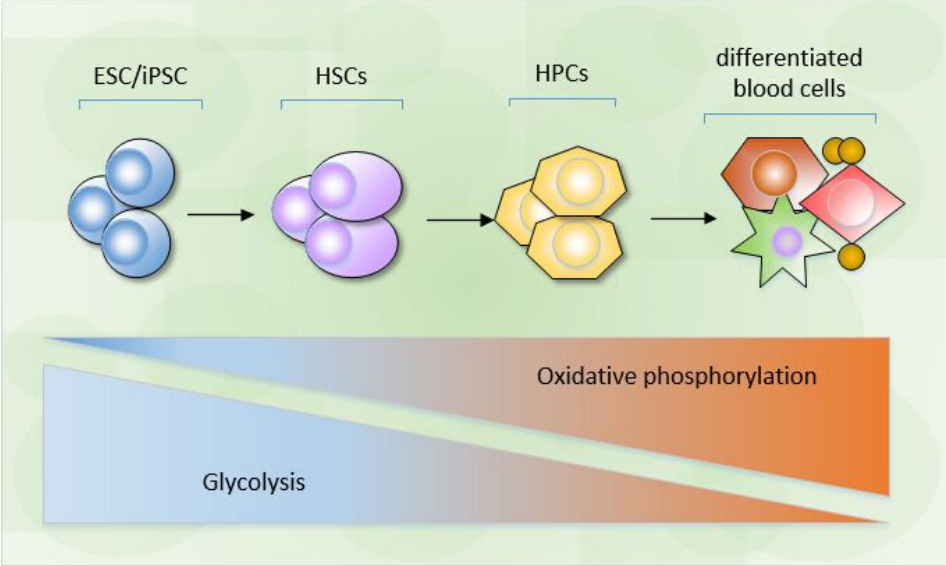

Figure 1.2. Differentiation hierarchy and metabolic features of HSCs. Stem cell fate may be directly modified by metabolism. The differentiation of stem cells is accompanied by a biogenic shift from glycolysis to mitochondrial oxidative phosphorylation. HSCs prefer to use glycolysis rather than mitochondrial oxidative phosphorylation as a main energy source. Following differentiation, terminally differentiated cells lose colony-forming capacity and shift from glycolysis to mitochondrial oxidative phosphorylation. Abbreviations: ESC, embryonic stem cells; iPSC, induced pluripotent stem cells; HSCs, hematopoietic stem cells; HPCs, hematopoietic progenitor cells.

Interestingly, recent studies have demonstrated unique mitochondrial bioenergetic features associated with leukemia and drug resistance (Farge et al., 2017; Kao et al., 2019). Namely, increased oxidative phosphorylation was observed in acute myeloid leukemia (AML) under chemotherapeutic selective pressure but was not exclusive to the leukemia stem cell compartment (Farge et al., 2017). This increase in oxidative phosphorylation accompanies increased mitochondrial biogenesis and so has been postulated to be due to an increased reliance on this metabolic pathway (Farge et al., 2017). It has therefore been postulated that targeting mitochondrial metabolism may represent a strategy to overcome this new hallmark of AML drug resistance (Farge et al., 2017). However, another study has suggested that increased reliance on oxidative phosphorylation may be representative of mitochondrial inefficiency due to other energetic and metabolic changes (Kao et al., 2019). Intriguingly, alterations in mitochondrial bioenergetics were observed to be associated with and possibly secondary to alterations in sphingolipid metabolism (Kao et al., 2019). Not surprisingly, dysfunctional sphingolipid metabolism has also been associated with AML and therapy resistance (Barth et al., 2019; Kao et al., 2019). Overall, this highlights the importance of metabolic changes that occur during hematologic malignancy and demonstrates the relevance of metabolic-targeting therapeutic strategies.

Malignant transformation of HSCs

Hematologic malignancies such as leukemia do not propagate efficiently in vivo outside of the bone marrow and are difficult to grow ex vivo. This suggests that supporting cells within the bone marrow niche may help to support or drive the malignant transformation of HSCs. Furthermore, malignant HSCs may remodel the niche to increase its own support, while

suppressing normal hematopoiesis (Calvi and Link, 2015; Krause and Scadden, 2015; Zhang and Sadek, 2014). Alteration of the marrow niche is an important and necessary step in leukemogenesis, which is a hypothesis supported by the observation that fewer than one in one million AML cells possess stem cell-like activity (Behrmann et al., 2018).

Notch signaling

Notch signaling in endothelial cells leads to the expansion of HSC niches by inhibiting Cxcl12 expression in bone, which increases capillary perivascular cells and arteriole formation and elevates cellular SCF levels (Kusumbe et al., 2016). Defects in this signaling pathway have been observed to lead to the development of myeloproliferative disease and have for many decades been linked to the development of T-cell neoplasms (Lampreia et al., 2017; Weber and Calvi, 2010). Further, it appears that constitutive Notch activation via transduction of HSCs with active Notch1 intracellular domain increases self-renewal of HSCs (Weber and Calvi, 2010). Constitutive Notch activation prevents HSC differentiation into hematopoietic progenitors (Weber and Calvi, 2010). To disrupt the cancer-stromal interactions in leukemia, CXCR4 antagonists have been clinically used to promote mobilization of leukemic cells from the protective microenvironment, making them more sensitive to conventional chemotherapy (Liu et al., 2016; Tsou et al., 2018).

Alterations in bone marrow stromal cells have been shown to be sufficient to initiate myeloproliferative disorders (Calvi and Link, 2015). In acute lymphoblastic leukemia and chronic myeloid leukemia, malignant cells were shown to have increased G-CSF production and reduced homing and retention in the bone marrow, which was related to decreased Cxcl12 expression in bone marrow stromal cells (van den Berk et al., 2014; Zhang et al., 2012).

Osteogenic differentiation has also been shown to be reduced in myelodysplastic syndrome (MDS)-derived MSC (Geyh et al., 2013). In fact, MSCs from a wide diversity of MDS subtypes are structurally, epigenetically, and functionally altered, which leads to impaired stromal support and may contribute to deficient hematopoiesis in MDS (Geyh et al., 2013).

An activating mutation of β-catenin in mouse osteoblasts has also been shown to alter the differentiation potential of myeloid and lymphoid progenitors, leading to development of AML. This demonstrates a link between genetic alterations in osteoblasts and the development of AML (Kode et al., 2014). Moreover, this shows that an altered microenvironment can serve as the inciting event in hematologic malignancy.

In addition, leukemic bone marrow infiltration in an MLL-AF9 AML model has been shown to be promoted by neuropathy of the sympathetic nervous system (Hanoun et al., 2014). Development of AML damages the sympathetic nervous system and disrupts the quiescence of perivascular MSCs. This can lead to increased osteoblastic differentiation at the expense of HSC-maintaining periarteriolar niche cells. Moreover, stromal β2-adrenergic receptors have been shown to regulate leukemia stem cells. This is evidenced by rescue of the healthy HSC niche by β2 agonist treatment, which otherwise limits leukemia stem cell expansion (Hanoun et al., 2014).

Malignant HSCs remodel the bone marrow niche

Just as the bone marrow niche may have profound effects on HSC behavior, malignant HSCs may also shape the microenvironment to selectively enhance their own support over normal HSCs (Calvi and Link, 2015). In chronic myeloid leukemia (CML), malignant cells release exosomes that stimulate bone marrow stromal cells to produce IL-8 to promote CML cell survival (Corrado et al., 2014). The secretion of IL-8 is part of the crosstalk between CML cells

and bone marrow stromal cells mediated by exosomes (Corrado et al., 2014). Furthermore, in myeloproliferative neoplasms, leukemic cells stimulate MSCs to overproduce functionally altered osteoblastic lineage cells, which accumulate in the bone marrow cavity as inflammatory myelofibrotic cells (Schepers et al., 2013). Malignant HSCs have also been found to produce IL-1β to trigger neural damage and Schwann cell death, which further causes MSC reduction (Arranz et al., 2014). Finally, healthy MSCs have been shown to adopt MDS-MSC-like molecular features when exposed to MDS cells, indicative of an instructive remodeling of the microenvironment (Ito and Suda, 2014).

Conclusions

This review has illustrated the contributions of multiple cell populations within the bone marrow microenvironment to the complex regulation of HSC function. There is a continuing and further need to understand how the niche controls HSC function during stress situations, including during infection, malignancy, and during therapy. Examining the crosstalk between HSCs and their niche may provide targetable pathways to alter the HSC niche in such a way that it becomes less hospitable to malignant cells (Krause and Scadden, 2015). In this manner, targeting the HSC niche itself is an attractive avenue for the treatment of hematologic malignancies. Future work utilizing high resolution single-cell methods, as well as endogenous labeling and tracking of stem cells, may further foster studies on the complex interplay occurring within the microenvironment of the bone marrow.

1.2. Brief Overview of Hematologic Disorders

Abstract

Hematologic disorders are diseases related to blood. They may affect the production of blood and its components, such as blood cells, hemoglobin, blood proteins, bone marrow, platelets, blood vessels, and the spleen. Common blood disorders include anemia, hemophilia, myelodysplastic syndrome, blood cancers such as leukemia (including acute myeloid leukemia, chronic myeloid leukemia, acute lymphocytic leukemia, and chronic lymphocytic leukemia), multiple myeloma, and lymphoma (Hodgkin lymphoma and non-Hodgkin lymphoma).

Anemia

Anemia occurs when there are not enough red blood cells in the body or when red blood cells do not function properly (Table 1.1). It is diagnosed when the hemoglobin value is less than 13.5 gm/dl in a man or less than 12.0 gm/dl in a woman by a blood test (Conrad, 1990). Depending on the size of the red blood cells, anemia can be classified as microcytic anemia, macrocytic anemia, normocytic anemia. Based on the amount of hemoglobin in each cell, anemia can be classified by severity as mild, moderate, or severe (Powell and Achebe, 2016).

Table 1.1. Brief description for major hematologic disorders

	Brief disease description	Treatment options
Anemia	Absolute decrease in red blood cell mass	Changing diet, taking dietary supplements, treating the underlying disease, immunosuppressive medications, blood transfusions
Hemophilia	An inherited genetic defect that impairs the body's ability to clot properly	Commercially prepared clotting factors
Myelodysplastic syndrome	A group of cancers in which immature blood cells in the bone marrow do not become healthy blood cells	Blood products, hematopoietic growth factors, chemotherapy, hypomethylating agents, allogeneic stem cell transplantation
Acute myeloid leukemia	A type of cancer that originates from immature myeloid cells	Chemotherapy, hematopoietic stem cell transplant, CARTs
Chronic myeloid leukemia	A form of leukemia characterized by the proliferation of mature granulocytes	Tyrosine-kinase inhibitors, allogeneic bone marrow or stem cell transplantation
Acute lymphocytic leukemia	A type of cancer that originates from the immature lymphoid line of blood cells	Chemotherapy, tyrosine-kinase inhibitors, immunotherapy, radiation therapy, one marrow transplant
Chronic lymphocytic leukemia	A form of leukemia characterized by the proliferation of mature lymphocytes	Chemotherapy, targeted therapy, biological therapy
Multiple myeloma	A cancer of plasma cells	Chemotherapy, autologous hematopoietic stem cell transplant
Hodgkin lymphoma	A type of lymphoma that originates from a specific type of lymphocytes (Hodgkin cells)	Chemotherapy, radiation therapy, immunotherapy, stem cell transplant
Non-Hodgkin lymphoma	A diverse group of blood cancers that includes all types of lymphomas except Hodgkin lymphomas	Radiation therapy, chemotherapy, immunotherapy, targeted therapy, stem cell transplant

Causes

Causes of anemia include decreased production of red blood cells, increased destruction of red blood cells, blood loss, and body fluid overload. Conditions that decrease the production of red blood cells include poor diet, intestinal disorders, and kidney failure. Conditions that increase the destruction of red blood cells include thalassemia, chronic disease, cancer, and infections. Blood loss may arise by trauma, surgery, or menstruation. Pregnancy or hypervolemia caused by excessive sodium or fluid intake can lead to fluid overload (Powell and Achebe, 2016).

Pathophysiology

Anemias arising from different conditions have different pathophysiological processes. For anemia caused by poor diet lacking iron, decreased availability of iron leads to progressive depletion of bone marrow iron stores and eventually impairs erythropoiesis. For anemia caused by poor diet lacking vitamin B12 or folate, defective DNA synthesis leads to delayed nuclear maturity compared to the cytoplasm. This dyspoiesis results in intramedullary cell death and ineffective erythropoiesis (Cuskelly et al., 1996). For anemia caused by thalassemia, decreased production of one or more globin polypeptide chains leads to an abnormal form of hemoglobin being synthesized and, consequently, large numbers of red blood cells being destroyed (Marengo-Rowe, 2007).

Signs and symptoms

Since red blood cells carry hemoglobin, an iron-rich protein that transports oxygen throughout the body, patients with anemia may experience symptoms such as weakness,

shortness of breath, dizziness, irregular heartbeat, headache, cold hands or feet, and pale skin. Anemia often starts slowly without any symptoms. As the disease progresses, more symptoms may appear (Lewis, 1976).

Treatment

Treatment for anemia depends on cause and severity. For anemia caused by malnutrition, treatment varies from changing diet to taking dietary supplements. For anemia caused by chronic disease, cancer, or infections, treating the underlying disease is the solution. For aplastic anemia, which is caused by bone marrow failure, immunosuppressive medications and blood transfusions may be used for treatment (Stauder et al., 2018).

Hemophilia

Hemophilia is a genetic disorder that impairs the body's ability to clot properly (Table 1.1). This results in external bleeding after an injury or surgery, or internal bleeding inside the brain or joints. There are two main types of hemophilia: hemophilia A, which occurs due to low levels of clotting factor VIII; and hemophilia B, which occurs due to low levels of clotting factor IX (CDC, 2020). Both genes are on the X chromosome. Since females have two X chromosomes and males have one, males are more likely to have hemophilia. A female with one affected X chromosome is a carrier of hemophilia and may pass it on to her son.

Causes

Hemophilia is mainly caused by X-linked recessive genetic mutations. Hemophilia A is caused by a genetic mutation on clotting factor VIII. Hemophilia B is caused by a genetic

mutation on clotting factor IX. As with all genetic disorders, it is also possible to acquire the disease through spontaneous mutation (Peyvandi et al., 2016).

Pathophysiology

When an injury disrupts the endothelial lining of a blood vessel, coagulation begins. Exposure of blood to the subendothelial space initiates two processes: changes in platelets to form a plug; and the exposure of subendothelial tissue factor to clotting factors, which eventually leads to fibrin formation to strengthen the platelet plug. When clotting factors cannot work properly or are missing, people cannot form fibrin stands to strengthen the platelet plug. Thus, patients with hemophilia tend to have longer bleeding time or even bleed spontaneously (Furie and Furie, 2005).

Signs and symptoms

Symptoms caused by hemophilia vary with severity. Bleeding is the most common symptom, which can happen in the skin (bruise), mouth, muscle, soft tissue (hematoma), brain, and joints. The bleeding can damage organs and tissues, and it can even be life-threatening (CDC, 2020).

Treatment

There is no cure for hemophilia yet. The goal of current treatment is to prevent bleeding episodes. The mild and moderate severity patients rarely need treatment. When the disease becomes severe, commercially prepared clotting factors should be used to decrease the damage to the body. The factors can either be isolated from human blood serum, recombinant, or a

combination of the two. 20% of patients with hemophilia may gradually develop antibodies to the clotting factors, which makes the treatment more difficult (Peyvandi et al., 2016). However, in recent years patients can be diagnosed since infants during circumcision, there is a shift from treatment to prevention (Commissioner, 2020).

Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is a group of clonal bone marrow neoplasms that arise from the expansion of abnormal hematopoietic stem cells (Table 1.1). It is characterized by morphologic dysplasia in hematopoietic cells and peripheral cytopenia (Arber et al., 2016). Patients with MDS have an increased risk of AML transformation. It is most commonly diagnosed in people in their 70s in the US. The risk increases as a person gets older (Garcia-Manero et al., 2020).

Risk factors

There are several known risk factors for MDS. Older age is one of the most important risk factors for MDS. Men are more common to get MDS than women. Prior treatment with chemotherapy, certain inherited syndromes, some environmental exposures and smoking increase the risk of MDS (Poynter et al., 2017).

Pathophysiology

The pathophysiology of MDS is characterized by a multi-step process involving cytogenetic changes and/or gene mutations, abnormalities of the bone marrow microenvironment and widespread gene hypermethylation at advanced stages (Fenaux et al., 2020). A set of

recurrently mutated genes are central to the pathogenesis of MDS. These genes are organized into a limited number of cellular processes, including RNA splicing, epigenetic and traditional transcriptional regulation, and signal transduction (Fenaux et al., 2020).

Signs and symptoms

Signs and symptoms are generally related to the decrease of blood cells. Low red blood cell counts are related to chronic tiredness, shortness of breath, chilled sensation, and sometimes chest pain. Low counts of white blood cells, which fight diseases, can increase susceptibility to infection. Low numbers of platelets, which function in clotting, may lead to increased susceptibility to bleeding and bruising, as well as subcutaneous hemorrhaging (Hasserjian, 2019).

Treatment

The goals of current therapies are to control symptoms and improve overall survival. The initial step is assessing a patient's eligibility for allogeneic hematopoietic stem cell transplantation, which is the only potentially curative approach but is associated with substantial morbidity and graft-versus-host disease (Cazzola, 2020). For patients who are not eligible for transplantation, risk stratification can help inform clinical decision making. For patients with lower-risk MDS, the choice of a medical treatment is largely based on the specific disease subtype. Administration of erythropoiesis-stimulating agents can increase red-cell production and ameliorate anemia. Patients with higher-risk MDS have a median life expectancy of less than two years. For such patients, treatment is aimed at ameliorating cytopenia as well as at preventing evolution to AML and thus prolonging survival (Malcovati et al., 2013).

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a type of blood malignancy that originates from differentiation arrest of immature myeloid progenitor cells (Table 1.1). These cells grow rapidly and interfere with normal blood cell production in the bone marrow. AML is the most common form of acute leukemia in adults. The disease is fatal within weeks or months if left untreated, with a median of 4.3 months (Estey, 2021). The median age at diagnosis is 68 years in the US (Shallis et al., 2019).

Risk Factors

A number of risk factors for developing AML have been identified. "Preleukemic" blood disorders such as MDS and myeloproliferative neoplasms, hereditary defects such as Down Syndrome, exposure to chemicals such as benzene, and exposure to ionizing radiation have been identified to increase the risk of developing AML (Belson et al., 2007).

Pathophysiology

AML can arise in patients with an underlying hematological disorder or as a consequence of prior therapy, but in majority of cases, it appears as a *de novo* malignancy (Sill et al., 2011). The pathogenesis of AML involves the abnormal proliferation and differentiation of a clonal population of myeloid stem cells, which depends heavily on the interactions between different somatic alterations and chromosomal rearrangements (De Kouchkovsky and Abdul-Hay, 2016). Actually, chromosomal rearrangements and genetic mutations are identified in more than 97% of cases (Patel et al., 2012). For example, chromosomal translocation t(8:21) in core-binding factor AML results in the formation of chimeric proteins, which alter the normal maturation process of

myeloid precursor cells. This will eventually result in the accumulation of poorly differentiated myeloid cells and entity of AML (De Kouchkovsky and Abdul-Hay, 2016).

Signs and symptoms

Most of the clinical manifestations of AML reflect the lack of normal mature blood cell production as leukemic cells accumulate and replace normal cells. The early signs of AML are often vague and nonspecific. The majority of patients presents with a combination of leukocytosis and signs of bone marrow failure such as shortness of breath, easy bruising and bleeding, and susceptibility to infections. Fatigue, anorexia and weight loss are common complaints (De Kouchkovsky and Abdul-Hay, 2016).

Treatment

AML requires different therapeutic interventions since it is a heterogeneous group of disorders. There is a shift from "one size fits all" approach towards more personalized treatment based on the patients' genomic signature (Nair et al., 2020). Nonchemotherapy regimens consisting of all trans-retinoic acid and arsenic trioxide in acute promyelocytic leukemia resulted in cure rates exceeding 80% (Lo-Coco et al., 2013). Using known drugs in improved regimens (fludarabine, high-dose cytarabine, anthracyclines, gemtuzumab, ozogamicin) increased the cure rate in core binding factor AML to 80% (Kantarjian, 2016). Newly approved drugs provide fresh hope for other types of AML patients, presenting a steppingstone into a new era of precision treatment (Tiong and Wei, 2019). These include a new liposomal formulation of cytarabine and daunorubicin, and a B-cell lymphoma 2 inhibitor in combination with hypomethylating agents, among others (Nair et al., 2020).

Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a form of leukemia characterized by the proliferation of immature and maturing granulocytes in the bone marrow (Table 1.1). In Western countries, CML accounts for 15% of all leukemias affecting adults. The average age at diagnosis is around 64 years (Turkina et al., 2020).

Risk factors

The exact cause of CML is still unknown, but exposure to ionizing radiation from events such as atomic bombing has been implicated (Corso et al., 1995). Other agents such as benzene are also possible causes (Vlaanderen et al., 2012).

Pathophysiology

CML is known to be caused by a single, specific genetic mutation that results from a chromosomal translocation called the Philadelphia chromosome. The cytogenetic aberration consists of a reciprocal translocation between the long arms of chromosomes 22 and 9 [t (9; 22)], which results in a *BCR/ABL* fusion gene encoding a chimeric protein with strong tyrosine kinase activity. The continuous expression of this protein leads to the development of the CML phenotype (Quintás-Cardama and Cortes, 2009).

Signs and symptoms

CML progresses through three phases: chronic, accelerated, and blast. In the chronic phase, mature granulocytes proliferate. Most patients are diagnosed in this phase, which is most often asymptomatic. If left untreated, patients progress to the accelerated phase after 3-5 years, in

which additional cytogenetic abnormalities occur. Symptoms during this phase include bleeding, petechiae, and ecchymosis. Blast is the terminal phase of CML in which cells with additional mutations proliferate and the disease progresses rapidly. Symptoms in this stage include fever, bone pain, and a rapidly enlarging spleen (Bonifacio et al., 2019). Young patients with CML may already have a more aggressive form of CML by the time they are diagnosed (Tefferi, 2006).

Treatment

The goals of treatment are to achieve hematologic, cytogenic, and molecular remission. The first line of treatment is tyrosine-kinase inhibitors such as imatinib and dasatinib, which specifically target BCR-ABL, the fusion protein caused by the Philadelphia chromosome. With the current use of these inhibitors, there is no statistically significant difference between the survival rates of CML patients and the general population (Gambacorti-Passerini et al., 2011). Allogeneic bone marrow or stem cell transplantation is a therapeutic option particularly for advanced phase CML.

Acute lymphocytic leukemia

Acute lymphocytic leukemia (ALL) is a cancer of the lymphoid line of blood cells characterized by large numbers of immature lymphocytes (Table 1.1). It is the most common pediatric malignancy. In the past ALL progressed rapidly and untreatable, but now the survival probability is as high as 80-90% in children and 30-40% in adults (Kato and Manabe, 2018; Paul et al., 2016).

Risk factors

Although most ALL arises in healthy individuals, inherited genetic susceptibility and environmental risk factors have been identified in some patients (Malard and Mohty, 2020). People carrying inherited genetic abnormalities such as Down syndrome, Li-Fraumeni syndrome, or neurofibromatosis type 1 have increased risk of developing ALL, although ALL is not hereditary. Common infections such as influenza during pregnancy may also indirectly lead to ALL by triggering abnormal immune responses (Cazzaniga et al., 2017).

Pathophysiology

The genetic changes that lead to ALL include chromosomal translocations such as the translocation of *C-MYC*, intrachromosomal rearrangements such as the generation of *ETV6-RUNX1* fusion gene, changes in the number of chromosomes in leukemic cells such as trisomy 4, and additional mutations in individual genes (Hunger and Mullighan, 2015).

Signs and symptoms

The clinical presentation of ALL is nonspecific. Symptoms may include weight loss, fever, night sweats, loss of appetite, and enlarged liver or spleen. The T-cell subtype of ALL often causes an enlarged thymus, which can press on the trachea or the superior vena cava, causing coughing or headaches (Malard and Mohty, 2020).

Treatment

Current treatment options include chemotherapy, tyrosine-kinase inhibitors, immunotherapy, radiation therapy, and bone marrow transplant. Chemotherapy is the main

treatment for patients with ALL and consists of three phases: remission induction, consolidation, and maintenance. Tyrosine-kinase inhibitors such as imatinib are often incorporated into the treatment plan for people with the Philadelphia chromosome. Immunotherapies used for ALL include chimeric antigen receptors (CARs) T cells. They are genetically modified and patient-derived in order to bind to leukemia cells that express specific antibodies (Mardiana and Gill, 2020). In 2017, the U.S. FDA approved the CAR-T cell therapy Novartis' Kymriah for treatment of ALL. The therapeutic CAR-T cells are created from a patient's own cells and reprogrammed to detect CD19, which target and destroy the B cells (Liu et al., 2017). Bone marrow transplant is often suggested for relapsed patients (Nagler et al., 2020).

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is caused by the monoclonal expansion and progressive accumulation of mature-appearing neoplastic B lymphocytes (Table 1.1). These have a distinct immune phenotype characterized by expression of B cell markers such as CD19 and CD20, which are not usually expressed on non-malignant B cells (Hallek et al., 2018a). CLL is the most common leukemia overall with a median age at diagnosis of 71 (Woyach, 2018). Many patients experience an indolent disease course and never require therapy, but some patients, especially the very elderly and those with medical comorbidities, will die from their disease (Strati et al., 2017).

Risk factors

A limited number of risk factors for CLL have been identified. Having a relative with CLL has been found to contribute to disease risk, indicating that inherited predisposition is a risk

factor (Cerhan and Slager, 2015). Reduced sun exposure, allergic immune dysfunction, exposure to herbicides and pesticides, and hepatitis C virus infection can also be associated with an increased risk (Slager et al., 2014).

Pathophysiology

Genetic alterations including chromosomal alterations, somatic mutations, and alterations in miRNA have been found to be associated with the development of CLL by promoting the proliferation of B lymphocytes (Calin et al., 2005). These CLL cells follow chemokine gradients into lymph nodes and form proliferation centers in which they are exposed to chemokines, integrins, and cytokines from surrounding cells (Hallek et al., 2018a). CLL cells usually display an unusual but characteristic pattern of molecules, including surface antigen CD5, B-cell antigens CD19, CD20, and CD23. In addition, all the CLL cells within one individual are clonal and genetically identical (Hallek et al., 2018b).

Signs and symptoms

Patients with CLL usually don't have any symptoms for at least a few years since the lymphocytes grow and spread very slow. The most common presentation of CLL is the incidental discovery of lymphocytosis on a complete blood count. Then as the B cells infiltrate the bone marrow, patients develop more symptoms such as lymphadenopathy, B symptoms (fever, night sweats, weight loss, fatigue) or cytopenia (anemia, thrombocytopenia, neutropenia) (Hallek et al., 2018b).

Treatment

Since CLL progresses slowly in most cases, it is not treated until the onset of symptoms. Current treatment for CLL has changed dramatically over the last 10 years with first the addition of monoclonal antibodies to chemotherapy, and subsequently the introduction of targeted therapies for this disease (Woyach, 2018). Current targets drugs used for CLL include Bcl-2 inhibitor venetoclax, CD20 antibody rituximab, and CD52 antibody alemtuzumab. Risk-based individualized therapy is now possible, ranging from chemoimmunotherapy for low-risk CLL to novel molecularly targeted therapy primarily with BTK inhibitors such as ibrutinib for high-risk disease (Burger and O'Brien, 2018). For patients who are too frail to be considered candidates for the novel agents, allogenic stem cell transplant has been considered the treatment choice of these patients and the only approach offered with curative intent in the disease (Gribben, 2018).

Multiple myeloma

Multiple myeloma (MM) is a neoplastic plasma-cells disorder, which is characterized by clonal proliferation of malignant plasma cells in the bone marrow, secreted monoclonal protein in the blood or urine, and associated organ dysfunction (Palumbo, 2011) (Table 1.1). It is preceded by monoclonal gammopathy of uncertain significance that progresses to smoldering myeloma and, finally, to symptomatic myeloma (Kuehl and Bergsagel, 2002). MM accounts for 13% of hematological cancers. The median age at diagnosis 69 years and the five-year survival rate is 53.9% in the US (Padala et al., 2021)**.**

Risk factors

A few risk factors could affect someone's chance of getting MM. Someone who has a sibling or a parent with MM and has African ancestry is more likely to get MM than someone who does not. Actually, several susceptibility regions have been identified in MM (Koura and Langston, 2013). Being overweight or obese also increases a person's risk of developing myeloma (Roberts et al., 2010).

Pathophysiology

MM is characterized by the proliferation of plasma cells, which are differentiated from B cells. B cells are a type of white blood cell of the lymphocyte subtype. They originate from the bone marrow and migrate into the spleen to complete development. They are then activated in the secondary lymphoid organs such as the spleen and lymph nodes by receiving a constant supply of T cell-dependent antigens through circulating lymph. Most of these B cells become plasma cells and begin producing and secreting large volumes of antibodies. In MM, genetic or epigenetic mutations happen in a plasma cell, which establishes an abnormal clone of bone marrow plasma cells. If left uncontrolled, the disease progresses from monoclonal gammopathy of undetermined significance to smoldering multiple myeloma, multiple myeloma, and eventually to plasma cell leukemia (Dutta et al., 2017).

Signs and symptoms

Presenting symptoms of MM are vague and nonspecific. The most common clinical manifestations of MM are anemia, infections, bone disease, and renal failure. Back pain, weight

loss, or abnormal lab results are indications for the presence of MM, especially in older patients (Goldschmidt et al., 2016).

Treatment

Different types of treatment may be combined at the same time or used after one another depending on the stage of the cancer. If the disease is in an early stage like smoldering multiple myeloma, no immediate treatment is needed as patients can do well for years. When patients develop end-organ damage, it is the indication for treatment (Röllig et al., 2015). Patients are often given a combination of 2 or 3 drugs containing bortezomib, lenalidomide, or dexamethasone. The drugs chosen depend on the patient's health including their kidney function, and whether a stem cell transplant is planned. Some patients are given additional cycles of treatment after transplant. This is called consolidation treatment and increases the chance of a complete response (Mohty et al., 2015). Supportive treatments such as blood transfusions may also be used as part of the treatment plan.

Hodgkin lymphoma

Lymphoma is cancer that begins in cells of the lymph system. Hodgkin lymphoma (HL) originates from a specific type of lymphocyte and accounts for about 10% of all lymphomas (Table 1.1). In 1832, Thomas Hodgkin described a case series of enlarged lymph nodes, later to be known as HL (Cirillo et al., 2019). There are two main types of HL: classical HL (cHL), which accounts for 90% of all cases; and nodular lymphocyte predominant HL (NLPHL), which accounts for less than 10%. HL is most common in two different age groups: young adults between 15 to 35 years old and older adults over age 50 (Thyss et al., 2014).

Risk factors

The etiology of HL is not well understood. People who have been infected with Epstein–Barr virus or human immunodeficiency virus are at greater risk (Shanbhag and Ambinder, 2018). The incidence of HL also increases after solid organ transplantation and in patients with a history of autoimmune conditions, such as rheumatoid arthritis, systemic lupus erythematosus, and sarcoidosis (Landgren et al., 2006).

Pathophysiology

In around 1900, Dorothy Reed and Carl Sternberg characterized the abnormal mononucleated Hodgkin and multinucleated Reed-Sternberg cells that are pathognomonic for the disease (Küppers, 2009). HL results from the clonal transformation of B cells, giving rise to pathognomonic Reed-Sternberg cells in cHL or popcorn cells in NLPHL. Reed–Sternberg cells are usually CD30 and CD15 positive, but negative for CD20 and CD45 (Cirillo et al., 2019). The popcorn cells in NLPHL are variants of Reed-Sternberg cells and have a popcorn-like appearance. Unlike classic Reed-Sternberg cells, these cells are CD15 and CD30 negative while positive for the B cell marker CD20 (Saini et al., 2011).

Signs and symptoms

The most common symptom of HL is the painless enlargement of cervical or axillary lymph nodes (Ansell, 2015). As the disease spreads through internal lymph nodes, liver, or bone marrow, systematic symptoms (B symptoms) such as fever, night sweats, and weight loss develop. Other possible symptoms include itching skin, fatigue, and loss of appetite (Shanbhag and Ambinder, 2018).

Treatment

HL is considered a prime example of treatment success with more than 80% cure rates using modern combined modality therapies (Mottok and Steidl, 2018). The exact choice of treatment depends on the precise stage of the disease. Patients in early stages are effectively treated with combination chemotherapy followed by involved-field radiation therapy. Patients in advanced-stage disease are often offered chemotherapy together with radiation therapy either for a longer duration or of a different type. For patients who are not cured with the first-line therapy, multiple second-line therapies such as immunotherapy or stem cell transplant are considered acceptable (Ansell, 2015).

Non-Hodgkin lymphoma

Non-Hodgkin lymphoma (NHL) is a diverse group of blood cancers that includes all types of lymphomas except HL (Table 1.1). It represents a wide spectrum of illnesses that vary from the most indolent to the most aggressive types. They arise from lymphocytes that are at various stages of development, and the characteristics of the specific disease subtype reflect those of the cell from which they originate (Armitage et al., 2017). NHL is the most common hematologic malignancy and the fifth most common type of cancer in more developed regions of the world (Morton et al., 2014). It represents a wide spectrum of illnesses that vary from the most indolent to the most aggressive malignancies (Armitage et al., 2017).

Risk factors

Various factors have been found to affect an individual's risk of developing NHL. Some of these factors are common while some of them are subtype specific (Morton et al., 2014).

Common risk factors include family history of NHL, recreational sun exposure, hay fever, allergy, and socioeconomic status (Morton et al., 2014). Take immunosuppressant drugs or having any medical conditions that weakens the immune system, such as autoimmune diseases, HIV infection, or undergoing organ transplantation is also related to higher risk of getting NHL (Shiels et al., 2013; Zintzaras et al., 2005). Obesity is a risk factor for diffuse large B-cell lymphoma (Castillo et al., 2014). The Epstein-Barr virus is closely associated with both Burkitt lymphoma and nasal NK–T-cell lymphoma (Kwong, 2005; Saha and Robertson, 2011). Hepatitis C virus has been associated with splenic marginal zone lymphoma and diffuse large B-cell lymphoma (Giordano et al., 2007).

Pathophysiology

NHL is a cancer with a number of subgroups. It arises from the clonal transformation of lymphocytes. B cell lymphomas account for more than >85% of cases of NHL, while the others are derived from T cells or natural killer cells (Chaudhari et al., 2019). The classification of NHL is complex and ever evolving, reflecting new insights into the cells of origin and the biologic bases of these heterogeneous diseases. Currently, there are more than 50 different subtypes listed in the latest World Health Organization classification (Swerdlow et al., 2016). For non-specialists, NHL is most usefully categorized as low grade (indolent) or high grade (aggressive) lymphoma. Follicular lymphoma and diffuse large B cell lymphomas are the most common indolent and aggressive subtypes, respectively (Chaudhari et al., 2019).

Signs and symptoms

The presenting features of NHL are diverse. Patients may be entirely asymptomatic. The most common symptom of NHL is the painless enlargement of cervical or axillary nodes (Al-Naeeb et al., 2018). Patients may later have systemic symptoms such as fevers, drenching night sweats, weight loss, pruritis, and fatigue. Since NHL can involve any organ in the body, a myriad of presentations are possible, mimicking a wide range of other conditions (Armitage et al., 2017).

Treatment

The principles of treatment differ for high-grade and low-grade lymphoma. For patients with low grade lymphomas and no significant signs or symptoms, a "watch and wait" approach can be used (Al-Naeeb et al., 2018). High grade NHL may progress rapidly and requires urgent treatment. Standard treatment includes radiation therapy, chemotherapy, immunotherapy, targeted therapy, or stem cell transplant. Patients who fail to respond to first line chemotherapy or who relapse after chemoimmunotherapy may consider clinical trials as they are the only way to get state-of-the-art cancer treatment (Chiappella et al., 2017).

1.3. Epigenetics and Sphingolipid Metabolism in Health and Disease[2]

Abstract

Sphingolipids represent one of the major classes of bioactive lipids. Studies of sphingolipids have intensified in the past several years, revealing their roles in nearly all cell biological processes. In addition, epigenetic regulation has gained substantial interest due to its role in controlling gene expression and activity without changing the genetic code. In this review, we first introduce a brief background on sphingolipid biology, highlighting its role in pathophysiology. We then illustrate the concept of epigenetic regulation, focusing on how it affects the metabolism of sphingolipids. We further discuss the roles of bioactive sphingolipids as epigenetic regulators themselves. Overall, a better understanding of the relationship between epigenetics and sphingolipid metabolism may help to improve the development of sphingolipid-targeted therapeutics.

Sphingolipid biology

Sphingolipids are a class of bioactive lipids that contain a sphingoid base backbone, they are a class of aliphatic amino alcohols that include sphingosine and are found in all cellular and subcellular membranes (Borodzicz et al., 2015). There are variety of sphingolipids including ceramides and dihydroceramides, phytoceramides, sphingosine and dihydrosphingosine (sphinganine), various phosphorylated sphingolipids, sphingomyelins, glycosphingolipids, gangliosides, cerebrosides, and sulfatides. Sphingolipids can be formed by *de novo* synthesis or

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generated through salvage pathways from existing sphingolipids via various catabolic enzyme activities (Figure 1.3). Ceramide can be deacetylated to form sphingosine, and subsequently phosphorylated to form sphingosine-1-phosphate (S1P). Ceramide can also be glycosylated to form glycosphingolipids or acquire a phosphocholine group to form sphingomyelin. The synthesis and degradation of sphingolipids are finely tuned to maintain cellular homeostasis. On the cell surface, they can function as adhesion sites for extracellular proteins and serve both as structural lipids and signaling molecules (Iqbal et al., 2017). In 1996, the "sphingolipid rheostat" model was proposed. It postulated that ceramide and S1P differentially regulate cellular signaling pathways involved in cellular death and proliferation/survival, respectively. Factors including growth factors, cellular stress and inflammatory mediators may alter the balance between ceramide and S1P to control cell fate (Evangelisti et al., 2016).

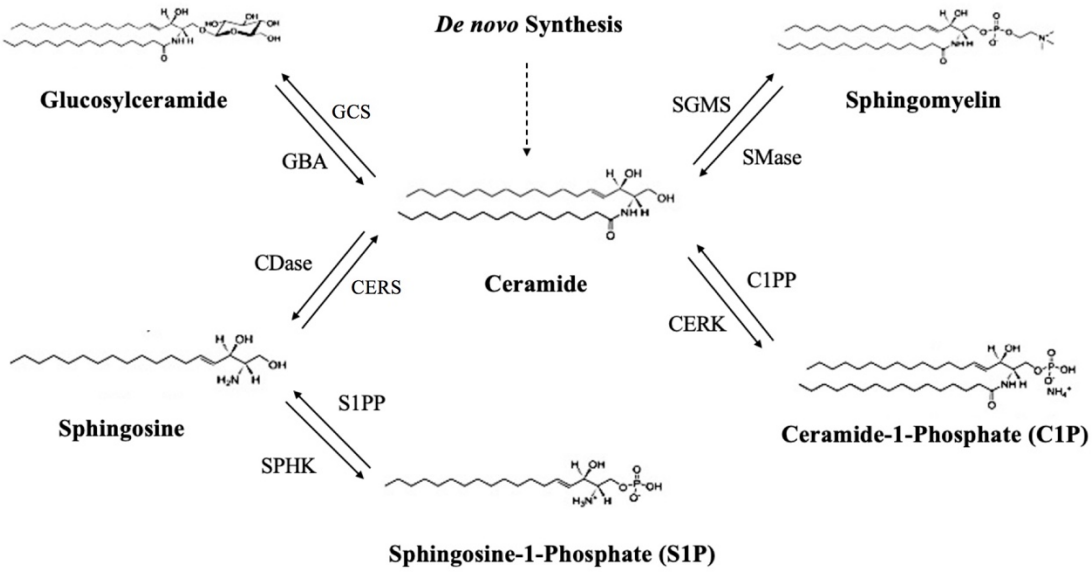


Figure 1.3. Ceramide is at a hypothetical center of sphingolipid metabolism. De novo ceramide synthesis initially generates dihydro-sphingoid species prior to desaturation. Salvage ceramide synthesis, or turnover by the successive activities of acidic/lysosomal-localized catabolic enzymes, generates sphingosine that escapes the lysosome to be re-acylated to ceramide. Glucosylceramide synthase (GCS); cerebrosidase (GBA); sphingomyelin synthase (SGMS); ceramide kinase (CERK), ceramide-1-phosphate phosphatase (C1PP); ceramidases (CDase); ceramide synthases (CERS); sphingosine kinases (SPHK); S1P phosphatase (S1PP).

Pathophysiological roles of sphingolipids

Structurally diverse sphingolipids are bioactive and have roles in multiple biological processes. which include cell growth, cell death, cell differentiation, inflammation, apoptosis and angiogenesis (Hannun and Obeid, 2018). Consequently, abnormalities in sphingolipid homeostasis can lead to or contribute to aberrant pathophysiological processes such as metabolic disorders, cancer, inflammation, neurological syndromes and cardiovascular disease (Schneider-Schaulies and Schneider-Schaulies, 2015).

Metabolic disorders

Sphingolipids influence glucose metabolism in a variety of tissues. Changes in sphingolipid levels have consequential pathophysiological effects in metabolic syndromes. It has been observed that diabetic patients have elevated plasma ceramide levels (Borodzicz et al., 2015). Furthermore, increased levels of ceramide have been shown to result in attenuation of insulin action, most likely via inhibition of AKT (Hannun and Obeid, 2018; Hla and Dannenberg, 2012). In a similar manner, other sphingolipids, including sphingomyelin, glucosylceramide, and other glycosphingolipids, have been shown to inhibit insulin action (Aerts et al., 2011; Chavez et al., 2014). On the contrary, extracellular S1P carried by high-density lipoprotein can increase insulin levels by protecting pancreatic islet cells from apoptosis (Rütti et al., 2009).

Cancer

Sphingolipids have a strong influence in the development and progression of cancer and have gained attention for their roles in cell death, proliferation, and multi-drug resistance.

Ceramide can function as a tumor suppressor lipid by potentiating signaling events that drive apoptosis, autophagic responses and cell cycle arrest (Morad and Cabot, 2013). Total plasma ceramide has been shown to be a promising biomarker of metastatic tumor response to radiation therapy (Dubois et al., 2016). However, defects in ceramide generation and metabolism in cancer cells have been shown to contribute to tumor cell survival and resistance to chemotherapy likely by neutralizing ceramide or generating pro-survival and mitogenic sphingolipids such as S1P (Morad and Cabot, 2013). In addition, increased S1P signaling in endothelial cells has been associated with induction of angiogenesis and inflammatory pathways, thereby contributing to tumor invasion and metastasis (Hannun and Obeid, 2018; Hla and Dannenberg, 2012).

Inflammation

During inflammation, immune cells are recruited to sites of infection or injury and activate cytokine networks to protect the host. However, unchecked inflammation can lead to numerous pathophysiological states such as autoimmune disorders. It has been demonstrated that circulating S1P is a key regulator of lymphocyte egress (Cyster and Schwab, 2012). S1P is enriched in lymph and blood compared to interstitial fluids. What's more, the majority (65%) of plasma S1P is complexed with Apolipoprotein M (ApoM) (Blaho et al., 2015). ApoM–S1P is dispensable for lymphocyte trafficking yet restrains lymphopoiesis by activating the S1P1 receptor on bone marrow lymphocyte progenitors. Thus, the signaling axis of ApoM–S1P–S1P1 regulates the lymphocyte emigration from the graining lymph node and adaptive immune responses (Blaho et al., 2015). High levels of S1P have also been found in patients with rheumatoid arthritis and with ulcerative colitis (Kitano et al., 2006; Maceyka and Spiegel, 2014).

Altogether, these roles for S1P signaling in inflammatory and immune disease has led to clinical trials and FDA-approval of various S1P receptor modulators (Maceyka and Spiegel, 2014).

Neurological disease

In humans, sphingolipid concentrations are highest in the nervous system where their dysfunction contributes to neurological diseases. Key sphingolipid species are critical for the normal development and function of brain. Gangliosides, which are heavily glycosylated sphingolipids, are major components of neuronal membranes and account for 10–12% of the lipid content in the nervous system (Posse de Chaves and Sipione, 2010). There is evidence that gangliosides can contribute to the initiation and progression of Alzheimer's disease by facilitating plaque formation (Olsen and Færgeman, 2017). Conversely, defective ganglioside biosynthesis is associated with the development of epilepsy through a loss-of-function mutation in the monosialodihexosylganglioside 3 (GM3) synthase gene (Fragaki et al., 2013).

Cardiovascular disease

Recent data suggests that specific ceramide species are linked to cardiovascular disease (Chen et al., 2011). Higher plasma levels of sphingomyelin have been associated with increased atherosclerosis and have been proposed as independent risk factors for coronary heart disease in humans (Jiang et al., 2000). Similarly, plasma ceramides have been linked to atherogenesis and can predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes (Laaksonen et al., 2016). These types of findings have led to the idea that inhibition of ceramide *de novo* synthesis could serve as a post-ischemic strategy to reduce myocardial reperfusion injury (Reforgiato et al., 2016). In contrast, plasma S1P is believed to be

cardioprotective. Low S1P levels have been associated with impaired cell signaling and ischemic heart disease, although the exact relation between the two has not been conclusively addressed (Polzin et al., 2017).

Epigenetic regulation

The human genome is an expanse of genomic loci that contain protein-coding genes and their regulatory elements, such as promotors and enhancers. Genes are only actively expressed when they are accessible to regulatory factors and transcriptional machinery, or they are transcriptionally suppressed within compact and inaccessible structures (Mann et al., 2007). Epigenetics are inherited traits that are functionally relevant changes to the genome which cannot be identified by changes in the nucleotide sequence (Figure 1.4). Covalent modifications to histones and DNA without altering the DNA nucleotide sequence facilitate how cells maintain "memories" for gene expression patterns (Ronnekleiv-Kelly et al., 2017). These changes can be maintained through multiple cell divisions or may persist for multiple generations as non-genetic factors leading to permanent changes in gene expression (McCarrey, 2015). Epigenetic regulation of gene expression is essential for normal development and cellular function. Changes in chromatin configuration may cause a wide range of diseases by altering the activity of specific genes (Brookes and Shi, 2014). Currently, three major mechanisms are thought to be involved in the epigenetic regulation of gene expression patterns: DNA methylation, histone modification and non-coding RNAs.

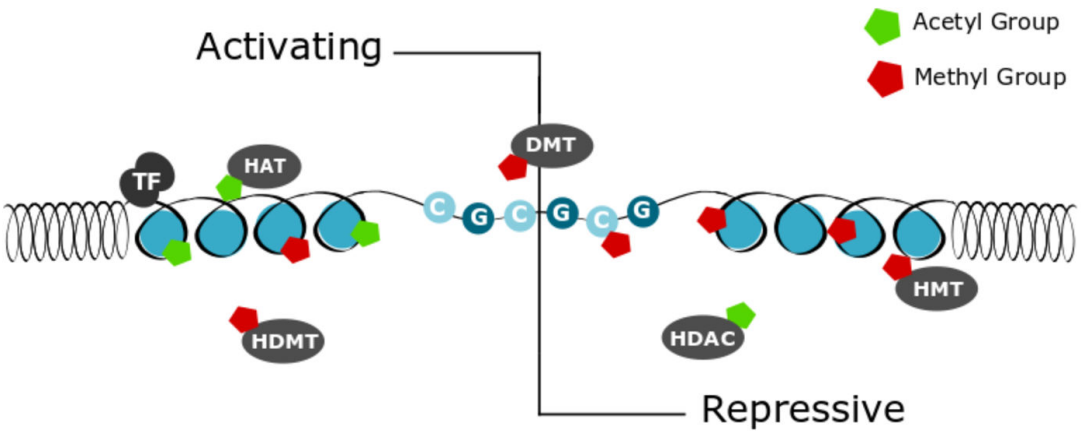


Figure 1.4. Epigenetic regulation is a dynamic process that occurs above the level of traditional base pair alterations. Common modifications include acetylation, methylation, phosphorylation, and ubiquitination. Chromatin remodeling events can be either activating or repressive and occur at the histone level. Common activating events at the chromatin level include acetylation of histone tails by histone acetyl transferases (HATs) and removal of methyl groups by histone demethyltransferases (HDMTs). However, addition of methyl groups by histone methyl transferases (HMT) can also be activating. These modifications produce an open chromatin state, in which transcription factors can more easily access the DNA and facilitate gene expression. Common repressive events at the histone level include removal of acetyl groups by histone deacetylases (HDACs) and addition of methyl groups by HMTs. Epigenetic control can also be exhibited by direct modifications to nucleotides in certain sequences, such as CpG islands. Addition of methyl groups by methyltransferases is a repressive mark and reversal of methylation by demethyltransferases (DMTs) is frequently activating.

DNA methylation

DNA methylation refers to the addition of a methyl group to cytosine at the 5′-position, converting it to 5-methylcytosine. DNA methylation is mainly catalyzed by three DNA methyltransferases (DNMTs): DNMT3A and DNMT3B primarily establish new sites of methylation whereas DNMT1 maintains existing methylation patterns (Ahuja et al., 2016). Cytosine methylation within DNA sequences that serve as recognition sites for transcription factors (TFs) directly can interfere with TF binding or recruit transcriptional repressor complexes, both of which can repress transcription of genes. Most of our DNA is heavily methylated, thus DNA methylation is critical for silencing of transcription and resulting in changes to numerous cellular processes (Azad et al., 2013). Abnormalities in DNA methylation play a role in pathological processes such as carcinogenesis and oncogenesis. As a result, DNMT inhibitors are being explored as potential cancer therapies. These inhibitors are cytidine analogues and work by incorporating into replicating DNA, covalently binding to the catalytic sites, and inhibiting the enzymatic activities of DNMTs (Azad et al., 2013). To date, two DNMT inhibitors — azacitidine (also known as 5-azacytidine) and decitabine (also known as 5-aza-2′-deoxycytidine), have been approved by the FDA for the clinical treatment of myelodysplastic syndrome (Pfister and Ashworth, 2017).

Histone modification

Histones are the chief protein components of chromatin that act as spools for DNA to wind around. Histones affect gene transcription by changing the accessibility of chromatin to transcription factors and gene expression machinery. They are subject to a wide range of modifications, including methylation, acetylation, phosphorylation, and ubiquitination (Tan et

al., 2011). Most studies regarding transcriptional changes with respect to histone modification have focused on acetylation and methylation (Tough et al., 2016). Histone acetylation primarily occurs at lysine residues H3K18 and H3K27. The acetylation alters the net charge of histone and weakens its interaction with DNA leading to a more open chromatin state. The enzymes responsible for this type of modification are histone acetylases (HATs) and antagonized by histone deacetylases (HDACs). Overexpression of HDACs is observed in many cancer types, including prostate, gastric, and endometrial cancers (Tough et al., 2016). Currently, three HDAC inhibitors are approved by the FDA: vorinostat (also known as SAHA) for the treatment of cutaneous T cell lymphoma, belinostat for the treatment of peripheral T cell lymphoma, and romidepsin for the treatment of both cutaneous and peripheral T-cell lymphoma (Campbell and Thomas, 2017; Smolewski and Robak, 2017).

Histone methylation occurs on both lysine and arginine residues. Methylation of residue H3K4 acts as a gene enhancer whereas methylation of residues H3K9 or H3K27 represses gene promoters. Methylation of histones are catalyzed by histone methyltransferases and are removed by histone demethylases. Specifically, methylation of H3K27 is regulated by Polycomb-group proteins (PcGs), which are important in many aspects of development like homeotic gene regulation and X chromosome inactivation (Kassis et al., 2017; Maclary et al., 2017). The catalytic enzyme in the PcG, EZH2, is upregulated in numerous cancers. Currently, inhibitors of several histone methyltransferases are being tested in clinical trials, such as inhibitors of EZH2 and DOT1L, although no drugs have been approved in clinical use (Hock, 2012).

Non-coding RNAs

Non-coding RNA transcripts, RNA transcripts that do not code for translated proteins, that include microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), circular RNAs (circRNAs), and long noncoding RNAs (lncRNAs), have a profound epigenetic influence on transcriptional output of the genome (Wang et al., 2013b). Approximately 50% of miRNA genes are repressed by epigenetic methylation of their own promoter while other miRNAs are derived from spliced intronic sequences of coding mRNA (Wang et al., 2013b). After being processed into mature miRNA and incorporated into the RNA-induced silencing complex (RISC) miRNAs bind to the 3′-UTR of target mRNAs for degradation. A single miRNA is capable of binding and degrading multiple mRNAs (Bamezai et al., 2012). Like miRNAs, piRNA associate with the RISC complex and function as gene silencing mechanism, especially silencing of transposons. Originally, piRNAs were thought to be expressed exclusively in germline tissue and only play a biological role in development. Recently evidence has implicated piRNA to be a functional epigenetic modifier in both germ and somatic stem cell self-renewal as well as tumorigenesis (Bamezai et al., 2012). Conversely, ciRNA antagonize gene silencing by acting as miRNA "sponges "or a decoy to miRNA normal target genes and play a key role in disease progression, especially cancer (Di Ruscio et al., 2013). Lastly, lncRNA have the most dynamic range of epigenetic regulatory mechanisms that have been characterized to date. Evidence has demonstrated that lncRNAs have functional roles in chromatin remodeling, X-chromosome inactivation, RNA splicing, genomic imprinting and DNA methylation (Ahuja et al., 2016; Di Ruscio et al., 2013; Lee and Bartolomei, 2013; Mercer et al., 2009; Rinn et al., 2007).

Epigenetic regulators of sphingolipid metabolism

Beyond being lipid components of cellular membranes, sphingolipids mediate signaling functions in both physiological and pathophysiological processes. Multiple factors including but not limited to development, environmental chemicals, drugs and diet affect sphingolipid homeostasis through epigenetic mechanisms (McCarrey, 2015; Wegner et al., 2016).

Development

Gangliosides are a class of glycosylated sphingolipids with one or more sialic acids bound to a carbohydrate and are particularly abundant in the nervous system (Yu et al., 2009). During brain development there is a pattern shift of gangliosides from simple to complex species that is mainly regulated by the stage expression of genes that encode the glycosyltrasferase enzymes (Tsai and Yu, 2014). A study found that the developmental alteration of gangliosides is regulated through histone acetylation of the glycosyltransferase enzymes in the mouse brain. Furthermore, HDAC inhibitors can effectively change the expression patterns of gangliosides and glycosyltransferases in neuroepithelial cells (Suzuki et al., 2011).

Environmental factors

Hypoxia occurs from stress at high altitude or an otherwise oxygen-depleted environment. In tissues and cells, hypoxia can occur where blood flow is limited due to various physiological conditions (Salminen et al., 2016). During hypoxia, hypoxia-inducible transcription factor-1α (HIF-1α) is expressed to boost energy metabolism and autophagy. Recent studies found that hypoxia induces the expression of HIF-1α through increased activity of sphingosine kinase (SPHK). This effect can be blocked by administration of a SPHK inhibitor or

S1P receptor antagonists (Sanagawa et al., 2016). Stabilization of HIF-1α further increases the expression of distinct histone lysine demethylases to promote gene expression (Salminen et al., 2016). Thus, sphingolipid metabolism is closely intertwined with epigenetic changes in chromatin during hypoxia. Similarly, cigarette smoke has long been proven to induce epigenetic modifications such as aberrant methylation (Zöchbauer-Müller et al., 2003). It is also the major cause of chronic obstructive pulmonary disease (COPD) where alveolar macrophages are defective in their ability to phagocytose apoptotic cells (Barnawi et al., 2017). A study identified that cigarette smoke extract significantly increased expression of S1P receptor 5 (S1PR5). In alveolar macrophages from COPD patients, the expression levels of S1PR5 is significantly increased and is correlated with defective efferocytosis (Barnawi et al., 2015). They further found that this dysregulation of S1P signaling system is caused by reduced DNA methylation of the S1PR5 gene. In this way, decreased promoter methylation released gene transcription of S1PR5, which is essential for S1P-mediated clearance of apoptotic cells (Barnawi et al., 2015).

Drugs

Neutral sphingomyelinase 2 (nSMase2) is a key ceramide-producing enzyme in cellular stress responses. Tretinoin, also known as all-trans retinoic acid (ATRA), is medication used for the treatment of acute promyelocytic leukemia (APL) (Abaza et al., 2017). Although the mechanism of action is still unknown, studies have demonstrated that tretinoin encourages progenitor cells in APL patients to differentiate terminally and thereby ameliorating the progression of the disease (Nitto and Sawaki, 2014). A recent study identified nSMase2 as an early ATRA-induced gene and implicated nSMase2 having a role in growth arrest after ATRA treatment (Clarke et al., 2016). Furthermore, it was found that ATRA regulates nSMase2 directly

through modulation of histone acetylation in a CREB-binding protein and p300 dependent manner (Clarke et al., 2016).

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can be used to improve tissue repair. Current MSC therapies are often primed with bioactive lipids such as S1P and ceramide-1-phosphate, which can enhance in vivo engraftment and survival of transplanted cells (Ratajczak et al., 2010). A recent study found that the HDAC inhibitor valproic acid (VPA) enforced the priming effect of S1P at a low dosage in human umbilical cord derived MSCs through the activation of the MAPK and AKT signaling pathways (Kim et al., 2013). This study indicated that the combinatory effects of epigenetic modulators and S1P-priming strategies can improve the therapeutic potency of stem cells. Similarly, VPA has been found to induce the generation of glycosphingolipid GM3, a known suppressor of epidermal growth factor receptor phosphorylation to suppress cancer cell proliferation (Kawashima et al., 2016). Additionally, another HDAC inhibitor, AR-42, has been shown to exert an anti-colon cancer activity through a mechanism of ceramide production (Xu et al., 2015).

Toxins

Fumonisin B1 (FB1) is a Fusarium mycotoxin frequently occurring in maize-based food and feed. Importantly, FB1 is a well-recognized inhibitor of *de novo* ceramide synthesis due to its ability to inhibit (dihydro)ceramide synthase activity. Intriguingly, FB1 may lead to liver tumorigenesis by disrupting DNA methylation and chromatin modifications which causes chromatin instability in HepG2 cells (Chuturgoon et al., 2014; Demirel et al., 2015).

Sphingolipids as epigenetic regulators

As mentioned, bioactive sphingolipids serve both as structural lipids and signaling molecules. Aside from prominent roles associated with being located on the plasma membrane, sphingolipids and their metabolizing enzymes are also found in the nucleus and associated with the nuclear matrix and chromatin. Their metabolism in the nucleus has been linked to remodeling of chromatin and epigenetic regulation of gene expression (Spiegel et al., 2012).

S1P

S1P was the first identified endogenous nuclear regulator of HDACs. In human MCF-7 breast cancer cells, S1P in the nucleus was found to specifically bind to the HDAC1 and HDAC2 and inhibited their enzymatic activity (Hait et al., 2009). Another study in dystrophic mice revealed that expression of S1P increased histone acetylation of specific genes, in which inflammatory genes are downregulated while metabolic genes are upregulated, helping muscle cells maintain energy metabolism (Nguyen-Tran et al., 2014). Additionally, in drosophila of Duchenne muscular dystrophy, S1P was found to increase the capacity of the muscle cell to use fatty acids as an energy source by inhibiting the acetylation of specific histone residues (Nguyen-Tran et al., 2014). Thus, nuclear S1P can be directly linked to epigenetic regulation of gene expression by modulating histone acetylation. In a somewhat contrasting manner, the degradation of S1P by the S1P lyase has also been attributed to the regulation of sepsis-mediated inflammation. This was attributed to S1P degradation products serving as regulators of histone acetylation (Ebenezer et al., 2017).

Ceramide

Inhibitor 2 of protein phosphatase 2A (I2PP2A) is a biological inhibitor of the cellular serine/threonine protein phosphatase PP2A (Dent, 2013; Mukhopadhyay et al., 2013). I2PP2A has been suggested to be oncogenic and has been shown to be overexpressed in many tumor cell types. I2PP2A also has other targets besides PP2A, e.g., DNA exonucleases and the modification of histone acetylation. It has been shown that ceramide can disrupt the association between PP2A and I2PP2A. Additionally, ceramide inhibits I2PP2A's upregulation of c-Myc and its downregulation of histone acetylation in prostate cancer cells, blocking the epigenetic action of I2PP2A (Mukhopadhyay et al., 2013). Conversely, in Madin-Darby canine kidney cells, sphingomyelin-derived ceramide has also been shown to inhibit the deacetylation of microtubules which induces formation of a ciliogenic lipid-protein complex that can sustain primary cilia (He et al., 2012). This may not be directly epigenetic in nature but involves the regulation of a post-translational modification that also can play a role in epigenetics.

Gangliosides

Gangliosides are expressed primarily on the outer leaflet of the plasma membrane of cells in all vertebrates, and are most abundant in the nervous tissues (Tsai et al., 2016). Studies have provided evidence that ganglioside GM1 is associated with gene regulation in neuronal cells through an interaction with active chromatin via acetylated histones (Itokazu et al., 2017). Ganglioside GM1 was found to bind to acetylated histones H3 and H4 on the promoters of the *GalNAcT* and *NeuroD1* genes. This interaction was shown to activate neuronal differentiation in neural stem cells, and was further supported by exogenous supplementation of ganglioside GM1 (Itokazu et al., 2017; Tsai et al., 2016).

Sulfatide

Sulfatide also is a glycosphingolipid. In hepatocellular carcinoma cells, it has been found to suppress *miR-223* expression in association with reduced recruitment of acetylated histone H3 and C/EBPα to the *pre-miR-223* gene promoter. Sulfatide was also shown to stimulate the expression of histone deacetylases HDAC9 and HDAC10, and enhance their recruitment to the *miR-223* gene promoter (Dong et al., 2014).

Concluding thoughts

Sphingolipid metabolism and epigenetic modulation are closely intertwined with one another. Epigenetic regulation is an important regulatory mechanism for controlling the expression pattern of sphingolipids. Conversely, sphingolipids in the nucleus can directly modulate transcriptional activity via epigenetic modulation. An understanding of both processes is critical as they play an integral role in both normal development and pathological processes. A deeper investigation into how sphingolipid metabolism influences epigenetic regulation, and the reciprocal relationship, in the context pathophysiology of disease development can potentially lead to new diagnostics and therapeutic targets (Hannun and Obeid, 2018).

Future studies of the relationships between epigenetics and sphingolipid metabolism should address the following questions: 1) what other sphingolipids can directly influence epigenetic regulation, 2) how can aberrant epigenetic regulation and sphingolipid metabolism contribute to the development of cancer and other diseases, and 3) what are the mechanisms that dictate the interplay between epigenetic and sphingolipid metabolic regulation? Investigation of these questions may yield results that could contribute to the advancement of biomedical

research and the development of the next generation of molecular and sphingolipid-targeted therapies.

1.4. Therapeutic Potential of Ceramides in Cancer Treatment[3]

Abstract

Ceramides are a family of wax-like lipids that fall under the broader category of sphingolipids. A ceramide molecule is composed of a sphingosine side chain linked to a fatty acid via an amide group. Distinct from other sphingolipids, the head of the ceramide sphingosine side group is a simple alcohol rather than a sugar or phosphocholine linkage. The fatty acid chains of ceramide can vary in chain length and degree of saturation. The degree of saturation determines the biological activity of the ceramide (Ventura et al., 2019). Shorthand nomenclature for these backbones include a "d" or "t" to indicate the number of hydroxyl groups followed by the acyl chain length and number of double bonds as seen in fatty acids. For instance, sphingosine can be written as d18:1 (Pruett et al., 2008). Ceramides are highly abundant within the cell membrane of eukaryotic cells and are classically appreciated for their role as structural elements. More recently, ceramides are being appreciated for their biological activity as well. They have been found to participate in a variety of cellular pathways including apoptosis, cell senescence, the cell cycle, and cell differentiation (Holthuis et al., 2001). This review discusses pathways of ceramide synthesis and metabolism, roles of ceramide in different diseases, targeting ceramide metabolism in the treatment of cancer, as well as ceramide-delivering nanotechnologies.

[3] Weiyuan Wang, Bert F. Prince, Alexander J. Thorpe, Brian M. Barth. Therapeutic Potential of Ceramides in Cancer Treatment. In preparation for submission.

Ceramide Metabolism

Ceramide metabolism is a complex network of interconnected pathways with many origins (Figure 1.5). Broadly, ceramide metabolism can be classified into *de novo* synthesis and catabolism, including recycling, salvage, and degradation.

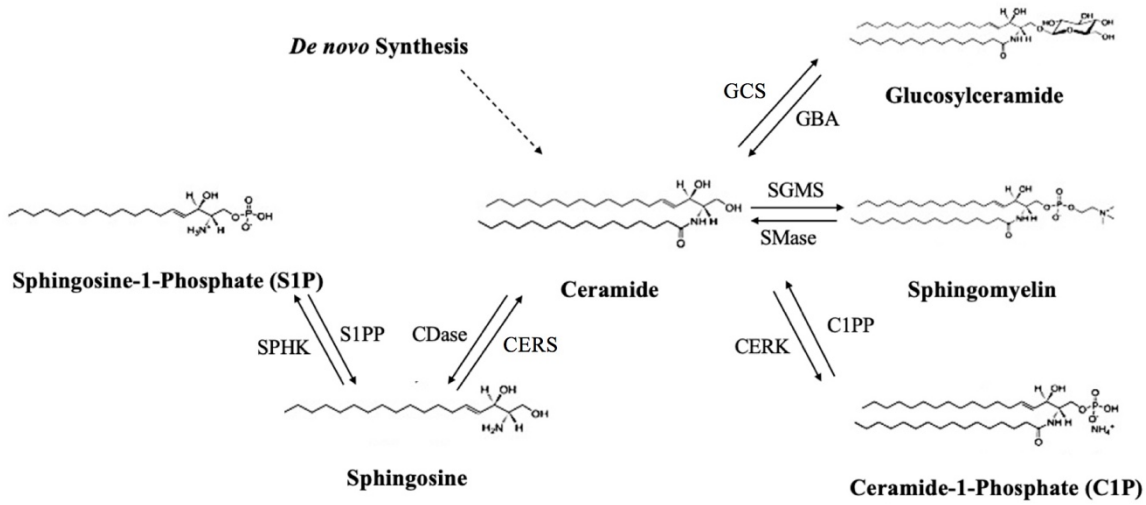


Figure 1.5. Pathway for ceramide synthesis and metabolism. *De novo* ceramide synthesis initially generates dihydrosphingoid species prior to desaturation. Salvage ceramide synthesis, or turnover by the successive activities of acidic/lysosomal-localized catabolic enzymes, generates sphingosine that escapes the lysosome to be re-acylated to ceramide. Glucosylceramide synthase (GCS); cerebrosidase (GBA); sphingomyelin synthase (SGMS); ceramide kinase (CERK), ceramide-1-phosphate phosphatase (C1PP); ceramidases (CDase); ceramide synthase (CERS); sphingosine kinase (SPHK); S1P phosphatase (S1PP).

De novo synthesis

De novo synthesis of ceramide occurs in the endoplasmic reticulum. It starts with the condensation of a serine residue and a palmitoyl-CoA by serine palmitoyltransferase, which produces 3-keto-dihydrosphingosine. This unstable intermediate is rapidly reduced to dihydrosphingosine (also known as sphinganine) by 3-ketosphinganine reductase. The ceramide synthase (CERS) family catalyzes the N-acylation of dihydrosphingosine into dihydroceramide. Desaturase then introduces a 4,5-trans-double bond into the sphinganine backbone of dihydroceramide, producing a complete ceramide molecule.

Six ceramide synthases (CERS1-6) have been identified in humans (Table 1.2). Each CERS enzyme prefers different chain lengths of the fatty acyl-CoA substrate involved in the N-acylation of dihydrosphingosine. The resulting dihydroceramide will contain a fatty acid of varying lengths depending on the CERS enzyme used. The chain lengths of the fatty acyl-CoA used will vary from 14 to 36 carbons. Ceramides with different chain compositions impact cell physiology differently (Pinto et al., 2014). In addition, different ceramide synthases have different tissue distribution patterns. For example, CERS1 is most highly expressed in the brain and skeletal muscle, which correlates with an abundance of C18 ceramide in those tissues. CERS2 is most highly expressed in the liver and kidneys and favors long chain fatty acids (C22:0-24:0) (Wattenberg, 2018). CERS3 is highly expressed in the skin and testis and is highly selective toward very-long (C22:0-C24:0) and ultra-long (more than C26:0) chains. CERS4 is highly expressed in the skin and leukocytes and catalyzes formation of ceramide with a high selectivity towards long and very-long chains (C18:0-C22:0) (Kihara, 2012). CERS5 is more commonly found in the muscle and brain. It prefers short chain fatty acyl-CoA and mainly produces ceramides incorporating C16:0 fatty acyl-CoA (Wattenberg, 2018). CERS6 also uses

C16:0 fatty acyl-CoA as a substrate and is most commonly found in the brain (Kitatani et al., 2008).

Table 1.2. Classification and distribution of ceramide synthases

Ceramide synthase	Chain length of the fatty acyl-CoA substrate	Tissue distribution
CERS1	C18	Brain and skeletal muscle
CERS2	C22-C24	Liver and kidneys
CERS3	C22-C24, > C26	Skin and testis
CERS4	C18-22	Skin, leukocytes
CERS5	C16	Muscle and brain
CERS6	C16	Brain

Salvage, recycling, and degradation

In addition to the *de novo* synthesis, ceramide can be "salvaged" from complex sphingolipid reservoirs, recycled into other sphingolipids, or degraded into simple molecules. These three pathways, alongside with *de novo* synthesis, regulate the cellular levels of ceramide.

The salvage of ceramide from larger sphingolipids provides for more than half of ceramide biosynthesis. One such example is the catabolism of glycosphingolipids. During the breakdown of large glycosphingolipids, sugar groups are cleaved off which leads to the formation of either glucosylceramide or galactosylceramide. In turn, specific β-glucosidases and galactosidases hydrolyze these lipids to form ceramide (Kolter and Sandhoff, 2005). In addition to the formation of ceramide from larger sphingolipids such as glycosphingolipids, ceramide can be formed via the hydrolysis of sphingomyelin by acid sphingomyelinase (aSMase) (Wattenberg, 2018). Ceramide can also act as a substrate to build more complex sphingolipids. For instance, ceramide can be converted into sphingomyelin, a large component of the fatty myelin sheath, via

the action of sphingomyelin synthase (SGMS). Similarly, ceramide can be acted on by glucosylceramide synthase (GCS) to form glucosylceramide. GCS is an enzyme that in humans is encoded by the *UGCG* gene. These new substrates can go on to form more complex sphingolipids in the Golgi apparatus, an example of which would be gangliosides. These products are then transported to the plasma membrane, the major reservoir of these lipids (Hanada et al., 2003; Maceyka and Spiegel, 2014). The degradation of ceramide is also a necessary part of maintaining lipid homeostasis. Ceramides present in endothelial cells are deacylated by acid ceramidase to form sphingosine, the center of sphingolipid metabolism. It eventually results in the hydrolysis of triglycerides and phospholipids and shedding of some of the surface components (Hussain et al., 2012).

Ceramide and Sphingosine-1-phosphate

In addition to ceramide, sphingosine-1-phosphate (S1P) is an important sphingolipid to highlight. Ceramide can be converted into S1P via two enzymatic reactions. First, ceramidase (CDase), typically acid CDase, converts ceramide to sphingosine and then sphingosine kinase (SPHK), typically SPHK1, phosphorylates sphingosine to S1P. Although S1P and ceramide share structural similarities and are easily interconverted, the two exhibits opposite effects. Whereas ceramide is well appreciated for its role in the stimulation of apoptosis, S1P is appreciated as a pro-survival signal, leading to increased cell growth and viability (Ogretmen, 2018). S1P's pro-survival effects are beneficial to tumors and thus an excess of S1P in tissues is associated with tumor neogenesis. S1P signals through G-protein coupled receptors (GPCRs) known as S1P receptors (S1PRs). Activation of S1PRs often leads to subsequent activation of the Raf-Mek-Erk pathway, a classic mitogenic pathway. Thus, activation of these GPCRs leads to

increased tumor cell viability. The control of the titer of ceramide and S1P in tumors is thought to be a potential therapeutic target (Wang et al., 2018).

Roles of ceramides in diseases

Structurally diverse ceramides can provide platforms for signaling events and protein/lipid trafficking along cellular membranes (Bieberich, 2018). Thus, changes in ceramide levels can alter the biophysical properties of the membrane. In this way, ceramide functions as a sensor and affects the distribution of transmembrane proteins and other cellular responses (Ventura et al., 2019). Consequently, abnormalities in ceramide homeostasis can lead to various diseases.

Skin diseases

Skin ceramide composition, which plays crucial roles in maintaining the barrier function of the stratum corneum, is changed in patients with atopic dermatitis (Shen et al., 2018). It is accompanied by a change of ceramide synthase isoform expression from CERS3 to CERS4, leading to a barrier repair response (Peters et al., 2020). Topical treatment with pseudo-ceramide lotion, which contains 3% of Cetyl-PG hydroxyethyl palmitamide, has been found to significantly reduce skin symptoms. Additionally, a significant decrease in trans-epidermal water loss and increased water content has been shown with pseudo-ceramide treatment. Those changes are associated with a distinct switch of the ceramide profile from an atopic dermatitis to a healthy skin phenotype without any increased level of endogenous ceramides (Ishida et al., 2020). Increased ceramide content results in acceleration of epidermal barrier restoration after acute skin damage (Shin et al., 2020).

Metabolic syndrome

Ceramides contribute to lipotoxicity that underlies metabolic syndrome and related diseases such as type 2 diabetes and cardiovascular disease (Fang et al., 2019). The species of ceramide that are associated with cardiovascular disease progression are C16 ceramide (d18:1/16:0), C18 ceramide (d18:1/18:0), and C24:1 ceramide (d18:1/24:1). These ceramides lead to abnormalities in glucose metabolism by blocking insulin signaling. Additionally, these ceramides dysregulate lipid metabolism by promoting adipose inflammation. The C24 ceramide (d18:1/24:0) species has been associated with good cardiovascular health (Poss et al., 2019). It has been found that daily oral treatment with P053, which specifically targets CERS1, promotes skeletal muscle fatty acid oxidation and reduces whole-body fat accumulation without causing insulin resistance (Turner et al., 2018).

Infections and Inflammation

Different properties of structural domains formed by ceramides can cause changes in membrane curvature. These changes in membrane morphology may be exploited by pathogens, leading to easier host cell infection (Prévost et al., 2015). Bile acids and ceramides have been found to allow for the entry of viruses and to facilitate their subsequent replication in jejunal enteroids. Some viral pathogens invade the epithelial barrier and replicate in the human small intestine. Ceramide assists their release into the cytoplasm by acting as an initial binding factor for norovirus strains (Murakami et al., 2020). Conversely, it has also been found that ceramide can mediate a neutrophil extracellular trap formation to boost bactericidal capacity in neutrophils (Corriden et al., 2015).

Cancers

Ceramides of specific chain lengths have been found to be dysregulated in various cancer types, either mediating apoptosis via mitochondrial membrane perturbation and/or influencing cell death signaling or having protective roles against apoptosis (Ogretmen, 2018). The exact effect of ceramide on cancer depends on the specific ceramide structure and the cellular context (Jeffries and Krupenko, 2018). For example, C16 ceramide generation is decreased in human ovarian carcinoma cells (Prinetti et al., 2006). Overexpression of CERS2, which increased the generation of C24 ceramide, protected Hela cells from ionizing radiation (IR)-induced apoptosis (Mesicek et al., 2010). However, C14 ceramide, C16 ceramide, and C18 ceramide levels are significantly increased in breast cancer tissue compared to the normal tissue suggesting a contrast in function of ceramide in different cancers (Moro et al., 2018). Notably, various anticancer therapeutics have been shown to alter the levels of cellular ceramides (Table 1.3).

Table 1.3. Anti-cancer therapeutics that affect ceramide generation

Name of the anti-cancer therapeutics	**Mechanism of action**	**Effects on ceramide expression**	**Cancer types**	**Citations**
Chemotherapy agents				
Sorafenib	Kinase inhibitor against VEGFR, PDGFR and Raf kinases.	Increase CERS1 expression	Acute myeloid leukemia	(Dany et al., 2016)
Quizartinib	Tyrosine kinase inhibitor against FLT3/STK1, CSF1R/FMS, SCFR/KIT, and PDGFRs	Increase CERS1 expression	Acute myeloid leukemia	(Dany et al., 2016)

Table 1.3. Continued

Name of the anti-cancer therapeutics	Mechanism of action	Effects on ceramide expression	Cancer types	Citations
Chemotherapy agents				
Crenolanib	Tyrosine kinase inhibitor against FLT3, PDGFRα and PDGFRβ	Increase CERS1 expression	Acute myeloid leukemia	(Dany et al., 2016)
Imatinib	Tyrosine kinase inhibitor against Bcr-Abl	Increase CERS1 expression	Chronic myelogenous leukemia	(Baran et al., 2007)
Dasatinib	Tyrosine kinase inhibitor against Bcr-Abl	Increase CERS1, CERS5, CERS6 expression	Chronic myelogenous leukemia	(Gencer et al., 2011)
Doxorubicin	Interfering with the function of DNA	Increase CERS1, CERS2, CERS5 expression	Embryonic kidney cells, Bladder cancer, Breast cancer	(Fan et al., 2013; Huang et al., 2018; Min et al., 2007)
Cinobufotalin	Inducing growth inhibition and apoptosis	Increase CERS1 expression	Hepatocellular carcinoma	(Cheng et al., 2015)
Aburs agglutinin	Inactivating Type II ribosome	Increase CERS1 expression	Glioblastoma	(Panda et al., 2018)
Teniposide	Inhibitor of topoisomerase II	Increase CERS1 expression	Glioma	(Wang et al., 2017)
Cisplatin	Interfering with DNA replication	Increase CERS1, CERS6 expression	Embryonic kidney cells, Oral squamous carcinoma	(Li et al., 2018; Min et al., 2007)

Table 1.3. Continued

Name of the anti-cancer therapeutics	Mechanism of action	Effects on ceramide expression	Cancer types	Citations
Chemotherapy agents				
Carboplatin	Interfering with DNA replication	Increase CERS1 expression	Embryonic kidney cells	(Min et al., 2007)
Vincristine	Inhibitor of mitosis at metaphase	Increase CERS1, CERS5 expression	Embryonic kidney cells	(Min et al., 2007)
Oxaliplatin	Inhibiting DNA synthesis	Increase CERS5 expression	Colon cancer	(Brachtendorf et al., 2018)
Etoposide	Inhibiting DNA synthesis	Increase CERS2, CERS3, CERS4 expression	Embryonic fibroblasts	(Siddique et al., 2012)
Fluorouracil (5-FU)	Inhibiting DNA replication	Decrease CERS5 expression	Colon cancer	(Brachtendorf et al., 2018)
Dorsomorphin	Inhibitor of BMP signaling	Increase CERS5 expression	Breast cancer	(Jin et al., 2009)
Methotrexate	Inhibiting the synthesis of DNA, RNA, thymidylate, and protein	Increase CERS6 expression	Liver cancer, Lung cancer	(Fekry et al., 2016)
Daunorubicin	Inhibiting DNA synthesis	Increase CERS expression	Leukemia	(Bose et al., 1995)
Fludarabine	Inhibiting DNA synthesis	Increase CERS2 expression	Chronic lymphocytic leukemia	(Biswal et al., 2000)

Table 1.3. Continued

Name of the anti-cancer therapeutics	Mechanism of action	Effects on ceramide expression	Cancer types	Citations
Chemotherapy agents				
Gemcitabine	Inhibiting DNA synthesis	Increase CERS2 expression	Pancreatic cancer	(Modrak et al., 2009)
Camptothecin	Inhibiting DNA synthesis	Increase *de novo* ceramide synthesis	Follicular thyroid carcinoma	(Rath et al., 2009)
Irinotecan	Inhibiting DNA synthesis	Block formation of glucosyl-ceramide	Colorectal cancer	(Litvak et al., 2003)
Radiation				
UV-B radiation	Damaging DNA	Increase CERS1 expression	Lung cancer	(Sridevi et al., 2009)
Ionizing radiation	Damaging DNA	Increase CERS2, CERS5, CERS6 expression	Cervical cancer	(Mesicek et al., 2010)
Alternative medicine				
Staurosporine	Inhibitor of protein kinases against PKC	Increase CERS2, CERS6 expression	Glioma	(Jensen et al., 2014)
Hormone inhibitors				
Anastrozole	Blocking the production of estrogens	Decrease CERS4, CERS5 expression	Endometrial cancer	(Mojakgomo et al., 2015)

Table 1.3. Continued

Name of the anti-cancer therapeutics	Mechanism of action	Effects on ceramide expression	Cancer types	Citations
Hormone inhibitors				
Tamoxifen	Selective estrogen receptor modulator	Inhibit ceramide glycosylation	Breast cancer	(Wang et al., 2003)
Adjuvant drugs				
Tetrahydrocannabinol (THC)	Partial agonist activity at cannabinoid receptors	Increase CERS5, CERS6 expression	Glioma	(Hernández-Tiedra et al., 2016)
R(+)-methanandamide	Synthetic long-lasting anandamide analog	Increase CERS6 expression	Mantle cell lymphoma	(Gustafsson et al., 2009)
Anti-inflammatory drugs				
Celecoxib	COX-2 inhibitor	Increase CERS6 expression	Colon cancer	(Schiffmann et al., 2010)
Drugs in clinical trials				
Stichoposide D	Inducing apoptosis	Increase CERS6 expression	Leukemia	(Yun et al., 2015)
Cladososide C2	Inducing apoptosis	Increase CERS6 expression	Leukemia	(Yun et al., 2015)
TRAIL(Apo-2L)	Inducing TNF-related apoptosis	Increase CERS6 expression	Colon cancer	(White-Gilbertson et al., 2009)

Table 1.3. Continued

Name of the anti-cancer therapeutics	Mechanism of action	Effects on ceramide expression	Cancer types	Citations
Drugs in clinical trials				
IL-24	Inducing apoptosis and angiogenesis	Increase CERS6 expression	Glioblastoma	(Yacoub et al., 2010)
Resveratrol	Not yet clear	Inhibition of dihydroceramide desaturase	Gastric cancer	(Signorelli et al., 2009)
Fenretinide	Inducing apoptosis	Activate serine palmitoyl transferase	Prostate cancer	(Wang et al., 2003)

Targeting ceramide metabolism for cancer therapy

Considering the known effects of ceramide and its dysregulation in many cancers, developing ceramide-based therapies for cancers is very attractive. Various therapies have aimed to target ceramide metabolism as a method to control apoptosis in cancer cells (Table 1.4). These therapies upregulate endogenous production of ceramides or downregulate ceramide neutralization enzymes. Enzymes that neutralize ceramide convert ceramide to non-ceramide sphingolipids. Examples include SGMS and CDase (Figure 1.5). The goal of these therapies is to raise the intracellular concentration of ceramide to direct cancer cells towards apoptosis. In addition to augmenting endogenous ceramide synthesis via pharmacological interventions, increasing ceramide levels through exogenous analogs has been widely explored in various cancer models. Various analogs or inhibitors of ceramide signaling have also shown efficacy in

clinical trials. Ceramides are not the only sphingolipids that are being explored for their potential to treat cancers. For example, Fingolimod (FTY720), a structural analog of S1P, has been approved for clinical use in the treatment of multiple sclerosis and has also shown efficacies in treatments of cancer (Chun and Hartung, 2010). Mechanistically, Fingolimod suppresses tumor growth via S1PR-dependent or receptor-independent mechanisms in colon and lung cancer, respectively (Liang et al., 2013; Saddoughi et al., 2013). It has also shown potential to treat imatinib-refractory chronic myeloid leukemia by inhibiting stem cell proliferation and expansion *in vitro* (Neviani et al., 2013).

Table 1.4. Synthetic drugs that target ceramide metabolism

Synthetic drugs	**Mechanism of action**	**Effective cancer types**	**Citations**
Analog 315 and 403	Ceramide analog	Primary effusion lymphoma, breast cancer	(Chen et al., 2020)
Analog 406	C8-Ceramide analog	Breast cancer, ovarian cancer	(Ponnapakam et al., 2014)
LCL30	C16-Ceramide analog	Squamous carcinoma	(Separovic et al., 2011)
C16-serinol	C16-Ceramide analog	Neuroblastoma	(Bieberich et al., 2000)
LCL85	C16-Ceramide analog	Colon carcinoma	(Paschall et al., 2014)
LCL29	C6-Ceramide analog	Squamous carcinoma	(Separovic et al., 2011)
LCL85	C16-Ceramide analog	Colon cancer, breast cancer	(Paschall et al., 2014)
LCL124	C6-Ceramide analog	Pancreatic cancer	(Beckham et al., 2013)

Table 1.4. Continued

Synthetic drugs	Mechanism of action	Effective cancer types	Citations
LCL204	Inhibitor of ceramidase	Head and neck squamous cell cancers	(Elojeimy et al., 2007)
D-MAPP, B13	Inhibitors of ceramidase	Colon cancer	(Selzner et al., 2001)
Ceranib-2	Inhibitor of ceramidase	Mammary adenocarcinoma	(Draper et al., 2011)
LCL385	Inhibitor of ceramidase	Prostate tumors	(Mahdy et al., 2009)
LCL521	Inhibitor of ceramidase	Squamous cell carcinoma	(Korbelik et al., 2016)
Fingolimod	Structural analog of S1P	Colon cancer, lung cancer, chronic myeloid leukemia	(Liang et al., 2013; Neviani et al., 2013; Saddoughi et al., 2013)l
SK1-I	Inhibitor of SPHK1	Glioblastoma	(Kapitonov et al., 2009)
PF-543	Inhibitor of S1P	Head and neck carcinoma, colorectal cancer	(Ju et al., 2016; Schnute et al., 2012)
VPC03090	S1PR 1/3 antagonist	4T1 mammary carcinoma	(Kennedy et al., 2011)
AB1	S1PR 2 antagonist	Neuroblastoma	(Li et al., 2015)
Sonepcizumab	S1P antibody	Renal cell carcinoma	(Pal et al., 2017)
ABC294640	Inhibitor of sphingosine kinase 2	Pancreatic cancer, prostate cancer, advanced solid tumors, multiple myeloma, lymphoma	(Britten et al., 2017; Lewis et al., 2016; Qin et al., 2014; Venant et al., 2015; Venkata et al., 2014)

Nanotechnology for ceramide delivery

One of the issues with ceramide-based therapies is the delivery of the drug to appropriate body tissues. Since ceramide is highly hydrophobic, a suitable carrier is needed for its successful delivery without restricting its pharmacological effects. Recently, delivering drugs via nanotechnology has become more appreciated. Nanoparticles, measuring up to 400 nm in size, have demonstrated efficacy for carrying and delivering therapeutic molecules with diverse physiological properties. Several delivery materials have been explored to increase the drug performance (Table 1.5). Importantly, the use of nanoparticles may allow for selective targeting of tumors due to the enhanced permeation retention (EPR) effect. The EPR effect is the propensity of tumor vasculature to be leaky. This is largely due to the disorganized manner in which tumor-mediated neovascularization occurs. Gaps between vascular endothelium provides potential for targeting of anti-cancer therapies to specific tissues. Nanoparticles are small enough to leave the vasculature at points of disorganization and leakiness. In theory, this facet of tumors would allow for specific targeting of cancerous tissues by nanomedicines (Kester et al., 2015). A particularly optimistic facet of nanotechnology usage in ceramide delivery is the potential for non-toxic therapies. One of the largest issues in cancer therapeutics is the toxic nature of many of the drugs used to treat cancer. Unlike many standards of care therapeutics, the C6-ceramide nanoliposome has shown promising results in terms of toxicity. In dog and murine models, the C6-ceramide nanoliposome was minimally toxic (Kester et al., 2015). The use of nanotechnology in drug delivery may lead to a new generation of cancer therapeutics, one in which toxicity is not the norm for standard of care therapies.

Table 1.5. Nanotechnology for ceramide delivery

Delivery materials	Advantages	Citations
C6-Ceramide Nanoliposome	Overcome the lack of solubility in physiological solutions, limited cell permeability and circulating catabolic enzymes	(Stover and Kester, 2003)
Poly(D,L-lactide-co-glycolide) PLGA	Exhibit favorable performances in anticancer drug delivery but insufficient tumor targetability	(Graf et al., 2012)
Polyethyleneglycol-chitosan-ceramide	Have sustained release and higher cellular uptake but lower cytotoxicity	(Battogtokh and Ko, 2014a)
Solid lipid nanoparticles	Provide large surface area and high loading content for hydrophobic drugs, have better stability	(Balakrishnan et al., 2016)
Chitosan-ceramide graft copolymer	Improve the stability and reduce toxicity under physiological conditions	(Battogtokh and Ko, 2014b)

Combined usage of ceramide and other drugs in the treatment of cancer

With the application of nanotechnology, exogenous ceramides are being explored in various cancers. C6-ceramide has been found to prevent metastasis and recurrence of anaplastic thyroid carcinoma cell lines (Fujiwara et al., 2020). The C6-ceramide nanoliposome slows the growth of liver tumors in mice by boosting T-cell activity (Bai and Guo, 2018). In addition to being administered alone, exogenous ceramide can also be incorporated with other drugs into innovative cancer treatments (Moro et al., 2019). The nanoliposome shows potential as a delivery mechanism of such therapies because ceramide can be easily distributed into the hydrophobic membrane of the nanoliposome, and hydrophilic therapies can be placed into the aqueous interior of the nanoliposome. As emerging studies have shown that ceramide can

influence other drugs through nanoparticles, ceramide will probably have more significant clinical application in combination with chemotherapy (Table 1.6).

Table 1.6. Combination of ceramide and other drugs in the treatment of cancer

Combination usage	**Cancer types**	**Citations**
Curcumin + C6-ceramide	Melanoma	(Yu et al., 2010)
Curcumin + C6-ceramide	Osteosarcoma	(Dhule et al., 2014)
Vinblastine + C6-ceramide	Hepatocarcinoma and colorectal cancer	(Adiseshaiah et al., 2013)
Sorafenib + C6-ceramide + C8-ceramide	Melanoma and breast cancer	(Tran et al., 2008)
Safingol + C2-ceramide	Acute myeloid leukemia	(Tan et al., 2014)
Doxorubicin + C6-ceramide	Breast cancer and melanoma	(Fonseca et al., 2014)
Myrisplatin + C6-ceramide	Ovarian cancer	(S et al., 2014)
Paclitaxel + C6-ceramide	Melanoma	(Carvalho et al., 2017)
Gemcitabine + C6-ceramide	Pancreatic cancer	(Jiang et al., 2011)
PDMP + C6-ceramide	Pancreatic cancer	(Jiang et al., 2011)

Conclusions

Sphingolipids, and other lipids as well, have been classically appreciated as being structural elements of cells. While this is a truism, it is not the whole picture. Biologically active

lipids play vital roles in the development and prevention of diseases. This is highlighted through an examination of ceramides, their metabolic pathways, and their demonstrative physiological effects. Ceramides play large roles in many disease processes. One disease that is highly associated with ceramides is cancer. Many cancers experience a downregulation of ceramides and the enzymes responsible for the biosynthesis of ceramides. This is because the presence of large quantities of endogenous ceramides leads to the induction of apoptosis. Interestingly a highly-related sphingolipid, S1P, is highly bioactive but acts to achieve the opposite physiological effect of ceramide, that is an increase in mitogenic and cell survival signaling. Ceramides have antitumor therapeutic potential through their role in the stimulation of both mitochondrial mediated and death-receptor mediated apoptosis. The question of how to exploit this effect of endogenous ceramides is yet to be definitively answered. Some drugs, as mentioned above, have aimed to control the metabolism of ceramide. The result of these therapies is an increased abundance of intracellular ceramide. They do this primarily by downregulating ceramide neutralization enzymes, which are responsible for the conversion of ceramide to non-ceramide sphingolipids. Within this category of therapeutics, therapies that aim to downregulate the activity of sphingosine-1-phosphate, such as AB1 and Sonepcizumab should be considered as well because they act to manipulate sphingolipid metabolism to combat cancers. As S1P's activity yields an opposite physiological effect of ceramide, the antagonism of S1PRs has therapeutic potential. Other drugs have instead focused on introducing exogenous ceramides to cancer tissues. Although the goal of the therapies is similar, these do not involve control of the metabolism of tumor cells. In many ways this is a more direct way of raising the intracellular levels of ceramide, but there are issues that need to be solved before these therapies can be brought to the bedside. Such therapies have had troubles finding effective and targeted methods

to deliver exogenous ceramides. The development of delivery systems such as the nanoliposome provide optimism for the next generation of cancer therapeutics. Through such delivery methods, there is real potential for less toxic and more efficacious therapies such as novel ceramide-based drugs. Although a promising field, ceramide-based therapies have a way to go until they are standard of care. New approaches to delivery will need to be developed to solve existing issues such as drug loading and non-specific tissue targeting. It is hoped that in time these novel ceramide-based therapies will efficiently treat a variety of malignancies.

1.5. Regulation of Growth/Differentiation Factor 1 in Human and Animals

Abstract

Growth/differentiation factor 1 (GDF1) is a protein that in humans is encoded by the *GDF1* gene. It is a member of the transforming growth factor-beta (TGFβ) superfamily, which encompasses a large group of structurally related polypeptides that regulate cell growth and differentiation. GDF1 is mainly found in the brain, spinal cord and peripheral nerves of embryos. It has a role in left-right patterning and mesoderm induction during embryonic development. This review further discusses GDF1's structure, signaling pathway, and function across different species.

Human

Structure

GDF1 encodes a secreted ligand of the TGFβ superfamily of proteins. The GDF1 protein is a 12-15kDa secreted protein. It is synthesized as a preproprotein that contains a 29 amino acid signal peptide, a 224 amino acid propeptide, and a 119 amino acid mature peptide which is released following proteolysis at a dibasic cleavage site (Lee, 1991). It is mainly found in the brain of embryos and is absent from most normal adult tissue (Figure 1.6).

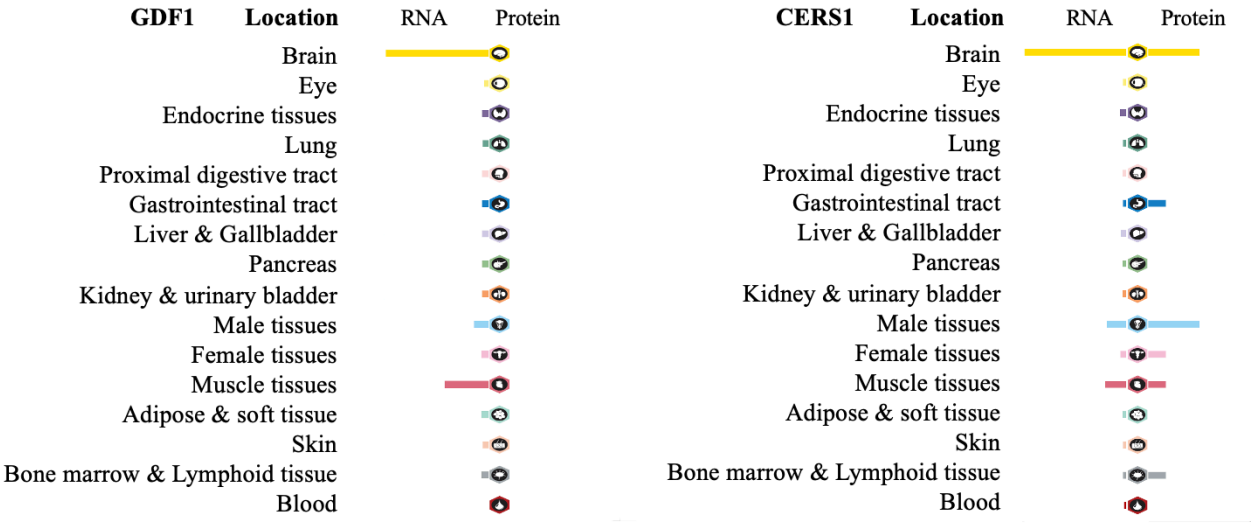


Figure 1.6. Tissue-specificity of GDF1 and CERS1 mRNA and protein. RNA-seq was performed using tissue samples from healthy human individuals in order to determine tissue-specificity of all protein-coding genes. GDF1 and CERS1 are both predominantly expressed at high levels in human and murine brain and male tissues and are nearly absent in most other tissues. Image copied and modified from The Human Protein Atlas.

Interestingly, *GDF1* is located on a bicistronic gene on chromosome 19 which also produces the ceramide synthase 1 (CERS1) protein from a non-overlapping reading frame (Figure 1.7). CERS1 catalyzes the synthesis of C18 ceramide and is primarily expressed in the brain and male tissues (Ginkel et al., 2012) (Figure 1.6). The polycistronic structure of mRNAs is an important aspect of translational control in prokaryotes, in which genes belonging to the same functional pathways are often packaged into operons, which are transcribed into a single mRNA (Jacob and Monod, 1961; Kozak, 2005) (Figure 1.8). However, they are rare in eukaryotes such as mouse and human. The clusters of co-expressed genes may be conserved by natural selection (Singer et al., 2005). A mammalian bicistronic transcript of *SNURF-SNRPN* has been found to encode two independent proteins in human and mouse, and a polycistronic cDNA

that encodes two functional enzymes is found in tomato plant too (García-Ríos et al., 1997; Gray et al., 1999). Therefore, *CERS1* and *GDF1* may have functional connections in evolution.

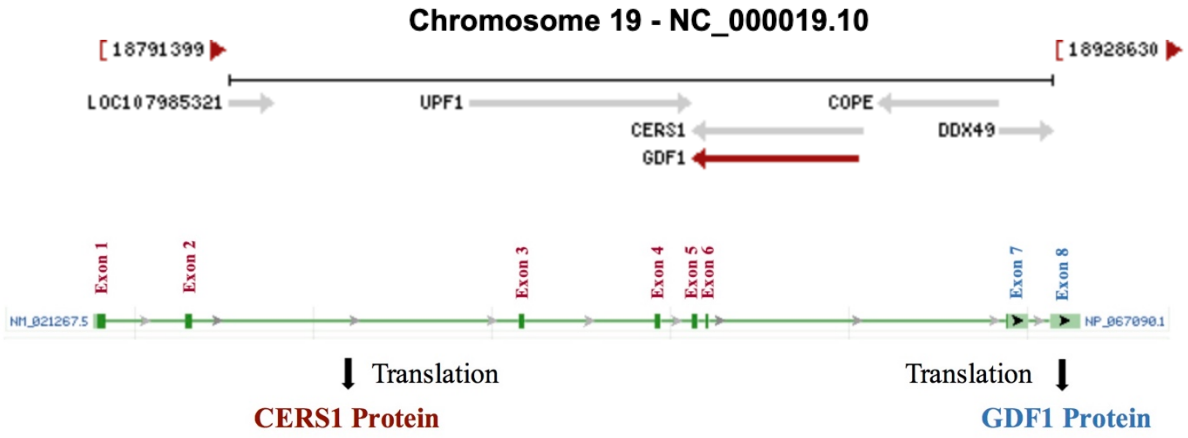
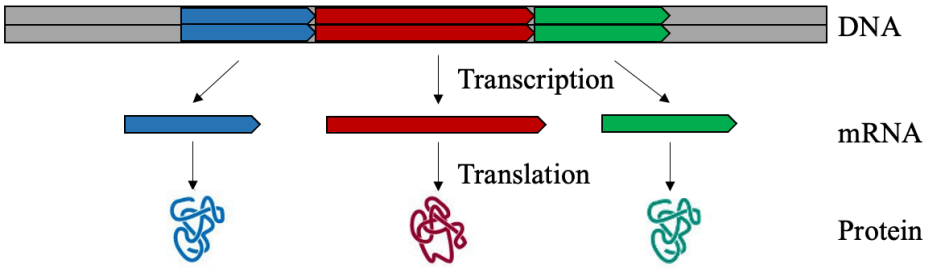


Figure 1.7. Genomic context of *Homo sapiens* chromosome 19 -NC_000019.10 and schematic representations of human *GDF1* and *CERS1* mRNA transcript. Upper: The human *GDF1* and *CERS1* genes are both located on 19p13.11. They are transcribed to a single bicistronic mRNA. Lower: The mRNA transcript of *GDF1* and *CERS1* has eight exons, with the first six encoding for CERS1 and the last two encoding for GDF1. Each vertical green line in the figure represents one mRNA transcript isoform, with green block representing the exon and grey arrow representing the translating direction, respectively. Image copied and modified from NCBI Database.

Monocistronic transcription



Polycistronic transcription

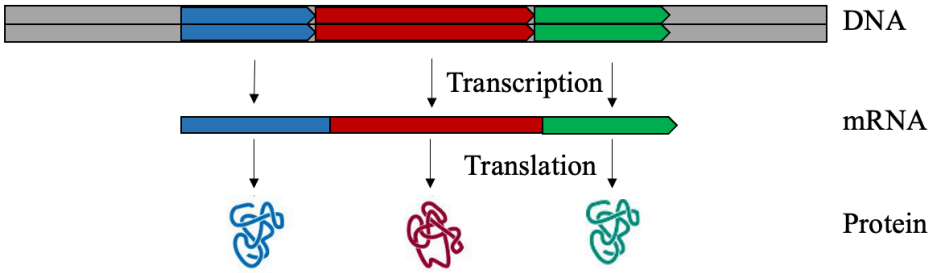


Figure 1.8. Monocistronic vs polycistronic transcription in eukaryotes. A cistron is an alternative term for "gene", which is a region of DNA that encodes function. An mRNA molecule is said to be monocistronic when it contains the genetic information to translate only a single protein. This is the case for most of the eukaryotic mRNAs. On the other hand, polycistronic mRNA carries several open reading frames, each of which is translated into a polypeptide. These polypeptides usually have a related function, and their coding sequence is grouped and regulated together in a regulatory region, containing a promoter and an operator. Most of the mRNA found in bacteria and archaea is polycistronic, as is the human mitochondrial genome.

Signaling pathway

The encoded preproprotein of GDF1 is proteolytically processed to generate each subunit of the disulfide-linked homodimer. GDF1 potentiates NODAL activity by stabilizing a low molecular weight fraction (Fuerer et al., 2014). The NODAL-GDF1 heterodimer can bind to the serine/threonine kinase receptors ACVR2B and ACVR1B and the EGF-CFC coreceptor CRYPTO on the cell surface. Activated ACVR1B recruits and phosphorylates SMAD2 or SMAD3 in the cytoplasm. Two SMAD3 (or two SMAD2) then bind to SMAD4 and forms a heterodimeric complex. This complex then enters the cell nucleus and binds directly to DNA where it acts as a transcription factor for various genes (Sandomenico and Ruvo, 2019) (Figure 1.9).

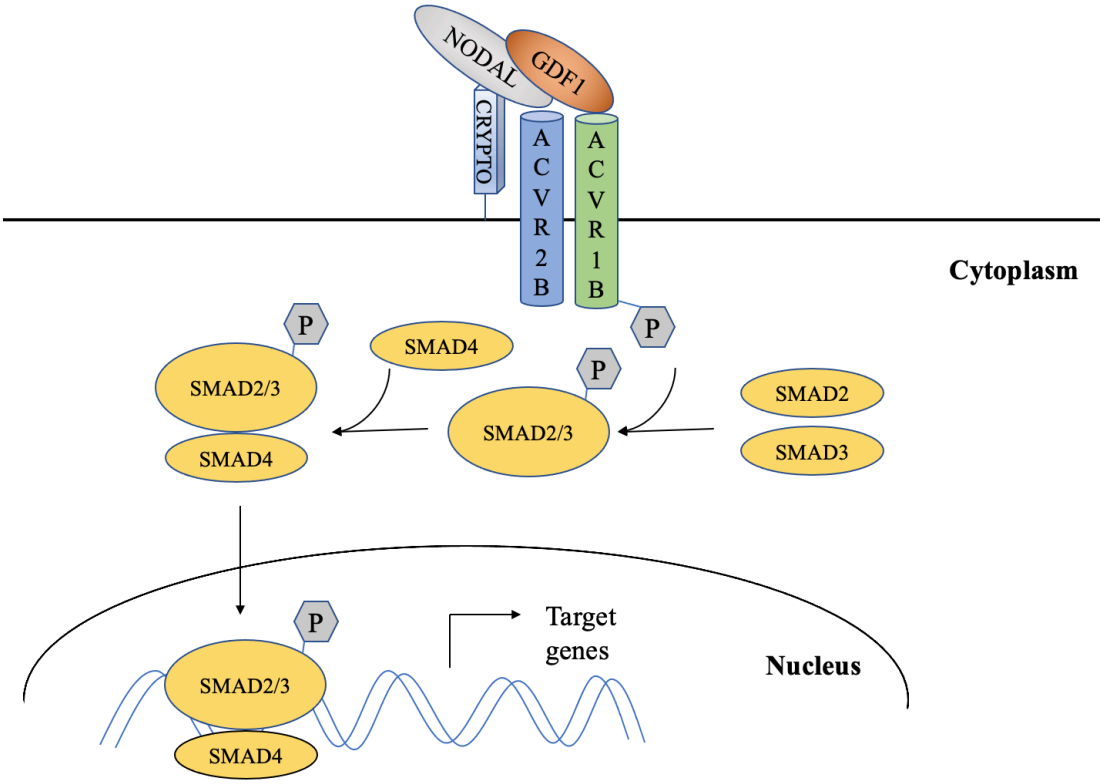


Figure 1.9. Schematic diagram of GDF1 signaling pathway. GDF1 and NODAL form a dimer which binds to ACVR1B and ACVR1B as well as the coreceptor CRYPTO on the cell surface. The ligand binding triggers the phosphorylation of the type I receptor by the type II kinase, as well as the phosphorylation of SMAD2 (or SMAD3) on the inner surface of the cell membrane. Activated SMAD2/3 then forms a complex with Smad4 and translocate into the nucleus to affect the expression of various target genes.

Function

GDF1 is involved in the establishment of left-right asymmetry in early embryogenesis and in neural development in later embryogenesis. During early embryonic development, GDF1 controls the formation of organizing centers that are necessary for normal forebrain and branchial arch development in the anterior primitive streak (Olov Andersson et al. 2006). In the axis patterning of the embryo, the node is the central initiator of left-right asymmetry (Figure 1.10). NODAL and GDF1 secreted from the ventral node define the left side of the embryo through interactions with LEFTY2 and PITX in the lateral plate mesoderm. Meanwhile, a leftward flow induced by active cilia prevents NODAL from signaling on the right side of the embryo, in conjunction with the right side-specific expression of Cerberus (Quail et al., 2013). Mutations in this gene are associated with several congenital cardiovascular malformations (Marek- Yagel et al., 2020). For example, deletion of the *GDF1* gene causes heterotaxy with varied complex heart malformations of left–right patterning (Jin et al., 2017). In the adult, the expression of *GDF1* is almost exclusively restricted to the central nervous system. Recent studies revealed that *GDF1* has a protective role in pathological cardiac hypertrophy. Cardiac-specific *GDF1* overexpression markedly attenuated cardiac hypertrophy, fibrosis, and cardiac dysfunction, whereas loss of *GDF1* in cardiomyocytes exaggerated the pathological cardiac hypertrophy and dysfunction in response to pressure overload (Zhang et al., 2014).

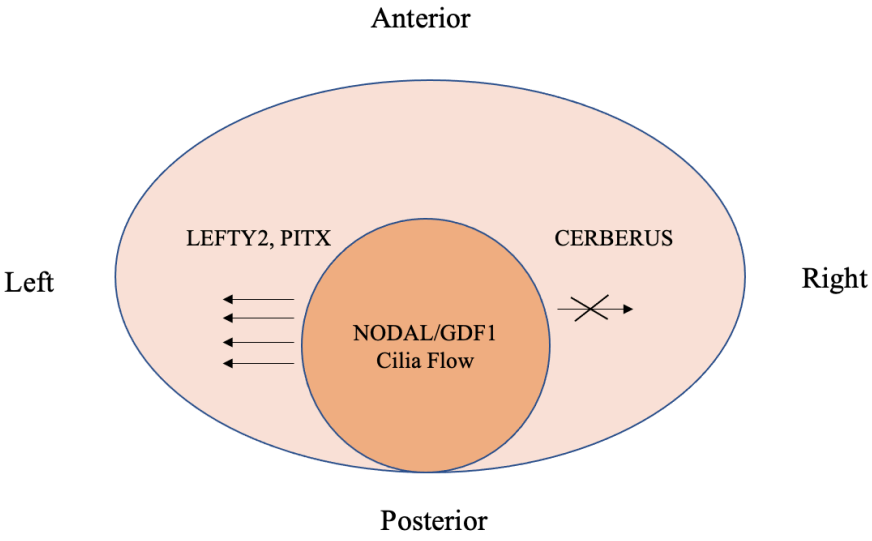


Figure 1.10. Schematic outline of the NODAL/GDF1 signaling pathway during establishment of left-right asymmetry. During gastrulation, the node is the central initiator of left-right asymmetry. GDF1 and NODAL from the node define the left side of the embryo through interactions with LEFTY2 and PITX in the lateral plate mesoderm. Meanwhile, a cilia leftward flow and CERBERUS signal prevent NODAL from signaling on the right side of the embryo.

Although GDF1 is absent from most normal adult tissue, its expression re-emerges during cancer progression. It was found that *GDF1* has a novel functional role in tumor suppression. Abnormal promoter hypermethylation is the major mechanism causing the loss of GDF1 in gastric cancer. Loss of GDF1 is associated with poor survival in stomach cancer patients. GDF1 functions to stimulate p-SMAD2/3 and p21, but it inhibits p-Rb in human gastric cells (Yang et al., 2016). Based on this notion, it is plausible that GDF1 may exert comparable antitumor functions in other cancers, which warrants future investigation.

Mouse

Structure

Mature mouse and rat *GDF1* shares an 81% sequence identity with human *GDF1*. The mouse *GDF1* gene is located on chromosome 8 upstream and in close proximity to the gene coding for CERS1 with which it is expressed on the same bicistronic mRNA (Ginkel et al., 2012). In the early embryonic stage, a 1.4kb mRNA fragment that expresses only *GDF1* is present. From embryonic day 9 and onwards, an additional 3kb fragment is detected which consists of *CERS1* and *GDF1* in a bicistronic mRNA. In the adult mouse, only the 3kb fragment is expressed; the 3-kb species is also found in adult spinal cord, cerebellum, and brain stem as well as in fetal brains from various developmental stages (Lee, 1991) (Figure 1.11).

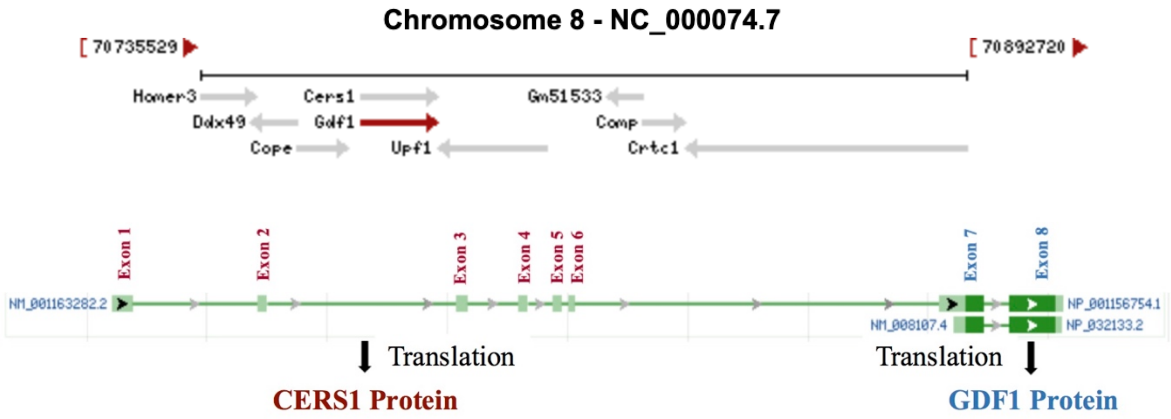


Figure 1.11. Genomic context of *Mus musculus* chromosome 8 – NC_000074.7 and schematic representations of mouse *GDF1* and *CERS1* mRNA transcripts. Upper: The *Mus musculus CERS1* and *GDF1* gene are both located on 8; 8 B3.3. *GDF1* is translated from a monocistronic mRNA early in development, and from a bicistronic mRNA in later stages that also encodes *CERS1*. Lower: The bicistronic mRNA of *GDF1* and *CERS1* has eight exons, with the first six encoding for CERS1 and the last two encoding for GDF1. The monocistronic mRNA of *GDF1* has two exons. Each vertical green line in the figure represents one mRNA transcript isoform, with green block representing the exon and grey arrow representing the translating direction, respectively. Image copied and modified from NCBI Database.

Function

Similar as in human, *GDF1* in mouse is involved in the establishment of left-right asymmetry in early embryogenesis and in neural development in later embryogenesis. At early stages of mouse development, *GDF1* is expressed initially throughout the embryo node and then most prominently in the primitive node, ventral neural tube, and intermediate and lateral plate mesoderm (Shen, 2007). It is crucial for mouse embryonic development, particularly the left-right patterning, by acting as an upstream regulator of multiple symmetry-associated genes such as *Lefty2*, *Nodal*, and *Pitx*. Deletion of the *GDF1* gene leads to embryonic lethality in mice

(Rankin et al., 2000). In late-stage embryos and adult mice, it is exclusively expressed in the nervous system such as the brain, spinal cord, and the peripheral nerves (Lee, 1991). The loss-of-function of GDF1 in mice exhibited a variety of defects with disturbance in the left-right axis formation, including heterotaxy and heart defects such as abnormal atrium and ventricle (Rankin et al., 2000; Tanaka et al., 2007). In addition, mice with cardiac-specific overexpression of the *GDF1* gene are resistant to cardiac remodeling via inhibition of MEK–ERK1/2 and SMAD signaling, indicating that GDF1 alleviates pressure overload-induced cardiac hypertrophy and dysfunction (Yang et al., 2016).

Other animals

GDF1 is highly conserved across species (Andersson et al., 2007; Lee, 1990). It is the functional equivalent of *Xenopus* and chicken *VG1,* or zebrafish *DVR1,* or yeast *Longevity-assurance gene 1* (*Lag1*) (Table 1.6). *GDF1* has been proposed to be a mammalian ortholog of *Xenopus*, zebrafish, and chicken *Vg1*, due to their sequence similarity and the fact that both *GDF1* and *Vg1* regulate left–right patterning (Helde and Grunwald, 1993; Joseph and Melton, 1998; Shah et al., 1997; Wall et al., 2000). It also requires Activin as receptors and EGF-CFC proteins as coreceptors (Cheng et al., 2003). *Hyl-1*, a worm homolog to yeast LAG1, represents a novel mechanism for affecting lifespan in *C. elegans* (Tedesco et al., 2008). *GDF1* is the mammalian homologue of yeast *Lag1* which is differentially expressed during the yeast replicative life span and was shown to play a role in determining yeast longevity (Wang et al., 2007).

Table 1.7. Homologs of *GDF1* in different species

Species	Homologs
Mammalian	*GDF1*
Xenopus, Zebrafish, and chicken	*Vg1*
C. elegans	*Hyl-1*
Yeast	*Lag1*

1.6. Rationale and Significance of Thesis Research

Rationale

Acute myeloid leukemia (AML) is a cancer of the myeloid lineage of blood cells. The disease can progress quickly and be fatal within days or months if left untreated. Currently, there are no viable curative treatments for AML besides bone marrow transplant, which has potentially life-threatening side effects.

Significance

Chapters 2 and 3 explore the role of growth/differentiation factor 1 (GDF1) as a regulator of ceramide metabolism, as well as its therapeutic efficacy in preclinical models of AML. Experimental data indicates that GDF1 has the potential to promote ceramide buildup by downregulating the expression of genes encoding for ceramide de-toxifying enzymes. In addition, GDF1 was observed to regulate hematopoiesis, including specific regulation of erythropoiesis, as well as exert combinatorial efficacy with cytarabine *in vivo*. These results rationalize further investigation of GDF1 as a potential therapeutic modality for AML.

Chapter 4 evaluates the role of obesity in the progression of AML, where it facilitates a ceramide-dependent pathway. That obesity directly accelerates the development of AML emphasizes the necessity of elucidating mechanisms linking cancer and obesity to better address and reduce the burden of both diseases.

1.7. Aims and Hypotheses of Individual Chapters

CHAPTER 2

GDF1 REGULATES CERAMIDE DETOXIFICATION IN ACUTE MYELOID LEUKEMIA

Hypothesis - GDF1 regulates ceramide detoxification in acute myeloid leukemia (AML).

Aim 1 - To study GDF1 regulation of ceramide metabolism in AML.

Aim 2 - To evaluate SMAD2/3 and STAT3 signaling as GDF1-mediated pathways in AML.

CHAPTER 3

REGULATION OF HEMATOPOIESIS BY GDF1 IN ACUTE MYELOID LEUKEMIA

Hypothesis - GDF1 promotes hematopoiesis in AML.

Aim 1 - To investigate GDF1 regulation of hematopoiesis and erythropoiesis.

Aim 2 - To evaluate the anti-AML therapeutic efficacy of GDF1 alone or in combination with cytarabine.

CHAPTER 4

OBESITY PROMOTES ACUTE MYELOID LEUKEMIA PROGRESSION BY UPREGULATING THE CERAMIDE-MEDIATED NADPH OXIDASE 2

Hypothesis - Obesity promotes AML progression by upregulating the ceramide-mediated NADPH oxidase 2.

Aim 1 - To demonstrate that obesity mediates the progression of AML.

Aim 2 - To show that obesity in AML upregulates the ceramide-medicated NADPH oxidase 2.

CHAPTER 2

GDF1 REGULATES CERAMIDE DETOXIFICATION IN ACUTE MYELOID LEUKEMIA[4]

2.1. Abstract

Growth/differentiation factor 1 (GDF1) is primarily known for its role in development, especially that of the heart. GDF1 is a member of the transforming growth factor beta (TGFβ) superfamily. The gene encoding for GDF1 is encoded from a rare bicistronic transcript that also encodes for ceramide synthase 1 (CERS1). Due to its connection to CERS1, it was speculated that GDF1 may play a role in sphingolipid metabolism or signaling. Therefore, the regulation of *CERS1/GDF1* was investigated in acute myeloid leukemia (AML) in addition to its effects on the expression of sphingolipid-modifying enzymes. In this study, it is reported for the first time that GDF1 can alter sphingolipid metabolism in a manner that may restore AML sensitivity to nanoliposomal C6-ceramide (Lip-C6). Gene expression analysis revealed that *CERS1/GDF1* is variably expressed in AML. Treatment of leukemia cells with recombinant GDF1 reduced the expression of ceramide-neutralizing enzymes encoded by *UDP-glucose ceramide glycosyltransferase* (*UGCG*) and *sphingomyelin synthase 1* (*SGMS1*). It was further shown that GDF1 exerted divergent transforming growth factor beta receptor 1 (TGFβR1)-dependent upregulation of SMAD2/3 and downregulation of STAT3. Altogether, these results identify GDF1 as a novel regulator of ceramide metabolism in AML. Moreover, this study suggests that GDF1 may be able to restore anti-AML therapeutic efficacy of therapeutics such as Lip-C6.

[4] Weiyuan Wang, Paul T. Toran, Andrea L. Cote, Emma J. Arsenault, Alexander J. Thorpe, Vasiliki Papakotsi, Emily C. Sullivan, Rachel J. Sabol, Mary A. Hurley, Brian M. Barth. GDF1 Regulates Ceramide Detoxification in Acute Myeloid Leukemia. In preparation for submission.

2.2. Introduction

Acute myeloid leukemia (AML) is a heterogenous malignancy characterized by the expansion of immature myeloid progenitors, primarily in the blood and bone marrow (Grimwade et al., 2016). Genetic abnormalities and molecular abnormalities such as chromosomal changes are common in AML (Kumar, 2011). This cancer can occur in several different ways. AML that arises from myelodysplastic syndrome (MDS) is known as AML with MDS-related changes (AML-MRC) (Arber et al., 2016; Papaemmanuil et al., 2016). Other patients that do not have clinical history of prior MDS are classified as *de novo* AML (DN-AML). In general, AML-MRC occurs in older patients and has more unfavorable outcomes (Arber and Erba, 2020). The poor prognostics and increasing incidence of this disease demand novel therapies for the treatment of AML (Baldus et al., 2007). One potential therapy of particular interest is ceramide, a proapoptotic sphingolipid which, when formulated into a nanoliposome, becomes a potent anticancer drug called nanoliposomal C6-ceramide (Lip-C6) (Barth et al., 2011). The AML-MRC variant of AML appears to be predominantly sensitive to Lip-C6 due whereas enhanced ceramide metabolism in DN-AML predominates as a mechanism of resistance (Barth et al., 2019). For this reason, increasing sensitivity to ceramide-regulating therapeutics is an adequate strategy to combat drug resistance in cancer.

Growth/differentiation factor 1 (GDF1) is a member of the transforming growth factor beta (TGFβ) superfamily. It is primarily known for its role in development and stem cell biology, including anterior axis development, left-right patterning, and cardiac development (Andersson et al., 2006; Karkera et al., 2007a). GDF1 is a ligand of the transforming growth factor beta receptor 1 (TGFβR1), a receptor serine/threonine kinase which functions through receptor

heterodimerization and activation of downstream SMAD signaling (Moore-Smith and Pasche, 2011).

GDF1 is also a factor in AML, wherein it regulates sphingolipid metabolism through downregulation of genes that produce enzymes to break down ceramide (Wang et al., 2019). The gene encoding for GDF1 is a rare bicistronic gene that also encodes for ceramide synthase 1 (CERS1). Both genes are under control of the same promoter, which is found upstream of *CERS1* (Meyers-Needham et al., 2012). CERS1 promotes the formation of C18:0 dihydroceramide from dihydrosphingosine, as well as C18:0 ceramide from sphingosine in salvage ceramide biosynthetic pathways (Mullen et al., 2012).

The present study explores a hypothesis that GDF1 regulates ceramide detoxification in AML, which would enhance the sensitivity of AML to ceramide-regulating therapies such as Lip-C6. This study first demonstrates that the *CERS1/GDF1* transcript is variably expressed in AML, with a slight upregulation in AML-MRC. Furthermore, treatment with recombinant GDF1 was shown to reduce the expression of enzymes that break down ceramide, while also upregulating SMAD2/3 phosphorylation and downregulating STAT3 phosphorylation in a TGFβR1-dependent manner.

2.3. Results

The efficacy of Lip-C6 was first evaluated in murine models of AML. Both DN-AML ($FLT3^{ITD}$ and MLL-AF9) and AML-MRC (Nup98-HoxD13) models were used. Lip-C6 therapy was more effective in reducing the colony forming ability of bone marrow from AML-MRC mouse models as compared with bone marrow from DN-AML or wildtype mouse models (Barth et al., 2019) (Figure 2.1A). Similarly, *in vivo* treatment with Lip-C6, but not the nanoliposomal

ghost control (Lip-Ghost), significantly decreased CD11b^{+}/Gr-1^{+} myeloid leukemia cells in AML-MRC mice but not DN-AML mice (Barth et al., 2019) (Figure 2.1B).

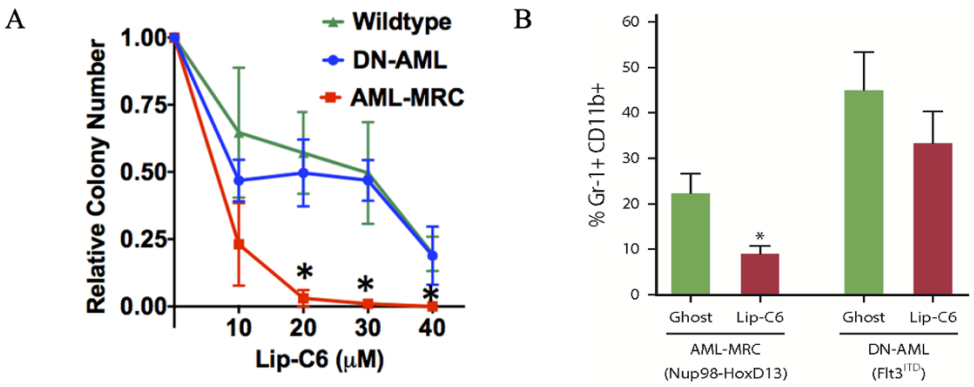


Figure 2.1. AML-MRC is more sensitive to Lip-C6 compared with DN-AML. (A) Bone marrow of DN-AML (FLT3^{ITD} and MLL-AF9) and AML-MRC (Nup98-HoxD13) mice was harvested and treated with varying concentrations of ceramide nanoliposome (Lip-C6) in a colony-forming assay. (*p<0.05, ANOVA) (B) DN-AML (FLT3^{ITD}) and AML-MRC (Nup98-HoxD13) transgenic mice models were treated daily for 10 days with Lip-C6 (11.6 mg/kg) or Lip-Ghost, which is a nanoliposome without ceramide. Mice were euthanized, and bone marrow was collected, prepared, and analyzed via flow cytometry to evaluate Gr-1^{+}CD11b^{+} cells, representing leukemia burden. *P = .045 (n = 4 per group; unpaired Student t test with Welch's correction) comparing leukemia burden in AML-MRC mice treated with Lip-C6 versus Lip-Ghost. Lip-C6: Nanoliposomal C6-ceramide, Lip-Ghost: Nanoliposomal Ghost. Adapted from published manuscript (Barth et al., 2019).

A role for GDF1 was next evaluated as a possible explanation for the observed differences in sensitivity to Lip-C6 between AML-MRC and DN-AML. It was speculated that GDF1 may increase the sensitivity to ceramide-based therapies in AML by impacting ceramide metabolism. *CERS1/GDF1* expression was initially evaluated by analyzing RNA sequencing data from the BEAT AML dataset (Tyner et al., 2018). Patient samples (n=404) from the BEAT AML dataset have varying expression of *CERS1/GDF1* (Figure 2.2A) (Tyner et al., 2018). *CERS1/GDF1* is also variably expressed in the bone marrow of murine transgenic mouse models of AML and other myeloid hematologic disorders, with the data trend mirroring that of the BEAT AML patient data (Figure 2.2B). More specific comparison of bone marrow from AML-MRC and DN-AML mouse models indicated that *GDF1* is expressed at different levels in these two variants of AML. Despite a high amount of variability, *GDF1* expression was generally observed to be greater in AML-MRC when compared to DN-AML (Figure 2.2C). Therefore, these differences in *CERS1/GDF1* expression may be responsible for distinct sensitivity to Lip-C6 between AML-MRC and DN-AML.

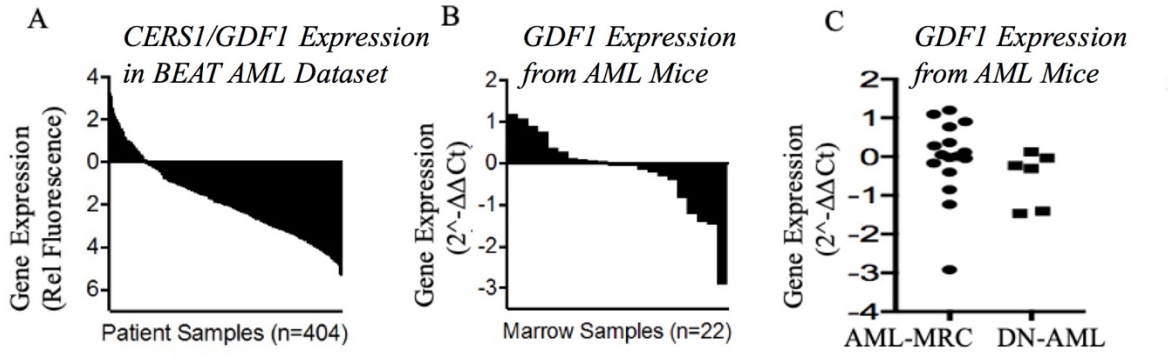


Figure 2.2. C*ERS1/GDF1* is differentially expressed in AML. (A) RNA sequencing data from AML patients (n=404) in the BEAT AML dataset was analyzed for expression of *CERS1/GDF1* and found to be mostly downregulated (samples with matched transcriptomic and genomic sequencing) (Tyner et al., 2018). (B, C) Bone marrow samples from various transgenic mouse models of both DN-AML ($FLT3^{ITD}$ and MLL-AF9) and AML-MRC (Mx1-Asxl1+/-; Mx1-$Srsf2^{P95H}$**;** Mx1-Tet2+/-; Mx1-Asxl1+/-Tet2+/-; Nup98-HoxD13; Vav1-Tert+/-) (n=22) were analyzed via RT-qPCR. Wildtype and leukemic murine tissue samples were age- and sex- matched and housekeeping gene normalization was performed. Not all subtypes cluster, indicating that distinct molecular evolution of the disease may contribute to differential regulation of *CERS1/GDF1*.

Leukemia where *CERS1/GDF1* is downregulated may benefit from treatment with recombinant GDF1 to gain a possible therapeutic effect. *CERS1/GDF1* was observed to be downregulated in the bone marrow of $FLT3^{ITD}$ transgenic mice, a model of DN-AML (Figure 2.3A). In contrast, the expression of *UGCG* and *SGMS1* was augmented in the bone marrow of these DN-AML mice (Figure 2.3A). Interestingly, *in vivo* treatment with the hypomethylating agent decitabine augmented *CERS1/GDF1* expression while downregulating *UGCG* and *SGMS1* expression (Figure 2.3A). These results indicate an inverse correlation between *CERS1/GDF1* and *UGCG* and *SGMS1* expression in DN-AML. The ability of recombinant GDF1 to regulate

the expression of *UGCG* and *SGMS1*, genes encoding enzymes responsible for ceramide neutralization, was further evaluated by *in vitro* treatment of AML cell lines. Recombinant GDF1 treatment of HL-60, Kasumi-1, and Kasumi-3 cells led to a downregulation of expression for both *UGCG* and *SGMS1* (Figure 2.3B).

Phosflow was used to further evaluate GDF1-mediated signaling in AML cell lines. Recombinant GDF1 treatment increased phosphorylation of SMAD2/3 while simultaneously decreasing tyrosine phosphorylation of STAT3 (Figure 2.3C-D). The TGFβR1 inhibitor IN-1130 blocked GDF1-stimulated SMAD2/3 phosphorylation while also preventing the GDF1-mediated decrease in tyrosine phosphorylation of STAT3 (Figure 2.3C-D). This demonstrated a TGFβR1-dependent mechanism for GDF1 in AML cells. In contrast, TGFβ and growth/differentiation factor 3 (GDF3), which are related to GDF1 and can also engage TGFβR1, did not fully recapitulate the effects of GDF1 on SMAD2/3 and STAT3 signaling. Specifically, TGFβ similarly increased SMAD2/3 phosphorylation but did not impact STAT3 tyrosine phosphorylation (Figure 2.3C-D). More so, GDF3 similarly downregulated STAT3 tyrosine phosphorylation but had no effect on SMAD2/3 phosphorylation (Figure 2.3C-D). This is despite GDF1 and GDF3 having recently been suggested to be evolutionarily linked and shown to have partially redundant functions in the pre-gastrulation embryo (Opazo and Zavala, 2018). The unique ability of GDF1 to both increase SMAD2/3 phosphorylation and decrease STAT3 tyrosine phosphorylation may be due to the engagement of a separate co-receptor from other related ligands in addition to its TGFβR1 engagement. Overall, these results suggest that GDF1 may have a unique impact on leukemia cells compared to other ligands of the TGFβR1 system.

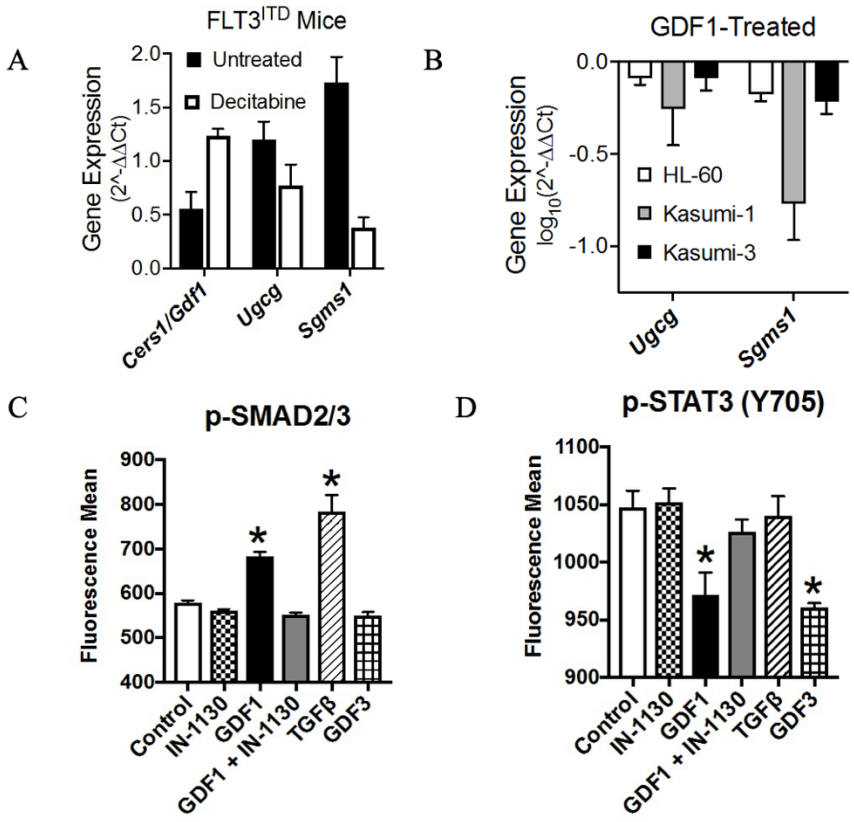


Figure 2.3. Treatment with recombinant GDF1 triggers multiple cellular effects. (A) FLT3^{ITD} mice were treated with the hypomethylating agent decitabine. Bone marrow samples were obtained and RT-qPCR was used to analyze the expression change of genes encoding for ceramide neutralizing enzymes. Decitabine treatment led to an increased expression of *CERS1/GDF1* and a decreased expression of *UGCG* and *SGMS1* (B) Recombinant GDF1 was administered *in vitro* to three leukemia cell lines: HL-60, Kasumi-1, and Kasumi-3 (200 ug/ml, 48 hours). RT-qPCR was performed to analyze the expression of *UGCG* and *SGMS1*. Both *UGCG* and *SGMS1* were downregulated following treatment. (C, D) Kasumi-1 cells were treated with the TGFβR1 inhibitor IN-1130, recombinant GDF1, a combination of recombinant GDF1 and IN-1130, TGFβ, or GDF3. Phosflow was used to evaluate phosphorylation of SMAD2/3 and tyrosine phosphorylation of STAT3 (residue Y705). In a TGFβR1-dependent fashion, GDF1 both (C) upregulated SMAD2/3 signaling and (D) downregulated STAT3 signaling. * $p > 0.05$, n=3.

2.4. Discussion

These findings highlight a role for GDF1 as a regulator of ceramide metabolism in AML. AML-MRC is uniquely sensitive to Lip-C6 as compared with DN-AML (Barth et al., 2019), which further highlights the need to increase DN-AML sensitivity to ceramide-based therapeutics. It was observed that the expression of *CERS1/GDF1* is highly variable in both AML patient samples and murine models of AML. Interestingly, *GDF1* expression was generally lower in the bone marrow of DN-AML transgenic mouse models as compared with AML-MRC transgenic mouse models. These discrepancy in *GDF1* expression may help explain differential sensitivity to Lip-C6 in AML. Increasing *GDF1* expression or administering recombinant GDF1 may enhance AML sensitivity to Lip-C6 and other ceramide-elevating therapeutics.

Ceramide is a powerful proapoptotic sphingolipid with the potential to treat many different cancers (Barth et al., 2011). Unfortunately, AML can resist Lip-C6 by upregulating ceramide-neutralizing enzymes (Barth et al., 2019). Therefore, strategies to inhibit these enzymes, or to downregulate their expression, are of interest in the treatment of AML and other ceramide-resistant cancers. Recombinant GDF1 was observed to lower the expression of ceramide-neutralizing enzymes encoded by *UGCG* and *SGMS1*. These enzymes prevent apoptosis by neutralizing proapoptotic ceramide to nonapoptotic sphingolipid metabolites.

This study further indicated that GDF1 promoted the phosphorylation of SMAD2/3, while simultaneously inactivating STAT3 by decreasing its tyrosine phosphorylation. Both these effects occurred in a TGFβR1-dependent fashion and distinct from the single SMAD2/3-regulating or STAT3-regulating effects of the related ligands TGFβ and GDF3, respectively. This suggests that GDF1 has a unique capacity to regulate leukemia cells in by engaging

TGFβR1 to regulate both SMAD2/3 and STAT3 capability to shift sphingolipid metabolism towards ceramide accumulation by downregulating the expression of genes encoding for ceramide detoxification enzymes (Figure 2.4).

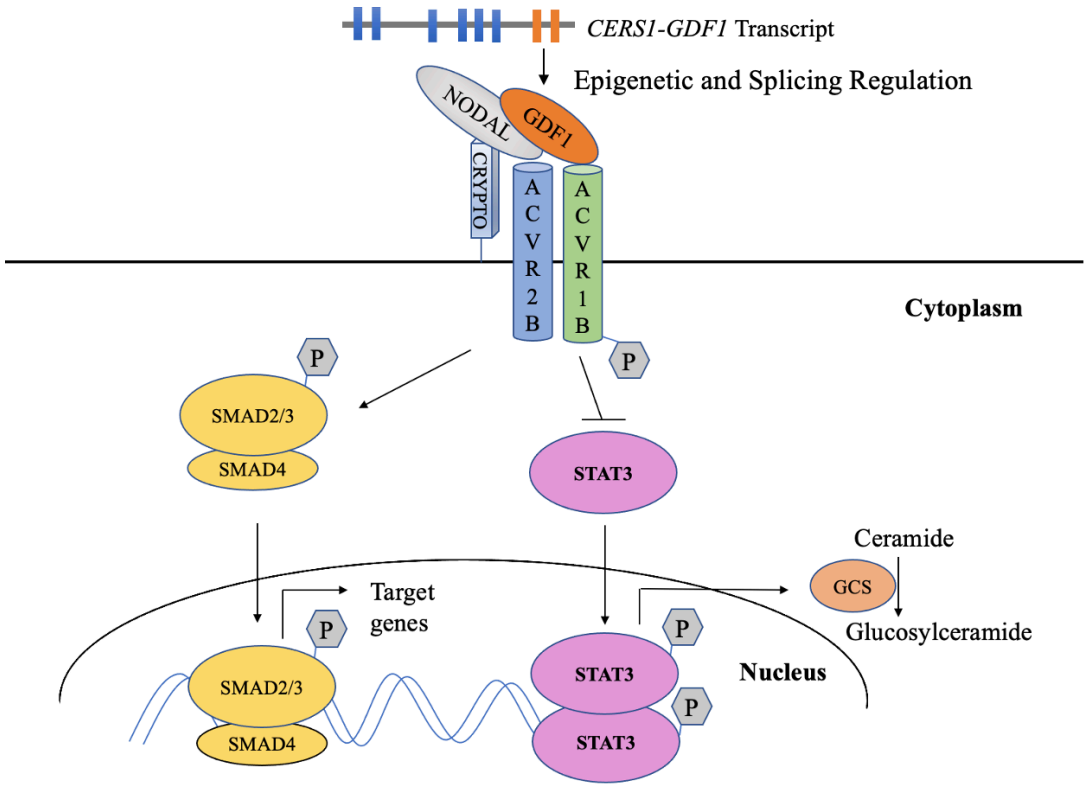


Figure 2.4. Schematic diagram of GDF1 signaling pathway. GDF1, translated from a bicistronic gene, signals through a receptor serine/threonine kinase system that includes TGFβR1. Upon ligand binding, the receptor system triggers phosphorylation of SMAD2/3, which induces the expression of target genes. Simultaneously, the receptor system inactivates STAT3 by decreasing its tyrosine phosphorylation. Collectively, the effects of GDF1 is to block ceramide neutralization to glucosylceramide by binding to the promoter region of genes such as *UGCG*, which encodes for GCS. In this way, GDF1 can help to increase intracellular ceramide levels. *CERS1*, *ceramide synthase 1*; GCS, glucosylceramide synthase; *UGCG*, gene encoding for GCS.

2.5. Materials and Methods

BEAT AML dataset

The BEAT AML program is a collaborative program for functional genomic data integration involving numerous academic medical centers working collectively to accrue and characterize a large cohort of AML patient specimens. Information available in the BEAT AML dataset includes whole exome sequencing and RNA-sequencing, which is available through recent publication (Tyner et al., 2018). For the present study, samples from the BEAT AML dataset that have paired genomic and transcriptomic data were analyzed.

Cell culture

The human Kasumi-1 and Kasumi-3 cell lines were maintained in RPMI-1640 supplemented with 20% FBS. Similarly, the human HL-60 cell line was maintained in Isocove's Modified Dulbecco's Medium supplemented with 20% FBS. All cultures were incubated at 37°C and 5% CO2.

Animal studies

C57BL/6-Tg(Vav1-NUP98/HOXD13)G2Apla/J (Nup98-HoxD13), B6.Cg-Tg(Mx1-cre)1Cgn/J-Asxl1$^{tm1.1Iaai}$/J (+/-)(Mx1-Asxl1+/-), B6.Cg-Tg(Mx1-cre)1Cgn/J-B6J.B6NTac(SJL)-Srsf2$^{tm1.1Oaw}$/J (+/-)(Mx1-Srsf2^{P95H}), B6.Cg-Tg(Mx1-cre)1Cgn/J-B6J.B6NTac(SJL)-B6;129S-Tet2$^{tm1.1Iaai}$/J(+/-)(Mx1-Tet2+/-), B6.Cg-Tg(Mx1-cre)1Cgn/J-B6J.B6NTac(SJL)-Asxl1$^{tm1.1Iaai}$/J-B6;129S-Tet2$^{tm1.1Iaai}$/J(+/-)(Mx1- Asxl1+/-Tet2+/-), B6.Cg-Commd10$^{Tg(Vav1-icre)A2Kio}$/J-B6.129S-Tert^{tm1Yjc}/J (+/-)(Vav1-Tert+/-) transgenic mice are commonly used murine models of MDS and other myeloid hematologic disorders, many which evolve to AML-MRC (Chou et al., 2010; Hou

et al., 2016; Kirwan et al., 2009; Nibourel et al., 2010; Slape et al., 2008). In contrast, B6.129-Flt3^{tm1Dgg}/J (Flt3^{ITD}) and Kmt2a$^{tm2(MLLT3)Thr}$/KsyJ (MLL-AF9) transgenic mice were used as models of DN-AML (Chen et al., 2019; Loghavi et al., 2014).

Bone marrow cells were isolated from the femurs and tibias of 4–8-month-old (male and female) transgenic mice and their respective wildtype littermates for colony-forming assay, flow cytometry, and RT-qPCR analysis. These transgenic mice include AML-MRC mouse models such as Nup98-HoxD13, Mx1-Asxl1+/-, Mx1-Srsf2^{P95H}, Mx1-Tet2+/-, Mx1-Asxl1+/-Tet2+/-, Vav1-Tert+/-, as well as DN-AML mouse models such as MLL-AF9 and FLT3^{ITD}. Mice were submitted to the New Hampshire Veterinary Diagnostic Laboratory (NHVDL) at the University of New Hampshire for histopathological evaluation. Leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of New Hampshire.

Ceramide Nanoliposome (Lip-C6) formulation and treatment

Lip-C6 and Lip-Ghost were generously prepared and provided by Dr. Mark Kester of the University of Virginia. Briefly, lipids dissolved in chloroform, were combined in specific molar ratios, dried to a film under nitrogen, and then hydrated by addition of 0.9% NaCl. Solutions were sealed, heated at 60°C for 60 minutes, subjected to vortex mixing, and sonicated until light no longer diffracted through the suspensions. Lipid vesicle-containing solutions were then extruded at 60°C by passing the solutions 10 times through 100 nm polycarbonate filters using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Size and charge were validated using a Malvern Zetasizer Nano ZS at 25°C, and C6-ceramide encapsulation into anionic

formulations was quantified by mass spectroscopy. Liposomal suspensions were stored at room temperature until use.

Therapeutic efficacy of Lip-C6 as a standalone treatment was evaluated using transgenic mouse models. 4–8-month-old (male and female) AML-MRC (Nup98-HoxD13) and DN-AML ($Flt3^{ITD}$) transgenic mice were injected with Lip-C6 (11.6 mg/kg) or Lip-Ghost (volume matched) daily for 10 days. One day after the final injection, mice were euthanized and bone marrow was collected from femurs and tibias, spleens were removed and measured, and blood was collected by cardiac puncture for blood smears.

Recombinant GDF1 formulation and treatment

Recombinant human GDF1 was purchased from R&D Systems. It arrived in a lyophilized form with BSA as a carrier protein and was re-suspended according to the manufacture's direction. Therapeutic efficacy of recombinant GDF1 as a standalone treatment was evaluated using HL-60, Kasumi-1, and Kasumi-3 cell lines. These cell lines were first cultured in T25 flasks, then 1.2 x 10^6 cells were seeded into each well of a six-well plate and treated with recombinant GDF1 reagent (200 ug/ml, 48 hours).

Colony-forming assays

Murine bone marrow mononuclear cells were isolated and prepared from transgenic mice and their wildtype counterparts and cleaned of debris using Ficoll-Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation. Prior to colony-forming assays, leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. Murine samples were cultured

in triplicate in 12-well plates at a density of 2.5 x 10^4 cells per well in Murine MethoCult GF (#M3434) (StemCell Technologies, Vancouver, BC). The plating density was selected to yield typical wildtype or transgenic mouse colony out-growth of ~30-150 colonies per well in the absence of drug treatment. Sterile water was added into empty wells, and space between wells, to maintain optimal humidity necessary for colony growth. During assay set-up, murine bone marrow cells were added simultaneously to the culture media with Lip-C6 or controls. The cultures were mixed vigorously and then dispensed to multi-well plates and incubated for 10-14 days. Blinded investigators manually counted total colonies, including blast or miscellaneous colonies.

RT-qPCR analysis

Total cellular RNA from mouse bone marrow samples or cell lines was extracted and cleaned using Zymo Research kits. RNA quality was evaluated using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific), and reverse transcribed with Taq polymerase to generate cDNA. Quantitative polymerase chain reaction (qPCR) was performed using PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies) on a CFX96 Real-Time PCR System (Biorad). Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method using cycle threshold (Ct) values for target genes as well as two endogenous reference genes (*ACTB* and *TBP*). Primer and probe assays were obtained from Integrated DNA Technologies.

Flow cytometry and Phosflow

Isolated bone marrow cells from transgenic mice were prepared and stained with Mouse Fc Block, fluorophore-conjugated anti-mouse Gr-1 (clone RB6-8C5) and fluorophore-conjugated

anti-mouse CD11b (clone M1/70) monoclonal antibodies from BD Biosciences (San Jose, CA). Gr-1+ CD11b+ cells were evaluated and quantified as representative of leukemia burden. Phosflow was performed to evaluate phosphorylation of SMAD2/3 on serine/threonine residues and tyrosine phosphorylation of STAT3 (residue Y705). For Phosflow studies, Kasumi-1 cells were fixed, permeabilized, and stained with specific fluorophore-conjugated antibodies, all with reagents from BD Biosciences. Flow cytometry was performed at the University of New Hampshire's University Instrumentation Center using a Sony SH800Z sorting flow cytometer.

Statistical analysis

GraphPad Prism (GraphPad Software, La Jolla, CA) was used to perform data analyses. For dose-response colony-forming assays, a 2-way ANOVA with a Tukey's post hoc multiple comparisons test was performed. Comparisons of colony-forming, RT-qPCR, or Phosflow data were made using a student t-test or 1-way ANOVA followed by a Tukey's multiple comparisons test.

CHAPTER 3

REGULATION OF HEMATOPOIESIS BY GDF1 IN ACUTE MYELOID LEUKEMIA[5]

3.1. Abstract

In acute myeloid leukemia (AML), hematopoiesis is altered such that maturation arrests at the myeloid blast phase, leading to the expansion and proliferation of myeloid progenitors. Consequently, restoration of normal hematopoiesis may be a viable strategy to treat AML and other related blood disorders. Growth/differentiation factor 1 (GDF1) is a transforming growth factor beta (TGFβ) superfamily member protein. This ligand, which is an important factor in embryonic development, may also be effective in re-establishment of normal hematopoiesis in AML. Earlier GDF1 was identified as a potential treatment to enhance sensitivity to ceramide-based therapeutics, due to a role downregulating ceramide-neutralizing enzymes. In the present study, therapeutic efficacy is reported for recombinant GDF1 as a standalone agent as well as in combination with cytarabine (AraC). It was determined that recombinant GDF1 treatment promotes blood cell differentiation in cells exhibiting abnormal hematopoiesis, while not impacting normal hematopoiesis. More interestingly, recombinant GDF1 demonstrated an ability to promote erythropoiesis. Collectively, these studies highlight the potential efficacy of recombinant GDF1 as a hematopoietic-regulating anti-AML therapy.

[5] Weiyuan Wang, Paul T. Toran, Alexander J. Thorpe, Emma J. Arsenault, Andrea L. Cote, Emily C. Sullivan, Vasiliki Papakotsi, Brian M. Barth. Regulation of Hematopoiesis by GDF1 in Acute Myeloid Leukemia. In preparation for submission.

3.2. Introduction

Acute myeloid leukemia (AML) is characterized by expansion of immature myeloid cells within the peripheral blood and bone marrow (Grimwade et al., 2016). In AML and related myeloid hematologic malignancies, such as myelodysplastic syndrome (MDS), the differentiation of the myeloid lineage is arrested so that there is an accumulation of blasts expressing markers for monocytes, erythroid cells, and megakaryocytes (Saultz and Garzon, 2016). AML can be classified as either AML with MDS-related changes (AML-MRC) or as *de novo* AML (DN-AML) if there is no prior history of MDS or MDS-related changes (Arber and Erba, 2020; Barth et al., 2019).

The transforming growth factor-beta (TGFβ) superfamily member growth/differentiation factor 1 (GDF1) has a profound role in embryonic development and stem cell biology. In particular, it is involved in body plan and cardiac development (Andersson et al., 2006; Karkera et al., 2007b). GDF1 can bind to TGFβR1, which is a receptor serine/threonine kinase (Moore-Smith and Pasche, 2011). The gene encoding for GDF1 is a rare bicistronic gene that also encodes for ceramide synthase 1 (CERS1). Both genes are under control of the same promoter, which is found upstream of *CERS1* (Meyers-Needham et al., 2012). CERS1 promotes the formation of C18:0 dihydroceramide from dihydrosphingosine, as well as C18:0 ceramide from sphingosine in salvage ceramide biosynthetic pathways (Mullen et al., 2012).

Ceramide-based therapeutics are under investigation for the treatment of AML and other cancers due to their apoptosis-promoting abilities (Barth et al., 2011). In particular, nanoliposomal C6-ceramide (Lip-C6) has shown efficacy in AML-MRC but not DN-AML, due to enhanced metabolism of ceramide in DN-AML (Barth et al., 2019). Recently, recombinant

GDF1 was shown to downregulate ceramide metabolism and signal in a TGFβR1-dependent fashion in AML cells (Wang et al., 2019).

In the present study, anti-AML therapeutic efficacy of recombinant GDF1 was more closely evaluated by focusing on its regulation of hematopoiesis. It was found that recombinant GDF1 alters malignant hematopoiesis by promoting differentiation, as well as by specifically promoting erythropoiesis (red blood cell development). Interestingly, recombinant GDF1 did not appear to have an impact on normal blood cell differentiation. Additionally, recombinant GDF1 treatment exerted therapeutic efficacy in combination with the standard of care drug cytarabine (AraC). Taken together, this study provides evidence for the use of recombinant GDF1 as a potential therapy for AML due to its unique ability to regulate malignant hematopoiesis.

3.3. Results

The physiological impact of recombinant GDF1 was first assessed using bone marrow from transgenic mouse models of AML. When bone marrow from DN-AML transgenic mice was treated with recombinant GDF1 *in vitro*, blood cell differentiation was improved. This was evident by an increase in CFU-M, BFU-E, and CFU-E and reduction in CFU-GM. Recombinant GDF1 treatment effectively promoted differentiation while decreasing the number of immature cells in this model (Figure 3.1). Notably, these results also demonstrated that recombinant GDF1 promoted erythropoiesis (red blood cell development). Importantly, recombinant GDF1 treatment did not significantly affect blood cell differentiation of bone marrow from wildtype mice (Figure 3.1). Therefore, recombinant GDF1 appears to only affect abnormal, malignant hematopoiesis, while not impacting normal hematopoiesis.

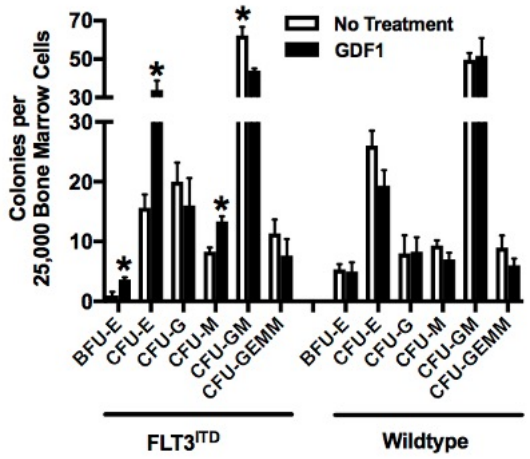


Figure 3.1. GDF1 treatment promotes hematopoiesis in leukemic mice alone. Bone marrow from $FLT3^{ITD}$ transgenic and C57BL/6J wildtype mice were treated with recombinant GDF1 in colony forming assays for 10 days. GDF1 caused a decrease in CFU-GM (immature myeloid cells) and an increase in maturation of monocytes (CFU-M) and erythrocytes (BFU-E and CFU-E) from $FLT3^{ITD}$ mice but not wildtype mice, suggesting GDF1 only impacts abnormal hematopoiesis (*$p<0.05$, 1-way ANOVA).

Next, the Nup98-HoxD13 transgenic mouse model of AML-MRC was treated with recombinant GDF1, as well as with Lip-C6 and its control nanoliposomal Ghost (Lip-Ghost). Flow cytometry of harvested bone marrow revelaed that recombinant GDF1 promoted erythropoiesis, as evident by the increase in erythroid progenitors to a similar degree of that triggered by Lip-C6 (Figure 3.2). This treatment also reduced the amount of immature myeloid cells (myeloid leukemia cells), again in a similar effect of that of Lip-C6, suggesting that recombinant GDF1 also regulates myelopoiesis in AML-MRC (Figure 3.2).

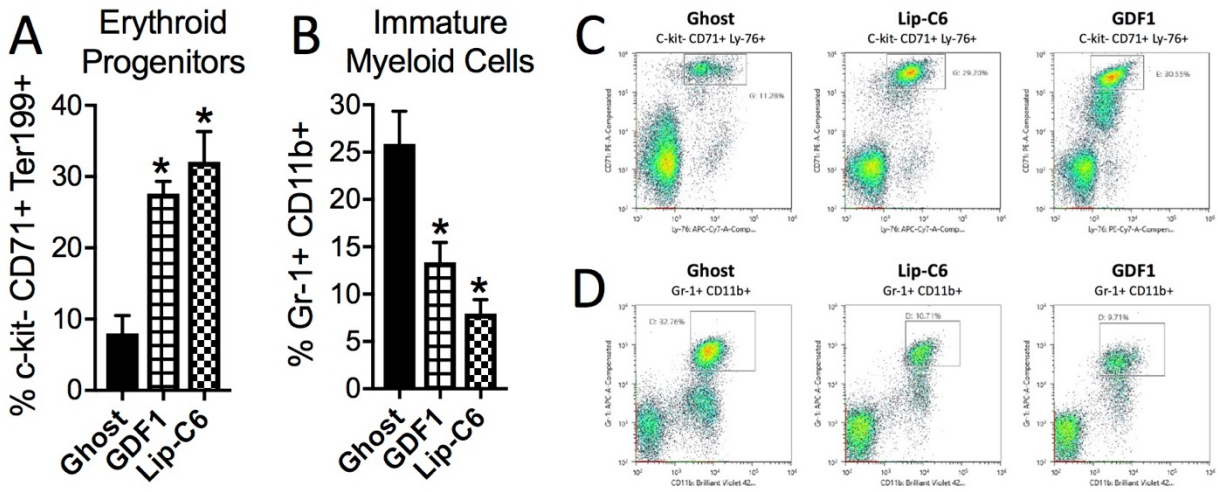


Figure 3.2. GDF1 regulates hematopoiesis in mice with AML-MRC. Nup98-HoxD13 transgenic mice were injected with recombinant GDF1, Lip-C6, or Lip-Ghost for 10 days. Following treatment, mice were euthanized, and bone marrow erythroid progenitors (A, C) and immature myeloid cells (B, D) were assessed using flow cytometry. Erythroid progenitors were upregulated while immature myeloid cells (myeloid leukemia blasts) were downregulated by GDF1 or Lip-C6 treatment (*$p<0.05$, 1-way ANOVA).

The effects of recombinant GDF1 treatment on the survival of mice engrafted with C1498 AML cells was also assessed. Overall survival was not significantly impacted by treatment with recombinant GDF1 or the standard of care agent AraC alone. However, combinatorial treatment with recombinant GDF1 and AraC significantly extended overall survival in this highly aggressive DN-AML model (Figure 3.3). These results suggest that recombinant GDF1 may be an effective agent to be combined with standard of care anti-AML chemotherapy, which can elevated endogenous ceramide levels, as well as other ceramide-based therapeutics.

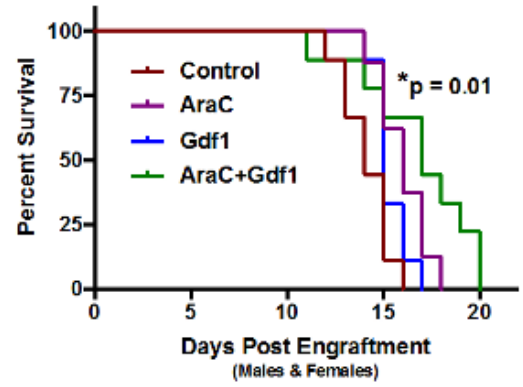


Figure 3.3. GDF1 improves the survival rate of mice engrafted with AML and has a combinatorial effect with AraC. C57BL/6J wildtype mice were engrafted with C1498 AML cells, and then treated with recombinant GDF1 +/- AraC. Logrank analysis was used to evaluate survival. AraC: cytarabine.

3.4. Discussion

These findings substantiate recombinant GDF1 as a potential treatment for AML. Current therapies for AML include drugs such as AraC, as well as novel therapies under development, such as Lip-C6 (Barth et al., 2019; Galmarini et al., 2002). Unfortunately, AML often develops resistance to these therapies. For that reason, the development of strategies to combat resistance is a pressing challenge. Previously, GDF1 was shown to regulate sphingolipid metabolism by downregulating genes associated with ceramide neutralization (Wang et al., 2019). In addition, it was previously shown that GDF1 signaled uniquely by stimulating SMAD2/3 phosphorylation while downregulating STAT3 tyrosine phosphorylation in a TGFβR1-dependent manner (Wang et al., 2019). The present study further demonstrates, for the first time, that recombinant GDF1 can impact malignant hematopoiesis by promoting differentiation along the myeloid and erythroid lineages. This may be beneficial as recombinant GDF1 only impacted malignant hematopoiesis and not that from normal controls, highlighting the reduced possibility of off-target effects. However, it is important to note that the present study focused solely on

hematopoiesis in the bone marrow, and so its impacts on hematopoiesis in other tissues remains in question. For instance, another group has found that a GDF15, another member of the TGFβ superfamily, regulates stress erythropoiesis in the spleen (Hao et al., 2019). Therefore, future research may focus on the hematopoietic-regulatory role of GDF1 in other tissues.

The promotion of erythropoiesis (red blood cell development) by GDF1 could have further applications beyond AML. In low-risk MDS, incomplete erythropoiesis leads to macrocytic anemia (Sibon et al., 2019). Other forms of anemia with red blood cell deficiency including congenital hyperplastic anemia, also known as Diamond-Blackfan anemia as well as anemia occurring with chronic illnesses such as infection and inflammatory disorders are also triggered by failed differentiation of erythroid progenitors (Lipton et al., 1986; Means and Krantz, 1992). It is possible, then, that the promotion of erythropoiesis by recombinant GDF1 may be useful in the treatment of anemia in a variety of other conditions.

These results further showed that recombinant GDF1 exerted combinatorial anti-AML efficacy with AraC. In addition to acting as a nucleoside analog to promote DNA damage, AraC also promotes the generation of endogenous ceramide (Galmarini et al., 2002; Saddoughi et al., 2008). Therefore, these two treatments may work together by AraC triggering ceramide production and recombinant GDF1 preventing the breakdown of ceramide. Recombinant GDF1 and AraC combined to significantly extend leukemic mouse survival, whereas single-agent treatments had no beneficial effect on lifespan. Strikingly, this was a significant increase in lifespan using a highly aggressive murine model of DN-AML.

In summary, this study demonstrated that recombinant GDF1 is a powerful regulator of leukemic (abnormal) mouse hematopoiesis that does not affect wildtype (normal) mouse hematopoiesis. This is an exciting finding, as it demonstrates that recombinant GDF1 may be

unlikely to trigger side effects impacting normal, noncancerous hematopoiesis and erythropoiesis. Increased survival due to combinatorial treatment with recombinant GDF1 and AraC further indicates that recombinant GDF1 may be a suitable treatment to combine with ceramide-based therapeutics. Future work may focus on the use of recombinant GDF1 in other models of AML, as well as the combination of recombinant GDF1 with Lip-C6 or other ceramide-based therapeutics.

3.5. Materials and Methods

Cell culture

Murine C1498 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cultures were incubated at 37°C and 5% CO2.

Animal studies

C57BL/6-Tg(Vav1-NUP98/HOXD13)G2Apla/J (Nup98-HoxD13) transgenic mice are a murine model of MDS, which fully recapitulates the cellular features of MDS and predictably evolves to AML-MRC (Slape et al., 2008). In contrast, B6.129-Flt3^{tm1Dgg}/J (Flt3^{ITD}) transgenic mice were used as models of DN-AML (Loghavi et al., 2014). Alternatively, wildtype C57BL/6J mice were engrafted with 1 x 10^6 C1498 AML cells for combinatorial therapeutic studies.

Bone marrow cells were isolated from the femurs and tibias of 4–8-month-old (male and female) transgenic mice and their respective wildtype littermates for colony-forming assay or flow cytometry analysis. Mice were submitted to the New Hampshire Veterinary Diagnostic Laboratory (NHVDL) at the University of New Hampshire for histopathological evaluation. Leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on

spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of New Hampshire.

Ceramide Nanoliposome formulation and treatment

Lip-C6 and Lip-Ghost were generously prepared and provided by Dr. Mark Kester of the University of Virginia. Briefly, lipids dissolved in chloroform, were combined in specific molar ratios, dried to a film under nitrogen, and then hydrated by addition of 0.9% NaCl. Solutions were sealed, heated at 60°C for 60 minutes, subjected to vortex mixing, and sonicated until light no longer diffracted through the suspensions. Lipid vesicle-containing solutions were then extruded at 60°C by passing the solutions 10 times through 100 nm polycarbonate filters using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Size and charge were validated using a Malvern Zetasizer Nano ZS at 25°C, and C6-ceramide encapsulation into anionic formulations was quantified by mass spectroscopy. Liposomal suspensions were stored at room temperature until use.

Therapeutic efficacy of Lip-C6, Lip-Ghost, or recombinant GDF1 as a standalone treatments were evaluated using transgenic mouse models. 4–8-month-old (male and female) Nup98-HoxD13 transgenic mice were injected with Lip-C6 (11.6 mg/kg), Lip-Ghost (volume matched), or recombinant GDF1 (2 μg/kg) daily for 10 days. One day after the final injection, mice were euthanized and bone marrow was collected from femurs and tibias, spleens were removed and measured, and blood was collected by cardiac puncture for blood smears.

Recombinant GDF1 formulation and treatment

Recombinant human GDF1 was purchased from R&D Systems. It arrived in a lyophilized form with BSA as a carrier protein and was re-suspended according to the manufacture's direction. Therapeutic efficacy of recombinant GDF1 as a standalone treatment was evaluated *in vitro* with colony-forming assays using bone marrow from $FLT3^{ITD}$ transgenic or C57BL/6J wildtype mice as described below. In addition, therapeutic efficacy of recombinant GDF1 as a standalone treatment was evaluated *in vivo* using Nup98-HoxD13 transgenic mice as described above. Lastly, therapeutic efficacy of recombinant GDF1 in combination with AraC was evaluated using 4–8-month-old (male and female) C1498-engrafted wildtype mice. Mice were treated with recombinant GDF1 (2 μg/kg) +/- AraC (50 ng/kg), three times per week for two weeks and survival was monitored.

Colony-forming assays

Murine bone marrow mononuclear cells were isolated and prepared from transgenic mice and their wildtype counterparts and cleaned of debris using Ficoll-Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation. Prior to colony-forming assays, leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. Murine samples were cultured in triplicate in 12-well plates at a density of 2.5×10^4 cells per well in Murine MethoCult GF (#M3434) (StemCell Technologies, Vancouver, BC). The plating density was selected to yield typical wildtype or transgenic mouse colony out-growth of ~30-150 colonies per well in the absence of drug treatment. Sterile water was added into empty wells, and space between wells, to maintain optimal humidity necessary for colony growth. During assay set-up, murine bone

marrow cells were added simultaneously to the culture media with recombinant GDF1 (200 ug/ml) or controls. The cultures were mixed vigorously and then dispensed to multi-well plates and incubated for 10-14 days. Blinded investigators manually counted each type of colonies and total colonies, including blast or miscellaneous colonies. Other types of colonies counted were CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM colonies.

Flow cytometry

Isolated bone marrow cells from transgenic mice were prepared and stained with Mouse Fc Block, fluorophore-conjugated anti-mouse Gr-1 (clone RB6-8C5), fluorophore-conjugated anti-mouse CD11b (clone M1/70), fluorophore-conjugated anti-mouse CD117 (clone 2B8), fluorophore-conjugated anti-mouse CD71 (clone C2), and fluorophore-conjugated anti-mouse TER-119/LY-76 (clone TER-119) monoclonal antibodies from BD Biosciences (San Jose, CA). Gr-1+ CD11b+ cells were evaluated and quantified as representative of leukemia burden (immature myeloid cells). CD117- CD71+ Ter119+ cells were evaluated and quantified as representative of erythroid cells. Flow cytometry was performed at the University of New Hampshire's University Instrumentation Center using a Sony SH800Z sorting flow cytometer.

Statistical analysis

GraphPad Prism (GraphPad Software, La Jolla, CA) was used to perform data analyses. Comparisons of colony-forming and flow cytometry data were made using 1-way ANOVA followed by a Tukey's multiple comparisons test. The Mantel-Cox Logrank test was used to determine survival significance between *in vivo* treatment groups.

CHAPTER 4[6]

OBESITY PROMOTES ACUTE MYELOID LEUKEMIA PROGRESSION BY UPREGULATING THE CERAMIDE-MEDIATED NADPH OXIDASE 2

4.1. Abstract

Acute myeloid leukemia (AML) is a type of blood cancer of the myeloid cell lineage. Obesity is characterized by an increase in body weight that results in excessive fat accumulation. Obesity has been associated with an increased incidence of many cancers, including blood cancers. This study evaluated the role obesity in AML progression in a novel transgenic mouse model developed by crossing $Flt3^{ITD}$ mice with $Lep^{ob/ob}$ mice. Leukemia burden was augmented in obese AML mice. In addition, it was determined that obesity upregulated the ceramide-mediated and ceramide-1-phosphate-mediated NADPH oxidase 2. Notably, increased oxidative pathways has been attributed to disease progression in AML. Taken together, this study demonstrates a direct link between obesity and the progression of AML in part by augmenting the ceramide-mediated NADPH oxidase 2.

4.2. Introduction

Cancer is a group of diseases involving abnormal cell growth that has the potential to invade into other parts of the body (Font-Burgada et al., 2016). Acute myeloid leukemia (AML) is a type of blood cancer of the myeloid cell lineage. It most commonly occurs in older adults

[6] [6] Weiyuan Wang, Paul T. Toran, Rachel J. Sabol, Tamara G. Hathorn, Emma J. Arsenault, Andrea L. Cote, Emily C. Sullivan, Vasiliki Papakotsi, Brian M. Barth. Obesity Promotes Acute Myeloid Leukemia Progression by Upregulating the Ceramide-Mediated NADPH Oxidase 2. In preparation for submission.

over 60 years old and accounts for the largest number of annual deaths from leukemia in the United States (O'Donnell et al., 2013). In recent years, obesity has been found to be an important risk factor for the development of cancer, including blood cancers. Obesity can promote the progression of cancer through several systemic mechanisms, such as causing chronic inflammation; producing excess amounts of estrogen, insulin, adipokines; as well as changing the mechanical properties of the scaffolding surrounding cancer cells (Gallagher and LeRoith, 2015; Gregor and Hotamisligil, 2011; Seo et al., 2015). Chronic inflammation takes place at particular organ sites, including the liver, pancreas, and gastrointestinal tract (Font-Burgada et al., 2016). Obesity-associated inflammation can dramatically alter tissue composition, thereby creating a fertile environment for cancer development (Olson et al., 2017).

Sphingolipids are a class of lipids composed of sphingoid base and fatty acids with ceramide being a hypothetical central metabolite. Sphingolipid metabolism is a complex network of interconnected pathways. Sphingolipids are formed via the metabolism of sphingomyelin, a constituent of the plasma membrane, through metabolism of other complex sphingolipids, or by *de novo* synthesis. In addition to functioning as structural elements, sphingolipids also participate in a variety of cellular pathways including apoptosis, cell senescence, the cell cycle, and cell differentiation. Several sphingolipid mediators including ceramide and ceramide-1-phosphate (C1P) have been found to play an integral role in inflammation (Nixon, 2009).

This research study evaluated a link between obesity and AML in a novel transgenic mouse model. The impact of obesity on the progression of AML was further explored by studying the expression of genes encoding sphingolipid-metabolizing enzymes involved in inflammation and oxidative stress. Increased leukemia burden was observed in obese AML mice, which also upregulated inflammatory sphingolipid metabolism and the NADPH oxidase 2.

4.3. Results

Initially, a transgenic obese AML mouse model was developed by crossing the $Flt3^{ITD}$ and $Lep^{ob/ob}$ mice. The $Lep^{ob/ob}$ mouse is a mjaor model of obesity that arises due to a spontaneous mutation in the gene encoding the hormone leptin (Lutz and Woods, 2012). Leptin is produced predominantly by adipocytes (Mohamed-Ali et al., 1998). It helps regulate energy balance by inhibiting hunger and diminishing fat storage in adipocytes. In obesity, the body develops insensitivity to leptin, thus is unable to detect satiety (Pan et al., 2014). Homozygous leptin-deficient mice are phenotypically indistinguishable from their unaffected littermates at birth, but gain weight rapidly throughout their lives, reaching a weight over twice that of unaffected mice. The $Flt3^{ITD}$ transgenic mouse is a model where a myeloproliferative neoplasm evolves to AML. FMS-like tyrosine kinase-3 (FLT3) is a receptor tyrosine kinase and proto-oncogene involved in crucial steps of hematopoiesis such as proliferation, differentiation and survival. The $FLT3^{ITD}$ mutation has been strongly associated with oor prognosis, leukocytosis, high blast counts, increased risk of relapse and shorter overall survival of AML patients (Lagunas-Rangel and Chávez-Valencia, 2017). These two mouse models were crossed to develop the $Flt3^{ITD}$ x $Lep^{ob/ob}$ mouse model to further study the role of obesity in AML development and progression. Notably, the $Flt3^{ITD}$ x $Lep^{ob/ob}$ mouse model had similar weight gain as the $Lep^{ob/ob}$ mouse model and they were indistinguishable in appearance (Figure 4.1). In addition, peripheral blood smears demonstrated that the transgenic obese AML model ($Flt3^{ITD}$ x $Lep^{ob/ob}$ mice) developed a more robust leukemia as evidenced by the substantial appearance of blasts as compared with the $Flt3^{ITD}$ transgenic AML mouse model (Figure 4.2).

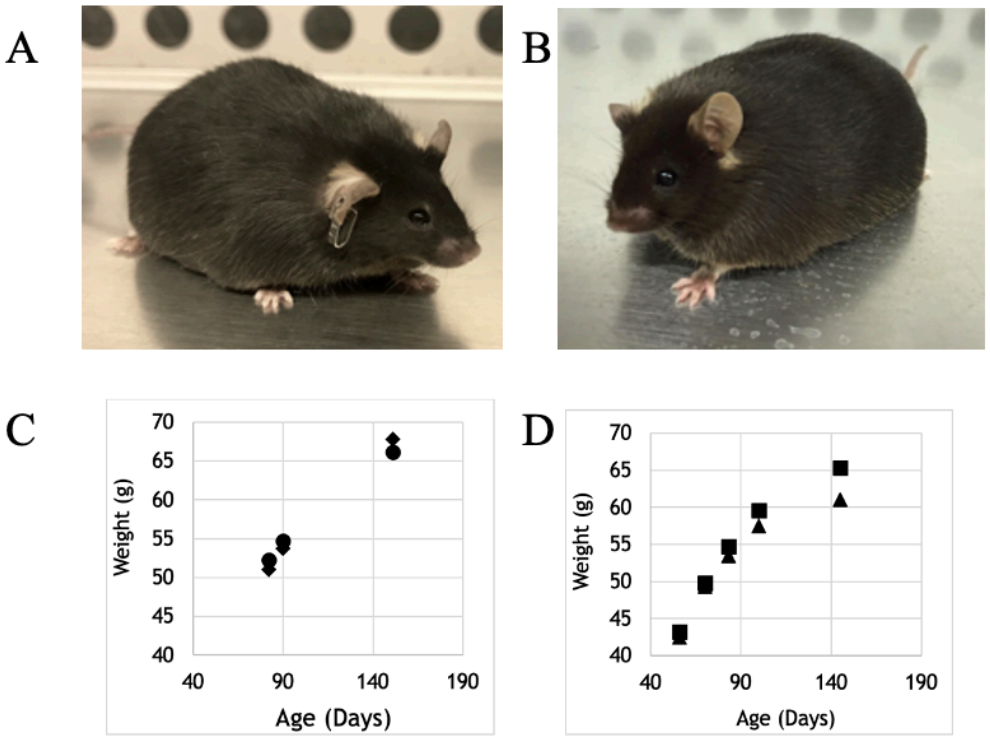


Figure 4.1. Flt3^{ITD} x Lep$^{ob/ob}$ mice have similar weight gain as Lep$^{ob/ob}$ mice. An aged Flt3^{ITD} x Lep$^{ob/ob}$ mouse (A) and an aged Lep$^{ob/ob}$ mouse (B) are demonstrated visually. Weight gain patterns of Flt3^{ITD} x Lep$^{ob/ob}$ mice (C) and Lep$^{ob/ob}$ mice (D). As the age of these transgenic mice approaches 160 days, their weight plateaus around 65 grams.

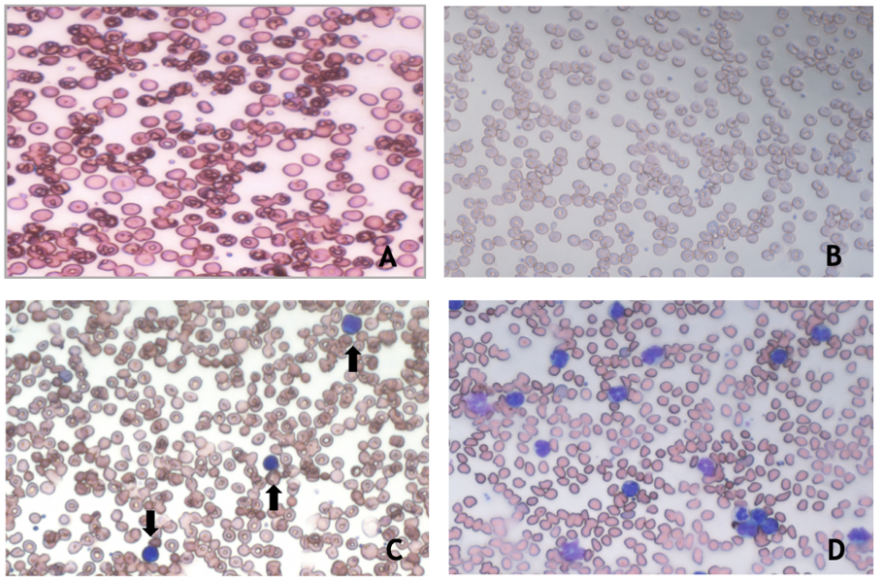


Figure 4.2. Flt3^{ITD} x Lep$^{ob/ob}$ mice developed robust AML compared with Flt3^{ITD} mice. A peripheral blood smear from a wildtype mouse (A) displays all small, pink, and normal red blood cells, indicative of normal and nonmalignant cells. Similarly, a peripheral blood smear from a Lep$^{ob/ob}$ mouse (B) displays a similar appearance, indicative that obese mice likewise do not have notable phenotypic changes in the blood system. In contrast, a peripheral blood smear from a Flt3^{ITD} mouse (C) displays a handful of blasts, indicative that the mouse has leukemic transformation. Most notably, a peripheral blood smear from a Flt3^{ITD} x Lep$^{ob/ob}$ mouse (D) shows considerably greater number of blasts than that of the Flt3^{ITD} mouse (C), indicative that the malignancy has more robustly developed.

The impact of obesity on ceramide metabolism and signaling in AML was next investigated to further explore a possible mechanism where obesity can promote AML progression. Obesity is known to induce several pathological conditions including insulin resistance, in which glucose metabolism and ceramide metabolism play key pathological roles (Longato et al., 2012). Obesity-driven increases in ceramide have been linked to glucose intolerance (Turpin et al., 2014). Acid sphingomyelinase (SMPD1) is an enzyme that hydrolyzes sphingomyelin to liberate ceramide, modulating membrane properties and signal transduction

pathways including those mediated by inflammation (Gorelik et al., 2016). Ceramide kinase (CERK) is a lipid kinase that is responsible for the phosphorylation of ceramide to ceramide-1-phosphate (C1P) at the plasma membrane and in Golgi complex (Boath et al., 2008). NADPH oxidase 2 is a superoxide generating enzyme. Superoxide is crucial in killing foreign bacteria in the human body. Decreased activity of NADPH oxidase 2 can lead to an increased susceptibility to organisms such as catalase-positive microbes, while its increased activity can lead to oxidative stress (Fuentes et al. 2018). Both ceramide and C1P have been found to activate NADPH oxidase 2 to participate in redox-mediated signaling processes (Barth et al., 2012).

In the present study, obesity in the $Flt3^{ITD}$ x $Lep^{ob/ob}$ mouse model was shown to lead to aberrant gene expression involving aspects of sphingolipid metabolism and redox signaling, reflected increased gene expression of *Smpd1*, *Cerk*, and *Nox2* (encodes primary membrane-bound subunit of NADPH oxidase 2) (Figure 4.3). Collective upregulation of these genes enables the ceramide-mediated NADPH oxidase 2 (Figure 4.4), where inflammatory stress can provoke ceramide and C1P generation that promotes activation of NADPH oxidase 2. Activation of this pathway would enable redox-mediated progression of AML, similar as observed in the $Flt3^{ITD}$ x $Lep^{ob/ob}$ obese AML transgenic mouse model.

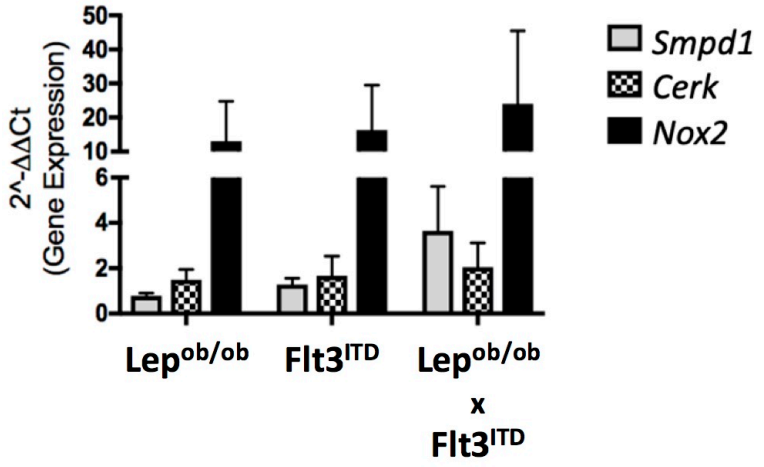


Figure 4.3. *Smpd1*, *Cerk*, and *Nox2* are upregulated in Flt3^{ITD} x Lep$^{ob/ob}$ mice. Bone marrow from age-matched wildtype, Lep$^{ob/ob}$, Flt3^{ITD}, and Flt3^{ITD} x Lep$^{ob/b}$ mice was harvested and analyzed by RT-qPCR (wildtype mice were used as a fully-normal control). Expression of *Smpd1*, *Cerk*, and Nox2 was elevated in all mice and was highest in the Flt3^{ITD} x Lep$^{ob/b}$ obese AML mouse model. n=3 per mouse.

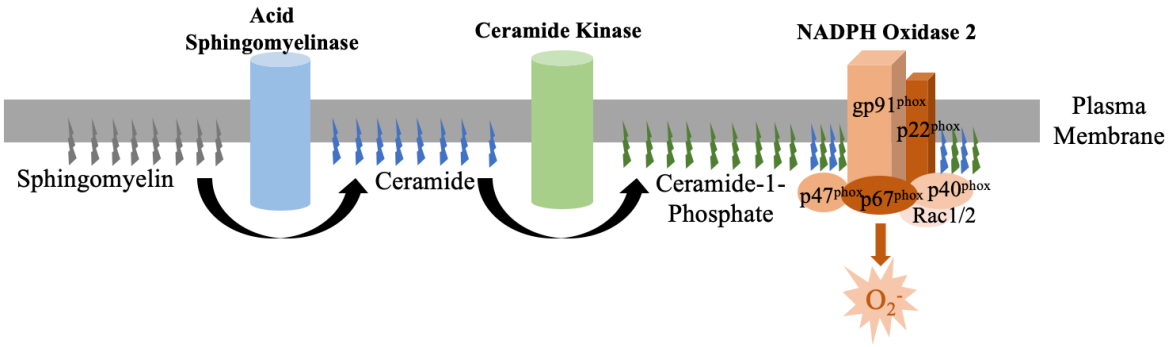


Figure 4.4. Schematic diagram of the ceramide- and C1P-mediated NADPH oxidase 2. Acid sphingomyelinase generates ceramide from sphingomyelin, including in response to obesity-driven inflammation. Ceramide kinase coverts ceramide into ceramide-1-phosphate. Both ceramide and ceramide-1-phosphate activate NADPH oxidase 2, which generates superoxide (O_2^-). Membrane subunits of NADPH oxidase 2 include gp91^{phox} and p22^{phox}. Cytosolic subunits of NADPH oxidase 2 include p47^{phox}, p67^{phox}, p40^{phox}, and Rac1/2.

4.4. Discussion

Obesity is an important risk factor for the development of cancer, although the relationship is not as clear as that between tobacco use and lung cancer (Zitvogel et al., 2017). Nearly 25% of the relative contribution to cancer has since been ascribed to being overweight and obese (Nimptsch and Pischon, 2016). It is widely agreed that obesity promotes both primary tumor growth and metastatic progression through several systemic mechanisms (Olson et al., 2017). In particular, obesity-associated oxidative stress has been found to cause genetic damage, facilitating the transformation of premalignant cells (Manna and Jain, 2015). Moreover, NADPH oxidase-dependent redox-signaling has been shown to promote proliferative signaling.

In this study, the role of obesity in the progression AML was evaluated in a novel transgenic obese AML mouse model ($Flt3^{ITD}$ x $Lep^{ob/ob}$ mice). Interestingly, these mice had similar weight gains as their obese littermates but developed a more robust AML than non-obese $Flt3^{ITD}$ transgenic AML mice, indicative that obesity augments the progression of AML. Mechanistically, obesity upregulates the expression of genes encoding for the: 1) ceramide-generating enzyme SMPD1, 2) C1P-generating enzyme CERK, and 3) primary membrane-bound NADPH oxidase 2 subunit. Both ceramide and C1P can activate the NADPH oxidase 2 to promote redox signaling and oxidative stress. Therefore, these results indicate that transgenic obese AML mice have a high oxidative burden. By increasing oxidative stress and redox-signaling within the AML cells obesity may accelerate the molecular evolution of AML (Marseglia et al., 2014), as well as promote a more proliferative cellular state.

There are other mechanisms through which obesity can promote cancer progression. For example, at a primary tumor site, obesity increases myofibroblast content, which stiffens extracellular matrices and enhances cancer cell growth. Inflammasome activation in tumor-

infiltrating myeloid cells has also been shown to drive increased IL-1β signaling in tumors. In response, adipocytes express more VEGF-A, which increases tumor angiogenesis (Kolb et al., 2016). Additionally, cholesterol metabolites have been shown to promote metastasis of cancer by promoting the epithelial-mesenchymal transition (Nelson et al., 2013). Moreover, at the metastatic site, IL5Ralpha+ monocytes generate GM-CSF and promote neutrophil expansion (Quail et al., 2017). Circulating neutrophilia occurs in response to the increased GM-CSF and can promote seeding and outgrowth of disseminated cancer cells (Olson et al., 2017).

Altogether, interventions are being investigated to mitigate the effects of obesity in cancer. Caloric restriction is the most widely studied strategy. Caloric restriction has been found to reverse breast inflammation associated with obesity in women (Bhardwaj et al., 2013). Second, lifestyle interventions, such as exercise and fasting, have been found to improve immune function in preclinical studies. Fasting increases the levels of lymphoid progenitor cells and cytotoxic CD8+ lymphocytes, enhancing the targeted killing of cancer cells (Di Biase et al., 2016). In conclusion, it is imperative to understand mechanisms by which obesity affects cancer progression. In this way, better personalized approaches to treat obese cancer patients can be developed.

4.5. Materials and methods

Animal studies

B6.129-Flt3^{tm1Dgg}/J (Flt3^{ITD}) transgenic mice develop a myeloproliferative neoplasm that evolves to AML (Loghavi et al., 2014). FLT3 is a receptor tyrosine kinase. It is important for the development of the hematopoietic and immune systems. The FLT3^{ITD} mutation is one of the most common molecular abnormality in acute myeloid leukemia (Levis and Small, 2003). Mice

that are homozygous for this targeted mutation develop a progressive myeloproliferative disease, exhibiting increased myeloid and monocytic cell populations and reduced B and T cell lymphoid cell numbers. These FLT3-mutant transgenic mice were crossed with B6.Cg-Lep^{ob}/J (*Lep^{ob}*, commonly referred to as *ob* or *ob/ob*) mice. Homozygous *Lep$^{ob/ob}$* mice have a spontaneous mutation in the gene encoding for the hormone leptin, which normally regulates energy homeostasis and appetite. These homozygous leptin-deficient mice exhibit obesity, hyperphagia, transient hyperglycemia, glucose intolerance, and elevated plasma insulin. Therefore, crossing FLT3-mutant mice with leptin-deficient mice resulted in a new transgenic mouse that develops AML in the context of obesity.

Bone marrow cells were isolated from the femurs and tibias of 4–8-month-old (male and female) transgenic mice and their respective wildtype littermates for RT-qPCR analysis. Mice were submitted to the New Hampshire Veterinary Diagnostic Laboratory (NHVDL) at the University of New Hampshire for histopathological evaluation. Leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of New Hampshire.

Peripheral blood smear preparation

Peripheral blood smears are prepared by placing a drop of blood on one end of a slide and using a spreader slide to disperse the blood over the slide's length. The slide is left to air dry and stained with Wright-Giesma stain to distinguish the cells from each other. The eosin Y dye stains red blood cells a faint pink color, while the methylene blue and azure B dyes stains lymphocytes

a purple-blue color (Schulte et al., 1989). The slide is left to air dry for roughly 24 hours before it is observed under a light microscope.

RT-qPCR analysis

Total cellular RNA from mouse bone marrow samples was extracted and cleaned using Zymo Research kits. RNA quality was evaluated using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific), and reverse transcribed with Taq polymerase to generate cDNA. Quantitative polymerase chain reaction (qPCR) was performed using PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies) on a CFX96 Real-Time PCR System (Biorad). Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method using cycle threshold (Ct) values for target genes as well as two endogenous reference genes (*ACTB* and *TBP*). Primer and probe assays were obtained from Integrated DNA Technologies.

Statistical analysis

GraphPad Prism (GraphPad Software, La Jolla, CA) was used to perform data analyses. Comparisons of RT-qPCR data were made using student's t-tests as well as 1-way ANOVA followed by a Tukey's multiple comparisons test.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5.1. General Conclusions

Growth/differentiation factor 1 (GDF1) is transcribed from a bicistronic gene and can engage transforming growth factor beta receptor 1 (TGFβR1) to mediate cellular effects including regulation of gene expression by the SMAD2/3 pathway. Currently, the understood role of GDF1 is limited to left-right patterning, including during cardiac development, as well as mesoderm induction during embryonic development. Little else is known about the role of GDF1, especially outside of embryonic development, although many related members of the transforming growth factor beta (TGFβ) superfamily have been attributed roles in stem cell biology. Previously, nanoliposomal C6-ceramide (Lip-C6) was shown to exert preclinical therapeutic efficacy in acute myeloid leukemia (AML) with myelodysplastic syndrome (MDS)-related changes (AML-MRC) whereas other types of AML were resistant due to enhanced ceramide metabolism. Intriguingly, *GDF1* expression is likewise low in these Lip-C6-resistant AMLs. Therefore, it was postulated that GDF1 may have anti-AML therapeutic efficacy due to an ability to regulate ceramide metabolism.

In the first research study (Chapter 2), *GDF1* was observed to be variably expressed in AML, and mostly downregulated in Lip-C6-resistant AML. More so, it was shown that GDF1 downregulated the expression of *UGCG* and *SGMS1*, both of which encode enzymes that metabolize ceramide into non-toxic sphingolipids. Lastly, GDF1 was observed to uniquely promote SMAD2/3 phosphorylation while downregulating STAT3 tyrosine phosphorylation,

both in a TGFβR1-dependent manner. In the second research study (Chapter 3), it was found that recombinant GDF1 promotes hematopoiesis by decreasing immature myeloid cells while increasing the maturation of monocytes and erythrocytes. Moreover, it was found that recombinant GDF1 exerted combinatorial anti-AML efficacy with cytarabine (AraC). Collectively, the studies reported in Chapters 2 and 3 demonstrate an anti-AML effect for GDF1 that is inherently linked to its ability to regulate ceramide metabolism. In the final research study (Chapter 4), a transgenic obese AML mouse model was developed to study links between obesity and the development and progression of AML. These obese AML mice develop a more robust AML than non-obese counterparts and upregulate elements of the ceramide-mediated NADPH oxidase 2. In that way, obesity can activate a sphingolipid-mediated pathway that is responsive to obesity-driven inflammation to augment AML development and progression.

5.2. Future Directions

In the future, drug discovery efforts are planned to uncover compounds that regulate ceramide metabolism including regulators of GDF1. These studies are further planned to evaluate anti-AML therapeutic efficacy in combination with ceramide-based therapeutics such as Lip-C6. Previous studies have demonstrated an anti-AML therapeutic ability of bioactive compounds found in a plant called Devil's Club (*Oplopanax horridus*). This is a medicinal plant used among indigenous people of Southeast and Southcentral Alaska and the coastal Pacific Northwest. Future studies are planned to extract bioactive compounds of Devil's Club, and other medicinal plants, to uncover compounds that can regulate ceramide metabolism, GDF1, as well as exert anti-AML efficacy. This will shed more light on the potential of medicinal plants for the development of novel AML therapeutics.

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