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The Association Between Oxidative Stress, Cellular Differentiation And Galectins In Human Promyelocytic Leukemia Cells (HL-60)

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Abstract

Galectins are a group of β-galactoside-binding proteins involved in different cellular processes including stress responses and differentiation. The role and expression of galectins under oxidative stress and during neutrophilic differentiation was examined in HL-60 cells. Galectin gene (*LGALS*), and galectin protein expression were determined using RT-qPCR and immunoblotting, respectively. Neutrophilic differentiation was measured via a spectrofluorometric assay. DNA methylation and JNK signaling were investigated as galectin regulatory mechanisms. Menadione-induced oxidative stress, DMSO-induced differentiation, DNA hypomethylation and JNK signaling all promoted similar galectin expression profiles. Antioxidant N-acetylcysteine attenuated the menadione-induced galectin expression but only partially attenuated DMSO-induced galectin expression. Galectin inhibitory sugars decreased cell proliferation and inhibited differentiation. Finally, correlative analysis suggests galectins are biomarkers of oxidative stress and cellular differentiation. My findings indicate that galectins represent novel therapeutic targets in the context of acute myeloid leukemia and their expression profiles can be considered biomarkers of oxidative cell stress and differentiation.

Keywords

Galectins, oxidative stress, hydrogen peroxide, cellular differentiation, decitabine, menadione, JNK

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List of Abbreviations

ANOVA	Analysis of variance
AP-1	Activator protein 1
AzaC	5-azacytosine
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CRD	Carbohydrate recognition domain
DAPI	4', 6-diamidino-2-phenylindole
IMDM	Iscove's modification of Dulbecco's Modified Eagles medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ERK	Extracellular regulated kinase
FBS	Fetal bovine serum
fMLP	N-formylmethionyl-leucyl-phenylalanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hanks balanced salt solution
HL-60	Human promyelocytic leukemia cells
ho-1	Heme oxygenase 1
H-Ras	V-Ha-Ras Harvey Rat Sarcoma Viral Oncogene Homolog
IL-2	Interleukin 2
IL-10	Interleukin 10
JNK	c-Jun N-terminal kinase
K_d	Dissociation constant
LacNac	N-acetyl-D-lactosamine
LGALS	Lectin, galactoside binding, soluble
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mRNA	Messenger ribonucleic acid
NAC	N-acetylcysteine

NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NQO1	NADPH quinone oxidoreductase 1
PBS	Phosphate-buffered saline
PMSF	phenylmethane sulfonyl fluoride
p47*phox*	Neutrophilic cytosolic factor 1
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sp1	Specificity protein 1
TBST	Tris buffered saline with Tween
TDG	Thiodigalactoside
TGF-β	Transforming growth factor beta
Tris	Trisaminomethane
α-MM	α-methyl-D-mannoside

Chapter 1

1 Introduction

1.1 Galectins – an overview

Galectins are a family of glycan-binding proteins with binding specificity toward β-galactosides (Barondes et al., 1994). Originally, discovered in 1975 by Teichberg et al. the first vertebrate galectin from *Electrophorus electricus* (eel) was originally named "electrolectin", and was found to be inhibited by β-D-galactosyl residues while having lectin like activity within mammalian cell culture (Teichberg et al., 1975). Shortly thereafter other research groups started purifying related lectins from other vertebrate tissues. Galectin-1 was purified in 1976 from calf heart and lung (de Waard et al., 1976), and the first avian galectin from chicken muscles (Nowak et al., 1976). Some years later galectin-3 was identified in fibroblasts (Roff and Wang, 1983), and then purified a year later from mouse lung (Crittenden et al., 1984). Galectins are relatively small in size and have specific binding toward N-acetyllactosamine-enriched glycoconjugates. They are part of a larger family of proteins known as lectins that are found more predominately in plants and exhibit high specificity toward many different sugar moieties (Varki et al., 2009). Since their original discovery galectins have undergone various name changes including: galaptin, S-Lac and S-type lectins before finally being designated as "galectin" in 1994 to avoid name discrepancies (Allen et al., 1990; Barondes et al., 1994; Thiemann and Baum, 2016). Today, 16 galectin genes have been identified in mammals with 12 galectins being characterized and sequenced in humans (Thiemann and Baum, 2016; Timoshenko, 2015) however, these proteins have been identified across all eukaryotic taxa. Others, for example, galectin-5 and -6 are found in rodents, while galectin-11 has been identified in sheep, but is not found in humans (Varki et al., 2009). Galectin activity has been well reported across many tissues and their differential regulation is important for maintaining cellular functions (Laderach et al., 2013). Since their original discovery, galectins have been identified in both vertebrates and invertebrates including: mammals, amphibians, fish, birds, reptiles, nematodes, sponges and even fungi (Gupta, 2012).

Galectins may be secreted into the extracellular matrix where they will bind their appropriate glycoreceptor at the cell surface; initiating a signal transduction pathway, which drives gene expression of factors important for various biological functions. Specifically, galectins serve as mediators of several fundamental biological processes including: cell growth (Manzi et al., 2016), apoptosis (Stillman et al., 2006), immune function (Chung et al., 2013), differentiation (Abedin et al., 2003), migration and adhesion (Perillo et al., 1998). Although all galectins display affinity toward lactose – N-acetyllactosamine and poly-N-acetyllactosamine residues are located on different glycoconjugates and modification of these sugar moieties can define galectin specific binding/functionality (Ahmad et al., 2004). Even though galactose is the basic recognition element for many galectins; actual glycans present on different proteins/lipids at the cell surface are more complicated *in vivo*. Galectins typically exist as dimers (also multimers) allowing a single galectin to bind multiple sites. Lectin-carbohydrate interactions are relatively weak; therefore, structural orientation of the peptide sequence of the galectin can allow for multiple galectin binding domains to bind their respective ligand at the same time, creating a stronger interaction and lowering the dissociation constant. For example, generally, galectins bind glycoreceptors containing N-acetyllactosamine residues with a dissociation constant (K_d) of 90 – 100 μM but galectins can also bind glycoreceptors containing polylactosamine sequences with stronger affinity: K_d value of 1 μM (Rabinovich et al., 2002). In a biological context, galectins do not bind the simple disaccharides alone but rather lactosamine sequences that are present on complex N-glycans and O-glycans on numerous branches leading to high-avidity binding since the affinity of each galectin binding site for its respective ligand is additive in a multimeric complex (Thiemann and Baum, 2016). Therefore, it is important to understand the structural differences of galectins to better define their roles in different tissues and explain why certain galectins are only expressed within specific tissues while others are not.

1.2 Structural features and classification

Galectins are grouped according to their structural domain composition (Hirabayashi and Kasai, 1993). Specifically, all galectins are characterized, identified and often classified according to their respective carbohydrate recognition domain(s) (CRD). The CRD

consists of about 135 amino acids, composing two protein β-sheets, which are formed by five and six β-strands each folded antiparallel to each other forming a pocket for the respective glycan to bind (Cooper, 2002; Thiemann and Baum, 2016). All galectins contain either one or multiple CRDs, distinguishing homo-dimeric from hetero-dimeric galectins. Galectins were once originally referred to as S-type lectins; this was to denote that many galectins contained free cysteine residues (Varki et al., 2009). In fact, it has been stated that certain galectins required the inclusion of β-mercaptoethanol in buffers to maintain their activity, perhaps because of the disulfide linkage of cysteine residues – important for proteins structure (Varki et al., 2009). More recent studies have shown that, mouse galectin-2 for example, only require a certain cysteine (Cys75) residue present in the amino acid motif of the CRD for this domain to exhibit lectin-binding capabilities while mutagenesis of other conserved free cysteine residues had no effect (Tamura et al., 2016). Galectin-1 for example, contains six cysteine residues (Cys2-Cys16, Cys42-Cys60, Cys88-Cys130), which are required to be in a reduced state to allow for proper carbohydrate binding activity and biological function (Kadoya and Horie, 2005; Klyosov, 2008). However, other studies have shown that site-directed mutagenesis of these cysteine residues into serine residues did not inhibit sugar binding (Liao et al., 1994) but that mutagenesis of other conserved hydrophilic residues abolished carbohydrate binding activity (Hirabayashi and Kasai, 1991). Interestingly, the amino acid sequences of the CRD are highly homologous across and within species (90% of the 134 amino acids are identical between humans and mice) (Kadoya and Horie, 2005). The structural features and organization of the CRD categorize galectins into the following 3 sub-families: proto-type, tandem-repeat and chimeric galectins.

Despite the CRD playing a dominant role in galectin function; other non-lectin domains also play functional roles. In fact, non-lectin domains of galectins are often crucial for specific protein-protein interactions – despite being less frequent than CRD-dependent interactions. Park et al. (2001) demonstrate that galectin-1 and -3 are factors required for pre-mRNA splicing within HeLa cells through a non CRD-dependent interaction (Park et al., 2001). Additionally, intracellular galectin-1 has been reported to interact with H-Ras, a gene frequently mutated in tumours to promote malignant differentiation, in multiple cell lines including HeLa, HEK293, Rat1 and Molt4 in a CRD-independent fashion (Camby et

al., 2006; Yu et al., 2015). Interestingly, Liu et al. (1996) used murine monoclonal antibodies to bind epitopes along the amino-terminal region of galectin-3, which inhibited galectin-mediated superoxide production (Liu et al., 1996). In fact, most galectins have features typical of cytoplasmic proteins including an acetylated N-terminus, free thiol groups and absence of glycosylation, which inherently affects their localization and functional capacity.

Galectin localization is important for determining their function. These proteins can work both intracellularly by interacting with various intracellular ligands or extracellularly by mediating cell-cell, cell-matrix interactions and by binding cell surface glycans to activate a signal transduction pathway (Gupta, 2012; Varki et al., 2009). Galectin-1 shows multiple functions depending on its subcellular localization. Intracellularly, galectin-1 can arrest cell growth while extracellular galectin-1 can induce proliferation or apoptosis (Stillman et al., 2005). Contrasting roles in galectin-1 localization function are likely due to CRD-dependent interactions happening exclusively in the extracellular matrix. Interestingly, however, galectins lack any signal sequence for classical secretion into the extracellular space despite the regulation of galectin secretion being markedly changed during different cellular processes (Hirabayashi and Kasai, 1993; Sato and Hughes, 1994). Additionally, structural characteristics of galectins including an acetylated N-terminus, absence of both disulfide bonds and glycan chains indicate that these proteins would normally reside within the cytoplasm despite being biologically active in the extracellular space and on the cell surface. It is surprising that galectins are secreted at all considering the oxidizing environment of the extracellular space (which would affect galectin folding). Therefore, the biological function of galectins in the oxidative extracellular space may be dependent on their immediate binding to glycoreceptors, which would prevent oxidation of free cysteine residues (Vasta, 2009). It has been proposed that galectins may exit cells according to the structure of the protein, the cell type and its polarity (Hughes 1999). Interestingly, different cell types secrete different levels of total produced galectin. CHO-K1 cells have been reported to secrete all expressed galectin-3 (Sato et al., 1993) while BHK-21 cells secrete only 12% (Lindstedt et al., 1993). Identification of secreted galectins and their localization within different tissues is clinically important especially when designing drugs that bind galectins as therapeutic targets particularly those that are overexpressed in

different cancers. Despite the accumulation of data on individual galectins, there is still no galectin "signature/profile" that identifies their function across cell types (Laderach et al., 2013). This is, in part, due to galectins displaying multifaceted biological functions. For example, galectin-3 has been reported to display both anti-apoptotic and pro-apoptotic effects (Fukumori et al., 2003; Nakahara et al., 2005). Often many differences in galectin expression can be seen across various cancer lines (Satelli et al., 2008), and even altered galectin transcript levels, which are dependent on specific cellular differentiation events (Abedin et al., 2003).

Prototypical galectins

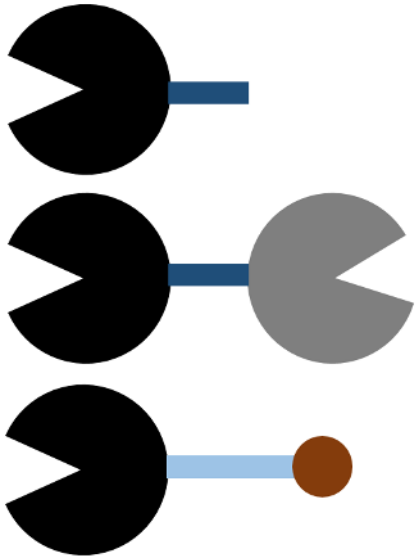
The galectins in this family are galectin-1, -2, -5, -7, -10, -11, -13, -14, -15 and -16. Prototypical galectins are generally smaller (14-16 kDa) than others and have a single galactoside binding domain with a short peptide region followed by an N-terminal sequence. These proteins can exist either as a galectin monomer or more typically as a homodimer. There are 8 conserved amino acids in the CRD that allow for proper binding of prototypical galectins (Hirabayashi and Kasai, 1991; Lobsanov et al., 1993). Monomeric galectins consist of galectin-5, -10 and -14 while galectins-1, -2, -7, -11, -13, -15 and -16 exist as non-covalent homodimers.

Tandem-repeat galectins

Galectin-4, -6, -8, -9 and -12 fall under this subfamily and are generally larger (between 35-39 kDa) than other galectins. These galectins contain two non-identical CRDs: one being located at the N-terminus with the other at the C-terminus, linked together by a short linker peptide region that is roughly 25 – 35 amino acids in size – highly rich in proline and glycine residues. There is evidence that this linker region increases susceptibility of these proteins to proteolysis and that truncated versions increase resistance toward degradation (Nishi et al., 2005). As a result, these galectins are typically less stable than the others.

Chimeric galectins

Currently, galectin-3 is the only galectin classified as a chimeric type and it is 26 kDa in size. This monomeric galectin contains two functional domains: the N-terminal regulatory domain and the C-terminal CRD. The N-terminal regulatory domain contains many repeated collagen-like sequences (7-14 regions with 8-11 amino acids in each) (Rapoport et al., 2008; Weis and Drickamer, 1994), and shares high homology with the heterogeneous nuclear ribonucleoprotein complex (Jia and Wang, 1988). The regulatory domain of galectin-3 is also phosphorylated on at least two different residues and is susceptible to cleavage by enzymes that impact its ability for both lectin binding and self-oligomerization (Balan et al., 2012; Rapoport et al., 2008). Oligomerization is a unique trait of galectin-3, which allows this protein to form galectin-glycan structures between and across cell surfaces.



Proto-type
- Galectin 1, 2, 7, 10, 13, 14, and 16

Tandem-repeat-type
- Galectin 4, 8, 9, and 12

Chimera-type
- Galectin 3

Figure 1. Classification of galectins based on their molecular structure. Galectins are classified based on their carbohydrate recognition domains (*black* and *grey*), non-lectin linker domain (*blue* and *light blue*) and N-terminal end domains (*brown*).

Illustration above shows the basic molecular characteristics of human galectins (adapted from Timoshenko et al. [2016]). Prototype galectins may form homodimers (not shown). Tandem-repeat galectins contain two non-identical CRDs joined together by a short linker peptide region. Chimeric galectin-3 is monomeric, however, may oligomerize using its N-terminal end domain (*brown circle*) to form pentamers.

1.3 Cellular functions of galectins

Numerous studies have shown that galectins play prominent roles in cell signaling, crosslinking and interactions with a variety of ECM molecules. This is achieved by galectins through their ability to interact with glycolipids (Ahmed et al., 2002), glycoprotein receptors (Stillman et al., 2006), integrins (Friedrichs et al., 2008) and other ligands to mediate cellular functions (Boscher et al., 2011). Further, galectins often regulate other molecular targets to facilitate these biological functions. For example, exposure treatment of galectin-1, -2, -3, -4 and -7 promotes adhesion of human breast cancer cells accompanied by increased production of the lymphangiogenic factor: vascular endothelial growth factor-C (VEGF-C) (Timoshenko et al., 2010). Further, galectins induce a variety of functional cellular responses such as aggregation of blood and immune cells, activation of neutrophils (production of ROS and degranulation), and an increase in cytoplasmic Ca^{2+} availability (Timoshenko et al., 1995, 1997, 2003). Each galectin has slightly different affinity toward galactose, dependent on glycan structural organization on the cell surface with other complex oligosaccharides. Moreover, structural differences between galectins allow each galectin to maintain a certain role within different tissues despite significant homology.

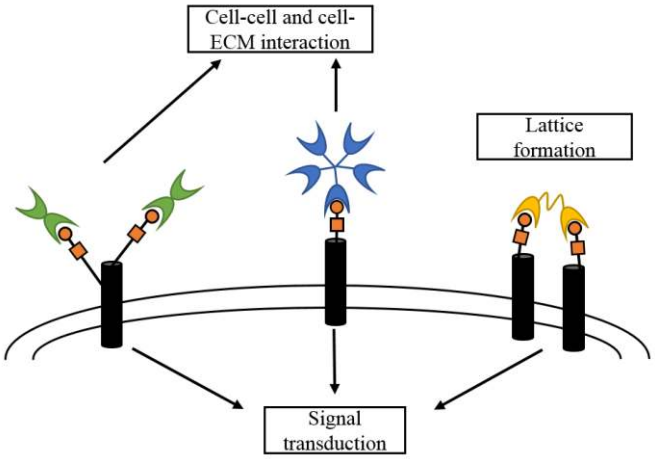

Cell-cell and cell-ECM interaction
Lattice formation
Signal transduction

Figure 2. Interactions between cell surface glycoconjugates (*black cylinders* represent transmembrane receptors, *orange circles* and *squares* represent galactose-containing oligosaccharides) with various galectin family members (*green* proto, *blue* chimeric, *yellow* tandem-repeat) which facilitates cellular functions.

The illustration above demonstrates the facilitation of galectin binding which can mediate signal transduction and cell adhesion events, among others (adapted from Liu and Rabinovich [2005]).

1.3.1 Galectin-1

Generally, galectin-1 is differentially expressed by both tumourigenic and normal tissues, mediating of a wide variety of biological functions that are dependent on protein-carbohydrate and protein-protein interactions (Camby et al., 2006). Galectin-1 is typically associated with initiating and augmenting inflammatory responses including apoptosis, adhesion and migration. This galectin is capable of inducing cell death of both resting (Perillo et al., 1995), and activated (Matarrese et al., 2005) T-cells. Similarly, upregulation of galectin-1 expression promotes apoptosis in rat macrophages and activated T-cells, suppress *in vitro* T-cell activation and inhibits cellular proliferation (Blaser et al., 1998; Rabinovich et al., 1998). Cells overexpressing galectin-1 are able to increase cell migration by binding, in a CRD-dependent manner, to fibronectin, laminin and collagen (Horiguchi et al., 2003) through cross-linking glycoproteins exposed on the cell surface. Interestingly, galectin-1 can differentially impact the effect of cell proliferation depending on cell type. For example, galectin-1 has been shown to inhibit proliferation in lymphocytes, neuroblastoma and prostate cancer while increasing cell proliferation in cervical, ovarian and pancreatic tumour cells (Vladoiu et al., 2014). More interestingly, galectin-1 has been shown to elicit contrasting effects on the same cell type. Yamaoka et al. (2000) demonstrated that inhibition of the galectin-1 gene (*LGALS1*) decreases rat brain tumour cell proliferation while Kopitz et al. (2001) showed that administration of recombinant galectin-1 inhibited brain tumour growth (Kopitz et al., 2001; Yamaoka et al., 2000).

Most evidence, however, suggests galectin-1 as a master regulator of various immune responses including T-cell survival and immune disorders, inflammation and allergies (Gupta, 2012) achieved by binding of various glycoconjugate receptors (CD45, CD32 and CD7) (Pace et al., 1999). Most interestingly, it has been shown that secreted galectin-1 is able to promote tumour cell evasion of the immune response; likely mediated by binding CD4 and CD8 receptors of immune T-cells to promote apoptosis (Rubinstein et al., 2004), an effect that can be attenuated by the addition of lactose. Typically this galectin works in an anti-inflammatory manner by suppressing the secretion of the pro-inflammatory cytokine interleukin-2 (IL-2) (Rabinovich et al., 1999), and promoting the release of anti-inflammatory cytokine interleukin-10 (IL-10) (van der Leij et al., 2004). Kiss et al. (2007)

provide evidence of reduced swelling through edema assay testing providing more evidence of an anti-inflammatory role for this galectin (Kiss et al., 2007). Today, many CD receptors have been recognized as galectin-1 binding partners (Elola et al., 2005), however, it has been shown that different galectins have a specific preference for separate glycoprotein CD receptors despite high CRD homology (Stillman et al., 2006).

1.3.2 Galectin-3

There is a considerable amount of literature on galectin-3 highlighting its biological activity, which is strongly influenced by its localization, cell type, neoplastic processes, cellular proliferation status and others (Dumic et al., 2006). The localization of galectin-3 can strongly affect its biological activity. Generally, intracellular galectin-3 acts as an anti-apoptotic factor while extracellular galectin-3 is pro-apoptotic (Dumic et al., 2006). Galectin-3 has been shown to work intracellularly with multiple different ligands in the cytoplasm including Bcl-2 (Akahani et al., 1997; Yang et al., 1996), APO-1/Fas (Fukumori et al., 2004), Nucling (Liu et al., 2004), and AIPI (Liu et al., 2002) to mediate apoptotic events (either pro- or anti-apoptotic).

Galectin-3 is able to work in the nucleus to mediate gene transcription that affects cell proliferation (Paron et al., 2003), and cell cycle regulation (Kim et al., 1999). Similar to the only documented function of nuclear galectin-1; galectin-3 is also capable of binding Gemin-4 subsequently affecting post-transcriptional regulation of mRNA processing (Paushkin et al., 2002).

Extracellular galectin-3 is able to mediate cell-ECM interaction through binding to laminin, fibronectin, elastin, collagen IV and mediate cell-cell interactions through preferential binding to the ligand N-acetyllactosamine located on CD receptors and glycoreceptors (Dumic et al., 2006). In this way, galectin-3 regulates cell adhesion and migration primarily through interaction with integrins in a CRD-dependent manner (Ochieng et al., 1998).

Similar to galectin-1, galectin-3 is also predominately involved in immune function. Galectin-3 can bind CD receptors to suppress apoptosis or increase proliferation (Akahani et al., 1997; Yang et al., 1996). It has been proposed that the role of galectin-3 is to

oligomerize with itself and then bridge monocyte activated macrophages to apoptotic neutrophils in order to increase their uptake (Karlsson et al., 2009) suggesting that the structural characteristics (divalent vs multivalent), and glycan specificity (receptor selectivity) provide contrasting roles for similar galectins.

The maintenance of endogenous galectins is crucial in their ability to carry out biological roles. For example, phosphorylation of the regulatory domain of galectin-3 is critical for protecting it from subsequent cleavage by the prostate specific antigen (Balan et al., 2012). This provides evidence of the phosphorylated/non-phosphorylated galectin-3 ratio as a prognostic marker of prostate cancer considering intact galectin-3 increases levels of angiogenesis and metastasis. Galectin-3 may also be used as a marker of cellular stress responses considering many tumourigenic processes are involved with different types of cell stress.

Additionally, galectin-3 expression is vastly upregulated near sites of inflammation. Galectin-3 is typically described as a pro-inflammatory galectin, in contrast to galectin-1. Galectin-3 can potentiate inflammatory responses by increasing superoxide anion production, increase adhesion of neutrophils to components of the ECM and work in a similar fashion to that of cytokines to affect both innate and adaptive immunity. Another interesting quality of this galectin during immune response is that it is able to recognize glycoreceptors present on foreign pathogens (Mandrell et al., 1994). Although the specific function for galectin-3 binding foreign pathogens is not well understood, galectin-3 knockout murine models display a reduced ability to clear infection (Beatty et al., 2002), and develop fewer white blood cells than wild type mice (Zuberi et al., 2004).

1.3.3 Galectins-8, -9, -10 and -12

Galectin-8 is a tandem-repeat galectin that effects a wide variety of functions including cell adhesion, cell proliferation, apoptosis and others (Tribulatti et al., 2007; Zick et al., 2004). Interestingly, this galectin has also elicited opposing roles dependent on cell type. For example, galectin-8 has been shown to induce apoptosis in thymocytes (Tribulatti et al., 2007) while preventing apoptosis in brain cancer (Metz et al., 2016). This galectin is well characterized in cancer and its expression levels correlate with the degree of malignancy

and differentiation of different tumours (Bidon-Wagner and Le Pennec, 2004). In fact, a galectin finger-printing study revealed that galectin-8 was detected in all but two of 61 different human tumour cell lines and was more highly expressed than galectins-1, -2, -3, -4, -7 and -9 (Lahm et al., 2001).

Galectin-9 is also a tandem-repeat galectin classically known to be a regulator of both adaptive and innate immunity and functions by inducing secretion of a variety of cytokines from different blood cells (Kojima et al., 2014; Matsuura et al., 2009). Recently, increased galectin-9 expression has been suggested as a marker of distinct cancer types where other galectins are notably downregulated (Muniz et al., 2015).

Galectin-10, also known as Charcot-Leyden crystal protein, is a major component of eosinophils (Dyer and Rosenberg, 1996). Originally found to have affinity for galactosyl residues; this galectin shows higher affinity toward mannosyl residues (Dyer and Rosenberg, 1996; Swaminathan et al., 1999). This galectin is believed to be a mediator and potential biomarker of eosinophilic airway inflammation based on its accumulation within sputum (Chua et al., 2012), and in peripheral blood from patients suffering from bronchial inflammation (Devouassoux et al., 2008).

Galectin-12 is a tandem-repeat galectin, typically expressed in leukocytes and adipose tissue that was cloned originally by Yang et al. (2001), and was found to impact the cell cycle of cancer cells. Although galectin-12 expression across most tissues is relatively weak, it is reported to have relatively high expression in acute promyelocytic leukemia (Timoshenko et al., 2016; Xue et al., 2016), and is involved with regulating adipocyte differentiation (Yang et al., 2004).

1.4 Oxidative stress

Oxidative stress can be defined as a disturbance in the balance between reactive oxygen species (ROS) production and antioxidant detoxification, leading to tissue damage (Betteridge, 2000). Maintaining a proper physiological redox status in a cell is crucial for cell survival and homeostasis. ROS may be produced by dedicated enzyme complexes i.e. NOX family of NADPH oxidases, or as by-products of oxidation-reduction reactions

(Janssen-Heininger et al., 2008; Murphy, 2009; Tochhawng et al., 2013). Induced oxidative stress can be achieved *in vitro* through exogenous administration of H_2O_2 or specific chemical inducers. For instance, 2-methyl-1,4-naphthoquinone (menadione) is a redox cycling compound that undergoes reduction via intracellular enzymes reducing molecular oxygen to a free radical superoxide anion (Criddle et al., 2006). Menadione has been shown to induce significant levels of H_2O_2 (Timoshenko et al., 1996). Additionally, the hypoxia mimetic agent, cobalt chloride ($CoCl_2$) has been shown to induce ROS via a mechanism that is non-mitochondrial (Chandel et al., 1998; Kotake-Nara and Saida, 2007). Free radicals consist of one or more unpaired electrons making them extremely reactive with various cellular components including lipids, proteins, nucleic acids and carbohydrates in comparison to non-radicals (Halliwell, 1994; Tsukahara, 2007). Damage often occurs when the antioxidant defense system is inefficient or when ROS levels rise excessively, leading to free radical induced apoptosis. Often anti-apoptotic genes in certain cells appear to encode free radical scavengers i.e. glutathione or antioxidant enzymes i.e. glutathione peroxidase (GPX) or heme oxygenase 1 (*ho*-1) as a means to counter the stress (Dobashi et al., 2001; Sarafian and Bredesen, 1994). *ho*-1 catabolizes cellular heme into biliverdin carbon monoxide and free iron. It is strongly upregulated during oxidative stress and is documented as one of the sensitive and reliable markers of oxidative stress (Choi and Alam, 1996; Poss and Tonegawa, 1997). Although an abundance of ROS may lead to oxidative damage; appropriate levels of ROS must be present to maintain physiological conditions. In fact, rapid ROS production known as oxidative "burst" is an important function of immune cells in which case large amounts of ROS are produced by oxidation of NADPH in order to combat foreign pathogens. Neutrophils and other phagocytes are often responsible and present at sites of inflammation in order to maintain healthy conditions. At the site of infection, neutrophils are the earliest of granulocytes recruited and release antimicrobial compounds from their granules in addition to the production of ROS in order to kill invading pathogens (van Gisbergen et al., 2005).

Oxidative stress is clinically relevant and has been implicated in the development of various diseases/disorders, a short list of which include: cancer (Reuter et al., 2010), Alzheimer's (Christen, 2000), Parkinson's (Blesa et al., 2015), Huntington's (Gil-Mohapel et al., 2014) cardiovascular diseases (Dhalla et al., 2000), autism (Chauhan and Chauhan,

2006), and depression (Michel et al., 2012). In the event of oxidative stress, the cell has numerous stress response mechanisms that have evolved in order to deal with increased ROS. Often, oxidative stress activates numerous signaling pathways that are involved with gene expression changes that ultimately govern the cells fate of either survival or death. ROS are known to activate pro-inflammatory signaling pathways causing the activation of mitogen-activated protein kinase (MAPK) dependent transcription factors including, NF-κB, which following activation is translocated to the nucleus to modulate gene expression changes of several cytokines to induce apoptosis, inflammation and fibrosis (Bierhaus et al., 1997; Pugliese et al., 2015), and is well characterized within immune cells (Dumic et al., 2006). In particular, activation of MAPKs include a large number of serine/threonine kinases falling under the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 subfamilies, which are responsible for activation of various genes relevant for antioxidant defenses (Martindale and Holbrook, 2002). Further, oxidative stress has also been found to regulate transcription factors directly including: NF-κB (Gloire et al., 2006), p53 (Liu et al., 2008a), Nrf2 (Ma, 2013), HSF1 (Ahn and Thiele, 2003), and others. In certain diseases, these factors promote cellular proliferation, apoptosis, transformation, angiogenesis, metastasis and activation of other signaling networks (Reuter et al., 2010). Another redox stress sensitive cascade includes the PP1-JNK-Sp1 signaling pathway (Chu and Ferro, 2006; Ryu et al., 2003). Following activation via phosphorylation, the transcription factor Sp1 is translocated to the nucleus and binds GC-rich motifs of promoters augmenting transcription of genes relevant for stress responses (Ryu et al., 2003), cellular differentiation (Gong et al., 2014), proliferation (Zhang et al., 2014). Interestingly, galectins-1, -3, -10 and -12 have been reported to contain multiple potential Sp1 binding sites in their promoter region: indicating a common redox-sensitive regulatory factor and the potential for expression of these galectins to be influenced by oxidative stress (Dyer and Rosenberg, 2001; Hotta et al., 2001).

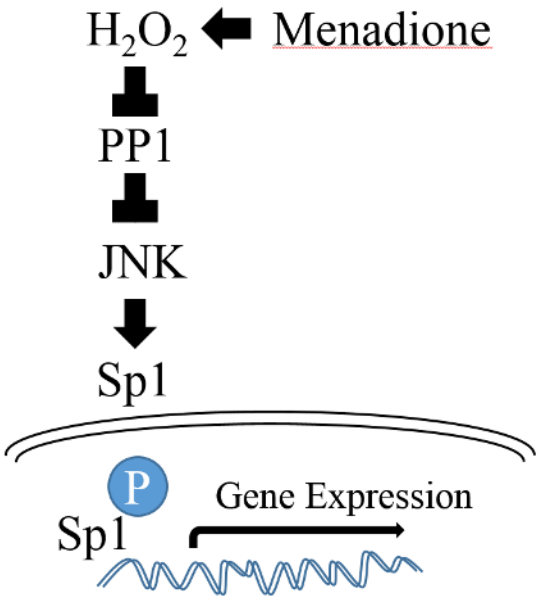

H_2O_2
Menadione
PP1
JNK
Sp1
P
Sp1
Gene Expression

Figure 3. Redox sensitive PP1-JNK-Sp1 signaling pathway. Hydrogen peroxide inhibits PP1, which leads to the phosphorylation and subsequent activation of the Sp1 transcription factor. Inducer of reactive oxygen species – menadione – is proposed to increase galectin gene expression via JNK signaling and Sp1 activation/binding.

The above schematic demonstrates the PP1-JNK-Sp1 signaling pathway (interpreted from Chu and Ferro [2006]). Many galectin promoter regions have potential Sp1 binding sites and therefore this transcription factor is proposed in regulating galectin transcript expression.

Only recently, have galectins been analyzed more critically regarding their involvement with oxidative processes, redox status and related signaling mechanisms. Although many galectins share high sequence homology indicating similar biological function, there appears to be different regulatory mechanisms controlling their expression. This level of complexity makes it difficult to discern the precise function of each galectin across many cell types and conceptually as a whole considering there is often galectin functional redundancy.

1.4.1 Galectin-1 – role in oxidative stress

Galectin-1 is expressed in many tissues, primarily of mesodermal origin including: skeletal and smooth muscle, heart, liver, lung, spleen, kidneys and others (Klyosov, 2008). As mentioned previously galectin-1 has been reported to be redox-sensitive whereby its structure and subsequent function is altered via oxidation of free cysteine residues. This potentially explains why galectin-1 is mitogenic for various human cells including lymph, vascular and liver cells, while being an inhibitor of cell growth in neuroblastoma and connective tissue of bone marrow (Camby et al., 2006). A specific example includes oxidation of the three intramolecular disulfide bonds of galectin-1 allowing for a gain of CRD-independent effects leading to increased axonal regeneration in peripheral nerve but at the same time losing lectin binding associated functionality (Inagaki et al., 2000). A similar effect is demonstrated by Yu et al. (2015) whereby exogenously added oxidized human galectin-1 confers resistance toward oxidative stress in Molt4 cells compared to the reduced form of this galectin (Yu et al., 2015). Further, mouse galectin-2 is susceptible to oxidation by ROS resulting in loss of CRD-dependent function (Tamura et al., 2015). These data indicate that the manner in which galectin-1 confers its resistance may be dependent on its redox state.

Aside from galectin-1 being structurally affected by the redox environment, this protein can also play a direct role in various oxidative processes. In fact, early studies demonstrated the potency of human and animal galectin-1, in addition to other galactoside-specific lectins to induce a respiratory burst from neutrophils (Timoshenko and Gabius, 1993; Timoshenko et al., 1997). Elola et al. (2005) reported that exogenously added pig spleen galectin-1 to white blood cells was able to activate polymorphonuclear leukocytes (PMN)

to produce significant levels of $O_2^{\bullet}$- when combined with other priming agents (Elola et al., 2005). Similarly, galectin-1 alone is able to activate extravasated human neutrophils to produce significant levels of $O_2^{\bullet}$-. Lin et al. (2015) demonstrated that galectin-1 can produce ROS by activating NADPH oxidase, which is important for wound healing in myofibroblasts (Lin et al., 2015). Interestingly, galectin-1 has been shown to promote angiogenesis and protect cancer cells from oxidative stress in a CRD-dependent fashion, which is attenuated by the disaccharide thiodigalactoside (TDG) (Ito et al., 2011).

In addition to direct redox interactions, galectin-1 has also been reported playing a role within various oxidative stress/redox-sensitive signaling pathways. One example includes the interaction between galectin-1 and transforming growth factor (TGF-β). This growth factor is able to promote ROS by activating NADPH oxidase (Thannickal et al., 1998), and has been found to increase galectin-1 expression in a dose dependent manner (Daroqui et al., 2007). Further, galectin-1 has been reported to increase p38 and ERK expression *in vitro* (Chung et al., 2012; Maeda et al., 2004). Similarly, galectin-1 gene expression is down-regulated through the redox-sensitive ERK pathway evident by inhibition of MEK1/2 with inhibitors U0126 and SB202190 in activated T-cells (Fuertes et al., 2004). These findings show how galectin-1 expression is affected by both up- and down-stream components of these MAPK pathways. mTOR signaling is also oxidative stress sensitive (Heberle et al., 2015), and its downstream target molecule, $P70^{S6}$ has been shown to increase galectin-1 expression (Fuertes et al., 2004). Galectin-1 has been shown to activate the JNK/c-Jun/AP-1 signaling pathway leading to apoptosis in T-cells (Brandt et al., 2010). This apoptotic effect was attenuated with the inhibitor SP600125, however, the role of JNK signaling on regulating galectin-1 gene expression has yet to be demonstrated. Evidence suggests JNK as a potential mediator of galectin-1 expression given its strong association with T-cell apoptosis (Zhu et al., 1999), and oxidative stress (Wang et al., 2008).

Considering the strong association between oxidative stress and the pathogenesis of many diseases, galectin-1 is suggested as being a strong candidate biomarker of various stress responses. The interaction between galectin-1 and different signaling pathways suggest this protein as a major player within develop of diseases and its importance in mediating gene transcription of factors relevant for host defenses.

1.4.2 Galectin-3 – role in oxidative stress

The role of galectin-3 regarding oxidative processes has been well documented. In fact, galectin-3 has been listed as a biomarker for many different diseases/conditions implicated with oxidative stress including thrombosis (Madrigal-Matute et al., 2014), heart failure (Erkilet et al., 2013; Medvedeva et al., 2016), various cancers (breast, kidney, thyroid and prostate) (Balan et al., 2012; Idikio, 2011), cardiac fibrosis (Martínez-Martínez et al., 2014), and Alzheimer's disease (Wang et al., 2015). It is no wonder then, why galectin-3 is well studied considering its appeal across so many clinical paradigms.

Similar to galectin-1, exogenously administered galectin-3 is also able to promote superoxide production by human neutrophils demonstrating the direct role of this galectin in oxidative processes (Almkvist et al., 2001; Karlsson et al., 1998). This oxidative burst by human neutrophils is induced following galectin-3 binding primarily the receptors CD66a and CD66b (Feuk-Lagerstedt et al., 1999). A similar effect has been demonstrated in mast cells whereby galectin-3 promotes oxidative stress-induced mast cells death via a CRD-dependent mechanism (Suzuki et al., 2008). Interestingly, this effect could not be replicated by galectin-1, indicating glycoreceptor-specific affinities for different galectin isoforms; dependent on cell type. Interestingly, galectin-3 has also been shown to potentially attenuate superoxide production via downregulation of priming factors suggesting that the specific role of this protein in either eliciting or inhibiting oxidative burst is cell context dependent (Cortegano et al., 1998).

Galectin-3 regulation of and by redox signaling pathways has also been characterized. The promoter region of *LGALS3* contains several regulatory elements including binding sites for AP-1 and NF-κB (Vasta and Ahmed, 2009). Interestingly, galectin-3 is has also been reported to stimulate the ERK/p38 and JNK pathways indicating galectin-3 can regulate and be regulated by these pathways (Filer et al., 2009). Further, Chen et al. (2006) report that mice knockout models for galectin-3 show decreased JNK1 protein and mRNA levels and a concurrent decrease in cytokine production, indicating the potential for galectin-3 to regulate this pathway at the transcriptional level (Chen et al., 2006).

1.4.3 Galectins-8, -9, -10 and -12 – role in oxidative stress

Although the literature primarily indicates a strong association between galectins-1 and -3 with oxidative processes and redox regulation, there is evidence of other galectins participating in these processes.

Galectin-8 has also been shown to elicit superoxide anion production in primed human neutrophils, both intracellularly and extracellularly in a CRD-dependent manner (Carlsson et al., 2007). Furthermore, the levels of superoxide production are comparable to that of the potent chemotactic factor formylmethionyl-leucyl-phenylalanine (fMLP), which often serves as a positive control for neutrophilic oxidative burst (Nishi et al., 2003). The redox-sensitive factors ERK and Akt act downstream of galectin-8 signal transduction (Romaniuk et al., 2010); however, the relation of this pathway to galectin-8 is not studied in the context of cellular stress. Additionally, little is known about the regulatory methods of this galectin both transcriptionally and translationally in the context of oxidative stress. However, many transcriptional isoforms of galectin-8 exist indicating the potential involvement of stress-dependent splicing mechanisms. Considering widespread galectin-8 cell expression, further details should be identified for better understanding of this galectins role in a cellular stress context.

Originally discovered in as a potent eosinophilic chemoattractant, galectin-9 has been reported as a key regulatory of autoimmune disease, in particular, by shifting both adaptive and innate immunity to an anti-inflammatory profile (Wiersma et al., 2013). Similar to galectin-8, galectin-9 is also a tandem-repeat galectin that consists of different spliced isoforms (Chabot et al., 2002), indicating its potential in having stress dependent splice variants. Vega-Carrascal et al. (2014) provide the only evidence of ROS production by human neutrophils, induced by galectin-9. The authors report that while galectin-9 alone was only able to promote superoxide production at supra-physiological levels, neutrophils primed prior to exposure to galectin-9 are more sensitive to superoxide production via NADPH oxidase (Vega-Carrascal et al., 2014). Redox-sensitive pathways have also been implicated in the regulation of galectin-9, and vice versa. In particular, inhibition of p38, but not JNK/c-Jun, significantly reduced expression of galectin-9 (Hsu et al., 2015). In contrast, JNK-induced galectin-9 expression has been detected in astrocytes indicating cell

type specific galectin-9 regulation (Steelman et al., 2013). Additionally, there is evidence of JNK pathway activation induced by galectin-9, indicating a role of galectin-9 working both up- and down-stream of this redox signaling pathway (Kobayashi et al., 2010).

Prototypical galectin-10 has been reported in few molecular processes regarding oxidative stress and redox signaling. Selective binding preference of mannose by galectin-10 perhaps indicates receptor specific stress mediation. A deletion of the galectin-10 encoding gene in *Caenorhabditis elegans* revealed a protective role of this protein against oxidative stress (Nemoto-Sasaki and Kasai, 2009). However, no molecular signaling factors regarding galectin-10 gene expression nor factors impacted by galectin-10 have been identified.

The more recently discovered galectin-12 has also been implicated in oxidative processes. Involvement of galectin-12 with adipocyte regulation and diminished insulin resistance suggests this tandem-repeat galectin may be a potential therapeutic target for metabolic syndrome and related disorders – all of which are highly impacted by elevation in reactive oxygen species. Unfortunately, few molecular mechanisms regulating or responding to galectin-12 have been documented. Galectin-12 has been reported to induce adipogenic signaling through increased expression of C/EBPs (Yang et al., 2004). Interestingly, these C/EBP transcription factors regulate the ROS producing enzyme NADPH oxidase, indirectly implicating the association of galectin-12 with oxidative burst (Manea et al., 2014). Yang et al. (2004) also report galectin-12 expression is upstream of the oxidative stress sensitive ERK and Akt pathways (Yang et al., 2004). Additionally, ablation of galectin-12 expression was shown to diminish levels of nitric oxide secretion while reducing NF-κB and AP-1 expression, in macrophages (Wan et al., 2016).

1.5 Cellular differentiation

Cellular differentiation is a developmental process whereby cells become more specialized. For example, early stem cells may divide further into pluripotent stem cells, which can then give rise to various cell types within an organism. Mesenchymal stem cells (MSCs) from the bone marrow give rise to adipocytes and stromal cells (Beyer Nardi and da Silva Meirelles, 2006) whereas neural stem cells (NSCs) give rise to the nervous system and differentiate into neurons, astrocytes and other specialized neural cell types (Gage, 2000).

In particular, embryonic stem cells may further divide into hematopoietic stem cells (HSCs), which are stem cells that can give rise to over 10 distinct mature blood cell types through the process of hematopoiesis (Seita and Weissman, 2010). Although hematopoiesis occurs at embryonic development, this process is still present in adulthood to replenish the blood system (Jagannathan-Bogdan and Zon, 2013). Blood cells are divided into three lineages based on their respective differentiation patterns: erythrocytes consisting of oxygen carrying red blood cells, lymphocytes consisting primarily of T and B-cells important for immune function and promyelocytes, which give rise to granulocytes (basophils, eosinophils and neutrophils), and macrophages. Production of fully differentiated granulocytes and macrophages is dependent on induction by hematopoietic cytokines (Miranda and Johnson, 2007). Major cytokines responsible for differentiation primarily consist of colony-stimulating factors (CSFs), which are lineage specific glycoproteins that function as cytokines and interact with their cognate receptors on the surface of early progenitor cells within bone marrow (Barreda et al., 2004). Binding of cytokines to their respective receptors elicits multiple signaling pathways including: p38, JNK, ERK, JAK/STAT and PI3K/Akt. These pathways then activate transcription factors such as C/EBPs, cyclins, c-Myc and others relevant for myeloid differentiation (Miranda and Johnson, 2007). Differentiation is accompanied by selective increased expression of specific proteins. In particular, p47*phox* is one of many cytosolic components of superoxide producing enzyme, NADPH oxidase, that is upregulated in response to neutrophilic differentiation of HL-60 cells, and can therefore be considered a marker (Ellison et al., 2015). The expression of NADPH oxidase is important for fully functioning differentiated HL-60 (Chen et al., 2012). Further, knockout studies of this subunit have shown to decrease oxidative stress implicating its potential as a marker for both differentiation and oxidative processes (Landmesser et al., 2002; Pal et al., 2014).

Interestingly, galectins have also been reported in playing a role within hematopoietic cellular differentiation. In fact, evidence suggests that elevated galectin expression occurs during the processes of differentiation and may act as a potential secondary mediator (Acosta-Rodríguez et al., 2004). The involvement of each galectin with differentiation is discussed below.

1.5.1 Galectin-1 – role in cellular differentiation

Galectin-1 gene expression is implicated in myeloid cellular differentiation (Abedin et al., 2003). Interestingly, the change in galectin-1 gene expression can vary depending on cell type and the inducer of differentiation. For example, treatment of different cells with differentiating agents have reported to decrease (Chiariotti et al., 1994), have no effect on (Abedin et al., 2003) or increase (Ohannesian et al., 1994) galectin-1 expression. Interestingly, exogenously added recombinant galectin-1 was shown to have a biphasic effect on hematopoietic cells by augmenting differentiation of promyelocytes into the granulocytic lineage at a low dose or inhibiting differentiation at a high dose (Vas et al., 2005). This data indicates the complexity of galectin-1 as the low dose effect was dependent following CRD binding activity while the high dose was CRD-independent. Other studies have shown high galectin-1 expression during differentiation of HSCs along with other well-known factors (Silva et al., 2003).

Although little information is present on regulatory mechanisms associated with galectin-1 and hematopoietic cell differentiation; Liu et al. (2008) report that galectin-1 contributes to thymocyte differentiation and cell fate through transient activation of the ERK pathway (Liu et al., 2008b). Other results indicate that epigenetic mechanisms may contribute to galectin-1 expression. Methylation of the galectin-1 promoter at CpG clusters indicates one molecular mechanism governing modulation over the transcriptional activity of the galectin-1 gene (Benvenuto et al., 1996). Interestingly, inhibitors of DNA methyltransferases including 5-azacytidine (AzaC), and the less toxic 5-aza-2'-deoxycytidine (decitabine) have been shown to increase cellular differentiation via removal of methyl groups on candidate promoters (Borodovsky et al., 2013; Turcan et al., 2013). In fact, lymphocytes treated with AzaC show a concomitant increase in galectin-1 gene expression followed by induction of hematopoietic cellular differentiation (Poirier et al., 2001).

1.5.2 Galectin-3 – role in cellular differentiation

One unique feature of galectin-3 is its involvement in regulating cellular differentiation. This chimeric galectin mediates cellular differentiation of the hematopoietic lineage,

during both myeloid (Abedin et al., 2003), and lymphoid (Oliveira et al., 2009) hematopoiesis. Generally, it has been shown that endogenous levels of galectin-3 are strongly upregulated on the cell surface, binding primarily CD34, during myeloid cell differentiation (Le Marer, 2000). Increased galectin-3 expression has been observed during monocytic differentiation by human promyelocytes (Abedin et al., 2003), primary human monocytic differentiation into macrophages (Liu et al., 1995), and during T-cell differentiation (Vasil'eva et al., 2013). However, galectin-3 has also been shown to directly inhibit differentiation in non-hematopoietic cell types (Nakajima et al., 2014) making the function of this galectin during differentiation cell context dependent. In particular, differentiation of dendritic-cells decreased galectin-3 expression (Dietz et al., 2000). These opposing findings make galectin gene expression difficult to utilize as prognostic markers of general cell function (in this case differentiation). More evidence is needed to understand the role of galectin expression in response to or as possible mediators of differentiation.

Galectin-3 has also been reported to be a regulator of differentiation. It's expression/function directly inhibits osteoblast differentiation (Nakajima et al., 2014) while augmenting oligodendrocyte differentiation (Pasquini et al., 2011). Similarly, galectin-3 has been reported to inhibit granulocyte-macrophage colony stimulating factor, a cytokine important for inducing early stages of differentiation (Krugluger et al., 1997). In fact, the expression of galectin-3 may not only be important for inducing/augmenting differentiation into specific lineages: but by also inhibiting differentiation into other final cell fates. Acosta-Rodríguez et al. (2004) provide one example whereby knockdown of galectin-3 not only induced B-cell differentiation but also reduced the percentage of plasma cell differentiation (Acosta-Rodríguez et al., 2004). The opposing effects of galectin-3 demonstrate its complexity during hematopoietic cellular differentiation and potential to be regulated by different networks suggesting different cell types maintain different regulatory methods for mediating galectin-3 expression.

Epigenetic regulation has also been reported for galectin-3. Malignant prostate epithelial cells were shown to be sensitive to AzaC treatment displaying a marked increase in galectin-3 gene expression that was not seen within benign and malignant tissue (Ahmed et al., 2007). Similarly, various CpG islands within the transcription start site of the

LGALS3 gene suggest that the DNA methylations state of the galectin-3 gene is a potential biomarker for cancer (Keller et al., 2013; Ruebel et al., 2005).

1.5.3 Galectins-8, -9, -10 and -12 – role in cellular differentiation

Despite there being substantially less data on the galectins-8, -9, -10 and 12, there is literature indicating their involvement with differentiation.

Similar to galectin-3, galectin-8 is able to promote T-cell differentiation by modulating cytokine activity through TGF-β signaling (Sampson et al., 2016). Although there is little literature on the involvement with galectin-9 and cellular differentiation, there is some evidence of transcript downregulation during monocytic differentiation (Abedin et al., 2003). In contrast, upregulation of galectin-9 mRNA during monocytic differentiation has been reported making the role of this galectin during differentiation unclear (Harwood et al., 2016). Unrelated to hematopoietic differentiation, galectin-9 promotes MSC differentiation into chondrocytes through TGF-β signaling (Arikawa et al., 2009).

Abedin et al. (2003) also reported an increase in galectin-10 transcript levels following neutrophilic differentiation of promyelocytes *in vitro* despite this galectin being expressed predominately in eosinophils (Abedin et al., 2003). Knockdown of galectin-10 expression during differentiation of eosinophil progenitors significantly inhibited granulogenesis indicating a novel role for galectin-10 during eosinophil development (Doyle and Ackerman, 2009). This galectin has large sequence and structure homology to galectins-1 and -3 suggesting that it may also play a prominent role in hematopoietic cell differentiation (Ackerman et al., 1993).

Galectin-12 has been reported in playing contrasting roles during cell differentiation. In particular, galectin-12 expression promotes adipocyte differentiation (Yang et al., 2004) while inhibiting neutrophilic differentiation of promyelocytes (Xue et al., 2016). Interestingly, however, ablation of galectin-12 in bone marrow cells did not affect their capability in differentiating into macrophages indicating the redundant function of other galectins in myeloid cell differentiation (Wan et al., 2016). Various factors thought to be involved with galectin-12 during cellular differentiation include vacuolar protein sorting

13 homolog C (VPS13C), C/EBP transcription factors and ERK1/2 pathways effecting NF-κB and AP1 (Wan et al., 2016; Yang et al., 2004, 2016).

1.6 Hypothesis

The existing literature shows contrasting roles for galectins during cell differentiation and in response to oxidative stress. Interestingly, many of these galectins are expressed within certain tissue/cell types and most studies typically only examine a single galectin. Therefore, understanding galectin family expression patterns/profiles across different tissues is important for elucidating the overall synergistic, additive or antagonistic functions of galectins. Further, often altered galectin expression patterns are observed in response to different types of cell stress including: oxidative stress, hypoxia, nutrient deprivation, radiation and others (reviewed in Timoshenko, 2015). These findings suggest an importance of galectins in response to many types of pathological stress positioning galectins as possible therapeutic intervention targets. The overarching goal of my study is determine if galectins may be used as biomarkers of different types of cellular stress found within the tumour microenvironment by comparing galectin expression profiles in a leukemic cell line.

The Timoshenko lab has recently demonstrated that the human promyelocytic leukemic HL-60 cell line is an excellent model to investigate stress-sensitive galectins (Timoshenko et al., 2016). HL-60 cells express 6 of the 12 known mammalian galectins and are easily differentiated in neutrophils when exposed to dimethyl sulfoxide (DMSO). My study specifically aims to identify the galectin expression profile of HL-60 cells exposed to both oxidative stress and DMSO-induced neutrophilic differentiation in an effort to establish a link between these two processes and identify potential molecular mechanisms responsible. Interestingly, Ogino et al. (2010) have reported that H_2O_2 is able to enhance neutrophilic differentiation of HL-60 cells despite not investigating any molecular mechanisms potentially responsible (Ogino et al., 2010). Thus altered expression of galectins in response to either oxidative stress or during differentiation may reveal overlapping regulatory mechanisms of galectin expression.

I hypothesize that oxidative stress responsive galectins are involved with HL-60 cell neutrophilic differentiation. The rationale behind this study is that both oxidative stress and cell differentiation are capable of producing altered galectin expression profiles.

Objective 1: to determine the galectin expression profile of HL-60 under menadione-induced oxidative stress and DMSO-induced neutrophilic differentiation. This will be done by RT-qPCR and immunoblotting techniques.

Objective 2: To investigate the effect of galectin inhibitors on HL-60 cellular proliferation and neutrophilic differentiation. This will be done by determining the cell population doubling time through direct counts. Neutrophilic differentiation will be determined using a spectrofluorometric assay used to measure ROS production.

Objective 3: To investigate the effect DNA methyltransferase inhibition and JNK signaling on galectin expression and during oxidative stress and neutrophilic differentiation. This will be done by RT-qPCR and immunoblotting techniques.

Chapter 2

2 Materials and methods

2.1 Cell culture

Human promyelocytic leukemia suspension cell line HL-60 (ATCC® CCL-240™) was obtained from ATCC. Cells were cultured in Iscove's Modified Dulbecco's Eagles medium (IMDM) (Corning™) supplemented with 10% charcoal stripped fetal bovine serum (FBS) (Wisent Bioproducts), 100 IU/mL penicillin, 100 µg/mL streptomycin, in a humidified incubator at 37°C and 5% CO_2. Cells were maintained under confluent conditions ($1x10^6$ cells/mL) for all treatments and passaged accordingly. Cell viability was determined using a Leitz Z0124 microscope using a trypan blue (0.2%) exclusion test.

2.2 Cell treatments

To induce oxidative stress, HL-60 cells were treated with 10 µM menadione sodium bisulfite (Sigma-Aldrich) or 100 µM $CoCl_2$ (Sigma-Aldrich) for 24 hrs. HL-60 neutrophilic differentiation was accomplished by treating cells with 1.3% DMSO for 72 hrs unless otherwise stated. Either 1 or 2.5 mM NAC (Sigma-Aldrich) was administered as an antioxidant for 24 hrs and 72 hrs for either oxidative stress or differentiation experiments, respectively. Global hypomethylation was accomplished using 50 nM DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine; Cayman Chemical) for 72 hrs. Treatment with NAC or decitabine for 72 hrs included treating 3 times every 24 hrs from initial passage. Inhibition and activation of JNK was accomplished using 25 µM SP600125 (LG Labs), and 400 nM anisomycin (StressMarq), respectively for 24 hrs. Galectin inhibitors used included lactose (Sigma-Aldrich), N-acetyl-D-lactosamine (LacNac; Carbosynth), thiodigalactoside (TDG; Carbosynth), and galectin-1 specific inhibitor OTX008 (Axon Medchem). Mannose (Sigma-Aldrich), and α-methyl-D-mannoside (α-MM; Sigma-Aldrich) were used as non-inhibitory sugars except for their specific affinity to galectin-10.

2.3 RNA extraction and cDNA synthesis

Following cell treatment, cells were centrifuged and resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+}. Cells were then centrifuged, lysed with TRIzol® (Ambion), and RNA was isolated according to the manufacturer's protocol. RNA was dissolved in water and quantified using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher). Purity was assessed considering $A_{260/280}$ of > 1.8 as the threshold. cDNA was reverse transcribed from 2 µg of RNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (K1671; Thermo Fisher) according to the manufacturer's protocol. All primers were synthesized by *Bio*Corp UWO *Oligo*Factory (Western University, Department of Biochemistry).

2.4 RT-PCR and RT-qPCR

Primer information including sequence, amplicon size, cycling conditions and referencing is located in Appendix A: supplementary material. RT-PCR reaction mixtures consisted of 2X Taq master mix (FroggaBio) mixed with nuclease–free water, 800 nM forward primer, 800 nM reverse primer and 1 µL of cDNA template diluted 25 times. RT-PCR products were separated on a 2% agarose gel made with a 40 mM Tris, 20 mM acetic and 1 mM EDTA (TAE). Nucleic acids were stained using SYBR® Safe (Invitrogen), and imaged using a ChemiDoc XRS system (Bio-Rad).

RT-qPCR for relative mRNA transcript levels were quantified using a SensiFAST™ SYBR® No-ROX Kit (Bioline) mixed with nuclease–free water, 400 nM forward primer, 400 nM reverse primer, 0.5 µL of undiluted cDNA template and amplified with a CFX96™ Real-Time PCR Detection System (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Quantification of relative fold transcript levels was calculated using Livak's $2^{-\Delta\Delta Ct}$ or Pfaffl methodology to predict accurate gene expression levels based on realistic PCR amplification efficiencies. RT-qPCR using the Oxidative Stress RT^2 Profiler PCR Array (PAHS-065Z; Qiagen) was performed as described by the manufacturer.

2.5 Antibodies

Primary antibodies listed below were diluted in 50 mM Tris, 150 mM NaCl and 0.05% Tween-20 (TBS-T) supplemented with 5% bovine serum albumin (BSA), and 0.05% sodium azide. For western blotting purposes, the following primary antibodies were used: galectin-1 (1:200; sc-28248; Santa Cruz), galectin-3 (1:200; sc-20157; Santa Cruz), galectin-10 (1:10,000; ab-157475; AbCam), galectin-12 (1:200; sc-67294; Santa Cruz), SAPK/JNK (1:1000; 9252; Cell Signaling Technology), Phospho-SAPK/JNK (1:1000; 9251; Cell Signaling Technology), and β-actin (1:200; sc-47778; Santa Cruz). Secondary antibodies include anti-rabbit IgG-HRP (1:10,000; sc-2004; Santa Cruz), and anti-mouse IgG HRP (1:10,000; sc-2005; Santa Cruz).

2.6 Protein isolation and western blotting

Following cell treatment, cells were centrifuged and resuspended in DPBS. Cells were then homogenized in a 2% SDS extraction buffer containing 50 mM Tris pH 7.5 supplemented with 1 mM PMSF and protease inhibitor cocktail containing 1 mM AEBSF, 5 mM EDTA, 50 µM leupeptin and 1 µM pepstatin. Samples were then incubated on ice for an additional 10 minutes before further homogenization via sonication with a Microson™ XL-2000 Ultrasonic Liquid Processor (Qsonica) for 15 seconds at amplitude setting 2. Total protein concentration was determined using the *DC* Protein Assay™ (5000111; Bio-Rad) as per the manufacturer's protocol and absorbance was measured at 595 nm by a Model 3550 Microplate Reader (Bio-Rad). Protein extract (25 µg) was resolved by 15% SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). Membranes were blocked with 5% BSA and 1% milk in TBS-T at room temperature for 1 hr before being probed with primary antibody overnight at 4°C. Membranes were then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz), amplified with SuperSignal™ West Pico Chemiluminescent Substrate (34080; Thermo Fisher), and imaged with a ChemiDoc XRS system (Bio-Rad). Densitometry was performed using Image Lab™ software and significant differences were determined using SPSS considering $p < 0.05$ as the statistical threshold.

2.7 Measurement of fMLP-induced ROS production

Cells were seeded at $0.5x10^6$ cells/mL in 24 well plates and treated with 1.3% DMSO for 72 hrs in order to achieve fully neutrophil-like differentiated HL-60 cells. Cells were then collected, centrifuged and resuspended in Hanks' Balanced Salt Solution (HBSS) pre-warmed to 37°C. Cells were loaded into a cuvette and absorbance was read at 600 nm in order to standardize cell concentration using NanoDrop 2000c spectrophotometer (Thermo Fisher). The cuvette was then loaded into an AMINCO-Bowman Series 2 Luminescence Spectrometer reading excitation and emission wavelengths at 380 and 460 nm, respectively. Cells were then exposed to 1 µM scopoletin (Sigma-Aldrich); a fluorescent substrate for 20 µg/mL horseradish peroxidase (HRP; Sigma) that is oxidized into non-fluorescent products. A "cocktail" containing scopoletin and HRP was then added to the cuvette; producing fluorescence until a stable level is observed. To induce oxidative burst, 100 nM fMLP was added to the cuvette. Scopoletin oxidation was measured as a decrease in fluorescent intensity. The subsequent kinetic data points were run through R using the algorithm described in Appendix A: Supplementary material to determine the point at which the slope (rate of H_2O_2 production) is greatest. Rate of H_2O_2 production was calculated as the maximal slope of recorded traces of scopoletin oxidation normalized to cell concentration and original fluorescent maximum (F_{max}) presented as pmol/sec/$1x10^6$ cells.

2.8 Microscopy and nuclei staining

HL-60 cell cultures were observed and imaged using an inverted Leica DM IL LED microscope in integrated modulation contrast (IMC) mode. To investigate cell nuclei, cytospins were prepared. Firstly, cells were resuspended in IMDM at $0.5x10^6$ cells/mL, then centrifuged onto glass slides for 4 mins at 500 rpm using a Shandon's Cytocentrifuge 2. Cells were then fixed for 10 minutes using methanol and nuclei staining was performed using 2 µg/mL Hoechst-33342. Imaging of segmented nuclei to confirm cellular differentiation was done using an AxioImager A.1 fluorescent microscope (Carl Zeiss), and images were taken with a CCD camera Model XCD-X700 (Sony) using Northern Eclipse 8.0 software (Emprix Imaging Inc.). Scale bars were added using Northern Eclipse 8.0 software.

2.9 Cell proliferation assay

Cells were seeded at $0.1x10^6$ cells/mL in 24 well plates and treated with different galectin inhibitors at various concentrations for 96 hrs. Cell count and viability were determined using a Leitz Z0124 microscope and using a trypan blue (0.2%) exclusion test every 24 hours from initial seeding in order to determine cell doubling time.

2.10 Statistical analysis

All experiments were performed in at least triplicate unless otherwise stated and data are expressed as mean ± SD (unless stated otherwise). A one-way analysis of variance (ANOVA) followed by a Tukey's HSD test were used to determine statistical significant differences across means. An independent samples t-test was used to compare means of treated groups vs. their respective control. Correlation analysis between genes was performed using a Pearson's correlation test. Significant differences were determined using SPSS and GraphPad Prism 7 software considering $p < 0.05$ as the statistical threshold.

Chapter 3

3 Results

3.1 The galectin expression profile was differentially regulated in response to menadione-induced oxidative stress

To determine the galectin expression profile, end-point PCR was performed to amplify 6 of the 12 known human galectin genes expressed in HL-60 cells (Figure 4) (for reference see Appendix A: Supplementary material). The galectin mRNA expression profile was then determined for HL-60 cells treated with oxidative stress inducers: 10 μM menadione with or without 1 mM NAC and 100 μM $CoCl_2$ for 24 hrs, using RT-qPCR (Figure 5). There was a statistically significant difference between treatment groups as determined by one-way ANOVA for *LGALS1* ($F_{4,1} = 21.6$, $p < 0.001$), *LGALS3* ($F_{4,1} = 139.14$, $p < 0.001$), *LGALS9* ($F_{4,1} = 13.77$, $p < 0.001$), *LGALS10* ($F_{4,1} = 11.93$, $p < 0.01$), and *LGALS12* ($F_{4,1} = 8.74$, $p < 0.01$). $CoCl_2$ significantly increased *LGALS1* ($p < 0.05$), and *LGALS10* ($p < 0.05$) transcript expression only (Figure 5A, E) while significantly decreased ($p < 0.001$) expression of *LGALS9* (Figure 5D). Similarly, transcript expression of *LGALS1*, *LGALS3* and *LGALS10* was significantly up-regulated ($p < 0.01$, $p < 0.001$, $p < 0.01$ respectively) in response to menadione (Figure 5A, B, E), which was attenuated by the antioxidant NAC. Similar to the effect of $CoCl_2$, menadione significantly decreased ($p < 0.01$) *LGALS9* expression that was also rescued by the addition of NAC (Figure 5D). Although treatment with menadione did not significantly increase *LGALS12* transcript expression relative to the control; expression between menadione alone and menadione with NAC, was significantly different ($p < 0.05$) (Figure 5F), similar to the trend seen with *LGALS1*, *LGALS3* and *LGALS10*. Interestingly, no significant differences in *LGALS8* transcript levels were noticed across all treatments ($F_{4,1} = 0.79$, $p > 0.05$).

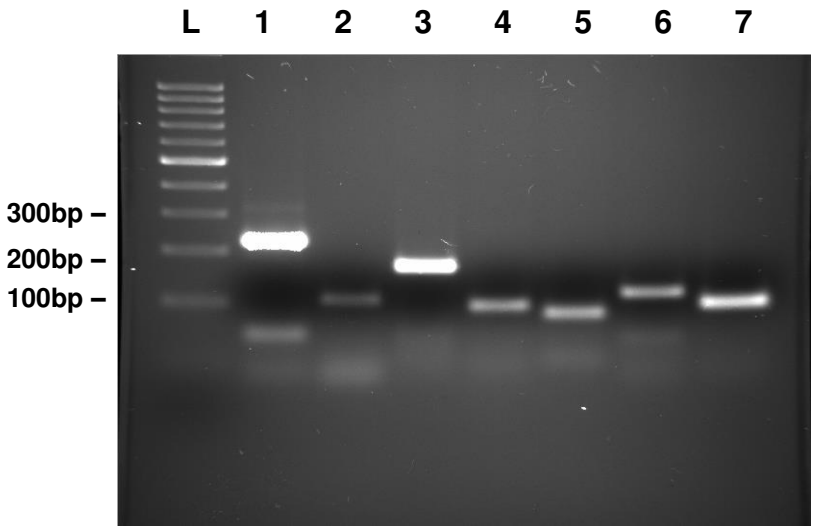

L
1
2
3
4
5
6
7
300bp –
200bp –
100bp –

Figure 4. Verification of galectin gene PCR amplicons following RT-PCR by agarose gel electrophoresis.

PCR products were resolved by a 2% agarose gel via electrophoresis and visualized by SYBR® Safe staining. Amplified products were of the expected sizes. Lane L (ladder), lane 1 *(LGALS1*, 220 bp), lane 2 (*LGALS3*, 108 bp), lane 3 (*LGALS8*, 172 bp), lane 4 (*LGALS9*, 91 bp), lane 5 (*LGALS10*, 82 bp), lane 6 (*LGALS12*, 111 bp), and lane 7 (GAPDH, 95 bp).

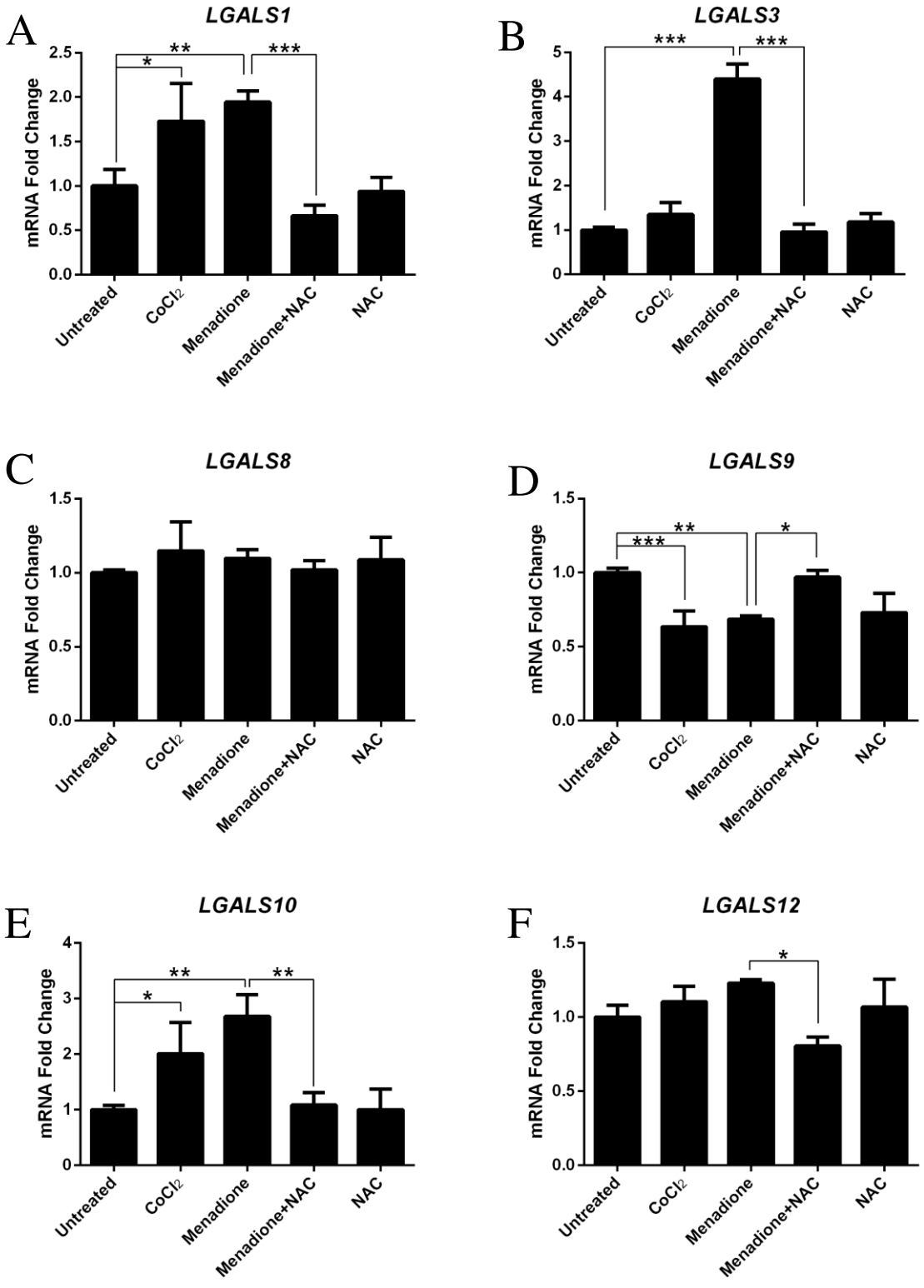

A
LGALS1
mRNA Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*
**
***
Untreated
CoCl2
Menadione
Menadione+NAC
NAC
B
LGALS3
mRNA Fold Change
5
4
3
2
1
0
***
***
Untreated
CoCl2
Menadione
Menadione+NAC
NAC
C
LGALS8
mRNA Fold Change
1.5
1.0
0.5
0.0
Untreated
CoCl2
Menadione
Menadione+NAC
NAC
D
LGALS9
mRNA Fold Change
1.5
1.0
0.5
0.0
***
**
*
Untreated
CoCl2
Menadione
Menadione+NAC
NAC
E
LGALS10
mRNA Fold Change
4
3
2
1
0
*
**
**
Untreated
CoCl2
Menadione
Menadione+NAC
NAC
F
LGALS12
mRNA Fold Change
1.5
1.0
0.5
0.0
*
Untreated
CoCl2
Menadione
Menadione+NAC
NAC

Figure 5. Oxidative stress altered HL-60 galectin transcript levels.

ΔΔCt relative galectin transcript expression in HL-60 cells treated with 100 µM $CoCl_2$, 10 µM menadione and/or 1 mM NAC for 24 hrs relative to untreated cells, using RT-qPCR. (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10* and (F) *LGALS12*. GAPDH was used as an internal control. The data represent the mean ± SD, n = 3-6. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

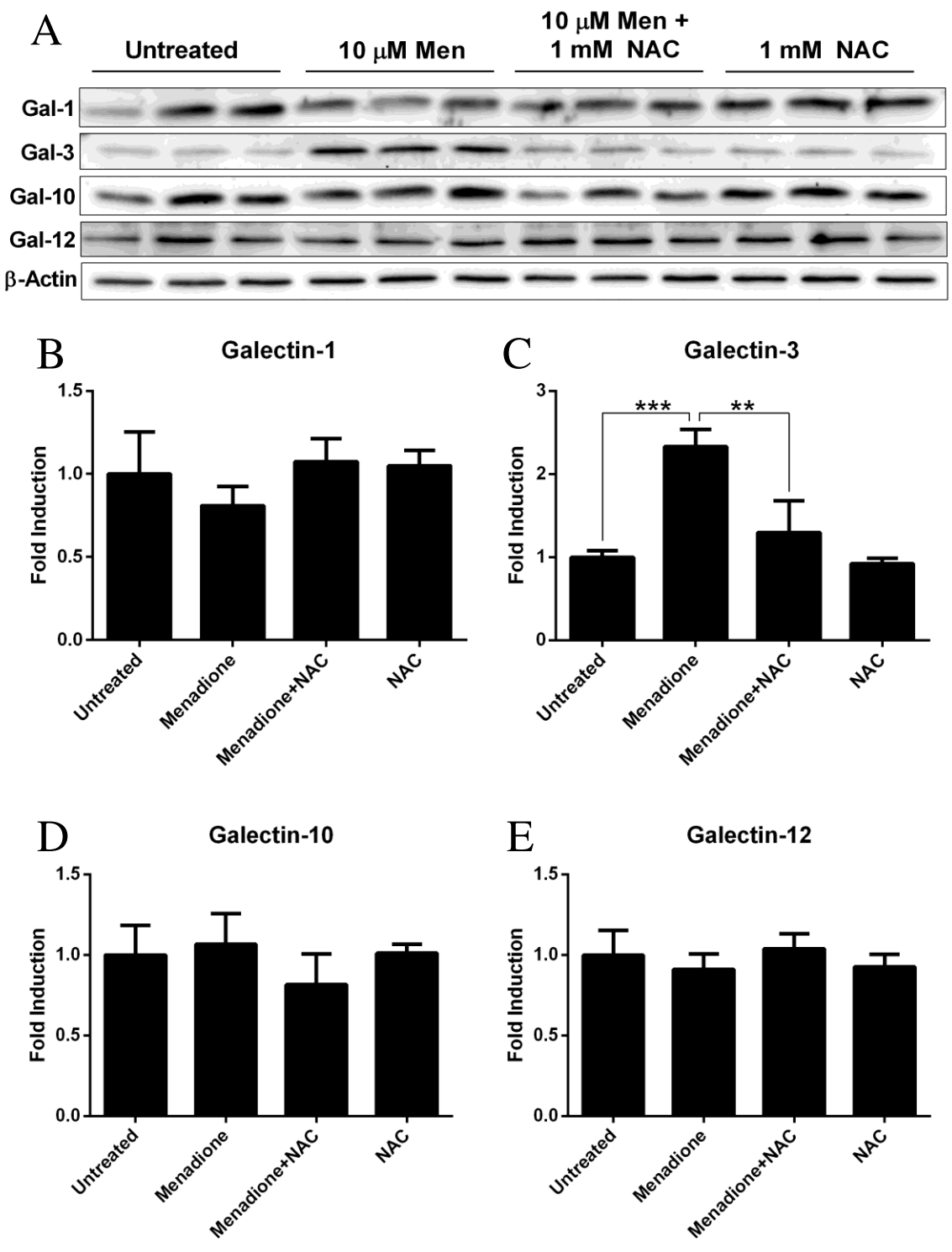
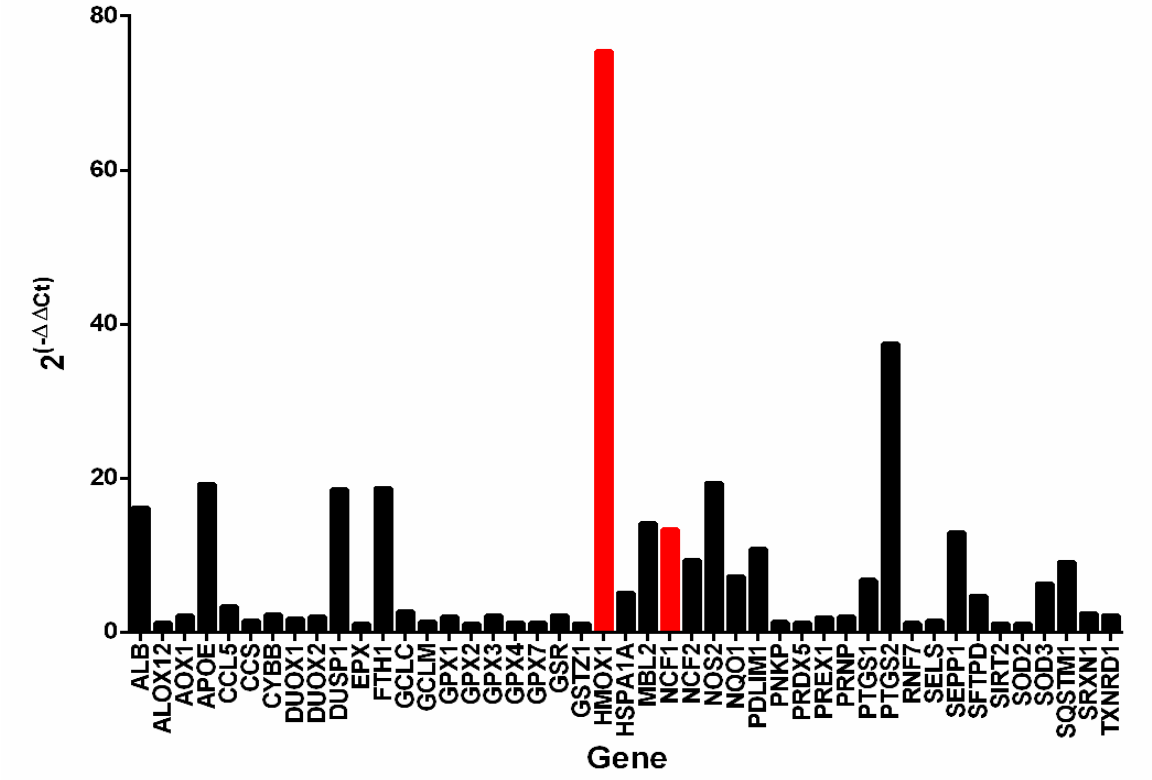

A
Untreated
10 μM Men
10 μM Men + 1 mM NAC
1 mM NAC
Gal-1
Gal-3
Gal-10
Gal-12
β-Actin
B
Galectin-1
Fold Induction
1.5
1.0
0.5
0.0
Untreated
Menadione
Menadione+NAC
NAC
C
Galectin-3
Fold Induction
3
2
1
0
***
**
Untreated
Menadione
Menadione+NAC
NAC
D
Galectin-10
Fold Induction
1.5
1.0
0.5
0.0
Untreated
Menadione
Menadione+NAC
NAC
E
Galectin-12
Fold Induction
1.5
1.0
0.5
0.0
Untreated
Menadione
Menadione+NAC
NAC

Figure 6. Oxidative stress altered HL-60 galectin protein levels.

Immunoblot for galectin protein expression in (A) HL-60 cells treated with 10 μM menadione and/or 1 mM NAC for 24 hrs. Quantification of (B) galectin-1, (C) galectin-3, (D) galectin-10 and (E) galectin-12 protein levels by densitometric analysis of the immunoblot in (A). β-actin was used as a loading control. 25 μg of protein were loaded per well. The data represent the mean ± SD, n = 3. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

A


80
60
40
20
0
2(-ΔΔCt)
ALB
ALOX12
AOX1
APOE
CCL5
CCS
CYBB
DUOX1
DUOX2
DUSP1
EPX
FTH1
GCLC
GCLM
GPX1
GPX2
GPX3
GPX4
GPX7
GSR
GSTZ1
HMOX1
HSPA1A
MBL2
NCF1
NCF2
NOS2
NQO1
PDLIM1
PNKP
PRDX5
PREX1
PRNP
PTGS1
PTGS2
RNF7
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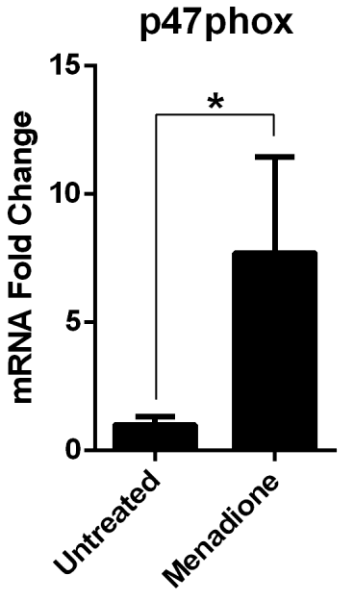
SEPP1
SFTPD
SIRT2
SOD2
SOD3
SQSTM1
SRXN1
TXNRD1
Gene

B


p47phox
15
10
5
0
mRNA Fold Change
*
Untreated
Menadione

C

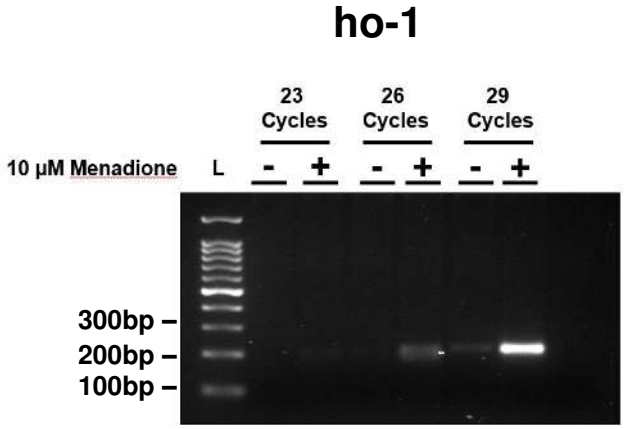

ho-1
23 Cycles
26 Cycles
29 Cycles
10 μM Menadione
L
-
+
-
+
-
+
300bp
200bp
100bp

Figure 7. Expression profiling of oxidative stress markers in HL-60 cells following menadione exposure.

Cells were treated with 10 µM menadione for 24 hrs. (A) Oxidative Stress RT^2 Profiler Array Kit using RT-qPCR. The data represent relative fold transcript levels ($2^{-\Delta\Delta Ct}$) of 84 genes (only candidate genes are shown) sensitive to oxidative stress, n = 1. (B) RT-qPCR for oxidative stress marker, p47*phox*, expression following treatment with 10 µM menadione for 24 hrs, relative to control cells. GAPDH was used as an internal control. The data is presented as the mean ± SD n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (C) Gel electrophoretic analysis of RT-PCR product for oxidative stress marker *ho*-1 (220 bp).

To confirm differences observed at the transcript expression, western blotting was performed to analyze protein levels (Figure 6). Significant differences were determined after densitometry analysis of bands. There was a statistically significant difference between treatment groups as determined by one-way ANOVA for galectin-3 protein levels ($F_{3,1} = 25.44$, $p < 0.001$), only. Densitometry analyses revealed a significant increase ($p < 0.001$) in galectin-3 expression, which was attenuated by NAC exposure (Figure 6C). No significant differences were observed with galectin-1 ($F_{3,1} = 1.64$, $p > 0.05$) galectin-10 ($F_{3,1} = 1.3$, $p > 0.05$), and galectin-12 ($F_{3,1} = 0.9$, $p > 0.05$) (Figure 6B, D, E).

To further determine the menadione-induced oxidative stress response, RNA from HL-60 cells treated with 10 μM menadione was analyzed using an RT-qPCR array kit for 84 genes related to oxidative stress (Figure 7). From this array, *ho*-1 and neutrophil cytosolic factor 1 (p47*phox*) were picked as strong oxidative response candidates. *ho*-1 is a classic antioxidant protein product that cleaves heme while p47*phox* – typically a marker of oxidative stress – is more classically known as a marker of cellular differentiation. HL-60 cells treated with 10 μM menadione exhibited p47*phox* expression as detected by RT-qPCR (Figure 7B). Gel electrophoresis following RT-PCR revealed a *ho*-1 amplicon of the correct size (Figure 7C), indicating that menadione-induced oxidative stress in HL-60 cells.

3.2 The galectin expression profile was differentially regulated in response to DMSO-induced HL-60 neutrophilic differentiation.

Before determining the galectin expression profile for differentiated HL-60 cells, the neutrophilic phenotype was first confirmed. In order to differentiate HL-60 cells into neutrophil-like cells, cells were treated with 1.3% DMSO for 72 hrs. Differentiation was confirmed using 3 different techniques. Firstly, the rate of H_2O_2 production and cell viability was plotted versus time for 6 days (Figure 8A). Following cellular differentiation, HL-60 expresses fMLP receptors that bind fMLP subsequently activating oxidative burst, which was measured using a fluorometer. An increase and saturation of H_2O_2 production was observed at day 3 while cell viability remained ~80% (Figure 8A). Differentiation was further confirmed using both: 2 μg/mL Hoechst staining for segmented nuclei, characteristic of neutrophils (Campbell et al., 1995), and by inverted light microscopy to

reveal smaller cell and more rounded cell morphology (Figure 8B). Both smaller/rounded cells and multi-lobed nuclei were visible following 5 day exposure to 1.3% DMSO: indicative of cellular differentiation (Figure 8B). Finally, RT-qPCR analyses of the differentiation marker p47*phox* (Ellison et al., 2015) following 3 day treatment with 1.3% DMSO revealed a significant increase ($p < 0.01$) in transcript expression. These three techniques indicated prominent cellular differentiation following at least 72 hr exposure to 1.3% DMSO.

Following 72 hr treatment with 1.3% DMSO total RNA was isolated and analyzed for galectin transcript expression using RT-qPCR (Figure 9). There was a statistically significant difference between treatment groups as determined by one-way ANOVA for *LGALS1* ($F_{3,1} = 10.337$, $p < 0.01$), *LGALS3* ($F_{3,1} = 76.5$, $p < 0.001$), *LGALS9* ($F_{3,1} = 9.99$, $p < 0.01$), *LGALS10* ($F_{3,1} = 75.69$, $p < 0.001$), and *LGALS12* ($F_{3,1} = 16.16$, $p < 0.001$). A significant increase in *LGALS3* ($p < 0.001$), and *LGALS10* ($p < 0.001$) (Figure 9B, E) transcript expression during differentiation was observed while *LGALS12* (Figure 9F) was significantly downregulated ($p < 0.01$) compared to control cells. Interestingly, NAC partially attenuated DMSO-induced *LGALS3* and *LGALS10* transcript expression ($p < 0.001$ and $p < 0.01$, respectively) (Figure 9B, E), indicating a redox dependent component during cell differentiation, which subsequently impacts galectin transcript expression. Similar to the result observed during oxidative stress; no significant differences were detected across all treatments for *LGALS8* ($F_{3,1} = 2.76$, $p > 0.05$). Interestingly, *LGALS1* expression was significantly upregulated ($p < 0.01$) while *LGALS9* expression was significantly downregulated ($p < 0.01$) when both 1.3% DMSO and NAC were administered together, however, induced no significant expression changes when either treatment was added alone (Figure 9A, D).

To confirm differences seen at the transcript levels, western blotting was performed against individual galectins to analyze protein levels following 72 hr treatment with 1.3% DMSO (Figure 10). There was a statistically significant difference for galectin-1 ($F_{3,1} = 12.01$, $p < 0.01$), galectin-3 ($F_{3,1} = 103.41$, $p < 0.001$), galectin-10 ($F_{3,1} = 47.59$, $p < 0.001$), and galectin-12 ($F_{3,1} = 7.36$, $p < 0.05$) between treatment groups as determined by one-way

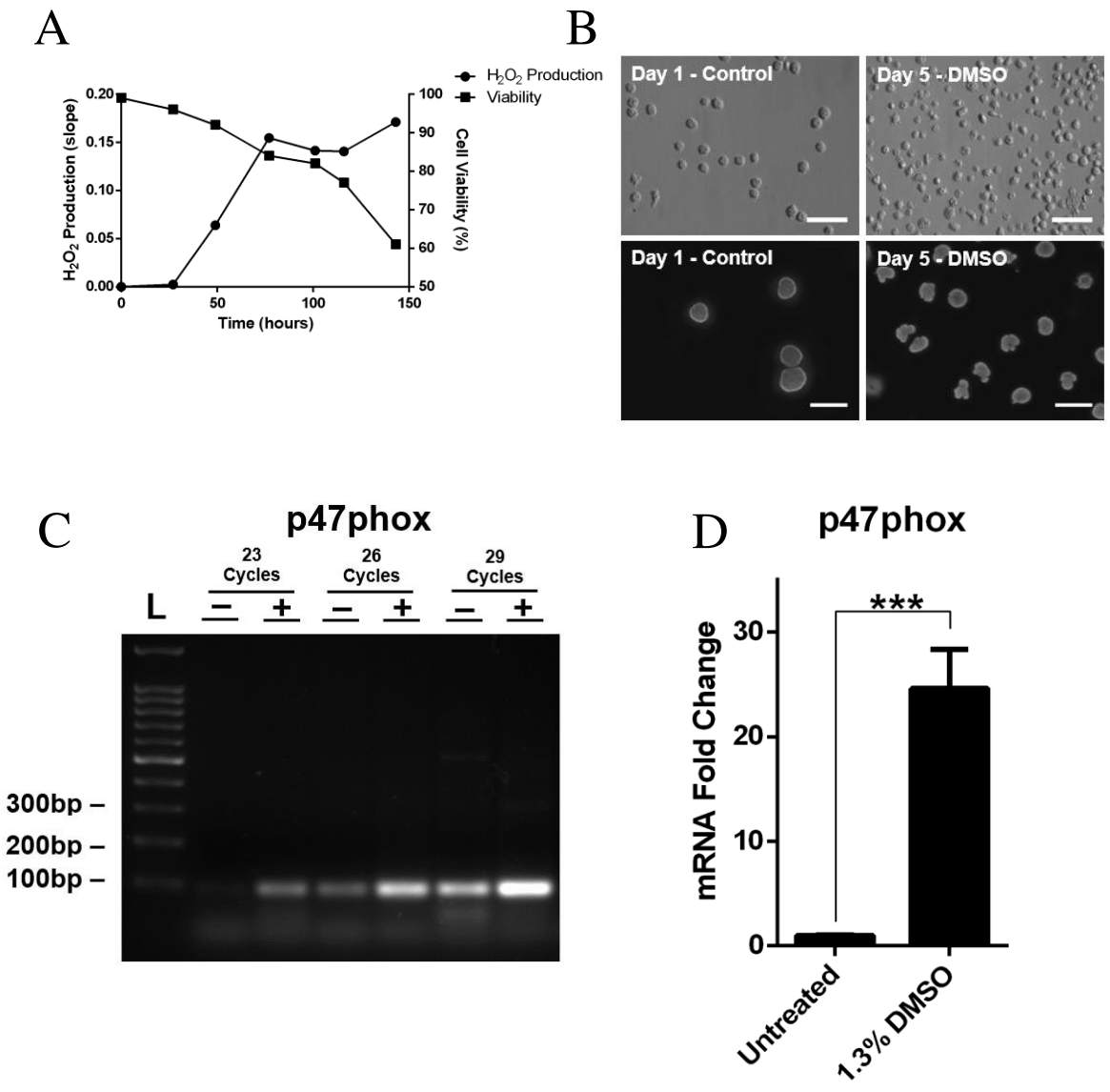

A
B
C
D
H_2O_2 Production
Viability
H_2O_2 Production (slope)
Cell Viability (%)
Time (hours)
Day 1 - Control
Day 5 - DMSO
Day 1 - Control
Day 5 - DMSO
p47phox
23 Cycles
26 Cycles
29 Cycles
L
300bp –
200bp –
100bp –
p47phox
mRNA Fold Change
***
Untreated
1.3% DMSO

Figure 8. Confirmation of DMSO-induced neutrophilic differentiation in HL-60 cells.

Verification of HL-60 cell differentiation induced by 1.3% DMSO through: (A) fMLP induced H_2O_2 production 72 hrs post treatment, (B) cell morphology (upper panels), and nuclei staining (lower panels) with 2 µg/mL Hoechst of HL-60 control cells plated for 24 hrs vs HL-60 cells following treatment with 1.3% DMSO for 120 hrs. Scale bars, 100 µm (light microscopy), and 25 µm (fluorescent microscopy). (C) RT-PCR and (D) RT-qPCR for differentiation marker, p47*phox* (93 bp) following treatment with 1.3% DMSO for 72 hrs. GAPDH was used as an internal control. The data represent the mean ± SD, n = 3. Significant differences among treatments were determined using an independent samples t-test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

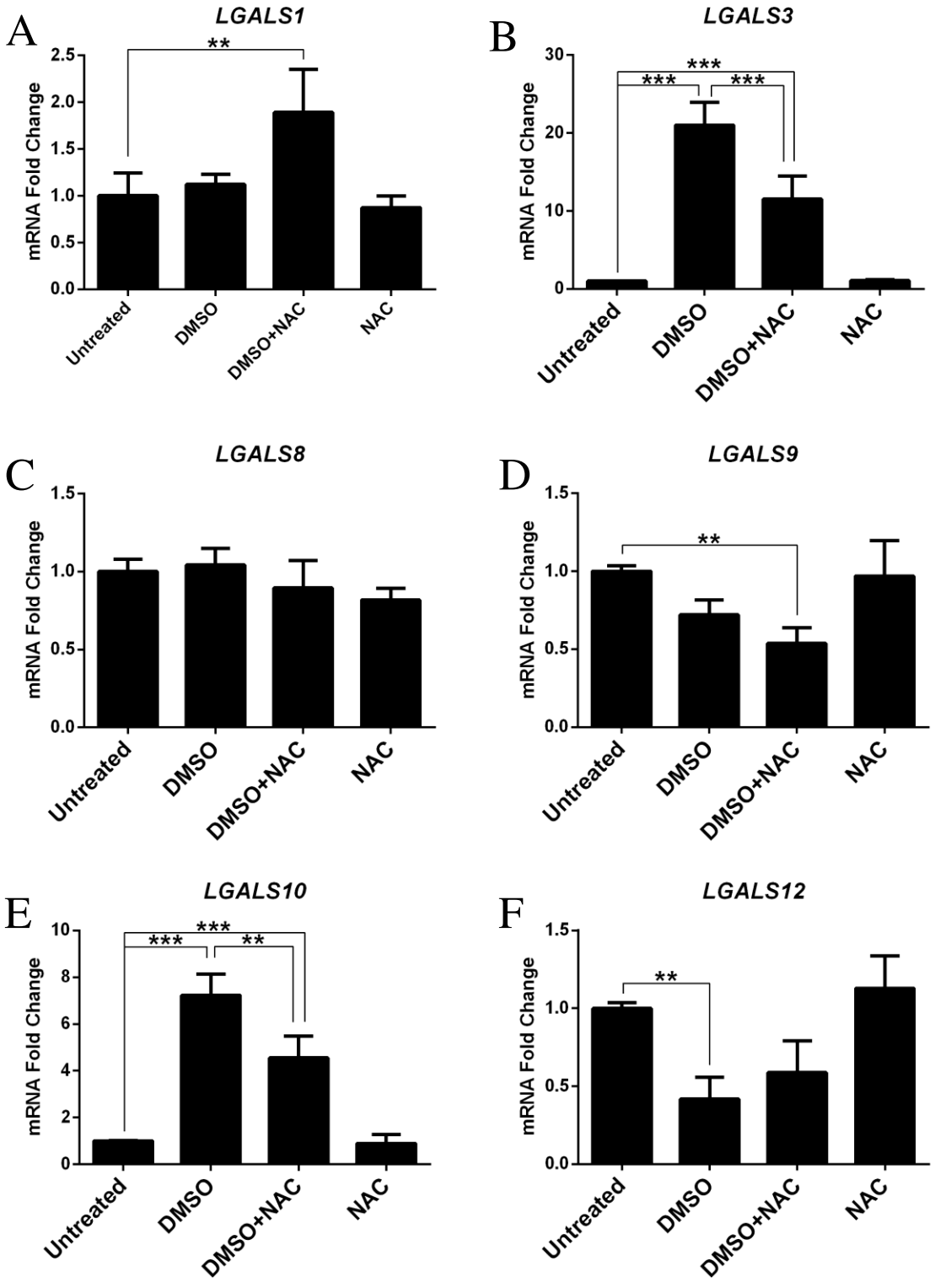
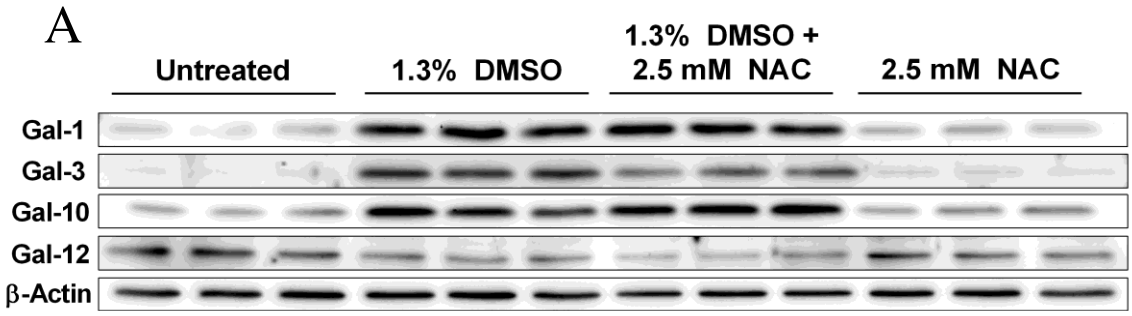
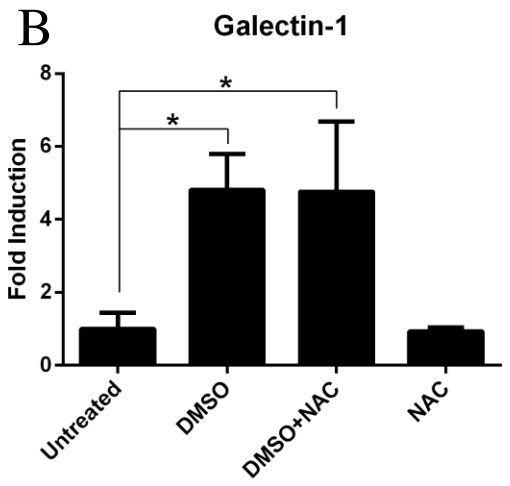
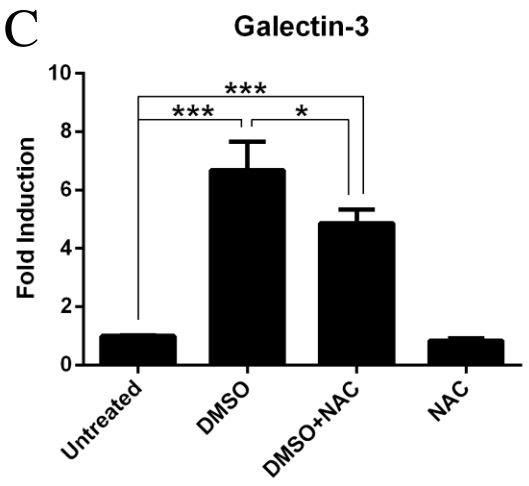

A
LGALS1
mRNA Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
**
Untreated
DMSO
DMSO+NAC
NAC
B
LGALS3
mRNA Fold Change
30
20
10
0
***
***
***
Untreated
DMSO
DMSO+NAC
NAC
C
LGALS8
mRNA Fold Change
1.5
1.0
0.5
0.0
Untreated
DMSO
DMSO+NAC
NAC
D
LGALS9
mRNA Fold Change
1.5
1.0
0.5
0.0
**
Untreated
DMSO
DMSO+NAC
NAC
E
LGALS10
mRNA Fold Change
10
8
6
4
2
0
***
***
**
Untreated
DMSO
DMSO+NAC
NAC
F
LGALS12
mRNA Fold Change
1.5
1.0
0.5
0.0
**
Untreated
DMSO
DMSO+NAC
NAC

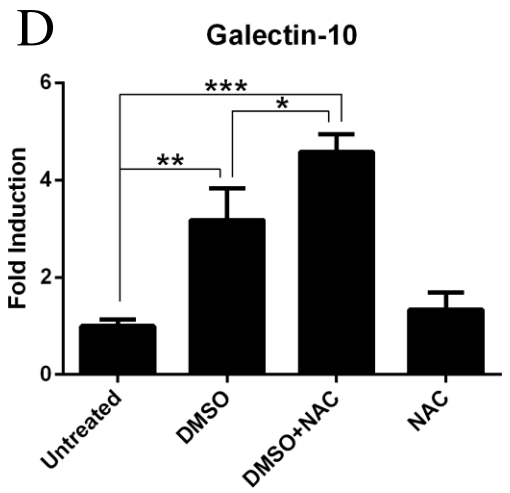
Figure 9. Neutrophilic differentiation of HL-60 cells induced altered galectin transcript levels.

ΔΔCt relative galectin transcript expression in HL-60 cells treated with 1.3% DMSO and/or 2.5 mM NAC for 72 hrs relative to untreated cells, using RT-qPCR. (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10* and (F) *LGALS12*. GAPDH was used as an internal control. The data represent the mean ± SD, n = 3-5. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.


A
Untreated
1.3% DMSO
1.3% DMSO + 2.5 mM NAC
2.5 mM NAC
Gal-1
Gal-3
Gal-10
Gal-12
β-Actin


B
Galectin-1
Fold Induction
Untreated
DMSO
DMSO+NAC
NAC


C
Galectin-3
Fold Induction
Untreated
DMSO
DMSO+NAC
NAC


D
Galectin-10
Fold Induction
Untreated
DMSO
DMSO+NAC
NAC

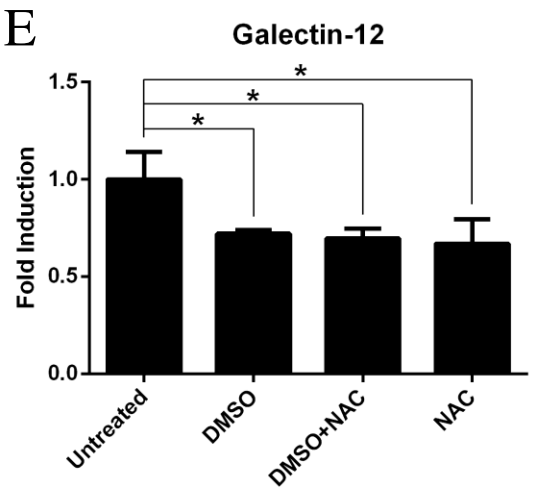

E
Galectin-12
Fold Induction
Untreated
DMSO
DMSO+NAC
NAC

Figure 10. Neutrophilic differentiation of HL-60 cells induced altered galectin protein levels.

Immunoblots for galectin protein expression in (A) HL-60 cells treated with 1.3% DMSO and/or 2.5 mM NAC for 72 hrs. Quantification of (B) galectin-1, (C) galectin-3, (D) galectin-10 and (E) galectin-12 protein levels by densitometric analysis of the immunoblot in (A). β-actin was used as a loading control. 25 μg of protein were loaded per well. The data represent the mean ± SD, n = 3. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

ANOVA. DMSO-induced neutrophilic differentiation significantly increased protein levels of galectins-1, -3, and -10 ($p < 0.05$, $p < 0.001$ and $p < 0.01$, respectively) (Figure 10B, C, D) while significantly decreasing galectin-12 protein levels ($p < 0.05$) (Figure 10E). Interestingly, only galectin-3 levels (Figure 10C) showed significant partial attenuation ($p < 0.05$) whereas the other galectins showed no attenuation.

3.3 Inhibition of galectins suppressed cell proliferation and differentially affected neutrophilic differentiation of HL-60 cells.

To determine the effect galectins have on cellular proliferation of HL-60, competitive inhibition of galectins was achieved using both non-specific and specific CRD binding galectin inhibitors – using direct counts of cells in suspension cell culture (Figure 11). Inhibitors used included: lactose, LacNac, lactobionic acid, TDG, and OTX008. In addition, α-MM and mannose were used as negative controls considering these sugars are absent of β-galactosyl residues. There was a statistically significant difference between treatment groups for TDG ($F_{4,1} = 12.6$, $p < 0.001$), and OTX008 ($F_{3,1} = 11.53$, $p < 0.001$). Treatment with 40 mM TDG ($p < 0.001$), and 2.5 ($p < 0.05$) or 5 μM OTX008 ($p < 0.001$) significantly decreased HL-60 cell proliferation indicated by increased cell doubling times (Figure 11E, F). Although treatments with other inhibitors did not significantly slow cell proliferation, values showed a trend toward increased doubling time (Figure 11A, B, C, D). Inhibitors used at the indicated concentrations had no significant effect on cell viability

Treatment with the same inhibitors was used to determine the role of galectins during neutrophilic differentiation (Figure 12) measured as the fold change in fMLP induced rate of H_2O_2 production. Cells were incubated with both differentiation agent 1.3% DMSO for 72 hrs and a specific galectin inhibitor at the time of original seeding. There was a statistically significant difference across galectin inhibitor treatment groups when measuring H_2O_2 production ($F_{6,1} = 82.039$, $p < 0.001$). Although, treatment with lactose ($p < 0.05$), and lactobionic acid ($p < 0.001$) showed a significant increase in H_2O_2 production indicative of increased cellular differentiation; treatment with OTX008 significantly decreased ($p < 0.001$) H_2O_2 production indicative of decreased cellular differentiation (Figure 12).

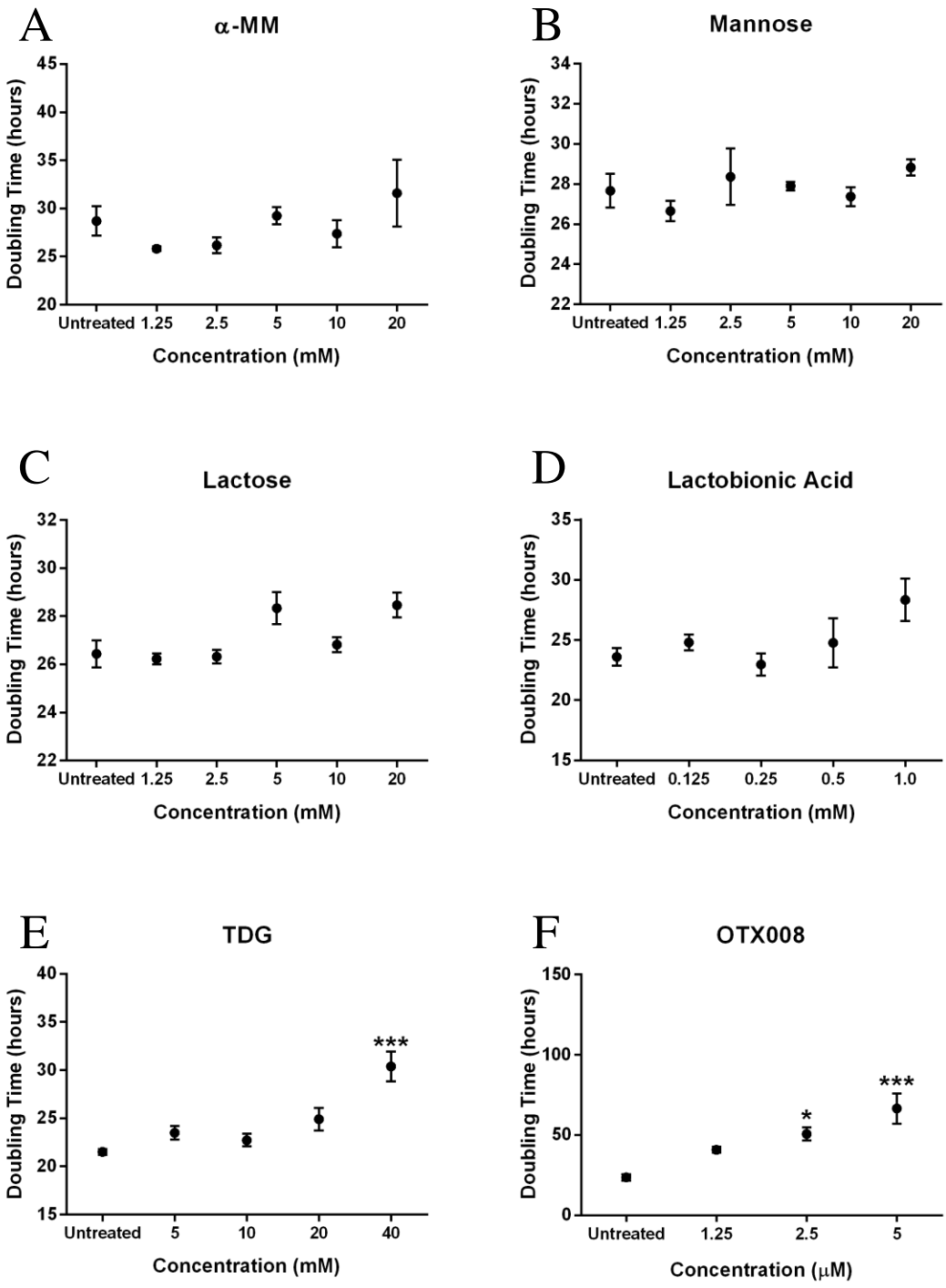

A
α-MM
Doubling Time (hours)
45
40
35
30
25
20
Untreated 1.25 2.5 5 10 20
Concentration (mM)
B
Mannose
Doubling Time (hours)
34
32
30
28
26
24
22
Untreated 1.25 2.5 5 10 20
Concentration (mM)
C
Lactose
Doubling Time (hours)
32
30
28
26
24
22
Untreated 1.25 2.5 5 10 20
Concentration (mM)
D
Lactobionic Acid
Doubling Time (hours)
35
30
25
20
15
Untreated 0.125 0.25 0.5 1.0
Concentration (mM)
E
TDG
Doubling Time (hours)
40
35
30
25
20
15
***
Untreated 5 10 20 40
Concentration (mM)
F
OTX008
Doubling Time (hours)
150
100
50
0
*
***
Untreated 1.25 2.5 5
Concentration (μM)

Figure 11. Galectin-specific and non-specific inhibitory sugars increased HL-60 cell doubling time.

Cell proliferation of HL-60 cells represented as the doubling time over 5 days treated with non-specific sugars such as (A) α-MM and (B) mannose and specific galectin inhibitors including (C) lactose, (D) lactobionic acid, (E) TDG and (F) OTX008. The data represent the mean ± SE n = 4-5. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

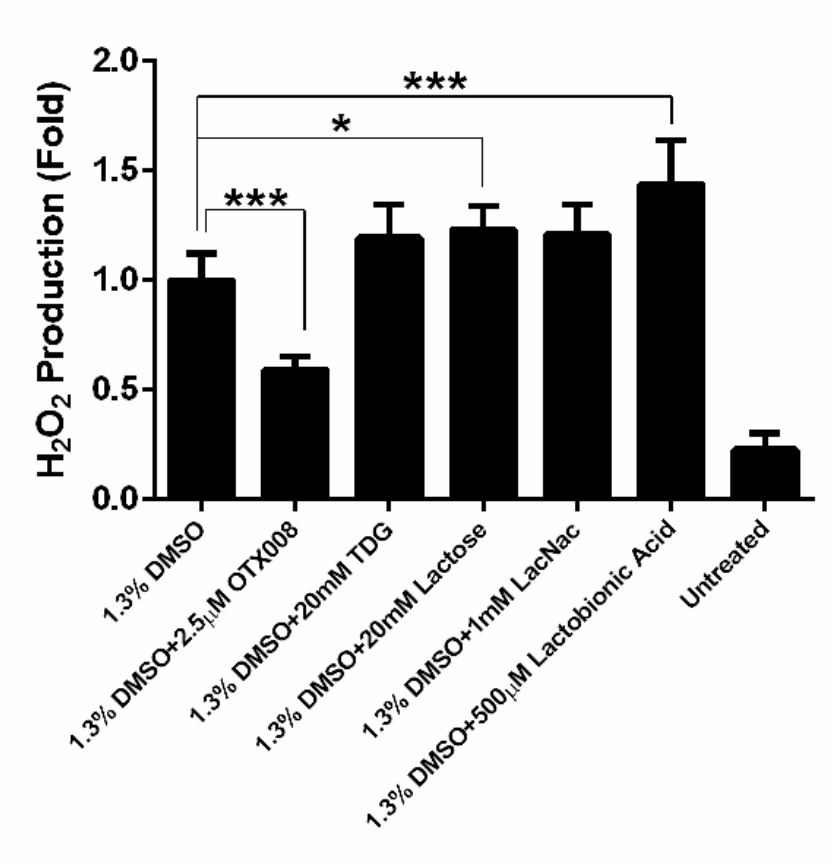

2.0
1.5
1.0
0.5
0.0
H2O2 Production (Fold)
***
*
***
1.3% DMSO
1.3% DMSO+2.5μM OTX008
1.3% DMSO+20mM TDG
1.3% DMSO+20mM Lactose
1.3% DMSO+1mM LacNac
1.3% DMSO+500μM Lactobionic Acid
Untreated

Figure 12. Galectin-specific and non-specific inhibitory sugars differentially affected DMSO-induced HL-60 neutrophilic differentiation.

Rate of H_2O_2 production (pmol/sec/1E6 cells, standardized to fold expression), as a measure of cellular differentiation induced by the chemotactic peptide 100 nM fMLP by HL-60 cells 72 hrs post treatment with 1.3% DMSO alone and in combination with various galectin inhibitors. The data represent the mean ± SD, n = 5-9. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

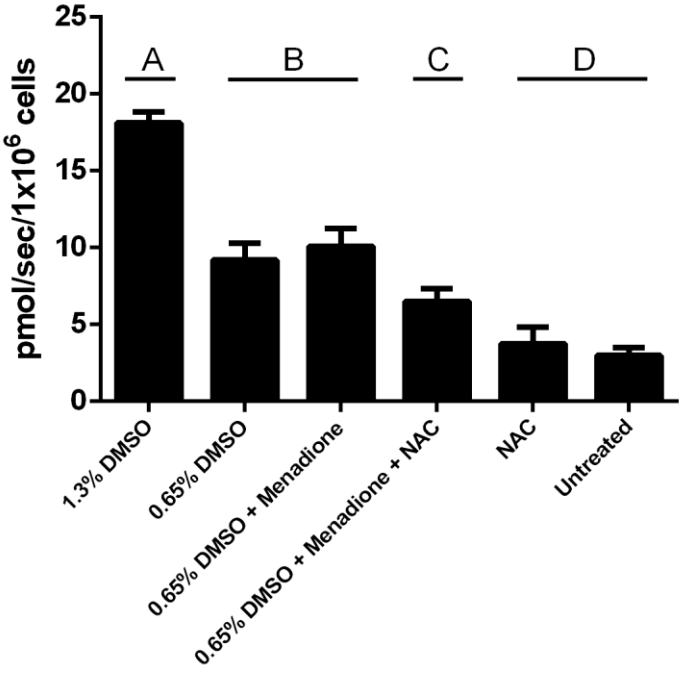
3.4 Modification of the redox environment impacted DMSO-induced neutrophilic differentiation.

In light of the observation that the antioxidant NAC impacted galectin expression during neutrophilic differentiation; I sought to test the effect of both oxidative stress inducer menadione and NAC on HL-60 neutrophilic differentiation as measured by fMLP induced H_2O_2 production (Figure 13A, B). There was a statistically significant reduction in DMSO-induced differentiation following NAC treatment (Figure 13A) ($F_{3,1} = 41.54$, $p < 0.001$), (Figure 13B) ($F_{5,1} = 125.73$, $p < 0.001$) as determined by one-way ANOVA. DMSO (1.3%) administered in conjunction with 2.5 mM NAC for 72 hrs showed a significant decrease ($p < 0.001$) in H_2O_2 production rates in comparison to the positive control indicating that quenching ROS during differentiation was able to diminish levels of the neutrophilic phenotype (Figure 13A). Interestingly, adding 0.65% DMSO in conjunction with 10 μM menadione did not significantly increase levels of H_2O_2 compared to 0.65% alone (Figure 13B). However, the antioxidant NAC was still capable of significantly decreasing levels of H_2O_2 produced by 0.65% DMSO alone ($p < 0.05$), and in conjunction with 10 μM menadione ($p < 0.01$) (Figure 13B). Complete attenuation of H_2O_2 production similar to untreated levels was not observed, indicating a small synergistic effect of 0.65% DMSO with 10 μM menadione (Figure 13B).

3.5 DNA methylation regulated galectin transcript and protein levels in HL-60 cells.

DNA demethylation has been reported to induce cellular differentiation of HL-60 cells (Schwartsmann et al., 1987); therefore, I sought to investigate the effect of DNA methylation on galectin gene expression by using decitabine – an inhibitor of DNA methyltransferases. HL-60 RNA was isolated for RT-qPCR following treatment with 50 nM decitabine and/or 0.65 – 1.3% DMSO for 72 hrs to determine relative p47*phox* transcript levels (Figure 14B). Treatment with 50 nM decitabine showed a significant increase ($p < 0.05$) in p47*phox* expression indicating the potential for this drug to increase HL-60 cell differentiation (Figure 14A). Interestingly, co-treatment with 0.65% DMSO and 50 nM decitabine displayed a synergistic effect on p47*phox* induction in comparison

A


pmol/sec/1x10^6 cells
25
20
15
10
5
0
A
B
C
D
1.3% DMSO
0.65% DMSO
0.65% DMSO + Menadione
0.65% DMSO + Menadione + NAC
NAC
Untreated

B

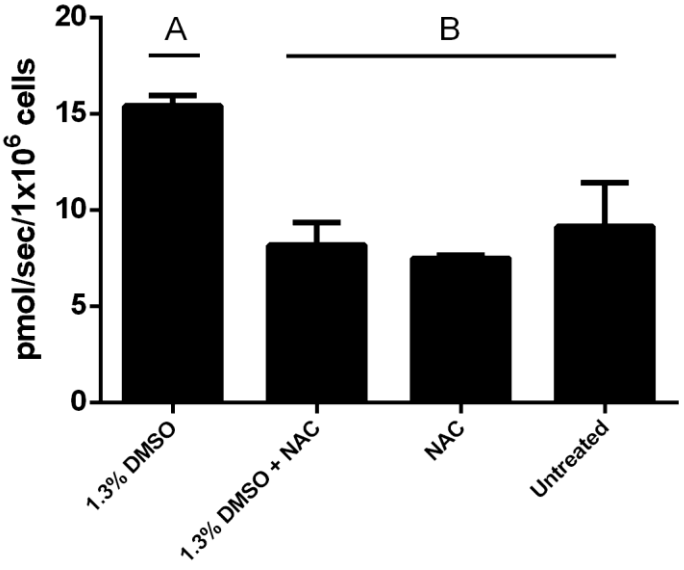
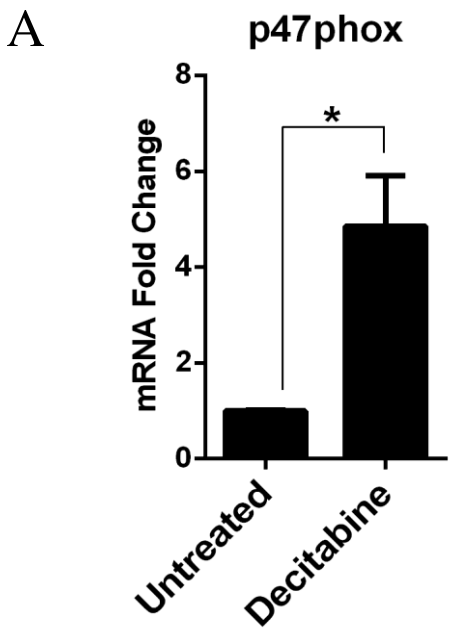

pmol/sec/1x10^6 cells
20
15
10
5
0
A
B
1.3% DMSO
1.3% DMSO + NAC
NAC
Untreated

Figure 13. Modification of the redox environment changes the HL-60 final differentiated phenotype.

Rate of H_2O_2 production as a measure of cellular differentiation induced by the chemotactic peptide 100 nM fMLP by HL-60 cells 72 hrs post treatment with 0.65 – 1.3% DMSO alone and in combination with 10 μM menadione or 2.5 mM NAC, or both. Panels (A) and (B) represent individual experiments under different treatments. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test and are represented as different letters, considering $p < 0.05$. The data represent the mean ± SD, n = 3-5.


A
p47phox
mRNA Fold Change
8
6
4
2
0
*
Untreated
Decitabine

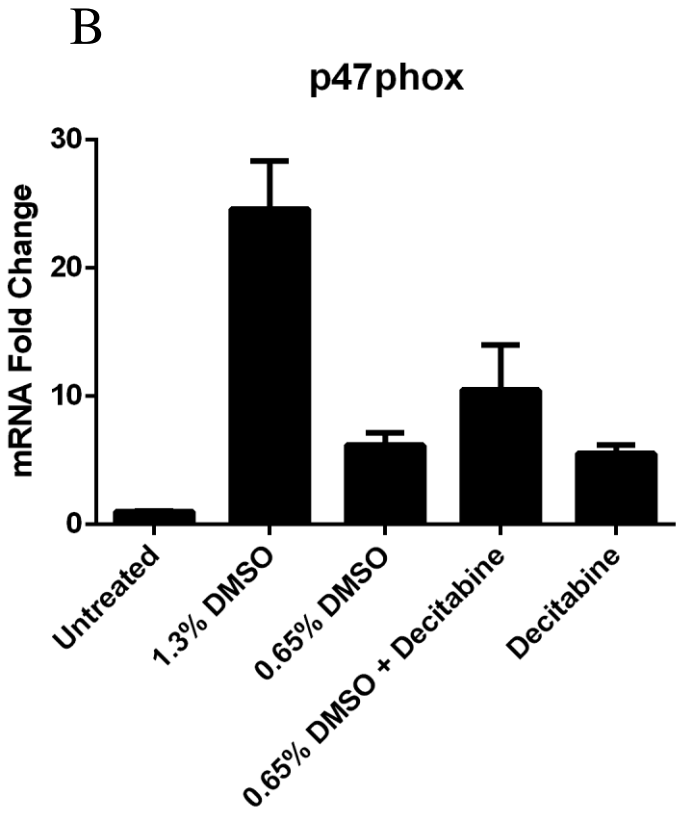

B
p47phox
mRNA Fold Change
30
20
10
0
Untreated
1.3% DMSO
0.65% DMSO
0.65% DMSO + Decitabine
Decitabine

Figure 14. Inhibition of DNA methyltransferases upregulated the differentiation marker, p47*phox*.

ΔΔCt relative p47*phox* transcript levels in HL-60 cells treated with (A) 50 nM decitabine for 72 hrs or (B) 0.65 – 1.3% DMSO with or without 50 nM decitabine relative to untreated cells, using RT-qPCR. GAPDH was used as an internal control. The data represent the mean ± SD, panel (A) n = 3, (B) n = 1. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

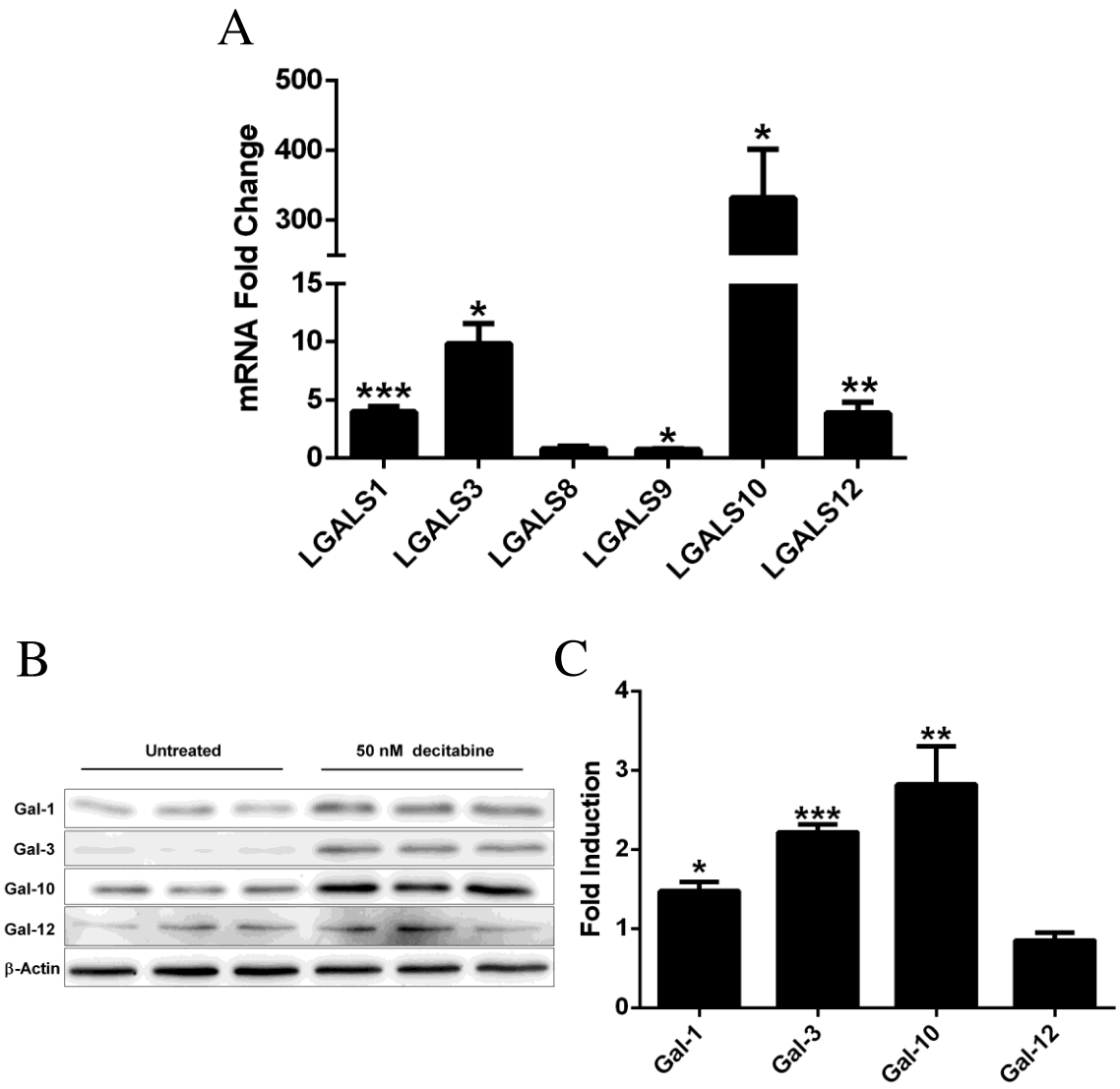

A
mRNA Fold Change
500
400
300
15
10
5
0
***
*
*
*
**
LGALS1
LGALS3
LGALS8
LGALS9
LGALS10
LGALS12
B
Untreated
50 nM decitabine
Gal-1
Gal-3
Gal-10
Gal-12
β-Actin
C
Fold Induction
4
3
2
1
0
*
***
**
Gal-1
Gal-3
Gal-10
Gal-12

Figure 15. Inhibition of DNA methyltransferases induced changes in galectin transcript and protein levels.

(A) ΔΔCt relative galectin transcript expression in HL-60 cells treated with 50 nM decitabine relative to untreated cells, using RT-qPCR. The data represent the mean ± SD, n = 3-4. Significant differences between treatments were determined using an independent samples T-test considering *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. GAPDH was used as an internal control. (B) Immunoblots for galectin protein expression in HL-60 cells treated with 50 nM decitabine for 72 hrs. β-actin was used as a loading control. The data represent the mean ± SD, n = 3. (C) Quantification of galectin levels by densitometric analysis. Significant differences among treatments were determined using ANOVA followed by a Tukey's HSD test considering *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

to treatment with either 0.65% DMSO or 50 nM decitabine, alone (Figure 14B). Therefore, decitabine works synergistically with DMSO to induce the differentiated phenotype.

The HL-60 galectin expression profile was determined following treatment with 50 nM decitabine at both the transcript and protein level (Figure 15). There was a statistically significant difference between galectins ($F_{5,1} = 67.14$, $p < 0.001$) following treatment with 50 nM decitabine as determined by one-way ANOVA. Treatment with decitabine significantly increased *LGALS1, LGALS3, LGALS10* and *LGALS12* transcript levels ($p < 0.001$, $p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) (Figure 15A). Additionally, a significant decrease in expression ($p < 0.05$) was observed with *LGALS9* (Figure 15A). There was a statistically significant change across galectin protein expression levels ($F_{3,1} = 33.56$, $p < 0.001$) following treatment with 50 nM decitabine as determined by one-way ANOVA. Western blots showed a significant increase of galectin-1, -3, and -10 expression ($p < 0.05$, $p < 0.001$ and $p < 0.01$, respectively) (Figure 15B, C). Interestingly, an increase in galectin-12 expression was not observed at the protein level (Figure 15B, C).

3.6 The JNK signaling pathway was differentially regulated in response to oxidative stress and HL-60 neutrophilic differentiation and regulates galectin transcript levels.

The effect of JNK signaling on galectin transcript levels was investigated using the JNK inhibitor SP600125 (Figure 16A), and activator anisomycin (Figure 16B) followed by RT-qPCR analysis. Significant differences in galectin transcript levels were determined following treatment with SP600125 ($F_{5,1} = 22.579$, $p < 0.001$), and anisomycin ($F_{5,1} = 112.39$, $p < 0.001$) as indicated by one-way ANOVA. Interestingly, inhibition of JNK signaling increased *LGALS1* and *LGALS12* transcript levels ($p < 0.01$ and $p < 0.05$, respectively), and significantly decreased *LGALS8*, *LGALS9* and *LGALS10* levels ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) (Figure 16A). Activation of JNK signaling increased expression of *LGALS1*, *LGALS3* and *LGALS8* ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) while significantly decreased *LGALS9* and *LGALS12* expression ($p < 0.05$ and $p < 0.05$, respectively) (Figure 16B). These data indicate that galectins are differentially regulated by the JNK signaling pathway and that other signaling pathways (i.e. MAPKs) may also regulate galectin expression as a compensatory response.

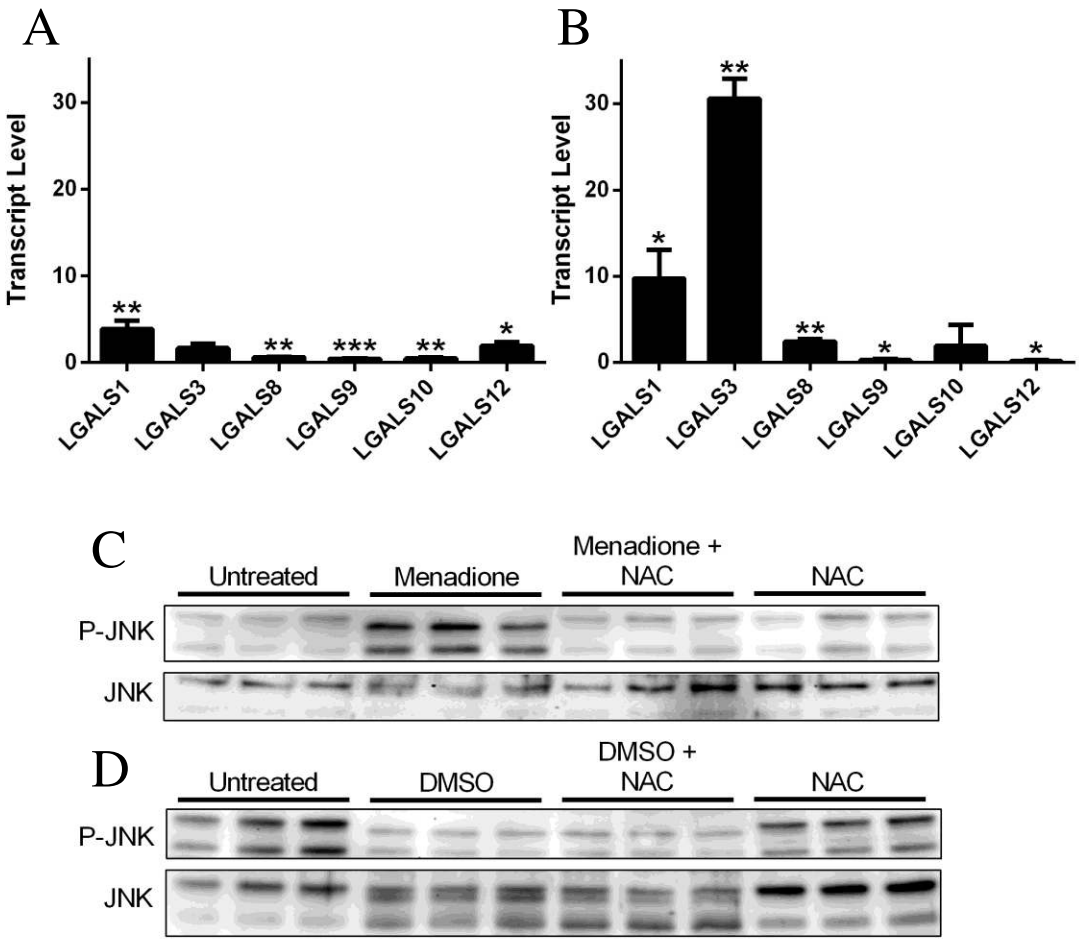

A
B
Transcript Level
30
20
10
0
**
**
***
**
*
LGALS1
LGALS3
LGALS8
LGALS9
LGALS10
LGALS12
*
**
**
*
*
C
Untreated
Menadione
Menadione +
NAC
NAC
P-JNK
JNK
D
Untreated
DMSO
DMSO +
NAC
NAC
P-JNK
JNK

Figure 16. JNK signaling altered galectin levels and was differentially regulated during oxidative stress and DMSO-induced neutrophilic differentiation.

ΔΔCt relative galectin transcript levels in HL-60 cells treated with either JNK inhibitor (A) 25 μM SP600125 or activator (B) 400 nM anisomycin, respectively for 24 hrs, relative to untreated cells using RT-qPCR. The data represent the mean ± SD, n = 3. Significant differences between treatments were determined using an independent samples T-test considering $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Immunoblots for phosphorylated JNK and total JNK levels in HL-60 cells treated with (C) 10 μM menadione with or without 1 mM NAC for 24 hrs (D) 1.3% DMSO with or without 2.5 mM NAC for 72 hrs, n = 3.

To determine endogenous phosphorylated JNK levels during oxidative stress and DMSO-induced neutrophilic differentiation, western blotting for phosphorylated JNK and total JNK was performed. Oxidative stress increased phosphorylated JNK levels, which were attenuated with NAC (Figure 16C). In contrast, differentiation of HL-60 was accompanied by decreased phosphorylated JNK levels (Figure 16C).

3.7 Correlative analysis between galectins, oxidative stress and cellular differentiation suggest galectins as biomarkers of cellular stress responses.

To identify a specific correlation between galectins, oxidative stress and cellular differentiation a Pearson's correlation was computed based on treatments with 1.3% DMSO, 10 μM menadione, 50 nM decitabine, 25 μM SP600125 and 400 nM anisomycin. This test measures the strength of linear association between two genes. *ho*-1 was used as a marker of menadione-induced oxidative stress while p47*phox* was used as marker of cellular differentiation. The Pearson's correlation test revealed a significant positive correlation in the expression of oxidative stress marker *ho*-1 with *LGALS1* ($p < 0.05$, $R^2 = 0.400$, n = 15), *LGALS3* ($p < 0.001$, $R^2 = 0.756$, n = 15), and *LGALS8* ($p < 0.01$, $R^2 = 0.570$, n = 15) while a significant negative correlation was observed with *LGALS12* ($p < 0.01$, $R^2 = 0.426$, n = 15) (Figure 17A, B, C, F).

Correlations were also determined between transcript levels of the cellular differentiation marker p47*phox* and galectins (Figure 18). In particular, there was a significant positive correlation of expression of p47*phox* with *LGALS3* ($p < 0.001$, $R^2 = 0.723$, n = 12), *LGALS9* ($p < 0.05$, $R^2 = 0.424$, n = 12), and *LGALS10* ($p < 0.001$, $R^2 = 0.868$, n = 12) while a significant negative correlation was observed with *LGALS1* ($p < 0.001$, $R^2 = 0.691$, n = 12), and *LGALS12* ($p < 0.05$, $R^2 = 0.456$, n = 12) (Figure 18A, B, D, E, F).

Correlations between transcript levels of individual galectins were also tested to determine expression patterns and the possibility for expression redundancy, across all treatments (Figure 19). Pearson's correlation analyses identified a significant correlation between *LGALS1* with *LGALS3* ($p < 0.05$, $R^2 = 0.302$, n = 15), *LGALS8* ($p < 0.01$, $R^2 = 0.547$, n = 15), and *LGALS12* ($p < 0.01$, $R^2 = 0.641$, n = 12) while a significant negative correlation

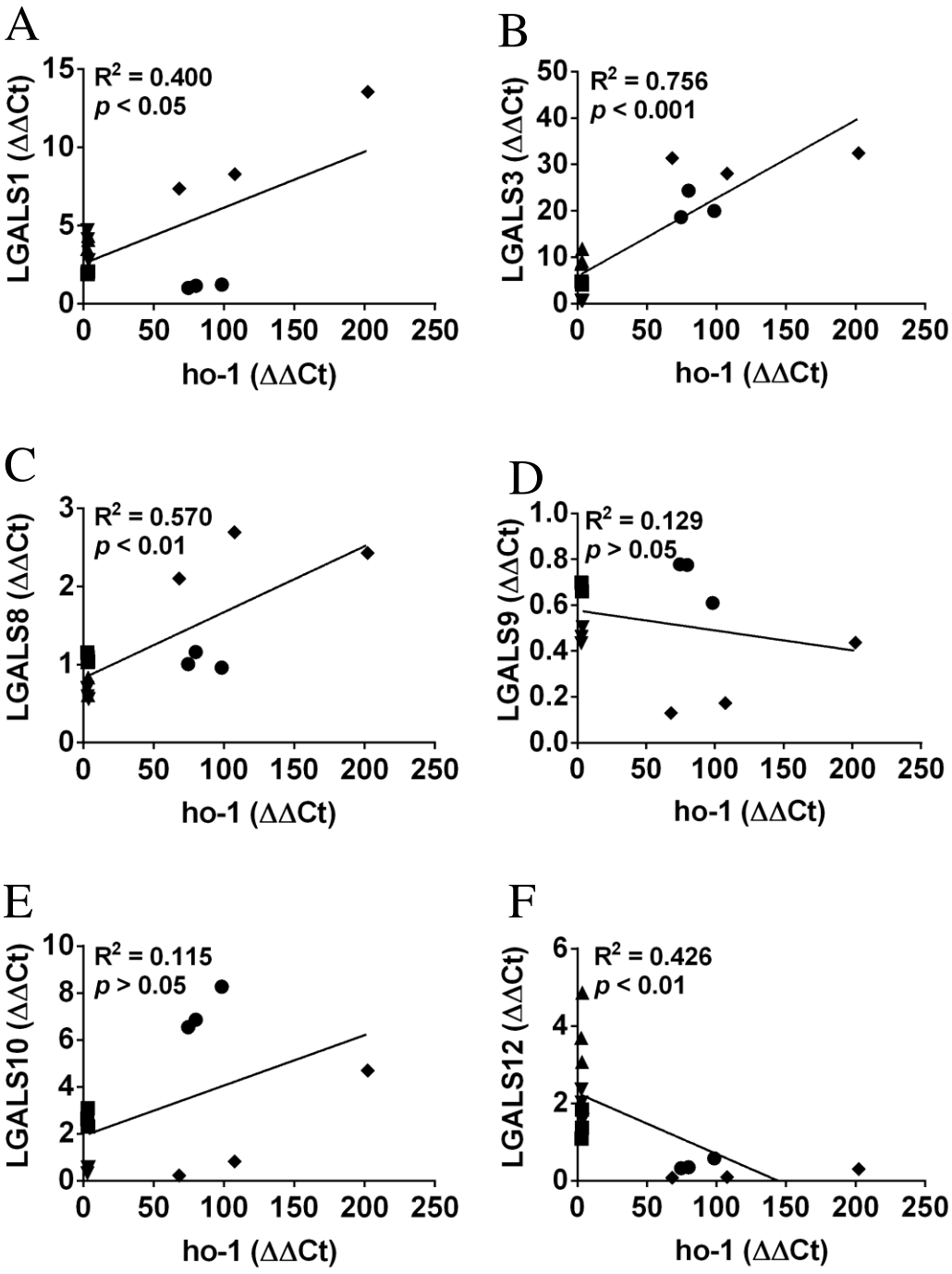

A
R² = 0.400
p < 0.05
LGALS1 (ΔΔCt)
ho-1 (ΔΔCt)
B
R² = 0.756
p < 0.001
LGALS3 (ΔΔCt)
ho-1 (ΔΔCt)
C
R² = 0.570
p < 0.01
LGALS8 (ΔΔCt)
ho-1 (ΔΔCt)
D
R² = 0.129
p > 0.05
LGALS9 (ΔΔCt)
ho-1 (ΔΔCt)
E
R² = 0.115
p > 0.05
LGALS10 (ΔΔCt)
ho-1 (ΔΔCt)
F
R² = 0.426
p < 0.01
LGALS12 (ΔΔCt)
ho-1 (ΔΔCt)

Figure 17. Correlation in transcript levels between galectins and the oxidative stress marker *ho*-1.

ΔΔCt relative galectin and *ho*-1 transcript levels in HL-60 cells treated with (●) 1.3% DMSO, (■) 10 µM menadione, (▲) 50 nM decitabine, (▼) 25 µM SP600125 and (◆) 400 nM anisomycin, (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10* and (F) *LGALS12*. GAPDH was used as an internal control. Significant correlation between genes was determined using the Pearson's correlation test considering $p < 0.05$, n = 3 for each treatment.

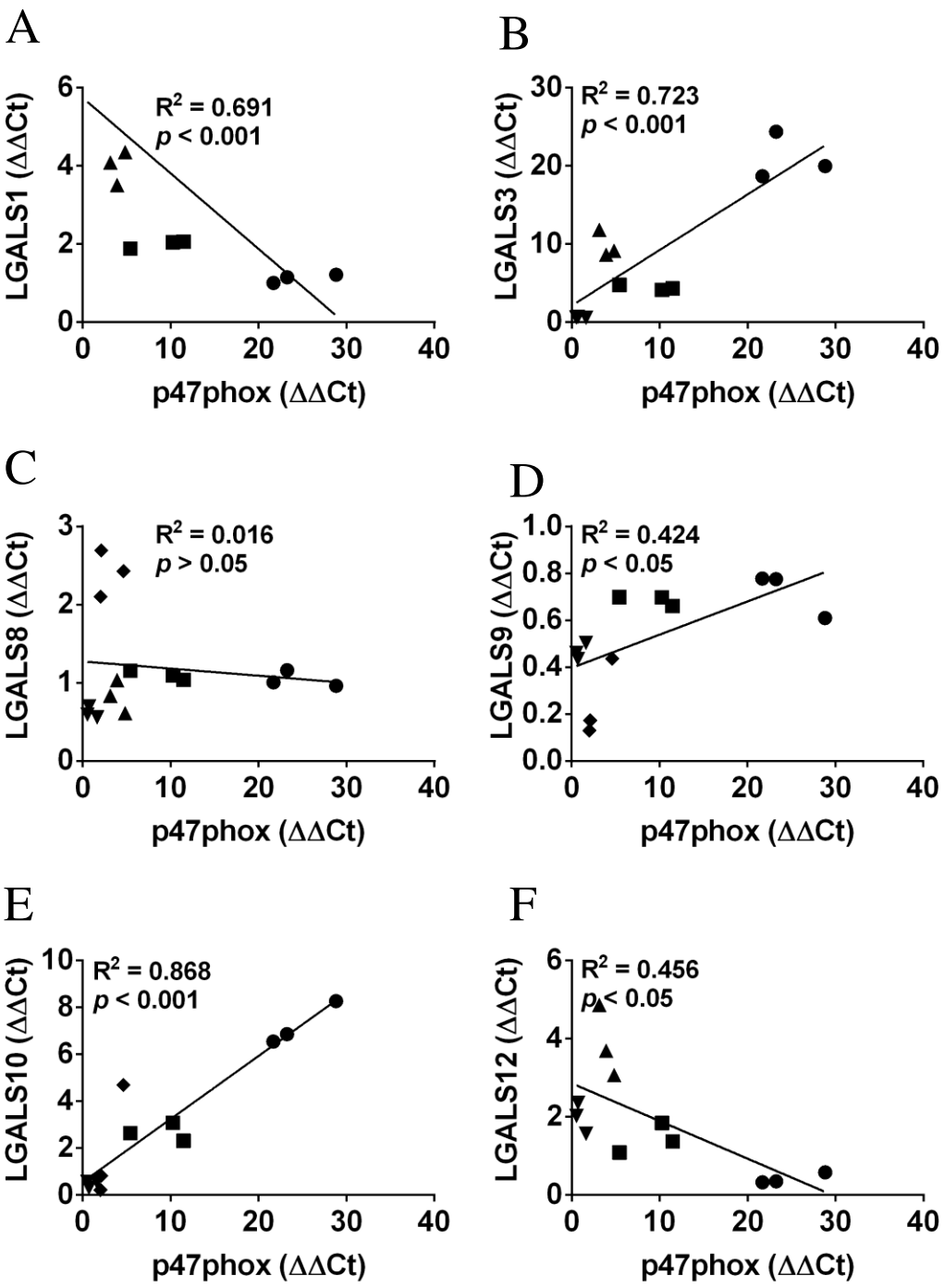

A
LGALS1 (ΔΔCt)
R² = 0.691
p < 0.001
p47phox (ΔΔCt)
B
LGALS3 (ΔΔCt)
R² = 0.723
p < 0.001
p47phox (ΔΔCt)
C
LGALS8 (ΔΔCt)
R² = 0.016
p > 0.05
p47phox (ΔΔCt)
D
LGALS9 (ΔΔCt)
R² = 0.424
p < 0.05
p47phox (ΔΔCt)
E
LGALS10 (ΔΔCt)
R² = 0.868
p < 0.001
p47phox (ΔΔCt)
F
LGALS12 (ΔΔCt)
R² = 0.456
p < 0.05
p47phox (ΔΔCt)

Figure 18. Correlation in transcript levels between galectins and marker of HL-60 cell neutrophilic differentiation p47*phox*.

ΔΔCt relative galectin and p47*phox* transcript levels in HL-60 cells treated with (●) 1.3% DMSO, (■) 10 µM menadione, (▲) 50 nM decitabine, (▼) 25 µM SP600125 and (◆) 400 nM anisomycin. (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10* and (F*) LGALS12*. GAPDH was used as an internal control. Significant correlation between genes was determined using the Pearson's correlation test considering $p < 0.05$, n = 3 for each treatment.

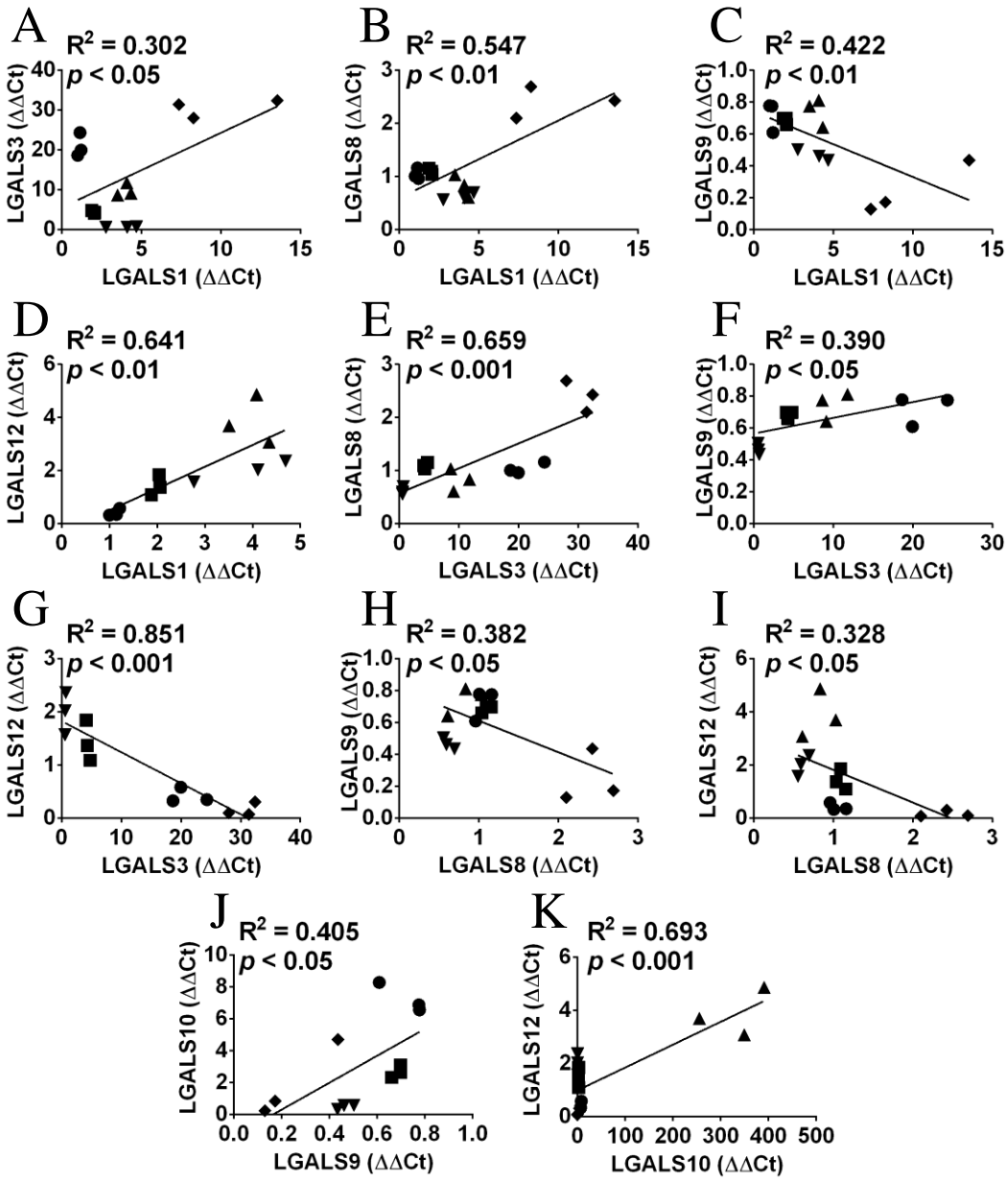

A
R² = 0.302
p < 0.05
LGALS3 (ΔΔCt)
LGALS1 (ΔΔCt)
B
R² = 0.547
p < 0.01
LGALS8 (ΔΔCt)
LGALS1 (ΔΔCt)
C
R² = 0.422
p < 0.01
LGALS9 (ΔΔCt)
LGALS1 (ΔΔCt)
D
R² = 0.641
p < 0.01
LGALS12 (ΔΔCt)
LGALS1 (ΔΔCt)
E
R² = 0.659
p < 0.001
LGALS8 (ΔΔCt)
LGALS3 (ΔΔCt)
F
R² = 0.390
p < 0.05
LGALS9 (ΔΔCt)
LGALS3 (ΔΔCt)
G
R² = 0.851
p < 0.001
LGALS12 (ΔΔCt)
LGALS3 (ΔΔCt)
H
R² = 0.382
p < 0.05
LGALS9 (ΔΔCt)
LGALS8 (ΔΔCt)
I
R² = 0.328
p < 0.05
LGALS12 (ΔΔCt)
LGALS8 (ΔΔCt)
J
R² = 0.405
p < 0.05
LGALS10 (ΔΔCt)
LGALS9 (ΔΔCt)
K
R² = 0.693
p < 0.001
LGALS12 (ΔΔCt)
LGALS10 (ΔΔCt)

Figure 19. Correlation between galectin transcript levels.

ΔΔCt relative galectin transcript levels in HL-60 cells treated with (●) 1.3% DMSO, (■) 10 μM menadione, (▲) 50 nM decitabine, (▼) 25 μM SP600125 and (◆) 400 nM anisomycin. Correlations were made between each individual galectin vs.: (A–D) *LGALS1*, (E–G) *LGALS3*, (H–I) *LGALS8*, (J) *LGALS9* and (K) *LGALS12*. GAPDH was used as an internal control. Significant correlation between gene expressions was determined using the Pearson's correlation test considering $p < 0.05$, n = 3 for each treatment.

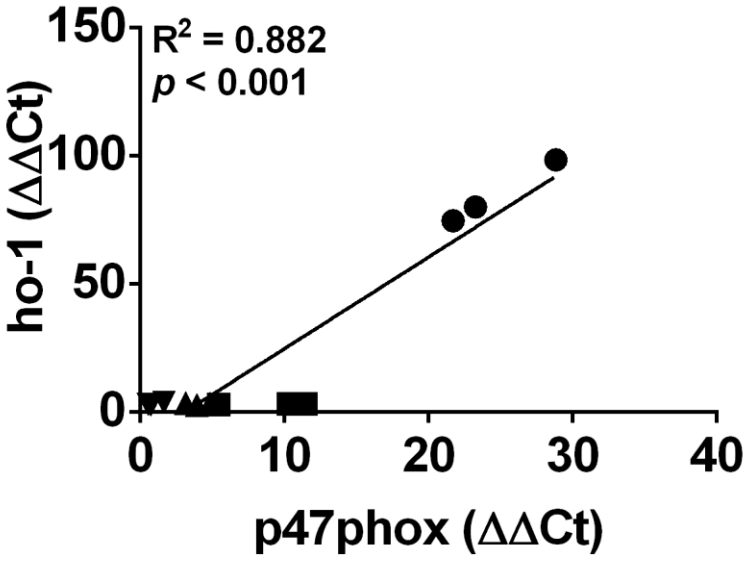

150
100
50
0
R² = 0.882
p < 0.001
ho-1 (ΔΔCt)
0
10
20
30
40
p47phox (ΔΔCt)

Figure 20. Correlation between oxidative stress and cellular differentiation.

ΔΔCt relative *ho*-1 and p47*phox* transcript levels in HL-60 cells treated with (●) 1.3% DMSO, (■) 10 µM menadione, (▲) 50 nM decitabine and (▼) 25 µM SP600125. GAPDH was used as an internal control. Significant correlation between genes was determined using the Pearson's correlation test considering $p < 0.05$, n = 3 for each treatment.

was observed with *LGALS9* ($p < 0.01$, $R^2 = 0.422$, n = 15) (Figure 19A, B, C, D). When correlating galectin transcript levels with *LGALS3*, there was a significant positive correlation with *LGALS8* ($p < 0.001$, $R^2 = 0.659$, n = 15), and *LGALS9* ($p < 0.05$, $R^2 = 0.390$, n = 12) while a significant negative correlation was observed with *LGALS12* ($p < 0.001$, $R^2 = 0.851$, n = 12) (Figure 19E, F, G). Further, the Pearson's correlation analyses identified a significant negative correlation between *LGALS8* with *LGALS9* levels ($p < 0.05$, $R^2 = 0.382$, n = 15), and *LGALS12* ($p < 0.05$, $R^2 = 0.328$, n = 15) (Figure 19H, I). This test also revealed a significant positive correlation between *LGALS9* with *LGALS10* expression ($p < 0.05$, $R^2 = 0.405$, n = 12) (Figure 19J). Finally, the Pearson's correlation analyses identified a significant positive correlation between *LGALS10* and *LGALS12* expression ($p < 0.001$, $R^2 = 0.693$, n = 15) (Figure 19K).

Lastly, to identify a link between oxidative stress and cellular differentiation, a Pearson's correlation test was computed to determine their relationship (Figure 20). This test identified a significant positive correlation between oxidative stress marker, *ho*-1 and the cellular differentiation marker, p47*phox* ($p < 0.001$, $R^2 = 0.882$, n = 12) (Figure 20).

Chapter 4

4 Discussion

The findings of my study demonstrate that oxidative stress sensitive galectins are involved with HL-60 neutrophilic differentiation. The first objective utilized immunoblotting and RT-qPCR techniques to define galectin-1, -3 and -10 as key stress-sensitive galectins that are similarly regulated under oxidative stress and neutrophilic differentiation. Additionally, the other galectins indicate minor similarities and differences between expression profiles of HL-60 including differential regulation of galectin-9 and -12. Competitive galectin inhibition with inhibitory sugar TDG and the selective small-molecule inhibitor OTX008 were used to investigate the second objective and demonstrate the relevance of galectins in promoting cellular proliferation. Similarly, both galectin-specific and non-specific inhibitory sugars differentially effected H_2O_2 production by HL-60 cells indicating galectins are involved with both the promotion and potential inhibition of DMSO-induced neutrophilic differentiation. Investigation of the third objective with immunoblotting and RT-qPCR techniques revealed the direct effect of DNA hypomethylation and JNK signaling on upregulating galectin transcript levels. Inhibition of DNA methyltransferases increased the same stress-sensitive galectins (*LGALS1*, *LGALS3*, and *LGALS10*) regulated during oxidative stress and differentiation. Immunoblotting for JNK levels indicate that this pathway is differentially regulated under oxidative stress and neutrophilic differentiation despite these two processes inducing similar galectin expression profiles.

4.1 Interpretation

4.1.1 Oxidative stress and neutrophilic differentiation induce similar galectin expression profiles.

There is a precedent for increased galectin expression during oxidative stress. Specifically, galectin-1 and -3 have been shown to display a protective role against the harmful effects of increased ROS production (Ito et al., 2011; Martínez-Martínez et al., 2014). However, the role of increased galectin expression following oxidative stress may also be related to these proteins being involved with apoptotic pathways. Galectin-3, for example, has been reported to both induce (Xue et al., 2013), and inhibit (Hoyer et al., 2004) apoptosis in

lymphocytic cell lineages. However, Matarrese et al. (2000) showed that overexpression of galectin-3 in breast cancer cells provides a protective effect from menadione-induced ROS, suggesting antioxidant-like properties of galectin-3 (Matarrese et al., 2000). The decreased expression of *LGALS9* is probably related to its often down-regulated expression within tumour cells (which undergo cellular stress including oxidative stress) in comparison to normal cells (Heusschen et al., 2013). However, signals related to inflammatory responses such as LPS have been reported to elevate galectin-9 mRNA levels, indicating stress-specific expression of this galectin (Kasamatsu et al., 2005).

Increased expression of *LGALS1*, *LGALS3* and *LGALS10* during oxidative stress indicate a common regulatory method amongst these galectins (Figure 5). In particular, Sp1 has been suggested to regulate transcription of these galectins considering the many possible Sp1 transcriptional binding spots amongst their promoters (Dyer and Rosenberg, 2001). Additionally, Sp1 has been identified as a redox-sensitive transcription factor (Chu and Ferro, 2006). Specifically, activation of the PP1-JNK-Sp1 signaling pathway via ROS promotes the phosphorylation and subsequent activation (and translocation to the nucleus) of transcription factor Sp1 (Chu and Ferro, 2006). Although *LGALS9* was generally downregulated in my study (Figure 5 and Figure 9); this galectin has been reported being involved with many of the same stress sensitive pathways that increase galectin transcript levels indicating galectin regulatory mechanism overlap (Laderach et al., 2010). The stable expression of *LGALS8* is interesting considering its vast differential expression across many different normal and tumour lines (Elola et al., 2014; Lahm et al., 2001). Unfortunately, little is known about *LGALS8* regarding the transcriptional and translational machinery with respect to stress-sensitive models (Timoshenko, 2015). Similarly, despite evidence suggesting involvement of *LGALS12* redox-sensitive pathways (Yang et al., 2004), little is known about this galectin with respect to regulatory methods involved with cell stress. Although this galectin is reported to play a role within adipose tissue development, its expression is influenced among insulin-signaling, oxidative stress and inflammatory-related signaling pathways (Li et al., 2010).

The expression changes associated with DMSO-induced neutrophilic differentiation of HL-60 cells are interesting for two reasons. First, the expression pattern observed during

differentiation was markedly stronger than that of oxidative stress (Figure 9). And second, generally, the expression pattern – in regards to down- and up-regulation of galectins – was similar to that of oxidative stress. Specifically, there is an upregulation with *LGALS3* and *LGALS10* that was also observed at the protein level with galectin-1. This indicates the potential for an association between oxidative stress and neutrophilic differentiation based on galectin expression patterning. Partial attenuation of *LGALS3* and *LGALS10* expression following NAC administration with DMSO-induced differentiation is in accordance with another study showing NAC supplementation reduces differentiation marker expression during HL-60 differentiation (Krance et al., 2010). Complete attenuation was not achieved suggesting that other molecular mechanisms are at play for galectin expression. For example, both galectin-1 and galectin-3 are involved with regulating apoptosis in T-cells (Stillman et al., 2006), a process that is commonly associated with the development of immune cells (Opferman, 2008). The similar expression pattern observed between galectin-1, -3 and -10 can be expected considering the high level of amino acid sequence homology specifically between these galectins (Leonidas et al., 1995). Non-varying expression of *LGALS8* during cellular differentiation is expected considering its expression has been shown to be stable during eosinophil-, monocyte- and neutrophil-like differentiation of HL-60 cells (Abedin et al., 2003). The following expression profiles indicate the potential for galectins as targets in disease diagnosis, prognosis and clinical therapeutic models. Unfortunately, therapeutic strategies associated with acute myeloid leukemia (AML) have reached their limit of effectiveness (Zhou et al., 2013). Considering the strong association between ROS and hematopoietic stem cell differentiation, galectins may represent ideal candidates for targeted treatment against AML.

4.1.2 Galectin-specific and non-specific inhibitory sugars and modification of the redox environment using NAC decreased cellular proliferation and impacted the HL-60 cell differentiated phenotype.

The use of galectin inhibitors on HL-60 cell proliferation showed varied results. Although all sugars showed a trend toward decreased cell proliferation in a dose-dependent manner; only TDG and OTX008 significantly increased the cell doubling time (Figure 11E, F). Both of these inhibitors are known to inhibit galectin-1 suggesting this galectins involvement

with cellular proliferation. Galectin-1 has been show to demonstrate conflicting roles during cell proliferation depending on its localization and cell type. In particular, extracellular galectin-1 has been reported to bind α5β1 integrin in order to inhibit cellular proliferation through increased Sp1 transactivation in carcinoma cell lines (Fischer et al., 2005), while intracellular galectin-1 can enhance cell proliferation through protein-protein interactions with RAS (Prior et al., 2003). The effect on cell proliferation observed with the galectin-1 inhibitor OTX008, has also been reported to inhibit endothelial cell proliferation (Dings et al., 2006). OTX008 is unable to affect galectin-1s interaction with ECM components but rather its proposed mechanism of action is through regulating galectin-1 interaction with intracellular components involved with cell cycle components directly (Astorgues-Xerri et al., 2014). Since lactose exhibited no inhibitory effect despite its reported relatively low dissociation constant value (K_d) with galectin-1 at 12.4 μM in HL-60 cells and 64 μM in bovine tissue and with galectin-3 at 55.2 μM (Schwarz et al., 1998; Stowell et al., 2008), these galectins then may mediate cellular proliferation through CRD-independent interactions. Interestingly, Cedeno-Laurent et al. (2012) demonstrate that lactose attenuates the modulatory effect of galectin-1 on cell proliferation in T-cells further indicating that the effect of galectin-1 on cell proliferation is cell type dependent (Cedeno-Laurent et al., 2012). TDG demonstrated an inhibitory effect on HL-60 cellular proliferation (Figure 11E). TDGs effect on HL-60 cell proliferation can be attributed to – unlike lactose – its stronger binding affinity to galectin-1 and -3 with K_d values of 24 μM and 43 μM, respectively, and increased metabolic stability (van Hattum et al., 2013). Maeda et al. (2003) report similar results whereby galectin-1 and -3 stimulate hepatic cell proliferation through activation of the ERK pathway and this effect was attenuated by TDG (Maeda et al., 2003). α-MM and mannose were used to assess their potential inhibition of galectin-10 during cellular proliferation. My results indicate that despite galectin-10s preferential binding for mannosyl residues over galactosyl residues, galectin-10 does not contribute to cellular proliferation of HL-60 in a CRD-dependent way (Figure 11A, B). Galectin-8 maintains a lower binding avidity (K_d = 130 μM–150 μM) toward lactose compared to the other galectins (Hirabayashi et al., 2002; Ideo et al., 2003). Interestingly, Cattaneo et al. (2011) provide evidence that both lactose and TDG inhibited galectin-8 in peripheral blood mononuclear cells (PBMCs) at concentrations of 50 mM and 30 mM,

respectively (Cattaneo et al., 2011). Similarly, 50 mM lactose was used to inhibit recombinant galectin-3 stimulated cell proliferation in fibroblasts (Inohara et al., 1998), and preadipocytes (Kiwaki et al., 2007). Thus, HL-60 cell proliferation may not be inhibited by lactose at my experimental concentrations despite relatively low dissociation constants whereas TDG falls within my tested range (Figure 11C, E).

OTX008 was able to decrease fMLP induced H_2O_2 production indicating that the expression of galectin-1 is important for the process of differentiation (Figure 12). Galectin-1 expression has been reported to play a role in the differentiation of dendritic cells (Fulcher et al., 2009), thymocytes (Liu et al., 2008b), and both B- and T- cells (Espeli et al., 2009; Liu et al., 2009). Interestingly, TDG did not have an effect on neutrophilic differentiation despite its ability to inhibit galectins, which mediate cellular proliferation. Thus, the expression of other galectins including -3, -10 and -12 may be associated with the direct effect of differentiation including apoptosis, cycle arrest and the indirect effect of cytotoxicity induced by DMSO. Interestingly, ~30% of cells are apoptotic following cellular differentiation through the activation of caspases and MAPK pathways (Duval et al., 2006). My findings indicate no change in differentiation following the addition of TDG after DMSO-induced differentiation (Figure 12) despite an inhibitory effect of this drug on T-cell differentiation (de la Fuente et al., 2014). T-cell differentiation is reported to be dependent on galectin-1 binding to CD69 (de la Fuente et al., 2014). Considering OTX008 inhibited differentiation while TDG did not, HL-60 neutrophilic differentiation may be, in part, mediated through CRD-independent function of galectin-1. Surprisingly, lactose and lactobionic acid treatment induced increased levels of ROS production indicating "enhanced" neutrophilic differentiation and the potential for galectins to have an inhibitory effect on differentiation (Figure 12). One similar example includes galectin-3s ability to inhibit osteoblast differentiation, which was attenuated via the addition of lactose (Nakajima et al., 2014). Additionally, galectin-12 is downregulated following neutrophilic differentiation of promyelocytes indicating that lactose and lactobionic acid may be inhibiting galectin-12 expression and subsequently increasing differentiation (Xue et al., 2016). The extent of which some inhibitors work while others do not remains elusive; however, differences between the binding affinities for these inhibitors toward all galectins may provide an explanation for the varying effect on differentiation. Additionally,

determining the glycoreceptor content/profile/structure on the cell surface of HL-60 would provide more details as to which glycans represent a significant binding partner to certain galectins over others.

To better understand the role of oxidative stress and its potential association with neutrophilic differentiation, modification of the redox environment using menadione and NAC during differentiation was investigated. My results indicate that menadione, a redox cycling compound was unable to "enhance" neutrophilic differentiation. Ogino et al. (2010) reported an enhanced effect during differentiation when using the direct oxidant: H_2O_2 (Ogino et al., 2010). Typically, reductive enzymes including NADPH-ubiquinone oxidoreductase and NADPH-cytochrome P450 metabolize menadione by one electron reduction, which subsequently undergoes redox cycling in the presence of oxygen to produce superoxide anion. However, other reductive enzymes including NADPH quinone oxidoreductase 1 (NQO1) metabolize menadione via a 2 electron reduction step, which creates a stable hydroquinone that is unable to enter redox cycling and generate ROS. NQO1 is thought to be a cellular detoxifying mechanism (Criddle et al., 2006), and therefore the oxidative stress induced by menadione may be quickly reversed via increased NQO1 expression. Criddle et al. (2007) reported the effect of menadione is highly dependent on NQO1 expression (Criddle et al., 2007). Further, increased NQO1 expression is observed during cellular differentiation alone indicating the abundance of this enzyme present during this process and therefore negating the effect of any quinones present (Zhao et al., 2009).

Administration of NAC decreased DMSO-induced neutrophilic differentiation as measured by fMLP induced ROS production (Figure 13A, B). Further, NAC is able to inhibit both adipocyte differentiation of mouse embryonic fibroblasts and macrophagic differentiation of HL-60, via downregulation of ERK and JNK phosphorylation (Chien et al., 2013; Pieralisi et al., 2016). Similarly, inhibition of ROS production decreases monocytic differentiation into macrophages through the ERK pathway (Zhang et al., 2013). Interestingly, Chen et al. (2012) report that ROS produced from NADPH oxidase during HL-60 cell differentiation are essential for this process (Chen et al., 2012). Moreover, inhibition with antioxidants is able to inhibit the final differentiated phenotype, while the

opposite is also true. Specifically, the differentiation of promyelocytic leukemia cells (NB4) is enhanced when superoxide dismutase is inhibited with siRNA while differentiation is inhibited through endogenous ROS quenching with antioxidant, NAC (Yang et al. 2015). Perhaps then, only endogenous levels of ROS production are required for differentiation and exogenous chemical inducers of ROS are redundant. This may explain menadiones inability to significantly increase levels of differentiation alone (Figure 13A).

4.1.3 Inhibition of DNA methyltransferases induced a similar galectin expression pattern to that produced by oxidative stress and neutrophilic differentiation.

DNA methylation was investigated as a regulatory mechanism of galectin expression considering inhibition of DNA methyltransferases is associated with increased differentiation in leukemic cell lines (Qin et al., 2007). My results indicate increased differentiation following DNA hypomethylation with decitabine considering the marked significant increase in differentiation marker, p47*phox* (Figure 14A, B). Interestingly, the expression profile of HL-60 cells in response to decitabine (Figure 15) is similar to expression profiles induced by oxidative stress and neutrophilic cellular differentiation. Specifically, treatment with decitabine increased expression of galectin-1, -3 and -10 at both the transcript and protein levels (Figure 15). This suggests that promoter demethylation is, in part, responsible for regulating *LGALS1*, *LGALS3* and *LGALS10* levels during the oxidative stress response and HL-60 cell differentiation. Epigenetic changes including promoter hypermethylation are early events in neoplastic processes and contribute by silencing tumour suppressor genes (Jones and Baylin, 2002). In fact, methylated genes have been evaluated as biomarkers for detection of cancer (Fackler et al., 2004). In particular, hypermethylation of the *LGALS3* promoter has been validated as a marker for early diagnosis of prostate (Ahmed et al., 2009), and thyroid cancers (Keller et al., 2013). Similarly, the *LGALS1* promoter is hypermethylated in colorectal adenocarcinoma cells and re-expression of this gene induces apoptosis (Satelli and Rao, 2011). My study provides the first evidence of *LGALS10* promoter methylation and its marked expression change over other galectins following decitabine treatment (Figure 15A). Additionally, my study provides evidence for galectin-10 as a marker for oxidative

stress and cellular differentiation in the context of AML. Although no DNA methylation regulatory mechanism has been reported to transcriptionally regulate *LGALS10*, the promoter region shares many sequence similarities to that of *LGALS1* and *LGALS3*, specifically, Sp1 binding sites (Dyer and Rosenberg, 2001). Interestingly, the transcription factor Sp1 has been reported to bind promoter regions sensitive to methylation and therefore protect genes from transcription repression (Höller et al., 1988). The decrease in *LGALS9* gene expression following DNA demethylation (Figure 15A) indicates the potential for this galectin to be regulated by transcription factors that are repressed via methylation. Most interesting, the increase in *LGALS12* expression following treatment with decitabine (Figure 15A) is not associated with the decrease observed following neutrophilic differentiation. Xue et al. (2016) report an inhibitory function for galectin-12 during neutrophilic differentiation suggesting multiple and complex regulatory mechanisms for this galectin (Xue et al., 2016).

4.1.4 JNK signaling is impacted by oxidative stress and differentiation and induces galectin expression.

Perhaps most interesting is the contrasting regulatory effect of JNK signaling observed during oxidative stress and neutrophilic differentiation. Increased phosphorylated JNK levels were observed during oxidative stress while the opposite effect was seen during differentiation (Figure 16C, D). Further, the addition of NAC with menadione was able to rescue basal protein levels while the addition of NAC with DMSO had no effect (Figure 16D). This indicates that JNK signaling during neutrophilic differentiation is not redox-sensitive and that the *LGALS3* and *LGALS10* expression attenuated with the addition of NAC (Figure 9) may be done through other redox-sensitive MAPK pathways, i.e. p38 and ERK signaling. This effect may explain the differences in the galectin expression profiles of both processes. Differential phosphorylation of JNK in HL-60 cells is most likely dependent on the stimulus used and the incubation time, following treatment. For example, Wang and Studzinski (2001) showed that 96 hr DMSO-induced differentiation of HL-60 is accompanied by a decrease in phosphorylated JNK levels while the inhibition of the p38 MAPK pathway increases levels of phosphorylated JNK but only in response to 1,23 D_3-induced monocytic differentiation (and not neutrophilic). Further, TPA-induced

differentiation of HL-60 shows only a transient (~1 hr) increase in phosphorylated JNK before returning to control levels while still showing prominent levels of phosphorylated ERK (Chien et al., 2013). In fact, most studies show – through the use of JNK inhibitor SP600125 – that increased signaling through this pathway is not involved in HL-60 differentiation (Chung et al., 2005; Yu et al., 2008). Therefore, it is probable that differential JNK levels during differentiation is a result of the inhibition or augmentation of other MAPK pathways. Increased phosphorylated JNK during oxidative stress, however, has been reported in a number of other studies (Kaneto et al., 2004; Wang et al., 2008). Interestingly, JNK signaling has been reported as a protective mechanism against oxidative stress by inducing expression of protective genes (Wang et al., 2003; Wu et al., 2009). Therefore, this may explain the observed downregulation of JNK signaling following neutrophilic differentiation – these genes are not necessary considering oxidative stress is needed in the promotion of differentiation. Additionally, this also helps explain the associated induction of apoptosis during differentiation. These results provide more evidence of the importance of JNK signaling during neutrophilic differentiation and insight into the treatment of AML differentiation through MAPK pathways associated with galectin expression.

Both an inhibitor (SP600125), and activator (anisomycin) were used to investigate the role of direct JNK signaling on galectin expression (Figure 16A, B). Inhibition of JNK signaling led to decreased galectin expression while augmentation of this pathway increased galectin expression. Increased *LGALS1* expression may be a result of an indirect (off/non-target) effect caused by inhibition of the JNK signaling pathway. For example, inhibition of specific MAPK pathways can drastically increase the expression of others (Wang and Studzinski, 2001). Specifically, inhibition of the p38 MAPK pathway potentiated the JNK pathway leading to activation of transcription factors implicated in HL-60 differentiation (Wang and Studzinski, 2001). Many MAPK pathways crosstalk with each other and often affect the same set of target genes. Therefore *LGALS1* may be indirectly regulated through either ERK or p38 signaling as a compensatory response (Shen et al., 2003). A similar effect of decreased *LGALS9* expression has been reported following inhibition of JNK signaling with SP600125 (Steelman et al., 2013); however, a subsequent decrease following exposure to anisomycin has not been investigated. Activation of *LGALS10* has

been reported being reliant on AP-1 and Oct1 transcription factors binding to the GC box (Dyer and Rosenberg, 2001). AP-1 is heavily regulated through MAPK signaling pathways indicating the reported downregulation of *LGALS10* is likely through decreased JNK signaling (Karin, 1995). The increase in *LGALS12* expression following treatment with SP600125 (Figure 16A) is interesting considering its downregulation following neutrophilic differentiation, which is accompanied by decreased phosphorylated JNK levels. Thus, in the context of cellular differentiation *LGALS12* is regulated through various networks besides JNK signaling. These data indicate the potential for off-target effects on galectin expression following regulation of JNK and the specificity for JNK signaling on only certain galectins.

4.1.5 Galectin gene expression correlates with oxidative stress and differentiation.

The correlation analysis indicates that there is both a strong positive and negative correlation of galectins with oxidative stress. Correlations between galectins with *ho*-1 and p47*phox* are indicative of galectins participating in both oxidative stress responses and differentiation, respectively. In particular, *LGALS1*, *LGALS3* and *LGALS8* were positively correlated with oxidative stress marker *ho*-1 (Figure 17A, B, C) whereas *LGALS3*, *LGALS9* and *LGALS10* positively correlated with cellular differentiation marker p47*phox* (Figure 18B, D, E). These findings suggest galectins as biomarkers of oxidative stress in the context of leukemia. Both *LGALS1* and *LGALS3* have been proposed as biomarkers of other cancers such as renal cell carcinoma – a malignant tumour typically associated with levels of oxidative stress (Ganesamoni et al., 2012; von Klot et al., 2014). More generally, galectin-3 has been proposed as a marker of fibrosis, which is a process highly associated with oxidative stress in the context of cardiovascular diseases (Hrynchyshyn et al., 2013). Additionally, *LGALS12* negatively correlated with both *ho*-1 and p47*phox* (Figure 17F and Figure 18F). Decreased *LGALS12* transcript levels is suggested as an additional biomarker of cellular stress responses. Decreased *LGALS12* transcript levels during neutrophilic differentiation may be due to this proteins effect on cell cycling (Yang et al., 2001), as it has been shown that *LGALS12* can inhibit human NB4 promyelocytic leukemia cell differentiation (Xue et al., 2016). Interestingly, *LGALS10* only positively correlated with

p47*phox* (Figure 18E) while showing no correlation with *ho*-1 (Figure 17E) indicating the expression of this galectin is specific for neutrophilic differentiation and its function is potentially unrelated to its oxidative stress sensitivity. Additionally, galectin-10 has been proposed as a potential biomarker for eosinophilic airway inflammation based on its correlation with sputum eosinophil levels (Chua et al., 2012). My findings suggest that galectin-10 transcript levels may be a ubiquitous marker for other granulocytes in the context of differentiation. *LGALS8* expression did not correlate with differentiation marker p47*phox* (Figure 18C). This is interesting considering *LGALS8* overexpression has been shown to enhance osteoclast differentiation (Vinik et al., 2015), and T-cell differentiation (Sampson et al., 2016). Potentially, *LGALS8* role in differentiation is cell type/context specific. These findings provide insight to expression patterns of galectins in a leukemic model under oxidative stress and DMSO-induced differentiation.

In order to further analyze galectin expression patterns; correlations between galectin isoform expressions were performed to determine potential galectin redundancy. Generally, galectin-8 expression across treatments remained invariable; however, there was a significant positive correlation between *LGALS1* and *LGALS8* indicating the potential for redundancy between these proteins (Figure 19B). Other studies have shown functional redundancy between galectin-1 and galectin-8 when promoting plasma cell differentiation (Tsai et al., 2011), and eliciting T-cell responses (Tribulatti et al., 2012). My results also indicate a significant positive correlation between *LGALS1* and *LGALS3* transcript levels (Figure 19A). There is a precedent for correlated expression between *LGALS1* and *LGALS3* in cancer lines (Wang et al., 2000), however, the function of these two proteins is likely related to their cellular localization/distribution rather than their correlation being functionally redundant (Sanjuán et al., 1997). The significant negative correlation between *LGALS8* and *LGALS9* transcript levels (Figure 19H) is interesting considering these two proteins have been shown to exist as multiple splice variants (Nishi et al., 2006). These variants contain structural similarities allowing these proteins to be affected by other molecular factors in similar fashion. For example, isoforms of galectin-8 and galectin-9 containing the longest linker peptide regions are more susceptible to cleavage (Nishi et al., 2006). The correlation existing between transcript levels of these two galectins is likely due to structural features as a consequence of alternative splicing.

Future studies should identify expression patterns of individual splicing transcript variants to confirm my findings or determine new correlations. Interestingly, there was a correlation with *LGALS10* and *LGALS12* transcript levels (Figure 19K) indicating the potential for similar expression patterns and redundancy. No transcript level correlation between these galectins has been investigated; therefore, future studies should aim to identify similar expression patterns across other treatments to identify new correlations considering both galectins are involved within differentiation.

The positive correlation between *ho*-1 and p47*phox* transcript levels (Figure 20) indicates a strong association between oxidative stress and neutrophilic differentiation considering many galectins correlate with both markers; galectins may be considered biomarkers and potentially mediators of these two processes. In particular, the link between oxidative stress and its contribution in promoting differentiation is, in part, through upregulation of galectins. Although JNK signaling is differentially affected by differentiation and oxidative stress; galectin expression remains variable indicating not only are galectins biomarkers for both processes, but galectins may also feedback to promote the differentiation of promyelocytes in HL-60 cells.

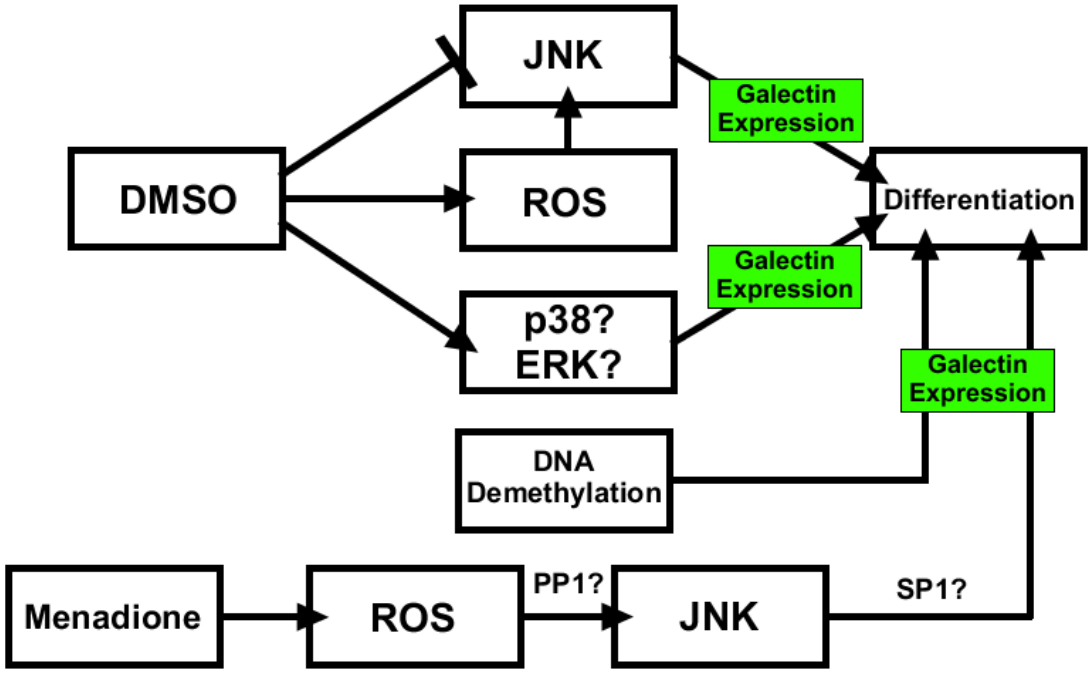

JNK
Galectin Expression
DMSO
ROS
Differentiation
Galectin Expression
p38? ERK?
Galectin Expression
DNA Demethylation
Menadione
ROS
PP1?
JNK
SP1?

Figure 21. Suggested pathway of menadione-induced oxidative stress and DMSO-induced neutrophilic differentiation of HL-60 cells.

The above schematic is the proposed method for galectin contribution in DMSO-induced HL-60 neutrophilic differentiation (Figure 21). It is speculative that JNK signaling works to increase Sp1 nuclear translocation and activation to increase galectin expression responsible in promoting cellular differentiation. Additionally, galectin expression by other MAPK pathways following DMSO treatment is also speculative. However, my study indicates that increased galectin gene expression following JNK activation, oxidative stress, and inhibition of DNA methyltransferases, is prominent. ROS signaling must precede differentiation considering treatment with NAC was able to successfully inhibit differentiation in all cases. Future studies should include in-depth individual galectin analyses to identify specific differences between galectins, helping to eliminate the assumption of functional redundancy.

4.2 Conclusions and application

In conclusion, I have determined the galectin expression profile of HL-60 cells under oxidative stress and during DMSO-induced neutrophilic differentiation. Similarities in galectin expression including upregulating of galectin-1, -3 and -10 at either the transcript or protein levels indicates an association between cellular differentiation and the redox environment with respect to galectins. The differential expression of galectins following oxidant exposure suggests these proteins may represent markers of the cellular stress response. It also suggests the potential for galectin-1, -3 and -10 as clinical targets when developing therapeutic treatment for AML.

Using different galectin inhibitors, galectin-1 is, at least, suggested to play a role in cellular proliferation and the progression of HL-60 cell differentiation. Considering other galectin inhibitory sugars (besides TDG) displayed no effect on either process, it is likely that these galectins either are not significantly involved, or are working in a CRD-independent fashion intracellularly to facilitate their biological role. Additionally, the upregulation of galectin-1, -3 and -10 following DMSO-induced expression may be an indirect effect of differentiation, including: apoptosis or cell cycle arrest. These data suggest the complex role of galectins in HL-60 cell stress response and differentiation despite their widespread expression and often structural redundancy.

Investigation of the redox environment was accomplished using the oxidant menadione and antioxidant NAC. My results suggest the addition of NAC is sufficient to reduce the levels of DMSO-induced neutrophilic differentiation of HL-60 cells while menadione was unable to enhance this effect. Understanding the role of ROS during differentiation is important considering the crucial roles of free radicals in the pathogenesis of many diseases (e.g. cancer due to loss of differentiation) (Ye et al., 2015). Further, considering galectins are involved with many types of cellular differentiation, the regulatory mechanisms associating ROS and cellular differentiation may be, in part, mediated through galectin expression. For example, ROS production has been known to facilitate adipocyte differentiation, which also requires galectin-12 expression (Lee et al., 2009; Yang et al., 2004). These results suggest an association between oxidative stress and cellular differentiation with respect to both up and down regulation of galectins. Finally, these data

suggest galectins may act as major players in the pathogenesis of diseases attributed to ROS production.

JNK signaling and DNA methylation are two distinct regulatory methods of galectin expression during oxidative stress and differentiation. The galectin expression profile generated from demethylation shows similarities to that of oxidative stress and DMSO-induced neutrophilic differentiation including upregulation of galectins-1, -3 and -10. Therefore, it is possible that other epigenetic mechanisms are involved with these two processes with respect to galectin expression. Interestingly, neutrophilic differentiation and oxidative stress produced distinctly opposite expression patterns with decreased phosphorylated JNK levels following differentiation and increased levels following the addition of menadione. This suggests the potential for different MAPK pathways or other regulatory mechanisms to be involved with galectin expression during these two processes despite the similarities in expression.

The correlation analysis revealed coordinated galectin expression as a potential biomarker for oxidative stress and differentiation of HL-60 cells. Specifically, *LGALS1*, *LGALS3* and *LGALS8* may be considered biomarkers of oxidative stress while *LGALS3*, *LGALS9* and *LGALS10* may be considered biomarkers for HL-60 neutrophilic differentiation. Downregulation of *LGALS12* may be considered a negatively correlated biomarker for both oxidative stress and neutrophilic differentiation. Correlation between marker genes of each process suggests that galectins act as mediators responsible in promoting differentiation following oxidative stress. Finally, correlated expression amongst different galectin indicate the potential for functional redundancy; however, further analysis of individual galectins is required to draw specific and definite conclusions.

In conclusion, this study demonstrates an association between oxidative stress and neutrophilic differentiation of HL-60 cells with respect to upregulation of galectins. Distinct similarities and differences in galectin expression profiles provide insight to different regulatory mechanisms associated with these processes and the potential for galectin expression as biomarkers of different biological processes and stress responses. In particular, galectins and their expression associated with AML and differentiation may be

considered a clinical target when developing various drug treatments/therapeutic medication.

4.3 Study limitations and future directions

One major project limitation was the inability for genetic manipulation: specifically knockdown experiments utilizing RNAi based techniques. Like many leukemic cell lines, HL-60 cells have been reported as being transfection-incompetent in comparison to other lines such as K562 (Esendagli et al., 2009). Despite this, more sophisticated methods of gene delivery such as lentiviral techniques have proven useful for RNAi in the context of studying HL-60 cellular differentiation (Jian et al., 2011). More recent techniques including the powerful gene editing tool CRISPR/Cas9 system has proven useful for manipulating gene expression for studying myeloid leukemia in the context of differentiation (Valletta et al., 2015). Specifically, CRISPR/Cas9 directed gene knockdown is very specific and eliminates indirect/off-target effects that may be associated with the galectin inhibitors used in this study. In this way, I could study the effect inhibiting galectins that are typically upregulated during differentiation and then test for the neutrophilic phenotype.

Another method to examine the effect of individual galectins on cellular proliferation other than genetic manipulation using RNAi would be to utilize glycan modification enzymes/ drugs to alter glycan structure, i.e. remove terminal sialic acid glycan residues. Different galectins, for example, have shown similar specificity toward LacNac binding; however, the addition/removal of 2–3-sialic acid on terminal LacNac structures can significantly change specificity of only specific galectins (Stowell et al., 2008). This method allows for analyses of only endogenously expressed galectins. Exogenously added recombinant galectins are often used to assess the role of individual galectins on cellular functions when added in abundance.

Another study limitation is the use of DMSO as a “specific” inducer of neutrophilic differentiation. DMSO was originally described to induce neutrophilic differentiation based on morphological changes (Collins et al., 1979). It has been reported that HL-60 cells induced with DMSO can undergo defective neutrophil maturation evident by failure

of secondary granule gene expression; therefore, these cells may only represent an atypical phenotype of proper granulocytes (Khanna-Gupta et al., 1994). In fact, DMSO-induced differentiation increases CD14 expression: a common marker of monocytic differentiation into mature macrophages (Zamani et al., 2013). Therefore, future studies should include – in addition to multiple promyelocytic cell lines – multiple neutrophilic differentiation stimuli such as all-trans retinoic acid (ATRA) when comparing galectin expression profiles. This is especially important considering lineage specific differentiation using various chemical inducers enables different galectin expression profiles (Abedin et al., 2003). Additionally, investigation of specific transcription factors affected by JNK signaling should be investigated. In particular, chromatin immunoprecipitation analyses could be utilized to identify specific interactions between galectin DNA sequences and relevant transcription factors such as Sp1. Considering expression of specific galectins are changed during oxidative stress and differentiation, identification of transcription factors responsible for this increase would offer insight to potentially many signaling pathways.

Another study barrier was the limitation of immortalized cell lines. Animal models would provide a more physiologically relevant system in order to identify the specific roles of galectins. The inclusion of primary cultures may also offer a better model for examination of galectins. Further studies using these more physiological models to confirm the findings presented here would be the next step to further validate galectins as key regulators of neutrophilic differentiation and possible regulators of leukemic progression.

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Appendix A: supplementary material

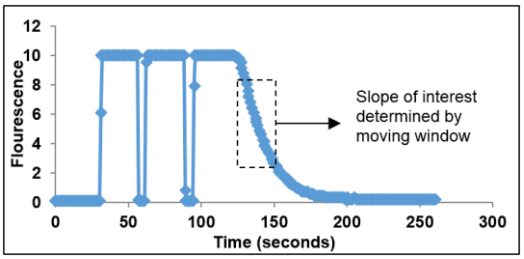
Gene Name & RefSeq		Sequence, 5' – 3'	Size	2 Step Cycling (RT-qPCR) 3 Step Cycling (RT-PCR)	PCR efficiencies & quantification method	PMCID or PMID for reference (if applicable)
LGALS1	F	CCTGGAGAGTGCCTTCGAGTG	220bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 60°C for 25 Sec	100%	23108139
NM_002305	R	CTGCAACACTTCCAGGCTGG			ΔΔCt	
LGALS3	F	CAGAATTGCTTTAGATTTCCAA	108bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 60°C for 25 Sec	113%	18202194
NM_002306	R	TTATCCAGCTTTGTATTGCAA			Pfaffl	
LGALS8	F	TGGGGACGGGAAGAGATCAC	172bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 62°C for 25 Sec	81.4%	Designed
NM_006499	R	TGCCATAAATGCCCAGAGTGTC			Pfaffl	
LGALS9	F	CTTTCATCACCACCATTCTG	91bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 62°C for 25 Sec	86.8%	18202194
NM_009587	R	ATGTGGAACCTCTGAGCACTG			Pfaffl	
LGALS10	F	GGATGGCCAAGAATTTGAACTG	82bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 62°C for 25 Sec	112.6%	Designed
NM_001828	R	GGTGTAAGAGGATTGGCCATTG			Pfaffl	
LGALS12	F	TGTGAGCCTGAGGGACCA	111bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 65°C for 25 Sec	83.6%	18202194
NM_001142535	R	GCTGAGATCAGTTTCTTCTGC			Pfaffl	
p47*phox*	F	GTCAGATGAAAGCAAAGCGA	93bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 60°C for 25 Sec OR Extend: 72°C for 60 Sec	ND*	23147401
NM_000265	R	CATAGTTGGGCTCAGGGTCT			ΔΔCt	
***ho*-1**	F	CTCAAACCTCCAAAAGCC	220bp	Denaturation: 95°C for 30 Sec Anneal/Extend: 60°C for 30 Sec OR Extend: 72°C for 60 Sec	-	PMCID: PMC2175339
NM_002133	R	TCAAAAACCACCCCAACCC			-	
GAPDH	F	GAGTCAACGGATTTGGTCGT	95bp	Denaturation: 95°C for 5 Sec Anneal/Extend: 62°C for 25 Sec	93.3%	PMCID: PMC3489664
NM_002046	R	AATGAAGGGGTCATTGATGG			Pfaffl	

Table 1. Primer sequences and RedSeq, amplicon size, cycling conditions, efficiencies, method of quantification and primer references.

A

```
slope <- function (data,n){
 j.max <- n-1
 b1 <- list()
 max <- length(data$V1) - n + 1
 for (i in 1:max){
  d.max <- n+i-1
  sub.data <- data[i:d.max,]
  b <- list()
  m <- 0
  for (j in 1:j.max){
   k.min <- j+1
   for (k in k.min:n){
    m <- m + 1
    b[m] <- (sub.data[j,2] - sub.data[k,2]) / (sub.data[j,1] - sub.data[k,1])
}
}
  b1[i] <- median(unlist(b))
}
 b1
 x <- which.min(unlist(b1))
 r1 <- data[x + round(n/2),1]
 r2 <- data[x + round(n/2),2]
 r3 <- b1[x]
 result <- cbind(r1, r2, r3)
}
```

B



Algorithm 1. (A) R code used to determine the minimum first derivative of the negative slope indicative of the time point at which the slope is steepest. Algorithm describes a moving window of 10 units (seconds) from initial time of reading (0 seconds) to 900 seconds. (B) Example kinetic from fluorometric assay. Illustration shows the moving window used to determine the steepest point of the slope.

Curriculum Vitae

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Manuscripts:

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Abstracts and Posters:

Vinnai JR, Cumming RC, Timoshenko AV (2016) The association of oxidative stress and cellular differentiations status with upregulation of galectins in HL-60 cells. 13th Annual Department of Oncology Research & Education day, London, Ontario June 17, 2016, p. 50.

Vinnai J, Lewis J, Cuthbert TN, Timoshenko AV (2015) Differential expression of galectins in cancer cells treated with stress stimuli mimicking the tumour microenvironment. The Canadian Cancer Research Conference, Montreal, Canada, November 8-10, 2015. Abstract # NP73, p. 347.

Vinnai J, Lewis J, Cuthbert TN, Timoshenko AV (2015) Differential expression of galectins in cancer cells treated with stress stimuli mimicking the tumour microenvironment. Program and Abstracts Bok, abstract #85. 12th Annual Department of Oncology Research & Education Day. Schulich School of Medicine & Dentistry, Western University, London, Canada, June 26, p. 54.