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Galectin-3: A Harbinger of Reactive Oxygen Species, Fibrosis, and Inflammation in Pulmonary Arterial Hypertension

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Abstract

Significance: Pulmonary arterial hypertension (PAH) is a progressive disease arising from the narrowing of pulmonary arteries (PAs) resulting in high pulmonary arterial blood pressure and ultimately right ventricle (RV) failure. A defining characteristic of PAH is the excessive and unrelenting inward remodeling of PAs that includes increased proliferation, inflammation, and fibrosis.

Critical Issues: There is no cure for PAH nor interventions that effectively arrest or reverse PA remodeling, and intensive research over the past several decades has sought to identify novel molecular mechanisms of therapeutic value.

Recent Advances: Galectin-3 (Gal-3) is a carbohydrate-binding lectin remarkable for its chimeric structure, composed of an N-terminal oligomerization domain and a C-terminal carbohydrate-recognition domain. Gal-3 has been identified as a regulator of numerous changes in cell behavior that contributes to aberrant PA remodeling, including cell proliferation, inflammation, and fibrosis, but its role in PAH has remained poorly understood until recently. In contrast, pathological roles for Gal-3 have been proposed in cancer and inflammatory and fibroproliferative disorders, such as pulmonary vascular and cardiac fibrosis. Herein, we summarize the recent literature on the role of Gal-3 in the development of PAH. We provide experimental evidence supporting the ability of Gal-3 to influence reactive oxygen species production, NADPH oxidase enzyme expression, and redox signaling, which have been shown to contribute to both vascular remodeling and increased pulmonary arterial pressure.

Future Directions: While several preclinical studies suggest that Gal-3 promotes hypertensive pulmonary vascular remodeling, the clinical significance of Gal-3 in human PAH remains to be established. *Antioxid. Redox Signal.* 31, 1053–1069.

Keywords: pulmonary hypertension, PAH, galectin-3, vascular remodeling, reactive oxygen species, inflammation, fibrosis

Pulmonary Arterial Hypertension

PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive disease of the lung vasculature that is characterized by a sustained elevation of pulmonary arterial pressure (46). PAH is currently defined as a mean resting pulmonary artery pressure (mPAP) of ≥25 mmHg (84) and results from increased pulmonary vascular resistance that leads to compensatory right ventricular hypertrophy (45, 46). Medial hyperplasia of pulmonary artery (PA) smooth muscle cells is a hallmark feature of PAH (64), which ranges from hypertrophy to vessel occlusion (150). In most forms of PAH, muscularization of small distal PA occurs (152) and is further characterized by excessive vascular cell proliferation, fibrosis, and inflammation, leading to medial remodeling, rarefaction, and a loss of compliance of the pulmonary blood vessels (55, 131, 150, 160).

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Increased resistance to blood flow and more rigid blood vessels (loss of vascular compliance) contribute to the failure of the right ventricle (RV) (104, 169). The response of the RV to the increased afterload associated with PAH is both temporal and multifaceted with increases in end-diastolic volume, hypertrophy, alterations in contractility, dilation, cardiac fibrosis, and eventual decompensation (37). Increased RV volume (diastolic and systolic) combined with increased intraluminal pressure lead to an unsustainable increase in wall stress that culminates in right heart failure and ultimately death (15, 49, 165).

Within the pulmonary vasculature, endothelial cells become dysfunctional and fail to maintain homeostasis. Vascular smooth muscle cells undergo a phenotypic switch from a quiescent contractile phenotype to a ''synthetic'' phenotype that is characterized by a decrease in contractile smooth muscle genes, increased secretion of matrix and proteases, and increased proliferation (70, 121). The subsequent increase in proinflammatory and profibrotic molecules drives increased fibrosis, inflammation, and the deposition of extracellular matrix (106, 127, 136, 141, 168).

The molecular triggers of altered cellular behavior in PAH remain incompletely defined. Endothelin, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs), hypoxic signaling, altered metabolism, and reactive oxygen and nitrogen species have all been identified as contributing factors (11, 44, 102, 106), but how these seemingly disparate signals are integrated into the complex pathology underlying PAH is an increasingly important question.

A substantial body of evidence exists in support of the role of increased levels of reactive oxygen species (ROS) in both human and experimental models of pulmonary hypertension (PH) (16, 24, 34, 63, 74, 133, 184). The major ROS that are produced in the pulmonary vasculature are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and hydroperoxyl radical ($HO\cdot_2$) (40). Numerous mechanisms have been proposed to account for increases in ROS, including altered endothelial nitric oxide synthase activity, increased NADPH oxidase (NOX) enzyme expression and activation, and altered mitochondrial function. However, steady-state levels of ROS also reflect the balance between ROS generation and elimination or scavenging, and there is evidence to support alterations in both pathways in PH (44).

We have previously shown that ROS contribute to the development of PAH (10) and have shown in Figure 1A that vascular contraction to angiotensin II is enhanced in PAs from rats with monocrotaline (MCT)-induced PAH. Treatment of precontracted vessels with the antioxidant Tempol elicits a greater relaxation of induced tone in MCT-treated vessels compared with control (Fig. 1B), suggesting that increased oxidant tone in PAs from MCT-induced PAH augments vascular contraction. Of the ROS, O_2^- and H_2O_2 activate multiple signaling pathways promoting cell proliferation and apoptosis, elevated vascular tone, fibrosis, and inflammation, which are all hallmark signs of PAH (40). However, the cellular origin and functional significance of ROS in PAH remain poorly delineated.

The human genome encodes five Nox isoforms, and four of these isoforms, NOX1, NOX2, NOX4, and NOX5, are expressed in pulmonary vascular cells. NOX4 is regarded as a constitutively active enzyme that produces levels of H_2O_2 that are primarily controlled by changes in gene expression (20, 117). Increased expression of NOX4 has been reported in human PAH (105), and several lines of evidence support an important role for NOX4 in the pathogenesis of PAH in rats and humans (10, 105), but this remains controversial in mice (50, 115, 166). NOX4 has been reported to be a major NADPH oxidase homolog expressed in human pulmonary arterial smooth muscle cells (PASMCs) (155), and its expression both at the mRNA level and at the protein level is significantly increased in the lungs from patients with idiopathic PAH compared with healthy lungs (105), which suggests a correlation between NOX4 and the onset of PAH.

NOX4 mediates the hypoxia-induced growth of human PASMCs (75). However, others have shown that the NOX4 expression is highest in endothelial cells and perivascular fibroblasts (1, 10, 149). Endothelial cell NOX4 is thought to be protective and supports endothelial nitric oxide synthase function (25, 143), whereas fibroblast NOX4 is highly upregulated by TGF-β and is profibrotic (10, 58). Collectively, these findings support the premise for NOX4 expression being inherently involved in pulmonary vascular remodeling by promoting arterial medial smooth muscle proliferation, endothelial proliferation, and adventitial fibroblast activation in PAH. NOX4 is also upregulated in cardiac hypertrophy and remodeling (185).

Epidemiologically, PAH is more frequent in women than in men, and untreated PAH has a survival time of 5–7 years after diagnosis (14). From a therapeutic standpoint, there are a number of vasodilator drugs that are indicated for the treatment of PAH, but none of the current therapeutics offers long-term success for survival due to limited effectiveness and unwanted side effects (142), and more importantly focus is being increasingly placed on the underlying causes of the vascular remodeling that is a hallmark of the disease (130).

Galectin-3

Galectin-3 (Gal-3; *LGALS3*, Mac-2) is a member of the lectin family of proteins, which recognize and bind to specific carbohydrate motifs on glycosylated proteins as well as lipids (31). Gal-3 protein was first identified in the 3T3 mouse fibroblast cell line (134) and found to be highly expressed in the lungs (26). The gene encoding Gal-3 was cloned in 1987, and changes in mRNA expression in fibroblasts were observed in response to growth factors (77). Gal-3 protein was detected in both the cytoplasm and the nucleus of cells, and higher expression levels were observed in the nucleus of proliferating cells (107). Gal-3 expression was found to be readily induced by growth factors in young cells, but aged cells or those with replicative senescence were unresponsive (23).

In the late 1980s/early 1990s, the macrophage surface antigen, Mac-2, was determined to be identical to Gal-3 and shown to be expressed in high concentrations by subsets of proinflammatory macrophages and secreted into the extracellular space (21, 176). Mac-2 has been used extensively in tissue staining applications to identify or mark macrophages (109), and this practice continues, although to a lesser extent. Gal-3 expression, however, is not confined to macrophages, and it is found to be expressed in fibroblasts (where it was

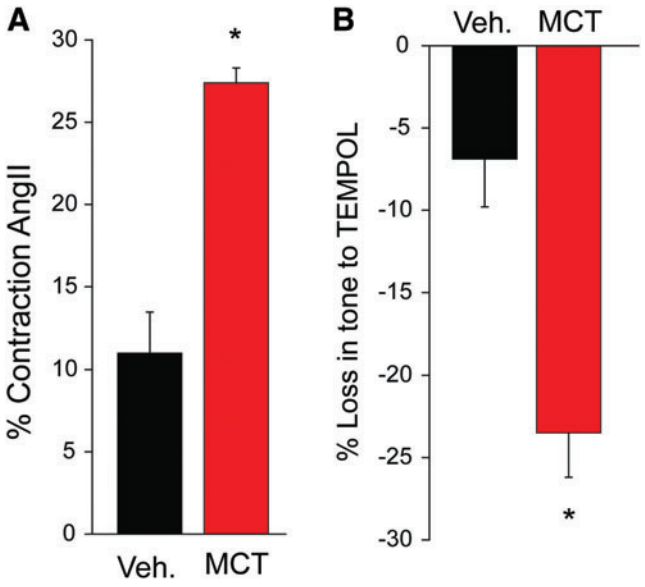

FIG. 1. Hypertensive PAs produce greater contractile force with greater dependency on ROS. (A) PA from control (vehicle) and MCT rats were mounted on a myograph (1 g passive tension) and contracted with low-dose angiotensin II (100 n*M*). **(B)** Drop in tension following the addition of ROS scavenger TEMPOL (100 m*M*). $n = 3$–4, $^*p < 0.05$. MCT, monocrotaline; PAs, pulmonary arteries; ROS, reactive oxygen species. Color images are available online.

originally discovered), smooth muscle cells (5), endothelial cells (111), activated T cells (78), epithelial cells (68, 79), and selected types of tumor cells (86).

Gal-3 belongs to a family of 16 related members that all share an evolutionarily conserved carbohydrate recognition domain (CRD) that can bind β-galactosides and lactose, but they differ in their ability to bind more complex saccharides. They can be broadly classified into three types: the prototypes that contain one CRD and are monomers or homodimers (includes galectin-1, 2, 5, 7, 10, 11, 13, 14, 15, and 16), the chimeras (Gal-3 is the only member) that contain one CRD and a self-association domain, and the tandem-repeat galectins (galectin-4, 6, 8, 9, and 12) that have two CRDs connected by a linker peptide. As the only chimeric galectin, Gal-3 is composed of C-terminal CRD that is present in all members of the galectin family and a unique N-terminal domain that contains glycine- and proline-rich domains that enable Gal-3 to oligomerize with other Gal-3 molecules (Fig. 2A) or to engage in protein–protein interactions with other proteins.

Gal-3 is initially expressed as a monomer but can self-assemble into dimers and higher order structures in response to diverse stimuli. Cysteine 173 (previously referred to as cysteine 186) has been proposed as a critical residue that enables disulfide bonds between homodimers (175). Carbohydrate binding to the C-terminal CRD of Gal-3 triggers a structural change in the N-terminus to enable oligomerization into pentamers (Fig. 2B) (52, 87). Specific monoclonal antibodies targeting the N-terminus of Gal-3 have been shown to facilitate the multimerization of Gal-3, whereas others can induce disruption of the self-association process, thus highlighting the important role of the N-terminus in regulating the formation of Gal-3 oligomers (89).

There are also reports that the C-terminal CRD can initiate self-assembly, although more weakly *via* ''F-faces'' within the CRD (73, 87), and that this can be modified by the N-terminal domain and also impact oligomerization and substrate binding (156). Tissue transglutaminase can also directly transamidate and promote Gal-3 oligomerization, which may increase and stabilize interactions with substrates (103, 164).

Gal-3 can be found in the cell cytosol, the nucleus, and the extracellular space. How Gal-3 traffics to these different intracellular locations remains poorly understood, and it may involve post-translational modifications or through protein binding or vesicular traffic. Cytosolic Gal-3 can regulate intracellular signaling and apoptosis/cell survival (4), in the nucleus, Gal-3 has been shown to influence RNA processing (galactose/lactose-specific lectin found in association with ribonucleoprotein complexes), and in the extracellular space, Gal-3 binds to numerous ligands including receptors and integrins to influence signaling, cell:cell and cell:matrix interactions. Gal-3 does not contain a signal peptide and its secretion to the extracellular space, while polarized, has been shown to be unaffected by chemical inhibition of the classical secretory pathway (8, 69). Instead, secretion of Gal-3 is inhibited by methylamine and increased by heat shock and

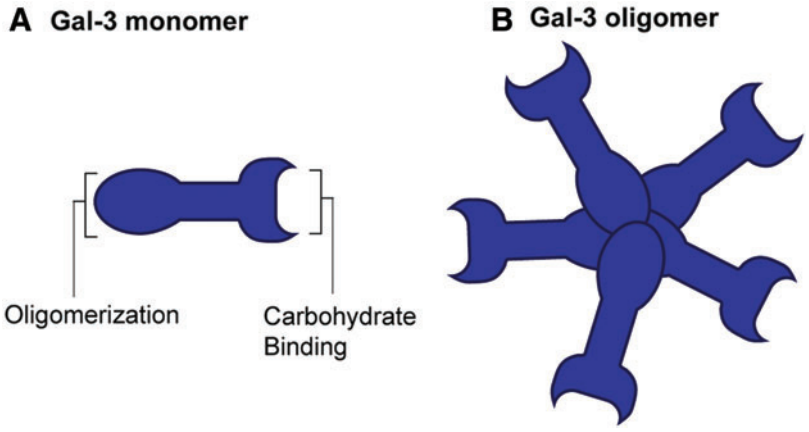

FIG. 2. Schematic illustration of Gal-3 monomer (A) and oligomer (B). Gal-3 is understood to be initially expressed as a monomer that then assembles into a larger multimers in response to carbohydrate binding and other post-translational modifications. Gal-3, galectin-3. Color images are available online.

calcium-mobilizing agents, suggesting that exocytosis is the major export pathway (139). Recent studies support this hypothesis and show that Gal-3 can be incorporated into exosomes, which are then released into the extracellular space (8). However, several important questions remain including whether this pathway accounts for the export of both free and encapsulated Gal-3 as secreted Gal-3 is reported to be predominantly free and not packaged into extracellular vesicles (153), and how and whether Gal-3 that is present within exosomes can be released. Based on a CRISPR-Cas9 genomic screen, another proposed mechanism is that Gal-3 may bind to N-linked glycosylated proteins with signal peptides that are en route to the plasma membrane.

While inhibition of N-linked glycosylation reduced surface expression of Gal-3, it did not reduce its presence in the extracellular media, indicating that N-linked glycosylation is not required for secretion but essential for extracellular membrane binding (153). An alternative mechanism for secretion is the reported ability of Gal-3 to penetrate lipid bilayers, which may enable it to exit (as well as enter) cells and traffic to the nucleus and other intracellular organelles (93).

Gal-3 is subjected to several post-translation modifications. It is cleaved by matrix metalloproteinases 2 and 9 between Ala62 and Tyr63 to yield an intact CRD and N-terminal peptides, which results in increased carbohydrate binding but reduced oligomerization (120). Gal-3 is also a substrate for other proteases including MMP-7, MMP-13, MT1-MMP, PSA, and proteases encoded by parasites (48). Gal-3 is primarily phosphorylated on Ser6 and to a lesser extent Ser12 (68) and Tyr107 (6, 7), although this may be signal dependent. Phosphorylation can impact the subcellular localization of Gal-3 by promoting the translocation from the nucleus to the cytoplasm (158), thus influencing its ability to regulate apoptosis in the cytoplasm (181). Ser6 phosphorylation can impact the ability of Gal-3 to recognize carbohydrate motifs, and the phosphorylation of Tyr107 may impair protease-dependent cleavage (48).

Gal-3 Ligands

Ligand-binding specificity is encoded by the CRD of Gal-3, and while there are overlapping substrates, it has been shown to bind to distinct subsets of glycoproteins than other galectin family members (154). Gal-3 binds to numerous substrates including (but not limited to) signaling molecules (Ras, TGF-β), transcriptional regulators (β-catenin), ribonucleoproteins (RNA splicing), cell surface receptors (integrins [β1], TGF-β, deleted in malignant brain tumors 1, vascular endothelial growth factor (VEGF), epidermal growth factor receptor [EGFR]), lysosomal proteins, and matrix proteins (fibronectin, collagen, laminin) (19, 35, 62, 100, 114, 119, 135). In addition to glycosylated proteins, Gal-3 can also bind to glycosphingolipids present on mammalian cells, which may enable interaction with ABO blood group antigens and the HNK-1 antigen in the neurons and leukocytes (22).

Gal-3 influences a variety of processes including RNA splicing proliferation, altered signaling, migration, apoptosis, fibrosis, and inflammation (4, 18, 28, 59, 60, 72). As a result, a pathogenic role for Gal-3 has been proposed in numerous diseases such as cancer (76, 183) and inflammatory (113, 122) and fibroproliferative disorders such as pulmonary, cardiac, and hepatic fibrosis (54, 60, 116, 148, 161, 162).

How Gal-3 alters signaling depends on its intracellular location and also the ability of its CRD to recognize specific glycosylation motifs on substrates and the ability to form oligomers. Gal-3 binds to the EGFR in mesenchymal cells, which results in a mitogenic response and increased collagen (85). Gal-3 also binds with high avidity to advanced glycosylation end-product binding proteins in a variety of cell types, including macrophages and endothelial cells (129, 167). A notable marker for cell adhesion, CD98, has also been reported to be a receptor for Gal-3 (29), and there is support for a mechanistic link between CD98, interleukin-4, and Gal-3 to elicit macrophage activation and drive both inflammatory and fibrotic diseases (33). Furthermore, studies show that Gal-3 can bind to CD45, which induces cellular apoptosis (177).

With regard to ligands, extracellular matrix glycoproteins (laminins and integrins) have been identified as matrices that interact with Gal-3 (99, 180). Specifically, increased Gal-3 expression and subsequent binding increase β_1 integrin-mediated cell adhesion to both laminin and fibronectin (99), and other studies show that Gal-3 may be involved in an integrin-induced epithelial–mesenchymal transition process that upregulates fibronectin and cellular migration (99). LGALS3BP (Gal-3 binding protein) is another Gal-3 ligand that mediates the induction of VEGF to promote angiogenesis (128), alters signal transduction in cancer cells to promote cell survival, migration, proliferation (128), and cell adhesion (71), and enhances antiviral response/innate immunity (91). LGALS3BP forms ring-shaped oligomers of varying sizes (mostly decamers) that interact with Gal-3 and also fibronectin, collagens, and integrins (108).

In the nucleus, Gal-3 has been detected as part of the spliceosome complex where it is important for pre-mRNA splicing. This is mediated *via* a specific interaction with the U1 small nuclear ribonucleoprotein (snRNP), which facilitates pre-mRNA splicing (56). Lysosome membrane permeabilization (LMP) occurs in response to excessive lipids, osmotic changes, and increased ROS (145). Following lysosomal damage, β-galactosides containing proteins, normally protected within the lumen of the lysosomes, are exposed to the cytosol where they are recognized by galectins including Gal-3 (3). Autophagy can prevent excessive cell death following LMP by sequestering membrane remnants (145).

The tripartite motif (TRIM) family of proteins are regulators of autophagy that assemble activated ULK1 and Beclin 1 on target complexes. Gal-3 binds to TRIM16 enabling it to recognize endomembrane damage and connect LMP to the autophagic response (19). Elevated ROS, which can induce LMP, have been shown to increase the lysosomal localization of Gal-3 (125).

Gal-3 Inhibitors

Some of the earliest used, and least specific, approaches to inhibiting the actions of Gal-3 have included the use of high concentrations (m*M*) of sugars including lactose and N-acetyllactosamine (83), which overwhelm the ability of the CRD on Gal-3 to recognize substrates. Major limitations with the use of high concentrations of sugars as Gal-3 inhibitors are the high potential for off-target effects through inhibition

of other galectin family members as well as other molecules and mechanisms that may be affected.

Complex plant-derived polysaccharides including modified citrus pectins and galactomannans can also inhibit Gal-3. They are complex carbohydrate polymers that can offer greater structural complexity and diversity that enable the specific binding/absorption of different galectin isoforms with differing affinities. Inhibitors from Galectin Therapeutics including GR-MD-02 (galactoarabino-rhamnogalaturonan) and GM-CT-01 (galactomannan) are part of this class of molecules that are more specific for Gal-3 (9, 54, 162). Glycomimetics are another class of inhibitor that contain chemical substitutions (*i.e.*, aromatic amido or 4 amido-1,2,3-triazolyl groups) that can mimic the structure of carbohydrates. They also offer advantages with regard to specificity and potency as well as the potential for intracellular actions (30).

Highly specific small-molecule inhibitors of Gal-3 have recently been developed including TD139 (95), which is a potent inhibitor that binds specifically to the galactoside-binding pocket of Gal-3. TD139 has very attractive pharmacokinetics including intracellular actions and can be inhaled, which provides advantages with lung targeting and minimization of systemic actions. Neutralizing Gal-3 antibodies have also been developed that are highly isoform specific and can bind extracellular Gal-3 and prevent many of its actions (179). The ability of neutralizing antibodies to interfere with the intracellular actions of Gal-3 may be a limitation. Finally, genetic approaches to targeting Gal-3 include standard silencing approaches using siRNA and the germline knockout of Gal-3 in mice and rats (9, 66).

The Role of Gal-3 in Inflammation

Inflammation underlies the pathology of many if not most chronic diseases. Vascularized tissue and individual cells respond to injury, infection, and irritation by initiating an inflammatory response. Acute inflammation is the early response, which usually resolves within a short period of time to enable the transition to the process of healing. In contrast, chronic inflammation is the failure of acute inflammation to resolve itself, resulting in harmful or deleterious conditions usually through persistence of an inflammatory stimulus (112).

Gal-3 is an important regulator of the immune system, and it modulates many immune reactions and directly impacts immune cell function *via* both autocrine and paracrine actions. Gal-3 is highly expressed in myeloid cells including monocytes, macrophages, dendritic cells, and neutrophils and has been shown to have important roles in regulating innate immunity as well as contributing to both acute and chronic inflammations. Gal-3 binds directly to CD11b on macrophages (33) and CD66 on neutrophils and can regulate inflammatory cell extravasation (140). Gal-3 regulates immune cell differentiation as well as the binding to numerous pathogens including lipopolysaccharide (LPS), the endotoxin from gram-negative bacteria (38), *Helicobacter pylori* (123), pathogenic fungi, and *Trypanosoma cruzi* (27) to name but a few.

Gal-3 can also function as a pattern-recognition receptor and a danger-associated molecular pattern (32) that can promote the assembly of inflammasomes, which produce interleukin (IL)-1β and IL-18 and activate the unfolded protein response that can amplify inflammatory responses by potentiating nuclear factor kappa light chain enhancer of activated B cells (NFκB) as well as other pathways. Gal-3 is generally regarded as a proinflammatory molecule and has been reported to activate T and B lymphocytes (65), mast cells, (43) monocytes and macrophages (138), and neutrophils (178). Gal-3 is expressed on the surface of human monocytes, and differentiation to macrophages is accompanied by increased expression levels. Moreover, Gal-3 is important in regulating macrophage polarization toward the M2 phenotype, and macrophages lacking Gal-3 show an impaired ability to express M2 gene sets in response to IL-4 (96).

Gal-3 is important for phagocytosis, and macrophages lacking Gal-3 exhibit reduced ability to remodel actin fibers post, suggesting that intracellular Gal-3 contributes to macrophage phagocytosis (137). Gal-3 can also function as chemoattractant, and high levels can promote the inward migration of monocytes and macrophages (138). The effects of Gal-3 on the behavior of immune cells can be mediated by extracellular Gal-3 binding to membrane receptors on the cell surface or by intracellular Gal-3 modulating the activity of intracellular proteins. To recognize intracellular bacterial pathogens that reside and replicate with specialized vacuoles, the innate immune system has to be able to distinguish pathogen-containing vacuoles from endogenous vesicles.

Gal-3 can recognize the carbohydrate modifications on the luminal side of vacuolar membranes and enable the delivery of antimicrobial GTPases to pathogen-containing vacuoles (36). While these actions of Gal-3 are important in defending from pathogens and maintaining the health of an organism, not all are helpful. Gal-3 expression is increased by influenza, and while helpful for an antiviral response, it has been shown to facilitate the binding of *Streptococcus pneumoniae* to the pulmonary epithelium resulting in increased susceptibility of influenza patients to pneumonia (118). Similarly, Gal-3 has numerous roles in inflammatory diseases such as atherosclerosis, sepsis, arthritis, asthma, and systemic sclerosis, which are reviewed in more details elsewhere (144). Another mechanism by which Gal-3 regulates the function of the immune system is through the regulation of ROS production, which is discussed below.

The Role of Gal-3 in Fibrotic Disease

Fibrosis refers to the deposition of excessive amounts of connective tissue as part of a reparative process, often secondary to inflammation, that results in the scarring or hardening of a tissue or organ, which impairs the ability to function efficiently. Gal-3 has long been identified as a driver of fibrosis (81). By activating fibroblasts, Gal-3 induces secretion of collagen leading to fibrosis (59, 60). In PAH, fibrosis occurs in both the lung vasculature and the right ventricle (13). Vascular fibrosis results from a diverse range of stimuli including oxidative stress, inflammatory cells and the release of inflammatory cytokines, compromised endothelial function, and the production of endothelium-derived vasoactive substances including the renin–angiotensin aldosterone system (17).

Elevated aldosterone can increase Gal-3 expression in vascular smooth muscle cells and drive vascular fibrosis (18). Collagen I expression is increased by Gal-3 in rat vascular smooth muscle cells, and in hypertensive aldosterone-treated rats, Gal-3 expression is increased along with the onset of

vascular hypertrophy, inflammation, and fibrosis, which is reversed in the presence of Gal-3 inhibitors and absent in Gal-3 knockout (KO) mice (18). Wang *et al.* investigated the effect of Gal-3 on pulmonary vascular fibrosis in the MCT-treated rat model of PAH and found evidence of increased vascular fibrosis and *in vitro* that Gal-3 mediated TGF-β1-induced vascular fibrosis *via* the STAT3 and MMP9 signaling pathways (171). Myocardiac fibrosis is best understood in the left ventricle (LV) and occurs in response to injury; ischemia, chronic stress, or excessive deposition of matrix reduces tissue compliance, limits contractility, and accelerates the progression to heart failure.

In the setting of PAH, the RV, under conditions of prolonged increases in volume overload and excessive afterload, undergoes a plethora of compensatory and eventually decompensatory pathophysiological and morphological remodeling changes that eventually lead to failure. Among these alterations in cardiac morphology is the development of fibrosis (124). While fibrosis of the right ventricle can be commonly detected in human and animal models of PAH, the relationship between fibrosis and *cor pulmonale* is not as clearly defined as for the LV.

Recent studies have observed increased circulating levels of Gal-3 in cardiac fibrosis, which may provide utility as a clinical biomarker providing diagnostic information for the potential onset of heart failure (61, 92). Increased levels of Gal-3 are seen in fibrotic hearts, and multiple lines of evidence suggest that it contributes to myocardial fibrosis. In mice, knockout or pharmacological inhibition of Gal-3 reduces LV fibrosis and improves function (182). In rats, infusion of recombinant Gal-3 for 4 weeks promotes cardiac fibroblast proliferation, collagen production, and cyclin D1 expression leading to LV dysfunction (146). Mechanistically, hyaluronic acid has been reported to be a major component of myocardial fibrosis, and Gal-3 upregulates CD44, which increases the levels of hyaluronic acid (67, 170).

PAH Is Associated with Increased Levels of Gal-3

Increasing evidence supports a role for Gal-3 in the development of PAH. In humans with PAH, circulating Gal-3 is elevated and correlates with RV ejection fraction, end diastolic and systolic volumes (37), and this is supported by other studies showing that Gal-3 levels correlate with the severity of PAH, are a biomarker of disease progression (17), and are a strong predictor of mortality (101) in PAH.

Circulating levels of Gal-3 correlate with RV dysfunction (2). These data are in agreement with an already recognized role of Gal-3 as a promising indicator of left-sided cardiac failure (12, 41). Gal-3 expression has also been shown to be upregulated in different established experimental rat models of PAH. Luo *et al.* (94) have reported that Gal-3 is upregulated in lung tissue from the hypoxia-induced rat model of PAH, and we have reported increased Gal-3 expression in the MCT rat model and the Sugen 5416/hypoxia rat model of PAH (9). Elevated Gal-3 expression has also been reported in the hypoxia-induced mouse model of PAH (53).

Gal-3 Has a Functional Role in the Development of PAH

Hao *et al.* reported that chronic hypoxia increased both RV hypertrophy and right ventricle systolic pressure (RVSP) in wild-type (WT) mice (53); however, in Gal-3 KO mice, both these indices were not elevated by hypoxia, suggesting an amelioration of PAH. Similarly, in the hypoxia-induced rat model of PAH, where both mPAP and RVSP as well as the Fulton Index (RV/LV+S, an index of RV hypertrophy) were increased by hypoxia but inhibited by N-Lac, a nonselective galectin inhibitor (94). Furthermore, Luo *et al.* found that Gal-3 inhibition by N-Lac attenuated the medial hypertrophy as well as collagen deposition in the PA, suggesting that Gal-3 expression is involved in both PA proliferation and fibrosis, possibly *via* a TGF-β signaling pathway (94). While intriguing, a caveat of these studies is the reversibility of hypoxia models of pulmonary hypertension upon return to normoxia, which is distinct from the progressive remodeling seen in human PAH.

The rat MCT and Sugen/hypoxia models are irreversible leading to *cor pulmonale* and are more inflammatory and fibrotic (82, 152, 159, 174). Barman *et al.* observed an increase in Gal-3 expression in isolated PAs from both the MCT rat models of PAH, Sugen/hypoxia model and human PAH, which was found primarily within the medial smooth muscle layer (9). Inhibitors of Gal-3, which have been used in animal models of fibrosis and human nonalcoholic steatohepatitis (NASH) (54, 161, 162), were used to assess a functional role of Gal-3 in these models. Inhibition of Gal-3 in prevention studies reduced PA vascular remodeling and ameliorated *in vivo* indices of PAH. Inhibition of Gal-3 in reversal studies also showed significant efficacy at slowing disease progression (9).

To provide a complementary genetic approach that is more selective, Gal-3 was knocked out in the Sprague-Dawley rat (SDR) using CRISPR Cas9 technology. In rats, noninvasive indices of PAH were assessed *in vivo* using high-resolution digital ultrasound in both WT and Gal-3 KO rats treated with or without MCT. MCT-treated WT rats exhibited a time-dependent increase in PAH that was absent in Gal-3 KO rats (9). In addition, while RVSP was significantly increased in WT rats exposed to Sugen/hypoxia, there was no difference between control WT rats and Sugen/hypoxia-exposed Gal-3 KO rats (9). Collectively, these results advance the hypothesis that Gal-3 expression is increased in PAH from rodent models and human PAH, and it contributes to the vascular remodeling of PAs and the development of PAH in multiple models.

Gal-3 Promotes the Development of PAH Through Multiple Mechanisms

The ability of Gal-3 to regulate cell proliferation has been well documented (35, 72, 126). Gal-3 levels are higher in some proliferating cancer cells (76, 132, 144, 172). In PAs from rodents and humans with PAH, Gal-3 expression was detected within the hyperproliferative smooth muscle layer. This correlated with increases in numerous cellular markers of proliferation in isolated PAs, and proliferation was significantly decreased in PAs isolated from MCT-treated rats in which Gal-3 function was suppressed through pharmacological inhibition using GR or from Gal-3 KO rats. In addition, isolated PASMCs from knockout rats have reduced the capacity to proliferate, and this deficit is rescued by recombinant Gal-3, and increased expression of Gal-3 *via* adenoviral-mediated gene transfer stimulates human PASMC proliferation (9).

The ability of PDGF to stimulate PASMC proliferation has been shown to be dependent on increased expression of Gal-3 and is reduced by silencing of Gal-3 (51). Inhibition of Gal-3 in human pulmonary arterial vascular smooth muscle cells (HPASMCs) reduces proliferation by decreasing cyclin D1 expression and increasing p27 expression and promoting a contractile phenotype (53). Gal-3 has also been shown to mediate the ability of TGF-β to increase the proliferation of pulmonary fibroblasts (94).

As discussed above, ROS contribute to the development of PAH. Gal-3 has been shown to promote ROS generation in a range of cells. Recombinant Gal-3 stimulates dose-dependent ROS production in neutrophils (80, 178) and monocytes (90). In mast cells, extracellular Gal-3 but not Gal-1 induces apoptosis *via* the release of superoxide (157). While the ability of Gal-3 to induce ROS production appears to be mediated by its extracellular actions, the mechanisms by which Gal-3 promotes ROS production are not completely understood.

In the MCT model of PAH, we found increased expression of NOX1, NOX2, and NOX4 mRNA in isolated PAs (Fig. 3A–C). Pretreatment with an inhibitor of Gal-3 that ameliorates PAH (9) leads to significant reductions in NOX1, NOX2, and NOX4 expression (Fig. 3A–C). Increased intracellular and extracellular Gal-3 can contribute to superoxide production. Transduction of mouse peritoneal macrophages with a Gal-3 adenovirus resulted in increased phorbol myristate acetate-stimulated superoxide production. Alternatively, extracellular recombinant Gal-3 increased superoxide production in mouse peritoneal macrophages, which was accompanied by increased expression of NOX2, the major oxidoreductase in immune cells (Fig. 3E).

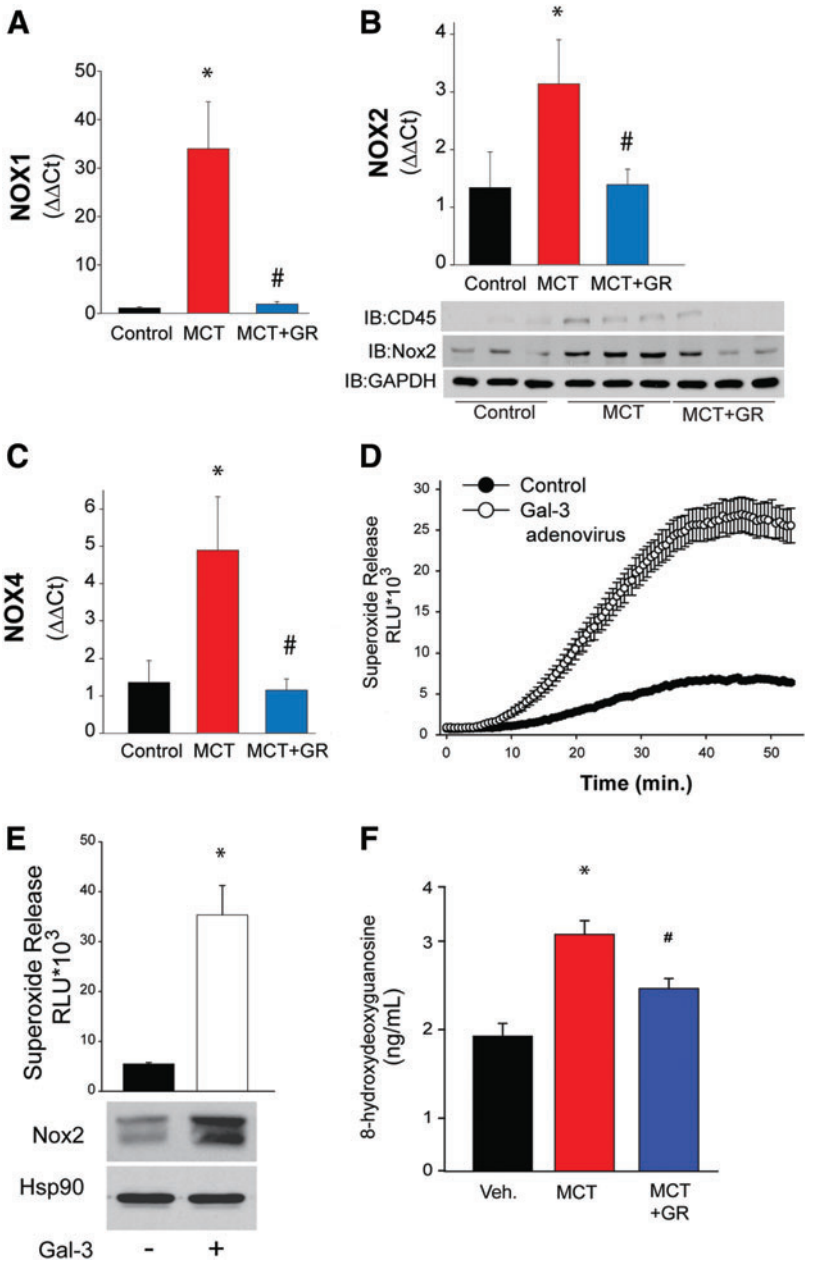

FIG. 3. Gal-3 increases the expression of NOX enzymes and reactive oxygen-specific production in PAs from a rat model of pulmonary hypertension. The expression of NOX enzymes was determined in PAs isolated from rats treated with MCT for 4 weeks. Relative expression of **(A)** NOX1 mRNA, **(B)** NOX2 mRNA and protein, and **(C)** NOX4 mRNA was determined in PAs isolated from control, MCT-treated, and MCT-treated with the Gal-3 inhibitor GR by real-time PCR ($n=3$–4). In **(D)**, mouse peritoneal macrophages were transduced with control (GFP) or Gal-3 adenovirus, and the ability to generate ROS was determined using enhanced L-012 chemiluminescence ($n=8$). In **(E)**, mouse peritoneal macrophages were incubated with recombinant Gal-3 (10 μg/mL), and 24 h later, basal superoxide production was determined using L-012 *versus* NOX2 expression ($n=8$). In **(F)**, the levels of 8-hydroxydeoxyguanosine, a molecular footprint of ROS production *in vivo*, were measured by ELISA in lung tissue isolated from control, MCT-treated rats, and MCT-treated rats plus the Gal-3 inhibitor GR ($n=5$). $^{*}p<0.05$ *versus* vehicle/control, $^{\#}p<0.05$ *versus* MCT. NOX, NADPH oxidase; PCR, polymerase chain reaction. Color images are available online.

To assess whether Gal-3 contributes to vascular ROS production in PAH, we measured the expression levels of 8-hydroxy deoxyguanosine, a molecular footprint of DNA damage due to ROS, in the lungs from control rats and rats treated with MCT and MCT plus a Gal-3 inhibitor. We found that MCT increased the levels of ROS as estimated by 8-hydroxy deoxyguanosine and that pretreatment with a Gal-3 inhibitor reduced these levels to control (Fig. 3F). Collectively, these results suggest that *in vivo* Gal-3 contributes to the elevation of ROS *via* the upregulation of multiple NOX isoforms that contribute to aberrant vascular remodeling.

Others have shown a relationship between Gal-3 and oxidative stress in blood vessels (39). For example, monocytes treated with phorbol myristate acetate, which induces NADPH-oxidase-dependent ROS, increased both Gal-3 mRNA and protein expression, which was inhibited by the putative NADPH inhibitor, apocynin (98). Evidence also shows a possible relationship between Gal-3 and Nox4 to promote RV remodeling. He *et al.* found that a positive correlation exists between serum Nox4 and Gal-3 levels in PAH patients (57). Furthermore, in the MCT-induced rat model of PAH, both Gal-3 and Nox4 expressions were upregulated in the right ventricular myocardium with specific staining of both moieties in the intracellular myocardial matrix (57).

In specific cell types, it has been proposed that Gal-3 stimulates cardiac fibroblasts to promote RV fibrosis *via* interacting with Nox4, and it has been observed that knockdown of Gal-3 can inhibit Nox4 protein expression and Nox4-derived production of ROS, which is greatly increased in cardiac fibrosis (57). While we found that the inhibition of Gal-3 robustly decreased the expression level of NOX4 (Fig. 3C), we did not observe an ability of Gal-3 to upregulate NOX4 expression in fibroblasts (Fig. 5D), suggesting that other cell types or mechanisms are involved.

Pulmonary hypertension is accompanied by increased vascular inflammation (55, 151, 163) and recruitment of

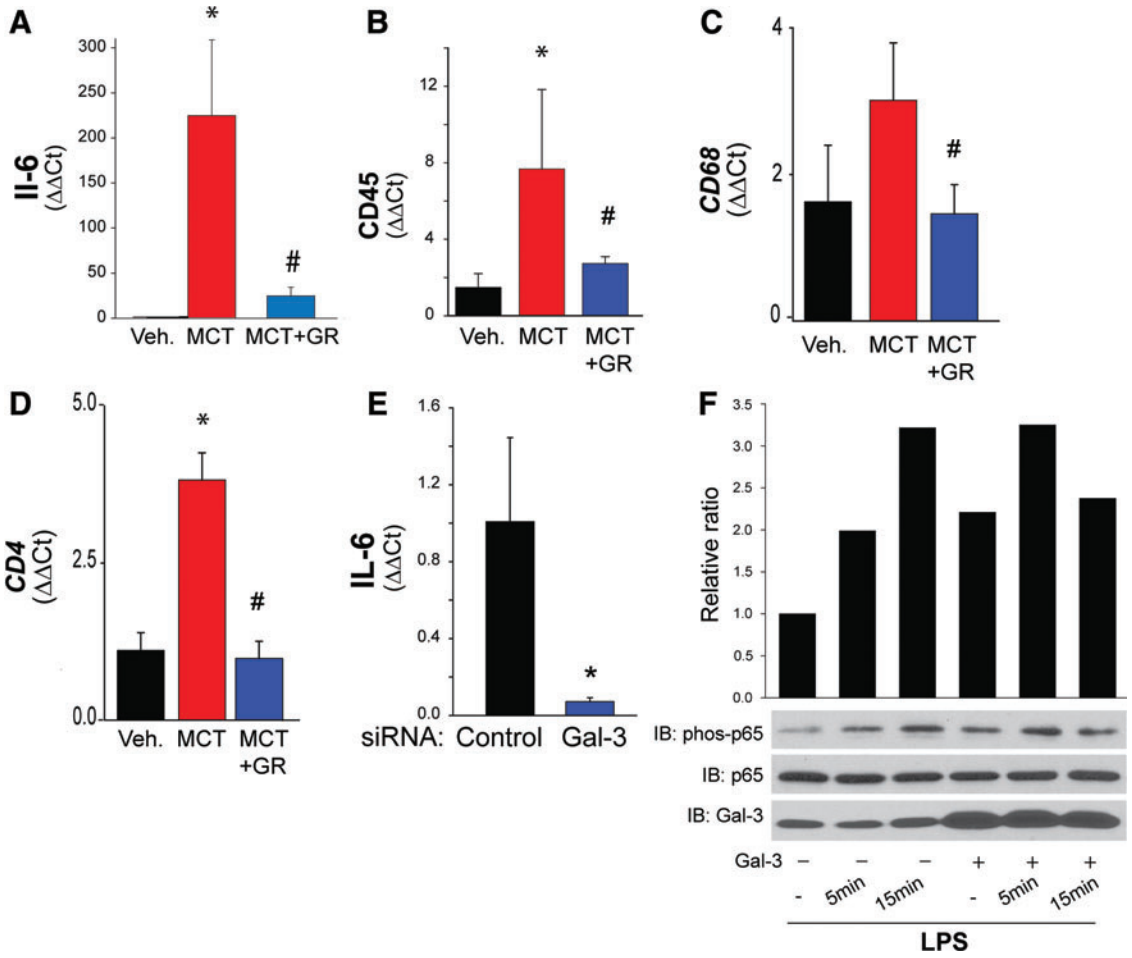

FIG. 4. Gal-3 promotes inflammation in hypertensive PAs. The expression of proinflammatory genes was determined in PAs isolated from rats treated with MCT for 4 weeks. Relative expression of **(A)** IL-6 mRNA, **(B)** CD45 mRNA **(C)** CD68 mRNA, and **(D)** CD4 mRNA was determined in PAs isolated from control, MCT-treated, and MCT-treated with the Gal-3 inhibitor GR by real-time PCR. In **(E),** silencing Gal-3 in HPASMCs reduced IL-6 mRNA expression. In **(F)**, Gal-3 regulates the NF-κB activity. HPASMCs were pretreated with recombinant Gal-3 (10 μg/mL) and then exposed to vehicle or LPS and time-dependent changes in the levels of phosphorylated p65, total p65, and Gal-3 determined by Western blot. $n = 2$–4, $^{*}p < 0.05$ *versus* vehicle/control, $^{\#}p < 0.05$ *versus* MCT. HPASMCs, human pulmonary arterial vascular smooth muscle cells; IL, interleukin; LPS, lipopolysaccharide; NFκB, nuclear factor kappa light chain enhancer of activated B cells. Color images are available online.

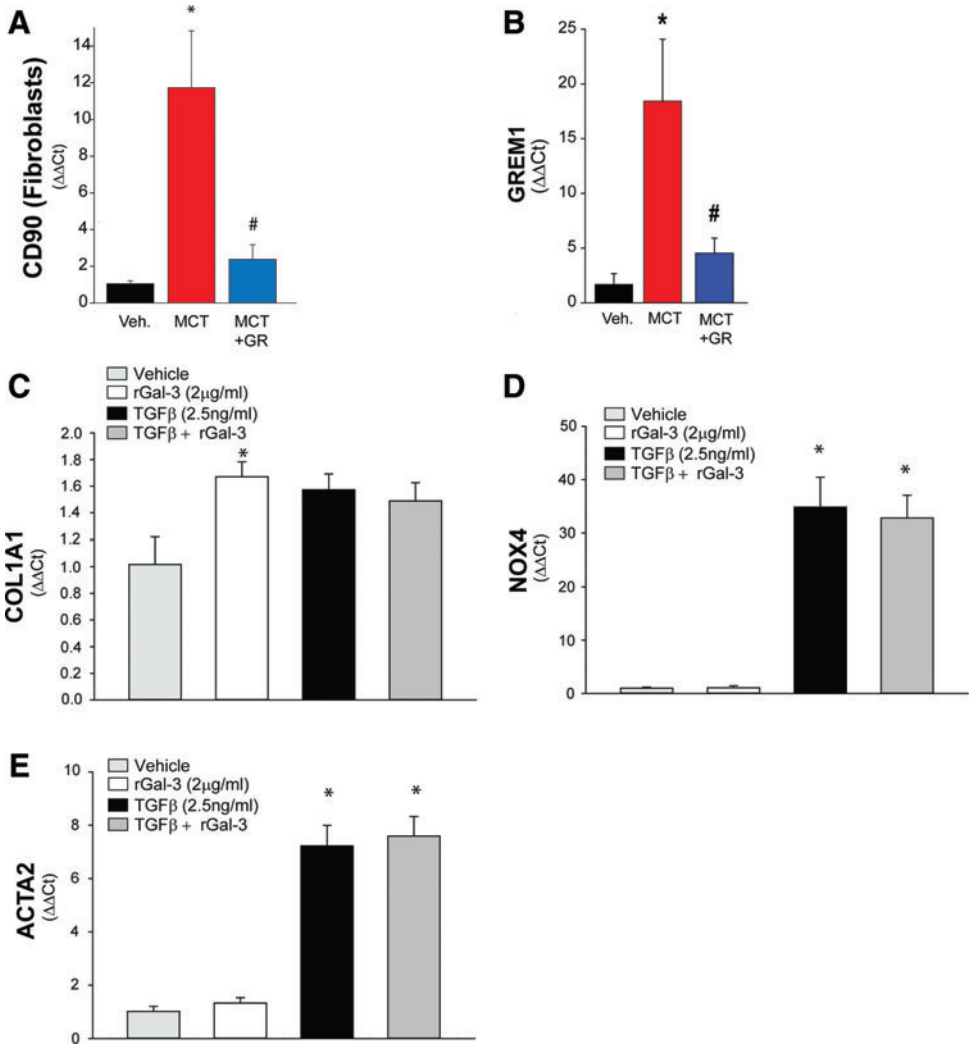

FIG. 5. Gal-3 promotes vascular fibrosis in hypertensive PAs. The expression of profibrotic markers was determined in PAs isolated from rats treated with MCT for 4 weeks. Relative expression of **(A)** CD90 (Thy1, fibroblast marker) and **(B)** GREM1 mRNA was determined in PAs isolated from control, MCT-treated, and MCT-treated with fibroblasts. In **(C),** recombinant Gal-3 (2 μg/mL) increased collagen expression in fibroblasts but did not modify the ability of TGF-β1 (2.5 ng/mL). In **(D)**, recombinant Gal-3 did not increase NOX4 expression or alter the ability of TGF-β1 to robustly increase NOX4 expression. In **(E)**, recombinant Gal-3 did not increase the expression or smooth muscle actin or alter the ability of TGF-β1 to robustly increase expression. $n = 3$–4, $^{*}p < 0.05$ *versus* vehicle, $^{\#}p < 0.05$ *versus* MCT. TGF-β1, transforming growth factor beta 1. Color images are available online.

inflammatory cells (42). As discussed above, Gal-3 is intimately involved in the function of immune cells. To assess the role of Gal-3 in regulating vascular inflammation, we measured the expression level of inflammatory markers in isolated PAs from control, MCT-treated, and MCT plus Gal-3 inhibitor-treated rats. We found that MCT-induced pulmonary hypertension was associated with increased expression of IL-6 (proinflammatory cytokine), CD45 (pan leukocyte marker), CD68 (monocytic cell marker), and CD4 (T cell marker) in isolated PAs, and the inhibition of Gal-3 significantly attenuated MCT-induced vascular inflammation (Fig. 4A–D). Silencing Gal-3 resulted in reduced expression of the proinflammatory cytokine, IL-6 (Fig. 4E).

To determine the mechanism by which Gal-3 impacts vascular inflammation, we treated human PASMC with LPS with and without recombinant Gal-3. LPS induced the phosphorylation of p65, a transcription factor that orchestrates many aspects of inflammatory signaling. In cells pretreated with recombinant Gal-3, the phosphorylation of p65 was increased in unstimulated cells, suggesting that priming and the response to LPS were enhanced (Fig. 4F).

Fibrosis contributes to the stiffening and compromised function of organs and blood vessels. Pulmonary hypertension is accompanied by increased PA stiffness (47, 173), increased deposition of matrix (160), and increased numbers of vascular fibroblasts (88). Gal-3 is a potent regulator of fibrosis and has been identified as a contributing factor to idiopathic pulmonary fibrosis (116), liver fibrosis (162), renal fibrosis (59), cardiac fibrosis (61), and vascular fibrosis (18).

To investigate a possible pathogenic role of Gal-3 in regulating vascular fibrosis in a model of PAH, we measured the indices of fibrosis in PAs from control, MCT-treated, and MCT plus a Gal-3 inhibitor-treated rats. We found that MCT-induced PAH resulted in increased expression of CD90 (a marker of fibroblasts) and Grem1 (a marker of fibrosis). Pretreatment with the Gal-3 inhibitor significantly reduced the markers of vascular fibrosis (Fig. 5A, B). In isolated lung fibroblasts, recombinant Gal-3 and TGF-β increased collagen expression. However, there was no significant interaction between Gal-3 and the actions of TGF-β (Fig. 5C). Recombinant Gal-3 failed to increase the expression of fibroblast Nox4 and ACTA2 (a marker of myofibroblasts), which were robustly increased by TGF-β. These data suggest that Gal-3 contributes to the vascular fibrosis seen in hypertensive PAs, but that its actions on fibroblasts are distinct from those of TGF-β.

Gal-3 is expressed to varying degrees in a number of cell types, and as discussed above, Gal-3 can have a variety of effects, depending on the cell type involved. Our group has shown that the majority of Gal-3 protein expression was

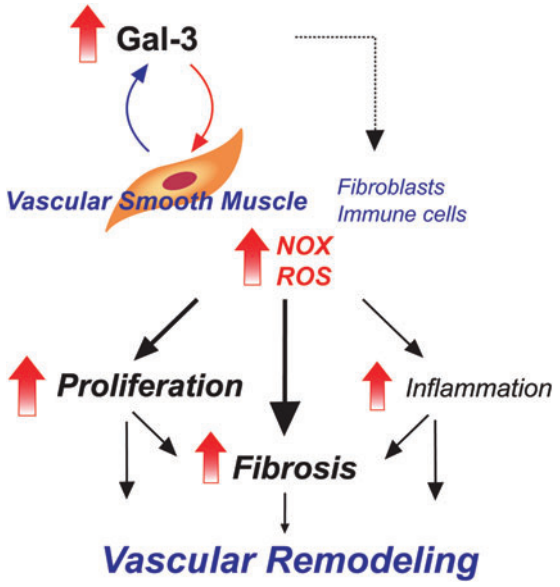

FIG. 6. Summary of the proposed mechanisms by which Gal-3 promotes vascular remodeling in PA to induce pulmonary hypertension. Gal-3 increases cell proliferation, inflammation, and fibrosis (matrix deposition) *via* paracrine and autocrine actions on different cell types. Color images are available online.

found in the smooth muscle-rich media area of PAs where it regulates proliferation (9). Others have shown that Gal-3 is expressed in perivascular fibroblasts (94) in models of pulmonary hypertension. However, given prominent roles of macrophage Gal-3 in atherosclerosis (5, 97, 110) and fibrosis (116), it would not be surprising if this cell type also played a significant role as a source of Gal-3 in either the PA or the RV. The relative ability of Gal-3 to influence ROS, inflammation, and fibrosis (as shown in Figs. 3–5) may depend heavily on the cell type expressing Gal-3. Function delineation of the various autocrine *versus* paracrine actions of Gal-3 in different cell types awaits further investigation.

Summary and Clinical Perspectives

Studies thus far support the premise that Gal-3 expression is increased in both rodent and human PAH. Circulating levels of Gal-3 likely derive from increased expression in the right ventricle, but increases in expression in isolated PAs suggest local effects of Gal-3 to promote PA remodeling through changes in cell proliferation, increased ROS, inflammation, and fibrosis (Fig. 6). Gal-3 is expressed in many cell types and influences a variety of mechanisms to alter cell function. The sum of these actions contributes to the changes in cellularity and altered vascular and RV function seen in PAH.

Given that PAH is a complex disease originating from diverse mechanisms in multiple cell types, it suggests that targeting Gal-3 may be a useful therapeutic approach. Recent studies suggest that Gal-3 serves as a circulating biomarker in humans that tracks PAH severity and progression. The ability of Gal-3 inhibitors to impact multiple pathways (*i.e.*, silver shotgun as opposed to silver bullet) may be advantageous in the approach to treating complex diseases such as PAH and may also have utility in combinatorial strategies that have significantly greater potential to delay the progression of pulmonary vascular disease (147). Most of the therapies targeting Gal-3 bind in the extracellular space and whether small molecules that can suppress the actions of both extracellular and intracellular Gal-3 are more efficacious in disease states awaits further development.

Materials and Methods

Cell culture and reagents

HPASMCs were obtained from Lonza and grown in smooth muscle growth media and supplemented with fetal bovine serum to 5% final concentration (Lonza). HPASMCs stained positive for smooth muscle α-actin and negative for von Willebrand (factor VIII) as indicated by the supplier. All chemicals were purchased from Sigma, unless indicated otherwise.

Rat model of PH

The MCT model results from a single i.p. injection of MCT (60/mg/kg), which induces a severe PH 4 weeks after exposure. Adult, age-matched male SDR (250–300 g) were used as controls for both rat models of PH. The Animal Care and Use Committee at the Augusta University approved all procedures and protocols, and this study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996). All groups of rats were housed under temperature-controlled conditions (21–23°C), maintained on standard rat chow, allowed free access to food and water, and exposed to a 12:12-h light–dark cycle. Gal-3 inhibitor (Galectin Therapeutics, courtesy of Peter Traber) was administered to MCT-treated rats: (GR-MD-02; 60 mg/kg i.v.) was delivered twice weekly for 4 weeks starting at the time of the single MCT injection in SDR.

Analysis of gene expression

PAs (down to fourth order) were dissected from surrounding parenchyma, snap frozen in liquid nitrogen, pulverized, and RNA extracted using TRIZOL or proteins solubilized in 2×Laemmli buffer. cDNA was synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) and used to assess relative PA gene expression using real-time reverse transcription polymerase chain reaction (iQ SYBR Green; Bio-Rad). Proteins were size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and expression determined by Western blotting.

Measurement of ROS

Peritoneal macrophages were seeded into 96-well plates and transduced with Gal-3 adenovirus (100 multiplicity of infection) or treated with recombinant Gal-3 (10 μg/mL). Twenty-four hours later, the media was changed from RPMI to phenol-free Dulbecco's modified Eagle's medium (Sigma) containing L-012 (400 μ*M*, Wako) and incubated for 30 min before the addition of agonists. Luminescence was quantified over time using a Lumistar Galaxy (BMG) luminometer. The specificity of L-012 for ROS was confirmed by co-incubation with the superoxide scavenger, SOD (100 U/mL).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics and GraphPad InStat. Data sets were assessed for normal distribution, reported as mean ± standard error of the mean, and statistical significance was determined either by the unpaired *t*-test (for two groups) or by two-way analysis of variance (ANOVA) (for more than three groups). In data sets analyzed by ANOVA, the Bonferroni *post hoc* test was employed. A value of $p < 0.05$ was considered statistically significant.

Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ANOVA = analysis of variance
CRD = carbohydrate recognition domain
EGFR = epidermal growth factor receptor
Gal-3 (or *LGALS3*, or Mac-2) = galectin-3
H_2O_2 = hydrogen peroxide
HPASMCs = human pulmonary arterial vascular smooth muscle cells
IL = interleukin
KO = knockout
LMP = lysosome membrane permeabilization
LPS = lipopolysaccharide
LV = left ventricle
MCT = monocrotaline
mPAP = mean resting pulmonary artery pressure
NFκB = nuclear factor kappa light chain enhancer of activated B cells
NOX = NADPH oxidase
O_2^- = superoxide
PAs = pulmonary arteries
PAH = pulmonary arterial hypertension
PASMCs = pulmonary arterial smooth muscle cells
PCR = polymerase chain reaction
PDGF = platelet-derived growth factor
PH = pulmonary hypertension
ROS = reactive oxygen species
RV = right ventricle
RVSP = right ventricle systolic pressure
SDR = Sprague-Dawley rats
TGF-β = transforming growth factor beta
TRIM = tripartite motif
VEGF = vascular endothelial growth factor
WT = wild-type