An Investigation of BMAL1 and Pulmonary Hypertension

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Abstract

Pulmonary hypertension (PH) is a chronic disease with poor prognosis, decreased quality of life, and high mortality risks. The W.H.O. Group 3 PH is caused by presence of lung disease or hypoxia and when present with interstitial pulmonary fibrosis (IPF), outcomes are severely worsened. In addition to costly hospital expenses, there is a lack of therapeutic remedies for Group 3 PH, thus the investigation of PH and its related pathways are beneficial to identify a possible drug target. A specific group of myeloid cells termed myeloid-derived suppressor cells (MDSCs) have been determined to be necessary for the development of PH. This study aims to define a correlation between circadian genes and pulmonary hypertension through manipulation of the circadian transcription factor, brain and muscle ARNT-like 1 (BMAL1) in murine models with normoxia, hypoxia, and bleomycin-induced conditions. BMAL1 has been shown to be altered in hypoxic conditions, with global over-expression of BMAL1 being protective against PH. Specific deletion of BMAL1 in myeloid cells have been shown to have the reverse effect. In BMAL1 OE models, PD-L1 is down-regulated in hypoxia when compared to control models, suggesting a potential therapeutic target for future studies.
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Background

Pulmonary Hypertension

Pulmonary hypertension (PH) is a chronic disease defined by pulmonary arterial pressures greater than or equal to 25 mmHg. The World Health Organization (W.H.O.) classifies PH into five subcategories based on cause or origin. In W.H.O. Group 3, PH is caused by presence of lung diseases or hypoxia. When PH arises with interstitial pulmonary fibrosis (IPF), the prognosis of IPF is severely worsened, quality of life is significantly decreased, and mortality risks are greatly increased. The hospital expenses that result from patients with PH total to about $2 billion annually. There are currently no targeted therapeutic solutions to Group 3 PH and IPF, therefore, further investigation about the molecular and cellular pathways involved can prove to be useful in finding a drug target (1).

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are myeloid cells that are distinct from the classical myeloid cells that arise from strong pathogen signals of short duration such as toll-like receptor (TLR) ligands, damage associated molecular patterns (DAMP), and pathogen-associated molecular pattern (PAMP) molecules. In comparison, MDSCs result from weak pathogenic signals of long duration. Under these conditions, MDSCs exhibit immature phenotype and morphology and weak phagocytic activity. These characteristics lead to an inhibition of adaptive immunity, and failure to eliminate the threat. MDSCs are marked by their immunosuppressive capability which occurs primarily through the inhibition of T-cell function and can lead to vascular remodeling and pathogenesis of disease (2). MDSCs have been found to contribute to the development of pulmonary hypertension. In murine models, trafficking of granulocytic-MDSCs (G-MDSCs), which are a subpopulation of MDSCs, to the lung were shown to contribute to PH. Specifically, C-X-C chemokine receptor 2 (CXCR2) inhibition in myeloid cells was shown to prevent recruitment of MDSCs to the lung. This lack of MDSCs demonstrated an absence of vascular remodeling and thus prevention of pulmonary hypertension. However, removal of CXCR2 in endothelial cells demonstrated worsened vascular remodeling and increase in MDSC migration (3). Although the responses differ in myeloid and endothelial cells, the outcomes prove that CXCR2 plays a role in the regulation of MDSCs and therefore immunosuppression.

Circadian Rhythm and Immunity

Circadian rhythms are present in almost all organisms on earth due to the earth’s rotation which produces a 24-hour light and dark cycle. Certain metabolic functions switch on or off throughout the day in response to light, controlled by the suprachiasmatic nuclei (SCN) in the hypothalamus (4). This response is evolutionary and inherently adapted to avoid damage caused by radiation exposure by operating certain processes during different times of day. Brain and muscle ARNT-like 1 (BMAL1) and circadian locomoter output cycles protein kaput (CLOCK) are circadian transcriptional factors that create a heterodimer to act on the E-box elements. The CLOCK-BMAL1 complex amplifies target genes for circadian transcription factors period circadian protein homologue 1 (PER1) and PER2, cryptochrome 1 (CRY1) and CRY2, REV-ERBα and REV-ERBB. The transcription of these genes serves as a feedback loop for regulation of CLOCK-BMAL1 through subsequent repression throughout the day.

Immune functions are under circadian control and include trafficking of immune cells, host-pathogen interactions, and activation of adaptive and innate immunity. This temporal regulation ensures that activation occurs when needed and not activated when not needed to avoid
excess expenditure of energy (5). This mechanism is shown through the example of a neutrophil’s ability to defend the host when needed but also avoiding over-activation which can cause damage to the vascular system. Adrover et al. explains how a neutrophil timer controlled by BMAL1, CXCR2, and CXCR4 creates a diurnal timer to create a balance between the neutrophil’s benefits and disadvantages. Specifically, BMAL1 and CXCR2 drive neutrophil aging, a term used to describe the process of neutrophil entering circulation until they exit circulation, and CXCR4 antagonizes it (6). Another example of BMAL1 control of immune function is exemplified in myeloid specific deletion of BMAL1. BMAL1 was deleted in myeloid cells in BMAL1^[floxP/floxP]^; LysMCre mice and demonstrated increased trafficking of Ly6c^[hi] inflammatory monocytes to atherosclerosis lesions and increased lesion sizes. The presence of Ly6c^[hi] promotes monocyte progression into M1 monocytes which then favors atherosclerosis progression. The same mice exhibited an increase in innate and adaptive immune cell numbers shown through an increase in CD4^[+^] and CD8^[+^] T-cells in the plaque after flow cytometry analysis (7, 8).

The examples of neutrophil and Ly6c^[hi] monocyte control by BMAL1 manipulation and resulting progression or repression of disease can be applied to pulmonary hypertension. Myeloid-derived suppressor cells can then be investigated as they are also a critical part of the immune system. Analyzing BMAL1 effect on MDSCs should give us more knowledge on how the pathway works and how the circadian pathway links to pulmonary hypertension through a T-cell suppression assay.

Clinical Relevance

Given that the circadian clock and immunity have been shown to be closely correlated, investigating specific pathways in immune checkpoints will provide a potential target for pharmaceutical therapy. The programmed cell death 1 (PD-1) expression and programmed cell death ligand 1 (PD-L1) checkpoint pathway is an existing and widely used therapeutic target which inhibits tumor progression and the evasion of apoptosis (9). An exploration of varying expression of BMAL1 as a transcriptional factor on PD-L1 will prove to be useful in correlating the known literature and a clinical perspective.
Methods

Mice strains
Mice (tetO-Bmal x R26M2rtTA KI) were bred with the Tet-On expression system, a binary transgenic system in which expression from a target transgene is dependent on the presence of an inducible transcriptional activator. The Tet-On system is based on the reverse tetracycline-controlled transactivator (rtTA) which activates the tetracycline-responsive promoter element (TRE) controlled gene in the presence of doxycycline (Dox) (Jackson Laboratories) (10). The breeding scheme is outlined in Figure 1. Groups intended for inducible expression of over expression of BMAL1 (BMAL1 OE) in all tissue types received doxycycline diet for at least two weeks. Groups are outlined in Figure 2. All procedures were approved by and performed in accordance with the University of Florida Institutional Animal Care and Use Committee (IACUC) under Protocol 202008502.

Figure 1. Cre-Lox breeding scheme.

Hypoxia Chamber
Mice were exposed to chronic hypoxia in a normobaric chamber that maintained carbon dioxide levels at < 0.1%. Oxygen and carbon dioxide levels were continuously monitored over a period of 4 weeks. Normoxia models were kept in room air of FiO₂ at 21% and hypoxia models were kept at FiO₂ at 10% (11).

Intraperitoneal injections
After exposure to doxycycline to induce BMAL1 OE, mice were injected with 0.018 U·g⁻¹ bleomycin (Thermo Fisher Scientific) or vehicle twice a week for 28 days. Five days after the last injection, pulmonary hemodynamic measurements were measured, and tissues were collected. After two weeks of doxycycline diet, mice intended for T-cell suppression assay for bleomycin exposure underwent intraperitoneal injection of 0.018 U·g⁻¹ bleomycin twice a week for 14 days. One day after the last injection, mice were euthanized with isoflurane and harvested for spleen.
Pulmonary hemodynamic assessments
To measure right ventricular systolic pressure (RVSP), invasive surgery was performed on anesthetized mice. An incision is made at the right internal jugular vein and a Millar 1.4-French pressure-volume microtip catheter transducer (SPR-839; Millar Instruments) connected to a Power-Lab/8s (ADInstruments) was inserted and threaded down into the right ventricle. RVSP (mmHg) recordings were collected using Chart 5 (ADInstruments). After completion of the measurements, the heart was removed with excision of the atria, and the RV and left ventricle (LV) plus septum (LV+S), isolated for measurement of the RV:LV + S. Left lung was placed in 10% formalin for histology. The right lung was divided into upper, middle, and lower lobes and snap frozen in liquid nitrogen for protein and RNA processing. This procedure was performed by various members of the Brantly and Bryant lab because of its complexity and requirement of a higher level of skill (3).

MDSC and T-cell Isolation
After two weeks of biweekly bleomycin injections or vehicle, the spleen was harvested for MDSC isolation using Stemcell kit #19867. The spleen was harvested from C57BL/6 wildtype mouse to obtain T-cells then isolated using Stemcell kit #19851. MDSCs and T-cells were isolated using CD3 monoclonal antibody-coated magnetic beads.

T-Cell Suppression Assay
T-cell inhibition is a standard method in assessing MDSC function. A non-specific suppression was performed using CD3 and CD28 antibody activation. Cell Trace Violet (Thermo Fisher #C34557) was used for staining of T-cells, including a control group without stain. Both cells were counted for each sample using 50 μL cell suspension and 50 μL tryphan blue. MDSCs were titrated to a concentration of 4x10^6 cells/mL. T-cells were titrated to a concentration of 1x10^6 cells/mL. Cells were cultured in wells using 1:1 and 1:2 ratios of T-cells to MSDCs. Each well used 2 μL anti CD3/CD28 mouse beads (Thermo Fisher) to stimulate the cells. Cells were incubated in 37°C with 5% CO2 for 4 days. Proliferation was assessed by Cell Trace Violet dilution using flow cytometry using FlowJo program.

Western Blot
Protein concentrations were quantified following the Thermo Scientific Pierce BCA Protein Assay Kit protocol. Gel electrophoresis was run on a 7.5% TGX Precast gel. Protein was transferred onto a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad). Primary antibodies included rabbit BMAL1, pBMAL1, PD-L1, and β-actin, incubated in 5% non-fat milk or bovine serum albumin (Sigma-Aldrich) solution overnight in 4°C. HRP conjugated anti-goat IgG and HRP-linked anti-rabbit IgG were used as secondary antibodies (Cell Signaling Technology) and incubated for 1 hour at room temperature. Blots were imaged using ChemiDoc imaging system (Bio-Rad) and analyzed in Image Lab software.

Immunohistology
Masson’s Trichrome
Slides of lung tissue were analyzed using the Keyence BZ-X microscope and software. Images of slides were captured at 10X magnification. Each sample was analyzed using 10 randomized and non-overlapping high-powered fields (HPF) with each HPF given a fibrosis score from 0-4 (0 = normal, 1 = <50% of airway wall thickness, 2 = >50% airway wall thickness, 3 = small
inflammatory patch partially in field, 4 = large inflammatory patch majority of field). The scores for each sample are then averaged out of 10 to obtain an average score for each specimen.

α-Smooth Muscle Actin
Slides of lung tissue stained for α-smooth muscle actin muscularization were scanned and analyzed using the Keyence BZ-X microscope and software. Each HPF was examined for small, medium, or large muscularized vessels with each size categorized as partial or complete. 10 randomized HPFs were used per sample to obtain a mean score for each of the six categories.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc.). Values of P < 0.05 were considered to be statistically significant.

Results
BMAL1 Expression is Different in Normoxia and Hypoxia
BMAL1 is a circadian gene, however, investigation of BMAL1 as a transcription factor can identify its correlated pathways in hypoxic conditions for potential targets. In a western blot analysis of phosphorylated BMAL1 (pBMAL1) and BMAL1 in wildtype mice under normoxia and hypoxia conditions, there is a clear difference in level of protein expression (Figure 2). In hypoxia, pBMAL1 has decreased expression compared to BMAL1 with an increased expression.

![Figure 2](Image)

Figure 2. Western blot for wildtype (WT) mice (n=2), treated with antibodies pBMAL1, BMAL1 in normoxia (Nx) and hypoxia (Hx) conditions. Blot with β-actin antibody ensured equal loading.

BMAL1 OE is Protective Against Hypoxia
To understand the effects of over expression of BMAL1 in all tissue types, mice were fed doxycycline chow for at least two weeks then anesthetized for hemodynamic measurements and tissue collection. Mice induced with BMAL1 OE have lower measured RVSP (mmHg) and RV:LV+S (%) measurements compared to control mouse models in chronic hypoxia (Figure 3A, 3B). Values are considered significant with a p-value of 0.02. In α-SMA staining, BMAL1 OE mice have a decreased complete to partial ratio of muscularized vessels compared to control models in hypoxia (Figure 3C). A decreased complete to partial ratio signifies a decrease in phenotype alteration of endothelial cells to α-SMA producing mesenchymal cells. BMAL1 OE protects against the endothelial-to-mesenchymal transition (EndoMT) known to contribute to vascular remodeling and chronic tissue scarring in PH (12, 13).
**Figure 3.** Global over expression of BMAL1 are protected against the development of pulmonary hypertension.  
(A) Right ventricular systolic pressure (mmHg) of WT mice and BMAL1 OE mice in Nx and Hx. (B) Right ventricular (RV) to left ventricle (LV) plus septum mass (RV:LV+S) percentage of WT and BMAL1 OE mice in Nx and Hx. (C) Immunohistochemical (IHC) staining for α-SMA of lung sections from hypoxia-induced mice at 10X magnification. Complete and partially muscularized vessels were counted and averaged from 10 high-powered fields (HPF) and ratio calculated.

**mBMAL1 KO is Deleterious in Hypoxia**

To investigate the effects of BMAL1 deletion is myeloid cells, termed “mBMAL1 KO”, mice were exposed to normoxia and chronic hypoxia and anesthetized for hemodynamic measurements and tissue collection. mBMAL1 KO mice in hypoxia have worsened development of pulmonary hypertension. RVSP (mmHg) measurements are higher in mBMAL1 knockout mice compared to control mice in hypoxia, \( p = 0.001 \) (Figure 4A). RV:LV+S (%) measurements are higher in mBMAL1 knockout mice compared to control mice in hypoxia (Figure 4B). In α-SMA staining, mBMAL1 in hypoxia have increased complete to partial ratio of muscularized vessels compared to control models in hypoxia (Figure 4C).

**Figure 4.** mBMAL1 KO develops worsened PH. (A) Right ventricular systolic pressure (mmHg) of WT mice and mBMAL1 KO mice in Nx and Hx. (B) Right ventricular (RV) to left ventricle (LV) plus septum mass (RV:LV+S) percentage of WT and mBMAL1 KO mice in Nx and Hx. (C) Immunohistochemical (IHC) staining for α-SMA of lung sections from hypoxia-induced mice taken at 10X magnification, complete and partially muscularized vessels were counted and averaged from 10 high-powered fields (HPF) and ratio calculated.
Gating Strategy of MDSCs

BMAL1 OE and its effect on MDSC function is unknown, so the T-cell suppression assay was performed to investigate the correlation in both vehicle and hypoxia treatment. Results for the TCSA are inconclusive and cannot be considered to be significant due to human error, inexperience, and lack of uniformity in the procedures. However, the following gating strategies were used to for assessing MDSC function as shown with the presence of the CD11b+ marker. Specific MDSC subsets polymorphonuclear (PMN-MDSC) and monocytic (M-MDSC) are important to define as they have distinct biochemical traits, phenotypic and morphological features. Their definition is important as they have different roles depending on the pathological condition they exist in (14).

**Figure 5.** (A) Gating strategy shown for CD11b+ cells, a marker for MDSCs in WT and BMAL1 OE mice in vehicle and hypoxia administered conditions. (B) Representative plots for PMN-MDSCs and M-MDSCs for WT and BMAL1 OE mice in vehicle and hypoxia.

**BMAL1 OE Mice Have Lower PD-L1 Expression**

To identify potential therapeutic targets for BMAL1 models, PD-L1 expression was analyzed in a western blot analysis. BMAL1 OE models have decreased expression of the PD-L1 protein in both hypoxia and bleomycin models (Figure 8).

**Figure 6.** BMAL1 OE shows down-regulation of PD-L1. Wildtype mice (n=3) and BMAL1 OE mice (n=3), from left to right, incubated with PD-L1 antibody and β-actin as a loading control in normoxia and hypoxia.
Discussion

BMAL1 expression was shown to have phenotypic changes for murine models in pulmonary hypertensive conditions. To confirm that BMAL1 was affected in hypoxia, protein expression levels of phosphorylated BMAL1 and BMAL1 was investigated to identify these changes. The western blot showed that there was an increase in BMAL1 expression as opposed to pBMAL1, showing that hypoxia has a correlation to BMAL1 expression, which is an important distinction to make, knowing that hypoxia defines W.H.O. Group 3 pulmonary hypertension.

Global BMAL1 over expression was induced in mouse models and was found to be protective in hypoxia in physiologic data collected in RVSP measurements, RV:LV+S ratios and \( \alpha \)-SMA analysis. Specific deletion of BMAL1 in myeloid cells showed the opposite effect in repeated measurements of RVSP, RV:LV+S, and \( \alpha \)-SMA analysis. These changes identify a correlation with the circadian role in lung-specific hemodynamic and phenotypic remodeling, which is a characteristic important in considering pulmonary hypertension. These findings reveal future potential targets to consider such as modifications to the microenvironments of cells associated with PH with a circadian perspective. Global BMAL1 deletion models have not been investigated because survivability rates for murine models are low along with the presence of severe pathological conditions and age associated weight decline (15). A myeloid-specific BMAL1 overexpression model (mBMAL1 OE) is currently being performed and results have not been concluded to be mentioned in the timeline of this thesis.

As described in previous literature, circadian rhythm genes BMAL1 and CLOCK act as a heterodimer to regulate metabolic functions throughout the day to limit energy expenditure. One of these circadian controls is the immune system. Myeloid-derived suppressor cells have been shown to be necessary for the development of pulmonary hypertension, so an investigation on how circadian rhythm influences MDSCs as a part of immunity was an interesting aspect to look at. Unfortunately, due to time constraints and a lack of uniformity in procedures, the T-cell suppression assay data to determine MDSC function as a result of BMAL1 OE was insignificant and needs to be repeated.

Considering that there are no therapeutic targets specific for Group 3 pulmonary hypertension, investigation of a widely used drug target and its application in circadian models in PH was attractive. Anti-PD-1/PD-L1 therapy is a promising and established treatment for cancer patients which functions by using the PD-1/PD-L1 axis to surpass immune checkpoints and support tumor proliferation. The BMAL1 OE mice show that up-regulation of BMAL1 can contribute to decreasing programmed death-ligand 1 expression and therefore, evasion of apoptosis for cells that are detrimental to the development of pulmonary hypertension.
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References


