Loss of skeletal muscle Bmal1 impairs limb perfusion and muscle regeneration in a mouse model of peripheral arterial disease

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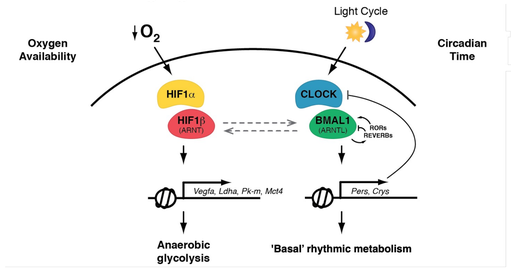
Introduction

Atherosclerotic plaque buildup narrows arteries, reducing blood supply to the limbs. Peripheral artery disease (PAD) is a manifestation of lower limb atherosclerosis beyond the aortic bifurcation. PAD patients experience symptoms like calf cramping or fatigue while walking. Critical limb ischemia, a severe PAD stage, necessitates amputation due to significant blockage of blood flow to the limbs[1]. Aging is a well-established risk factor for PAD and impedes reperfusion.

Mammalian circadian clocks regulate behavior and metabolism by controlling gene expression through an auto-regulatory transcription-translation feedback loop[2]. The clock network includes master pacemaker neurons reset by light and peripheral clocks influenced by the master pacemaker and nutrients. *Bmal1* deficiency or *Clock* gene mutations disrupt circadian behavior, gene expression patterns[3], and accelerate aging in mice[4]. Shift work, sleep loss, and jet lag lead to desynchrony among brain and peripheral clocks, associated with increased mortality and poorer health in older age[5].

Hypoxia-inducible factors (HIFs) activate gene transcription pathways in response to low oxygen levels. HIF α and β heterodimer initiates transcription under hypoxia by binding to hypoxia-response elements and regulates genes crucial for hypoxic growth and survival. The circadian clock plays a critical role in modulating HIF activity in hypoxia. *Bmal1*-deficient myofibers exhibit impaired hypoxic HIF1α accumulation, affecting the induction of HIF target genes[6].



Crosstalk of Oxygen Sensing and Clock Pathways

Methods

- To investigate the role of muscle BMAL1 in ischemia limb perfusion and muscle regeneration, we performed femoral artery ligation (FAL) surgery in adult-life inducible skeletal muscle-specific Bmal1 knockout (*Bmal1^{musc}*) and control mice.
- Limb perfusion was measured with laser doppler imaging. At 30 days post-surgery, angiogenesis and muscle recovery were assessed with CD31/α-SMA co-immunohistochemistry, hematoxylin and eosin staining, and Laminin immunohistochemistry, respectively.
- Additionally, RNA-sequencing, quantitative real-time PCR, and Western blotting assays were performed on both ligated and non-ligated contralateral hindlimb muscles to investigate key genes and signaling pathways involved in *Bmal1*-mediated regulation of limb perfusion and muscle regeneration.

Results

➢ **Loss of skeletal muscle *Bmal1* impairs hindlimb ischemic reperfusion and intramuscular angiogenesis**

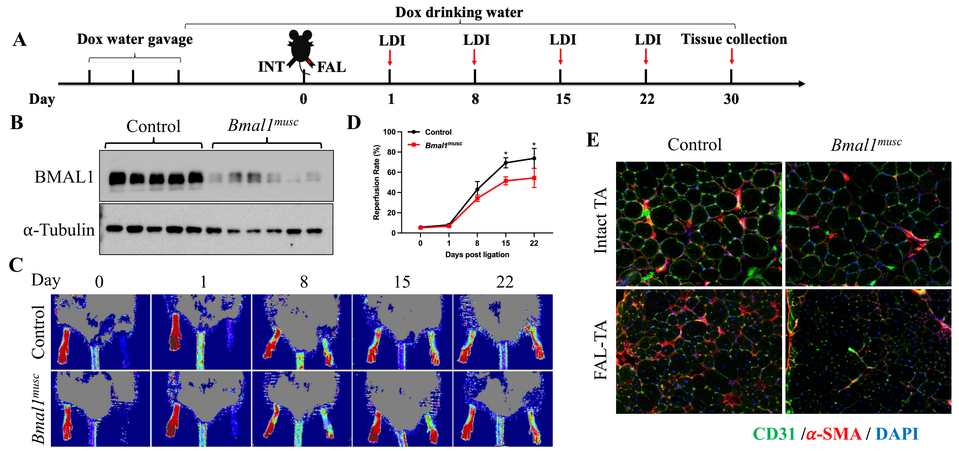


Figure 1 Blood reperfusion in *Bmal1^{musc}* hindlimb ischemia injury model. A, Experimental design: Control and *Bmal1^{musc}* mice were administered oral doxycycline (Dox) water for 3 days, followed by continuous Dox-drinking water throughout the experiment. FAL surgery was performed on one side of lower limb, with the contralateral side remained intact. On day 30 post-surgery, TA muscles from both Control and *Bmal1^{musc}* mice were collected. **B**, Immunoblot of BMAL1 and α-Tubulin in gastrocnemius (Gas) muscle of Control and *Bmal1^{musc}* mice after induction by doxycycline. **B**, LDI of limb perfusion recovery in Control and *Bmal1^{musc}* mice after FAL. **C**, Quantification of limb perfusion over time. * $p < 0.05$, ** $p < 0.01$ by multiple *t*-test. **D**, CD31 immunohistochemistry in TA muscle of Control and *Bmal1^{musc}* mice on 30 days post FAL.

➢ **Loss of skeletal muscle *Bmal1* reduces the number of newly formed myofibers, while promoting the formation of enlarged regenerating myofibers**

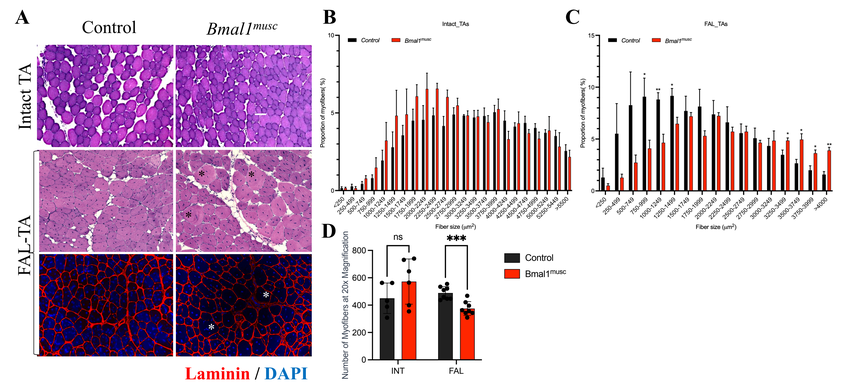


Figure 2 Skeletal muscle repair in *Bmal1^{musc}* hindlimb ischemia injury model. A, Representative H&E- and Laminin/DAPI stained sections of FA-ligated and the contralateral intact control tibialis anterior (TA) muscles from Control and *Bmal1^{musc}* mice on day 30 post-surgery. asterisk indicates the hypertrophic myofibers in *Bmal1^{musc}* mice. **B**,**C**, Quantification of muscle fiber size (cross-sectional area) distribution in the contralateral intact (**B**) and FA-ligated (**C**) TA muscles. **D**, Quantification of muscle fiber number. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by multiple *t*-test.

➢ **Loss of skeletal muscle *Bmal1* reduces the expression of the pro-angiogenic gene *Vegfα* and the anti-myogenic gene *Myostatin* while inducing activation of TGFβ signaling pathway**

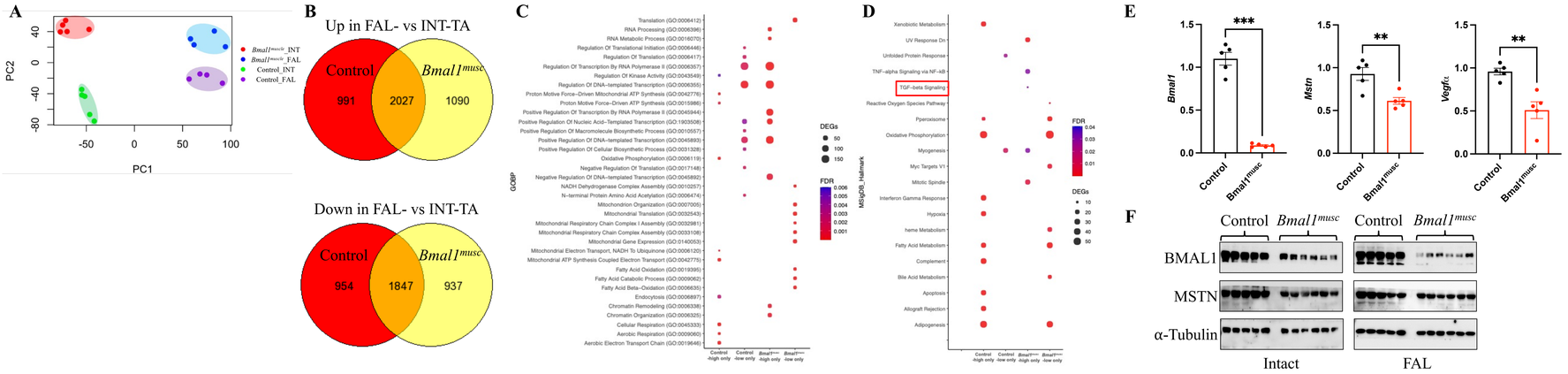


Figure 3 RNA-sequencing analysis of skeletal muscle in *Bmal1^{musc}* hindlimb ischemia injury model. A, Principal component analysis. **B**, Venn diagrams illustrating the significantly up-regulated (left) and down-regulated (right) genes in FA-ligated vs intact TAs in Control (shown in red) and *Bmal1^{musc}* (shown in yellow) mice, respectively. **C&D**, Enrichment analysis of GOBP (**C**) and MSigDB_hallmark pathways (**D**). **E&F**, validation of gene expression by qPCR (**E**) and Western blot (**F**)., ** $p < 0.01$, *** $p < 0.001$ by multiple *t*-test.

Conclusions

The skeletal muscle expression of the circadian activator BMAL1 plays a critical role in determining the severity of ischemic muscle injury in a mouse PAD model. Together, our data lead us to hypothesize that induction of circadian dysfunction leads to impaired muscle reperfusion during hindlimb ischemia. This impairment is partially attributed to the reduction in the expression of HIF1α-targeted pro-angiogenic gene *Vegfα,* and the activation of Tgfβ signaling pathway. Furthermore, we hypothesize that reduced myostatin (Mstn) production results in a decrease in the number of newly formed myofibers, while promoting the formation of enlarged regenerating myofibers. However, such enlarged myofibers are reported to be associated with compromised muscle force production[7]. Therefore, we anticipate that circadian disruption may accelerate muscle dysfunction in PAD, and that methods to target muscle circadian function could be a promising strategy to preserve muscle health.

References

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