

Supplementary Material

Bmal1 downregulation worsens critical limb ischemia by promoting inflammation and impairing angiogenesis

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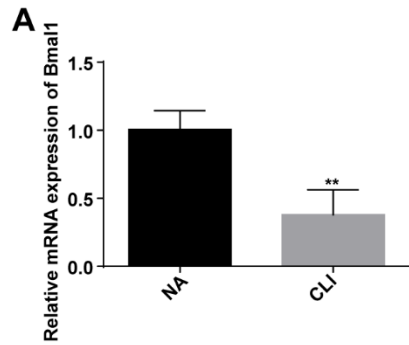
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Figure S1 to S6

Table S1 to S4

Figure S1**Figure S1 Bmal1 expression is decreased in the femoral artery of CLI patients**

(A) Relative mRNA expression of Bmal1 in the femoral artery of CLI patients and normal artery specimens detected by real-time PCR. Data presented as mean \pm SEM (n=3, unpaired t test). **p<0.01 CLI vs NA.

Figure S2

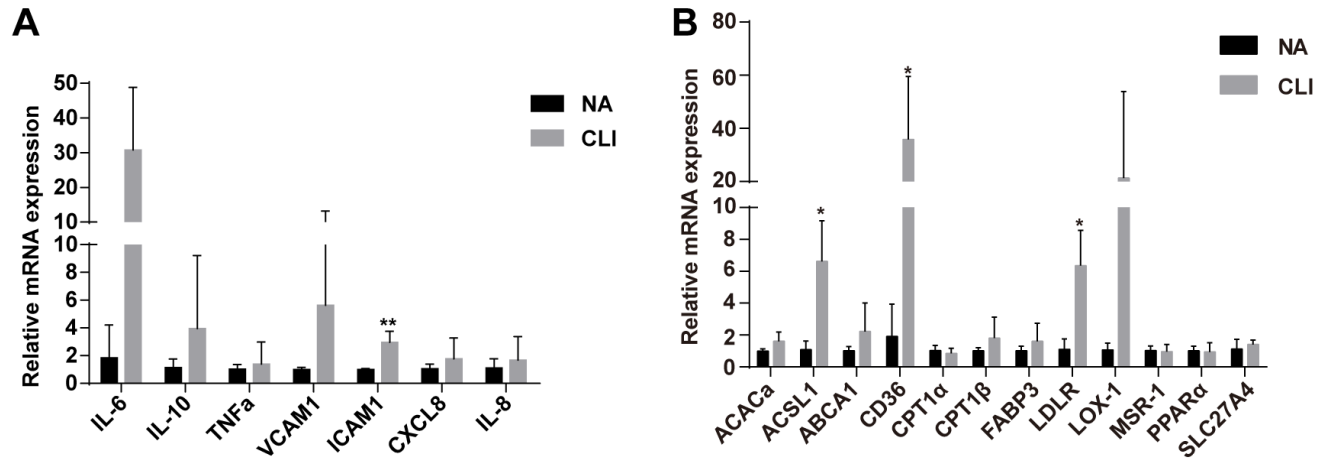


Figure S2 Expression of genes involved in inflammation and lipids uptake are increased in CLI femoral artery

(A-B) Relative mRNA expression of genes involved in inflammation and lipids metabolism in femoral artery. Data presented as mean ± SEM (n=3, unpaired t test). *p<0.05 and **p<0.01 CLI vs NA.

Figure S3

A

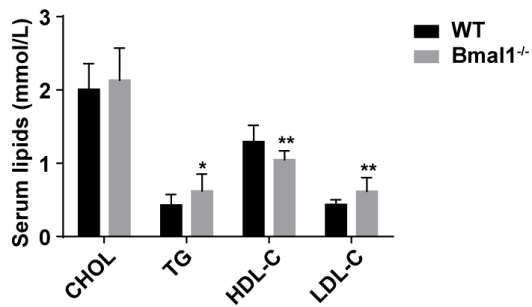


Figure S3 Serum lipids content of Bmal1^{-/-} mice and their littermates of WT mice

(A) Serum lipids content of Bmal1^{-/-} mice and their littermates of WT mice. Data presented as mean \pm SEM (n=5, unpaired t test). *p<0.05 and **p<0.01 Bmal1^{-/-} vs WT.

Figure S4

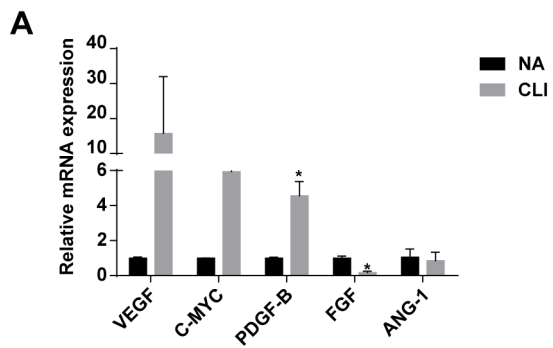


Figure S4 Angiogenesis is elevated in CLI patients

(A) Relative mRNA expression of genes involved in angiogenesis in the femoral artery of CLI patients and normal artery specimens detected by real-time PCR. Data presented as mean \pm SEM (n=3, unpaired t test). *p<0.05 CLI vs NA.

Figure S5

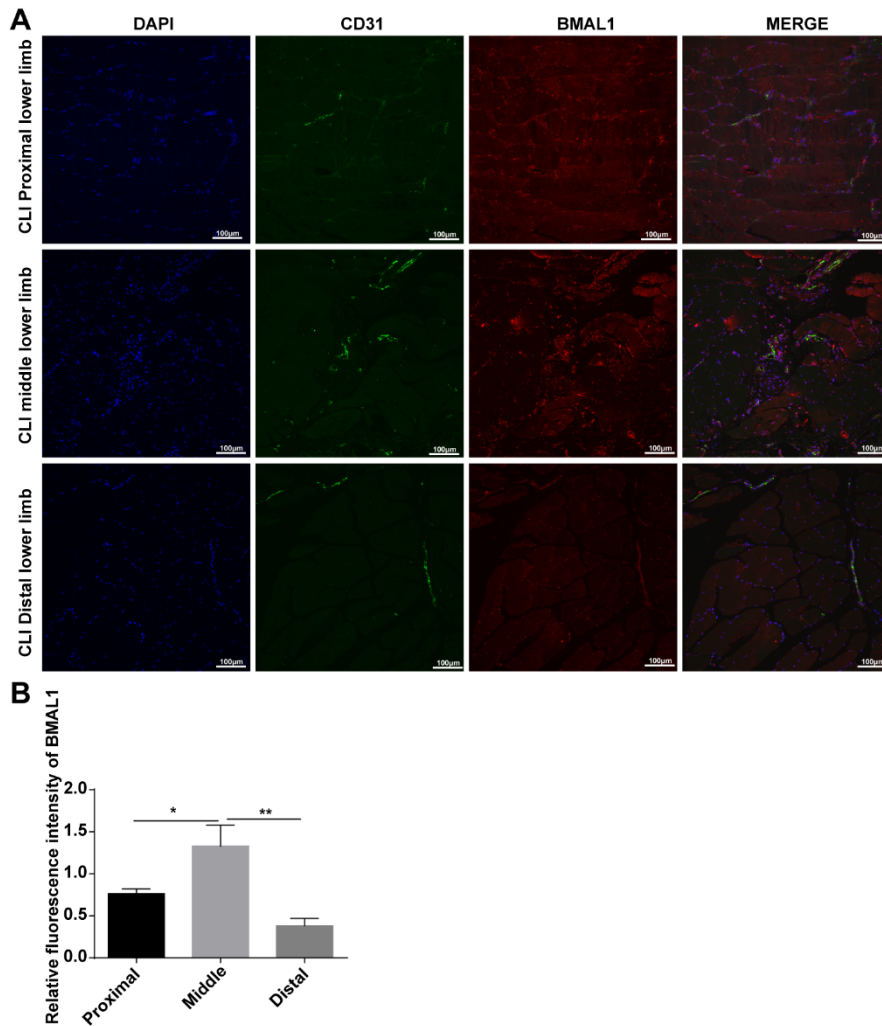
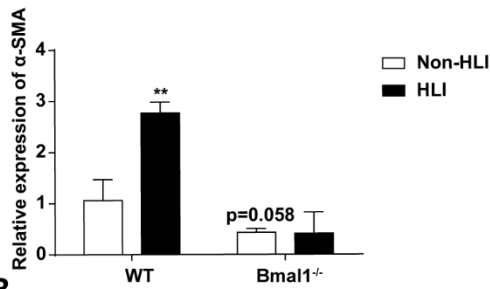


Figure S5 Bmal1 is down-regulated in the endothelial cells in the distal lower limb muscle of CLI patients.

(A-B) Immunofluorescence co-staining of BMAL1 and CD31 conducted in the lower limb muscle of CLI patients with specific antibodies. Relative fluorescence intensity of BAML1 was determined with image J. Data presented as mean \pm SEM (n=9, one way ANOVA with post-hoc Turkey test). *p<0.05 middle lower limb vs proximal lower limb; **p<0.01 distal lower limb vs middle lower limb.

Figure S6

A



B

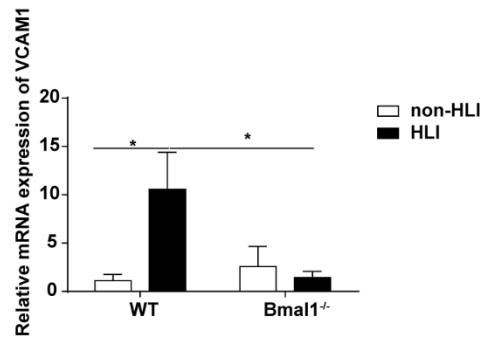
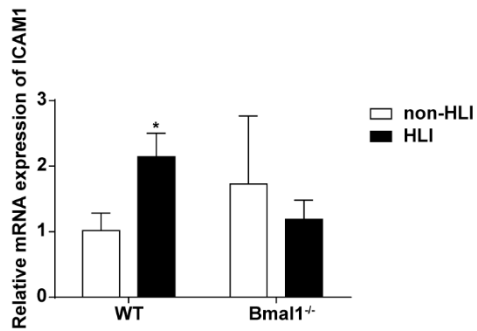
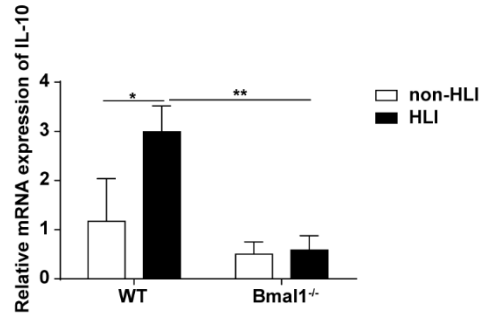
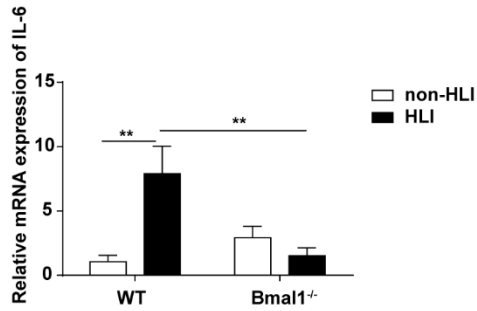


Figure S6 Angiogenesis is stimulated by HLI in WT mice

(A) Relative mRNA expression of α -SMA in HLI and Non-HLI mice. Data presented as mean \pm SEM (n=4, unpaired t test). **p<0.01 WT HLI vs WT Non-HLI.

(B) Relative mRNA expression of inflammatory factors in HLI and Non-HLI mice. Data presented as mean \pm SEM (n=4, unpaired t test). *p<0.05 and **p<0.01 WT HLI vs WT Non-HLI, *p<0.05 and **p<0.01 *Bmal1*^{-/-} HLI vs WT HLI.

Table S1 Primers used in the research

h-GAPDH	Forward: CCATCTTCCAGGAGCGAGATC
	Reverse: GCCTTCTCCATGGTGGTGAA
h-BMAL1	Forward: TGGATGAAGACAACGAACCA
	Reverse: TAGCTGTTGCCCTCTGGTCT
h-VEGF	Forward: ATCTTCAAGCCATCCTGTGTGC
	Reverse: CAAGGCCACAGGGATTTTC
h-CD31	Forward: AACAGTGTTGACATGAAGAGCC
	Reverse: TGTA AACAGCACGTCATCCTT
h-c-Myc	Forward: ACAGCGTCTGCTCCACCT
	Reverse: CCTCATCTTCTTGTTCCCTCCT
h-PDGFB	Forward: CTTTAAGAAGGCCACGGTGA
	Reverse: CTAGGCTCCAAGGGTCTCCT
h-MCP-1	Forward: CCCAGTCACCTGCTGTTAT
	Reverse: TGGAATCCTGAACCCACTTC
h-FGF	Forward: TGCCTCCAGGAATTACAAG
	Reverse: TATAAAAGCCCGTCGGTGTC
h-ANG-1	Forward: AGCGCCGAAGTCCAGAAAAC
	Reverse: TACTCTCACGACAGTTGCCAT
h-IL-6	Forward: GCTCCCTACTTCACAAGTCC

	Reverse: GCAGGTTTGCCGAGRAGATC
h-IL-10	Forward: GACTTTAAGGGTTACCTGGGTTG
	Reverse: TCACATGCGCCTTGATGTCTG
h-TNF α	Forward: CTGGGCAGGTCTACTTTGGG
	Reverse: CTGGAGGCCCCAGTTTGAAT
h-VCAM1	Forward: TCAGATTGGAGACTCAGTCATGT
	Reverse: ACTCCTCACCTTCCCGCTC
h-ICAM1	Forward: GGCCGGCCAGCTTATACAC
	Reverse: TAGACACTTGAGCTCGGGCA
h-CXCL8	Forward: TTTTGCCAAGGAGTGCTAAAGA
	Reverse: AACCTCTGCACCCAGTTTTTC
h-IL-8	Forward: CTCTCTTGGCAGCCTTCCTGATT
	Reverse: AACTTCTCCACAACCCTCTGCAC
h-ACACa	Forward: TCACACCTGAAGACCTTAAAGCC
	Reverse: AGCCCACTGCTTGTACTG
h-ACSL1	Forward: CTTCTGGTACGCCACGAGAC
	Reverse: GTCGCTGTCAAGTAGTGCG
h-ABCA1	Forward: ACCCACCTATGAACAACATGA
	Reverse: GAGTCGGGTAACGGAAACAGG
h-CD36	Forward: CGAAAGTCACTGCGACATGA
	Reverse: CCTTGGATGGAAGAACGAATC

h-CPT1 α	Forward: ATCAATCGGACTCTGGAAACGG
	Reverse: TCAGGGAGTAGCGCATGGT
h-CPT1 β	Forward: GAGGCCTCAATGACCAGAATGT
	Reverse: GTGGACTCGCTGGTACAGGAA
h-FABP3	Forward: CACTCACCCACGGCACTGCA
	Reverse: TCCCGGTCAGTGGCACCTGA
h-LDLR	Forward: ACGGCGTCTCTTCCTATGACA
	Reverse: CCCTTGGTATCCGCAACAGA
h-LOX-1	Forward: GCAGAAGAAGCTTCACAGGAGTCAG
	Reverse: TGGAGATTCAGATTCTGGTGGTGAAGT
h-MSR1	Forward: GCAGTGGGATCACTTTCACAA
	Reverse: AGCTGTCATTGAGCGAGCATC
h-PPAR α	Forward: ACTTATCCTGTGGTCCCCGG
	Reverse: CCGACAGAAAGGCACTTGTGA
h-SLC27A4	Forward: TGGACCCCTCGCTCAGCCTC
	Reverse: CAGCCCTGTGGTGCCGGATG
m-GAPDH	Forward: TGAAGGTCGGTGTGAACGG
	Reverse: CGTGAGTGGAGTCATACTGGAA
m-Bmal1	Forward: GGCTGTTTCAGCACATGAAAAC
	Reverse: GCTGCCCTGAGAATTAGGTGTT
m-Kc	Forward: AGCCACCCGCTCGCTTCTCT

	Reverse: TCGGTTTGGGTGCAGTGGGG
m-ACTA2	Forward: CCCAGACATCAGGGAGTAATGG
	Reverse: TCTATCGGATACTTCAGCGTCA
m-VEGF	Forward: GCACATAGAGAGAATGAGCTTCC
	Reverse: CTCCGCTCTGAACAAGGCT
m-CD31	Forward: GGTGCATGGCGTATCCAAG
	Reverse: TGGAGGTCTTATCTATCCTTCGC
m-IL-10	Forward: TCAAACAAAGGACCAGCTGGACAACATACT
	Reverse: TCTACCAGGTAAAACCTGGATCATTTC
m-IL-6	Forward: GCTTAATTACACATGTTCTCTGGGAAA
	Reverse: CAAGTGCATCATCGTTGTTCATAAC
m-VCAM-1	Forward: AGTTGGGGATTTCGGTTGTTTC
	Reverse: CATTCTTACCACCCCATTTG
m-ICAM-1	Forward: GTGGCGGGAAAGTTCCTG
	Reverse: CGTCTTGCAGGTCATCTTAGGAG
m-ACSL1	Forward: ATCTGGTGGAAACGAGGCAAG
	Reverse: TCCTTTGGGGTTGCCTGTAG
m-CPT1 β	Forward: CTGAGACACATCACCGTCTGGAA
	Reverse: CACCCCTAAGGATGCCATTCT
m-CD36	Forward: AGATGACGTGGCAAAGAACAG
	Reverse: CCTTGGCTAGATAACGAACTCTG

m-FABP3	Forward: GAA TAG AGT TCG ACG AGG TGA
	Reverse: GAA TAG AGT TCG ACG AGG TGA
m-LDLR	Forward: CTCCTGCATTCACGGTAGCC
	Reverse: CCCACTGTGACACTTGA ACTTG
m-MSR-1	Forward: TGAACGAGAGGATGCTGACTG
	Reverse: TGTCATTGAACGTGCGTCAAA
m-SLC27A4	Forward: ACTGTTCTCCAAGCTAGTGCT
	Reverse: GATGAAGACCCGGATGAAACG
VEGF-ChIP- -906/-725	Forward: AGCTCCACAACTTGGTGCCAAATT
	Reverse: CTGGAGTTGCTTCATGTACAGAGAG
VEGF-ChIP- -729/-574	Forward: TGGGCTCTCTGTACATGAAGCAACT
	Reverse: CGAAGACGCTGCTCGCTCCATTCAC
VEGF-ChIP- -382/-175	Forward: CTCTCCGCCTTCCCCTGCCCCCTTC
	Reverse: GACGCTCAGTGAAGCCTGGCCCGCA
IL10-ChIP- -1986/-1750	Forward: CTAACACAGACACAGCCCAGAAAAC
	Reverse: CAGGGAGTGTC AATGTAGAAACCTA
IL10-ChIP- -888/-600	Forward: AGGTTTCATTCTATGTGCTGGAGAT

	Reverse: CTTTACCCCGATTTCATTAGGATTC
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Table S2 Antibodies used in the research

Target antigen	Vendor or Source	Catalog #	Working concentration
BMAL1	CST	14020	For WB 0.5 $\mu\text{g/ml}$
BMAL1	Novas	NB100-2288	For IF 10 $\mu\text{g/ml}$ For ChIP 3 μg
CD31	Abcam	ab9498	For IF 1 $\mu\text{g/ml}$
VEGF	Proteintech	66828-1-Ig	For WB 0.4 $\mu\text{g/ml}$
GAPDH	Proteintech	60004-1-Ig	For WB 0.02 $\mu\text{g/ml}$
β -ACTIN	Proteintech	66009-1-Ig	For WB 0.0427 $\mu\text{g/ml}$

Table S3 primers used for luciferase reporter constructs

hVEGF-2000-luciferase	Forward: ggtaccgagctcttacgcgtgctagcaggaaagttagtggcttcct
	Reverse: ccaacagtaccggaatgccaagcttgcggacgctcagtgaagc
hVEGF-1216-luciferase	Forward: ggtaccgagctcttacgcgtgctagcgtgatagaagccttgcc
hVEGF-971-luciferase	Forward: ggtaccgagctcttacgcgtgctagcctccaacaggtcctctcc
hVEGF-785-luciferase	Forward: ggtaccgagctcttacgcgtgctagcagggtccagatggcacat
hVEGF-736-luciferase	Forward: ggtaccgagctcttacgcgtgctagcccagccctgggctctctgtacat
hVEGF-540-luciferase	Forward: ggtaccgagctcttacgcgtgctagcgagcagcagcgtcttcgagagt
hVEGF-47-luciferase	Forward: ggtaccgagctcttacgcgtgctagcagtcggctggtagcggggaggatcgc
	Reverse: Ccaacagtaccggaatgccaagcttaaccggatcaatgaatatcaa
hIL10-2000-luciferase	Forward: ggtaccgagctcttacgcgtgctagcgttagacctgcaggctaacaca
	Reverse: Ccaacagtaccggaatgccaagcttagaccttcacctctctgtccc
hIL10-1753-luciferase	Forward: ggtaccgagctcttacgcgtgctagcctgaagagttggaagagacacc
hIL10-1185-luciferase	Forward: ggtaccgagctcttacgcgtgctagcgtagagcaaacctcctcgccgcaa
hIL10-677-luciferase	Forward: ggtaccgagctcttacgcgtgctagcccaagcacagttgggggtgggggac

hIL10-324-luciferase	Forward: ggtaccgagctcttacgcgtgctagcaatgagaaccacagctgagggcc
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Table S4 Characteristics of patients

CLI patients(n=11)	Mean \pm SD or n (%)
Age (y; mean \pm SD)	73.36 \pm 8.15
Male gender	8 (72.7)
Hypertension	7 (63.6)
Smoker	3 (27.3)
Diabetes mellitus	6 (54.5)
Ischemic heart disease	4 (36.4)
Antiplatelet drugs	5 (45.5)
Lipid-lowering drugs	3 (27.3)
systolic blood pressure (mmHg)	128 \pm 15.40
Total cholesterol (mmol/L)	3.96 \pm 0.66

HDL cholesterol	0.90±0.21
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(mmol/L)

LDL cholesterol	2.37±0.59
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(mmol/L)

Triglycerides (mmol/L)	1.51±0.41
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Fasting glucose	7.83±2.92
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(mmol/L)

HDL, high-density lipoprotein; LDL, low-density lipoprotein