

**Supplementary Table 1. Primer sets used in this study**

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<b>Primer name</b>	<b>Cat</b>	<b>Base sequence (5'-3')</b>	
M-GAPDH	NM_008084.3	Sense	TGAAGGGTGGAGCCAAAAG
		Antisense	AGTCTTCTGGGTGGCAGTGAT
M-CollagenI	NM_007742	Sense	GGTATGCTTGATCTGTATCTGCCAC
		Antisense	GAGTTTGGTGATACGTATTCTTCCG
M-OCN	NM_007541.3	Sense	TTCTGCTCACTCTGCTGACCC
		Antisense	CTGATAGCTCGTCACAAGCAGG
M-Runx2	NM_001145920.2	Sense	CGCCACCACTCACTACCACAC
		Antisense	TGGATTTAATAGCGTGCTGCC
H-ACTIN	NM_001101	Sense	GTCCACCGCAAATGCTTCTA
		Antisense	TGCTGTCACCTTCACCGTTC
H-Areg	NM_001657	Sense	ATTGGACCTCAATGACACCTACT
		Antisense	GACAATATAGCCAGGTATTTGTGG

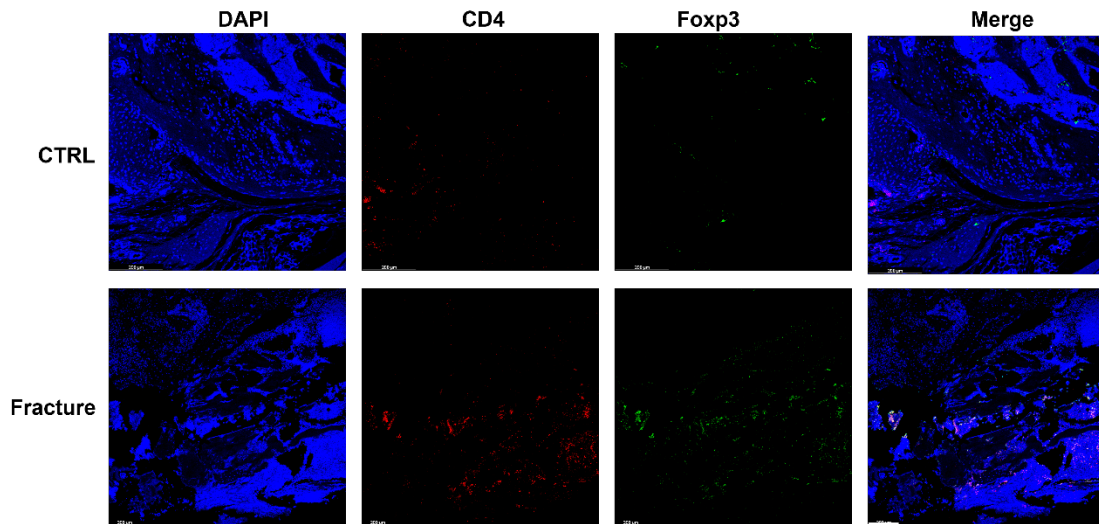
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**Supplementary Table 2. Baseline characteristics of patients for Treg expression in peripheral blood and bone callus**

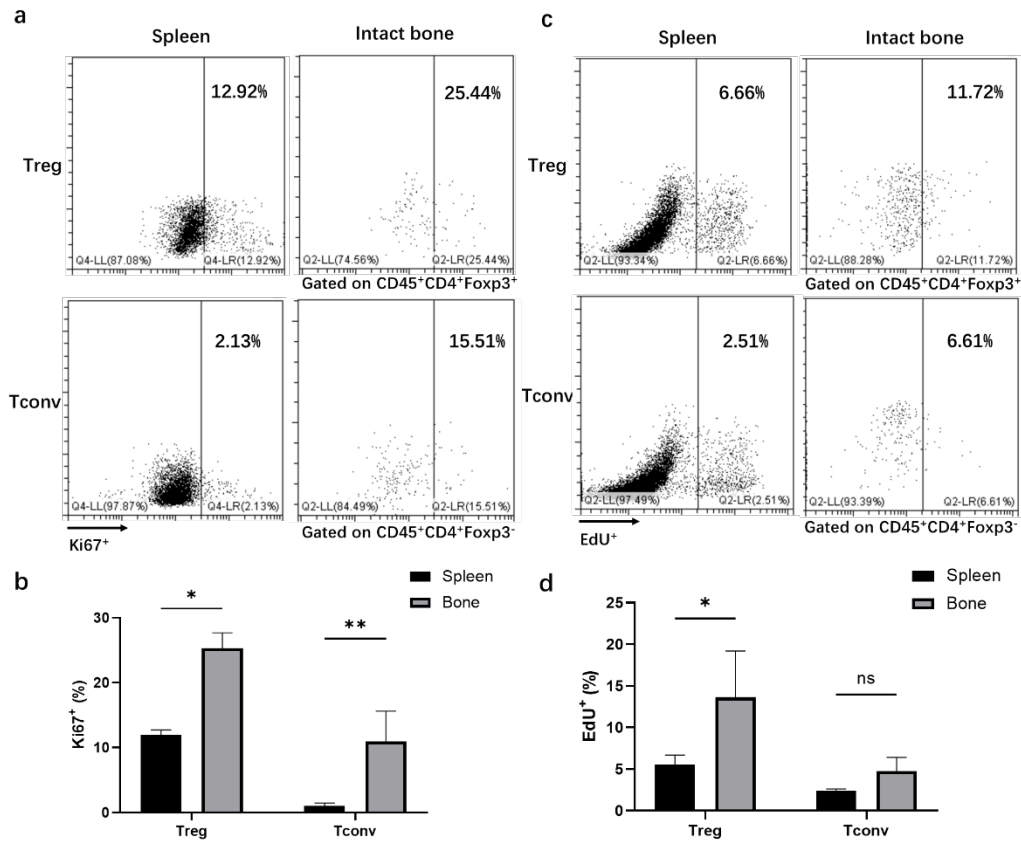
Characteristic		Peripheral Blood (n=15)	Bone callus (n=15)	Total (n=30)	<i>P</i>
Gender, n (%)	Male	7 (46.7%)	10 (66.7%)	17	0.224
	Female	8 (53.3%)	5 (33.3%)	13	
				46.5 (35.75,	
Age, Median (IQR)		39 (27, 50)	50 (42, 57)	52.00)	0.327
Body weight, Median (IQR)		61 (58, 74)	65 (60, 75)	63 (59, 75)	0.339
Days after fracture, Median (IQR)		7 (2, 8)	5 (5, 7)	6 (4, 8)	0.695
WBC, Median (IQR)		6.87 (5.98, 8.32)	6.65 (5.01, 8.67)	6.71 (5.88, 8.41)	0.361
Monocytes, Median (IQR)		0.46 (0.36, 0.73)	0.52 (0.43, 0.58)	0.48 (0.40, 0.65)	0.242
Lymphocytes, Median (IQR)		1.50 (1.06, 1.70)	1.40 (0.80, 2.00)	1.42 (1.02, 1.72)	0.008

**Supplementary Table 3. Baseline characteristics of patients for amphiregulin expression in Tregs**

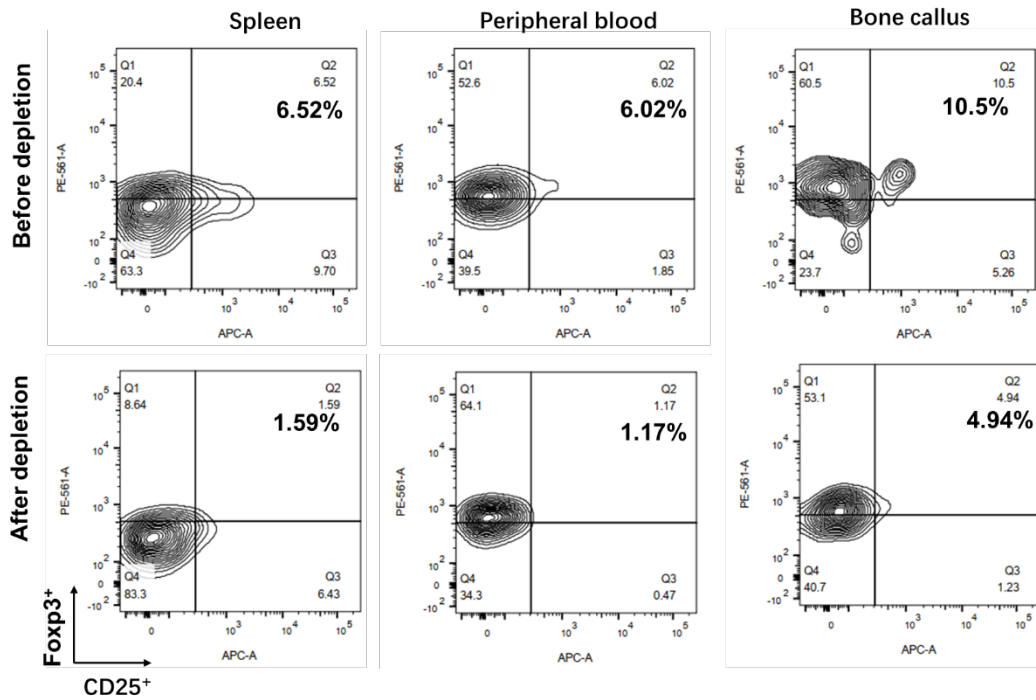
Characteristic		Peripheral Blood (n=8)	Bone callus (n=8)	Total (n=16)	<i>P</i>
Gender, n (%)	Male	6 (75%)	5 (62.5%)	11 (68.8%)	0.334
	Female	2 (25%)	3 (37.5%)	5 (31.2%)	
Age, Median (IQR)		41.5 (37.8, 46.3)	45.0 (35.5, 50.8)	41.5 (37.0, 49.8)	0.373
Body weight, Median (IQR)		71.0 (57.0, 78.5)	57.5 (54.1, 67.5)	60.0 (54.6, 73.5)	0.195
Days after fracture, Median (IQR)		4 (2.25, 6.5)	5 (5, 6)	5 (3, 6)	0.399
WBC, Median (IQR)		7.41 (6.89, 7.60)	6.97 (6.34, 8.05)	7.24 (6.51, 7.62)	0.496
Monocytes, Median (IQR)		0.67 (0.53, 0.70)	0.70 (0.48, 0.70)	0.70 (0.53, 0.70)	0.132
Lymphocytes, Median (IQR)		1.97 (1.55, 2.67)	1.70 (1.23, 1.81)	1.75 (1.53, 2.34)	0.115



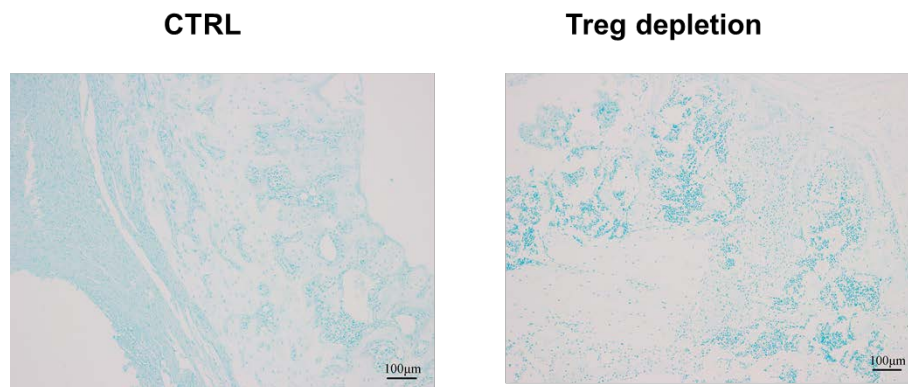
**Supplementary Figure 1.** Immunofluorescence images of Tregs in bone. The normal bone was used as a control. The fractured femur was dissected 5 days post fracture and conducted with decalcification and immunofluorescence staining. Red fluorescence represented CD4 (ab183685, Abcam) while the green fluorescence represented Foxp3 (sc-53879, Santa). Scale bar = 200  $\mu\text{m}$ .



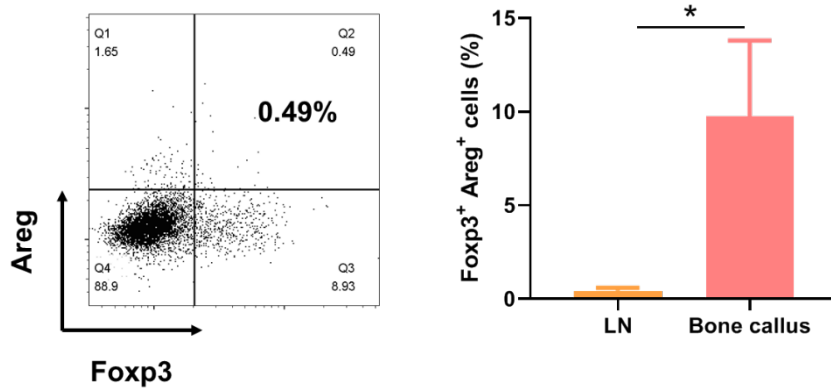
**Supplementary Figure 2.** **a** FCM graphs of Ki67 expression in spleen and intact bone Tregs and Tconvs. Numbers indicate the proportion of cells in the frame. **b** Quantitative analysis of Ki67 in spleen and intact bone Treg and Tconv cell subsets (n = 4). **c** Flow cytometry graphs of EdU expression in spleen and intact bone Tregs and Tconvs. Numbers indicated the proportion of cells in the frame. 1 mg EdU were intraperitoneally injected to mice and FCM analysis was performed on 24 h post EdU administration. **d** Quantitative analysis of EdU in spleen and intact bone Tregs and Tconvs (n = 4). Data are shown as means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ .



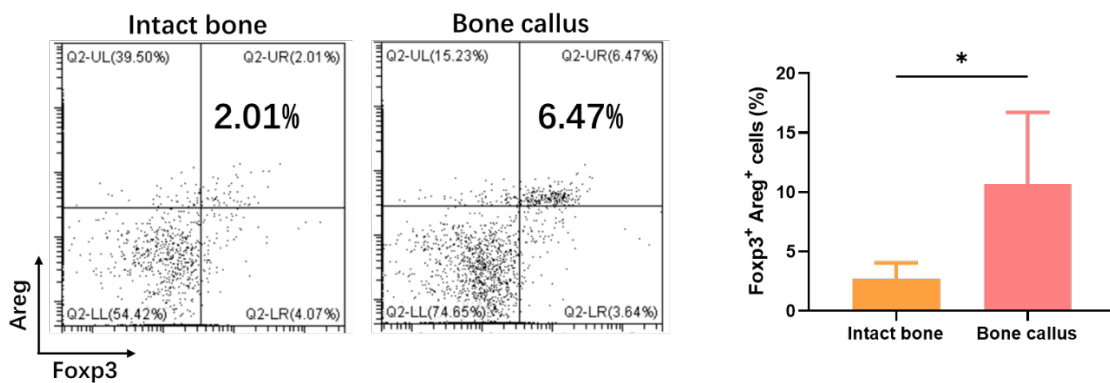
**Supplementary Figure 3.** The proportions of Tregs in spleen, peripheral blood and bone callus were detected by flow cytometry before and after Treg depletion. 200  $\mu\text{g}$  anti-CD25 mAb or isotype control was injected to C57BL/6 mice intraperitoneally 3 days before and 3, 4 days after fracture to manipulate Treg levels.



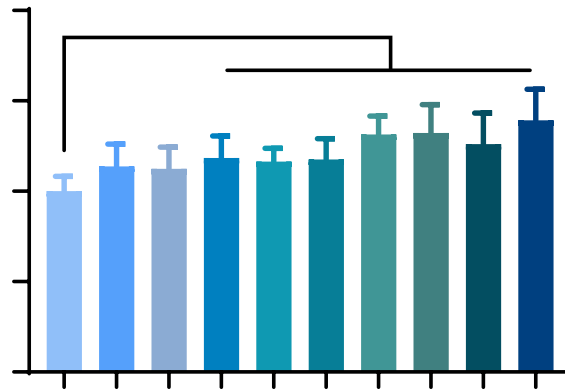
**Supplementary Figure 4.** The representative Alcian-blue staining images of bone callus on day 21 post fracture in Treg depletion group and control group. The fractured femurs in control and Treg depleted groups were dissected and subjected to fixation by 4% paraformaldehyde, decalcification by 5% nitric acid working solution, and Alcian-blue staining, scale bar = 100  $\mu\text{m}$ .



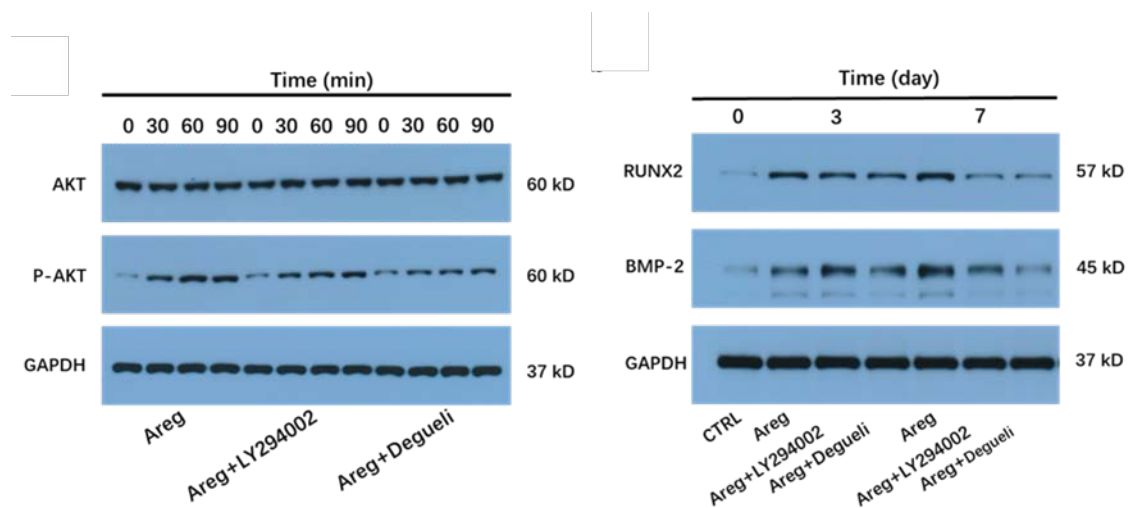
**Supplementary Figure 5.** The expression of amphiregulin in non-draining lymph node on day 5 post fracture (n = 4). The mesenteric lymph nodes were away from fractured femur and used as non-draining lymph node. Amphiregulin was abbreviated as Areg. Data are shown as means  $\pm$  SD. \* $P < 0.05$ .



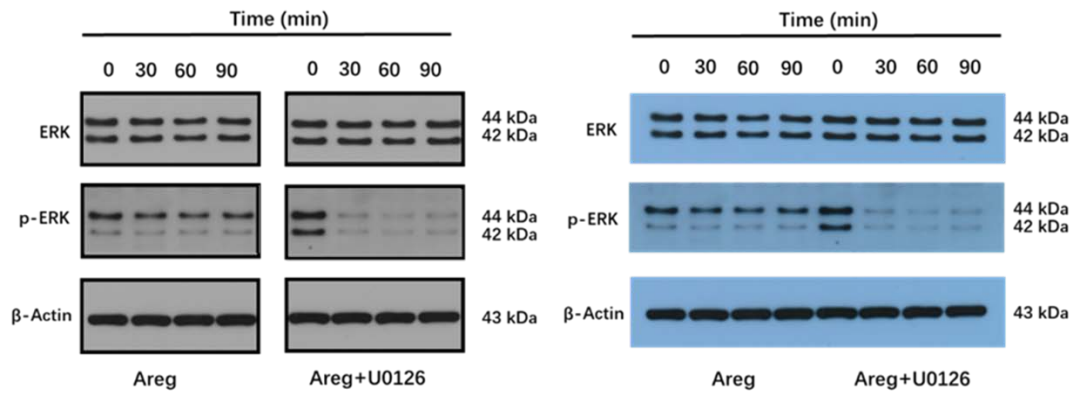
**Supplementary Figure 6.** The expression of amphiregulin in bone callus and intact bone on day 5 post fracture (n = 4). Data are shown as means  $\pm$  SD. \* $P < 0.05$ .



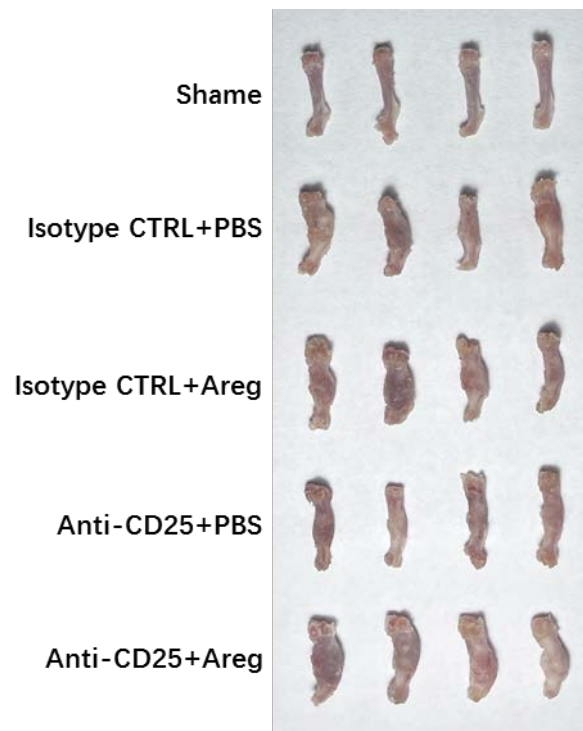
**Supplementary Figure 7.** Different concentrations of amphiregulin promoted the proliferation of BMSCs tested by CCK-8 assay after co-incubation for 48 hours (n = 5). Amphiregulin was abbreviated as Areg. Data are shown as means  $\pm$  SD. \* $P < 0.05$ .



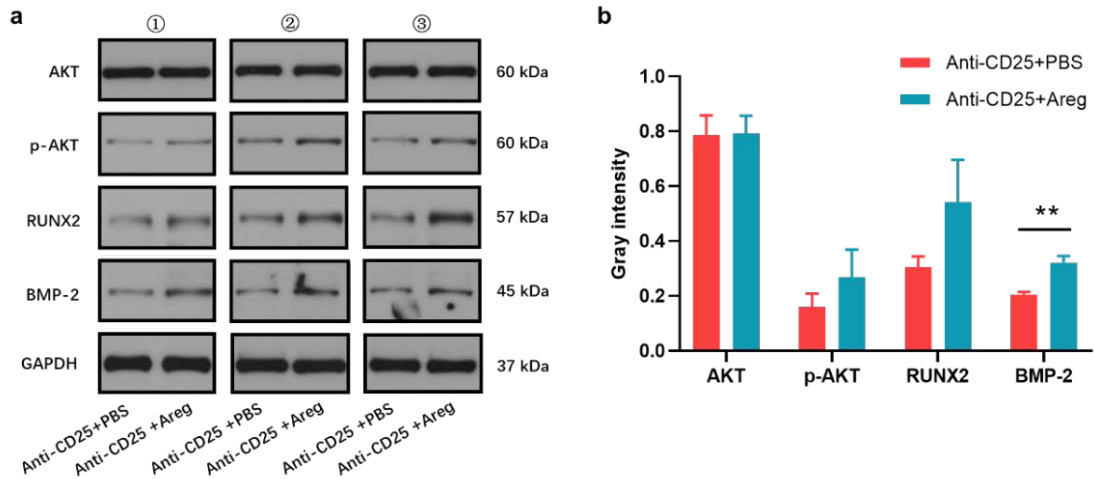
**Supplementary Figure 8.** The original western blots of Fig. 6G and Fig. 6H



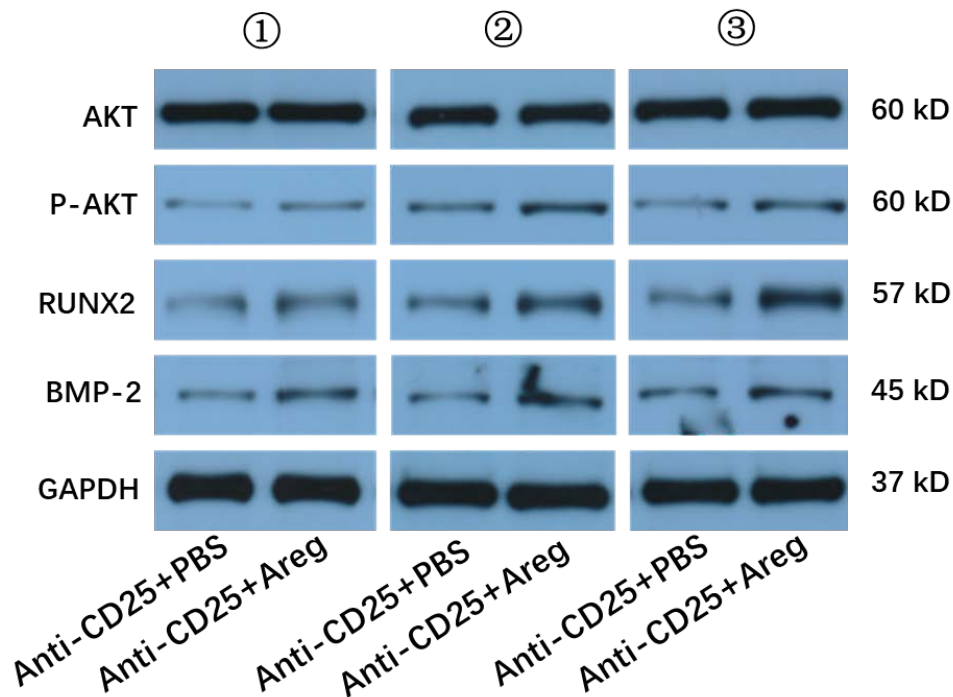
**Supplementary Figure 9.** Protein levels of ERK and p-ERK in BMSCs treated with amphiregulin with or without ERK inhibitor U0126 at different time points (0, 30, 60, and 90 min) and the original western blots.



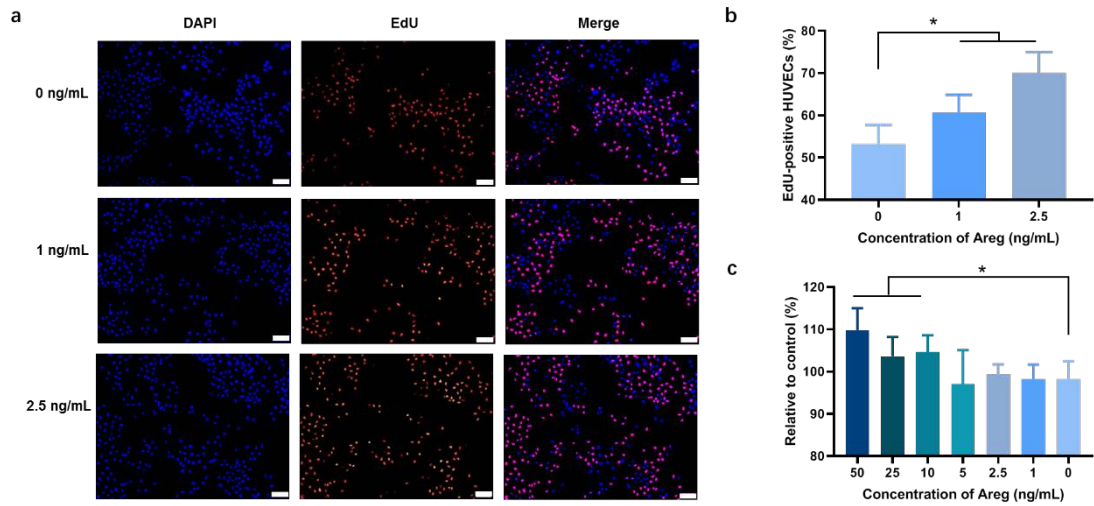
**Supplementary Figure 10.** Photos of femurs obtained on day 21 after fracture (n = 4). The mice were sacrificed and femurs were dissected. The muscle tissue was removed by medical gauze as possible, the femurs with callus were directly photographed by camera.



**Supplementary Figure 11. Amphiregulin accelerated murine femoral fracture healing.** **a** Protein expression of AKT, p-AKT, RUNX2 and BMP-2 shown by Western blotting in skeletal muscle. **b** Quantitative analysis of **a** (n = 3). Data are shown as means ± SD. \*\*P < 0.01.



**Supplementary Figure 12.** The original western blots of Supplementary Figure.11a.



**Supplementary Figure 13. The role of amphiregulin on HUVECs.** **a** Treated with different concentration of Areg from 0 to 2.5 ng/mL, EdU assay was performed in 48 hours. Scale bar = 200  $\mu$ m. **b** Quantification analysis of percentage of EdU-positive cells (n = 5). **c** Different concentration of Areg promoted the proliferation of BMSCs tested by CCK-8 assay in 48 hours (n = 5). Data are shown as means  $\pm$  SD. \* $P < 0.05$ .