

# Systemic administration of an ApoE-neutralizing antibody improves aged bone fracture healing

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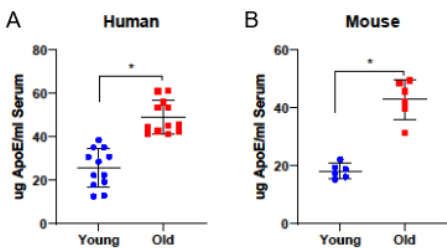
**Introduction:** Bone fracture leads to several complications due to delayed union or nonunion in older adults. Importantly, there is limited knowledge regarding manipulation of circulating factors to modulate bone fracture healing in the elderly. Apolipoprotein E (ApoE) is a circulating protein primarily produced by the liver to regulate lipid transportation and metabolism. In this study, we observed an increase in ApoE level with aging, and we found improved healing process when we inhibited its production from hepatocytes. Furthermore, we investigated the mechanism of ApoE inhibition on osteoblast differentiation to suppress bone fracture healing. Finally, we used a neutralizing antibody to sequester ApoE which enhanced fracture healing in aged mice.

**Methods:** *Mice* - All animal experiments were approved by the Duke IACUC and IRB. ApoE floxed mice and Albumin-Cre mice were purchased from the Jackson laboratory. *Analysis of Fracture Callus* - Midshaft tibial fracture was induced in 24-month-old mice. After 21 days of healing, the calluses were dissected, fixed and scanned using a Scanco vivaCT 80 (resolution=8µm). Calluses were decalcified, paraffin embedded, cut, and stained. *Cell Culture* - BMSCs from the bone marrow of femurs and tibiae of unfractured mice were isolated based on the adherence to plastic tissue culture flasks. For osteoblast differentiation, BMSCs were induced in osteogenic medium with or without recombinant ApoE (100ng/mL). *Gene Expression Analysis* - RNA was extracted using TRIzol Reagent and reversed transcribed to cDNA. Bulk RNA sequencing was performed based on Illumina's standard protocol. Realtime PCR was performed using the SYBR Green PCR Master Mix with normalization to GAPDH. Proteins were extracted with RIPA lysis buffer and separated using SDS-PAGE gels and transferred to a PVDF membrane. Next, the PVDF membrane was incubated with specific antibodies to target the proteins of interest. *Neutralizing Antibody Treatment* - anti-ApoE neutralizing antibody HJ6.3 or isotype IgG was intraperitoneally injected 3 days post fracture surgery. Fracture calluses were harvested for subsequent analysis after 21 days post injury.

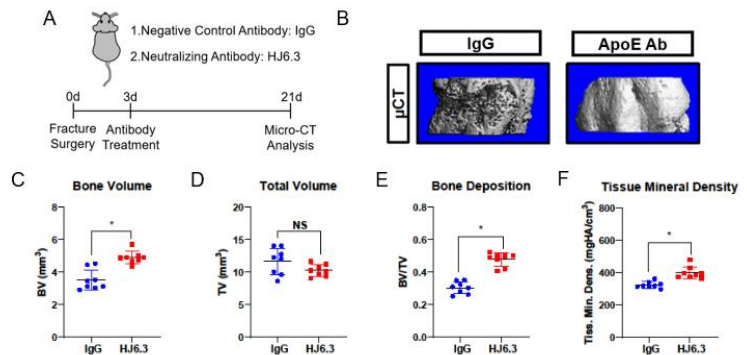
**Results:** We collected serum samples from young and aged patients, as well as from young and aged mice, to measure ApoE concentration by ELISA. We found that aged patients and mice had significantly higher levels of serum ApoE compared with young patients and mice (Fig 1). We next chose to investigate the impact of circulating ApoE on bone fracture healing in aged mice. As the liver is the primary (>90%) source of serum ApoE, we bred Albumin-Cre mice to ApoE floxed mice to generate ApoE<sup>Alb</sup> mice which deleted ApoE expression in the hepatocytes. Indeed, ELISA analysis revealed that the ApoE<sup>Alb</sup> mice exhibited a substantial 90% reduction in plasma ApoE concentration. Subsequently, the 21-day fracture callus of ApoE<sup>Alb</sup> mice contained 30% more bone tissue compared with littermate controls. To understand whether ApoE affects osteoblast differentiation, BMSCs were cultured in osteogenic medium with vehicle or recombinant ApoE (100ng/ml). Cell staining experiments and RT-PCR test revealed decreased mineralization and a decrease in osteogenic markers following ApoE treatment. To better understand the mechanism by which ApoE inhibits osteogenic activity, we performed bulk RNA sequencing (RNA-seq) to compare differentiated cells treated or untreated with recombinant ApoE. GO analysis revealed that recombinant ApoE affected several biological processes including Ossification, Bone development, Osteoblast differentiation and Bone mineralization. KEGG analysis and GSEA analysis showed that the two most enriched pathways among the differentially expressed genes were the Wnt signaling pathway and Hippo signaling pathway. Western blot and immunostaining demonstrated protein levels of β-catenin (a key effector of Wnt signaling) and YAP (a key effector of Hippo signaling) were decreased by ApoE treatment. The Wnt signaling pathway and the Hippo signaling pathway exhibit strong crosstalk and can mutually influence each other. However, by using gene modulation methods we were able to prove that β-catenin serves as the primary mechanism through which ApoE inhibits osteoblast differentiation. Finally, to determine the efficacy of targeting ApoE to improve aged fracture healing, we administered ApoE neutralizing antibody (HJ6.3) at 3-dpf using systemic injection (IP) to aged mice with bone fracture. Fracture calluses from mice treated with ApoE neutralizing antibody exhibited elevated levels of bone volume, bone deposition and tissue mineral density compared to IgG (Fig 2).

**Discussion:** ApoE acts as an aging factor, impairing bone healing with age. Using ApoE<sup>Alb</sup> mice we were able to reduce ApoE concentration in circulation which improved aged fracture healing. Most importantly, administration of ApoE-neutralizing antibody enhanced aged bone fracture healing.

**Significance/Clinical Relevance:** Our work validates ApoE as a therapeutic target to promote aged bone regeneration. Indeed, in our work here neutralization of circulating ApoE increased bone deposition within the fracture callus and accelerated bone repair. Thus, ApoE could be regarded as both a diagnostic biomarker for assessing fracture healing rate in older adults and a therapeutic target to improve fracture healing.



**Figure 1 – Circulating levels of ApoE in increase with age.** Serum collected from A) humans (young, 35-45 years; old 75-85 years) and from B) mice (young, 4 months; old, 24 months) was assessed for total ApoE protein using ELISA. Young patients, n = 12; old patients, n = 12; young mice, n = 7; old mice, n = 7. Data are expressed as mean ± 95% confidence interval. \*Statistically significant, p<0.05.



**Figure 2 – ApoE neutralizing antibody enhances fracture healing in aged mice.** A) Schematic diagram, 24-month-old mice underwent tibial fracture surgery. Mice were intraperitoneally injected with anti-ApoE neutralizing antibody HJ6.3 or the negative control antibody three days post fracture surgery. B) 21-day fracture calluses were assessed using µCT for C) bone volume, D) total volume, E) bone deposition within the callus and F) for tissue mineral density. n=6. Data are expressed as mean ± 95% confidence interval. \*Statistically significant, p<0.05.