Augmenting SOD2 Levels in *Apoe*$^{-/-}$ Mice with Peptide-mRNA Nanoparticles Preserves Atherosclerotic Plaque Stability in Advanced Atherosclerosis

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ABSTRACT

Erosion or rupture of the advanced atherosclerotic plaque is the leading cause of atherosclerosis complications such as acute coronary syndrome and stroke. Atherosclerotic plaques in aging hypercholesterolemic mice have decreased SOD2 levels and increased mitochondrial oxidative stress, which leads to cell apoptosis, necrotic core expansion, and fibrous cap thinning and rupture. We hypothesized that increasing SOD2 expression in advanced atherosclerotic plaque cells in mice using mRNA polyplex would protect mitochondria from oxidative damage and preserve plaque stability.

Macrophages isolated from *Sod2*$^{+/-}$ mice treated with amphipathic cationic peptide p5RHH:*Sod2* mRNA polyplexes had markedly increased levels of *Sod2* mRNA and mitochondrial SOD2 ($p<0.01$), higher SOD activity, which resulted in lower levels of mitochondrial and cellular reactive oxygen species (ROS) and improved mitochondrial function. RNA-Seq analysis showed that macrophages treated with *Sod2* nanoplexes had broadly decreased inflammatory cytokines and immune activation pathways. In *Apoe*$^{-/-}$ mice, atherosclerotic lesions with vulnerable plaque features were induced in the carotid artery by placing a perivascular collar over the carotid artery and 4-months Western diet. Mice were treated for 4 weeks with saline or with 0.5 mg/kg p5RHH:*Sod2* mRNA polyplexes which selectively accumulated in atherosclerotic lesion macrophages. The carotid plaques of the mice treated with *Sod2* mRNA differed significantly from the plaques of the control *Apoe*$^{-/-}$ mice by higher SOD2 expression, lower mitochondrial ROS and oxidative DNA damage, and reduced expression of CCL2, IL1, IL6, MMP 2 and 9 ($p<0.01$). *Sod2* polyplexes-treated mouse plaques exhibited 25% fewer inflammatory macrophages ($p<0.01$) and 98% more smooth muscle cells ($p<0.001$) with a reduced lipid core, preserved fibromuscular cap, and decreased plaque vulnerability index ($p<0.05$).

The results suggest that selective overexpression of *Sod2* in plaque macrophages using nanoplexes reduces mitochondrial stress and preserves plaque stability without adverse effects. Using nanoparticle-based mRNA therapeutics to modulate plaque morphology has great potential in the prevention and treatment of atherosclerosis complications.

BACKGROUND

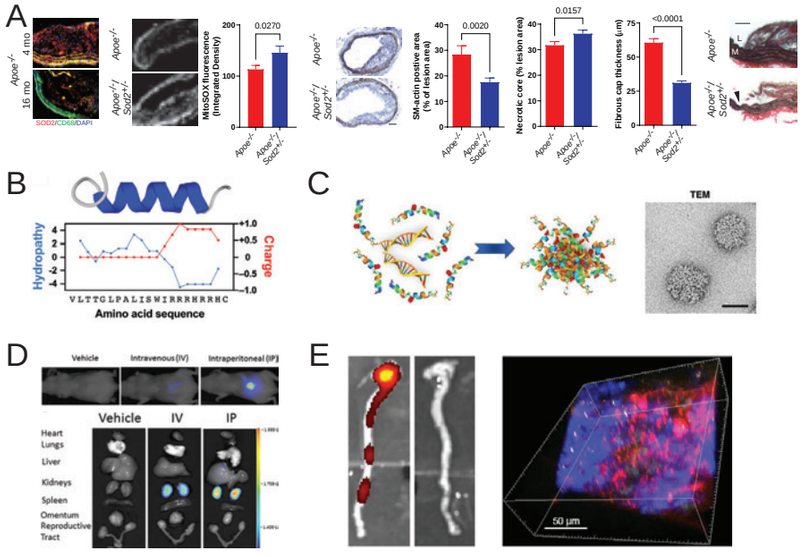

Figure 1. **Decreased SOD2 levels underly the unstable plaque phenotype (A).** Image credit: *J Am Heart Assoc* 6(11):e006775
p5RHH targeted delivery in atherosclerosis. (B) Image: *Mol Ther* 29(5):1744-1757. (C) Image: *Biomater Adv* 139:213009. (D) Image: *Sci Rep* 9, 4762. (E) Image: *Int J Nanomedicine* 6;13:5187-5205

RESULTS

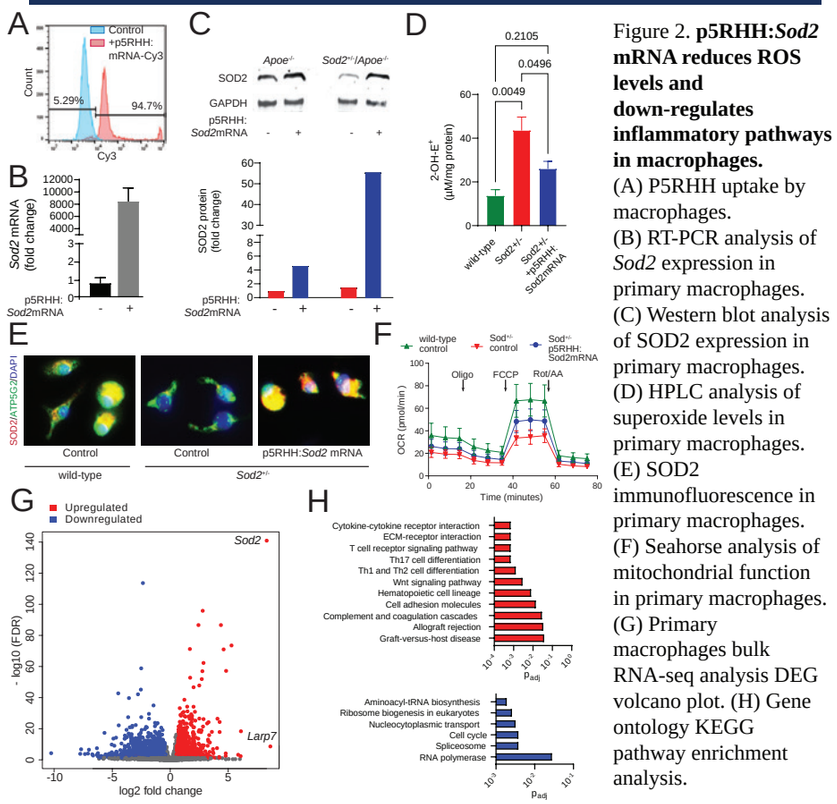

Figure 2. **p5RHH:*Sod2* mRNA reduces ROS levels and down-regulates inflammatory pathways in macrophages.** (A) P5RHH uptake by macrophages. (B) RT-PCR analysis of *Sod2* expression in primary macrophages. (C) Western blot analysis of SOD2 expression in primary macrophages. (D) HPLC analysis of superoxide levels in primary macrophages. (E) SOD2 immunofluorescence in primary macrophages. (F) Seahorse analysis of mitochondrial function in primary macrophages. (G) Primary macrophages bulk RNA-seq analysis DEG volcano plot. (H) Gene ontology KEGG pathway enrichment analysis.

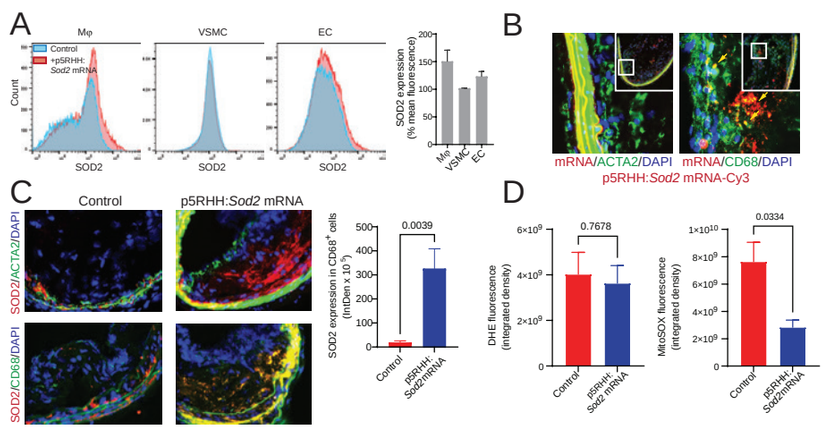

Figure 3. **p5RHH:*Sod2* mRNA treatment in *Apoe*$^{-/-}$ mice with advanced atherosclerotic lesions.** (A) Flow cytometry analysis of SOD2 expression. (B) Fluorescence images of carotid artery atherosclerotic lesions from p5RHH:*Sod2* mRNA-Cy3 treated mice. (C) Immunofluorescence microscopy analysis of SOD2 expression. (D) Cellular (DHE) and mitochondrial (MitoSOX) superoxide generation in carotid artery atherosclerotic lesions from control and p5RHH:*Sod2* mRNA treated mice.

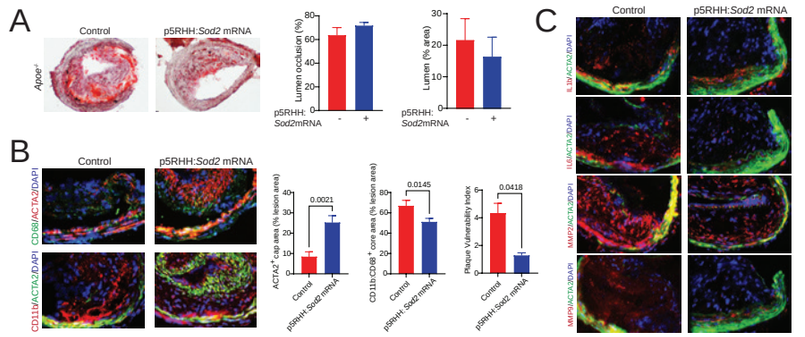

Figure 4. **Plaque vulnerability and inflammation are reduced after p5RHH:*Sod2* mRNA treatment in *Apoe*$^{-/-}$ mice with advanced atherosclerotic lesions.** (A) Oil red O staining of carotid artery sections. (B) Immunofluorescence staining for macrophage and SMC markers. (C) Inflammatory and remodeling markers expression in carotid artery lesions.

Figure 6. **Pro-inflammatory plaque macrophage populations were depleted after p5RHH:*Sod2* mRNA treatment in *Apoe*$^{-/-}$ mice with advanced atherosclerotic lesions.** (A) Flow cytometry analysis of plaque cells phenotype. (B) t-SNE clustering of spectral flow cytometry data from carotid artery atherosclerotic lesions. (C) Heat-map representation of macrophage markers relative expression for each distinct cluster.

CONCLUSIONS

- Treatment with p5RHH nanoplexes selectively targeted to plaque macrophages increases SOD2 levels
- SOD2 augmentation modulates macrophage inflammatory phenotype through mitochondrial oxidative stress reduction and improved function
- p5RHH:*Sod2* mRNA treatment in *Apoe*$^{-/-}$ mice with advanced atherosclerosis reduces inflammation and preserves plaque stability