ONLINE SUPPLEMENT

CHBPR: AT_{1A} RECEPTORS IN VASCULAR SMOOTH MUSCLE CELLS DO NOT INFLUENCE AORTIC REMODELING IN HYPERTENSION

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SUPPLEMENT MATERIALS AND METHODS

Generation of experimental animals- A mouse line with a conditional *Agtr1a* allele was generated using homologous recombination in embryonic stem cells.[1] Two *loxp* sites were placed in positions flanking exon 3 of the *Agtr1a* gene (Supplemental Figure 1A), which contains the entire protein encoding sequence, 3' untranslated region, and poly-adenylation signal, such that *Cre*-mediated recombination renders the gene completely non-functional. The targeting vector was introduced into ES cells by electroporation as described previously[2] and the transfected ES cells were cultured in media containing G418 for positive selection. Correctly modified ES cells were identified by Southern analysis. Prior to injection of the correctly modified ES cell lines into blastocysts, the neomycin cassette was removed by transient transfection with plasmid vectors expressing Flp recombinase (pCAGGS-Flpe-IRES) and CMV-GFP. GFP-expressing cells were identified, sorted by FACS, colonies plated, expanded and split into duplicate 12-well plates. Clones that had undergone the appropriate Flp-mediated recombination with removal of the neomycin cassette were identified by Southern analysis (supplemental Figure 1D). Appropriately modified ES cell lines were then expanded and injected into blastocysts to generate chimeras. Germ-line transmitting chimeras were bred with wild-type C57Bl/6 mice to generate inbred C57Bl/6 *Agtr1a*$^{flox/+}$ mice. In order to delete AT_{1A}-receptors from VSMCs, we used a *Sm22*α*-Cre* transgenic mouse wherein *Cre* expression is driven by the Sm22α promoter.[3]

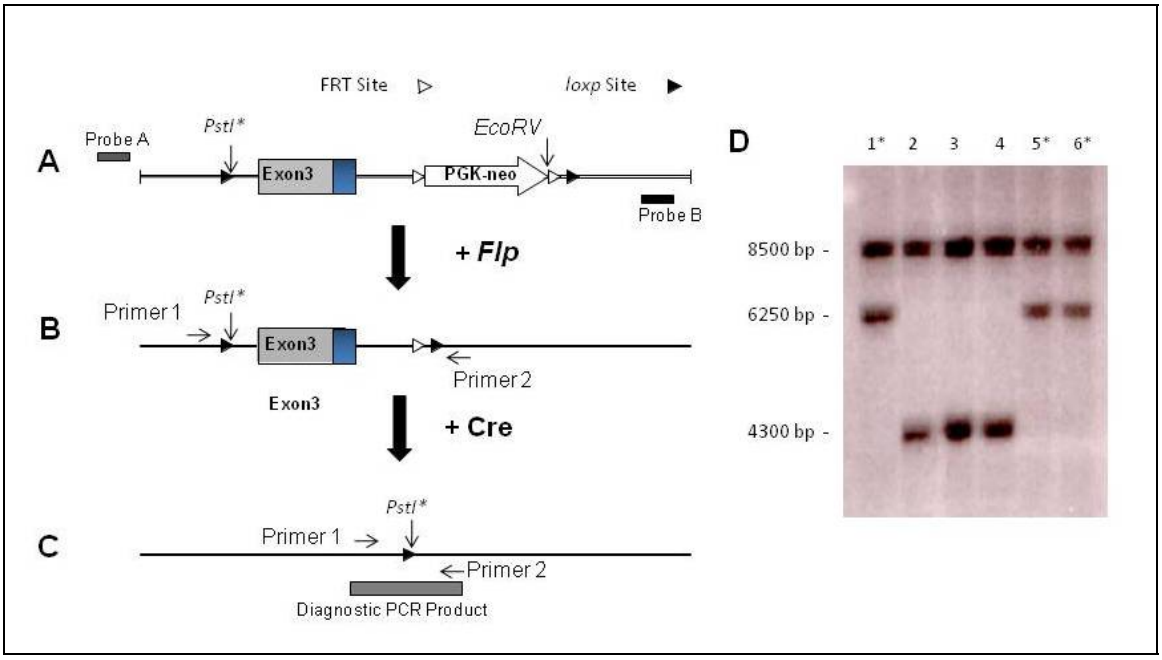
X-galactosidase staining- In order to verify appropriate, tissue-specific expression of *Cre* recombinase, SM22α-*Cre* transgenic mice were inter-crossed with *Rosa26-lacZ* reporter mice. Tissues, except mesentery, were harvested and fixed in formalin and sectioned at 10μm. Mesentery was cleaned in xylenes and mounted with Feramount. Tissues were stained for β-galactosidase activity using a *LacZ* Staining Kit (InvivoGen) according to manufacturer's instructions. As shown in Supplemental Figure 2A-C, X-gal stained tissues from *SM22α-Cre*$^{+}$*-Rosa26-lacZ*$^{+}$ mice demonstrated robust *LacZ*-staining in smooth muscle cells in the aorta, as well as mesenteric and medium-sized branches of the renal artery, compared to the absence of any specific staining in *Cre*$^{-}$ controls or in non-smooth muscle cell lineages (supplemental Figure 2D-F).

REFERENCES

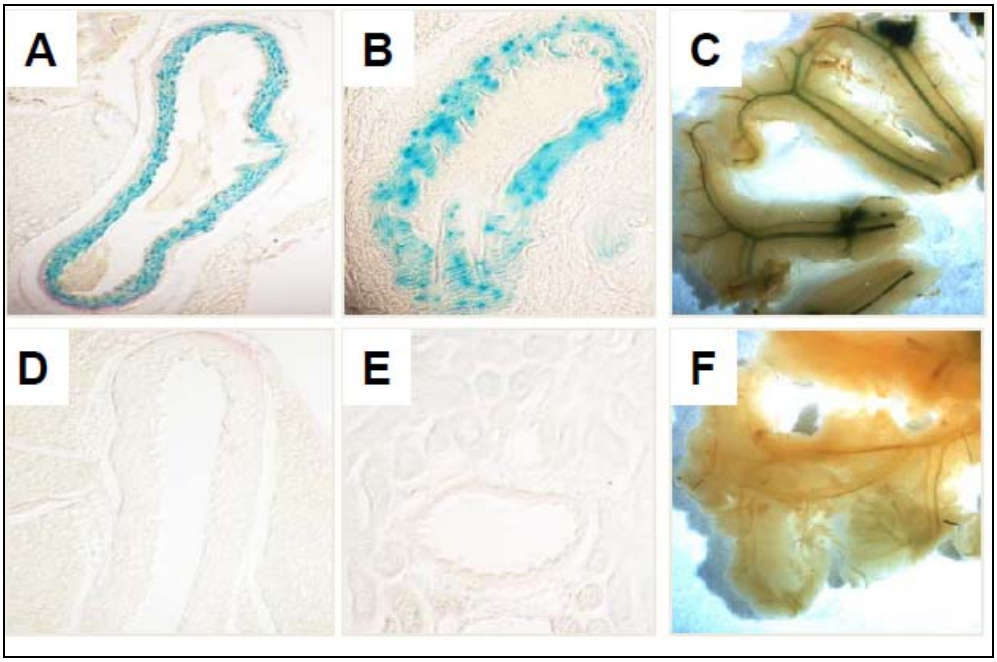
1. Kim HS, Smithies O. Recombinant fragment assay for gene targetting based on the polymerase chain reaction. *Nucleic Acids Res*. 1988;16:8887-8903.

2. Thomas DW, Mannon RB, Mannon PJ, Latour A, Oliver JA, Hoffman M, Smithies O, Koller BH, Coffman TM. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane a2. *J Clin Invest.* 1998;102:1994-2001.

3. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. Lrp: Role in vascular wall integrity and protection from atherosclerosis. *Science*. 2003;300:329-332.



Supplemental Figure S1. Generation of mice with a conditional *Agtr1a* allele. (A) Targeting vector containing PGK-Neo cassette for positive selection with *loxp* sites engineered in positions flanking exon 3 of the *Agtr1a* gene (black triangles). FRT sites are depicted by open triangles and novel *PstI* and *EcoRV* recognition sites are shown. Probes A and B were used for Southern analysis. (B) Correctly targeted *Agtr1a*^{flox} allele after exposure to *Flp* recombinase to remove PGK-Neo cassette. (C) Null allele is generated after exposure of *Agtr1a*^{flox} to *Cre* recombinase. Positions of diagnostic PCR fragments are shown. (D) Confirmation of genomic structure of *Agtr1a*^{flox} allele by Southern analysis. Genomic DNA from ES cell clones was digested with *BamH1* and probed with Probe B. The 8.5 kb band is the endogenous *Agtr1a* locus. The 4.3 kb band is the targeted floxed locus containing neo. The 6.25 kb band is the targeted floxed locus with neo removed by *Flp*. *Indicates clones with correct rearrangement. Chimeric mice were generated from correctly targeted ES lines using standard techniques.



Supplemental Figure S2. Verification of smooth muscle specific *Cre* recombinase expression. Specific expression of the *SM22α-Cre* transgene was verified by inter-crossing with *Rosa26-lacZ* reporter mice. Specific x-gal staining was seen in the smooth muscle layer of aorta (A), large vessels of the kidney (B) and throughout the first branches of the mesentery (C) from Cre^{+} *Rosa26-lacZ* $^{+}$ mice. However no x-gal staining was seen in aorta (D), kidney (E) or mesentery (F) of Cre^{-} *Rosa26-lacZ* $^{+}$ mice.