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Genetic loss of proadrenomedullin N-terminal 20 peptide (PAMP) in mice is compatible with survival

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Abstract

Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are small peptides derived from a common precursor, pre-proadrenomedullin. Although AM and PAMP share hypotensive effects in the cardiovascular system, the peptides also exert diverse and distinct effects on endocrine physiology, innate immunity, cytoskeletal biology and receptor signaling pathways. Tremendous knowledge has been gleaned from the study of several genetic animal models of AM deletion or overexpression, some of which also simultaneously delete the coding region for PAMP peptide. However, deletion of PAMP without concurrent deletion of AM in an animal model is not currently available for the study of PAMP function. Here, we present the generation of $Adm^{PAMP/\ PAMP}$ and $Adm^{PAMP/-}$ mice, which lack the coding sequence for PAMP while preserving the coding sequence for AM. $Adm^{PAMP/\ PAMP}$ mice survive to adulthood without any obvious abnormalities and are fertile, though $Adm^{PAMP/-}$ females have small litters. Interestingly, these animals express lower levels of *Adm* mRNA and AM peptide than wild type animals, but these levels are still compatible with survival. Importantly, despite reduced levels, the spatiotemporal expression of AM peptide within the hearts of $Adm^{PAMP/-}$ mice remains similar to wild type animals. $Adm^{PAMP/\ PAMP}$ mice are now a publicly available tool for future investigations of PAMP function.

Keywords

PAMP; adrenomedullin; genetic model; mouse

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Introduction

Proadrenomedullin N-terminal 20 peptide (PAMP) is a 20 amino acid byproduct of pre-proadrenomedullin, which is cleaved into signal peptide, PAMP, mid-regional proadrenomedullin (MR-proADM), and adrenomedullin (*Adm*, AM) [1]. AM is the best characterized of these byproducts due to the availability of animal models, and MR-proADM is gaining traction as a biomarker primarily of cardiovascular disease but also of pulmonary disease and pregnancy complications [2–4]. Far fewer studies have addressed the role of PAMP in physiology and pathophysiology.

Like AM, PAMP is potently expressed and secreted by adrenal chromaffin cells but is also found widely throughout the body, including in plasma and urine [5]. PAMP is hydrolyzed by neprilysin, and like other neprilysin targets, exerts a hypotensive effect [6]. For example, a bolus of human PAMP or PAMP [9–20] exerts a five to ten minute hypotensive effect in rats [7, 8]. In a hypertensive rat model, PAMP overexpression blunts blood pressure increases and lessens the severity of myocardial hypertrophy [9]. Evidence suggests that this PAMP hypotensive effect is secondary to inhibition of norepinephrine release from peripheral sympathetic nerves, due in part to a decrease in catecholamine synthesis enzyme expression [10–12]. A bona fide PAMP receptor remains elusive, but one study has suggested that PAMP activates the G-protein-coupled receptor MrgX2 via $G_{\alpha q}$ and $G_{\alpha i}$ [13], while another study has suggested that the PAMP receptor is $G_{\alpha i3}$-coupled [14].

An assortment of biological roles for PAMP beyond blood pressure regulation have been identified. PAMP has been associated with angiogenesis in humans, mice, chicken, and fish. It also possesses anti-microbial activity in humans [15]. PAMP is also anti-nicotinic and inhibits aldosterone secretion [16, 17]. More recently, a role for PAMP as a microtubule-associated protein has emerged. In this context, PAMP binds to tubulin, leading to its destabilization, and increases kinesin velocity [18, 19].

A small body of literature has examined changes in plasma PAMP in several disease states in humans. Elevated plasma PAMP has been found in essential hypertension [20], impaired renal function [21], rheumatoid arthritis [22], and congestive heart failure [23], the severity of which correlates with PAMP levels [24]. In contrast, decreased plasma and urinary PAMP has been found in glomerulonephritis [25].

Collectively, although several roles for PAMP have emerged since its discovery in the early 1990s, its primary biological function remains obscure. Moreover, it is still unknown whether PAMP is necessary for survival. In this regard, the more widely-studies protein product of pre-proadrenomedullin, AM peptide, is necessary for embryonic development and survival based on 3 independent genetic mouse models. One of these models exclusively deletes the coding sequence for AM peptide [26], while two others delete the entire proadrenomedullin sequence and therefore both PAMP and AM peptides [27, 28]. Here, in a departure from previously published animal models, we present a novel gene targeting approach in which PAMP is deleted exclusively, while retaining the coding sequence for AM peptide, and demonstrate that mice lacking PAMP are viable and fertile.

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Materials & Methods

Generation of Mice with Deletion of PAMP Coding Sequence

Briefly, a gene targeting vector was generated containing the *Adm* promoter and exon 1, intron 1, part of exon 2, part of exon 3, intron 3, and exon 4 of the *Adm* gene upstream of a BamH1 site and a neomycin resistance cassette (Figure 1A). The targeting vector was electroporated into 129S6/SvEv embryonic stem cells, which were then subjected to neomycin selection. Homologous recombination in neomycin-resistant clones was confirmed by Southern blot after BamH1 digestion and/or PCR. Male chimeras were bred with wild type SvEv females to establish a genetically-pure, isogenic 129S6/SvEv line. PCR-based genotyping of the targeted allele was accomplished using two primers: P1 – 5-GTGCTGACGGGATCGTGCTG-3’; P2 – 5’-CATGCAGTACCCGAGGGACCT-3’. These primers amplify a 603 bp wild type allele and a 380 bp targeted allele (Figure 1C). $Adm^{PAMP/ \ PAMP}$ mice are available at the Mutant Mouse Resource and Research Center (MMRRC) at UNC-CH. $Adm^{+/-}$ animals [27] and $Adm^{hi/hi}$ animals [29] have been described elsewhere.

Gene Expression Analysis

Total RNA from $Adm^{PAMP/+}$, $Adm^{PAMP/-}$ and $Adm^{PAMP/ \ PAMP}$ adult hearts was isolated with TRIzol. Complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase. For cDNA from wild type hearts, a commercially available stock of pooled wild type mouse heart cDNA was utilized. cDNA from all genotypes was then used in quantitative real-time reverse transcriptase PCR (qRT-PCR) on a Stratagene MXP3000 and MxPro Software (Stratagene). Mouse GAPDH was used as an endogenous reference gene. qRT-PCR was performed in biological triplicate or quadruplicate and in technical triplicate and analyzed using the $2^{- \quad Ct}$ method.

Western Blotting

Hearts from adult $Adm^{+/+}$, $Adm^{PAMP/ \ PAMP}$, and $Adm^{hi/hi}$ mice were homogenized and lysed. Lysate protein concentration was determined with a BCA Protein Assay Kit (Pierce). Protein was loaded onto a SDS-PAGE gel, subjected to gel electrophoresis, and then transferred to a nitrocellulose membrane. The membrane was blocked and probed overnight in anti-adrenomedullin antibody (Novus Biologicals) at 4°C. The membrane was washed in tris-buffered saline/0.1% Tween 20 (TBST), incubated in secondary antibody at room temperature, and then washed again in TBST. Finally, the blot was imaged on an Odyssey CLx (LI-COR).

Immunohistochemistry

Tissue sections were permeabilized, blocked with 5% normal donkey serum, and incubated overnight at room temperature with the primary antibody, rabbit anti-mouse adrenomedullin (1:100; Novus Biological), and then incubated with appropriate secondary antibody (1:200; Jackson ImmunoResearch Laboratories) and DAPI (1:1000; Sigma-Aldrich). Images were acquired on a Nikon E800 microscope (Nikon) with a Hamamatsu camera (Hamamatsu Photonics).

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Results

To delete the coding sequence for PAMP, we generated a targeting vector that eliminated the PAMP-encoding 3' region of exon 2 and the PAMP-encoding 5' region of exon 3 as well as the interceding intron within the *Adm* gene locus (Figure 1A). Therefore, because exons 2 and 3 are genetically fused, the resulting targeted allele, Adm^{PAMP}, contains only 3 exons. In contrast to the 2 previously described AM knockout mouse models that concurrently delete PAMP, we discovered that $Adm^{PAMP/ PAMP}$ animals survived to adulthood without any apparent abnormalities. We detected the targeted allele in adult heterozygous and homozygous mice by Southern blot (Figure 1B) and by PCR (Figure 1C).

To confirm appropriate embryonic development and survival of $Adm^{PAMP/ PAMP}$ animals, we determined the genotypes of 51 pups from 8 litters born to $Adm^{PAMP/+}$ intercrosses. The genotype ratios observed in our colony were consistent with the expected Mendelian ratios, as shown by an observed/expected ratio of approximately 1 for each genotype (Table 1).

Due to the unavailability of commercial antibodies for PAMP, we were unable to confirm the absence of PAMP protein expression as expected. Nevertheless, we assessed changes in *Adm* mRNA in wild type, heterozygous, and homozygous mice by qRT-PCR. We were surprised to find that *Adm* mRNA expression in hearts from $Adm^{PAMP/+}$ and $Adm^{PAMP/ PAMP}$ mice was 76% and 50%, respectively, of *Adm* expression in wild type animals (Figure 2A). To further confirm that the reduced expression of *Adm* mRNA originated from the Adm^{PAMP} allele, we crossed $Adm^{PAMP/+}$ mice to those with a full genetic knockout of the *Adm* locus, $Adm^{+/-}$ [27], to generate a compound heterozygous mouse line, $Adm^{PAMP/-}$. Consistent with the expected genetic titration, these animals expressed approximately 2% of *Adm* mRNA, yet survived to adulthood, indicating that even a very low level of AM peptide is sufficient for embryonic survival. We observed a similar pattern of *Adm* mRNA expression in lungs from animals of these genotypes as well (data not shown).

Compared to wildtype animals, we confirmed the retained expression of AM peptide in $Adm^{PAMP/ PAMP}$ homozygous animals (Figure 2B), indicating that the genetically engineered allele remained in frame for appropriate translation. Consistent with reduced mRNA expression levels, we observed a decrease in AM protein expression in hearts of $Adm^{PAMP/ PAMP}$ homozygous animals by western blot (Figure 2B). This reduced expression stands in contrast to increased AM protein expression in hearts from $Adm^{hi/hi}$ mice, which overexpress AM via stabilization of the 5' untranslated region of the *Adm* gene [29].

To confirm that the spatiotemporal regulatory expression of AM peptide was not altered by the Adm^{PAMP} allele, we performed immunohistochemistry for AM peptide in postnatal day 21 mouse hearts from an allelic genetic series of animals that have graded levels of AM expression. Figure 2C shows that the expression of AM peptide is predictably localized in the epicardium (arrows) and endothelial cells of coronary vessels (arrowheads). Semi-quantitative staining intensity analysis of the epicardium revealed graded expression of AM

peptide that was roughly equivalent between $Adm^{+/-}$ mice (22.1% mean intensity/area) and $Adm^{PAMP/-}$ mice (18.1% mean intensity/area), when normalized to wildtype animals (100%) and compared to the overexpression in $Adm^{hi/hi}$ mice (141% mean intensity/area). Importantly, the cell-specific pattern of expression, albeit reduced in $Adm^{PAMP/-}$ mice compared to wildtype and $Adm^{hi/hi}$ mice, is fully conserved in $Adm^{PAMP/-}$ mice, indicating that the expression of AM peptide from the Adm^{PAMP} allele is spatiotemporally conserved.

Since the $Adm^{PAMP/-}$ mice express levels of AM protein equivalent to that observed in $Adm^{+/-}$ mice, we expected and found that $Adm^{PAMP/-}$ females bred to wildtype males exhibited smaller litter sizes at postnatal day 1 (2.8 pups/litter, N=5) compared to the number of conceptuses observed at embryonic day e13.5 (9.7 pups/letter, N=3), similar to what has been previously reported for $Adm^{+/-}$ female mice [27].

Although PAMP peptide has been primarily characterized as a hypotensive factor [7, 8], we failed to observe a statistically significant compensatory cardiac hypertrophy response in adult $Adm^{PAMP/ PAMP}$ or $Adm^{PAMP/-}$ animals, compared to wildtype animals, reflected by equivalent heart weight:body weight ratios, 4.76+/−0.32 mg/g (n=4), 4.31+/−0.14 mg/g (N=6), 4.12+/−0.14 mg/g (N=7), respectively. These data imply that under basal conditions, there is unlikely to be a hypertensive phenotype in animals lacking PAMP.

Discussion

Here, we have described the generation of a genetically targeted mouse line in which portions of exon 2, exon 3 and intron 2 of the *Adm* gene are deleted, thereby eliminating the coding sequence for PAMP while preserving the coding sequence for AM peptide. We have also crossed this Adm^{PAMP} allele to a knockout allele of the *Adm* gene to generate a $Adm^{PAMP/-}$ mouse line in which the expression of AM peptide only occurs from the Adm^{PAMP} allele. To our knowledge, this is the first genetic mouse model that deletes PAMP exclusively, in contrast to previously generated mouse models that delete AM and PAMP concurrently [27, 28]. Given that the genotypes of offspring from $Adm^{PAMP/+}$ intercrosses are born in expected Mendelian ratios and survive to adulthood, we conclude that $Adm^{PAMP/ PAMP}$ mice are viable and without any apparent developmental abnormalities. Once $Adm^{PAMP/ PAMP}$ mice reach adulthood, they are fertile and can therefore be intercrossed. Thus, in contrast to the essential functions of AM peptide in mammalian embryogenesis and fertility, PAMP peptide is dispensable.

The lack of commercial antibodies for PAMP precludes our ability to directly confirm deletion of PAMP by traditional western blot analysis. However, we assessed changes in *Adm* mRNA and protein by qRT-PCR, western blotting and immunohistochemistry, which consistently demonstrated a positive correlation between PAMP genotype and AM dosage in the heart. Interestingly, AM expression in the heart of $Adm^{PAMP/ PAMP}$ animals was approximately 50% of that of wild-type animals, suggesting that there may exist intronic regulatory elements within deleted intron 2 of the *Adm* gene, or as yet unidentified epigenetic control mechanisms, which may control the overall mRNA transcription. Nevertheless, this reduction in AM expression did not impair embryonic development. Therefore, in light of these findings and the embryonic lethality of $Adm^{-/-}$ embryos by

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e12.5, we conclude that a very small dosage of AM peptide is sufficient for ensuring survival.

We previously showed that genetic titration of *Adm* does not affect basal blood pressure [30]. Therefore, we do not expect basal blood pressure to change with alterations in PAMP expression, especially given that the PAMP hypotensive effect appears to be less potent than the AM hypotensive effect. Consistent with this rationale, we observed no compensatory cardiac hypertrophy in $Adm^{PAMP/\ PAMP}$ or $Adm^{PAMP/-}$ animals. Nevertheless, it will be the goal of future studies to determine whether $Adm^{PAMP/\ PAMP}$ or $Adm^{PAMP/-}$ animals mice reveal any phenotypes, cardiovascular or otherwise, when challenged, aged and further studied. This new genetic mouse model is currently available at the Mutant Mouse Resource and Research Centers at the University of North Carolina at Chapel Hill for use by the biomedical research community.

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Highlights

- -First described genetic allele in mice resulting in deletion of PAMP peptide, a proteolytic by-product of the adrenomedullin gene.
- -PAMP deletion is compatible with embryonic survival.
  - *Adm□PAMP/□PAMP* mice have no overt phenotypes and survive into adulthood.
  - *Adm□PAMP/□PAMP* mice are available to the scientific community through the Mutant Mouse Regional Resource Center.

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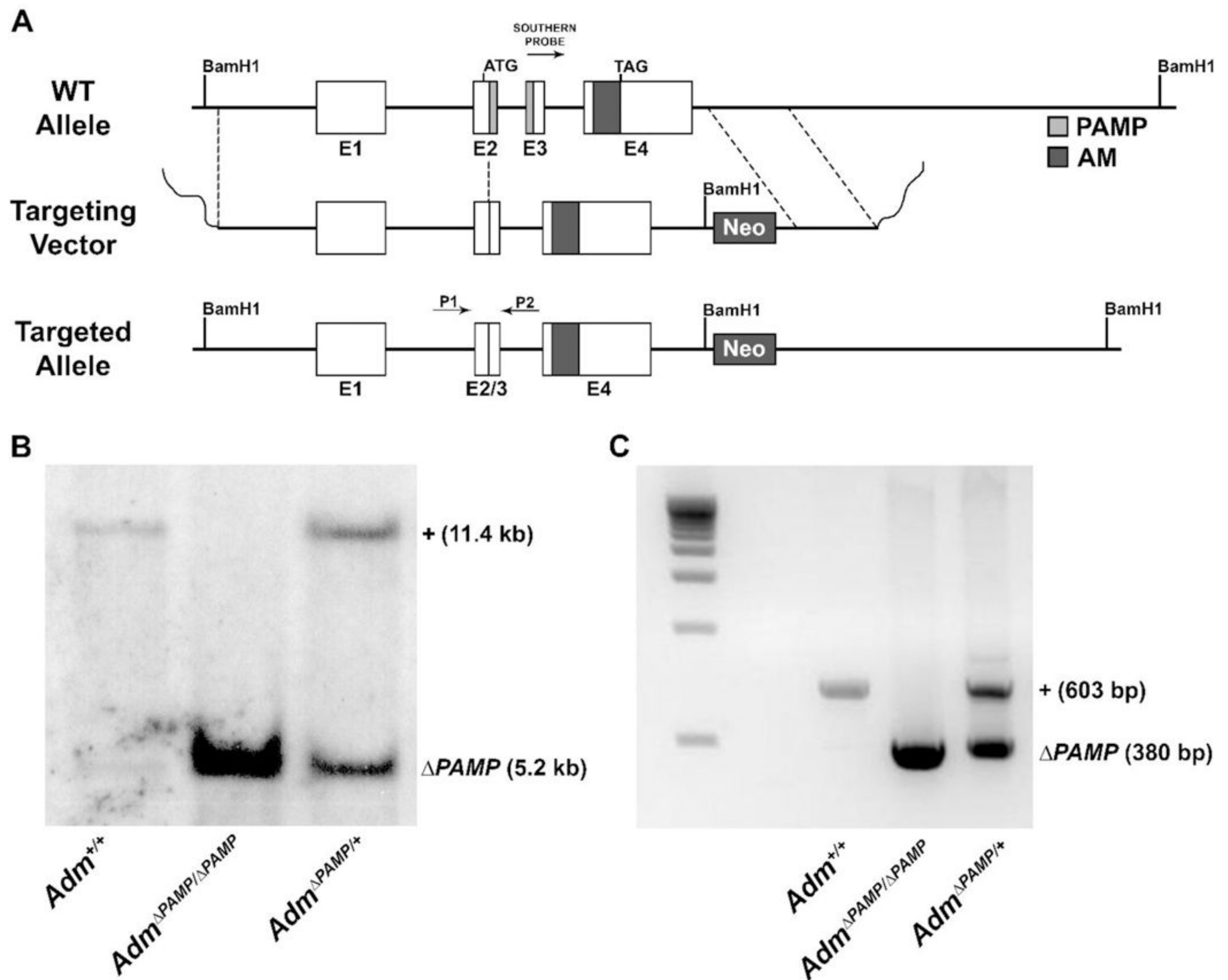

Figure 1.

Generation of *Adm* $^{PAMP/\ PAMP}$ animals. (A) Illustration of wild type *Adm* allele; targeting vector; and targeted *Adm* allele after homologous recombination. E1–4, exons 1–4; ATG, initiator methionine; P1 and P2, primers to detect and differentiate between wild type and targeted alleles by PCR; TAG, stop codon. (B) Detection of wild type and targeted alleles by Southern blot. (C) Detection of wild type and targeted alleles by PCR.

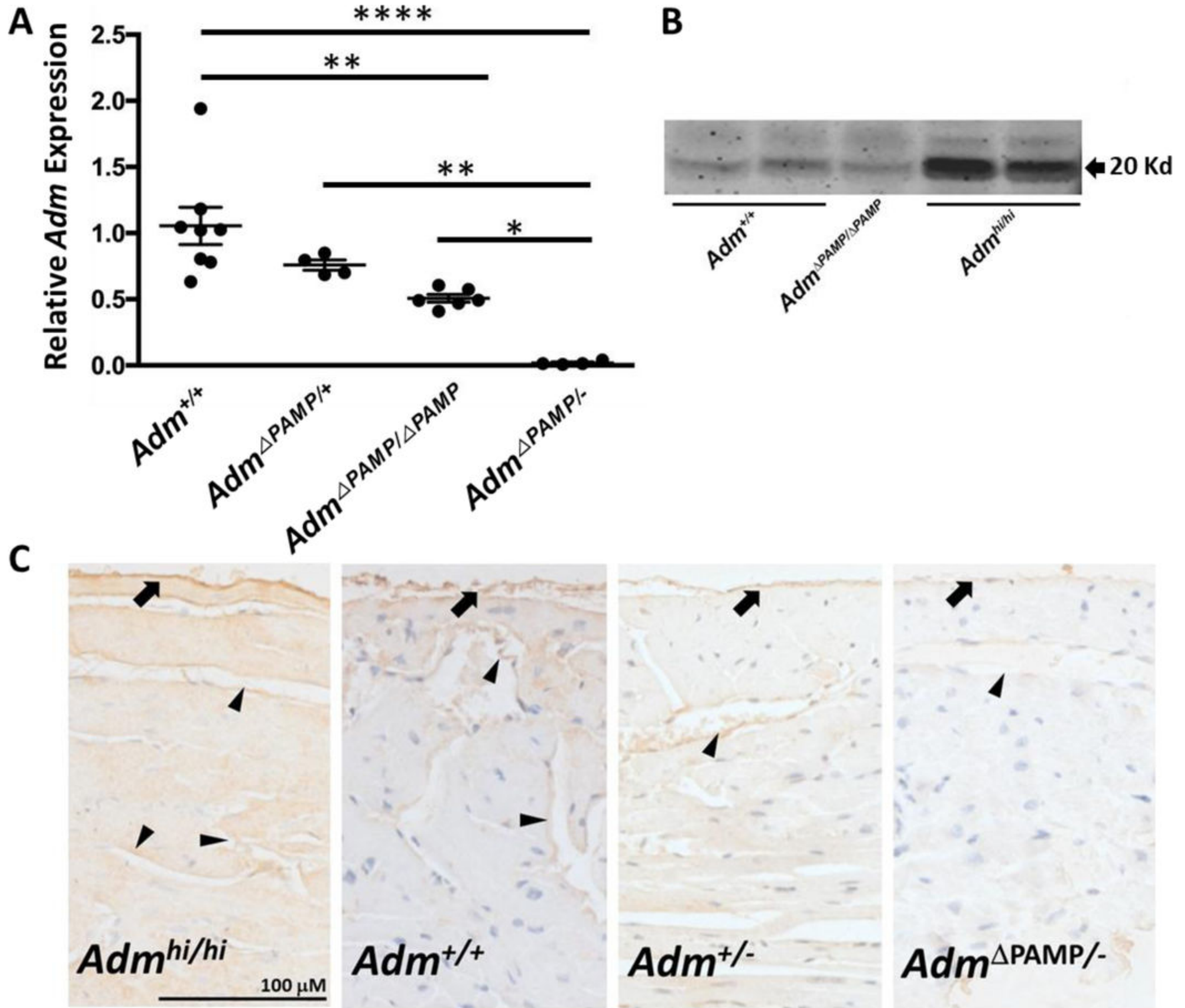

Figure 2.

Reduced but sufficient levels of *Adm* mRNA and protein levels in *Adm* $^{PAMP/\ PAMP}$ and *Adm* $^{PAMP/-}$ animals. (A) Relative *Adm* mRNA expression in adult mouse hearts by qRT-PCR, where each data point represents an individual animal. Data are normalized to *Gapdh* expression. *p<0.05, **p<0.01, ****p<0.0001, one-way ANOVA Tukey's Multiple Comparison's test. (B) AM protein expression in adult mouse hearts by western blot. Dash beneath lanes 1,2 and 3,4 indicate individual animals of the same genotype. (C) Immunohistochemistry for AM protein in postnatal day 21 mouse hearts, counterstained with DAB (brown) and DAPI (blue). Arrows indicate high level of basal AM secretion from epicardial cells. Arrowheads indicate enriched AM protein in endothelial cells of coronary vessels. N > 3 animals/genotype. Scale bar = 100 microns.

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Table 1

Breeding table displaying actual and expected Mendelian ratios from a *Adm* $^{PAMP/+}$ intercross

	Adm$^{+/+}$	***Adm*** $^{PAMP/+}$	***Adm*** $^{PAMP/\ PAMP}$
Expected (n=51)	12.75	25.5	12.75
Observed (n=51)	12	27	12
Observed/Expected, %	106.25	94.44	106.25

n=51 pups from 8 litters.