PROJECT TITLE: Does loss of AKAP121 precede induction of cardiac hypertrophy?

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SUMMARY:

The A-kinase anchor proteins (AKAPs) are diverse regulators of cell function, acting as scaffolds to co-localize the protein kinase A regulatory sub-unit with target proteins, such as other kinases and phosphatases. We have previously demonstrated that over-expression of AKAP121 in cardiomyocytes attenuates hypertrophy induced by isoproterenol. Furthermore, knockdown of AKAP121 expression induced spontaneous cardiomyocyte hypertrophy, suggesting that AKAP121 behaves as a potent anti-hypertrophic regulator. In this study, mice underwent transverse aortic constriction (TAC), resulting in increased cardiac afterload and progressive hypertrophy. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting was used to examine expression of AKAP121 in banded and sham control animals. The degree of hypertrophy was assessed by heart weight to body weight or tibia length ratios. We hypothesized that AKAP121 expression will decrease soon after banding, prior to frank hypertrophy. Our current results demonstrate a correlation between loss of AKAP121 expression and induction of the hypertrophic gene program in response to pressure overload induced by thoracic aortic banding, thus indicating a potential role for AKAP121 as a regulator of the hypertrophic response *in vivo*. Our data also suggests that a sex-specific mechanism may be involved in regulation of hypertrophic gene expression.

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Student's Signature **Supervisor's Signature**

1. INTRODUCTION

Cardiac hypertrophy is believed to be an adaptive process that attempts to compensate for increased demands placed on the heart. The hypertrophic response occurs not only during the normal process of development and growth of the fetal heart, but also in response to pathological stresses such as hypertension and myocardial infarction[1]. Although the hypertrophic response is compensatory initially, it ultimately results in pathologic remodeling leading to heart failure and sudden death [2]. Understanding of the processes that underlie hypertrophy is critical to the development of anti-hypertrophic therapies.

Cardiac hypertrophy is an enlargement of the heart associated with an increase in the size of individual cardiomyocytes, as well as upregulation of proteins and in pathologic hypertrophy, fetal cardiac genes [3,4]. The molecular mechanisms that couple the cell membrane initiated pro-hypertrophic signals to reprogramming of cardiomyocyte gene expression for the most part remains unclear. Over the past 15 years, a variety of intracellular signaling pathways and molecular mechanisms involved in driving the cardiac hypertrophic gene program have been identified. Among these is one of the most potent hypertrophic regulatory mechanisms involving a calcium-dependent phosphatase, calcineurin, and its downstream effector, the nuclear factor of activated T cells (NFAT) family of transcription factors [2,3]. In response to pro-hypertrophic signals, calcineurin dephosphorylates NFAT, permitting its translocation to the nucleus and transactivation of the hypertrophic gene program [2]. Despite the importance of this pathway, the mechanisms regulating its activation and inhibition remain only partially understood, and important modulators of calcineurin and NFAT activity likely remain to be identified.

A-kinase anchor proteins (AKAPs) have recently come to attention as potential regulators of cardiomyocyte cell growth. AKAPs function as scaffold proteins that act to localize multiple interacting proteins to subcellular environments, thus acting as mediators for required biological outcomes [5]. While all AKAPs appear to act to localize signaling to provide precise spatial control over cellular responses, the specific interactions regulated are unique to each AKAP. Other researchers have demonstrated that AKAP-Ibc/AKAP13 plays a role in cardiomyocyte hypertrophy by interacting with protein kinases PKC/PKD, with knockdown of AKAP-Ibc suppressing PKD activated hypertrophy [4,6]. In addition, mAKAP/AKAP6 has also been reported to have a similar role in the hypertrophy of neonatal rat cardiomyocytes through a pathway involving ERK5, with knockdown of mAKAP inhibiting the hypertrophic response[6]. Thus the interaction of AKAPs with its substrates affects downstream cardiac hypertrophic gene expression in ways unique to each AKAP. Other AKAPs have been reported as being expressed in the heart, including AKAP350/AKAP9, AKAP95/AKAP8, AKAP121/AKAP1 [5,7]. It is unknown whether any of these AKAPs play a role in cardiac hypertrophy. It is not easy to predict the way in which each AKAP may affect hypertrophy and each AKAP must thus be individually tested in order to determine its role in cardiac hypertrophy.

We previously showed that knockdown of AKAP121 results in significant hypertrophy of cardiomyocytes *in vitro* [1]. Loss of AKAP121 was accompanied by a decrease in phosphorylated

NFATc3, leading to a net translocation of NFATc3 from the cytosol to the nucleus. These results are consistent with activation of the calcineurin/NFAT hypertrophic pathway. We further found that AKAP121 binds calcineurin, suggesting that knockdown of AKAP121 results in the release of an active pool of calcineurin and downstream activation of hypertrophic genes. We also demonstrated that over-expression of AKAP121 resulted in a decrease in cardiomyocyte cell size and rendered cells resistant to isoproterenol-induced hypertrophy. AKAP121 thus appears to attenuate hypertrophy and may play a role as a negative regulator of hypertrophic cell remodeling.

In this study we explore the *in vivo* response of AKAP121 expression to pro-hypertrophic signals induced by transverse aortic constriction (TAC), a pressure overload model that mimics hypertension-induced cardiac hypertrophy in humans. To date, the only examination of this issue in the literature is a report that transverse aortic banding in male rats leads to decreased AKAP121 protein expression at 3 days post surgery [8]. In this study we report preliminary findings that indicate that AKAP121 is down-regulated prior to induction of cardiac hypertrophy. As the previously mentioned study only considered male animals, we also examined the potential differences that exist in the hypertrophic response of males and females.

2. MATERIALS AND METHODS

2.1 Animal Studies

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following the guidelines established by the Canadian Council on Animal Care. Trained staff performed surgeries in the R.O. Burrell animal facility at St. Boniface Hospital Research Centre.

2.2 Mouse model of pressure overload

The study consisted of a total of 60 C57BL/6 mice (30 males, 30 females) aged 6 to 8 weeks that were randomized to either undergo a sham procedure or to undergo transverse aortic constriction (TAC). The surgeries were performed in sets of 10 consisting of 5 sham and 5 banded (TAC) animals. Hearts were harvested at 3 days or 8 weeks post surgery.

After induction of a surgical plane of anesthesia by ketamine/xylazine injection, increased cardiac afterload was induced by TAC, a procedure in which a suture is tied around the transverse aorta. To ensure that the overload induction was similar between animals, a guide (27 gauge) was used for suture application. Analgesia was supplied by sub-cutaneous administration of buprenorphine (0.03-0.04 mg/kg) 1 hour prior to surgery and every 12 hours post-op (total of 3 injections) and as needed. Control mice underwent a sham procedure in which thoracotomy was performed, but the suture was not placed around the aorta.

The animals were sacrificed at 3 days (40 mice total) or 8 weeks (20 mice total) post surgery by injection of ketamine:xylazine (150:10 mg/kg). Induction of a surgical plane of anesthesia was

confirmed by toe pinch/pedal reflex before the heart was removed. The hearts were rinsed in ice cold phosphate-buffered saline (PBS). Hearts selected for molecular analysis were flash-frozen in liquid nitrogen, as a whole heart for the 8 week time and one 3 day time point, or after sectioning into left and right sides for the other 3 day time point. The tissues were stored at -80° C for mRNA and protein analysis. The 8 week time point hearts selected for histological analysis were placed in 4% formaldehyde for fixation. After termination, the heart and body weight and tibia length were determined for confirmation of cardiac hypertrophy.

2.3 Quantitative Real-Time PCR (RNA Analysis)

Total cardiac RNA was isolated from hearts using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). RNA concentration was quantified spectrophotometrically in TE (Tris + EDTA) buffer and appropriate amounts of RNA, based on primer optimization (25 ng for all primers, except BNP for which 100 ng was used), used for quantitative real-time PCR reactions performed using B-R One-Step SYBR® Green qRT-PCR kit (Quanta Biosciences). Expression of AKAP121 and internal control GAPDH was assayed along with markers of cardiac hypertrophy: natriuretic peptides (ANP, BNP) and myocardial proteins (cardiac α-actin, cardiac β-actin). The primers (Sigma-Aldrich) used for amplification of the genes of interest were as follows: AKAP121 (forward) 5'-AGGCTCCAACCCTAAGAAGG- 3'; AKAP121 (reverse) 5'-GTCTGCTTCAGAAAACTCACGTAC-3'; ANP (forward) 5'- ATCTGATGGATTTCAAGAACC-3'; ANP (reverse) 5'-TCTCAGAGGTGGGTTGACCT-3'; BNP (forward) 5'-CAGAGCAATTCAAGATGCAG-3'; BNP (reverse) 5'-AATTTTGAGGTCTCTGCTGGA-3'; Cardiac-α actin (forward) 5'-CTGCAGTCAGCCCTGGAG-3'; Cardiac-α actin (reverse) 5'-TCCTTTCGATCTCTGCCTTG-3'; Cardiac-β actin (forward) 5'-GAGCCTCCAGAGTCTGCTGAAGGA-3'; Cardiac-β actin (reverse) 5'-TTGGCACGGACAGCATCATC-3'; GAPDH (forward) 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH (reverse) 5'-GGCATGGACTGTGGTCATGAG-3'.

A Bio-Rad iQ5 real-time PCR machine was used for the amplification reactions. The thermocycler program conditions used were as follows: initial cDNA synthesis step at 50°C for 10 minutes, followed by a denaturing step at 95°C for 5 minutes, 45 cycles of 10 seconds denaturing at 95°C and 30 seconds of annealing at 60°C (data collection point). Finally, a 1 minute denaturing step at 95°C and an annealing step of 1 minute at 55°C.

2.4 Western Blot Analysis of Proteins

Protein was isolated from hearts by homogenization in Radio-Immunoprecipitation Assay (RIPA) lysis buffer containing a protease inhibitor cocktail. The lysate was centrifuged at 14 000 rpm for 15 mins at 4°C and the supernatant collected. Protein concentration was determined using a Coomassie stain-based assay (Thermo Scientific) with optical density being read in a plate reader. Proteins were resolved on SDS-polyacrylamide gels and wet-transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Blots were immuno-labeled with anti-AKAP 149 (BD Biosciences) primary antibody (the human homologue of AKAP121; antibody cross reacts with mouse AKAP121) and goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Labeled bands were detected

using Western Blotting Luminol Reagent (Santa Cruz Biotechnology), visualized by exposure to clear blue CL-X Posure™ film (Thermo Scientific), and analyzed using a GS-800 calibrated densitometer (Bio-Rad) and Quantity One 4.5.2 (Bio-Rad) image analysis software. Anti-α-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa) was used for gel loading controls.

2.5 Histological Analysis

For light microscopy, the hearts were fixed for 3 days in 4% formaldehyde after which it was rinsed in phophate-buffered saline (PBS) and dehydrated in an ethanol series. The next day, tissue was placed in xylene for clearing, allowed to dry completely, and wax embedded. After solidification the tissue was sectioned at 5 μm and stained with hematoxylin/eosin for visualization.

2.6 Statistical Analysis

All data are expressed as means ± SE. Statistical significance of results was determined by two-tailed Student's *t* test. Mann-Whitney test was used where appropriate. Significance was defined by a P-value < 0.05. P-value < 0.01 is indicated where applicable.

3. RESULTS

3.1 Confirmation of cardiac hypertrophy

As an indicator of cardiac hypertrophy we determined the heart weight-to-body weight (HW/BW) ratio in all animals. As body weight is susceptible to many confounding factors, the heart weight-to-tibia length (HW/T) ratio was also determined. At 3 days post surgery an increase in the HW/BW and HW/T ratios are seen in the TAC males, but no significant change is noted in the TAC females (Table 1). At 8 weeks post surgery both ratios are significantly increased in both sexes, indicating that hypertrophic changes have taken place at this time point.

For visual confirmation of hypertrophy at the 8 week time point we analyzed H&E stained heart tissue sections from the sham control and banded group for hypertrophic changes. Increased cardiomyocyte size is noted (Fig.1). Hypertrophic changes if present at 3 days are not expected to be obvious enough to be recognized on histology sections; therefore we did not perform histological analysis at the 3 day time point.

3.2 Transcriptional response of AKAP121 in relation to hypertrophic gene expression

Although knockdown of AKAP121 has been demonstrated to induce hypertrophy in cardiomyocytes *in vitro,* the *in vivo* regulation of AKAP121 in response to hypertrophic signals has not yet been fully explored [1]. To determine whether loss of AKAP 121 expression precedes cardiac hypertrophy, we analyzed changes in AKAP121 expression at the transcriptional level

using quantitative RT-PCR. Changes in natriuretic peptides atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), as well as myocardial proteins cardiac α-actin and cardiac β-actin were analyzed as indicators of the hypertrophic response.

At 3 days post surgery we expected to see downregulation of AKAP121 in the absence of induction of hypertrophic marker expression in the TAC animals. In the first 3 day time point where we analyzed whole heart lysates, we found no significant difference between the sham control and banded group in terms of AKAP121 expression (results not shown). Thus considering that the left and right heart might have different responses to hypertrophic signals we designed a second 3 day time point in which we analyzed the left and right hemispheres of the hearts separately for changes in AKAP121 expression. This time we observed a significant decrease in AKAP121 mRNA in both the left and right hemispheres when considering the pooled data of males and females (Fig.2). Although some hypertrophic markers were up-regulated at this point (BNP and cardiac β-actin in the right and left sides; ANP in the left side only), others showed no difference from the sham control group (Fig.2). Separate analysis of males and females in terms of AKAP121 expression did not show a significant difference at this time point, but a significant increase in the expression of BNP was noted in the banded males. Although statistical significance is not achieved, the data demonstrates a trend towards a greater hypertrophic response to pressure-overload in the males as compared to the females.

At 8 weeks post surgery, all the cardiac hypertrophic markers were induced in the banded group but AKAP121 expression did not show a statistically significant difference between the sham and banded groups, except in the female animals alone (Fig.2).

3.3 AKAP121 protein levels decrease early in the response to hypertrophy

We analyzed the mouse hearts for changes in AKAP121 protein levels by western blotting (Fig.3). At 8 weeks post surgery a 20% decrease AKAP121 protein levels is seen, but without significance. At 3 days post surgery a significant decrease in AKAP121 levels occurred in the left heart of the females (57%), but not in the males (43%). There is no significant decrease in AKAP121 protein levels in the right heart, although a 10% decrease is seen.

4. DISCUSSION

Cardiac hypertrophy can have both physiological and pathological consequences for heart function. Concentric remodeling in response to normal physiologic hypertrophic signals, as occurs in cardiac development and high levels of exercise, improves the efficiency of the heart. On the other hand, the eccentric pattern of myocardial remodeling that occurs in response to pathologic hypertrophic signals, compromise cardiac function and is itself a risk factor for heart failure and sudden death. The underlying mechanisms that drive the heart towards either of these processes are still unclear. Identification of new regulators of cardiomyocyte hypertrophy is of imperative interest clinically, both in terms of gaining a better understanding of the underlying processes and in development of novel therapies to decrease the patient mortality and morbidity associated with pathological remodeling.

A complex network of signaling pathways control the induction of cardiomyocyte hypertrophy. These include cAMP-dependent protein kinase A (PKA) pathways, mitogen-activated protein kinase A (MAPK) pathways, and calcineurin-NFAT transcription factor pathway[9]. The complex nature of signaling networks such as these provide a challenge for understanding exactly how connections between the different pathways involved are formed. Developments in the understanding of these networks has lead us to believe that they exist as integrative frameworks that contain areas where signaling pathways converge and share common molecules, functionally acting as nodes that allow crosstalk between pathways and integration of signals[4,10] . Therapeutically, these centers of integration may be important for modulating, through fewer targets, multiple pathways involved in a specific cellular response.

The A-kinase anchor proteins (AKAPs) have recently come to attention as one such protein class that acts as a node in the signaling network controlling induction of the hypertrophic gene program. AKAPs are a family of proteins that although structurally diverse, share the common function of acting as scaffolds that localize interacting proteins such as phosphotases and kinases in subcellular compartments, such as the mitochondria, with all AKAPs interacting with PKA [5,11]. This gives AKAPs the ability to regulate spatial and temporal signaling, providing precise control over cellular responses. We recently demonstrated the role of AKAP121 as a negative regulator of cardiomyocyte hypertrophy in cell culture studies [1]. Whether AKAP121 has the same role *in vivo* remains unanswered.

Our current results demonstrate a significant decrease in AKAP121 protein and mRNA levels in 3 days post TAC animals upon consideration of the sex-pooled data (Fig.2)(Fig.3). Interestingly, when the males and females were looked at separately, the AKAP121 mRNA levels showed a downward trend, but did not reach statistical significance (Fig.2). The AKAP121 protein levels, however, were significantly decreased in the females, and showed a similar trend in the males (Fig.3). A recent study reported a decrease in AKAP121 protein levels, but not mRNA levels, of left ventricles occurring at 3 days post aortic banding in male rats [8]. Both results demonstrate a similar percentage (~40%) decrease of AKAP121 protein levels in the male TAC animals as compared to the sham controls. The difference in the statistical findings in relation to this data may be due to the fact that the sample size of the Perrino et al. study was much greater (n=16) than the sample size of the males used in our study (n=2). An increased sample size is therefore needed to confirm these results.

Our hypothesis that AKAP121 has causative role in the initiation and progression of hypertrophy requires us to demonstrate that a decrease in AKAP121 expression precedes the induction of the hypertrophic gene program. Our data shows that although some markers of hypertrophy are already induced at 3 days post TAC, other markers show no change, reflecting the early time point (Fig.2). The upward trend of the hypertrophic genes are more prevalent in the male animals, with a significant increase in the expression of BNP occurring in both the left and right sides of the hearts of the males, but no significant changes in any of the markers analyzed were seen in the females at the 3 day time point. At 8 weeks however, there appears to be a similar degree of induction in both sexes with almost all the markers being up-regulated. The data

collected from the HW/BW ratios supports these findings in that the males demonstrate a greater increase in heart size at 3 days post TAC as compared to the females (Fig.1)(Table 1). A study by Skavdahl et al. also reported a significantly higher increase in HW/BW ratio in males than females was seen 2 weeks post TAC [12]. Therefore, the hypertrophic response appears to be more rapidly induced in males, both in terms of transcriptional hypertrophic marker expression and HW/BW ratios.

A link between sex and the prevalence and severity of cardiovascular disease has been reported in human clinical studies [13,14]. Although well documented, this link remains poorly understood. What is known is that the nature of the hypertrophy that occurs in females results in greater functional preservation than that which occurs in males [13,15]. Although females typically have greater hypertrophy, they also have more maintained LV function compared to males in response to both hypertension and aortic stenosis [16,17]. Krumholz et al. observed concentric hypertrophy in females in response to isolated systolic hypertension, whereas the same stimulus resulted in eccentric hypertrophy in males[16]. A recent study demonstrated a sex-dependent regulation of physiological cardiac hypertrophy in mice, with females showing an enhanced exercise-induced hypertrophic response [15]. This data supports the idea that females tend to have an enhanced adaptive response to hypertrophic signals as compared to males. Although female hormones such as estrogen are known to have a protective effect in females, with more interest developing in the genetics and molecular regulation of the hypertrophic response, the role of sex in hypertrophic changes has been revealed to be much larger than this.

The sex dichotomy in the hypertrophic response is further demonstrated in our data in that even though a greater hypertrophic response was noted in males, AKAP121 protein levels decreased more in females at 3 days post surgery (Fig.1)(Fig.3). At 8 weeks this difference starts to even out with both sexes showing a similar increase in HW/BW ratio as well as a more similar pattern of hypertrophic marker expression, but with a greater drop in AKAP121 mRNA expression in the females (Fig.2)(Fig.1)(Table 1). Thus, there appears to be a mechanism in place that acts to delay the hypertrophic response in females that is not present in the males.

In relation to the sex difference in hypertrophic response, overexposed film of AKAP149 probed membranes revealed bands of ~60kDa and 50kDa that were present at significantly larger concentrations in the female hearts (Fig.4). Previously we had reported that calcineurin consistently pulled down a band of ~50kDa in co-immunoprecipitation experiments [1]. This band immunoreacted with an AKAP121 antibody, suggesting it may be a proteolytic cleavage product. At this time we do not know if these bands correlate to the same protein, but a band of this size matches the approximate predicted molecular mass of splice variant S-AKAP84 and may thus represent a structural variant. Calcineurin inhibitors are able to attenuate hypertrophy in cardiomyocytes in which AKAP121 had been knocked down. As calcineurin is known to be involved in pathologic, but not physiologic hypertrophy, one could consider that if an inhibitor of calcineurin exists in one sex but not the other, this may in part explain the why females appear to demonstrate more protection to pathological hypertrophy than males [18]. As these bands are also present in the female sham controls, one could consider that basal expression of this protein could be a mechanism by which hypertrophy is delayed in females.

As demonstrated by alterations in intracellular NFATc3 localization, knockdown of AKAP121 results in activation of the calcineurin/NFAT pathway, a potent transducer of cardiac hypertrophy. Activation of calcineurin results in dephosphorylation of its downstream effector, NFAT, which upon dephosphorylation translocates from the cytoplasm to the nucleus where it transactivates genes involved in the cardiac hypertrophic program[1,3,19]. The formation of an AKAP121-calcineurin complex has been demonstrated by co-immunoprecipitation experiments [1]. This interaction provides a potential mechanism through which AKAP121 may regulate hypertrophic changes. In humans, AKAP79 inhibits the phosphatase activity of calcineurin by directly binding to it [20]. If AKAP121 binds to calcineurin in a similar manner, loss of AKAP121 would release an active pool of calcineurin with the ability to induce expression of the hypertrophic gene program. Whether this direct interaction is significant in regards to the *in vivo* hypertrophic response requires further experimentation.

AKAP121 has the potential to regulate hypertrophic changes, not only through the calcineurin/NFAT pathway, but also through the cAMP-induced PKA pathway. As demonstrated by the ability of inhibitors of these pathways to block hypertrophy induced by AKAP121 knockdown, activation of both of these pathways are critical to induction of hypertrophy[1]. How these pathways converge however remains unclear. We had previously proposed a model in which a phosphorylated target of PKA complexes with NFAT in order to drive hypertrophic gene expression. The existence of such a mechanism would not require direct interaction with calcineurin and could explain the significance of both pathways to the induction of hypertrophy. Due to the ability of PKA to directly phosphorylate and activate GATA4 in gonadal cells, the transcription factor GATA4 has been hypothesized as the putative PKA target connecting the two pathways[1].

At this time we cannot explain the sex differences in the hypertrophic response. Considering that AKAP121 levels may be regulated by degradation mechanisms such as proteolytic cleavage and ubiquitination[21], a reasonable consideration may be that an early response degradation mechanism is triggered in the females. A selectively activated degradation process could also explain the differences in the response of the left and right hearts. Another possibility is that AKAP121 undergoes a transformative process to a protein that acts as an inhibitor of hypertrophy. Such a mechanism would be consistent with a drop in AKAP121 protein levels along with a decreased hypertrophic response. On the other hand, the drop in AKAP121 protein levels may merely be a consequence of a sex-specific early adaptive process that delays hypertrophic changes in the females.

Future Directions

Our current results show preliminary data that suggest a causative role of AKAP121 in cardiac hypertrophy *in vivo*. Our results demonstrate that loss of AKAP121 *in vivo* is related to induction of the hypertrophic gene program. As the hypertrophic response is already active at 3 days post TAC, earlier time points (2 days and 1 day) will have to be explored to delineate the exact temporal relationship between loss of AKAP121 and hypertrophic gene expression. Thus

although the lack of induction of all hypertrophic markers is suggestive of a causative role for AKAP121, no conclusions can be drawn from our current results as some parts of the hypertrophic program are already activated at 3 days.

The main challenge we faced in this study was a lack of statistical power due to a small sample size. In many cases we are able to achieve statistical significance in the pooled male and female data (n=5), but not in the separate male and female data (n=3 or n=2). Thus in situations where we are currently not seeing any differences between the sexes, real differences might be revealed if we increase the sample size of the males and females in future studies aimed at exploring the sex differences in the hypertrophic response.

The appearance of the 50kDa band in females is an intriguing finding that will require further inquiry into the identity of this band, including sequencing and study of its interactions with potential activators of the hypertrophic gene program. A potential interaction of this band with calcineurin could suggest a role as an inhibitor the hypertrophic gene program. It could however also be a coincidental occurrence as a result of the hypertrophic response, or an activator of an inhibitory pathway.

Although we had previously demonstrated that AKAP121 knockdown in cardiomyocytes *in vitro* resulted in hypertrophy, future studies of the *in vivo* role of AKAP121 is required to fully explore the role of AKAP121 in hypertrophy. AKAP121 knockout mice studies are required to explore whether compensatory mechanisms are present *in vivo* that do not have the appropriate time frame to develop in the *in vitro* model. In the same way animals in which AKAP121 is overexpressed should be analyzed for attenuation of hypertrophy induced by TAC. These studies will give more insight into the role of AKAP121 in cardiac hypertrophy in the whole organism and allow exploitation of this protein as a potential therapeutic target.

Conclusion

In an earlier study, we had identified AKAP121 as a novel negative regulator of cardiomyocyte hypertrophy. In this study, we show preliminary data that suggests a role for AKAP121 as a negative regulator of cardiac hypertrophy *in vivo.* We also demonstrate the potential for the existence of a sex-specific mechanism in regulation of the hypertrophic response.

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FIGURES

A

	Males	Females
3 Days	12.25 ± 1.78 #	3.24 ± 2.99
8 Weeks	73.73 ± 22.38*	69.56 ± 19.72*

B

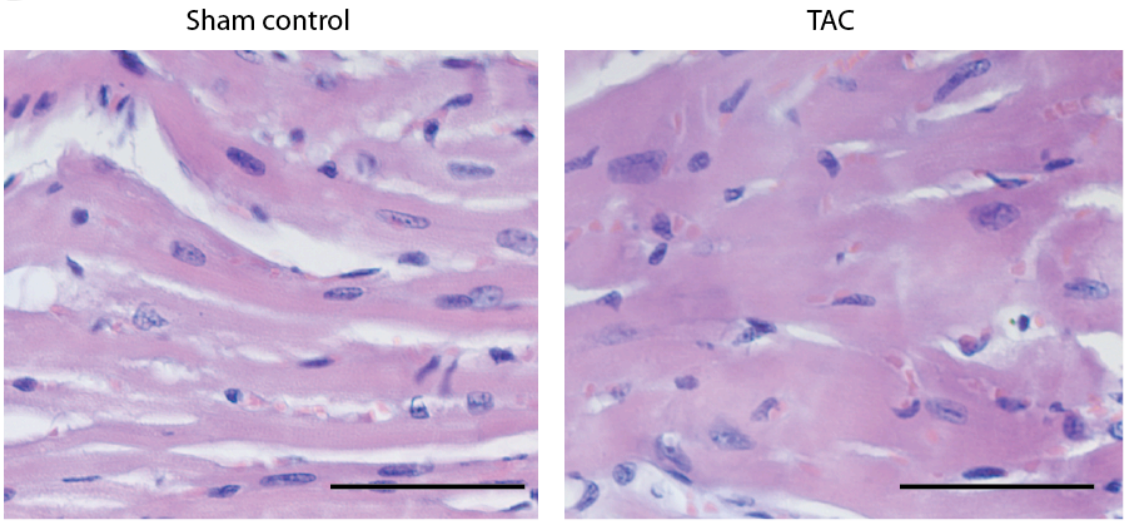
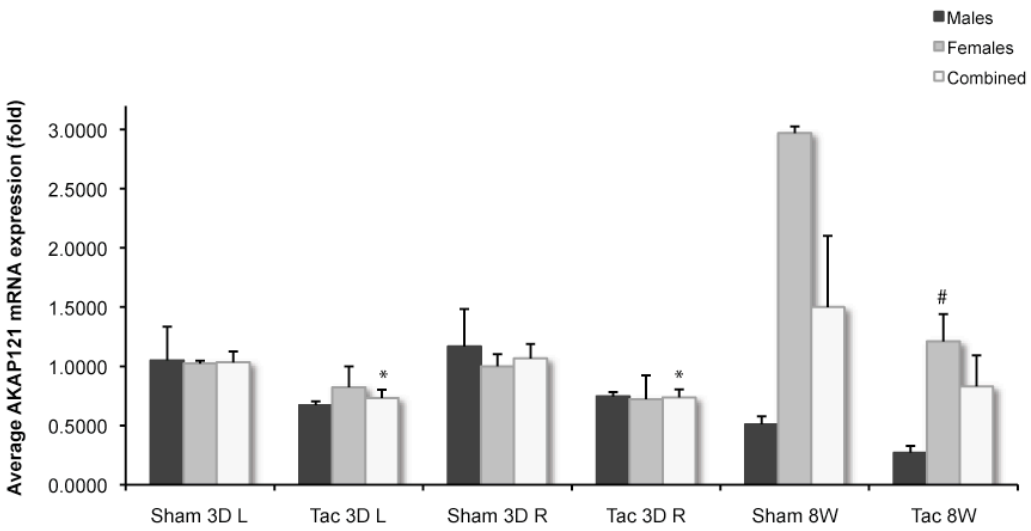


Figure 1. Cardiac hypertrophy induced by transverse aortic constriction (TAC) in C57BL/6 mice. **(A)** The increase in heart weight-to-body weight ratio in TAC males are higher at 3 days post-surgery as compared to females. At 8 weeks both sexes show a similar degree of hypertrophy (as measured by HW/BW ratio). The results represent the average percentage increase in HW/BW ratio in TAC animals above the sham controls ± S.E.; *P<0.05; # P<0.01 **(B)** Representative tissue sections of the hearts of the 8 week post surgery animals stained with H&E to visualize the hypertrophic changes. Scale bars indicate 50 μm.

A

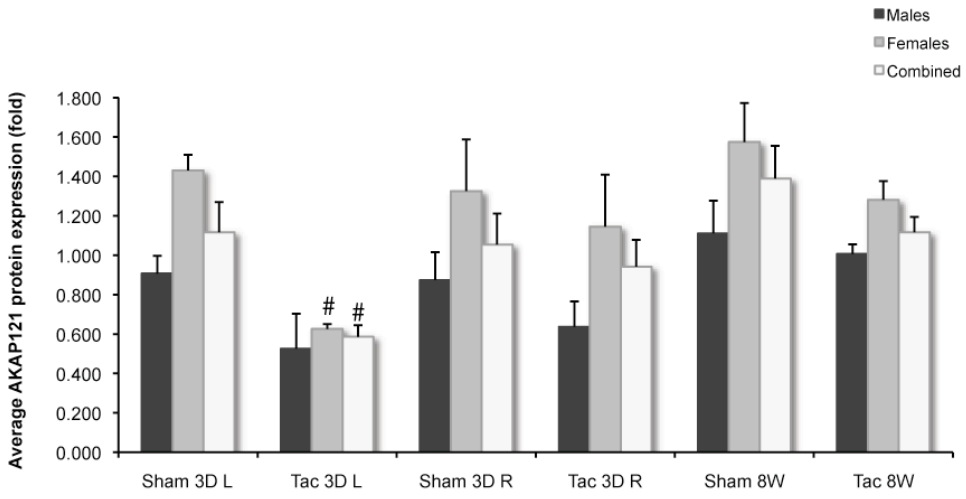


B

		ANP	BNP	Card-α actin	Card-β actin
Males	3D L	+	++ *	-	+
	3D R	-	++ *	-	+
	8W	+	++ #	-	+++*
Females	3D L	↔	+	↔	+
	3D R	↔	++++	↔	++
	8W	+*	++ #	-*	++ #

Figure 2. Transcriptional response of AKAP121 and markers of cardiac hypertrophy to pressure overload induced by transverse aortic banding (TAC). (**A)** Analysis of AKAP121 expression in sham control and banded (TAC) animals at 3 days and 8 weeks post surgery through quantitative real-time PCR. Results are shown as the pooled data for males and females, as well as separately for each sex. Results represent mean ± SE. *P<0.05 or #P<0.01 for TAC vs Sham. (**B)** Analysis of markers of cardiac hypertrophy through quantitative real-time PCR in all treatment groups. Results are shown according to the directional trend of each marker. Results represent mean ± SE. *P<0.05 #P<0.01 for TAC vs Sham. 3D, 3 days post surgery; 8W, 8 weeks post surgery; L, left heart; R, right heart; + upward trend; - downward trend; ↔ no change.

A



B

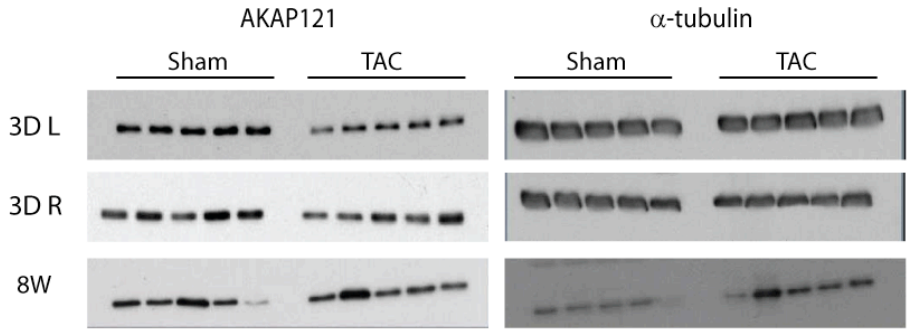


Figure 3. Regulation of AKAP121 protein levels in response to hypertrophic signals in transverse aortic banded (TAC) animals. **(A)** Quantitative analysis of AKAP121(110 kDa) protein levels comparing sham control and banded (TAC) animals at 3 days and 8 weeks post-surgery. #P<0.01. 3D, 3 days post surgery; 8W, 8 weeks post surgery; L, left heart, R, right heart. **(B)** Lysates of cardiac tissue were processed for western blotting using antibodies against AKAP121 or α-tubulin in 10 separate experiments (5 Sham, 5 TAC) at each time point.

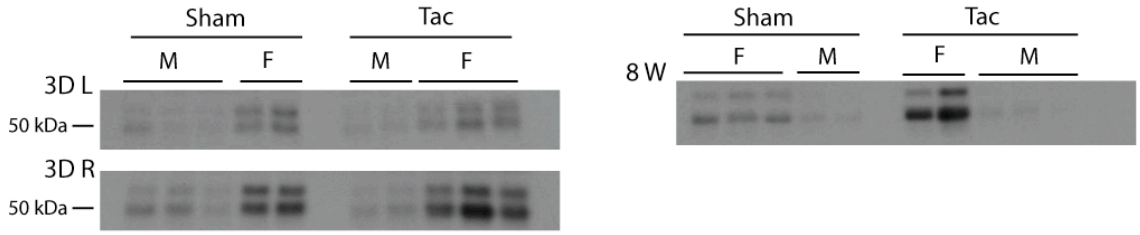


Figure 4. Increased expression of ~60kDa and 50kDa bands in female mouse hearts compared to males upon probing with AKAP149 primary antibody. Immunoblots for this region are shown for 3 days (3D) and 8 weeks (8W) post surgery. L, left heart; R, right heart; M, males; F, females.

TABLES

Table 1. Morphometric analysis of sham control and TAC C57BL/6 mice for assessment of TAC-induced cardiac hypertrophy at **(A)** 3 days and **(B)** 8 weeks post surgery. *P<0.05; #P<0.01.

A

3 DAYS	Males		Females	
	Sham (n=5)	Tac (n=5)	Sham (n=5)	Tac (n=5)
BW (g)	23.04 ± 0.96	22.24 ± 0.73	19.80 ± 0.45	18.48 ± 0.29
T (mm)	16.80 ± 0.74	18.80 ± 0.45 *	16.60 ± 0.25	17.40 ± 0.40
HW (mg)	0.106 ± 0.002	0.102 ± 0.005	0.102 ± 0.002	0.103 ± 0.002
HW/BW	5.04 ± 0.08	5.66 ± 0.09 #	5.35 ± 0.12	5.53 ± 0.16
HW/T	6.94 ± 0.30	6.70 ± 0.26 *	6.39 ± 0.16	5.88 ± 0.20

B

8 WEEKS	Males		Females	
	Sham (n=5)	Tac (n=5)	Sham (n=5)	Tac (n=5)
BW (g)	26.70 ± 0.71	29.48 ± 0.70 *	24.26 ± 0.43	24.64 ± 0.27
T (mm)	16.90 ± 0.33	17.10 ± 0.10	17.00 ± 0.00	17.40 ± 0.25
HW (mg)	0.13 ± 0.01	0.25 ± 0.03 #	0.12 ± 0.01	0.19 ± 0.03
HW/BW	4.87 ± 0.26	8.46 ± 1.09 *	5.12 ± 0.31	7.90 ± 1.11 *
HW/T	7.71 ± 0.49	14.47 ± 1.59 #	7.29 ± 0.40	11.18 ± 1.55 *