Exploring AKIP1 function in the heart

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Chapter 7

Summary and Future perspectives

SUMMARY

Heart failure (HF) is the common end stage syndrome of many cardiac disorders, including valve insufficiency (volume overload), hypertension (pressure overload), myocardial infarction and cardiomyopathies. These different initial events all result in altered ventricular wall stress triggering cardiac remodeling. One of the main processes that contributes to cardiac remodeling is cardiomyocyte growth (hypertrophy). Induction of hypertrophy is accompanied by marked changes in gene expression profiles of which the re-expression of fetal genes, the so called "fetal gene program" is well known[1,2]. The aim of this thesis was to generate an inventory of gene expression changes specific for cardiomyocyte hypertrophy and to further investigate the role of these changes in hypertrophy development (**chapter 1**). In particular, we explored the function of A kinase interacting protein 1 (AKIP1), which was identified in the inventory as a novel hypertrophy associated gene.

That cardiomyocyte growth and changes in cardiomyocyte function are the underlying causes of cardiac hypertrophy and HF development has been recognized for ages. Unravelling the molecular mechanism underlying these processes has, however, only started in the last decades and it has become clear that these changes are very complex. In **chapter 2**, we reviewed the current knowledge on gene expression regulation during hypertrophy development. We described how neurohormonal and biomechanical signals are transduced to the nucleus and affect a set of transcription factors and chromatin modulators. Calcium signaling and protein (de)phosphorylation plays an essential role in this transduction process. Multiple transcription factors, including MEF2, GATA, Nkx-2.5 and NFAT, cooperate in controlling cardiomyocyte gene expression and binding sites for these proteins have been found in promoters of many hypertrophy associated genes. However, even for an extensively explored gene like ANP (atrial natriuretic protein) the regulation of its expression *in vivo* is still mysterious[3]. Genetic modulation of signal transduction pathways and transcription factors in *in vitro* studies and in animal models has shown that targeting these systems allows modulation of hypertrophy development. However, due to their central role, not only in cardiomyocytes, but also in many other cells it is unlikely that targeting these central systems will provide therapeutic opportunities. We therefore postulated that it might be more promising to investigate the potential downstream targets of these pathways. Several studies that target downstream genes are currently underway and provide promising results, like BNP[4], SERCA[5], and myosin[6]. Generating an inventory of hypertrophy associated genes may therefore allow the identification of new potential targets against HF.

In order to identify novel HF associated genes, we performed a whole genome analysis on several cardiac hypertrophy/HF models and compared the gene expression profiles with

their corresponding control groups in **chapter 3**. *In vitro* models included PE, Iso and ET-1 stimulated neonatal rat ventricular cardiomyocytes (NRVCs) and *in vivo* animal models consisted of post-myocardial infarction (MI) and spontaneous hypertensive Ren-2 rat models. In addition to the identification of established hypertrophy associated genes like ANP, BNP, XIRP2 and FHL1, we also identified a number of genes not previously reported to be associated with hypertrophy/HF. Also *in vitro* and *in vivo* specific gene sets were identified as well as genes specific for the underlying aetiologies. Together these results showed that combining multiple models, both *in vivo* and *in vitro*, could provide a robust set of hypertrophy/HF associated genes and provided insight in the differences between the used models. Amongst others we identified Dhrs7c[7] and AKIP1 to be differentially expressed in most models. The latter was also one of the strongest upregulated genes and hence was further investigated as described in the next chapters.

In **chapter 4**, we investigated the role of AKIP1 in hypertrophy development in NRVCs. We first confirmed the gene array data and showed that AKIP1 expression was elevated in the used hypertrophy/HF models both at the mRNA level and at the protein level. Gain of function experiments by overexpressing AKIP1 revealed that AKIP1 overexpression was sufficient to induce hypertrophy as determined by an increase in cell size, protein synthesis and cytoskeletal reorganization. This hypertrophy effect was, however, not associated with re-expression of the "fetal gene program". Further analysis revealed that Akt kinase pathway was activated and essential for AKIP1-induced hypertrophy effect. Moreover, downstream targets controlling protein synthesis, including rpS6 and eEF2, were activated by AKIP1 overexpression. Although, AKIP1 was not essential for PE induced hypertrophy in NRVCs, it did potentiate neurohormonal induced protein synthesis. In conclusion, our results showed that AKIP1 was induced in hypertrophic/HF hearts and could stimulate adaptive cardiomyocyte growth *in vitro* via Akt signaling.

During our studies on hypertrophy it was reported by others that besides nucleus/cytoplasmic localization, AKIP1 also localized to mitochondria[8]. Given the strong association between bioenergetics and hypertrophy we decided to investigate the role of AKIP1 in metabolic respiration. In **chapter 5**, the effect of AKIP1 on the oxygen consumption rate (OCR) in cardiomyocytes was described. Using a Seahorse flux analyzer the OCR in cultured cardiomyocytes was investigated and it was shown that PE significantly stimulated the OCR. Silencing of AKIP1 attenuated this increase indicating that this was partially mediated via AKIP1 upregulation. Interestingly, AKIP1 overexpression was sufficient to stimulate OCR. This increase of OCR was independent of glycolytic flux, since a similar increase was observed by using pyruvate instead of glucose as a substrate. Mitochondrial biogenesis was not enhanced in AKIP1 overexpressing cells. Instead, we observed lower levels of the electron transport chain (ETC) complexes,

suggesting that these complexes worked more efficient. Despite increased OCR, less superoxide was generated, and this was not a result of increased expression of reactive oxygen species (ROS) scavenging systems. This further supports the notion that the ETC worked more efficiently in AKIP1 overexpressing cells. In contrast silencing of AKIP1 resulted in enhanced production of reactive oxygen species (ROS) production without changes of ETC complexes, suggesting less efficient electron transport coupling. Together, these results show that AKIP1 is able to modulate mitochondrial function and limit ROS production in cardiomyocytes. This more efficient respiration could be important under conditions of stress, and hence might be a compensatory effect. In line with this idea is the recent observation that mouse hearts with overexpressed AKIP1 are more resistant against ischemia/reperfusion *ex vivo*[8]. Thus, AKIP appears to modulate mitochondrial function resulting in improved respiration and less production of toxic ROS.

To translate the *in vitro* findings to the *in vivo* situation, we generated a cardiac specific AKIP1 transgenic (Tg) mouse line as described in **chapter 6**. Based on our *in vitro* studies, we first evaluated cardiac hypertrophy in these mice. However, we did not observe a spontaneous hypertrophy phenotype in AKIP1-Tg mice at 4 and 12 month of age. Subsequently, these mice were subjected to a transverse aortic constriction (TAC) pressure overload model for chronic hypertrophy. Sham-operated mice were used as controls. TAC induced considerable hypertrophy in both Wt and AKIP1-Tg groups, as determined by increased heart weight, cardiomyocyte size and hypertrophic markers. Moreover, both aortic and LV pressures were higher in the TAC groups and cardiac contractility and relaxation functions decreased in TAC mice, resulting in reduced cardiac function as assessed by echocardiography and cardiac MRI measurement. Unexpectedly, we did not observe any differences between Wt and AKIP1-Tg animals. Thus, whereas *in vitro* in neonatal rat cardiomyocytes AKIP1 induced profound cardiomyocyte hypertrophy this was absent in the *in vivo* situation in transgenic mice. As discussed in chapter 6, there might be multiple reasons for this observed difference, like rat versus mouse, neonatal versus adult and of course the different *in vitro* context. Thus, although AKIP1 expression is clearly elevated in multiple hypertrophy/HF models our current data do not support a role for AKIP1 in mediating cardiomyocyte growth *in vivo*.

FUTURE PERSPECTIVES

AKIP1 is upregulated upon multiple forms of cardiac stress and although it could induce cardiomyocyte growth *in vitro* it did not appear to have a similar effect *in vivo* in a pressure overload model. Further investigations into other hypertrophy models (adrenergic- and exercise-induced hypertrophy) will be required and may show that this effect could be stimulus dependent. During our studies, another group reported that AKIP1 expression was

controlled by oxidative stress and that AKIP1 could attenuate cardiac ischemia/reperfusion damage *ex vivo*[8,9]. We also observed changed oxidative consumption rates in AKIP1 overexpressing cardiomyocytes and reduced ROS formation in these cells *in vitro*. This is highly interesting because under normal conditions increased oxidative metabolism is accompanied by increased ROS production. Moreover, AKIP1 silencing in cardiomyocytes had an opposite effect and resulted in increased ROS production. Thus, AKIP1 could be an important target in controlling toxic ROS radicals. Therefore investigating mitochondrial respiratory function and ROS production in our transgenic mouse may be a more rewarding avenue. Preliminary results showed already that the AKIP1 transgenic mice are less prone to *in vivo* cardiac ischemia/reperfusion injury (unpublished data from Harmen G. Booij and B. Daan Westenbrink), confirming and extending the *ex vivo* data. Whether this is a result of diminished ROS production has still to be determined, but it does support the notion that AKIP1 upregulation may have a stress adaptive function. Finally, it will be interesting to investigate whether an AKIP1 knock out mouse would be more prone to oxidative damage and show advanced aging.

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Nederlandse samenvatting

SAMENVATTING

Hartfalen (HF) is het eindstadium van veel verschillende cardiovasculaire aandoeningen, waaronder een verminderde werking van de hartkleppen (volume belasting), hoge bloeddruk (drukbelasting), hartinfarct en andere hartspier aandoeningen. Deze verschillende oorzaken resulteren allemaal in een verhoogde wandspanning van de kamer, en kunnen leiden tot remodelering van het hart. Een van de belangrijkste processen die bijdraagt aan deze remodelering is de groei van de hartspiercellen, zogenoemde hypertrofie. Hypertrofie gaat gepaard met veranderingen in de expressie van specifieke genen. De activatie van het "fetal gene program" is hiervan een bekend fenomeen, waarbij genen die normalerwijze in het foetale hart tot expressie komen weer tot re-expressie komen in het adulte zieke hart[1,2]. Het doel van dit proefschrift was het genereren van een overzicht van deze specifieke genexpressie veranderingen en om de functie van deze genexpressie veranderingen te onderzoeken (Hoofdstuk 1). Hierbij hebben we ons specifiek gericht op het gen coderend voor A kinase interacting protein 1 (AKIP1), die prominent naar voren kwam in onze screen naar hypertrofie geassocieerde genexpressie veranderingen.

Het is reeds bekend dat de groei van hartspiercellen en veranderingen in de functie van deze cellen belangrijke onderliggende oorzaken zijn van de ontwikkeling van hartfalen. De laatste decennia, wordt in toenemende mate onderzoek gedaan naar de complexe moleculaire mechanismen die hieraan ten grondslag liggen. In hoofdstuk 2 hebben we de huidige stand van zaken over de regulatie van genexpressie tijdens de ontwikkeling van hypertrofie en hartfalen beschreven. We beschrijven hierin hoe neuro-hormonale en biomechanische signalen worden overgebracht naar de celkern en daar effect hebben op de activiteit van transcriptiefactoren en chromatine modulatoren. Bij deze signaaloverdracht spelen calcium en eiwit (de)defosforylatie een belangrijke rol. In de celkern werken verschillende transcriptiefactoren, zoals MEF2, GATA, NRX2.5 en NFAT, samen om de genexpressie te reguleren. Bindingsplaatsen voor deze transcriptiefactoren zijn veelvuldig aanwezig in promotorgebieden van hypertrofie geassocieerde genen. Echter, zelfs voor uitvoerig onderzochte genen zoals ANP (atrial natriuretic peptide) is de precieze regulatie van de expressie door deze transcriptiefactoren nog steeds niet opgehelderd[3]. Uit *in vitro* studies en diermodellen is gebleken dat de ontwikkeling van hypertrofie beïnvloed kan worden door het manipuleren van deze signaaltransductie routes en transcriptiefactoren. Klinisch is dit echter moeilijk toe te passen, omdat deze factoren een essentiële functie in hartspier- en vele andere cellen hebben. Het is daarom wellicht zinvoller om de rol van downstream genen die een minder centrale functie innemen te onderzoeken. Zo worden er momenteel meerdere studies gedaan waarbij downstream genen zoals BNP[4], SERCA[5] en myosine[6] worden gemanipuleerd, waarmee hopelijk postieve resultaten behaald kunnen

worden. Het identificeren van genen die met hypertrofie geassocieerd zijn kan daarom mogelijk nieuwe kandidaten opleveren voor het behandelen van HF.

Om genen die geassocieerd zijn met hypertrofie en HF te identificeren hebben we in hoofdstuk 3 een analyse gemaakt van de genexpressie van het hele genoom in verschillende hypertrofie en HF modellen en vergeleken met de bijbehorende controlegroepen. De *in vitro* modellen die we hierbij hebben gebruikt waren neonatale rat hartspiercellen die waren gestimuleerd met fenylefrine (PE), isoprenaline (Iso) en endotheline (ET-1). De *in vivo* diermodellen bestonden uit ratten met een hart infarct (MI) en REN2 ratten.Deze laatste ontwikkelen een hoge bloeddruk wat leidt tot HF. Naast de identificatie van bekende met hypertrofie geassocieerde genen zoals ANP, BNP, Xirp2 en FHL1 hebben we ook een aantal genen gevonden waarvan de relatie met hypertrofie en HF niet eerder beschreven was. Daarnaast werden clusters van genen gevonden die specifiek waren voor de *in vitro* en *in vivo* situatie, evenals genen die specifiek waren voor de onderliggende oorzaak. Deze resultaten laten zien dat door verschillende modellen, zowel *in vitro* als *in vivo,* te combineren, een robuuste lijst met hypertrofie en HF geassocieerde genen verkregen kan worden die ook inzicht geeft in de verschillen tussen de gebruikte modellen. We hebben onder andere Dhrs7c[7] en AKIP1 geïdentificeerd als genen die in de meeste modellen differentieel tot expressie kwamen. Laatstgenoemde was één van de genen waarvan de expressie sterk omhoog ging in verschillende modellen. Daarom hebben we AKIP1 verder onderzocht zoals beschreven wordt in de volgende hoofdstukken.

In hoofdstuk 4 hebben we de rol van AKIP1 in de ontwikkeling van hypertrofie onderzocht in neonatale hartspiercellen. We hebben eerst de data van de genanalyse uit hoofdstuk 3 bevestigd door te laten zien dat de expressie van AKIP1 verhoogd was in de gebruikte hypertrofie en HF modellen zowel op mRNA als op eiwit niveau. Studies waarbij AKIP1 tot overexpressie werd gebracht, lieten vervolgens zien dat AKIP1 overexpressie resulteerd in de ontwikkeling van hypertrofie. Onder deze condities werd een duidelijke toename in celgrootte gemeten, aslwel een verhoogde eiwitsynthese en een veranderde organisatie van het cytoskelet. Activatie van het “fetal gene program bleef echter achterwege. Verdere analyse liet zien dat de Akt kinase signaalroute werd geactiveerd en dat dit essentieel was voor het door AKIP1 geïnduceerde effect. Ook andere eiwitten die de eiwitsynthese beïnvloeden, waaronder rpS6 en eEF2 werden geactiveerd door AKIP1 overexpressie. AKIP1 was niet nodig voor de door PE geïnduceerde hypertrofie in neonatale rat hartspiercellen, maar versterkte wel de eiwit synthese. Onze resultaten laten zien dat AKIP1 wordt geïnduceerd in harten met hypertrofie en HF en dat AKIP1 de groei van hartspiercellen *in vitro* stimuleert via de Akt signaalroute.

Tijdens onze studies kwam er een publicatie uit, waarin beschreven werd dat AKIP1 naast de reeds bekende lokalisatie in het cytoplasma en de kern, ook gelokaliseerd is in de mitochondriën[8]. Doordat er een sterke relatie is tussen de bio-energetische regulatie en

hypertrofie hebben we vervolgens gekeken naar de rol van AKIP1 in het metabolisme in hartspiercellen. In hoofdstuk 5 hebben we het effect van AKIP1 op de zuurstof opname snelheid (OCR) in hartspiercellen beschreven. Door gebruik te maken van een Seahorse flux analyzer hebben we de OCR in hartspiercellen onderzocht en hebben we laten zien dat PE de OCR significant stimuleert. Deze toename in OCR kon worden verlaagd door de AKIP1 expressie te verlagen (AKIP1 silencing). Dit geeft aan dat deze toename in OCR na PE toediening gedeeltelijk werd veroorzaakt door een verhoging van AKIP1. Overexpressie van AKIP1 liet vervolgens zien dat dit resulteerde in een verhoogde OCR. Deze toename was onafhankelijk van de glycolyse, omdat een vergelijkbare toename werd gemeten met pyruvaat als substraat. Er werd echter geen toename van het aantal mitochondriën in cellen met AKIP1 overexpressie gemeten en het bleek dat de complexen van de electron transport chain (ETC) zelfs verlaagd waren. Dit suggereerde dat de ETC onder deze condities efficiënter functioneerde. Inderdaad werd er ondanks de verhoging van de OCR minder superoxide gemeten in de mitochondriën wat niet het resultaat was van veranderingen in expressie van antioxidant systemen in de cel. Dit bevestigt een efficiëntere werking van de ETC in cellen met AKIP1 overexpressie. In cellen met verlaagde AKIP1 expressie werden daarentegen verhoogde niveaus van reactieve zuurstof moleculen (ROS) gemeten, zonder veranderingen in de ETC niveaus. Dit duidt op een minder efficiënte ETC koppeling in afwezigheid van AKIP1. Deze resultaten laten zien dat AKIP1 de mitochondriële functie moduleert en de ROS productie verminderd in hartspiercellen. Deze efficiëntere cellulaire respiratie kan vooral belangrijk zijn onder stressvolle situaties, en kan daarom een compenserend mechanisme zijn. De recente observatie dat harten van muizen met AKIP1 overexpressie meer beschermd zijn tegen ischemie/reperfusie schade *ex vivo* sluit aan bij deze bevindingen[8]. AKIP1 lijkt de mitochondriële functie te beïnvloeden wat leidt tot een verbeterde cellulaire respiratie en verminderde productie van toxische ROS.

Om de bevindingen van de *in vitro* experimenten te bevestigen *in vivo,* hebben we een transgene muis (Tg) gemaakt die AKIP1 specifiek in het hart tot expressie brengt (Hoofdstuk 6). Gebaseerd op ons *in vitro* onderzoek hebben we eerst gekeken naar hypertrofie in de harten van deze muizen. We zagen geen spontaan hypertroof fenotype in deze muizen op een leeftijd van 4 en 12 maanden tenopzichte van de niet transgene wildtype (Wt) muizen. Daarom hebben we vervolgens een drukbelastingsmodel voor chronische hypertrofie gebruikt. We hebben een transverse aorta constrictie (TAC) toegepast, waarbij sham geopereerde dieren werden gebruikt als controle groep. TAC induceerde een duidelijke hypertrofie in zowel de Wt als de Tg groepen , zoals bepaald door een toename in hartgewicht, hartspiercel grootte en hypertrofie markers. Zowel de druk in de aorta als in de LV waren hoger in de TAC groepen en de contractie en de relaxatie van het hart waren verminderd. Dit resulteerde in een verminderde functie van het hart dat bepaald werd d.m.v. echocardiografie en MRI. Helaas zagen we geen verschillen

tussen de Wt en Tg dieren. Dus, waar AKIP1 in *in vitro* experimenten in neonatale hartspiercellen duidelijk hypertrofie induceerde, zagen we dit effect niet in de transgene muizen. Zoals we beargumenteren in hoofdstuk 6 kunnen hier meerdere redenen voor zijn, zoals verschillen in species (rat versus muis), verschillen in leeftijd (neonataal versus adult) en natuurlijk de bijzondere *in vitro* context. Samengevat, hoewel AKIP1 expressie duidelijk verhoogd is in meerdere hypertrofie en HF modellen kunnen onze data de rol van AKIP1 in het moduleren van de groei van de hartspiercel *in* vivo momenteel niet ondersteunen.

TOEKOMST PERSPECTIEVEN

AKIP1 expressie is verhoogd in meerdere modellen van cardiale stress en hoewel het de groei van hartspiercellen *in vitro* induceerde bleek AKIP1 niet hetzelfde effect te hebben in een drukbelastingsmodel *in vivo*. Omdat we momenteel niet kunnen uitsluiten dat dit context afhankelijk is, is verder onderzoek in andere hypertrofie modellen nodig, zoals adrenerge of inspanning (training) geïnduceerde hypertrofie. Tijdens onze experimenten werd door anderen gepubliceerd dat de expressie van AKIP1 beïnvloed werd door oxidatieve stress en dat AKIP1 de schade die ontstaat bij ischemie/reperfusie *ex vivo* kan verminderen[8,9]. Onze metabole studies lieten zien dat AKIP1 overexpressie de zuurstof consumptie snelheid in cellen verhoogde en tegelijkertijd de ROS niveaus in deze cellen verminderde. Dit is een interessante bevinding, omdat onder normale omstandigheden een verhoging van de zuurstof consumptie gepaard gaat met een verhoging van de ROS productie. In cellen met verminderde AKIP1 expressie zagen we een tegenovergesteld effect, namelijk een verhoging van de ROS niveaus. AKIP1 kan dus een belangrijke functie hebben bij het reguleren van de toxische ROS productie. Daarom zou het onderzoeken van de mitochondriële functie en de ROS productie in de transgene AKIP1 muizen een belangrijke volgende stap moeten zijn. Voorlopige resultaten laten zien dat de AKIP1 transgene muizen minder vatbaar zijn voor schade die wordt geïnduceerd door ischemie/reperfusie *in vivo* (niet gepubliceerde data van Harmen G. Booij en B. Daan Westenebrink). Dit is een bevestiging van de *ex vivo* data. Of dit een resultaat is van een verminderde ROS productie moet nog onderzocht worden, maar het ondersteund wel de stelling dat het verhogen van de expressie van AKIP1 mogelijk een aanpassing is van de cel op stress. Tot slot zou het interessant zijn om te onderzoeken of een AKIP1 knock-out muis meer vatbaar is voor oxidative schade en mogelijk versnelde veroudering.

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Curriculum Vitae

Hongjuan Yu was awarded her Masters degree in Medical Sciences in 2008 after 7 years of study at Harbin Medical University (HMU). Under the supervision of Prof. dr. Jin Zhou and Prof. dr. Jialan Shi, she investigated the relationship between phosphatidylserine exposure and coagulant activity of blood cells during blood storage. Following her graduation, she was trained as a resident physician at the First Affiliated Hospital of HMU. To improve her scientific skills, she began her PhD project in January 2010 at the department of Experimental Cardiology, University Medical Center Groningen, under the supervision of Prof. dr. W.H. van Gilst and Dr. H.H.W. Silljé. Her PhD thesis focused mainly on investigating the role of novel heart failure-associated genes in the heart. During her research period, she learned multiple experimental techniques, especially in the field of molecular and cellular biology, and learned to work as an independent researcher. After 4 years of research, she completed her thesis writing and will obtain her PhD degree in December 2013. Thereafter, she will return to China and continue her research and clinical training at the First Affiliated Hospital of HMU.