Chapter 6

General discussion

GENERAL DISCUSSION

Although MEF2 has been described as a common end-point for several prohypertrophic signaling cascades in the heart [1-3], the direct consequences of MEF2 activation in cardiac muscle remained to be demonstrated. The aim of this thesis was to dissect the functional role of MEF2 in the development of cardiac hypertrophy and heart failure.

One central pathway that transduces intracellular prohypertrophic signals in heart muscle employs the Ca^{2+} activated phosphatase calcineurin [4]. MEF2 transcription factors are activated downstream of cardiac calcineurin activity, but the precise mechanism of this activation remained to be determined [5, 6]. In this thesis, we demonstrated that MEF2 transcriptional activity in the heart is induced by recruiting the well-described calcineurin target NFAT (Chapter 2), a process earlier identified in T-cells [7]. This observation is reminiscent of the cooperative interaction between MEF2 proteins and myogenic basic helix-loop-helix factors in skeletal muscles [8] and between MEF2 proteins and GATA factors in cardiac muscles [9], and it suggests that MEF2 factors interact with a range of transcription factors to optimize gene expression. Given the overlapping expression patterns of MEF2 and NFAT factors in smooth muscle, skeletal muscle, cardiac muscle, and the central nervous system, the NFAT/MEF2-dependent pathway described in this work suggests the existence of this molecular paradigm for Ca^{2+}/calmodulin signaling in excitable tissue.

We analyzed MEF2 transcription factor function in adult cardiac muscle, starting from the premise that MEF2 would be primarily involved in the initial hypertrophic remodeling phase of the heart muscle [10]. However, a Cre/LoxP-dependent approach to antagonize cardiac MEF2 transcriptional activity prevented calcineurin induced ventricular chamber dilation and cardiac dysfunction, with minor effects on cardiac growth (Chapter 2). Furthermore, transgenic mice overexpressing MEF2A in the heart displayed ventricular chamber dilation, an observation recently confirmed by others [11]. These results are in line with our data obtained from *in vitro* experiments, as we observed an elongated morphology of cultured cardiomyocytes associated with disassembly of myofibril components, which affected both M-band and Z-disc components, and redistribution of focal adhesion sites upon increasing MEF2 transcriptional activity (Chapter 3).

Many cardiomyopathic conditions share the common characteristic of myofibril degeneration, which contrasts the promotion of sarcomere organization during early phases of cardiac hypertrophy [12-14]. Alterations in myofibrillar architecture and focal adhesion sites are also observed in cardiomyocytes from MLP knockout mice and tropomodulin-overexpressing transgenic mice, two models with severe chamber dilation [15]. Cardiomyocytes from these mice present striking alterations

of their cell-matrix as well as their cell-cell contacts. Isolated myocytes from these cardiomyopathic models elicit a more irregular shape compared with their wild-type counterparts [16], which may serve to increase the number of focal contacts between neighboring cells. Specifically, the costameric protein vinculin showed patchy staining near the plasma membranes in MLP null cardiomyocytes compared with the regular well-ordered striations that are observed at the membrane of wildtype cardiomyocytes [16]. These differences in vinculin subcellular distribution resemble the changes observed in our study on vinculin localization in hypertrophic and elongated myocytes following MEF2 activation. Increased expression of extracellular matrix components, and a general upregulation of vinculin has also been described in patients with DCM [13, 17], suggesting that this phenomenon might not be restricted to mouse models for this disease.

Although overexpression of (truncated mutants of) transcription factors harbors the risk of unwanted transcriptional squelching, the consistency in opposite phenotypes we observed using our combinatorial dominant-negative versus gain-of-function models for MEF2 transcriptional activity justifies the overall conclusions drawn in this thesis. These results suggest that MEF2 promotes symptoms that are more compatible with later manifestations of cardiac disease, which extend beyond and stem from initial pathological cardiac growth, namely chamber dilation, mechanical dysfunction, and end-stage heart failure. This conclusion is further underlined by microarray analyses described in Chapter 2 and 3, which were performed to gain mechanistic insights into the cardiomyopathic remodeling processes. Namely, the classification profiles of MEF2 target genes secondary to cardiac calcineurin stress signaling correlate well with those observed in gene chip analysis of human end-stage idiopathic dilated cardiomyopathy [18] and with the most characteristic pathological features of human end-stage dilated cardiomyopathy, which include a dynamic redistribution of cytoskeletal structures, extensive fibrosis, and extracellular alterations in mitochondria number and morphology [13]. In contrast, the classification profiles of MEF2 target genes identified revealed surprisingly little overlap with those observed with massive hypertrophic remodeling in early pressure overload (http://cardiogenomics.med.harvard.edu). The mere existence of a genetic pathway specifically underlying ventricular dilation will be of fundamental clinical and therapeutic interest.

One very interesting gene identified in this thesis by microarray analysis, using an inducible MEF2 activity assay in a novel cardiomyocyte cell line [19] to screen for early target genes, is myotonic dystrophy protein kinase (DMPK). We demonstrated that DMPK is a direct transcriptional target of MEF2 and, more importantly, that knockdown of DMPK expression was sufficient to inhibit cardiomyocyte elongation and sarcomere disassembly in response to elevated MEF2 activity (Chapter 3). The progression of the inherited neuromuscular disease

myotonic dystrophy 1 involves an expansion of untranslated CTG repeats in the 3' prime UTR of *dmpk*, which is believed to result in a pathological accumulation of RNA in the nucleus [20, 21]. However, the cardiac DM1 pathology may be more directly due to increased DMPK protein levels, rather than RNA toxicity [22]. In line, transgenic overexpression of DMPK in murine muscle evokes a heart failure phenotype associated with extensive myocyte disarray and interstitial fibrosis [23]. In this thesis, we identify DMPK as a direct transcriptional target of MEF2, promoting myocyte elongation, myofibril degeneration and polarized focal adhesion redistribution, changes of the heart muscle, which are characteristic of dilated cardiomyopathy, and we postulate that DMPK may serve as a promising therapeutic target for cardiomyopathic conditions.

Given the similarities between the aspects of MEF2 induced cardiomyocyte remodeling and the cardiac phenotype of MLP knockout mice [16, 24], we examined the activation levels of MEF2 in MLP deficient hearts (Chapter 4). Expression of a MEF2 reporter transgene [25] was significantly enhanced in the absence of MLP protein. Although the molecular mechanism underlying this activation is unclear, plausible explanations do exist. Disruption of the gene for MLP leads to impaired cytoskeletal organization in cardiomyocytes [24]. Although the mechanistical link between disturbances of the cytoskeleton in individual cardiomyocytes and the progression to dilated cardiomyopathy is unclear, several studies have reported the involvement of impaired intracellular calcium homeostasis [26-28]. This could underlie the observed increase in MEF2 activity, as MEF2 is a well-known downstream effector of calcium signaling [1, 3]. In addition, calsarcin-1 has been found to interact with calcineurin at the Z-disc and mice lacking calsarcin-1 protein are sensitized to calcineurin signaling and display an accelerated progression to cardiomyopathy [29]. Calcineurin is known to activate MEF2 transcriptional activity in the heart [30]. Loss of Z-disc integrity by MLP deficiency has also been described to result in decreased calcineurin compartmentalization, although this was thought to result in inhibition of calcineurin activity [31].

Since cardiac MEF2 activity was strongly enhanced in homozygous MLP knockout mice, we analyzed whether MEF2 inhibition strategies would attenuate the severe dilated cardiomyopathy in this relevant mouse model. As our approach to inhibit MEF2 activity did not result in improvement of the dilated cardiac phenotype, we conclude that pathological cardiac remodeling in MLP deficient mice is independent of MEF2. Mechanistically, expression levels of DMPK, a MEF2 transcriptional target involved in maladaptive cardiomyocyte remodeling (Chapter 3), are unchanged in hearts from MLP knockout mice. Nevertheless, we cannot fully exclude that the inactivation potential of the dominant-negative transgene is insufficient to cope with the dramatic increase in MEF2 activity observed in hearts

of MLP deficient mice and that MEF2 target genes, other than DMPK, may be responsible for the onset of dilated cardiomyopathy in the MLP deficient myocardium.

Next, we tested whether MEF2 would be involved in the genesis of dilation and severe cardiac dysfunction following a pathophysiological trigger, such as sustained biomechanical stress (Chapter 5). Therefore, we used our *in vivo* MEF2 inhibitory strategy in pressure overload conditions (TAC). Unexpectedly, we observed a clear decrease in cardiac function in DNMEF2 mice at 5 weeks of pressure overload. Examination of PGC-1α transcript levels provided mechanistical insights into this phenomenon, as this demonstrated a decrease in PGC-1α expression after TAC and a lower level of PGC-1α transcripts in sham-operated DNMEF2 mice when compared to wildtype counterparts. PGC-1α expression is often upregulated in response to increased energy demand. In skeletal muscle, for example, swimming exercise results in activation of PGC-1α expression [32]. Nevertheless, earlier studies have reported decreased PGC-1α expression in response to aortic banding or in other models for cardiac disease [33-38]. MEF2 is known to bind and activate the PGC-1α promoter, especially when coactivated with PGC-1α itself [39]. Disruption of this autoregulatory loop by inhibition of MEF2 transcriptional activity would be a plausible explanation for the lower PGC-1α transcript levels observed in our DNMEF2 mice at basal conditions. Furthermore, this basal reduction in PGC-1α expression can explain the accelerated heart failure development in DNMEF2 mice after aortic banding, as it has been shown that a certain threshold level of PGC-1α is critical for the heart to cope with enhanced stress [34, 40]. As PGC-1α is known to be the key regulator of mitochondrial biogenesis, the impaired cardiac adaptation in DNMEF2 mice could be caused by a decrease in functional mitochondria.

Conclusively, data in Chapter 5 demonstrate that MEF2 inhibition, although it is clearly beneficial downstream of exclusive calcineurin activation, results in enhanced cardiac dysfunction due to impaired mitochondrial adaptation in the broad setting of pressure overload, and questions the use of MEF2 as a therapeutic target for cardiac disease.

REFERENCES

1. Frey N., Katus H.A., Olson E.N., Hill J.A. Hypertrophy of the heart: a new therapeutic target? Circulation 2004;109(13):1580-9.
2. McKinsey T.A., Olson E.N. Toward transcriptional therapies for the failing heart: chemical screens to modulate genes. J Clin Invest 2005;115(3):538-46.
3. Heineke J., Molkentin J.D. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 2006;7(8):589-600.

4. Wilkins B.J., Molkentin J.D. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun 2004;322(4):1178-91.
5. Zhang C.L., McKinsey T.A., Chang S., Antos C.L., Hill J.A., Olson E.N. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 2002;110(4):479-88.
6. Passier R., Zeng H., Frey N., Naya F.J., Nicol R.L., McKinsey T.A., Overbeek P., Richardson J.A., Grant S.R., Olson E.N. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest 2000;105(10):1395-406.
7. Youn H.D., Chatila T.A., Liu J.O. Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. Embo J 2000;19(16):4323-31.
8. Molkentin J.D., Olson E.N. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci U S A 1996;93(18):9366-73.
9. Morin S., Charron F., Robitaille L., Nemer M. GATA-dependent recruitment of MEF2 proteins to target promoters. Embo J 2000;19(9):2046-55.
10. Olson E.N., Schneider M.D. Sizing up the heart: development redux in disease. Genes Dev 2003;17(16):1937-56.
11. Xu J., Gong N.L., Bodi I., Aronow B.J., Backx P.H., Molkentin J.D. Myocyte enhancer factors 2A and 2C induce dilated cardiomyopathy in transgenic mice. J Biol Chem 2006;281(14):9152-62.
12. Mann D.L., Urabe Y., Kent R.L., Vinciguerra S., Cooper G.t. Cellular versus myocardial basis for the contractile dysfunction of hypertrophied myocardium. Circ Res 1991;68(2):402-15.
13. Schaper J., Froede R., Hein S., Buck A., Hashizume H., Speiser B., Friedl A., Bleese N. Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. Circulation 1991;83(2):504-14.
14. Beltrami C.A., Finato N., Rocco M., Feruglio G.A., Puricelli C., Cigola E., Sonnenblick E.H., Olivetti G., Anversa P. The cellular basis of dilated cardiomyopathy in humans. J Mol Cell Cardiol 1995;27(1):291-305.
15. Sussman M.A., Welch S., Cambon N., Klevitsky R., Hewett T.E., Price R., Witt S.A., Kimball T.R. Myofibril degeneration caused by tropomodulin overexpression leads to dilated cardiomyopathy in juvenile mice. J Clin Invest 1998;101(1):51-61.
16. Ehler E., Horowits R., Zuppinger C., Price R.L., Perriard E., Leu M., Caroni P., Sussman M., Eppenberger H.M., Perriard J.C. Alterations at the intercalated disk associated with the absence of muscle LIM protein. J Cell Biol 2001;153(4):763-72.
17. Wilke A., Schonian U., Herzum M., Hengstenberg C., Hufnagel G., Brilla C.G., Maisch B. [The extracellular matrix and cytoskeleton of the myocardium in cardiac inflammatory reaction]. Herz 1995;20(2):95-108.
18. Grzeskowiak R., Witt H., Drungowski M., Thermann R., Hennig S., Perrot A., Osterziel K.J., Klingbiel D., Scheid S., Spang R., Lehrach H., Ruiz P. Expression profiling of human idiopathic dilated cardiomyopathy. Cardiovasc Res 2003;59(2):400-11.
19. Rybkin, II, Markham D.W., Yan Z., Bassel-Duby R., Williams R.S., Olson E.N. Conditional expression of SV40 T-antigen in mouse cardiomyocytes facilitates an inducible switch from proliferation to differentiation. J Biol Chem 2003;278(18):15927-34.
20. Davis B.M., McCurrach M.E., Taneja K.L., Singer R.H., Housman D.E. Expansion of a CUG trinucleotide repeat in the 3' untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts. Proc Natl Acad Sci U S A 1997;94(14):7388-93.
21. Mahadevan M., Tsilfidis C., Sabourin L., Shutler G., Amemiya C., Jansen G., Neville C., Narang M., Barcelo J., O'Hoy K., et al. Myotonic dystrophy mutation: an unstable

CTG repeat in the 3' untranslated region of the gene. Science 1992;255(5049):1253-5.
22. Mahadevan M.S., Yadava R.S., Yu Q., Balijepalli S., Frenzel-McCardell C.D., Bourne T.D., Phillips L.H. Reversible model of RNA toxicity and cardiac conduction defects in myotonic dystrophy. Nat Genet 2006;38(9):1066-70.
23. O'Cochlain D.F., Perez-Terzic C., Reyes S., Kane G.C., Behfar A., Hodgson D.M., Strommen J.A., Liu X.K., van den Broek W., Wansink D.G., Wieringa B., Terzic A. Transgenic overexpression of human DMPK accumulates into hypertrophic cardiomyopathy, myotonic myopathy and hypotension traits of myotonic dystrophy. Hum Mol Genet 2004;13(20):2505-18.
24. Arber S., Hunter J.J., Ross J., Jr., Hongo M., Sansig G., Borg J., Perriard J.C., Chien K.R., Caroni P. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell 1997;88(3):393-403.
25. Naya F.J., Wu C., Richardson J.A., Overbeek P., Olson E.N. Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. Development 1999;126(10):2045-52.
26. Su Z., Yao A., Zubair I., Sugishita K., Ritter M., Li F., Hunter J.J., Chien K.R., Barry W.H. Effects of deletion of muscle LIM protein on myocyte function. Am J Physiol Heart Circ Physiol 2001;280(6):H2665-73.
27. Rockman H.A., Chien K.R., Choi D.J., Iaccarino G., Hunter J.J., Ross J., Jr., Lefkowitz R.J., Koch W.J. Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. Proc Natl Acad Sci U S A 1998;95(12):7000-5.
28. Minamisawa S., Hoshijima M., Chu G., Ward C.A., Frank K., Gu Y., Martone M.E., Wang Y., Ross J., Jr., Kranias E.G., Giles W.R., Chien K.R. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell 1999;99(3):313-22.
29. Frey N., Barrientos T., Shelton J.M., Frank D., Rutten H., Gehring D., Kuhn C., Lutz M., Rothermel B., Bassel-Duby R., Richardson J.A., Katus H.A., Hill J.A., Olson E.N. Mice lacking calsarcin-1 are sensitized to calcineurin signaling and show accelerated cardiomyopathy in response to pathological biomechanical stress. Nat Med 2004;10(12):1336-43.
30. van Oort R.J., van Rooij E., Bourajjaj M., Schimmel J., Jansen M.A., van der Nagel R., Doevendans P.A., Schneider M.D., van Echteld C.J., De Windt L.J. MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. Circulation 2006;114(4):298-308.
31. Heineke J., Ruetten H., Willenbockel C., Gross S.C., Naguib M., Schaefer A., Kempf T., Hilfiker-Kleiner D., Caroni P., Kraft T., Kaiser R.A., Molkentin J.D., Drexler H., Wollert K.C. Attenuation of cardiac remodeling after myocardial infarction by muscle LIM protein-calcineurin signaling at the sarcomeric Z-disc. Proc Natl Acad Sci U S A 2005;102(5):1655-60.
32. Baar K., Wende A.R., Jones T.E., Marison M., Nolte L.A., Chen M., Kelly D.P., Holloszy J.O. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. Faseb J 2002;16(14):1879-86.
33. Garnier A., Fortin D., Delomenie C., Momken I., Veksler V., Ventura-Clapier R. Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. J Physiol 2003;551(Pt 2):491-501.
34. Arany Z., Novikov M., Chin S., Ma Y., Rosenzweig A., Spiegelman B.M. Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. Proc Natl Acad Sci U S A 2006;103(26):10086-91.
35. Czubryt M.P., Olson E.N. Balancing contractility and energy production: the role of myocyte enhancer factor 2 (MEF2) in cardiac hypertrophy. Recent Prog Horm Res 2004;59:105-24.

36. Finck B.N., Kelly D.P. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest 2006;116(3):615-22.
37. Sano M., Wang S.C., Shirai M., Scaglia F., Xie M., Sakai S., Tanaka T., Kulkarni P.A., Barger P.M., Youker K.A., Taffet G.E., Hamamori Y., Michael L.H., Craigen W.J., Schneider M.D. Activation of cardiac Cdk9 represses PGC-1 and confers a predisposition to heart failure. Embo J 2004;23(17):3559-69.
38. van den Bosch B.J., van den Burg C.M., Schoonderwoerd K., Lindsey P.J., Scholte H.R., de Coo R.F., van Rooij E., Rockman H.A., Doevendans P.A., Smeets H.J. Regional absence of mitochondria causing energy depletion in the myocardium of muscle LIM protein knockout mice. Cardiovasc Res 2005;65(2):411-8.
39. Handschin C., Rhee J., Lin J., Tarr P.T., Spiegelman B.M. An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. Proc Natl Acad Sci U S A 2003;100(12):7111-6.
40. Czubryt M.P., McAnally J., Fishman G.I., Olson E.N. Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. Proc Natl Acad Sci U S A 2003;100(4):1711-6.