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The CNC gene *PRKAR1A* Plays an Essential Role in Cardiac Development and Myxomagenesis

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Abstract

Cardiac myxomas are the most common primary tumors of the heart, although little is known about their etiology. Mutations of the protein kinase A (PKA) regulatory subunit gene PRKAR1A cause inherited myxomas in the setting of the Carney complex tumor syndrome, providing a possible window for understanding their pathogenesis. We recently reported that cardiac-specific knockout of this gene causes myxomatous changes in the heart, although the mice die during gestation from cardiac failure. In this review, we discuss these findings and place them in the larger understanding of how PKA dysregulation might affect cardiac function and cause myxomagenesis.

Introduction

Cardiac myxoma is the most common primary tumor affecting the heart, accounting for nearly half of cardiac neoplasms (Reynen 1995). Its name derives from its appearance as a cell-poor, myxoid neoplasm with a mucopolysaccharide-rich extracellular matrix. Myxomas usually arise from the inter-atrial septum and more frequently involve the left side of the heart. Although cardiac myxomas are histopathologically benign, they cause a chronic inflammatory-like syndrome, and can cause catastrophic morbidity due to embolism or intracardiac obstruction.

Inherited myxomas are observed in the setting of the Carney complex (CNC), a dominantly inherited syndrome characterized by cardiac and extracardiac myxomas in the setting of spotty skin pigmentation, endocrine overactivity, and schwannomas (Carney et al. 1985). Approximately 30% to 60% of CNC patients will develop cardiac myxomas (Bertherat 2006), usually at much younger ages than the sporadic tumors. In this setting, the myxomas can be seen in any chamber of the heart, can be multiple, and can recur after initial resection. As a cardinal feature of CNC, cardiac myxomas are responsible for the death of more than 50% of patients, either from tumors themselves, or from post-surgical complications (Stratakis et al. 2001).

Molecular genetic analysis has revealed that mutations in the *PRKAR1A* gene, encoding the type 1A regulatory subunit of the cAMP-dependent protein kinase (PKA), are the cause of

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CNC in at least 50% of cases (Kirschner et al. 2000a; Kirschner et al. 2000b). In tumors from CNC patients, PKA activity is elevated (Bertherat 2006; Kirschner et al. 2000a), a finding also seen in mouse cells lacking the Prkar1a protein (Nadella and Kirschner 2005).

The cAMP-PKA Signaling Pathway

The cAMP-PKA signaling pathway regulates numerous important biological processes under both physiological and pathological conditions, including diabetes, heart failure, and cancer. At the cellular level, cAMP-PKA signal transduction is critically involved in numerous cellular processes, including the regulation of metabolism, gene expression and cell growth. At the structural level, PKA is composed of two catalytic (C) and two regulatory (R) subunits which form a tetrameric holoenzyme, R_2C_2. In the presence of cAMP, the holoenzyme dissociates into an $R_2(cAMP)_4$ dimer and two free (active) C subunits, which can phosphorylate a diverse number of target proteins both in the cytoplasmic and the nuclear compartments, including enzymes, structural proteins, and transcription factors.

In higher organisms, there are two isoforms of PKA, Type 1 and Type 2, based on the pattern of enzymatic elution in anion-exchange chromatography. The isoform specificity is determined by the identity of the regulatory subunits in the holoenzyme, and humans (and mice) have two genes encoding Type I regulatory subunits (*PRKARIA* and *PRKARIB*) and two encoding Type 2 subunits (*PRKAR2A* and *PRKAR2B*). The R subunits differ in molecular weight, tissue specificity, subcellular distribution, and expression pattern during development and cell cycle (ChoChung et al. 1995). *PRKAR1A* is ubiquitously expressed, whereas *PRKAR1B* is expressed primarily in brain, testis and lymphocytes. Similarly, *PRKAR2A* has ubiquitous expression, while *PRKAR2B* is expressed mainly in brain, adipose, and some endocrine tissues. The catalytic subunits are encoded by three functionally equivalent genes in humans (*PRKACA, PRKACB, PRKACG*), whereas mice only have two of these (*Prkaca* and *Prkacb)* (Foss et al. 1994). The catalytic subunits exhibit differential tissue expression, although most tissues show a predominance of the Cα catalytic subunit (Beebe et al. 1990). The type 1 PKA isoforms appear to have a higher affinity for cAMP, making them the major source of regulated cytoplasmic PKA activity within the cell. (Tasken et al. 1997). Type 2 PKA appears to exist more commonly anchored in multiprotein complexes, suggesting that it may a play a role in producing localized cAMP-dependent effects in specific cellular subdomains (Ruehr et al. 2004).

In addition, cAMP-dependent responses may be mediated by PRKX, an X-chromosome (located at Xp22.3) encoded protein that appears to function as a novel catalytic subunit of Type I PKA (Klink et al. 1995; Zimmermann et al. 1999). In mice, Prkx is ubiquitously and highly expressed in the central nervous system and in the heart during early developmental stages, but the status of older embryos is unclear. In humans, low levels of *PRKX* mRNA were detected in mRNA from embryonic heart, but no protein was detected by Western blotting of either fetal or adult heart (Li et al. 2005). Thus, the role of PRKX in cardiovascular function is unknown. A related kinase on the Y-chromosome, *PRKY*, (located at Yp11.2 has been studied even less.

PKA in Cardiac Function and Development

The cAMP-PKA pathway is known to play a critical role in mediating the effects of adrenergic stimulation on the heart (Rockman et al. 2002). The classic paradigm of cAMP-PKA-mediated signal transduction in cardiomyocytes involves ligand-dependent activation of the β-adrenoceptors (β-AR), including β_1– and β_2– ARs, which triggers the interaction of guanine nucleotide-binding protein (G- proteins) with adenylyl cyclases (ACs), and then ACs catalyze the conversion of ATP to second messenger cAMP. The increase in intracellular cAMP level in turn activates PKA. PKA then phosphorylates cardiac target proteins such as Ca^{2+} channels,

phospholamban, troponin I and C. These biochemical responses result in increases in contractility, rate of cardiac relaxation, heart rate, and impulse conduction (Wang and Dhalla 2000). The physiological functions and regulation of the key components of the β-adrenergic receptor-mediated PKA signal pathway in cardiac function have been extensively studied in human patients and by genetic manipulation in mice (Wang and Dhalla 2000). The cAMP/PKA system can also be activated by other G-protein-coupled receptors in the heart, including multiple isoforms of the adenosine receptor (Dobson and Fenton 1997).

In addition to its role as a mediator of normal physiology, PKA protein level and activity are enhanced in the failing heart (Wang et al. 1999). In studies of human patients with heart failure, the increased PKA phosphorylation of ryanodine receptor (RyR2) resulted in leakage of Ca^{2+} from the sarcoplasmic reticulum, contributing to impaired contractility and ventricular arrhythmias causing sudden cardiac death (Marks 2003). Taken together, these data demonstrate that PKA is a critical regulator of cardiac function.

Ablation of *Prkar1a* from the Heart Leads to Cardiac Failure

Very little information regarding the role of PRKAR1A in cardiac development is available. Unlike other PKA-R subunit knockouts, mice lacking *Prkar1a* do not survive embryogenesis and die before embryonic day 9.5 (e9.5) (Amieux et al. 2002; Kirschner et al. 2005). Amieux et al. demonstrated that ablation of Prkar1a leads to enhanced PKA activity and a developmental failure of the mesoderm (Amieux et al. 2002). Because the mesoderm gives rise to essential structures, including the embryonic heart tube, the *Prkar1a* knockout (KO) embryos cannot be used to study cardiac development.

To circumvent this generalized developmental failure, we utilized a tissue-specific knockout approach to delete *Prkar1a* from the developing heart. Using the promoter of the α-myosin heavy chain (αMHC) to drive expression of the cre recombinase, we generated mice lacking *Prkar1a* in the developing heart (Prkar1a-CKO mice) (Yin et al. 2008). The αMHC promoter is active at low levels by e10.5 (Ng et al. 1991), which is after the major cardiac specification events have taken place. As might be predicted, these animals developed normal cardiac structures, with clearly identifiable atria, ventricles, and pericardial structures. However, cardiac failure became evident at e11.5, with marked thinning of ventricular walls characterized by both hypoplasia of the compact layer and severely reduced trabeculation. These ventricular changes were associated with atrial dilation, heart failure, and embryonic demise before e12.5.

Examination of the hearts demonstrated that the Prkar1a-CKO animals had reduced cardiomyocyte proliferation which could be detected at e10.5, when the hearts appeared structurally normal. Ablation of *Prkar1a* was not associated with changes in the abundance of the other regulatory subunits, indicating a lack of compensation. In concordance with the reduced levels of the R1a regulatory subunit, hearts from Prkar1a-CKO mice exhibited enhancement of both free and total PKA activity, similar to the observation in null cells *in vitro* (Nadella and Kirschner 2005; Yin et al. 2008).

Because one of the well-known targets of PKA is the transcription factor cAMP-response element binding (CREB) protein, we sought to determine the effects of *Prkar1a* ablation on the expression of cardiac-specific proteins. In Prkar1a-CKO hearts, there was significant downregulation of all cardiac transcription factors investigated, including Nkx2–5, Gata4, serum response factor (Srf), and myocardin (Myocd). As has been thoroughly reviewed by other authors (Bruneau 2002; Nemer 2008; Olson 2004; Srivastava 2006), cardiogenesis occurs by means of an intricate transcriptional program requiring the precise expression of cardiac-specific genes directed by a complex array of transcription factors. A detailed discussion of this process is beyond the scope of the present review, but some general comments are worthwhile. Through inductive interactions in the developing heart field, the production of

transcription factors such as Nkx2-5, Myocd, Gata family members (likely Gata4, -5, and -6) and possibly Srf, begins in the cardiac crescent as early as e7.5 in the mouse embryo (Buckingham et al. 2005). Although it is dispensable for establishment of cardiac lineage (Lyons et al. 1995; Tanaka et al. 1999), Nkx2-5 is required for proper left ventricular chamber specification (Yamagishi et al. 2001). In human, mutations in *NKX2-5* are associated with septal and conduction defects (Benson et al. 1999; Schott et al. 1998). The analysis of *Nkx2-5*-deficient embryos indicates that it plays a crucial role in transcriptional regulation of several cardiac-specific genes and for the embryonic myocardium to differentiate beyond the stage of heart looping (reviewed by (Akazawa and Komuro 2005). Moreover, mice lacking Nkx2-5 fail to express the eHAND transcription factor in the heart, suggesting that eHAND and its downstream targets depend on Nkx2-5 in the left ventricle (Biben and Harvey 1997).

Both transcript and protein of Gata4 are found during formation of the heart tube at E8.0 in endocardium, myocardium and precardiac mesoderm (Heikinheimo et al. 1994). The importance of Gata4 in heart development is evidenced by the *Gata4* knockout mouse, which develop bilateral heart tubes (*cardia bifida*), and have a reduced number of cardiac myocytes (Kuo et al. 1997; Molkentin et al. 1997). Similar to *NKX2-5*, mutations in *GATA4* cause atrial and ventricular septal defects in human (Garg et al. 2003), and *GATA4* and the T-box transcription factor TBX5 also form a complex to regulate downstream genes, such as myosin heavy chain. Moreover, Srf is a MADS-Box family transcription factor, which broadly expressed in the embryo and essential for mesoderm formation. Srf interacts with Nkx2-5, GATA factors and myocardin (Myocd) to synergistically activate various transcriptional target genes (Bruneau 2002).

Because the cre transgene is not expressed until after cardiac differentiation is underway, the excess PKA activity caused by ablation of *Prkar1a* must interfere with the actions of these transcription factors that are necessary for the maintenance of the developing heart, rather than its initial specification. To look at the downstream effects of this interference, we examined the expression of structural proteins required for cardiac function, including members of the actin and myosin families. All of the structural proteins exhibited lower message levels, and significant reductions were observed for β-MHC and for actins A1 and A2. The effects of excess PKA activity resulting from *Prkar1a* ablation may either be direct effects on transcription (via CREB) or may be due to interference with other transcription factors, as has previously been demonstrated to be the case for Srf (Bruneau 2002; Davis et al. 2003; Nguyen et al. 2004). The fact that transcription factors function in combination and hierarchical networks (Bruneau 2002; Durocher et al. 1997) may explain why small reductions in individual factors, as observed here, causes synergistic and deleterious effects during cardiogenesis. This hypothesis is corroborated by our data: reductions in both *Gata4* and *Nkx2–5* lead to significant reductions in *Nppa*. Also, as mentioned, reduction in Nkx2-5 may cause loss of eHAND/HAND1, which works in concert for the expression of the structural proteins. In addition, we observed consistent downregulation of the *Slc8a1* (Ncx1) which is required for Ca^{2+} extrusion (Schulze et al. 1993) and plays a pivotal role in the regulation of intracellular Ca^{2+} homeostasis in cardiac myocytes.

The importance of proper regulation of transcription is underscored by the similarity among other mouse knockouts that have targeted various transcription factors. For example, the phenotype of embryonic demise resulting from a thinning of the myocardium has previously been observed by mice lacking either Srf (Miano et al. 2004; Parlakian et al. 2004) or Gata4 (Oka et al. 2006; Zeisberg et al. 2005). In *Srf* knockout hearts, increased apoptosis caused the decrease in cell number, whereas in our model, the defect appeared to lie in reduced proliferation. This observation is likely due to a direct antiproliferative effect of elevated PKA activity which has been well described in many cell systems, including cultured smooth muscle myocytes (Indolfi et al. 2001).

One of the striking aspects of the cardiac phenotype exhibited in the *Prkar1a*-CKO hearts was the disorganization of the sarcomeres. Z disks were easily seen, but they were not properly assembled, and myofilaments appeared loose and disarrayed. Defective expression of the Z-disk actins Acta1 and Acta2 likely underlies this ultrastructural defect. In addition, the significantly diminished expression of actins and Myh7 (β-MHC) may result in a deficiency in both thin (actin) and thick (myosin) myofilament components and disruption of both M lines and I bands. PKA is also known to phosphorylate myosin associate proteins such as the myosin binding protein C (MyBP-C) and Troponin I (cTnI) (Kentish et al. 2001; Yang et al. 2001), although the role of potential altered phosphorylation of these proteins in the observed phenotype has not yet been investigated. Most likely, the disorganized Z disks and myofilaments contribute to poor cardiac contraction and heart failure, causing demise of the growing embryo.

Prkar1a Ablation as a Cause of Cardiac Myxoma Development

Although cardiac myxomas are common in CNC patients, no cardiac tumors have been observed in mice heterozygous for *Prkar1a* mutations, made as the exact genetic model for human patients (Kirschner et al. 2005; Veugelers et al. 2004). These observations shed doubt on the notion that haploinsufficiency of *Prkar1a* is sufficient for myxomagenesis, but suggest that the mouse lifespan may be too short for the development of additional genetic hits (at the *Prkar1a* locus or elsewhere) that cause these tumors. In general, Prkar1a-CKO mice did not survive long enough to develop *bona fide* myxomas. However, analysis of Prkar1a-CKO mice showed that approximately 50% of *Prkar1a*-CKO hearts exhibited stroma-rich lesions suggestive of human cardiac myxomas. At dissection of e11.5 mice, typical myxoid material was frequently noted in the endocardial cushion. This structure is composed of a subset of cells that undergo remodeling to form the valvular structures and membranous septa of the mature heart after cardiac looping (Person et al. 2005). This observation may help explain the observation that myxomas arise predominantly in the interatrial septum (Reynen 1995).

Histologically, the majority of Prkar1a-CKO hearts exhibited the characteristic features of cardiac myxomas, including the presence of small numbers of polygonal or stellate cells surrounded by abundant loose stroma rich in acid mucopolysaccharides. Most strikingly, they also exhibit epithelium- or gland-like changes, which are rare but diagnostic features of human cardiac myxomas (Goldman et al. 1987; Johansson 1989). Finally, the tumors showed evidence of nuclear polymorphism and cellular atypia, as is ordinarily seen only in neoplastic cells. Thus, the evidence is convincing that this mouse model develops early phases in the development of cardiac myxoma.

There has been considerable debate regarding the histogenesis of cardiac myxoma. Various tumor cells have been identified in cardiac myxomas, including endothelial, fibroblastic, and muscle cells along with the typical stromal cells and glands (Abenoza and Sibley 1986). Most authors favor that myxomas arise from the endocardium (Fine et al. 1968) and are derived from subendocardial multipotent mesenchymal cells (Ferrans and Roberts 1973; Lie 1989). Our findings support the hypothesis that cardiac myxomas originate from primitive pluripotent mesenchymal cells, such as those arising from epithelial mesenchymal transition (EMT) in the endocardial cushion (Kinsella and Fitzharris 1980; Person et al. 2005). This tissue origin also may shed insight on why PKA activation, with its anti-proliferative effect in differentiated cardiomyocytes, may cause the formation of tumors. Specifically, it is known, from our work and the work of others, that the effects of PKA activation are cell- and differentiation-state specific. In this model, PKA activation in these pluripotent cells may lead to cell division, whereas the overall effect in the heart is anti-proliferative.

Integration of the Current Data with Prior Studies

The Prkar1a-CKO model demonstrates that Prkar1a is required for proper cardiac development and function. Depletion of Prkar1a causes excess PKA activity, with resultant downregulation of the transcriptional activity of cardiac transcription factors and their downstream targets, including key cardiac structural proteins and proteins involved in calcium handling. The similarities of the Prkar1a-CKO model with other cardiac transcription factor knockouts suggest that there is a common downstream mechanism for these pathways in the production of a functioning heart (Figure 1).

Intriguingly, *Prkar1a*-CKO mutants developed atypia and myxoma-like changes in the myocardium. Neither of these findings has been reported in conventional *Prkar1a* knockouts or in any other genetically modified mouse, suggesting that *Prkar1a*-CKO mice may serve as the first good model with which to study the formation of cardiac myxomas. Inherited cardiac myxomas have also been observed in a single family with the trismus-pseudocamptodactyly syndrome (sometimes called a CNC variant), caused by inactivating mutations in *MYH8*, encoding perinatal myosin (Veugelers et al. 2004). The fact that ablation of *PRKAR1a/ Prkar1a* causes disruption of myosin expression suggests that the two mechanisms may also be related. Although PKA is not known to directly phosphorylate myosins, its effects on myosin-associated proteins is well described and has important functional consequences (Kentish et al. 2001; Yang et al. 2001), providing a plausible link between the two genetic defects. Although there are no data to indicate that other transcription factor knockouts produce the predicted myxomatous changes, it would be interesting to reexamine the hearts of those mice.

Regardless of the outcome, examining the interrelationships of these proteins both for cardiac development and for myxomas formation should provide for an interesting set of future investigations.

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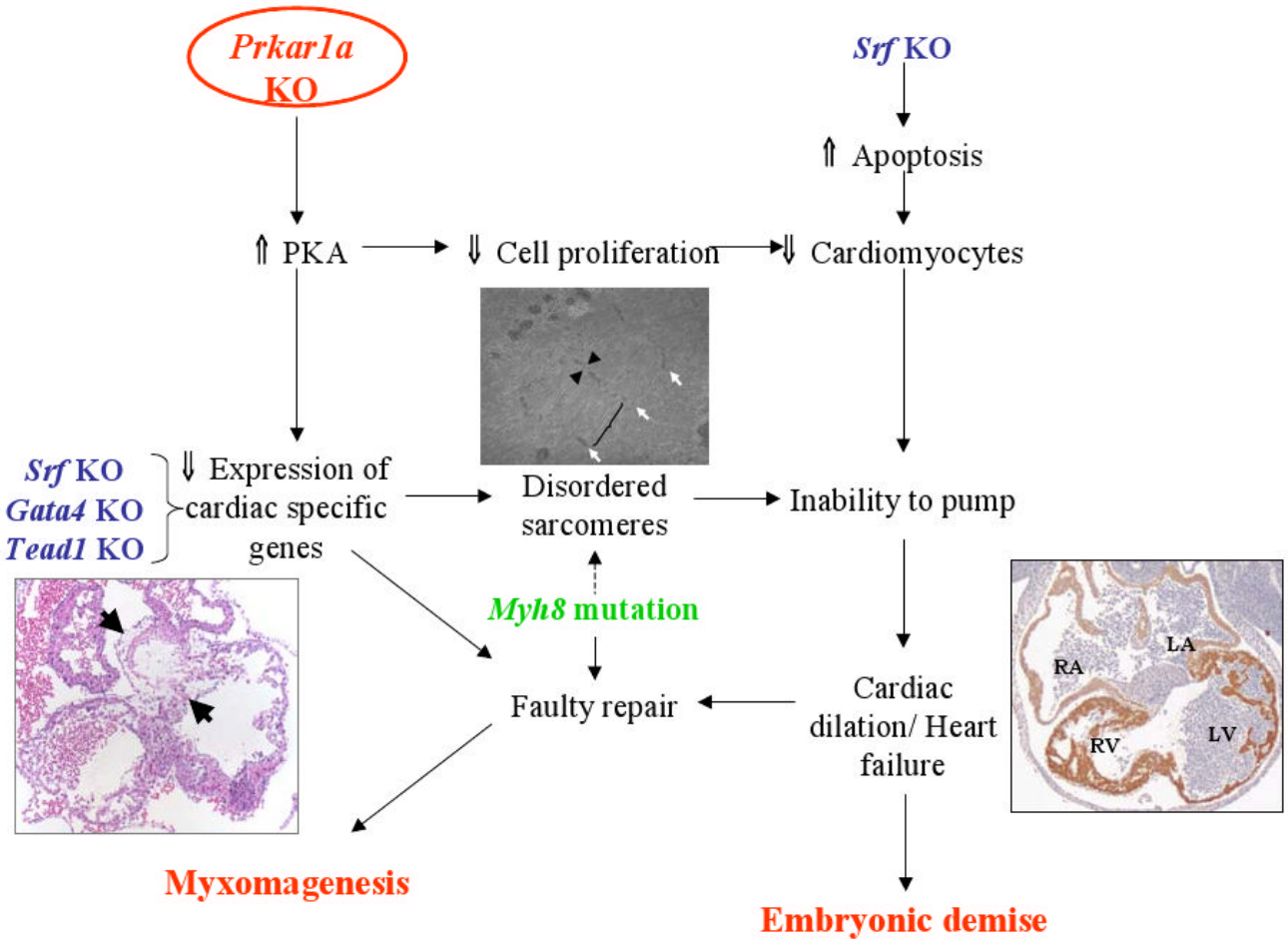

Figure 1. Model for the interactions of Prkar1a with other genes sharing a cardiac phenotype

Prkar1a KO (red text) causes reduction of cardiac-specific genes and a reduced number of cardiomyocytes with aberrant sarcomeres (Middle figure: white arrow: residual Z-disk; arrowhead: residual I-band)), eventually leading to a thin-walled, dilated heart (Right figure). At the end, these changes cause myxomagenesis (Left figure, arrows), and embryonic demise. These same pathways may be affected by knockout of other cardiac-specific transcription factors (blue text). A mechanism to account for myxomagenesis associated with mutation of *MYH8* is also proposed in the same scheme. See text for details and references.