Myocardial KRAS<sup>G12D</sup> expression does not cause cardiomyopathy in mice

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Received 24 May 2013; revised 10 October 2013; accepted 8 November 2013; online publish-ahead-of-print 20 November 2013

Time for primary review: 46 days

**Aims**

Germ-line mutations in genes encoding components of the RAS/mitogen-activated protein kinase (MAPK) pathway cause developmental disorders called RASopathies. Hypertrophic cardiomyopathy (HCM) is the most common myocardial pathology and a leading cause of death in RASopathy patients. KRAS mutations are found in Noonan and cardio-facio-cutaneous syndromes. KRAS mutations, unlike mutations of RAF1 and HRAS, are rarely associated with HCM. This has been attributed to the fact that germ-line KRAS mutations cause only a moderate up-regulation of the MAPK pathway. Highly bioactive KRAS mutations have been hypothesized to cause severe cardiomyopathy incompatible with life. The aim of this study was to define the impact of KRAS<sup>G12D</sup> expression in the heart.

**Methods and results**

To generate mice with endogenous cardiomyocyte-specific KRAS<sup>G12D</sup> expression (cKRAS<sup>G12D</sup> mice), we bred mice with a Cre-inducible allele expressing KRAS<sup>G12D</sup> from its endogenous promoter (Kras2LSL) to mice expressing Cre under control of the cardiomyocyte-specific α-myosin heavy chain promoter (α-MHC-Cre). cKRAS<sup>G12D</sup> mice showed high levels of myocardial ERK and AKT signalling. However, surprisingly, cKRAS<sup>G12D</sup> mice were born in Mendelian ratios, appeared healthy, and had normal function, size, and histology of the heart.

**Conclusion**

Mice with cardiomyocyte-specific KRAS<sup>G12D</sup> expression do not develop heart pathology. These results challenge the view that the level of MAPK activation correlates with the severity of HCM in RASopathies and suggests that MAPK-independent strategies may be of interest in the development of new treatments for these syndromes.

**Keywords**

KRAS • RASopathies • Noonan syndrome • Cardio-facio-cutaneous syndrome • Cardiomyopathy

1. Introduction

Germ-line mutations in KRAS, HRAS, RAF1, BRAF, and PTPN11 increase the activity of the RAS/mitogen-activated protein kinase (MAPK) pathway and lead to developmental syndromes called RASopathies. RASopathies, including Noonan, Costello, and cardio-facio-cutaneous syndromes, are characterized by facial dysmorphism, reduced growth, cognitive deficits, skeletal defects, and different types of heart disorders. The most common heart disorder is hypertrophic cardiomyopathy (HCM), which is associated with poor prognosis in RASopathy patients. Furthermore, mutations in HRAS cause Costello syndrome and lead to HCM in the majority of patients, and mice with germ-line or cardiomyocyte-specific Hras mutations also develop HCM. KRAS mutations, however, found in patients with Noonan and cardio-facio-cutaneous syndromes, are rarely associated with HCM. A potential explanation for why HRAS mutations are associated with HCM to a higher degree than KRAS mutations is that KRAS mutations in RASopathies cause a relatively weak increase in MAPK signalling. Consistent with this notion, KRAS mutations that cause RASopathies are rarely observed in cancer, whereas RASopathy HRAS mutations are often observed in cancer. Furthermore, cancer-causing KRAS mutations, such as KRAS<sup>G12D</sup>, are believed to be too powerful if they occurred in the germ-line and have been hypothesized to cause severe cardiomyopathy or even heart malignancy. Indeed, germ-line expression of the Kras<sup>G12D</sup>
mutation in mice results in cardiomegaly and embryonic lethality.\textsuperscript{19} It is not known, however, whether cardiomegaly is the result of KRAS\textsuperscript{G12D} expression in cardiomyocytes or in non-cardiac cells. Thus far, the impact of cardiomyocyte-specific KRAS\textsuperscript{G12D} activation has never been evaluated. To address this issue, we generated mice with cardiomyocyte-specific expression of KRAS\textsuperscript{G12D} and evaluated MAPK signalling and heart pathology.

2. Methods

2.1 Mouse breeding and genotyping

Mice heterozygous for a conditional oncogenic KRAS allele, Kras\textsuperscript{2+/-\textsubscript{MHC}}\textsuperscript{Cre} were bred with mice hemizygous for the \textalpha-myosin heavy chain (MHC) Cre transgene,\textsuperscript{21} to produce Kras\textsuperscript{1+/-\textsubscript{MHC}\textsuperscript{Cre}} mice with the cardiomyocyte-specific expression of KRAS\textsuperscript{G12D}, here designated cardiomyocyte-KRAS\textsuperscript{G12D} (cKRAS\textsuperscript{G12D}) mice. Littermate Kras\textsuperscript{2+/+\textsubscript{MHC}}\textsuperscript{Cre} mice were used as controls. Mouse experiments were approved by the Research Animal Ethics Committee in Gothenburg (approval number 322-2010) in accordance with the European Parliament directive 2010/63/EU. Mice were killed at 3 or 18 months of age for heart, liver, and lung tissue of adult mice, and from heart tissue of embryos. The Kras\textsuperscript{2+/-\textsubscript{MHC}}\textsuperscript{Cre} and the Cre-activated Kras\textsuperscript{2+/+\textsubscript{MHC}}\textsuperscript{Cre} alleles were detected as described.\textsuperscript{22} The \textalpha-MHC-Cre allele was detected with forward primer 5'-'ATGACAGACAGATCCCTCTATCTCC-3' and reverse primer 5'-CTCATCCTCGTTGACATCGAC-3', yielding a 300 bp fragment.

2.2 Echocardiography

Hair removal gel was applied to the chest of mice 1 day before echocardiography (ECG). ECG examination was performed using a VEVO 770 system with a linear RMV704 transducer (Visualsonics, Inc., Toronto, Canada) on mice anaesthetized with isoflurane inhalation (Canada) on mice anaesthetized with isoflurane inhalation (1.0% for 3–18 months of age by inhalation with 4% isoflurane followed by excision of the heart. Genotyping was performed by PCR amplification of genomic DNA from tail, heart, liver, and lung tissue of adult mice, and from heart tissue of embryos. The Kras\textsuperscript{2+/-\textsubscript{MHC}}\textsuperscript{Cre} and the Cre-activated Kras\textsuperscript{2+/+\textsubscript{MHC}}\textsuperscript{Cre} alleles were detected as described.\textsuperscript{22} The \textalpha-MHC-Cre allele was detected with forward primer 5'-ATGACAGACAGATCCCTCTATCTCC-3' and reverse primer 5'-CTCATCCTCGTTGACATCGAC-3', yielding a 300 bp fragment.

2.3 Western blotting

Heart tissue from the left ventricle was homogenized in lysis buffer containing 9 M urea (U0631; Sigma-Aldrich, MO, USA) and complete protease inhibitor cocktail (11836170001; Roche Applied Science, IN, USA), sonicated, and centrifuged for 10 min at 14 000 × g. Protein extracts were size-fractionated on 4–12% polyacrylamide Bis-Tris gels (Nupage, Life Technologies, CA, USA), transferred onto nitrocellulose membranes, and incubated with antibodies to phosphorylated ERK1/2 (4377), total ERK1/2 (9102), phosphorylated AKT (4060), total AKT (9727), Bclin-1 (3495), Atg5 (8540), Atg7 (8558), and LC3A (4599, Cell Signaling Technology, MA, USA). Protein bands were visualized with a secondary antibody IRDye 800CW Goat Anti-Rabbit (Li-Cor, NE, USA) and scanned in the Li-Cor Odyssey Imager with the Odyssey software, version 3.0 (Li-Cor). Band densities were analysed with ImageJ 1.45S (National Institute of Health, MD, USA).

2.4 Histology and immunohistochemistry

Hearts were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned horizontally 5 mm from the apex, and stained with Masson’s trichrome. For TUNEL and CD31 staining, ventricular wall tissue was frozen in OCT compound (Sakura Finetek, Sweden). Cryosections were stained with antibodies to the antiapoptotic marker cleaved caspase-3 (C590, Cell Signaling Technology, MA, USA) and CD31 (AF3628, R&D Systems, MN, USA). Histology and immunohistochemistry sections were scanned with a Mirax Scanner (Zeiss, Germany), and quantification

<table>
<thead>
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<th>Table 1</th>
<th>ECG analysis</th>
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<tr>
<td>Rest</td>
<td>Dobutamin</td>
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<tr>
<td>Ctrl (n = 6)</td>
<td>Kras\textsuperscript{G12D} (n = 6)</td>
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<tr>
<td>HR, bpm</td>
<td>423 ± 27</td>
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<tr>
<td>SV, µL</td>
<td>35.4 ± 2.3</td>
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<tr>
<td>CO, mL/min</td>
<td>15.1 ± 1.6</td>
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<td>Cl, mL/min/g</td>
<td>0.45 ± 0.05</td>
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<tr>
<td>LVEDV, µL</td>
<td>66.7 ± 6.3</td>
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<tr>
<td>LVEF, %</td>
<td>54.1 ± 2.7</td>
</tr>
<tr>
<td>FS, %</td>
<td>38.0 ± 6.3</td>
</tr>
<tr>
<td>LVAWd, mm</td>
<td>0.914 ± 0.06</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.817 ± 0.05</td>
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Values are mean ± SEM. P-values are from Student’s t-test (unpaired, two-tailed).

HR, heart rate; bpm, beats per minute; SV, stroke volume; CO, cardiac output; Cl, cardiac index; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; FS, fractional shortening; LVAWd, left ventricle anterior wall diameter during diastole; LVPWd, left ventricle posterior wall diameter during diastole.
of heart size, cardiomyocyte cross-sectional area, fibrosis and capillary density were done with the BioPix iQ 2.1.8 software (Biopix AB, Sweden).

2.5 Statistical analysis

The values are mean ± SEM. Differences between groups were analysed with Student's t-test (unpaired, two-tailed) and considered significant when \( P < 0.05 \).

3. Results

3.1 Cardiomyocyte-specific KRAS^{G12D} expression does not affect heart size and contractility

The Kras^{2LSL/+} αMHC-Cre^{+/0} (cKRAS^{G12D}) mice were born at Mendelian ratios and developed no visible signs of disease. ECG parameters were similar in 12-month-old cKRAS^{G12D} and littermate Kras^{2/+}/αMHC-Cre^{+/0} (control) mice. The response of hearts to dobutamine-induced stress was also similar in the two groups of mice, suggesting that the cardiac reserve was unaffected in cKRAS^{G12D} mice (Table 1). The mice were killed at 3 or 18 months of age for heart analyses. Relative heart weight and body weight were similar in cKRAS^{G12D} and control mice (Figure 1A and B, and see Supplementary material online, Figure S1). Moreover, the size of the left ventricular lumen and the thickness of the left and right ventricular walls were not different, as assessed by image analyses of haematoxylin and eosin-stained heart sections (Figure 1C–F).

3.2 Expression of KRAS^{G12D} is heart specific and starts late in embryonic development

To confirm the tissue specificity of KRAS^{G12D} expression, DNA was extracted from heart, lung, and liver tissue of cKRAS^{G12D} and control mice. As expected, the unrecombined Kras^{2LSL} allele was found in all tissues of cKRAS^{G12D} mice, but in none of control mouse tissues. The recombined {Kras^{2G12D} allele in adult cKRAS^{G12D} mice was readily detected in DNA from heart tissue, but was undetectable in the lung and liver (Figure 2A). Analyses of DNA from hearts of developing embryos revealed that the recombined {Kras^{2G12D} allele was detected at embryonic day 20, but not at embryonic days 12–18 (Figure 2B). Thus, KRAS^{G12D} expression in cKRAS^{G12D} mice is heart specific and begins at embryonic day 19 or 20.

To confirm bioactivity of the inducible {Kras^{2LSL} allele in this strain of mice, we administered a Cre-adenovirus by inhalation to lungs of {Kras^{2LSL} mice as described earlier. As expected, 8 weeks after Cre-adenovirus administration, {Kras^{2LSL} mice exhibited advanced lung tumour lesions, demonstrating that the allele produces bioactive {KRAS^{G12D} (see Supplementary material online, Figure S2).
3.3 Hyperactivation of MAPK signalling pathways in the myocardium of cKRAS\textsuperscript{G12D} mice

To test whether signalling downstream of KRAS was affected in hearts of cKRAS\textsuperscript{G12D} mice, we performed western blots of lysates of left ventricular tissue. The levels of phospho-ERK1/2 and phospho-AKT were higher in cKRAS\textsuperscript{G12D} hearts than in control hearts at 18 months of age (Figure 2C and D), indicating a significant hyperactivity of two important pathways downstream of KRAS. Similar results were observed in hearts of 3-month-old mice (Figure 2C and D), suggesting that cKRAS\textsuperscript{G12D} mice exhibit life-long hyperactivation of MAPK signalling in the myocardium.

3.4 Myocardial KRAS\textsuperscript{G12D} expression does not induce heart fibrosis and cardiomyocyte hypertrophy, and does not affect capillary density and protein degradation pathways

Although heart function in cKRAS\textsuperscript{G12D} mice was unaffected, we argued that the increased MAPK signalling might induce subclinical levels of interstitial fibrosis, cardiomyocyte hypertrophy, or apoptosis—well-known signs of myocardial pathology in humans and mice. However, this was not the case. Analyses of Masson’s trichrome-stained left ventricle sections revealed similar low levels of fibrosis in cKRAS\textsuperscript{G12D} and control mice (Figure 3A). Cardiomyocyte diameter was
also unaffected in cKRASG12D mice, ruling out cell hypertrophy, and levels of apoptosis, evaluated with TUNEL staining, were similar in the two groups of mice (Figure 3B and C). Based on previous studies, we hypothesized that the increased phospho-AKT levels in hearts of cKRASG12D mice might reduce myocardial capillary density. However, capillary density was similar in cKRASG12D and control hearts at 18 months of age, as assessed by quantification of CD31 immunostaining (Figure 3D).

A potential explanation for the absence of hypertrophy in cKRASG12D hearts is that activation of protein degradation pathways, such as ubiquitination or autophagy, counteracts the hypertrophic signalling. However, mRNA levels of the two main cardiomyocyte ubiquitin ligases Atrogin1 and MuRF1 and protein levels of the well-established autophagy markers Beclin-1, Atg5, Atg7, and LC3A were similar in cKRASG12D and control hearts (Figure 4A and B).

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**Figure 3** Cardiomyocyte-specific expression of KRASG12D does not affect myocardial fibrosis, cardiomyocyte diameter, cardiomyocyte apoptosis, or myocardial capillary density in mice. (A–D) Representative photographs (left) and quantification (right, n = 6/genotype) of myocardial sections from control and cKRASG12D mice sacrificed at 18 months of age. (A) Masson’s trichrome (collagen) staining showing low levels of myocardial fibrosis in control and cKRASG12D mice. (B) Masson’s trichrome staining showing similar cardiomyocyte size in control and cKRASG12D mice (scale bar = 20 μm). (C) TUNEL staining showing low levels of apoptosis control and cKRASG12D mice. Negative and positive controls are lung and thymus from a wild-type mouse sacrificed at 3 weeks of age. (D) CD31 staining (brown) showing similar capillary density in the myocardium of control and cKRASG12D mice (scale bar = 30 μm).
4. Discussion

In this study, we generated mice with cardiomyocyte-specific expression of KRAS<sup>G12D</sup>. Surprisingly, despite significant hyperactivation of the MAPK and AKT pathways in the myocardium, these mice did not develop heart disease.

In an earlier study, Tuveson et al. showed that widespread expression of endogenous KRAS<sup>G12D</sup> (using the same Kras<sup>LSL</sup> allele as in the current study) leads to embryonic cardiomegaly and death. Based on this finding, it has been suggested that myocardial KRAS<sup>G12D</sup> expression is detrimental for heart function. The absence of heart disease in cKRAS<sup>G12D</sup> mice in the current study questions this conclusion. However, there are two main differences between the mice of the two studies. Firstly, KRAS<sup>G12D</sup> expression was turned on early during embryonic development in the study by Tuveson et al. and late in the current study, suggesting that KRAS<sup>G12D</sup> expression in cardiomyocytes might be harmful for early heart development, but is well tolerated during late embryonic development and throughout life. Secondly, KRAS<sup>G12D</sup> was expressed in all tissues of the embryos in Tuveson’s study and only in cardiomyocytes in the current study. Consequently, we cannot rule out the possibility that the cardiomegaly in embryos with widespread KRAS<sup>G12D</sup> expression is caused by KRAS<sup>G12D</sup> expression in cell types other than cardiomyocytes, such as heart fibroblasts or endothelial cells.

The basal levels of phospho-ERK1/2 were increased in the myocardium of cKRAS<sup>G12D</sup> mice. Previously published RASopathy mouse models have not exhibited increased myocardial phospho-ERK1/2 levels under basal conditions. There is uncertainty in the literature as to whether increased MAPK activation causes HCM in RASopathies or if it is mediated by other mutation-specific mechanisms. Our data clearly demonstrate that life-long hyperactivation of the ERK–MAPK pathway in cardiomyocytes does not cause HCM or other heart pathologies in mice. A potential implication of these results is that ERK–MAPK-independent pathways cause HCM in RASopathy patients, which has been suggested earlier. An alternative explanation is that highly bioactive mutations, such as KRAS<sup>G12D</sup>, stimulate myocardial defence mechanisms, making them less harmful than moderately activating mutations. Two of the most important defence mechanisms that suppress pathological cardiomyocyte hypertrophy are ubiquitination and autophagy, leading to protein degradation. Markers of ubiquitination and autophagy were unaffected in cKRAS<sup>G12D</sup> hearts, suggesting that the expression of KRAS<sup>G12D</sup> does not activate known hypertrophy defence mechanisms in the heart.

The cKRAS<sup>G12D</sup> mice also exhibited increased levels of myocardial phospho-AKT. AKT signalling in myocardial biology is complex. On one hand, increased AKT signalling promotes survival of cardiomyocytes and is thereby cardioprotective in mouse models of pressure-overload-induced heart failure and myocardial infarction. On the other hand, overexpressing AKT in cardiomyocytes results in hypertrophy and heart failure in mice. Our data demonstrate that life-long activation of AKT signalling by expression of physiological levels of KRAS<sup>G12D</sup> is harmless.

Expression of KRAS<sup>G12D</sup> from the endogenous promoter in mouse embryonic fibroblasts leads to reduced levels of phospho-ERK and -AKT compared with wild-type MEFs. Here, we found significantly increased levels of phospho-ERK and -AKT in KRAS<sup>G12D</sup>-expressing cardiomyocytes, demonstrating that the impact of endogenous KRAS<sup>G12D</sup> on downstream signalling is cell type-specific.

There were no signs of heart malignancy in the cKRAS<sup>G12D</sup> mice, as assessed by macroscopic examination, histology, and ECG. KRAS mutations have been found in patients with cardiac rhabdomyosarcoma, a rare form of heart malignancy. The likeliest explanation for the absence of malignancy in cKRAS<sup>G12D</sup> mice is that whereas endogenous KRAS<sup>G12D</sup> is sufficient to initiate tumours in many different organs in the mouse, additional genetic events are required for heart tumour formation.

We conclude that mouse hearts are resistant to cardiomyocyte-specific expression of KRAS<sup>G12D</sup> despite hyperactivation of downstream ERK and AKT signalling. A clinical implication of this surprising result is that treatment of HCM in RASopathies should not only focus on targeting MAPK signalling, but also on identifying other mutation-specific downstream effects.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Mohammad Bohlooly (AstraZeneca) for αMHC-Cre transgenic mice and B. Thorisson for technical assistance.

Conflict of interest: none declared.
Funding
This work was supported by a Starting Investigator Grant from the European Research Council and by grants from the Swedish Cancer Society; the Swedish Research Council; the Swedish Children’s Cancer Fund; Västra Götalandsregionen; the Göran Gustafsson Foundation; and the Ingabritt and Arne Lundberg’s Research Foundation (to M.O.B.) and the Gothenburg Medical Society (to M.G.D).

References