Replacement of K-Ras with H-Ras supports normal embryonic development despite inducing cardiovascular pathology in adult mice

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Ras proteins are highly related GTPases that have key roles in regulating growth, differentiation and tumorigenesis. Gene-targeting experiments have shown that, out of the three mammalian *ras* genes, only K-*ras* is essential for normal mouse embryogenesis, and that mice deprived of H-*ras* and/or N-*ras* show no major phenotype. We generated mice (HrasKI) in which the K-*ras* gene had been modified to encode H-Ras protein. HrasKI mice produce undetectable amounts of K-Ras but—in contrast to mice homozygous for a null K-*ras* allele—they are born at the expected mendelian frequency, indicating that H-Ras can be substituted for K-Ras in embryonic development. However, adult HrasKI mice show dilated cardiomyopathy associated with arterial hypertension. Our results show that K-Ras can be replaced by H-Ras in its essential function in embryogenesis, and indicate that K-Ras has a unique role in cardiovascular homeostasis.

Keywords: K-*ras*; H-*ras*; embryonic development; cardiomyopathy; blood pressure

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INTRODUCTION

The three mammalian *ras* genes, H-, N- and K-*ras*, encode highly related 21 kDa proteins that regulate cell growth, differentiation, proliferation, and apoptosis (Bar-Sagi, 2001; Malumbres & Barbacid, 2003). Furthermore, Ras proteins have important roles in malignant transformation (Downward, 2003).

An as yet unresolved issue is whether the different Ras proteins have individual roles or are functionally interchangeable

(reviewed by Shields *et al*, 2000). Targeting of *ras* family genes in mice has shown that only K-*ras* function is essential for normal mouse development. K-*ras*-deficient embryos die between embryonic day 12 and term (Johnson *et al*, 1997; Koera *et al*, 1997). In contrast to the severe phenotype of K-*ras*-null mice, H-*ras*, N-*ras* and K-*ras* 4A knockout mice are viable and show no obvious abnormalities (Umanoff *et al*, 1995; Ise *et al*, 2000; Plowman *et al*, 2003). Even the concurrent removal of both H-*ras* and N-*ras* results in viable mice, indicating that K-*ras* is not only essential, but also sufficient for normal mouse development (Esteban *et al*, 2001). However, it remains to be clarified whether the unique biological function of K-*ras* is because of a specific function of its gene products or because of its distinctive expression pattern (Esteban *et al*, 2001).

We aimed to address this question by the knock-in of H-*ras* coding sequence at the K-*ras* locus. We show that such mice (referred to as HrasKI) produce undetectable amounts of K-Ras, but are born at the expected mendelian frequency. These results indicate that H-Ras can substitute for K-Ras in its essential function during embryogenesis. Furthermore, adult HrasKI mice are affected by dilated cardiomyopathy associated with arterial hypertension, which suggests that K-Ras has a unique role in cardiovascular homeostasis.

RESULTS

Generation of knock-in mice

The K-*ras* locus encompasses about 30 kb and the gene consists of five exons, with a noncoding exon at the 5' end (exon 0) and two alternative (4A and 4B) coding exons at the 3'-terminal region (Fig 1A). K- and H-*ras* exon 1 and part of exon 2 encode identical amino-acid sequences. The rearranged locus (*Kras*^(Hras)) was designed to exclude the potential synthesis of any K-*ras* gene product and to direct the synthesis of the H-Ras protein with the same spatial and temporal distribution as that of K-Ras. Therefore, the H-*ras* coding sequence was fused in-frame with the exon 2 of K-*ras*, followed by the 3' untranslated

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Fig 1 | Targeting strategy of knock-in of H-ras (or K-ras) cDNA in K-ras locus. (A) Wild-type K-ras targeted region with targeting vector and the predicted mutant locus. The coding exons are represented by open boxes. The HSV-TK (herpes simplex virus thymidine kinase) and neo cassettes are indicated. The cloned coding sequences for H- or K-ras are represented by dashed boxes. The 3' untranslated region is represented by a grey box. The probes used for Southern hybridization are shown by black boxes and the expected sizes of the signals are indicated by dashed lines. EI, *EcoRI*; EV, *EcoRV*; S, *SacI*; X, *XhoI*. (B) Southern blot analysis of genomic DNA from ES clones using 3' (left) and 5' (right) probes. +/+, wild type; +/-, HrasKI heterozygous.

region of K-ras. As a result of homologous recombination, this cassette was inserted between K-ras exons 2 and 3. To control for phenotypes induced merely by the genomic rearrangement at the K-ras locus, we applied the same strategy to insert the K-ras complementary DNA in the K-ras locus (*Kras*^(Kras)). We refer to these control mice as KrasKI. In this case, the rearranged locus was designed to express only the main K-Ras 4B isoform and not the K-Ras 4A protein, which has already been shown to be dispensable (Plowman *et al*, 2003). We targeted one allele of K-ras gene in mouse embryonic stem (ES) cells using the positive-negative selection method. Appropriate homologous recombination was achieved with both constructs (Fig 1B).

The K-Ras protein is not essential for embryogenesis

Chimeric animals, produced by injecting recombinant ES cells into C57/BL6 blastocysts, were bred to 129Sv or C57/BL6 mice to determine germline contribution. Mice heterozygous for either the *K-ras*^(Hras) or the *K-ras*^(Kras) allele were obtained and mated. Among the offspring, wild-type, heterozygous and homozygous mice for either mutated allele were present in the expected mendelian ratios (as supported by χ^2 statistic), indicating that all mice develop normally (supplementary Table 1 online). They were

Fig 2 | Expression of the Ras isoforms in different tissues from wild-type (WT), KrasKI and HrasKI adult mice. The protein extracts were probed with antibodies specific for each Ras protein. Pan-Ras antibody was used to detect the total expression levels of Ras proteins. Tubulin antibody was used to normalize the amount of loaded proteins.

morphologically normal and were indistinguishable from their wild-type littermates. To verify that the rearranged K-*ras*^(Hras) locus was not producing any K-Ras protein, western blot analyses were carried out on different adult tissues of wild-type, HrasKI and the control KrasKI mice (Fig 2).

These experiments showed that K-Ras is undetectable in HrasKI mice, whereas it is normally expressed in the wild-type and KrasKI lines with minor changes. No significant changes were observed in either N-Ras or total Ras protein levels, excluding the occurrence of any compensatory changes in expression of other members of the family.

Next, we investigated whether H-Ras can substitute for K-Ras in the functions shown to be altered in the K-*ras* knockout embryos (Koera *et al*, 1997; Johnson *et al*, 2001). All analyses were performed comparing HrasKI with both KrasKI and wild-type mice. We found no difference among the three genotypes in liver structure, peripheral blood smears and complete blood count (CBC) analyses, apoptosis of spinal cord motoneurons and thickness of the ventricular walls or size of the embryonic heart (data not shown).

HrasKI mice show hypertension and dilated heart

When the heart was examined by transthoracic echocardiography in 10- to 12-week-old male mice, the HrasKI mice showed increased left-ventricular end-diastolic diameter and reduced fractional shortening as compared with the wild-type mice, indicating that HrasKI mice have a typical pattern of dilated cardiomyopathy with a depressed systolic function (Fig 3A; supplementary Table 2 online). The echo results were supported by the histological analysis showing both an increased cardiomyocyte area and fibrosis in the left ventricle of HrasKI mice (Fig 3B,C). Importantly, KrasKI mice showed no difference from the wild type, suggesting that rearrangement of the K-*ras* locus *per se* did not determine any significant phenotype and that the Kras 4A isoform is dispensable also for this function.

To evaluate whether cardiac dilation and dysfunction were associated to an altered cardiac loading condition, arterial blood pressure (BP) was evaluated non-invasively in conscious mice by tail-cuff measurements and invasively by a radio-telemetric catheter chronically implanted in the femoral artery (Vecchione *et al*, 2002). Interestingly, the results obtained with both measurements showed that the HrasKI mice have both systolic and diastolic BP higher than either wild-type or KrasKI mice (Fig 4A,B), indicating that the dilated cardiomyopathy observed in the HrasKI mice might be secondary to a hypertensive condition.

DISCUSSION

The data reported in this paper address the question of whether the specific function of K-*ras* is a property of its expression pattern or of exclusive features of the encoded protein, which are implied by the results obtained in knockout mice (Johnson *et al*, 1997; Koera *et al*, 1997). We generated a knock-in model, HrasKI, where K-Ras protein production is abolished and substituted by H-Ras, expressed under the control of the regulatory regions of K-*ras* gene. Analysis of HrasKI mice shows that the H-Ras protein is capable of replacing the essential function of K-Ras in development, as HrasKI mice are born at the expected mendelian

Fig 3 | Left ventricular remodelling. (A) Representative M-mode left ventricular echocardiographic recordings of wild-type (+/+), KrasKI and HrasKI mice. All measurements were determined in a short-axis view at the level of papillary muscles. (B) Collagen deposition (light blue), indicative of fibrosis, evaluated by Masson's trichrome staining in the left ventricle. (C) Left ventricles processed for histology to measure the cardiomyocyte cross-sectional area from +/+, KrasKI and HrasKI mice. (D) Mean values from 200 cardiomyocyte area determinations per mouse. **P* < 0.05 versus +/+.

frequency and do not show any of the defects reported in K-*ras* knockout embryos. These results show that H-Ras can substitute for K-Ras and indicate that the essential function of K-Ras is a property of its specific expression pattern in development (Shields *et al*, 2000). However, HrasKI adult mice show a pathological cardiovascular phenotype, characterized by dilated cardiomyopathy associated with, and probably a consequence of, high systolic and diastolic BP. This phenotype is not simply a consequence of the genomic rearrangement at the K-*ras* locus in HrasKI mice, as a similar rearrangement inserting K-*ras*4B cDNA in the K-*ras* gene (KrasKI mice) shows no cardiovascular phenotype. Thus, it is the expression of the H-Ras in place of K-Ras that is responsible for the altered cardiovascular parameters.

Ras proteins are highly homologous, except for the 25 carboxy-terminal amino acids of the hypervariable region (HVR). The HVR contains protein sequences that are necessary for Ras to associate with the inner plasma membrane (PM); membrane localization of Ras is, in turn, necessary for Ras signalling (Willumsen *et al*, 1984). The first steps in post-translational modification of the Ras proteins are directed by the conserved C-terminal tetrapeptide of the HVR (the CAAX motif) and are common for all isoforms. These

Fig 4 | BP measurements. (A) Systolic BP evaluated non-invasively by tail-cuff measurements in conscious mice ($n=6$ for each group). $*P<0.01$ versus wild type (+/+) and KrasKI. Data are mean $\pm$ s.e.m. (B) Systolic and diastolic BP evaluated invasively by radiotelemetry in conscious mice ($n=3$ for each group). $*P<0.02$ versus +/+ and KrasKI. Data are mean $\pm$ s.e.m.

steps include farnesylation of the cysteine residue of the CAAX motif, proteolytic cleavage of the AAX tripeptide and methylation of the farnesylated cysteine (reviewed by Hancock, 2003). After these modifications, the fate of K-Ras diverges from that of the other isoforms because of the exclusive presence of a polybasic, lysine-rich region in its HVR. Farnesylated K-Ras is directly targeted to the PM by an as yet unknown mechanism, whereas all other Ras isoforms undergo palmitoylation of cysteine residues (one in N-Ras and K-Ras 4A, two in H-Ras) in their HVRs and travel through the Golgi secretory pathway to the PM (Apolloni *et al*, 2000). The differences between K-Ras and H-Ras HVR structure and lipid addition are responsible for both their different kinetics of membrane association and their distinct targeting to subcellular compartments and/or PM subdomains. The K-Ras polybasic region is responsible for its stable association to the PM, where it localizes to non-raft lipid microdomains (Jackson *et al*, 1990; Prior *et al*, 2001). H-Ras, in contrast, is dynamically associated with the PM because of the unstable nature of palmitoylation, and prevalently localized to lipid rafts, in its inactive state, moving to non-raft subdomains when activated (Magee *et al*, 1987; Lu & Hofmann, 1995; Prior *et al*, 2001). Moreover, it has been recently shown that the depalmitoylation–repalmitoylation cycle drives the rapid exchange of H-Ras between the PM and the Golgi (Rocks *et al*, 2005), whereas K-Ras is constitutively PM associated (Apolloni *et al*, 2000). Overall, the differences in PM microdomain localization, as well

as the different distribution between subcellular compartments of K- and H-Ras, can generate distinct output signals from the two isoforms *in vivo*, despite their apparently similar *in vitro* ability to interact with effectors. Our data indicate that the K-Ras-mediated signals required for the completion of embryonic development can also be transduced by H-Ras, whereas the physiology of the cardiovascular system seems to specifically require a K-Ras-generated signal.

Several investigations have already placed Ras signalling at the centre of the cardiovascular disease pathway. In addition, many studies have suggested a role for Ras proteins in heart functioning both *in vitro* and *in vivo* (reviewed by Sugden & Clerk, 2000). Furthermore, a lethal dilated cardiomyopathy is developed by heart-specific knockout of Rce1, an endoprotease responsible for clipping off the last three residues of CAAX sequence after prenylation, a post-translational modification essential for targeting Ras proteins to membranes (Bergo *et al*, 2004). Moreover, it has been reported that calmodulin binds to K-Ras, but not to H- or N-Ras, modulating its downstream signalling (Villalonga *et al*, 2001), causing dissociation of only K-Ras 4B from membranes in a Ca^{2+} -dependent manner that could result in its translocation to distinct regions of the cell and activation of diverse signalling pathways (Sidhu *et al*, 2003). These findings argue for a specific role of K-Ras 4B in the heart, as calcium is a key molecule in the maintenance of cardiac contractility through the actin–myosin complex (Chien *et al*, 2003). A recent study has linked Ras to a novel hyperplasia suppressor gene (HSG, renamed rat mitofusin-2), which shows a central function in the regulation of vascular smooth muscle cell (VSMC) proliferation in the case of chronic hypertension and associated vascular injury (Chen *et al*, 2004; Chien & Hoshijima, 2004). In particular, it has been shown that overexpression of rHSG induces cell-cycle arrest in the G0/G1 phase mainly by inhibiting the Ras–Raf–MEK–ERK1/2 pathway in VSMCs (Chen *et al*, 2004).

Heart growth occurs primarily through cardiomyocyte proliferation. After birth, cardiomyocytes withdraw from the cell cycle and the heart grows predominantly through hypertrophy rather than myocyte hyperplasia. When the pathological hypertrophy occurs in response to abnormal stress such as hypertension, pressure overload or myocardial infarction, it progresses to dilated cardiomyopathy and subsequently heart failure (reviewed by Olson & Schneider, 2003). Although the HrasKI mice show ventricular dilatation and thinning of cardiac muscle during adult life, we have not been able to identify an intermediate hypertrophy stage. This may imply that the hypertrophy stage occurs early or that the substitution of K-Ras by H-Ras renders cardiomyocytes more susceptible to elevated BP. It also remains unclear as to the primary cause of the elevated BP and whether other organs such as the kidney are involved. Experiments are in progress to investigate further the mechanisms leading to hypertension in these mice.

Our results show that there is no specific requirement for K-Ras protein in mouse development, as H-ras expression, driven by the regulatory sequences of the K-ras gene, is able to rescue the wild-type embryonic phenotype in the absence of K-Ras. However, during the adult life, K-Ras function is not entirely rescued by H-Ras, as HrasKI mice develop hypertension and dilated cardiomyopathy, showing a novel biological function of K-Ras protein in the control of cardiovascular homeostasis.

METHODS

Gene targeting and generation of mutant mice. A genomic DNA clone containing the entire K-ras locus was isolated from a 129/Sv genomic PAC library (YAC Screening Centre, DIBIT-HSR) by PCR screening. Targeting vectors used for knock-in of either the H-ras or the K-ras cDNA in the K-ras locus were constructed in pBluescript KS. The DNA fragments used as homology arms were a 4.9 kb K-ras genomic DNA fragment derived from intron 2 up to the start of exon 2, and a 3.1 kb fragment containing exon 3 and flanking regions. The H-ras and K-ras coding sequences were amplified by reverse transcription-PCR from total mouse kidney RNA. We used a PCR-based strategy to fuse either cDNA to the beginning of K-ras exon 2 in the 5' homology arm. In brief, a sequence spanning from intron 2 to the start of K-ras exon 2 was amplified using the oligonucleotides 5'-CTTAGTCTCTAGAGGAACTTCTGTTG-3' and 5'-TCTTGACCTGCTGTGTCGAG-3' (PCR product A). Two diverse PCR reactions were carried out to amplify the coding sequence of either H-ras (5'-GACACAGCAGGTCAAGAAGAGTAT-3' and 5'-TATCTCGAGTCAGGACACACATTTGC-3'; PCR product B) or K-ras (5'-CTCGACACAGCAGGTCAAGA-3' and 5'-TATCTCGAGTCACATAACTGTACACCTTGT-3'; PCR product C); *XhoI* restriction site is in italics. As the PCR product A shares an identical sequence at its 3' end with the 5' portion of PCR products B and C, recombinant cistrons, containing either the H-ras or the K-ras cDNA fused to the beginning of K-ras exon-2, were obtained using the PCR products A + B or A + C as templates in two subsequent PCR reactions. The products of amplification were verified by sequencing.

Addition of the 3' homology arm and insertion of a herpes simplex virus thymidine kinase (HSV-TK) and a Neo cassette were achieved by standard techniques. The constructs thus obtained were denoted *Kras*^(Hras) or *Kras*^(Kras), depending on the nature of the inserted cDNA.

Mouse R1 ES cells (gift from Dr A. Nagy) were cultured and electroporated as described elsewhere (Matise et al, 2000). Chimeric mice, generated by microinjection of targeted ES cell clones into C57/BL6 blastocysts, were crossed with either C57/BL6 or 129/Sv female mice. Genotyping was carried out on tail DNA preparations by Southern blot analysis. Heterozygous progeny were interbred to obtain mice homozygous for either the *Kras*^(Hras) or the *Kras*^(Kras) construct.

Protein extraction and western blot analyses. Tissues isolated from 4-week-old mice were immediately homogenized in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulphonyl fluoride and a cocktail of protease inhibitors; Sigma, St Louis, MO, USA). A 50 µg portion of total protein from different tissues was separated by a gradient 4–12% SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon membrane (Millipore, Billerica, MA, USA).

Immunoblots were probed with monoclonal anti-K-Ras (F234), anti-N-Ras (F155), anti-H-Ras (F235), anti-pan Ras and anti-tubulin antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and visualized by enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Histological and immunohistochemical analysis. Paraffin-embedded tissue and embryo sections were prepared as described previously (Parlato et al, 2004). Apoptosis was monitored using an

antibody that detects activated caspase 3 (cleaved caspase 3 (Asp175) antibody; Cell Signaling Technology, Beverly, MA, USA). Paraffin-embedded hearts were cut into 5 µm slices and stained with haematoxylin and eosin for morphological analysis or with Masson's trichrome (Sigma) for detection of fibrosis. To measure the myocyte area, suitable cross-sections were defined as those having nearly circular capillary profiles and nuclei.

Haematological analysis. Peripheral blood smears were stained with May-Grunwald-Giemsa. CBC analyses were performed using the COULTER® MAXM Hematology Analyzer (Beckman Coulter, Fullerton, CA, USA).

Transthoracic echocardiography. Echocardiographic analysis was performed using a commercially available echocardiograph (System Five Performance, General Electric Vingmed, Waukesha, WI, USA) equipped with a 10 MHz imaging transducer. End-diastolic and end-systolic interventricular septum (IVSTd, IVSTs), posterior wall thickness (PWTd, PWTs) and left ventricular internal diameters (LVEDD, LVESD) were measured using a computed NIH image analysis system (National Institutes of Health, Bethesda, MD, USA) as described previously (Vecchione et al, 2002; Brancaccio et al, 2003).

Evaluation of blood pressure. Non-invasive BP was evaluated by tail-cuff measurements (Visitech Systems, Apex, NC, USA) as described previously (Jung et al, 2004). In another group of mice, BP was evaluated invasively by radiotelemetric analysis. In particular, in anaesthetized mice, the left femoral artery was exposed and cannulated with a catheter 0.4 mm in diameter, connected to a radiotelemetric device (TA11PA-C20, Data Sciences International, St Paul, MN, USA) anchored subcutaneously. Each analysed mouse was housed in a separate cage and allowed to recover from surgical procedures for 10 days, before recording BP and heart rate continuously for 4 h daily (from 0800 to 1200), in basal conditions (4 days) and during the period of active treatment (18 days). From a receiver, placed underneath each cage, the telemetered pressure signals were consolidated by the multiplexer, and stored in a dedicated computerized data acquisition system (Dataquest Acquisition and Analysis System, DQ ART 1.1 Gold, Data Sciences International). A dedicated software analysis (Dataquest Acquisition and Analysis System, DQ ART 1.1 Gold, Data Sciences International) calculated the mean value during the acquisition time (Vecchione et al, 2002).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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