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Signaling to Cardiac Hypertrophy: Insights from Human and Mouse RASopathies

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Cardiac hypertrophy is the heart's response to a variety of extrinsic and intrinsic stimuli, some of which might finally lead up to a maladaptive state. An integral part of the pathogenesis of the hypertrophic cardiomyopathy disease (HCM) is the activation of the rat sarcoma (RAS)/RAF/MEK (mitogen-activated protein kinase kinase)/MAPK (mitogen-activated protein kinase) cascade. Therefore, the molecular signaling involving RAS has been the subject of intense research efforts, particularly after the identification of the RASopathies. These constitute a class of developmental disorders caused by germline mutations affecting proteins contributing to the RAS pathway. Among other phenotypic features, a subset of these syndromes is characterized by HCM, prompting researchers and clinicians to delve into the chief signaling constituents of cardiac hypertrophy. In this review, we summarize current advances in the knowledge of the molecular signaling events involved in the pathogenesis of cardiac hypertrophy through work completed on patients and on genetically manipulated animals with HCM and RASopathies. Important insights are drawn from the recognition of parallels between cardiac hypertrophy and cancer. Future research promises to further elucidate the complex molecular interactions responsible for cardiac hypertrophy, possibly pointing the way for the identification of new specific targets for the treatment of HCM.

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INTRODUCTION

Rat sarcoma (RAS) belongs to the family of small G proteins, low molecular weight (20–40 kDa) guanine nucleotide-binding proteins. These proteins were identified in 1980 in a sarcoma virus (1) and constitute molecular switches involved in transmitting extracellular stimuli downstream into the cell. Small G proteins exist in two conformations: an inactive form, in which they bind guanosine diphosphate (GDP), and an active form, interacting with guanosine triphosphate (GTP). Extracellular stimuli induce dissociation of GDP and binding of GTP, which prompts a conformational change and consequent binding with downstream effectors. Since both the spontaneous exchange GDP/GTP and the hydrolysis of GTP are inefficient processes, regulatory proteins are needed for them to proceed: guanine nucleotide exchange factors (GEFs) activate the small G protein, while GTPase activating proteins (GAPs) (such as neurofibromin 1 and RASA1 [RAS p21 protein activator (GTPase activating protein) 1]) stimulate the hydrolysis of GTP. Induction of GEF activity is promoted by growth factors, which activate tyrosine kinase receptors (RTKs). Activation of RTKs may be negatively regulated by casitas b-lineage lymphoma (CBL) ubiquitin ligase. Phosphotyrosines are binding sites for adapter proteins, like SHC/growth factor receptor-bound 2 (GRB2) complex, which bind the cytosolic protein son of sevenless (SOS), the most important GEF of RAS. SH2 domain-containing protein tyrosine phosphatase (SHP2) is also a RAS regulator; in fact it interacts with RTKs and adapter proteins like GRB2 and GRB2-associated-binding protein (GAB), inducing RAS signaling. RAS activates, in turn, a cascade of downstream kinases: RAF, mitogen-activated protein kinase kinase (MEK) and mitogen-activated protein kinase (MAPK). Scaffold proteins, like SHOC2, link the RAS to the RAF family. RAF activity can be prevented by SPRED1 (sprouty-related, EVH1 domain containing 1) binding. RAFs, when induced, phosphorylate MEK, which finally activates MAPKs. The molecular targets of the MAPK family are usually nuclear transcription factors, cytoplasmic factors responsible for the initiation of translation and apoptosis regulators (2,3).

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Hypertrophic Cardiomyopathy

The response of the heart to meet the change in demands of circulation is dependent primarily on myocardial

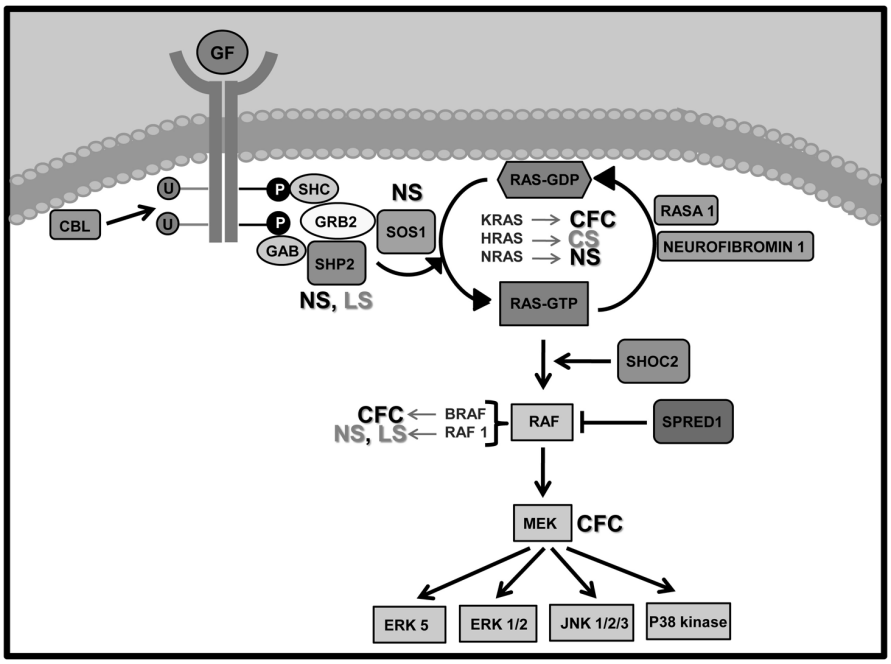

Figure 1. Schematic representation of RAS pathway and its correlation with RASopathies. A growth factor activates its RTK causing phosphorylation of tyrosines, which are bound by adapter proteins, SHC/GRB2 and GAB. These interact with the activators of RAS, SOS1 and SHP2, and induce the dissociation of GDP and binding of GTP. RAS-GTP activates a cascade of downstream kinases: the MAPK pathway. On the other hand, the inhibition of RAS signaling is mediated by GAPs: RASA1 and neurofibromin 1. The RASopathies are developmental pathologies due to mutations on genes encoding proteins of the RAS pathway. HCM is uncommon in NS and CFC patients. However, nearly all NS patients with mutated *RAF1* develop HCM. *PTPN11* (encoding SHP2) loss-of-function mutations were observed in the majority of LS patients who developed HCM. Mutations in *RAF1* have also been identified in a small percentage of LS with HCM. CS patients harboring *HRAS* mutations consistently show cardiac hypertrophy. Light gray shading indicates the mutation-specific syndromes accompanied by HCM; dark gray shading indicates those syndromes in which cardiac hypertrophy is less common.

growth. The heart enhances its contractile function in response to increased workload by increasing cardiomyocyte size and force production (4). For example, physiological hypertrophy is often developed by athletes: the increase of heart muscle mass reduces ventricular wall stress and compensates for the increased hemodynamic demand, improving heart contractility. In contrast, pathological signals may lead to maladaptive hypertrophy, with diminished cardiac output and enhanced risk of sudden death, arrhythmia and heart failure. Pathological signals may be extrinsic, like arterial hypertension, myocardial infarction and aortic stenosis, or intrinsic to the cardiomyocyte, like genetic hypertrophic cardiomyopathy (HCM). Usually the genetic causes of HCM are autosomal dominant mutations on proteins of the sarcomere. A secondary form of HCM may arise as part of a series of dysfunctions in a congenital syndrome. Irrespective of the origin of the pathological hypertrophy, typical features are reexpression of fetal genes and sarcomeric remodeling. In most cases, ventricular hypertrophy is detectable as ventricular wall thickening; however, hypertrophy can also lead to dilation of the ventricular chambers and cause contractile dysfunction and apoptosis.

RAS Pathway Mutations in RASopathies Associated with HCM

Over the past three decades, much has been learned about both RAS GTPase activity and the consequences of somatic mutations in RAS signaling, which occur in cancer (5), with RAS and cancer accounting for more than 20,000 publications in public web libraries. Only recently, the appreciation that RAS activation has important effects on development has emerged: it was the discovery of germline mutations in the genes neurofibromin 1 (*NF1*) (6) and protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*) (which encodes SHP2) (7). These genes provided the first indication that aberrant RAS signaling might contribute to the pathogenesis of human developmental diseases. Germline mutations in genes encoding molecules in the RAS/RAF/MEK/MAPK cascade were shown to cause RASopathies, many of these mutations affect SHP2, SOS1, RAS, RAF and MEK proteins (Figure 1). RASopathies include a number of phenotypically similar diseases such as Noonan syndrome (NS, MIM #163950), Costello syndrome (CS, #218040), cardio-facio-cutaneous syndrome (CFC, #115150) and Noonan syndrome with multiple lentigines, also known as LEOPARD syndrome (acronym for lentigines [multiple brown-black spots on the skin], electrocardiographic conduction defects, ocular hypertelorism, pulmonary stenosis, abnormalities of genitals, retarded growth resulting in short stature and deafness or hearing loss due to inner ear malfunction) (LS, #151100, #611554). All of these mutations perturb the intrinsic biochemical properties of the encoded proteins. Germline mutations are usually dominant gain-of-function mutations; LS seems to be an exception, because it is caused by autosomal dominant-negative *PTPN11* mutations (8). However, gain-of-function mutations of v-raf-1 murine leukemia viral oncogene homolog 1 (*RAF1*) have been identified in a small percentage of LS patients (9).

The common underlying pathogenetic mechanism involved in RASopathies brings about a significant overlap in phenotype, which includes craniofacial dysmorphology and heart defects. The cardiac abnormalities in CS, NS and CFC syndromes are quite similar, although they vary in severity and frequency. Congenital cardiac defects include pulmonary valve and aortic stenosis, atrial and ventricular septal defects and mitral insufficiency. Among the RASopathies, congenital heart defects are the most common in NS (*PTPN11* or *SOS1)*. They also occur in CFC syndrome (v-raf murine sarcoma viral oncogene homolog B1 [*BRAF*]) and NS (*RAF1*). Conduction abnormalities in patients with an *RAF1* mutation, CS and LS are also well described. Vascular abnormalities (that is, aortic dilation, coronary artery dilation and peripheral aneurysms) have also been reported in patients with NS and LS (10). HCM occurs with similar frequency in CS (10,11) and LS (12), whereas it is less common in CFC syndrome (13–15) and NS patients (16–19). In approximately one-third of CS patients, HCM is chronically severe, worsened or stable. However, the resolution or regression of HCM on echocardiographic follow-up was also reported (10).

The fundamental role of several RTKs, like epidermal growth factor receptor (EGFR) (also known as ErbB), fibroblast GFR and vascular endothelial GFR, has been described in heart development, from the induction of cardiac mesoderm to the formation of cardiac cushions in the atrioventricular (AV) canal and the outflow tract (OFT) and the epithelial-to-mesenchymal transition process leading to valve formation (20). The RAS signaling mutations causing defective AV and OFT development and valvulogenesis (that is, atrioventricular septal defects and pulmonary valvular stenosis) in a subset of RASopathies (21) probably affect multiple populations of cell progenitors. Indeed, various cell lineages contribute at different stages to OFT and AV channels, including the so-called second heart field (22,23). Despite the clear involvement of RAS signaling in the prenatal development of the heart, important effects of this signaling should also be considered in the postnatal response to stress. In this review, we emphasize the role of the RAS pathway in HCM. The age at which HCM is diagnosed ranges from infancy, in patients with a severe form of the rare neonatal phenotype, to childhood, the more common age of presentation (10). In studies focused on fetuses, investigators failed to diagnose prenatal onset of HCM, at least in CS (24,25). So far, hypertrophy has seemed to develop as a counteraction of the cardiomyocyte to increased hemodynamic demands. However, it remains to be established whether the pathological phenotype may result from RAS signaling pathway alterations in fetal cardiomyocytes and, if so, how this occurs.

Pathophysiological Signaling: Experimental Relevance of the RAS/RAF/MEK/MAPK Pathway in HCM

The discovery of mutated *RAS*-related genes in RASopathy patients has underlined the relevance of this signaling to HCM (Figure 1). In addition to genetic lesions, pathological stimuli, like biomechanical stress and neurohumoral factors, lead to a hypertrophic state of the heart (26–28). The altered stimulation of membrane receptors, such as RTKs, is a crucial initiating step leading to the final activation of MAPKs, which thereby induce the hypertrophic response (27,29–33). By altering the levels and activities of cardiac transcription factors (for example, GATA binding protein 4 [GATA4], myocyte enhancer factor 2 [MEF2] and nuclear factor of activated T cells [NFAT]), the RAS/RAF/MEK/MAPK pathway leads to reexpression of fetal cardiac genes (34). In particular, the upregulation of *β-MHC* (β myosin heavy chain) and atrial natriuretic factor and the downregulation of *SERCA* (sarcoplasmic reticulum Ca^{2+} ATPase) genes occur in the pathological hypertrophy, with consequent loss of efficient contraction and calcium cycling (30,33,34). Such transcriptional remodeling correlates with the loss of cardiac function; conversely, the improvement of cardiac function in response to drugs or implantation of an assist device is accompanied by the normalization of gene expression (35,36). Studies using genetically engineered mice have suggested that direct targeting of the hypertrophic response itself is beneficial and may provide a suitable therapeutic option in such cases (4,37,38). Thus, strategies to normalize cardiac gene expression by controlling its upstream signaling offer an attractive approach for HCM therapy.

To this aim, a considerable number of animal models, mainly murine, have been generated to reproduce a hypertrophic phenotype due to hyperactivation of RTKs, RAS and RAS-downstream proteins. The attenuation of hypertrophic responses by the use of inhibitors of MEK1/2, dominant-negative RAF1 or MEK1 and antisense oligonucleotides against extracellular signal-regulated kinase 1 and 2 (ERK1/2) definitely substantiated the important role of this cascade for cardiomyocyte hypertrophy (27,30,33). All of the findings accumulated over the past couple of decades underscore the importance of both RAS-downstream ERK-mediated and ERK-independent pathways in cardiac hypertrophy. The earliest evidence of a correlation between RAS and hypertrophy came from experiments on cultured myocytes (39,40). Then, a link between RAS expression levels and the severity of hypertrophy was given by RAS mRNA measurements among HCM patients (41). However, the *in vivo* proof of its role in hypertrophy derives from transgenic mice in which oncogenic RAS is expressed in the cardiac ventricular chamber (42). Mutations on the v-Ha-ras Harvey rat sarcoma viral oncogene homolog (*HRAS*) gene have been found in the majority of patients affected by CS with signs of HCM (11). A mouse and a zebrafish model for *HRAS*-related CS have been produced so far. However, although the mouse model showed signs of cardiac hypertrophy (43), mutant fish did not (44), revealing an inconsistency with

the human phenotype. The involvement of aberrant RTK signaling in the pathogenesis of cardiac hypertrophy has been recently demonstrated by our group with the use of a transgenic conditional mouse model expressing the constitutively active Met tyrosine kinase (45). RTK activation engenders RAS signaling to be turned on through GRB2, which has been implicated in the development of hypertrophy (46). In turn, GRB2 mediates the activation of SOS1, which is mutated in NS patients and mouse models leading to HCM (47). Also, SHP2 is a key component of multiple RTK, cytokine receptor and integrin signaling cascades. The animal model carrying an NS-associated *Ptpn11* mutation does not develop cardiac hypertrophy (48), in line with the observation that HCM occurs in less than 5%–10% of NS patients with *PTPN11* mutations (19). In contrast, a dominant-negative mutation with catalytic loss of function affecting SHP2 leads to HCM in both LS patients and mice (49). As expected, the LS mice show reduced agonist-evoked ERK/MAPK signaling, with a concomitant enhancement of protein kinase B (AKT)–mammalian target of rapamycin (mTOR) activity.

The importance of excessive phosphoinositide 3-kinase (PI3K)-AKT-mTOR signaling in the pathogenesis of HCM in LS was further proved by rapamycin treatment, which reverses LS cardiac defects. Indeed, even though direct mutations on *PI3K* or *AKT* have not been found, it is known that the prolonged constitutive activation of PI3K in the heart results in hypertrophy (50). In contrast, temporally controlled overexpression of cardiac PI3K in adult transgenic mice does not induce hypertrophy, but results in increased contractility (51). In fact, in the cardiac context, PI3K plays important roles in cardiac growth and exercise-induced hypertrophy and exerts protective effects against pathological stimuli (52–54). Similarly, short-term activation of AKT in cardiomyocytes results in physiological adaptive hypertrophy, whereas chronic activation of AKT leads to pathological hypertrophy (55). Thus, the PI3K-AKT pathway shows a high degree of complexity, with different roles in regulating cardiac hypertrophy. RAS activation results in the stimulation of RAF family members, which have been implicated in the development of hypertrophy (56,57) and of HCM in RASopathies (9,13,14). *RAF1* mutations have been more frequently found to be associated with HCM, and nearly all NS patients with *RAF1* mutations exhibit HCM (58). Like NS patients, mice heterozygous for the NS-associated *RAF1* mutation exhibit eccentric cardiac hypertrophy (59). A mouse model of a constitutively active form of BRAF has also been created (60). However, mutant mice show an increased total number of cardiomyocytes, rather than alterations in size, in contrast to data from CFC patients with mutated *BRAF* who do develop HCM (61).

RAF activity results in the subsequent phosphorylation of MEKs. Mutated MEK1 was found in a few CFC patients with signs of HCM (13). Evidence from cultured cardiac myocytes exposed to a MEK1-specific inhibitor demonstrated the critical contribution of the ERK pathway to hypertrophy (62,63). *In vivo*, inhibition of MEK attenuated cardiac growth in both induced and genetic models of hypertrophy (48,59,64,65). Final activation of MAPK has also been documented in different heart diseases (66,67). Sprouty1, an endogenous inhibitor of ERK, was reported to be induced in human hearts during hypertrophy regression after implantation of an assist device (68). These observations are in agreement with reports about MAPK phosphatase-1 inhibiting effects on cardiac hypertrophy, *in vitro* and *in vivo* (69). However, mice lacking ERK1 and one ERK2 allele show a normal hypertrophic response to pressure overload and exercise (70), suggesting that ERK1/2 may not be mandatory for cardiomyocyte growth. Consistent results were raised in mice with cardiac-specific inhibition of ERK1/2 activity (70–72). These proofs underpin the hypothesis that cardiac hypertrophy can develop independently of ERK1/2.

Recently, a new regulatory mechanism for ERK2 in cardiac hypertrophy was described: Thr188 autophosphorylation on ERK2 directs it to the nucleus, leading to phosphorylation of nuclear targets, without an overall increase in the activity of ERK1/2 (73). Thus, Thr188 phosphorylation might represent a specific switch toward hypertrophic signaling, uncoupled from physiological signaling, and hence embody a good target for therapy. Moreover, because overall ERK1/2 activity remains unchanged, ERK1/2 functions other than the hypertrophic one might be unaffected, reducing massive side effects.

Cooperative Effects on the RAS Pathway: Cross-Links in Hypertrophy

A first suggestion that RAS is in front of a network rather than in a top-down pathway actually comes from genetic syndromes with HCM. For example, whereas both RAS and MEK1 gain-of-function approaches result in a hypertrophic response, only MEK activation has no cardiotoxic effects (74). This discrepancy might be explained by ERK-independent RAS pathways or by activated V12H RAS recruiting of modulatory proteins, for example, Gβγ-subunits, which could influence its downstream cascade (75). Accordingly, the autophosphorylation of ERK2 at Thr188 requires the integrated activation of the RAF/MEK/ERK1/2 cascade, as well as that of Gαq-coupled receptors (73). Also, activation of β-adrenergic receptors regulates the activity of small GTPases involved in cardiac hypertrophy (76,77): a novel family of exchange proteins directly activated by cyclic AMP (EPAC) (78) has been shown to link G protein–coupled receptors (GPCRs) and RAS signaling (Figure 2). EPAC acts as a GEF on RAS-like small GTPases Rap1/2 (79,80), participating in multiple cellular events initiated by GPCRs (76,81,82). In turn, Rap1 activates BRAF and has been involved in cardiac hypertrophy (77). In addition, signaling via GPCRs promotes cardiac hypertrophy via ERK (83,84). Likewise, other GPCRs, including α-adrenergic receptors (28,85),

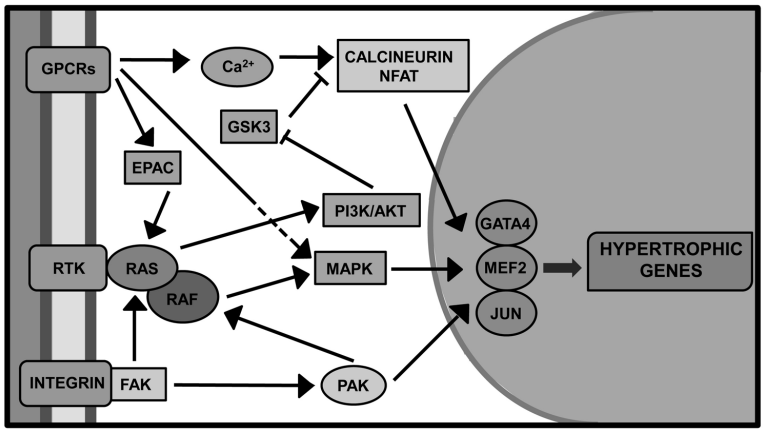

Figure 2. RAS Network: cross-links in hypertrophy. The cardiac hypertrophic response implicates signal transduction pathways initiated by ligand-stimulated membrane-bound receptors (RTKs, GPCRs) and biomechanical stress sensors (integrins). Various signaling effectors interact with the RAS/MEK/MAPK pathway. GPCR receptors activate RAS proteins through EPAC, induce release of internal Ca2+ stores and elicit pathological hypertrophy through calcineurin/NFAT. GPCRs also act through ERK activation. GSK3 kinase negatively regulates NFAT and, in turn, is inactivated by the PI3K-AKT pathway stimulated by RAS. Stimuli acting on integrins prompt cardiac hypertrophy through FAK activation and considerable cross-talk with RTK-mediated signaling. In addition, PAK regulates RAF1 activity. All these pathways converge on the modulation of transcriptional factors (MEF2, JUN and GATA4), which induce the expression of genes of the hypertrophic program.

angiotensin receptors (86) and endothelin receptors (63,87), signal through ERK to promote cardiomyocyte hypertrophy. Moreover, nuclear targeted α-adrenergic receptors might activate ERK located in caveolae (88).

Several studies have also implicated focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, in the first phase of the hypertrophic response to stretch in cardiomyocytes (89) by regulating the activation of MEF2 and JUN N-terminal kinase (JNK)-JUN pathways, which are early activators of the hypertrophic genetic program (90).

Irrespective of the original stimulus, these pathways converge to the activation in the nucleus of transcriptional factors, which have earlier roles during development, including GATA4 and MEF2 (Figure 2). In the normal adult myocardium, only basal MEF2 activity is required for the maintenance of contractile properties, whereas stress stimuli, through MAPK, stimulate its activity (Figure 2) (91,92). In the adult, these factors induce a fetal gene program, which might be beneficial in adapting to stress, in principle, but then leads to altered contractility, uncontrolled calcium transients and inadequate energetic and, finally, maladaptative changes (93–95).

Gene expression profiling from temporally regulated V12H RAS transgenic hearts has suggested that induction of early response genes, loss of mitochondrial function and altered ionic channel proteins are the likely culprits of the pathological changes in extracellular matrix remodeling, cardiac output and electrophysiological parameters (96). Recent work has suggested that the selective induction of Gαi in the RAS transgenic heart contributes to impaired sarcoplasmic reticulum calcium cycling (97), and a number of other studies have led to descriptions of RAS-induced alterations in calcium transients (98–100). Interestingly, abnormalities in delicate calcium homeostasis can trigger cardiac hypertrophy through mechanisms that have not been fully elucidated (101). The major link between calcium signaling and expression of fetal genes is represented by NFATs. NFATs are activated by calcineurin through its dephosphorylating action, then migrate into the nucleus, where they interact with MEF2 and GATA4 (102,103). Moreover, GSK3 mediates export of NFATs from the nucleus and termination of transcription. GSK3 is a known effector of the PI3K-AKT pathway, and activation of AKT leads to inhibition of GSK3, thus crossing calcineurin, RAS and PI3K pathways (Figure 2). Indeed, the involvement of PI3K signaling was underlined in cardiac pathogenesis (50) and hypertrophy (51,57). Finally, phosphorylation of RAF1 through PI3K and P21-activated kinase (PAK, Figure 2) provides a costimulatory signal, which, together with RAS, leads to strong activation of RAF1 kinase (104).

What is clear, in light of this exciting progress, is that an intricate web of interconnected signaling modules exists around the hub of RAS, in which RAS and fluctuations in calcium concentrations interplay and regulate each other.

Lessons from Cancer to Cardiac Hypertrophy

Interesting insights from the role of RAS mutations in tumorigenesis might provide a new perspective for considering the role of hyperactive RAS in the development of HCM in RAS syndromes. Parallels between cancer and hypertrophy have already been suggested, concerning both leading causes and therapy (105). Inadequately activated RTKs are implicated in many proliferative disorders and have therefore received considerable attention. More recently, the same pathway was shown to be involved in the pathogenesis of cardiac hypertrophy, prompting scientists to take advantage of the experience gained in cancer for the cardiovascular field. For example, an unanticipated role for mutated *CBL* in the pathogenesis of a clinically variable condition with features fitting or partially overlapping NS was suggested (17): CBL is a small E3 ubiquitin ligase that negatively regulates intracellular signaling downstream of RTKs. Moreover, cardiac-specific expression of

Tpr-Met, the oncogenic fusion protein of hepatocyte growth factor receptor, was shown to result in concentric hypertrophy and congestive heart failure (45).

A first important suggestion comes from the analysis of the strength in degree and duration of the activity of mutated proteins. Activating *RAS* mutations occur in ~30% of human cancers with a pattern skewed with respect to tissue type and isoform. Interestingly, the somatic mutations triggering cancer tend to be more malignant and less variable than the germline mutations found in RASopathies. Indeed, RAS-related developmental disorders seem to be caused by moderately hyperactivated proteins that can be tolerated in the germline, whereas some, if not most, of the mutations found in cancer are incompatible with development, as suggested by G12D v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) lethality during embryogenesis (106). This might be true also in the case of HCM, which develops in the presence of germline mutants that can be generally considered mild hypermorphs, whereas strong gain-of-function proteins would lead to lethal malformations of the heart, or even cancer, as happens in the Von Hippel-Lindau knock-out mouse (107).

Spontaneous regression is another interesting feature of RAS syndromes: the diagnosis of RASopathies primarily depends on clinical features, but the prevalence of the different characteristics among affected individuals greatly depends on age. The facial features, for example, generally become more difficult to detect in later adolescence and adulthood. The example of juvenile myelomonocytic leukemia is particularly intriguing in this respect, because myeloproliferative disorders that occur in NS infants frequently regress without treatment. Consistently, in some cases (14% of CS patients with HCM and one patient with mutated *KRAS*) a natural regression of HCM occurs with time (10). Elucidating the mechanisms of spontaneous regression in cancer, still not well understood, might be very useful to stimulate cardiomyocytes to escape from HCM as well.

Interestingly, the tissues that are perturbed in CS (the nervous and musculoskeletal systems) greatly overlap with the types of malignancies that are observed (rhabdomyosarcoma, neuroblastoma, ganglioneuroblastoma and bladder cancer). Because *HRAS* mutations account only for <1% of all cancer-associated *RAS* mutations, emerging considerations are that HRAS is transforming only in those tissues in which it exerts a major role and/or the gene is expressed at low levels in most types of cancer-initiating cells. The concept of tumor addiction might be easily transferred to RASopathies as well: specific tissues depend on the sustained activity of specific proteins to grow and survive, as it has been described for Met kinase (108). Accordingly, HRAS, which is mutated in CS, may create a dependence on cardiac cells for their proliferation and survival, so CS patients develop HCM more frequently than patients with other RASopathies. Moreover, for each tissue, or even cell type, there might be a threshold in the response to gain and loss in RAS signaling, dictated by the fact that oncogenes may convey both prosurvival and proapoptotic signals. This idea might explain why either decreasing or increasing SHP2 phosphatase activity has deleterious developmental consequences (109). Finally, cardiomyocytes might be less sensitive to a mutation for which the valves are more sensitive. This may explain why 40% of patients with mutated *RAS* do not develop HCM. For this reason, efforts still have to be addressed to study the role of each causative molecule not only in pathological but also in physiological conditions.

It is also notable that the kinase activities of some of the mutant BRAF proteins found in CFC syndrome are comparable to BRAF oncoproteins; yet the germline CFC mutations do not predispose to tumor formation at all. These observations suggest that the relatively low risk of cancer in many of these syndromes is not entirely due to the degree of biochemical activity of the mutant protein. Indeed, the observation that many individuals with CS do not develop cancer provides evidence that cooperating mutations or concurrent microenvironmental cues are needed for tumorigenesis. This observation should be kept in mind in analysis of models designed to mimic hypertrophy in RASopathies: these syndromes are diseases of complex systems, in which the paradigm one gene one phenotype might be far from being valid. In fact, in humans as well as in mice, different ERK1/2 functions are selectively switched on (for example, the protective and antiapoptotic functions of ERK1/2 more than their hypertrophic functions) in response to a combination of upstream signals that altogether would differently influence the cardiac phenotype. Furthermore, the constitutive activation of ERK1/2 in these transgenic models may be distinct from the carefully tuned activation pattern of endogenous kinases (33,71), even if one of them carries an activating mutation.

Considering all of these findings, why would germline mutations affecting single genes in RAS signaling be sufficient to confer an overt clinical phenotype? Once again, *cancer docet*. The phenomenon of drug resistance in target therapy has been extensively described, that is to say that cancer cells find new pathways to bypass a signaling blockade. Thus, because the germline mutations are present throughout development, there is a substantial amount of time for body cells to adapt to them, activating regulatory feedback loops as well. However, not all the cancers are able to find an escape and those that do not can be eradicated. Correspondingly, the tissues that are perturbed in RAS syndromes may be not only highly sensitive to the RAS/RAF/MEK/MAPK pathway to specify their cell fates, but also relatively incapable of adjusting to overthreshold quantitative variations. It is as if hypertrophic cardiomyocytes were like cancer cells that could not find an escape.

Future Directions for Therapy

Treatment of cardiac manifestations is generally the same in RAS syndrome pa-

tients as in the general population. Surgical and pharmacologic treatment (like verapamil, an inhibitor of calcium channels, and β-blockers, inhibitors of adrenergic receptors) are generally used to alleviate severe cardiac hypertrophy. The RAS/MAPK pathway has long been a drug target because of its involvement in malignant tumors (110). As pointed out in this review, efficient therapeutic strategies that directly target the RAF-MEK-ERK1/2 cascade might be the best tool against cardiac hypertrophy (30,33,111). Possibly, advanced therapies could be based on antisense oligonucleotides that inhibit the translation of the altered genetic portion, or on gene-targeted therapies.

Direct inhibitors of the pathway, which have already been identified in anticancer studies, might represent an alternative. Unfortunately, anticancer drugs designed to specifically target molecules in the RAS pathway have shown considerable side effects. Selective inhibitors of RAF, such as PLX4032, have remarkable clinical activity in patients with melanomas who carry mutated *BRAF* (112). However, as for other inhibitors of oncogenic kinases (113,114), response to PLX4032 is profound but often temporary, because of the loss of addiction and the onset of resistance (115). Moreover, the functional role of MAPKs in the heart itself presents a potential dilemma to any heart disease therapies targeting this pathway: the physiological role of RAS signaling should not be forgotten. In cultured myocytes, MAPK-inducing agents have protective effects against starvation, hypoxia or reoxygenation injury (116,117). MEK transgenic hearts are protected from ischemia/reperfusion and apoptosis; similarly, inactivation of ERK2 promotes damage and death in response to ischemia/reperfusion (118), whereas inhibition of RAF1 promotes myocyte apoptosis and heart failure (58,119). In fact, the RAS pathway modulates the activity of regulators of apoptosis (120–123), as well as of protein kinase Cε and p53 (124). Thus, although activation of RAS signaling may trigger HCM, inhibiting the pathway could render hearts more vulnerable to stress-induced myocyte death, considerably increasing the risk of worsening heart disease.

Clinical efficacy and feasibility would certainly be implemented whether maladaptive hypertrophic signals could be selectively blocked, without affecting the physiological positive effects. It follows that only detailed knowledge about the differential molecular mechanisms of ERK1/2 activation could lead to realistic therapeutic opportunities. The identification of a Thr188-phosphorylation site on ERK2 might be a first step in this direction: this phosphorylation can selectively activate hypertrophic ERK1/2 functions and, therefore, may be targeted without dangerous side effects. Mutant mice that lack this phosphorylation site show preserved cardiac structure and function and attenuated hypertrophic response, indeed demonstrating that ERK1/2 can be selectively targeted, at least in the adult patient (59).

The latest recommended goals for treating hypertrophy may require multiple drugs. Lower doses in combination may render more efficacy and safety than highest doses of single agents. The choice of added agents should greatly depend on both cost and compliance. Currently, generic statins offer several cost benefits. These inhibitors of the posttranscriptional lipid modifications of RAS are likely to be effective, because they have been shown to exert antitumoral action in different cancer cells with mutations on *RAS* (125,126). Indeed, statins reduced cardiac hypertrophy in a transgenic model of HCM (127,128). A combination of low doses of statins and specific inhibitors of pathological ERK1/2 signaling represents, at the moment, a pretty exciting possibility still to be explored.

CONCLUSION

The RAS/RAF/MEK/MAPK pathway is involved in both proliferation and differentiation of different cellular lines, the cardiac lineage included, and thus plays a pivotal role during both adulthood and embryogenesis; this characteristic finally excludes the possibility of completely inhibiting this signaling cascade in young patients (for example, pediatric patients with RASopathy-associated HCM). Although the need of introducing novel drugs in the pediatric clinics is evident, the balance between risk and benefit should be carefully considered. Finally, the dose used to treat children should be reduced as much as possible, considering the possible adverse impact of even a modest degree of toxicity. We suggest that the severity and degree of cardiovascular impairment in pediatric patients with RASopathies should be subjected to accurate stratification; this could enable the minimization of drug dosage to spare patients with mild manifestations of HCM from treatment-related morbidity and mortality. On the other hand, the most severe cases should be treated with targeted and rationally designed therapies aimed at chronicizing a disease that would be otherwise lethal.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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