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Targeting Mitogen-Activated Protein Kinase Signaling in Mouse Models of Cardiomyopathy Caused by Lamin A/C Gene Mutations

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Abstract

The most frequently occurring mutations in the gene encoding nuclear lamin A and nuclear lamin C cause striated muscle diseases virtually always involving the heart. In this review, we describe the approaches and methods used to discover that cardiomyopathy-causing lamin A/C gene mutations increase MAP kinase signaling in the heart and that this plays a role in disease pathogenesis. We review different mouse models of cardiomyopathy caused by lamin A/C gene mutations and how transcriptomic analysis of one model identified increased cardiac activity of the ERK1/2, JNK, and p38 α MAP kinases. We describe methods used to measure the activity of these MAP kinases in mouse hearts and then discuss preclinical treatment protocols using pharmacological inhibitors to demonstrate their role in pathogenesis. Several of these kinase inhibitors are in clinical development and could potentially be used to treat human subjects with cardiomyopathy caused by lamin A/C gene mutations.

1. INTRODUCTION

Lamin A and lamin C are two of the protein building blocks of the nuclear lamina, a meshwork of intermediate filaments on the inner aspect of the nuclear envelope inner membrane (Aebi, Cohn, Buhle, & Gerace, 1986; Fisher, Chaudhary, & Blobel, 1986; Goldman, Maul, Steinert, Yang, & Goldman, 1986; McKeon, Kirschner, & Caput, 1986). They are encoded by the lamin A/C gene (*LMNA*) on chromosome 1q21 and expressed in most differentiated somatic cells (Lin & Worman, 1993; Wydner, McNeil, Lin, Worman, & Lawrence, 1996). Human lamin A and lamin C are identical for the first 566 amino acids with lamin C having six unique carboxyl-terminal amino acids and prelamin A, the precursor of lamin A, having 98 unique carboxyl-terminal amino acids. Prelamin A contains a cysteine–aliphatic–aliphatic–any amino acid (CAAX) motif at its carboxyl-terminus,

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which signals the following series of posttranslational enzymatic modifications: (1) farnesylation of the cysteine, (2) cleavage of the –AAX, and (3) methylation of the carboxyl group of the newly exposed farnesylcysteine (Bergo, Wahlstrom, Fong, & Young, 2008). This modified protein is then recognized by the zinc metalloproteinase ZMPSTE24 and cleaved 15 amino acids from the farnesylated cysteine to yield lamin A (Rusiñol & Sinensky, 2006; Young, Meta, Yang, & Fong, 2006). Lamins and the nuclear lamina have been implicated in a wide range of functions from providing structural support to the nucleus to regulating transcription and DNA replication. More recent data also demonstrate that lamins are connected to the cytoskeleton via integral proteins of the inner and outer nuclear membranes, functioning in mechanotransduction and nuclear positioning (Chang, Worman, & Gundersen, 2015).

Scientific interest in lamin A and lamin C has significantly increased in the past decade and a half since mutations in *LMNA* have been connected to a broad range of inherited diseases often called laminopathies (Worman, Fong, Muchir, & Young, 2009). Depending upon the mutation, these diseases predominantly affect either (1) striated muscle, (2) adipose tissue, (3) peripheral nerve, or (4) multiple systems generating progeroid phenotypes. The most frequently occurring *LMNA* mutations lead to striated muscle diseases virtually always involving the heart. In 1999, Bonne et al. (1999) identified *LMNA* mutations causing autosomal dominant Emery–Dreifuss muscular dystrophy. Progressive muscle weakness and wasting, contractures of the elbows, ankles, and neck; and dilated cardiomyopathy with an early onset atrioventricular conduction block are the classical clinical features. Soon after, mutations in *LMNA* were shown to cause dilated cardiomyopathy without significant skeletal muscle involvement, limb-girdle muscular dystrophy type 1B, and cardiomyopathy with variable skeletal muscle involvement (Brodsky et al., 2000; Fatkin et al., 1999; Muchir et al., 2000). Based on the case series and reports published since these initial discoveries, we now know that the same mutations in *LMNA* can cause any one of these phenotypes, overlaps of these phenotypes and congenital muscular dystrophy, with dilated cardiomyopathy as a common feature (Lu, Muchir, Nagy, & Worman, 2011).

Various cellular signaling pathways are perturbed in diseases arising from mutations in genes encoding nuclear envelope proteins including *LMNA* (Dauer & Worman, 2009). We have used mouse models of cardiomyopathy caused by *LMNA* mutations to analyze alterations in cell signaling in affected heart. In particular, our research has focused on abnormal mitogen-activated protein (MAP) kinase signaling and AKT-mTOR signaling in the *Lmna*^{H222P/H222P} mouse model (Choi et al., 2012; Muchir et al., 2007; Muchir, Wu, et al., 2012). Here, we review methods underlying our discoveries of altered MAP kinase signaling in hearts of mice with *Lmna* mutations and its role in the pathogenesis of cardiomyopathy.

2. MOUSE MODELS OF CARDIOMYOPATHY CAUSED BY *LMNA* MUTATIONS

Several mouse models of human laminopathies, as well as mice with selective deletions of lamin A or lamin C and altered prelamin A processing, have been generated (Stewart, Kozlov, Fong, & Young, 2007; Zhang, Kieckhafer, & Cao, 2013). While the heart is secondarily affected in some models of progeria, several knockout and knockin mice

develop a primary dilated cardiomyopathy, sometimes with accompanying skeletal muscle disease resembling muscular dystrophy (Table 1).

The original *Lmna*^{-/-} mice generated by Sullivan et al. develop dilated cardiomyopathy and muscular dystrophy at an early age, dying by 6–8 weeks (Nikolova et al., 2004; Sullivan et al., 1999). One report has suggested that these mice may not be complete knockouts but rather express a truncated prelamin A (Jahn et al., 2012). Nonetheless, these mice and embryonic fibroblasts derived from them have been widely used to obtain important insights regarding the functions of lamin A and lamin C. Another gene trap *Lmna* knockout line has a shorter lifespan and does not develop left ventricular dilatation prior to death (Kubben et al., 2011).

Lmna^{N195K/N195K} mice, *Lmna*^{H222P/H222P} mice, and homozygous mice expressing nonfarnesylated prelamin A without lamin C all develop cardiomyopathy; however, their phenotypes vary. *Lmna*^{N195K/N195K} have a significantly shorter lifespan than *Lmna*^{H222P/H222P} mice or homozygous mice expressing nonfarnesylated prelamin A without lamin C (Arimura et al., 2005; Davies et al., 2010; Mounkes et al., 2005). *Lmna*^{H222P/H222P} mice also develop skeletal muscle disease. *Lmna*^{32/32} mice have a lifespan of less than 3 weeks and skeletal muscle defects but do not develop left ventricular dilatation prior to death (Bertrand et al., 2012). *Lmna*^{H222P/H222P} mice and homozygous mice expressing nonfarnesylated prelamin A without lamin C have sex differences in disease severity, with male mice more severely affected than female mice.

In humans, cardiomyopathy caused by *LMNA* mutations is virtually always an autosomal dominant disease. In contrast, heterozygous *Lmna* knockout and knockin mice generally have normal lifespans. An exception is *Lmna*^{32/+} mice, which die between 30 and 70 weeks of age and develop dilated cardiomyopathy (Cattin et al., 2013). Although they apparently have a normal lifespan, the *Lmna*^{+/-} mice generated by Sullivan et al. have been reported to develop cardiac conduction defects and late-onset left ventricular dilation at around 20 weeks (Chandar et al., 2010; Sullivan et al., 1999). It is unclear if other heterozygous lines develop late-onset heart abnormalities, as careful phenotyping of older mice has not been reported in the literature.

For most of our research, we have used *Lmna*^{H222P/H222P} mice generated by Arimura et al. (2005). We have found this to be a useful model, as the mice do not develop heart abnormalities until 8–10 weeks of age and grow to a size, when they are much easier to analyze than those with earlier-onset disease and shorter lifespans. By 16 weeks of age, male *Lmna*^{H222P/H222P} mice also have significant cardiac fibrosis, which occurs in humans with cardiomyopathy caused by *LMNA* mutations (Holmström et al., 2011; Raman, Sparks, Baker, McCarthy, & Wooley, 2007). In contrast to the mice expressing nonfarnesylated prelamin A without lamin C, *Lmna*^{H222P} corresponds to a naturally occurring human disease-causing mutation. Because of the sex differences in disease severity, we have mostly utilized male *Lmna*^{H222P/H222P} mice. The male mice develop clinically detectable cardiomyopathy at an earlier age than female mice, making it less time consuming and more cost-effective to carry out experiments. One drawback of male *Lmna*^{H222P/H222P} mice is that at 16–20 weeks of age they develop myopathy that involves the diaphragm as well as other

muscle groups, which makes it difficult to determine if cardiomyopathy is the only process responsible for their death.

3. ALTERED MAP KINASE SIGNALING IN HEARTS OF *Lmna*^{H222P/H222P} MICE

3.1 Cardiac Transcriptomic Analysis

To determine if altered signal transduction occurs in cardiomyopathy caused by *LMNA* mutation, we carried out a transcriptomic analysis of hearts of *Lmna*^{H222P/H222P} male mice at 10 weeks of age. This age is concurrent with the onset of left ventricular dysfunction (Muchir et al., 2007; Muchir, Wu, et al., 2012). We first examined similarities in transcription profiles between hearts from wild-type ($n=8$), *Lmna*^{H222P/+} ($n=7$), and *Lmna*^{H222P/H222P} ($n=6$) mice using hierarchical cluster analysis. This analysis revealed a strong consistency between replicates and yielded 104 probe sets in hearts from *Lmna*^{H222P/+} mice and 114 in hearts from *Lmna*^{H222P/H222P} mice with statistically significant differences in expressed compared to wild-type mouse hearts. There were 57 similar probe sets between hearts from *Lmna*^{H222P/H222P} and *Lmna*^{H222P/+} mice.

We then analyzed Gene Ontology terms applied to genes to identify functional related groups differentially expressed in hearts of mutant mice compared to control mice. This analysis revealed significant differences in expression of genes in several groups, including those encoding proteins in the extracellular-signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 α kinase branches of the MAP kinase pathway. This can be visualized using the KEGG-based pathway visualization tool (Arakawa, Kono, Yamada, Mori, & Tomita, 2005; Fig. 1). The stepwise protocol used for the transcriptomic analysis as follows:

- I. Left ventricles from wild-type, *Lmna*^{H222P/+}, and *Lmna*^{H222P/H222P} male mice were isolated after cervical dislocation at 10 weeks of age.
- II. A rotor-stator homogenizer (Omni International) was used to disrupt 20 mg of tissue in 350 μ l of buffer RNeasy Lysis Buffer (Qiagen) containing 1% β -mercaptoethanol until the tissue was uniformly homogeneous. We centrifuged the lysate for 3 min at 13,000 $\times g$ and transferred the supernatant to a microcentrifuge tube. We then added 1 volume of 70% ethanol and mixed immediately by pipetting. We transferred up to 700 μ l of the sample to an RNeasy spin column placed in a 2-ml collection tube, which was centrifuged for 15 s at 8000 $\times g$. We discarded the flow-through. We add 700 μ l of Buffer RNeasy Wash 1 to the RNeasy spin column and centrifuged for 15 s at 8000 $\times g$. We discarded the flow-through. We then added 500 μ l of Buffer RPE (Qiagen) and centrifuged for 15 s at 8000 $\times g$. We discarded the flow-through. We again added 500 μ l of Buffer RPE and centrifuged for 2 min at 8000 $\times g$. We discarded the flow-through. We placed the RNeasy spin column in a 1.5-ml tube and added 30 μ l of RNase-free water and centrifuged for 1 min at 8000 $\times g$. Adequacy and integrity of extracted RNA were determined by gel electrophoresis and concentrations were measured by ultraviolet absorption spectroscopy (OD 260 and 280 nm).

- III. To analyze RNA transcripts, we used GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix). Methods for target preparation, hybridization, fluidics station setup, array washing staining, and probe array scanning were those in the GeneChip Expression Analysis Technical Manual (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf).
- IV. Image files were obtained through Affymetrix GeneChip software and analyzed by robust multichip analysis using Affymetrix microarray “*cel*” image files and GeneTraffic 3.0 software (Stratagene). Genes were identified as being differentially expressed if they met a false discovery rate threshold of $q < 0.05$ in a two-tailed Student’s *t*-test and showed at least a twofold difference in expression independent of absolute signal intensity.
- V. Gene expression changes related to functional groups were analyzed using the class score method in ErmineJ (version 2.1.12; <http://www.bioinformatics.ubc.ca/ermineJ/>) (Lee, Braynen, Keshav, & Pavlidis, 2005) and the database for annotation, visualization, and integrated discovery program (<http://david.abcc.ncifcrf.gov/>) (Dennis et al., 2003). Alterations in expression of genes encoding component of the MAP kinase pathway, specifically the ERK1/2, JNK, and p38 α branches, were visualized using the KEGG-based pathway visualization tool (Arakawa et al., 2005).

3.2 Immunoblotting Assessment of MAP Kinase Activities in Hearts

Transcriptomic data suggesting that expression of genes in the MAP kinase signaling pathway are altered in hearts of *Lmna*^{H222P/H222P} mice at 10 weeks of age, led us to test whether the activities of the proteins were altered in heart tissue. We used immunoblotting analysis to directly demonstrate increased phosphorylation (activation) of ERK1/2, JNK, and p38 α using the following protocol.

- I. Hearts were excised from *Lmna*^{H222P/H222P} and *Lmna*^{+/+} mice. Left ventricles were dissected and snap-frozen in liquid nitrogen-cooled isopentane and then homogenized using a roto-stator homogenizer (Omni International) in extraction buffer (25 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1% SDS, 1 mM dithiothreitol) containing 25 mg/ml aprotinin and 10 mg/ml leupeptin.
- II. Proteins in homogenates (20 μ g) were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), transferred to nitrocellulose membranes (0.45 μ M, Invitrogen), and blotted with optimal dilutions of primary antibodies against ERK1/2 (No sc94, Santa Cruz Biotechnology), phosphorylated ERK1/2 (No 9101, Cell Signaling Technology), JNK (No sc474, Santa Cruz Biotechnology), phosphorylated JNK (No 9251, Cell Signaling), p38 α (No 9212, Cell Signaling), or phosphorylated p38 α (No 9216, Cell Signaling) in Tris-buffered saline containing 0.1% polysorbate 20 and incubated at room temperature overnight. Antibodies against β -tubulin, β -actin, or GADPH were used as internal controls to normalize the amounts of protein between immunoblots. Blots were then washed in Tris-

buffered saline containing 0.1% polysorbate 20, labeled with horseradish peroxidase-conjugated secondary antibodies, and washed again.

- III. Recognized proteins were visualized by enhanced chemiluminescence (GE Healthcare Life Sciences). Band densities were calculated using Scion Imaging software (version alpha 4.0.3.2), and kinase activity was estimated by calculating the ratio of phosphorylated MAP kinase/total MAP kinase.

This analysis showed increased ratios of phosphorylated to total JNK, ERK1/2, and p38 α in hearts of male *Lmna*^{H222P/H222P} mice compared to those from *Lmna*^{+/+} mice (Fig. 2). For ERK1/2, this increased activation occurs in mice as young as 4 weeks of age and for p38 α in mice as young as 8 weeks (Choi et al., 2012; Muchir, Wu, et al., 2012).

3.3 Activated/Phosphorylated MAP Kinases Translocate into the Nucleus of Cardiomyocytes

Activated/phosphorylated MAP kinases translocate to the nucleus where they modulate the activities of target genes (Plotnikov, Zehorai, Procaccia, & Seger, 2011). We therefore examined nuclear translocation of ERK1/2 in mouse hearts by using an antibody that recognized phosphorylated ERK1/2 in tissue sections. We showed that the phosphorylated form of ERK1/2 is predominantly located in the nucleus of cardiomyocytes. Immunofluorescence microscopic analysis of heart sections from *Lmna*^{+/+} mice using these antibodies revealed a faint, rather diffuse pattern, whereas in heart sections from *Lmna*^{H222P/H222P} mice there was intense and predominantly nuclear fluorescence (Muchir et al., 2007). The protocol we used to analyze nuclear localization of ERK1/2 is as follows:

- I. Hearts were excised from *Lmna*^{+/+} mice and *Lmna*^{H222P/H222P} mice and snap-frozen in liquid nitrogen-cooled isopentane; portions of these hearts can be stored in liquid nitrogen for other subsequent experiments.
- II. Immunofluorescence labeling of phosphorylated ERK1/2 was performed on transverse frozen sections (8 μ m) of left ventricular muscle. Sections were fixed in 3.7% formaldehyde in phosphate-buffered saline for 15 min and then blocked in 5% fetal goat serum in phosphate-buffered saline with 0.1% Triton X-100 for 1 h. This fixation was suitable for the anti-ERK1/2 antibodies used.
- III. Sections were incubated in blocking solution with antiphosphorylated ERK1/2 monoclonal antibody (Cell Signaling Technology) overnight at 4 °C followed by washing in phosphate-buffered saline and incubation with Texas red-conjugated goat anti-mouse immunoglobulin G secondary antibody (Invitrogen). Sections were counterstained with 0.1 μ g/ml 4',6-diamidino-2-phenylindole (Sigma-Aldrich) to label nuclei.
- IV. Images were analyzed using a planar immunofluorescence microscope and intensity of phosphorylated ERK1/2 labeling of cardiomyocytes was measured using Scion Imaging software (version alpha 4.0.3.2; Scion Corp.).

4. MAP KINASE INHIBITOR TREATMENT STUDIES IN *Lmna*^{H222P/H222P} MICE

The discovery that MAP kinase activities were abnormally elevated in hearts of *Lmna*^{H222P/H222P} mice led us to hypothesize that decreasing their activities would lead to clinical improvement in the living animals. We therefore examined the effects of treatment with mitogen-activated protein kinase kinase 1/2 (MEK1/2), JNK, and p38 α inhibitors on *Lmna*^{H222P/H222P} mice.

4.1 MEK1/2 Inhibitors

Allosteric inhibitors of MEK1/2, the MAP kinase kinase that phosphorylates ERK1/2, have been synthesized and tested in humans, primarily for oncology indications (Zhao & Adjei, 2014). Glaxo's trametinib was the first drug in this class to be approved by the U.S. Food and Drug Administration (Wright & McCormack, 2013). We have used several other MEK1/2 inhibitors to examine the effects of blocking ERK1/2 activity in hearts of *Lmna*^{H222P/H222P} mice. Our initial published studies utilized PD098059, which was one of the first small molecule inhibitors of MEK1/2 synthesized by scientists at Parke-Davis Pharmaceutical Research Division of Warner-Lambert Co. (now Pfizer) (Dudley, Pang, Decker, Bridges, & Saltiel, 1995). Although PD098059 inhibits MEK1/2 *in vitro* and *in vivo* (Alessi, Cuenda, Cohen, Dudley, & Saltiel, 1995), it never advanced in clinical development. In subsequent studies, we therefore switched to selumetinib, a potent and selective MEK1/2 inhibitor discovered by scientists at Array BioPharma and licensed to AstraZeneca for clinical development; it is currently being studied in phase III clinical trials (Ciombor & Bekaii-Saab, 2015; Yeh et al., 2007).

4.2 JNK Inhibitors

Pharmacological inhibitors of JNK have been synthesized but their clinical development has been rather limited (Bogoyevitch & Arthur, 2008). They have been hypothesized to be potentially therapeutically useful in a wide range of conditions including neurodegeneration, inflammation, diabetes, viral infections, and fibrosis; however, there are no human clinical data to support these indications. One, PGL5001, has been studied in a phase II clinical trial for inflammatory endometriosis (Barnes, 2013). In our studies of *Lmna*^{H222P/H222P} mice, we used the reversible ATP-competitive JNK inhibitor SP600125 (Bennett et al., 2001).

4.3 p38 α Inhibitors

Several small molecule inhibitors of p38 α have been synthesized (Coulthard, White, Jones, McDermott, & Burchill, 2009). Dilmapiomod has been used in phase II clinical trials for neuropathic pain and chronic obstructive pulmonary disease (Anand et al., 2011; Betts et al., 2015; Singh, Smyth, Borrill, Sweeney, & Tal-Singer, 2010). The p38 α inhibitor ARRY-371797, discovered at Array BioPharma, has been studied in a phase II trial for acute inflammatory pain (Coulthard et al., 2009). According to the company, its development for this indication was discontinued because the results were similar to other p38 inhibitors evaluated in rheumatoid arthritis (<http://investor.arraybiopharma.com/phoenix.zhtml?c=123810&p=irol-newsArticle&ID=1305781>). We used ARRY-371797 in our studies of *Lmna*^{H222P/H222P} mice. Based on its beneficial effects on left ventricular diameters and fractional shortening in these mice (Muchir, Wu, et al., 2012), Array BioPharma has started

a phase II trial of ARRY-371797 for patients with cardiomyopathy caused by *LMNA* mutations (<https://clinicaltrials.gov/ct2/show/NCT02057341>).

4.4 Treatment Protocols

- I. A summary of the MAP kinase inhibitors used is provided in Table 2.
- II. For MEK1/2 inhibition, PD098059 (Selleck Chemicals) or selumetinib (Selleck Chemicals) were delivered to male *Lmna*^{H222P/H222P} mice by intraperitoneal injection using a 27-gauge 5/8-in. needle and syringe. For JNK inhibition, SP600125 (Calbiochem) was similarly delivered. The drugs were dissolved in dimethyl sulfoxide (Sigma-Aldrich) at a concentration of 1.0 or 0.5 mg/ml. Doses were 3 mg/kg/day for PD098059, 1 mg/kg/day for selumetinib, and 1 mg/kg for SP600125, given 5 days a week. To study the effects of an angiotensin-converting-enzyme inhibitor, benazepril (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and similarly administered by intraperitoneal injection at a dose of 10 mg/kg/day. The placebo control consisted of dimethyl sulfoxide alone and was delivered similarly. During treatment, mice were fed a chow diet and housed in a barrier facility.
- III. For p38 α inhibition, ARRY-371797 (Array BioPharma) was delivered to male *Lmna*^{H222P/H222P} mice by oral gavage. It was dissolved in Water for Injection (Gibco) at a concentration of 0.5 mg/ml and dosed at 30 mg/kg twice a day. The placebo consisted of the same volume of Water for Injection.
- IV. To assess effects on heart, treatment was started either when mice were 8 weeks of age (asymptomatic) and continued until 16 weeks or, alternatively, started at 16 weeks of age (symptomatic) and continued until 20 weeks of age. At 16 or 20 weeks of age, mice were analyzed by echocardiography, then euthanized and hearts were excised for biochemical and histological analyses (see below).
- V. To assess the effects of treatment on survival, selumetinib dissolved in dimethyl sulfoxide was diluted in drinking water to a concentration of 0.2 mg/ml; the same volume of dimethyl sulfoxide diluted in drinking water was used as placebo. Treatment was begun at 16 weeks of age and continued until the endpoints were reached (see below).

5. ASSESSMENT OF *Lmna*^{H222P/H222P} MICE AFTER TREATMENT WITH MAP KINASE INHIBITORS

As summarized in Table 3, treatment of *Lmna*^{H222P/H222P} mice with MAP kinase inhibitors has various beneficial effects. Most of our work focused on MEK1/2 inhibitors because, as a class, they are further along in clinical development than JNK and p38 α inhibitors. When treatment with a MEK1/2 inhibitor is begun at 8 weeks of age, before the onset of symptoms, male *Lmna*^{H222P/H222P} mice have significantly smaller left ventricular end systolic and end diastolic diameters and a significantly greater left ventricular ejection fraction, with values similar to wild-type mice, at 16 weeks of age (Muchir et al., 2009). Treatment with the JNK inhibitor SP600125 and analysis at the same ages gives similar

results (Wu et al., 2010). Male *Lmna*^{H222P/H222P} mice treated with MEK1/2 inhibitors or a JNK inhibitor starting at 16 weeks of age, when heart function is already abnormal, have significantly greater left ventricular fractional shortening and ejection fraction, significantly smaller left ventricular diameters and significantly less left ventricular fibrosis than placebo-treated mice at 20 weeks of age (Muchir, Reilly, et al., 2012; Wu et al., 2011). MEK1/2 inhibitor treatment from 16 to 20 weeks also decreases serum concentrations of natriuretic peptide A, which is secreted in response to increased cardiac filling pressure (Muchir, Reilly, et al., 2012). Treatment with a MEK1/2 inhibitor also has synergistic benefits when combined with an angiotensin-converting enzyme inhibitor (Muchir, Wu, Sera, Homma, & Worman, 2014). Treatment with a p38 α inhibitor from 16 to 20 weeks has similar beneficial effects on left ventricular diameters and fractional shortening but does not decrease the expression of genes encoding collagens responsible for fibrosis (Muchir, Wu, et al., 2012). Finally, treatment with a MEK1/2 inhibitor starting at 16 weeks of age significantly prolonged survival of male *Lmna*^{H222P/H222P} mice (Muchir, Reilly, et al., 2012).

The protocols and methods we used to assess the effects of the MAP kinase inhibitors were as follows. Ideally, preclinical drug studies using such protocols such as these should, at a minimum, report on sample-size estimation, whether and how animals were randomized, whether investigators were blind to the treatment and the handling of data, as recommended by the U.S. National Institute of Neurological Disorders and Stroke (Landis et al., 2012).

5.1 Echocardiography

Echocardiography provides a convenient, noninvasive method to assess left ventricular diameters and functions in mice (Rottman, Ni, & Brown, 2007). Potential limitations have been related to the small size and rapid heart rate; however, the development of small probes operating at higher frequencies and faster frame rates has overcome these limitations. Care must be taken not to give too much anesthesia to maintain a physiologically relevant heart rate.

- I. Mice were anesthetized with 1.5% isoflurane by inhalation and placed on a heating pad at 37 °C. We aimed at maintaining a heart rate of greater than 500/s.
- II. Echocardiography was performed using a Visualsonics Vevo 770 ultrasound with a 30 MHz transducer applied to the chest wall. Cardiac ventricular dimensions were measured in two-dimensional mode and M-mode three times for each animal. A typical echocardiographic examination takes approximately 3–5 min.
- III. Fractional shortening was calculated using the following formula: fractional shortening (%) = [(left ventricular end diastolic diameter—left ventricular end systolic diameter)/left ventricular end diastolic diameter] $\times$ 100. Left ventricular ejection fraction was calculated using the modified Simpson rule (Folland et al., 1979).
- IV. The echocardiographer was always blind to mouse genotype and treatment.

5.2 Inhibition of MAP Kinase Activities in Hearts

After treatment with MAP kinase inhibitors, hearts were excised from drug-treated and placebo-treated mice to assess inhibition of ERK1/2 (which is activated by MEK1/2), JNK, and p38 α . As described in Section 3.2 above, we used immunoblotting to measure their activities. Treatment with inhibitors gave approximately 30–70% decreases in the ratio of the phosphorylated MAP kinase to the total MAP kinase compared to treatment with placebo (Muchir, Reilly, et al., 2012; Muchir et al., 2009; Muchir, Wu, et al., 2012; Wu et al., 2011, 2010).

5.3 Natriuretic Peptide A Secretion

Natriuretic peptide A, also known as atrial natriuretic factor and atriopeptin, is secreted from the heart in response to increased intravascular volume and right atrial pressure. Its concentration is elevated in the blood in congestive heart failure (Tikkanen, Fyhrquist, Metsärinne, & Leidenius, 1985). We measured natriuretic peptide A in serum from *Lmna*^{H222P/H222P} mice treated with selumetinib or placebo using the following methods.

- I. Natriuretic peptide A is detected in mouse serum samples using a commercial competitive enzyme immunoassay (RayBiotech).
- II. We added 100 μ l antinatriuretic peptide A antibody solution to each well of the 96-well plate coated with rabbit secondary antibody and incubate for 1.5 h at room temperature.
- III. Serum samples containing biotinylated natriuretic peptide A (10 pg/ml) was added to wells of the microplate and incubated for 2.5 h at room temperature.
- IV. After washing four times in phosphate-buffered saline, we added 100 μ l of horseradish peroxidase–streptavidin solution to each well and incubated for 45 min at room temperature. After washing and incubation for 30 min with 100 μ l of RayBiotech 3,3',5,5'-tetramethylbenzidine One-Step substrate reagent in the dark, absorbance was measured at 450 nm.

5.4 Cardiac Fibrosis

We assessed left ventricular fibrosis in excised hearts of *Lmna*^{H222P/H222P} treated with either a MAP kinase inhibitor or a placebo using histological and biochemical methods. Histological assessment was a semiquantitative analysis of collagen staining. Biochemical analysis measured the expression of two genes encoding collagens.

- I. Sections of left ventricles from *Lmna*^{H222P/H222P} mice treated with a MAP kinase inhibitor or placebo were fixed in 4% formaldehyde for 48 h, embedded in paraffin, sectioned at 5 μ m, and stained with Gomori trichrome, which stains collagen blue.
- II. Representative stained sections were photographed using a Microphot SA (Nikon) light microscope attached to a Spot RT Slide camera (Diagnostic Instruments).
- III. To quantify fibrosis, micrographs were processed (JMicroVision software) and blue-stained fibrotic tissue measured (ImageJ64 software).

- IV. For biochemical analysis of collagen gene expression, primers corresponding to mouse *Colla1* and *Colla2* gene cDNAs (*Colla1* forward 5'-agacggacagtactggatcg-3' and reverse 5'-gtctctttccttgggggttc-3'; *Colla2* forward 5'-ccgtgcttctcagaacatca-3' and reverse 5'-gagcagccatcgactaggac-3') are used from real-time RT-PCR.
- V. Reactions contained HotStart-IT SYBR Green qPCR Master Mix (Affymetrix), 200 nM of each primer, and 0.2 ml of template in a 25-ml reaction volume.
- VI. Amplification was carried out using the ABI 7300 Real-Time PCR System (Applied Biosystems) with an initial denaturation at 95 °C for 2 min followed by 50 cycles at 95 °C for 30 s and 62 °C for 30 s. Relative levels of mRNA expression were calculated using the C_T method (Ponchel et al., 2003) and normalized to calculated Gapdh mRNA expression level.

5.5 Survival

We demonstrated a significantly prolonged survival of male *Lmna*^{H222/H222P} mice treated with the MEK1/2 inhibitor selumetinib. We actually used a combined endpoint of survival and required euthanasia for mice that are in distress. This is a more compassionate protocol to limit pain and distress and was encouraged by our institution's Institutional Animal Care and Use Committee.

- I. Selumetinib dissolved in dimethyl sulfoxide was diluted in drinking water to a concentration of 0.2 mg/ml; the same volume of dimethyl sulfoxide diluted in drinking water was used as placebo.
- II. Treatment was started when mice were 16 weeks of age and continued until death or until a veterinarian blind to treatment group recommended euthanasia because of undue distress.
- III. Criteria for euthanasia were (1) difficulty with normal ambulation, (2) failure to eat or drink, (3) loss of body mass of >20%, (4) rough or unkempt coat, and (5) respiratory distress.
- IV. Data were analyzed using the Kaplan–Meier estimator (Kaplan & Meier, 1958) followed by a log-rank test with $p < 0.05$ considered statistically significant; GraphPad Prism software was used for this analysis.

6. CONCLUSIONS

The experiments and methods described in this review have demonstrated the utility of a mouse model of cardiomyopathy caused by *LMNA* mutation in identifying dysregulated cardiac MAP kinase signaling in the hearts of these animals. Pharmacological inhibitors of kinases in the ERK1/2, JNK, and p38 α pathways were used to demonstrate a role for their abnormally increased signaling activity in pathogenesis. Several of these kinase inhibitors are in clinical development for other indications and could potentially be developed to treat human subjects with cardiomyopathy caused by *LMNA* mutations. Abnormally elevated ERK1/2 and p38 α activity has indeed been demonstrated in hearts from affected human

subjects (Muchir, Reilly, et al., 2012; Muchir, Wu, et al., 2012). A small clinical trial of a p38 α inhibitor is already underway based on this research and others could be anticipated.

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ABBREVIATIONS

ERK1/2	extracellular-signal-regulated kinase 1/2
JNK	c-Jun N-terminal kinase
MAP	mitogen-activated protein
MEK1/2	mitogen-activated protein kinase kinase 1/2

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Figure 1. Genes in the MAP kinase signaling pathway (MAPK signaling pathway) identified using the KEGG-based pathway visualization tool. These include the ERK1/2 (ERK signaling), JNK (JNK signaling), and p38α (p38 signaling) branches within this GO term. Black asterisks indicate genes in the ERK1/2 and JNK branches with statistically significant differences ($q < 0.05$) in expression detected on Affymetrix Mouse Genome 430 2.0 Arrays in hearts of *Lmna*^{H222P/H222P} mice compared to wild-type controls. Red asterisks (dark gray in the print version) indicate genes with statistically differences in expression genes in the p38α branch. From Muchir, Wu, et al. (2012), by permission of Oxford University Press.

Figure 2.

Increased phosphorylation of total ERK1/2, JNK, and p38α in hearts of male *Lmna*^{H222P/H222P} mice. (A) Immunoblot showing phosphorylated JNK (pJNK) and total JNK in protein extracts of hearts from 10-week-old *Lmna*^{+/+}, *Lmna*^{H222P/+}, and *Lmna*^{H222P/H222P} male mice. (B) Immunoblot showing phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 in protein extracts of hearts from 10-week-old *Lmna*^{+/+}, *Lmna*^{H222P/+}, and *Lmna*^{H222P/H222P} male mice. (C) Immunoblot showing phosphorylated p38α (p-p38) and total p38α (p38) in protein extracts of hearts from 8-week-old *Lmna*^{+/+} and *Lmna*^{H222P/H222P} male mice; samples from two hearts of each genotype are shown. (A) and (B) are from Muchir et al. (2007), by permission of the American Society for Clinical Investigation and (C) is from Muchir, Wu, et al. (2012), by permission of Oxford University Press.

Table 1**Knockout and Knockin Mouse Models of Cardiomyopathy Caused by *LMNA* Mutations**

Strain	Homozygous	Heterozygous	References
<i>Lmna</i> ^{-/-} (deletion of exons 8–11)	Lifespan 6–8 weeks, growth retardation at 2 weeks, cardiac conduction defects and left ventricular dilatation at 4–6 weeks, regional muscular dystrophy	Apparently normal lifespan, cardiac conduction defects at 10 weeks, and left ventricular dilatation at 20 weeks (Wolf et al., 2008)	Sullivan et al. (1999), Nikolova et al. (2004), and Chandar et al. (2010)
<i>Lmna</i> ^{GT-/-} (promoter trap construct inserted into intron 2)	Lifespan 2–3 weeks, postnatal growth retardation, left ventricular hypertrophy but no dilatation or conduction defects, muscular dystrophy, decreased subcutaneous adipose tissue	Apparently normal lifespan, no significant cardiac abnormalities	Kubben et al. (2011)
<i>Lmna</i> ^{H222P/H222P}	Male mice median survival 28 weeks, growth retardation at 12 weeks, progressive left ventricular dilatation starting at 8–10 weeks, conduction defects at 12 weeks, regional muscular dystrophy 16–20 weeks; female mice live longer and have later onset of abnormalities	Apparently normal lifespan, no reported cardiac abnormalities	Arimura et al. (2005)
<i>Lmna</i> ^{N195K/N195K}	Lifespan 12–16 weeks, growth retardation at 4 weeks, left ventricular dilatation at 8 weeks (when diameter normalized to body mass), conduction defects by 8 weeks, mild skeletal muscle disease	Apparently normal lifespan, no reported cardiac abnormalities	Mounkes, Kozlov, Rottman, and Stewart (2005)
<i>Lmna</i> ^{hPLAO/nPLAO} (expresses nonfarnesylated lamin A only and no lamin C)	Male mice median survival 38.5 weeks, left ventricular dilatation (age of onset not specified), conduction not analyzed, no apparent skeletal muscle disease; female mice live longer and have less severe disease	Not characterized	Davies et al. (2010)
<i>Lmna</i> ^{32/ 32}	Median survival 15 days (all die by 19 days), growth retardation at 5 days, decreased heart weight but no left ventricular dilatation at 14 days, skeletal myopathy disease at birth	Lifespan 35–70 weeks, left ventricular dilatation by 30–40 weeks, no conduction defects, no skeletal muscle disease	Bertrand et al. (2012) and Cattin et al. (2013)

Table 2

MAP Kinase Inhibitors Used in Treatment Studies of *Lmna*^{H222P/H222P} Mice

Drug	Target	Source	Stock (mg/ml)	Stability	Dose	References for Dose
PD98059	MEK1/2	Calbiochem	1.0	Up to 4 months −20 °C (protect from light)	3 mg/kg/day ip	Dudley et al. (1995)
Selumetinib	MEK1/2	Selleck Chemicals	0.5	Up to 8 months −80 °C	1 mg/kg/day ip ~1 mg/kg/day po ^a	Denton and Gustafson (2011)
SP600125	JNK1/2/3	Calbiochem	1.0	Up to 2 months −20 °C (protect from light)	3 mg/kg/day ip	Bennett et al. (2001)
ARRY-371797	p38α	Array BioPharma	0.5	Not disclosed by source	30 mg/kg/bid po ^b	Array BioPharma personal communication

^aDrug added to drinking water to provide estimated dose of 1 mg/kg/day.

^bBy gastric gavage.

Ip, intraperitoneal; po, oral; bid, twice a day.

Table 3
Summary of Beneficial Effects of MAP Kinase Inhibitor Treatment on *Lmn^{ΔH222P/H222P}* Mice

Target	Drug	Treatment	LV Function ^a	Serum ANF ^b	Fibrosis	Survival	References
MEK1/2	PD98059	8–16 weeks	Significantly improved	NA	NA	NA	Muchir, Shan, Bonne, Lehnart, and Worman (2009)
MEK1/2	PD98059	16–20 weeks	Significantly improved	NA	Significantly decreased	NA	Wu, Muchir, Shan, Bonne, and Worman (2011)
MEK1/2	Selumetinib	16–20 weeks	Significantly improved	Decreased	Significantly decreased	NA	Muchir, Reilly, et al. (2012)
MEK1/2	Selumetinib	16 weeks to death/euthanasia		NA	Significantly decreased	Prolonged	Muchir, Reilly, et al. (2012)
JNK	SP600125	8–16 weeks	Significantly improved	NA	Significantly decreased	NA	Wu, Shan, Bonne, Worman, and Muchir (2010)
JNK	SP600125	16–20 weeks	Significantly improved	NA	Significantly decreased	NA	Wu et al. (2011)
P38α	ARRY-371797	16–20 weeks	Significantly improved	NA	No change	NA	Muchir, Wu, et al. (2012)

^aLeft ventricular function compared to placebo based on fractional shortening/ejection fraction determined by echocardiography.

^bNatriuretic peptide A serum concentration.

NA, not assessed.