A Novel Role for Tumor Necrosis Factor–Like Weak Inducer of Apoptosis (TWEAK) in the Development of Cardiac Dysfunction and Failure

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Background—Tumor necrosis factor–like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor superfamily, is a multifunctional cytokine known to regulate cellular functions in contexts of injury and disease through its receptor, fibroblast growth factor–inducible molecule 14 (Fn14). Although many of the processes and downstream signals regulated by the TWEAK/Fn14 pathway have been implicated in the development of cardiac dysfunction, the role of TWEAK in the cardiovascular system is completely unknown.

Methods and Results—Herein, we demonstrate that mouse and human cardiomyocytes express the TWEAK receptor Fn14. Furthermore, we determine that elevated circulating levels of TWEAK, induced via transgenic or adenoviral-mediated gene expression in mice, result in dilated cardiomyopathy with subsequent severe cardiac dysfunction. This phenotype was mediated exclusively by the Fn14 receptor, independent of tumor necrosis factor-α, and was associated with cardiomyocyte elongation and cardiac fibrosis but not cardiomyocyte apoptosis. Moreover, we find that circulating TWEAK levels were differentially upregulated in patients with idiopathic dilated cardiomyopathy compared with other forms of heart disease and normal control subjects.

Conclusions—Our data suggest that TWEAK/Fn14 may be important in regulating myocardial structural remodeling and function and may play a role in the pathogenesis of dilated cardiomyopathy. (Circulation. 2009;119:2058-2068.)

Key Words: cardiomyocytes ■ heart failure ■ hypertrophy ■ Fn14 TWEAK receptor ■ Tweak protein, mouse

Members of the tumor necrosis factor (TNF) superfamily of cytokines represent a diverse group of signaling molecules that function as essential mediators of human disease, including the pathogenesis of cardiovascular disease.1 TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily of ligands,2 is first synthesized as a type II transmembrane homotrimer and functions primarily as a soluble cytokine with diverse biological roles including proinflammatory activity, angiogenesis, and the regulation of cell survival, proliferation, and death.3 TWEAK mediates these effects through its receptor Fn14,4 a tightly regulated and inducible receptor, and has been suggested to signal through a variety of downstream signaling cascades, including via the nuclear factor-κB, mitogen-activated protein kinase, and AKT pathways.5–7 Whereas the expression of TWEAK has been identified across a range of tissues, including primary inflammatory cells, endothelial cells, and neurons, as well as numerous primary tumors and tumor cell lines,3,8 Fn14 is expressed at relatively low levels in normal tissue. Importantly, Fn14 expression is highly upregulated in contexts of tissue injury and regeneration and chronic inflammatory disease, supporting a role for this pathway in physiological and pathological tissue remodeling.3

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Although many of the processes and downstream signals regulated by TWEAK/Fn14 have been implicated in the...
pathogenesis of cardiomyopathy and heart failure, the role of TWEAK/Fn14 in the cardiovascular system is poorly understood. Therefore, the goal of the present study was to investigate the role of TWEAK in the development of cardiac dysfunction and failure. Herein, we demonstrate that adult mouse and human cardiomyocytes express the TWEAK receptor Fn14. Furthermore, we show that TWEAK overexpression in mice results in dilated cardiomyopathy (DCM), with subsequent cardiac dysfunction and early mortality. This phenotype was mediated exclusively by the Fn14 receptor, independent of TNF-α. Moreover, we find that circulating TWEAK levels were differentially upregulated in patients with idiopathic DCM compared with other forms of heart disease and normal control subjects. Taken together, our data, for the first time, reveal a previously unrecognized role for TWEAK/Fn14 signaling in the development of cardiac dysfunction.

**Methods**

**TWEAK Transgenic and Fn14-Deficient Mice**

Briefly (for expanded Methods, please see the online-only Data Supplement), a murine TWEAK transgenic construct containing an apolipoprotein E enhancer/human α-antitrypsin promoter, the full-length murine TWEAK coding sequence (aa 1 to 249), and the SV40 polyA addition site was generated. C57BL/6xDBA/2 F1 mice expressing full-length TWEAK (fl-TWEAK) were produced and maintained by backcrossing to the C57BL/6 strain, as described previously. A soluble murine TWEAK transgenic construct was generated by cloning of a transgene composed of a murine IgG-k signal sequence followed by murine TWEAK cDNA encoding amino acids 101 to 249 into an expression vector downstream of the α-antitrypsin promoter and β-globin intron and upstream of the human growth hormone polyA sequence (plasmid was obtained from Sigma, St. Louis, Mo). Fn14−/− mice were generated as described on the 129 strain background and backcrossed onto the C57BL/6 strain. All mice were in accordance with Institutional Animal Care and Use Committee–approved protocols.

**Serum TWEAK Analysis**

Circulating TWEAK was detected in human and mouse serum by solid-phase enzyme-linked immunosorbent assay with the use of anti-TWEAK monoclonal antibody as described previously (for detailed methods, please see the online-only Data Supplement).

**Fn14 Protein Expression in Cardiomyocytes and Heart Tissue**

Fn14 expression was confirmed in adult cardiomyocytes with the use of immunocytochemistry with the P3D8 antibody against murine Fn14 (Biogen Idec, Cambridge, Mass) and costained with α-actin (Abcam, Cambridge, Mass) to delineate myofilament proteins. Cardiomyocytes or heart tissue homogenate was used to perform SDS-PAGE and was followed by Western blotting analysis with a polyclonal rabbit antibody against Fn14 (Biogen Idec).

**TWEAK Adenovirus and In Vivo Gene Delivery**

Adenoviral vector—expressing soluble murine TWEAK (AdTWEAK) and adenoviral control vector—expressing GFP (adCon) were generated as described. A total of 10^5 viral particles was delivered intravenously to adult wild-type (WT) and Fn14−/− mice and in other studies to TNF-deficient mice (strain B6;129S6-Tnf−/−) or their WT counterparts (strain B6129SF2J 101045), both obtained from the Jackson Laboratory (Bar Harbor, Me).

**Murine Echocardiography**

Transthoracic echocardiograms were obtained in conscious mice, as described previously. The heart was imaged in the 2-dimensional parasternal short-axis view, and an M-mode measurement was recorded at the midventricle at the level of the papillary muscles. Heart rate, wall thickness, and end-diastolic and end-systolic dimensions were measured from the M-mode image with analysis software in Sequoia, Acuson (Sonoma Health Products, Forestville, Calif) or Vevo 770, Visual Sonic (Toronto, Ontario, Canada). Diastolic left ventricular (LV) chamber dimension at diastole was used as a marker of cardiac chamber enlargement. Wall thickness is the average of anterior and posterior wall thickness at diastole. Fractional shortening, which was defined as the end-diastolic dimension minus the end-systolic dimension normalized for the end-diastolic dimension, was used as an index of cardiac contractile function.

**Cardiomyocyte Analysis and Contractile Function**

Cardiomyocytes were isolated from adult mice, and cell contractile function was determined, as described previously. Cardiomyocytes were perfused with 1.8 mmol/L CaCl₂ Tyrode solution at 37°C and were electrically paced at 300 bpm via platinum wires. Cell shortening and relengthening were measured through video edge detection (IonOptix, Milton, Mass). Percent cell shortening was calculated as diastolic cell length minus systolic cell length normalized to diastolic cell length. The duration of cardiomyocyte relaxation was determined by commercially available acquisition software (IonOptix). Cell length and cell width measurements were determined in ≈500 WT or fl-TWEAK cardiomyocytes from each heart (n = 3 to 6 hearts per group), and cell histograms were generated.

**Gross Tissue and Histopathological Analysis**

Total heart weight and body weight in mice were determined at the time of euthanasia, and the ratio was used as an index of cardiac hypertrophy. Lung samples were also obtained to determine the ratio of wet-to-dry weight, as an indicator of pulmonary congestion. Hearts were arrested in diastole by 30 mmol/L KCl, followed by perfusion fixation with 10% buffered formalin. Hearts were stained with trichrome for histological assessment of fibrosis. Apoptotic cells were detected in midpapillary section with a commercially available terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay kit (In Situ Cell Death kit, Roche Applied Science, Indianapolis, Ind). TUNEL-positive nuclei were counted by a blinded observer, at 5 to 6 fields per section at ×20 magnification of a total of ≈2000 nuclei counted per heart (n = 3 hearts per group). Sections were costained with α-actin (Abcam) to delineate cardiomyocytes from other cell types in the heart.

**Quantitative Polymerase Chain Reaction and In Situ Hybridization**

Quantitative polymerase chain reaction (PCR) for TWEAK, Fn14, α-myosin heavy chain, atrial natriuretic peptide, brain natriuretic peptide, and sarcoendoplasmic reticulum Ca^{2+}-ATPase as well as in situ analysis for Fn14 on paraffin-embedded E12.5 mouse heart sections was performed by the methods described previously.

**Human Studies**

A total of 56 patients with diagnosed heart failure who were seen in the Cardiomyopathy Clinic at Boston Medical Center and 14 healthy, age-matched control subjects were included in this study. Patients with heart failure were subdivided into 3 groups, according to the cause of their cardiomyopathy, as follows: (1) coronary artery disease (CAD) or ischemic cardiomyopathy, defined as documented prior myocardial infarction or angiographically demonstrated CAD; (2) hypertensive cardiomyopathy, defined as documented chronic hypertension (systolic blood pressure >140 mm Hg) in the absence of CAD; or (3) idiopathic DCM, defined as cardiomyopathy in the absence of an underlying cause, including CAD (excluded via coronary angiography), hypertension, toxin exposure, chronic alcohol/drug use, thyroid disease, myocarditis, and infiltrative disease. Additionally, patients with familial idiopathic DCM, known as
large-scale whole genome expression analysis across 80 adult human tissues and cells\(^{18}\) (http://symatlas.gnf.org) revealed a high relative expression of Fn14 in cardiomyocytes, 3-fold greater than the overall tissue mean, with the level of expression second only to bronchial epithelial cells, smooth muscle cells, colorectal adenocarcinoma, and placenta (Figure 1 in the online-only Data Supplement).

### Circulating TWEAK Results in Profound Cardiomegaly, Heart Failure, and Early Mortality in Mice

To determine the role of the TWEAK/FN14 axis in the development of cardiovascular pathology, transgenic mice were generated through α-antitrypsin promoter–driven overexpression of truncated, soluble TWEAK (sTWEAK) or fl-TWEAK (Figure 2A).\(^{11}\) A noncardiac transgene promoter was utilized to specifically increase systemic circulating elevations of TWEAK and to avoid nonspecific cardiac dysfunction that may have resulted from a elevated local cytokine concentrations at the level of the cardiomyocyte. Ten independent sTWEAK founders and 1 fl-TWEAK founder were generated with detectable circulating TWEAK, and transgene expression was confirmed in the liver of TWEAK transgenic mice, without expression in the heart (Figure 2B).\(^{11}\) Circulating TWEAK in sTWEAK founders was \(\approx1000\)-fold relative to WT littermate control mice (Figure 2C). Within 3 to 4 months of age, 5 of the 10 sTWEAK founders died unexpectedly, and another 5 had to be euthanized because of signs of end-stage heart failure, including labored breathing, anorexia, and anasarca. Autopsy analysis of sTWEAK founders revealed the presence of grossly enlarged hearts in all animals, consistent with profound DCM (Figure 2D). Pathological analysis ruled out congenital cardiac abnormalities, including atrial or ventricular septal defects, malposition of the great vessels, and valvular pathology (data not shown).

To determine the functional consequences of chronic exposure to lower circulating levels of TWEAK (Figure 2C), we employed fl-TWEAK transgenic mice. Similar to findings seen with sTWEAK mice, fl-TWEAK transgenic mice developed progressive DCM and subsequent heart failure, as marked by cardiomegaly and biventricular chamber enlargement (Figure 3A), with a corresponding increase in heart weight–to–body weight ratio and wet-to-dry lung weights relative to WT controls (Figure 3B and 3C). The development of DCM in fl-TWEAK mice resulted in increased mortality, with only 50% survival at 6 months (Figure 3D), in stark contrast to WT littermate controls, which had 100% survival.

Histopathological analysis revealed frequent mural thrombi in enlarged cardiac atria and ventricles in fl-TWEAK mice and inflammation only in association with thrombi. No evidence of myocarditis was found in fl-TWEAK hearts. The progressive development of myocardial and perivascular fibrosis was noted, with marked fibrosis present in end-stage failing hearts (Figure II in the online-only Data Supplement). Given the prior association of TWEAK with vasculogenesis,\(^{4,8,15}\) fl-TWEAK mice were further examined for vascular malformation. Hearts from fl-TWEAK mice exhibited no disparity in vascular organization, large-vessel concentration, or capillary density. Associated with the development of cardiomyopathy, fl-TWEAK mice demonstrated profound in vivo contractile failure and adverse structural remodeling with lung congestion (Figure 3C), as well as reduced fractional shortening and increased ventricular dilation, as evidenced by echocardiography (Figure 3E and 3F).

To exclude any confounding effects of TWEAK on cardiac development and to determine whether short-term exposure to higher circulating TWEAK was sufficient to result in DCM, elevated circulating levels of TWEAK were induced in adult mice through intravenously delivered adenovirus encoding the murine sTWEAK gene (adTWEAK). After in vivo adenoviral delivery, circulating TWEAK levels were elevated markedly compared with fl-TWEAK mice (Figure III in the online-only Data Supplement), with the rapid development of progressive cardiac dysfunction and dilatation within 2 to 3 weeks. Similar to the phenotype observed in fl-TWEAK mice, adTWEAK mice exhibited marked cardiac enlargement and increased heart weight–to–body weight ratio, progressive in vivo ventricular dilatation, and impaired contractile function by echocardiography as well as alteration in fetal gene program (Table).

### TWEAK Results in the Development of Cardiomyocyte Hypertrophy and Reduced Cellular Contractility

Because TWEAK may affect several cell types within the heart in vivo, including cardiac endothelial and smooth...
muscle cells, we examined the direct effects of circulating TWEAK on cardiomyocyte size and function. Cardiomyocytes isolated from adult fl-TWEAK mice revealed the development of cellular hypertrophy with cellular elongation and minimal change in cell width (Figure 4A, 4B, and 4C). fl-TWEAK cardiomyocytes also exhibited decreased cellular shortening and prolonged cellular relaxation (Figure 4D and 4E).

TWEAK-Induced Cardiac Dysfunction Is Not Associated With Cellular Apoptosis
To determine whether programmed cell death plays a causal role in TWEAK-mediated DCM, cellular apoptosis was measured with the TUNEL assay in heart sections from adTWEAK- and adCon-treated animals at 1 and 3 weeks after viral injection. Evaluation of programmed cell death in...
adTWEAK hearts revealed infrequent TUNEL-positive cells (<0.1% of total nuclei counted), with no detectable increase compared with adCon counterparts at 1 week after adenoviral TWEAK. A significant increase occurred, however, in TUNEL-positive cells in adTWEAK hearts at 3 weeks after adenovirus delivery compared with adCon hearts at the same time point (Figure 5A). Importantly, costaining for the cardiac-specific protein α-actin revealed that TUNEL-positive cells were not cardiac in origin (Figure 5B and 5C). Similar results were found in fl-TWEAK mice (data not shown). Given the lack of TUNEL-positive cardiomyocytes in adTWEAK hearts, our data suggest that ventricular enlargement and contractile dysfunction in adTWEAK mice were not secondary to increased apoptotic cell death.

**Fn14 Is Required for TWEAK-Induced Cardiac Dysfunction and Dilation**

TWEAK has previously been suggested to signal through a cell membrane–bound receptor, Fn14,4,19 as well as through Fn14 receptor–independent means.20–22 To further investigate the mechanisms underlying TWEAK-induced cardiomyopathy, we determined whether Fn14 is required for the development of contractile dysfunction and structural remodeling after treatment with TWEAK. Mice lacking the Fn14 receptor (Fn14<sup>−/−</sup>)<sup>11,12</sup> were examined and demonstrated normal survival (data not shown) and cardiac function. Mice were subsequently subjected to the tail vein injection with adTWEAK or adCon. Strikingly, mice lacking Fn14 were protected against TWEAK-induced cardiac dysfunction and dilation, with preservation of baseline heart weight-to-body weight ratio (Figure 6A and 6B). Importantly, LV chamber dimension and contractile function (Figure 6C) as determined by echocardiography were also maintained in Fn14<sup>−/−</sup> mice receiving adTWEAK compared with adCon. Thus, these data suggest that TWEAK-induced cardiac dysfunction is mediated via the Fn14 receptor.

**TWEAK-Induced Cardiac Hypertrophy Is Independent of TNF Signals**

Because TWEAK and TNF are related cytokines with similar pleiotropic activity and TNF overexpression has previously been shown to induce DCM in mice, we investigated whether TWEAK mediates its effect indirectly through TNF by adTWEAK injection into TNF knockout mice. We found comparable DCM induced by adTWEAK in WT and TNF knockout mice, as shown by the increased heart weight-to-body weight ratios (Figure 7). Thus, our data demonstrate that TNF does not mediate TWEAK-induced cardiac hypertrophy.

**Elevated Circulating TWEAK Levels in Patients With Idiopathic DCM**

To determine whether elevated circulating TWEAK is associated with the development of cardiomyopathy in humans, serum samples from patients with cardiomyopathy and...
healthy controls were obtained, and circulating TWEAK levels were determined. Patients with cardiomyopathy were subdivided according to the causal pathway of cardiac dysfunction into CAD (as determined by prior myocardial infarction or angiographically proven atherosclerosis), hypertension (as determined by the presence of chronic hypertension), and idiopathic DCM (heart failure in the absence of a known cause including CAD, hypertension, toxin exposure, drug or alcohol use, thyroid disease, myocarditis, or infiltrative disease). Patients with familial DCM or known genetic cardiomyopathy were excluded from this investigation (patient classification is further described in Methods). All patients exhibited New York Heart Association class I to III symptoms, consistent with a mild to moderate degree of heart failure, were without evidence of acute cardiac decompensation or acute renal failure, and were matched for age (patient demographics are provided in the Table in the online-only Data Supplement). All heart failure patients were maintained on established cardiac regimens (Table in the online-only Data Supplement), and all serum samples were obtained in hemodynamically stable patients on an outpatient basis. The degree of contractile failure and ventricular dilation, as determined by echocardiography, was similar among CAD, hypertensive, and idiopathic DCM patients, as evidenced by

Figure 3. Development of DCM and heart failure in fl-TWEAK mice and cardiac enlargement in fl-TWEAK mice. A, Representative whole hearts and trichrome-stained longitudinal sections from WT and fl-TWEAK animals, demonstrating LV, right ventricular (RV), and left atrial (LA) enlargement in transgenic hearts. B, Heart weight-to-body weight ratios, suggesting the development of cardiac hypertrophy in fl-TWEAK animals. C, Wet-to-dry lung weights in WT and fl-TWEAK mice. *P<0.01 vs WT. D, Survival plots for WT and fl-TWEAK animals, showing increased mortality in fl-TWEAK mice. *P<0.01 vs WT. E, Representative M-mode conscious transthoracic echocardiograms in WT and fl-TWEAK animals. F, Diastolic and systolic ventricular chamber dimensions and fractional shortening in WT and fl-TWEAK mice, as assessed by echocardiography, showing the development of in vivo idiopathic DCM and heart failure with TWEAK overexpression. *P<0.01 vs WT.
the significantly reduced ejection fraction and elevated ventricular diastolic dimensions (Table in the online-only Data Supplement) relative to control matched patients.

Circulating TWEAK levels were assessed in serum samples from all patients. Patients with DCM exhibited elevated circulating TWEAK levels relative to controls (mean circulating TWEAK, 161 ± 35 versus 100 ± 6 pg/mL; P < 0.01) and patients with CAD-associated (107 ± 9; P < 0.05) or hypertension-associated (109 ± 14; P < 0.05) cardiomyopathy. TWEAK levels were <150 pg/mL in all controls, whereas approximately a third of patients with DCM exhibited circulating TWEAK levels >150 pg/mL. Circulating TWEAK levels in patients with DCM did not correlate with clinical functional parameters, including New York Heart Association class, ejection fraction, or LV diastolic chamber dimensions (data not shown).

Discussion

This report provides the first evidence suggesting that the TWEAK/Fn14 axis may play an important role in the development of cardiac dysfunction. We demonstrate that cardiomyocytes express the TWEAK receptor Fn14 and that elevated circulating levels of TWEAK are sufficient to cause DCM, cardiac dysfunction, and early death in mice. TWEAK-induced cardiomyopathy is also found to be dependent on the Fn14 receptor, but not TNF, and is associated with cardiomyocyte elongation and cardiac fibrosis but not increased premature apoptosis. Moreover, circulating TWEAK is found to be differentially increased in patients with idiopathic DCM relative to other forms of cardiomyopathy and normal controls.

We employed 2 genetic approaches to specifically determine the consequence of elevated circulating levels of TWEAK in mice. Using both transgenic mice and adenoviral-mediated delivery, we showed that elevated circulating TWEAK is sufficient to cause cardiac dilation and progression to heart failure, with characteristic functional and histopathological features. Interestingly, the time to onset of cardiac dysfunction appeared to correlate with the levels of circulating TWEAK, with cardiomyopathy developing most rapidly in adTWEAK mice with high levels of circulating TWEAK, followed by sTWEAK mice with intermediate levels of circulating TWEAK, and more slowly in fl-TWEAK, which exhibit lower circulating levels of TWEAK. It is noteworthy that although we have reported previously that overexpression of TWEAK in mice stimulates oval cell proliferation, serum liver enzymes were normal in fl-TWEAK mice, suggesting the lack of any significant liver dysfunction that may have contributed to cardiac dysfunction.

The mechanisms underlying the development of TWEAK-induced DCM in mice remain to be thoroughly understood. Although TWEAK has previously been suggested to signal through both Fn14†‡ and Fn14 receptor–independent mechanisms,20–22 our studies indicate that signaling through the TWEAK receptor Fn14, which is expressed in cardiomyocytes, is required. TWEAK does not appear to act by inducing cardiomyocyte apoptosis because no significant increase in apoptosis was found in TWEAK-overexpressing mice before the development of heart failure, suggesting that apoptosis is not a cause but rather a consequence in this context. We also show that TWEAK-induced hypertrophy in mice is independent of TNF.10 Fn14 has been shown to be associated with TNF receptor–associated factors and may signal through nuclear factor-κB, mitogen-activated protein kinase, and AKT pathways. Consistent with this signaling potential, we found that TWEAK activates nuclear translocation of p65 as well as p50 in different time courses in isolated adult cardiomyocytes (data not shown). Additionally, in agreement with the suggested ability of TWEAK to promote cell growth in other cell types,3 we found that circulating TWEAK led to cardiomyocyte hypertrophy by increasing cell length and upregulation of the fetal gene program. Future efforts to delineate the molecular mechanisms by which the TWEAK/Fn14 pathway acts on the heart are certainly warranted.

The TWEAK/Fn14 pathway is generally expressed at low levels in normal animals and is highly upregulated with injury or disease.3 Consistent with this, no homeostatic role of the pathway is apparent, and TWEAK- and Fn14-deficient mice are healthy and have a normal life span (L.C. Burkly, PhD, unpublished data, 2009). Once upregulated by acute injury, TWEAK/Fn14 plays a beneficial role in coordinating cell expansion and acute inflammation contributing to tissue repair, as exemplified in models of acute liver and skeletal

### Table. Animal Characterization After adTWEAK Administration

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<td><strong>1 Week after injection</strong></td>
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<tr>
<td>adCon (n=10)</td>
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<tr>
<td>adTWEAK (n=10)</td>
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3 Weeks after injection

| adCon (n=6) | 3.97 ± 0.04 | 0.86 ± 0.04 | 2.90 ± 0.19 | 51.5 ± 1.3 | 689 ± 11 | 2.1 ± 0.5 | 1.4 ± 0.2 | 5.3 ± 0.6 | 1.4 ± 0.1 |
| adTWEAK (n=5) | 5.65 ± 0.28†‡ | 0.74 ± 0.03† | 4.06 ± 0.28†‡ | 12.6 ± 4.1†‡ | 457 ± 58†‡ | 32.2 ± 8.3†‡ | 4.6 ± 1.1†‡ | 12361 ± 849†‡ | 0.6 ± 0.1†‡ |

**HW** indicates heart weight; **BW**, body weight; **FS**, fractional shortening; **HR**, heart rate; **ANP**, atrial natriuretic peptide; **BNP**, brain natriuretic peptide; **MHC**, myosin heavy chain; **SERCA**, sarcoendoplasmic reticulum Ca2+ ATPase; and **GADPH**, glyceraldehyde-3-phosphate dehydrogenase.

*P < 0.05 vs adCon 1 week; †P < 0.05 vs adCon 3 weeks; ‡P < 0.05 vs adTWEAK 1 week.
Figure 4. Cellular remodeling and cardiomyocyte dysfunction in fl-TWEAK hearts. A, Representative cardiomyocyte isolates from WT and fl-TWEAK hearts. Histograms and corresponding average number of cell length (B) and cell width (C) measured in isolated WT and fl-TWEAK cardiomyocytes are shown (n=3 animals each and >500 cardiomyocytes counted per heart; *P<0.05 vs WT). D, Representative cellular tracings for paced WT and fl-TWEAK cardiomyocytes. E, Percent cell shortening and time to relaxation for WT and fl-TWEAK cardiomyocytes, demonstrating impaired cardiomyocyte contraction and prolonged relaxation with TWEAK overexpression (n=4 animals each and 20 to 30 cardiomyocytes per heart; *P<0.05 vs WT).
Sustained expression of the TWEAK/Fn14 pathway, however, in the context of pathological stress may contribute to maladaptive cardiac remodeling, as modeled in our TWEAK-overexpressing mice. Our studies highlight the potential contribution of TWEAK-induced cell growth in mediating cardiac structural and functional changes, although it is possible that TWEAK-induced proinflammatory cytokines, chemokines, matrix metalloproteinases, and regulation of cell death may contribute under more complex pathophysiological conditions.

Figure 5. Limited apoptosis in adTWEAK hearts. A, Percent TUNEL in adTWEAK and adCon hearts at 1 and 3 weeks after TWEAK or control viral injection via tail vein (see Methods). Greater than 2000 nuclei were counted from 5 to 6 random areas from midpapillary section of each heart. *P<0.05 vs adTWEAK (n=3 each). B, Representative TUNEL staining at 3 weeks after viral injection adTWEAK hearts at ×40 magnification and ×63 magnification (C). α-Actin stains for cardiac myocytes (red), and DAPI stains for nuclei (blue).

Figure 6. Fn14 mediates the development of TWEAK-induced DCM. A, Representative whole heart pictures from WT and Fn14 knockout (Fn14−/−) animals treated with control adenovirus (adCon) or adenovirus encoding murine soluble TWEAK (adTWEAK). B, Heart weight-to-body weight ratios from WT and Fn14−/− animals after intravenous injection with adCon or adTWEAK, demonstrating attenuated TWEAK-mediated cardiac enlargement in animals that lack the Fn14 receptor. C, In vivo LV diastolic chamber dimension and cardiac fractional shortening, as determined by conscious transthoracic echocardiography, in WT and Fn14−/− animals after intravenous delivery with adTWEAK, showing lack of TWEAK-mediated DCM and contractile failure in mice that lack the Fn14 receptor. n=3 to 5 per group; for B, *P<0.01 vs WT adCon, †P<0.01 vs WT adTWEAK; for C, *P<0.05 vs WT adTWEAK.
Elevated levels of cytokines have previously been associated with heart failure in general and correlate with severity of symptomatic disease.²⁰²⁴ Interestingly, circulating TWEAK appears to be differentially upregulated in patients with idiopathic DCM compared with patients with heart failure of known cause and healthy controls, suggesting that in at least a subset of patients with DCM, the TWEAK/Fn14 axis may contribute to the pathogenesis of heart failure. In addition to our findings of elevated circulating TWEAK in idiopathic DCM patients, circulating TWEAK was found to be altered in other human diseases associated with elevated inflammation, including rheumatoid arthritis, stroke, atherosclerosis, type 2 diabetes, and end-stage renal disease.²⁵–²⁸ Although the levels of circulating TWEAK achieved in mice, through either transgenesis or viral-mediated overexpression, were significantly higher than those measured in DCM patients, the time course for the development of cardiac dysfunction in mice was condensed, occurring in weeks to months compared with years in humans. As observed in our AdTWEAK-treated mice, cardiac dysfunction may persist after circulating TWEAK levels have returned to lower values. Importantly, our human samples were obtained in patients with stable cardiomyopathy rather than new-onset cardiomyopathy, and, as such, it is unknown whether patients exhibited higher circulating TWEAK levels at the time of initial cardiac dysfunction. Our data, however, suggest that TWEAK may trigger the molecular events sufficient to induce cardiac dysfunction, which may persist even after TWEAK levels return to normal. Although we acknowledge the potential caveats associated with the difference in absolute concentration of circulating TWEAK between humans and mice, our studies provide a “proof of concept” for TWEAK in the development of cardiac dysfunction. Note that the source of systemic TWEAK secretion in patients with DCM remains unclear, although prior reports have detailed the expression and secretion of TWEAK by activated circulating immune cells and vascular cells.³

Our study reveals for the first time the importance of a relatively unstudied signaling pathway, TWEAK/Fn14, in the cardiovascular system. Further studies are warranted to delineate the potential for TWEAK as a biomarker and/or therapeutic target for the treatment of patients with DCM.

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**Disclosures**

A. Jakubowski, J.S. Thompson, T. Crowell, M.Z. Wang, and Drs Su, Ambrose, Parr, Lincecum, Hsu, Zheng, Michaelson, and Burky are past or present employees of Biogen Idec Inc. The remaining authors report no conflicts.

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**CLINICAL PERSPECTIVE**

Cardiomyopathy and heart failure remain the only cardiovascular diseases in which the incidence and mortality continue to rise. Despite intensive investigation, the mechanisms underlying the pathophysiology of heart failure and the progression of cardiac dysfunction remain poorly understood. In the present report, we identify a novel cytokine, TWEAK, and its cellular receptor Fn14 as important mediators of cardiac dysfunction and cardiomyopathy. Augmenting circulating TWEAK levels in mice through genetic approaches resulted in the development of dilated cardiomyopathy, with subsequent cardiac dysfunction and early mortality, a process dependent on the Fn14 cellular receptor. Moreover, we found that circulating TWEAK levels are elevated in patients with idiopathic dilated cardiomyopathy relative to other forms of heart disease and normal control subjects. Taken together, our data suggest that TWEAK/Fn14 may play an important role in the pathogenesis of cardiomyopathy.
A Novel Role for Tumor Necrosis Factor–Like Weak Inducer of Apoptosis (TWEAK) in the Development of Cardiac Dysfunction and Failure

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SUPPLEMENTAL MATERIAL
Expanded Supplemental Methods

TWEAK Transgenic and Fn14 Deficient Mice

The murine TWEAK transgenic construct containing an apolipoprotein E (ApoE) enhancer/human alpha antitrypsin (AAT) promoter, the full-length murine TWEAK coding sequence (aa 1-249), and the SV40 polyA addition site were generated. The AAT promoter primarily drives expression in the liver without expression in the heart.

C57BL/6 × DBA/2 F1 mice expressing full-length TWEAK (fl-TWEAK) were produced, typed, and maintained by backcrossing to the C57BL/6 strain, as previously described.1 The cardiomyopathy phenotype was analyzed in transgenic mice from the fourth to the seventh backcross generations and nontransgenic littermate mice from the same backcross were used as wild-type (WT) controls. A soluble murine TWEAK transgenic construct was generated by cloning of a transgene composed of a murine IgG-κ signal sequence followed by murine TWEAK cDNA encoding amino acids 101 to 249 into an expression vector downstream of the AAT promoter and beta globin intron, and upstream of the human growth hormone polyA sequence (plasmid was obtained from Kimi Araki and Masatake Araki, Department of Pathology, CMU, Geneva, Switzerland). The expression cassette was excised from the vector with HindIII + XhoI prior to microinjection. Soluble TWEAK transgenic (sTWEAK) (C57BL/6 X SJL) F2 mice were generated at Xenogen (Cranbury, NJ) through standard procedures. Fn14−/− mice were generated as described 1,2 on the 129 strain background and then were backcrossed five times onto the C57BL/6 strain. The Fn14−/− was backcrossed five times onto the C57BL/6 background and WT and knockout mice were intercrossed to generate cohorts for
experiments. All mice were used in accordance with Institutional Animal Care and Use Committee approved protocols.

**Serum TWEAK Analysis**

Circulating TWEAK was detected in human serum by solid phase enzyme-linked immunosorbent assay (ELISA) as follows. Anti-TWEAK antibody BEB3 was coated at 2 µg/mL in 0.1 mol/L carbonate, pH 9.5, onto 96 well plates (Corning Costar 3590; Corning Life Sciences, Cambridge, Mass) overnight at 4°C. Plates were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 6 hours at 4°C and then were washed four times with 0.1% Tween 20 in PBS. Human serum was diluted 20-fold in PBS with 3% BSA and loaded at 100 µL per well. The standard was his-tagged recombinant soluble human TWEAK, starting at a concentration of 20 ng/mL titrated down to 0.1 pg/mL with a 3-fold dilution factor. Both serum samples and standard were incubated on the plate overnight at 4°C. The plate was washed six times with 0.1% Tween 20 in PBS, and the signal detected by the addition of 100 µL of premixed biotinylated anti-TWEAK antibody P5G9 at 0.5 µg/mL final dilution and HRP-Avidin at 1:250 final dilution (BD Biosciences, San Jose, Calif) for 1 hour. The plate was then washed six times with 0.1% Tween 20 in PBS, followed by the addition of 100 µL substrate solution (BD Biosciences) over 1 hour at room temperature. The reaction was stopped by the addition of 100 µl 2 N H₂SO₄, and absorption read at 450 nm. For the ELISA to detect circulating TWEAK in mouse serum, plates were coated with the anti-TWEAK mAb BC.B10 at 5 µg/mL in 50 mmol/L sodium carbonate buffer and blocked with 1% BSA in PBS for 1 hour. The standard his-tagged recombinant soluble murine
TWEAK and diluted samples were incubated for 1 hour; this was followed by washing and addition of anti-TWEAK mAb AB.G11 at 5 µg/mL for 30 minutes. For detection, biotin mouse anti-hamster mAb G94-56 (BD PharMingen, San Diego, Calif) was added at 2 µg/mL, followed by alkaline phosphatase/streptavidin at 1:1000 (Jackson ImmunoResearch Laboratories, West Grove, Pa) and substrate solution at 5 mg/mL (Sigma, Natick, Mass). The mAbs BE.B3, BC.B10, AB.G11 and P5G9 have been previously described,3-5 Recombinant soluble human TWEAK was as previously described,5 except that it was His- rather than myc-tagged. Murine recombinant soluble TWEAK was an aglycosyl form with an N-terminal six times his followed by amino acid residues A106-H249, with the N139Q substitution at the N-linked glycosylation site of murine TWEAK (Genbank accession number AF030100). Human and murine forms of soluble TWEAK were expressed by the yeast Pichia pastoris and were purified by conventional methods.

References


### Supplementary Table 1. Patient Demographics

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<th>DCM</th>
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<td>14 (8/6)</td>
<td>25 (16/9)</td>
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HTN, hypertension; CAD, coronary artery disease; DCM, dilated cardiomyopathy; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic dimension; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker.
Supplemental Figure 1

Tissue Fn14 Expression Pattern

Cardiomyocyte
Heart
Supplemental Figure 2

WT

10X

40X

fl-TWEAK
Supplemental Figure 3

TWEAK, μg/mL

Day 1    Day 3  Day7  Day14

0 50 100 150 200 250 300
Supplemental Figure Legends


**Supplemental Figure 2: Myocardial fibrosis in fl-TWEAK mice.** Representative Trichrome stained cross-sectional histology sections, from wild type (WT) and transgenic mice overexpressing full-length TWEAK (fl-TWEAK), demonstrating the development of myocardial fibrosis in end-stage failing fl-TWEAK hearts.

**Supplemental Figure 3: Circulating TWEAK levels following injection with TWEAK adenovirus.** Circulating TWEAK levels over time in mice following tail vein injection of adenoviral vector (1011 viral particles) expressing soluble murine TWEAK.