Reduced EGFR causes abnormal valvular differentiation leading to calcific aortic stenosis and left ventricular hypertrophy in C57BL/6J but not 129S1/SvImJ mice

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Barrick CJ, Roberts RB, Rojas M, Rajamannan NM, Suitt CB, O’Brien KD, Smyth SS, Threadgill DW. Reduced EGFR causes abnormal valvular differentiation leading to calcific aortic stenosis and left ventricular hypertrophy in C57BL/6J but not 129S1/SvImJ mice. Am J Physiol Heart Circ Physiol 297: H65–H75, 2009. First published May 15, 2009; doi:10.1152/ajpheart.00866.2008.—Epidermal growth factor receptor (EGFR) signaling contributes to aortic valve development in mice. Because developmental phenotypes in Egfr-null mice are dependent on genetic background, the hypomorphic Egfrwa2 allele was made congenic on C57BL/6J (B6) and 129S1/SvImJ (129) backgrounds and used to identify the underlying cellular cause of EGFR-related aortic valve abnormalities. Egfrwa2wa2 mice on both genetic backgrounds develop aortic valve hyperplasia. Many B6-Egfrwa2wa2 mice die before weaning, and those surviving to 3 mo of age or older develop severe left ventricular hypertrophy and heart failure. The cardiac phenotype was accompanied by significantly thicker aortic cusps and larger transvalvular gradients in B6-Egfrwa2wa2 mice compared with heterozygous controls and age-matched Egfrwa2wa2 homozygous mice on either 129 or B6129F1 backgrounds. Histological analysis revealed cellular changes in B6-Egfrwa2wa2 aortic valves underlying elevated pressure gradients and progression to heart failure, including increased cellular proliferation, ectopic cartilage formation, extensive calcification, and inflammatory infiltrate, mimicking changes seen in human calcific aortic stenosis. Despite having congenitally enlarged valves, 129 and B6129F1-Egfrwa2wa2 mice have normal lifespans, absence of left ventricular hypertrophy, and normal systolic function. These results show the requirement of EGFR activity for normal valvulogenesis and demonstrate that dominantly acting genetic modifiers curtail pathological changes in congenitally deformed valves. These studies provide a novel model of aortic sclerosis and stenosis and suggest that long-term inhibition of EGFR signaling for cancer therapy may have unexpected consequences on aortic valves in susceptible individuals.

Epidermal growth factor receptor aortic sclerosis; hypothyroidism; aortic stenosis; valvulogenesis

DEGENERATIVE AORTIC STENOSIS (AS) is an age-dependent process in which calcification and fibrosis of the aortic valve leaflets progress slowly over several decades before causing clinically relevant AS and significant obstruction of left ventricular outflow. Although investigations are underway to determine whether statin therapy may prevent or delay disease progression, at present, the mainstay of treatment for severe calcific AS is valve replacement surgery (5). AS is already the most common indication for valve replacement surgery in the US (5), and, given the aging US population, calcific AS is predicted to become an increasing health burden (1, 21, 22, 45). Although several risk factors have been associated with increased prevalence and/or progression of AS, growing evidence suggests that genetic factors also modify susceptibility to congenital and calcific valvular disease (11, 16, 17, 26, 27, 36, 41, 42). Moreover, genetic factors may predispose to both developmental defects such as bicuspid aortic valve and valvular calcification (18). Even though the incidence of AS is increasing, there is a paucity of useful experimental models for development of AS caused by calcific valves. In mice, most genetic defects associated with valvular abnormalities have either drastically shortened lifespans and/or other significant organ defects (12, 14, 25, 28, 53, 60).

Studies in mice have revealed a role of bone morphogenetic protein (BMP) and epidermal growth factor receptor (EGFR) signaling in valve development (11, 15, 28). Mice homozygous for the hypomorphic Egfrwa2 mutation develop semilunar valve thickening and AS on hybrid (C57BL/6J×C3H/HeSnJ) or mixed (B6EiC3H×129SvJ) genetic backgrounds (10, 25). The hypomorphic Egflwa2 mutation is a single-nucleotide transversion resulting in the substitution of a glycine for a conserved valine residue near the amino terminus of the tyrosine kinase domain. As a consequence, ligand-dependent autophosphorylation of EGFRwa2 is diminished 5- to 10-fold in vitro, and the ability of EGFRwa2 to phosphorylate an exogenous substrate is reduced by >90% compared with that of the control receptor (30). Similar to Egfrwa2 homozygotes, mice lacking Egfr have semilunar valve thickening on a mixed genetic background (10) but also pre- or perinatal lethality depending on the genetic background (55, 57, 58).

To understand genetic susceptibility to AS, we created congenic lines by backcrossing the Egfrwa2 allele onto the commonly used C57BL/6J (B6) and 129S1/SvImJ (129) mouse strains that have different susceptibilities to atherosclerosis (26) and responses to pressure-induced cardiac overload (2). On all backgrounds, Egfrwa2wa2 mice have significantly thickened aortic valves compared with Egfrwa2+/heterozygous
littermates, yet the severity of valvular abnormalities, development of left ventricular hypertrophy (LVH), and progression to cardiac failure differs dramatically by genetic background. Phenotypic analysis of Egfrwa2 homozygous mice shows that the 129 strain harbors dominant, protective genetic modifiers that prevent progression to calcific AS and cardiac failure in the context of reduced EGFR activity.

METHODS

Generation of congenic lines. Stock B6EiC3H-a/A-Egfrwa2 Wnt3a+/ mice were obtained from The Jackson Laboratory. The Egfrwa2 allele was backcrossed to the 129 and B6 inbred strains. After 10 generations, animals carrying the Egfrwa2 allele were freely intercrossed. Segregation and loss of the Wnt3a mutation, which resides ∼20 cM distal to Egfr on chromosome 11 and is maintained in cis with Egfr in the progenitor stocks, was verified by PCR genotyping. An F1 population (B6129F1) was created by outcrossing B6-Egfrwa2+/ females to 129-Egfrwa2+/males. Homozygous pups were identified by their curly whisker phenotype and confirmed by PCR genotyping as previously described (32). Adult Egfrwa2 homozygous mice and wild-type controls were housed and analyzed in same-sex littermate pairs to allow for paired statistical comparisons. All animal experiments were performed under National Institutes of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Echocardiography. Transthoracic echocardiography (TTE) was performed using a 30-MHz probe and the Vevo 660 ultrasonograph (VisualSonics) as previously described (2). Doppler tracings were obtained at the level of the aortic root, and mean maximum velocities were obtained over three cardiac cycles. Pressure gradients were calculated from the modified Bernoulli equation $P = 4v^2$, where $v$ is the maximum transvalvular velocity in m/s.

Blood pressure measurements. Systolic blood pressure was measured in conscious mice using a blood pressure analysis tail cuff system (Hatteras Systems) as previously described (20).

Arterial cannulation and ventricular pressure measurements. Mice were anesthetized with 50–80 mg of pentobarbital given intraperitoneally and were intubated. Mouse body temperature was monitored with a rectal probe and maintained at 37 ± 1°C throughout the surgical procedure. The mouse was positioned on a “mouse pad” (THM-100 board, Indus Instruments) that contains EKG electrodes and a built-in surface mounted semi-conductor temperature sensor (THM-100 board, Indus Instruments) that contains EKG electrodes. All procedures were done aseptically.

Surgery was performed with the aid of an OPMI-DFC surgical stereomicroscope (Carl Zeiss). An 1-cm midline neck incision from the lower mandible to the sternum was made. The right common carotid artery was exposed and isolated by blunt dissection of the thin layer around the trachea. A secure suture was placed around the distal end of the artery, and a loose suture was placed around the proximal end of the artery. A small vascular clip was placed on the proximal end to minimize bleeding. A 1- to 2-mm incision near the distal end of the artery was made, and the incision extended longitudinally. After removal of the clip, a 1.4-Fr catheter (Millar Instruments) was quickly advanced beyond the proximal suture. The catheter was then advanced retrograde down the ascending aorta, and pressure readings were obtained. The shape of the pulmonary vein loops was used to determine the optimal placement of the catheter within the left ventricle.

Histology. After the mice were weighed, the hearts, lungs, liver, and kidneys were dissected, rinsed in PBS, and weighed individually. Hearts were cut in cross-section just below the level of the papillary muscle. For assessment of cardiomyocyte size and fibrosis, the top half of the heart was fixed in neutral buffered formalin and embedded in paraffin wax. Sections of 5-µm thickness were prepared at 200-µm intervals. The sections were stained with hematoxylin and eosin (H & E) for examination of gross appearance, while Masson’s Trichrome (MT) or Periodic Acid-Schiff counterstained with hematoxylin (PAS-H) was employed to facilitate quantification of fibrosis, valve size, and cardiomyocyte size. Cardiomyocyte hypertrophy was assessed by measuring cross-sectional area of 10 cardiomyocytes/field in 10 randomly selected fields in at least three sections (resulting in area measurements of ∼300 cardiomyocytes/sample). Only cardiomyocytes having nearly circular capillary profiles and centered nuclei in the left ventricular free wall were measured. Histological images were analyzed using Nova Prime 6.75.10 software (BioQuant Image Analysis).

For measurement of cardiac valve size, morphology, and immunohistochemical analysis, serial sagittal sections were collected from at least four littermate pairs on each genetic background. Aortic cusp thickness was only measured from sections where the aortic outflow tract and aortic walls were clearly visible and in similar orientation. Immunostaining of the valves for osteopontin was detected using anti-osteopontin (Abcam) and a streptavidin-biotin method following manufacturer’s instructions (DakoCytomation, Universal LSAB). Proliferating nuclear cell antigen (PCNA), a DNA polymerase, and phospho-ERK1/2 (pERK) were used as markers for cellular proliferation. PCNA and pERK staining was performed using antibodies from Cell Signaling and visualized with ABC Detection Kit (Vector). Histological images were analyzed using MetaMorph software. Positively stained cells per cross section were manually counted in three sections per heart.

Alizarin Red S and Von Kossa’s method were used as markers for calcification (9), whereas Movat’s pentachrome stain was used to assess extracellular matrix (ECM) composition. DAPI and Rat anti-mouse MOMA2 (monocyte/macrophage) antibody followed by a Cy3-labeled goat anti-mouse secondary was used to detect inflammatory cells within the aortic valves. Histological images were analyzed using ImageJ software to quantify percent positive stained area for Alizarin Red S and Masson’s trichrome stained sections. At least three sections per heart were quantified.

Gene expression. Total RNA was extracted from the lower half of the left ventricle using TRIzol (Invitrogen). After DNase treatment, 500 ng of total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). The expression of the genes coding for α-myosin heavy chain (Myh7), β-myosin heavy chain (Myh7), atrial natriuretic peptide (Nppa), brain natriuretic peptide (Nppb), and medium chain acyl dehydrogenase (Acadm) was determined by real-time quantitative PCR (qPCR) using Taqman Universal Master Mix and Assays-On Demand primers and probes (Applied Biosystems). Primers targeting the gene coding for β-actin (Actb) were used as an internal control. Results are represented as mean fold changes relative to Egfrwa2+/+ expression. Reactions were run on a Stratagene MX3000P machine with analysis software. Threshold cycles (7) were determined by an in-program algorithm that assigns a fluorescence baseline based on readings before exponential amplification. Fold change in expression was calculated using the 2-ΔΔCt method (29), using Actb as an endogenous control. Similar results were obtained using GusB as an endogenous control.

In vivo phosphorylation assays. Neonatal pups at postnatal day (PD) 7 were injected subcutaneously with 5 mg/g body wt of EGF (R & D Systems) in PBS. After 10 min, liver and heart were harvested, frozen in liquid nitrogen, and stored at −80°C. The frozen tissues were sonicated in 5–10 ml/g tissue of lysis buffer consisting of 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 mg/ml of leupeptin, 10 mg/ml of aprotinin, 1 mM sodium vanadate, and 10 mM β-glycerophosphate at 4°C. The tissue lysates were cleared by centrifugation for 10 min at 4°C, and protein concentrations were determined by the Bradford assay (Bio-Rad). An equal amount of protein lysate (15 mg liver or 30 mg heart) was separated by denaturing 7.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad). Protein blots were incubated overnight at 4°C with poly-
clonal rabbit EGFR antibody (Neomarker), polyclonal rabbit phospho-EGF receptor (Tyr1045) antibody (Cell Signaling), or polyclonal rabbit pERK (Thr202/Tyr204) antibody (Cell Signaling) followed by incubation goat anti-rabbit horseradish peroxidase conjugated antibody and detected with an enhanced chemiluminescence system (Amersham Pharmacia). All images were imported into Adobe Photoshop and underwent global brightness and contrast adjustment to improve quality.

Statistical analysis. Data are presented as means ± SE. The nonparametric Wilcoxon rank sum test or the two sided t-test was used for statistical analysis, pairing data from litters. The Kruskal-Wallis test or analysis of variance (ANOVA) was used to detect significance by genotype or genetic background. Statistical analyses were performed using StatView (SAS). A P value of <0.05 was considered significant.

RESULTS

B6 mice homozygous for Egfrwa2 have reduced postnatal survival due to enlarged hearts. Although B6-Egfrwa2wa2 mice were born close to the expected Mendelian ratios from B6-Egfrwa2/+ female by B6-Egfrwa2wa2 male matings (43%, n = 8 litters, 43 pups), at weaning, <30% of surviving mice were homozygous for the Egfrwa2 allele compared with 44% and 46% from similar crosses on an enhanced Chlamydia system (Amersham Pharmacia). All images were imported into Adobe Photoshop and underwent global brightness and contrast adjustment to improve quality.

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C57BL/6J-Egfrwa2/wa2 mice have pathological, molecular, and functional evidence of cardiac hypertrophy and failure. Consistent with the increase in heart weight, cardiomyocyte size, as measured by mean cross-sectional area, was enlarged by almost threefold in adult B6-Egfrwa2/wa2 mice compared with heterozygous littermates (n = 8 sibling pairs, P < 0.01; Fig. 3A). By 12 wk of age, B6-Egfrwa2/wa2 mice demonstrated dilated left ventricles and thickened septal and chamber walls (Figs. 1B and 3A). No difference in cardiomyocyte size was detected among 129 or B6129F1-Egfrwa2/wa2 and heterozygous littermates, even at 15 mo of age (data not shown). Hyperchromasia and karyomegaly, hallmarks of cardiac hypertrophy, were observed in histological sections from adult B6-Egfrwa2/wa2 hearts. Additionally, multifocal lesions with vacuolar degeneration, inflammatory infiltrate, and necrosis were observed in the hearts of moribund B6-Egfrwa2/wa2 mice. These pathological changes were accompanied by significant interstitial and perivascular fibrosis (Fig. 3B, arrows). B6-Egfrwa2/wa2 hearts had a fourfold increase in fibrotic area compared with B6-Egfrwa2/+ hearts and more than twice the fibrotic area as 129-Egfrwa2/wa2 hearts [B6-Egfrwa2/wa2, 11.00 ± 3.11% vs. B6-Egfrwa2/+; 2.79 ± 0.99% vs. 129-Egfr wa2/wa2 4.96 ± 1.00% (P < 0.05)]. Consistent with previous reports (10), histological examination of other tissues that were severely affected in Egfr-null mice (brain, liver, lung, kidney, gastrointestinal tract) revealed no significant morphological defects in Egfrwa2/wa2 mice.

Fig. 2. Expression and activity of EGFR. Analysis of total EGFR, phospho-EGFR, and phospho-ERK1/2 heart (A) and liver lysates (B). Left: Western blots. Right: quantification by densitometry of signal relative to B6-Egfrwa2/+ values. All values were normalized to the β-tubulin loading control. Values represent the means from at least three samples. Phospho-EGFR was not detected in heart lysates. Images were imported into Adobe Photoshop and underwent global brightness and contrast adjustment to improve quality. C: Egfr transcript levels in total RNA extracted from hearts of adult Egfrwa2 littermates. Fold change is relative to B6-Egfrwa2/+ samples. **P < 0.01.
The reexpression of a fetal gene program, which occurs in response to a variety of stimuli, is a consistent and sensitive marker of cardiac hypertrophy in humans and laboratory animals. The expression of the atrial and brain natriuretic peptide genes (Nppb and Nppa, the products of which induce vasodilation and sodium excretion) and the gene for the fetal isoform of myosin heavy chain (Myh7) were significantly or suggestively upregulated in B6-Egfrwa2/wa2 left ventricles compared with wild-type (mean fold transcript increases in B6-Egfrwa2/wa2 vs. Egfrwa2/+ hearts: Nppb, 6.5 ± 7.5, P < 0.07; Nppa, 50 ± 2.5, P < 0.05; Myh7, 20 ± 2.5, P < 0.05; 4 sibling pairs; data not shown). Consistent with altered cardiac metabolism, expression of the medium chain acyl dehydrogenase gene (Acadm) was reduced by ~50% in B6-Egfrwa2/wa2 left ventricle (data not shown). In agreement with histological measurements, there were no significant differences in expression of these markers in the left ventricle among 129 or B6129F1-Egfrwa2/wa2 and heterozygous litters.

Cardiovascular function was evaluated by TTE in 3- to 5-mo-old sex-matched littermates on all three genetic backgrounds (Fig. 3; Table 1). Compared with their sex-matched B6-Egfrwa2/+ littermates, B6-Egfrwa2/wa2 mice had larger left ventricles and reduced fractional shortening (FS; a measure of ventricular systolic function) consistent with dilation of the left ventricle and a thickened chamber wall. By contrast, 129-Egfrwa2/wa2 mice had smaller left-ventricular end-diameter diastole (LVEDD) and left-ventricular end-diameter systole (LVEDS) measurements with thinner chamber walls compared with 129-Egfrwa2/+ littermate controls, matching their proportionately smaller heart and body size; there was no significant difference in FS among 129 or B6129F1-Egfrwa2/wa2 and heterozygous litters. To determine whether systemic hypertension was the cause for cardiac hypertrophy in B6-Egfrwa2/wa2, systolic blood pressures were measured by tail cuff in conscious mice and by arterial catheterization in anesthetized mice (Table 2); no significant differences by genetic background or genotype were observed. These findings eliminate hypertension as a cause for differences in cardiac hypertrophy.

C57BL/6J-Egfrwa2/wa2 mice develop calcific AS due to abnormal cellular differentiation within the valves. In performing echocardiographic analysis, elevated average peak velocities as
measured by Doppler were observed in Egfr<sup>wa2/wa2</sup> mice compared with age- and sex-matched littermate controls (Table 1), suggesting the presence of a gradient across the aortic valve in mice with the <i>Egfr</i><sup>wa2</sup> mutation. Because genetic modifiers that impact the development of AS could lead to background-dependent differences in cardiac hypertrophy, left-ventricular failure, and sudden cardiac death, the effects of background on the aortic valve function and homeostasis was examined in more detail. Aliasing occurred on Doppler traces from many B6-Egfr<sup>wa2/wa2</sup> mice; aliasing is an artifact that is recorded when peak velocities are above the instrument threshold (i.e., >4 m/s for the VisualSonics system). Thus the gradients in the most severely affected B6-Egfr<sup>wa2/wa2</sup> mice calculated from peak Doppler velocities may under represent the true peak velocities. Direct pressure measurements obtained by cardiac catheterization confirmed higher left-ventricular peak systolic pressures (PSP) in B6-Egfr<sup>wa2/wa2</sup> mice compared with littermate controls (Table 2). Although the catheter cannot simultaneously monitor left ventricle and aortic PSP, peak-to-peak gradients were calculated by measuring aortic PSP and then advancing the catheter into the left ventricle to measure left-ventricular PSP. B6-Egfr<sup>wa2/wa2</sup> mice consistently exhibited the largest pressure gradients, indicating severe AS.

To understand why B6-Egfr<sup>wa2/wa2</sup> but not 129-Egfr<sup>wa2/wa2</sup> mice developed a significant gradient across the aortic valve, histological assessment of the effects of background on the cellular composition of the aortic valve was performed. Aortic valve cusp thickness, estimated by cusp diameter, was significantly increased in Egfr<sup>wa2</sup> homozygous mice on all three genetic backgrounds compared with heterozygous controls (Fig. 4A). B6-Egfr<sup>wa2/wa2</sup> mice had the thickest cusps. Transvalvular gradients showed a positive linear relationship with aortic valve thickness (R<sup>2</sup> = 0.89; Fig. 4B), suggesting that, in this mouse model as in human patients, there may be a physiological threshold for AS that must be reached before significant pressure gradients and cardiac disease symptoms appear. A positive linear relationship also existed between aortic valve thickness and heart weight in B6-Egfr<sup>wa2/wa2</sup> mice (R<sup>2</sup> = 0.77; Fig. 4C), suggesting an inverse relationship between hypertrophy and aortic systolic opening. However, some 129-Egfr<sup>wa2/wa2</sup> and B6129F1-Egfr<sup>wa2/wa2</sup> mice had cusp diameters that overlapped with B6-Egfr<sup>wa2/wa2</sup> mice, and yet the 129-Egfr<sup>wa2/wa2</sup> mice and B6129F1-Egfr<sup>wa2/wa2</sup> mice did not develop cardiac hypertrophy. Therefore, histological analysis was performed to determine whether there were also alterations in the cellular composition of the valves in the B6-Egfr<sup>wa2/wa2</sup> mice that would make them more prone to stenosis.

Studies of diseased aortic valves have shown cusp and leaflet thickening, inflammatory infiltrate, collagen fiber disorganization, increased valvular interstitial cell (VIC) proliferation, and calcification. Examination of the thickened aortic valves demonstrated distinct genetic background differences in cellular morphology, differentiation, and cellular composition by immunohistochemical analysis using markers for cellular proliferation (PCNA, pERK1/2), extracellular matrix composition (ECM) (Movat’s pentachrome), macrophage infiltration (MOMA2), calcification (Alizarin Red S and von Kossa), and mature osteoblasts (osteopontin). B6-Egfr<sup>wa2/wa2</sup> valves had elevated cellular proliferation, as indicated by significantly increased numbers of PCNA-positive nuclei (B6-Egfr<sup>wa2/wa2</sup>; 41.14 ± 13.5, vs. F1-Egfr<sup>wa2/wa2</sup> mice; 8.00 ± 2.53 vs. 129-Egfr<sup>wa2/wa2</sup> mice; 0.60 ± 0.40; P < 0.05) and ERK1/2 activation (Fig. 5, A and B). Altered ECM composition (Fig. 6A), unique to B6-Egfr<sup>wa2/wa2</sup> valves, was revealed by the intense teal color with Movat’s pentachrome stain, indicating close proximity.

<table>
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<tr>
<th>Genetic Background</th>
<th>B6</th>
<th>129</th>
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<tr>
<td></td>
<td>wa2/+</td>
<td>wa2/wa2</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>4</td>
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<tr>
<td>Sys, mmHg</td>
<td>119±9</td>
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<td>Dias, mmHg</td>
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<tr>
<td>MAP, mmHg</td>
<td>102±15</td>
<td>110±15</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>757±40</td>
<td>734±24</td>
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</table>

Values are means ± SE. Sys, systolic blood pressure; Dias, diastolic blood pressure; MAP, mean arterial blood pressure. No significant differences were detected in any parameters by genotype or genetic background.

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**Table 1. Measurements of cardiac function from 3- to 5-mo-old Egfr<sup>wa2</sup> littermates**

<table>
<thead>
<tr>
<th>Genetic Background</th>
<th>B6</th>
<th>129</th>
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<tbody>
<tr>
<td></td>
<td>wa2/+</td>
<td>wa2/wa2</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>11</td>
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<tr>
<td>LVEDD, mm</td>
<td>3.52±0.06</td>
<td>4.88±0.15‡</td>
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<tr>
<td>LVEDS, mm</td>
<td>2.11±0.08</td>
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<td>LVPWTh,d, mm</td>
<td>0.97±0.07</td>
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<tr>
<td>LVPWTh,s, mm</td>
<td>1.34±0.08</td>
<td>2.16±0.10‡</td>
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<tr>
<td>%FS</td>
<td>40.23±1.70</td>
<td>29.32±1.50‡</td>
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<tr>
<td>RWT</td>
<td>0.56±0.05</td>
<td>0.63±0.05</td>
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<tr>
<td>HR, beats/min</td>
<td>437±14</td>
<td>430±9</td>
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<td>Pressure gradient, mmHg (catheterization)</td>
<td>−0.40±1.33</td>
<td>22.13±2.87†</td>
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<tr>
<td>Mean peak Ao velocity, cm/s</td>
<td>108±13</td>
<td>371±100*</td>
</tr>
<tr>
<td>N</td>
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<td>5</td>
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</tbody>
</table>

Values are means ± SE. LVEDD, left-ventricular end-diastolic volume; LVEDS, left-ventricular end-systolic volume; %FS, percent fractional shortening; HR, heart rate. Comparison by genotype: *P < 0.05; †P < 0.01; ‡P < 0.001. LVPWTh,d, left-ventricular posterior wall thickness, diastole; LVPWTh,s, left-ventricular posterior wall thickness, systole; RWT, right ventricular wall thickness.

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**Table 2. Blood pressure measurements from 3- to 5-mo-old Egfr<sup>wa2</sup> littermates**

<table>
<thead>
<tr>
<th>Genetic Background</th>
<th>B6</th>
<th>129</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wa2/+</td>
<td>wa2/wa2</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Sys, mmHg</td>
<td>102*</td>
<td>100*</td>
</tr>
<tr>
<td>Dias, mmHg</td>
<td>90*</td>
<td>92*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>3.57*</td>
<td>3.57*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.56*</td>
<td>0.56*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>437±14</td>
<td>430±9</td>
</tr>
<tr>
<td>Pressure gradient, mmHg (catheterization)</td>
<td>−0.40±1.33</td>
<td>22.13±2.87†</td>
</tr>
<tr>
<td>Mean peak Ao velocity, cm/s</td>
<td>108±13</td>
<td>371±100*</td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sys, systolic blood pressure; Dias, diastolic blood pressure; MAP, mean arterial blood pressure. No significant differences were detected in any parameters by genotype or genetic background.
of glycosaminoglycans and collagen. B6-EGfrwa2/wa2 valves also stained positive for osteopontin, had significantly greater calcification, and showed evidence of inflammatory infiltrate (Fig. 6, B–D). These pathological changes were completely absent or very mild in aortic valves of F1- or 129-EGfrwa2/+ and EGfrwa2/wa2 mice. Taken together, these data suggest that the B6 genetic background has sustained postnatal proliferation and differentiation of valvular interstitial cells residing in cardiac valves, resulting in severe calcific AS.

In summary, histopathological and echocardiographic analysis confirmed that B6-EGfrwa2/wa2 mice displayed increased chamber size, myofiber hypertrophy, and decreased fractional shortening, signs of severe cardiac dysfunction. Taken together, the data support a model where congenitally thickened aortic valves become progressively stiff and dysfunctional in the B6-EGfrwa2/wa2 mice, resulting in increased afterload, cardiac hypertrophy, and, ultimately, cardiac failure and premature death.

**Fig. 4. Aortic valve assessment.** A: comparison of mean aortic valve cusp diameter by genotype and genetic background (B6, n = 8 sibling pairs; B6129F1, n = 4 sibling pairs; 129, n = 6 sibling pairs). **P < 0.01. A representative hemotoxylin and eosin stained section of the thickest region of two cusps of the aortic valves is also shown. B: simple linear regression analysis for correlation of peak systolic pressure (PSP) and mean cusp thickness. Solid line is the regression line for all data points (R² = 0.84), whereas the broken line represents the regression line for B6-EGfrwa2/wa2 mice (R² = 0.77). C: simple linear regression analysis of heart weight and mean cusp thickness by genotype and genetic background. Solid line is the regression line for all data points (R² = 0.51), whereas the broken line represents best fit for B6-EGfrwa2/wa2 mice (R² = 0.77).

**DISCUSSION**

EGfrwa2 mutant mice on a mixed genetic background have semilunar valve thickening and mild to moderate AS that is enhanced when combined with a mutation in Ptpn11 (10). Since previous studies established that phenotypes resulting from reduced or ablated EGFR activity are highly dependent on genetic background (57, 58), we generated and examined B6 and 129-EGfrwa2 congenic lines and their B6129F1 hybrids. Similar to findings in other organ systems, genetic background significantly modifies the cardiovascular phenotype in the EGfrwa2 mouse model of AS, although there are no detectable background-dependent differences in residual EGFR activity. B6-EGfrwa2/wa2 mice have significantly decreased survival secondary to congestive heart failure. In contrast, mice homozygous for the EGfrwa2 mutation on the 129 inbred or B6129F1 backgrounds have normal lifespans and cardiac function with no histological or molecular evidence of LVH, implying the
presence of dominant 129 protective modifiers. Although aortic valves were thickened in 
\textit{Egfr}\textsuperscript{wa2/wa2} mice on all genetic backgrounds relative to littermate controls, only adult B6-
\textit{Egfr}\textsuperscript{wa2/wa2} mice developed a significant pressure gradient across the aortic valves. Compared with 
\textit{Egfr}\textsuperscript{wa2} homozygous mice on other genetic backgrounds, the B6-\textit{Egfr}\textsuperscript{wa2/wa2} mice 
have significantly more thickened aortic valves. Aortic valve thickness correlated highly with pressure gradients and cardiac 
weight in \textit{Egfr}\textsuperscript{wa2} mutant mice. We also found degenerative cellular changes unique to B6-\textit{Egfr}\textsuperscript{wa2/wa2} cardiac valves, such 
as increased valvular interstitial cell proliferation and differentiation, altered ECM composition, calcification, and inflammation. 
Our observations are consistent with the development of aortic sclerosis in 129-\textit{Egfr}\textsuperscript{wa2} and hemodynamically 
significant AS in adult B6-\textit{Egfr}\textsuperscript{wa2/wa2} that likely relate to differences in cellular changes in the valves.

In humans, significant hemodynamic or clinical changes are rare in AS until the valve orifice has narrowed to approxi-
mately one-third of normal (4, 6). Studies using a hypercho-
lesterolemic mouse model of AS showed that an almost iden-
tical threshold of decreased aortic valve orifice must be 
achieved before significant hemodynamically induced trans-
valvular gradients and pathophysiological responses are de-
tected in mice (59). Our morphometric, immunohistochemical, 
and hemodynamic analyses also support a threshold effect in 
the \textit{Egfr}\textsuperscript{wa2} model of AS, whereby congenitally thickened aortic valves of B6-\textit{Egfr}\textsuperscript{wa2/wa2} mice become progressively 
larger and stiffer than those of 129 or B6129F\textsubscript{1}-\textit{Egfr}\textsuperscript{wa2/wa2} mice, leading to a critical reduction in systolic valve area, valve 
flexibility, and elevated afterload. The simplest explanation for 
our observations is that dominant protective 129 genetic mod-
ifiers influence cardiac valve development and homeostasis 
promoted by the \textit{Egfr}\textsuperscript{wa2} mutation. Genetic studies using 
knockout mouse models established that endocardial outflow 
track cushion growth is largely controlled by the amount of 
BMP signaling, with diminished and excessive signaling cor-
relating to hypo- and hyperplastic cushions, respectively (13, 
19). Defective cardiac valvulogenesis in mice lacking \textit{Hbegf} 
(coding for the EGFR ligand heparin-binding EGF), \textit{Egfr}, or 
\textit{Adam17} (coding for a metalloprotease required to process 
mature EGFR ligands) results from abnormal valvular mesen-
chymal cell proliferation, whereas \textit{Erbb3}-deficient embryos 
have hypoplastic cushions that completely lack mesenchymal 
cells. Because the thickened valves of \textit{Hbegf-null} embryos 
showed dramatic increases in activated BMP signaling effec-
tors SMAD1/5/8 (24, 25) and since EGFR has been shown to 
downregulate BMP signaling by inactivating SMAD1 in vitro, 
it was postulated that EGFR activation by TACE-derived 
soluble HBEGF normally limits BMP signaling during the 
transition from cushion formation/growth to valve remodeling 
(25). Although we did not find overt genetic background-
dependent differences in cardiac valve size from 15.5 dpc 
through PD\textsubscript{1} \textit{Egfr}\textsuperscript{wa2/wa2} mice (C. J. Barrick and D. W. 
Threadgill, unpublished observations), modifiers may have 
affected the balance of these signaling pathways postnatally. 
The cellular changes we observed in adult B6-\textit{Egfr}\textsuperscript{wa2/wa2} 
semilunar valves mimic those seen in degenerative AS (8, 38, 
43, 49, 51, 52), suggesting that EGFR activity is also required 
to repress abnormal growth and differentiation in mature aortic 
valves. Several mediators of BMP signaling like TGFB\textsubscript{1} 
(transforming growth factors beta 1), BMP2, and BMP4 are 
detected in aortic valve lesions and promote differentiation of 
isolated valvular interstitial cells to osteoblast-like cells (31); 
moreover, osteoblasts express EGFR, and EGF stimulates 
osteoblast proliferation in vitro. Additionally, primary osteo-
blast cultures isolated from \textit{Egfr-null} mice have increased 

Fig. 5. Cellular proliferation in aortic valves. Aortic 
valve sections stained for phospho-ERK1/2 (A) and 
PCNA (B) [magnification \( \times 200 \), scale bar \( 100 \mu m \) (A) or \( 50 \mu m \) (B)]. Significantly more positive 
nuclei (arrow) were counted in B6-\textit{Egfr}\textsuperscript{wa2/wa2} aortic 
valves compared with F1 or 129S1-\textit{Egfr}\textsuperscript{wa2/wa2} aor-
tic valves \((P < 0.05)\). At least three sections per 
aortic valve were analyzed; data points represent the 
mean value.
differentiation and formation of bone nodules relative to controls (54). Since EGFR normally suppresses BMP signaling, attenuation of EGFR signaling may predispose differentiation of valvular interstitial cells to a calcifying cell phenotype on the B6 background. Thus we speculate that genetic modifiers altering the balance of these signaling pathways underlie strain-specific differences in cardiac valve disease.

Accumulating evidence from epidemiological, clinical, and animal studies supports the hypothesis that calcific AS and atherosclerosis have common cardiovascular risk factors and pathological processes (8, 37, 39, 47, 48, 50, 54, 56). B6 and 129 inbred mouse strains are known to differ markedly in susceptibility to atherosclerosis and calcific AS (23). Recently, it was shown that B6 mice chronically fed a high-fat/high-carbohydrate diet acquire aortic valve abnormalities, with decreased corrected valve orifice area, histological evidence of calcification and inflammation, and increased mean aortic outflow tract peak systolic velocities (15). Since we detect similar pathological changes in the cardiac valves of wild-type B6 mice subjected to surgically induced pressure overload (C. J. Barrick and D. W. Threadgill, unpublished observations), we speculate that the B6 background is predisposed to valve disease that can be triggered by a variety of insults. Moreover, the predisposition of B6 mice to atherosclerosis and calcific AS may be mediated by common mechanisms. If true, our observations have important implications for medical therapy of AS and might suggest that pharmacological approaches for the management of atherosclerosis may also prevent the development of AS.

EGFR signaling is also crucial to cardiomyocyte function and survival. EGF increases cardiomyocyte contractility via EGFR-mediated elevation in cAMP levels (33), leads to positive inotropic effects in perfused rat hearts (34), and is cardioprotective against the harmful effects of chronic sympathetic stimulation (35, 44). Using the ecdysone-inducible gene expression system, Rajagopalan et al. (46) expressed humanized cardiomyocyte-specific dominant-negative EGFR mutant receptors (hErbB-1-mut), resulting in selective reduction of endogenous EGFR activity in cardiomyocytes. hErbB-1-mut induction resulted in cardiac hypertrophy, compromised cardiac function, and decreased survival with stress induced by aortic banding in young adult mice (46). We recently reported that B6 mice are more susceptible to LVH and heart failure induced by aortic banding than 129S1 mice (2) and also found that chronic exposure of B6 mice to EGFR inhibitors depressed cardiac function and increased cardiac apoptosis compared with controls (3). Thus we cannot exclude the possibility that 129 protective modifiers sustain cardiomyocyte function and survival despite chronic pressure overload and reduced EGFR signaling. However, we did not detect differences in cardiomyocyte apoptosis in the hearts of age- and sex-matched B6 and 129-Egfwa2/wa2 hearts (C. J. Barrick and D. W. Threadgill, unpublished observations). Additionally, the mild pressure gradients across the aortic valve (8–10 mmHg) in the 129-Egfwa2/wa2 mice suggest that mechanisms curtailting aortic valve disease progression are largely responsible for strain differences in heart failure and mortality.

Most models of aortic valve deformities caused by gene disruptions have a drastically shortened lifespan and/or do not
have isolated valve defects (12, 14, 25, 28, 53, 60). Consequently, it has been difficult to conduct detailed phenotypic analysis. Herein, we have extensively phenotyped two Egfr<sup>wa2</sup> congenic lines that are susceptible or resistant to valvular degeneration with subsequent development of LVH and cardiac failure, despite having similar congenital valvular disease. Humans with acquired or congenital AS have considerable variability in onset of symptoms, cardiac response and function, and disease progression. A subset of patients with AS remain asymptomatic despite rapid disease development and have a heightened risk of sudden death. Thus far, the only reliable predictor for these high-risk patients is age and the extent of valvular calcification (40). The development of therapeutic approaches that will halt or retard valve calcification should be aided by the Egfr<sup>wa2</sup> model of AS. Finally, many cancers are now being managed as chronic rather than terminal diseases, and since targeted inhibition of EGFR activity is becoming a widely used cancer therapeutic, our results suggest that a subset of patients with congenital valve defects or who are predisposed to valvular defects may be at risk for developing drug-induced valvulopathy with prolonged EGFR inhibition.

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