Today we're going to address the use of animal models to study human cardiovascular disease. Some people believe that the role of animal models is simply to recapitulate the human condition. I want to stress the use of animal models to study the pathogenesis of disease with an earmarked view of identifying new treatment strategies.

As our example, we are going to discuss a common human condition called Marfan syndrome. Marfan syndrome is a disorder of the body's connective tissue that typically involves the eye, the skeleton, and the heart. Typical manifestations in the ocular system include early and severe myopia, or nearsightedness, but more specifically, upward and temporal dislocation of the ocular lens due to failure of supporting structures called ciliary zonules. The skeleton shows a so-called dolichostenomelia , or long-bone overgrowth. Other manifestations include curvature of the spine and anterior chest deformity. The combination of long fingers and loose joints leads to the typical thumb sign and wrist sign. In the thumb sign, the distal phalanx of the thumb can protrude from the closed fist. In the wrist sign, the pinky and thumb will overlap when wrapped around the contralateral wrist. Other findings include flat feet and knees that curve backward, so-called genu recurvatum .

The most important manifestations in Marfan syndrome occur in the cardiovascular system. These are clearly associated with morbidity and early mortality. Findings include thickening and floppiness of the mitral valve, mitral valve prolapse, that can lead to valve dysfunction and heart failure. But, more importantly, people with Marfan syndrome show progressive enlargement of the beginning portion of the aorta at the level of the sinuses of Valsalva that predisposes to aortic tear, rupture, and sudden death.

This slide shows a typical dissection in a patient with Marfan syndrome that fortunately was able to be repaired surgically. This slide summarizes what was known about the cause of Marfan syndrome prior to 1990. It is an elastin stain of the aortic media from a normal individual and an individual with Marfan syndrome. In the normal situation, you see densely packed and parallel elastic fibers in the aortic matrix. In the sample from someone with Marfan syndrome, you see loss of elastin content, fragmentation and disarray of elastic fibers, and the accumulation of amorphous matrix between the fibers.

In 1991, we were able to show that mutations in the gene encoding fibrillin-1, an essential component of the extracellular matrix, was beside a primary mutation in the Marfan syndrome. There is no common mutation in the Marfan syndrome. Basically, each individual has a private mutation which has frustrated attempts at widespread molecular diagnosis. We knew that fibrillin 1 monomers aggregate to form complex extracellular structures called microfibrils and that microfibrils cluster around the edges of a maturing elastic fiber during embryogenesis. These simple observations led to the absolute conclusion that microfibrils are needed to form an elastic fibroid during embryogenesis. This boded poorly for the development of treatment strategies. It suggested that an individual with Marfan syndrome is born with an obligate predisposition for tissue failure later in life.

In retrospect, I think that this view needed to be challenged. For example, the Keating group had shown that a deficiency of elastin leads to narrowing of the ascending aorta, so-called supravalvar aortic stenosis rather than aortic aneurysm. Even more informative information was demonstrated by the Chien and Olson groups when they studied a molecule called fibulin-5. Fibulin-5 is known to occur at the interface between elastic fibers and neighboring smooth muscle cells. These investigators found that a deficiency of fibulin-5 leads to complete failure of elastic fiber formation during embryogenesis, exactly what we were proposing for the pathogenesis of Marfan syndrome. These animals developed very loose skin, pulmonary emphysema, and narrowing of the aorta, again in contrast to the enlargement of the aorta seen in Marfan syndrome.

We had an opportunity to directly test this hypothesis upon the creation of animal models of Marfan syndrome using gene targeting. Fortunately, we found that these genetic manipulations faithfully recapitulated all aspects of the human phenotype, including long bone overgrowth and curvature of the spine, as you see in the fibrillin-1 deficient animal at the bottom of the slide.

We found that these animals also showed aortic enlargement in dissection, but that this was an acquired event later in development after birth. What you see here is an aortic segment from an animal that makes no normal fibrillin-1. What we observe is normal elastin content and normal formation of elastic fibers during development. At a later time point, you can see focal disruption of elastic fibers and degradation of other aspects of the extracellular matrix. We were able to define a precise pathogenetic sequence for breakdown of the aortic wall.

The earliest event that we could observe was calcification of elastic fibers in the wall of the aorta. This was followed by migration and proliferation of smooth muscle cells in the intima of the aorta leading to intimal hyperplasia. A final event was the accumulation of inflammatory cells, first at the adventitial surface that ultimately consumed the vascular wall leading to structural collapse and aneurysm formation.

In order to determine what the precise event was that triggered this response, we used ultra-structural analysis to look at the aortic wall of fibrillin-1 deficient mice. In wild-type mice, you can see that the elastic fibers have a very ruffled irregular border that manifests streaming projections between elastic fibers and neighboring smooth muscle cells. In the fibrillin-1 deficient mutant mice, you can see that the elastic fibers are abnormally smooth in appearance without connections to neighboring cells. Instead, these cells simply abut against the elastic fibers. These streaming projections are actually bundles of microfibrils that are called connecting filaments, here abbreviated CF.

In the absence of connecting filaments, the smooth muscle cells adopt a very abnormal synthetic repertoire that includes multiple matrix elements, including collagen, elastin, and proteoglycans . Informatively, these cells also begin to secrete matrix degrading enzymes, such as matrix metalloproteinase-2 and -9. Ultimately, these very abnormal cells in the mutant mice

associate with zones of elastic fiber breakdown due to the production of these matrix degrading enzymes.

So, our current view is that a smooth muscle cell is normally connected to the elastic matrix via connecting filaments that are composed of microfibrils. In individuals with Marfan syndrome that have a deficiency of connecting filaments, we believe that these smooth muscle cells begin to secrete proteins in an abnormal developmental context that ultimately do damage to the neighboring matrix. This damage initiates an inflammatory response that results in complete breakdown of the structural support of the vessel wall culminating in aneurysm.

So, our conclusion is that fibrillin-1 and microfibrils are needed for elastic fiber homeostasis rather than formation, and that therapeutic strategies aimed at blunting secondary events such as inflammation or electrolysis by matrix degrading enzymes may hold promise in the treatment of individuals with Marfan syndrome.

The second belief was that Marfan syndrome is a classic dominant negative disorder. This means that production of mutant protein by the abnormal copy of the fibrillin-1 gene leads to abnormal aggregation of mutant and wild-type protein that effectively eliminates all fibrillin-1 from the extracellular matrix, leading to clinical manifestations. Evidence for this dominant negative effect included autosomal dominance inheritance, aggregation of fibrillin-1 monomers during microfibrillar assembly, and the dramatic paucity of extracellular protein in cultured cells and tissues derived from patients that are heterozygous for *FBN1* mutations as seen in the fibroblast cell cultures on the lower portion of this slide.

In order to test this dominant negative hypothesis, we introduced a human gene encoding a natural recurring fibrillin-1 mutation on the mouse background. So these mice had two normal copies of the fibrillin-1 gene but, in addition, had a mutant copy as a third copy of this gene. The expectation from the dominant negative hypothesis would be that the production of mutant protein should lead to functional inactivation of all fibrillin-1 and, hence, manifestations of Marfan syndrome. Surprisingly, we found that mice that harbored either a wild-type transgene or a mutant transgene, had the ability to effectively deposit microfibrils in the extracellular matrix. Also of note was the fact that these mice are entirely normal; they show no clinical or histologic manifestations of Marfan syndrome throughout a normal life span.

In order to determine whether the mouse was an adequate system to observe a dominant negative effect, we then targeted a naturally occurring FBN1 mutation in one of the endogenous mouse copies of the fibrillin-1 gene. This fully recapitulates the genetic mechanism seen in people. Here, again, there should be a one-to-one ratio between the production of wild type and mutant protein. We observe that heterozygous mice that carried one abnormal copy of the fibrillin-1 gene lacked the ability to effectively deposit fibrillin-1 in the extracellular matrix. We found that these mice showed the typical skeletal manifestations of Marfan syndrome, including bone overgrowth. And we also saw that they demonstrated

very definitive abnormalities of the aortic wall, including elastic fiber breakdown, smooth muscle cell proliferation, and aortic enlargement.

In order to determine whether we had given the proper opportunity to observe a dominant negative effect in our transgenic mice, we had to demonstrate that the protein derived from the transgene, from a human transgene, was able to interact with the protein derived from the endogenous mouse alleles. This was achieved in many ways. First, we demonstrated co-localization of human and mouse protein using immunohistologic techniques. Second, using immunoprecipitation, we showed that human and mouse protein interacts with high efficiency. And, finally, using electron microscopy, we could show that the mutant human protein was widely distributed in all microfibrillar beds in these mice. So, if there was a dominant negative interfering effect of the mutant protein, we should have been able to observe phenotypic consequences which we did not.

So, this has led us to consider a so-called haploinsufficiency-driven dominant negative effect. In this view, a normal cell that has two normal copies of the fibrillin-1 gene makes a 2x complement of normal protein, which is effectively secreted from the cell, aggregates in the extracellular matrix, and forms normal microfibrils. A cell that has one normal copy and one abnormal copy of the fibrillin-1 gene may never achieve a sufficient concentration of wild-type protein to trigger deposition of protein in the extracellular matrix, leading to the paucity of fibrillin-1 in the matrix that we observe in both people and mice with heterozygous fibrillin-1 mutations.

In our transgenic models that have two normal copies of the gene and one abnormal copy, they do achieve this threshold for deposition of protein and, in this view, the mutant protein becomes less relevant, or irrelevant, to microfibrillar assembly and the pathogenesis of disease.

So, what are the predictions of this dosage-dependent deposition model. Well, one prediction should be that, if we introduce an additional normal copy of the gene on a heterozygous mutant background, we should be able to rescue the phenotype. This is exactly what we observed. So, a wild-type animal has the indicated width of the aortic wall; an animal that has one mutant copy of the fibrillin-1 gene marked C1039G/+ has a greatly thickened aortic wall that manifests disease. In our transgenic animals that carry a third either normal or mutant copy of the gene, we see no impact on the thickness of the aortic wall. In our heterozygous mice, when we add a normal copy of the gene as a third copy, we see complete rescue of aortic wall thickness in architecture, exactly as we would predict from our dosage-dependent deposition model. Interestingly, even addition of a third mutant copy of the gene had some benefit, suggesting that the mutant protein has some residual function.

So, in the old view, if you increase the expression of fibrillin-1, say using a drug intervention, the dominant negative effect would effectively neutralize all of the protein, preventing any therapeutic benefit. In the new view, if you non-specifically increase expression of both normal and mutant protein, you will allow the cell to reach the threshold

for deposition of fibrillin-1, you'll have a functional complement of microfibrils that will either abbreviate or prevent disease.

So, the lesson is that half normal amounts of normal fibrillin-1 contributes to the complex pathogenesis of Marfan syndrome and that therapeutic strategies aimed at increasing expression of fibrillin-1 may benefit individuals heterozygous for *FBN1* mutations.

The final belief was that fibrillin-1 is strictly a structural protein. We had the ability to test this hypothesis when studying pulmonary disease in the Marfan syndrome which is rarely discussed. These individuals are described as having emphysema with upper lobe bullae and fibrosis and diffuse hyperinflation, indications of chronic obstructive pulmonary disease. This commonly presents as spontaneous lung rupture, or pneumothorax, that's seen in about 10 percent of patients overall. Interestingly, 90 percent of patients that have one pneumothorax will have recurrent disease, and 55 percent of individuals that have a pneumothorax on one side will go on to show a pneumothorax on the other side, suggesting that a specific population of individuals with Marfan syndrome are uniquely predisposed. The frequency is increased in the neonatal presentation of severe and rapidly progressive disease and is not dependent upon smoking or other environmental stress.

So, the prevailing view was that lung disease in Marfan syndrome manifests a structural deficiency of the tissue; these weakened tissues are acted upon by physiologic stress over time, and ultimately fail. What we would have expected to see with this hypothesis is that very young fibrillin-1 deficient mice would not show lung disease. In contrast, when we look at 9 days after birth, we saw that the lungs of fibrillin-1 deficient mice demonstrated homogeneous distal air space widening. This was also seen in heterozygous fibrillin-1 deficient mice as seen in the central panel here.

Precise morphometric analysis of these lungs documented that this abnormality could be traced back to one day after birth. So this was immediately inconsistent with the hypothesis that the lung is gradually failing over time due to structural destruction of the lung wall. If you look at a higher power view, we also notice that in the mutant lung, we do not see any evidence of inflammation or tissue destruction. In contrast, as indicated by the arrowheads, we see a paucity of primordial septae. So, this was most consistent with a failure of a signal for the lung to septate during development rather than classic emphysema.

So, how could a deficiency of a structural protein lead to developmental failure of lung septation? In considering this question, we recognized that the fibrillin-1 protein is very similar to a second family of proteins called the latent transforming growth factor beta binding proteins, or LTBPs. What was known is that TGF beta, a multi-potential growth factor, is secreted from the cell in the context of a large latent complex that includes its processed end terminal propeptide called latency associated peptide, or LAP, and is one of three LTBPs. It was also known that this large latent complex binds to the extracellular matrix, but the binding partner and the physiologic importance of this event were unknown. We reason that, perhaps, microfibrils serve as the binding platform for this large latent complex of TGF beta. In this

view, individuals with an abnormal content or character of microfibrils, such as people with Marfan syndrome, may show inefficient binding of this latent complex and, hence, promiscuous activation of TGF beta, which could then have downstream effects, plausibly including perturbation of development.

In order to test this hypothesis, we first used an antibody that is specific for free and active TGF beta. As you can see in the right hand panel, there is a great increase in the amount of immunostaining in the fibrillin-1 deficient lung. Now, this could either manifest increased synthesis of TGF beta or increased activation of TGF beta. If it were simple increased production of TGF beta, you'd expect to see a corresponding increase in the small latent complex that includes latency associated peptide. But when we immunostain these tissues with an antibody specific for LAP, we saw a decrease rather than an increase in immunostaining in the mutant lung. This was also evident by quantitative western blot analysis. So, all of these findings, taken together, are most consistent with increased activation of TGF beta in the fibrillin-1 deficient state.

Well, we have too much activation of TGF beta, but the question remains whether this can support increased TGF beta signaling in this developmental context. In order to address this question, we made a designer reporter gene that expresses a jellyfish protein called green fluorescent protein in proportion to the amount of TGF beta signaling using a TGF beta responsive promoter element from the PAI1 gene. Once we had constructed these reporter mice, we then bred them to our fibrillin-1 deficient mice to ask whether there was, indeed, increased TGF beta activity in the lungs of these animals. The results were quite striking.

In the upper left panel, you see a non-transgenic mouse that doesn't carry this reporter allele. Any signal that you see just represents auto-fluorescence of the tissues. In the upper right panel, we have a reporter mouse that has two normal copies of the fibrillin-1 gene. The glowing that you see represents the normal amount of TGF beta signaling in the developing lung. In the lower left panel, you see a mouse that has one targeted disrupted copy of the fibrillin-1 gene, and we observe a four-fold increase in the amount of TGF beta signaling. In the lower right panel, you see a mouse that has no normal production of fibrillin-1 and observe a twenty-five-fold increase in the amount of TGF beta signaling.

So, now we have too much TGF beta signaling, and a lung phenotype. We have yet to develop a cause and effect relationship. In order to accomplish this, we asked whether we could rescue lung development by antagonizing TGF beta signaling in this mouse model. To achieve this, we injected pregnant mice with a TGF beta neutralizing antibody and then assayed the lung of the resulting pups after birth. As you can see, administration of five milligrams per kilogram of neutralizing antibody led to a subtle, but significant, improvement in lung development. Administration of ten milligrams per kilogram of neutralizing antibody resulted in a complete rescue of lung development. This is further illustrated with morphometric analysis that shows that we have achieved a normal level of lung septation in mice that have no fibrillin-1 production simply by antagonizing TGF beta one signaling.

Well, we're talking about mice here. I think a valid question would be, is there any relevance to people with Marfan syndrome? Here, we're looking at lung tissue from a very young child with typical Marfan syndrome. We observe diffuse widening of the distal air space and the absence of inflammation or tissue destruction, exactly what we observed in our mouse model, suggesting that there is direct relevance of these observations to the clinical development of Marfan syndrome in people.

So, our conclusion is that TGF beta is a physiologic negative regulator of alveolar septation, that extracellular matrix sequestration regulates local cytokine activation in signaling and, finally, that perturbation of this process can contribute to the pathogenesis of disease, including Marfan syndrome.

What is the broader relevance of this finding? Well, we hypothesized that dysregulation of cytokine signaling may underlie other manifestations of Marfan syndrome that are difficult, or impossible, to reconcile with models that singularly invoke structural failure of the tissues. These would include long-bone overgrowth, thickening of the mitral valve with dysfunction, so-called myxomatous changes, muscle hypoplasia and reduced fat that is seen in Marfan syndrome, and even cranofacial abnormalities.

To begin to address this hypothesis, we have now looked at the level of TGF beta activation in some of these tissues. We have observed a dramatic increase in the amount of immunoreactive TGF beta and TGF beta signaling in the growth plate of fibrillin-1 deficient mice as compared to their wild-type litter mates. We've also observed an increase in the amount of free TGF beta and TGF beta signaling in the dramatically abnormal skeletal muscle of fibrillin-1 deficient mice that shows both decreased cell number and muscle cell hypoplasia.

Of greatest relevance to the cardiovascular system, we have observed increased TGF beta activation and signaling in the wall of the aorta that is most prominent at the adventitial surface, but that extends deep into the vessel wall. Finally, we have observed TGF beta activation in the heart myocardium and in the AV valves as shown in the right panel.

Normally, TGF beta interacts with its receptor at the cell surface and initiates a signaling cascade that is dependent upon downstream effector molecules called the receptor activated SMADS , or R-smads. The activated R smads translocate to the nucleus and stimulate expression of TGF beta responsive genes. To regulate this process, the cell normally produces inhibitory smads, or I-smads 6 and 7 that blunt the level of signaling.

Knowledge of this pathway presents many opportunities to antagonize TGF beta signaling. For example, we could use our simple strategy of administering TGF beta neutralizing antibody. There is some concern that this may not be effective to study some phenotypes because they are slow in development and only emerge over the course of many months to years. As an alternative, we have developed numerous genetic strategies to antagonize TGF beta signaling in fibrillin-1 deficient mice. These include targeting one copy of the gene that encodes the critical downstream effector smad 4. So, our hypothesis is that if we

deprive the cell of smad 4 and blunt TGF beta signaling, we may be able to improve manifestations of Marfan syndrome that show relevance to abnormal cytokine activation.

Another strategy that we are pursuing is to increase the expression of the naturally occurring inhibitory smads 6 or 7, specifically in the cardiovascular system. Finally, there are pharmacological strategies that can be considered. For example, the drug losartan , which is an angiotensin II Type I receptor antagonist, has been shown to lower blood pressure, a desirable effect in individuals with Marfan syndrome, but has also been shown to decrease both the production and activation of TGF beta. This appears to be an ideal pharmacologic candidate for further scrutiny.

As preliminary evidence of the benefit of these manipulations, I'm showing you the effect on lung development of introduction of one targeted copy of the downstream effector smad 4. On the left, you see the amount of septation in a normal wild-type animal. In the middle, you see the effect of a mutation in one copy of the fibrillin-1 gene that leads to failed lung development in widened distal air space. If you introduce a targeting of smad 4 on this fibrillin deficient background, as you see on the right, by introduction of one targeted copy of the smad 4 gene, we have greatly improved lung development, which should have therapeutic benefit in individuals with Marfan syndrome.

We also began to study the impact of antagonism of TGF beta signaling on the mitral valve. In the left hand panels, you can see that in homozygous targeted FBN 1 deficient mice, there are gross abnormalities of AV valve architecture that include lengthening of the valve, thickening of the valve, and folding of the valve such that it fuses back to itself, leading to incompetence of both the mitral and tricuspid valves. Administration of a single dose of TGF beta neutralizing antibody results in dramatic normalization of mitral valve architecture, as shown in the middle panels. On the right, for comparison, I'm showing the normal mitral valves in mice that have the normal amounts of fibrillin-1.

Finally, we have observed a dramatic benefit of antagonizing TGF beta signaling in the aortic wall of fibrillin-1 deficient mice. On the left, you can see that mice that have one targeted copy of the fibrillin-1 gene show thickening and disorganization of the aortic wall in association with increased production of matrix degrading enzymes, such as matrix metalloprotenase, or MMP9. By introduction of one targeted copy of Smad 4, the essential effector of TGF beta signaling, we have both rescued aortic wall architecture and dramatically decreased the expression of matrix degrading enzymes.

So, in conclusion, these data document that the evolution of Marfan syndrome manifests a dynamic interplay between genetic predisposition, physiologic stress, time, and both productive and deleterious compensatory events that are faithfully recapitulated in the mouse system. Novel targets for therapeutic intervention include mediators of inflammation, matrix degradation, and cytokine activation and/or signaling. We believe that our mouse models are ideal for testing new treatment strategies.

I'd like to end by acknowledging the people in my laboratory who did this work and the essential collaborations of the laboratories of Lynn Sakai and Checco Ramirez .

Thank you.