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Hi. My name is Dan Judge. I'm an associate at the Johns Hopkins D.W. Reynolds Center.

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The purpose of this lecture is to explain how genetic engineering in mouse models can be used to improve our understanding of cardiovascular diseases. By way of an overview, I will describe why to use mouse models; we'll first talk about simple transgenesis as well as YAC transgenics, which are little more complicated. We'll talk about conditional expression of genes of interest. We'll talk about homologous recombination which is a little more complicated, requires a bit more time. And finally, reporter alleles which can provide an enormous amount of information through use of many of the technologies described previously. We'll finish with some work that's on the horizon and soon to be available more broadly in this area.

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Starting with, why to use mouse models, there are some disadvantages which I will briefly go over. They include the fact that physiologic variation *in vivo* can become quite complicated and if you're looking at a simple cellular event like transcription, *in vitro* study may be simpler using a cell model instead. Age-dependent phenotypes in the time required for the design and construction of a genetically altered mouse can be quite long in some models and the cost of housing mice is certainly a concern.

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Advantages include the fact that the cardiac function, rather than the cellular function is largely dependent on variables that cannot be controlled *in vitro*. Cell-based studies may not test for the functional effects at the organ level, and animal models allow testing of drug therapies to respond to genetically induced disorders modeling human diseases.

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When you're designing an animal model, why choose a mouse? There are certainly other species that could be used. Mice, however, are perhaps the easiest rodent to manipulate genetically. They have a short gestation, only 21 days. Large litter sizes, on average at 10 pups per litter, depending on the strain. The lifespan of a mouse is about two years and is appropriate for assessment of both early developmental and late age-dependent effects. However, other good options exist such as zebrafish and drosophila, though mice may be more physiologically relevant to the human condition for some diseases.

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First, talking about simple transgenesis. The purpose of this strategy would be to simply express your gene of interest and to understand its physiologic relevance. Before delving into a project like this, you should answer several simple questions, such as where would you like this gene expressed, do you want the human ortholog expressed or the endogenous mouse gene over-expressed, recognizing sometimes of course that the human gene may not interact with the endogenous mouse proteins that may be required for the

effects. Do you want to express a mutant form of your gene of interest or the wild type form of your gene of interest?

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This is an example of a plasmid used for transgenic construction. You'll note at the top, the gene of interest is inserted into a plasmid which contains a promoter, in this case shown is a CMV promoter which is a constitutively-expressing system. That can be varied. A poly-A tail follows it, and it's important for stable mRNA. A gene of interest is put into the middle of those. Other critical components of this transgene vector are ampicillin resistance which allows you to expend this DNA piece in bacteria. And you may need to test this in mammalian cells, and the ability to test cells that have been transfected is facilitated by the use of a neomycin resistance cassette, as an example.

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Following along the lines of the technical approach, after one designs the ideal targeting vector or the transgene vector, one isolates fertilized eggs from a donor mouse.

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Subsequently pro-nuclear injection of the gene of interest. The plasmid containing your transgene is inserted into male pro-nuclei.

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Subsequently, as shown here, integration of your gene of interest and your transgene plasmid may result in only part of this mouse becoming transgenic, because the DNA is inserted after a few cell divisions have taken place.

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So subsequent mice that are created need to be confirmed for germline transmission, meaning that the gene of interest or the transgene is expressed and passed on to subsequent progeny by germline transmission.

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One also needs to consider before designing or before producing the transgene, a system to genotype the mice. The fastest and easiest way to do this is with polymerase chain reaction or PCR, identifying mice that bear this copy of the transgene from DNA isolated from a tail clipping or a cheek swab. Southern analysis, however, is quite good for comparing copy number among transgenics, confirming PCR results if contamination is suspected, and it is a good idea to have something like a this designed and set up for your gene of interest. For instance, if you are over producing an endogenous mouse gene and it is not different from the surrounding, from the endogenous gene in any way it would be difficult to detect that by PCR whereas if you are expressing or over-expressing human gene in a mouse system it's easier, based on the differences, to do so.

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I want to emphasize the importance of isolating multiple founders. These are mice that contain the transgene at different sites of incorporation. Remember that the gene of

interest and the transgene plasmid are going to be incorporated randomly into the genome, and the site of incorporation can influence the phenotype. The phenotype may be due to effects on genes near the site of incorporation. I will talk about that in a subsequent example. Varying copy numbers are taken in by founders and variable effects are seen accordingly. So you test each for mRNA expression and protein expression in each strain from different founders.

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Here is an example of Southern blot showing copy number. In lanes two and three, you can see by the intense band that there is a high number of your transgene incorporated into the strain. Lines four and five have medium copy number in comparison to two and three, but a little bit more than one and certainly more than six.

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The importance of an insertional effect is really highlighted by work done by Kuro-o, et al. and published in 1997. They were making a transgenic and their gene of interest was the sodium proton exchanger; the rabbit type-one sodium proton exchanger, specifically, is what they used. They made 28 distinct strains of mice from different founders, and only three had stable expression of their gene of interest. They mated the remainder to homozygosity, hoping to see expression of their gene of interest.

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In fact, none of them did, but one did result in a profound prematurely aged appearance, shown in this picture. Random insertion of the transgene, in fact, had disrupted a never previously described gene, which they termed *Klotho* for the Greek goddess of spinning, and subsequently much of their work is focused on this endogenous gene which is randomly decreased in its expression by the site of insertion by their transgene.

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The importance of variation in the background strain is also something to consider. The background strain can be used for breeding and can influence results, so confirming the phenotype in multiple strains is ideal when you're creating a transgenic. A common inbred strain for mice is "C57 black six" or C57BL/6. These mice are genetically identical except for gender, and are inbred since 1921, depending on their site of breeding. And they differ from other strains, much like one person differs from the next. These mice have a particular preference for ethanol and sweets, whereas another mouse may not. Findings in a transgenic need to be confirmed in this strain as well as another strain, if you were to use this strain, to make sure that it's not a modifier effect. Different strains can be, are well described, actually, on the Jackson laboratory web site which is shown here. http://www.jax.org

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Next we will talk about YAC transgenesis.

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YAC transgenics are used to study "normal" expression of your gene of interest with:

- an endogenous promoter, not a promoter that is constitutive, or designed in the earlier experiments that I mentioned with simple transgenics.
- an enhancer element
- the three prime untranslated region
- intronic elements

All of these remain intact with your gene of interest. Large pieces of DNA that would be required for all of this information need to be incorporated into something called an artificial chromosome, like a YAC or a BAC. Smaller pieces of DNA, over 100 kilobases, can be put into something called a P1 but if you are over that size, either a BAC or a YAC is required for the larger pieces of DNA to be shuttled, to be inserted into the mice.

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By way of describing the technical approach, I will talk about the need to identify a large genomic fragment containing the full-length gene of interest, all exons, introns, large portions of the five-prime, and three-prime untranslated regions. It seems easiest to work with something that is not mouse, typically human because there is much better characterization of human YACs than other species. But you can imagine if you're putting in this large piece of mouse DNA it would be very difficult to discern it from endogenously expressed mouse gene of interest. One needs again a resistance cassette such as neomycin resistance for identifying cells that have been successfully transfected with your YAC transgene. In order to insert this large piece of DNA one can microinject fertilized pro-nuclei as described earlier, with the simple transgenics. Or one can transfect embryonic stem cells, as we will be describing and talking about in the next section, or subsequent sections about homologous recombination. Suffice it to say that transfecting embryonic stem cells in this way does not require homologous recombination with a specific site of insertion. One then, as in prior experiments with simple transgenics, should test the level of expression of your gene of interest.

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For example, *FBN1* is a very large gene and mutations, heterozygous mutations in *FBN1* cause Marfan syndrome in humans. The gene is large, it is widely expressed, and the promoter is not well-characterized. In order to test the hypothesis that expression of mutant forms of fibrillin-1 would cause disease in mice modeling Marfan syndrome we created YAC transgenics. We proposed that dominant negative interference, based on the human disease, would lead to, with adequate expression of the mutant transgene, would lead to the same findings of Marfan syndrome in transgenic mice.

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Here's an example of the schematic showing a non-transgenic, with two endogenous mouse alleles. This model is of the fibrillin-1 gene, with the surrounding five-prime and three-prime regions, and each of the 65 exons. The introns, which are not drawn in, can be imagined between each of the small boxes. A transgenic mouse bearing the wild type fibrillin-1 human transgene is shown above. The two endogenous mouse alleles and the transgene bearing a mutant form is shown below.

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Here we characterized different strains of transgenics harboring the human fibrillin-1 transgene, both the wild-type, which is shown on the left, and three strains with the mutant transgene, shown to the right. A non-transgenic is included for control to show that human fibrillin-1 mRNA is not present. This is a northern blot so this is mRNA. All the way to the right is a human mRNA sample, again no endogenous mouse background. The beta-actin signal is used to compare the loading from one sample to the next.

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Here's a look at the protein level. We used an antibody that was specific to the human form of fibrillin-1 protein. A non-transgenic has no green microfibrils. The transgenic produced by the wild type transgene shows green microfibrils, which are specifically human, and all the way to the right is highest level expressing mutant transgenic and the important finding here is that these are stable microfibrils produced from a mutant form of fibrillin-1.

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In creating the mice we were surprised to find that there was in fact no aortic pathology, there was no bone pathology, and no lung pathology – many sites that we would expect for there to be pathology if this was recapitulating the dominant negative effects in humans.

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We also looked for co-localization of the human and mouse fibrillin-1 with immuno-electron-microscopy, shown here. To the left is a non-transgenic strain. In the center is a transgenic producing wild-type fibrillin-1 protein. And to the right is a transgenic strain producing high levels of the mutant fibrillin-1 protein. In each of these developing microfibrils we can see antibody recognition of the human protein present, and a spacing that suggests that both human and mouse are present.

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Our preliminary conclusions for these experiments said that mutant fibrillin-1 can be stably secreted and incorporated into the extracellular matrix. There's no apparent consequence of overexpression of mutant human fibrillin-1 in a normal mouse background, and mouse and human proteins appear to interact based on co-immunoprecipitation and immunolocalization studies that we've performed.

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Let's move our attention to conditional transgenics, next, as another example. These are tools that allow you to turn on or turn off your transgene, either permanently or transiently in response to these following systems. First we will discuss the cre-loxP system, where conditional deletion or conditional activation by the removal of interfering DNA can lead to your effects desired.

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Cre refers to the 38 kilodalton recombinase that mediates recombination at loxP sites. LoxP refers to "locus of crossing over," two 13-base pair inverted repeats separated by an 8-base pair asymmetric spacer region.

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The inverted repeats when placed over each other in reverse show homology. The gene of interest is flanked by loxP sites. If you'd like to delete it, when cre recombinase is expressed, the triangles which are homologous, lead to homologous recombination again and a looping out of the gene of interest, resulting in a single loxP site remaining, and a removed gene of interest.

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Here is an example of how it would be used in mice. At the top we have a gene of interest, diagramed and flanked by loxP sites with triangles. To the right, mating with this mouse would be a mouse with cre-recombinase only in its myocytes, in its heart. And when these two mice are mated, the gene of interest is deleted only in the heart, and remains floxed, or flanked by loxP sites in all other tissues where cre-recombinase is not present.

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The tetracycline-responsive transgenic uses a different system to make conditional transgenics. It utilizes a trans-activating protein, referred to as tTA, which prevents transcription. In other words, with tetracycline it is off or it is referred to as the "tet-off" system. tTA is a fusion protein consisting of the VP16 trans-activation domain fused to the E. coli tetracycline repressor protein; tetR is how it's abbreviated. In the absence of tetracycline or doxycycline, in this case, transcription of the gene of interest is activated. In the presence of tetracycline or doxycycline the gene of interest transcription is activated.

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Here's an example where in the tet-off system with an alpha-MHC promoter driving production of the trans-Tet activator there is no phenotype. In the other mouse with the Tet-O promoter driving the gene of interest, again there is no phenotype. When they are mated and you have both, (with proper screening, if you find mice that have both transgenes), without tetracycline in this system, (this is the Tet-off system), without tetracycline or doxycycline the gene of interest is expressed and when you give the mouse doxycycline which is usually easier, it turns the gene off.

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Tetracycline responsive transgenics: This is sort of the mirror analogue, using a modification of the system previously described, called a reverse trans-Tet activator. When this system is used, similar to the last system, the reverse trans-Tet activator binds DNA only when doxycycline is present. In this one, one has to be careful not to use tetracycline because it doesn't respond quite as well as doxycycline.
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In the setting of doxycycline, transcription of the gene of interest is activated, shown here Tet-on, again. When these mice are mated, the presence of both transgenes leads to the expression of your gene of interest with doxycycline, and absence of expression without doxycycline.

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Next we will turn our attention to targeting of the endogenous mouse genes. This is what people refer to as "knocking out" your gene of interest. This requires a large piece of homologous DNA from a mouse, usually about 7 kb; it can be larger, maybe 10 kilobases of fragment of your murine gene of interest. When choosing the targeting vector around a critical site like the start codons, all start codons in the early portion, or alternate start sites, must be contained within your targeting vector if your are knocking out expression of your gene of interest. Your targeting vector should contain a positive selection cassette, such as neomycin-resistance, to determine which cells carry this, and may contain a negative selection cassette such as the thymidine-kinase gene.

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First, one creates the targeting vector, as shown here. This is the region of interest that you are targeting. The first four exons and a five-prime UTR are shown from your gene of interest. The targeting vector is homologous, although the first two exons here are replaced by the neomycin-resistance cassette. The homology at these sites leads to recombination and substitution here now with the endogenous gene, after recombination, substitution of the first two exons with the neomycin-resistance cassette preventing transcription of that allele.

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This is a heterozygous mouse but when mated to homozygosity for the neomycin resistance cassette substituting for the first two exons, one would have no expression of your gene of interest.

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Targeting of the endogenous mouse genes requires first transfecting embryonic stem cells and isolating stably expressing clones by their neomycin resistance.

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At that point the cells are screened for stable clones that are in the correct position. About 2 to 3%, actually, of cells that incorporate your targeting vector will end up having it in the right position, so rather a low number. When you find those that are correctly targeted, one microinjects these correctly targeted embryonic stem cells into blastocysts and then implants them into pseudo-pregnant females which are prepared to carry a blastocyst.

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The resulting mouse is called chimera, which means that some of its cells are isolated and have resulted from the embryonic stem cell that you have targeted, and some of them are not. They generally have a marbled coat color, as shown in the diagram. When you mate

that with a wild-type, or a non-targeted mouse, those that have offspring containing your targeted gene of interest have germline incorporation and are subsequently targeted for that copy of the gene in subsequent generations.

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Here is how one completes the experiment to delete the gene of interest, mating two heterozygous knockouts, or haploinsufficient mice, can result in 25% without targeting, 50% which are heterozygous for the targeting event, and 25% which are homozygous for your targeting event, or completely deleted for the critical starting points of the gene of interest.

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Targeting of the endogenous mouse genes can be used to engineer mice with subtle alterations in a gene of interest as well. This is called "knocking-in" a mutation. For instance, in Marfan syndrome, fully expressed heterozygous missense mutations in humans result in disease. We chose a typical human mutation, and engineered a mouse with this mutation. In order to prevent transcriptional interference by the neomycin resistance cassette, that portion of the gene was removed by cre-loxP technology, and I will describe that as well.

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Here's our targeting vector. It contains several flanking exons, and the introns as well, around exon 25, to result in a C1039G mutation. The neomycin resistance cassette is in intron 24 and flanked by loxP sites. We were able to correctly target embryonic stem cells with neomycin resistance. Prior experiments in multiple targeting events with other mice have shown the neomycin resistance cassette can decrease the expression that allele. In order for us to have full expression of this mutant allele, much like in the human condition, we used a CMV or ubiquitously expressing cre-transgenic to remove the loxP-flanked neomycin resistance cassette, resulting in a single base pair mutation in the coding sequence and only a 34 base-pair loxP site in the intron, which did not interfere with transcription.

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By Southern analysis one can see before cre-loxP that the targeted piece of DNA is higher up. With northern analysis, which is mRNA which is electrophoresed on a gel, the fibrillin-1 gene is fully expressed in comparison to a beta actin control, in both the heterozygous and homozygous strains.

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These mice did, in fact, recapitulate Marfan-aortic disease in heterozygosity. And, I want to remind you of the work we did with transgenics which really shows it can happen, that mice are not inherently resistance to the disease seen in humans. Further work is ongoing to characterize this interaction better.

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Let's move our attention next to reporter systems. Transgenic mice can be used for reporter systems to see where your gene of interest is expressed, (anomaly in video acknowledged – will repair) to identify the activity of endogenous responses to genetic manipulations, or responses to therapy, in fact. Reporter constructs include production of some protein that can be assayed easily. Beta-galactosidase by the LacZ gene produces blue when properly treated. Green fluorescent protein can be easily identified with fluorescence or by antibodies. Firefly luciferase can be assayed specifically, and again provides a nice report of where activity is. The promoter for a construct like this determines where the reporter will be turned on.

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Transgenic reporters include, for example, if you are wondering where and when the gene of interest is expressed, you can add GFP and cDNA to your targeted alteration to produce a fusion protein, shown here. This is how the cDNA, as well how the protein, would look, with your gene of interest immediately fused to a green fluorescent protein. That way when you assay for your green fluorescent protein presumably that site is, as well, where your gene of interest is. This is targeting of the endogenous gene of interest. Here an example of a mouse with then just left ventricular expression of your gene of interest based on the left ventricular highlighting of the green portion of the heart.

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Alternatively if you'd like to demonstrate where an endogenous gene of interest is active, you can create a ubiquitously expressed transgenic with a promoter responsive to your gene of interest. Shown here, this promoter is driving production of green fluorescent protein. This, by itself, is simply inserted into a mouse rather than homologous recombination in the last experiment. With activity of your gene of interest, which your promoter is responsive to, the left ventricle again lights up suggesting that your gene of interest is active in the heart. This requires a promoter that is specific and responsive to and your gene of interest, or to the protein of interest.

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Several new things are on the horizon that I'll explain briefly.

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One of which is a new technology within the past few years of using RNAi to suppress a gene of interest. Initial work along these lines was done in cultured cells and has recently been done *in vivo*. By way of background, RNA interference is the use of double stranded RNA-dependent post-transcriptional gene silencing. It's recently been shown to potently knock down and occasionally completely silence endogenous mRNA transcripts. Carmel et al. recently demonstrated the ability of a transgene engineered with a U6 snRNA promoter driving production of double-stranded RNA to knockdown expression of *Neil1*, which happened to be their gene of interest, in murine embryonic stem cells.

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The majority of the cell lines demonstrated 80% reduction of *Neil1* expression and protein. Chimeric mice were produced, and subsequent progeny actually demonstrated

persistent reduction in *Neil1*. The reference for this is shown here. *Nat Struct Biol.* 2003;10:91-2

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Finally a consortium of research groups in San Francisco has recently, through NIH funding and the NHLBI Program for Genomic Applications, has set out to target a number of genes at random and to make those targeted embryonic stem cells available to scientists, to researchers. Here is a web site which describes their techniques and availability of their expanding, successfully targeted, embryonic stem cells. This greatly enhances the ability or improves the ability for a researcher to go through what might be a year, otherwise, to have a correctly targeted embryonic stem cell.

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In summary I will say that mouse models can provide critical *in vivo* opportunities for modulation of your gene of interest. Simple transgenic expression can be useful with proper design and characterization. Large genes, with endogenous promoter and intronic segments, can be expressed with a yeast artificial chromosome, or YACs. Conditional transgenics can be engineered with cre-loxP or tetracycline-responsive systems.

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Targeted alterations, knock-out or knock-in, occur by homologous recombination in murine embryonic stem cells. And reporter systems can be used to follow production of your gene of interest by homologous recombination or transgenesis. New technologies continue to expand the horizons for use of genetic engineering in this field. Thank you.