Lecture 23
Transgenes and Gene Targeting in Mice I

In the next two lectures I will be telling you about some of the ways in which we can study gene function in higher eukaryotes, more specifically in the laboratory mouse **Mus Musculus**. I will be doing this by telling you about a remarkable number of manipulations that have been made to the mouse genome in order to generate an experimental mouse model system for human **Sickle Cell Disease.**   The mouse that was developed to explore this human disease turns out to be one of most genetically modified mice on the planet…and so it gives us an interesting framework in which to tell you about making **transgenic** and **knockout** mice.   To set the scene for genetically modifying mice to mimic human **sickle cell disease** we need to step back a bit and consider this devastating human disease and some of its features.

Human Sickle Cell Disease (a.k.a. sickle cell anemia): Sickle cell disease is a human blood disorder that is caused by a single mutation in a gene that encodes one of the subunits of **hemoglobin** (**Hb)**, namely β-**globin.**

Sickle Cell Disease – An autosomal Recessive disorder of Hemoglobin

· Red blood cells (RBCs) make up 40% of the blood volume

· Hemoglobin makes up 70% of the proteins in RBCs

A single mutation in the sixth amino acid of the β-globin chain (Glutamine -> Valine) causes Sickle Cell Disease

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Hemoglobin is a tetrameric protein made up of two α-**globin** proteins, and two β-**globin** proteins; ααββ. Each of the 4 globin proteins embrace an iron-containing heme molecule (iron is what makes hemoglobin and Red Blood Cells red) whose function is to bind oxygen in the lungs and release it in all the tissues of the animal. The very simple change of the sixth amino acid in β-**globin** (glutamine is substituted with a valine) causes devastating consequences. It turns out that **Hb** containing β-**globin** subunits with the sickle mutation (known as **HbS**) does not directly interfere with the ability of hemoglobin to store or release oxygen, but rather this amino acid change bestows a novel property on the hemoglobin molecule; in its deoxygenated state the **HbS** molecules aggregate together to form polymeric fibers, and the presence of these fibers grossly distort the shape of Red Blood Cells **(RBCs)**. Instead of being shaped almost like a doughnut (without the actual hole) and having tremendous flexibility to squeeze through tiny capillaries within tissues, the aggregated **HbS fibers** cause the **RBCs** to become curved (like a sickle), rigid, prone to rupture and prone to clumping; rupture causes anemia and clumping clogs small blood vessels, leading to tissue damage.

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Freq. sickle cell disease in US born children

Ethnicity	HbS
African American	1/500
Hispanic	1/14,000
Middle Eastern	0/22,000
Native American	1/17,000
Caucasian	1/160,000
Asian	0/200,000

1/12 African Americans are carriers (heterozygous) for the HbS allele

Heterozygotes for the β–globin sickle cell mutation turn out to be resistant to MALARIA infection; the malaria parasite does not grow well in RBCs in heterozygous individuals. You will consider such issues in the population genetics lectures.

It turns out that **Sickle Cell Disease** is very common in many parts of the world, especially sub-Saharan Africa, and even among parts of the US population, in particular African Americans and Hispanic Americans. The prevalence of such a devastating disease allele is actually quite surprising since one would expect it to be selected against as the human population expanded. However, it turns out that people who are heterozygous for the sickle mutation in the β-**globin** gene are **resistant to malaria**, and so this gives a survival advantage for people who are carriers of the mutant allele; they are said to have the **sickle cell trait** but they do not have **sickle cell disease**.

Organization and Expression of the Human globin genes: It turns out that mammals have a number of different β-**globin**-like genes, and a number of α-**globin**-like genes, i.e., a β-**globin** family and an α-**globin** family of genes. These two gene families are found on separate chromosome; some of the family members are **pseudogenes** (genes that do not produce functional proteins), and the functional family members turn out to be expressed at different times during development. How did all of these globin genes appear in mammalian genomes, and what are they doing there.
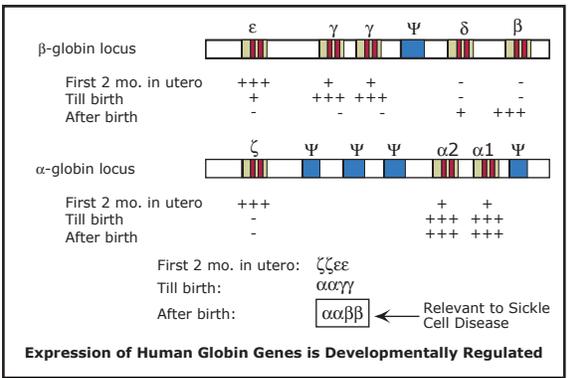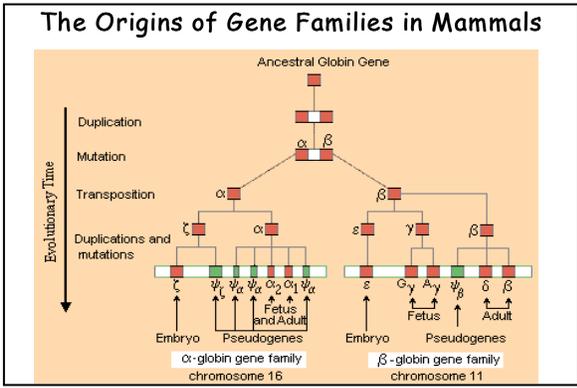

Figure by MIT OCW.

Many genes in mammals exist as multi-gene families, and the globin genes are a good example of this. During mammalian evolution it appears that gene duplication was a common event, and this has allowed the duplicated genes to accumulate mutations that sometimes inactivate the gene (leading to pseudogenes that are non-functional) and sometimes to genes that produce proteins that can carry out a slightly different function. For the globin genes, soon after duplication of an ancestral gene to create the α-**globin** and β-**globin** ancestral genes, these two genes were segregated to separate chromosomes where they evolved their own gene families through further duplication and mutations during thousands of years.

Healthy people: $\alpha\alpha\beta\beta$

Sickle Cell Trait: $\alpha\alpha\beta\beta_s$

Sickle Cell Disease: $\alpha\alpha\beta_s\beta_s$

$\alpha\alpha\beta_s\beta_s$ is soluble when oxygenated, but precipitates in low oxygen

It is the $\alpha\alpha\beta_S\beta_S$ hemoglobin molecule expressed after birth that is responsible for aggregating and causing **sickle cell disease.** The $\alpha\alpha\beta\beta_S$ hemoglobin tetramers expressed in people heterozygous for the sickle mutation do not aggregate to form fibers, and so do not cause disease; however, should such heterozygous people live at high altitude some sickling can occur.

It is sobering to note that almost 50 years since the molecular basis of this disease was discovered there still does not exist a really effective therapy for the disease. Hemoglobin was one of first proteins to be purified, it's gene was one of the first to be cloned, and the globin proteins were among the first to have their structure determined by x-ray crystallography…and although some progress has been made in therapy, much more still needs to be done. This is precisely why having a robust **mouse model for sickle cell disease** to test experimental therapies is absolutely critical. Tremendous strides have been made in generating a **mouse model for sickle cell disease**.

How do we Genetically modify the mouse genome?

(1) Transgenes
 • adding genes by pronuclear injection
 • random insertion with no replacement

(2) "Knock-outs"
 • subtracting or deleting genes
 • gene targeting
 • specific insertion with replacement

There are two general ways to specifically modify the genetic makeup of a mouse. One involves the random integration of a cloned gene somewhere into the mouse genome (i.e., the introduction of a "**transgene**"). The other involves precisely targeting a specific gene in the mouse and introducing a know alteration of that gene, usually the deletion of the gene and the insertion of a marker gene in its place (a gene **knock-out** by targeted homologous recombination).

Introduction of the Human β-globin gene with the sickle cell mutation (β_S^H) into the mouse genome: In the 1980's and early 1990's several groups tried to make a mouse with **sickle cell disease** by introducing the Human β-globin gene with the sickle mutation **(β_S^H)**, in the hope that if the protein was expressed at high levels it would precipitate **Hb** fibers that would cause sickling of RBCs, thus mimicking **sickle cell disease**. How does one make a **transgenic mouse**?

Mice are treated with a hormone to make them super-ovulate and then mated. Soon after mating, the fertilized eggs are retrieved from the uterus. Eggs that contain two pronuclei (one from the mother and one from the father) indicating that the embryo is still at the one-cell stage, are identified under the

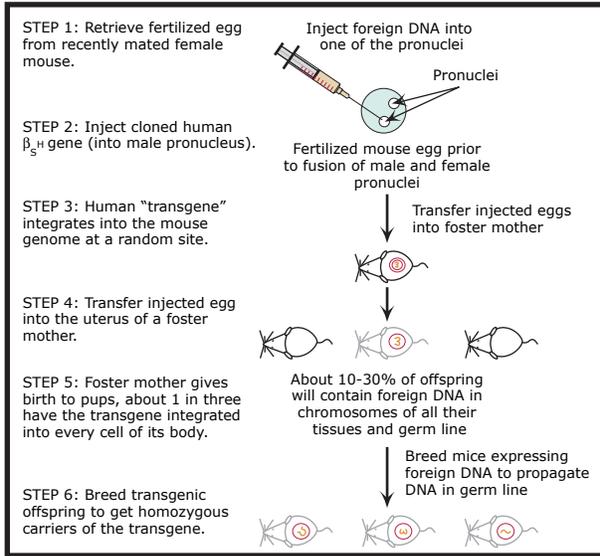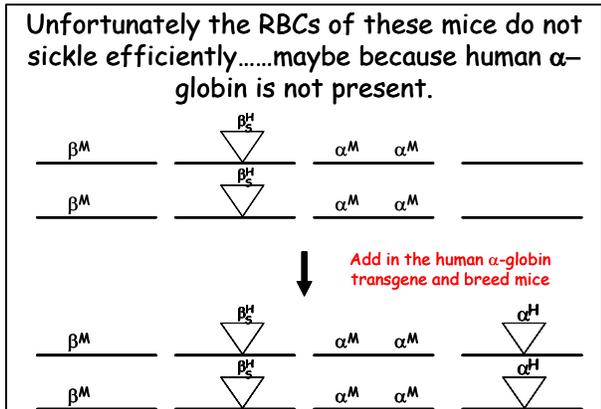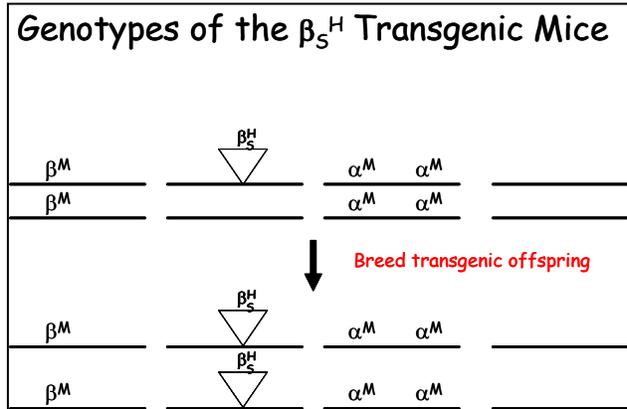microscope. The male pronucleus is injected (still under the microscope) with

STEP 1: Retrieve fertilized egg from recently mated female mouse.

Inject foreign DNA into one of the pronuclei

Pronuclei

STEP 2: Inject cloned human β_S^H gene (into male pronucleus).

Fertilized mouse egg prior to fusion of male and female pronuclei

STEP 3: Human "transgene" integrates into the mouse genome at a random site.

Transfer injected eggs into foster mother

STEP 4: Transfer injected egg into the uterus of a foster mother.

STEP 5: Foster mother gives birth to pups, about 1 in three have the transgene integrated into every cell of its body.

About 10-30% of offspring will contain foreign DNA in chromosomes of all their tissues and germ line

Breed mice expressing foreign DNA to propagate DNA in germ line

STEP 6: Breed transgenic offspring to get homozygous carriers of the transgene.

Figure by MIT OCW.

purified DNA fragments that contain the β_S^H gene along with an appropriate promoter region to give it a good chance of being expressed in once integrated into the genome. The injected DNA quite often gets incorporated into the genome, and about one three eggs that are implanted into a foster mother mouse will have the β_S^H gene integrated, and will go on to produce a baby mouse. Animals that score positive for the human **transgene**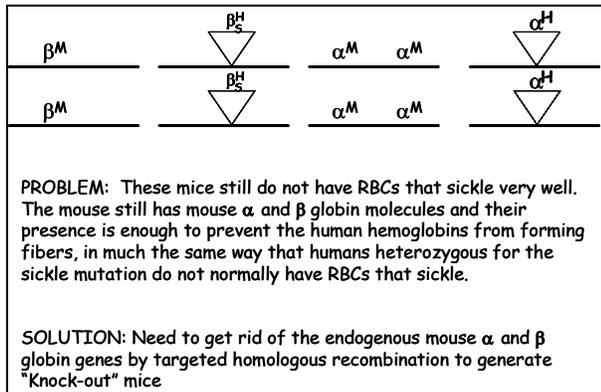 are mated to generate mice homozygous for the transgene. Among these progeny one is likely to contain the mutated human $\beta-$globin protein in its RBCs.

This was indeed achieved, BUT, this mouse did not prove to be a good model for **sickle cell disease**. It turns out that the human β-globin protein does not complex well with the **mouse α-globin protein (α^M)** and so the cloned gene encoding the **human α-globin protein (α^H)** was introduced into fertilized mouse eggs to create a new transgenic mouse line, which was then mated with the β_S^H transgenic mouse to produce a mouse expressing both β_S^H and α^H human proteins.

Genotypes of the β_S^H Transgenic Mice

β^M β_S^H α^M α^M

β^M α^M α^M

Breed transgenic offspring

β^M β_S^H α^M α^M

β^M β_S^H α^M α^M

Unfortunately the RBCs of these mice do not sickle efficiently......maybe because human $\alpha-$globin is not present.

β^M β_S^H α^M α^M

β^M β_S^H α^M α^M

Add in the human α-globin transgene and breed mice

β^M β_S^H α^M α^M α^H

β^M β_S^H α^M α^M α^H

Note that the α^H gene is almost certain to integrate into different location than the β_S^H gene did, and probably in a different chromosome. These alleles will therefore sort independently when the two transgenic mouse lines are bred together. The strong expectation was that the presence of the $\alpha^H \alpha^H \beta_S^H \beta_S^H$ hemoglobin tetramer in mouse RBCs would lead to the precipitation of fibers

and the sickling of the mouse RBCs. However, much to the disappointment of the research teams involved, this was simply not the case. It turns out that the presence of the normal mouse hemoglobin proteins is enough to prevent the mutant hemoglobin tetramers from precipitating into fibers, and so these mice do not make a good model for human **sickle cell disease.**

β^M _____

β^M _____

β^H_S ▽

β^H_S ▽

α^M α^M

α^M α^M

α^H ▽

α^H ▽

PROBLEM: These mice still do not have RBCs that sickle very well. The mouse still has mouse α and β globin molecules and their presence is enough to prevent the human hemoglobins from forming fibers, in much the same way that humans heterozygous for the sickle mutation do not normally have RBCs that sickle.

SOLUTION: Need to get rid of the endogenous mouse α and β globin genes by targeted homologous recombination to generate "Knock-out" mice

It was decided that the only solution to this problem would be to eliminate the endogenous mouse α and β globin genes.

This will be the topic of the next lecture.