NEUROSCIENCE

LECTURE 11: Development of transgenic mice

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it

- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.

- Transgenic sheep and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesize human proteins in the "white" of their eggs.

These animals should eventually prove to be valuable sources of proteins for human therapy.

In July 2000, researchers from the team that produced Dolly reported success in producing transgenic lambs in which the transgene had been inserted at a specific site in the genome and functioned well.

Transgenic **mice** have provided the tools for exploring many biological questions.

An example:

Normal mice cannot be infected with polio virus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus. So normal mice cannot serve as an inexpensive, easily-manipulated model for studying the disease. However, transgenic mice expressing the human gene for the polio virus receptor

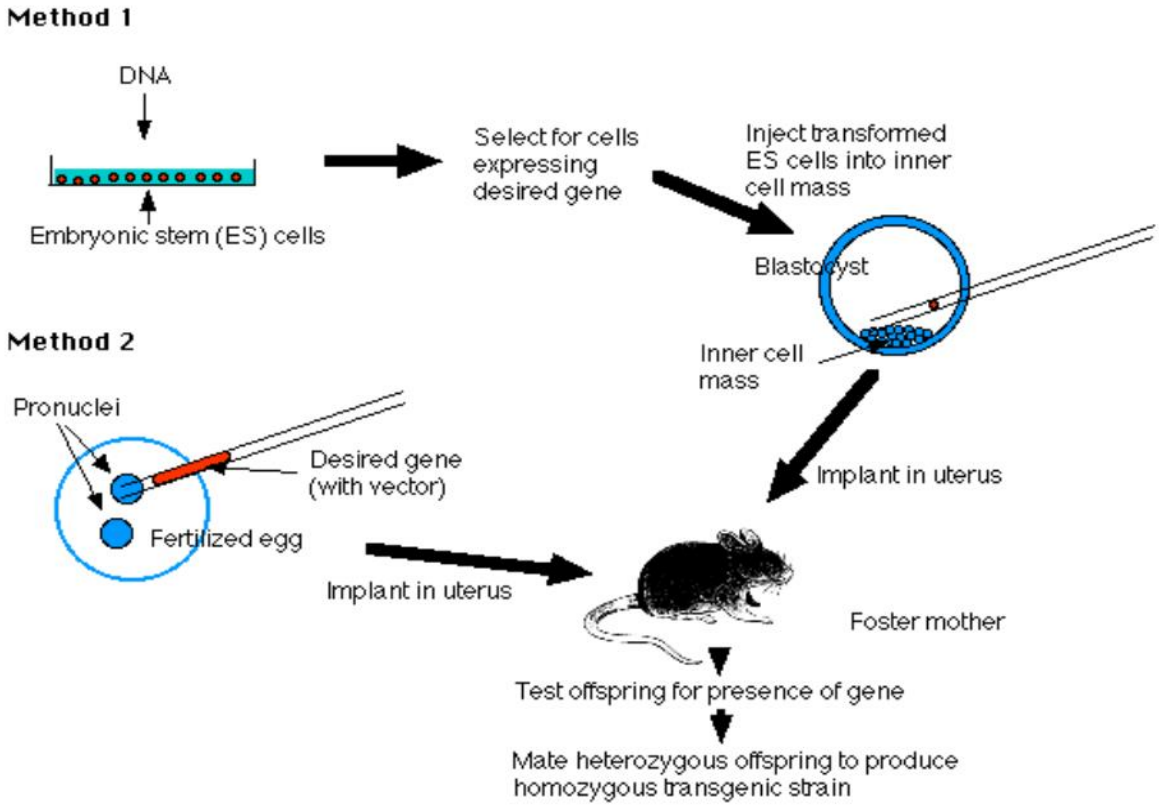
- can be infected by polio virus and even
- develop paralysis and other pathological changes characteristic of the disease in humans.

Two methods of producing transgenic mice are widely used:

- transforming **embryonic stem cells** (**ES** cells) growing in tissue culture with the desired DNA;
- injecting the desired gene into the **pronucleus** of a fertilized mouse egg.

The Embryonic Stem Cell Method (Method "1")

Embryonic stem cells (**ES** cells) are harvested from the **inner cell mass** (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, **including its gametes**.



1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing

- the gene you desire (e.g., the insulin gene);
- **vector** DNA to enable the molecules to be inserted into host DNA molecules;
- **promoter and enhancer sequences** to enable the gene to be expressed by host cells.

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells.

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

- Prepare a **pseudopregnant** mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they **implant** successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

- Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10–20% will have it, and they will be heterozygous for the gene.

7. Establish a transgenic strain

- Mate two heterozygous mice and screen their offspring for the 1 in 4 that will be **homozygous** for the transgene.
- Mating these will found the transgenic strain.

The Pronucleus Method (Method "2")

1. Prepare your DNA as in Method 1

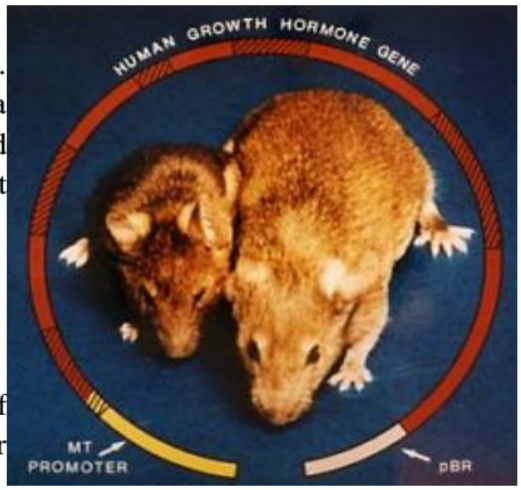
2. Transform fertilized eggs

- Harvest freshly fertilized eggs before the sperm head has become a pronucleus.
- Inject the male pronucleus with your DNA.
- When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo.

3. Implant the embryos in a pseudopregnant foster mother and proceed as in Method 1.

An Example

This image (courtesy of R. L. Brinster and R. E. Hammer) shows a transgenic mouse (right) with a normal littermate (left). The giant mouse developed from a fertilized egg transformed with a recombinant DNA molecule containing:



- the gene for **human growth hormone**
- a strong mouse gene **promoter**

The levels of growth hormone in the serum of some of the transgenic mice were several hundred times higher than in control mice.

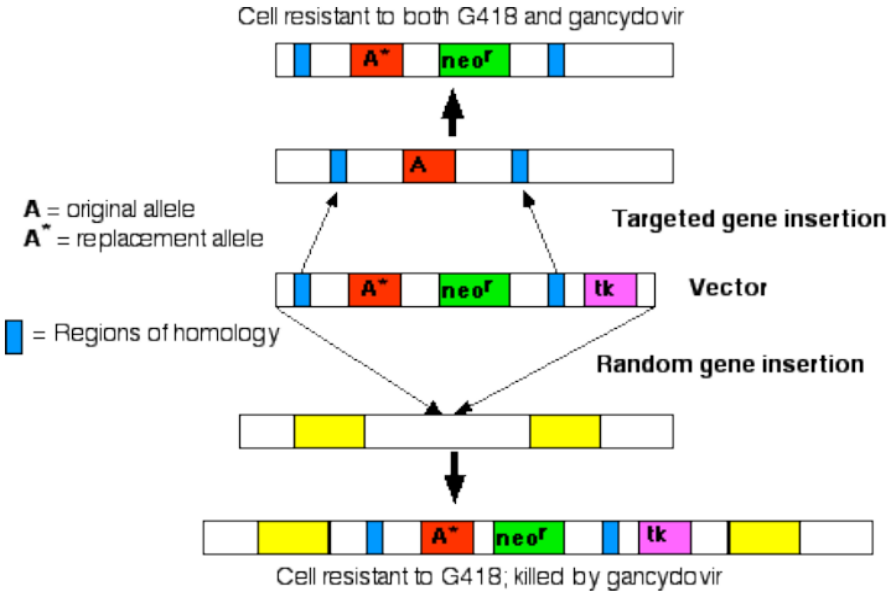
Random vs. Targeted Gene Insertion

The early vectors used for gene insertion could, and did, place the gene (from one to 200 copies of it) anywhere in the genome. However, if you know some of the DNA sequence flanking a particular gene, it is possible to design vectors that replace that gene. The replacement gene can be one that

- restores function in a mutant animal or
- knocks out the function of a particular locus.

In either case, targeted gene insertion requires

- the desired gene
- ***neo***^{r}, a gene that encodes an enzyme that inactivates the antibiotic neomycin and its relatives, like the drug G418, which is lethal to mammalian cells;
- ***tk***, a gene that encodes **thymidine kinase**, an enzyme that phosphorylates the nucleoside analog **ganciclovir**. **DNA polymerase** fails to discriminate against the resulting nucleotide and inserts this nonfunctional nucleotide into freshly-replicating DNA. So ganciclovir kills cells that contain the *tk* gene.



Step 1

Treat culture of ES cells with preparation of vector DNA.

Results:

- **Most cells** fail to take up the vector; these cells will be killed if exposed to G418.
- In a **few cells**: the vector is inserted randomly in the genome. In random insertion, the entire vector, including the ***tk*** gene, is inserted into host DNA. These cells are resistant to G418 but killed by gancyclovir.
- In **still fewer cells**: **homologous recombination** occurs. Stretches of DNA sequence in the vector find the homologous sequences in the host genome, and the region between these homologous sequences replaces the equivalent region in the host DNA.

Step 2

Culture the mixture of cells in medium containing both G418 and ganciclovir.

- The cells (the majority) that failed to take up the vector are killed by G418.
- The cells in which the vector was inserted randomly are killed by gancyclovir (because they contain the *tk* gene).
- This leaves a population of cells transformed by homologous recombination (enriched several thousand fold).

Step 3

Inject these into the inner cell mass of mouse blastocysts.

Knockout Mice: What do they teach us?

If the replacement gene (**A*** in the diagram) is nonfunctional (a "null" allele), mating of the heterozygous transgenic mice will produce a strain of "**knockout mice**" homozygous for the nonfunctional gene (both copies of the gene at that locus have been "knocked out").

Knockout mice are valuable tools for discovering the function(s) of genes for which mutant strains were not previously available. Two generalizations have emerged from examining knockout mice:

- Knockout mice are often surprisingly unaffected by their deficiency. Many genes turn out not to be indispensable. The mouse genome appears to have sufficient redundancy to compensate for a single missing pair of alleles.
- Most genes are **pleiotropic**. They are expressed in different tissues in different ways and at different times in development.

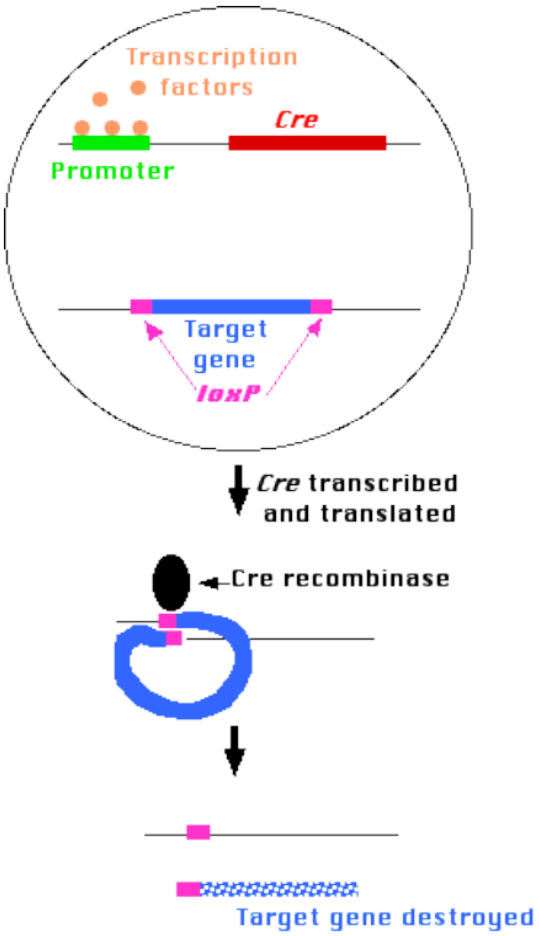
Tissue-Specific Knockout Mice

While "housekeeping" genes are expressed in all types of cells at all stages of development, other genes are normally expressed in only certain types of cells when turned on by the appropriate signals (e.g. the arrival of a hormone).

To study such genes, one might expect that the methods described above would work. However, it turns out that genes that are only expressed in certain adult tissues may nonetheless be vital during embryonic development. In such cases, the animals do not survive long enough for their knockout gene to be studied.

Fortunately, there are now techniques with which transgenic mice can be made where a particular gene gets knocked out in only one type of cell.

The Cre/*loxP* System



One of the bacteriophages that infects E. coli, called P1, produces an enzyme — designated Cre — that cuts its DNA into lengths suitable for packaging into fresh virus particles. Cre cuts the viral DNA wherever it encounters a pair of sequences designated *loxP*. All the DNA between the two *loxP* sites is removed, and the remaining DNA ligated together again (so the enzyme is a recombinase).

Using "Method 1" (above), mice can be made transgenic for

- the gene encoding Cre attached to a promoter that will be activated only when it is bound by the same transcription factors that turn on the other genes required for the unique function(s) of that type of cell;
- a "target" gene, the one whose function is to be studied, flanked by *loxP* sequences.

In the adult animal,

- those cells that
  - receive signals (e.g., the arrival of a hormone or cytokine)
  - to turn on production of the transcription factors needed
  - to activate the promoters of the genes whose products are needed by that particular kind of cell

  will also turn on transcription of the Cre gene. Its protein will then remove the "target" gene under study.

- All other cells will lack the transcription factors needed to bind to the Cre promoter (and/or any enhancers) so the target gene remains intact.

The result: a mouse with a particular gene knocked out in only certain cells.

Knock-in Mice

The Cre/*loxP* system can also be used to

- remove DNA sequences that block gene transcription. The "target" gene can then be turned **on** in certain cells or at certain times as the experimenter wishes.
- replace one of the mouse's own genes with a new gene that the investigator wishes to study.

Such transgenic mice are called "knock-in" mice.

Transgenic rodent models of Parkinson's disease

Parkinson's disease is emerging as a complex interplay of the environment and genetic risk factors. Overall, PD is primarily idiopathic with a subset (<15% of cases) with a family historyof PD. In pedigrees with a pattern of inherited PD, genetic linkage studies have identified 13PARK loci to date (OMIM 168600). Molecular genetics studies have identified genesassociated with 7 of 13 PARK loci and we will be describing the current and possible transgenicanimals for three of these genes (i.e. Parkin, DJ-1 and PINK1). We will also discuss a recentlydeveloped transgenic animal (MitoPark) that focuses on mitochondrial dysfunction as apathogenic mechanism of PD.

Parkin (PARK2)

Studies of autosomal recessive inheritance pattern of early-onset PD in a group of Japanese families led to the identification of the Parkin gene at the PARK2 locus . Additional studieshave confirmed that mutations in Parkin are linked to autosomal recessively inherited PD. Unlike α-synculein that has few identified mutations, more than a 100 mutations have beenidentified in the Parkin gene. Parkin has E3 ubiquitin-protein ligaseactivity and targets proteins for degredation by the proteosome [8, 21, 46].

Several laboratories have generated Parkin knockout mice by targeting different exons of the Parkin gene [13, 22, 30, 33, 37, 45]. In mice missing exon 3, striatal dopamine levels are increased; synaptic excitability in striatal spiny neurons and DAT levels are decreased. However, the number of nigral dopaminergic neurons remains normal for up to 2 years. The mice exhibit behavioral deficits that are associated with the basal ganglia function and have decreased DA release in response to amphetamine [13, 22]. The exon 3 knockout mice show reduced mitochondrial respiration and increased oxidative damage [30]. Similar to exon 3 deletion, exon 7 deletion did not affect the nigral neuron numbers, but decreased TH-producingcells in the locus coeruleus [45]. In contrast, mice without exon 2 of Parkin exhibited noalterations in behavior, catecholamine levels or altered sensitivity to methamphetamine or 6-OHDA [32, 33]. Sato *et al*. [37] generated mice with a knockout of exon 2 and identified agerelateddeclines in striatal dopamine and increase in D1/D2 receptor binding using ex-vivoPET imaging. Behavioral testing and immuno-labeling of dopaminergic nigral neuronsrevealed no abnormalities compared to wild-type mice [37]. Overall, Parkin knockout micefail to develop a Parkinsonian phenotype, but the different knockout models may provide ameans to examine the role of Parkin in protein turnover, oxidative stress and mitochondrialdysfunction.

DJ-1 (PARK7)

The DJ-1 gene was identified at the PARK7 locus [44] with a point mutation (L166P) that cosegregated with the disease allele in an Italian family [5]. PARK7, like PARK2 is inherited in an autosomal recessive manner. Many mutations in the DJ-1 gene have been associated withearly onset PD [2,3,5,15, 17]. DJ-1 is involved in multiple cellular processes including oxidative stress and cellular transformation [27].

Based on a mutation observed in human DJ-1 by Bonifati*et al*. [5], a transgenic mouse missingthe first 5 exons and part of the promoter of DJ-1 was created [7]. No observable expressionof DJ-1 was observed in the homozygous null mice which did show a progressive decline inselected motor tests. There was increased striatal dopamine and evoked dopamine overflow inthe striatum. There was no change in the number of nigral dopaminergic neurons or markersof these neurons [7]. Similarly, by disrupting exon 2 of DJ-1, Goldberg *et al*. [14] generatedmice with decreased evoked dopamine overflow in the striatum and lower locomotor activitycompared to wild-type mice. No change in the number of dopaminergic neurons of thesubstantianigra was observed [14]. A third study by Kim *et al*. [23] generated a DJ-1 knockoutby also disrupting exon 2, the first coding exon of DJ-1, and found no change

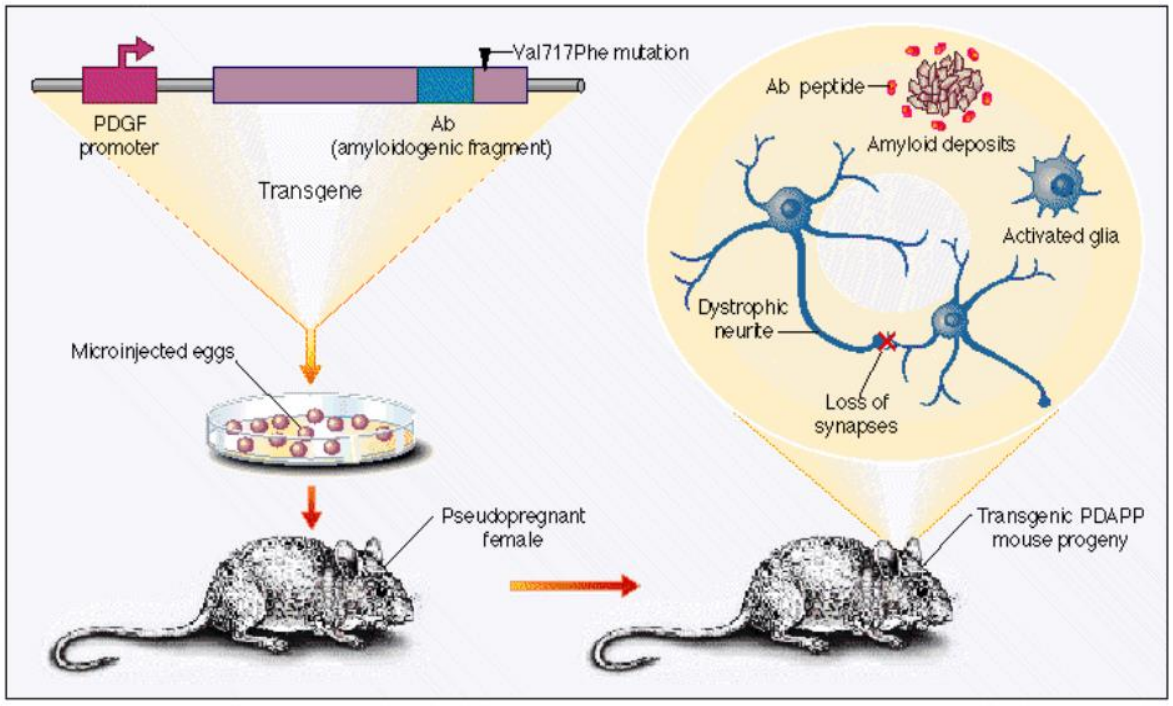
in striataldopamine levels or nigral dopaminergic neuron numbers. These mice did exhibit decreasedlocomotion in response to amphetamine and increased sensitivity to MPTP which could berestored by viral vector delivery of DJ-1 to the striatum. Cortical neurons derived fromembryonic brain of DJ-1 knockout mice were more sensitive to oxidative stress [23]. Overall,these studies demonstrate that absence of DJ-1 expression 1) decreases motor functions and 2)alters dopamine function in the nigrostriatal pathway. The DJ-1 knockout mice may provide auseful platform for testing gene therapeutic strategies for patients carrying deletion mutationsand offer opportunities to study the function of DJ-1 in oxidative stress.

PINK1 (PARK6)

Analysis of the PARK6 locus on chromosome 1 [43] led to the identification of point mutationsin the PINK1 gene, a putative mitochondrial kinase [42]. Mutations in PINK1 are the secondmost frequently occurring cause of autosomal recessively inherited early-onset PD [6,16, 20,35, 36, 40]. PINK1 is localized throughout the brain and colocalizes to mitochondria where itis thought to prevent mitochondrial dysfunction [12].

Recently, two studies describe knockdown [47] and knockout [25] of PINK1 gene in mice. First, Zhou *et al*. [47] used RNAi and the Cre-loxP system to induce expression of a PINK1 shRNA in the presence of Cre. Using CMV-Cre transgenic animals crossed with inactive PINK1 shRNAespression, they observed widespread silencing of the PINK1 gene in brain and other tissues. Despite decreased PINK1 mRNA and protein, no change in striatal dopamine,nigral dopaminergic neurons numbers and motor activity (rotarod test) was observed in thePINK1 knockdown mice compared to wild-type mice [47]. In the second study, Kitada*et al*.[24] created a PINK1 knockout mouse by deleting exons 4–7 (kinase domain) and introducinga nonsense mutation starting in exon 8. Mice deficient in PINK1 expression had normal levelsof striatal dopamine and nigral dopaminergic neurons. Similar to observations of DJ-1knockout mice, evoked dopamine overflow in the striatum is reduced in PINK1 knockout mice[25]. Mutations in both DJ-1 and PINK1 genes have been identified in subset of patients withearly onset PD. Biochemical studies suggest DJ-1 stabilizes PINK1 and works cooperativelyto protect cells against oxidative stress [41]. Studies with Drosophilia found that PINK1 mayfunction through a similar pathway as Parkin as well [9, 31] Future studies examining theinteractions of PINK1, Parkin and DJ-1 may lead to the development of a mouse model thatmore closely resembles the pathology of PD.

Transgenic rodent models of Alzheimer's disease



The transgene consists of the human *APP* gene containing a mutation causing a rare form of early-onset familial Alzheimer's disease (Val717Phe). The transgene, whose expression is driven by the platelet-derived growth factor (PDGF) promoter, is microinjected into mouse eggs and implanted in a pseudopregnant female mouse. After the progeny are screened for the presence of the transgene, they are bred and their offspring are analyzed for pathologic features characteristic of Alzheimer's disease. The brains of the transgenic PDAPP (PDGF promoter expressing amyloid precursor protein) mice have abundant -amyloid deposits (made up of the A peptide), dystrophic neurites, activated glia, and overall decreases in synaptic density.