Developmental Biology Midterm 1 Name :
November 12, 2012

1. (15 pts) John Gurdon won the 2012 Nobel Prize in Physiology or Medicine for work he did in the 1960's. What was the major developmental hypothesis he set out to test? What techniques did he development to test this hypothesis? What result of his experiments led to the Nobel Prize?

John Gurdon was testing the hypothesis of genomic equivalence or that when cells divide they retain a full genomic compliment.

Gurdon developed the technique of nuclear transfer. In his first experiments he transplanted nuclei from several developmental stages into an activated enucleated oocyte. Nuclei derived from early blastula stage were the best at supporting normal development to the tadpole stage (80% of the time). Late embryonic and larval nuclei support development to the tadpole stage in less than 1% of the transplantations. These nuclear transplantation experiments supported the hypothesis of Genome Equivalent, but also suggested that the changes occurring in the genome during development and differentiation were difficult to undo. Further nuclear transplantations studies were done with another more primitive frog (Xenopus laevis) that develops at a much faster rate than the leopard frog. In addition, the idea that the egg cytoplasm reprograms the nuclei was further tested by serially transplanting nuclei from somatic cells to eggs, allowing them to divide to the blastula stage, and then transplanting them back to an enucleated egg. This significantly improved the success rate and led to the development of adult frogs from transplanted intestinal nuclei. Nuclei from intestinal epithelial cells transplanted into enucleated Xenopus eggs gave rise to feeding tadpoles in about 1% of the transplantations. But, when serial nuclear transplantations were performed the success rate increased to about 10%.

Gurdon showed that when he transplanted a nucleus from a differentiated cell into an activated enucleated egg that the transplanted nuclei harbored all the genes necessary for supporting normal development. He therefore concluded that cellular differentiation occurs through reversible changes to DNA and that exposure to egg cytoplasm is able to cause nuclear reprogramming of a differentiated somatic cell nucleus.

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2. (10 pts) What 1997 announcement was a direct result of Gurdon's experiments and "shook" society and the world of developmental biology? Why did it take so long? What was the technical breakthrough that made it successful after 30 year of trying by others?

In 1997 Dolly the sheep was the first mammal to be cloned from an adult somatic cell, using the technique of nuclear transfer.

It had taken so long to repeat Gurdons experiments in a mammal because most scientists assumed that using the nucleus from a rapidly dividing cell would work best since nuclei in early embryos normally undergo rapid divisions.

The key breakthrough that allowed for the cloning of dolly was to culture donor mammary cells in a media adjusted to starve the cells into the G_0 stage of the cell cycle prior to transfer.

This technique allowed the donor DNA (mammary cell) to be exposed to the oocyte cytoplasm for a greater period of time and is thought to be important for reprogramming.

3. (20 pts) Shinya Yamanaka shared the 2012 Nobel Prize with John Gurdon. What was the logic of Yamanaka's experiment? Make sure to include a detailed description of his selection technique. What was the outcome of his experiments? How did he prove that he was successful? What are the ethical and medical implications that led to the awarding of the Nobel Prize?

Yamanaka's logic for generating induced pluripotent stem cells (iPSC's) was based on evidence that the developmental state of a differentiated somatic cell could be reprogrammed. This was demonstrated via nuclear transfer and other cell fusion experiments in which embryonic stem cells (ESC's) were fused to adult somatic cells and shown that these ESC's also harbor reprogramming activity. Therefore, leveraging the knowledge that all cells have the same DNA and ESC's have reprogramming abilities, Yamanaka supposed that the forced expression of ESC specific genes in somatic cells might induce them to take on a more embryonic character.

To induce reprogramming Yamanaka co-transduced fibroblasts with retroviral vectors, each carrying a unique cDNA encoding a candidate gene. In total, retroviral vectors encoding 24 genes previously implicated in the biology of ESC's were tested. The retrovirus also encoded the drug resistance gene *neomycin* to allow for positive selection of cells that had incorporated the cDNA sequence. Yamanaka also used an ESC specific promoter (*Fbx15*) to express beta-galactosidase, which will turn cells blue if they start to express embryonic genes.

By repeating this experiment with pools of retroviruses that lacked just one of the 24 candidate genes, Yamanaka identified a set of 4 genes that when introduced together could induce the formation of embryonic stem cell-like colonies (*Oct3/4*, *Sox2*, c-*Myc*, *Klf4*).

Yamanaka used several techniques to demonstrate the pluripotency of iPS cells. He showed that mouse iPS cells were able to form embryoid bodies *in vitro* and teratomas *in vivo*. When injected into mouse blastocysts iPS cells were able to contribute to a variety of distinct cell types, demonstrating their pluripotent developmental potential.

The development of iPS cells does not require the use of fertilized oocytes for the creation of embryonic stem cells and therefore sidesteps the ethical debate over the generation of embryonic stem cells.

In the future, this technique will allow for the derivation iPS cells from easily accessible tissues, like skin cells, which can then be differentiated into various organs for transplantation and bypass issues with rejection. We can also develop cell lines from patients with debilitating diseases by taking a skin cell and generating iPS cell lines for studying that disease.

4. (20 pts) Who developed gene targeting by homologous recombination in the mouse? Briefly explain the technique and its significance for medical research on human diseases. What key contribution did Martin Evans provide that made this technique practical and why? Want was the technical roadblock Evan's discovery got around?

Mario Capecchi and Oliver Smithies independently developed gene targeting by homologous recombination in the mouse.
Embryonic stem cells (ESC's) are grown in culture to high numbers and electroporated to allow for the introduction of a linearized targeting vector. The construct can be specifically targeted in the genome using regions of homology that flank the cDNA that will be inserted in the genome. The general strategy to enrich for cells in which homologous recombination has occurred is known as positive–negative selection. The positive selectable gene, *neomycin* is used to select for recipient cells that have incorporated the targeting vector anywhere in their genomes. The negative selectable gene *HSV-tk* is located at the end of the linearized targeting vector and used to select against cells containing random insertions of the target vector. Cells that insert this construct by homologous recombination are rare, (1/1000) but can easily be selected for and subsequently enriched. These ESC's are then injected into mouse blastocysts and implanted in to a host uterus to generate chimeric mice. To facilitate isolation of the desired progeny, the ESC's and recipient blastocysts are derived from mice with distinguishable coat color alleles. This permits evaluation of the degree of chimerism by coat color chimerism and evaluation of ESC contribution to the formation of the germ line by the coat color of the progeny derived from the chimeric animals.

This technique has allowed scientists to model human development and disease in the mouse by making transgenic animals that can knock-out, overexpress or modify a genes function.

Martin Evans developed a technique to culture embryonic stem cells that were capable of giving rise to germ cells when transplanted to mouse blastocysts. This was important because it provided a more efficient system for targeting and selecting for cells with a single targeted recombinant event, which could then be transmitted to the germ line for the generation of transgenic animals.

The low frequency of homologous recombination relative to random integration of the targeting vector (1/1000) into the recipient cell genome made it impractical to attempt gene targeting directly in one-cell mouse zygotes. Instead, gene targeting in was done in cultured ESC's, from which the relatively rare targeted recombinants could be selected and purified. These purified cells, when subsequently introduced into a preimplantation embryo and allowed to mature in a foster mother, would contribute to the formation of all tissues of the mouse, including the germ line.

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5. (15 pts) Assuming the Nobel Prizing winning techniques that we have discussed become safe and routinely applicable to humans, describe an experimental protocol that would lead to the safe and permanent cure of SCID-X1 without the use of bone marrow extraction or transplantation. (Not a germ cell cure, just curing kids already born.)

Using Yamanaka's findings one could use a skin fibroblast from a SCID-X1 patient and dedifferenetiate the cell back to an induced pluripotent stem cell (iPS cell). Then using Capecchi and Smithies technique of homologous recombination and selection we could replace the defective *interluken-2-receptor* (*il2r*) gene with a functional copy. The cells harboring a functional copy of *il2r* could now be enriched for, and differentiated into hematopoietic stem cells (HSC) for injection back into the patient. This technique would lead to a repopulation of their bone marrow with HSC's harboring a functional copy of *il2r* and cure the individual. This method would also avoid the possibility of rejection routinely seen with organ transplantation.

6. (20 pts) OK, you cured two kids of SCIDs in Question 5. Now they grow up to adulthood, marry and want to have kids of their own. They decide they want to have kids that don't have to deal with SCIDs at all, that is they want a germ line cure. Again, assume the techniques described in recently published papers become safe and routine. Describe your experimental protocol in detail, from beginning to birth of a healthy baby.

Using Yamanaka's technique you could harvest a skin fibroblast from both the father and mother of the baby to be, and dedifferentiate these cells to induced pluripotent stem cells (iPSC's). Then using Capecchi and Smithies technique of homologous recombination and selection we could replace the defective *interluken-2-receptor* (*il2r*) gene with a functional copy. The cells harboring a functional copy of *il2r* could now be enriched for and differentiated into a sperm cell from the father and an oocyte from the mother using the techniques developed by Mitinori Saitou's group. These germ cells would then be fertilized *in vitro* using IVF techniques developed by Robert Edwards and implanted back into the mother's uterus and carried to term. This combination of techniques could theoretically result in a child that carries a normal genetic complement of their parent's genome but has replaced the defective *il2r* gene with a functional copy.