1) You are interested in establishing the beginning and end of the period in the cerebral cortex of the Tasmanian devil (a fierce marsupial found in northeastern Australia) when cerebral cortex neurons are first born and when neurogenesis is complete. You would also like to establish whether the pattern of cell migration and settling is the same in the Tasmanian devil and in mammals such as the ferret and rhesus monkey. Consequently, using bromodeoxyuridine you pulse label Tasmanian devils in utero beginning on embryonic day 24, another litter is pulse-labeled at embryonic day 29, another set of newborns are pulse labeled on the day of birth and a final set of animals are pulse labeled on postnatal day 4. You find the following pattern of bromodeoxyuridine labeled cells when the cortices of these ferrets are examined as young juveniles.

- a) When are cortical layer 6 cells born? **Would be E29**

- b) When are cortical layer 2/3 cells born? **On the day of birth PO**
c) Which cells have the longest migration path? The cells born on P0

You compare your pulse labeling pattern with that of ferrets pulsed at comparable stages of development.

d) You find that young juvenile ferrets have no labeled cells above layer 2/3 or below layer 6 when pulsed at a stage comparable to E24 in the tasmanian devils. However you are surprised to find when you examine younger (P6) ferrets pulsed at the “E24” stage that they show the same bilayer pattern of expression as the tasmanian devil. Can you suggest an explanation for what is going on between the P6 and young juvenile stage in the ferret cerebral cortex that is not going on in the young tasmanian devils cortex?

One of 2 things either the top and bottom cells are dying between P6 and the young juvenile or they start to divide in early postnatal life and dilute out their label.

e) Suggest an experiment that would test your hypothesis.

If they started to divide between P6 and the young juvenile a pulse given at say P12 should produce some heavily labeled cell wen the cortex is examined a day later. If cells were undergoing programmed cell death they might express vertebrate orhologs of c elegans cell death genes.

f) Would you have detected the bilayer pattern of cell genesis after and E24 pulse of BrdU if you had used a retrovirus with a reporter gene to label a few progenitors at E24? No because the retrovirus will label the progeny of dividing cells but because it will not become diluted out with subsequent cell division all progeny of the stem cell will be labeled not just those cell that leave the mitotic cycle soon after labeling.

2)a) In the Bixby et al paper that you read what two characteristics of the NCSCs were the investigators dealing with?

Age and location within the embryo’s body

b) Which of these characterisitcs were they manipulating?

Position or location in which differentiation takes place and level of factors that may be involved in biasing the multipotent stem cells to favor one differentiation pathway over another

c) Why do they subclone from the NCSCs they have isolated bt cell sorting.

To assure that the progeny of these cells can themselves produce a range of neural crest derived cell types .ie to prove that the NCSCs are truly multipotential.

The use of antibody markers for a particular cell type is critical to many experiments in development
d) What markers did Bixby et al use to identify post-migratory neural crest stem cells?
Neurotrophin receptor p75 positive (p75+); myelin basic protein negative (P0-); a4 integrin.

e) What markers did they used to show differentiation into a neuron phenotype?
Mash-1 and neuron-specific B-tubulin

3) In mammals the germ line cells arise from the extra-embryonic endoderm and migrate while still dividing to the gonadal ridge where the cells begin to undergo meiosis and assume the phenotypes of either male or female gametes. Using experimental paradigms similar to those in Bixby et al how might you determine if the male or the female gonadal ridge provides a permissive or instructive environment for germ cells that had recently migrated to the ridge. You can assume that you have available for this experiment a mouse line in which all cells express the green fluorescent protein.

You can karyotype cells and determine by their sex chromosomes whether they are male or female. Dissect out the gonadal ridge with germ line stem cells from a GFP donor male mouse and inject them into the gonadal region of host female mice. Allow the embryos to develop for several days and then histologically examine the host gonad determine whether the green donor cells differentiate as oogonia or spermatogonia. Then perform the converse experiment with green stem cells from a female into the gonadal ridge region of a male host. If in both these experiments the germ cell differentiation according to the phenotype of the host then the gonad is instructing the phenotype of the germ cells. If you never get green donor cells differentiating into oogonia or sperm outside of the gonad but the gamete phenotype is always that of the donor animal then the gonad is simply permissive. In fact the former case has been shown to be true it is the genotype of the gonad that determines whether sperm or eggs will differentiate.

4) There are four mouse FGFs (4, 8, 9, 17) known to be expressed in the apical epidermal ridge. Only FGF 8 is expressed throughout the AER and is expressed earlier than the other genes. FGF10 on the other hand is expressed throughout the lateral plate mesoderm and becomes restricted to hindlimb and forelimb lateral plate mesoderm immediately before the appearance of limb buds. When a bead soaked in FGF8 is inserted into the lateral plate mesoderm in the trunk region FGF10 also becomes restricted to the region surrounding the bead and an ectopic limb bud begins to form. Thus it was postulated that FGF8 initiates limb bud development by retaining the expression of FGF10 in respective limb regions.

a) Cite a loss of function experiments one could perform to determine if FGF10 is critical to the FGF8 effect on limb bud initiation.

KO FGF by homologous recombination and see if limb buds still form.
b) How would you perform this experiment given data that FGF10 plays a critical role in gastrulation.

**Use a cre/lox slicing system in which the cre-recombinase removes the floxed FGF10 gene from the lateral plate mesoderm after gastrulation takes place.**

c) The regulatory region of what gene might one used to to activate the cre recombinase in LPM?

**The regulatory region of one of the TBX transcription factors either TBX 4 or 5 might be useful for this purpose.**

Recent evidence has shown that Wnt signaling may play multiple roles in limb development and that early Wnt signalling may be critical to the initiation of limb formation. A retroviral vector has been used to express activated B-catenin in the chick embryo interlimb region and to produce an ectopic limb.

d) How might you determine if the Wnt pathway was functioning upstream or downstream of FGF10.

**Determine if FGF10 expression is maintained in the interlimb region by the activated catenin expressing cells.**

**Overexpress a b-catenin down-regulator like GSK3B via a retrovirus vector in the normal hindlimb or forelimb region and determine if limb bud formation is blocked.**

5) A very powerful but technically difficult technique of creating conditional mutations is now being used to investigate the later developmental role of vertebrate genes that cannot simply be “knocked out” because they are crucial to successful completion of earlier developmental stages. The technique involves placing splicing sites for a recombinase (either flip or cre) in the regulatory region of, or surrounding critical exons of a gene of interest. Consequently, when the appropriate recombinase is expressed at the time or in the tissues of interest, transcription of the particular gene you are interested in stops because a critical region of the gene or its regulatory region is “spliced” out. In order to accomplish this conditional KO the recombinase that does the splicing has to have high and selective expression at the time and ideally only in the cells where you want to KO the gene. Consequently, you must first make a transgenic animal using a positive regulatory region of a second gene that is coexpressed with your gene of interest at the time of interest to drive recombinase expression in those cells.

**BMP4 is a gene of considerable interest in limb bud development since it may be a mediator of shh polarizing activity. However, BMP4 is also very important in early axial patterning.**

a) Using what you know about the molecules involved in the autonomous development of the limb bud can you design a cre or flip transgenic mouse that could be bred with a mouse line carrying splicing sites that would allow you to knockout BMP4 in the region adjacent to the zone of polarizing activity?
You could put the cre recombinase with a powerful promoter under the control of the Hox gene regulatory sequences for one of the Hox genes that is expressed for a relatively long duration in the founder cells laid down by the progress zone say Hox gene 9 or 10. The choice of a regulatory sequence for a gene that is not expressed at critical early times or positions is important. This expression of Cre recombinase should splice out BMP4 in the founder cells expressing the Hox gene and if BMP4 is critical to the polarizing effects of the ZPA, anterior posterior patterning should be abnormal in the region expressing Hox 10 for example.

b) What important control would you have to perform to make sure your recombinase transgenic has the appropriate expression pattern?

You would have to check for appropriately high levels of Cre activity in the expected cells perhaps by breeding your Cre mouse to the “Cre” reporter mouse described in the answer to question 8 below. Note localization of Cre transcript would not be sufficient because Cre protein may be inactivated or degraded rapidly in some cells.

c) How might you check to make sure that the expected KO has occurred at the correct time or in the correct place so that you can attribute any phenotypic change to loss of BMP4 function in the limb bud?

You would perform in situ hybridization for BMP4 transcript on limb buds over the interval in which you expect BMP4 transcription to stop.

6) In the developing vertebrate, cells within rhombomeres form tight borders and will not cross those borders once they are specified. One possible mechanism for this border maintenance is through repulsive interactions between Eph Receptors and the ephrin ligands, as described in class for axon pathfinding. EphA4 (an Eph receptor) is expressed in rhombomeres r3/r5, while ephrinB is expressed in rhombomeres r2/r4/r6.

EphA4 expressed in Cell on r3 boundary
ephrinB expressed in Cell on r4 boundary

You decide to do experiments in zebrafish to test whether Eph signaling is important for cell sorting into specific rhombomeres.
If Eph signaling is important for maintenance of these boundaries, what might you expect to see if you expressed truncated EphA4 in rhombomeres r3/r5 (it no longer has a cytoplasmic domain, only the extracellular domain)?

**Truncate EphA4 would act as a dominant negative (it can bind ephrinB but nothing happens in the receiving cell) and these cells will not necessarily stay in r3/r5. If you stain for these cells you will find them in all of the rhombomeres.**

If full length ephrinB is expressed at random in a zebrafish embryo, all cells that express it in r3/r5 are found at the borders, while in r2/r4/r6 they can be seen throughout the rhombomere (black staining is ephrin B, white arrows show staining in even rhombomeres and black arrows show staining in odd rhomboeres):

What does this suggest about the role of ephrinB in cell sorting? Describe two alternatives you might expect if you expressed truncated ephrinB (ephrinB has extracellular and cytoplasmic domains). Explain what each might mean for Eph/ephrin signaling.

**This result suggests that there is signaling downstream of ephrinB as well as the Eph receptor, as ephrin B expressing cells try to sort out to be near other ephrinB cells. If you expressed truncated ephrinB, the cells might be found everywhere, instead of at the borders, which would support signaing downstream of ephrinB. If expressing truncated ephrinB had the same effect as expressing full length ephrinB, in that those cells in r3/r5 are still found at the borders, it would suggest that signaling downstream of ephrinB is not necessary for cell sorting.**