Work 2. Mouse - cell lineage reporters, embryology

Introduction

During development, different cell types and tissues are formed under genetic regulation. This regulation manifests in a differential gene expression, that can be analyzed at the level of mRNA or protein. The differential gene expression in certain cell types can also serve as a proxy to track the cell type of interest in the tissue or in the developmental time. In this case, the cell type specific gene can be called a *marker gene*. During development, genes can also be turned off. For understanding the developmental history of cell lineages, in an embryo or a renewing organ, it is highly useful to track cells that once expressed a specific lineage-defining gene. For this, somatic DNA recombination can be used to leave a permanent label in the genome of a cell and its descendants.

The gene products can be detected in tissue using probes against the transcribed RNA molecules as in the case of in situ mRNA hybridization (ISH) method, or by using antibodies that bind specific epitopes on the proteins or other molecules of interest, as in the case of immunohistochemistry (IHC). These methods allow analyses of tissue distribution of gene expression, but they are limited to a small number of known target gene products. To analyse cell-specific gene expression in a genome wide manner, new RNA-sequencing based methods have recently been developed.

Aims and workflow

In this work, we will learn how **site-specific recombinase** driver and reporter mouse lines can be used to mark specific regions of an embryo, to study developmental origins of cell types, and how **single cell mRNA sequencing (scRNAseq**) can be used to characterize cell types in a tissue.
In this project, we will focus on developing **serotonin (5-hydroxytryptamine, 5-HT) neurons** in the anterior brainstem. These neurons are highly important for regulation of mood and behavior, and are targets of the most widely used anti-depressant medications. Your aim is to find:

1. **Where do the serotonergic neurons come from during brain development?**
2. **What are the gene products the embryonic serotonergic neuron precursors express?**

The experimental work includes 3 parts:

1, Cell lineage reporters: collection and phenotyping of reporter mouse embryos
We will dissect the mouse embryos from uterus and select the embryos having a desired Cre-recombinase driver and Cre-reporter genotype based on the expression of fluorescent markers.

2, Cell dissociation
We will use the cell dissociation protocol suitable for sample preparation for scRNAseq on 10xGenomics Chromium platform. We will use reporter mouse embryos (if available).

3, scRNAseq: analysis of gene expression in single cells (Work 3)
We will analyse scRNAseq data from the embryonic anterior brainstem in order to find the serotonergic cell groups and analyse the gene products they express.

1.1. Cell lineage reporters

Background

Reporter mouse lines

In addition to the detection of native gene products, it is also possible to use genome engineering to label the molecules or cell types of interest genetically. A large number of reporter mouse lines have been developed, directing reporter gene expression in specific cellular compartments, cell types or tissues. Genetic tools are also available for temporally controlled genetic manipulation, such as the activation of the reporter expression upon drug administration, or for optogenetic cell manipulation, where enzymes or other markers are activated by specific wavelength of light. Reporter mouse lines greatly facilitate the detection of gene products and cell types in mouse tissues, are useful for cell lineage tracing, and importantly, enable in vivo detection of gene expression or activity.

Cre-loxP system for genetic engineering in mouse

One of the most widespread genetic tools in mouse is the Cre-loxP system. This technique is based on the insertion of short target sequences (34-bp *loxP* sequences) of a site-specific DNA recombinase *Cre* into the mouse genome. Upon expression, the Cre-recombinase recognizes the *loxP* sequences, and mediates a recombination between the *loxP* . The outcome: deletion or inversion of the DNA flanked by the *loxP* sequences *('floxed'* DNA) - depending on the orientation of the *loxP* sequences.

Depending on the placement of *loxP* sequences, it is possible to inactivate or activate genes. Cre-LoxP system allows conditional mutagenesis: spatially and/or temporally controlled gene inactivation. For this aim, Cre-recombinase sequence is typically inserted downstream of a promoter with desired spatial or temporal activity. The choice of a promoter then defines the specificity of Cre expression and enables for example tissue- or cell type-specific recombination. In the target gene to be inactivated, the LoxP sites are placed around an important region, that will be deleted by Cre-recombinase activity.

The Cre-LoxP system is also a great tool for cell lineage tracing. For this, Cre -expressing mouse lines are combined with reporter lines, where the expression of an easily detectable reporter gene is

activated by the Cre-mediated recombination. Common reporter genes are for example *LacZ* (β-galactosidase), *GFP* (green fluorescent protein) or *RFP* (red fluorescent protein). One of the most widely used genomic loci for the insertion of reporter genes is the constitutively active mouse *Rosa26* locus *(R26)*. The reporter lines also are tools for validation of the recombinase activity of the Cre-driver lines, that then can be used in other applications (such as conditional mutagenesis).

As the *R26* locus is active in every single cell of the mouse, Cre-dependent activation of a reporter gene in this locus results in the permanent reporter expression in the recombined cells and their descendants (i.e. it will not fade after the possible silencing of the promoter driving Cre expression). This is useful for example in cases where Cre has been inserted under the promoter of a gene that is expressed early in development, but downregulated later. In this case, the permanent reporter will allow lineage tracing of all the cells that develop from the cells that once expressed Cre.

In this work, we will use different Cre-expressing mouse strains and analyze the recombination patterns in the reporter mouse *R26* ^{tdTomato} (**Fig.1**) *R26* ^{tdTomato} mice carry a variant of the RFP gene after a transcriptional stop-sequence, that in turn is flanked by *loxP*-sequences. When Cre is not present, RFP is silent. Upon Cre-mediated recombination, floxed stop-sequence is removed, and RFP is expressed.

In the fresh tissue, the activated fluorescent protein expression can be directly detected under fluorescent light. In fixed tissue, the expression of GFP and RFP can be detected indirectly, with specific antibodies. With indirect detection, co-labelling for other proteins or features of interest is possible. The mouse lines used in this work are *En1*^{Cre} and *Pax7*^{Cre}.

En1 and Pax7 are transcription factors expressed early in specific regions in the neural tube (*where*?). When combined with the reporter allele (*R26* ^{tdTomato}*)*, we should see the reporter gene activation in the domains and cells corresponding to the expression pattern of the respective driver.

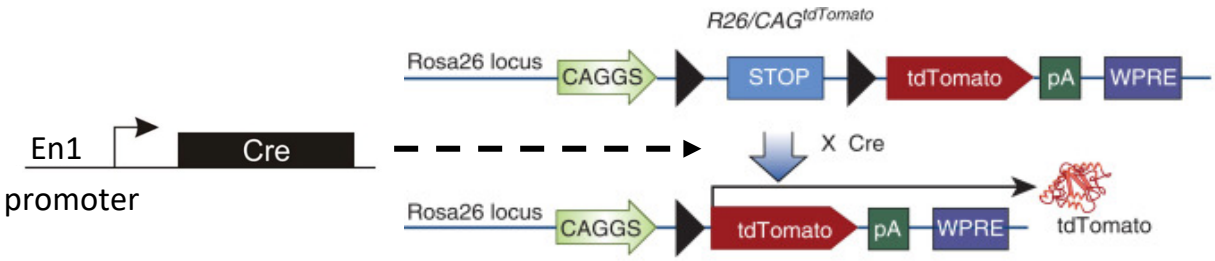

Figure 1. Expected recombination patterns in the *En1*^{Cre}*; R26*^{tdTomato} reporter mouse line

Cre is inserted under the promoter sequences of the gene of interest (*Engrailed-1, En1*). RFP coding sequence is inserted into the R26 locus.
Cells, where En1 promoter is not active, do not express *Cre.* RFP is not produced from the reporter allele.
Cells, where En1 promoter is active, express *Cre.* RFP is produced from the reporter allele.

Practical work: dissecting embryos and collecting genotyping samples

Day 1.

Materials for the dissection

- Ice in a styrofoam box
- Petri dishes filled with sterile 1x PBS (keep on ice)
- 70% ethanol in a spray bottle
- Forceps, scissors – cleaned with 70% ethanol and dried
- A small spoon or a disposable plastic pipette with the end cut off (for transferring the embryos)

Fixation

- 4 % PFA (paraformaldehyde) in 1 x PBS
- 12- or 24 well plates

  NB! PFA solution is a fixative and thus harmful to all living cells. **Avoid inhaling PFA** and keep the plates, PFA bottles and tubes closed when not pipetting from them. You can also use the fume hood for the PFA handling steps!

Mice

Pregnant females (stages between E9.5 - E14.5) from the following crosses:

male: *En1*$^{Cre/+}$ x female: *R26R*$^{tdT/tdT}$

male: *En1*$^{Cre/+}$*;R26R*$^{tdT+}$ x female: *wild-type (WT)*

male: *Pax7*$^{Cre/+}$ x female: *R26R*$^{tdT/tdT}$

Work protocol I: dissection of embryos

1. Dissect embryos as demonstrated. Be careful, they are very fragile! Place each embryo in a separate well of 24-well plate in cold 1xPBS. Mark the wells, for example with numbers and your group code.
2. Tissue piece such as tail of the embryo or yolk sac can be collected for genotyping the embryos. Genotyping sample should be collected with clean, sterile forceps and placed in a sterile 1.5ml Eppendorf tube. It is important to clean the scissors/forceps (e.g. wiping with 70% ethanol) before collecting every genotyping sample, to avoid cross-contamination.
3. After all embryos are dissected, inspect them for the fluorescent protein expression under the microscope. Check the embryos for RFP expression and write down the results. Take pictures of both RFP -positive and negative embryos.
4. Return to the course lab and place all the embryos in 4% PFA for fixation. Leave the embryos in the fixative in RT with a gentle shaking, the plates covered with tin foil.
   After 1-2 days fixation in 4%PFA, fixed samples can be further processed for gene expression analyses (whole-mount IHC, ISH or dehydration and embedding for longer term storage and the histochemistry, ISH, or IHC on paraffin or cryosections, for example).

Cell dissociation (suitable for scRNAseq)

Day 2.

Prepare for the dissection and dissect the embryos as in the previous day. Fixation will not be used today. Today, we will dissect rhombomere 1 area of the neural tube and dissociate the live cells.

1. Before starting the dissection, set up a water bath to 37C. Prepare the papain mixture, by adding 5 ml of EBSS to the papain vial. Warm the papain solution on the water bath for 10 minutes, then store at RT (room temperature, 23±3 °C) until use.

2. Dissect the embryos in 1x PBS. Transfer to a plate with Neurobasal supplemented with 5% FCS (Fetal calf serum). Extract the rhombomere 1 piece and transfer it into a clean Eppendorf tube with 100 µl of EBSS (Earl's Balanced Salt Solution). Wash once in EBSS. Store the tube on ice until the next step.

3. Prepare the Ovomucoid protease inhibitor, DNAse I and the dissociation solutions. For dissociation solution, add 500 µl of EBSS into the DNAse I vial. After DNAse is dissolved, add 250 µl of the DNAse solution to the papain vial. For Ovomucoid inhibitor solution, mix 2.7 ml EBSS, 300 µl of Ovomucoid inhibitor, and 150 µl of DNAse I in 15 ml Falcon tube. Store at RT.

4. Remove the EBSS and add 500 µl of dissociation solution to the tissue piece. Mix by pipetting 3-5 x and place the sample on the water bath for 30 minutes.

5. Trituration: using a glass pipette, aspirate all the solution into the capillary for 5-10 times. The tissue piece should disintegrate. After trituration, no tissue pieces should be visible.

6. Spin at 300 x g for 4 minutes to pellet the cells. Remove supernatant and add 500 µl of ovomucoid protease inhibitor solution. Resuspend the pellet gently.

7. Spin at 300 x g for 4 minutes. Remove supernatant and add 500 µl of EBSS+0,04%BSA solution. Resuspend the pellet gently. Filter through a 40 µm filter.

8. Spin at 300 x g for 4 minutes to collect the cells. Remove supernatant and add 100 µl of EBSS+0,04%BSA. Inspect the cells under the microscope. Viable cells are round, with clearly visible cell membrane and round nucleus. Membrane wrinkling, cell or nucleus disintegration and many darkly coloured, grayish cells in the sample are signs of cell death.

   In case of fluorescent reporters, the reporter expressing cells can be detected under fluorescence microscope. However, the cells are encapsulated randomly in the 10xGenomics Chromium chips and are not imaged after encapsulation.

Test - Labwork 2

2.1. Identify the main regions of the developing central nervous system (forebrain, midbrain, hindbrain). Where do you see the RFP/GFP signal?

2.2. Does the pattern of expression of the reporter gene match the expression of the gene driving Cre expression (for example En1 gene expression at E12.5, please use MGI resources for this)? Why this may not always be the case?

2.3. Were all the embryos positive for the reporter protein expression? Does the observed number of fluorescent embryos match the frequencies expected by Mendelian genetics?

2.4. In single cell suspension, were all cells positive for the reporter expression? Do you expect the serotonergic neurons to be negative or positive for reporter expression when using En1-Cre driver? What about when using Pax7-Cre?