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NON-TECHNICAL SUMMARY

Cell competition and the dynamics of tumour development in epithelial tissues

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Epithelial tissues, Development, Maintenance, Repair, Cancer

Animal types	Life stages
Mice	adult, embryo, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To define the mechanisms that regulate stem and progenitor cell fate in epithelial tissues, and how these programmes become subverted during tumour initiation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In cancer, tumours develop through a multi-stage Darwinian-like process, evolving from a phase of neoplasia (i.e. abnormal growth of tissue) to invasive carcinoma (where tumour cells infiltrate into underlying tissue) and metastatic transformation (where cancer spreads to different parts of the body). Entry into this programme is often preceded by phase of “field cancerization” in which the acquisition of mutations in certain cancer genes, known as driver genes, enable cells to outcompete their neighbours, expanding the pool of cells on which further damaging mutations can impact. To resolve the biochemical pathways that lead to cancer formation, emphasis has been placed on resolving the mutational landscape of tumour types – the “cancer genome”. However, while the repertoire of cancer genes is becoming increasingly well-characterized, the impact of mutations on the behaviour of individual cells – notably, how they balance cell proliferation through division and differentiation in functional cell types – remains underexplored. To probe the earliest stages of tumour formation, methods based on the use of transgenic animal models have been developed to trace the lineages of mutant cells and their daughter cell progeny -known as “clones”- following the activation of mutations in specific cancer-associated genes (Sánchez-Danés et al., 2016). However, current cell labelling strategies struggle to resolve how the fate of neighbouring wild type cells are influenced by the activation of mutations in neighbours, and how these changes can drive field cancerization and cancer progression, with consequences for our understanding of early cancer-detection and risk management strategies.

To understand the mechanisms that drive tumour development, there is a pressing need to understand the pathways that regulate cell fate decision making in healthy tissues, and how these programmes become subverted during the earliest stages of tumour initiation and progression.

What outputs do you think you will see at the end of this project?

This project will provide insights into the cellular and biomolecular mechanisms that regulate cell fate decision-making in the epithelial lining of the mouse gastrointestinal tract (including the small intestine, colon and stomach), skin epidermis and oesophagus, both under normal healthy conditions and following the activation of mutations in specific cancer-associated genes.

Using a genetic cell lineage tracing strategy developed by our lab – known as the red2cDNA series – we will quantify at single-cell resolution the fate behaviour of mutant epithelial cells (marked by a red fluorescent reporter gene) as well as wild type cells (marked by a fluorescent reporter of a different colour). This will allow us to study in parallel the clonal evolution of mutant epithelial cells and the effect they have on the clonal dynamics of neighbouring wild type cells as well as the surrounding tissue. By combining this approach with gene expression profiling, this novel experimental design will allow us to:

- resolve both the cellular and biomolecular basis of epithelial cell fate decision-making during the development, maintenance and repair of healthy epithelial tissues;
- establish a new standard in the study of the mechanisms of pre-neoplastic transformation and field cancerization in columnar and epithelial tissues, which can inform new strategies of early cancer-detection and risk management;
- provide an exemplar for how quantitative modelling-based approaches can impact on cancer research.

Our novel red2cDNA mouse models will provide a valuable and versatile resource that can be readily generalised to the study of other tissue types and cancer-associated mutations, and used by other groups for the identification of novel biological markers, testing immuno-detection methods, intravital imaging as well as investigating other cancer-related biological process, such as metabolism and inflammation.

Who or what will benefit from these outputs, and how?

The outputs of the proposed research will have immediate impact on the research community through publications, resources and conference presentations. In the medium term, all transgenic mouse lines generated through this project will be made available to the research community, where they can be used to study mechanisms of epithelial cell fate in healthy and diseased conditions, and readily adapted and applied to other tissue types not considered in this programme. In the longer term, this research is expected to provide potential actionable targets for use in regenerative medicine, as well as early cancer diagnostics and therapeutics.

How will you look to maximise the outputs of this work?

As a collaborative group, we will remain alert to opportunities to extend our work by sharing our material and intellectual resources. In particular, we will be ready to share our transgenic mouse models, building collaborative partnerships with researchers whose interests extend to other tissue types, or with technological skills (such as intravital imaging) not yet available to our laboratory. Even within the scope of the current project, lineage tracing studies of transgenic mouse models may sometimes provide data associated with other tissue types. In this case, we will readily share tissue samples and data with researchers who have expertise and interest in these areas.

Species and numbers of animals expected to be used

- Mice: 23950

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The activation of cancer-associated DNA mutations can have a dramatic effect on the fate behaviour of epithelial cells, allowing mutant cells to outcompete their non-mutant (wild type) neighbours during tissue turnover, creating pre-neoplastic lesions that can undergo subsequent transformation to more aggressive phases of disease. These changes in cell fate may be driven by direct crosstalk between neighbouring mutant and wild type cells, or they may be mediated by mutation-driven changes to the local tissue environment. To develop a comprehensive knowledge and understanding of the factors that drive epithelial cell competition during the earliest phases of tumour development, it is necessary to study cell fate behaviour in their native tissue environment, making animal studies unavoidable.

While some insights can be obtained from in vitro culture systems, the knowledge accumulated from the investigation of the mouse is incomparable. The mouse is the most appropriate animal model for the proposed studies since: (i) it is a mammal; (ii) its physiology is more extensively characterised than that of other mammalian model species; (iii) mice are amenable to transgenic manipulation allowing oncogenic mutations to be conditionally induced; and (iv) a large number of relevant transgenic and gene knock-out lines are already available. The experiments detailed here will involve creating and analysing transgenic mice, and are classified as mild to moderate with respect to potential discomfort, stress or suffering.

To contrast changes in cell competition during phases of development, we will study the action of oncogenic mutations in epithelial tissues in embryonic, postnatal (0 to 3 months) and adult (3 to 15 months) mouse tissues. As injury-induced cellular plasticity is considered as a key driver of pre-neoplasia, we will also study the effect of oncogenic mutations on cell fate behaviour during mild tissue regeneration models.

Typically, what will be done to an animal used in your project?

The majority of animal experiments involve the activation of DNA mutations in cancer-associated genes targeted on specific epithelial cell populations using genetically-engineered mouse models. To activate such oncogenic mutations coupled to fluorescent reporter genes, we will use drug-inducing agents (such as Tamoxifen) administered orally or delivered by injection. Animals will then be allowed to age before being killed and tissues collected at various time points following cell labelling, allowing the size and cell composition of mutant cells and their progeny –clones – to be quantified and compared to those of neighbouring non-mutant (normal) clones. To study cellular dynamics in the embryo, pregnant females will be treated with the same drug-inducing agent, administered orally or delivered by injection, and pups recovered and killed before delivery. In studies of postnatal development and adult, mice will be treated with the same drug-inducing agent, administered orally or delivered by injection, and tissue collected over a minimal range of time points, from days and weeks to months and up to one year post-induction.

In some experiments involving adult mice (3 months of age), two drug treatments may be required, one to activate the oncogenic mutation and fluorescent reporter gene, and another to induce mild tissue "damage" in an injury-like model involving the targeted genetic ablation of specific cell types resulting in the rapid regeneration of tissue. The mice will then be aged, killed and tissues collected at various time points.

In other injury models involving adult mice (3 months of age), the activation of the oncogenic mutation and fluorescent reporter gene following treatment by a drug-inducing agent will be coupled with the administration of a chemical agent or an endoscopic biopsy to create mild tissue damage resulting in rapid tissue regeneration. The mice will then be aged, killed and tissues collected at various time points.

What are the expected impacts and/or adverse effects for the animals during your project?

To study the fate decisions made by cells during the normal development and maintenance of epithelia, including tissues of the gastrointestinal tract, skin epidermis and oesophagus, we will use the administration of a drug-inducing agent to induce the expression of fluorescent marker genes in cells. Although oral administration of the drug is our favoured route, consistency in the induction frequency of mutant cells across animals might require the use of intraperitoneal injection. In this case, around 50% of the animals might experience mild discomfort due to the injection, which will last <1 day.

From previous studies of tissue regeneration using the proposed procedures, we expect adverse effects on the animals to be mild. Once again, if required, we expect that around 50% of the animals may experience mild discomfort due to intraperitoneal injections of the agents that are used to mark cells and/or induce mild tissue damage to elicit a repair response. Since the epithelial tissues repair rapidly and efficiently, any discomfort associated with injury should be mild and should last no longer than 2 days.

To induce the earliest stages of tumour formation, we will make use of genetically modified mouse models that allow oncogenic mutations to be activated in individual cells. We will explore the activation of oncogenes both during tissue development, targeting the embryonic and early postnatal phase, and in adult. The early lesions that form through these studies will be small, leading to, at most, only slight discomfort even when the induction efficiency is relatively high because the tumours will grow in places where they don’t cause pain. We expect that tumours of moderate severity will only develop from lesions in adulthood in around 5% of mice. This might result in more discomfort to the mice because of the tumour size. Animals will be monitored daily, and should tumours develop, the animals will be humanly killed. Based on one or, maximum, two oncogenic mutations, the mouse models used in this study are not expected to reach a stage of metastasis.

Surgical procedures will be carried out aseptically under anaesthesia, and animals may receive analgesics during and after surgery, where possible. All animals will be monitored regularly and, if there are any concerns, animals will be examined and weighed. The majority of animals (>95%) are not expected to show adverse clinical signs. However, some weight loss with or without other clinical signs such as piloerection or hunched posture may be seen. In these cases, if the signs do not resolve within 24 hours or the animal deteriorates, they will be humanely killed.

In all cases, animals will be humanly killed after the experiments, either after induction of a tissue repair response or after generation of tumours. We will analyse the presence of particular phenotypes (e.g. appearance of tumours or the efficiency of tissue repair) by using the molecular, histological or culture techniques.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

93.6% of procedures are expected to result in mild severity and the remaining 6.4% are expected to be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The goal of the project is to understand how mutations in cancer-associated genes can drive cell competition in epithelial tissues, promoting the earliest stages of tumour growth. These changes in cell fate may be mediated by mutation-driven changes to the surrounding tissue – known as the tumour microenvironment. To study the pathways that drive early tumour development, it is important to study mutant cells in their native tissue environment. In this case, animal studies are unavoidable.

Which non-animal alternatives did you consider for use in this project?

Currently, there are excellent in vitro culture models - knowns as “organoids” - of the target epithelial tissues that have been developed for both mouse and human epithelia. Cultures of intestinal epithelial tissues can be grown and sequentially repassaged that recapitulate the glandular organization and pattern of tissue, while stratified cultures of skin interfollicular epidermis and oesophagus can also be maintained over the long-term.

Why were they not suitable?

Despite the promise of organoids, current technologies do not allow such culture systems to be grown and maintained together with supporting stromal and immune cells. As such, they cannot be used to resolve mutation-driven changes in the tumour microenvironment and how these changes may mediate cell competition.

However, where possible, we will make use of organoid culture systems as a preliminary screening platform to identify the potential effects of oncogenic mutations on epithelial cell fate and in follow-up studies to perturb signalling pathways identified by the in vivo analysis of the mouse model. Using this method, we will be able to focus on the targeted effects of candidate signalling pathways that will significantly reduce the number of animals required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Protocol 1: Superovulation. Superovulation is required for generating new transgenic strains, freezing lines, and importing new lines when rederivation is required. We anticipate needing up to 60 mice per year for cryopreservation (300 over 5 years). We will also need to generate at least 10 lines and/or import at least 15-20 different CreERT and floxed allele strains (translating to 350 mice in total). Total of 650 mice.

Protocol 2: Generation of founders, To generate 10 lines for Protocol 1, genetically modified embryos will be implanted into pseudo-pregnant females to produce “founder mice”. We anticipate needing around 60 founders per line, translating to a total of 600 mice.

Protocol 3: Embryo recipient. Embryo recipients are required when thawing embryos from frozen stocks, when generating new strains or when rederiving mice from other facilities. Total of 400 mice over 5 years.

Protocol 4: Vasectomy. For each allele, we will use around 6 males to mate with the females for use in Protocols 1 and 2. For 15 alleles, this translates to a total of 90 mice over 5 years.

Protocol 5: Breeding and maintenance of genetically altered animals. When designing the experiments, we will perform quantitative statistical analysis to ensure that the number of mice used per group is the minimal to be informative. To achieve sufficient statistical power, we will need n=5 mice per group when analysing lineage tracing models and when inducing tissue regeneration response. When using stem cell isolation by Fluorescence-activated cell sorting, it is crucial to obtain a sufficient number of cells to ensure statistical significance. To achieve this, we will need 10 mice for each group at each time point. We will breed a number of different mouse strains carrying transgenic lines, Cre-dependent reporters or floxed alleles (estimated at around 20 different lines). In addition, we will generate additional mouse lines. Some of the mice will be used for tissue harvesting and in vitro experimentation. Therefore, in planning to maintaining 10 lines at any one time and also perform necessary experiments, we estimate a requirement of around 2,000 mice per year, translating to a total of 10,000 mice over 5 years.

Protocol 6: Embryonic epithelial cell behaviour. We require at least 5 mouse embryos per group x2 groups (control and treated condition) x5 different conditions (viz. time of treatment) x4 time points x15 lines = 3,000 mice. For cell isolation experiments, we will need 10 embryos per group x2 groups x4 time points x15 lines = 1,200, translating to a total of 4,200 embryos over 5 years. Considering average number of pups from a pregnant mouse as 5, we require 840 pregnant mice, which also includes spontaneous stillbirth. For titrating the tamoxifen dose within these different mouse lines, we will need 60 more pregnant mice. There may also be circumstances where the status and stage of pregnancy

might be not assessed accurately requiring 100 more mice. We will therefore require 1,000 pregnant mice in total.

Amendment October 2020: Additionally, we will require 5 mouse embryos per group x2 groups (control and treated condition) x3 different conditions (viz. time of treatment) x2 chase time points x5 lines = 300 embryos. For the cell isolation experiments we will need 10 embryos per group x2 groups x2 time points x2 lines = 80 embryos, translating to a total of 380 embryos over 5 years. Considering the average number of pups from a pregnant mouse as 5, we require 80 pregnant mice, which also includes spontaneous stillbirth. Mice will also be harvested after birth. We will need 5 mice per group x2 groups (control and treated condition) x2 different conditions (viz. time of treatment) x4 chase time points x3 lines = 240 pups. Therefore, we will need an additional 240+80 = 320 mice. In total, for this Protocol, we will therefore require 1320 mice.

Protocol 7: Post-natal epithelial cell behaviour. We aim to analyse 5 mice per group x2 groups x3 different conditions (viz. time of treatment) x5 time points x10 lines = 1,500 mice. For the cell isolation experiments we will need 10 mice per group x2 groups x2 time points x10 lines = 400 mice, translating to a total of 1,900 mice over 5 years.

Amendment October 2020: Additionally, we will analyse 5 mice per group x2 groups x3 different conditions (viz. time of treatment) x3 chase time points x5 lines = 450 mice. For the cell isolation experiments, we will require 5 mice per group x2 groups x2 time points x2 lines = 40 mice, translating to a total of 490 mice over 5 years. In total, we require 2390 mice.

Protocol 8: Adult epithelial cell behaviour. We aim to have 2 groups x5 mice per group x6 different conditions (viz. time of treatment) x5 time points x10 lines = 3,000 mice. For cell isolation experiments, we will need 10 mice per group x2 groups x5 time points x10 lines = 1,000 mice, translating to a total of 4,000 mice over 5 years.

Amendment October 2020: We will analyse 2 groups x5 mice per group x2 different conditions (viz. time of treatment) x5 chase time points x5 lines = 500 mice. For the cell isolation experiments, we will require 5 mice per group x2 groups x5 time points x5 lines = 250 mice, translating to a total of 750 mice over 5 years. In total, we require 4750 mice.

Protocol 9: Adult epithelial cell manipulation by signalling pathway specific agent. We aim to have 2 groups x5 mice per group x5 different conditions (viz. different agent) x5 time points x4 lines = 1,000 mice, translating to a total of 1,000 mice over 5 years.

Amendment October 2020: We will require 2 groups x5 mice per group x5 different conditions (viz. different agent) x2 time points x2 lines = 200 mice, translating to a total of 200 mice over 5 years. In total, we require 1200 mice.

Protocol 10: Epithelial regeneration. We aim to have 2 groups x5 mice per group x6 different regenerative conditions x4 time points x5 lines = 1200 mice. For the cell isolation experiments we will need 10 mice per group x2 groups x3 time points x5 lines = 300, translating to a total of 1,500 mice over 5 years.

Protocol 11: Endoscopy and biopsy of colonic epithelium. We aim to have 2 groups x5 mice per group x5 different conditions x4 time points x4 lines = 800. For biopsy, we will need 10 mice per group x2

groups x5 time points x2 lines = 200, translating to a total of 1,000 mice over 5 years.

When designing the experiments, we perform statistical analysis to ensure that we use the minimum number of mice per group that will be informative. A strength of the current approach is its reliance on quantitative modelling-based approaches that allows cell fate behaviour to be inferred from the statistical analysis of clonal ensembles. Within this framework, we use the statistical distribution of clone sizes and compositions, and its evolution over the given time course, to abstract quantitative models of cellular hierarchy and cell fate choice. Statistical approaches based on Bayesian analyses or least-squares measures are used to obtain model parameter estimates with known statistical confidence.

Although the cohort size will depend on the complexity of the underlying cellular dynamics in the appropriate condition, with 50-100 clones per tissue sample, statistical significance can be achieved from a minimal number of mice (n=3-5 per group). Depending on the context and the complexity of fate behaviour (viz. the multiplicity/heterogeneity of cell states and repertoire of cell fate decisions), to trace the fate behaviour of cells in normal mice and mice harbouring oncogenic mutations, we anticipate quantifying at least 3-5 time points per mouse model. Indeed, the ability to abstract precise quantitative information from a minimal number of mice based on statistical clonal ensembles is a signature of our approach and major strength of the programme.

Although the induction frequency may vary between epithelial tissue types, lineage tracing assays based on ubiquitous or, in some cases, targeted-promoters (such as Keratin14 or Lgr5) will allow clonal data to be recovered from multiple tissues from the same animal significantly reducing the required number of mice.

The number of mice needed for isolation of tissue for organoid generation will depend on whether organoids are to be formed from dissociated tissue or from Fluorescence activated cell sorted cells. In general, each organoid isolation would require up to 10 animals from a minimal number of time points and conditions, and 3 separate isolations per genotype would be needed to ensure statistical reproducibility.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When working with different oncogenic Genetically Modified lines, we will conduct a pilot study to assess the phenotype of the oncogenic mutation based on quantitative clonal analysis over a limited number of timepoints. Only those lines that show an interesting phenotype, viz. driving non-neutral cell competition or inducing changes in the tumour microenvironment, will proceed to more in-depth analysis. This quantitative assessment of clonal fate will allow us to reduce considerably the number of mice used (estimated >75% for those lines that do not proceed beyond the pilot study).

The licence holder has a background in theoretical and statistical physics, and is conversant with the necessary mathematical and statistical expertise that will inform the experimental design. Nevertheless, the licence holder is ready to receive and respond to advice from local statisticians.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To reduce the number of breeding pairs, the mice will usually be kept as homozygous, whenever possible; although we are alert to the need to breed from heterozygotes too, to avoid the risk of mutation selection.

As appropriate, we will undertake pilot experiments, particularly with cancer models, seeking training from our experienced collaborators, so we can be assured that we can safely monitor tumour progression to stay within the moderate limit.

Agonists or inhibitors will be pre-screened in pilot experiments to obtain an indication of the dose that is likely to be effective. As we will only use previously-validated compounds, the starting dose will be the minimum to have an effect according to literature (taken to be at least the IC50). Specifically, we will refer to the resource ‘Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. *Morton DB et al. Lab Anim. 2001 Jan;35(1):1-41.*

Tissues other than those that named on the licence might be affected by the candidate gene perturbations since promoters might be expressed in the stem and/or progenitor cell compartments of other organs. To maximise the information from a single animal, we will collect samples from respective organs and share these with other scientists, mitigating the need for further breeding and experimentation.

We will remain alert to any advances that will enable the replacement of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most appropriate animal model for the proposed studies since: (i) it is a mammal; (ii) its physiology is more extensively characterised than that of other mammalian model species; (iii) mice are amenable to transgenic manipulation; and (iv) a large number of relevant transgenic and knock-out lines are already available. The experiments detailed here will involve creating and analysing transgenic mice, and are classified as mild to moderate with respect to potential discomfort, stress or suffering.

Mice will receive tamoxifen, doxycycline or Diptheria toxin administration in order to activate fluorescent reporter genes, oncogenes or to ablate targeted cell populations. To study the dynamics of tissue regeneration in the colon, we will use Dextran Sodium Sulfate treatment at low dose, which induces mild colitis, an inflammation of the inner lining of the colon.

Why can’t you use animals that are less sentient?

Currently, the mouse is the least sentient mammal for which transgenic technologies are available.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of procedures outlined in the project are ones in which the lab has years of experience and expertise. This expertise has allowed us to design experiments that minimise the welfare costs for the animals.

Mice will be maintained in a specific-pathogen free environment in individually ventilated cages. Mice will receive tamoxifen, doxycycline and Diptheria toxin administration, which normally do not harm mice. In cases where timing and dosing is not critical, compound administration such as doxycycline will be supplied in the food or drinking water. If timing and dosing is critical, as is typically the case for tamoxifen administration to induce cells at a defined clonal density, the choice of administration will be optimised against the balance of harm and benefit. Our preferred method will be oral gavage, a method of drug delivery which in our experience has sufficient labelling efficiency with our current genetically altered strains. However, in cases of new genetically altered strains, if the recombination efficiency is not optimal and consistent, we will try alternative routes such as intraperitoneal injection or subcutaneous injection, the pain of which is also considered to be mild. The ability to control the clonal induction rate in the experimental animal by precise dosing will reduce any variability in our quantitative results, which in turn will enable us to reduce total usage of mice. Similarly, alternative routes will be also preferred for labelling agents (such as Bromodeoxyuridine / 5-bromo-2'-deoxyuridine) if the labelling efficiency and timing is not optimal with oral gavage.

Diptheria toxin-mediated specific cell loss in the stomach and intestine may cause local inflammation. However, the affected tissue will heal rapidly without causing any clinical signs. Cell ablation methods based on the pharmacological agents DMP-777/L-635 and 5-FU-mediated cell ablation methods were shown by our lab and others to have a transient effect on the target cells in stomach, and therefore treated mice can remain healthy, without major adverse effects, when used with an appropriate dose. Moreover, tissue-specific and cell-specific ablation by Diptheria toxin administration will result in even milder effects on mice.

Dextran Sodium Sulfate is one of the most well-characterized agents in rodent models of colon epithelial cell regeneration. For each mouse strain, we will refine our model by determining the dose that is sufficient to cause reproducible partial epithelial loss with minimal clinical signs. For example, the general dose of Dextran Sodium Sulfate administration used in the literature is 3% Dextran Sodium Sulfate solution in drinking water. Based on previous studies, we will set this dose as the maximum, while aiming to use a lower dose, if suitable, to address our scientific questions.

For the gene knock-out studies, since we will use inducible conditional alleles, mice should not display a phenotype before induction. To avoid unexpected pain and suffering, animals will be first bred and analysed as heterozygous animals. We will only use well-established reagents and protocols to induce expression or deletion of candidate gene(s). Taken together, the overall harm to mice that can be caused by performing the experimental plan will be minimal.

Additionally, we will use endoscopic biopsy to induce regeneration in the colon, currently the most refined approach available. It represents a significant refinement compared to other techniques, such as carcinogen treatment. All endoscopic biopsy studies will use anaesthesia and peri- and post-operative analgesia as part of the protocol regime, as discussed with the Named Veterinary Surgeon. Surgery will be conducted using the aseptic technique, which meets at the least the standards set out in the Home Office Minimum Standards for Aseptic Surgery.

Amendment October 2020: When appropriate, explant cultures will be used to investigate mechanisms of cell competition. This will minimize the number of procedures that an animal will experience.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For general health monitoring of the animals, we will be guided by the “Working document on a severity assessment framework” prepared by the “National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes”.

We will assess ‘body condition’ of animal as defined by *Ullman-Culleré MH & Foltz CJ, Lab Anim Sci. 1999 Jun; 49(3):319-23.*

To assess the actual severity of our procedures on the mice, we will refer to the Grimace scale published by the NC3Rs (https://www.nc3rs.org.uk/grimacescales), which is itself based on the study by Langford DJ, et al. 2010. Coding of facial expressions of pain in the laboratory mouse. Nature Methods 7(6): 447-449.

For monitoring general health monitoring in the context of tumour burden, we will follow the ‘Guidelines for the welfare and use of animals in cancer research. *Workman P. et al. British Journal of Cancer (2010) 102, 1555–1577’.*

We will conduct surgery using aseptic technique in accordance with the following guidance: *Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2nd Edition 2017. http://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf*

For the breeding of Genetically Altered mice, we will refer to the guidelines provided by the Home Office, detailed in https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf

In estimating the correct dose of chemical agonists or inhibitors, we will refer to the resource: Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Morton DB et al. Lab Anim. 2001 Jan;35(1):1-41.

We will remain alert to any advances that will enable the replacement of animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institution remains a major centre in the fields of developmental and cancer biology, with many labs engaged in animal work and the study of transgenic mouse models. Importantly, there is a close and effective integration of expertise both at the level of academic researchers and the animal facilities, supported by the institutional biomedical services team. This network allows for the dissemination of best practice and information on advances in 3Rs. We are ready to receive and act upon advances as soon as we are made aware of changes in best practice.