Mechanisms driving regeneration and fibrosis.

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Applicant

Mekayla Storer

Primary establishment

University of Cambridge

Additional establishments

None

Introductory details

Introductory details

What's the title of this project?

Focus on your broad aims and use simple language. For example 'Genes and lifestyle influences on brain ageing'.

Mechanisms driving regeneration and fibrosis.

Licence holder

Mekayla Storer

Is this project for higher education and training purposes?

No

Which permissible purposes apply to this project?

- (a) Basic research

Project licence duration

5 Years 0 Months

Which types of animals will be used in this project?

- Mice

Non-technical summary

Aims

What's the aim of this project?

Keep this to a short one or two sentence summary.

We aim to understand how to prevent permanent scarring after serious injury and instead help tissues like the heart, bone, and skin repair themselves naturally. Our goal is to restore these tissues so they work and look as close as possible to their original healthy state.

Why is it important to undertake this work?

Many tissues in the human body, such as the heart, kidney and bones, have only a limited ability to repair themselves after injury. When tissues cannot heal properly, it can lead to serious illness or disability. Fibrosis, or harmful scarring, affects millions of people and contributes to conditions such as chronic kidney disease, liver disease, and heart failure, which is the leading cause of premature death in the UK. As people age, conditions like osteoporosis and slower tissue repair increase the risk of fractures, poor healing, and long-term disability. Current treatments for these problems are often limited and cannot restore normal tissue function. By studying how tissues repair, regenerate, and sometimes fail, this research will generate knowledge that could help develop new treatments to reduce scarring, improve healing, maintain tissue function in older people, and ultimately benefit millions of patients.

Key words that describe this project

Choose up to 5. For example: cancer, stem cells, therapy.

- Regeneration
- Scarring
- Heart Attack
- Bone Fracture
- Digit Tip

Benefits

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

Outputs can include new information, publications, or products.

At the end of this project, we expect to produce new knowledge that will help us better understand how tissues repair themselves, why this ability sometimes fails, and how it links to disease. This work will lead to scientific publications that can be shared with the wider research community, and in the longer term, it may support the development of new treatments.

One key output will be new information on how to reduce harmful scarring (fibrosis). Scarring happens inside the body after many different injuries and illnesses, and it can stop organs, like the heart, from working properly. Our cardiac injury model will help us to see how heart tissue heals and why it often forms too much scar tissue instead of repairing itself well. Understanding this process will point scientists toward new ways of improving healing and protecting heart function.

Another important output will be a better understanding of how bones repair themselves after fractures and why this process is sometimes slow or incomplete. Our bone fracture model will allow us to study how the bone's support structures are built. This will help us to learn why bone healing can fail, especially as we age, and may guide future strategies to help broken bones heal faster and stronger.

We also expect to learn more about why some tissues can repair themselves well, while others cannot. For example, the tip of a mouse digit (the scientific term for finger or toe) can regrow after injury, but this ability fades if the tissue is injured repeatedly, a process similar to ageing. By studying this process, we hope to find out why tissues lose their ability to repair themselves over time. In the long term, this knowledge could help identify new strategies to keep tissues healthier for longer and to slow down age-related decline.

Finally, our research may reveal why tissues that are good at regeneration are also less likely to develop cancer. In many tissues, faulty repair can lead to tumour growth, but in tissues like the fingertip, this does not happen. Understanding how these tissues protect themselves could provide valuable clues for new ways to prevent cancer while encouraging safe tissue repair.

Together, these outputs will provide valuable insights into wound healing, ageing, heart repair, broken bone recovery and cancer. The knowledge gained will be shared through scientific articles and presentations, and in the future, it could support the design of safer and more effective treatments for human disease.

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

The impact of these outputs may be seen in the short-term, or they may not be fully realised until you've completed the project. Consider all timescales in your answer.

This project aims to improve our understanding of how tissues repair themselves, how scarring can be reduced, and how regeneration can occur safely without increasing the risk of cancer. In the short term, the outputs of this work, such as new information about tissue repair and regeneration, will benefit the scientific community. This knowledge will be shared through publications, conference presentations, and collaborations, helping other researchers build on our findings and avoid repeating experiments unnecessarily.

In the longer term, this research could lead to the development of new treatments for a wide range of human diseases. One important area we are studying is the extracellular matrix, which is the scaffolding or support structure that surrounds and holds our cells in place. If we can learn how to control this scaffolding, we may be able to reduce harmful scarring (called fibrosis), which is a big problem in diseases of the kidney, liver and heart. Understanding how regenerative capacity declines with repeated injury may also help develop strategies to maintain tissue repair in aging tissues, potentially improving recovery from injury and degenerative diseases.

Additionally, insights into how certain tissues regenerate without forming cancer could inform new approaches to promote safe tissue repair in organs that are normally prone to tumours. Over time, this knowledge may guide the creation of therapies that enhance regeneration, reduce scarring, and protect against cancer, ultimately benefiting patients with chronic or life-threatening conditions.

Overall, this work will benefit researchers, clinicians, and, ultimately, patients who suffer from fibrosis, age-related tissue degeneration, heart disease, or conditions with a risk of tumour formation. It also has potential wider societal and economic benefits by supporting the development of new regenerative treatments.

Will this work be offered as a service to others?

No

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

For example, collaboration, dissemination of new knowledge, or publication of unsuccessful approaches.

We want to make sure that the knowledge gained from this project benefits as many people as possible, both inside and outside the scientific community. To do this, we will share our results openly. This includes publishing our findings in scientific journals, presenting them at conferences, and running workshops. Importantly, we will also publish results from experiments that do not work as expected, so that other researchers do not waste time or animals repeating the same work.

We will also share our findings with the public in an accessible way, so that people interested in tissue repair, regeneration and disease can learn more about the research and its potential benefits. Wherever possible, results will be shared before or at the same time as formal publication, allowing other scientists to use the information sooner.

Collaboration will be an important part of this project. We will work with other researchers and companies to share knowledge, skills, and techniques, and the tools and models we develop will be made available to others in the field. The data we generate will also be stored in publicly available databases, making it widely accessible to the research community. However, in some circumstances data may have to be kept confidential so that new discoveries can be protected with patents and eventually developed into new therapies.

Project harms

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

We are using mice in this study because their bodies and genes are very similar to humans, which means they react to injury in ways that closely match human responses. For example, the tip of a mouse's toe and the tip of a human finger are made up of the same parts including skin, blood vessels, nerves, and bone, and both can regrow after being injured. This makes mice a very good model for studying how tissues repair themselves, without needing to test directly on humans. Mice are also useful for studying illnesses or damage, like what happens in the heart after a heart attack or in bones after a break, because their tissues respond in a similar way. We are using adult mice because most injuries and diseases we want to study happen in adults, so the results will be more relevant to real-life medical problems. Mice are the least sentient mammals suitable for this kind of research, which means we can

answer important scientific questions while keeping animal suffering as low as possible.

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

For example, injections and surgical procedures. Include any relevant information about the duration of experiments and the number of procedures.

To study regeneration and fibrosis, animals will normally have one of three different surgical procedures. This will be either:

1) a surgery to remove 1-2 mm of the end of the digit, which will fully grow back. In a human this would be the same as removing the tip of your finger starting from midway through your nail. Otherwise, we will remove 3-4 mm of the end of the digit. This type of injury will not grow back but will heal and the mouse will have a shorter digit. In a human this would be the same as removing the tip of your finger up until the first joint. Some animals may have their digits amputated multiple times, and others may have small amounts of tumour cells injected into the toe tip so we can study how repair changes cancer.

2) a surgery on their heart, where a small thread is tied around blood vessels to cause a heart injury. This type of injury will mimic what happens when a person has a heart attack.

3) a surgery on their leg bone, where the bone is broken or drilled to study how it repairs. This type of injury will mimic what happens when a person has an accident and fractures their bone.

All these operations will always be done under general anaesthetic so the animals are asleep and cannot feel pain, and pain relief medicine will be given before the operation and afterwards. A scoring sheet will also be used to help decide when extra pain relief is needed, so the decision is clear and consistent and not based on opinion alone. In addition to surgery, some mice will be given injections of substances, or whenever possible will receive these substances mixed in with their food or water. These may include medicines that switch on or switch off certain genes, substances that label dividing cells, or treatments that may help healing. Mice may also be placed in scanners (like magnetic resonance imaging - MRI, or ultrasound) so we can take pictures of their organs while they are alive. For some scans, the mice may need to have food taken away for a few hours (but they will still have access to water). This short fasting period helps to make the images clearer and more accurate. We will also breed special types of mice that carry changes in their genes (called genetically altered mice). To activate these gene changes, we will inject a drug called tamoxifen into their belly once a day for four days. Sometimes mice will be placed in an incubator at 42^0C for up to 15 minutes. This short warming period helps to turn on certain special genes.

After surgery or other procedures, animals will usually be monitored for up to seven days, but sometimes longer if tissue samples are collected at later time points (up to 2 -3 months). When procedures are repeated, enough time (usually two weeks) will always be left between them to allow the mice to recover properly. Throughout all

experiments, animals will be housed in groups, given pain relief when needed, and carefully checked every day.

At the end of experiments, mice will be humanely killed so we can collect tissue samples that cannot be taken from a live animal. For example, we may take digit samples to study changes in the tissue, such as how cells look under the microscope (histology). These samples are essential for understanding healing and cannot be collected in any other way.

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

Examples can include pain, weight loss, tumours, or abnormal behaviour. State the estimated duration of these effects on an animal.

The mice in this project will be closely monitored, and we will work to reduce any discomfort as much as possible. Many animals will need surgery where the tips of the middle three toes on one or both back feet are removed. Although this is a moderate-severity procedure, regular checks show that this does not stop the mice from walking, moving around, or cleaning themselves normally. Their front feet will never be touched. All surgeries are carried out while the mice are asleep under anaesthetic, and they are given pain medicine so that they do not feel pain during or after the surgery. The mice usually wake up within 10 to 15 minutes and quickly return to normal behaviour. When 3-4 mm of the toe is removed, it does not grow back. There is often some swelling in the toe for 7 - 14 days after surgery. Because this swelling can last for up to two weeks, the mice will continue to receive pain medication for the full period. We will work with the veterinary team to make sure the medicine is the most effective it can be and helps the swelling go down faster. During daily checks, the mice behave normally and do not appear to be in pain.

In some studies, small numbers of tumour cells are placed into the toe. This can cause extra swelling and a tumour that may last for the whole experiment. We carry out daily scoring of the toe, the swelling and the mouse's ability to walk, stand and move around. These checks ensure that the tumour does not stop the mouse from moving normally or carrying out natural behaviours. The tumour cells stay only where they are placed and do not spread to other parts of the body.

Some genetically altered mice will be given a drug called tamoxifen. These mice may lose up to 10% of their body weight in the first one to two days after treatment. They usually regain the lost weight within 5 days and do not show other harmful effects.

In some cases, mice will have more complex surgeries. If we need to make a small bone fracture, the mouse will not be able to put weight on the operated leg immediately afterwards. This usually improves within 3 days. The mice are given pain medicine and are watched closely until they recover. For surgery to cause a heart attack, the risks are much greater. Some of the mice will not survive. Around half of the deaths happen during the operation itself, when the mouse is under anaesthetic and does not feel discomfort. The others may die suddenly at different times after the surgery. When sudden death occurs, the mouse becomes unconscious very quickly and dies within minutes, which means suffering is not prolonged. In humans, heart attacks can cause chest pain, breathing problems,

nausea and other symptoms. We cannot completely rule out the chance that mice may also feel some of these effects. Signs of heart failure in mice may include a blue colour to the skin, swelling in the feet and ankles, and trouble breathing. If these signs appear, the mouse will be put to sleep humanely to prevent further suffering.

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

20% Mild, 70% Moderate and 10% Severe.

Fate of animals

What will happen to animals used in this project?

Select all fates that apply across the whole project. You will be asked later about the fate of animals used in each protocol. Only the fates you select here will be presented as options in the Protocols section.

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

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Replacement

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

We need to use animals to achieve the aims of this project because they provide a living system that is essential for studying how tissues repair, regenerate, and sometimes fail to heal. While cells in a dish or computer models can provide some information, they cannot replicate the complex interactions between different cell types, tissues, and organs, or show how cells behave over time. For example, the mouse digit tip can fully regenerate after amputation, allowing us to study why this ability declines with repeated injury and to identify factors that support tissue repair, a process that occurs over several months and relies on signals from surrounding cells that are difficult to reproduce in a dish. Animals are also needed to study fibrosis, which is harmful scarring that occurs in diseases such as kidney, liver, and heart conditions, because healing involves interactions with the immune system that cannot be replicated properly outside an animal. They are also necessary for studying cancer, as tumour formation depends on interactions between multiple cell types and their surrounding tissues that only occur accurately in a whole organism. Most techniques that work with cells outside living animals do not fully reflect their normal behaviour over long periods, making animal studies essential for understanding these complex biological processes.

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

For our project we thought about different ways to studying regeneration and scarring without using animals. One exciting new method is called “organoids”. These are tiny 3D cell cultures that can be grown from human or mouse stem cells. They act a bit like mini organs such as the brain, kidney, lung, or liver, and can live for a long time in the lab. When looking at the heart, we can also make special heart muscle cells, called cardiomyocytes, from stem cells. These cells are useful because they behave like young heart cells and let us study how the heart works and responds to damage. For bone healing, we can grow bone cells such as osteoblasts, osteoclasts, and stem cells to study how bone is made and repaired. In our lab, we are also developing a non-animal model for digit tip regeneration using donated human embryonic tissue. This ‘ex vivo’ system keeps the tissue alive outside the body so we can watch how it repairs itself. It gives us the chance to study the same kinds of signals and processes that happen in people when fingertips regrow, which makes it a powerful alternative to animal experiments.

Why were they not suitable?

Although non-animal models are exciting and helpful, they are not always enough on their own. For example, scientists have not yet made an organoid that copies the human digit tip. These models also lack blood vessels and immune cells, which are important for guiding regeneration. For the heart, stem cell-derived heart cells behave like very young cells, not adult ones, so they do not show the same limits in healing that adults have. Adult heart cells, on the other hand, do not live long in culture, making them hard to study. For bone healing, cell cultures can teach us how certain bone cells work but they cannot copy the full environment of a broken bone inside a living body, where many types of cells including blood vessels and immune cells interact. In general, cells grown in the lab live in an artificial environment with unusual levels of oxygen and without the many signals they would normally get inside a body. Because of this, these models cannot yet replace animal studies. However, they are still very useful, and we can use them alongside animal models to give us more information while helping reduce the number of animals needed.

Reduction

Enter the estimated number of animals of each type used in this project.

Mice: 6500

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

Do not mention POWER calculations here. If relevant, there will be an opportunity to provide these details elsewhere.

We have carefully planned the number of animals we will need, to make sure we ask for enough to complete the work but do not use more than necessary. To do this, we have spoken with a trained statistician to help us design experiments properly, and we have based our numbers on the amount of work we expect to do each year.

We have also asked experienced researchers for advice and used our own past experience to guide our estimates. When working with genetically altered mice, we have thought about how best to keep the number of different strains as low as possible while still making sure we have enough animals for the experiments.

The natural breeding patterns of the mice we use have been taken into account. For example, some strains normally have smaller litters of around 6–8 pups, while others can have 10–15. To avoid producing more animals than we need, we will also buy in many of the mice from suppliers, as this gives us better control over numbers.

In some cases, we will first run small test studies (called pilot experiments) to find the lowest safe amount of a substance that still has an effect. This helps us plan later experiments with the right number of animals and avoids unnecessary use.

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

You may want to reference online tools (such as the NC3R's Experimental Design Assistant) or any relevant regulatory requirements.

We have carefully designed our experiments to make sure that we use the smallest number of animals possible while still collecting reliable and meaningful results. To support this, we used the NC3Rs Experimental Design Assistant, an online tool that helps researchers plan studies in the most efficient way. We also sought advice from a qualified statistician, who reviewed our plans to make sure the experiments are set up correctly and the data will be interpreted fairly.

Where possible, we have designed experiments to gather the maximum amount of information from each animal. For example, in some studies we can treat digits on one side of the body with a drug, while digits on the opposite side receive a control treatment. In this context, the control refers to an injection containing on the substance in which the drug is dissolved, but without the active drug itself. This allows us to compare the treated and untreated sides within the same animal. Because each animal provides both treated and control samples, fewer animals are needed overall.

We will also make use of non-invasive imaging methods, such as ultrasound and magnetic resonance imaging. These techniques allow us to take images before any treatment is given and then compare these 'before treatment' images with those taken afterwards. In this way, each animal acts as its own control. This reduces the natural variation that exists between animals and means fewer animals are required to detect meaningful changes.

The number of animals in each experimental group has been based on pre-existing data, ensuring we only use what is required. In many cases, especially when using imaging methods, each animal can act as its own control. This approach avoids repeating experiments unnecessarily and helps us keep numbers as low as possible.

Where it is possible, we will also use randomisation and blinding. Randomisation means that animals are assigned to treatment groups by chance rather than by any characteristic of the animal or researcher, which prevents unintentional bias. Blinding means that the person assessing the images or analysing the data does not know

which treatment group each animal belonged to. This avoids expectations influencing the results. Both practices increase the reliability of the findings and reduce the risk of needing to repeat experiments, which helps to keep animal use to a minimum.

We have also followed recognised standards such as the PREPARE guidelines, which give advice on how to plan animal research responsibly. Using these tools and recommendations helps us avoid wasting animals, minimise the numbers required, and ensure that every study provides useful results.

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

This may include efficient breeding, pilot studies, computer modelling, or sharing of tissue.

We have planned several steps to make sure we use the smallest number of animals needed while still carrying out meaningful research.

We will manage our breeding programmes carefully so that animals are not wasted. For genetically modified strains that are not currently in use, we will freeze embryos for future work instead of keeping live colonies. For strains with complex genetic backgrounds, we will plan breeding strategies so that the maximum number of suitable experimental animals and their control littermates are produced from each litter.

Where possible, we will carry out small test studies (pilot experiments). These will help us check the safety and effectiveness of treatments, such as the lowest amount of a substance needed to have an effect, before moving on to larger studies. We will also use information from substances and approaches that have already been tested by other researchers, which avoids unnecessary duplication.

We will increase the amount of information we gain from each animal. For example, non-invasive imaging techniques will allow us to monitor the same animal over time. When tissue is needed, we will divide samples so that one organ can be used for more than one type of test. Wherever possible, tissues will be shared within the research group, reducing the need to use additional animals.

We will also work with other scientists to share tissues, for example from mice used in digit removal studies, so that fewer animals need to be bred overall. In addition, we will stay up to date with new methods that may replace or reduce the use of animals in the future.

Refinement

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.

This project will use mice because they are the least sentient mammals in which heart, bone, and tissue repair can be studied, and their genes can be easily controlled to understand how healing happens. Three models will be used including regrowth of the digit tip, heart attack (called myocardial infarction), and bone fracture.

For **digit tip studies**, the very end of the toe is removed. This area if known to heal well in mice, and depending on how much is removed, the digit can either fully regrow or form a small scar. Care is taken to minimise the impact of this procedure, including the use of anaesthesia, pain relief, and regular monitoring to make sure the mice are comfortable and recovering normally.

For the **heart model**, a controlled heart attack is carried out under full general anaesthesia. This is the best established method for understanding how the heart repairs itself, and it cannot be studied safely in humans. Pain relief is given before and after the procedure, and animals are monitored closely during recovery.

For **bone healing**, a single surgery is used to create a small, controlled break in the thigh bone. This is also done under general anaesthesia, with pain relief and careful follow-up to ensure the animal's well-being.

Across all models, several refinements are used to minimise suffering. These include modern imaging methods, such as CT scans, which help researchers track healing in the same animal over time, reducing the need for extra procedures. Genetically altered mice also help limit interventions because genes can be switched on or off without additional surgery. Together, these refinements ensure that important questions about tissue repair can be answered while keeping discomfort and lasting harm to the lowest possible level.

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

For example, animals at a more immature life stage, species that are less sentient, or animals that have been terminally anaesthetised.

Less sentient species such as worms, fish, or amphibians cannot be used because the way they regenerate is very different from mammals. For example, animals like zebrafish and newts can repair their hearts throughout life using mechanisms and proteins that humans simply do not have. Studying these animals would not give us answers about why regeneration is so limited in adult humans, particularly in the heart and skeleton.

We also cannot use very young animals or animals under terminal anaesthesia. Young animals naturally repair injuries much more easily than adults, so they would not give results that match adult human healing. Animals under terminal anaesthesia are also unsuitable because regeneration and scarring take place slowly over many days or weeks, and these processes cannot occur normally in an animal that is not awake. Long periods of deep anaesthesia would also change how the body works and prevent normal healing from happening. For these reasons, adult mice are the simplest and least sentient mammals that can still model the adult human healing process.

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

I will stay informed about advances in the 3Rs (Replacement, Reduction and Refinement) by regularly consulting trusted resources such as the NC3Rs and Norecopa websites, signing up to the NC3Rs e-newsletter, and attending relevant workshops or events. I will also use my Institute's 3Rs search tool, which provides up-to-date information on ways to reduce animal numbers, replace animal use where possible, and refine techniques to minimise stress. In addition, I will keep up to date with the published scientific literature (in particular consulting the Alternatives to Laboratory Animals Journal) to identify new approaches that could improve animal welfare or reduce reliance on animal experiments.

To ensure that new methods are implemented effectively, I will discuss 3R opportunities in regular meetings with local experts in animal welfare, veterinary matters and information sourcing, and animal care technicians. Where new techniques are identified, I will work with colleagues who already have the expertise to ensure proper training and safe introduction of the method. Any refinements will be trialled first to confirm they do not cause additional harm. This approach ensures that any advances in the 3Rs are rapidly adopted while safeguarding both animal welfare and scientific quality.

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

Potential refinements include increased monitoring, post-operative care, pain management, and training of animals.

All methods in this project will use techniques that reduce animal stress and make sure the animal does not suffer as detailed below:

- **Planning and review:** Before each study begins, we will work closely with the veterinary team to plan the procedures and make sure the care and monitoring of the animals are as refined and safe as possible.
- **Anaesthesia and pain relief:** All surgeries, including digit tip amputations, heart procedures, and bone fractures, will be performed under anaesthesia so that the animals do not feel pain. All procedures will be done using clean techniques in line with Home Office Minimum Standards and LASA guidance (2017). Pain relief will be given before surgery and afterwards if needed. We will always consult the Named Veterinary Surgeon to make sure we are doing this correctly.
- **Monitoring and humane endpoints:** Animals will be carefully observed before and after procedures using health checks and scoring systems. If an animal becomes unwell or shows signs of distress that cannot be relieved, it will be humanely killed. Although a small number of animals may reach a higher level of illness due to the nature of the models, we will take every possible step to recognise problems early and act quickly so that animals do not remain in severe discomfort.

- **Non-invasive techniques:** Where possible, we will use non-invasive methods, such as imaging, to gather scientific information. This reduces the number of procedures and the total number of animals required.
- **Housing and enrichment:** Mice will be kept with other compatible animals and provided with bedding, nesting material, and shelters (such as cardboard tubes or small houses). This encourages natural behaviours, allows them to explore, and gives them a safe place to hide or rest.
- **Acclimatisation and handling:** Animals will have at least seven days to settle into their new surroundings and get used to handlers before experiments begin. We will use gentle handling techniques, such as tunnels and cupping, to reduce stress during procedures.
- **Palatable treatments:** Flavoured drinks, jelly, or spreads will be used to make medications and gene-activating substances easier for animals to take.
- **Least invasive routes and combining procedures:** When injections are required, we will use the least harmful route, the smallest needle possible, and only use the needle once. Where possible, more than one procedure will be carried out under a single anaesthetic to reduce repeated handling and recovery.

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

To ensure that all experiments are carried out to the highest possible standards, we will follow established guidance for planning, reporting, and performing animal research. At the planning stage, we will use the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, and for reporting results we will follow the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

For all surgical procedures, we will follow the Home Office Minimum Standards of Aseptic Surgery and the Laboratory Animal Science Association (LASA) Guidance on Preparing for and Undertaking Aseptic Surgery (2017). For the breeding and use of genetically altered mice, we will follow the Home Office guidelines and the NC3Rs resources on genetically altered mice, including the GAA Framework (2018) and guidance available at the NC3Rs website.

To ensure that substances are administered in the least harmful and most effective way, we will refer to recognised resources, including the BVAAWF/FRAME/RSPCA/UFAW report on refinement of substance administration, the article by Turner et al. (2011) on administration routes, the LASA Procedures with Care guidance, and recent publications on safe administration techniques. Following these guidelines ensures that experiments are conducted consistently, safely, and with the least possible impact on the welfare of the animals.

Applicant information

Experience

Have you managed similar work in this field before?

Yes

What were your, or your group's, main achievements that are relevant to this application?

My current research addresses the broad question of why adult mammals have largely lost the ability to regenerate complex tissues. One of the few exceptions to this general rule is the mammalian digit tip that, following amputation, undergoes remarkable multi-tissue regeneration. Regeneration, however, is level-dependent: removing more than 60% of the end of the digit fails to form a blastema, a transient tissue comprised of progenitor cells responsible for distal digit tip regeneration. Using this tractable mammalian model, my goal has been to identify the mechanisms that determine whether injury stimulates endogenous regeneration or pathological healing and fibrosis. My group has made a number of key achievements relevant to this application including:

1. Defining cell-environment interactions during regeneration. In our recent work, which is currently under review in Science (doi: https://doi.org/10.1101/2024.12.04.626830) we discovered that the physical and molecular environment surrounding injured cells plays a decisive role in determining the outcome of healing. Specifically, successful regeneration requires a soft and flexible extracellular matrix (ECM), which allows cells to respond to pro-regenerative cues. In contrast, a stiff ECM drives the secretion of proteins that promote fibrosis and scar tissue formation. Importantly, we demonstrated that this process can be manipulated whereby introducing the molecule HAPLN1 into non-regenerative injuries softened the ECM and enabled bone regrowth even when regeneration would normally fail. ***Our next objective is to identify ECM components that can be targeted to modulate fibrosis, with the intention of extending these findings to other pathological contexts, including pulmonary fibrosis and post-myocardial infarction remodelling.***

2. Uncovering epigenetic regulation of regeneration. While regenerative capacity declines with age, the digit tip retains this ability in both young and adult rodents (and even in humans). We hypothesized that this persistence might be due to epigenetic regulation, chemical modifications to DNA that control which genes are turned “on” or “off”. Our recent work, currently submitted to Science identified the enzyme PADI4 as a key player in this process. PADI4 is active during regeneration, and its removal resulted in shortened, abnormally shaped digits, demonstrating that it is essential for proper regrowth. Since PADI4 is also known to regulate embryonic stem cells, this finding connects developmental programmes with adult regeneration.

Beyond this, our work sets the stage for understanding why repeated injuries eventually exhaust regenerative potential. ***We are currently exploring whether***

accumulated epigenetic changes over multiple injury cycles may explain the eventual switch from regeneration to scarring and aim to complete this work within the duration of this licence. This work could provide critical insight into tissue resilience and regenerative decline.

What relevant scientific knowledge or education do you have?

I have over 15 years of research experience in developmental biology and tissue regeneration, with the past 10 years dedicated specifically to studying mammalian digit tip regeneration. My work has resulted in 22 international peer-reviewed publications, 13 of which were based on projects using in vivo rodent models. I hold a Bachelor of Biomedical Science with Honours from Griffith University, Australia, where my final year included a year-long laboratory placement that provided my initial training in conducting experiments with laboratory rodents. In 2011, I completed a Master's degree in Biomedicine, followed by a PhD in 2014 at the University of Pompeu Fabra, Spain—both of which involved extensive use of animal models in research.

What experience do you have of using the types of animals and experimental models stated in this licence application?

Throughout my scientific career (beginning in 2005), I have gained extensive experience working with laboratory-based animal models, particularly rodents, which have been central to my research activities. From 2007 to 2010, I worked as a Research Assistant at the Wellcome Sanger Institute, where I undertook rodent-based projects and held a UK personal licence. This period provided me with comprehensive training in experimental procedures, animal handling, and formed a strong foundation for my subsequent independent work.

During my PhD in Spain (2010-2014) and my post-doctoral fellowship in Canada (2015-2020), I continued to work extensively with rodents. In both countries, I completed the required national training programmes and held the equivalent of a UK personal licence, which enabled me to perform procedures independently and to high standards of welfare and compliance. These experiences provided me with broad exposure to different research environments, regulatory systems, and technical approaches.

Since establishing my own laboratory in 2021, I have maintained a UK personal licence and have focused primarily on the mouse digit tip regeneration model. Over the past ten years (from 2015 – present), my research has focused almost exclusively on this system in both neonatal and adult mice. This has enabled me to build considerable specialist expertise in the surgical, imaging, and analytical methodologies required for this model, and to establish it as a core platform for my group's work on tissue regeneration.

What experimental design and data analysis training have you had?

If you do not have this expertise, how will you access it?

I have received comprehensive training in experimental design and data analysis throughout my scientific career. As part of my PhD curriculum, I undertook formal coursework in the statistical analysis of experimental data, which provided a strong foundation in selecting appropriate statistical tests, designing robust experiments, and interpreting results rigorously. Since then, I have maintained a commitment to continued professional development in this area. At the Cambridge Stem Cell Institute, I participate in annual training on data integrity, which reinforces best practices in experimental design, selecting an appropriate cohort size, and statistical analysis. This training ensures I remain current with evolving standards in research reproducibility and data handling. Additionally, I have completed Modules 9, 10, and 11 of the Project Licence Holder training provided by University Biomedical Services (UBS), which further strengthened my understanding of the methodological considerations involved in animal research. Over the past 15 years, I have consistently applied this training in a practical research setting, refining my proficiency through hands-on experimental work.

Why are you the most suitable person to manage this project?

Your role, seniority or expertise in managing projects of this nature may be relevant.

I am well positioned to manage this project as a Group Leader at the Cambridge Stem Cell Institute, where I established my independent research laboratory nearly five years ago (2021). With over ten years of experience in the field of digit tip regeneration, my research has focused on understanding the cellular and molecular mechanisms underlying the regenerative process in mammals. This has enabled me to develop a broad range of technical expertise directly relevant to this project including surgical techniques such as digit tip amputation and injection procedures, as well as management of animal colonies. In addition to my technical capabilities, I have a strong track record in research leadership and supervision. I have successfully guided several MPhil and PhD students through regenerative medicine projects, supporting their scientific development while ensuring that research is conducted to the highest standards. I am fully competent in performing most of the experimental techniques required for this project and in cases, where I am not competent to do so (e.g. myocardial infarction and fracture healing models), I will ensure that team members are trained by suitably qualified and experienced personnel, drawing on established Institutional collaborations to support this.

What relevant expertise and staffing will be available to support you?

Include examples of practical or specialist support you'll be able to draw on. If anyone is going to help manage the project, explain how.

The proposed project will be conducted at the Cambridge Stem Cell Institute and the Anne McLaren Building (AMB), both of which provide world-class infrastructure to support advanced biomedical research. The Stem Cell Institute is equipped with state-of-the-art facilities that cater to the downstream analysis of mouse tissue samples, including advanced imaging, histology, flow cytometry, and genomics. Each of these core facilities is managed by dedicated institutional staff with specialist expertise who are committed to supporting the successful delivery of our research.

The Anne McLaren Building further houses exceptional animal facilities, including biomedical suites that are fully equipped for microinjection, surgery, non-invasive imaging, and cryopreservation. These facilities are supported by highly qualified and experienced animal technicians who offer a wide range of services to researchers. These services include assistance with re-derivation of mouse strains, colony establishment, and support for advanced experimental procedures such as surgery and post-operative care. The level of animal care provided in these facilities is exemplary, ensuring high standards of welfare for all animals involved in the project.

Within our research group, several members are trained in animal welfare, with the majority holding personal licences and possessing significant practical experience. These individuals will play a key role in monitoring the health of animals used in the project and ensuring that all procedures are conducted with minimal suffering. We have also appointed a dedicated laboratory technician whose responsibilities include colony management, surgical assistance, and maintaining high standards of animal welfare.

To support the implementation of new experimental techniques, our team will receive comprehensive training from the Wilson group (University of Cambridge, Department of Pharmacology) in myocardial infarction procedures and from the McCaskie group (University of Cambridge, Stem Cell Institute) in femoral fracture surgery. Both groups possess extensive expertise in their respective areas and have collaborated closely with our laboratory over the past three years, providing a strong foundation for continued knowledge exchange. In addition, a senior postdoctoral fellow in my group brings five years of prior experience working with the myocardial infarction model from a previous postdoctoral position. The combined expertise of our collaborators and internal staff will be instrumental in ensuring these techniques are introduced effectively and carried out to the highest scientific and welfare standards.

Funding

How do you plan to fund your work?

If you do not have full funding, explain how you will stage your work and the likelihood of you obtaining further funding.

This project will be initially supported by my Wellcome Career Development Award (2024-2029; £2,308,638, including a dedicated allocation of £220,628 for animal research) and a Cambridge Stem Cell Institute Seed Grant (£50,000). The early phase of the project will involve pilot studies to evaluate the effects of tumour cell injection into the digit tip, generating preliminary data to support future grant applications. During this period, additional funding will be actively sought from all eligible research councils, including the Medical Research Council (MRC), Biotechnology and Biological Sciences Research Council (BBSRC), and Cancer Research UK (CRUK). This funding will ensure that the experimental plans covered by the licence will be financially feasible.

Will this work support basic or translational research, or non-regulatory drug or device development?

Yes

Were any grant applications for this work peer reviewed? If so, by whom and what was the outcome?

All components of the work outlined in this project application have been submitted to external funding bodies and subjected to peer review. Objectives 1 and 2 formed part of my successful application for the Wellcome Career Development Award, which was granted in 2024 following rigorous evaluation by an external panel of experts and the Wellcome Trust's Scientific Advisory Board. Objective 3 was included in my application to the Lister Prize in Regenerative Medicine and was independently reviewed by a minimum of three external experts. Although the proposal received favourable reviews, it was ultimately not funded due to a lack of preliminary data. I aim to collect the necessary data and submit a new application to Cancer Research UK in the upcoming year.

Training

Training record

No training records found

Project location

Establishments

Will your project use any additional establishments?

No

Do the housing, husbandry, and care conditions at each establishment meet the requirements laid out in the Code of Practice for each type of animal you will be using?

Please read the Code of Practice for the housing and care of animals bred, supplied, or used for scientific purposes before you answer.

Yes

Places other than a licensed establishment (POLEs)

Will any part of your project be carried out in any places other than a licensed establishment (POLEs)?

No

Project plan

Scientific background

Will this work support basic or translational research, or non-regulatory drug or device development?

Yes

Briefly summarise the current state of scientific knowledge in this area of work to show how you arrived at the starting point of this project.

Be specific and relevant to your project aim - there's no need for a detailed overview of the entire field. Include any relevant non-animal research if it has contributed to the starting point of your project.

The capacity to regenerate complex tissues has largely been lost in mammals, where wound healing typically results in fibrosis and scar formation. Remarkably, in some species, including mice and humans, the distal portion of the digit tip can regenerate following amputation[1-2]. Regeneration, however, is level-dependent: removing more than 60% of the end of the digit leads to failure in the formation of a blastema, a transient tissue comprised of progenitor cells responsible for digit tip regeneration[3]. While previous studies have identified key molecular pathways involved in regeneration including Wnt signalling from the nail epidermis[4], or cytokine signalling from the overlying wound epithelium[5], what remains less understood are the mechanisms that determine whether an injury heals by fibrosis or by regeneration. This unresolved balance between scarring and regenerative healing represents a major obstacle for advancing regenerative medicine in mammalian systems.

Our recent work has addressed this by investigating the role of the extracellular matrix (ECM) and tissue mechanics in shaping regenerative outcomes. We identified hyaluronic acid, collagen and HAPLN1 as critical ECM factors that dictate whether a permissive microenvironment for regeneration is established, or whether healing defaults to fibrosis. Specially, we found that soft, hyaluronic acid-rich matrices enhance cellular responsiveness to pro-regenerative BMP signalling, whereas collagen-driven stiffness promotes scarring[6]. Importantly, stabilising the hyaluronic acid matrix with HAPLN1 can restore regeneration even in normally non-regenerative contexts6. In parallel, we have uncovered epigenetic mechanisms that regulate plasticity, showing that PADI4-mediated histone modifications link immune sensing to regenerative gene activation. Taken together, these discoveries show that

both ECM mechanics and epigenetic signalling are pivotal in determining whether healing proceeds via fibrosis or regeneration.

Interestingly, these same regenerative mechanisms may also provide a route to suppress cancer progression. Regeneration and tumorigenesis share overlapping initiation cues, yet highly regenerative species such as salamanders appear resistant to malignant transformation through mechanisms unknown[7-8]. In line with this, work with collaborators revealed that osteosarcoma cells introduced into regenerative digit tip environments were directed towards bone formation, whereas in non-regenerative contexts they gave rise to aggressive tumours. These findings suggest that regenerative tissues can harbour intrinsic tumour-suppressive properties. Building on this foundation, our project aims to harness these endogenous regenerative principles not only to promote healing in non-regenerative organs or alleviate fibrosis but also to develop novel therapeutic strategies against cancers.

1. Borgens RB. 1982 Mice regrow the tips of their foretoes. Science 217, 747-50.

2. Illingworth CM. 1974 Trapped fingers and amputated finger tips in children. J Pediatr Surg. 9(6):853-58.

3. Simkin J, Han M, Yu L, Yan M, Muneoka K. 2013 The mouse digit tip: from wound healing to regeneration. Methods Mol Biol. 1037:419-35.

4. Takeo M, Chou WC, Sun Q, Lee W, Rabbani P, Loomis C, Taketo MM, Ito M. 2013 Wnt activation in nail epithelium couples nail growth to digit regeneration. Nature. 499(7457):228-32.

5. Lehoczky JA, Robert B, Tabin CJ. 2011 Mouse digit tip regeneration is mediated by fate-restricted progenitor cells. Proc. Natl. Acad. Sci. USA. 108, 20609–20614.

6. Mui B, Wong JY, Bray T, Connolly L, Wang JH, Winkle A, Robey P, Franze K, Chalut K and Storer MA. Hyaluronic acid and emergent tissue mechanics orchestrate mammalian regeneration. bioRxiv 2024.12.04.626830; doi: https://doi.org/10.1101/2024.12.04.626830

7. Brockes JP. 1999 Regeneration and cancer. Biochim Biophys Acta. 1377(1):M1-11.

8. Okamoto M. 1997 Simultaneous demonstration of lens regeneration from dorsal iris and tumour production from ventral iris in the same newt eye after carcinogen administration. Differentiation. 61(5):285-292.

What new knowledge do you hope to discover that will address a gap in fundamental scientific knowledge or meet a clinical need?

Refer to the basis for any scientific hypotheses you plan to test during this project.

This project seeks to uncover new knowledge about how tissues decide to elicit a regenerative or fibrotic response following injury, with the ultimate aim of informing therapeutic strategies for conditions where healing is currently limited. Fibrosis is a hallmark of many chronic diseases, yet effective treatments remain scarce. Our preliminary findings suggest that the extracellular matrix (ECM) plays a decisive role in tipping the balance between scarring and regeneration. We, therefore, hypothesise that selective modulation of ECM composition and mechanics can

suppress fibrosis and promote regenerative healing. By screening ECM-modulating factors across diverse injury and fibrosis models, we aim to identify molecular targets with translational potential for anti-fibrotic therapies.

A second knowledge gap we aim to address is how tissues lose regenerative resilience with repeated injury or age. The mouse digit tip provides a tractable model to investigate this phenomenon of regenerative exhaustion. Although digit tips can regenerate after a single injury, repeated amputations eventually lead to regenerative failure. We hypothesise that this decline reflects progressive epigenetic and microenvironmental changes that limit cellular plasticity. By dissecting these changes at the molecular and cellular level, we hope to uncover pathways that preserve regenerative capacity. In the long term, such insights could guide the development of interventions to counteract age-related loss of tissue repair.

Finally, we will explore the fundamental links between regeneration and tumorigenesis. While some regenerative cues can accelerate tumour initiation in predisposed contexts, highly regenerative tissues, such as the digit tip, appear resistant to malignancy. We hypothesise that regenerative environments contain intrinsic tumour-suppressive mechanisms that constrain abnormal cell growth. Building on our observations that osteosarcoma cells are redirected towards bone formation in regenerative contexts, we will test the molecular basis of this protective effect. In doing so, we aim to identify endogenous mechanisms that both enable safe regeneration and suppress cancer, with the long-term goal of informing novel treatments for osteosarcoma and other cancers.

Does your project mainly involve translational or veterinary clinical applications?

No

Will you be producing data primarily for regulatory authorities that use standardised protocol frameworks?

No

Will you be undertaking non-regulatory testing or screening as a service to others?

No

Will you be producing genetically altered or surgically prepared animals/animal products using standardised protocol frameworks as a service to others?

This includes projects to create, breed, maintain and supply genetically altered animals to researchers within the establishment, projects taking blood and other tissues for researchers and other clients within and/or external to the establishment.

No

Will you be manufacturing vaccines and medicines for medical or veterinary use?

No

Do you need to transfer animals from a project that's due to expire?

Yes

Project licence number

PP2274970

Expiry date

For example, 13 06 2019

24 May 2026

Action plan

Objective 1

Objective title

To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.

Objective 2

Objective title

To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.

Objective 3

Objective title

To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

How do each of these objectives relate to each other and help you to achieve your aim?

Outline any interdependencies, stop:go points, and milestones. Include any key in vitro, ex vivo or in silico work, clinical findings, or results from epidemiological studies carried out under other projects that will enable you to achieve your objectives. Consider including images (.jpg and .png files) of annotated flow charts and decision trees in your action plan to illustrate how objectives relate to each other.

This project integrates *in silico* modelling, *in vitro* cell culture systems, and transgenic mouse models to investigate the mechanisms that drive fibrosis in lieu of tissue regeneration. Our primary goal is to define the cues that promote plasticity and growth, with the ultimate aim of manipulating these signals to enhance repair and regeneration in instances where it does not occur naturally. In parallel, we will examine how these same cues become dysregulated in disease states. To achieve these aims we have defined a series of objectives, each of which can be pursued independently.

Objective 1

To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.

This project aims to assess pharmacological agents that may modify the extracellular matrix (ECM) in the context of fibrosis. Initial analyses will be undertaken without the use of animals, drawing on existing proteomic datasets from three injury models and applying machine learning to identify key ECM components and suitable pharmacological agents (milestone one). Selected candidates will then be screened in *vitro* using engineered heart tissue, lung organoids or slice cultures, and digit tip fibroblasts (milestone two). Only agents showing the highest potential (no more than five) will progress to *in vivo* studies, where they will be administered in mouse models of myocardial infarction, bleomycin-induced lung fibrosis (in collaboration), and fibrosis following digit tip amputation. The results (**milestone 1**) are expected to identify druggable ECM targets with translational potential, thereby informing future development of therapies for fibrotic disease.

Objective 2

To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.

Understanding how regenerative resilience is maintained, particularly the capacity of tissues to regenerate following repeated injury, is essential for identifying the mechanisms that promote or limit repair. This process is influenced not only by intrinsic epigenetic regulation, which governs whether cells retain or lose their regenerative potential, but also by dynamic changes within the local tissue microenvironment. Deciphering how these factors interact is critical for understanding why regenerative ability is preserved in some contexts yet diminishes in others and will inform strategies to improve regenerative outcomes.

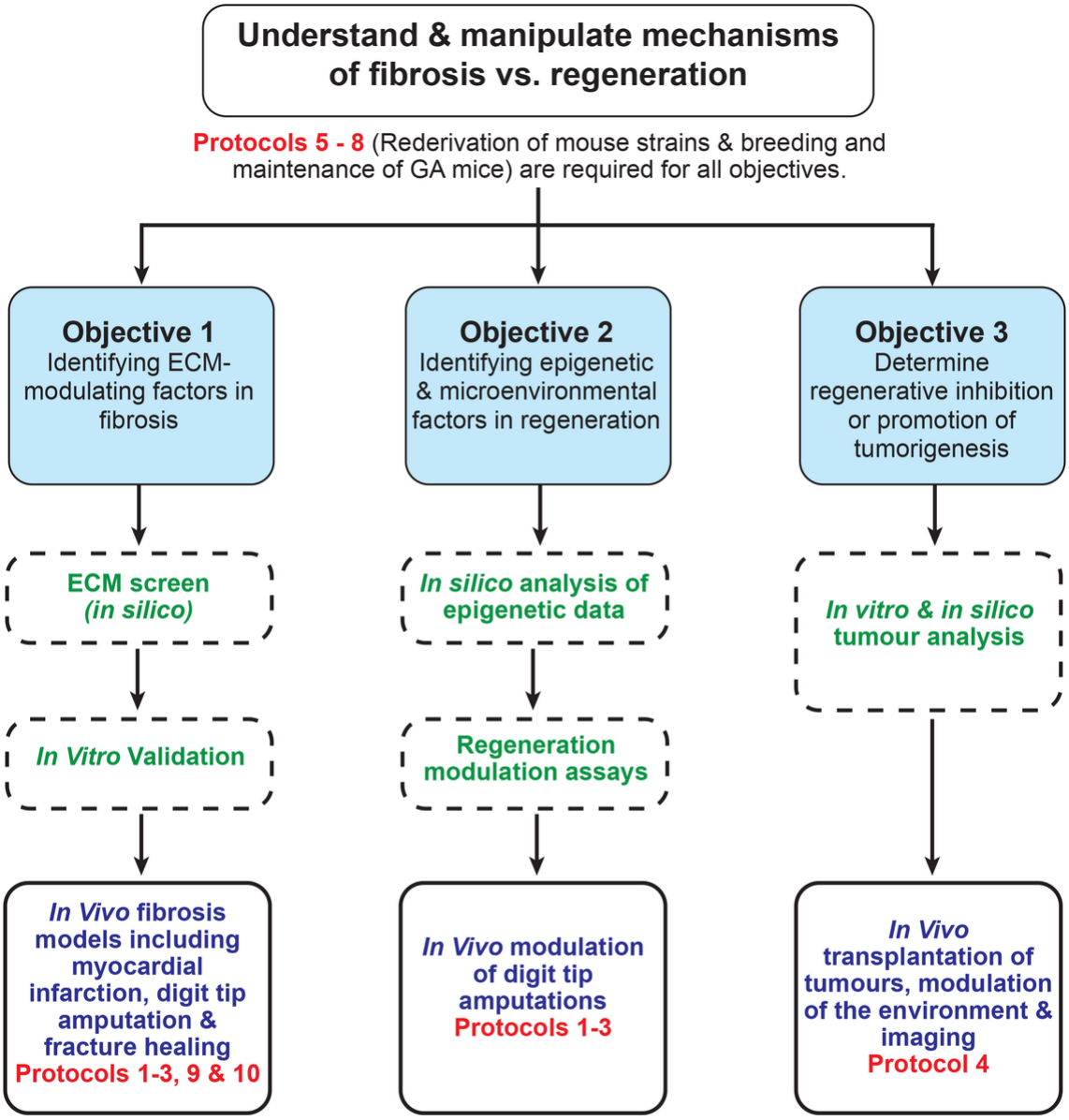
To address these questions, this work will define the genome-wide epigenetic and microenvironmental changes that occur during repeated amputation. We have already generated single-cell ATAC and RNA sequencing datasets from murine digits following one amputation (full regeneration), three amputations (partial impairment), and five amputations (regenerative failure). The **second milestone** of this programme will be to analyse these datasets to identify changes in chromatin accessibility and associated cellular responses. Findings from these analyses will then guide approaches to modulate regeneration using pharmacological agents that influence the epigenetic code or, where appropriate, through transgenic mouse models.

Objective 3

To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

Osteosarcoma is a highly aggressive bone tumour with poor survival rates, and current treatments have changed little over the past five decades. There is therefore a clear need for alternative therapeutic approaches. Evidence suggests that regeneration and tumour initiation share overlapping mechanisms, yet some highly regenerative species display resistance to malignancy through unknown processes. Our preliminary studies show that the regenerative microenvironment of the murine digit tip can suppress osteosarcoma growth, whereas non-regenerative sites promote tumour formation. To investigate this further we will use transplantation studies in combination with single-cell genomic assays, *in vitro* culture systems and *in vivo* imaging to map the cellular and molecular features of the regenerative versus non-regenerative injury environments. We will then test whether pharmacological modulation of these environments can reduce tumour growth. The results of these studies represent a **third milestone** and is expected to identify endogenous mechanisms of tumour resistance in mammals, providing insights that could inform the development of novel therapeutic strategies against cancer.

The suite of genetically altered (GA) protocols will not only be used to generate, breed and maintain GA animals, but may be used to re-derive and cryopreserve lines, when necessary. It is my expectation that GA animals may be needed for Objectives 1 & 2 enabling modification or tracking of particular cellular populations within the tissue microenvironment that contribute to fibrosis or regenerative failure.



Key To Flow Diagram
Green Text & Dotted Borders = Non-regulated procedures or previously generated data.
Blue Text = Regulated procedures undertaken in this licence.
Red Text = Protocol number.
Black Arrows illustrate the direction of the work flow.

Where relevant, how will you seek to use or develop non-animal alternatives for all or part of your work?

Wherever possible, I will seek to use or develop non-animal alternatives to address the questions in this project. I will remain actively informed of emerging technologies by attending conferences, conducting regular literature searchers, and making use of resources such as the NC3Rs website and the University of Cambridge UBS 3Rs Search Tool. I will also collaborate with colleagues across Cambridge and the wider research community to adopt or help develop new methodologies that reduce, refine, or replace animal use.

Promising *in vitro* systems are already available, including 3D organoid cultures and ‘organ-on-a-chip’ platforms, which increasingly capture aspects of tissue physiology. For example, human iPSC-derived cardiomyocytes combined with microfluidic systems can mimic some features of myocardial infarction, and bone-on-a-chip models incorporating osteoblasts, mesenchymal stem cells and mechanical loading can recapitulate aspects of fracture repair. Although these approaches cannot yet fully replicate the complex, multi-tissue interactions of regeneration, they are advancing rapidly, and I will actively explore their application to reduce reliance on *in vivo* models.

Will you be producing data primarily for regulatory authorities that use standardised protocol frameworks?

No

Will you be undertaking non-regulatory testing or screening as a service to others?

No

Will you be producing genetically altered or surgically prepared animals/animal products using standardised protocol frameworks?

This includes projects to create, breed, maintain and supply genetically altered animals to researchers within the establishment, projects taking blood and other tissues for researchers and other clients within and/or external to the establishment.

No

Will you be manufacturing vaccines and medicines for medical or veterinary use?

No

General principles

Unnecessary duplication of work must be avoided. Under what circumstances would you knowingly duplicate work?

Work would only be knowingly duplicated when it is scientifically necessary to ensure that results are reliable and valid. This could include confirming findings from other laboratories, particularly if previous studies were underpowered, validating new techniques or protocols against established methods, or addressing gaps or inconsistencies in existing data. In all cases, duplication would be carefully planned, with thorough experimental design, review of the literature, and sharing of data with

the scientific community to minimise the number of animals used and avoid unnecessary repetition.

Will all of your protocols or experiments use animals of both sexes?

No

Why will you use animals of a single sex in some protocols or experiments?

Not all protocols in this project can use animals of both sexes. Embryo-recipient and vasectomy procedures require females and males respectively for biological and technical reasons and therefore must be single-sex. However, the majority of experimental protocols will use animals of both sexes, and sex will be included as a biological variable wherever feasible.

Protocols

General constraints

Please note, constraints on procedures involving anaesthesia, surgery, substance administration and withdrawal of fluids apply to all protocols.

Anaesthesia

Induction and maintenance of general or local anaesthesia, sedation or analgesia to mitigate the pain, suffering or distress associated with the performance of other regulated procedures is indicated using the following codes in protocols:

- AA no anaesthesia
- ABL local anaesthesia
- AB general anaesthesia with recovery
- AC non-recovery general anaesthesia
- AD under neuromuscular blockade

General anaesthesia

If authorised in this licence and unless otherwise specified, all animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health should be humanely killed unless a programme of enhanced monitoring and care is instituted until the animal fully recovers.

Surgery

If authorised in this licence and unless otherwise specified:

- Surgical procedures should be carried out aseptically, to at least the published Home Office minimum;
- In the uncommon event of post-operative complications, animals will be humanely killed unless, in the opinion of a veterinary surgeon, such complications can be remedied promptly and successfully using no more than minor interventions. Minimally inflamed wounds without obvious infection may be re-closed on one occasion within 48 hours of the initial surgery. In the event of recurrence, NVS advice will be followed;
- Peri and post-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS;
- All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health will be humanely killed by a Schedule 1 method unless a programme of enhanced monitoring and care is instituted until the animal fully recovers;
- Any animal not fully recovered from the surgical procedure within 24 hrs (eating, drinking and return to normal behaviour) should be humanely killed.

Administration of substances and withdrawal of fluids

If authorised in this licence and unless otherwise specified, administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes, and frequencies that of themselves will result in no more than transient discomfort and no lasting harm using published guidelines on minimal severity.

Protocol 1

Title

Digit Tip Amputations

Protocol 1: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To induce regeneration and blastema formation or wound healing and scar formation in the distal digit tips of adult mice.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

This protocol was categorised as moderate severity when taking into account the animal's experiences and life stage (adult). Animals will undergo a minor superficial surgical procedure on up to 5 occasions performed under general anaesthesia. Anaesthesia will be brief with an expected duration of less than 10 minutes per animal. Animals are expected to recover rapidly and without the need for supportive treatment. Some animals may also be administered labelling reagents (such as EdU or BrdU) on a single occasion via a single systemic injection.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.

Protocol 1: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

1500

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

- Protocol 5: Breeding and maintenance of genetically altered mice (mild)
- Other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 1: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms driving regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes.

For example (but not limited to):

- $TLR4^{-/-}$
- $PADI4^{-/-}$

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 1: Steps

Step 1: Repeated Digit Tip Amputations (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Amputation surgeries will be performed, up to 5 occasions, with a minimum of 2 weeks recovery between amputations, on the second through fourth digits of either one or both hind limbs (AB) in one of the following planes:

a. amputation of the distal one third of the terminal phalanges (removing approximately 1-2mm)

b. amputation of the distal one third of the second phalanges (removing approximately 3-4mm)

The maximum number of digit amputations per animal = 30. Should digits be repeatedly amputated on more than one occasion, the same digits will receive the amputation.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Digit tip amputation surgeries remove a small amount of bone and will therefore cause the animal transient pain. Amputation surgeries of the distal one third of the second phalanges that remove 3 - 4mm of the digit are expected to display minimal swelling within the digit at 7 - 14 days post amputation. These effects have been observed in nearly all mice that receive this degree of digit tip amputation as part of the wound healing process and the swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the

natural behaviour of the mice, including grooming activities, mobility or balance in any way.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

All animals will be monitored closely for adverse surgical effects (including swelling of the digit tip outside of the expected range, changes in gait, reduced activity, or persistent licking of the affected digits) and pain controlled using both pre-emptive and post-operative analgesics as required and as recommended by the NVS. Animals will also be provided with soft bedding and additional warmth, and food and water placed on the cage floor so that mice can access them without needing to rear up.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions including administration of analgesics.

If post-operative swelling of the digit persists beyond the expected recovery period, worsens, or begins to impair normal behaviour or mobility, and does not improve with minor supportive measures within 48 hours, the animal will be immediately killed using an appropriate Schedule 1 method.

Step 2: Cell Labelling (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Cell labelling: Mice may be administered labelling agents (such as EdU or BrdU) via intra-peritoneal injection or sub-cutaneous (AA) on a single occasion. [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 3: Schedule 1. (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Animals will be killed by a Schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 1: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 1: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Most animals (90%) will undergo a single round of digit tip amputation surgery affecting up to six digits. This surgical procedure is carried out under general anaesthetic (less than 10 minutes per animal) which includes an injection of peri-operative analgesia by standard routes and dosages. Animals make a rapid recovery from surgery, having frequently been observed to start climbing on the bars of their cages, resume normal activities in less than 30 minutes and typically do not require post-operative analgesia. Upon recovery, animals will be returned to their cages (group housed) until the time of tissue collection (7 - 168 days following surgery). Animals that received non-regenerative amputations (removal of approximately 3-4mm of the distal digit tip) are expected to display minimal swelling within the digit at 7 - 14 days post amputation and this swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the natural behaviour of the mice or affect their gait in any way. Occasionally, in addition to the digit tip amputation surgery, some mice (10%) will also be administered cell labelling reagents (e.g. EdU) on a single occasion using standard routes and dosages.

In a minority of animals (approximately 5%), up to five rounds of digit-tip amputation may be required over the duration of the protocol, resulting in a maximum of 30 digit procedures in total. Each round is performed under general anaesthesia with peri-operative analgesia and no less than 28 days between procedures. Recovery characteristics remain similar to those described for the typical case above, with animals generally returning quickly to normal behaviours after each procedure. Occasionally, in addition to the repeated digit tip amputation surgery, some mice (10%) will also be administered cell labelling reagents (e.g. EdU) on a single occasion using standard routes and dosages.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Any animal will be immediately killed by a Schedule 1 method if it shows signs of suffering that in any way compromises normal behaviour but only when these are not those expected as step related adverse effects.

Examples of clinical signs that would indicate compromised welfare include, but are not limited to:

- Marked reduction in normal activity, exploration, or social interaction
- Reduced food or water intake, dehydration, or persistent weight loss (no improvement within 48 hours) that is greater than 15%
- Poor coat condition, pilo-erection, or hunched appearance
- Limping, lameness or reluctance to bear weight on the limp with amputated digits

- Excessive toe licking

Protocol 1: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Adult mice with regenerating or non-regenerative digit tips (from which tissue can be obtained for histology and molecular biology approaches posthumously).

Will this protocol generate quantitative data?

No

Protocol 1: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Murine digit tip regeneration mimics the regenerative process observed when the distal phalanx in humans is injured. This model is therefore the most appropriate scientific approach for studying *mammalian regeneration* in the lowest possible sentient species with the broadest applications (i.e. by studying this process in adult mice the findings will be applicable to a broader range of individuals as many mechanisms available in younger animals are silenced in adults).

This protocol will be used to obtain the necessary samples required for cellular and molecular biology approaches. The number of animals requested in this protocol have been calculated based on the anticipated experiments planned.

Animals may have up to six digits amputated per animal. This is because the digit tip only provides a very small amount of tissue and some experiments require dissociation of this tissue into a live single cell suspension. By amputating up to six digits per animal this will enable me to drastically reduce the number of mice needed for experiments, reducing experimental variability, while still ensuring that the well-being and natural behaviours of the animal are not compromised.

Some animals may have the same digits amputated repeatedly. This will allow us to investigate the limitations of regeneration whereby repeated amputation is predicted to lead to regenerative failure. Such studies are important to help us understand the mechanisms underlying regeneration and how these may change with age. Mice will be given sufficient time to regenerate their digits entirely (28 days) between individual amputations to ensure minimal suffering or an excessive inflammatory response.

b) the most refined for the purpose?

This experimental model is inherently refined because it represents a naturally regenerating system in which tissue removal heals without the need for more invasive surgical techniques, suturing, or procedures that would result in chronic tissue deficits. The rapid and complete functional recovery associated with digit tip regeneration minimises long term welfare impact compared with alternative injury models.

Surgical procedures will be carried out using aseptic technique which meets at least Home Office Minimum Standards of Aseptic Surgery and the LASA Guiding Principles.

Peri-operative analgesia will be provided and where necessary post-operative analgesia will be given; agents will be administered at doses and frequencies agreed upon in advance with the Named Veterinary Surgeon.

Post-surgical monitoring will be conducted following surgery, using an approved post-surgical monitoring chart as agreed upon in advance with the Named Veterinary Surgeon.

Amputation surgeries will be restricted to the hindlimb digits 2 through 4 to ensure normal grooming routines, movement or balance are not affected. This surgery does not appear to affect the animal's well-being or stop it from carrying out normal behaviour. In the event of repeated amputations, surgeries will be performed no less than 28 days apart to ensure the animal has had sufficient time to recover.

The removal of six digits is performed to control for biological variability during digit regeneration, which is essential to obtain robust and reproducible results. By standardising the number and location of digits removed, we reduce experimental variability between animals, allowing clearer interpretation of regenerative outcomes.

For each model and/or method, what is the scientific need for the expected clinical signs?

The majority of adult mice that undergo this procedure are not expected to display any clinical signs. Those animals that receive non-regenerative amputations (amputations that remove 3-4mm of the second phalanx) will present with minimal swelling of the digit as part of the natural wound healing process. This tissue response represents one of the main distinctions between regenerative and non-regenerative processes. It is not expected that repeated amputations of the same digits will present with additional clinical signs other than what is expected of a single amputation surgery.

In my previous institute and through my experience over the past five years in my current animal facility, I worked closely with veterinary surgeons to assess the response of adult mice undergoing digit tip amputation surgeries and observed the following parameters upon recovery from anaesthetic:

- Adult mice were active and gait was observed as normal (some mice have been observed to climb on the cage bars immediately following recovery).
- Adult mice did not exhibit audible vocalisation.
- Eyes were bright and open (did not observe ‘grimacing’) and posture was normal (not hunched).
- Respiratory rate was observed to be normal (this was ascertained by observation and no device was used to measure this).
- Prior to surgeries adult mice were given pre-emptive analgesia with analgesic effects lasting up to 12-24 hours. The mice were subsequently observed for a further seven days following surgery (daily observations).

We also observed the following:

- Food and water intake were normal and minimal weight loss was observed in some animals (less than 10%), often returning to baseline within 48 hours.
- Muscle tone was good and coat appeared well cared for (no grooming deficits).
- Animals remained social and continued to interact with each other.

Why scientifically do the animals need to suffer to this degree?

Adult mice that undergo this procedure appear to recover within 5 - 10 minutes following surgery and resume normal activities. Although suffering appears to be minimal the process of digit tip amputation is necessary in order to pursue my chosen scientific questions. In the event of repeated amputations, these studies are important to help us understand how regenerative process may change with age and what leads to regenerative failure. There is currently no other alternative approach to answer this question at present.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

Generally, clinical signs are not expected to be seen, even in animals that undergo repeated amputation surgeries, and animals appear to recover quickly from the surgery. In those cases where mice receive non-regenerative amputations, the associated inflammatory response and minimal swelling that accompanies the wound healing process is essential for pursing my chosen scientific questions.

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

Cell-labelling agents such as BrdU and EdU are widely used in mouse studies to assess cellular proliferation through incorporation into newly synthesised DNA. Their suitability will be confirmed by reviewing published toxicity data for the specific mouse strain used and by employing doses with established efficacy in the literature. All solutions will be prepared fresh and rendered sterile (e.g., by filtration) to prevent infection or injection-site complications.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

Dosing regimens will follow established guidance for mice, including recommended doses, routes, and frequencies. Volumes and frequency will be minimised in line with Home Office and institutional limits. For cell-labelling, EdU or BrdU will be administered as a single intraperitoneal or subcutaneous injection, not exceeding 20 ml/kg.

Protocol 2

Title

Administering Agents in Digit Tips

Protocol 2: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To investigate whether the administered agents modulate fibrotic remodeling or regenerative responses in the mammalian digit tip.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

This protocol was categorised as moderate severity when taking into account the animal's cumulative experiences and life stage (adult). The majority of animals will undergo a minor superficial surgical procedure on up to 5 occasions performed under general anaesthesia, followed by administration of substances (most often through a direct injection, usually on one occasion, into the digit tip). Animals will be allowed sufficient time to recover between the initial surgical procedure and the aseptic digit tip injection. For each surgical procedure or when needed for restraint purposes, animals will be administered anaesthesia whereby induction is rapid and the duration will be less than 10 minutes per animal. Some animals may also be administered labelling reagents (such as EdU, BrdU or tamoxifen) on up to four occasions. A small number of animals may also undergo imaging procedures that are non-invasive (e.g. MRI or CT), likely on three occasions.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.

Protocol 2: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

1250

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

- Protocol 5: Breeding and maintenance of genetically altered mice (mild)
- Other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 2: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms driving regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes. Examples include fluorescent reporter lines (e.g. Floxed tdTomato mice), Cre driver lines (e.g. PdgfraCreERT2 mice) or conditional alleles (e.g. Floxed Piezo1 mice).

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 2: Steps

Step 1: Repeated Digit Tip Amputation (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Amputation surgeries will be performed, up to 5 occasions, with a minimum of 2 weeks recovery between amputations, on the second through fourth digits of either one or both hind limbs (AB) in one of the following planes:

a. amputation of the distal one third of the terminal phalanges (removing approximately 1-2mm)

b. amputation of the distal one third of the second phalanges (removing approximately 3-4mm)

The maximum number of digits amputated per animal = 30. Should digits be repeatedly amputated on more than one occasion, the same digits will receive the amputation.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Digit tip amputation surgeries remove a small amount of bone and will therefore cause the animal transient pain. Amputation surgeries of the distal one third of the second phalanges that remove 3 - 4mm of the digit are expected to display minimal swelling within the digit at 7 - 14 days post amputation. These effects have been observed in nearly all mice that receive this degree of digit tip amputation as part of the wound healing process and the swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the natural behaviour of the mice, including grooming activities, mobility or balance in any way.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

All animals will be monitored closely for adverse surgical effects (including swelling of the digit tip outside of the expected range, changes in gait, reduced activity, or persistent licking of the affected digits) and pain controlled using both pre-emptive and post-operative analgesics as required and as recommended by the NVS. Animals will also be provided with soft bedding and additional warms, and food and water placed on the cage floor so that mice can access them without needing to rear up.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions including administration of analgesics.

If post-operative swelling of the digit persists beyond the expected recovery period, worsens, or begins to impair normal behaviour or mobility, and does not improve with minor supportive measures within 48 hours, the animal will be immediately killed using an appropriate Schedule 1 method.

Step 2: Substance Administration (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

With the exception of untreated controls, mice will receive substances or cells (e.g. growth factors, ligands, function-blocking antibodies, chemical inhibitors, or viral over-expression vectors). Control substances/cells may also be administered. Up to three agents may be given, in combination where possible, to minimise the number of dosing occasions. All administrations will be performed using the least invasive route available through:

a. Local administration: Direct injection into the second through fourth hindlimb digits (AB). [Max vol. 5ul/digit]. Direct injections into the digit will be performed no less than 3 days following a prior surgical procedure and on up to three occasions.

b. Systemic administration: Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

c. Systemic administration: Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

d. Systemic administration: Intravenously (AA/AB), maximum treatment = once a day up to 5 days [Max vol. 10ml/kg].

e. Systemic administration: Orally (e.g. in diet, drinking water) (AA), maximum treatment = 8 weeks.

Additional animals may be treated with administration vehicle only. In some instances a combination of routes may be needed to effectively deliver the substances.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 3: Transgene Induction (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Gene Induction : Except for untreated controls, mice may be administered trans-gene inducing or deleting agents (such as tamoxifen) or vehicle control, at any stage, by one or more of the following routes on up to 5 occasions (minimum 24h interval).

a. Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

b. Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Animals receiving the trans-gene inducing or deleting reagent, tamoxifen, may experience weight loss during the first 1-2 days of administration. The likely incidence of this occurring is dependent on the genetic strain of the mice but is not uncommon. These mice typically regain weight back to base-line within the next 2-5 days and display no harmful side effects.

Oral gavage may cause stress, transient oesophageal irritation, and, rarely, mortality (~5%) at high doses. IP injections may cause temporary abdominal or scrotal swelling.

In addition, animals receiving tamoxifen via oral gavage or intraperitoneal (IP) injection may show transient reduced activity, hunched posture, and a ruffled or greasy coat, typically within the first 1–7 days.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for the first two weeks, or until weight is stable. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

Male mice given tamoxifen will be monitored for testicular swelling daily during the days of administration and for 48 hours afterwards.

All procedures and monitoring will follow the UBS tamoxifen guidance developed by the NVS, ensuring that refinements, dosing, and welfare measures are implemented in accordance with expert veterinary advice.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Animals showing 15% weight loss (from the animal's initial weight measurement when dosing begins) measured for 2 consecutive days will be killed immediately by an appropriate Schedule 1 method.

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Testicular swelling causing more than mild discomfort (difficulty walking or changed activity) in male mice treated with tamoxifen will be killed immediately by an appropriate Schedule 1 method.

Step 4: Cell Labelling (optional)

Repeated from protocol 1

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Cell labelling: Mice may be administered labelling agents (such as EdU or BrdU) via intra-peritoneal injection or sub-cutaneous (AA) on a single occasion. [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 5: Fasting Prior To Imaging (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Animals will be weighed to establish a baseline body weight prior to any fasting (AA). Food restriction will then be applied for up to 4 hours before imaging, with no more than two such fasting periods per week. After each imaging session, animals will be provided with food ad libitum. A further fasting period will only be undertaken once

the animal's body weight has returned to its baseline level. Water will be available at all times. Food intake and body weight will be closely monitored to ensure that mice do not lose more than 15% of their baseline body weight.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Short-term food restriction may result in transient hunger and a temporary reduction in body weight (typically ≤10% of baseline). Minor weight loss is expected to be short-lived, with body weight returning to baseline within 24 hours following re-feeding. The incidence and severity of adverse effects are expected to be low due to the limited duration and frequency of fasting, continuous access to water, and close monitoring of body weight and food intake.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for a week, or until weight is stable following food restriction. During fasting, body weight will be recorded prior to each session to ensure weight loss does not exceed 15% of baseline. Fasting will be limited to a maximum of 4 hours and no more than two sessions per week, with a further session only undertaken once body weight has returned to baseline. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Mice exhibiting a 15% weight loss will be immediately killed by a schedule 1 method.

Step 6: Non-Invasive Imaging (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Non-Invasive Imaging: Animals may be fasted for up to 4 hours prior to imaging - See Step 'Fasting Prior To Imaging' (AA). Animals may be assessed by any or a combination of non-invasive imaging methods (for example, but not limited to):

• bioluminescence imaging (BLI) (AB),

• fluorescence imaging (FLI) (AB),

• MRI (AB/AC) with fasting,

• MSOT (AB/AC) with fasting,

• ultrasound (AB) with fasting,

• PET/SPECT (AB/AC) with fasting

BLI, FLI and ultrasound imaging typically takes a maximum of 15 min and may be conducted up to once per week or once every 2 weeks throughout some long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 12. In addition, BLI, FLI and ultrasound imaging may be used daily for short term monitoring of administered agents (including cells).

MSOT measurements typically take about 30 min and may be conducted up to twice per week or once every 2 weeks throughout long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 10.

MRI and CT measurements of tissue anatomy, where these do not involve injection of an imaging agent, typically take about 30 min and may be conducted weekly throughout experiments which can last up to 3 months. The total number of imaging examinations will not exceed 12. MRI, CT and PET/SPECT imaging following injection of an established or novel imaging agent usually take under 2 hours and may be conducted for a maximum of 5 times for a maximum period of 2 weeks and no more than twice per day (AB/AC). Imaging may be repeated on the same animal but typically, this will not exceed five sessions within any 2 week period, where each imaging session will not exceed 5 hours. Typically, there will be only one imaging session per day. Occasionally it may be necessary to conduct two imaging sessions in one 12 hour period, in which case each imaging session will not exceed 2 hours.

Imaging agents may be administered via one of the following routes prior to or during imaging:

a) Intra-peritoneal, with or without cannulation, maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

b) Intravenous, with or without cannulation, maximum of 20 ml/kg bolus injection or constant infusion via a cannulated vein, for a maximum of 3 hours [AA/AB/AC].

Maximum frequency of 5 times within any 2 week period, and a maximum of 10 injections in 3 months.

c) Subcutaneous, a maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

d) Oral gavage, a maximum of 20 ml/kg bolus injection [AA]. Maximum frequency of 5 times in any 2 week period and a maximum of 10 times in 3 months.

Animals will undergo a maximum of two imaging sessions per week in total, irrespective of imaging type or combination of imaging modalities. After each imaging session and recovery, animals will be assessed to ensure they are fully alert, behaving normally, and showing no adverse effects. Imaging on a consecutive day will only proceed if the animal meets all of these criteria.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 7: Killing by Schedule 1. (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing by a schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 2: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 2: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

The majority of animals in this protocol (~95%) will undergo digit tip amputation, a surgical procedure under general anaesthetic (less than 10 minutes per animal) which includes an injection of peri-operative analgesia by standard routes and dosages. Animals make a rapid recovery from surgery, having frequently been observed to start climbing on the bars of their cages, resume normal activities in less than 30 minutes and typically do not require post-operative analgesia. Upon recovery, animals will be returned to their group-housed cages. Animals that received non-regenerative amputations (removal of approximately 3-4mm of the distal digit tip) are expected to display minimal swelling within the digit at 7 - 14 days post amputation and this swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the natural behaviour of the mice or affect their gait in any way. All mice will be administered substances, typically in one of the following ways but on occasion through combined routes:

a. Typically (85% of animals), adult mice will be placed under general anaesthetic (less than 15 minutes per animal) for restraint purposes and substances will be injected on a single occasion directly into the digit tip. Animals are given peri-operative analgesia by standard routes and dosages. Animals make a rapid recovery from the anaesthetic and typically do not require post-operative analgesia.

b. Occasionally (in ~10% of animals), adult mice will be administered substances systemically by oral gavage, intravenous or intra-peritoneal injection on up to 5 occasions (minimum interval 24 hours).

c. Occasionally (in ~5% of animals), adult mice will be administered substances systemically through their diet or drinking water. When substances have been reported as being taste aversive, these will be masked (e.g. by adding the substance to chocolate flavoured food pellets or adding sucrose into the water). Additionally, all mice will be monitored throughout the duration of the experiment to ensure general health is not compromised (e.g. mice are eating and drinking sufficiently and that no other complications arise affecting general behaviour or health).

d. Occasionally (in ~5% of animals), adult mice will undergo non-invasive imaging of tissues on up to 12 occasions (minimum interval 12 hours). Animals may be fasted for up to 4 hours prior to imaging. This will typically include a single session of fluorescence imaging to visualise implanted cells, followed by weekly sessions of MRI to monitor changes in tissue architecture. At most, an animal may undergo up to two 4-hour fasting periods per week, each followed by an imaging session using the most demanding combinations of modalities, for example MRI, PET/SPECT, or MSOT, with any associated injections of contrast agents, within the overall limit of two imaging sessions per week. This defines the upper limit of procedural exposure for an individual animal.

At maximum, a single animal may receive substances via one direct route (e.g. direct injections into the digit tip) and one systemic route of administration (e.g. intra-peritoneal injection, oral gavage or via modified diet or drinking water).

Taking into account all relevant procedures, including imaging, surgical interventions, and any restraint procedures requiring anaesthesia (such as for injections), the maximum number of occasions on which an animal may be anaesthetised = 18.

In the instance when substances are administered systemically, agents will be administered by the least invasive route in the first instance when multiple routes are available (e.g. Administered in the food or drinking water as opposed to intravenous injection).

Occasionally (in ~5% of animals), in addition to the initial digit tip amputation surgery and administration of substances, some mice will also be administered cell labelling reagents (e.g. EdU) on a single occasion or tamoxifen (up to four times) to induce fluorescent reporters using standard routes and dosages.

Mice will remain in group-housed cages until the time of tissue collection (3 - 90 days following the initial digit tip amputation surgery).

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Any animal will be immediately killed by a Schedule 1 method if it shows signs of suffering that in any way compromises normal behaviour but only when these are not those expected as step related adverse effects.

Examples of clinical signs that would indicate compromised welfare include, but are not limited to:

- Marked reduction in normal activity, exploration, or social interaction
- Reduced food or water intake, dehydration, or weight loss of 15% that persists for more than 48 hours
- Poor coat condition, pilo-erection, or hunched appearance
- Limping, lameness or reluctance to bear weight on the limp with amputated digits
- Excessive toe licking

Throughout the protocol, a general humane endpoint of 20% body weight loss from an animal's baseline will be applied, independent of specific experimental steps. This is separate from the gene induction step, where animals showing ≥15% weight loss from the initial dosing weight on two consecutive days will be killed immediately using an appropriate Schedule 1 method.

Protocol 2: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Phenotypic data on the effects of specific agents in reducing fibrosis or enhancing regeneration in the murine digit tip.

Will this protocol generate quantitative data?

Yes

Will your experimental design be determined by a regulatory guideline?

No

Where relevant, explain how and when pilot studies will be used.

Pilot studies, using no more than 3 animals per condition, will be conducted to generate preliminary data and in order to allow for procedures and techniques to be perfected before embarking on large scale experiments. Specifically, we will perform pilot experiments when animals are to be injected with pharmacological agents or inhibitor substances to obtain an indication of the dose and the timing of the injections, that is likely to be effective.

How will you choose different experimental groups?

For example, controls, dose levels, satellites etc.

Animals will be randomly assigned to either the control or treatment group. The treatment, that is the pharmacological agents or inhibitor substances, will be selected according to preliminary data obtained from *in vitro* culture experiments and doses will be determined through pilot experiments, taking into account the effective reported dose levels in the literature.

How will you choose control groups?

Provide a robust scientific justification for controls with significant suffering such as sham surgery controls or untreated infected controls.

Each individual experiment will use a control group of mice that are contrasted directly to the experimental group of mice. For example, when injecting non-regenerative digits with a substance resuspended in 0.1% BSA, control non-regenerative digits will be injected with the same vehicle, 0.1% BSA. A sham surgery control will only be included in the event that the necessary vehicle control has not been tested in this system, or been reported in the literature to be innocuous. The inclusion of a sham surgery control would be limited to pilot experiments and is necessary to ensure that unknown vehicle controls alone cannot account for any observed effects and result in misleading or inaccurate conclusions.

How will experiments and data analysis be randomised and blinded?

Prior to each experiment, animals will be randomly allocated to treatment or control groups. For localised injections, the left or right digits will be assigned using a randomisation method (e.g. unique identifiers with group allocation determined by random number selection drawn out of a hat). For systemic administration, the entire animal will be randomly assigned to either experimental or control groups.

Treatments (e.g. agonists or inhibitors) will be coded by an independent individual not directly involved in the study. Codes will remain concealed (e.g. sealed envelope) until the experiment and data collection are complete.

Where possible, the researcher performing the surgical intervention or treatment will not be involved in subsequent data analysis. Data analysis will also be conducted under blinded conditions whereby samples will be re-coded by an independent individual not associated with the study prior to analysis. Analysts will only be informed of group structure when necessary for statistical comparison but will remain blinded to treatment identity. This approach minimises bias in both data collection and interpretation.

How will you minimise variables to ensure reproducibility?

We will aim to minimise variables in this protocol by using inbred mice from the same supplier such as Charles River Laboratories for all experiments and will avoid exposing the laboratory mice to unnecessary stress by employing good handling practises. Bias will be avoided through good experimental design including incorporation of randomisation and blinding practises both during experimental procedures and analysis of the data. Additionally, accurate records will be maintained of all work conducted under this project licence and methods will be reported responsibly including descriptions of all variables such as mouse age, number, strain, housing conditions and appropriate statistical analyses.

How will you determine group sizes?

You should reference POWER calculations you have made, if relevant.

Group sizes were determined using POWER calculations for an unpaired t-test whereby the biological effect size (using proportional differences) is expected to be 1. 5 to 2.0 fold and standard deviations approximately 20%, based on published literature. The power was set to 90% and alpha 5% (two sided) resulting in group sizes of n = 8 animals per condition. To accommodate occasional animal loss due to experimental complications and unexpected variability, a sample size of n = 10 was subsequently selected for each group (control and experimental). Additionally, advice on group size calculations and experimental design has been sought from a local bio-statistician.

How will you maximise the data output from the animals you use on this protocol?

To maximise the information obtained from each animal, experiments will be designed so that, where possible, digits from one hindlimb receive the treatment while digits on the contralateral hindlimb serve as vehicle-treated controls. This within-animal design reduces variability and the number of animals required.

As only hindlimb digits are required for these studies, surplus tissues of scientific value will be shared with other researchers, reducing the need for additional breeding or animal use.

Furthermore, all experimental approaches and resulting data will be disseminated to the wider scientific community. This ensures transparency, prevents unnecessary duplication of work, and maximises the impact of findings generated from each animal.

Protocol 2: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Murine digit tip regeneration mimics the regenerative process observed when the distal phalanx in humans is injured. This model is therefore the most appropriate scientific approach for studying *mammalian regeneration* in the lowest possible sentient species with the broadest applications (i.e. by studying this process in adult mice the findings will be applicable to a broader range of individuals as many mechanisms available in younger animals are silenced in adults). Additionally, regeneration is level dependent. Amputating more than 60% of the digit will result in regeneration failure thereby allowing us to study a regenerative and non-regenerative response in the same animal.

Individual digit tip amputations within the same mouse do show some variability in the rate of regeneration. This particular set of experiments also requires that very small volumes (usually 1ul) be administered into solid tissue which generates a second source of variability. Therefore, in order to minimise these effects, and allow for reproducibility, the measurements from 3 digits per condition are combined and

averaged. Experiments were designed so that control and experimental conditions (e.g. left hindlimb digits versus right hindlimb digits) can be performed in the same animal which once again minimises experimental variability between animals and allows for a reduction in the number of animals required.

The use of a dissecting scope when performing amputation surgeries ensures that the site for either amputation plane is readily detectable.

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b) the most refined for the purpose?

Surgical procedures will be carried out using aseptic technique which meets at least Home Office Minimum Standards of Aseptic Surgery and the LASA Guiding Principles.

Peri-operative analgesia will be provided and where necessary post-operative analgesia will be given; agents will be administered at doses and frequencies agreed upon in advance with the Named Veterinary Surgeon.

Post-surgical monitoring will be conducted following surgery, using an approved post-surgical monitoring chart as agreed upon in advance with the Named Veterinary Surgeon.

Amputation surgeries will be restricted to the hindlimb digits 2 through 4 to ensure normal grooming routines, movement or balance are not affected. This surgery does not appear to affect the animal's well-being or stop it from carrying out normal behaviour.

Substances will be pre-screened in pilot experiments to obtain an indication of the dose that is likely to be effective. As this study will only use previously-validated compounds, the starting dose will be the minimum to have an effect according to the literature. Additionally, we will refer to the resources *"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* and *"Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider". Turner PV et al. J Am Assoc Lab Anim Sci. 2011 50(5): 600–613,* to aid in further refining the appropriate dosing regime for administered substances.

In the instance when substances are administered systemically, agents will be administered by the least invasive route in the first instance when multiple routes are available (e.g. Administered in the food or drinking water as opposed to intravenous injection).

A pain monitoring and scoring system will be introduced for this protocol with the help of the Named Veterinary Surgeon. This will allow us to ensure the welfare of our animals and may help to inform the use of toe clipping for identification purposes which is used overseas.

For each model and/or method, what is the scientific need for the expected clinical signs?

The majority of adult mice that undergo this procedure are not expected to display any clinical signs. Those animals that receive non-regenerative amputations (amputations that remove 3-4mm of the second phalanx) will present with minimal swelling of digit as part of the natural wound healing process. This tissue response represents one of the main distinctions between regenerative and non-regenerative processes.

In my previous laboratory, we collaborated closely with a veterinary surgeon to assess the responses of adult mice undergoing digit tip amputation. Over the past five years, through our own experience, we have consistently observed the following parameters during recovery from anaesthesia:

- Adult mice were active and gait was observed as normal (some mice have been observed to climb on the cage bars immediately following recovery).
- Adult mice did not exhibit audible vocalisation.
- Eyes were bright and open (did not observe 'grimacing') and posture was normal (not hunched).
- Respiratory rate was observed to be normal (this was ascertained by observation and no device was used to measure this).
- Prior to surgeries adult mice were given pre-emptive analgesia with analgesic effects lasting up to 12-24 hours. The mice were subsequently observed for a further seven days following surgery (daily observations).

We also observed the following:

- Food and water intake were normal and no weight loss was observed.
- Muscle tone was good and coat appeared well cared for (no grooming deficits).
- Animals remained social and continued to interact with each other.

Why scientifically do the animals need to suffer to this degree?

Adult mice that undergo this procedure appear to recover within 5 - 10 minutes following surgery and resume normal activities. Although suffering appears to be minimal the process of digit tip amputation and administration of substances is necessary in order to pursue my chosen scientific questions. In some instances, administration of substances through the diet may result in initial transient weight loss as mice become accustomed to the change in taste. It is expected that mice will regain their weight back to their initial starting point within 3 days. All steps to minimise this circumstance have been employed including a slow acclimatization to the diet over a period of 2 weeks together with addition of flavouring (e.g. chocolate). Administration of substances is essential for pursing my chosen scientific questions

however we will choose a route of administration that results in the least amount of clinical signs and suffering.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

Clinical signs are generally not expected, and animals typically recover quickly from procedures. Following tamoxifen administration, a transient weight loss is occasionally observed dependent on the strain. In some animals this may exceed 15% for a short period but is reversible with supportive care, with animals stabilising within 24 hours. Immediate euthanasia at 15% weight loss would result in the unnecessary loss of animals that would otherwise recover fully, increasing animal use without welfare benefit. An upper limit of 20% weight loss is therefore set as an immediate humane endpoint, beyond which recovery is unlikely and welfare would be compromised.

In animals undergoing non-regenerative amputations, the associated inflammatory response and minimal swelling during wound healing are intrinsic to addressing the scientific objectives and cannot be avoided.

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

When using substances for the first time, doses will be chosen to minimise the risk of toxicity based on the available literature and validated in small pilot study. The starting dose used will be the minimum that was shown to have an effect according to the published literature.

All substances will be prepared in a Biosafety cabinet using sterile equipment and sterile solutions.

As this study will only use previously-validated compounds, all administered substances are not expected to cause adverse effects. However, if animals show, unexpectedly, any clinical signs such as hunched posture, pilo-erection or subdued behaviour, these animals will be kept under close observation. If the signs do not resolve within 24 hours they will be killed. In the event of worsening clinical signs animals will be immediately killed.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

For most substances, the dose, route, frequency, and duration in mice are well documented. Where data are lacking, short-term pilot studies using low doses in a small number of mice (typically 2–3) will be conducted to identify a safe and effective regimen. Dosing volumes and frequencies will be minimised and follow standard guidelines (UBS and LASA) for each route of administration (e.g., subcutaneous or intra-peritoneal) as documented below. Pilot studies allow adjustment of doses before treating the remainder of animals in each experimental group. Maximum volumes and frequencies exclude administration for rehydration, anaesthesia, or analgesia.

Mouse	Daily Max. Volume (ml/kg)	Max. No./Day <10 Days	Max. No./Day >10 Days	Max. No. Injections
S/C (Subcutaneous)	20 ml/kg	3	2	24
I/P (Intra-peritoneal)	20 ml/kg	3	1	30
I/V (Intravenous)	0.8 ml over 2 min	2	1	14
Oral Gavage	20 ml/kg	3	2	30

Protocol 3

Title

Gene Induction/ Editing in the Digit Tip

Protocol 3: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To lineage trace, ablate or modify genes/cells prior to or during digit tip regeneration to determine how important they are to this process.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

This protocol was categorised as moderate severity when taking into account the animal's cumulative experiences (typically 6 regulated procedures which are detailed in the following sentences) and life stage (adult). Typically, animals will experience digit tip amputation on up to 5 occasions. For each surgical procedure, animals will be administered anaesthesia whereby induction is rapid and the duration will be less than 10 minutes per animal. Except for untreated controls, all animals will be administered a trans-gene inducing or deleting agents by standard routes (oral gavage or intra-peritoneal injection) on up to four occasions. Occasionally, some animals may also be administered labelling reagents (such as EdU, BrdU) on a single occasion or undergo non-invasive imaging.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.

Protocol 3: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

500

Which life stages will be used in this protocol?

Select all that apply

- Pregnant adult

- Adult
- Embryo and egg

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

- Protocol 5: Breeding and maintenance of genetically altered mice (mild)
- Other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 3: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms driving regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes. Examples include fluorescent reporter lines (e.g. Floxed tdTomato mice), Cre driver lines (e.g. PdgfraCreERT2 mice) or conditional alleles (e.g. Floxed Piezo1 or Piezo2 mice).

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 3: Steps

Step 1: Repeated Digit Tip Amputation (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only non-pregnant adult mice will be used in this step.

Amputation surgeries will be performed, up to 5 occasions, with a minimum of 2 weeks recovery between amputations, on the second through fourth digits of either one or both hind limbs (AB) in one of the following planes:

a. amputation of the distal one third of the terminal phalanges (removing approximately 1-2mm)

b. amputation of the distal one third of the second phalanges (removing approximately 3-4mm)

The maximum number of digits amputated per animal = 30. Should digits be repeatedly amputated on more than one occasion, the same digits will receive the amputation.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Digit tip amputation surgeries remove a small amount of bone and will therefore cause the animal transient pain. Amputation surgeries of the distal one third of the second phalanges that remove 3 - 4mm of the digit are expected to display minimal swelling within the digit at 7 - 14 days post amputation. These effects have been observed in nearly all mice that receive this degree of digit tip amputation as part of the wound healing process and the swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the natural behaviour of the mice, including grooming activities, mobility or balance in any way.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

All animals will be monitored closely for adverse surgical effects (including swelling of the digit tip outside of the expected range, changes in gait, reduced activity, or persistent licking of the affected digits) and pain controlled using both pre-emptive and post-operative analgesics as required and as recommended by the NVS. Animals will also be provided with soft bedding and additional warmth, and food and water placed on the cage floor so that mice can access them without needing to rear up.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions including administration of analgesics.

If post-operative swelling of the digit persists beyond the expected recovery period, worsens, or begins to impair normal behaviour or mobility, and does not improve with minor supportive measures within 48 hours, the animal will be immediately killed using an appropriate Schedule 1 method.

Step 2: Timed Mating (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Sexual experience (AA).

Mating with an intact partner (confirmed by the presence of a plug or by subsequent pregnancy, or by visual inspection).

For females mating with intact males:

- Plug checking will be performed twice daily

- Once pregnancy is confirmed by clear plugs and/or abdomen swelling by the end of week 2, the male will be removed to prevent another mating after delivery.
- The mother may either be humanely killed for embryo collection between gestational age E9.50 - E18.50 or allowed to give birth. She will undergo only optional procedures that do not harm the embryos, and any resulting litter will be maintained until adulthood and used in this protocol (see Step 4).
- After parturition and weaning of the litter, the mother will not mate again and will be humanely killed by an appropriate Schedule 1 method.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 3: Transgene Induction (Adult Mice or Pregnant Female) (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Either non-pregnant adult mice or pregnant mice may be used in this step.

Gene Induction : Except for untreated controls, mice may be administered trans-gene inducing or deleting agents (such as tamoxifen) or vehicle control, at any stage, by one or more of the following routes on up to 5 occasions (minimum 24h interval).

a. Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

b. Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Animals receiving the trans-gene inducing or deleting reagent, tamoxifen, may experience weight loss during the first 1-2 days of administration. The likely incidence of this occurring is dependent on the genetic strain of the mice but is not uncommon. These mice typically regain weight back to base-line within the next 2-5 days and display no harmful side effects.

Doses of tamoxifen in pregnant female animals may induce abortion during the early stages of gestation.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for the first two weeks, or until weight is stable. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

Male mice given tamoxifen will be monitored for testicular swelling daily during the days of administration and for 48 hours afterwards.

Animals will be dosed later in the pregnancy where possible or co-administration of progesterone will be attempted where early dosing is required.

All procedures and monitoring will follow the UBS tamoxifen guidance developed by the NVS, ensuring that refinements, dosing, and welfare measures are implemented in accordance with expert veterinary advice.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Animals showing 15% weight loss (from the animal's initial weight measurement when dosing begins) measured for 2 consecutive days will be killed immediately by an appropriate Schedule 1 method.

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Testicular swelling causing more than mild discomfort (difficulty walking or changed activity) in male mice treated with tamoxifen will be killed immediately by an appropriate Schedule 1 method.

Pregnant female mice showing any signs of ill health such as subdued behaviour, hunched posture or pilo-erection will be killed immediately by an appropriate Schedule 1 method.

Step 4: Littering Down & Maintenance (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

In a small number of cases (less than 5%), pregnant females that have received substances during gestation (see Step 2) will be allowed to litter naturally, and their offspring will either be humanely killed for tissue collection or allowed to develop to adulthood and used within this protocol.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Typically we do not expect any adverse effects but due to the increased transient stress associated with substance administration we may have increased numbers of spontaneous pregnancy terminations.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

To minimise the potential for adverse effects, substances will be administered at low doses. Timed matings will be used to allow increased monitoring during the expected littering period, thereby reducing the risk of unanticipated birthing complications.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Dams and litters will be monitored closely during gestation and the peri-parturient period. The dam will be humanely killed using a Schedule 1 method if she shows signs of distress, complications during parturition, or deterioration in general condition that cannot be promptly alleviated. Signs of distress may include, but are not limited to: reduction in activity, abnormal posture or gait, reduced food or water intake, behavioural signs consistent with pain or discomfort (e.g. restlessness or reduced responsiveness).

Pups will be humanely killed using a Schedule 1 method if they show evidence of compromised welfare, including failure to suckle, hypothermia or failure to thrive (e.g. reduced activity compared to littermates or delayed weight gain).

Step 5: Tissue Biopsy (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Tissue biopsy to determine genetic status by one of the following methods:

• ear biopsy (AA/ABL)

• blood sampling (AA/ABL)

• hair sampling (AA/AB)

• mouth swabbing (AA)

Rarely, due to technical problems during analysis, a second sample may be taken using the least invasive method.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 6: Non-Invasive Imaging (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only non-pregnant adult mice will be used in this step.

Non-Invasive Imaging: Animals may be fasted for up to 4 hours prior to imaging - See Step 'Fasting Prior To Imaging' (AA). Animals may be assessed by any or a combination of non-invasive imaging methods (for example, but not limited to):

• bioluminescence imaging (BLI) (AB),

• fluorescence imaging (FLI) (AB),

• MRI (AB/AC) with fasting,

• MSOT (AB/AC) with fasting,

• ultrasound (AB) with fasting,

• PET/SPECT (AB/AC) with fasting

BLI, FLI and ultrasound imaging typically takes a maximum of 15 min and may be conducted up to once per week or once every 2 weeks throughout some long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 12. In addition, BLI, FLI and ultrasound imaging may be used daily for short term monitoring of administered agents (including cells).

MSOT measurements typically take about 30 min and may be conducted up to twice per week or once every 2 weeks throughout long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 10.

MRI and CT measurements of tissue anatomy, where these do not involve injection of an imaging agent, typically take about 30 min and may be conducted weekly

throughout experiments which can last up to 3 months. The total number of imaging examinations will not exceed 12. MRI, CT and PET/SPECT imaging following injection of an established or novel imaging agent usually take under 2 hours and may be conducted for a maximum of 5 times for a maximum period of 2 weeks and no more than twice per day (AB/AC). Imaging may be repeated on the same animal but typically, this will not exceed five sessions within any 2 week period, where each imaging session will not exceed 5 hours. Typically, there will be only one imaging session per day. Occasionally it may be necessary to conduct two imaging sessions in one 12 hour period, in which case each imaging session will not exceed 2 hours.

Imaging agents may be administered via one of the following routes prior to or during imaging:

a) Intra-peritoneal, with or without cannulation, maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

b) Intravenous, with or without cannulation, maximum of 20 ml/kg bolus injection or constant infusion via a cannulated vein, for a maximum of 3 hours [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period, and a maximum of 10 injections in 3 months.

c) Subcutaneous, a maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

d) Oral gavage, a maximum of 20 ml/kg bolus injection [AA]. Maximum frequency of 5 times in any 2 week period and a maximum of 10 times in 3 months.

Animals will undergo a maximum of two imaging sessions per week in total, irrespective of imaging type or combination of imaging modalities. After each imaging session and recovery, animals will be assessed to ensure they are fully alert, behaving normally, and showing no adverse effects. Imaging on a consecutive day will only proceed if the animal meets all of these criteria.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 7: Cell Labelling (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only non-pregnant adult mice will be used in this step.

Cell labelling: Mice may be administered labelling agents (such as EdU or BrdU) via intra-peritoneal injection or sub-cutaneous (AA) on a single occasion. [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 8: Fasting Prior To Imaging (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only non-pregnant adult mice will be used in this step.

Animals will be weighed to establish a baseline body weight prior to any fasting. Food restriction will then be applied for up to 4 hours before imaging, with no more than two such fasting periods per week. After each imaging session, animals will be provided with food ad libitum. A further fasting period will only be undertaken once the animal's body weight has returned to its baseline level. Water will be available at

all times. Food intake and body weight will be closely monitored to ensure that body weight does not fall below 85% of baseline.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Short-term food restriction may result in transient hunger and a temporary reduction in body weight (typically ≤10% of baseline). Minor weight loss is expected to be short-lived, with body weight returning to baseline within 24 hours following re-feeding. The incidence and severity of adverse effects are expected to be low due to the limited duration and frequency of fasting, continuous access to water, and close monitoring of body weight and food intake.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for a week, or until weight is stable following food restriction. During fasting, body weight will be recorded prior to each session to ensure weight loss does not exceed 15% of baseline. Fasting will be limited to a maximum of 4 hours and no more than two sessions per week, with a further session only undertaken once body weight has returned to baseline. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Mice exhibiting a 15% weight loss will be immediately killed by a schedule 1 method.

Step 9: Killing by Schedule 1. (mandatory)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing by a schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 3: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 3: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Animals will be administered trans-gene inducing or deleting reagents (tamoxifen) on up to 5 occasions (minimum interval 24 hours), typically via intraperitoneal injection (when trans-gene induction is time-sensitive) and occasionally by oral gavage (when timing is less critical). Some animals may experience temporary weight loss following administration; however, body weight typically returns to baseline within 2–5 days. In a small proportion of cases (less than 5%), pregnant females will be allowed to litter

naturally following tamoxifen administration, and offspring will either be humanely killed for tissue collection or allowed to develop to adulthood and used within this protocol.

Most (~95%) adult mice (excluding timed-pregnant females) will undergo a digit tip amputation procedure under general anaesthesia. Each surgery lasts less than 10 minutes per animal and includes an injection of peri-operative analgesia at standard doses and via standard routes. In some cases, the procedure may be repeated up to five times. Upon recovery, animals will be returned to their cages. Animals are expected to be active and display normal behaviour (normal posture, eyes bright and open, good muscle tone and social behaviour) following surgery. Animals that received non-regenerative amputations (removal of approximately 3-4mm of the distal digit tip) are expected to display minimal swelling within the digit at 7 - 14 days post amputation and this swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the natural behaviour of the mice or affect their gait in any way.

Occasionally (~5%), some animals may receive cell-labelling reagents (EdU) via a single intra-peritoneal injection, typically administered 24 hours before tissue collection (which occurs 14–84 days after surgery). This is expected to cause only brief discomfort and no lasting harm. In addition, adult mice may (~5%) undergo non-invasive tissue imaging on up to 12 occasions, with a minimum interval of 12 hours between sessions. This imaging schedule generally includes one fluorescence imaging session to visualise labelled cells, followed by weekly MRI sessions to assess changes in tissue architecture.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Any animal will be immediately killed by a Schedule 1 method if it shows signs of suffering that in any way compromises normal behaviour but only when these are not those expected as step related adverse effects. In the case of individual animals or particular scientific interest, advice will be sought promptly from the Home Office Inspector.

Examples of clinical signs that would indicate compromised welfare include, but are not limited to:

- Marked reduction in normal activity, exploration, or social interaction
- Reduced food or water intake, dehydration, or weight loss of 15% that persists for more than 48 hours
- Poor coat condition, pilo-erection, or hunched appearance
- Limping or reluctance to bear weight on the limp with amputated digits
- Excessive toe licking

Protocol 3: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Phenotypic data on how specific cell or gene modulation affects fibrosis and regeneration in the murine digit tip.

Will this protocol generate quantitative data?

Yes

Will your experimental design be determined by a regulatory guideline?

No

Where relevant, explain how and when pilot studies will be used.

Pilot studies involving no more than three animals per condition will be conducted to generate preliminary data and to optimise procedures and techniques before commencing large-scale experiments. Specifically, pilot experiments will be performed when modulating particular genes or cell populations to determine the effective dose and timing of transgene-deleting or -inducing reagents, ensuring that these conditions are both effective and do not compromise animal welfare. In addition, this study will use commercially available, previously validated genetically altered mouse lines. Accordingly, the initial doses of transgene modulating reagents will be set at the lowest levels reported in the literature to produce a biological effect.

How will you choose different experimental groups?

For example, controls, dose levels, satellites etc.

Animals will be assigned to either the control or experimental group based on their genotype.

How will you choose control groups?

Provide a robust scientific justification for controls with significant suffering such as sham surgery controls or untreated infected controls.

Each experiment will include a control group of mice that will be directly compared with the experimental group. The experimental group may carry up to three alleles of interest: a cell-specific Cre recombinase, a TdTomato reporter, and Diphtheria Toxin A (DTA). Multiple control groups including 1) untreated Cre recombinase; TdTomato reporter; DTA, 2) treated Cre recombinase; TdTomato reporter, and 3) treated DTA; TdTomato reporter) will be used only in pilot experiments. Including an untreated control group in pilot studies is essential to confirm that inducible gene expression is tightly regulated. The two treated control groups will then be used to verify that both yield equivalent results, enabling a reduction in the number of control groups required for full-scale experiments.

How will experiments and data analysis be randomised and blinded?

Prior to conducting experiments, animals will be assigned to either control or experimental group based on their consequent genotype. The control and experimental group of animals will then be uniquely coded (for example by assigning each group a number and obscuring genotyping information) by an independent person who is not associated with the study, and the coded information will be placed in an envelope until the experiment is completed. Where possible, the lab member conducting the surgical intervention and treatment will not participate in analysis of the collected data. Additionally, data analysis will be carried out in a blinded fashion by assigning further codes to the samples upon collection by an independent person not associated with the study. Those analysing the data will, when necessary, be informed as to which animals are grouped together in order to make group comparisons, but will not be aware if the group represents controls or experimental mice in order to avoid any subjective decisions about applying data transformation to the outcome or in handling outliers.

How will you minimise variables to ensure reproducibility?

We will aim to minimise variables in this protocol by using inbred, previously validated genetically altered mice for experiments and will avoid exposing the laboratory mice to unnecessary stress by employing good handling practises. Bias will be avoided through good experimental design including incorporation of blinding practises both during experimental procedures and analysis of the data. Additionally, accurate records will be maintained of all work conducted under this project licence and methods will be reported responsibly including descriptions of all variables such as mouse age, number, strain, housing conditions and appropriate statistical analyses.

How will you determine group sizes?

You should reference POWER calculations you have made, if relevant.

Group sizes were determined using POWER calculations for an unpaired t-test whereby the biological effect size (using proportional differences) is expected to be 1. 5 to 2.0 fold and standard deviations approximately 20%, based on published literature. The power was set to 90% and alpha 5% (two sided) resulting in group sizes of n = 8 animals per condition. To accommodate occasional animal loss due to experimental complications and unexpected variability, a sample size of n = 10 was subsequently selected for each group (control and experimental). Additionally, advice on group size calculations and experimental design has been sought from a local bio-statistician.

How will you maximise the data output from the animals you use on this protocol?

To maximise the information gained from a single animal, experiments will be designed in such a manner, that where possible, the digits from one hindlimb will be amputated and digits on the contralateral side will be utilised as uninjured control digits to verify that ablation of this specific cell population does not affect tissue homeostasis. Since these experiments only require the hindlimb digits, we will share any remaining tissues of interest with other scientists, alleviating the need for further breeding and animal usage. Additionally, all experimental approaches and data will be disseminated to the scientific community to avoid unnecessary duplication and to share any new findings.

Protocol 3: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Murine digit tip regeneration mimics the regenerative process observed when the distal phalanx in humans is injured. This model is therefore the most appropriate scientific approach for studying mammalian regeneration in the lowest possible sentient species with the broadest applications. To investigate the contribution of specific genes or cell lineages to adult regeneration, gene expression is induced during embryonic development via pregnant adults. This allows lineage tracing or functional manipulation of cells from development through to adulthood. Normally, the offspring are then used for regeneration experiments, capturing developmental influences on adult regenerative capacity and ensuring findings are relevant to a wider range of physiological states.

Additionally, the use of transgenic animals that carry a cytotoxic gene under the control of a tissue-specific promoter, currently represents the most efficient method of ablating target cells to analyse cell function. Inducible systems, such as tamoxifen-driven Cre recombination, permit precise temporal and spatial control of gene activation or lineage labelling during embryogenesis, enabling the study of developmental contributions to adult regeneration.

Individual digit tip amputations within the same mouse do show some variability in the rate of regeneration. Therefore, in order to minimise these effects, and allow for reproducibility, the measurements from 3 digits per condition are combined and averaged. Some mice may have both regenerative and non-regenerative amputations performed in the same animal (six digits amputated in total). This will allow for direct comparison of the role of targeted cell populations or genes in both conditions while minimising experimental variability between animals. Using offspring derived from pregnant adults in this protocol ensures that embryonic manipulations are incorporated while reducing the total number of animals required and maintaining the well-being and normal behaviours of the mice.

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b) the most refined for the purpose?

Surgical procedures will be carried out using aseptic technique which meets at least Home Office Minimum Standards of Aseptic Surgery and the LASA Guiding Principles.

Peri-operative analgesia will be provided and where necessary post-operative analgesia will be given; agents will be administered at doses and frequencies agreed upon in advance with the Named Veterinary Surgeon.

Post-surgical monitoring will be conducted following surgery, using an approved post-surgical monitoring chart as agreed upon in advance with the Named Veterinary Surgeon.

Amputation surgeries will be restricted to the hindlimb digits 2 through 4 to ensure normal grooming routines, movement or balance are not affected. This surgery does not appear to affect the animal's well-being or stop it from carrying out normal behaviour.

Trans-gene inducing or deleting substances will be pre-screened in pilot experiments to obtain an indication of the dose that is likely to be effective. As this study will only use previously-validated compounds (tamoxifen), the starting dose will be the minimum to have an effect according to the literature.

All animals receiving trans-gene inducing or deleting substances will be carefully monitored to ensure the health and well-being of the animals does not suffer. Supportive care will be given to any animals displaying weight loss and the animals carefully monitored.

For each model and/or method, what is the scientific need for the expected clinical signs?

The majority of adult mice that undergo this procedure are not expected to display any clinical signs. Those animals that receive non-regenerative amputations (amputations that remove 3-4mm of the second phalanx) will present with minimal swelling of digit as part of the natural wound healing process. This tissue response represents one of the main distinctions between regenerative and non-regenerative processes.

As mentioned in Protocols 1 & 2, in my previous laboratory we worked closely with a veterinary surgeon to ascertain the response of adult mice receiving digit tip amputation. Over the past five years, through our own experience, we have consistently observed the following parameters during recovery from anaesthesia:

- Adult mice were active and gait was observed as normal (some mice have been observed to climb on the cage bars immediately following recovery).
- Adult mice did not exhibit audible vocalisation.
- Eyes were bright and open (did not observe ‘grimacing’) and posture was normal (not hunched).
- Respiratory rate was observed to be normal (this was ascertained by observation and no device was used to measure this).
- Prior to surgeries adult mice were given pre-emptive analgesia with analgesic effects lasting up to 12-24 hours. The mice were subsequently observed for a further seven days following surgery (daily observations).

We also observed the following:

- Food and water intake were normal and no weight loss was observed.
- Muscle tone was good and coat appeared well cared for (no grooming deficits).

- Animals remained social and continued to interact with each other.

Why scientifically do the animals need to suffer to this degree?

Adult mice that undergo this surgical procedure appear to recover within 5 - 10 minutes following surgery and resume normal activities. Although suffering appears to be minimal the process of digit tip amputation combined with cell/gene specific ablation is necessary in order to pursue my chosen scientific questions.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

Clinical signs are generally not expected, and animals typically recover quickly from procedures. Following tamoxifen administration, a transient weight loss is occasionally observed dependent on the strain. In some animals this may exceed 15% for a short period but is reversible with supportive care, with animals stabilising within 24 hours. Immediate euthanasia at 15% weight loss would result in the unnecessary loss of animals that would otherwise recover fully, increasing animal use without welfare benefit. An upper limit of 20% weight loss is therefore set as an immediate humane endpoint, beyond which recovery is unlikely and welfare would be compromised.

In animals undergoing non-regenerative amputations, the associated inflammatory response and minimal swelling during wound healing are intrinsic to addressing the scientific objectives and cannot be avoided.

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

When using trans-gene inducing or deleting agents (i.e. tamoxifen) for the first time, doses will be chosen to minimise the risk of toxicity based on the available literature and validated in small pilot study.

As this study will only use previously-validated reagents, all administered substances are not expected to cause adverse effects. However, if animals show, unexpectedly, any clinical signs such as hunched posture, pilo-erection or subdued behaviour, these animals will be immediately killed by a schedule 1 method.

To ensure sterility, all trans-gene inducing or deleting agents (i.e. tamoxifen) will be prepared in a Biosafety cabinet using sterile equipment and sterile filtered solutions.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

For most substances, the dose, route, frequency, and duration in mice are well documented. Where data are lacking, short-term pilot studies using low doses in a small number of mice (typically 2–3) will be conducted to identify a safe and effective regimen. Dosing volumes and frequencies will be minimised and follow standard guidelines (UBS and LASA) for each route of administration (e.g., subcutaneous or intra-peritoneal) as documented below. Pilot studies allow adjustment of doses before treating the remainder of animals in each experimental group. Maximum volumes and frequencies exclude administration for rehydration, anaesthesia, or analgesia.

Mouse	Daily Max. Volume (ml/kg)	Max. No./Day <10 Days	Max. No./Day >10 Days	Max. No. Injections
S/C (Subcutaneous)	20 ml/kg	3	2	24
I/P (Intra-peritoneal)	20 ml/kg	3	1	30
I/V (Intravenous)	0.8 ml over 2 min	2	1	14
Oral Gavage	20 ml/kg	3	2	30

Protocol 4

Title

Transplantation of Tumour Cells in the Digit Tip

Protocol 4: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To determine how the regenerative capacity of the mammalian digit tip affects susceptibility to tumour formation.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

This protocol was categorised as moderate severity when taking into account the animal's cumulative experiences and life stage (adult). All animals will receive transplantation of tumour cells through direct injection into the digit tip, followed by a single superficial surgical procedure under general anaesthesia. 50% of our animals are likely to be administered substances (most often through direct injection, usually on one occasion, into the digit tip). Animals will be allowed sufficient time to recover between the initial surgical procedure and the aseptic digit tip injection. For each surgical procedure or when needed for restraint purposes, animals will be administered anaesthesia whereby induction is rapid and the duration will be less than 10 minutes per animal. Some animals may also be administered labelling reagents (such as EdU, BrdU or tamoxifen) on up to four occasions. A small number of animals may also undergo imaging procedures that are non-invasive (e.g. MRI or CT), likely on six occasions. A very small subset an animals may be placed in an incubator at 42 degrees for a short period of time (no longer than 15 minutes) to induce expression of heat sensitive proteins.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

Protocol 4: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

750

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

- Protocol 5: Breeding and maintenance of genetically altered mice (mild)
- Other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 4: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms driving regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes. Examples include fluorescent reporter lines (e.g. Floxed tdTomato mice), Cre driver lines (e.g. PdgfraCreERT2 mice) or conditional alleles (e.g. Floxed Cas9 mice).

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 4: Steps

Step 1: Digit Tip Amputation - Single (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Amputation surgeries will be performed, on a single occasion, on the second through fourth digits of either one or both hind limbs (AB) in one of the following planes:

a. amputation of the distal one third of the terminal phalanges (removing approximately 1-2mm)

b. amputation of the distal one third of the second phalanges (removing approximately 3-4mm)

The maximum number of digits amputated per animal = 6.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Digit tip amputation surgeries remove a small amount of bone and will therefore cause the animal transient pain. Amputation surgeries of the distal one third of the second phalanges that remove 3 - 4mm of the digit are expected to display minimal swelling within the digit at 7 - 14 days post amputation. These effects have been observed in nearly all mice that receive this degree of digit tip amputation as part of

the wound healing process and the swelling naturally resolves by 21 days post amputation without any intervention. This swelling does not appear to impede the natural behaviour of the mice, including grooming activities, mobility or balance in any way.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

All animals will be monitored closely for adverse surgical effects (including swelling of the digit tip outside of the expected range, changes in gait, reduced activity, or persistent licking of the affected digits) and pain controlled using both pre-emptive and post-operative analgesics as required and as recommended by the NVS. Animals will also be provided with soft bedding and additional warmth, and food and water placed on the cage floor so that mice can access them without needing to rear up.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions including administration of analgesics.

Any animals showing signs of pain or general distress such as subdued behaviour, lameness or excessive toe licking, in which the animals do not show improvement with mild interventions within 24 hours will be killed immediately by a Schedule 1 method.

If post-operative swelling of the digit persists beyond the expected recovery period, worsens, or begins to impair normal behaviour or mobility, and does not improve with minor supportive measures within 48 hours, the animal will be immediately killed using an appropriate Schedule 1 method.

Step 2: Tumourigenic Transplantation (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Except for untreated control animals, mice will receive cell injections (e.g. osteosarcoma or other tumourigenic cell lines, cells carrying oncogenic mutations, or control cells) directly into digits two to four of the hindlimbs (AB). Injections may be performed on up to two occasions, with a maximum volume of 5 µl per digit. Cell transplantation may occur before amputation, at the time of amputation, or during the healing phase.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

When tumourigenic cells are transplanted into non-regenerative digit amputations, tumours are likely to develop in approximately 80% of animals. These tumours can grow to a substantial size (approximately 0.5-1cm) and are typically associated with local swelling of the affected digit. The swelling and tumour mass may slightly impede the animal's gait, although normal mobility, grooming, and other behaviours are generally maintained. The degree of discomfort is expected to be mild to moderate, and suffering is anticipated to persist for as long as the tumour is present. Animals will be closely monitored, and any signs of excessive discomfort or distress will be managed according to the licence's humane endpoints.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

All animals receiving tumour cell transplants will be monitored closely for any signs of adverse effects, including abnormal swelling, tumour formation, changes in gait, or other indicators of pain or distress. Monitoring will be carried out at least daily, with increased frequency during periods of rapid tumour growth or if clinical signs suggest discomfort.

A scoring sheet will be used to systematically record observations for tumour size, swelling, gait, mobility, grooming, activity, and overall condition. These records will be used to determine whether additional interventions are required and whether humane endpoints have been reached. Score sheets will be maintained for a minimum of 7 days post-intervention. Animals reaching a cumulative score of 10 or more, or a score of 4 in any single category, will be humanely killed by a Schedule 1 method to prevent further suffering. An example of the score sheet criteria is provided below.

Pre-emptive and post-procedural analgesics will be administered as recommended by the Named Veterinary Surgeon (NVS) to minimise pain and distress. Animals will

also be provided with soft bedding, additional warmth and easy access to food (soft mash on the bottom of the cage) to lessen any potential suffering or discomfort.

Parameter	**0**	**1**	**2**	**4 (Endpoint)**
Weight Loss	≤5%	>5–10%	>10–15%	>15%
Posture	Normal	Mild hunching at rest	Hunching during movement	Persistent hunching; reluctance to move
Provoked Behaviour	Moves away quickly	Slower/reluctant movement	Moves after long pause	No movement away
Fur Condition	Normal	Mild ruffling	Moderate ruffling	Poor coat condition with evidence of reduced self-care
Mobility	Normal gait	Mild limp	Clear limp, partial weight avoidance	No weight bearing OR failure to improve by 72 hours
Swelling	None	Mild swelling	Moderate swelling	Swelling that interferes with normal behaviour or function that does not improve with treatment by 72 hours
Tumour Size	None	Small, localised	Moderate, measurable	Large (>1cm)
Feeding Behaviour	Normal intake	Some intake	Minimal intake for 12 hours	No intake for 24 hours

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of post-transplant complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions including administration of analgesics.

Following our post-surgical scoring system, if animals reach a cumulative score of 10 or above or when a score of 4 is reached for any individual criteria the animal will be immediately killed by a Schedule 1 method. Animals approaching, but not yet

reaching, these thresholds will be monitored at least twice daily, and if high scores persist for more than 48 hours without improvement, humane endpoints will be applied.

Tumours reaching a maximum size of 1 cm in diameter or those that impair mobility, ulcerate, or cause signs of systemic illness, will also trigger humane euthanasia immediately using a Schedule 1 method.

Step 3: Administration of Substances - Optional (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

With the exception of untreated controls, mice will receive substances or cells (e.g. growth factors, ligands, function-blocking antibodies, chemical inhibitors, or viral over-expression vectors). Control substances/cells may also be administered. Up to three agents may be given, in combination where possible, to minimise the number of dosing occasions. All administrations will be performed using the least invasive route available through either:

a. Local administration: Direct injection into the second through fourth hindlimb digits (AB). [Max vol. 5ul/digit]. Direct injections into the digit will be performed no less than 3 days following a prior surgical procedure.

b. Systemic administration: Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

c. Systemic administration: Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

d. Systemic administration: Intravenously (AA/AB), maximum treatment = once a day up to 5 days [Max vol. 10ml/kg].

e. Systemic administration: Orally (e.g. in diet, drinking water) (AA), maximum treatment = 8 weeks.

Additional animals may be treated with administration vehicle only.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 4: Transgene Induction (optional)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Gene Induction : Except for untreated controls, mice may be administered trans-gene inducing or deleting agents (such as tamoxifen) or vehicle control, at any stage, by one or more of the following routes on up to 5 occasions (minimum 24h interval).

a. Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

b. Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Animals receiving the trans-gene inducing or deleting reagent, tamoxifen, may experience weight loss during the first 1-2 days of administration. The likely

incidence of this occurring is dependent on the genetic strain of the mice but is not uncommon. These mice typically regain weight back to base-line within the next 2-5 days and display no harmful side effects.

Oral gavage may cause stress, transient oesophageal irritation, and, rarely, mortality (~5%) at high doses. IP injections may cause temporary abdominal or scrotal swelling.

In addition, animals receiving tamoxifen via oral gavage or intraperitoneal (IP) injection may show transient reduced activity, hunched posture, and a ruffled or greasy coat, typically within the first 1–7 days.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for the first two weeks, or until weight is stable. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

Male mice given tamoxifen will be monitored for testicular swelling daily during the days of administration and for 48 hours afterwards.

All procedures and monitoring will follow the UBS tamoxifen guidance developed by the NVS, ensuring that refinements, dosing, and welfare measures are implemented in accordance with expert veterinary advice.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Animals showing 15% weight loss (from the animal's initial weight measurement when dosing begins) measured for 2 consecutive days will be killed immediately by an appropriate Schedule 1 method.

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Testicular swelling causing more than mild discomfort (difficulty walking or changed activity) in male mice treated with tamoxifen will be killed immediately by an appropriate Schedule 1 method.

Step 5: Cell Labelling (optional)

Repeated from protocol 1

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Cell labelling: Mice may be administered labelling agents (such as EdU or BrdU) via intra-peritoneal injection or sub-cutaneous (AA) on a single occasion. [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 6: Fasting Prior To Imaging (optional)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Animals will be weighed to establish a baseline body weight prior to any fasting (AA). Food restriction will then be applied for up to 4 hours before imaging, with no more than two such fasting periods per week. After each imaging session, animals will be provided with food ad libitum. A further fasting period will only be undertaken once the animal's body weight has returned to its baseline level. Water will be available at all times. Food intake and body weight will be closely monitored to ensure that mice do not lose more than 15% of their baseline body weight.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Short-term food restriction may result in transient hunger and a temporary reduction in body weight (typically ≤10% of baseline). Minor weight loss is expected to be short-lived, with body weight returning to baseline within 24 hours following re-feeding. The incidence and severity of adverse effects are expected to be low due to the limited duration and frequency of fasting, continuous access to water, and close monitoring of body weight and food intake.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for a week, or until weight is stable following food restriction. During fasting, body weight will be recorded prior to each session to ensure weight loss does not exceed 15% of baseline. Fasting will be limited to a maximum of 4 hours and no more than two sessions per week, with a further session only undertaken once body weight has returned to baseline. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Mice exhibiting a 15% weight loss will be immediately killed by a schedule 1 method.

Step 7: Non-Invasive Imaging (optional)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Non-Invasive Imaging: Animals may be fasted for up to 4 hours prior to imaging - See Step 'Fasting Prior To Imaging' (AA). Animals may be assessed by any or a combination of non-invasive imaging methods (for example, but not limited to):

• bioluminescence imaging (BLI) (AB),

• fluorescence imaging (FLI) (AB),

• MRI (AB/AC) with fasting,

• MSOT (AB/AC) with fasting,

• ultrasound (AB) with fasting,

• PET/SPECT (AB/AC) with fasting

BLI, FLI and ultrasound imaging typically takes a maximum of 15 min and may be conducted up to once per week or once every 2 weeks throughout some long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 12. In addition, BLI, FLI and ultrasound imaging may be used daily for short term monitoring of administered agents (including cells).

MSOT measurements typically take about 30 min and may be conducted up to twice per week or once every 2 weeks throughout long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 10.

MRI and CT measurements of tissue anatomy, where these do not involve injection of an imaging agent, typically take about 30 min and may be conducted weekly throughout experiments which can last up to 3 months. The total number of imaging examinations will not exceed 12. MRI, CT and PET/SPECT imaging following injection of an established or novel imaging agent usually take under 2 hours and may be conducted for a maximum of 5 times for a maximum period of 2 weeks and no more than twice per day (AB/AC). Imaging may be repeated on the same animal but typically, this will not exceed five sessions within any 2 week period, where each imaging session will not exceed 5 hours. Typically, there will be only one imaging session per day. Occasionally it may be necessary to conduct two imaging sessions in one 12 hour period, in which case each imaging session will not exceed 2 hours.

Imaging agents may be administered via one of the following routes prior to or during imaging:

a) Intra-peritoneal, with or without cannulation, maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

b) Intravenous, with or without cannulation, maximum of 20 ml/kg bolus injection or constant infusion via a cannulated vein, for a maximum of 3 hours [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period, and a maximum of 10 injections in 3 months.

c) Subcutaneous, a maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

d) Oral gavage, a maximum of 20 ml/kg bolus injection [AA]. Maximum frequency of 5 times in any 2 week period and a maximum of 10 times in 3 months.

Animals will undergo a maximum of two imaging sessions per week in total, irrespective of imaging type or combination of imaging modalities. After each imaging session and recovery, animals will be assessed to ensure they are fully alert,

behaving normally, and showing no adverse effects. Imaging on a consecutive day will only proceed if the animal meets all of these criteria.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 8: Heat Shock (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Animals will be placed in an incubator at 42 degrees for up to 15 minutes to enable induction of heat-sensitive plasmids (AA).

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

The expected adverse effects of this step are mild and transient stress or discomfort resulting from brief exposure to a heat shock at 42°C for up to 15 minutes. Signs may include temporary increased activity, restlessness, or mild vocalisation. The incidence is anticipated to be low, and any discomfort is expected to resolve immediately once the animal is returned to normal conditions. No lasting harm is expected.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be closely observed throughout the procedure for any signs of distress, including abnormal behaviour, vocalisation, or attempts to escape. The duration and intensity of the heat exposure are minimised to limit discomfort. If any signs of undue stress or distress are observed, the animal will be immediately removed from the incubator, returned to normal conditions, and provided with supportive care as needed.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Animals exhibiting signs of distress that do not resolve upon removal from the heat exposure within 30 minutes, or any other indicators of suffering such as over-grooming or abnormal activity (hyper-active or lack of activity) will be humanely killed using an appropriate Schedule 1 method.

Step 9: Killing by Schedule 1. (mandatory)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing by a schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 4: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 4: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Typically, animals in this protocol will receive tumourigenic cell transplants via direct injection into the digit tip under general anaesthesia for restraint purposes. Tumour cells transplanted into the proximal regions of the digit (P2) are expected to grow to a moderate size within approximately two weeks and may be associated with local swelling; however, this does not appear to impede the natural behaviour of the mouse.

Subsequently, all mice will undergo digit tip amputation, a surgical procedure performed under general anaesthesia (typically less than 10 minutes per animal) with peri-operative analgesia administered according to standard routes and dosages. Animals are expected to make a rapid recovery, often resuming normal activities such as climbing within 30 minutes post-surgery, and typically do not require further post-operative analgesia. Following recovery, animals will be returned to their group-housed cages.

Animals undergoing non-regenerative amputations (removal of approximately 3–4 mm of the distal digit tip) are expected to display minimal digit swelling between 7 and 14 days post-amputation. This swelling typically resolves naturally by 21 days and does not appear to affect gait or normal behaviour.

Approximately 50% of mice will be administered additional substances, either by one of the following methods or occasionally by a combination of routes:

a. **Local administration:** Adult mice will be placed under general anaesthesia (typically less than 15 minutes per animal) for restraint, and substances will be injected directly into the digit tip on a single occasion. Peri-operative analgesia will be administered according to standard routes and dosages. Recovery from anaesthesia is expected to be rapid, and post-operative analgesia is typically not required.

b. **Systemic administration**: Substances may occasionally be administered systemically by oral gavage, intravenous, or intraperitoneal injection on up to five occasions, with a minimum interval of 24 hours between doses.

c. **Dietary or drinking water administration:** Substances may occasionally be administered in the diet or drinking water. Where substances are taste-aversive, they will be masked (for example, by incorporation into chocolate-flavoured pellets or by addition of sucrose to the drinking water). All animals will be monitored throughout the experiment to ensure general health and wellbeing, including adequate food and water intake and the absence of behavioural or health complications.

d. **Non-invasive imaging:** Adult mice may occasionally undergo non-invasive imaging on up to 12 occasions, with a minimum interval of 12 hours between sessions. This may include fluorescence imaging to visualise implanted cells, followed by weekly MRI sessions to assess tissue architecture.

The maximum number of anaesthetic events for any individual animal, combining imaging and surgical procedures, is 13 occasions. When multiple routes of systemic administration are available, the least invasive route will be used first (e.g., administration via food or water rather than intravenous injection).

In addition to the initial cell transplantation, digit tip amputation, and administration of substances, some mice (~5%) may also receive cell labelling reagents (e.g., EdU) on a single occasion or tamoxifen up to four times to induce fluorescent reporters, using standard routes and dosages. Some mice (~2%) may also be placed in an incubator at 42 degrees on a single occasion to induce expression of heat-sensitive plasmids.

Mice will remain in group-housed cages until tissue collection, which will occur between 3 and 56 days following the initial digit tip amputation surgery.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Any animal will be immediately killed by a Schedule 1 method if it shows signs of suffering that in any way compromises normal behaviour but only when these are not those expected as step related adverse effects (e.g. if tumours start to impede the animal's day to day activities).

Examples of clinical signs that would indicate compromised welfare include, but are not limited to:

- Marked reduction in normal activity, exploration, or social interaction

- Reduced food or water intake, dehydration, or persistent weight loss (no improvement within 72 hours) of 15%
- Poor coat condition, pilo-erection, or hunched appearance
- Limping or reluctance to bear weight on the limp with amputated digits
- Excessive toe licking

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Protocol 4: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Phenotypic data on the effects of regenerative capacity on tumour formation in the murine digit tip, including observations of tumour incidence, growth, and tissue responses following digit tip amputation.

Will this protocol generate quantitative data?

Yes

Will your experimental design be determined by a regulatory guideline?

No

Where relevant, explain how and when pilot studies will be used.

Pilot studies, using no more than 3 animals per condition, will be conducted to generate preliminary data and in order to allow for procedures and techniques to be perfected before embarking on large scale experiments. Specifically, we will perform pilot experiments when animals are to be injected with pharmacological agents or inhibitor substances to obtain an indication of the dose and the timing of the injections, that is likely to be effective.

How will you choose different experimental groups?

For example, controls, dose levels, satellites etc.

Animals will be randomly assigned to either the control or treatment group. The treatment, that is the pharmacological agents or inhibitor substances, will be selected according to preliminary data obtained from *in vitro* culture experiments and doses will be determined through pilot experiments, taking into account the effective reported dose levels in the literature.

How will you choose control groups?

Provide a robust scientific justification for controls with significant suffering such as sham surgery controls or untreated infected controls.

Each individual experiment will use a control group of mice that are contrasted directly to the experimental group of mice. For example, when injecting non-regenerative digits with a substance resuspended in 0.1% BSA, control non-regenerative digits will be injected with the same vehicle, 0.1% BSA. A sham surgery control will only be included in the event that the necessary vehicle control has not been tested in this system, or been reported in the literature to be innocuous. The inclusion of a sham surgery control would be limited to pilot experiments and is necessary to ensure that unknown vehicle controls alone cannot account for any observed effects and result in misleading or inaccurate conclusions.

How will experiments and data analysis be randomised and blinded?

Prior to each experiment, animals will be randomly allocated to treatment or control groups. For localised injections, the left or right digits will be assigned using a randomisation method (e.g. unique identifiers with group allocation determined by random number selection drawn out of a hat). For systemic administration, the entire animal will be randomly assigned to either experimental or control groups.

Treatments (e.g. agonists or inhibitors) will be coded by an independent individual not directly involved in the study. Codes will remain concealed (e.g. sealed envelope) until the experiment and data collection are complete.

Where possible, the researcher performing the surgical intervention or treatment will not be involved in subsequent data analysis. Data analysis will also be conducted under blinded conditions whereby samples will be re-coded by an independent individual not associated with the study prior to analysis. Analysts will only be informed of group structure when necessary for statistical comparison but will remain blinded to treatment identity. This approach minimises bias in both data collection and interpretation.

How will you minimise variables to ensure reproducibility?

We will aim to minimise variables in this protocol by using inbred mice from the same supplier such as Charles River Laboratories for all experiments and will avoid exposing the laboratory mice to unnecessary stress by employing good handling practises. Bias will be avoided through good experimental design including incorporation of randomisation and blinding practises both during experimental procedures and analysis of the data. Additionally, accurate records will be maintained of all work conducted under this project licence and methods will be reported responsibly including descriptions of all variables such as mouse age, number, strain, housing conditions and appropriate statistical analyses.

How will you determine group sizes?

You should reference POWER calculations you have made, if relevant.

Group sizes were determined using POWER calculations for an unpaired t-test whereby the biological effect size (using proportional differences) is expected to be 1. 5 to 2.0 fold and standard deviations approximately 20%, based on published literature. The power was set to 90% and alpha 5% (two sided) resulting in group

sizes of n = 8 animals per condition. To accommodate occasional animal loss due to experimental complications and unexpected variability, a sample size of n = 10 was subsequently selected for each group (control and experimental). Additionally, advice on group size calculations and experimental design has been sought from a local bio-statistician.

How will you maximise the data output from the animals you use on this protocol?

To maximise the information obtained from each animal, experiments will be designed so that, where possible, digits from one hindlimb receive the treatment while digits on the contralateral hindlimb serve as vehicle-treated controls. This within-animal design reduces variability and the number of animals required.

As only hindlimb digits are required for these studies, surplus tissues of scientific value will be shared with other researchers, reducing the need for additional breeding or animal use.

Furthermore, all experimental approaches and resulting data will be disseminated to the wider scientific community. This ensures transparency, prevents unnecessary duplication of work, and maximises the impact of findings generated from each animal.

Protocol 4: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Mice are selected as the experimental species because they are a well-established, genetically tractable model for studying both tumour biology and tissue regeneration, and they represent the lowest sentient species suitable for this work. Murine digit tip regeneration closely mimics the regenerative process observed when the distal phalanx is injured in humans, providing a relevant tissue environment in which to study tumour formation in regenerative versus non-regenerative contexts. Studying this process in adult mice ensures that the findings are applicable across a broad range of individuals, as many regenerative mechanisms present in younger animals are inactive in adults.

Tumour cell transplantation into the digit tip allows controlled initiation of tumour formation within a defined tissue context. The model is level-dependent: digits amputated proximally (non-regenerative) allow tumour development in a non-regenerative environment, whereas distal (regenerative) amputations provide a contrasting environment. This enables direct comparison of tumour incidence, growth, and morphology in regenerative versus non-regenerative tissue within the same animal.

Heat shock is applied by placing the mouse at 42°C for 15 minutes to induce the expression of genes that are temperature-sensitive. This method allows precise temporal control of gene activation, which is critical for dissecting the role of specific genes in tumour formation and regenerative processes. The use of heat shock in this

context provides experimental flexibility and mechanistic insight that cannot be achieved using constitutive gene expression models.

Individual digits may show some variability in regeneration or tumour growth, and small-volume injections (typically 1 µl) can contribute additional variability. To minimise these effects and ensure reproducibility, measurements from three digits per condition are combined and averaged. Using left versus right hindlimb digits for control and experimental conditions within the same animal further reduces inter-animal variability and allows a reduction in the total number of animals required.

Overall, the combination of mice, digit tip amputation, and tumour cell transplantation is the most appropriate scientific approach to investigate how regenerative capacity affects tumour formation under controlled, reproducible conditions, generating mechanistic insights that cannot be obtained in vitro or in less regenerative species.

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b) the most refined for the purpose?

Surgical procedures will be carried out using aseptic technique which meets at least Home Office Minimum Standards of Aseptic Surgery and the LASA Guiding Principles.

Peri-operative analgesia will be provided and where necessary post-operative analgesia will be given; agents will be administered at doses and frequencies agreed upon in advance with the Named Veterinary Surgeon.

Post-surgical monitoring will be conducted following surgery, using an approved post-surgical monitoring chart as agreed upon in advance with the Named Veterinary Surgeon.

Amputation surgeries will be restricted to the hindlimb digits 2 through 4 to ensure normal grooming routines, movement or balance are not affected. This surgery does not appear to affect the animal's well-being or stop it from carrying out normal behaviour.

Substances will be pre-screened in pilot experiments to obtain an indication of the dose that is likely to be effective. As this study will only use previously-validated compounds, the starting dose will be the minimum to have an effect according to the literature. Additionally, we will refer to the resources *"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* and *"Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider". Turner PV et al. J Am Assoc Lab Anim Sci. 2011 50(5): 600–613,* to aid in further refining the appropriate dosing regime for administered substances.

In the instance when substances are administered systemically, agents will be administered by the least invasive route in the first instance when multiple routes are available (e.g. Administered in the food or drinking water as opposed to intravenous injection).

A pain monitoring and scoring system will be introduced for this protocol with the help of the Named Veterinary Surgeon. This will allow us to ensure the welfare of our animals and may help to inform the use of toe clipping for identification purposes which is used overseas.

For each model and/or method, what is the scientific need for the expected clinical signs?

The scientific need for the expected clinical signs in this model arises from the objective of studying mechanisms that inhibit tumour development and testing novel therapies for cancer. Implantation of tumour cells into the digit tip provides a reproducible and accessible site for monitoring local tumour growth, vascularisation, and tissue response *in vivo*. This model allows the assessment of disease progression and treatment efficacy in a physiologically relevant context that cannot be replicated *in vitro* or in less severe models.

The expected clinical signs are directly related to local tumour growth and include mild to moderate swelling, redness, or thickening of the affected digit. These effects are necessary indicators of tumour establishment and progression, and provide measurable endpoints for evaluating therapeutic interventions. The changes are typically confined to the digit and do not impair mobility or normal behaviour. Systemic effects are not expected before the planned experimental endpoint, at which point animals will be humanely killed.

Animals will be monitored closely for signs of pain or distress, including swelling beyond the digit, ulceration, reduced mobility, or weight loss. Any animal showing signs of excessive tumour burden or systemic illness will be euthanised immediately in accordance with humane endpoints. Although this procedure is classified as moderate, the expected clinical signs are scientifically necessary to obtain meaningful data on tumour growth or impairment to growth and response to treatment, ultimately contributing to the development of new and more effective cancer therapies.

Why scientifically do the animals need to suffer to this degree?

Adult mice that undergo tumour cell transplantation into the digit tip followed by digit tip amputation typically recover quickly from anaesthesia and resume normal activities within a short period. Although local swelling and impaired gait may occur as a consequence of tumour growth, this level of clinical sign is necessary to achieve the scientific objectives of the study, which are to determine how regenerative capacity affects tumour formation and progression.

Some animals may experience mild discomfort due to swelling or the presence of the tumour, and this may occasionally require additional analgesia or anti-inflammatory support. All steps to minimise suffering have been employed, including careful surgical technique, peri-operative analgesia, and close monitoring to provide timely intervention if clinical signs worsen. The tumour growth and associated tissue changes are an inherent part of the model and cannot be avoided without compromising the experimental outcomes. The route and timing of administration of

cells and substances have been selected to minimise distress while still allowing the study to answer the scientific questions. The tumour lines chosen are not expected to metastasize.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

It is not possible to achieve the scientific outputs of this protocol using an earlier humane endpoint or without animals displaying any clinical signs, because the study requires the development and progression of tumour formation within the digit tip to assess the interaction between regenerative capacity and tumourigenesis. Tumour growth and the associated tissue changes, including swelling, are essential for evaluating how regeneration influences tumour incidence, size, and morphology.

While the presence of tumours may temporarily impair gait or cause local swelling, these effects are intrinsic to the biological processes under investigation and cannot be avoided without compromising the experimental objectives. Animals will be closely monitored, and analgesia or other supportive care will be provided as required to minimise discomfort. Humane endpoints have been defined to ensure that suffering is limited to the minimum necessary to achieve the scientific objectives.

Following tamoxifen administration, a transient weight loss is occasionally observed dependent on the strain. In some animals this may exceed 15% for a short period but is reversible with supportive care, with animals stabilising within 24 hours. Immediate euthanasia at 15% weight loss would result in the unnecessary loss of animals that would otherwise recover fully, increasing animal use without welfare benefit. An upper limit of 20% weight loss is therefore set as an immediate humane endpoint, beyond which recovery is unlikely and welfare would be compromised.

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

When using substances for the first time, doses will be chosen to minimise the risk of toxicity based on the available literature and validated in small pilot study. The starting dose used will be the minimum that was shown to have an effect according to the published literature.

All substances will be prepared in a Biosafety cabinet using sterile equipment and sterile solutions.

As this study will only use previously-validated compounds, all administered substances are not expected to cause adverse effects. However, if animals show,

unexpectedly, any clinical signs such as hunched posture, pilo-erection or subdued behaviour, these animals will be kept under close observation. If the signs do not resolve within 24 hours they will be killed. In the event of worsening clinical signs animals will be immediately killed.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

For most substances, the dose, route, frequency, and duration in mice are well documented. Where data are lacking, short-term pilot studies using low doses in a small number of mice (typically 2–3) will be conducted to identify a safe and effective regimen. Dosing volumes and frequencies will be minimised and follow standard guidelines (UBS and LASA) for each route of administration (e.g., subcutaneous or intra-peritoneal) as documented below. Pilot studies allow adjustment of doses before treating the remainder of animals in each experimental group. Maximum volumes and frequencies exclude administration for rehydration, anaesthesia, or analgesia.

Mouse	Daily Max. Volume (ml/kg)	Max. No./Day <10 Days	Max. No./Day >10 Days	Max. No. Injections
S/C (Subcutaneous)	20 ml/kg	3	2	24
I/P (Intra-peritoneal)	20 ml/kg	3	1	30
I/V (Intravenous)	0.8 ml over 2 min	2	1	14
Oral Gavage	20 ml/kg	3	2	30

Protocol 5

Title

Breeding and maintenance of GA mice (Mild)

Protocol 5: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To produce, maintain and provide genetically altered mice.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Mild

What proportion of animals will experience this severity?

Few – we expect the phenotype(s) to be sub-threshold and genotyping will generally be undertaken using surplus material from ear notching for identification.

Why are you proposing this severity category?

Animals are not expected to show harmful phenotypes that are more than minor or transient.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.
- To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

Protocol 5: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

5000

Which life stages will be used in this protocol?

Select all that apply

- Adult
- Embryo and egg
- Neonate
- Juvenile
- Pregnant adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

Genetically altered animals for use in this protocol may be obtained from other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 5: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms influencing regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes.

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 5: Steps

Step 1: Breeding (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

The production and birth of genetically altered offspring arising from:

- breeding by conventional breeding methods
- generation of genetically altered embryos and blastocysts by in vitro manipulation and/or fertilisation with implantation into an embryo recipient and intrauterine development.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 2: Tissue Biopsy (optional)

Repeated from protocol 3

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Tissue biopsy to determine genetic status by one of the following methods:

• ear biopsy (AA/ABL)

• blood sampling (AA/ABL)

• hair sampling (AA/AB)

• mouth swabbing (AA)

Rarely, due to technical problems during analysis, a second sample may be taken using the least invasive method.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 3: Maintenance (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Maintenance to the age of 15 months.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 4: Schedule 1 (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Terminal procedures:

Schedule 1 killing.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 5: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

- Continued use on another protocol in this project

Please state the relevant protocol.

Offspring may be moved to any protocols on this licence authorising the use of GA mice of the type produced on this protocol.

Adult mice may be used for superovulation under protocol 6.

Protocol 5: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Animals may be used for natural mating on a number of occasions.

Animals produced under this protocol are not expected to exhibit any harmful phenotype.

Offspring will be maintained by methods appropriate to their genetic alteration until they reach a maximum of 15 months of age.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Some animals may have an altered immune system making them more susceptible to infection. Animals with altered immune status will be housed in a barrier environment thereby minimising the likelihood of compromising health.

Any animal will be immediately killed by Schedule 1 method if it shows signs of suffering that is greater than minor and transient or in any way compromises normal behaviour unless moved on to another protocol for a specific purpose (continued use).

Animals exhibiting any unexpected harmful phenotypes will be killed (Schedule 1), or in the case of individual animals of particular scientific interest, advice will be sought promptly from a Home Office inspector. Other than those described in the strain-specific adverse effects above, animals are not expected to die because of any authorised genetic alteration. A small number of animals, living beyond the neonatal period (5 days – before which ASRU does not require you to report any mortality), may suddenly and unexpectedly die having shown no preceding clinical signs indicative of impending death. Unless otherwise indicated, such deaths, should they occur, are unlikely to be related to the genotype. However, as per the published ASRU Advice Note on Severity Assessment of GA animals, should the mortality rate (age-matched) of the genetically altered strain rise beyond that present in the background source breeding colony, this will be reported under PPL standard condition 18.

Protocol 5: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Genetically altered mice.

Will this protocol generate quantitative data?

No

Protocol 5: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Genetically altered mice of these types are required to achieve the scientific aim of this project.

---

b) the most refined for the purpose?

The act of natural mating is not regulated per se and no animals on this protocol are expected to show clinical signs that could exceed mild.

For each model and/or method, what is the scientific need for the expected clinical signs?

No significant clinical signs are expected.

Why scientifically do the animals need to suffer to this degree?

No significant clinical signs are expected.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

N/A

Will you be administering substances for experimental purposes?

No

Protocol 6

Title

Superovulation

Protocol 6: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

Superovulation to generate ova, blastocysts, or embryos for re-derivation, IVF and cryopreservation.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Mild

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

All animals will be administered substances to cause superovulation using standard routes which will cause mild, transient distress and no lasting harm.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.
- To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

Protocol 6: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

100

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

Genetically altered animals (with or without associated wild types) for use in this protocol may be obtained from:

• This licence: Protocol 5 'Breeding and maintenance of genetically altered animals' (mild); or

• Other project licences with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- Yes, from another protocol - in this project or another project

Describe the procedures that have been applied to them and why you are choosing to re-use them

Wild-type animals that have been genotyped using a regulated method and kept alive under the care of the NVS.

Protocol 6: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms influencing regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes.

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 6: Steps

Step 1: Superovulation (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Administration of agents to induce superovulation e.g., gonadotrophin, hormones or other agents administered typically twice but rarely up to four times by the following routes:

• intra-peritoneal (AA)

• subcutaneous (AA)

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 2: Killing by Schedule 1. (mandatory)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing by a schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 6: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 6: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

The mice will typically experience two injections, approximately 48 hours apart. If embryos are needed these animals will also be mated. Mice will be killed by a Schedule 1 method at the appropriate time to harvest oocytes/blastocysts/embryos post-mortem.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Genetically altered animals used in this protocol are not expected to exhibit any harmful phenotype but if they do, they will be immediately killed by a Schedule 1 method. Where the immune status of the animals might compromise health, they will be housed in a barrier environment. Females will be of an appropriate size if they are to be mated. Over vigorous males will be replaced. Any animal showing any deviation from normal health or wellbeing will be immediately killed by a Schedule 1 method.

Protocol 6: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Oocytes, blastocysts, or embryos harvested post-mortem.

Will this protocol generate quantitative data?

No

Protocol 6: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Injection of hormones, such as gonadotrophin, is required to stimulate a greater number of ova to develop in the mouse ovary, which will generate higher numbers of oocytes/blastocysts/embryos (if mated). This enables a larger number of

oocytes/blastocysts/embryos to be collected from a smaller number of females, which is a reduction.

---

b) the most refined for the purpose?

The doses and routes used are well validated and are not anticipated to produce any adverse effects, beyond transient discomfort due to the injections.

For each model and/or method, what is the scientific need for the expected clinical signs?

No clinical signs expected.

Why scientifically do the animals need to suffer to this degree?

No clinical signs are expected.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

N/A

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

Substances used are commercially available for pharmaceutical use and are known not to have toxic side effects.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

Routes, dosage volumes, frequencies and durations will be obtained from published literature. No more than 20ml/kg will be administered by either route.

Protocol 7

Title

Embryo recipients

Protocol 7: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To implant and grow embryos and blastocysts in recipients of an appropriate health status.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals will experience moderate severity.

Why are you proposing this severity category?

Animals undergoing surgical embryo transfer will experience short-lived post-operative pain and discomfort.

Animals undergoing non-surgical embryo transfer will experience no more than mild transient discomfort and no lasting harm.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.
- To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

Protocol 7: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

100

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

No

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- Yes, from another protocol - in this project or another project

Describe the procedures that have been applied to them and why you are choosing to re-use them

We may re-use wild-type mice that have been genotyped by a regulated method. We may re-use mice that have been implanted previously by a non-surgical method.

- Yes, within this protocol

What is the maximum number of times an animal will be used in this protocol?

Only enter numerals, for example 40

2

Protocol 7: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

No

Protocol 7: Steps

Step 1: Embryo/ Blastocyst Implantation (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Embryos and blastocysts will be implanted surgically (AB) or non-surgically (AA/AB) into the reproductive tract of a mouse rendered pseudo-pregnant by mating with a sterile male.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

The animals are expected to recover rapidly from the surgery within 2 hours.

There may be some mild weight loss.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

See general constraints.

Peri and post-operative analgesia will be provided; animals will be monitored post-surgery.

Incisions may be re-closed on one occasion if they should open within 48 hours of the surgery.

Significant weight loss is not expected but animals will be weighed daily until fully recovered from the procedure.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

See general constraints.

Any animal not fully recovered from the surgical procedure within 24 hrs (eating, drinking and return to normal behaviour and bodyweight) will be humanely killed.

Weight loss that reaches 10% compared to the pre surgery weight will be killed.

Step 2: Schedule 1 (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

All surgically implanted mice will be killed by a Schedule 1 method.

Non-surgically implanted mice will be killed by a Schedule 1 method or kept alive for potential re-use.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 7: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

---

Protocol 7: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Female mice will be rendered pseudo-pregnant by mating with a sterile male. Implantation will either be non-surgical, with or without brief anaesthesia, or by a surgical procedure under general anaesthesia. If embryos are required the dam will be humanely killed by a schedule 1 method, otherwise they will be allowed to give birth naturally and raise the litter.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

See General constraints.

Females will be of an appropriate size if they are to be mated.

Over vigorous males will be replaced.

Protocol 7: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Genetically altered mice.

Will this protocol generate quantitative data?

No

Protocol 7: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

The only method currently available for allowing GA embryos to develop is to implant them into the uterus of a pseudo-pregnant mouse.

---

b) the most refined for the purpose?

Non-surgical embryo transfer methods will be used where the success rate matches that of surgical embryo transfer methods or is satisfactory.

For each model and/or method, what is the scientific need for the expected clinical signs?

Animals are expected to make an uneventful recovery from any surgery. No other clinical signs are expected.

Why scientifically do the animals need to suffer to this degree?

This protocol follows current standard procedures.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

N/A

Will you be administering substances for experimental purposes?

No

Protocol 8

Title

Vasectomy

Protocol 8: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To produce sterile male mice for mating to obtain pseudo-pregnant female mice to be used for embryo transfer.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

All animals will undergo surgery for vasectomy and will experience short-lived post-operative pain and discomfort.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.
- To determine the mechanisms by which regenerative processes in the mammalian digit tip can inhibit or promote tumorigenesis.

Protocol 8: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

30

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

No

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- Yes, from another protocol - in this project or another project

Describe the procedures that have been applied to them and why you are choosing to re-use them

Wild type mice that have been genotyped by a regulated method.

Protocol 8: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

No

Protocol 8: Steps

Step 1: Vasectomy (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Vasectomy (AB).

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

The animals are expected to recover rapidly from the surgery within 2 hours.

There may be some mild weight loss.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

See general constraints.

Peri and post-operative analgesia will be provided; animals will be monitored post-surgery.

Incisions may be re-closed on one occasion if they should open within 48 hours of the surgery.

Significant weight loss is not expected but animals will be weighed daily until fully recovered from the procedure.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

See general constraints.

Any animal not fully recovered from the surgical procedure within 24 hrs (eating, drinking and return to normal behaviour and bodyweight) will be humanely killed.

Weight loss that reaches 10% compared to the pre surgery weight will be killed.

Step 2: Schedule 1 (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing using a Schedule 1 method.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 8: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

---

Protocol 8: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

After a post-op recovery period (minimum 2 weeks) sterility will be assessed, e.g., by mating the vasectomised male with normal females. Typically, mice are then kept alive under the direct supervision of a veterinary surgeon for mating with embryo

recipients to render them pseudo-pregnant until they are too large or too old, when they will be humanely killed.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

See General constraints.

Protocol 8: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Sterile male mice.

Will this protocol generate quantitative data?

No

Protocol 8: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

Sterile male mice are required to induce pseudopregnancy in females prior to embryo transfer. Surgical vasectomy is a reliable method to produce sterile males.

---

b) the most refined for the purpose?

Where possible genetically altered sterile males will be used instead of vasectomised males. Animals will not be used for mating until they are regaining weight and they are showing no adverse signs following surgery.

For each model and/or method, what is the scientific need for the expected clinical signs?

No clinical signs are expected, apart from non-eventful recovery from surgery.

Why scientifically do the animals need to suffer to this degree?

Where possible genetically altered sterile males will be used instead of vasectomised males. When this is not possible, surgical vasectomy using a scrotal approach is currently the most refined standard procedure.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

N/A

Will you be administering substances for experimental purposes?

No

Protocol 9

Title

Myocardial Infarction

Protocol 9: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

This protocol induces myocardial ischemia/necrosis to assess whether extracellular matrix modulation can reduce fibrosis and identify potential therapeutic strategies.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Severe

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

This protocol is categorised as severe, based on the cumulative experience of the animals and the expected physiological impact of inducing myocardial infarction (MI). Adult transgenic and wild-type mice will undergo ligation of the left anterior descending (LAD) coronary artery under general anaesthesia and aseptic conditions. Despite refined surgical techniques, peri-operative analgesia, and close monitoring, up to 20% of animals may die during or shortly after surgery due to the severity of the cardiac injury.

Surviving animals generally recover within several days. A small number (<5%) may develop progressive signs of heart failure during recovery, including cyanosis, peripheral oedema, or reduced food and water intake. These animals will be closely monitored and humanely killed upon reaching predefined humane endpoints to prevent unnecessary suffering.

Additional procedures (e.g. tamoxifen or EdU administration, non-invasive imaging) are expected to cause no more than mild, transient effects.

The severe classification therefore reflects the potential for peri-/post-surgical mortality and heart failure despite comprehensive refinement and welfare monitoring in consultation with the NVS and NACWO.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.

Protocol 9: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

500

Which life stages will be used in this protocol?

Select all that apply

- Adult

- Juvenile

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

- Protocol 5: Breeding and maintenance of genetically altered mice (mild)
- Other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 9: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms driving regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes. Examples include fluorescent reporter lines (e.g. Floxed tdTomato mice), Cre driver lines (e.g. PdgfraCreERT2 mice) or conditional alleles (e.g. Floxed Col1a1).

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 9: Steps

Step 1: MI Surgery (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Both juvenile and adult mice will be used in this step.

Animals will undergo an injury of a coronary artery (preferentially the left ventricular descending artery) or sham surgery on one occasion only via one of the following methods:

a) A physical injury (AB); or

b) A transient (in general, less than 2 hours) or permanent (for the duration of the experiment) ligature causing partial or total occlusion (AB).

Animals may be monitored by electrocardiogram (ECG) during the whole procedure.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Using our model of myocardial infarction (MI), the expected mortality rate is approximately 20% over the 28–56 day study period. Around half of these deaths occur during the surgical procedure to induce MI, while animals are under deep general anaesthesia and therefore do not experience pain or distress. The remaining deaths (up to 10%) typically occur suddenly at variable times post-operatively. Signs

that may precede cardiac failure include reduced movement, hunching, laboured breathing (breathlessness at rest), orbital tightening, and ear lowering.

In humans, acute myocardial infarction can cause chest tightness, shortness of breath, sweating, nausea, and pain. Although equivalent sensations cannot be confirmed in rodents, brief discomfort or distress in the minute before sudden death cannot be fully excluded. However, in these cases, loss of consciousness is expected to occur rapidly, and death follows within minutes, minimising the duration of suffering.

A small number of animals (<5%) may develop progressive signs of heart failure during the recovery period. These may include cyanosis, peripheral oedema, breathlessness, and reduced food or water intake. Such animals will be closely monitored and humanely killed if they reach predetermined humane endpoints to prevent unnecessary suffering.

Throughout all procedures, animals will be continuously monitored during anaesthesia and in the post-operative period. To minimise stress and potential adverse effects associated with long-term anaesthesia and social reintegration, experiments (MI or sham) will be carried out in small cohorts of 2–4 animals.

All animals will be humanely killed at the end of the experiment (28–56 days post-surgery).

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Before using the myocardial infarction (MI) model, anaesthetic, surgical, and post-operative monitoring procedures will be reviewed with the Named Veterinary Surgeon (NVS) to identify and implement any additional refinements aimed at reducing mortality and improving welfare.

All surgical procedures will be performed aseptically, following the *LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).* Appropriate analgesic agents will be administered pre-emptively and post-operatively as required to minimise pain. Analgesia will be routinely provided for 48–72 hours post-operatively, as animals are likely to experience pain beyond the first 24 hours.

Surgery will be conducted under general anaesthesia with intubation. If pneumothorax occurs during surgery and the animal is assessed to be suffering or unlikely to recover, the procedure will be immediately terminated, and the animal will be humanely killed using a Schedule 1 method.

Following surgery, animals will recover in a temperature-controlled recovery cabinet with easy access to softened food, recovery gel, and water for at least the first night. Mice will be closely monitored during the immediate post-operative period, with frequent checks in the hours following surgery. Analgesia will be administered to all animals for a minimum of 24 hours and extended if required based on clinical assessment.

If any animal displays signs of persistent distress or poor recovery, such as breathlessness at rest or failure to regain mobility within 24 hours, it will be humanely killed using a Schedule 1 method. Individual health monitoring scoresheets will be

maintained for at least 7 days post-surgery and animals scored daily. Animals reaching a cumulative clinical score of 10 or higher, or a score of 4 in any single category (weight loss, posture, provoked behaviour, fur condition, breathing, or feeding behaviour), will be euthanised using a Schedule 1 method to prevent further suffering.

Criteria	Score 0	Score 1	Score 2	Score 4
Weight loss	5% or less	>5% to 10%	>10% to 15%	>15%
Posture	Normal	Hunching noted only at rest	Moderate hunching during movement	Severe hunching during movement
Provoked behaviour (after cage is opened)	Moves away quickly	Moves away slowly/ reluctantly	Moves away slowly after an extended pause	Does not move away
Fur texture	Normal	Mild ruffling	Moderate ruffling	Severe ruffling/ poor grooming
Breathing	Normal	Mild increase in respiration	Moderate increase in respiration	Abnormal respiratory pattern, laboured breathing
Eating Behaviour	Eating food and supplements placed at the bottom of the cage	Some food and supplements have been eaten	Supplements and food remain untouched for 12 hours	Supplements and food remain untouched for 24 hours

As mortality is most likely to occur immediately after surgery, all procedures will be performed early in the day to allow intensive monitoring throughout the afternoon, evening, and following days, ensuring any adverse effects are detected and managed promptly.

After recovery, animals will be observed at least once daily for the remainder of the study period (28–56 days). Signs of imminent cardiac failure including cyanosis, oedema, reduced mobility, hunching, or breathlessness at rest, will prompt immediate humane killing by a Schedule 1 method. In addition, a validated mouse grimace scale will be used to detect subtle indicators of distress (orbital tightening, ear lowering), and animals displaying these signs will also be euthanised by a Schedule 1 method.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of post-operative complications (e.g., infection, pain, hunched posture, or inactivity), animals will be euthanised by a Schedule 1 method unless they show clear improvement within 48 hours. Re-suture may be performed on only one occasion up to 48 hours after surgery.

If animals show signs of imminent cardiac failure (cyanosis, oedema, restrain of movement, hunching, breathless at rest, orbital tightening and ear lowering), animals will be immediately killed by a Schedule 1 method.

Following our post-surgical scoring system, if animals reach a cumulative score of 10 or above or when a score of 4 is reached for any individual criteria the animal will be immediately killed by a Schedule 1 method. Animals approaching, but not yet reaching, these thresholds will be monitored at least twice daily, and if high scores persist for more than 48 hours without improvement, humane endpoints will be applied.

Step 2: MI Administration of Substances (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only adult mice will be used in this step.

With the exception of untreated controls, mice will receive substances or cells (e.g. growth factors, ligands, function-blocking antibodies, chemical inhibitors, modified RNA, or viral over-expression vectors). Control substances/cells may also be administered. Up to three agents may be given, in combination where possible, to minimise the number of dosing occasions. All administrations will be performed using the least invasive route available through either:

a. Local administration: Direct injection into the heart i.e. heart muscle or myocardium (AB). [Max vol. 20ul/injection with 1-3 injection locations = 60ul total]. Direct injections into the heart will be performed either immediately post-infarction/sham surgery or at later time points via ultrasound-guided intra-myocardial injection.

b. Systemic administration: Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

c. Systemic administration: Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

d. Systemic administration: Intravenously (AA/AB), maximum treatment = once a day up to 5 days [Max vol. 10ml/kg].

e. Systemic administration: Orally (e.g. in diet, drinking water) (AA), maximum treatment = 8 weeks.

Additional animals may be treated with administration vehicle only.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 3: Transgene Induction (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only adult mice will be used in this step.

Gene Induction : Except for untreated controls, mice may be administered trans-gene inducing or deleting agents (such as tamoxifen) or vehicle control, at any stage, by one or more of the following routes on up to 5 occasions (minimum 24h interval).

a. Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

b. Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Animals receiving the trans-gene inducing or deleting reagent, tamoxifen, may experience weight loss during the first 1-2 days of administration. The likely incidence of this occurring is dependent on the genetic strain of the mice but is not uncommon. These mice typically regain weight back to base-line within the next 2-5 days and display no harmful side effects.

Oral gavage may cause stress, transient oesophageal irritation, and, rarely, mortality (~5%) at high doses. IP injections may cause temporary abdominal or scrotal swelling.

In addition, animals receiving tamoxifen via oral gavage or intra-peritoneal (IP) injection may show transient reduced activity, hunched posture, and a ruffled or greasy coat, typically within the first 1–7 days.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for the first two weeks, or until weight is stable. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

Male mice given tamoxifen will be monitored for testicular swelling daily during the days of administration and for 48 hours afterwards.

All procedures and monitoring will follow the UBS tamoxifen guidance developed by the NVS, ensuring that refinements, dosing, and welfare measures are implemented in accordance with expert veterinary advice.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Animals showing 15% weight loss (from the animal's initial weight measurement when dosing begins) measured for 2 consecutive days will be killed immediately by an appropriate Schedule 1 method.

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Testicular swelling causing more than mild discomfort (difficulty walking or changed activity) in male mice treated with tamoxifen will be killed immediately by an appropriate Schedule 1 method.

Step 4: Fasting Prior To Imaging (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only adult mice will be used in this step.

Animals will be weighed to establish a baseline body weight prior to any fasting (AA). Food restriction will then be applied for up to 4 hours before imaging, with no more than two such fasting periods per week. After each imaging session, animals will be provided with food ad libitum. A further fasting period will only be undertaken once the animal's body weight has returned to its baseline level. Water will be available at all times. Food intake and body weight will be closely monitored to ensure that mice do not lose more than 15% of their baseline body weight.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Short-term food restriction may result in transient hunger and a temporary reduction in body weight (typically ≤10% of baseline). Minor weight loss is expected to be short-lived, with body weight returning to baseline within 24 hours following re-feeding. The incidence and severity of adverse effects are expected to be low due to the limited duration and frequency of fasting, continuous access to water, and close monitoring of body weight and food intake.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for a week, or until weight is stable following food restriction. During fasting, body weight will be recorded prior to each session to ensure weight loss does not exceed 15% of baseline. Fasting will be limited to a maximum of 4 hours and no more than two sessions per week, with a further session only undertaken once body weight has returned to baseline. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Mice exhibiting a 15% weight loss will be immediately killed by a schedule 1 method.

Step 5: Non-Invasive Imaging (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Only adult mice will be used in this step.

Non-Invasive Imaging: Animals may be fasted for up to 4 hours prior to imaging - See Step 'Fasting Prior To Imaging' (AA). Animals may be assessed by any or a combination of non-invasive imaging methods (for example, but not limited to):

• bioluminescence imaging (BLI) (AB),

• fluorescence imaging (FLI) (AB),

• MRI (AB/AC) with fasting,

• MSOT (AB/AC) with fasting,

• ultrasound (AB) with fasting,

• PET/SPECT (AB/AC) with fasting

BLI, FLI and ultrasound imaging typically takes a maximum of 15 min and may be conducted up to once per week or once every 2 weeks throughout some long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 12. In addition, BLI, FLI and ultrasound imaging may be used daily for short term monitoring of administered agents (including cells).

MSOT measurements typically take about 30 min and may be conducted up to twice per week or once every 2 weeks throughout long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 10.

MRI and CT measurements of tissue anatomy, where these do not involve injection of an imaging agent, typically take about 30 min and may be conducted weekly

throughout experiments which can last up to 3 months. The total number of imaging examinations will not exceed 12. MRI, CT and PET/SPECT imaging following injection of an established or novel imaging agent usually take under 2 hours and may be conducted for a maximum of 5 times for a maximum period of 2 weeks and no more than twice per day (AB/AC). Imaging may be repeated on the same animal but typically, this will not exceed five sessions within any 2 week period, where each imaging session will not exceed 5 hours. Typically, there will be only one imaging session per day. Occasionally it may be necessary to conduct two imaging sessions in one 12 hour period, in which case each imaging session will not exceed 2 hours.

Imaging agents may be administered via one of the following routes prior to or during imaging:

a) Intra-peritoneal, with or without cannulation, maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

b) Intravenous, with or without cannulation, maximum of 20 ml/kg bolus injection or constant infusion via a cannulated vein, for a maximum of 3 hours [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period, and a maximum of 10 injections in 3 months.

c) Subcutaneous, a maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

d) Oral gavage, a maximum of 20 ml/kg bolus injection [AA]. Maximum frequency of 5 times in any 2 week period and a maximum of 10 times in 3 months.

Animals will undergo a maximum of two imaging sessions per week in total, irrespective of imaging type or combination of imaging modalities. After each imaging session and recovery, animals will be assessed to ensure they are fully alert, behaving normally, and showing no adverse effects. Imaging on a consecutive day will only proceed if the animal meets all of these criteria.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 6: Cell Labelling (Stage Specified) (optional)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Both juvenile and adult mice will be used in this step.

Cell labelling: Mice may be administered labelling agents (such as EdU or BrdU) via intra-peritoneal injection (only adult) or sub-cutaneous (AA) on a single occasion. [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 7: Killing by Schedule 1. (mandatory)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing by a schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 9: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 9: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Typically, all mice will undergo a surgical procedure to induce a myocardial infarction under general anaesthesia. Each surgery lasts approximately 40 minutes per animal and includes an injection of peri-operative analgesia at standard doses and via standard routes. Upon recovery, animals will be returned to their cages and will be closely monitored throughout the entirety of the experiment. Some mice will be administered substances, typically in one of the following ways but on occasion through combined routes:

a. Typically (~80%), at the time of surgery substances will be injected on a single occasion directly into the heart. In some instances, substances may need to be injected post-surgery using ultrasound guided intra-myocardial injection under anaesthetic.

b. Occasionally (~10%), adult mice will be administered substances systemically by oral gavage, intravenous or intra-peritoneal injection on up to 5 occasions (minimum interval 24 hours).

c. Occasionally (5%), adult mice will be administered substances systemically through their diet or drinking water. When substances have been reported as being taste aversive, these will be masked (e.g. by adding the substance to chocolate

flavoured food pellets or adding sucrose into the water). Additionally, all mice will be monitored throughout the duration of the experiment to ensure general health is not compromised (e.g. mice are eating and drinking sufficiently and that no other complications arise affecting general behaviour or health).

d. Occasionally (10%), adult mice will undergo non-invasive imaging of tissues on up to 12 occasions (minimum interval 12 hours). This will typically include a single session of fluorescence imaging to visual implanted cells, followed by weekly sessions of MRI to visualise changes in tissue architecture.

Taking into account all relevant procedures, including imaging, surgical interventions, and any restraint procedures requiring anaesthesia (such as for injections), the maximum number of occasions on which an animal may be anaesthetised = 18.

In the instance when substances are administered systemically, agents will be administered by the least invasive route in the first instance when multiple routes are available (e.g. Administered in the food or drinking water as opposed to intravenous injection).

Occasionally (5%), in addition to the initial MI surgery and administration of substances, some mice will also be administered cell labelling reagents (e.g. EdU) on a single occasion or tamoxifen (up to four times) to induce fluorescent reporters/modify genes using standard routes and dosages.

Mice will remain group-housed until tissue collection, which will occur either at early time points (e.g., 4–96 hours post-surgery) or at later time points (e.g., 28–56 days post-surgery) when animals are culled.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

General humane endpoints will apply across all procedures in this protocol, in addition to those defined within individual steps. Any animal showing unexpected adverse effects, deteriorating clinical condition, or signs of pain or distress that cannot be rapidly and effectively alleviated within 48 hours will be humanely killed using a Schedule 1 method. Clinical signs triggering intervention may include: persistent weight loss (across 3 consecutive days) or failure to thrive, reduced mobility, sustained abnormal posture or behaviour, marked dehydration, impaired respiration, or any sudden or unexplained decline in condition.

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Where an individual animal exhibits an unforeseen phenotype of potential scientific interest, advice will be sought promptly from the Named Veterinary Surgeon and the Home Office Inspector before any decision is made.

Protocol 9: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Phenotypic data on how specific cell or gene modulation affects fibrosis and regeneration of the murine heart following myocardial infarction.

Will this protocol generate quantitative data?

Yes

Will your experimental design be determined by a regulatory guideline?

No

Where relevant, explain how and when pilot studies will be used.

Pilot studies, using no more than 3 animals per condition, will be conducted to generate preliminary data and in order to allow for procedures and techniques to be perfected before embarking on large scale experiments. Specifically, we will perform pilot experiments when animals are to be injected with pharmacological agents or inhibitor substances to obtain an indication of the dose and the timing of the injections, that is likely to be effective.

How will you choose different experimental groups?

For example, controls, dose levels, satellites etc.

Animals will be randomly assigned to either the control or treatment group. The treatment, that is the pharmacological agents or inhibitor substances, will be selected according to preliminary data obtained from *in vitro* culture experiments and doses will be determined through pilot experiments, taking into account the effective reported dose levels in the literature.

How will you choose control groups?

Provide a robust scientific justification for controls with significant suffering such as sham surgery controls or untreated infected controls.

Each individual experiment will use a control group of mice that are contrasted directly to the experimental group of mice. For example, when injecting hearts with a substance resuspended in 0.1% BSA, control hearts will be injected with the same vehicle, 0.1% BSA. A sham surgery control will only be included in the event that the necessary vehicle control has not been tested in this system, or been reported in the literature to be innocuous. The inclusion of a sham surgery control would be limited to pilot experiments and is necessary to ensure that unknown vehicle controls alone cannot account for any observed effects and result in misleading or inaccurate conclusions.

How will experiments and data analysis be randomised and blinded?

Prior to each experiment, animals will be randomly allocated to treatment or control groups using a randomisation method (e.g. unique identifiers with group allocation determined by random number selection drawn out of a hat).

Treatments (e.g. agonists or inhibitors) will be coded by an independent individual not directly involved in the study. Codes will remain concealed (e.g. sealed envelope) until the experiment and data collection are complete.

Where possible, the researcher performing the surgical intervention or treatment will not be involved in subsequent data analysis. Data analysis will also be conducted under blinded conditions whereby samples will be re-coded by an independent individual not associated with the study prior to analysis. Analysts will only be informed of group structure when necessary for statistical comparison but will remain blinded to treatment identity. This approach minimises bias in both data collection and interpretation.

How will you minimise variables to ensure reproducibility?

We will aim to minimise variables in this protocol by using inbred mice from the same supplier such as Charles River Laboratories for all experiments and will avoid exposing the laboratory mice to unnecessary stress by employing good handling practises. Bias will be avoided through good experimental design including incorporation of randomisation and blinding practises both during experimental procedures and analysis of the data. Additionally, accurate records will be maintained of all work conducted under this project licence and methods will be reported responsibly including descriptions of all variables such as mouse age, number, strain, housing conditions and appropriate statistical analyses.

How will you determine group sizes?

You should reference POWER calculations you have made, if relevant.

Group sizes were determined using POWER calculations for an unpaired t-test whereby the biological effect size (using proportional differences) is expected to be 1.44 and standard deviations approximately 20%, based on previous experiments and published literature. The power was set to 90% and alpha 5% (two-sided), resulting in group sizes of n = 10 animals per condition. To accommodate predicted animal loss due to myocardial infarction (~20%) and occasional experimental complications or unexpected variability, a sample size of n = 12 was subsequently selected for each group (control and experimental). Additionally, advice on group size calculations and experimental design has been sought from a local biostatistician and our collaborators who have extensive experience from the Wilson and Sinha laboratories.

How will you maximise the data output from the animals you use on this protocol?

Non-invasive imaging of the heart allows for serial assessment of regeneration and will maximise the data recorded from each animal.

As only hearts are required for these studies, surplus tissues of scientific value will be shared with other researchers, reducing the need for additional breeding or animal use.

Furthermore, all experimental approaches and resulting data will be disseminated to the wider scientific community. This ensures transparency, prevents unnecessary duplication of work, and maximises the impact of findings generated from each animal.

Protocol 9: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

The murine model of myocardial infarction (MI) produced by ligation of the left anterior descending (LAD) coronary artery is the most appropriate scientific approach for studying cardiac injury and regeneration in mammals. This model closely replicates the pathophysiological processes observed in human myocardial infarction, including ischaemia, necrosis, and post-injury remodelling, while using the lowest sentient species capable of reliably reproducing the relevant cardiovascular anatomy and physiological response.

The mouse model allows investigation of both acute and chronic stages of cardiac injury, as well as recovery and remodelling, using established and reproducible methods. The availability of well-characterised transgenic and knockout strains also enables mechanistic investigation of specific molecular and cellular pathways involved in repair and regeneration, which cannot be achieved using non-mammalian systems.

The surgical approach used to induce MI in mice has been refined extensively by our collaborators (Dr Sanjay Sinha and Dr Cathy Wilson) to ensure consistency and low variability, with mortality rates below 20%. The model permits either permanent ligation or ischaemia–reperfusion to be performed, enabling the study of both irreversible and reversible injury within a controlled and standardised framework.

To minimise inter-animal variability and reduce the number of animals used, imaging modalities such as echocardiography and magnetic resonance imaging (MRI) will be applied longitudinally to assess functional and structural changes in the same animal over time. This within-animal comparison design provides statistically robust data while significantly reducing the overall number of animals required.

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b) the most refined for the purpose?

All surgical procedures will be conducted using aseptic techniques that meet or exceed the Home Office Minimum Standards of Aseptic Surgery and the LASA Guiding Principles. Myocardial infarction will be induced in mice via ligation of the left anterior descending (LAD) coronary artery. This procedure is well established and reproducible, having been optimised in the laboratories of Dr Sanjay Sinha and Dr Cathy Wilson. Both complete ligation and ischaemia-reperfusion approaches via thoracotomy have been validated, achieving a procedure-related mortality of less than 20%.

Peri-operative and post-operative care will include administration of analgesics in accordance with doses and schedules agreed with the Named Veterinary Surgeon (NVS). Animals will be monitored post-operatively using an approved post-surgical monitoring chart, as agreed with the NVS.

Where transgene-inducing or -deleting substances are administered, doses will be determined through pilot studies to identify the minimum effective dose. Only previously validated compounds (e.g., tamoxifen) will be used. All animals receiving such substances will be carefully monitored to ensure that health and welfare are maintained, with supportive care provided in cases of weight loss or other signs of distress.

Substances will be administered using the least invasive route possible. Systemic administration will prioritise routes such as incorporation into food or drinking water over intravenous or intraperitoneal injection. Local administration at the time of myocardial infarction will be performed via direct surgical access through thoracotomy, a method that is well tolerated with minimal adverse effects. Where post-MI administration is required, ultrasound-guided intramyocardial injection will be employed. This technique, refined in collaboration with the Wilson laboratory and AstraZeneca, reduces surgical morbidity and allows delivery of substances at predetermined intervals.

All procedures involving the administration of substances will be further refined in line with the following guidance:

Morton DB et al., 2001. Refine procedures for administration of substances. BVAAWF/FRAME/RSPCA/UFAW Joint Working Group. Lab Anim. 35(1):1–41

Turner PV et al., 2011. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. J Am Assoc Lab Anim Sci. 50(5):600–613

For each model and/or method, what is the scientific need for the expected clinical signs?

The scientific need for the expected clinical signs in this model arises from the goal of studying heart regeneration and testing potential therapies to alleviate fibrosis following myocardial infarction (MI), a major cause of morbidity and mortality in humans. To achieve this, the left anterior descending (LAD) coronary artery is surgically ligated to induce MI in rodents. This method closely replicates the physiological and pathological cardiac responses observed in human patients following ischemic injury, allowing the study of tissue damage, fibrosis, and regeneration, which cannot be modelled *in vitro* or in less severe animal models.

The expected clinical signs, including reduced mobility, hunching, laboured breathing, and other indicators of cardiac stress, are a direct consequence of the coronary artery injury and are necessary to evaluate both the progression of disease and the effectiveness of experimental treatments such as gene therapy. In mice, the mortality rate is approximately 20% over the 28–56 day study period, with about half of deaths occurring during the initial surgery while animals are deeply anaesthetised and therefore do not experience discomfort. Most of the remaining deaths (up to

10%) occur suddenly post-operatively; in these cases, loss of consciousness is likely immediate, and death follows within minutes, limiting the duration of suffering.

Animals will be extensively monitored for signs of imminent cardiac failure, including cyanosis, oedema, reduced mobility, hunching, breathlessness at rest, orbital tightening, and ear lowering. Any animals displaying these signs will be humanely killed without delay.

Although the procedures are classified as severe, the expected clinical signs are scientifically necessary to provide a relevant and translational model of human MI. Inducing coronary artery injury in this way is essential to develop and evaluate novel therapies that have the potential to directly improve clinical outcomes in patients.

Why scientifically do the animals need to suffer to this degree?

Scientifically, some animals need to experience the effects of a heart attack in order to test and develop new treatments that could be used in patients. Myocardial infarction is a serious condition that frequently leads to death in humans, and the severity of the disease cannot be modelled adequately *in vitro* or in less severe animal models. Therefore, to generate meaningful data on the effectiveness of potential therapies, it is necessary for some animals to undergo the full physiological impact of a heart attack. The severity of these protocols reflects the significant burden of the condition in patients and is essential for identifying interventions that could ultimately reduce morbidity and mortality in humans.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

The scientific outputs cannot be achieved with an earlier humane endpoint or without animals showing clinical signs because the models are designed to study the heart's response to myocardial infarction, a complex pathology that cannot be fully reproduced *in vitro*. The progression of disease and the response to experimental interventions must be observed over defined periods to assess outcomes such as infarct size, fibrosis, and functional recovery.

While up to 10% of rodents may reach a severe category, death usually occurs suddenly and unpredictably, with only transient suffering. Any animals showing signs of suffering will be humanely killed, and all remaining animals will be humanely killed at the conclusion of the study. This approach is essential to obtain meaningful and reproducible data necessary for the development and testing of potential therapeutic interventions.

Following tamoxifen administration, a transient weight loss is occasionally observed dependent on the strain. In some animals this may exceed 15% for a short period but is reversible with supportive care, with animals stabilising within 24 hours. Immediate euthanasia at 15% weight loss would result in the unnecessary loss of animals that would otherwise recover fully, increasing animal use without welfare benefit. An upper limit of 20% weight loss is therefore set as an immediate humane endpoint, beyond which recovery is unlikely and welfare would be compromised.

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

When using substances for the first time, doses will be chosen to minimise the risk of toxicity based on the available literature and validated in small pilot study. The starting dose used will be the minimum that was shown to have an effect according to the published literature.

All substances will be prepared in a Biosafety cabinet using sterile equipment and sterile solutions.

As this study will only use previously-validated compounds, all administered substances are not expected to cause adverse effects. However, if animals show, unexpectedly, any clinical signs such as hunched posture, pilo-erection or subdued behaviour, these animals will be kept under close observation. If the signs do not resolve within 24 hours they will be killed. In the event of worsening clinical signs animals will be immediately killed.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

For most substances, the dose, route, frequency, and duration in mice are well documented. Where data are lacking, short-term pilot studies using low doses in a small number of mice (typically 2–3) will be conducted to identify a safe and effective regimen. Dosing volumes and frequencies will be minimised and follow standard guidelines (UBS and LASA) for each route of administration (e.g., subcutaneous or intra-peritoneal) as documented below. Pilot studies allow adjustment of doses before treating the remainder of animals in each experimental group. Maximum volumes and frequencies exclude administration for rehydration, anaesthesia, or analgesia.

Mouse	Daily Max. Volume (ml/kg)	Max. No./Day <10 Days	Max. No./Day >10 Days	Max. No. Injections
S/C (Subcutaneous)	20 ml/kg	3	2	24
I/P (Intra-peritoneal)	20 ml/kg	3	1	30

I/V (Intravenous)	0.8 ml over 2 min	2	1	14
Oral Gavage	20 ml/kg	3	2	30

Protocol 10

Title

Bone Fracture Healing

Protocol 10: Protocol details

Briefly describe the purposes of this protocol

Ensure that you state any relevant regulatory guidelines.

To characterise the dynamics of progenitor cells and the roles they play in bone repair and regeneration, mice will be subject to an induced fracture in the bone.

Given the controls and limitations in place, what is the highest severity that an animal could experience in this protocol?

Moderate

What proportion of animals will experience this severity?

All animals.

Why are you proposing this severity category?

This protocol was categorised as moderate severity when taking into account the animal's cumulative experiences and life stage (adult).The majority of animals are likely to undergo a single surgical procedure performed under general anaesthesia. Approximately 60% of animals will be administered substances including pharmacological inhibitors, biological molecules or cells which are not anticipated to harm the animal or negatively impact their welfare in any way. Approximately 50% of animals on the protocol will undergo non-invasive imaging under general anaesthesia for restraint purposes whereby induction is rapid and the duration will be less than 30 minutes per animal. Some animals may also be administered labelling reagents (such as EdU or BrdU) on a single occasion.

Locations where this protocol can be carried out

Select all that apply.

- University of Cambridge

Which of your objectives will this protocol address?

Select all that apply.

- To perform a selective screen of ECM-modulating factors across different models of fibrosis and injury.
- To identify the epigenetic programmes and tissue microenvironment that enable regenerative success.

Protocol 10: Animals used in this protocol

Mice

What is the maximum number of {{ values.speciesLabel }} that will be used in this protocol?

Only enter numerals, for example 40

300

Which life stages will be used in this protocol?

Select all that apply

- Adult

Will any {{ values.speciesLabel }} coming onto this protocol be classed as 'continued use'?

'Continued use' describes animals that are specifically genetically altered and bred for scientific use or animals that have had procedures applied to them in order to be prepared for use in this protocol.

Yes

How did these animals start their use?

Describe the procedures that have been applied to animals that will continue their use on to this protocol.

- Protocol 5: Breeding and maintenance of genetically altered mice (mild)
- Other projects with authority to breed and maintain genetically altered animals of a type authorised in this project, and to provide them for use on other projects.

Are you re-using any {{ values.speciesLabel }}?

'Re-use' describes using an animal for a new experiment when you could equally use a naive animal to get the same results. Select each that applies

- No

Protocol 10: Genetically altered animals (GAA)

Will this protocol use any genetically altered animals?

Yes

Which general types or strains will you be using and why?

GAA mouse strains associated with our investigation of the mechanisms driving regeneration and fibrosis.

GAA mice will include strains with mutations, reporters and/or conditional alleles in receptors and factors such as transcription factors, growth factors and enzymes. Examples include fluorescent reporter lines (e.g. Floxed tdTomato mice), Cre driver lines (e.g. PdgfraCreERT2 mice) or conditional alleles (e.g. Floxed Col1a1 mice).

Do you expect any of these GAAs to show a harmful phenotype with welfare consequences?

No

Protocol 10: Steps

Step 1: Fracture Surgery (mandatory)

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Mice will be administered a fracture surgery procedure by one of the two methods below:

Femoral Fracture Surgery: Through an open procedure the patellar tendon and the muscle surrounding the femoral shaft will be displaced to expose the bone. A pair of surgical scissors will be used to create a transverse, non-comminute fracture or a 0.8mm - 1.6 mm diameter hole will be created using a micro dental drill to expose the bone marrow. This procedure will be performed on one femur on a single occasion (AB).

OR

Digit Tip Fracture Surgery: A 0.8mm - 1.6mm diameter hole will be created using a micro dental drill. This procedure will be performed on up to six digits on a single occasion (AB).

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

We anticipate that 100% of animals used under this procedure will experience lameness of moderate severity in the immediate post-operative period. This will improve over 72 hours and in 95% of animals weight bearing will improve and no other clinical signs are expected. In 5% of animals, lameness may not improve after the initial perioperative 72 hour period or develop thereafter, in which case the general humane endpoints will apply.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Clinical signs of lameness will be a lack of weight bearing on the operated leg. Non-orthopaedic signs of clinically significant pain and discomfort would include pilo-erection, isolation, subdued behaviour and a hunched posture. To address the initial lameness experienced by the animals NSAIDs will be administered for 72 hours following surgery. Local anaesthetic (e.g. 0.25% bupivicaine) may also be injected into the limb at the end of the surgical procedure in order to provide specifically targeted analgesia. In addition, soft food will be provided on the floor of the cage during the perioperative period to reduce the impact of the surgery on feeding behaviour.

Post-operative monitoring will be carried out daily using a structured score sheet to record general health, weight, lameness, gait, weight-bearing and any swelling of the operated limb. These records will be used to determine whether additional interventions are required and whether humane endpoints have been reached. Score sheets will be maintained for a minimum of 7 days post-surgery. Animals reaching a cumulative score of 10 or more, or a score of 4 in any single category, will

be humanely killed by a Schedule 1 method to prevent further suffering. An example of the score sheet criteria is provided below:

Parameter	0	1	2	4 (End Point)
Weight Loss	≤5%	>5–10%	>10–15%	>15%
Posture	Normal	Mild hunching at rest	Hunching during movement	Persistent hunching; reluctance to move
Provoked Behaviour	Moves away quickly	Slower/reluctant movement	Moves after long pause	No movement away
Fur Condition	Normal	Mild ruffling	Moderate ruffling	Poor coat condition with evidence of reduced self-care
Mobility/Lameness	Normal gait	Mild limp	Clear limp, partial weight avoidance	No weight bearing OR failure to improve by 72 hours
Swelling (Limb or Digit)	None	Mild swelling	Moderate swelling	Swelling that interferes with normal behaviour or function that does not improve with treatment by 72 hours
Feeding Behaviour	Normal intake	Some intake	Minimal intake for 12 hours	No intake for 24 hours

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

In the event of complications animals will be killed by an appropriate Schedule 1 method immediately. Complications may specifically include:

- Unimproved lameness in the operated limb after 72 hour post-surgery. Clinical signs of lameness are a lack of weight bearing on the operated leg. Swelling may cause lameness and can be directly detected by handling the animal and identifying swelling visually or with careful digital palpation.
- Wound infections, recognised by pain, heat and swelling at the surgical incision.

- If weight loss (compared to pre-surgery values) reaches 15%.

Following our post-surgical scoring system, if animals reach a cumulative score of 10 or above or when a score of 4 is reached for any individual criteria the animal will be immediately killed by a Schedule 1 method. Animals approaching, but not yet reaching, these thresholds will be monitored at least twice daily, and if high scores persist for more than 48 hours without improvement, humane endpoints will be applied.

Step 2: Administration of Substances - Optional (optional)

Repeated from protocol 4

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

With the exception of untreated controls, mice will receive substances or cells (e.g. growth factors, ligands, function-blocking antibodies, chemical inhibitors, or viral over-expression vectors). Control substances/cells may also be administered. Up to three agents may be given, in combination where possible, to minimise the number of dosing occasions. All administrations will be performed using the least invasive route available through either:

a. Local administration: Direct injection into the second through fourth hindlimb digits (AB). [Max vol. 5ul/digit]. Direct injections into the digit will be performed no less than 3 days following a prior surgical procedure.

b. Systemic administration: Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

c. Systemic administration: Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

d. Systemic administration: Intravenously (AA/AB), maximum treatment = once a day up to 5 days [Max vol. 10ml/kg].

e. Systemic administration: Orally (e.g. in diet, drinking water) (AA), maximum treatment = 8 weeks.

Additional animals may be treated with administration vehicle only.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 3: Transgene Induction (optional)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Gene Induction : Except for untreated controls, mice may be administered trans-gene inducing or deleting agents (such as tamoxifen) or vehicle control, at any stage, by one or more of the following routes on up to 5 occasions (minimum 24h interval).

a. Oral Gavage (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

b. Intra-peritoneal (AA), maximum treatment = once a day up to 5 days [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Animals receiving the trans-gene inducing or deleting reagent, tamoxifen, may experience weight loss during the first 1-2 days of administration. The likely incidence of this occurring is dependent on the genetic strain of the mice but is not uncommon. These mice typically regain weight back to base-line within the next 2-5 days and display no harmful side effects.

Oral gavage may cause stress, transient oesophageal irritation, and, rarely, mortality (~5%) at high doses. IP injections may cause temporary abdominal or scrotal swelling.

In addition, animals receiving tamoxifen via oral gavage or intraperitoneal (IP) injection may show transient reduced activity, hunched posture, and a ruffled or greasy coat, typically within the first 1–7 days.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for the first two weeks, or until weight is stable. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

Male mice given tamoxifen will be monitored for testicular swelling daily during the days of administration and for 48 hours afterwards.

All procedures and monitoring will follow the UBS tamoxifen guidance developed by the NVS, ensuring that refinements, dosing, and welfare measures are implemented in accordance with expert veterinary advice.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Animals showing 15% weight loss (from the animal's initial weight measurement when dosing begins) measured for 2 consecutive days will be killed immediately by an appropriate Schedule 1 method.

Mice exhibiting a 20% weight loss will be immediately killed by a schedule 1 method.

Testicular swelling causing more than mild discomfort (difficulty walking or changed activity) in male mice treated with tamoxifen will be killed immediately by an appropriate Schedule 1 method.

Step 4: Cell Labelling (optional)

Repeated from protocol 1

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of

"penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Cell labelling: Mice may be administered labelling agents (such as EdU or BrdU) via intra-peritoneal injection or sub-cutaneous (AA) on a single occasion. [Max vol. 20ml/kg].

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 5: Fasting Prior To Imaging (optional)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Animals will be weighed to establish a baseline body weight prior to any fasting (AA). Food restriction will then be applied for up to 4 hours before imaging, with no more than two such fasting periods per week. After each imaging session, animals will be provided with food ad libitum. A further fasting period will only be undertaken once the animal's body weight has returned to its baseline level. Water will be available at all times. Food intake and body weight will be closely monitored to ensure that mice do not lose more than 15% of their baseline body weight.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

Yes

What are the likely adverse effects of this step?

State the expected adverse effect, including the likely incidence, and the anticipated degree and duration of suffering.

Short-term food restriction may result in transient hunger and a temporary reduction in body weight (typically ≤10% of baseline). Minor weight loss is expected to be short-lived, with body weight returning to baseline within 24 hours following re-feeding. The incidence and severity of adverse effects are expected to be low due to the limited duration and frequency of fasting, continuous access to water, and close monitoring of body weight and food intake.

How will you monitor for, control, and limit any of these adverse effects?

If adverse effects can't be prevented, how will you attempt to ameliorate their initial signs?

Animals will be monitored daily and body weights will be recorded for a week, or until weight is stable following food restriction. During fasting, body weight will be recorded prior to each session to ensure weight loss does not exceed 15% of baseline. Fasting will be limited to a maximum of 4 hours and no more than two sessions per week, with a further session only undertaken once body weight has returned to baseline. Additionally animals displaying weight loss will be provided with supportive care and receive wet mash or palatable diet to encourage weight gain.

What are the humane endpoints for this step?

This would be the point at which you would kill the animal to prevent further suffering.

Mice exhibiting a 15% weight loss will be immediately killed by a schedule 1 method.

Step 6: Non-Invasive Imaging (optional)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Non-Invasive Imaging: Animals may be fasted for up to 4 hours prior to imaging - See Step 'Fasting Prior To Imaging' (AA). Animals may be assessed by any or a combination of non-invasive imaging methods (for example, but not limited to):

• bioluminescence imaging (BLI) (AB),

• fluorescence imaging (FLI) (AB),

• MRI (AB/AC) with fasting,

• MSOT (AB/AC) with fasting,

• ultrasound (AB) with fasting,

• PET/SPECT (AB/AC) with fasting

BLI, FLI and ultrasound imaging typically takes a maximum of 15 min and may be conducted up to once per week or once every 2 weeks throughout some long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 12. In addition, BLI, FLI and ultrasound imaging may be used daily for short term monitoring of administered agents (including cells).

MSOT measurements typically take about 30 min and may be conducted up to twice per week or once every 2 weeks throughout long-term experiments, which can last up to 3 months. The total number of imaging examinations will not exceed 10.

MRI and CT measurements of tissue anatomy, where these do not involve injection of an imaging agent, typically take about 30 min and may be conducted weekly throughout experiments which can last up to 3 months. The total number of imaging examinations will not exceed 12. MRI, CT and PET/SPECT imaging following injection of an established or novel imaging agent usually take under 2 hours and may be conducted for a maximum of 5 times for a maximum period of 2 weeks and no more than twice per day (AB/AC). Imaging may be repeated on the same animal but typically, this will not exceed five sessions within any 2 week period, where each imaging session will not exceed 5 hours. Typically, there will be only one imaging session per day. Occasionally it may be necessary to conduct two imaging sessions in one 12 hour period, in which case each imaging session will not exceed 2 hours.

Imaging agents may be administered via one of the following routes prior to or during imaging:

a) Intra-peritoneal, with or without cannulation, maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

b) Intravenous, with or without cannulation, maximum of 20 ml/kg bolus injection or constant infusion via a cannulated vein, for a maximum of 3 hours [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period, and a maximum of 10 injections in 3 months.

c) Subcutaneous, a maximum of 20 ml/kg bolus injection [AA/AB/AC]. Maximum frequency of 5 times within any 2 week period and a maximum of 10 injections in 3 months.

d) Oral gavage, a maximum of 20 ml/kg bolus injection [AA]. Maximum frequency of 5 times in any 2 week period and a maximum of 10 times in 3 months.

Animals will undergo a maximum of two imaging sessions per week in total, irrespective of imaging type or combination of imaging modalities. After each imaging session and recovery, animals will be assessed to ensure they are fully alert, behaving normally, and showing no adverse effects. Imaging on a consecutive day will only proceed if the animal meets all of these criteria.

Is this step optional?

Yes

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Step 7: Killing by Schedule 1. (mandatory)

Repeated from protocol 2

Describe the procedures that will be carried out during this step.

Explain where one or more steps are repeated in one experiment, list any alternative techniques within a step (e.g. dosing routes), and include all procedures performed under terminal anaesthesia. When describing the technical aspects of a step, be broad enough to be flexible when the variation does not impact on animal welfare (e.g. use "antibiotic" instead of "penicillin"). Finally, avoid specifying volumes and frequencies when they do not impact on animal welfare.

Killing by a schedule 1 method.

Is this step optional?

No

Do you expect this step to have adverse effects for the animals that are more than mild and transient?

Do not list uncommon or unlikely adverse effects, or effects from procedures that will cause no more than transient discomfort and no lasting harm. For example, an intravenous injection of a small volume of an innocuous substance.

No

Protocol 10: Fate of animals

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

Select all that apply. These options are based on what you selected in the non-technical summary.

- Killed

Will you be using non-schedule 1 killing methods on a conscious animal?

No

Protocol 10: Animal experience

Summarise the typical experience or end-to-end scenario for an animal being used in this protocol.

Consider the cumulative effect of any combinations of procedures that you may carry out.

Adult mice will undergo femoral or digit fracture surgery, a single surgical procedure under general anaesthetic (approximately 30 minutes per animal) which includes administration of peri-operative and post-operative analgesia (for 72 hours) by standard routes and dosages. Upon recovery, animals will be returned to their cages. Animals are expected to experience lameness immediately after the surgery that will improve within the next 72 hours. Animals will gradually begin to bear more weight on the injured limb during the course of the fracture healing process which can take up to 8 weeks.

Animals that have an inducible genetic modification will receive trans-gene inducing or deleting reagents (tamoxifen) on up to 5 occasions (minimum interval 24 hours) typically via intra-peritoneal injection (when the induction of the trans-gene is time sensitive) and occasionally by oral gavage (when the induction of the trans-gene is not time sensitive). Approximately 60% of animals on this protocol will be treated with substances including pharmacological agents, biological molecules, cells or their products. This will be at any point in the experiment and may be via intra-peritoneal injection, sub-cutaneous injection, oral gavage, or through diet/water. The route and occasions will vary dependent on the substance.

Approximately, 50% of mice will undergo non-invasive micro-CT imaging under general anaesthesia. This will typically take 30 minutes and may be conducted weekly throughout the experiments (usually 8 sessions). Occasionally, some animals may also be administered cell labelling reagents (EdU or BrdU) on a single occasion via intra-peritoneal injection, typically 24 hours prior to tissue collection.

Describe the general humane endpoints that you will apply during the protocol.

These will be in addition to the endpoints stated for each step.

Any animal will be immediately killed by a Schedule 1 method if it shows signs of suffering that in any way compromises normal behaviour but only when these are not those expected as step related adverse effects.

Throughout the protocol, a general humane endpoint of 20% body weight loss from baseline will be applied, regardless of the experimental step. Animals reaching this threshold will be humanely killed immediately using a Schedule 1 method.

Protocol 10: Experimental design

What outputs are expected to arise from this protocol?

For example, test results, phenotypic information, or products.

Phenotypic information on the dynamics that skeletal cells play during, or in promoting bone fracture healing in the femur or digit.

Will this protocol generate quantitative data?

Yes

Will your experimental design be determined by a regulatory guideline?

No

Where relevant, explain how and when pilot studies will be used.

Pilot studies, using no more than 3 animals per condition, will be conducted to generate preliminary data and in order to allow for procedures and techniques to be perfected before embarking on large scale experiments. Specifically, we will perform pilot experiments when animals are to be injected with substances or cells to obtain an indication of the dose and the timing of the injections, that is likely to be effective. Additionally, this study will use genetically altered mice that have been previously generated and validated, therefore the starting dose of trans-gene deleting or inducing reagents used will be the minimum that was shown to have an effect according to the literature.

How will you choose different experimental groups?

For example, controls, dose levels, satellites etc.

After the initial surgery, wild-type animals will be randomly assigned to either the control or treatment group. The treatment (e.g. pharmacological substances) doses will be determined through pilot experiments, taking into account the effective reported dose levels in the literature.

When experiments use genetically modified animals, where appropriate, animals will be assigned to either the control or experimental group based on their genotype.

How will you choose control groups?

Provide a robust scientific justification for controls with significant suffering such as sham surgery controls or untreated infected controls.

Each individual experiment will use a control group of mice that are contrasted directly to the experimental group of mice. For example, when injecting mice that received fractures with a substance resuspended in 0.1% DMSO, control mice that received fractures will be injected with the same vehicle, 0.1% DMSO. A sham surgery control will only be included in the event that the necessary vehicle control has not been tested in this system, or been reported in the literature to be innocuous. The inclusion of a sham surgery control would be limited to pilot experiments and is necessary to ensure that unknown vehicle controls alone cannot account for any observed effects and result in misleading or inaccurate conclusions.

How will experiments and data analysis be randomised and blinded?

Prior to conducting experiments, the animal's legs (left side or right side) if receiving localised injections, or the entire mouse, if receiving systemic injections, will be randomly assigned to either the control or experimental group by standard methods. For example, for local injections, each side of the mouse will be identified with a unique identification number and numbers drawn out of a hat, randomly assigning them in a logical fashion to either control or experimental group. Treatments (substances or cells) will be uniquely coded by an independent person not associated with the study, and the coded information placed in an envelope until the experiment is completed. Where possible, the lab member conducting the surgical intervention and treatment will not participate in analysis of the collected data. Additionally, data analysis will be carried out in a blinded fashion by assigning further codes to the samples by an independent person not associated with the study. Those analysing the data will, when necessary, be informed as to which animals are grouped together in order to make group comparisons, but will not be aware what treatment these groups received in order to avoid any subjective decisions about applying data transformation to the outcome or in handling outliers.

How will you minimise variables to ensure reproducibility?

We will aim to minimise variables in this protocol by using inbred, previously validated genetically altered mice for experiments and will avoid exposing the laboratory mice to unnecessary stress by employing good handling practises. Bias will be avoided through good experimental design including incorporation of blinding practises both during experimental procedures and analysis of the data. Additionally, accurate records will be maintained of all work conducted under this project licence and methods will be reported responsibly including descriptions of all variables such as mouse age, number, strain, housing conditions and appropriate statistical analyses.

How will you determine group sizes?

You should reference POWER calculations you have made, if relevant.

Group sizes were determined using POWER calculations for an unpaired t-test whereby the biological effect size (using proportional differences) is expected to be 1. 5 to 2.0 fold and standard deviations approximately 20%, based on published literature. The power was set to 90% and alpha 5% (two sided) resulting in group sizes of n = 8 animals per condition. To accommodate occasional animal loss due to

experimental complications and unexpected variability, a sample size of n = 10 was subsequently selected for each group (control and experimental). Additionally, advice on group size calculations and experimental design has been sought from a local bio-statistician.

How will you maximise the data output from the animals you use on this protocol?

To maximise the information gained from a single animal, experiments will be designed in such a manner, that the femur or digits from one hindlimb will be fractured and the femur or digits on the contralateral side will be utilised as an uninjured control. Since these experiments only require the femurs, we will share any remaining tissues of interest with other scientists, alleviating the need for further breeding and animal usage. Additionally, all experimental approaches and data will be disseminated to the scientific community to avoid unnecessary duplication and to share any new findings.

Protocol 10: Protocol justification

Why is each type of animal, experimental model, and/or method selected for this protocol:

a) the most appropriate scientific approach?

My laboratory is specifically interested in understanding the behaviour of stem/precursor cells and the mechanisms that underlie complex tissue regeneration, including skeletal tissue, in mammals. The skeletal tissues that will be examined are extremely complex with numerous cell types that interact with each other. Currently, there is no adequate *in vitro* method to recapitulate the intricate series of events that unfolds during skeletal repair. Such a method would need to include combinations of bone, cartilage, muscle and tendon, as well as supporting cell types including immune and endothelial cells. Furthermore the geometric shape and forces applied to the bones and their joints during walking/ running are highly coordinated and use multiple tissue structures. For these reasons an animal model is an important part of the research programme, allowing us to study these complex processes and to identify clinically translatable therapies for bone regeneration in humans.

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b) the most refined for the purpose?

Surgical procedures will be carried out using aseptic technique which meets at least Home Office Minimum Standards of Aseptic Surgery and the LASA Guiding Principles.

Peri-operative and post-operative analgesia will be provided; agents will be administered at doses and frequencies agreed upon in advance with the Named Veterinary Surgeon.

Post-surgical monitoring will be conducted following surgery, using an approved post-surgical monitoring chart as agreed upon in advance with the Named Veterinary Surgeon.

The fracture protocol has been refined by including an open osteotomy as opposed to a closed fracture to ensure better reproducibility and precision. The impact of this refined method on the animal's health includes 1) faster healing, given that the fracture will not be comminuted (or broken in multiple locations); 2) fewer surgeries, given the greater precision of this technique; and 3) improved post-operative mobility, given the reduced damage to surrounding muscle and other soft tissues.

Trans-gene inducing or deleting substances will be pre-screened in pilot experiments to obtain an indication of the dose that is likely to be effective. As this study will only use previously-validated compounds (tamoxifen), the starting dose will be the minimum to have an effect according to the literature.

All animals receiving trans-gene inducing or deleting substances will be carefully monitored to ensure the health and well-being of the animals does not suffer. Supportive care will be given to any animals displaying weight loss and the animals carefully monitored.

For each model and/or method, what is the scientific need for the expected clinical signs?

The surgically created injury to the bone is a fundamental part of this protocol and provides the basis for us to understand the mechanisms of bone repair. Whilst the animal may initially exhibit moderate clinical signs these are expected to rapidly ease within 72 hours.

Why scientifically do the animals need to suffer to this degree?

The creation of a surgically induced fracture is essential to the scientific goal of identifying clinically translatable therapies to improve bone regeneration. Additional steps in the protocol (administration of substances, blood sampling and imaging) are only expected to cause transient mild discomfort.

Why can't you achieve your scientific outputs with an earlier humane endpoint, or without animals showing any clinical signs?

The healing of the joint surface after injury is a process that takes several weeks and our experiments are designed to understand specific aspects of the repair and regeneration events that occur during this time. Clinical signs are likely to be only observed in the first 72 hours following surgery, thereafter the animals are not expected to show any further issues.

Following tamoxifen administration, a transient weight loss is occasionally observed dependent on the strain. In some animals this may exceed 15% for a short period but is reversible with supportive care, with animals stabilising within 24 hours. Immediate euthanasia at 15% weight loss would result in the unnecessary loss of animals that would otherwise recover fully, increasing animal use without welfare benefit. An upper limit of 20% weight loss is therefore set as an immediate humane endpoint, beyond which recovery is unlikely and welfare would be compromised.

Will you be administering substances for experimental purposes?

Yes

How will you assess the suitability of these substances, and minimise the unnecessary harms arising from their administration given the particular strain or type of animal you will be using?

When assessing suitability, state how you will consider toxicity, efficacy, and sterility.

When using trans-gene inducing or deleting agents (i.e. tamoxifen) for the first time, doses will be chosen to minimise the risk of toxicity based on the available literature and validated in small pilot study.

As this study will only use previously-validated reagents, all administered substances are not expected to cause adverse effects. However, if animals show, unexpectedly, any clinical signs such as hunched posture, pilo-erection or subdued behaviour, these animals will be immediately killed by a schedule 1 method.

To ensure sterility, all trans-gene inducing or deleting agents (i.e. tamoxifen) will be prepared in a Biosafety cabinet using sterile equipment and sterile filtered solutions.

How will you determine an appropriate dosing regimen?

Include routes, dosage volumes, frequencies, and durations.

For most substances, the dose, route, frequency, and duration in mice are well documented. Where data are lacking, short-term pilot studies using low doses in a small number of mice (typically 2–3) will be conducted to identify a safe and effective regimen. Dosing volumes and frequencies will be minimised and follow standard guidelines (UBS and LASA) for each route of administration (e.g., subcutaneous or intra-peritoneal) as documented below. Pilot studies allow adjustment of doses before treating the remainder of animals in each experimental group. Maximum volumes and frequencies exclude administration for rehydration, anaesthesia, or analgesia.

Mouse	Daily Max. Volume (ml/kg)	Max. No./Day <10 Days	Max. No./Day >10 Days	Max. No. Injections
S/C (Subcutaneous)	20 ml/kg	3	2	24
I/P (Intra-peritoneal)	20 ml/kg	3	1	30
I/V (Intravenous)	0.8 ml over 2 min	2	1	14

Oral Gavage	20 ml/kg	3	2	30

Use of animals

Purpose bred animals

Will all schedule 2 animals used in your project be purpose bred?

This means animals that have been bred primarily to be used in regulated procedures or for the use of their tissues or organs for scientific purposes.

Yes

Endangered animals

Will you be using any endangered animals, apart from non-human primates?

Endangered animals are any of the species listed on Annex A of Council Regulation 338/97 and are not bred in captivity.

No

Animals taken from the wild

Will you be using any animals taken from the wild?

No

Feral animals

Will you be using any feral animals in your project?

A feral animal is an animal living in the wild but descended from domesticated individuals.

No

Other considerations

Neuromuscular blocking agents (NMBAs)

Will this project involve the use of neuromuscular blocking agents (NMBAs)?

No

Re-using animals

Why do you intend to re-use animals?

Explain how you will balance the needs of refining and reducing animal use before making your decision.

Animals will be re-used where this represents a refinement and contributes to a reduction in the total number of animals used, without compromising welfare or the scientific integrity of the work. Specifically, animals will be re-used for superovulation, as embryo recipients, or following vasectomy procedures. In some cases, wild-type mice previously genotyped by a regulated method or those that have undergone non-surgical implantation may also be re-used.

These procedures are of low severity and do not result in lasting adverse effects that would preclude re-use, as confirmed by the Named Veterinary Surgeon (NVS) and the Named Animal Care and Welfare Officer (NACWO) prior to any reallocation. Each animal's health, behaviour, and welfare status will be assessed to ensure full recovery before re-use is approved.

What are the limitations on re-using animals for this project?

For example, there may be a maximum number of times that an animal can be re-used, or a set of performance standards that requires a limit on re-use.

Re-use will be limited to animals that have fully recovered from previous mild or non-surgical procedures (e.g. superovulation, embryo transfer, vasectomy, or genotyping), as confirmed by the Named Veterinary Surgeon (NVS).

Re-use will not be permitted if the cumulative experience would exceed moderate severity, or if there is any evidence of lasting adverse effects. Normally, animals will be re-used only once, unless the NVS confirms that further use remains within welfare limits.

Commercial slaughter

Will you send any farm animals to a commercial slaughterhouse at the end of their use?

No

Animals containing human material

Do you intend to use animals containing human material in experiments classed as Category 2 or 3 by the Academy of Medical Sciences?

No

Keeping animals alive

What types of animals will you keep alive?

Only female animals used as embryo recipients under Protocol 7 and vasectomised male animals under Protocol 8 will be kept alive.

What criteria will the veterinary surgeon, or competent person trained by a veterinary surgeon, use to determine whether animals can be kept alive?

Non-surgically implanted female mice used in Protocol 7 will be assessed by the Named Veterinary Surgeon (NVS). They may be retained for potential re-use as embryo recipients only if they show no signs of ill health, are of suitable body weight, and are deemed to be in appropriate overall condition and fitness. The maximum number of uses for a non-surgically implanted female mouse is 2.

Vasectomised male mice used in Protocol 8 may be kept alive following consultation with the NVS, provided they have fully recovered from the procedure, do not exhibit excessive weight gain, and show no indicators of ill health. Where appropriate, and subject to veterinary approval, they may be re-used for pseudo-mating.

Are there any limitations on the period of time that animals that have been kept alive can be held under the supervision of the veterinary surgeon?

Up to 12 months.
