Home Office

NON-TECHNICAL SUMMARY

Defining the role of the senescent cells upon the cancer and pre-malignant microenvironment.

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Liver, Cancer, Senescence, Immunity, Endothelium

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Senescent cells are the earliest stage of cancer. We seek to understand the interaction between senescent cells, the cells that line blood vessels and cells of the immune system within the liver.

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

Why is it important to undertake this work?

Background

The development of cancer occurs when cells of the body accumulate damage to the genetic code that is present in every cell in our bodies. This generally occurs slowly, but can occur more quickly in parts of the body affected by long-term inflammation, such as liver cirrhosis or inflammatory bowel disease. Having these illnesses leads to damage to the genetic code and activation of cancer-causing genes. At the earliest stages, prior to our current ability to detect cancer, the body has defence mechanisms to suppress these cancer-causing genes and prevent cancer progressing. One such mechanism is called cellular senescence, where cells of the body sense this damage and respond by irreversibly preventing that cell from dividing any further. Senescent cells also signal to other cells around themselves preventing them from reproducing as well. Senescent cells also attract circulating immune cells that kill the senescent cell and the cancer-causing genes that it contains. Therefore, understanding how senescent cells are detected and killed by the immune system is important and could be a way of treating patients to prevent them getting cancer in the first place.

Immune cells, which circulate in our blood protect us from infection and cancer. They enter different parts of our body by sticking to the walls of the blood vessels when they detect a problem, such as inflammation. Within the liver the blood vessels are lined by sinusoidal endothelial cells, which can attract immune cells into the liver to kill bacteria and viruses. We know that immune cells enter the liver when cancer develops, but we do not know how this happens when cells of the liver become senescent. Nor do we understand whether the endothelial cell is important in this process. If we could understand the processes that cause immune cells to enter the liver to kill senescent cells, then we might be able to prevent cancer developing in the first place, through boosting the body's natural defences. Boosting the immune system to fight cancer has already been shown to be effective in other cancers, such as melanoma.

If we can find out whether it is possible to improve the functioning of the immune system and improve the ability to clear the earliest forms of cancer within the liver, we might be able to design treatments that prevent patients in the future from developing cancer.

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

If successful, this project has a number of significant outputs:

(A) Scientific advancement and publication

The aim of this project is to increase knowledge of the role of the endothelium (cells which line the blood vessels) and immune system (cells which attack infections and cancer) in combating the earliest forms of cancer. By the end of this project we hope to understand how pre-cancerous, senescent cells, signal to the endothelium and the immune system to prevent cancer from developing and how this system fails.

We will aim to promptly publish and disseminate the results in open access, peer-reviewed, high-impact scientific journals and regular presentations of data at high-profile meetings. Some data that is important for working with industry to develop new medicines may not be released straight-away, until intellectual property restrictions are sorted out.

(B) Drug-development and potential collaborations with industry

Through this work, we may identify drug targets to boost the immune system in the clearance of pre-cancerous cells with the ultimate goal of developing new drugs to work in patients. Any potential drugs or drug targets that we discover would be tested in subsequent projects.

(C) Public engagement

We will use well-established routes at our institution to engage with the wider public, explaining why we believe this research is important.

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

The following groups will likely be beneficiaries of this project:

(A) Other scientific researchers

This project will be directly of benefit to scientific researchers working in the fields of senescence, liver biology and liver cancer. Scientists will be able to access our newly developed knowledge through:

1. Publication of results in open access, peer-reviewed, high-impact scientific journals and regular presentation of data at national and international meetings. This will promote new scientific work from other groups around the world.

2. Sharing of our study data through online open access databases. Generation of publicly available data will drive discovery by other groups, but also prevent the need for repetition of expensive scientific studies.

(B) Future patients

Through development of knowledge about how senescent cells are cleared from the liver, we will be able to design novel immunotherapy treatments to prevent the development or progression of cancer.

(C) Society and the economy

If we are successful and find new treatments, these will be developed in partnership with pharmaceutical companies. In addition, if we can develop new treatments for cancer, this will improve people's health-span: their length of healthy life. Of course, if people live into dependent old age this may increase societal costs later.

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

We wholly support making our published findings available through Open Access. We intend to make data generated during this project open to the wider scientific community and the public, via multiple routes:

(A) Communication

New ideas from this research will be shared with scientists through talks at local, national and international scientific conferences; and educational talks for students. We hope that some of our findings will be of general interest and we will work with the public engagement teams at our institution to make the information interesting and digestible for lay readers.

(B) Dissemination

All the results arising from this project will be submitted to peer-reviewed journals and presented at national and international conferences.

Our published data will be deposited in publicly available databases, allowing access to our findings. Useful reagents derived from this study, will be distributed upon request or deposited in publicly accessible repositories, such as Addgene (https://www.addgene.org).

(C) Engagement

We have previously been active in explaining our work to the general public, both at fundraising events such as public tours of our institution, at science fairs, such as our institution's annual Science Festival and talks at local schools.

(D) Development and exploitation

It is possible that potential new treatments will be generated in this project. We will get advice and support from the Technology Transfer Team at our institution to help develop these further.

Species and numbers of animals expected to be used

- Mice: 14080

Predicted harms

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

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

Our project intends to study how senescent liver cells communicate with the cells lining the blood vessels and white blood cells of the immune system. The use of mice allows us to study several types of cell at the same time, in the development of cancer.

Mice have very similar biology to humans, particularly in their immune system. They have short development and generation times that will allow us to perform our experiments in a timely and efficient manner. Due to the widespread use of mice in cancer research there are a wealth of scientific tools, like antibodies available and drug doses already worked-out for experimental use in mice. To alter genes or pathways in the liver requires targeted genetic manipulation. There are many well known techniques to genetically-modify different cell types at different times in mice. In some cases this can be achieved through injected DNA molecules or viruses, but in the case of modification of the vascular endothelium will require the use of genetically altered mice. We have used all of these approaches before.

Therefore, the use of mice represents the best animal model for our experiments.

1) **Senescence.** We will make senescent liver cells through genetic modifications or using viruses. This is an established technique that we have used in mice that are 6-9 weeks old. It has not been demonstrated to work in other animals or in mice at other life stages.

2) **Chronic liver disease.** We are interested in understanding human liver disease; mice can get a similar form of liver disease. We will give the mice a modified high-fat diet or treat the mice with a drug (carbon tetrachloride), leading to the development of chronic liver disease in 6-9 months. Experiments studying liver disease in mice are in widespread use around the world. There are models of chronic liver disease that work faster or earlier in life, but they do not look like human liver disease

3) **Transplantation.** To look at the way different cell types communicate with each other, we can perform transplants of different types of cells together into mice that lack an immune system and then study their growth and interaction. The lack on an immune system is crucial to prevent rejection of the transplanted cells. This is a very straightforward process in mice, where animals lacking immune systems are commercially available.

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

To manipulate the biology of senescence and chronic liver disease requires targeted genetic manipulation. In some cases this can be achieved through injected DNA molecules or viruses, but in the case of modification of the endothelium will require the use of genetically altered animals. We will also use several other techniques:

1. **Liver-specific genetic alterations:** For these mice we will alter gene function within different cell types in the liver using combinations of inducing agents, viruses or drugs. These mice will be monitored by clinical signs or intermittent imaging / blood tests over the course of weeks or in a small minority of mice, months. We will not let the mice develop into old age.

2. **Hydrodynamic tail vein injection:** This involves the injection of genes in a large volume of saline into the mouse and results in delivery of the genes straight to the liver cells. We will use this technique to induce senescence within the liver cells before following these mice over the course of a month, or in a small minority of mice, months. We will not let the mice develop into old age.

3. **Induction of chronic liver disease:** We will induce chronic liver disease by giving these mice a high-fat diet and / or treating them with carbon tetrachloride (a chemical that damages the liver), whilst altering liver gene function using viruses or drugs. We will follow these mice for several months and monitor them by clinical signs, imaging or blood tests. We will not let the mice develop into old age.

4. **Transplantation:** These mice will be injected with complex mixtures of cells, either into the skin, spleen or liver. This allows us to study these cells for prolonged periods of time and see how they interact. These mice will be monitored by clinical signs, periodic imaging or blood tests. In mice with skin tumours, we can measure the tumour size directly with calipers. For mice with liver or spleen tumours, we will monitor the tumour using imaging, such as ultrasound. We will not let the mice develop into old age.

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

We are proposing a diverse plan of work that includes several procedures that the mice may find stressful. These can be divided into short-term and long-term impacts:

1. **Short-term impacts**

    1. **Anaesthesia for procedures**: Animals may find single or repeated general anaesthesia stressful. We will ensure that the mice are handled sensitively and keep repeated sessions to a minimum. Anaesthesia will last for less than 2 hours; it may be repeated, but not more than twice in a 24 hour period.

    2. **Blood taking / injections**: This requires handling and injection which may be painful. This will last only a few minutes.

    3. **Treatment with drug or inducing agents:** This requires handling which may be stressful. Some substances can be given in modified feed. Some substances need to be given by oral gavage which might be stressful. Some substances need to be given by injection which may be painful. Some agents need repeated dosing. We will ensure that we use the fewest number of doses to achieve our intended effect.

        1. Some inducing agents, like tamoxifen, can be painful when given by injection into the abdomen. We will ensure we use the fewest number of doses required. Tamoxifen can

cause weight loss in some mice. We will closely monitor this in the mice receiving this treatment.

4. **Hydrodynamic tail-vein injection**: Animals have to be placed in a restrainer, before intravenous injection of a large volume of saline. We inject mice whilst they are awake when they may suffer from pain or distress, as lots of scientific data around the world has been gained in this way before. It might be better to perform this in mice under anaesthetic, but we do not know whether this will give the same results or lead to more mice dying. We intend to compare the two methods during this project. After the injection the mice remain lethargic for around 30 minutes, but then recover without problems in most cases. Mice will only undergo this procedure once.

5. **Transplantation:**

    1. **Under the skin:** We will transplant mixtures of cells under the skin of some mice, to understand the effect of pre-cancerous cells upon endothelial cells. This is a quick procedure that involves one or two injections.

    2. **Into the liver or spleen:** We will transplant mixtures of cells transplanted in the liver or spleen of some mice. We will do this under general anaesthetic to ensure accurate placement and to minimise stress to the mice. Mice will only undergo this once.

2. **Longer-term impacts**

    1. **Induction of chronic liver disease:** To induce chronic liver disease, we will either:

        1. Give the mice a **modified high-fat diet**. The mice will get fat and so may move around less;

        2. with or without repeated **injections of carbon tetrachloride**. This can take up to a year to develop depending on the dietary modification that we use. This involves repeated injections in the abdomen that could be stressful or painful;

        3. We will not let mice reach the point where the mice start developing problems from liver failure.

    2. **Repeated investigations:** In some mice we will perform drug treatment or imaging several times over the course of an experiment. There is a tension here between performing more investigations on a small number of mice or fewer investigations on a larger number of mice. In our application we have set limits to the number of treatments or procedures that a single mouse can undergo, to prevent undue suffering.

    3. **Liver tumour or transplanted tumour development:** Some mice will develop liver tumours in the longer term after tail-vein injection or dietary modification. Mice undergoing transplantation will develop tumours in the skin, liver or spleen. We will closely monitor animals who might develop tumours and ensure that they do not develop signs of distress.

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

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

Mice	% of total
Mild	54.5%
Moderate	45.5%

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

- Killed
- Used in other projects

Replacement

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

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

When the liver is damaged, cancer of the liver can develop. Our goal is to understand how different cells within the liver interact when the liver is damaged and what goes wrong to allow cancer to develop. Crucially, we need to understand the activation of the immune system in long-term liver damage and whether the endothelium is involved in this process.

To understand the complex relationships between different cells in the liver, we need to study these cells in a living animal, as we cannot study these relationships using cells cultured in a laboratory. Importantly many of these processes are very slow and occur over a period of days or weeks. Animal experiments allow us to perform these experiments over longer time periods.

We can study some of these processes in liver samples obtained from patients, but these only allow us a snapshot. To manipulate or change the behavior of specific cell types in the liver means we need to use genetically-modified animals.

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

For some experiments we will use cells cultured in the laboratory, that allow us to study how two liver cell types communicate with each other. We have already utilised cultures of endothelial cells with either pre-cancerous senescent cells or cancer cells to determine the effect upon the endothelial cells.

In addition, we will make use of cutting-edge techniques, such as three dimensional (3D) cultures or liver-derived organoids that more closely resemble the normal liver, where possible. These will allow us to use human liver tissue in the laboratory, something that was not previously possible to do. We can also genetically modify these organoids, replacing some of the mouse experiments.

For experiments where we do not need to manipulate anything, we will study cell behavior in human liver samples removed as part of patients routine clinical care.

Why were they not suitable?

Study of cells grown in the laboratory allow us to develop ideas about which signalling pathways we will then study in living animals. However, these cell experiments do not allow the study of complex interactions, such as the immune system. This can only be studied in living animals:

1) Cell culture systems can work for two different cell types, but to study more than this is impossible, due to different growth rates and need for different culture conditions.

2) Cell culture systems cannot keep cells alive for long enough to study the effects of injury over prolonged time scales required to be representative of human chronic liver disease.

3) Introduction of the complete immune system into cell culture systems is nearly impossible due to the short lived nature of some cells of the immune system and the very slow growth of other white blood cells that protects us from infectious diseases and cancer. To study the effect of induced endothelial behaviours upon the immune system requires a living animal.

4) Studying human tissues is important to understand human health problems, but we are unable to manipulate specific cell types to see what effect these changes have. Experiments in mice allow us to do this.

Reduction

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

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

The numbers of animal used in experiments will be carefully predicted based upon data from our previous work and previous published work by other groups. In all cases we will use the minimum number of animals required, for the experiment to give us useful data. We will work with statisticians to make sure that a minimum number of mice are used to generate significant results.

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

Whenever we design experiments we are mindful of the 3Rs and the need to continually optimise our plans. We will reduce the number of mice required in our experiments by:

1) **Reducing breeding**. We are going to use cutting-edge techniques including specific viruses and genetic-editing technology to improve the efficiency of our breeding programme. These will reduce the number of mice that are bred with the wrong genotype.

2) **Improving experimental design.** We will follow the PREPARE guidelines and use experimental planning tools, such as the **NC3R's Experimental Design Assistant** to improve the design of our experiments. We will perform pilot experiments, where we are unsure as to the effect size. This will ensure that we use the minimum number of mice in our experiments. For complex experiments we will seek help from local statisticians to ensure that our experimental designs and mouse numbers are appropriate.

3) **Making sure we get the maximum information from each animal.** For each experiment we will try to get the maximum amount of information from each mouse. This will be through repeated scanning, where possible, and analysis of several organs after death. We will also share both samples and data with other scientists, so that experiments do not have to be repeated.

4) **Reporting our findings properly.** To report our scientific findings we will follow the ARRIVE guidelines, so that our findings are clearly set-out. This means that the experiments will not need to be repeated by other scientists.

5) **Use of previously generated tissues.** Through literature review, we will ensure that similar experiments have not been conducted before. If similar experiments have been conducted we will approach the study authors to ask for access to the banked tissue samples, potentially reducing the number of animals needed to be used in our study.

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

We will use a number of cutting-edge techniques that will allow a reduction in the numbers of mice that are required to be bred. Complex breeding programmes always generate large numbers of mice of the wrong genotype. Our techniques allow specific genetic modification of liver cells and include:

1) **Hydrodynamic tail-vein (HDTV) injection.** We currently use this technique on a regular basis, which allows delivery of small sections of DNA to liver cells, where it is cut and pasted into these cells DNA, altering their behaviour. Using this method we can take any mouse and express any gene that we have designed in the laboratory, within the liver, without the need for complex and wasteful breeding strategies.

2) **Adeno-associated viral (AAV**) **injection.** These viruses home to the liver, again allowing gene delivery to liver cells without the need for complex breeding strategies. This strategy is complementary to the HDTV above, but only allows certain genes to be expressed.

3) ***In vivo*** **CrispR-based DNA editing**. This novel technique allows us to use either HDTV or AAV to deliver molecular machines (Cas9 enzymes) allowing us to edit the DNA in liver cells. Therefore, this

technique allows us to create specific mutations in the liver cells in a live mouse, without the requirement for complex breeding strategies.

Refinement

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

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

We will use mice to study the interaction between senescent cells and blood vessel cells in liver disease. To do this we will induce liver cell senescence or chronic liver disease via:

1) **Modifying the DNA of liver cells.** Using either hydrodynamic tail-vein (HDTV) injection or injection of adeno-associated viruses (AAVs) will allow us to directly modify the DNA of liver cells, to induce senescence or modify specific genes or pathways. Both are standard techniques that we currently use in our laboratory. The main drawback of the HDTV injection technique is the success rate of delivery; currently about 70% in our hands. Although successfully-injected mice develop shortness of breath and lethargy shortly after the procedure, the mice return to normal within 30 minutes.

The HDTV injection has previously been delivered whilst the mice are awake to allow comparison with previous experiments. A refinement to the technique might be to perform HDTV injection under general anaesthetic, where the mice would experience less pain or distress. We intend to compare the two techniques to ensure that injection under anaesthetic works in the same way.

2) **Induction of chronic liver disease.** We will use well established techniques to induce chronic liver disease (CLD) in mice by:

1. Modifying the diet of the mice to include lots of sugar and fat. These modified diets will lead to chronic liver disease, that looks like human fatty liver disease within 6-9 months;

2. Treating the mice with a drug called carbon tetrachloride. This leads to liver damage and over time the mice will develop chronic liver disease, that looks like human liver cirrhosis

These techniques are well established and used in many laboratories around the world.

We have significant experience with these models of liver disease, including the problems which may develop. The mice will be closely monitored to ensure they remain in good clinical condition. These interventions will occur in normal or genetically modified mice. To target particular tissues at particularly timepoints we will use conventional transgenic modification:

3) **Genetically modified mice** will allow us to study particular genes or pathways, particularly at specific times. Where possible we will use inducible DNA modification (e.g. Cre-LoxP) to specifically

target particular tissues and limit the effects on the mouse. This will be particularly important to limit our modifications to the cells that line the blood vessels.

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

Mice develop liver disease, that looks very similar to liver disease in humans, but it occurs much more quickly. Lower species such as flies or worms do not develop chronic liver disease and do not have immune systems like humans. Indeed, their body organs are so different to humans that they are not suitable to study. Mice permit us to study senescence, chronic liver disease and the role of all components of the immune system. Many suitable genetically modified mice already exist and if they don't the tools are available to create them. Therefore, mice represent the most appropriate animal model for our experiments.

We are unable to study earlier stages of development as:

1) We are interested in senescence or pre-cancer. This has not been demonstrated or produced in embryos or juvenile animals in previous scientific studies.

2) Induction of senescence or chronic liver disease takes time, which would result in the animals being adults by the time that chronic liver disease had developed.

We are unable to perform experiments under terminal anaesthesia as senescence takes days and chronic liver disease weeks to develop. Performing this under anaesthesia is clearly unfeasible.

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

We will seek to constantly improve the experiments that we perform.

1) Staff development. This work will be performed by experienced doctors, scientists and technicians who will closely monitor the mice. A key refinement is ensuring that staff performing techniques are fully trained. This has been the key in our current facility to improve the success rate of delivery of hydro-dynamic tail-vein injection.

2) Housing and husbandry. We will ensure that we use environmental enrichment in order to promote normal animal behaviour in our mouse colony. We will plan to house the mice together where possible to avoid loneliness. We will ensure that mice have access to environmental enrichment, such as wooden chew sticks, nest-building materials and fun tunnels, in order to reduce stress. In our current mouse facility we identified that our mouse colony was on a diet too rich in fat and were developing fatty liver disease. On changing the diet to one lower in fat content, the weight of our mice does not increase as much over time and they do not develop fatty liver disease.

3) Improved experimental design. Pilot experiments will be performed when we try new techniques or models, allowing us to identify any unexpected problems and improve the full experiment. This approach will also mean that we will only use animals that are absolutely necessary.

4) Improved techniques. We set strict limits to minimise the harm or suffering to the animals used. We will only use a limited number of surgical procedures and ensure that pain is treated after these

procedures. We will use general anaesthesia for non-invasive imaging procedures, allowing us improved pictures of the liver. We will monitor the mice throughout the procedure and during the recovery period after anaesthetic. Close monitoring during longer-term experiments will ensure that animal suffering is kept to a minimum and clinical signs are picked up promptly.

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

We have extensive experience in studying senescence and chronic liver disease in mice. We will use this experience during this new project, but will make use of published guidelines to improve our experiments.

1) We will improve experimental design following the **PREPARE guidelines** and using tools, such as the **NC3Rs experimental design assistant**.

2) Our animal experimentation programme will be run according to the guidelines and position statements from the **Laboratory animal science association (LASA).** We will care for our mice according to the guidelines laid out in the **Workman et al** (British Journal of Cancer (2010) 102, 1555 – 1577)**,** on animal welfare in cancer research.

3) During our experiments we will collect data on mouse phenotype and clinical signs according to the **FELASA guidelines**.

4) When reporting our experimental results we will adhere to the **ARRIVE guidelines**, aiming to provide a comprehensive description of experiments and findings.

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

During this project I will ensure that I keep abreast of the latest developments in the 3Rs through several sources:

1) **Continuous professional development.** I will attend relevant training courses to keep my licence current, that will include developments in the 3Rs.

2) **Institutional 3Rs search tool.** Our establishment has a web-based search tool that allows me to keep up to date with the 3Rs.

3) **Online tools.** There are a number of websites for organisations that promote best practice in animal welfare that provide up-to-date information, such as the National Centre for the 3Rs (https://www.nc3rs.org.uk/) or the Norwegian National Consensus platform (https://norecopa.no/).

4) **Peers and colleagues.** In biomedical research I have extensive connections with other researchers throughout the UK and the world. I attend numerous conferences, where new findings are presented, allowing improvements in our techniques and experiments. An example of this is the use of AAVs to genetically alter liver cells; I learned this technique from scientific colleagues and have now brought this to our establishment, significantly reducing unwanted breedings.