Home Office

NON-TECHNICAL SUMMARY

Cytokine signalling in development and disease

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

Key words

zebrafish, marfan-related syndromes, cancer, therapy

Animal types	Life stages
Zebra fish	adult, juvenile, neonate, embryo, aged
Mice	adult, pregnant, juvenile, embryo, neonate

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand how cytokine signalling orchestrates early vertebrate development and how its deregulation results in human diseases, for example, cancer, fibrosis and Marfan syndromes.

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?

Understanding how cytokine signalling regulates early vertebrate development will aid in understanding the origin of human congenital abnormalities, and could help develop treatments. Cancer is a major cause of mortality in humans, accounting for about a quarter of all deaths and understanding how deregulated signalling results in cancer could directly result in new targeted treatments. Similarly, for other diseases such as fibrosis (idiopathic pulmonary fibrosis has a prevalence of 50/100,000) and Marfan syndromes (prevalence of 1 in 5000).

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

The outputs of this work include:

1. An understanding of the role of Activin in cancer and validation of anti-Activin therapy for different cancers, both as a monotherapy and in combination with other therapies. If successful, this could be developed into a cancer therapeutic for humans in the future.

2. A better understanding of the role of SMAD1/5 signalling in cancer, which could lead to new therapies, and to the development of diagnostic and prognostic markers.

3. Understanding how activating ACVR1 mutations result in DIPG could help with the development of treatments for this fatal disease.

4. An understanding of how mutations in Ski lead to disrupted TGF-β signalling in Shprintzen-Goldberg syndrome, which could lead to novel therapies. We anticipate that the zebrafish disease model we generate could be used to test novel therapeutics.

5. An understanding of how mutations in SMAD2 and 3 lead to disrupted TGF-β signalling in Loeys-Dietz syndrome, which could lead to novel therapies. The zebrafish disease model we generate could be used to test novel therapeutics.

6. Understanding how TGF-β family signalling dictates cell fate specification will be of great benefit to the basic research community and will inform tissue regeneration studies.

7. Transgenic lines of mice and zebrafish expressing ligand-dependent reporters or expressing biosensors for activated receptors, signalling pathways or transcription factors will be of enormous benefit to the research community as they can be used in numerous other projects where a visual readout of signalling is required.

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

There are likely to be multiple beneficiaries from the outputs above. These include:

1. The anti-Activin therapy we are developing, if successful, will be further developed in collaboration with a pharmaceutical company. We expect to know this within a year. Further development by a company would take of the order of 5 years, before first in human studies. The ultimate beneficiaries will be cancer patients, if we succeed in developing it as a drug.

2. From our other disease orientated projects, we anticipate that we might discover potential diagnostic or prognostic markers that could be developed for improved clinical management of patients. The time frame for this could be 3–5 years.

3. From our disease models, we may also identify possible treatments that rescue the effects of the mutations, that might ultimately be able to be developed into therapies. The time frame for this could be 5–10 years.

4. This work will benefit the basic research community by increasing our knowledge of embryonic development and cell biology.

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

A major mechanism to maximise the output of the work, alongside the publication of primary research papers, will be presentation of the work at both big international meetings and smaller workshops. Not only will the positive results be communicated, but where it is useful for other researchers, unsuccessful approaches will also be highlighted.

We collaborate widely, both internally and externally and this provides another avenue to share details of approaches that were ultimately sub-optimal and how methods were improved.

Where we develop new transformative methods, we will publish specific protocols papers and post on bioRxiv.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 77,000
- Mice: 13,120

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.

This project aims to understand how cell communication through cytokine signalling is involved in orchestrating early embryonic development and how its deregulation leads to human diseases such as cancer and Marfan-related syndromes.

For the embryonic development work we use zebrafish embryos that are less than 5 days old. These embryos develop ex utero and are transparent and thus excellent for imaging and we can generate 100 embryos from one set of parents that are synchronous.

For the cancer work, we want to understand how tumours develop and spread and to test candidate therapeutics to inhibit these processes. To do this, it is necessary to work with animals that have similar organs to humans – for example, lungs, pancreas and mammary glands. This leads to the choice of mice. The main cancer types that we study occur in adults, and occasionally, young adults. Therefore, we work with juvenile and adult mice.

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

For the mouse experiments, procedures will be performed that lead to the development of cancer in mice. In the majority of cases, this will be through injected cancer cells. In some cases, mice will develop tumours as an result of a particular breeding protocol. We either simply monitor tumour development and spread when we are investigating the effect of loss (or gain) of a particular protein/proteins, e.g. a component of TGF-β family signalling pathways. Alternatively, mice may receive therapies similar to those being used or being developed to treat patients. Where possible, advanced imaging methods will be used to monitor the spread of tumours and how they react to therapies. Extensive post-mortem tissue analysis will be performed to maximise the information obtained from each animal.

For the zebrafish work, we are predominantly generating either reporter lines expressing biosensors for different signalling pathways, or lines in which we have knocked out or mutated specific proteins in the TGF-β family pathways. These will mainly be studied at the level of embryos and larvae less than 5 days old. Thus the only procedures that the adults go through are breeding and genotyping. In some cases where we are modelling human diseases like cancer or Marfan-related syndromes, we may study the larvae up to juvenile and adult stages.

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

For the majority of the zebrafish experiments, we do not expect any adverse effects as we are mainly working with transgenics that have no negative effect on development or we are monitoring mutants only in larvae younger than 5 days old. For a small proportion of mutants, there may be some developmental abnormalities. If we observe abnormal behaviour, such as reduced swimming activity or retreat to dark tank corners, the fish will be killed.

In our project mice will develop tumours. Initially, these have little effect on the mice, but as they become larger they might affect mobility. Further, as tumours begin to spread they can affect weight, breathing, and behaviour. Depending on how much the animal is affected, the duration of the adverse effect may range from a small number of days to a small number of weeks.

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)?

For the zebrafish experiments, moderate severity is expected for about 10% of fish.

For the mouse experiments, moderate severity is expected for about a third of the animals.

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

- Killed

Replacement

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

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

We have been working on TGF-β superfamily signalling for 20 years and have gained tremendous insights into the mechanisms of signalling and the role of these signalling pathways in regulating cell behaviour from working in model tissue culture systems. However, in order to be able to investigate how these pathways regulate embryonic development, tumour formation and metastasis, and how mutations in pathway components lead to Marfan-related syndromes, we need to perform experiments in animals.

Our developmental work is performed on zebrafish embryos. We have chosen this system because of the rapid embryonic development ex utero and optical transparency allow a direct monitoring and visualization of development, morphology and physiology in the living organism. Recent development of novel genome editing tools (such as the CRISPR/Cas9) system has made it possible to generate mutant zebrafish lines in a highly efficient manner and the technology is easy to implement. Other advantages include ease of transient genetic manipulation using morpholino oligonucleotides for gene knockdown and sophisticated transgenesis tools (such as the Tol2 transposon system) to generate fluorescent reporter lines. For our zebrafish work, to minimise the number of procedures performed on animals deemed to be sentient, we do most of our work with zebrafish embryos and young larvae, before the onset of independent feeding. The only work that will be done on adult or juvenile fish is the fin clipping for genotyping, transient warming to induce expression of transgenes, and gamete harvesting. Concerning the genotyping, as often as possible mating will be performed followed by analysis of phenotype to determine the genotype of the fish as an alternative to fin clipping.

With regards to the mouse work, we only perform studies on mice that cannot be done in model tissue culture systems or in the zebrafish system. Moreover, we also study tumourigenicity in vitro. If our data show a good correlation between in vivo tumourigenicity and the in vitro properties of given cell lines and organoids, we may be able to further limit the number of tumourigenicity experiments.

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

We complement our in vivo analyses with tissue culture models and organoids.

Why were they not suitable?

We can gain a certain amount of information from these in vitro systems, but there are serious limitations. It is not currently possible to replicate the complexity of mammalian tissue structures in culture models. Moreover, the immune system only functions effectively in an organismal context with appropriate white blood cell movement and function within lymph nodes. Finally, in the process of metastasis the cancer cells move between organs. It is not possible to recreate this using in vitro models. One possibility to study tumour cell invasion is to perform some of these assays in young zebrafish larvae, before the onset of independent feeding. It is possible to gain information about the ability of particular tumour cells to exit the circulation and invade tissues. This may reduce the number of mice required in the project.

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?

For the zebrafish work we calculate the numbers of fish required based on the numbers of mutants and transgenics that we are currently maintaining, plus those that we need to generate to be able to achieve the aims of the project. Details of how many fish we need to raise to generate mutants and transgenics are given below. We currently maintain 30 mutant lines and 15 transgenic lines. We estimate that in the next 5 years we will generate around 20 new mutants and 7–10 new transgenics.

For the mouse work, the estimate is based on several factors. Firstly, we are using our previous experience of the last 5 years and the experience of our collaborators who use similar approaches with the same mouse lines. Secondly, we have taken into account the number of researchers within the group who perform mouse experiments (currently 3, and likely to be between 3 and 4 in the next five years). We also continually re-evaluate the numbers of mice required for each experiment using power calculations. For this we access help from an in house statistician when necessary. This will allow us to determine the number of animals required per experiment. Numbers of mice used for breeding are based on best practice and we maintain between 10-12 strains.

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

For the zebrafish work we regularly review our mutant and other stocks and cull any that are no longer required. For all zebrafish lines, we freeze sperm to archive the line. Thus, we will only maintain lines in the aquarium that we are actually using in on-going experiments. Through exchanges with other zebrafish labs, in the UK and elsewhere, we will be able to minimise the number of mutant and genetically modified strains that we keep in our own aquarium. Moreover, the stocks of adult mutant fish that we will keep will almost all be heterozygotes carrying recessive mutations and thus phenotypically normal. In addition, because we share the fish aquarium with a number of other labs, we ensure that multiple workers perform their experiments on the same day to maximise the use of the embryos from a given batch of fish. Any extras are fixed for subsequent experiments. We get on average 100 embryos per pair of fish.

For the mouse work experiments are designed to not falsely detect effects and not miss effects. Based on our previous experience of how variable our measurements are and how big the effect we are looking at is, then most experiments involve around 5-10 mice per group and 3-7 groups. If the necessary in vivo data does not exist, then experimental design will be informed by a combination of prior in vitro data generated in the laboratory, existing publications, and previous experience.

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

For the maintenance of zebrafish lines, we estimate that we use 400 fish per line over 5 years.

For generation of new transgenic lines, estimates of numbers of fish needed are based on statistics on the efficiency of transgenesis from ours and other laboratories. Typically for one transgenic line we will raise 100 F0s, which will be screened by crossing them to wild type and screening their progeny for the transgene. Typically 20-30 of the adult F0s will be founders. We will choose 2-3 of these and raise their progeny; the others will be culled. For each ‘family’ we will be raising between 50 and 80 fish which will be genotyped to identify heterozygous carriers of the transgene. The transgenic lines will be then maintained as for other wild type and mutant lines, which corresponds to about 4 tanks of around 20 fish each.

A similar strategy will be employed for the mutant fish generated by TALENs or CRISPR-Cas9 technology. To produce one mutant line we raise 200-250 (‘founder fish’), which are phenotypically normal after microinjection of the CRISPR/Cas9 constructs or TALENs. To screen for germline transmission and loss-of-function alleles the F0s will be crossed to wild type fish, and their progeny will be screened as embryos for the presence of the mutation. We will pick 2-3 positive founders and raise 200-250 progeny. The others will be culled. The F1s will be screened for the heterozygous mutation by fin clipping. Heterozygous F1s will be further outcrossed to wild type fish to segregate away any non-specific mutations and in-crossed to other transgenic lines (mutant lines to generate double mutants, GFP-transgenic reporter lines). We will raise 100-150 F2s from these crosses and screen them for the desired heterozygous mutations. The mutant line will be then maintained as for other wild type and transgenic lines, which corresponds to about 2-4 tanks of around 20 fish each.

For the mouse work we will try to keep as few mice as possible by careful monitoring our mouse colony and good practice. To minimise breeding, lines under sporadic use are maintained at lower levels, and frozen whenever practicable. Lines will be maintained in collaboration with other licensees wherever possible to minimise redundant breeding.

Our mouse lines are routinely maintained by keeping 2-3 breeding pairs, with around 3-4 litters/year - total 75-100 animals per strain/year. For crosses to enable characterisation of specific phenotypes, in general 5-6 breeding pairs will be kept with 6-8 litters/year - total 350-400 animals per strain/year. For mouse cancer models involving multiple alleles, such as the KPC mouse model of pancreatic cancer, where only approximately 1 in 4 mice will get tumours from an in-cross, then 10 breeding pairs will be kept with 6-8 litters/year – 700 mice per strain per year.

In our mouse experiments designed to test the effect of therapeutics on cancer development and spread, we are moving towards the use of transplantation models into syngeneic mice, rather than relying on spontaneous tumour models. Particularly in the case of the pancreatic cancer models, this reduces the numbers of mice that need to be bred for the experiment.

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 use the zebrafish model for the vertebrate developmental work, as these embryos develop ex utero and can be manipulated genetically and are transparent, and thus ideal for imaging.

All embryological work is preceded by in vitro or tissue culture studies to test the approach or activity of the biological entity to be introduced into the embryo. Failure at this preliminary stage is taken as final and no in vivo work will take place until this step is successful. In addition, for the zebrafish we will minimise suffering by taking pains to maintain the general health of the fish population, by attention to water quality, feeding regimes, and fish population density in each tank. We will check all breeding stock daily and cull any that show signs of significant illness or deformity. Where surgical or other potentially distressing procedures are required, e.g. fin clipping, we will perform them under general anaesthesia with analgesia both pre and post fin clip. Any fish or fish larvae showing signs of distress on recovery from a surgical or other procedure will be killed promptly by an approved method.

We will use mice in this project to investigate tumourigenesis in a mammalian system and to test new therapies. For the generation of breast tumours, we will most often employ sub-cutaneous injections or injections into the mammary fat pad. Using syngeneic transplant we have better control of tumour growth dynamics, than using a spontaneous genetic tumour model, where tumours arise at multiple sites sporadically. This reduces mouse numbers as 1 tumour from a genetic model can be used to generate tumours in around 15 mice. We use ultrasound-guided injection into the pancreas to study

pancreatic cancer. Similarly, this has advantages over a spontaneous genetic tumour model, as we have more control over the time of tumour development. For the study of lung metastases, intra-venous injections will be the route of choice. On rare occasion, we will study metastasis to other organs and therefore use other injection routes, such as intra-cardiac to give bone metastases. For skin tumours, we will use a topical carcinogen application. We are also using genetic models for breast cancer to mostly to generate tumours for transplantation assays. For the pancreatic cancer model, we are using spontaneous genetic tumour models. The parental lines are maintained at a minimal number and only bred in larger numbers for particular experiments.

For the mouse work we will check the animals every day and kill any exhibiting signs of significant illness. We will also reduce the overall tumour burden on mice and thus minimise possible adverse effects, by for example using the non-invasive imaging for metastasis assays.

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

We use zebrafish embryos for our developmental work, and most of this is on fry less than 5 days old, which are deemed non sentient and before the onset of independent feeding. For the tumour work, we will try to use zebrafish larvae to investigate tumour cell invasion and extravasation. This will inform our mouse experiments and should reduce the number of mice needed. We also use terminal anaesthesia to obtain very detailed information about tumours. However, to understand the efficacy of therapy it is necessary to analyse the same tumour before and after giving treatment. Where possible we will use on non-invasive longitudinal imaging.

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

We try to minimise any possible adverse effects. In particular, the use of fluorescently labelled cells coupled with microscopic analysis of tissues enables us to detect small metastases. This reduces the overall tumour burden needed to be able to detect metastasis from primary sites. Similar benefits are expected from the use of non-invasive fluorescent or bio-luminescent imaging. If the purpose of the experiment is simply to observe cellular behaviours in the primary tumour, then we would not grow the tumours to the larger size that some of the metastasis experiments require. Regular monitoring by BRF staff is in place and we strive to keep updated with the latest environment improvements, such as enhanced environmental stimulation.

Surgical procedures will be performed with suitable anaesthesia and animals monitored post-surgery to ensure that they recover well. We will also use suitable analgesia for all surgery.

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

We are aware of NC3Rs. We also discuss with colleagues in other research groups new improvements that lead to refinement.

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

We will stay up to date via regularly communication with BRF staff, other scientists in the field and regular visits to the following website https://www.nc3rs.org.uk/3rs-resources.