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NON-TECHNICAL SUMMARY

Dynamics in tissue mechanics, gene transcription and signalling during tissue formation and regeneration

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Tissue formation, Tissue dynamics, Tissue regeneration, Tissue mechanics, Regenerative medicine

Animal types	Life stages
Xenopus laevis	adult, embryo, neonate, juvenile
Xenopus tropicalis	adult, embryo, neonate, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to uncover key mechanisms that drive the formation, regeneration and repair of complex tissues.

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?

A primary goal in regenerative medicine is to identify and implement novel treatments aimed at improving our ability to regenerate injured, diseased, or aged tissues and organs. Because mammals exhibit poor regenerative capacities, there is an interest in understanding the regenerative mechanisms employed by organisms with higher regenerative capacity, such as planarians, Hydra, fish and amphibians, to help inform new possible regenerative therapies in humans.

The work undertaken in this project will advance our understanding of the molecular and cellular processes that underpin regeneration and repair. In turn, this work will provide critical information necessary for the design and eventual implementation of novel therapies aimed at promoting regenerative healing in humans.

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

The primary output of this project will be a greater understanding of the cellular and molecular mechanisms of tissue formation, repair and regeneration. More specifically, how dynamics in tissue mechanics, gene transcription and signalling drives the formation and repair of tissues in vertebrate organisms. Ultimately these outputs of new knowledge will be published in high quality, peer reviewed and open access journals.

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

Short term: the research community, in particular scientists interested in tissue mechanics and gene transcription dynamics during development and regeneration will benefit from the scientific publications generated during this project.

Medium term: the tools and techniques developed during this project (for example mutant and transgenic lines, imaging techniques) will be available to the scientific community to be applied to new research questions

Long term: this work will generate new hypotheses to improve repair and regenerative capabilities that can be tested first in mammalian models such as mice and then be translated to humans.

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

We will openly share our data with our collaborators and the scientific community through participation to meetings and conferences. All scientific results will be published in high quality, open access and peer reviewed journals in a timely manner. To avoid publication bias, we are committed to publishing all findings from this project, both positive and negative.

Transcriptomics datasets generated during this project will be made publicly available by being deposited at the European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI) repository. Software and scripts implementing workflows, algorithms, and macros will be available on GitHub. Mutant Xenopus lines will be deposited at the European Xenopus Research Centre (EXRC, https://xenopusresource.org/).

Our research aims to understand the mechanisms that underpins regeneration in vertebrates with the view to translate these findings in species with poor regenerative abilities (i.e. mammals), it is critical that we communicate our findings to clinicians and the public in general. We will continue existing collaborations with clinicians and participate in public engagement events.

Species and numbers of animals expected to be used

- Xenopus laevis: 5000
- Xenopus tropicalis: 14500

Predicted harms

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

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

The African clawed frog Xenopus was chosen for this study because of its low sentience (neuronal complexity) and because as a vertebrate, it shares many of the same genes and developmental pathways with humans. As tadpoles, Xenopus display great regenerative capabilities, being able to heal a wound without scarring or to regenerate a whole appendage like its tail.

Most of the experiments proposed in this project will be performed in early embryos or parts of embryos that can be cultured in vitro (explants) prior to the protected stage. These approaches allow us to investigate cellular/tissue mechanisms in the context of complex 3 Dimensional (3D) tissues while providing an excellent non-protected animal alternative to most in vivo studies. However, for the study of the formation and regeneration of complex tissues such as the spinal cord, it is necessary to perform some work in vivo in post-embryonic larval stage as it is not possible to recreate the full complexity of the tissue and its environment in culture.

Finally, we need to raise transgenic and mutant lines to adulthood to generate mutant embryos.

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

Most of the frogs used in this project will be used for breeding purposes. Typically, frogs will be injected with reproductive hormones that induce the maturation of oocytes and subsequent laying of eggs. This procedure can be repeated multiple times over the adult lifespan, with appropriate rest between procedures, as adult frogs replenish their stock of gametes over time.

Embryos obtained from these crosses will often be injected with genetic material that can express a fluorescent protein or compounds that prevent the expression of a particular genes. These embryos will mainly be used at pre-larval stage. At post-larval stages, tadpoles will mainly be subjected to tail amputation or spinal cord transection. This is done under anaesthetic and whilst it will cause discomfort, tadpoles regrow a functional tail, or repair their spinal cord within 7 days without long-lasting consequences.

Occasionally, we will raise embryos with modifications in their genomes (inactivation of a gene or expressing a transgene) to adulthood to generate genetically modified embryos. In this case, all animals will be carefully monitored to ensure that they are developing normally. Adult frogs will be genotyped by isolating genomic DNA from toe clippings or skin swabbing whichever is the most effective and humane. Frogs will then be identified either by their natural skin pigment pattern or microchipping.

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

The vast majority of our protected animals lead healthy lives. It is anticipated that only transient minor discomfort should occur in the adult animals during injection procedures but adverse reaction to the hormones is not expected.

Injected constructs may cause death or developmental abnormalities before tadpoles reach the protected stage. Harmful genetic alterations in embryos may be evident as altered morphology before free-feeding stage but some genetic alterations may result in a harmful phenotype later during development. Particular attention will be given to the morphology of tadpoles as well as their behaviour (for example ability to swim). Any larval tadpole showing signs deviating from normal development or behaviour will be euthanised immediately at pre-independent-feeding stage.

Any animal that will be raised to adulthood will be monitored at least 3x a week throughout their development to ensure that no deviation from normal development occurs. In particular, we will assess their ability to swim, their growth rate and any sign of ill-health (bloating, changes in activity level, skin discoloration). Any adult showing these signs will be euthanised immediately.

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)?

Adult frogs will experience only mild procedures (injection of hormones, generation of transgenic lines). Post-feeding stage tadpoles subjected to spinal cord transection or tail amputation will experience a

moderate level of pain. This will be the case for about 50% of the animals used under this project licence.

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

- Killed
- Kept alive

Replacement

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

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

The production of frog embryos require that adult animals are induced to ovulate or mate via the injection of reproductive hormones, and this is a regulated procedure. However, to study the formation and regeneration of complex tissues and organs, it is necessary to perform some work in vivo, as it is not possible to recreate fully the complex environment of tissues in culture. Therefore, experiments where we are investigating the multi-tissue events responsible for tissue repair and regeneration will necessitate working with post-embryonic larvae stages. However, we have chosen to pursue this work on a “lower” vertebrate (i.e. Xenopus frogs) with lower neurophysiological sentience.

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

We perform experiments using cell culture systems (for example neuronal or epithelial cell lines) for very specific purposes and we use them when appropriate. We also use cell lines for preliminary experiments (for example to test imaging probes) prior to using them in the Xenopus embryo, making our animal experiments more focused and reducing animal usage.

Why were they not suitable?

We are investigating the cellular mechanisms taking place in complex 3D tissue environments, that include multiple cell types and interactions between various tissues. Currently, this complexity cannot be accurately replicated using in vitro models. Furthermore, in vitro studies do not allow us to identify new signalling events from unexpected sources and surrounding tissues.

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 number of animals has been estimated based on previous licences covering our work. We have based our assumption that we will use a similar number of animals as in the previous 5 years.

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

In all our studies we aim at reducing animal numbers to a minimum by using the NC3R's Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) web application and the PREPARE guidelines (https://norecopa.no/PREPARE). We will also consult with our in-house statistical service for advice during the course of this project. More specifically, we have applied to all our experiments very stringent methods to obtain statistically meaningful results with the minimum number of animals possible.

Where possible, we calculate the precise number of embryos per time point per experimental condition based on previous experience or published data. If these data are not available, we will perform low number pilot experiment to estimate the magnitude of change and the intrinsic variability of the data to determine sample size. These numbers will be updated as more recent and relevant data becomes available.

Data analysis will be conducted according to a pre-specified statistical analysis plan drawn up in conjunction with establishment-based statisticians. Important experimental results will be designed with biological replicates and repeated and validated via an alternative follow-up experiment to minimise the likelihood of spurious non-replicable results.

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

To reduce the number of matings, we ensure that a maximum number of embryos are used. A single mating produces 1000s of eggs, generating multiple experimental units (i.e. a single frog embryo that has been subjected to a procedure) per conditions per mating. Experimental units not immediately used are banked (for example by fixing or freezing). We also co-ordinate between the different members of the group to share clutches of embryos to reduce the number of mating.

Recently, we have introduced means to identify individual frogs by microchipping (for Xenopus tropicalis) and by identification of pigment pattern via stored photographs for each individual (for Xenopus laevis). This allow us to ensure that animals are kept at the ideal density in each tank, for example by combining frogs from different genotypes. Furthermore, this allows us to monitor the health and reproductive capabilities of each frog to prevent keeping animals longer than necessary.

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 Xenopus laevis and tropicalis in our research. These are related species with distinct advantages: laevis embryos are bigger (about 1mm in diameter) making explant and grafting experiments much easier. However, the genome of Xenopus laevis is more complex and the generation time much longer that Xenopus tropicalis. Therefore, Xenopus tropicalis is much more suitable for genetic experiments such as generation of knockout or transgenic lines. Whilst zebrafish also has regenerative abilities), Xenopus has important advantages for our work: it is a tetrapod and therefore evolutionary closer to mammals, its genome (for tropicalis) is much closer to the mammalian genome, it is much easier to obtain explants, to perform graft experiments and Xenopus larvae are much more efficient at regenerating their tails. Amphibian embryos in general, and frog embryos more specifically, have been used to investigate the mechanisms responsible for tissue development for over a century. Indeed, much of what we currently know about how the vertebrate embryo develops has come from experiments initiated in frog embryos. Using an established experimental species reduces the use of animals, as one does not have to replicate accrued knowledge from other species.

Adult animals are used in this project for the purpose of generating embryos. The production of frog embryos require that adult animals are induced to ovulate or mate via the injection of reproductive hormones, and this is a regulated procedure. It is anticipated that only transient minor discomfort should occur in the adult animals during injection procedures but adverse reaction to the hormones is not expected.

As the embryos develop ex utero, experiments and harvesting embryos can be done without harming the parent. Xenopus is an extremely tractable system and techniques such as transplantation, injection of genetic material and imaging of fluorescent proteins are standard in the laboratory. The transparency of Xenopus tadpoles and the ease of culturing explants allows one to follow the behaviour of labelled cells (for example green fluorescent protein) over long periods of time. Chemical modifiers are easily applied by addition to the media. Some of our study involves analysis of regeneration. In our tail amputation assay, tadpoles are anaesthetised and the tail tips are removed (<50%, not exceeding 2mm width). Although the amputation damage is visible, it is still a very small amount when compared to the entire tadpole, the tissue is rapidly regenerated. Furthermore, these types of injuries occur regularly in nature.

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

Xenopus is a low-complexity vertebrate model that permits reliable and robust translation of findings to mammalian biology (including humans). Most obviously, the study of spinal column development and regeneration requires a vertebrate model.

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

Although extensive experience informs us that most of our regulated procedures (injection of reproductive hormones into adult frogs) are minimally-invasive, mild in severity and well-tolerated, we monitor frogs carefully by recording biological metrics like weight during procedures, and by constantly interacting with NACWOs and NVS to maximise welfare. Unusual events are flagged via our institutional recording system and we routinely review and refine our protocols. Procedures that may cause distress to the tadpoles or frogs are performed under deep anaesthesia. After procedures tadpoles will be closely observed and monitored for any signs of distress. During a procedure, any protected animal showing signs of distress will trigger the cessation of an experiment and subsequent protocol review.

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

There are many useful resources on the NC3Rs website (e.g. NC3Rs experimental design assistant). We are constantly reviewing and improving Xenopus husbandry by implementing best practice from the community (Slack channel with more than 120 participants, close contact with the European Xenopus Research Centre in Portsmouth and the National Xenopus Resource in Woods Hole, USA). Publications include the Xenopus book (Cold Spring Harbwour) and Xenopus protocols (Humana Press).

The new PREPARE Guidelines will also be strictly adhered to (https://norecopa.no/PREPARE.)

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

We have previously held funding from the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), and we maintain strong links with local and national advisors. NC3Rs regularly holds online workshops and institutional events aimed to improve experimental procedures with a focus on the 3Rs. We continually review and refine our protocols with discussion between our researchers, Named Animal Care & Welfare Officers (NACWOs) and NVS.