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NON-TECHNICAL SUMMARY

Regulation of RNA During Embryonic and Placental Development

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Pregnancy, Reproduction, Placenta, Embryo, RNA

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

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 factors regulating Ribonucleic Acid (RNA) molecules contribute to the establishment of a healthy pregnancy and the development of the embryo and placenta

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?

Establishment of a healthy pregnancy involves implantation of an embryo into the lining of the mother's uterus, and the formation of the placenta which provides the developing embryo with oxygen and nutrients. Researching how the uterus, placenta and embryo work together to establish pregnancy and support normal growth and development is crucial to understanding what may be going wrong in cases of early pregnancy loss, pregnancy complications, or infertility. This is important as there is still a significant gap in our knowledge in this area with around 30% of pregnancies being lost shortly after embryo implantation.

Most research into this topic has focused on which genes are switched on or off and how this may happen at different timepoints. My research aim is to understand how the molecule that is made when a gene is switched on, an RNA molecule, may be further processed to regulate its abundance or its function. RNA molecules carry the message encoded within our genome and direct the production of proteins that form our bodies and maintain their functions. There is emerging evidence that regulation of RNA molecules is important during embryonic development, but no comprehensive study of this phenomenon has been carried out to date.

My research involves studying mice with mutations in RNA processing genes. Mutations are changes in the DNA sequence that alter the function of gene. Mouse embryos with mutations in RNA processing genes are lost shortly after they have implanted in the uterus and they often have placental defects. I plan to analyse the uterus, oocytes (eggs), embryo, and placenta from these mice to identify which RNA molecules are being processed incorrectly. By also analysing closely what populations of cells and tissues are affected I will correlate molecular information with anatomical structure and thus identify precisely how key developmental events are affected by impaired RNA processing.

My work over the next five years will shed light on some of the mechanisms underpinning normal reproduction and will help work towards the longer term goal of improving outcomes for mothers and their babies.

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

The main outputs of this project will be:

- Better understanding about how factors that regulate RNA molecules contribute to the establishment of a healthy pregnancy

- New stem cell lines that can be used to model early developmental events in the lab
- Sequencing and imaging data sets that will be of value to the wider research field
- Publications in peer-reviewed journals and presentations at conferences

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

In the short term, understanding the role regulation of RNA plays in the establishment of pregnancy and the development of the embryo and placenta will benefit my research group and the wider fields of reproductive biology, embryology and placental biology, working across basic and clinical science. The knowledge generated will benefit us as we seek to understand the mechanisms of very early development and generate further research questions. The wider research field will also benefit from the sequencing data sets that will be made publicly available upon publication which they will be able to explore further to address their own research questions. In addition, stem cell lines that are generated from embryos will be shared upon reasonable request with other research groups who may be able to use them to address other research questions.

In the longer term, this work will represent an incremental step towards the much larger goals of preventing pregnancy complications, improving pregnancy outcomes and managing infertility.

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

- Dissemination of knowledge at national and international conferences
- Integration of data sets with those previously published to gain additional information
- Collaboration with other research groups, locally and internationally
- Social media activity to announce peer-reviewed publications
- Press releases describing significant findings
- Using new findings as preliminary data for new funding applications

Species and numbers of animals expected to be used

- Mice: 3240

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.

In order to study the establishment of pregnancy and the early development of the embryo and placenta, it is necessary to carry out analyses in a mammalian species (i.e. an animal that has a placenta). Mice are an appropriate model in this context as they share similarities with humans. Morphological changes associated with early embryonic development are similar in both species and they have similar types of placenta.

I will be using the C57Bl/6 strain of mice. This is the most common background for genetically altered mice. The genome of this strain has been most comprehensively sequenced, i.e. the entire DNA code for this strain is known. This means it will be the most appropriate for analysis of data sets containing DNA code information. and comparable to other data sets in the field.

I will evaluate several stages of development:

- The oocyte (egg): The egg contains a large amount of RNA molecules inherited from the mother and so it is an important stage to study. Several RNA processing factors have already been described as regulators of maternal RNA in the oocyte. Oocytes may be collected from young female mice aged 2-3 weeks or adult female mice

- The blastocyst (3.5 days after fertilisation): This is an embryo at the stage right before implantation. At this embryonic stage, I can assess if the embryo looks normal and if the expected genes are switched on. New stem cell lines can also be derived at this stage.

- Postimplantation stage (5.5 - 10.5 days after fertilisation): This is a critical window in development where all the structures of the embryo and placenta are developing and where I would expect to see many of the effects of carrying a mutation in an RNA processing gene. For some mutations it may be necessary to carry out analysis later in development.

All procedures used to generate embryos for research will be performed on adult mice only. To analyse embryos these will be collected from adults females following normal breeding and humane killing. To analyse oocytes these will be collected from adult females and 2-3 week old females following normal breeding and humane killing.

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

Some female mice (genetically altered or not) will be given a drug containing hormones that cause ovulation of an increased number of oocytes (eggs) than is typically observed in a natural ovulation. This is called superovulation and it will involve two injections, 48 hours apart, either into the body cavity (intraperitoneal) or under the skin (subcutaneous). For collection of oocytes, the animals will be humanely killed. If embryos are required, females will be mated first and at the desired developmental time point humanely killed and tissues collected.

Some female mice (genetically altered or not) will be mated with a sterile male to produce pseudo-pregnancy (i.e. making the uterus receptive for implantation of embryos) and then undergo either non-surgical or surgical embryo transfers. During non-surgical embryo transfer, embryos will be inserted directly into the uterus with a special device without any surgical intervention. Non-surgical embryo transfer will be used whenever possible as it will cause the least harm. In some cases, surgical transfer may be required, if for example there is persistent low efficiency of non-surgical transfers or if it is desirable to transfer embryos at very early stages. This will involve a surgical procedure to place the

embryos in the uterus. If surgical transfers are required the animals will have surgery under anaesthetic and be provided with post-operative care, including pain relief, to minimise pain and distress. When the aim is to generate a new mouse colony from frozen sperm or embryos, live offspring will be born following embryo transfer. When the aim is to study the pregnancy, embryos will be collected following implantation and a short period of development. Animals that were used for non-surgical transfers can be re-used once again. These animals will be humanely killed either following weaning of live offspring, or at an appropriate developmental stage for postimplantation embryo analysis.

Some male mice that have not been genetically altered will undergo surgery for vasectomy. They will have surgery under anaesthetic and be provided with post-operative care, including pain relief, to minimise pain and distress. These males will be used to mate with females to induce pseudo-pregnancy, (i.e. making the uterus receptive for implantation of embryos). These males may be kept alive to mate with other females. At the end of mating these animals will be humanely killed. Vasectomised males will be singly housed between matings.

Female and male mice will breed together to maintain a colony of mice containing genes with mutations for study. They will undergo no procedures other than mating and observational handling (e.g. checking health and signs of mating). Before weaning a small ear punch tissue biopsy will be taken to ascertain genetic status. Matings will be ongoing to maintain the colony, and timed to generate embryos at a particular developmental time point. Stud males will be singly housed in between timed matings. Pregnant females from timed matings will be humanely killed and tissues collected for analysis. For ongoing matings each female will have up to 6 litters and then both animals in the breeding pair humanely killed. Females will not be allowed to continue mating beyond 9 months.

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

Injections of hormones are expected to cause mild transient distress and no lasting harm.

Non-surgical embryo transfer is expected to cause mild transient discomfort and no lasting harm. Surgical embryo transfer is expected to cause short-lived (around 24 hours) post-operative pain and discomfort. Pain relief will be supplied.

Surgery for vasectomy is expected to cause short-lived (around 24 hours) post-operative pain and discomfort. Pain relief will be supplied.

No adverse effects of breeding genetically altered mice are expected. Mice with full loss of gene function will be assessed at embryonic stages only. Tissue biopsy may cause mild transient distress and no lasting harm.

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)?

Mild 95%

Moderate 5%

What will happen to animals used in this project?

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

Establishment of pregnancy requires interaction of the embryo with the maternal uterine tissue, and communication between the embryo and placenta is vital for normal growth and development. Studying human pregnancy at the earliest stages and at the molecular level is very challenging. There is no availability of human tissue within this very early developmental time window as these events begin before the detection of a clinical pregnancy, and genetic alteration is not possible. it is therefore necessary to use animal models to study these processes.

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

Human embryonic stem cells, placental stem cells and placental organoids (a mixture of different placental cell types that can form a "mini-organ") that recapitulate some aspects of development are available, and so I considered whether the project aim could be accomplished using these alone. Some researchers are also developing human "embryo-like" models from different stem cell types so I also considered if these models could be used.

Why were they not suitable?

While human embryonic stem cells, placental stem cells and placental organoids are very useful, they do not fully recapitulate all developmental processes. For example, they cannot form complex functional tissues. They also cannot yet be used to model the interactions between the embryo, placenta and maternal uterus that are required to establish pregnancy.

Human "embryo-like" models are still at the very early stages and much optimisation and improvement will be required before they can accurately recapitulate the interaction with the maternal uterus.

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?

Breeding and maintenance (3000): A large proportion of the animals used in this project will be in maintaining colonies of genetically altered animals to have continued production of animals carrying a copy of a mutant gene. These can then be used for timed matings to study the embryo and placenta and to carry out molecular analysis in full "knock-out" animals (carrying 2 copies of a mutant gene) that have completely lost the function of that gene. Maintenance of these lines will be achieved by mating genetically altered animals to unaltered animals. For experimental mating 2 animals carrying a single copy of the mutant gene will be mated together. The International Mouse Phenotyping Consortium (IMPC), an organisation that publishes recommendations on how best to study genetically altered mice, recommends studying a minimum of 28 embryos per timepoint. To achieve this, I will collect embryos from 4 litters per timepoint which should yield 32 embryos minimum (8-10 embryos/litter). This will slightly exceed the IMPC recommendation but will ensure good proportions of male and female embryos (roughly 4 each). To generate adequate numbers of animals for matings, following joint advice from NC3Rs and other organisations offering resources and advice on colony management, I have estimated that I will need 3 continual breeding pairs per mutant line generating 300 animals per year. I estimate that I will work with 2 lines at a time totalling 600 animals per year.

Superovulation (60): I estimate that I will use a maximum of 20 females for superovulation for production of blastocysts for stem cell derivations (4 females for a maximum of 5 mutant lines). This estimation is based on my previous experience of stem cell line derivation. I have also estimated 40 females will be required to produce blastocysts to freeze embryos (cryopreservation) to archive lines when no more experiments are planned or a long gap in experiments is anticipated. For cryopreservation I have estimated a maximum of 8 females for a maximum of 5 mutant lines. I have followed advice from other groups carrying out cryopreservation to estimate these numbers.

Female mice as recipients for embryo transfers (120): I estimate that I may need to carry out a maximum of 10 rederivations, that is the establishment of a mouse colony from frozen embryos or sperm. This is a combination of bringing in new lines and re-deriving lines that I may cryopreserve if necessary. I have allocated a maximum of 6 females per rederivation totalling 60 animals. I will also carry out embryo recipient experiments to study maternal effects on embryo implantation. 6 genetically altered females and 6 wild type females will be used per experiment. This type of experiment will be carried out a maximum of 5 times requiring a further 60 animals.

Vasectomy (60): This number is required to produce sterile males for mating with the females in the embryo recipients protocol described above.

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

I have followed advice from NC3Rs and the PREPARE guidelines. In particular the timed matings of animals carrying one copy of a mutant gene allows for the collection of embryos and placentas with 0, 1 or 2 copies from the same litter. This reduces the numbers of animals being used as it eliminates the

need for a separate unmodified colony. Using littermates in experiments also controls for the variability that may be seen between litters and thus reduces the number of animals needed to see consistent results. In addition, I was able to access data pilot studies carried out by the Wellcome Trust Deciphering the Mechanisms of Developmental Disorders (DMDD) project on placental phenotypes allowing me to more closely pinpoint which developmental timepoints to focus attention on in my studies and so use fewer animals.

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

I will follow published advice from NC3Rs and the Jackson Laboratory on Colony Management in order to optimise the number of animals used. I will avoid overbreeding and the production of surplus animals. During periods where the colony needs to be maintained but experiments are not ongoing I will use intermittent breeding (i.e. not continually breeding and therefore producing less litters). I will also periodically breed genetically altered mice with unmodified mice that are otherwise identical. This will prevents additional mutations being picked up over time that may cause variability in the analysis of embryos. Reducing variability will allow fewer animals to be studies to get significant results.

My project plan involves using some animals to generate new stem cell lines. Once derived, these lines will be used for RNA sequencing experiments which will yield much molecular data without using any animals and will complement the work that cannot be carried out in cell lines.

I will use both cell lines and surplus embryos produced in experimental timed matings for test molecular analysis experiments in the lab so that there is minimal new animal use for methods optimisation.

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.

I will use genetically altered mice. Live animals will only carry one copy of an altered gene and this will not have an adverse effect on the animal. Animals with two copies of an altered gene will only be assessed during the embryonic stage of life and will not be born.

I will also use some surgical methods on mice. Surgery will be carried out to vasectomise males. Vasectomised males will be used to generate pseudo-pregnancy in female mice. This is the only method available to rederive mouse lines and to confirm the effect of the maternal uterine environment on embryonic and placental. development. Genetically sterile male mice are not a suitable alternative to vasectomised males due to the large numbers of animals required to maintain a breeding colony producing the sterile males which will only be required sporadically. Some females will have surgical

embryo transfer carried out in situations where non-surgical transfers aren't appropriate (e.g. persistent low efficiency or when very early embryos are to be transferred). Surgical procedures will be carried out on a maximum of 5% of animals and the animals are not expected to experience any lasting harm.

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

To gain insight into human pregnancy it is necessary to use a mammalian species that carries young (rather than laying eggs) and develops a placenta. Mice are the most appropriate mammalian species to use as early embryonic development is similar to humans and they have a similar type of placenta. Less sentient species that are commonly used as experimental models such as flies, frogs, worms or fish do not carry young and do not develop a placenta and therefore cannot be used.

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

For embryo transfers non-surgical methods will be used where possible.

For timed matings, stud males may need to be housed singly between matings as they often show aggressive behaviour towards other males once they have mated with a female. I will keep the time they are housed individually to a minimum and ensure the use of environmental enrichment. Overly aggressive or large males will not be mated with females. Non-aversive handling of animals will be used, for example using a tunnel and cupped hand to handle animals, rather than tail capture which can cause stress.

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

I will follow PREPARE guidelines as well as the NC3Rs and Jackson laboratory's advice for breeding genetically altered animals. LASA guidance on aseptic technique for surgeries will also be followed. I will communicate frequently with the Named Animal Care and Welfare Officer and other staff in the animal facility to review refinement in our work.

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

I will stay up to date with 3Rs developments by checking the NC3Rs website as well as an internal 3Rs search tool. These can be implemented by adapting experimental plans or through discussion with animal facility staff. I will also maintain communication with the institution Named Information Officer and facility Named Animal Care and Welfare Officer who will be a source of information and can facilitate implementation.