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Introduction and project objectives

Introduction for the layman

There are many chronic human diseases that cause great suffering as a result of inherited or acquired mutations in our genetic material. Once present, these changes to the structure of DNA are passed from one cell to the next as cells divide. Inherited and acquired mutations are responsible for many diseases, common examples being cystic fibrosis and muscular dystrophy. In these cases, the debilitating consequences of mutations in DNA arise because of changes to the structure of the protein that is expressed from the mutated gene. In cystic fibrosis, for example, mutation leads to severe defects in the function of a large membrane protein (an ion channel) that maintains or balances the flow of chloride ions and water from the inside to the outside of the cell.

The concept of gene therapy is to overcome the damage caused by genetic mutations in human cells by providing, to the appropriate cells, a normal copy of the damaged gene. In principle, if the normal protein is then expressed in these target cells it will be possible to replace the malfunctioning protein with a fully functioning counterpart. However, this is technically very challenging, and systems that are currently being evaluated in clinical trials suffer from potential deficiencies that compromise safety.

The most challenging aspects of gene therapy arise from two main sources. First, available protocols for delivering DNA to the target cells are generally inefficient. The most efficient systems employ viral particles, but the best systems are also associated with immunological side-effects. Second, even when the DNA is delivered to target cells it is by no means certain that the gene will be faithfully expressed. In fact, all too often the protein is only made for a short time. One way of providing long-term expression is to use vectors that integrate into the chromosome of the target cell – this ensures that the new gene is not lost as cells divide. However a potential problem with this is that by interfering with the cell genome it is possible to alter the normal patterns of gene expression, and in some cases this can lead to cancer.

This project is designed to evaluate the possibility of developing extra-chromosomal gene delivery systems for gene therapy and evaluate protocols for their safe use in pre-clinical model systems. The episomal systems under study do not interfere with host cell chromosomes and so do not have any secondary genetic effects. The key features of Epi-vector project are to define the genetic elements that are required for persistent, long-term gene expression and maintenance of the extra-chromosomal DNA molecules in appropriate cells.

Scientific Objectives

The genetic elements that regulate chromatin function at natural chromosomal loci and the molecular mechanisms that regulate function are known. Even so, it has proved an immense challenge to understand how these genetic elements might be configured to provide regulated, efficient and sustained gene expression from extra-chromosomal DNA. The object of this project is to use knowledge of the best extra-chromosomal gene expression systems that are currently available to develop a new generation of DNA vectors for safe and efficient therapeutic application.

The optimal configuration of genetic elements will be established using a systematic approach that incorporates molecular and bioinformatic techniques to refine vector design. Critical features of extra-chromosomal DNA behavior will be evaluated in living cells to define design parameters, which ensure that the extra-chromosomal vectors are fully integrated into the host functional compartments. This will ensure efficient and regulated gene expression that can be sustained is both dividing and non-dividing cells. The refined, second-generation extra-chromosomal vectors will be validated for human gene therapy in a pre-clinical setting and protocols for clinical application established using myocytes, hepatocytes and haemotopoeitic stems cells as model systems.

The key objectives of EPI-vector are to provide:

- Extra-chromosomal gene delivery vectors for efficient and sustained gene expression in mammalian cells.
- Extra-chromosomal gene delivery systems that are composed entirely of DNA sequences of human origin and so minimize known risk factors that compromise safety during human gene therapy.
- Development of gene therapy protocols using ex-vivo technologies to develop sustained gene expression in cells in culture, including stem cells.
- Regulated gene delivery systems for a wide range of therapeutic applications.

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Work performed and results achieved during the project

The majority of gene delivery systems that have been evaluated in clinical trials are based on knowledge of natural properties of a variety of viruses that infect human cells. Adenovirus, retrovirus (eg lentivirus) and herpes virus genomes are the major models that have been developed as ectopic gene expression systems for human gene therapy. Because of safety concerns associated with the use of these systems, Epi-vector sets out to evaluate if an efficient and safe vector system can be developed without using components of viral origin. The starting point for the project was the design, by Partners 3 and 7, of an episomal gene expression system that was shown to deliver sustained gene expression over at least 100 cell divisions in the absence of selection pressure. At the time of submitting the Epi-vector proposal it was established that the efficient human S/MAR element was a key feature of the prototype vector – which was called pEPI-1. The proposal set out a series of experiments that would define the optimal configuration of genetic elements for sustained expression and test the use of 2nd generation vectors in model systems for gene therapy.

The key early steps in the project were to define how the different genetic components contributed to vector behavior. For efficient gene therapy 3 essential features ar required:

1) – Transcription of the therapeutic gene must be sustained in target cells and appropriately regulated to deliver as close as possible to the natural levels of expression;
2) – Replication – if the target is proliferating cells – should be efficient, to prevent vector loss;
3) – Segregation of the vector during cell division must also be efficient.

Since the proposal was submitted, substantial progress has been made in understanding how the genetic factors contribute to vector function. It has been shown that the dominant genetic element is the structure of the gene expression unit and the nuclear matrix attachment sequence – the S/MAR. The S/MAR in this context is essential for episomal maintenance and is believed that this element is responsible for targeting to active nuclear sites. Optimal S/MAR activity was demonstrated when transcription actually ran into the S/MAR. This was also true when a synthetic S/MAR was developed to replace the original element from the human interferon locus.

Extensive studies have shown that replication of the episomal vector is very efficient and that replication of the episomal DNA is regulated by the chromosomal systems, with DNA synthesis occuring during early S phase, as would be expected for an active gene domain. Each episome was shown to replicate from a single (or very few) initiation site, though specific sites of initiation – ie replication origins – were not seen. In fact, different sites were seen to be used in different episomes and these were distributed throughout the plasmid DNA. A small preference for initiation was seen in the S/MAR element, but this is probably consistent with the duplex unwinding properties of S/MARs. The use of known replication origins of chromosomal origin did not alter the patterns of episomal initiation, showing that a precise site of replication initiation is probably not needed in the extra-chromosomal context.

The most surprising property of the episomal vector system is its extremely high mitotic stability. Typically, a mitotic stability of >0.98 is seen, even though the vector does not contain any elements that would be expected to provide centromere function. In the proposal, it was argued that this key aspect is an epigenetic feature and that the best approach to undestand this behavior would involve analyzing the dynamic properties of the episomal DNAs using live cell imaging. Live cell imaging has prooved to be technically demanding because the systems available are only sufficiently sensitive when very large target sequences are used, and these have prooved to destabilise the vector sequence. Howvever, by optimising all possible aspects of the analysis we have been able to visualise the dynamic properties of the vector and shown that for the large part it becomes a spatially stable component of the nuclear chromatin. This implies that once cells stably maintain and express the Epi-vectors are established the vectors become

`fixed' into an active nuclear domain and acquire similar dynamic properties to the endogenous chromatin that is associated with the same nuclear compartment.

This view was supported by fixed cell experiments, where Epi-vectors were shown to be exclusively associated with the most active nuclear domains during interphase, remains associated, perhps throughout interphase with early S phase replication foci and also remains stably attached to equivalent locations on the chromatid arms during mitosis. These observations raise the fascinating possibility that even though Epi-vectors remain only loosely linked to chromatin the associations are sufficiently strong that the episomal DNA is able to behave as an endogenous genetic element that is replicated and segregated as a host sequence. Hence these studies suggest that while the plasmid DNAs are episomes genetically they are tightly associated with active nuclear compartments, in a way that allows them to mimic the behavior of chromosomal loci. In view of the association with the active nuclear domains we also see that the Epi-vectors establish a normal active chromatin configuration that is able to support long-term gene expression without silencing.

The original studies that provided proof of concept for Epi-vector behaviour were perform on cell lines that were adapted for continuous culture. During the Epi-vector project we have extended this to the analysis of numerous variant Epi-vectors, with different structures and tissue specific genes. The behaviour of Epi-vectors is seen to be remarkably robust and as well as providing regulated and tissue specific gene expression in model systems including primary and ES cells is also capable of providing appropriate expression during differentiation and throughout development development.

A crucial step in gene therapy is delivering the vector DNA to target cells. Using model systems, delivery can be achieved using routine cell transfection protocols, but for more specialized cells, this approach is often extremely inefficient. Plasmid delivery using pseudo-virus particles provides one possible route for efficient delivery to cells, such as stem cells, that are refractory to normal transfection methods. SV40 pseudo-virus delivery protocols are being developed. Early studies have begun to optimize the protocols for efficient packaging and delivery and progress is being made towards understanding the genetic design parameters that influence the efficiency of pseudo-virus assembly.

Epi-vector explores two general possibilities for delivering gene expression in a therapeutic environment:

1) – evaluating the behavior of Epi-vectors in a stem cell environment;
2) – evaluating the expression of Epi-vectors in model systems for gene therapy.

Good progress has been made towards both of these goals. Towards the first goal, we have shown that transgenic animals can be generated using Epi-vectors using both pig and mouse as model systems. In the pig system, extremely exciting experiments resulted from the use of sperm mediated gene transfer to deliver Epi-vectors during in vitro fertilization. Following implantation and development, Epi-vectors were shown to persist in about 50% of embryos with expression in many tissues and stable episomal propagation of the epi-vectors. Towards the second goal, significant progress is being made with respect to developing the gene expression systems that will be used to define the behavior of Epi-vectors in model systems for gene therapy, using myocytes and hepatocytes.

At the completeion of the project we have met the following key goals:

1 – define genetic parameters that drive regulated and stable gene expression from Epi-vectors.
2 – identified the epigenetic features that result in stable maintenance of Epi-vectors on prolifertaing cells.
3 – developed 2nd generation vectors that might have defined – artificial – S/MAR elements and have all bacterial components removed.
4 – established a variety of systems for regulated gene expression in primary cells including stem cells.
5- used Epi-vectors to develop transgeneic animals using pig and mouse as model systems.

6 – developed and characterised in vitro delivery systems for efficient Epi-vector delivery to a broad range of cell types.
7 – developed model systems to test the behaviour of Epi-vectors in a pre-clinical setting for gene therapy, using myocytes, hepatocytes and stem cells.

Intentions for use and impact

Results from the EPI-Vector project give insight into the fundamental relationship between the extra-chromosomal gene delivery systems and the human genome.

This information is beginning to add a new dimension to our understanding of vector behavior. The full impact of this study on the potential to further develop safe vectors for gene therapy should become visible in the second reporting period.

Plan for using and disseminating the knowledge

The major routes for disseminating knowledge obtained in the Epi-vector project are as follows:

1) All studies have been or will now be published in peer reviewed journals of the highest possible quality. Where appropriate members of the Epi-vector consortium are encouraged to also disseminate ideas related to Epi-vector in review articles.
2) Data provided by Epi-vector is regularly presented at international conferences.
3) Prior to publication, every effort is made to ensure that best possible use is made of any intellectual property that might be protected by patent application.
4) Partners to Epi-vector are encouraged to discuss the concepts of gene therapy beyond the normal academic audience, by attending appropriate public meetings.
5) Details relating to the aims of Epi-vector are freely available on the project web site at: www.ls.manchester.ac.uk/epivector/