final report

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Transient Production of therapeutic compounds

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1. AIMS OF PROJECT - PHASE 1

Objective: The ultimate aim of this project was to evaluate the feasibility of a process for transient production of recombinant therapeutic proteins at commercially competitive levels using *in vitro* culture of transfected primary cells from organs sourced from the meat processing industry.

In a previous project (A.BIT.0001; A.BIT.0008; A.BIT.0015) on "In-vitro organ culture", a technology was developed for keeping cells from bovine and ovine organs, collected post slaughter, alive for several weeks. The cells were shown to be able to support de-novo protein synthesis in response to chemical stimulants, resulting in up to 7 fold increases in the levels of some metabolites. This project sought to exploit this ability by producing specific target proteins through transient transformation with genes for the target protein. Organ culture often refers to the cultivation of whole organs ex vivo, however it is not a viable option for the efficient production of bioactives as proposed in this project. The primary reason is that intact organs of large size prevent efficient gaseous diffusion and the exchange of nutrients and metabolites, therefore the capacity for cell survival and the synthesis of bioactives will be largely negated. The dissociation and isolation of primary cells and minced cultures from organs was demonstrated to be the most efficient method for the maintenance of cell survival and functionality. Therefore, the term 'cell culture' is used throughout this report and will refer to primary cells and/or minced cultures derived from organs.

Transient production of recombinant proteins refers to the production of proteins by transient gene expression in host cells (cell lines or primary cells) transfected with recombinant DNA, rather than a stably transformed cell line. Transient recombinant protein production has become a popular technology for the production of sufficient quantity of recombinant proteins, such as monoclonal Antibodies (mAb), therapeutic proteins, for pre-clinical and clinical trials. Such proteins, derived through the recombinant process, can be high value products sought after by the pharmaceutical and medical industries for use in therapies and research. Global revenue on antibody products for example is currently about $33 billion.

The specific aims of this project were:

- To critically and systematically review the literature and patents in transient production of recombinant proteins by transfected primary cells from mammalian organs and cell culture, providing due diligence on both the production technology and the selected recombinant proteins as R&D targets;

- To establish protocols from organ collection and dissociated cell preparation, to cell transfection and protein production through in vitro culture of abattoir-derived primary cells or minced cultures, with an emphasis on protocol development for cell transfection and protein production;

- To demonstrate the ability to transfect primary cells derived from animal organs for transient production of two recombinant proteins: one being a model green fluorescent protein (GFP), and the other being a recombinant antibody or therapeutic proteins with commercial potential identified by the literature, patent and market survey;

- To identify a production target of a therapeutic protein in micrograms per gram of primary cells per week that would provide a basis for the commercial evaluation of the technology.

- To understand the transfection efficiency and recombinant protein productivity in vitro

with the culture of abattoir-derived primary cells, providing data towards quantifying the commercial potential of this technology and improving processes in subsequent phases.

In essence therefore the project aimed to demonstrate the proof of concept of increasing the value of bovine and ovine organs through the use of these tissues as cell factories. The technical objective was therefore proof of concept of transfecting primary cells or minced cultures derived from abattoir-harvested animal organs for transient production of commercially viable levels of therapeutic recombinant proteins.

Summary of Outcomes

Literature review

A comprehensive literature and patent review was completed of the transient production of recombinant proteins by transfected primary cells from mammalian organs and cell culture. This literature review specifically examined the following areas: Transfection Techniques, Vectors, Selection of a target protein, Assays and identification of a suitable outline of the experimental procedure.

Target proteins

Through a systematic analysis of the literature and patent database a list of potential targets was defined

- Monoclonal Antibodies.
- Human α-galactosidase A (GLA).
- Recombinant factor VIII.
- Erythropoietin (EPO).
- Virus Vaccines.
- Glycoproteins

Model protein selected

Green fluorescent protein was chosen to be used as a model protein for rapidly optimising transfection efficiency and establishing that the primary cells could be transfected and produce recombinant protein.

Target protein selected

The mouse monoclonal antibody Muromonab-CD3 (Othoclone, OKT-3) was selected as a target protein for the following reasons:

- Represented the first marketed monoclonal antibody therapy.
- IP position of OKT-3 was seen to allow freedom to operate in research and development capacity without infringing any patent
- It was a clinically relevant molecule with known market potential for establishing a business case.
- The sequence was available from patents and drugbank.
- It was a secreted molecule.
- Functional and quantitative assays had been previously described in the literature.

Cloning of target protein

The light and heavy chains of the mouse monoclonal antibody OKT-3 were inserted into the Gene-Art cloning program (Invitrogen) to design two separate vectors. The mammalian expression vector pcDNA3.1 featuring a viral cytomegalic virus (CMV) promoter was chosen for cloning to promote high transcription. Codon usage was optimised for bovine. A Kozak consensus sequence was included at the 5' of the gene to achieve the maximum initiation of translation. The commercially available Interleukin-2 signal peptide (IL-2) was cloned into the 5' region of the antibody sequence to allow secretion of the recombinant protein. The production of 10mg of each vector was out sourced to Invitrogen's Gene-Art service.

The successful development of an analytical method for the quantification of OKT-3
An ELISA-based assay was prepared using commercially available recombinant OKT-3 and antibodies.
The background reading of the assay was low (Ab_{450nM} 0.0205) and had a large dynamic range (Ab_{450nM} 0.027 and 1.665). The limit of detection was extremely sensitive at 32ng/ml.

Optimisation of cell preparation and transfection
Transfecting cells were prepared according to the literature and manufacturer's instructions. Four transfection modes were evaluated:

- Lipofection – lipid.
- Nucleofection – physical electric shock.
- Polyethyleneimine - chemical polymer.
- Calcium Phosphate –chemical.

The transfection techniques were tested using the liver cell line HEPG2 and primary rat hepatocyte. This ensured that the methodologies chosen were working in these model systems; established a proof of concept for the transient production of therapeutic and provided a model system that could be used to estimate transfection efficiency and production levels for preliminary business case and process models.

Extensive optimisation regimes were performed using a range of different methods to prepare bovine livers cell and mince organs viz.

- Chop and shake disaggregation.
- Perfusion disaggregation.
- Disaggregation of tissue while transporting.
- Mince organ Culture

Results

Cells that adhered to the well were found to be able to be transfected and produce protein for at least 120hrs after they were prepared and transfected. Cell out-growths from minced spleen cultures were also successful in producing transfectable cells.

Minced bovine liver organ cultures were transfected with the plasmid pEGFP that expresses green fluorescent protein (GFP) to determine whether minced organ cultures could be used in the production of recombinant proteins. Minced organ cultures were transfected using two different transfection methods; lipofection and polyethyleneimine. Minced organ cultures were then either directly analysed for recombinant GFP production using fluorescent microscopy or the samples were processed. Processing involved chopping the tissue into fine particles with a scalpel blade and examining the cells that were released into the supernatant and re-suspending the fine particles into fresh medium. Mince tissue were also processed for microscopic examination after two rounds of disaggregation. None of the transfected cells showed any GFP production above the control back ground levels.

Proof-of-principle experiments had previously been performed using the liver cell line HEPG2 and rat hepatocytes. The successful transient production of GFP as a model recombinant protein was demonstrated in these liver cells (Milestone 8). The preliminary transfection of cells derived from the bovine liver was also demonstrated (Milestone 8). In this milestone a new method of disaggregating while transporting was examined and an attempt was made to further optimise the chop and shake method which had successfully produced transfectable cells in preliminary studies. The production of attached and transfectable cells was found to be difficult, with only small patches of cells obtained. Further optimisation and replication of the preparation and transfection of these bovine liver cells may help to define the population of cells which readily attach and are transfected.

The cells produced by the chop and shake method grew in patches with the well never reaching confluence. Generally the wells seeded will HAM had more cells than when the alternative media were used; therefore HAM were used for all further experiment when testing the transfection of cells.

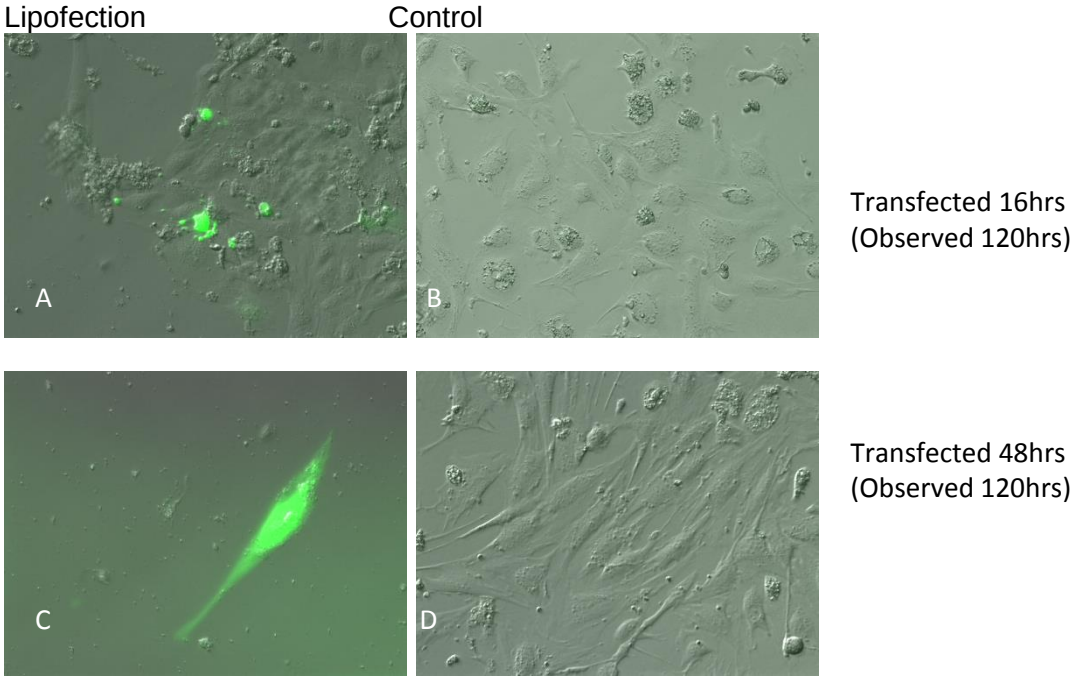

Figure 1. Transfections of bovine liver cells prepared by the chop and shake method A. Cells were prepared using the chop and shake method A (ROP 15). 20g of minced bovine liver was disaggregated in 20ml Collagenase type II in stages of 4x30min at 37°C. Cells were isolated by centrifugation. Cells were seeded at 2x105 cell /wells in HMM for 16hr prior to having their medium changed. Cells were transfected with 2ug per well of pEGFP using Lipofectamine 2000 at 16hrs (A), 48hrs (C) or maintained as no-transfected controls (B and D). The ratio of transfection reagent to DNA was 2.5:1. A composite picture of the bright field microscopy and GFP was prepared from microscopic analyses of the cells performed 120hrs after the initial seeding of the cells.

Figure 1 demonstrates that transfection of cells prepared by the chop and shake method A (ROP 15) resulted in the production of GFP in a few patches of cells. This proves that the production and transfection of these cells is reproducible. However the number of cells prepared and the transfection of cells was low. Only a few patches of cells were present in each well and within the transfected wells there was only 1-3 GFP expressing cells within these patches.

Conclusion

This project was designed to proceed through to large laboratory scale proof of concept of the process of transient production of target proteins in cells from bovine and ovine organs post slaughter. In light of the low transfection efficiency, the low likelihood of eventual technical success and a parallel assessment of the commercial feasibility of large scale organ cell culture and protein production, this project was terminated at the first Go/No Go point.