Institution: University of Kent

Unit of Assessment: 5, Biological Sciences

Title of case study: The improvement of recombinant protein production using the yeast *Saccharomyces cerevisiae* [Short title: ICS1_PDI]

1. Summary of the impact

This case study describes the impact of the discovery by Tuite and Freedman that elevating the levels of the enzyme protein disulphide isomerase (PDI) significantly increases the efficiency with which eukaryotic cells secrete disulphide-bonded proteins. This discovery led to the development of a patented, generic technology for improving both the yield and authenticity of high value, recombinant protein-based biopharmaceuticals. The patent has been used in the safe, animal free production of several FDA and EMEA approved biopharmaceuticals (e.g. recombinant human albumin; Recombumin®), generating multi-million dollar revenues. It has been sub-licensed to four major pharmaceutical companies (Novozymes, Pfizer, Glaxo, Repligen) to aid the safe production of biopharmaceuticals for a range of major human diseases (e.g. Type 2 diabetes).

2. Underpinning research

Many of the commercially-produced, protein-based biopharmaceuticals are secreted proteins that must undergo one or more post-translational modifications. Amongst these key modifications is the introduction of covalent linkages between spatially separated cysteine residues, to form stabilising disulphide bridges. The enzyme protein disulphide isomerise (PDI) catalyses the breakage and formation of such disulphide bonds in secreted proteins in all eukaryotic cells.

Collaboration between Tuite & Freedman at Kent led to the first cloning and characterisation of the yeast (*Saccharomyces cerevisiae) PDI1* gene encoding PDI. Subsequently, and with funding from Merck & Co (then known as Merck, Sharp & Dohme), Zeneca (ICI), Pfizer and the Biotechnology and Biological Sciences Research Council (BBSRC), the Kent team focussed on developing a safe yeast biocatalyst to produce secreted recombinant proteins that were correctly folded, i.e. to improve the yield of correctly folded and functional secreted recombinant proteins. In parallel, underpinning fundamental work on PDI led by Freedman (then at Kent but moved to the University of Warwick in 2002) had shown, for example, that peptides bound to PDI do so at a site distinct from the active site [*see 3.1*]. Due to their expertise in protein folding (Freedman) and yeast expression systems (Tuite), this research led to strains of yeast being engineered at Kent that expressed a range of folding catalysts with the focus being on PDI from yeast and humans. Their research on cell engineering for the efficient production of recombinant proteins in eukaryotic cells was supported by a significant level of Research Council and industrial funding for Tuite and Freedman in the period 1991-1995: Pfizer (1991-94), £851k; SERC-AFRC-DTI-Zeneca (1991-94), £72k; SERC-Merck, Sharp & Dohme (1992-1995, £183k).

The experimental strategy developed was to clone human and yeast DNA sequences encoding PDI into expression cassettes or vectors comprising a yeast promoter and transcription terminator [*see 3.2*]. The resulting expression cassettes were integrated into the genome of the host cell which, as a result, resulted in elevated levels of PDI protein in the correct cellular compartment, i.e. the endoplasmic reticulum. The engineered PDI-overproducing cells were then used as hosts for the expression of heterologous, disulphide-bonded proteins. The first report of the experimental finding that overexpression of PDI in yeast significantly increased the secretion of multiply disulphide bonded proteins was in Schultz et al [*see 3.3*], a paper on which Tuite was senior author. Schultz et al. [see *3.3*] demonstrated that this approach resulted in elevated levels of secreted antistasin, an inhibitor of the blood coagulation factor X, that has 10 disulphide bridges. Subsequently Tuite and Freedman [*see 3.4*] proposed that the rational engineering of yeast and mammalian cells through the overexpression of enzymes, such as PDI, that mediate rate-limiting steps in post-translational modification and/or secretion, would improve the efficiency of these expression systems.

Since 1993, in excess of an additional £1 million of competitive funding for PDI research was awarded to Freedman, Tuite and other collaborators at Kent, including 6 BBSRC/SERC/AFRC/EU project grants [*see Section 3 above*]. More recently, with funds provided by the University of Kent's 'Ideas Factory' scheme, Tuite has engineered novel yeast-human PDI molecules with improved substrate recognition properties (publication pending filing of patent application).

3. References to the research (Kent- based authors in **bold**)

3.1. Noiva, R., **Freedman, R.B.,** Lennarz, W.J. (1993) Peptide binding to protein disulfide isomerase occurs at a site distinct from the active sites. *Journal of Biological Chemistry* **268**:19210-19217.

3.2. **Luz, J.M.,** Markus, H., **Farquhar, R.**, Schultz, L.D., Ellis, R.W., **Freedman, R.B.** and **Tuite MF**. (1994) Expression and secretion of human protein disulphide isomerase in *Saccharomyces cerevisiae. Biochemical Society Transactions* **22:**76S.

3.3. Schultz, L.D., Markus, H.Z., Hofmann, K.J., Montgomery, D.L., Dunwiddie, C.T., Kniskern, P.J., **Freedman, R.B.**, Ellis, R.W. and **Tuite MF**. (1994) Using molecular genetics to improve the production of recombinant proteins by the yeast *Saccharomyces cerevisiae*. *Annals of the New York Academy of Science* **721**:148-157.

3.4. **Tuite, M.F.** and **Freedman, R.B.** (1994) Improving secretion of recombinant proteins from yeast and mammalian cells: rational or empirical design? *Trends in Biotechnology* **12**:432-434.

3.5. Patent family examples: **Tuite, M.F., Freedman, R.B.**, Schultz, L.D., Ellis, R.W. and Markus, H.Z. Method for increasing production of disulphide-bonded recombinant proteins by *Saccharomyces cerevisiae*. US 6291205 (B1) – US patent*, 2001;* EP 0746611 (B1) – European patent *2003* JP 2008271976 (A) – Japanese patent *2008*

Major grants awarded for PDI research at Kent, 1993-2002:

AFRC (BBSRC), 1993-95, Protein folding factors in the ER lumen of wheat, £113k (Freedman with P. Shewry);
SERC (BBSRC) 1993-96, Studies of post-translational processing mechanisms in insect cells, £250k (Freedman with B.C. Rooney and N. Jenkins);
SERC (BBSRC), 1993-96, Enzyme-catalysed folding and disulphide bond formation in secreted proteins; studies with periplasmic DsbA, £176k (Freedman with T.R. Hirst);
BBSRC, 1995-98, Structural basis of the polypeptide-binding and chaperone activities of protein disulphide-isomerase, £130k (Freedman);
EU (Framework IV) 1996-99 Cellular factors which facilitate protein folding: improved protein production by cell factories, £206k (Freedman, co-ordinator);
BBSRC, 1998-2002, Folding and assembly of bacterial enterotoxins: analysis of the roles of folding factors £195k (Freedman with L.W. Ruddock).

4. Details of the impact

A patent based on the research undertaken at Kent by Tuite and Freedman was jointly filed by the University of Kent and Merck & Co Inc and eventually granted in the USA in 2001 (US 6291205) and subsequently worldwide including Europe (2003; EP 0746611) and Japan (2008: JP 2008271976), Korea, Canada, Australia and New Zealand. The patent covers the generation and exploitation of genetically engineered eukaryotic cells (e.g. the yeast, *Saccharomyces cerevisiae)* that regularly and excessively produce human PDI or yeast PDI. The resulting intellectual property has been (and continues to be) licensed and sub-licensed to major US and UK companies for the commercial production of high value 'next generation' biopharmaceuticals.

The patented PDI-based technology developed by Freedman and Tuite has had its greatest impact on the animal-free commercial production of high value biopharmaceuticals. Technological

developments such as the one developed at Kent that improve the efficiency and authenticity of such drugs, are crucial to maintaining a healthy society. The global significance of such technologies is reflected in the strategy of national funding bodies such as the Technology Strategy Board (TSB) and the Biotechnology and Biological Sciences Research Council (BBSRC). Approvals for use of biopharmaceutical drugs have been steadily rising: there are now around 200 biopharmaceutical products on the market with approximately 15 having individual sales in the USA of greater than $1bn. By 2014, eight of the top ten pharmaceuticals are expected to be proteins. Many of these high value proteins are secreted from the host cell which facilitates downstream purification, but also typically requires a range of post-translational modifications such as disulphide bond formation, for authenticity, stability and function. The impact of the PDI-based technology developed at Kent has come through it facilitating significant increases in the levels and authenticity of a range of high value, secretory proteins produced commercially. To further extend the impact of the technology, second generation derivatives are currently being researched and developed by Tuite, a project strongly supported by Novozymes Biopharma UK Ltd [*see 5.1*].

Current licenses and applications

The PDI technology was originally developed in collaboration with Merck & Co (formerly Merck, Sharp and Dohme) and they were able to demonstrate that it significantly increased secretion of a multiply disulphide-bonded protein of therapeutic value, namely antistasin, an anticoagulant protein (Schultz et al 1994). The intellectual property is presently non-exclusively licensed to two companies, Novozymes Biopharma UK Ltd and Pfizer and both licensees have the rights to sub-license to third parties. In each case the licenses are domain limited with respect to the manufacture of certain types of proteins.

Novozymes Biopharma UK Ltd (formerly Delta Biotechnology Ltd and acquired by Novozymes A/S in July 2006) is one of the largest producers of industrial enzymes and biopharmaceuticals in Europe and the parent company Novozymes has global sales in excess of £1 billion (2012 figures). Novozymes Biopharma UK Ltd is a world leader in yeast-based expression systems and the main business of the company is the production of recombinant human serum albumin (rHA) which has 17 disulphide bridges. The commercially produced form of rHA is called Recombumin® that has both FDA and EMEA approval and is manufactured at Novozymes' own large-scale compliant facility using a yeast-based expression system. Recombumin® has global sales that exceed £10 million annually [*see 5.2*] and the market for Recombumin® is expected to grow due to its increasing demand as the primary protein drug excipient for use in pharmaceutical and vaccine product formulation. Delta Biotechnology said of the licensing agreement "*This patented technology has enhanced Delta's comprehensive technical capability. It has also allowed the company to further optimise its expression systems to produce recombinant proteins in exceptionally high yield which are correctly folded and able to conform to the rigorous standards required in the manufacture of high value biopharmaceuticals*." [*see 5.3*]

Biorexis Pharmaceutical Corporation (acquired by Pfizer in February 2007 and now operates as a subsidiary of Pfizer, Inc) has also taken out a license to use the PDI technology. The main business of Biorexis is the production of recombinant transferrin and transferrin-based protein fusions with the latter enhancing bioavailability and targeting *in vivo* [*see 5.4*]. In 2011 Novozymes Biopharma sub-licensed the PDI technology to two other companies for applications outside its use to generate recombinant human albumin [*see 5.5*] and principally for use in the manufacture of Novozymes' Albufuse® products for protein therapeutics with an extended circulatory half-life [*see 5.6*].

Wider impact

The PDI technology has generated revenues to the University of Kent and inventors of over £750K to date of which £245k has been received since January 2008. Furthermore, the University continues to review, update and seek appropriate licensees in the USA and elsewhere. This technology additionally underpins the IP portfolio that is offered within the newly formed Centre for Molecular Processing at Kent. Several studies building on Kent's research have demonstrated the potential impact of the PDI technology for other multiply disulphide-bonded proteins, including human serum albumin and transferrin [*see 5.7*].

The impact of the research was further recognised by the inclusion of Freedman and Tuite in the 2008 publication of Bioscience:Biomillions by the BBSRC. This publication sought to '*celebrate the success of close to 50 researchers who, with funding from BBSRC and others, are translating the UK's world-leading bioscience research into real outcomes".* [*see 5.8*]

5. Sources to corroborate the impact

5.1: Letter to Tuite and Freedman from the Business Development Manager and the Molecular Biology Manager of Novozymes Biopharma UK Ltd indicating strong support for further developments of PDI-based technology and dated 24/5/2011.

5.2: Novozymes brochure (dated 2011) describing the recombinant human albumin product Recombumin®, produced using yeast expression technology
www.biopharma.novozymes.com/en/products/albumin/recombumin/Pages/default.aspx

5.3: A case study on the Kent-Delta Biotechnology license agreement, produced by the University of Kent Innovation and Enterprise (KIE) department and including a quote from Delta Biotechnology on the value of the licensing agreement to them.
www.kent.ac.uk/enterprise/files/case-studies/delta-biotech-case-study-2012.pdf

5.4: Repligen brochure detailing the production of recombinant transferrin in yeast. biopro.repligen.com/rtransferrin_download_sample/?gclid=COCH-f3AwboCFbMbtAodiSgA_Q

5.5: Letter dated 22/12/2011 from the IPR manager of Novozymes Biopharma DK confirming that Novozymes Biopharma DK has granted sub-licences to two other companies for use to produce recombinant proteins other than human serum albumin.

5.6: Novozymes brochure describing the Albufuse® technology
www.biopharma.novozymes.com/en/products/albumin-fusion/Pages/albufuse-half-life-extension.aspx

5.7: A peer-reviewed publication authored by Novozymes staff. Reference: Finnis CJ, Payne T, et al., (2010) High-level production of animal-free recombinant transferrin from *Saccharomyces cerevisiae. M*icrobial Cell Factories **9**: 87-96. [doi: 10.1186/1475-2859-9-87]

5.8: BBSRC-produced brochure "*Bioscience Biomillions Delivering impact from research*", published in 2008 and includes Tuite and Freedman in the group of 50 researchers who "with funding from BBSRC and others are translating the UK's world-leading bioscience research into real outcomes". www.bbsrc.ac.uk/web/FILES/Publications/bioscience_biomillions.pdf