Hepatocyte generation from pancreatic acinar cell lines.

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Declaration

I hereby declare that this thesis has been composed by myself and has not been submitted in any previous application for a degree. The work presented has been performed by myself, unless otherwise stated. All sources of information have been appropriately acknowledged by means of reference.

Emma Fairhall
Abstract

The transdifferentiation of pancreatic acinar cells towards hepatocytes is an event that occurs in vitro and in vivo in rodents. The B-13 cell line is a model for studying this phenomenon in vitro; it readily transdifferentiates into hepatocyte-like cells in response to glucocorticoids such as dexamethasone (DEX). The transdifferentiation event is dependent on a transient suppression of Wnt signalling followed by induction of Serine/threonine-protein kinase 1 (SGK1) via interactions with the glucocorticoid receptor. This thesis has aimed to further explore pancreatic to hepatic transdifferentiation, using the B-13 cell as a model and also investigated the phenomenon in human cells.

As hepatic stellate cells are involved in liver regeneration and may support the progenitor niche in liver, coculture experiments were conducted to assess their effects on B-13 transdifferentiation. Transdifferentiation was enhanced in cocultures and found to be dependent on cell-cell interaction that resulted in further suppression of the Wnt signalling pathway by myofibroblasts.

B-13 transdifferentiation was shown to be able to take place in vivo for the first time; cells were found to engraft only into the liver and pancreas of NOD/SCID mice. Interestingly, only cells within the liver environment showed expression of hepatocyte-specific genes.

B-13 cells were also cultured in 3D bioreactor devices where they transdifferentiated into functional hepatocyte-like cells with gene expression at levels comparable to primary rat hepatocytes.

Elucidating the mechanisms involved during B-13 transdifferentiation will support the isolation of an equivalent human pancreatic cell. Studies with a human cell line and primary exocrine cells demonstrated that glucocorticoids also induce hepatocyte-gene expression, and thus the generation/isolation of a human equivalent to the B-13 is a realistic goal.
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Table of contents

Declaration--------------------------------------------------------------- ii

Abstract------------------------------------------------------------------- iii

Acknowledgements---------------------------------------------------------- iv

List of figures----------------------------------------------------------- xi

List of tables------------------------------------------------------------ xviii

Abbreviations------------------------------------------------------------- xix

SI units----------------------------------------------------------------- xxiii

1 Introduction------------------------------------------------------------- 1

1.1 Stem cells-------------------------------------------------------------- 3
  1.1.1 Embryonic Stem Cells----------------------------------------------- 3
  1.1.2 Adult Stem Cells---------------------------------------------------- 5
  1.1.3 Induced Pluripotent Stem (iPS) Cells----------------------------- 8

1.2 Endodermal origins of the adult liver and pancreas------------------- 9
  1.2.1 Liver development----------------------------------------------- 9
  1.2.2 Pancreas development-------------------------------------------- 11

1.3 The adult pancreas; anatomy and physiology-------------------------- 12

1.4 The adult liver; anatomy and physiology------------------------------- 15
  1.4.1 Liver structure and functional units----------------------------- 16
  1.4.2 Functions of the liver-------------------------------------------- 18
  1.4.3 Cells of the liver----------------------------------------------- 19
  1.4.4 Liver disease and current therapies----------------------------- 22
# Table of contents

1.4.5 Regenerative capacity of the liver

1.4.6 Stem cells in the liver

1.5 Alternative cell types for generation of hepatocyte cells

1.5.1 Oval cells/hepatic progenitor cells

1.5.2 Hepatoblasts

1.5.3 Embryonic stem cells

1.5.4 iPS cells

1.5.5 Extra-hepatic adult stem cells.

1.6 Transdifferentiation of the pancreas to liver

1.6.1 The AR42J-B-13 (B-13) cell line

1.6.2 Wnt signalling in transdifferentiation of pancreas to liver

1.6.3 Glucocorticoids in transdifferentiation of pancreas to liver

2 Materials and Methods

2.1 Animal work

2.1.1 Ethics

2.1.2 Housing

2.1.3 C57B16 (wild type) mice

2.1.4 Green Fluorescent Protein/ C57B16 mice

2.1.5 Non-Obese Diabetic Severe Combined immune-deficient (NOD-SCID) mice

2.2 Cell Culture

2.2.1 AR42J-B-13 (B-13) Cells

2.2.2 Induction of a hepatic phenotype in B-13 cells

2.2.3 Primary human hepatic stellate cells (hHSCs)

2.2.4 Primary mouse and rat hepatic stellate cells (m/rHSCs)

2.2.5 Primary mouse and rat hepatocytes

2.2.6 HPAC cells

2.2.7 Primary human pancreatic acinar cells

2.2.8 HepG2 Cells

2.2.9 Cell passage

2.2.10 Cell storage and revival
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.11 Cell viability and number</td>
<td>47</td>
</tr>
<tr>
<td>2.2.12 Coculture of B-13\textsuperscript{red} cells with GFP activated HSCs (myofibroblasts)</td>
<td>48</td>
</tr>
<tr>
<td>2.2.13 GFP myofibroblast conditioned media and its treatments</td>
<td>48</td>
</tr>
<tr>
<td>2.3 Cell transfection</td>
<td>48</td>
</tr>
<tr>
<td>2.3.1 Transfection using Effectene reagent (QIAGEN)</td>
<td>48</td>
</tr>
<tr>
<td>2.3.2 Production of stable transformed cell line</td>
<td>49</td>
</tr>
<tr>
<td>2.3.3 TOP/FLOP flash transfection and dual luciferase assay (Promega)</td>
<td>49</td>
</tr>
<tr>
<td>2.4 Plasmid DNA constructs</td>
<td>50</td>
</tr>
<tr>
<td>2.4.1 TCF/LEF (TOPFLASH and FLOPFLASH) transcriptional activity reporter gene.</td>
<td>50</td>
</tr>
<tr>
<td>2.4.2 pDsRed2-C1</td>
<td>51</td>
</tr>
<tr>
<td>2.4.3 pCAG2LMKOSimO</td>
<td>52</td>
</tr>
<tr>
<td>2.4.4 Transformation of TOP10 competent cells</td>
<td>52</td>
</tr>
<tr>
<td>2.4.5 Storage of DNA plasmids and glycerol stocks</td>
<td>52</td>
</tr>
<tr>
<td>2.4.6 Miniprep purification of plasmid DNA</td>
<td>53</td>
</tr>
<tr>
<td>2.4.7 Maxiprep purification of plasmid DNA</td>
<td>53</td>
</tr>
<tr>
<td>2.5 Cytogenetic analysis of B-13 cells</td>
<td>54</td>
</tr>
<tr>
<td>2.5.1 Preparation of metaphase spreads</td>
<td>54</td>
</tr>
<tr>
<td>2.6 Soft agar anchorage assay</td>
<td>54</td>
</tr>
<tr>
<td>2.6.1 Culture in soft agar</td>
<td>54</td>
</tr>
<tr>
<td>2.7 RNA and DNA isolation/ quantification</td>
<td>55</td>
</tr>
<tr>
<td>2.7.1 RNA isolation</td>
<td>55</td>
</tr>
<tr>
<td>2.7.2 Quantification of RNA concentration and integrity</td>
<td>55</td>
</tr>
<tr>
<td>2.7.3 DNase treatment of RNA</td>
<td>56</td>
</tr>
<tr>
<td>2.7.4 DNA isolation</td>
<td>56</td>
</tr>
<tr>
<td>2.7.5 Quantification of DNA concentration and integrity</td>
<td>56</td>
</tr>
<tr>
<td>2.7.6 Reverse transcription, 1\textsuperscript{st} strand DNA synthesis (cDNA)</td>
<td>57</td>
</tr>
<tr>
<td>2.7.7 Polymerase Chain Reaction (PCR)</td>
<td>57</td>
</tr>
<tr>
<td>2.7.8 PCR primer design</td>
<td>58</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.7.9 Agarose gel electrophoresis</td>
<td>58</td>
</tr>
<tr>
<td>2.7.10 Real-time PCR (SYBR Green qRT-PCR)</td>
<td>59</td>
</tr>
<tr>
<td>2.8 Protein isolation and quantification</td>
<td>64</td>
</tr>
<tr>
<td>2.8.1 Preparation of whole tissue samples</td>
<td>64</td>
</tr>
<tr>
<td>2.8.2 Preparation of cell extracts</td>
<td>64</td>
</tr>
<tr>
<td>2.8.3 Lowry protein assay</td>
<td>64</td>
</tr>
<tr>
<td>2.8.4 Sodium-dodecyl sulphate polyacrylamide gel electrophoresis</td>
<td>65</td>
</tr>
<tr>
<td>(SDS-PAGE)</td>
<td></td>
</tr>
<tr>
<td>2.8.5 Sample preparation and electrophoresis</td>
<td>65</td>
</tr>
<tr>
<td>2.8.6 Electrophoresis</td>
<td>66</td>
</tr>
<tr>
<td>2.8.7 Immunodetection</td>
<td>66</td>
</tr>
<tr>
<td>2.9 Immunohistochemistry and immunocytochemistry</td>
<td>67</td>
</tr>
<tr>
<td>2.9.1 Immunohistochemistry</td>
<td>67</td>
</tr>
<tr>
<td>2.9.2 Haematoxylin and eosin staining (H&amp;E)</td>
<td>67</td>
</tr>
<tr>
<td>2.9.3 Sodium citrate antigen retrieval</td>
<td>68</td>
</tr>
<tr>
<td>2.9.4 Fluorescent immunocytochemistry</td>
<td>68</td>
</tr>
<tr>
<td>2.9.5 DAPI staining of DNA</td>
<td>69</td>
</tr>
<tr>
<td>2.9.6 Double staining</td>
<td>69</td>
</tr>
<tr>
<td>2.10 Fluorescent in situ hybridisation (FISH) staining</td>
<td>70</td>
</tr>
<tr>
<td>2.10.1 FISH staining</td>
<td>70</td>
</tr>
<tr>
<td>2.11 Culturing B-13 cells in bio-artificial liver device</td>
<td>73</td>
</tr>
<tr>
<td>2.11.1 Bioreactor and perfusion equipment</td>
<td>73</td>
</tr>
<tr>
<td>2.11.2 Running of bioreactor</td>
<td>74</td>
</tr>
<tr>
<td>2.11.3 Inoculation of B-13 cells</td>
<td>75</td>
</tr>
<tr>
<td>2.11.4 Assessment of CYP450 activity and other parameters</td>
<td>75</td>
</tr>
<tr>
<td>2.11.5 Opening of Bioreactor</td>
<td>79</td>
</tr>
<tr>
<td>3 Results</td>
<td>80</td>
</tr>
<tr>
<td>3.1 Investigating the effects of B-13 and activated HSC (myofibroblasts) cocultures on the transdifferentiation towards the B-13/H phenotype</td>
<td>81</td>
</tr>
<tr>
<td>3.1.1 Generation of the B-13\textsuperscript{ed} cell line</td>
<td>84</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Coculture of B-13\textsuperscript{red} cells with myofibroblasts</td>
</tr>
<tr>
<td>3.1.3</td>
<td>Affects of coculture on Wnt signalling in B-13 cells</td>
</tr>
<tr>
<td>3.1.4</td>
<td>Identifying factors influencing enhanced transdifferentiation in coculture experiments</td>
</tr>
<tr>
<td>3.1.5</td>
<td>Chapter discussion</td>
</tr>
<tr>
<td>3.2</td>
<td>Investigations into cytogenetics and tumour-like behaviour of B-cells</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Cytogenetic analysis of B-13 cells</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Agar anchorage assay</td>
</tr>
<tr>
<td>3.2.3</td>
<td>In vivo assessment of tumour forming potential</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Chapter discussion</td>
</tr>
<tr>
<td>3.3</td>
<td>Investigations for the \textit{in vivo} potential of the B-13 cell line as a liver progenitor cell</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Determination of paracetamol and B-13\textsuperscript{red} cell dosage</td>
</tr>
<tr>
<td>3.3.2</td>
<td>The B-13 cell is able to engraft to the liver independent of paracetamol treatment</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Amylase positive cells within the livers are of B-13 origin</td>
</tr>
<tr>
<td>3.3.4</td>
<td>B-13 cells engraft to the liver and pancreas but show hepatocyte-like phenotype in the liver only.</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Chapter discussion</td>
</tr>
<tr>
<td>3.4</td>
<td>Culture of B-13 cells in 3D bio-artificial liver devices</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Establishing optimal culture conditions for bioreactor cultures</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Pilot study in bioreactor (BR)</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Identifying factors affecting B-13 culture in BR176</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Culturing of B-13 and B-13/H cells in 3D environment</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Chapter discussion</td>
</tr>
<tr>
<td>3.5</td>
<td>Culture of human pancreatic acinar cells for generation of human hepatocyte-like cells</td>
</tr>
<tr>
<td>3.5.1</td>
<td>The human pancreatic acinar cell (HPAC) line shows slowed proliferation in response to DEX</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Expression of liver-specific markers is induced in HPACS following DEX treatment and is dependent on activation of the GR.</td>
</tr>
</tbody>
</table>
3.5.3 Expression of liver specific markers through DEX treatment is inhibited in cocultures with liver myofibroblasts. 186
3.5.4 Expression of pluripotency inducing factors in HPACS 189
3.5.5 Culture of human primary acinar cells. 194
3.5.6 Chapter discussion 197

4 General Discussion 200
5 References 207
6 Publications and abstracts 222
7 Published manuscripts 228
List of figures

Figure 1.1: The cell lineage of a typical pluripotent stem cell ........................................ 5
Figure 1.2: Pathways involved in tissue regeneration in the mammalian adult. 6
Figure 1.3: The intestinal stem cell niche ........................................................................ 7
Figure 1.4: The early development of the liver and pancreas from the endodermal germ layer ......................................................................................................................... 10
Figure 1.5: Basic anatomy of the pancreas ...................................................................... 12
Figure 1.6: Basic anatomy of the liver .............................................................................. 15
Figure 1.7: Schematic diagrams representing the hepatic lobule (A) and the liver acinus (B) .......................................................................................................................... 17
Figure 1.8: Fibrosis during liver injury .............................................................................. 23
Figure 1.9: The potential uses of stem cell-derived hepatocytes for treatment of liver disease and in vitro toxicity testing ................................................................. 28
Figure 1.10: The typical morphology of B-13 cells before and after DEX treatment ................................................................................................................................. 35
Figure 1.11: The canonical Wnt signalling pathway ......................................................... 37
Figure 1.12: Crosstalk between the Wnt and glucocorticoid signalling pathways in the induction of B-13 to B-13/H transdifferentiation ......................................................... 40

Figure 2.1: Luciferase reaction .......................................................................................... 50
Figure 2.2: Vector map for (TOPFLASH catalog # 21-170) ........................................ 51
Figure 2.3: Vector and restriction map for pDsRed2-C1 .................................................. 51
Figure 2.4: Vector map for pCAG2L KoSimO .................................................................. 52
Figure 2.5: Images to show A) a typical jellyfish bioreactor employed for 3D culture studies; B) the set up of the bioreactor and the perfusion tubing within the processor-controlled perfusion device with electronic pressure, media flow regulation and waste pump operation .................................................................................... 73
Figure 2.6: A schematic diagram to show the organisation of the bioreactor devices used for 3D-culture .............................................................................................................. 74
Figure 2.7: Images to show the metabolism pathways for the drugs Phenacetin\textsuperscript{[194]}, Bupropion\textsuperscript{[195]}, Diclofenac\textsuperscript{[196]} and Midazolam\textsuperscript{[197]} which were
used in metabolism assays during the pilot bioreactor run. Images are adapted from the cited references.

**Figure 3.1 1:** The effect of various G418 disulphate concentrations on B-13 cell viability. ................................................................. 85

**Figure 3.1 2:** The expression of red fluorescent protein in the B-13red cell line. Cells are treated with and without DEX treatment, with B-13 cells as controls. 85

**Figure 3.1 3:** RT-PCR and Western blot analysis for the expression of liver specific markers in B-13red with or without DEX treatment ........................................... 86

**Figure 3.1 4:** The expression of the pancreatic marker amylase and liver markers albumin and CYP2E1 in B-13red and B-13/Hred cells shown by immunocytochemistry. ........................................................................ 87

**Figure 3.1 5:** The average number of hepatocyte-like cells per random field of view comparing B-13red cells alone, activated HSCs (myofibroblasts) alone or cocultures with or without DEX treatment for 7 days ................................................................ 88

**Figure 3.1 6:** Haematoxylin and eosin (H&E) staining of B-13red, activated HSCs (myofibroblasts) and cocultures after 7 days of culture with or without DEX. ......................................................................................... 89

**Figure 3.1 7:** Cultures of B-13red activated HSCs (myofibroblasts) and cocultures with or without DEX treatment for 7 days ................................................ 91

**Figure 3.1 8:** RT-PCR and Western blot analysis for the expression of liver specific markers in coculture experiments with or without DEX after 7 days .... 92

**Figure 3.1 9:** Wnt signalling activity in B-13 cells cultured with or without myofibroblasts after 24 or 48 hours of DEX treatment .................................................. 94

**Figure 3.1 10:** The effects of myofibroblast conditioned media on the rate of B-13 transdifferentiation ......................................................................................... 95

**Figure 3.1 11:** The average number of hepatocyte-like cells per random field of view comparing B-13red cells alone, B-13red cells treated with myofibroblast conditioned media or cocultures, with or without DEX treatment for 7 days ..... 96

**Figure 3.1 12:** RT-PCR and Western blot analysis for the expression of liver specific markers in coculture experiments with dead myofibroblasts with or without 10µM DEX for 7 days ......................................................................................... 97
Figure 3.1 13: The average number of hepatocyte like cells per random field of view comparing B-13red cells alone, B-13red cells with dead myofibroblasts or cocultures with or without DEX treatment for 7 days ......................................................... 98

Figure 3.1 14: The expression of amylase and CYP2E1 in B-13 cells after cocultures shown by immunohistochemistry ................................................................. 100

Figure 3.2 1: A typical G-banded metaphase spread of B-13 chromosomes. ........................................................................................................................................ 107

Figure 3.2 2: Metaphase spreads from B-13 cells, rat myofibroblasts and the human HepG2 cell line ........................................................................................................ 108

Figure 3.2 3: A typical G-banded karyotype of the B-13 cells .................................................. 109

Figure 3.2 4: PCR for the sex chromosomes in B-13 cells ................................................................. 109

Figure 3.2 5: Crystal violet staining to show colony formation after culture in agar ........................................................................................................................................ 111

Figure 3.2 6: The presence of cells or cell colonies after growth in soft agar for 14 days ........................................................................................................................................ 112

Figure 3.2 7: Colony formation after growth in soft agar ........................................................................ 112

Figure 3.2 8: RT-PCR and Western blot analysis of the expression for the pancreatic marker amylase in organs from NOD-SCID mice injected with B-13 cells, HepG2 cells and PBS ........................................................................................................................................ 114

Figure 3.2 9: The expression of amylase and CYP2E1 in tumours formed in B-13 injected NOD/SCID mice shown by immunohistochemistry .................................................. 115

Figure 3.3 1: Levels of alkaline phosphatase and alanine transaminase in paracetamol treated mice ........................................................................................................................................ 120

Figure 3.3 2: Morphology of livers from paracetamol treated mice and a non-treated control ........................................................................................................................................ 121

Figure 3.3 3: B-13red cells were visualised under the DsRed wavelength using an in vivo imaging system (ivis) ........................................................................................................................................ 122

Figure 3.3 4: Total fluorescence detected in varying numbers of B-13red cells under DsRed wavelengths using an in vivo imaging system (ivis) .................................................. 122

Figure 3.3 5: Western blot analysis and immunohistochemistry for the pancreatic marker amylase in pilot studies for injections of B-13red cells into NOD/SCID mice ........................................................................................................................................ 123
Figure 3.3 6: The expression of the pancreatic marker amylase in WT rat pancreas and liver and B-13red injected NOD/SCID mouse pancreas, liver, heart and lung shown by immunohistochemistry ..................................................125
Figure 3.3 7: The expression of the pancreatic marker amylase detected within the livers of NOD/SCID mice after intravenous injections of B-13red cells shown by immunohistochemistry ..........................................................126
Figure 3.3 8: The expression for the pancreatic marker amylase in B-13red injected NOD/SCID mice shown by RT-PCR ..........................................................126
Figure 3.3 9: Expression of amylase at the protein level in various organs of NOD/SCID mice after B-13 injections and non injected controls shown by Western blot ..................................................................................127
Figure 3.3 10: Organs from NOD/SCID mice injected with Cellvue® NIR815 conjugated B-13 cells were visualised under the near infrared wavelength using an in vivo imaging system (ivis) ........................................................................128
Figure 3.3 11: Total radiance detected from the organs of Cellvue® NIR815 B-13 cell injected NOD/SCID mice and non-injected controls ........................................129
Figure 3.3 12: Fluorescent in situ hybridisation (FISH) staining performed on rat liver (male and female), mouse liver (male) and B-13 cells ........................................131
Figure 3.3 13: Fish and amylase staining in serial sections from B-13 injected NOD/SCID mice livers ........................................................................................................132
Figure 3.3 14: Sections of liver and pancreas from B-13red injected NOD/SCID mice visualised under the DsRed wavelength show evidence of B-13 cell engraftment .......................................................................................133
Figure 3.3 15: Rat specific amylase and CYP2E1 mRNA was detected in the livers and pancreata of B-13red injected NOD/SCID mice ........................................135
Figure 3.3 16: DsRed and albumin expression in B-13red injected NOD/SCID mice .........................................................................................................................136

Figure 3.4 1: Typical cell morphology of B-13 cells treated with 10µM DEX in media containing 10% or 2.5% FCS ........................................................................143
Figure 3.4 2: Proliferation of B-13 cells in 10% or 2.5% FCS containing media with or without 10µM DEX treatment .................................................................143
List of Figures

Figure 3.4 3: RT-PCR and Western blot analysis for the expression of liver specific markers after culture with 10% or 2.5% FCS containing media ±10µM DEX .........................................................................................................................................................144

Figure 3.4 4: Cytochrome P450-carbon monoxide complexes in B-13/H cells and primary rat hepatocytes .........................................................................................................................145

Figure 3.4 5: The timescale of the pilot bioreactor study (BR 176) and the 2D parallel cultures ..............................................................................................................................................146

Figure 3.4 6: Cell metabolism of the B-13 cells cultured in bioreactor 176 and parallel 2D cultures ..............................................................................................................................................147

Figure 3.4 7: Enzyme release from B-13 cells cultured in bioreactor 176 and parallel 2D cultures ...............................................................................................................................................150

Figure 3.4 8: Urea production by B-13 cells cultured in bioreactor 176 and parallel 2D cultures ..............................................................................................................................................151

Figure 3.4 9: H&E stains of B-13 cells cultured in BR176 shown at x10 and x40 magnification ..................................................................................................................................................152

Figure 3.4 10: Typical DAB staining for samples from BR176 and parallel 2D cultures .........................................................................................................................................................153

Figure 3.4 11: RT-PCR analysis of mRNA samples from BR176 and parallel 2D cultures .........................................................................................................................................................154

Figure 3.4 12: CYP450 metabolism assay of B-13 cells in BR176 and parallel 2D cultures at 8 days .................................................................................................................................155

Figure 3.4 13: RT-PCR analysis for the expression of liver specific markers CYP2E1, CPS-1, and albumin, and the pancreatic marker amylase, at the mRNA level in B-13 cells cultured with a range of different 10µM DEX solutions. ..............................................................................................................................................157

Figure 3.4 14: Comparison of absolute levels of DEX (nM) measured in media samples from BR176 and parallel 2D cultures of B-13 cells on days 2, 4 and 6 of culture. ..............................................................................................................................................157

Figure 3.4 15: The effect of seeding density on the rate of B-13 transdifferentiation. .......................................................................................................................................................................158

Figure 3.4 16: The timescale of bioreactor cultures 196-199 and the 2D parallel cultures ..................................................................................................................................................159
List of Figures

Figure 3.4 17: Cell metabolism of bioreactors 196, 197, 198 and 199 over 8/15 days. Bioreactors 196 and 197 were inoculated with B-13 cells, bioreactor 198 with B-13/H cells and bioreactor 199 with rat hepatocytes ...........................................160

Figure 3.4 18: The enzyme release in bioreactors 196, 197, 198 and 199 over 8/15 days ........................................................................................................................163

Figure 3.4 19: Urea production in bioreactors 196, 197, 198 and 199 over 8/15 days. ........................................................................................................................164

Figure 3.4 20: H&E staining of bioreactors 199, 196, 197 and 198 .............165

Figure 3.4 21: Typical images of DAB immunohistochemistry for bioreactors 196-199 after 8/15 days culture .................................................................166

Figure 3.4 22: RT-PCR and Western blot analysis for the expression of liver specific markers in bioreactors 196-199. .........................................................168

Figure 3.4 23: Fold changes of CYP2E1 mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells ......169

Figure 3.4 24: Fold changes of albumin mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells ......169

Figure 3.4 25: Fold changes of CPS-1 mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells ......170

Figure 3.4 26: Fold changes of C/EBPβ mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells ......170

Figure 3.4 27: Fold changes of amylase mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells. ......171

Figure 3.4 28: EROD CYP450 assay for bioreactors 196, 197, 198 and 199 performed on day 1, day 8 and day 15 of bioreactor cultures ......................172

Figure 3.5 1: HPAC cell proliferation with 10μM DEX treatment or ethanol vehicle controls. ......................................................................................................179

Figure 3.5 2: Typical morphology of HPACs treated with ethanol vehicle and HPACs treated with 10μM DEX for 7 days ........................................179

Figure 3.5 3: RT-PCR and Western blot analysis for the expression of liver specific markers in HPACs and HPACs treated with DEX ..................181

Figure 3.5 4: Immunocytochemistry for CYP2E1 in B-13 and HPAC cells with or without DEX ..................................................................................182
Figure 3.5 5: RT-PCR analysis for the expression of nuclear receptors in HPAC cells

Figure 3.5 6: Immunocytochemistry for the glucocorticoid receptor (GR) in control and DEX treated HPACs

Figure 3.5 7: RT-PCR analysis for the expression of liver specific markers in HPACs treated with various nuclear receptor activators

Figure 3.5 8: Cocultures of control and DEX treated HPACs and liver myofibroblasts

Figure 3.5 9: RT-PCR analysis for the expression of liver-specific markers in HPAC coculture experiments

Figure 3.5 10: Immunocytochemistry for CYP2E1 and albumin in HPACs and cocultures of myofibroblasts and HPACs ± 10μM DEX

Figure 3.5 11: Visualisation of HPAC and pCAG HPAC cells under excitation/emission 549 and 565nm respectively

Figure 3.5 12: Bright field images of pCAG HPACS in standard or DEX treated cultures

Figure 3.5 13: RT-PCR analysis for the expression of liver specific markers and pluripotency markers in WT and pCAG HPACS

Figure 3.5 14: Immunocytochemistry in HPAC and pCAG HPAC cells with and without DEX treatment

Figure 3.5 15: Typical morphology of human primary acinar cells cultured over 21 days

Figure 3.5 16: RT-PCR analysis for the expression of liver, pancreatic and fibroblast specific markers in primary acinar cells

Figure 3.5 17: Co-expression of amylase and CYP2E1 in primary acinar cells shown by immunohistochemistry
List of tables

Table 2. 1: DNA oligonucleotide sequences employed in RT-PCR or genomic PCR. .................................................................63

Table 2. 2: Primary and secondary antibody specifications for Western blot (WB) and immunohistochemistry/immunocytochemistry (IHC/ICC). ...............72

Table 2. 3: Specifications of the bioreactors and running conditions used for 3D culture of B-13 cells ..............................................................75

Table 2. 4: A summary of the substrates and their concentrations used for assessment of CYP450 activity in bioreactor cultured B-13 cells and their 2D parallels. ...........................................................................77

Table 2. 5: A summary of the drugs, their products and the CYP450 enzymes involved for the CYP450 assays to test liver functionality in bioreactor metabolism studies. .........................................................77

Table 3.2 1: The average number of chromosomes per metaphase spread analysed from each cell type.....................................................108

Table 3.2 2: Summary of the B-13 cell karyotype.................................................110

Table 3.4 1: A summary of the bioreactor cultures carried out with details of the length of culture, cell type and cell number.......... Error! Bookmark not defined.
Abbreviations

AFF- 2-acetylaminofluorene
ALT- Alanine transaminase
AP- Alkaline phophotase
APC- Adenomatous polyposis coli
α-sma- Alpha-smooth muscle actin
AST- Aspartate transaminase
B-13- AR42J-B13 cell
BMP- Bone morphogenic protein
BMPRII- type II bone morphogenic receptor
BR- bioreactor
BSA- Bovine serum albumin
CAR- constitutive adrostane receptor
CBC- Comparative biology centre
cDNA- complementary Deoxyribonucleic acid
C/EBP- CCAAT/enhancer-binding protein
CPS-1- Carbamoyl-phosphate synthetase 1
CYP450- Cytochrome P450
DAB- 3,3'-Diaminobenzidine
DAPI- 4',6-Diamidino-2-phenylindole
DEX- Dexamethasone
DMEM- Dulbecco’s modified eagle media
DMSO- Dimethyl sulfoxide
DNA- Deoxyribonucleic acid
ds DNA- double stranded deoxyribonucleic acid
Dsh- Dishevelled
DTT- Dithiothreitol
EB- Embryonic bodies
EBSS- Earl’s balanced salt solution
ECM- Extra-cellular matrix
ECOD- 7- ethoxycoumarin O-deethylase
EDTA- Ethylenediamine tetraacetic acid
EGTA- Ethylene glycol tetraacetic acid
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<tr>
<td>EPCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>ERB</td>
<td>Electrode running buffer</td>
</tr>
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<td>EROD</td>
<td>7-ethoxyresorufin-O-deethylase</td>
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<tr>
<td>ESCs</td>
<td>Embryonic Stem Cells</td>
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<tr>
<td>Fah-//-</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FISH</td>
<td>Fluorescent in situ hybridisation</td>
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<tr>
<td>ICC</td>
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<td>Inner cell mass</td>
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Abbreviations

i.p.- Intraperitoneal
iPS- Induced pluripotent stem
ivis- in vivo imaging system
KLF4- Krüppel-like factor-4
k/o- knockout
LARII- Luciferase assay reagent
LB- Luria broth
LDC- Liver dendritic cell
LDH- Lactic acid dehydrogenase
Lgr5+- leucine-rich repeat-containing G protein-coupled receptor 5+
LRP5/6- Low density lipoprotein receptor-related protein 5 or 6
MAPK- Mitogen- activated protein kinase
MET- metyrapone
MMLV- moloney murine leukemia virus
MMPs- Matrix metalloproteinase’s
MNAR- modulator of non-genomic action of the estrogen receptor
MR- Mineralcorticoid receptor
mRNA- Messenger Ribonucleic acid
MSCs- Mesenchymal stem cells
NK- Natural killer cells
NCAM- Neural cell adhesion molecule
Neurog3- Neurogenin-3
NOD/SCID- Non-obese diabetic/severe combined immunodeficient
OCT4- Octamer-4
PB- phenobarbital
PBS- phosphate buffered saline
PCR- Polymerase chain reaction
Pdx1- Pancreatic and duodenal homeobox 1
PDGF- Platelet derived growth factor
PH- Partial heptectomy
Ptf1a- Pancreas transcription factor 1-alpha
PXR- pregnane X receptor
RA- Retinoic acid
RIF- rifampicin
Abbreviations

rmh- Rat/mouse/human
RNA- Ribonucleic acid
ROS- Reactive oxygen species
RT-PCR- Reverse transcription polymerase chain reaction
SD- standard deviation
SDS- Sodium dodecyl sulfate
SDS-PAGE- Sodium dodecyl sulphate- polycrylamide gel electrophoresis
SECs- Sinusoidal endothelial cells
SGK1- Serine/threonine-protein kinase 1
Shh- Sonic hedgehog
siRNA- small interfering RNA
S.O.C- Super Optimal broth with Catabolite repression
SOX2- Sex determining region Y-box 2
SSC- standard saline citrate
TEMED- Tetramethylethylenediamine
TGFβ1- Transforming growth factor beta
TNFα- Tumour necrosis factor alpha
TRITC- Tetramethyl rhodamine iso-thicyanate
VEGF- Vascular endothelial growth factor
WME- William’s Medium E
WT- wildtype
## SI Units

### Units:

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Chapter 1: Introduction
Chapter 1: Introduction

With increases in over 25% in the last decade, liver disease is now the fifth biggest killer in England and Wales and is the only major cause of death that is still rising year-on-year. Due to the liver's complexity and numerous metabolic roles it is susceptible to a variety of diseases including hepatitis, cirrhosis, fatty liver, liver cancer and genetic diseases. Although the occurrence of liver disease is growing, liver transplantation remains the only effective treatment for end stage liver disease, however treatment is limited due to the lack of available organs for transplantation. In addition hepatocytes, the main cell of the liver, are difficult to maintain in culture and consequently alternative sources are desperately required for liver cell replacement. Advances in the field of regenerative medicine bring hope of novel treatments through the use of stem cell-derived hepatocytes in transplantation procedures and extracorporeal liver devices. The generation of a limitless supply of stem cell-derived hepatocytes for uses in clinic and in vitro toxicity testing would hopefully provide successful therapeutics to reduce the impending threat of liver disease.
Chapter 1: Introduction

1.1 Stem cells

Stem cells have been identified in both embryonic tissue and in numerous adult tissues including the bone marrow, muscle and skin\(^1\). Stem cells are distinct from any other cell in that they possess the unique capacity to give rise to multiple cell types through a process termed differentiation, and have the ability of ‘self-renewal’; a method of limitless duplication without losing the potential to differentiate into other cell types (figure 1.1). The potential range of progeny that can arise from a stem cell population is known as the ‘potency’ where embryonic stem cells (ESCs) that can give rise to all body cell types are termed pluripotent while adult stem cells are often more restricted and so are described as multipotent or unipotent\(^2\). This restriction corresponds to the idea that development is hierarchical, where cells of the early blastula can give rise to all cell types and are then progressively restricted in their potency by numerous inductive signals\(^3\). Stem cells from both adult tissues and embryonic sources have proven to be powerful research tools in recent years alongside new exciting research in induced pluripotent stem (iPS) cell technologies\(^4\). The multi-lineage potential of stem cells, along with their capacity for self-renewal makes them a promising tool for regenerative medicine and cellular therapies as well as advancing our understanding of the underlying molecular mechanisms of stem cells and developmental biology.

1.1.1 Embryonic Stem Cells

Following the formation of the blastula, there is a phase in embryonic development termed gastrulation, here the cells undergo a series of movements that converts the simple ball of cells, termed the inner cell mass (ICM), into a three layered structure known as the gastrula. The three layers formed are termed the ‘germ layers’ which are comprised of the ectoderm, mesoderm and endoderm; at this stage of development the general body plan is set with regions of committed cells although no differentiation has yet taken place\(^5\).

During development a population of pluripotent cells appear as a cell cluster within the blastocyst known as the inner cell mass, which is the source of embryonic stem cells (ESCs) (figure 1.4). ESCs are true pluripotent cells as they are capable of differentiating into all three tissue lineages of the germ
layer, thus they have the ability to generate any cell of the body. Extra-embryonic tissues (i.e. the placenta), however can only be derived from the zygote; the one true totipotent cell.

Through explanting the ICM of mice, a number of groups created the first ESCs in 1981\textsuperscript{[6, 7]} and since the advent of human ESCs in 1998\textsuperscript{[8]} research in the field has advanced at a great pace. ESCs have now been established from a number of species including the rat, hamster, rabbit, dog and chicken, however most ESCs are different to mouse ESCs in that they have flatter morphologies and different growth factor demands\textsuperscript{[9]}. Research has now advanced so that standard protocols for future applications have been developed including maintenance in culture in the absence of animal-derived components\textsuperscript{[10]}. Due to the pluripotency of these cells there is a great interest in their potential for clinical use as they could be employed in regenerative medicine, and also provide models for basic developmental biology. Clinical trials have already taken place which involved replacing lost functionality through ESCs for disorders such as Parkinson’s disease\textsuperscript{[11]} and spinal cord injury\textsuperscript{[12]}. However many limitations for the use of these cells exist. Firstly as they are derived from the blastocyst many ethical issues primarily concerning the creation, treatment, and destruction of human embryos are raised. Secondly as ESCs are not derived from genetically identical sources to the patients, then problems with immuno-compatibility may be common.
1.1.2 Adult Stem Cells

In contrast to ESCs, adult stem cells are discrete cell populations found in specialised niches (controlled microenvironments) specific to the tissue in which they reside. They are often developmentally restricted to the tissues/organs in which they reside such as hematopoietic stem cells (HMSCs), which contribute to the blood system only\textsuperscript{[13]}. Adult stem cells show some advantages over working with ESCs as they are derived from adult tissue and so evade the ethical issues associated with ESCs and can also be patient-specific and thus avoid immuno-rejection concerns.

In the mammalian adult there are a number of mechanisms employed for the normal homeostasis and maintenance of organs and tissues as well as for replacing lost or damaged cells (figure 1.2). Depending on the tissue type, these mechanisms can be highly dependent on adult stem cells, for example high
Chapter 1: Introduction

turnover tissues such as the skin, intestine and blood have well established stem cell sources generating mature cells. Other tissues such as the pancreas and liver have less active stem cell populations, for example within the pancreas, mature cells have been shown to transiently de-differentiate into precursor cells before re-differentiating into mature cells during pancreatitis\(^{[14]}\) and in the liver the primary source of regeneration and repair is primarily through the replication of mature resident cells, the hepatocytes.

Figure 1.2: Pathways involved in tissue regeneration in the mammalian adult. Tissues can regenerate new cell populations upon damage or during normal tissue turnover through several mechanisms. 

A: Transient de-differentiation of resident mature cells into precursor cells followed by re-differentiation to generate new cells. This mechanism has been reported to occur in pancreatitis\(^{[14]}\). 

B: Resident adult stem/progenitor cells can be signalled to expand and differentiate upon damage and injury to the tissue. This method of tissue regeneration is established in high turnover tissues such as the intestine and blood system \(^{[15]}\). 

C: Recovery from injury within a tissue is also possible through expansion of pre-existing differentiated cells. This method is found in the liver where mature hepatocytes are responsible for the majority of repair in response to injury. Figure adapted from Dor and Stanger\(^{[15]}\).
Chapter 1: Introduction

Adult stem cells are known to reside in a microenvironment known as the niche which was first described by Schofield in 1978 as a ‘physiologically-limited environment that supports stem cells’[16]. The niche is thought to be critical for the maintenance of stem cell populations through a number of factors and signals secreted from neighbouring ‘companion’ cells. For example cycling leucine-rich repeat-containing G protein-coupled receptor 5+ (Lgr5+) stem cells found at the bottom of the intestinal crypt are maintained in a microenvironment with high Wnt activity[17], whilst bone morphogenic protein (BMP) signalling is inhibited by production of noggin and gremlin from tissue below the crypts[18] (figure 1.3). It is evident that a large number of adult stem cell populations exist within the mammalian adult and are very important for tissue maintenance and repair, however the availability of stem cells between tissues is highly variable and thus repairing damage to some tissues is a much greater challenge.

Figure 1. 3: The intestinal stem cell niche. Cycling Lgr5+ crypt base columnar stem cells are maintained in a microenvironment of high Wnt signalling surrounded by companion paneth cells. BMP signalling is inhibited by noggin and gremlin produced by submucosa tissues below the crypts. Figure adapted from Li et al[19].
1.1.3 Induced Pluripotent Stem (iPS) Cells

In 2006, a revolutionary study by Takahashi and Yamanaka successfully reprogrammed a somatic cell into a pluripotent cell using a retro-viral transduction technique; these cells were termed induced pluripotent stem (iPS) cells\[^4\]. The cells were reprogrammed by forced over-expression of four genes octamer-4 (OCT4), sex determining region Y-box 2 (SOX2), krüppel-like factor-4 (KLF4) and c-Myc. In a second set of experiments through reselection with master pluripotent factors OCT4 and Nanog, a second generation of iPS cells were formed\[^20\] which demonstrated true pluripotency by contributing to chimera formation. Since the first iPS cells were reported, many groups have replicated the process and they have now even been generated from several patients with genetic disorders\[^21\]; this new technology brings hope for future individualised cell based therapy. Nevertheless much caution is still needed as there have been many reported problems with regards to genome integrity due to the two additional phases where genetic alterations may occur, these are the original somatic differentiation and the reprogramming events\[^22\]. Another risk associated with iPS cells involves the pro-viral integrations of oncogenes particularly c-Myc which has previously been connected to many forms of cancer\[^23\] and chimeras with iPS cells have been shown to be prone to tumour formation\[^24\]. Currently, efforts are focused on finding methods that do not require permanent transgene integration such as via adenovirus (replication incompetent) or piggybac transposon technologies\[^25\]. Although the initial iPS work has been very exciting and promising, much work is needed to deal with the current risks before the research can advance further into the clinic.
1.2 Endodermal origins of the adult liver and pancreas

It is from the endoderm, the innermost of all the three germ layers, in which the liver and pancreas originate along with the epithelial lining of the gut and the respiratory system (figure 1.4). The development of the endoderm into both liver and pancreas is tightly regulated through a number of signalling pathways and transcription factors. It is evident that the liver and pancreas show a close developmental relationship and this may explain the observed transdifferentiation of cell types in adulthood discussed in section 1.6[26].

1.2.1 Liver development

The liver bud arises from the ventral diverticulum of the foregut through endoderm-mesoderm signalling. Fibroblast growth factor (FGF) is secreted from the adjacent cardiac mesoderm and has been shown to be essential in the induction of hepatic gene expression[27]. Albumin, transthyretin and α-fetoprotein are amongst the earliest genes expressed during mammalian hepatic differentiation; the induction of hepatic gene expression through FGF signalling acts specifically through the mitogen- activated protein kinase (MAPK) pathway[28]. Later on in development, the septum transversum develops between the liver primodium and the cardiac mesoderm which acts as a barrier to high FGF levels; where the higher levels of FGF lead to lung development[29]. Elevated FGF levels also suppress the development of the pancreas by activating expression of sonic hedgehog (Shh) which in turn suppresses the pancreatic growth factor pancreatic and duodenal homeobox 1 (Pdx1)[30]. Other signals, including BMP4 have also been shown to be important in liver development and the zinc finger transcription factor GATA-4 is highly expressed in the septum transversum which is regulated by BMP4[31].

After the formation of the liver bud through FGF and BMP4 signalling, further hepatic development has been shown to be dependent on Wnt signalling [5]. The contribution of Wnt signalling in liver development is complex and dependent on the stage of development, however it has been shown to be critical for the initiation of liver bud formation in zebra fish where Wnt22b mutants have inhibited liver bud development[32].
Chapter 1: Introduction

Figure 1.4: The early development of the liver and pancreas from the endodermal germ layer. During gastrulation the inner cell mass of the blastula undergoes rearrangements into the three germ layers, the ectoderm, mesoderm and endoderm. The liver and pancreas are both of endodermal origin with the key molecular mechanisms to drive their development shown. Figure adapted from Katsumoto et al.[9].

Many transcription factors are also essential in liver development; for example Forkhead box proteins, Foxa1 and Foxa2 have been shown to be responsible for establishing the competence of the foregut to respond to hepatic-inducing signals, and double knockout mice showed no liver bud formation[33]. Other transcription factors such as Sox17, Gata4, Gata6 and Hnf1b have also been shown to be essential in liver initiation and development[9].

In the post natal liver, hepatocyte growth factor (HGF) is produced by non-parenchymal cells and is responsible for liver growth through the induction of hepatocyte maturation and proliferation[34],[35].
1.2.2 Pancreas development

The pancreas originates from the duodenum as a large dorsal and a small ventral bud, which expand before fusing to form the pancreas. The earliest pancreatic marker expressed is the transcription factor Pdx1 and lineage tracing experiments have shown that all pancreatic cells are derived from Pdx1 expressing precursor cells. The dorsal bud of the pancreas has been shown to arise in an area where the notochord contacts the gut roof, within this region Shh and Indian hedgehog (Ihh) levels are suppressed, allowing the expression of Pdx1 to drive pancreatic specification. The ventral bud of the pancreas is formed in areas adjacent to the liver bud too distant from the cardiac mesoderm to receive FGF signals that hepatic promote development. Once pancreatic bud formation is initiated there is a continued outgrowth which is dependent on the close proximity of the pancreatic mesenchyme with expressed islet-1 and FGFs. Retinoic acid (RA) is also essential for early pancreatic development along with vascular endothelial growth factor (VEGF), a well-known maturation signal for the dorsal pancreas.

Endocrine precursors from the pancreas express Neurogenin-3 (Neurog3) and all pancreatic endocrine cells have been shown to originate from Neurog3 positive cells. High levels of Neurog3 cause suppression of endocrine development in neighbouring cells through notch signalling and thus lead to exocrine development in the majority of the pancreas. In comparison to endocrine development fewer transcription factors have been found to be involved in acinar development, although pancreas transcription factor 1-alpha (Ptf1a) has been shown to be essential. To date the Wnt signalling pathway is the only reported extrinsic cue with an explicit role in acinar specification, and exocrine development fails early when Wnt signalling is blocked through k/o of the intracellular Wnt signal protein β-catenin.
1.3 The adult pancreas; anatomy and physiology

Figure 1.5: Basic anatomy of the pancreas.

The pancreas is a large, elongated, aciniform organ that is located in the left hypochondrium of the abdomen behind the stomach, between the spleen and duodenum. The right side of the organ termed the head lies in close proximity to the curve of the duodenum whilst the body of the pancreas extends slightly upwards resulting in the tail of the organ being situated close to the spleen. The pancreas consists of two functionally and morphologically distinct cell populations, the exocrine and endocrine cells. The exocrine cells make up around 95-99% of the total pancreas whilst the endocrine cells, which compose the islets of Langerhans, scattered in the exocrine tissue, make up about 1-5% of the pancreas[^40].

The exocrine portion of the pancreas is composed of acinar cells and duct cells that play essential roles in the secretion of digestive enzymes; acinar cells are dedicated to synthesis, storage and the regulated release of approximately 24 enzymes and accessory proteins[^37]. The acini secrete a number of digestive
enzymes including, lipase, amylase, trypsinogen, and phospholipase, which cleave peptides, lipids and large carbohydrates before they are subsequently secreted in a ‘pro-form’ into the intestine through the ductal system. Alongside the secretion of digestive enzymes from the pancreas, the mature duct cells leading from the acini secrete bicarbonate and mucins[41]. The combined product, pancreatic juice, is then transported through a ductal network towards the duodenum where the bicarbonate ions act to neutralise the stomach acid in the duodenum. Due to their role in synthesis, storage and secretion of a large number of enzymes, acinar cells are packed with rough endoplasmic reticulum, have a large Golgi apparatus and mitochondrial compartment[42].

The endocrine compartment of the pancreas is composed of five different hormone secreting cell types which are difficult to distinguish through standard staining techniques, but can be classified by their secretion. The five cell types include the glucagon secreting α-cell, insulin secreting β-cells, somatostatin releasing δ-cells, ghrelin-producing ε-cells and the pancreatic polypeptide secreting PP-cells which all aggregate into the islets of Langerhans; where each islet is made up from a central core of beta cells surrounded by the other endocrine cells[9]. Islets are networked by a rich blood supply as all of the endocrine hormones play roles in the regulation of nutrient metabolism and glucose homeostasis[41, 43].

Insulin, produced by the β-cells, and glucagon, produced by the α-cells of the islets are the main hormones involved in glucose homeostasis. During periods of elevated blood glucose, insulin is secreted from the pancreas in order to increase the uptake of glucose into insulin-responsive tissues such as adipose tissue, muscle and liver. Glucose is exploited by these organs as a source of cellular energy via glycolysis or is stored as glycogen or fat in the liver and muscle cells, and adipose tissue[44]. During periods of low blood glucose, the hormone glucagon is released to raise glucose levels back to normal physiological levels. Glucagon acts principally on the liver where it acts to increase the breakdown of glycogen to glucose in a process known as glycogenolysis, resulting in the release of glucose back into the bloodstream alongside gluconeogenesis which involves the production of glucose from non-carbohydrate carbon substrates such as glucogenic amino acids, glycerol,
lactate and pyruvate\cite{45}. The δ-cells of the islets secrete the inhibitory hormone somatostatin which inhibits the secretion of both glucagon and insulin in addition to suppressing the exocrine secretory action of pancreas.

The failure of the islets of Langerhans to produce sufficient amounts of insulin results in the condition known as diabetes mellitus which leads in the failure to maintain blood glucose within the normal physiological levels. There are two major forms of diabetes mellitus, type I results from the body’s failure to produce insulin, usually due to the loss of β-cells, and type II is insulin resistance characterised by a defective responsiveness of body tissues to insulin. As well as disorders in the endocrine functions, exocrine pancreatic disorders can lead to serious digestive problems such as pancreatitis where the pancreatic juice digests the tissue of the organ resulting in necrosis, inflammation and fibrosis.
1.4 The adult liver; anatomy and physiology

The liver is both the largest internal organ and gland of the body, weighing between 1300-1700g in the adult depending on gender and body size and is situated in the right upper quadrant of the abdominal cavity. The liver is multi-lobed in most mammalian species; in humans the liver is divided into two major lobes, the left and right, which are divided by the falciform ligament. To reduce friction against other organs the liver is encapsulated within a thin double layered membrane termed the visceral peritoneum which folds back on itself to form the falciform ligament.

The liver receives a dual blood supply from the hepatic portal vein and the hepatic artery. The portal vein supplies the liver with approximately 75% of its blood which is drained from the spleen, pancreas and gastrointestinal tract thus the liver receives blood enriched in nutrients and toxins. Arterial blood is supplied to the liver via the hepatic artery, and blood from both sources flow through the liver sinusoids (hepatic capillaries) towards the efferent central
Chapter 1: Introduction

veins. Bile produced by the liver is collected in bile canaliculi (bile capillaries) which merge into bile ducts to allow the collection for storage in the gall bladder or drainage into the duodenum.

1.4.1 Liver structure and functional units

The liver possesses a unique vascular pattern in which afferent and efferent blood vessels are arranged within the parenchymal tissue and are connected by smaller capillary sized vessels known as the liver sinusoids. The homogenous landscape of hepatocytes infiltrated with vascular tissue and bile ducts masks the complex architecture of the liver. The two widely recognised units of the liver are the ‘lobule’ which was first described by Kiernan and the liver ‘acinus’ proposed by Rappaport[46] (figure 1.7).

The liver lobule is described as a hexagonal plate of hepatocytes which is lined by the sinusoidal capillaries radiating towards the efferent central vein. The lobules are perfused with blood in each of the corners known as the portal triads which consists of the portal vein, bile ducts and hepatic arteries; blood flows from the portal vein and hepatic arteries through the sinusoidal capillaries before reaching the central vein. The sinusoids of the liver are lined by specialised endothelial cells which control the flow of materials to and from the space between the hepatocytes and endothelial cells known as the space of Disse[47]. Whilst the portal lobule is descriptive of the afferent blood supply and bile drainage by the vessels of the portal tract, the acinus model emphasises the secretory functions of the liver and is favoured by histo-pathologists as it aids in the explanation of many pathological lesions[46]. The liver acinus is a smaller functional unit than the lobule and in contrast to the lobule, blood entering the acinus is thought to remain within the acinar units with nutrient and oxygen levels falling as the blood flows from zone 1 to zone 3 as its progresses towards the central vein.
Figure 1. 7: Schematic diagrams representing the hepatic lobule (A) and the liver acinus (B). A: Blood enters the lobule through the portal vein and hepatic artery at the portal triad and flows through the sinusoids, with nutrient and oxygen levels falling as the blood flows towards the central vein. Note that blood may flow into other adjacent lobules. B: In contrast to the lobule, blood entering the acinus is believed to remain within the acinar unit with nutrient and oxygen levels falling as it flows through zones 1-3 towards the efferent central vein. Adapted from Wallace et al[46].

Within the liver there is heterogeneous expression of genes along the sinusoid resulting in metabolic zonation along the lobule/acinus. The lobule is often divided into three zones based on gene expression. The periportal zone (zone 1) surrounds the afferent vessels and the portal triad where hepatocytes are first exposed to the nutrient and oxygen rich blood entering from the portal vein and hepatic artery. The intermediate zone (zone 2) lies between the periportal and the centrilobular zone (zone 3). The expression of cytochrome P450 (CYP450) genes is a model example for this heterogeneous expression, where CYP450 expression is highest in hepatocytes surrounding the central vein.
within the centrilobular zone\textsuperscript{[48]}, consequently although hepatocytes within the periportal zone may be first exposed to xenobiotics, it is the centrilobular zone where damage is observed as it is here the specific enzymes needed for the metabolism are expressed. Due to the fact that the majority of toxin exposure is through pro-toxins that require subsequent conversion mediated by specific enzymes, the damage is observed in the zones where the enzyme is expressed\textsuperscript{[46]}.

1.4.2 Functions of the liver

The liver exhibits both endocrine and exocrine properties. Endocrine functions include the secretion of hormones such as the insulin-like growth factors, angiotensinogen and thrombopoietin, and the major exocrine secretion is in the form of bile. Other crucial roles of the liver include drug detoxification, glycogen storage, metabolic control, urea metabolism, regulation of cholesterol and secretion of a number of plasma proteins such as albumin as well as playing a role in digestion and the body’s immune defence.

One of the pivotal functions in intermediary metabolism by the liver is the clearance of xenobiotics which regularly exposes the liver to high levels of toxins and pathogens which can trigger inflammatory responses. Drug metabolism involves the conversion of lipophilic chemical compounds into more readily excreted hydrophilic products through a series of phase I and phase II reactions. Phase I metabolism includes oxidation, reduction, hydrolysis and hydration reactions and often involves the CYP450 family of enzymes in oxidation reactions\textsuperscript{[49]}. In general, CYP450 enzymes use molecular oxygen to monoxygenate xenobiotics, also producing H\textsubscript{2}O\textsuperscript{[50]}. Phase II reactions, also known as conjugation reactions, are usually detoxicating in nature, and involve conjugation of phase I metabolites with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. Due to the high levels of drug metabolism in the liver and the high number of enzymes needed for these roles the smooth endoplasmic reticulum is the most abundant organelle of the hepatocytes\textsuperscript{[51]}.

Another essential function of the liver is the maintenance of blood ammonia levels within narrow limits. Ammonia is the product of amino acid metabolism
and the removal of it from the liver is a tightly regulated process via two pathways. The first is the urea cycle, which is a cycle of biochemical reactions to convert ammonia into urea through five different catalysed steps. The initial two steps of the cycle occur within the mitochondria of the hepatocytes with the remaining three in the cytosol\cite{52}. During liver injury the urea cycle can become disrupted and high levels of ammonia can be detected within the blood. In addition to the urea cycle glutamine synthetase is involved in the metabolism of ammonia by catalysing the condensation of glutamate and ammonia to form glutamine.

The synthesis of bile acids occurs exclusively within the liver and is localised within the centrilobular hepatocytes\cite{53}, where through a series of enzymatic reactions hydrophobic cholesterol is converted into more hydrophilic and lipophilic compounds catalysed by the CYP450 enzyme CYP7A1\cite{54}. Bile acids have also been shown to play numerous other roles and are now recognised as hormones in various metabolic pathways including triglyceride, glucose and energy homeostasis\cite{55}.

1.4.3 Cells of the liver

The liver is composed of at least 8 cell types, the hepatocytes, sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells, pit cells, liver dendritic cells, cholangiocytes and hepatic progenitor cells, all of which are essential for the normal function and turnover of the organ.

**Hepatocytes** are polarised epithelial cells and are the most populous cell found within the liver, they make up around 80% of the livers total mass and the average human liver is predicted to contain around $10^{11}$ hepatocytes\cite{56}. Hepatocytes are responsible for the majority of synthetic and metabolic functions of the liver; due to their numerous metabolic roles they contain a large number of mitochondria, peroxisomes, lysosomes, endoplasmic reticulum and Golgi complexes\cite{57}. As a result of the numerous and crucial roles of hepatocytes they are strategically positioned between two different environments: the blood plasma on the sinusoidal side and the bile on the caninicular side, giving the hepatocyte surface asymmetry and polarisation. Hepatocytes are arranged in cords which are held together by intercellular
adhesion complexes at the apical membranes, these form a permeability barrier between the perisinusoidal space of Dissè and the bile canaliculi. Around 35% of the hepatocyte surface faces the sinusoids and this surface area is greatly enhanced by microvilli which facilitates the transfer of secretions into the blood stream\cite{58}. Around 13% of the hepatocyte surface faces the bile canaliculus which is responsible for the collection of bile acids and bile salts which are then transported through the bile ducts towards the portal triad and subsequently the gall bladder and intestine. Hepatocytes also show a remarkable capacity to proliferate and regenerate the liver upon injury, this remarkable ability is discussed further in section 1.4.5.

**Sinusoidal endothelial cells (SECs)** constitute the sinusoidal wall, also known as the endothelium. SECs play an active and central role in regulating the exchange of macromolecules, solutes and fluid between the blood and surrounding hepatocytes. SECs also play a central role in the delivery of oxygen to the relatively hypoxic environment, and clearance of drugs, macromolecules and antigens. SECs have a morphological phenotype that is unique to mammalian endothelial cells, and are the only endothelial cells with open fenestrae which allow the easy passage of molecules and oxygen to the hepatocyte surface, thus the liver microcirculation is the most porous of all endothelial barriers\cite{47}. As well as fenestrae the high permeability is also reflected by the presence of special transporting systems including numerous vesicles and channels.

**Hepatic stellate cells (HSCs)** reside within the perisinusoidal space (space of Dissè) between the hepatocytes and the sinusoidal endothelial cells. In the normal liver they are described as quiescent and their roles include storage of vitamin A and modulation of the hepatic microcirculation in response to endothelial signalling\cite{59}. Upon liver injury, HSCs transdifferentiate into a myofibroblast like cell or ‘activated’ HSC which are the main contributors of liver fibrosis. Activated HSCs (myofibroblasts) lose vitamin A droplets and begin to express alpha-smooth muscle actin (α-sma), an actin isoform expressed in smooth muscle cells only\cite{60}. This transdifferentiation to a myofibroblast-like cell phenotype is triggered by a release of reactive oxygen species (ROS) and cytokines and chemokines such as platelet derived growth factor (PDGF) and
transforming growth factor beta (TGFβ1). Once activated, HSCs are pivotal to collagen synthesis, a major component of scar tissue, and HSCs play a central role in both the development and resolution of the fibrotic response\[61\].

**Kupffer cells** are resident macrophages of the liver located in the sinusoids. Kupffer cells are activated in response to liver injury, leading to the release of a number of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) which promotes collagen synthesis in activated stellate cells (myofibroblasts)\[62\]. Although Kupffer cells have been shown to have a major role in fibrosis they have also been shown to play a role in the disease reversal as Kupffer cell depletion inhibits the normal reversal of the disease\[63\].

**Pit cells** are the liver specific natural killer (NK) cells and belong to the group of sinusoidal cells together with SECs and Kupffer cells. They contain specific granules and so are classed as larger granular lymphocytes and are dependent on Kupffer cells\[64\].

**Liver dendritic cells (LDCs)** are the antigen presenting cells of the liver, although the phenotype and function of liver dendritic cells is still poorly understood. LDCs are immature and tolerogenic in normal liver, however during chronic liver injuries a proinflammatory population prevails\[65\].

**Cholangiocytes (biliary epithelial cells)** are found lining the intrahepatic bile ducts which are a complex network that function to deliver bile to the gall bladder and intestine. The cells play a role in the formation of bile which involves a number of secretory and absorptive processes contributing to the final composition of bile\[54\]. Cholangiocytes also play a role in inflammatory responses and interact with other liver cell types through the release of a number of growth factors, peptides and pro-inflammatory and chemotactic cytokines\[66\].

**Hepatic progenitor cells/oval cells** are a population of stem cells that reside within the canals of Hering within the terminal bile ductules\[67\]. This population of stem cells is thought to act as a second line of defence when the resident hepatocytes are no longer able to proliferate and aid in liver regeneration, these cells are discussed further in section 1.4.6.
1.4.4 Liver disease and current therapies

A wide range of insults injure the liver leading to inflammation and fibrosis including alcohol, viruses, poor diet and autoimmune diseases. Irrespective of the cause, liver damage regularly results in a wound healing response characterised by an accumulation of extra-cellular matrix (ECM) proteins. The build-up of scar tissue encapsulating the area of injury inhibits the normal functions of the liver and the gradual accumulation of scar tissue leads to cirrhosis and eventual hepatic failure.

During liver injury, hepatocytes, the functional cells of the liver, are targeted by hepatotoxins and undergo both necrosis and apoptosis. Signals released from dying cells and intracellular fragments lead to Kupffer cell activation in areas of damage, thus resulting in activation of both resident and circulating leucocytes by the release of cytokines and chemokines. The liver architecture is drastically altered in response to injury such as the loss of microvilli on hepatocyte surfaces and loss of fenestrae in the sinusoidal endothelial lining of the sinusoids which all result in impaired function of the liver (figure 1.8). In addition, the accumulation of ECM proteins, synthesised by myofibroblasts, leads to a distorted hepatic architecture and vascular structure by the formation of fibrous scar tissue, eventually leading to cirrhosis and liver failure.
Figure 1.8: Fibrosis during liver injury. **A:** The normal cellular architecture of a healthy liver sinusoid. The microvilli of hepatocytes are important for their normal cellular function to increase surface area for uptake. Hepatocytes are separated from the sinusoidal endothelium by the space of Dissè where quiescent hepatic stellate cells (HSCs) reside, fenestrae of the endothelial cells permits the passage of large macromolecules towards the hepatocytes. Tissue macrophages, Kupffer cells, are found within the sinusoidal space. **B:** During liver injury a fibrotic response is seen where HSCs lose their vitamin A and become activated where they are the primary source of ECM, leading to an accumulation of scar matrix. This leads to a widening of the space of Dissè and loss of endothelial fenestrae, consequently reducing transport across the sinusoidal wall. Activation of Kupffer cells contributes to activation of HSCs. Figure adapted from Hui and Friedman[68].
Chapter 1: Introduction

The process of fibrosis is reversible upon the removal of the primary injury, where the liver has the great capacity for regeneration to repair any damage. In the rat model of fibrosis caused by treatment with carbon tetrachloride the hepatic architecture appeared to be normal within weeks after cessation of carbon tetrachloride dosing\[^{70}\]. Reversal of fibrosis in animal models is often associated with the apoptosis of HSCs, and the stimulation of HSC apoptosis using a fungal toxin (gliotoxin) resulted in rapid recovery from fibrosis, thus HSC apoptosis may be an effective therapeutic approach to liver injury\[^{71}\].

There are currently no therapeutics indicated for the treatment of fibrosis although a number of treatments are in clinical trials. At the cirrhotic level of organ damage the liver loses the ability to regenerate and the only option at present is organ transplantation. The UK has one of the lowest organ donation rates in Europe with only 24% of the population registered as donors, thus a lack of organs limits the number of transplants available for those suffering from liver damage/disease\[^{72}\]. Currently there are numerous studies dedicated to finding new therapies for the treatment of liver diseases. Drugs targeting specific cells such as HSCs have been proposed however many of the proposed targets are expressed on other cells and so problems of drug specificity arise\[^{46}\]. Multiple candidate cells have been explored for use in regenerative medicine where the key goal would be to repopulate the damaged liver with progenitor cells\[^{73}\]. As well as cell transplantation, extracorporeal liver devices containing hepatocytes or stem cell-derived hepatocytes could be used to bridge the gap between liver failure and organ transplantation\[^{74}\].

1.4.5 Regenerative capacity of the liver

Regeneration involves re-establishing the normal form, function and size of an organ that has been impaired or lost through damage\[^{15}\]. Within the mammalian system, regeneration often requires a pre-existing scaffold allowing healthy regrowth after diffuse injury; however this cannot take place after amputation in organs such as the skin, intestine and pancreas. The liver is exceptional to this and has the unique capacity to regenerate and re-grow after removal of up to two thirds of the liver, where the remaining hepatocytes are able to proliferate and reconstitute the organ\[^{75}\]. Depending on the form of injury to the liver, there
Chapter 1: Introduction

are two different physiological forms of regeneration. In the majority of injuries such as exposure to hepatotoxins, resections or viral diseases, hepatocytes undergo replication to regenerate the liver. Following severe liver injury hepatocytes reach cellular senescence and can no longer proliferate and contribute to the regeneration of the liver, thus a second line of defence against liver failure is employed. A reserve population of liver stem cells which reside within a quiescent compartment of the liver can contribute to re-growth and maintenance of the organ [75, 76].

1.4.6 Stem cells in the liver

The concept of a progenitor cell population within the liver has been recognised since 1956 where Farber noted the presence of small cells with a high nuclear to cytoplasmic ratio and termed them ‘oval’ cells[77]. Since the observation of oval cells within the rodent, the human equivalent has been identified and were termed hepatic progenitor cells (HPCs); these cells are reported to reside within the terminal bile ductules known as the canals of Hering[67]. Liver progenitor cells have been shown to be bipotent where they can give rise to both hepatocytes and bile duct cells similarly to hepatoblasts during liver development[78].

As the liver has a great capacity to regenerate, the liver progenitor population is thought to act as a reserve rather than an active stem cell population for normal tissue homeostasis. HPCs are required only when the resident hepatocytes are unable to repair and regenerate the liver either due to senescence or cell cycle arrest caused by some hepatotoxins[79]. In animal models of liver injury, oval cell populations have been demonstrated to actively proliferate[77, 80] after treatment with the carcinogens ethionine 2-acetylamino-fluorene, and 3’-methyl-4-dimethylaminoazobenzene. Despite the increasing evidence for liver progenitor cells the extent of their contribution to liver repair and how they are activated to proliferate is still not known. One theory known as the ‘streaming hypothesis’ is that upon activation, the progenitor cells proliferate within the smallest branches of the intrahepatic biliary tract before migrating towards the central vein as progressively differentiated daughter hepatocytes,[81] and this concept is
supported by mitochondrial DNA mutation tracking experiments within normal and diseased human liver[82].

The stem cells niche for HPCs is found within the canals of Hering and here the companion cells of the niche are likely to be mesenchymal cells such as the portal fibroblasts, stellate cells or vascular endothelial cells[83]. In vitro studies have shown evidence that cells co-expressing CD117 and VEGF2 such as angioblasts may be responsible for maintaining HPCs in their native stem cell state whereas stellate cells support their differentiation[84], however there is little in vivo evidence for this process. Identification and isolation of HPC populations has been difficult due to the large variation of markers that have been reportedly expressed within the isolated populations. A number of groups have reported a common human derived progenitor cell population expressing EPCAM, CK19 and CD44[85-87], and all of these cells have been shown to differentiate into mature hepatocytes and bile duct cells in injured livers[83].

The origin of liver progenitor cells has been much debated and early investigations suggested that they may originate from bone marrow stem cells, as early studies demonstrated that bone marrow derived stem cells are capable of regenerating the damaged liver[88]. Oval cells also express a number of markers that are typically associated with hematopoietic stem cells such as CD34 and Thy-1 thus supporting evidence for a bone marrow origin[89]. However more recent work has shown that the previous observations were likely due to fusion with bone marrow derived cells and the weight of evidence now suggests that the contribution of bone marrow stem cells during liver regeneration is insignificant[90]. Another origin for hepatic stem cells proposed is of a mesenchymal lineage due to the expression of both epithelial and mesenchymal markers. Fate mapping experiments have failed to show any evidence of mesenchymal to epithelial transition during liver injury and so this suggested origin is still highly debated[91, 92].
Chapter 1: Introduction

1.5 Alternative cell types for generation of hepatocyte cells

The key goal of stem cell therapy for liver disease is the repopulation of the damaged liver with healthy, functional hepatocytes. Hepatocytes have been utilised in a number of clinical trials, and to date over 20 patients have received hepatocyte transplants\[^93\]. Although modest improvements have been observed in both biochemical and clinical parameters, definitive clinical benefits such as improved patient survival are still lacking\[^93\]. The long term effects of hepatocyte transplant are unclear and the integration of cells into the liver with beneficial outcomes are needed to provide the proof of concept that cellular therapy is a viable alternative to organ transplantation. Although some success has been shown through hepatocyte transplantation, the modality of treatment is limited by the availability of human hepatocytes as any available healthy liver grafts are used for organ transplantation\[^94\]. In addition to a limited supply, hepatocytes are notoriously difficult to maintain in \textit{ex vivo} cultures as they are unable to proliferate and de-differentiate into non hepatic cells. The cells also do not tolerate cryopreservation\[^95\]. These characteristics add further incentive for finding an alternative source of hepatocytes. Currently a number of cell lines are used as alternatives to primary hepatocytes which include the hepatoblastoma derived HepG2 cell line and the human hepatoma derived HepaRG cell line. Although HepaRG cells demonstrate liver-specific functions and overall better performances than many other cell alternatives, they still fail to predict human toxicology accurately\[^96\] and due to their origin are not suitable for use in clinical therapy.

There are multiple candidate cell sources that could be used in place of hepatocytes. Isolation of a functional and proliferating hepatocyte-like cell source could provide a limitless supply of cells for cellular transplantation, culture in bioartificial liver devices, gene therapy, drug testing and insights into the mechanisms of normal liver development (figure 1.9).
Figure 1. 9: The potential uses of stem cell-derived hepatocytes for treatment of liver disease and in vitro toxicity testing. Stem cell-derived hepatocytes could be isolated and expanded from a number of sources including embryonic stem cells, adult stem cells and reprogrammed iPS cells. The therapeutic potential would be great including use in cell transplants, seeding into bioartificial liver devices and gene therapy. Figure adapted Zhou et al\cite{97}.

### 1.5.1 Oval cells/hepatic progenitor cells

Oval cells or HPCs have been shown to differentiate by inhibiting the replication of resident hepatocytes through chemical injury such as retrorsine\cite{98} and 2-acetylaminofluorene (AFF)\cite{99}, upon which the progenitor cell populations can then be isolated for culture. Isolated HPCs from both rodents and humans have displayed bipotency, both in vitro and in vivo\cite{100}. Using epithelial cell adhesion molecule (EPCAM) and neural cell adhesion molecule (NCAM) as markers, Schmelzer et al, isolated human HPCs which were capable of repopulating the livers in animal models of injury\cite{86, 101}. In addition the success of hepatic differentiation has been shown to be enhanced through 3-D cultures\cite{80} or cultures with hepatic stellate cells\cite{102} which may mimic the stem cell niche or liver micro-environment, thus promoting the hepatic phenotype. HPCs show promise for cellular therapy, however the lack of distinct markers for their isolation coupled with the scarcity of the cells available and their dedifferentiation in culture makes progress challenging.
1.5.2 Hepatoblasts

Hepatoblasts are derived from foetal liver and have the same bipotency as HPCs and are able to generate both hepatocytes and cholangiocytes\textsuperscript{103, 104} and therefore represent another source of hepatocyte-like cells. Bipotent cell lines have been established from both rats\textsuperscript{105} and mice\textsuperscript{106} which are AFP, CK19 and albumin positive. \textit{In vivo} studies have shown that hepatoblasts from both rodents\textsuperscript{107} and humans\textsuperscript{108} are capable of repopulating livers upon transplantation after cryopreservation. Even more promising, 6 months post transplantation in rats, hepatoblasts were shown to make up to 5-10\% of the liver and generate bile structures which became integrated into the host biliary system\textsuperscript{107}. Although hepatoblasts and HPCs show similar potency, hepatoblasts may be more attractive for clinical use as adult-derived cells may have accumulated mutations and shortened telomeres. Yet, this argument has to be counterbalanced by the ethical issues associated with the use of foetal-derived cells along with the limitations of the low cell number and poor expansion \textit{in vitro}.

1.5.3 Embryonic stem cells

ESCs have been demonstrated to differentiate into endodermal lineages by several groups using both \textit{in vitro} and \textit{in vivo} techniques. The first reported differentiation into hepatocyte-like cells from ESCs was in 2002 where Jones \textit{et al}, utilized a liver-specific marker to demonstrate that murine ES cells can differentiate into hepatocytes \textit{in vitro}.\textsuperscript{109} Since 2002 more established and robust procedures have been developed to differentiate ESCs into hepatocyte-like cells, including the formation of embryonic bodies (EB) or monolayer adherent culture systems with subsequent directed differentiation towards the hepatic lineage\textsuperscript{110-114}. ESCs cultured as EBs have been shown to express AFP, transferin, and albumin\textsuperscript{115-117} and this work has been successfully repeated using human ESCS\textsuperscript{118, 119}. Directly differentiating ESCs, thus avoiding the EB stage has proved more successful by producing high yields of hepatocyte-like cells exhibiting improved hepatic function both \textit{in vitro} and \textit{in vivo} \textsuperscript{110}. \textit{In vivo} studies have provided evidence that hepatocyte-like cells derived from ESCs can engraft into the damaged liver of mice\textsuperscript{117} and rats\textsuperscript{120} and improve liver function. Although promising, there are still many concerns
that undifferentiated ESCs transplanted could give rise to teratomas, yet recent studies have shown that selection and removal of undifferentiated cells with oct3/4 expression, reduced teratoma formation\cite{121}. In addition the ethical issues associated with ESCs cannot be escaped and the differentiation procedures remain inefficient with low yields and large cellular heterogeneity.

1.5.4 iPS cells

Since the development of iPS technology, many groups have successfully generated hepatocyte-like cells from somatic cells and have shown high hepatic function\cite{110,122}. Rashid et al have developed hepatocyte-like cells from iPS cells that exhibit disease mutations to model metabolic disorders that affect the liver such as tyrosinemia, glycogen storage disease and alpha-1-antitrypsin deficiency, which have accurately reflected elements of the disease process\cite{123}.

In two separate studies, mouse fibroblast cells were directly converted into hepatocyte-like cells without induction of cellular potency first. This induction was directed by Gata4, HNF1α and foxa3\cite{124} or HNF4α with Foxa1, Foxa2 and Foxa3\cite{125}; cells exhibited hepatic gene expression and function and rescued over 50% of fumarlyacetate-acetate-hydrolase-deficient (Fah-/-) mice after transplantation\cite{124}. Although iPS derived hepatocyte-like cells have been shown to proliferate extensively they are unable to contribute significantly to liver regeneration and carry the threat of tumour formation\cite{126}. Another risk associated with iPS cell therapy is that given their adult origin the cells may contain an epigenetic ‘memory’ of the donor tissue and therefore this may restrict their differentiation potential and utility\cite{127,128}.

1.5.5 Extra-hepatic adult stem cells.

Adult stem cells, from tissues other than the liver, represent a large source of available cells that could be used to generate an unlimited supply of hepatocyte-like cells. There are numerous sources of stem cells throughout the adult body, however the two most accessible and highly available sources are from the bone marrow which contains both hematopoietic stem cells (HMSCs) and mesenchymal stem cells (MSCs).
Chapter 1: Introduction

Several reports have shown that HMSCs can be transplanted and contribute to partial correction of hepatic function\(^{129}\). There has been much deliberation over the contribution of HMSCs as many reports have shown that the repair process was primarily due to fusion and not transdifferentiation of the bone marrow cells\(^{130}\). Cre-lox studies have shown that it is possible for bone marrow to contribute to the liver however they have minimal contribution in normal physiology\(^{131}\). Phase I clinical trials have now taken place for patients with end stage liver disease and have reported improvements in bilirubin and albumin levels\(^{132-134}\), yet no overall survival was observed. More recently studies have found evidence to suggest that HMSCs may contribute significantly to the myofibroblast and stellate cell population and thus may accelerate the fibrotic process rather than resolve it\(^{135}\), raising concern for HMSCs use in liver therapy.

MSCs are multipotent and are capable of mesodermal, neuro-ectodermal and endodermal differentiation depending on the surrounding environment\(^{136}\). MSCs are derived from a number of sources including adipose tissue, umbilical cord blood and the bone marrow, and MSCs from all of these sources have been successfully differentiated into hepatocyte-like cells and integrated into animal models of injury\(^{137, 138}\). Similar to HMSCs, MSCs have been successfully used in patients with end stage liver disease in phase I and II clinical trials\(^{139-141}\), where treatment was reportedly well tolerated and hepatic function was shown to improve during the follow up\(^{139}\). However long term hepatic function has not been rescued and particular diseases such as hepatitis B show no improvements suggesting that MSCs may not be applicable for the treatment for all forms of liver disease.

The use of extra hepatic-derived hepatocyte-like cells is currently very promising with clinical trials undergoing, still the need for safety and clinical improvements was highlighted when a phase I clinical trial was prematurely stopped due to a patient developing radiocontrast nephropathy and liver failure following bone marrow transplantations\(^{142}\).
1.6 Transdifferentiation of the pancreas to liver

The phenomenon of metaplasia is essentially the reversible transformation of all cell types and occurs almost always in tissues that have been subjected to traumas such as infection. Many examples of metaplasia have been recognised for several decades and in the 1980s Slack proposed a theory that metaplasia involves a combination of changes in expression of regulatory genes\textsuperscript{143}. With advances in molecular biology in recent times, the mechanisms been have investigated and now several examples are well understood such as transdetermination in \textit{Drosophila melanogaster}, intestinal metaplasia, B-lymphocyte into macrophage and exocrine pancreas to hepatocytes\textsuperscript{144}. Transdifferentiation is a form of metaplasia that involves the switching of one differentiated cell type directly into another without undergoing an intermediate pluripotent state \textsuperscript{145}. Transdifferentiation was first described by Selman and Kafatos\textsuperscript{146} who observed a switch in cell type during metamorphosis in the silk moth.

The transdifferentiation of exocrine pancreas to hepatocytes has been frequently observed where foci of hepatocytes appear within the pancreas during regeneration following toxic damage\textsuperscript{147, 148}. The ability to switch cell types between the two tissues likely reflects the close developmental relationship\textsuperscript{143}, thus changes in the expression of only a few or just one transcription factor may be required to promote transdifferentiation\textsuperscript{149}. One of the first experiments that showed the potential for pancreatic to hepatic transdifferentiation was carried out by Scarpelli and Rao (1981)\textsuperscript{147} during induction of pancreatic regeneration in the hamster. Hepatocyte-like cells were observed within the pancreas that expressed the hepatocyte marker albumin and showed a variety of hepatic morphological features such as well developed bile canaliculi. Since these findings, a number of methods have been established to induce hepatocytes within the pancreas \textit{in vivo} which include treatment with peroxisome proliferators\textsuperscript{150}, treatment with carcinogens\textsuperscript{151, 152} and transplantation of epithelial cells into the liver\textsuperscript{153}. To date the most studied \textit{in vivo} model of pancreatic to hepatic transdifferentiation is through copper depletion in rats. This model is highly reproducible and results in an almost complete loss of acinar cells after copper depletion, followed by the
Chapter 1: Introduction

development of numerous hepatocytes during the recovery phase; in some studies over 60% of the pancreatic volume has been shown to be hepatocytes\(^{[154]}\). Pancreatic hepatocytes from copper-depleted rats are reported to express several hepatocyte transcription factors such as HNF-1, HNF3\(\alpha\), HNF3\(\beta\), HNF4, and C/EBP\(\alpha,\beta\) and \(\gamma\)^{[155, 156]} which suggests that they may be involved in the mechanisms of transdifferentiation. Some of these transcriptions factors are also expressed within the normal pancreas and thus may not be important in transdifferentiation, however C/EBP-\(\beta\) is not found in the normal pancreas and has been shown to be essential in transdifferentiation in \textit{in vitro} models\(^{[157]}\). Furthermore, in support of rodent studies, the appearance of pancreatic hepatocytes has also been observed in humans with pancreatic tumours\(^{[158]}\).

A number of cellular origins have been suggested for the pancreatic hepatocytes, including exocrine acinar cells, endocrine cells or ductular cells, of which the most widely accepted origin for pancreatic hepatocytes is an exocrine origin\(^{[159]}\). An acinar cell origin is supported by observations where the forced expression of the pancreatic specific transcription factor Pdx1\(^{[160]}\) resulted in hepatic to exocrine pancreas cell switching.

The transdifferentiation of pancreas to hepatocytes shows potential as another source of hepatocyte cells. As well as a wide range of \textit{in vivo} studies, a cell line known as the AR42J-B-13 (B-13) cell line has provided an invaluable cell model for studying the transdifferentiation of pancreas to hepatocytes \textit{in vitro}.

\textbf{1.6.1 The AR42J-B-13 (B-13) cell line}

The AR42J-B-13 (B-13) cell line is a pancreatic acinar cell line that is able to transdifferentiate into hepatocyte-like cells, termed B-13/H cells, upon treatment with glucocorticoids such as dexamethasone (DEX). The B-13 cell line was originally derived from the AR42J parent cell line which was generated by administering azaserine to Wistar/Lewis rats in 1979\(^{[161]}\). Here pancreatic adenocarcinomas were minced before re-transplantation into the same strain of rats. The cells isolated from the tumours were then seeded into culture vessels and one cell line, the AR42J cell line, showed high expression of exocrine
enzymes such as amylase. The B-13 cell line was subsequently cloned by the Kojima lab when they observed that some cells were able to convert into insulin producing cells after culture with Activin A and beta-cellulin\cite{162}. The first observations of B-13 transdifferentiation into hepatocyte-like cells was reported in 2000 by Shen et al\cite{157}.

The B-13 cell is of exocrine pancreas origin which supports in vivo studies showing that pancreatic hepatocytes originate from acinar cells. Furthermore following a combined treatment of DEX and EGF some B-13 cells have been reported to have duct cell-like features\cite{163}. Thus the B-13 cell shows similar potency to hepatoblasts and may even be less developmentally restricted as they also have pancreatic potential and so may possess an endodermal progenitor phenotype.

Transdifferentiation of B-13 cells into B-13/H cells can be promoted under relatively simple culture conditions requiring no cocktails of growth factors or monolayers which makes them an ideal in vitro model. Upon treatment with DEX, B-13 cells stop proliferating and alter their morphology to resemble hepatocyte-like cells with a high cytoplasm to nuclear ratio (figure 1.10). The expression of a number of hepatocyte-specific genes can be detected within ~3 days of DEX treatment including transferrin, UGT and CYP2E1, whilst albumin expression is not observed until later, at around day 9, showing the full transdifferentiation event takes time\cite{164}. Co-localisation of amylase and albumin following the conversion into B-13/H cells has suggested the change to be a true transdifferentiation event which has been further supported by cell lineage experiments. The acinar cell-specific elastase promoter was used to drive green fluorescent protein (GFP) expression in B-13s and after treatment with DEX it was possible to detect GFP and liver-specific proteins within the same cells, showing that the B-13/H cells were originally derived from exocrine pancreatic cells\cite{157}.

A number of liver-enriched transcription factors have been associated with conversion to B-13/H cells, however the key component identified thus far is CCAAT/enhancer-binding protein beta (C/EBPβ) which has also been identified
as playing an important role in transdifferentiation \textit{in vivo} \cite{106}. The increase in C/EBPβ expression is detectable after 4-5 weeks of copper depletion which is just before the appearance of hepatocytes suggesting a critical early role \cite{159}. To verify the action of C/EBPβ, transfection of the dominant negative form into the B-13 cells was carried out; these experiments resulted in a failure of the B-13 cells transdifferentiating upon DEX treatment which highlights the vital role of C/EBPβ induction to drive the hepatocyte phenotype \cite{157}.

The role of Wnt signalling in transdifferentiation has also been examined as nuclear receptors such as glucocorticoid receptors are known to cross-talk with the Wnt signalling pathway \cite{165}. During hepatic development the protein Wnt3A is critical and this protein was shown to be highly expressed by B-13 cells \cite{166}. Wallace et al \cite{167} showed that Wnt3A expression was repressed following DEX treatment of B-13s, leading to a rise in β-catenin phosphorylation and thus reductions in β-catenin localisation to the nucleus and Tcf/Lef transcriptional activity. The roles of Wnt signalling in this process were further confirmed when a small molecule Wnt agonist was shown to block glucocorticoid dependent transdifferentiation \cite{167}. Furthermore Wnt signalling was shown to play an upstream role to C/EBPβ as knockdown of β-catenin expression resulted in the induction of the transcription factor and conversion to B-13/H cells.

Elucidating the basic mechanisms that allow the B-13 cell to transdifferentiate is of key importance with the hope of finding a human equivalent which provides the basis of this thesis.

\textbf{Figure 1. 10: The typical morphology of B-13 cells before and after DEX treatment.} B-13 cells have a high nucleus to cytoplasm ratio and have rounded morphology. After DEX treatment B-13/H cells show typical hepatocyte morphology with a large cytoplasm to nuclear ratio. Figure taken from Wallace et al \cite{168}.
1.6.2 Wnt signalling in transdifferentiation of pancreas to liver

Wnt signalling was first identified in *Drosophila melanogaster* and is a highly conserved pathway found in all phyla across the animal kingdom\[169\]. Wnt signalling regulates cell fate decisions, has critical roles in creating normal body patterns during embryonic development, and is also important in the maintenance of tissue homeostasis in the adult. In addition Wnt signalling interacts with many other pathways during development; the Wnt/β-catenin cascade integrates signals from pathways including retinoic acid, FGF, TGF-β, and BMP in a range of cell-types and tissues. Wnts are a family of secreted glycoproteins which bind their receptors at the cell membrane leading to intracellular Wnt signalling. The signals are transduced via two distinct pathways termed the canonical pathway and non-canonical pathway. The canonical pathway is centred around regulating levels of intracellular β-catenin, a transcriptional co-regulator\[170\], and it is this pathway that shall be focused on as it has been recognised to play a role in transdifferentiation of B-13 cells\[167\].

In the absence of Wnt ligands, β-catenin is sequestered within the cytoplasm by the axin destruction complex composed of axin, adenomatous polyposis coli (APC), and GSK3β. CK1 and GSK3β phosphorylate β-catenin targeting it for ubiquitination and proteasomal degradation\[171\]; resulting in no free cytoplasmic β-catenin to reach the cell nucleus and drive expression of transcription factors through Tcf/Lef signalling. In the presence of Wnt ligands, Wnts bind to the transmembrane receptor, frizzled, and the frizzled co-receptor low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) where they form a Wnt-frizzled-LRP5/6 complex. The complex is able to recruit the scaffolding protein dishevelled (Dsh), which is a phosphoprotein and leads to the phosphorylation of LRP5/6. Next the axin degradation complex is recruited displacing GSK3β preventing the binding and subsequent phosphorylation of β-catenin. Consequently in the presence of a Wnt ligand, levels of β-catenin stabilise and accumulate within the cytoplasm where it can translocate via Rac-dependent mechanisms to the nucleus where it forms a complex with Tcf/Lef transcription factors and activates target genes for expression (figure 1.11).
Chapter 1: Introduction

Wnt signalling is known to be involved in both pancreatic and hepatic development, but in addition Wnts have been found to be essential in many aspects of the postnatal liver homeostasis\(^\text{[172]}\). Several Wnt ligands have been shown to be expressed by various liver cells including hepatocytes, Kupffer cells, stellate cells, SECs and BECs suggesting potential cross-talks between different cell types of the liver\(^\text{[173]}\). Wnt signalling plays roles in many of the key functions of the liver including ammonia and nitrogen metabolism, bile acid homeostasis and drug detoxification. Hepatic zonation has been shown to be dependent on Wnt signalling, as mice lacking APC expression exhibited extensions of the normal perivenous zone of glutamine synthetase (GS) expression towards the portal region\(^\text{[174]}\). In addition to normal liver homeostasis, Wnt signalling has been associated with progenitor-mediated liver

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**Figure 1.11: The canonical Wnt signalling pathway.** In the absence of a Wnt ligand, β-catenin is held in the cytoplasm by the axin destruction complex. GSK3β and CK1 phosphorylate β-catenin targeting it for ubiquitination and proteasomal degradation. In the presence of a Wnt ligand, Wnts bind to the transmembrane receptor frizzled where they form a Wnt-frizzled-LRP5/6 complex which is able to recruit dishevelled (Dsh) followed by the axin destruction complex displacing GSK3β. β-catenin then stabilises and accumulates within the cytoplasm before translocating to the nucleus and activating gene expression of target genes.
regeneration, where a role has been observed both experimentally and clinically\textsuperscript{[175]}. These observations are further supported \textit{in vivo} where a loss of β-catenin resulted in the delayed liver regeneration in partial heptectomy (PH) rats, and expression of a stable β-catenin resulted in accelerated recovery\textsuperscript{[176]}. Within the pancreas, canonical Wnt signalling has been shown to be critical for the development of the exocrine portion of the pancreas but not for the endocrine cell population\textsuperscript{[39, 177]}. Mice lacking β-catenin showed a loss of early exocrine cell differentiation and loss of exocrine specific markers such as amylase.

It has been shown that the transdifferentiation into B-13/H like cells is dependent of a transient repression of Wnt signalling which is actively repressed upstream of C/EBPβ\textsuperscript{[167]}. When Wnt agonists were used the glucocorticoid mediated transdifferentiation was inhibited and a si-RNA mediated knockdown of β-catenin could substitute glucocorticoid effects and lead to the B-13/H phenotype. Furthermore, Wnt signalling was shown to be upstream of C/EBPβ induction as knockdown of β-catenin using siRNA resulted in the induction of C/EBPβ. Once the transdifferentiation events have taken place Wnt expression is regained, showing that only a transient repression is needed for a conversion from pancreas to liver, before Wnt signalling is restored, possibly for normal hepatic function.

As discussed previously the Wnt signalling pathway has been shown to interact with many other cell signalling pathways and some nuclear receptors, including the glucocorticoid receptors, have been show to interact with components of the Wnt signalling pathway\textsuperscript{[165]}. Therefore the role of glucocorticoid signalling and its interaction with the Wnt signalling pathway are of interest with regards to the transdifferentiation of pancreas to liver, especially within the B-13 model system which is dependent on glucocorticoid treatment.

\textbf{1.6.3 Glucocorticoids in transdifferentiation of pancreas to liver}

Glucocorticoids are a class of steroid hormones that are able to bind to and modulate the transcriptional function of nuclear receptors such as the glucocorticoid (GR) and mineralcorticoid (MR) receptors\textsuperscript{[178]}. Glucocorticoids
are primarily secreted from the adrenal gland and play many roles including regulation of intermediary metabolism such as gluconeogenesis in the liver\textsuperscript{179}. During development, glucocorticoid levels are maintained at low levels by placental 11-beta hydroxysteroid dehydrogenase type 2, and at high levels they have potent effects upon tissue development, most notably in the lung\textsuperscript{180-182}.

The transdifferentiation of B-13 to B-13/H cells has been shown to be a glucocorticoid-dependent mechanism, and other classes of steroids do not result in a hepatocyte phenotype\textsuperscript{183}. In addition the response to DEX has been shown to act through the GR and not the MR\textsuperscript{184}. B-13 cells have been reported to express high levels of GR mRNA in contrast to low levels of MR mRNA and when treated with the GR antagonist, mifepristone, transdifferentiation was prevented\textsuperscript{184}, however treatment with the MR antagonist, spironolactone, had no effect. Further analysis at the mRNA level by microarray has shown a marked increase in the glucocorticoids regulated kinase, Serine/threonine-protein kinase 1 (SGK1), mRNA transcripts in B-13/H cells, which are barely detectable in B-13 cells. Inhibition of SGK1 through transfection with siRNA, resulted in a loss of the transdifferentiation response suggesting that the conversion into B-13/H cells is dependent on a SGK1 induction after glucocorticoid interactions with the GR. B-13 cells were transfected with human SGK1 isoforms to further confirm a role of SGK1 in B-13 to B-13/H transdifferentiation. Expression of either C or F isoforms significantly inhibited Wnt signalling resulting in Tcf/Lef transcriptional activity at levels similar to DEX treated B-13 cells. Mutant versions of the isoforms were also transfected that lacked kinase activity and resulted in no altered phenotype, demonstrating that the kinase function of SGK1 is essential for promoting the transdifferentiation events.

Observations have shown an importance for both glucocorticoid and Wnt signalling pathways in the conversion of pancreatic to hepatocyte phenotypes in the B-13 cell line after treatment with glucocorticoids. Nuclear receptors such as GR are able to crosstalk with the Wnt signalling pathway and are able to interact directly with components of the Wnt cascade\textsuperscript{165}. SGK1 has also been shown to phosphorylate β-catenin \textit{in vitro} providing a potential mechanisms of
crosstalk between the two pathways which both act to direct the transdifferentiation, and one possible route is the direct phosphorylation of \( \beta \)-catenin by SGK1 upon its induction.

![Diagram of Wnt and glucocorticoid signalling pathways in the induction of B-13 to B-13/H transdifferentiation](image)

**Figure 1.12:** Crosstalk between the Wnt and glucocorticoid signalling pathways in the induction of B-13 to B-13/H transdifferentiation. Glucocorticoid signals such as DEX interact with the GR and lead to an induction of SGK1 expression. In turn the SGK1 acts to phosphorylate \( \beta \)-catenin through a kinase dependent mechanism targeting it for proteosomal degradation. \( \beta \)-catenin is then unable to translocate to the nucleus and activate Tcf/Lef transcriptional activity allowing instead transcription and expression of hepatocyte specific transcription factors such as CEBP/\( \beta \) which drive the hepatocyte phenotype.

The role of glucocorticoids in the transdifferentiation of pancreas to hepatocytes has been investigated *in vivo* and upon treatment with glucocorticoids pancreatic hepatocytes have been observed in both rodents \([154]\) and humans\([183]\). Treatment of mouse embryonic pancreatic buds with
glucocorticoids was reported to induce the transdifferentiation towards hepatocytes\(^\text{[157]}\) which demonstrates that this effect is not simply an artefact of \textit{in vitro} B-13 studies. Rats treated with DEX for 25 days were found to have expression of hepatocyte markers such as CYP2E1, CPS-1 and albumin within occasional acinar cells of the pancreas\(^\text{[166]}\) and rats showed an increase in liver weight and decrease in pancreas weight. The expression of hepatocyte-specific markers was restricted to single or small clusters of cells providing evidence of pancreatic to hepatocyte transdifferentiation. To further test the effects of glucocorticoids, a transgenic mouse Tg(Crh) that over expresses the rat corticotrophin releasing hormone under the control of the mouse metalothienin-I-promoter\(^\text{[185]}\) was examined\(^\text{[186]}\). Mice with high circulating endogenous levels of glucocorticoids were found to have significantly smaller pancreata than wild type mice and extensive areas of the exocrine pancreas were shown to express hepatocyte-specific markers. Furthermore the observations from these studies were proven to be glucocorticoid dependent as Tg(Crh) adrenolectomised mice failed to develop the appearance of hepatocyte-like cells within the pancreas\(^\text{[186]}\). In addition to the evidence shown in rodent work, pancreas biopsies from patients undergoing long term glucocorticoid treatments have also shown hepatocyte-specific gene expression\(^\text{[183]}\). Together the evidence shows that the response of the B-13 cells to glucocorticoids is a real pathophysiological response in adult cells and thus these observations could have important clinical implications in striving to find an alternative hepatocyte-like cell for the use in treating end stage liver disease.
Chapter 2: Materials and Methods

2.1 Animal work

2.1.1 Ethics

All animal work was carried out adhering to Home Office regulations as outlined in both the project license (PPL 60/3907) and personal license (PIL 60/12598). Protocols were individually designed for each study and the senior animal technician consulted before the study commenced.

2.1.2 Housing

Animals were housed in the Comparative Biology Centre (CBC) at Newcastle University. All animals were cared for as per Home Office regulations and were kept at 20 ± 3°C with a humidity of 50±10 % with 12 hour light/dark cycles.

2.1.3 C57B16 (wild type) mice

Mice were purchased from Harlan and housed in the CBC at Newcastle University. Mouse were bred in house.

2.1.4 Green Fluorescent Protein/ C57B16 mice

The GFP/C57B16 transgenic strain was bred within house at the CBC at Newcastle University. All cell types in this animal express GFP. Animals were housed as described in 2.1.3.

2.1.5 Non-Obese Diabetic Severe Combined immune-deficient (NOD-SCID) mice

NOD-SCID mice were purchased from Harlan and housed within the CBC at Newcastle University. As the mice were immune-deficient, they were housed in a separate area to other animals where all food, water and equipment was autoclaved for sterilisation before animals were exposed. Mice were kept in an air conditioned environment of 20 ± 3°C with a humidity of 50 ± 10% and a 12 hour light/dark cycle. Animals were cared for as per Home Office regulations.
2.2 Cell Culture

2.2.1 AR42J-B-13 (B-13) Cells

B-13 cells were routinely cultured in Dulbecco’s Modified Eagle Media (DMEM) (Sigma D5546) 1000mg/L glucose, supplemented with 10% (v/v) foetal calf serum (FCS), and 80U/ml of penicillin and streptomycin and 2mM L-glutamine in 75cm² nunc culture flasks or 6-well plates. Cells were maintained in a humidified atmosphere at 37°C, 5% CO₂ in air. Cell culture media was changed every 2-3 days.

2.2.2 Induction of a hepatic phenotype in B-13 cells

B-13 cells were cultured as described in 2.2.1 with the addition of 10nM or 10μM DEX (Sigma). 10μM or 10mM stock of DEX in 100% ethanol was diluted 1:1000 into media to give a final working concentration of 10nM or 10μM respectively. After 5 days of culture with DEX the appearance of hepatocyte-like cells was observed within cultures. Fresh media and DEX was given to the cells every 2-3 days. Control cells were grown in parallel with the supplementation of 0.1% EtOH.

2.2.3 Primary human hepatic stellate cells (hHSCs)

Hepatic stellate cells were prepared from human liver samples received after surgical resection. The liver samples were perfused with Hank’s Balanced Salt Solution (HBSS)- 1L contains: 900ml dH₂O, 100ml 10X HBSS w/o Ca²⁺/Mg²⁺ (NaCl, 80g/L; KCl, 4g/L; glucose, 10g/L; KH₂PO₄, 600mg/L; Na₂HPO₄, 475mg/L and phenol red, 170mg/L), 6mls of 1M sterile 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 4.6mls of 7.5%(w/v) NaHCO₃ to remove any remaining blood. The tissue was then cut into smaller fragments and incubated with 40mg pronase, 10mg collagenase B and 1mg of DNasel, suspended in 50ml of (HBSS)+ (HBSS- with 1ml/L of 1M CaCl₂). After cutting up the liver, it was transferred into the enzyme solution and left at 37°C, 220rpm for 30 minutes. Once digested the samples were centrifuged at 400g for 7 minutes before re-suspending the cells in culture medium. Cells were then plated out into 6 well plates and grown in DMEM with 4.5g/L glucose, 110mg/L sodium pyruvate, and 110mg/L sodium bicarbonate, supplemented with 20%
(v/v) FCS, 10mg/L penicillin/streptomycin and 2mM L-glutamine. Cells were incubated in a humidified atmosphere at 37°C and 5% CO₂.

2.2.4 Primary mouse and rat hepatic stellate cells (m/rHSCs)

HSCs were prepared from the livers of mice and rats. After killing by schedule 1 the liver of each rodent was removed and cut into small fragments to ease digestion. 40mg pronase, 10mg collagenase B and 1mg of DNasel were suspended into 50ml of HBSS+. After cutting up the liver it was transferred into 5ml of the enzyme solution and left at 37°C, 220rpm for 30 minutes. Once digested the samples were centrifuged at 400g for 7 minutes before re-suspending the cells in culture medium. Cells were then plated out into 6 well plates and maintained in conditions described in 2.2.3.

2.2.5 Primary mouse and rat hepatocytes

Rat hepatocytes were prepared by collagenase perfusion; in brief, rats were anaesthetised by Intraperitoneal (i.p.) injection with 60mg sodium phenobarbitone per kg of body weight, the animal was then cut open and the internal organs exposed. A cannula was inserted into the hepatic portal vein and held in place using surgical suture. The liver was perfused with Ca²⁺Mg²⁺ free Earl’s Balanced Salt Solution (EBSS) supplemented with 500μM ethylene glycol tetraacetic acid (EGTA) pH 7.4 followed by EBSS⁺. The perfusate was maintained at 37°C and passed through a bubble trap before entering the liver. Once all blood was cleared from the liver it was dissected from the body and further perfused with EBSS⁺ (with 1.8mM Ca²⁺ and 0.8mM Mg²⁺) supplemented with 10mg collagenase A for 20mins or until digested (no longer than 30mins). The liver was dispersed in EBSS⁺ containing 2% bovine serum albumin (BSA) and filtered through 75μm sterile nylon mesh. The homogenate was centrifuged at 50g for 4 minutes at 4°C, the pellet was re-suspended in fresh William’s Medium E (WME) to wash and centrifuged at 50g for 4 minutes at 4°C. Cells were re-suspended in WME supplemented with 10% FCS, 1μg/ml insulin, 80U/ml penicillin and 80μg/ml streptomycin and plated on to collagen type I coated plates (bio-coat). Cells were incubated for 2 hours to allow attachment before the medium was replaced with serum - and insulin-free medium. Cells were routinely maintained in culture for 24 to 48 hours.
Chapter 2: Materials and Methods

2.2.6 HPAC cells

The Human Pancreatic Acinar Cell line (HPACs) were purchased from American Type Culture Collection (ATTC®) Catalogue No. CRL-2119. The cells originate from a pancreatic adenocarcinoma from a 64 year old Caucasian female. The cell line was derived in 1985 from a nude mouse xenograft of the primary tumour[187]. HPAC cells were cultured in the same media used for B-13 culture i.e. DMEM (Sigma D5546) 1000mg/L glucose, supplemented with 10% (v/v) FCS, and 80U/ml of penicillin and 2mM L-glutamine with a media change every 2-3 days. Dexamethasone treatment of the cells was also carried out as described in 2.2.2.

2.2.7 Primary human pancreatic acinar cells

Acinar cell samples were received from Guo Cai Huang at the Department of Diabetes and Endocrinology; Kings College London after islet cells had been prepared for transplantation. The exocrine cell suspension was filtered through sterilised nybolt fabric before centrifugation at 50g for 4 minutes at 4°C. The cells were then re-suspended into DMEM media containing 1000mg/L glucose, supplemented with 10% (v/v) FCS, and 80U/ml of penicillin and 2mM L-glutamine. Cells were cultured in high volumes of media to reduce enzymatic levels in the culture vessels and media was changed daily.

2.2.8 HepG2 Cells

The HepG2 (ATCC® HB8065 ™) cell line is a continuous human hepatoma cell line derived from the liver tissue of a fifteen year old male with differentiated hepatocellular carcinoma[188]. The cell line is a suitable in vitro model for polarised human hepatocytes. HepG2 cells were cultured in DMEM- (Sigma D5546 1000mg/L glucose) supplemented with 10% FCS and 80U/ml of penicillin and streptomycin and 2mM L-glutamine. Media was changed every 2-3 days and cells were passaged when 90% confluent using 2x Trypsin-Ethylendiamine tetraacetic acid (EDTA) (Sigma) as described in section 2.2.9.

2.2.9 Cell passage

Once cultured cells reached a confluency of 80-100% they were passaged. The cell media was aspirated from the cells before washing twice with sterile 1x
phosphate buffered saline (PBS) (8.0g/L NaCl; 0.2g/L KCl; 0.2g/L KH₂PO₄; 1.15g/L Na₂HPO₄) pH 7.4. Cells were incubated with 1 x Trypsin-EDTA (Sigma), diluted in 1x PBS until the cells began to detach from the culture vessel. Trypsin was inactivated by adding an equal volume of FCS-containing culture media to the cell suspension (4mls per 75² flask and 0.5ml per well of a 6-well plate). The cell suspension was then transferred to a 50ml Falcon tube and centrifuged at 2000rpm for 5 minutes. Supernatant was discarded and the cell pellet was re-suspended in fresh media. The cells were seeded accordingly for cell maintenance or frozen down for cell storage.

2.2.10 Cell storage and revival

Cell stocks for all cell lines were routinely frozen down and stored long term in liquid nitrogen. Cells were detached and pelleted by centrifugation as previously described (2.2.9), before re-suspending in freezing media (90% FCS 10% Dimethyl sulfoxide (DMSO)), typically 3mls per pellet from a confluent t75 flask. The suspension was then aliquoted out into sterile cryovials (1.5ml/cryovial). Aliquots were placed in an isopropanol filled cooling aid (Mr Frosty™, Nalgene) and left in the -80°C overnight; cells were cooled at a rate of approximately 1°C per hour and then transferred to liquid nitrogen for long term storage.

For revival, cells were removed from liquid nitrogen and rapidly thawed at 37°C, re-suspended in 50ml of fresh warmed media and transferred to a 50ml falcon tube. The cell suspension was then centrifuged for 4 minutes at 600rpm and the supernatant was discarded. The cell pellets were re-suspended in fresh media and transferred to a culture vessel. Cells were allowed to attach overnight before the media was changed after a minimum of 12 hours. Cells were initially seeded into small culture vessels e.g. 6-well plates.

2.2.11 Cell viability and number

Cell viability was assessed by the ability of a cell to exclude trypan blue. Freshly isolated cells were re-suspended in 100μl of fresh media, 100μl of 0.4% trypan blue (Gibco) solution and 200μl of media was added giving a final concentration of 0.1% (w/v) trypan blue. The cell suspension was then placed into a haemocytometer and the determination of non-viable cells was made based on their ability to exclude trypan blue. Total cell number and the number of non-
viable cells in a known volume of cell suspension were calculated and used to determine the percentage viability of the freshly isolated cells along with the total cell number.

2.2.12 Coculture of B-13\textsuperscript{red} cells with GFP activated HSCs (myofibroblasts)

For coculture experiments activated liver HSCs derived from GFP mice were cultured together with B-13\textsuperscript{red} cells (see section 2.3.2) to investigate whether they have any effects on the transdifferentiation into B-13/H cells. 6-well plates were used for the coculture experiments and were initially seeded with the mouse myofibroblasts which were left to reach around 60-70% confluency before B-13\textsuperscript{red} cells were seeded onto the HSC layer. During coculture cells were all treated with standard B-13 media composed of DMEM (Sigma D5546) 1000mg/L glucose, supplemented with 10% (v/v) FCS, and 80U/ml of penicillin and streptomycin and 2mM L-glutamine. Cultures were either treated with 10nM DEX or control 0.1% ethanol for 7 days, before RNA and protein samples were collected for analysis. Cultures of B-13\textsuperscript{red} and GFP myofibroblasts were also cultured separately as controls.

2.2.13 GFP myofibroblast conditioned media and its treatments

To confirm whether or not myofibroblasts are required for the effects observed in coculture or whether a secreted factor was being produced, the media used to culture myofibroblasts was collected and used for B-13 culture. The conditioned media was collected from the cell cultures and centrifuged to remove any cell debris before supplementing with 10% FCS. The media was then added to the B-13 cells which were cultured for 7 days as described in section 2.2.12.

2.3 Cell transfection

2.3.1 Transfection using effectene reagent (QIAGEN)

B-13 cells were transfected as detailed in the manufacturer’s protocol and cultured under conditions described in 2.2.1. After seeding the cells into fresh 75\textsuperscript{2} flasks or 6-well plates the cells were left for 24 hours to adhere to the surface. Effectene (Qiagen, Southampton, UK.) was routinely used for B-13 cell transfections. DNA (plasmid of interest), EC buffer and enhancer were then
mixed as a ratio of 1μg DNA: 150μl EC buffer: 10μl enhancer. The mixture was vortexed to mix, and left to incubate at room temperature for 10 minutes, after which effectene was added at a ratio of 15μl per 1μg of DNA. The solution was left at room temperature for a further 20 minutes before the master mix was added to the flasks/6-well plates in a drop wise manner, along with fresh media and left for 24-48 hours as optimised.

2.3.2 Production of stable transformed cell line

Cells were transfected with the relevant plasmid using effectene as described in 2.3.1 and left for 36 hours, after which cells were washed with 1xPBS. Fresh media supplemented with 250μg/ml of G418 was then given to the cells to start selection for positive cells. Selection was carried out for 1 month to ensure all surviving cells were stably transfected. To confirm cells were DsRed/pCAG2LMKOSimO positive cells were routinely checked under a fluorescent microscope.

2.3.3 TOP/FLOP flash transfection and dual luciferase assay (Promega)

A dual luciferase assay was performed to assess the activity of Wnt signalling in various experimental setups. Dual reporter systems work to increase experimental accuracy where two individual reporter enzymes are simultaneously expressed. The dual luciferase® assay (Promega) utilises the luciferase activities of both firefly (photinus pyralis) and renilla (renilla reniformis, sea pansy) detecting the activities of both sources respectively (figure 2.1). A difference in the structure of the two luciferase enzymes allows the discrimination between their respective bioluminescence. A transfection ratio of no less than 6:1 for experimental to control plasmid DNA vectors was employed as standard, in order to ensure independent genetic expression and suppress any trans-effects between the promoter elements (i.e TOPFLASH and Luciferase containing reporter gene: RL-TK). Both firefly and renilla luciferase follow michaelis-menten kinetics, maximum light output is not achieved until substrates (above Km) and cofactor are present in a large excess.
Chapter 2: Materials and Methods

Figure 2.1: Luciferase reaction.

Transfected cells were washed twice in 1 x PBS before 1 x passive lysis buffer (5 x stock diluted 1:4 with dH2O) was added to each well (500μl/well of 6 well plate, 100μl/well of 24 well plate) and shaken on an orbital shaker for 15 minutes to disrupt the cell membrane and lyse the cells. Luciferase assay reagent (LAR II) was prepared by resuspending the luciferase assay substrate with the luciferase assay buffer. 100μl of LAR II was then pipetted into a polypropylene tube and 100μl of cell lysate was added and mixed. The luciferase activity was measured using a luminometer. Firefly luciferase activity was then quenched using a pre-diluted stock of stop and glo reagent containing the substrate for renilla luciferase. The luciferase activity of the RL-TK was subsequently measured and recorded. For all transfections, the relative luciferase of the experimental plasmid DNA construct (e.g. TOPFLASH) was normalised to RL-TK levels by dividing the firefly measurement by the renilla value. All values were then further normalised to the control cells (FLOPFLASH) and data expressed as a fold change in luciferase activity in response to individual treatments.

2.4 Plasmid DNA constructs

2.4.1 TCF/LEF (TOPFLASH and FLOPFLASH) transcriptional activity reporter gene.

T cell factor (TCF) reporter plasmid (TOPFLASH catalog # 21-170) and control plasmid (FLOPFLASH catalog # 21-169) were purchased from Upstate (Millipore)
Chapter 2: Materials and Methods

Laboratories (New York, USA). The TOPFLASH reporter plasmid contains 2 sets (second set in reverse orientation) of three copies of the wild type TCF binding site upstream of a thymidine kinase (TK) minimal promoter and a luciferase open reading frame. FOPFLASH was used as negative control and contains two full and one incomplete mutated copy of the TCF binding site followed by 3 copies in the reverse orientation upstream of the TK minimal promoter and luciferase open reading frame.

Figure 2.2: Vector map for (TOPFLASH catalog # 21-170). Upstate (Millipore) Laboratories.

2.4.2 pDsRed2-C1

The pDsRed2-C1 vector was purchased from Clontech Laboratories (USA). The vector contains red fluorescent protein derived from Discosma sp. to allow cells transfected with the vector to be detected by fluorescent microscopy excitation/emission maxima: 558/583nm.

Figure 2.3: Vector and restriction map for pDsRed2-C1. (Clontech Laboratories, Inc.)
2.4.3 pCAG2LMKOSimO

The pCAG2LMKOSimO vector was purchased from Addgene (plasmid 20866). The vector contains the four mouse pluripotent transcription factors c-Myc, KLF4, OCT4 and SOX2 along with the ires-mOrange gene to allow the transfected cells to be detected by fluorescent microscopy, excitation and emission maxima are 548 and 562 nm, respectively.

Figure 2.4: Vector map for pCAG2LMKOSimO (Addgene plasmid 20866)\textsuperscript{[189]}

2.4.4 Transformation of TOP10 competent cells

Top10 cells from Invitrogen (Paisley, UK) were transformed with the plasmid of interest to generate DNA. Bacterial cell stocks were stored at -80°C before being thawed on ice prior to use. To each vial 50-100ng of plasmid DNA was added before gently tapping, to mix, and incubating on ice for 30 minutes. Vials were then incubated at 42°C for 30 seconds to heat shock, before being returned to ice. 250µl of S.O.C medium was added to the vials and were shaken at 37°C for 1 hour at 225rpm to allow cells to recover. A sample of the cells (50-200 µl) was spread on LB (Luria broth-10g NaCl, 10g Tryptone, 5g yeast in 1litre dH₂O) agar plates containing the required selection antibiotics, and colonies were left to grow overnight at 37°C. Colonies were selected and grown up for glycerol stocks and miniprep cultures as described in 2.4.4 and 2.4.5.

2.4.5 Storage of DNA plasmids and glycerol stocks

Stocks of plasmid DNA were prepared from overnight cultures before performing mini or maxipreps. 500µl of culture stock was mixed with 500µl of
Chapter 2: Materials and Methods

glycerol before immediately mixing them and placing into -80°C for storage.
Plasmid was produced from the stocks, if needed, by taking a small sample and
adding it to 5ml of LB medium with the correct antibiotic and grown up overnight
at 37°C and shaken at 220rpm.

2.4.6 Miniprep purification of plasmid DNA

The Qiagen miniprep protocol was followed using 1ml of overnight culture (see
2.4.4). In brief the culture was centrifuged at 10,000 rpm for 1 minute and the
supernatant was discarded. The bacterial pellet was then re-suspended in 250μl
of buffer P1 (containing RNase), 250μl of buffer P2 (highly concentrated salt
buffer containing SDS) and mixed before buffer N3 was added and mixed
immediately. The samples were centrifuged at 13,000 rpm for 10 minutes which
resulted in a white pellet. The supernatant was carefully removed and added to
a Qiaprep spin column and centrifuged again for 1 minute. The column was
again centrifuged after discarding the flow through and adding 750μl of buffer
PE to remove excess salt. 50μl of buffer EB was added to the column to elute
the DNA into a sterile eppendorf. The DNA was quantified as described in 2.7.5
and stored at -20°C.

2.4.7 Maxiprep purification of plasmid DNA

Maxipreps work on the same principle as minipreps but on a larger scale.
Overnight culture of 500ml culture flasks was carried out to produce high yields
of the DNA of interest. The Qiagen plasmid plus maxi protocol was followed.
After centrifugation the bacteria pellet was lysed by adding buffer P1, P2 and
after mixing, buffer S3. The lysate was then transferred to a QIAfilter cartridge
and incubated at room temperature for 10 minutes. The sample was filtered
through the cartridge with a plunger into a new tube before the addition of buffer
BB. The lysate was mixed and transferred to a QIagen plasmid plus midi spin
column and the solution was drawn through using a vacuum source. The
column was washed through with 0.7ml buffer ETR followed by buffer PE using
the vacuum source, followed by centrifugation for 1 minute with buffer EB to
elute the DNA into a clean eppendorf. DNA was quantified as described in 2.7.5
and stored at -20°C.
2.5 Cytogenetic analysis of B-13 cells

2.5.1 Preparation of metaphase spreads

For karyotyping, cells were expanded in culture to approximately 75% confluency before metaphase-arrest was induced by adding 0.1 μg ml⁻¹ colchicine in fresh media for 1 hour. The cells were harvested by trypsinisation in EDTA (SigmaChem Co, Poole, UK) and washed with 1× PBS (137 mM NaCl, 2.7 mM of KCl, 10 mM phosphate (pH 7.4)) and pelleted by centrifugation at 400 g for 5 minutes. The cell pellet was resuspended in 0.075 M KCl, added dropwise up to the volume of 10 ml before incubating the solution at 37 °C for 10 minutes. The cells were pelleted by centrifugation and resuspended in 5 ml of ice cold fixative (3:1 methanol–acetic acid) with continuous gentle mixing to avoid clumping. Centrifugation was repeated and the cells were re-suspended in 2 ml of fixative; this step was repeated twice. The cells were incubated at −20 °C overnight, re-suspended in fresh fixative and then dropped onto an angled glass slide under humidified conditions and left to dry in air overnight. The slides were incubated in trypsin solution for 15 seconds before immediate transfer to saline solution (0.9% (w/v) NaCl) followed by two 5 minute washes prior to staining with Wright-Giesma staining solution (Sigma Chem Co, Poole, UK) for 4 minutes. The slides were rinsed in distilled water, blot dried and mounted with coverslips using DPX. Images were taken on a Zeiss Axioskop and analysed using CytoVision®(Leica) software. Cells were karyotyped with the help of cytogenetic specialists Claire Schwab and Christine Harrison

2.6 Soft agar anchorage assay

2.6.1 Culture in soft agar

The soft agar assay for colony formation is considered the most stringent in vitro assay for detecting malignant cell growth[190]. Anchorage-independent cell growth of B-13 cells was measured by assessing colony formation in a soft agar suspension using the human hepatoma HepG2 cell line and primary rat HSCs as positive and negative controls respectively. A base layer of 1% w/v agar was mixed with an equal volume of culture media (see section 2.2.1), resulting in a 0.5% agar base. 1ml of the mixture was put into each well of a 6-well plate and left to set overnight at 4°C. Cells were suspended into 0.7% w/v agar with an
equal volume of cell culture media to give an upper layer of 0.35% agar. 2,500 cells were seeded per well and 0.5ml of culture media was added to the top of each well. Plates were incubated at 37°C, in 5% CO₂ for 21 days, with media changes every 2-3 days. Following the incubation period, each well was stained with 0.5ml of 0.005% w/v crystal violet for one hour at room temperature. Images were taken using BUC2-500c camera (Bestscope International Ltd) on an Optika microscope and colonies were counted.

2.7 RNA and DNA isolation/ quantification

2.7.1 RNA isolation

After washing cells twice with 1xPBS, 1ml of trizol was added to each well of a 6-well plate and cells were removed and transferred to a clean eppendorf tube with the aid of a cell scraper; for tissue samples, the tissue was homogenised in trizol using a sonicator. 200µl of chloroform was added to the tube and mixed by vortexing before centrifuging at 12,000 rpm at 4°C for 15 minutes. The upper aqueous layer formed was transferred to a clean eppendorf along with 500µl of isopropanol, samples were incubated on ice for 10 minutes before centrifuging at 12,000rpm, 4°C for 10 minutes. Supernatant was removed, and the pellet was washed with 70% ethanol and centrifuged at 12,000 rpm, 4°C for 10 minutes. Ethanol was discarded and the pellet was air dried before being re-suspended in an appropriate volume of sterile dH₂O. RNA samples were quantified and stored at -80°C.

2.7.2 Quantification of RNA concentration and integrity

To quantify RNA, samples were diluted 1:200 and absorbance was measured at 260 and 280nm, with 200µl of dH₂O used as a background. RNA concentration was calculated based on 1 absorbance unit at 260nm being equal to 25µg of RNA/ml. Purity of RNA samples was checked by calculating the 260/280 ratio with values close to 2.0 suggesting pure RNA and values lower than 2.0 suggesting DNA contamination.
2.7.3 DNase treatment of RNA

RNA was DNase treated to remove any genomic DNA contamination. RNA samples were diluted in 50µl of dH₂O; 0.1 volume of RQ1 10x DNase I reaction buffer and 0.1 volume of DNase I were added to the samples (e.g. if re-suspended in 50µl H₂O, add 5µl DNase I buffer and 5µl DNase I). The samples were mixed gently and incubated at 37°C for 30 minutes before the addition of 0.1 volume of RQ1 DNase stop solution (equal volume to DNase I). The sample was mixed and incubated at 65°C for 10 minutes to terminate the reaction, samples were stored at -20°C or -80°C.

2.7.4 DNA isolation

After washing cells twice with 1xPBS, 1ml of 1x PBS was added to each well of a 6-well plate and cells were removed and transferred to a clean eppendorf tube. The cells were pelleted before 200µl of genomic DNA preparation buffer (50mM Tris-HCl, 100mM NaCl, 10mM EDTA, 0.5% NP-40, pH8) was added along with 20µl of 20mg/ml proteinase K (Promega V320B in 10Mm Tris-HCl, pH7.5), mixing occasionally. Samples were incubated at 55°C overnight and allowed to cool before 50µg (5µl of a 10mg/ml stock) of RNase A (DNase free) was added and incubated at room temperature for 20 minutes. 200µl of phenol/chloroform/isoamyl alcohol (25/24/1 v/v/v) was added to the mixture before vortexing and pulse centrifugation. The upper aqueous phase (approx 200µl) was carefully removed and 20µl of 3M sodium acetate pH5.2 and 450µl of ice cooled 100% ethanol were added and the sample was incubated on dry ice for 1 hour. The precipitated DNA was centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatant was discarded before washing the pellet with 500µl of 70% ethanol and incubated for 1 hour on dry ice followed by centrifugation at 13,000rpm at 4°C for 10 minutes. The supernatant was again discarded and the DNA pellet was re-suspended in sterile pharmacy grade H₂O.

2.7.5 Quantification of DNA concentration and integrity

To quantify DNA, samples were diluted 1:200 and absorbance was measured at 260 and 280nm, with 200µl of dH₂O used as a background. DNA concentration was calculated based on 1 absorbance unit at 260nm being equal to 20µg of DNA/ml. Purity of DNA samples was checked by calculating the 260/280 ratio.
with values close to 1.8 suggesting pure DNA. Values below suggest protein contamination, whereas values close to 2.0 suggest RNA contamination.

### 2.7.6 Reverse transcription, 1st strand DNA synthesis (cDNA)

Moloney murine leukemia virus (MMLV) reverse transcriptase RNAse H minus point mutant is a RNA dependent DNA polymerase used for the synthesis of complementary DNA (cDNA). The −H mutant lacks RNase activity for the optimal generation of full length cDNA from long RNA templates. MMLV (-H) is thermostable, reducing any problems associated with secondary structure and gives higher yields of cDNA than MMLV. To create cDNA, RNA was diluted to a starting concentration of 200ng/µl. 4µl of RNA (800ng) was incubated with 1µl (50ng/µl) of random primers (Promega) at 95°C for 3 minutes. Samples were placed on ice. Master mix containing (per tube) 4µl of 5x RT buffer (50mM Tris-HCl (pH 8.3 @ 25°C), 75mM KCl,3mM MgCl2 and 10mM DTT), 8µl dH2O, 2µl of 10mM dNTP’s and 1µl of MMLV was prepared on ice and added to each tube (15µl total volume per reaction). The RNA was incubated for 1 hour at 42°C for optimal cDNA synthesis. Samples were stored at -20°C.

### 2.7.7 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) is a widely used molecular technique which allows the exponential amplification of specific DNA sequences from target DNA. PCR relies on thermal cycling which involves a number of cycles of repeated heating and cooling allowing for DNA melting, primer annealing and polymerase enzymatic replication of the DNA templates. Primers are designed specific to target genes of interest (see 2.7.8) allowing selective amplification of the target DNA. During the reaction, the DNA generated from earlier cycles can act as a target itself allowing the DNA to be exponentially amplified. Taq polymerase is the DNA polymerase enzyme that was used in the PCR reaction to amplify short segments of DNA. Taq was originally isolated from the bacterium *Thermus aquaticus* and performs in optimum temperatures of 70-80°C.

For PCR reactions 1µl of cDNA (40ng) was added to each reaction tube along with 20µl of PCR master mix which contained (per tube): 10µl 2x go-Taq green master mix (Taq DNA Polymerase, 1.6mM dNTPs, 3mM MgCl2 and reaction
buffers, 6μl dH₂O, 2μl of 10pmol/μl of up and downstream primers. PCR programs were optimised for each set of primers (table 2.1). The standard PCR program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Repetition</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>1 minute</td>
<td>x1</td>
</tr>
<tr>
<td>95°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>40-64°C</td>
<td>1 minute</td>
<td>x35</td>
</tr>
<tr>
<td>73°C</td>
<td>1.5 minutes</td>
<td></td>
</tr>
<tr>
<td>73°C</td>
<td>8 minutes</td>
<td>x1</td>
</tr>
<tr>
<td>4°C</td>
<td></td>
<td>Hold</td>
</tr>
</tbody>
</table>

Denature step  
Annealing step (dependent on primer design)  
Elongation step

PCR products were then visualised by running them out on an agarose gel, (1-2% dependent on amplicon size), as described in 2.7.9 and viewed under U.V light.

2.7.8 PCR primer design

Primer pairs were routinely designed to amplify specific DNA sequences of interest. DNA sequences were designed using the NCBI database (www.ncbi.nlm.nih.gov). Nucleotide BLAST was used to check the specificity of chosen primer sequences and also to assess the probability of dimer formation. The following guidelines were generally followed: primers should contain 40-60% G and C and have a length of approx 18-30 bases. Primer pairs were not complimentary to one another to avoid the formation of primer dimers. The G and C content of each primer should be similar in order to keep the Tm values within the same region. Tm = 2 C x (A + T) + 4 C x (G + C). An optimal annealing temperature for each set of primer pairs was determined with a starting point being made at 5°C below the Tm.

2.7.9 Agarose gel electrophoresis

Gel electrophoresis was used to separate and identify nucleic acids based on charge migration. Larger nucleic acid fragments migrate slower through the polymerised agarose gel, thus samples can be efficiently separated according to size. A DNA ladder (New England Biolabs® Inc. 100bp N3231; 1kb N3232) was also ran so the fragment size of the samples could be identified. An
electric field was applied across the agarose gel causing the negatively charged DNA molecules to be pulled through the agarose gel matrix and separated according to size. The percentage of agarose used for each gel was dependent upon the size of the nucleic fragment of interest, higher percentage gels were used for smaller fragments. In general 1.5% Agarose w/v powder was diluted in 1 x Tris acetate- Ethylenediaminetetraacetic acid (TAE) pH 8.0. The agarose was dissolved by heating and allowed to cool to ~50°C before 1μl/100ml of ethidium bromide (10mg/mL) was added. Ethidium bromide is a nucleic acid intercalating agent; upon exposure to UV light it fluoresces and allows nucleic acid samples to be visualised. The gel was poured into a cast with an appropriate comb and allowed to set at room temperature. Once set, the gel was placed into the gel tank which was filled with 1 x TAE buffer and samples were loaded into the wells. Agarose gels were run for approximately 45-60 minutes at 90 volts. Gels were visualised under UV light and images were taken.

2.7.10 Real-time PCR (SYBR Green qRT-PCR).

The relative expression of the genes of interest was compared between samples using SYBR Green qRT-PCR for quantitative analysis. qRT-PCR works in a similar manner to standard PCR by amplifying sequences of interest with primers designed for the target, however qRT-PCR also includes the addition of a probe i.e. SYBR Green, which fluoresces once bound to double stranded DNA (dsDNA). After each cycle, the level of fluorescence is determined and used to calculate the amount of dsDNA present, thus allowing the amount of transcript of the target gene to be determined, relative to other samples. Reaction conditions for each set of primers were optimised individually and performed in a 20µl reaction. For each well of a 96-well PCR plate the reaction volumes were: 10µl of 2X SYBR Green Master Mix, both upstream and downstream primers to a final concentration of 250nM and a sufficient volume of nuclease free H₂O to make a master mix, and 10ng of each cDNA sample. Each reaction was repeated in triplicate. The plates were sealed using optical film, vortexed gently and centrifuged at 250g to collect the reaction mixture into the bottom of each well. qRT-PCR reactions were performed in an Applied Biosystems 7500 fast thermocycler. The gene expression was calculated
relative to control groups and normalised against 18S ribosomal RNA to account for any variations in RNA template between samples. Fold change in gene expression was calculated as $2^{(-\Delta \Delta Ct)}$ where $\Delta Ct$ is the change in Ct relative to 18S and experimental control.
## Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>5’-3’ sequence</th>
<th>Annealing conditions (35 cycles)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT-PCR</strong></td>
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</tr>
<tr>
<td>rmCYP2EUS</td>
<td>TCGACTACAATGACAAGAAGTGT CAAGATTTGAGAATCTGTCGCGATCTC</td>
<td>42°C</td>
<td>Will amplify a rat CYP2E (NM_031543) cDNA sequence of 525bp</td>
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<td>rmCYP2EDS</td>
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<tr>
<td>rmhGAPDHUDS</td>
<td>TGACATCAAGAAGTGTGAAGGCTTACTCTTTGGAGCCATGT</td>
<td>50°C</td>
<td>Will amplify rat (NM_017008), human (NM_002046) or mouse (NM_008048) glyceraldehyde 3</td>
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<tr>
<td>rmhGAPDHDS2</td>
<td></td>
<td></td>
<td>phosphate dehydrogenase cDNA sequence of 243bp</td>
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<td>rCPS1US</td>
<td>ATACAACGGCAGTGATAGCACTTAACTAGCGAG</td>
<td>55°C</td>
<td>Will amplify rat CPS (NM_017072.1) cDNA sequence of 390bp</td>
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<tr>
<td>rCPS1DS</td>
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<td></td>
</tr>
<tr>
<td>rmAMYLASEUS</td>
<td>CAAAATGTGTTTCTCCCAAGGA CAAAATGTGTTTCTCCCAAGGA</td>
<td>57°C</td>
<td>Will amplify rat pancreatic amylase 2 (NM_031502.1) cDNA sequence of 224bp</td>
</tr>
<tr>
<td>rmAMYLASEDS</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>rmhVimentinUS</td>
<td>CGATGTGGACGTTTCGCAAGCC ATCCACTTTGAGTGAGTG</td>
<td>58°C</td>
<td>Will amplify rat vimentin sequence (NM_031140.1) cDNA sequence 226bp</td>
</tr>
<tr>
<td>rmhVimentinDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rAlbuminUS</td>
<td>CGTCAGAGGATGAAGTCTC CAGTTAGCGAGCAGCGG</td>
<td>47°C</td>
<td>Will amplify rat albumin (NM_134326) cDNA sequence of 471bp</td>
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<td>rAlbuminDS</td>
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<tr>
<td>rC/EBP8US</td>
<td>ACACGTGTAATCTGACGCGGCGAAGTTCAGGTTCTCA</td>
<td>55°C</td>
<td>Will amplify rat (NM_024125.4) cDNA sequences of 196bp</td>
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<tr>
<td>rC/EBP8DS</td>
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<tr>
<td>rCYP2C11US</td>
<td>CTGCCCAGCAGTCTCTCAGGATTGCTTCCTCCTGCCCTCTCTCCTCCTC</td>
<td>55°C</td>
<td>Will amplify rat (NM_019184.2) cDNA sequence of 88bp</td>
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<tr>
<td>rCYP2C11DS</td>
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<tr>
<td>rCYP4A1US</td>
<td>GAGTGGCTGCCCATGACACGCTGACGCTTACCTGACAGGATTGT</td>
<td>55°C</td>
<td>Will amplify rat CYP4a1 (NM_175837.1) cDNA sequence of 446pb</td>
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<td>rCYP4A1DS</td>
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<tr>
<td>rWIFIUS</td>
<td>GTGTGATCTCCGAAACTCTGTGAGCCGCTGC</td>
<td>42°C</td>
<td>Will amplify rat wnt inhibitory factor 1 (NM_053738.1) cDNA sequence of 105bp</td>
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<tr>
<td>rWIFIUS</td>
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<tr>
<td>mWIFIUS</td>
<td>CTCCCTCCTCTCTCCTCTCTCCTCTCTCCTCTCTCTCTCTCTCTCTCTCTCTCT</td>
<td>62°C</td>
<td>Will amplify mouse Wnt inhibitory factor 1 (NM_011915.2) cDNA sequence of 290bp</td>
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### Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Primer Sequence</th>
<th>Temperature</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>moSmoothUS</td>
<td>ACGTTTGTGGAATCGACGCCTCC</td>
<td>58°C</td>
<td>Will amplify mouse smooth muscle actin (NM_007392.2) cDNA sequence of 251bp</td>
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<tr>
<td>moSmoothDS</td>
<td>TGATGCCATCCATGCTCCGGGAAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rAmylase US</td>
<td>TGTGACTGGCGCAATAGTGCA</td>
<td>62°C</td>
<td>Selectively amplifies rat pancreatic amylase 2 (NM_031502.1) 497bp fragment over mouse pancreatic amylases (NM_001190403.1, NM_001042711.2, NM_001160150.1, NM_001160152.1, NM_001160151.1, XM_003084551.2) – at least 5 and up to 12 nucleotide mismatches between rat and mouse transcripts in primer hybridisation regions</td>
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<tr>
<td>rAmylase DS</td>
<td>CCATTCCATTTGGCAATAACTGTCGCA</td>
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<tr>
<td>rCYP2E1US</td>
<td>CTTAATTTCTCTTGATATC</td>
<td>60°C</td>
<td>Selectively amplify rat Cyp2e1 (NM_031543.1) 86bp fragment over mouse cyp2e1(NM_021282.2) – 13 nucleotide mismatches between rat and mouse transcripts in primer hybridisation regions</td>
</tr>
<tr>
<td>rCYP2E1 DS</td>
<td>TGATAGGAACTCTATATGCTTGGGT</td>
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<td></td>
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<tr>
<td>hPXR US</td>
<td>ACATGGGTGACATFTCAACCTACA</td>
<td>64°C</td>
<td>Will amplify the same size amplicon from all 3 human PXR reference transcripts (NM_003889, NM_022002 and NM_033013) cDNA sequence of 475bp</td>
</tr>
<tr>
<td>hPXR DS</td>
<td>AGCTCAGGTTACATAGCCATGAT</td>
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<td></td>
</tr>
<tr>
<td>hCAR US</td>
<td>AGAACAGTCAGCAAAAGCAT</td>
<td>50°C</td>
<td>Will amplify all 15 human reference transcripts of CAR (NM_001077469 to NM_001077482 and NM_005122) cDNA sequence of 293bp</td>
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<tr>
<td>hCAR DS</td>
<td>TGCACAAAATGTTCAACAT</td>
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<tr>
<td>hFXR US</td>
<td>TGTGTGTGTGTGAGAA</td>
<td>41°C</td>
<td>Will amplify human FXR (NM_005123) cDNA sequence of 701bp</td>
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<td>hFXR DS</td>
<td>ATCAGAGATCCACTATTTC</td>
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<tr>
<td>hGR US</td>
<td>GAAGCTATTTTTAATGTC</td>
<td>42°C</td>
<td>Will amplify all human GR transcripts (NM_000176, NM_001018074 - NM_001018077, NM_001020825 and NM_00102409) cDNA sequence of 607bp</td>
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<tr>
<td>hGR DS</td>
<td>ATCATATCCGATATAAC</td>
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<tr>
<td>hCYP2E1 US</td>
<td>CCCGAGACACCATTTTCAGAG</td>
<td>55°C</td>
<td>Will amplify human CYP2E1 (NM_000773.3) cDNA sequence of 228bp</td>
</tr>
<tr>
<td>hCYP2E1 DS</td>
<td>AGAAACAATCCATGCGGCAAG</td>
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<tr>
<td>hAlbumin US</td>
<td>AGCTCCCTGCTGTGAGCAGAAA</td>
<td>55°C</td>
<td>Will amplify albumin (NM_000477.5) cDNA sequence of 134bp</td>
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<tr>
<td>hAlbumin DS</td>
<td>AGCCAGGTACCTGGCCATGC</td>
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<td></td>
</tr>
<tr>
<td>hAmylase US</td>
<td>ACATGGGGCTGGAGGAGCCT</td>
<td>60°C</td>
<td>Will amplify all human pancreatic amylase transcripts (NM_020978, NM_000699.2) cDNA sequence of 152bp</td>
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<td>hAmylase DS</td>
<td>TGGTCGAGGAACCATGCTTGC</td>
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<tr>
<td>hCYP3A4 US</td>
<td>TGTCCTACCATAAGGCTTGTGTAT</td>
<td>60°C</td>
<td>Will amplify human CYP3A4 (NM_017460) cDNA sequence of 136bp</td>
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<td>hCYP3A4 DS</td>
<td>TGTCAGAAGACAGCTGCTGATGTC</td>
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<tr>
<td>hINS v1 US</td>
<td>GGCACATCAAGGATCACTGTCCT</td>
<td>64°C</td>
<td>Will amplify the human insulin transcript variant 1 (NM_000207.2) cDNA of sequence 377bp</td>
</tr>
<tr>
<td>hINS v1 DS</td>
<td>CCTGGCGGCTGCCTTCTAGTT</td>
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### Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Table 2.1: DNA oligonucleotide sequences employed in RT-PCR or genomic PCR.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hC/EPBβUS</strong></td>
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<tr>
<td><strong>rXChromUS</strong></td>
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<tr>
<td><strong>rYChromUS</strong></td>
</tr>
<tr>
<td><strong>genomCYP2E1US</strong></td>
</tr>
</tbody>
</table>
2.8 Protein isolation and quantification

2.8.1 Preparation of whole tissue samples

Tissue samples were snap frozen upon dissection and stored at -80°C until required. After thawing the tissue samples were homogenised in 1xPBS using a sonicator and pelleted by centrifugation at 13,000rpm. The pellet was re-suspended into a known volume of 20mM Tris pH 7.4. 5µl of each protein sample was taken for determining the concentration by the Lowry method described in 2.8.3. Samples were stored at -80°C.

2.8.2 Preparation of cell extracts

Cells were washed twice with 1xPBS before they were scraped into 1ml (per well of a 6-well plate) and transferred to a clean eppendorf tube. The cells were centrifuged at 13,000 rpm for 10 minutes and the supernatant discarded. The cell pellet was re-suspended in a known volume of 20mM Tris pH 7.4, 5μl of each protein sample was taken for determining the concentration by the Lowry method described in 2.8.3. Samples were stored at -80°C.

2.8.3 Lowry protein assay

To determine the concentration of protein in each sample a Lowry assay was carried out using BSA as a standard. Standard concentrations were prepared which ranged from 0-20mg/ml from a 20mg/ml stock diluted into dH₂O. 5µl of each standard and sample was diluted with 50µl of dH₂O and 1ml of ABC buffer and left at room temperature for 10 minutes. ABC buffer was prepared fresh on the day of use and consisted of Lowry A (2% w/v Na₂CO₃/ 4% w/v NaOH), Lowry B (2% w/v sodium tartrate) and Lowry C (1% w/v copper sulphate) mixed at a ratio of 100:1:1 (v:v:v). After incubation for 10 minutes at room temperature 100µl of Folins reagent (Fluca, Switzerland), diluted 1:1 with dH₂O was added to each sample. Further incubation for 30 minutes occurred before absorbance at 750nm was determined for each sample by a spectrophotometer.

The absorbance of the standards was used to produce a calibration curve from which an equation for the line of best fit was derived. This equation was re-arranged to give concentration values for the unknown samples which were
then made up to stocks of known concentration as described in section 2.8.5.

2.8.4 Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method commonly used to allow proteins to be separated by their molecular weight, and was first described in 1970 by Laemmli\[191\]. Proteins are first denatured by heating them in the presence of a reducing agent, Dithiothreitol (DTT), which breaks any disulphide bonds in the tertiary structure. The proteins are then coated in negatively charged SDS which linearises them, allowing the proteins to migrate towards a positive electrode through the gel matrix. Smaller proteins will travel further, thus separating them according to their size.

For SDS-PAGE, gels were cast between two glass plates held together with clamps and sealed at the bottom. Separating gels (9%) were made by mixing, 9% bis-acrylamide, 375mM Tris buffer pH 8.8, 0.1% w/v SDS, 0.05% w/v ammonium persulphate, and 0.05% v/v Tetramethylethylenediamine (TEMED). After pouring the separating gel 100μl of isopropanol was layered on top to eliminate air bubbles and to produce a straight edge between the separating and stacking gel. Once set, the isopropanol was washed off before the 4% stacking gel was cast. The stacking gel was composed of 4% w/v acrylamide, 125mM Tris buffer pH 6.8, 0.1% w/v SDS, 0.05% w/v ammonium persulphate and 0.1% v/v TEMED. Combs were placed into the gel casts to create wells for the protein samples to be loaded into. Once the gels had set the wells were rinsed with dH\textsubscript{2}O before placing the gels into a gel tank and filled with electrode running buffer (ERB) comprising of 20mM Tris, 160mM glycine and 0.08% w/v SDS, pH 8.3.

2.8.5 Sample preparation and electrophoresis

Before carrying out gel electrophoresis the protein samples were diluted to 1-2μg/μl in reducing loading buffer (62.5mM Tris buffer pH 6.8, 10% v/v glycerol, 2% w/v SDS, 100mM DTT and 0.02% w/v bromophenol blue). The protein samples were denatured by heating to 90°C for 3 minutes. Once denatured, samples were loaded into the wells of the gel (10-30μg per well). The gels were
run at 100V until the samples passed into the separating gel when the voltage was increased to 150V. Once the samples had passed through the entire length of the gel, the protein was electrotransfered onto nitrocellulose for immunodetection

2.8.6 Electrotransfer

Proteins were transferred onto positively charged nitrocellulose membranes. Before transfer began the gels were equilibrated in ice-cooled transfer buffer to allow any gel shrinkage to occur. Transfer buffer comprised of 25mM Tris, 192mM glycine and 20% v/v methanol, with a final pH of 8.3 ±0.3. Transfer of the proteins onto the nitrocellulose was carried out by running at 100v for 2 hours on ice. Once transfer had occurred, nitrocellulose membranes were washed twice with 1 xTBST (0.2M NaCl, 20mM Tris, and 0.05% v/v Tween 20, pH7.4) to remove any excess methanol. The membranes were blocked for 1 hour at room temperature in 3% (w/v) milk powder in 1xTBST buffer. After blocking the membranes were washed 3x in TBST, before immunodetection.

2.8.7 Immunodetection

The nitrocellulose membranes were incubated with a range of primary antibodies for 1 hour at room temperature. Primary antibodies were diluted in incubation buffer (0.05% milk powder w/v in 1xTBST) for dilutions see table 2.2. After primary incubation nitrocellulose membranes were washed thrice in 1xTBS-T buffer before incubation with the relevant secondary (HRP conjugated) antibody, diluted in incubation buffer. Secondary incubation was left for 1 hour at room temperature before the nitrocellulose membrane was washed again thrice in 1xTBS-T buffer.

To visualise the proteins of interest, ECL (Fisher) was applied to the membrane and incubated for 30 seconds. ECL is a chemillumenscent, able to bind to the HRP conjugated on the secondary antibody allowing detection of proteins. Any excess ECL was removed from the membrane before wrapping them in saran wrap. Xray films were exposed to the nitrocellulose membrane (hypefilm GE healthcare) in a dark room. The films were developed using an X-omat developer.
Chapter 2: Materials and Methods

2.9 Immunohistochemistry and immunocytochemistry

2.9.1 Immunohistochemistry

All tissues samples were fixed in 10% formalin diluted in 1 x PBS for a minimum of 24 hours before processing. Once processed (sequentially through increasing concentrations of ethanol), samples were mounted in paraffin and 4-6μm sections were cut and mounted onto super frost plus slides. Sections were de-waxed in xylene before re-hydrating through a series of ethanol washes (100% → 90% → 70%) and finally into dH₂O. Antigen retrieval was carried out using 0.01M citrate buffer (see section 2.9.3). Tissue sections were incubated with 3% H₂O₂ for 10 minutes at room temperature to quench endogenous peroxide activity and washed for 5 minutes in 1 x PBS. Non-specific binding of antibodies was reduced by incubating tissue sections in 20% (v/v) FCS in 1 x PBS for 20 minutes at room temperature prior to incubation with primary antibody diluted in 0.05% FCS in 1xPBS (for dilutions see table 2.2) for 1 hour at room temperature or at 4°C overnight. Sections were washed 3-4 times each for 5 minutes in 1 x PBS and incubated with the appropriate HRP conjugated secondary antibody diluted in 0.05% (v/v) FCS in 1 x PBS for 1 hour at room temperature followed by 3-4 5 minute washes in 1 x PBS. HRP activity was visualised using diaminobenzidine (DAB) chromogen (Dako) to develop colour essentially as outlined according to the manufacturer's instructions. DAB was applied to the slide and incubated from 1-15 minutes as optimised for individual antibodies. In all cases, control sections were stained without primary antibody incubation for each individual antibody used. After incubation with DAB, slides were rinsed briefly in dH₂O before being counterstained for 30 seconds in haematoxylin, washed in acidified water for 10 seconds and then sequentially dehydrated through 50%, 75%, 95%, 100% ethanol and finally into xylene before mounting in depex™.

2.9.2 Haematoxylin and eosin staining (H&E)

Sections were de-waxed by immersion into xylene before rehydrating through a series of ethanol washes (100%→90%→70%) and finally into dH₂O. Sections were stained with haematoxylin, a nuclear stain, for 1 minute before rinsing with water and differentiated in 1% acidified water for 30 seconds. Next sections
were counter stained with eosin, a cytoplasmic stain, for 1 minute and rinsed in water. To finish, sections were dehydrated through a series of ethanol washes (50%→70%→95%→100%) and finally into xylene before cover slips were mounted with depex™.

### 2.9.3 Sodium citrate antigen retrieval

Tissue sections were microwaved at full power for 2 x 10 minutes in 0.01M sodium citrate buffer pH 6.0 and then allowed to cool in the buffer for 30 minutes at room temperature before beginning with the immunohistochemistry staining protocol.

### 2.9.4 Fluorescent immunocytochemistry

Cells were cultured in NUNC® chamber slides to allow staining directly on the slide, alongside controls. Chamber slides were removed from the incubator before media was removed and cells were washed twice in 1x PBS. 1ml of cell fixative (2% v/v formaldehyde/ 0.2% v/v glutaraldehyde in 1 x PBS pH 7.4) was added to each chamber and cells were incubated for 15 minutes at room temperature. Fixative was removed and cells were washed twice for 5 minutes in 1x PBS. (If not staining immediately cells were left at this stage in 1 x PBS and stored at 4°C). After washing, non-specific protein binding was blocked by incubation with 2mls 20% (v/v) FCS in 1 x PBS per well for 20 minutes at room temperature whilst being gently rocked. The blocking solution was discarded and primary antibody added at the required dilution (as detailed in table 2.2) in 0.05% (v/v) FCS in 1 x PBS. Cells were incubated in primary antibody for 1 hour at room temperature with gentle agitation. Primary antibody was removed and cells were washed twice for 5 minutes in 1 x PBS. Secondary antibody was added at the required dilution in antibody incubation buffer and cells were allowed to incubate for 1 hour at room temperature with gentle agitation (for fluorescent secondary antibodies, incubation was carried out in the dark, i.e. slides were covered in foil). The secondary antibody was discarded and cells were washed once in 1 x PBS before being washed a further 4 times in 1 x PBS (5 minutes per wash). Finally, chambers were removed from the slides and coverslips were mounted using vectashield (anti-fade) and sealed with clear nail varnish, slides were visualised by fluorescence microscopy.
2.9.5 DAPI staining of DNA

The nuclei of cells was stained using DAPI (4',6-diamidino-2-phenylindole) which binds to AT rich regions of DNA within the minor groove of the double stranded helix. To stain, media was removed from the cell plates before washing twice with 1xPBS. The cells were fixed with 0.4% (v/v) formaldehyde in 1xPBS for 15 minutes at room temperature. Fixative was removed and cells were again washed with PBS. 6μg/mL of DAPI in 1xPBS was added to the cells and incubated for 10 minutes at room temperature. Cells were washed with 1xPBS and then analysed under a fluorescent microscope.

2.9.6 Double staining

Dual staining for CYP2E1 and amylase was carried out on both tissue and cells. Tissue samples were prepared for staining by de-waxing in xylene before rehydration through a series of ethanol washes (100% → 90% → 70%) and finally into dH2O. Antigen retrieval was carried out using 0.01M citrate buffer. Tissue sections were then incubated with 3% H2O2 for 10 minutes at room temperature (RT) to quench endogenous peroxide activity and washed for 5 minutes in 1xPBS. Cells were prepared by permeabilising in ice cooled methanol for 10 minutes before washing in 1xPBS and fixing in 2% w/v formaldehyde and 0.2% gluteraldehyde in 1xPBS pH 7.4.

Non-specific binding of antibodies was prevented in all samples by incubating in 20% FCS in 1xPBS for 20 minutes at RT.

Primary antibodies diluted in 0.05% FCS were applied to the samples and left to incubate overnight at 4°C. Sections were washed twice in 1xPBS before applying the appropriate secondary antibodies for 1 hour at RT. Samples were washed twice in 1xPBS before colour development was carried out using BCIP®/NBT substrate system followed by Liquid DAB+ substrate chromogen system for CYP2E1 (Alkaline peroxidase activity) and amylase (HRP activity) expression respectively.

In all cases, control sections were stained without primary antibody incubation for each individual antibody used. After colour development slides were rinsed briefly in dH2O and counterstained using nuclear fast red (Vector laboratories) when required. Samples were sequentially dehydrated through 50%, 75%, 95%, 100% ethanol and finally into xylene before mounting in depex™.
Chapter 2: Materials and Methods

2.10 Fluorescent in situ hybridisation (FISH) staining

2.10.1 FISH staining

Hybridisation was carried out essentially as described by Shearer et al[19] with some modifications. Paraffin embedded sections were mounted onto super frost coated slides and baked overnight at 55°C. Slides were deparaffinised in 3 changes of xylene at RT for 10 minutes before dehydrating in two changes of 100% ethanol for 5 minutes and air drying. Sections were incubated in 0.2M HCl for 20 minutes and subsequently washed in 2 changes of 2X standard saline citrate (SSC) for 5 minutes, followed by incubation in 1M NaSCN at 80°C for 10 minutes before repeating the wash steps in 2X SSC. Tissue digestion was performed by incubating sections at 37°C overnight with 0.4% pepsin in 0.001M HCl. After overnight digestion, sections were washed twice in 2X SSC and air dried before re-fixation in 10% buffered formalin for 15 minutes. Slides were washed twice with 2X SSC and air dried before 10µl of RatIDetect™ Chr Y FISH paint probe (Cambio) was applied to sections and sealed. Slides and probe were co-denatured by heating to 90°C for 5 minutes and hybridized overnight at 37°C in a humidified environment. After hybridisation coverslips were removed by soaking in 2x SSC/0.1%Tween20 at 45°C followed by two washes in 0.5 X SSC/0.1% Tween 20 at 45°C. Slides were briefly rinsed in dH2O and air dried out of direct sunlight. Samples were stained with DAPI using antifade compound (Vectashield), coverslips were applied and sealed. Analysis was performed on a Zeiss AxioImager microscope.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Mwt (kDa)</th>
<th>Dilution</th>
<th>Comments and Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>57</td>
<td>1:3000 WB</td>
<td>Rabbit polyclonal to Pancreatic Amylase (abcam ab21156) for WB 200μg goat polyclonal IgG from Santa Cruz (sc-12821) for IHC/ICC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:200 IHC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:200 IHC/ICC</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>60</td>
<td>1:3000 WB</td>
<td>Chicken polyclonal to liver albumin (abcam ab106582)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:500 IHC</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>50</td>
<td>1:5000 WB</td>
<td>Rabbit polyclonal to CYP2E1 (abcam ab28146)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:500 IHC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>44</td>
<td>1:4000 WB</td>
<td>Mouse monoclonal IgG. (Sigma Aldrich A5441).</td>
</tr>
<tr>
<td>CYP4A</td>
<td>50</td>
<td>1:1000 WB</td>
<td>Rabbit polyclonal to Cytochrome P450 4A (abcam ab 3573) detects all of CYP4A family, isoforms CYP4A1 and 4A3 to a lesser extent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:200 IHC/ICC</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>58</td>
<td>1:2000 WB</td>
<td>Rabbit polyclonal to CYP3A43 (abcam ab155029)</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>95</td>
<td>1:1000 WB</td>
<td>Mouse monoclonal to Glucocorticoid Receptor (abcam ab 2768)</td>
</tr>
<tr>
<td>receptor</td>
<td></td>
<td>1:100 IHC</td>
<td></td>
</tr>
<tr>
<td>CPS-1</td>
<td>165</td>
<td>1:5000 WB</td>
<td>Rabbit polyclonal to CPS1 - Liver Mitochondrial Marker (abcam ab 3682)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:300 IHC/ICC</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>57</td>
<td>1:2000 WB</td>
<td>Mouse monoclonal anti-Vimentin antibody clone VIM 3B4 (chemicon CBL202)</td>
</tr>
<tr>
<td>α smooth muscle</td>
<td>42</td>
<td>1:2000 WB</td>
<td>Monoclonal anti actin smooth muscle antibody produced in mouse (Sigma A7607)</td>
</tr>
<tr>
<td>anti rabbit-AP</td>
<td>N/A</td>
<td>1:200 IHC</td>
<td>Polyclonal to rabbit produced in donkey. Alkalike phosphatase conjugated (Abcam ab97061)</td>
</tr>
<tr>
<td>2 °</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Table 2.2: Primary and secondary antibody specifications for Western blot (WB) and immunohistochemistry/immunocytochemistry (IHC/ICC).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Method</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-goat-HRP 2°</td>
<td>N/A</td>
<td>1:200 IHC 1/3000</td>
<td>Polyclonal to goat produced in rabbit, horseradish peroxidise conjugated. (Sigma A5420)</td>
</tr>
<tr>
<td>anti-rabbit-HRP 2°</td>
<td>N/A</td>
<td>1:200 IHC 1:3000 WB</td>
<td>Polyclonal to rabbit produced in goat, horseradish peroxidise conjugated (Dako P0448)</td>
</tr>
<tr>
<td>anti-mouse-HRP 2°</td>
<td>N/A</td>
<td>1:200 IHC 1:4000 WB</td>
<td>Polyclonal to mouse produced in goat, horseradish peroxidise conjugated (Dako P0447)</td>
</tr>
<tr>
<td>anti-rabbit-FITC 2°</td>
<td>N/A</td>
<td>1:150 ICC</td>
<td>Polyclonal to rabbit produced in sheep. FITC conjugated (Sigma F7512)</td>
</tr>
<tr>
<td>anti-rabbit-TRITC 2°</td>
<td>N/A</td>
<td>1:400 ICC</td>
<td>Polyclonal to rabbit produced in swine. TRITC conjugated (Dako R0156)</td>
</tr>
</tbody>
</table>
2.11 Culturing B-13 cells in bio-artificial liver device

2.11.1 Bioreactor and perfusion equipment

Bioreactors composed of three independent capillary membrane systems, with two systems for medium perfusion (inflow and outflow) and the third for cell oxygenation-carbon dioxide removal which are interwoven to create an extra-capillary space were used (figures 2.5 and 2.6). The design of the bioreactors was considered to support the viability and function of primary liver cells, as the three dimensional spatial structure created by the interwoven capillary system represents an artificial equivalent of the hepatic vasculature at the lobular level\(^{[192]}\). The bioreactors employed in these studies contained 40 layers of interwoven capillaries which were made of polyethersulphone for media flow (Membrana, Wuppertal, Germany) and hydrophobic multilaminate hollow-fibre membrane for gas flow (MHF, Mitsubishi, Tokyo, Japan).

The perfusion tubing used to feed into the bioreactor device consisted of standard medical grade dialysis polyvinyl chloride (PVC) (B.Braun Melsungen, Germany) and was fitted with bubble traps to remove excess gas. Sterilization was performed by formaldehyde gas sterilisation at 60°C followed by degassing prior to cell inoculation.

Figure 2. 5: Images to show A) a typical jellyfish bioreactor employed for 3D culture studies; B) the set up of the bioreactor and the perfusion tubing within the processor-controlled perfusion device with electronic pressure, media flow regulation and waste pump operation.
Chapter 2: Materials and Methods

Figure 2.6: A schematic diagram to show the organisation of the bioreactor devices used for 3D-culture. The three interwoven, independent capillary systems are shown: Red and blue capillaries show the counter current flow of culture media and yellow capillaries serve to provide oxygen supply to the cells in the extra-capillary spaces. Cells are inoculated via a separate tubing system leading directly into the extra-capillary space (grey).

2.11.2 Running of bioreactor

Each bioreactor was integrated into a processor-controlled perfusion device with electronic pressure, media flow regulation and waste pump operation (figure 2.5). The perfusion systems contain modular pump units for recirculation and fresh media feed, with multichannel flow heads to allow constant pH levels to be maintained (between 7.3 and 7.6) and allow the rates of compressed air and CO$_2$ to be regulated. Each bioreactor was run under the same conditions with a media flow rate into the bioreactor of 1.5ml/hour with the same amount of waste medium leaving the circuit through an outflow valve. Medium was recirculated within the bioreactor at a rate of 10ml/hour. A constant temperature of 37°C was maintained by a heating unit within the perfusion circuit and flow rates of air, CO$_2$, O$_2$ and N$_2$ were all regulated by a gas mixing unit at a rate of 100ml/min consisting of 95% air and 5% CO$_2$ by volume. Daily measurements
of pH, pO2, pCO2, and buffer capacity were used to adjust medium perfusion and gas supply rates. Each bioreactor was maintained in the perfusion system for either 8 or 15 days before being shut down for analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioreactor</td>
<td>Jellyfish, 2mL</td>
</tr>
<tr>
<td>System volume</td>
<td>20mL</td>
</tr>
<tr>
<td>Feed media speed</td>
<td>1.5mL/h</td>
</tr>
<tr>
<td>Recirculation speed</td>
<td>10mL/h</td>
</tr>
<tr>
<td>Gas mix</td>
<td>20mL/min, 5% CO2</td>
</tr>
<tr>
<td>Serum supplementation</td>
<td>2.5%</td>
</tr>
<tr>
<td>Cells</td>
<td>B-13, (40-100 million cells)</td>
</tr>
</tbody>
</table>

Table 2.3: Specifications of the bioreactors and running conditions used for 3D culture of B-13 cells

2.11.3 Inoculation of B-13 cells

Once the bioreactors had been conditioned for 24 hours with re-circulating culture medium DMEM (Sigma D5546) 4500mg/L glucose, supplemented with 2.5% (v/v) FCS, 80U L-glutamine, 80U/ml of penicillin and 10µM dexamethasone. B-13 or B-13/H cells were inoculated into the bioreactors (40-100 million B-13 cells in 1.5ml media, or 50million B-13/H cells in 1.5ml of media) through the inoculation tubing system. After inoculation the bioreactors were shaken to evenly distribute cells and to remove excess gas from the cell compartment. Cells were also cultured in 2D systems, as outlined in 2.2.2, in parallel for a direct comparison to 3D culture.

2.11.4 Assessment of CYP450 activity and other parameters

Samples from the perfused medium were taken daily from each bioreactor for biochemical analysis. The metabolic activity of the cells was determined by measuring a number of parameters including glucose consumption and lactate production. Other parameters were also measured such as urea secretion, galactose uptake, and release of lactic acid dehydrogenase (LDH), glutamate dehydrogenase (GLDH), alanine transaminase (ALT) and aspartate aminotranferase (AST). All parameters measured were also assessed in the parallel
2D cultures. All evaluations were performed with assays adapted for using an automated clinical analyser.

During the pilot experiments drug metabolising activity of cells in culture was determined through the addition of phenacetin, diclofenac (both from Sigma-Aldrich, St. Louis, MO), bupropion (Toronto Research Chemicals, North York, Canada) and midazolam (Roche, Mannheim, Germany) as a cocktail with final concentrations in the medium of 26µM, 9µM, 100µM and 3µM respectively for both bioreactor and 2D cultures (see table 2.4). Samples of media were taken at 1 and 3 hours after addition of substrates and frozen at -80°C until analysis. The concentrations of metabolites were analysed at Pharmacelsus GmbH, Saarbrücken, Germany, as previously described\[193\]. The formation rate of metabolites was calculated from the slope of the regression line of the concentration-time curve\[193\]. A summary of the drugs and their metabolites can be seen in tables 2.4 and 2.5 and the metabolism pathways are shown in figure 2.7.

On days 1, 8 and 15 of the bioreactor runs CYP450 activity assays were also performed for each bioreactor and their 2D culture equivalents. Assays for 7-ethoxyresorufin O-deethylase (EROD) and 7-ethoxycoumarin O-deethylase (ECOD) were carried out by culturing the cells with 20µM of the relevant CYP450 substrate. During the running of the CYP450 assays the culture media for the 2D cultures was replaced with CYP450 media whereas the bioreactors were switched into recirculation mode before 1ml of media was replaced with 1ml of CYP450 media (to give final concentration of 20µM of ethoxyresorufin or ethoxycoumarin) at time point 0 hours. Samples were taken from the cultures every 30 minutes for 2 hours after which the bioreactor system was washed through with 40-60ml of media and returned into normal mode (feed and waste). The 2D cultures were washed twice before fresh media was added. The cells were left for 2 hours before the second CYP450 assay (ECOD) was carried out. For analysis standard concentrations of the EROD and ECOD products; hydroxyresorufin/dydroxycoumarin, respectively were prepared to create a standard curve for comparison and measured along with the samples in a black 96-well plate in duplicate. Measurements were made using a FLUOstar reader.
Chapter 2: Materials and Methods

(OPTIMA, MBG LABTECH). Two measurements were taken for each assay, first was for the free product which was measure directly from the media samples taken during the assay run. The second was for the absolute product produced by phase II reactions representing the total CYP450 activity within the cell cultures. For absolute product measurement 150µl of the sample or standard was mixed with 150 µl Na-Acetate buffer (1M, pH 5.5) + 20 µl of the enzyme, beta-glucuronidase/arylsulfatase, (Roche 10127698001-10 ml), samples were then incubated at 37°C overnight before readings were taken.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration of stock solution</th>
<th>Final concentration in culture vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin</td>
<td>10mM</td>
<td>26μM</td>
</tr>
<tr>
<td>Bupropion</td>
<td>10mM</td>
<td>100μM</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>5mM</td>
<td>9μM</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3mM</td>
<td>3μM</td>
</tr>
</tbody>
</table>

Table 2. 4: A summary of the substrates and their concentrations used for assessment of CYP450 activity in bioreactor cultured B-13 cells and their 2D parallels.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>CYP450 human/rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>CYP1A2</td>
</tr>
<tr>
<td>Bupropion</td>
<td>Hydroxybupropion</td>
<td>CYP2B6/1</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>4-Hydroxydiclofenac</td>
<td>CYP2C9/11</td>
</tr>
<tr>
<td>Midazolam</td>
<td>1’-Hydroxymidazolam</td>
<td>CYP3A4/2</td>
</tr>
</tbody>
</table>

Table 2. 5: A summary of the drugs, their products and the CYP450 enzymes involved for the CYP450 assays to test liver functionality in bioreactor 176 metabolism studies.
Figure 2.7: Images to show the metabolism pathways for the drugs Phenacetin\textsuperscript{194}, Bupropion\textsuperscript{195}, Diclofenac\textsuperscript{196}, and Midazolam\textsuperscript{197} which were used in metabolism assays during the pilot bioreactor run. Images are adapted from the cited references.
2.11.5 Opening of Bioreactor

After running of the bioreactors was complete, the devices were shut down and all tubing was disconnected. The cell compartment was accessed by drilling through the upper covering of the bioreactor and lifting off carefully so as to minimise disturbance to the cells. The cell mass (including the capillary layers) was cut from the cell chamber whilst being held together to maintain cell organisation. Samples were fixed in 10% formalin for 24 hours before processing and staining as described in section 2.9. Samples for protein and RNA analysis were also collected from the opened bioreactor and stored at -80°C until needed.
Chapter 3: Results
3.1 *Investigating the effects of* B-13 and activated HSC (myofibroblast) cocultures on the transdifferentiation towards the B-13/H phenotype
Chapter 3: Results 3.1

The establishment of a hepatocyte-like cell from stem cell sources has been successfully carried out by a number of groups with a range of approaches\cite{85}. The B-13 cell and the transdifferentiation into the B-13/H cell phenotype requires very simple culture methods where the addition of DEX to the culture media results in hepatocyte-like morphology and functionality within as little as 7 days. As the transdifferentiation of B-13 to B-13/H cells is relatively simple other methods commonly used to enhance stem cell differentiation into hepatocyte-like cells have never been assessed. Approaches such as feeder layers, cocultures and 3D culture in bioreactor systems are routinely used to promote hepatocyte maintenance and stem cell differentiation. Studies have shown that primary hepatocytes are able to survive for longer periods and maintain specific functions when they are cocultured with other cell types, such as the non-parenchymal liver cells\cite{198, 199}. The cell-cell interactions between resident cells of the liver may therefore be essential in maintaining and supporting hepatocytes. The role of hepatic stellate cells (HSCs) in liver regeneration is much debated, depletion of this cell population leads to a slower recovery\cite{200} suggesting they play an essential role in liver regeneration, however more recently HSCs were reported to act as negative regulators of hepatocyte regeneration\cite{201}. Furthermore HSCs have been associated with the hepatic progenitor cell niche where their synthesis of ECM has been suggested to facilitate in the appropriate expansion and specification of the cells\cite{202} alongside liver macrophage cells. Boulter et al\cite{203} have shown close associations between macrophages, HSCs and HPCs during liver injury where the HSCs were capable of influencing the progenitor cell response. The cross-talks between the liver cell types may therefore have important roles for modulating cell growth, migration, differentiation and proliferation.

As much attention has focused on the role of HSCs for the enhancement of hepatocyte survival and functions\cite{204} they prove a good model for assessing the effects of cocultures with B-13 cells. As activated HSCs (myofibroblasts) have been shown to have important functions both in liver regeneration and as support cells within the liver stem cell niche, activated HSCs may be able to interact and promote the transdifferentiation of B-13 cells. Thus the effect on B-13 transdifferentiation in the presence of myofibroblast cells was assessed to
elucidate whether cocultures can enhance the hepatic phenotype of B-13/H cells.
3.1.1 Generation of the B-13\textsuperscript{red} cell line

To allow tracking of both B-13 cells, and myofibroblasts in coculture, myofibroblasts were isolated from Tg(GFP) mice and B-13 cells were stably transfected with a construct, pDsRed2-C1 (Clontech) that encodes the constitutive expression of a red fluorescent protein, to generate the B-13\textsuperscript{red} cell line (figure 2.1). Before transfecting B-13 cells with the DsRed vector the ideal concentration of the antibiotic, G418, needed for selection was determined. B-13 cells were grown with a range of G418 concentrations before cell viability was determined, figure 3.1.1 shows the ideal concentration was found to be 250μ/ml as cells started dying after 5 days in this condition, a time point at which G418 has been shown to be effective previously\textsuperscript{[205]}. To generate the B-13\textsuperscript{red} cell line, B-13 cells were transfected with the DsRed vector, using effectene, for 48 hours and subsequently treated with G418 for selection. Confirmation of successful transfection was shown by B-13\textsuperscript{red} cells fluorescing red under the tetramethyl rhodamine iso-thiocyanate (TRITC) wavelength (541-572nm), a varying level of red fluorescence was observed within the population of cells since a mixed clone, rather than a single clone of the cell line was produced.

Before coculture experiments were performed it was necessary to ensure transfection with the DsRed construct had not altered the B-13 response to DEX, thus B-13\textsuperscript{red} cells were treated with DEX for 14 days. Figure 3.1.2 shows that the B-13\textsuperscript{red} cells showed a typical morphological change in response to DEX treatment and upon transdifferentiation, DsRed expression was still detectable although the fluorescence was present at a greater degree in the cytoplasm in the B-13/H\textsuperscript{red} cells when compared to B-13\textsuperscript{red} cells. As DsRed is still detectable within B-13/H\textsuperscript{red} cells after transdifferentiation, the B-13\textsuperscript{red} cells could be cocultured with liver myofibroblasts derived from GFP mice allowing both cell lineages to be easily traced.
Chapter 3: Results 3.1

Figure 3.1 1: The effect of various G418 disulphate concentrations on B-13 cell viability. Cell viability of B-13 cells was assessed over 192 hours to find an effective concentration for selection after transfection.

Figure 3.1 2: The expression of red fluorescent protein in the B-13red cell line. Cells are treated with and without DEX treatment, with B-13 cells as controls.
Figure 3.1.3 shows that B-13\textsuperscript{red} cells also retained their ability to differentiate into functional hepatocyte-like cells in response to DEX treatment at the level of mRNA and protein. As a direct comparison, non transfected B-13 cells were DEX treated in parallel to B-13\textsuperscript{red} cells and RT-PCR and Western blotting results showed similar levels of hepatocyte-specific gene expression from both cell populations. These data indicate that the presence of the DsRed construct within the cell line does not impair their ability to become functional B-13/H\textsuperscript{red} cells.

The ability of B-13\textsuperscript{red} to B-13/H\textsuperscript{red} cells to transdifferentiate in response to DEX was also confirmed by immunocytochemistry. After transdifferentiation towards hepatocyte-like cells the expression of the liver-specific markers albumin and CYP2E1 were detectable in B-13/H\textsuperscript{red} cells compared to undetectable levels in B-13\textsuperscript{red} cells (figure 3.1.4). B-13/H\textsuperscript{red} cells also retained a degree of pancreatic marker amylase expression, however it was observed at much higher levels in the B-13\textsuperscript{red} cells.
Figure 3.14: The expression of the pancreatic marker amylase and liver markers albumin and CYP2E1 in B-13\textsuperscript{red} and B-13/H\textsuperscript{red} cells shown by immunocytochemistry.
3.1.2 Coculture of B-13\textsuperscript{red} cells with myofibroblasts

Once it had been established that B-13\textsuperscript{red} cells were still able to transdifferentiate in response to DEX treatment, coculture experiments were set up in order to determine if activated HSCs (myofibroblasts) derived from GFP mice modulate the transdifferentiation response of B-13 cells to DEX. After 7 days of coculture the number of hepatocyte-like cells was counted in each treatment. When treated with DEX, B-13\textsuperscript{red} cells alone showed signs of transdifferentiation towards the hepatocyte-like cells at a normal rate, however in the presence of myofibroblasts the transdifferentiation of B-13\textsuperscript{red} cells was greatly enhanced with around 95% of cells showing B-13/H\textsuperscript{red} phenotype after only 7 days. Thus the number of hepatocyte-like cells per random field of view after 7 days of DEX treatment was significantly higher in coculture than B-13\textsuperscript{red} cells alone (figure 3.1.5) suggesting that the presence of myofibroblasts may promote the transdifferentiation towards the B-13/H phenotype.

![Graph showing the average number of hepatocyte-like cells per random field of view comparing B-13\textsuperscript{red} cells alone, activated HSCs (myofibroblasts) alone or cocultures with or without DEX treatment for 7 days. Data are expressed as mean of hepatocyte-like cells observed± SD ***P<0.001, and are typical of three separate experiments](image-url)

Figure 3.1 5: The average number of hepatocyte-like cells per random field of view comparing B-13\textsuperscript{red} cells alone, activated HSCs (myofibroblasts) alone or cocultures with or without DEX treatment for 7 days. Data are expressed as mean of hepatocyte-like cells observed± SD ***P<0.001, and are typical of three separate experiments
Chapter 3: Results 3.1

Haematoxylin and eosin (H&E) staining was performed to assess the cell morphologies and cell-cell interactions that may have formed between the different cell populations in culture. Figure 3.1.6 shows that DEX treatment on stellate cells alone had no effect, as with or without DEX treatments looked similar. After 7 days of DEX treatment for the B-13\textsuperscript{red} cells, a few hepatocyte-like cells could be observed however the majority of cells still displayed a B-13 phenotype similar to the non-DEX treated control. In coculture conditions with no DEX treatment both cell types appeared to show normal morphology. In coculture conditions treated with DEX a significant proportion of B-13 cells showed evidence of transdifferentiation displaying B-13/H morphologies. This observation supports findings of the cell counts where coculture with DEX was shown to enhance the transdifferentiation rate. Interestingly the myofibroblast cell population from cultures in the presence of B-13/H cells was lower whereas myofibroblast cultures without B-13/H cells were confluent. As the myofibroblast cells alone with DEX and the cocultures without DEX were found to be healthy this observation must be due to the presence of B-13/H cells after promoting their transdifferentiation.

Figure 3.1.6: Haematoxylin and eosin (H&E) staining of B-13\textsuperscript{red} activated HSCs (myofibroblasts) and cocultures after 7 days of culture with or without DEX.
The cell cultures were stained with DAPI and imaged under the GFP and DsRed wavelengths to show the two individual cell populations and to ascertain whether any cell fusion had taken place. Using fluorescence to view the two cell populations it was observed that no cell fusion had taken place where only DsRed positive cells appeared as hepatocyte-like cells and only GFP positive cells showed a fibroblastic morphology (figure 3.1.7). Although through H&E staining the stellate population appeared to be depleted in cocultures treated with DEX the fluorescent images clearly show that the stellate population is not completely lost.

The observations were further confirmed by examining the mRNA and protein levels by RT-PCR and Western blotting respectively (figure 3.1.8). Both RT-PCR and Western blotting showed that the expression of liver markers including CYP2E1, CPS-1 and albumin were detectable in cocultures treated with DEX after just 7 days, however no liver-specific genes were detected in the B-13\textsuperscript{red} cells treated with DEX after this culture time. The levels of liver markers within the cocultures were comparable to control B-13/H cells that had been DEX treated for the standard 14 days, thus this shows that the coculture of B-13\textsuperscript{red} cells with myofibroblasts is able to promote the transdifferentiation of the cell line towards the hepatic phenotype. There were no differences observed when comparing myofibroblast cells cultured alone with or without DEX treatment. Although the presence of myofibroblasts promotes transdifferentiation, the process is still DEX dependent as the coculture with no DEX treatment shows no liver marker gene expression and no change in B-13 cell morphology.

Furthermore the myofibroblast cell markers vimentin and alpha smooth muscle actin (\(\alpha\)-sma) were shown to decrease in the cocultures treated with DEX, supporting the observations made from H&E staining. Myofibroblast depletion has been shown to not be dependent on DEX or B-13 cells as myofibroblast cells are present in both conditions and with B-13 cells before the transdifferentiation event, thus the presence of B-13/H cells within the coculture is most likely to be the cause for the myofibroblast cell depletion.
Figure 3.1 7: Cultures of B-13red activated HSCs (myofibroblasts) and cocultures with or without DEX treatment for 7 days. Cells were DAPI stained to show cell nuclei before being viewed under GFP and DsRed wavelengths to show the individual cell populations and any cell-cell interactions.
Figure 3.1: RT-PCR and Western blot analysis for the expression of liver specific markers in coculture experiments with or without DEX after 7 days. A) RT-PCR analysis comparing the expression of liver specific markers CYP2E1, CPS-1 and albumin and myofibroblast markers α-sma and vimentin, at the mRNA level in coculture experiments. Results shown are typical of three separate experiments. B) Western blot analysis comparing the expression of liver specific markers CYP2E1, CPS-1 and albumin and myofibroblast markers α-sma and vimentin at the protein level in coculture. Results shown are typical of three separate experiments.
3.1.3 Affects of coculture on Wnt signalling in B-13 cells

It has previously been shown that the transdifferentiation of B-13 cells towards the B-13/H phenotype is dependent on the transient repression of canonical Wnt signalling\textsuperscript{[167]}. Thus the activity of Wnt signalling in B-13 cells in different coculture conditions was assessed to see whether the presence of myofibrobalsts had any effect on B-13 Wnt signalling activity.

B-13 cells were plated out and transfected with either the T cell factor reporter plasmid (TOPFLASH) or the control plasmid (FLOPFLASH). The transfections were left for 24 hours before coculture conditions were set up by the addition of myofibroblasts and DEX treatments to the relevant cultures. The firefly luciferase and thus Wnt signalling activities of each condition was measured at both 24 hours and 48 hours after DEX treatment in triplicate and values were normalised to the renilla luciferase control (figure 3.1.9). Results confirmed that in the presence of DEX there is a repression of Wnt signalling activity compared to the normal high activity observed in B-13 cell cultures. Interestingly in the presence of myofibroblasts the Wnt signalling activity in B-13 cells was also reduced and this effect was independent of DEX. Both coculture conditions showed a loss in Wnt activity, although with the addition of DEX, Wnt signalling appeared to be even further repressed. This suggests that myofibroblasts are able to repress Wnt signalling in B-13 cells although the presence of DEX may still be required to drive the transdifferentiation as no evidence of B-13/H cells was found in the coculture conditions with no DEX.
Figure 3.1 9: Wnt signalling activity in B-13 cells cultured with or without myofibroblasts after 24 or 48 hours of DEX treatment. Wnt signalling activity was determined by transfection with the Wnt signalling reporter TOPFLASH construct (or control FOPFLASH) and co-transfection with RL-TK as described in methods 2.3.3. Data is typical of four separate experiments.
3.1.4 Identifying factors influencing enhanced transdifferentiation in coculture experiments

To identify whether myofibroblast cells are releasing soluble factors during cocultures that are causing the promoted rate of transdifferentiation seen, coculture experiments were repeated but B-13 cells were also cultured with myofibroblast conditioned media with or without DEX. The cells were treated with conditioned media for 7 days, as in previous experiments before hepatic markers were analysed at the protein level by Western blotting.

![Figure 3.1 10. The effects of myofibroblast conditioned media on the rate of B-13 transdifferentiation. Western blot analysis comparing the expression of liver specific markers CYP2E1, CPS-1 and albumin, the pancreatic marker amylase and myofibroblast markers α-sma and vimentin at the protein level in coculture and myofibroblast conditioned media. Conditioned media was shown to have no effect on the rate of B-13 transdifferentiation after 7 days culture. Results shown are typical of three separate experiments.](image)

Protein expression showed that myofibroblast conditioned media had no effect on the rate of B-13 transdifferentiation compared to cocultures (figure 3.1.10). This suggests that direct cell-cell contacts may be involved in promoting the hepatic phenotype of B-13 cells rather than a soluble factor being released. These observations were further supported by counting the number hepatocyte-
like cells in each condition (figure 3.1.11). In conditioned media treated B-13 cells the number of hepatocyte like cells observed was similar to the control B-13 cells with DEX, in contrast to the high number of hepatocyte-like cells observed in cocultures treated with DEX.

Figure 3.1 11: The average number of hepatocyte-like cells per random field of view comparing B-13red cells alone, B-13red cells treated with myofibroblast conditioned media or cocultures, with or without DEX treatment for 7 days. Data are expressed as mean of hepatocytes-like cells observed± SD ***P<0.001, and are typical of three separate experiments.

To assess whether direct cell-cell contacts between the myofibroblasts and B-13 cells is required to enhance the rate of B-13 transdifferentiation observed in coculture experiments, B-13 cells were cultured with membrane fractions from dead myofibroblasts. Membrane fractions were created by treating stellate cells with gliotoxin (1.5 μmol/L[71]) for 48 hours before a viability test, using trypan blue, was carried out to confirm cell death and the cell fractions were pelleted and resuspended in fresh cell media. As in all coculture experiments, B-13s were cultured with the membrane fractions for 7 days with or without DEX alongside coculture and B-13 cell controls. Analysis of the mRNA and protein expression by RT-PCR and Western blotting respectively showed that culture of
the B-13 cells with myofibroblast cell membrane fragments and DEX treatment was able to promote the rate of transdifferentiation to levels comparable to the cocultures (figure 3.1.12). The expression of liver-specific markers such as CYP2E1, CPS-1 and albumin were detectable within B-13 cells treated with DEX and membrane fragments compared to B-13 cells treated with DEX alone for 14 days. These observations were also found at the protein level for CPS-1 although no evidence for CYP2E1 was found.

Figure 3.1 12: RT-PCR and Western blot analysis for the expression of liver specific markers in coculture experiments with dead myofibroblasts with or without 10µM DEX for 7 days. A) RT-PCR analysis comparing the expression of liver specific markers albumin, CYP2E1, and CPS-1, at the mRNA level in cocultures of B-13 cells and dead myofibroblasts. Results shown are typical of three separate experiments. B) Western blot analysis comparing the expression of liver specific markers CYP2E1 and CPS-1 at the protein level in cocultures of B-13 cells with dead myofibroblasts. Results shown are typical of three separate experiments.
Cell counts were performed for cultures with myofibroblast cell membrane fragments compared to cocultures and B-13 cells alone. The findings supported results from the RT-PCR and Western blot results with high numbers of B-13/H cells observed compared to the numbers observed in B-13 cells alone treated with DEX (figure 3.1.13). These observations suggest that a myofibroblast membrane bound signal is likely to be the cause of the enhanced rate of transdifferentiation observed as the presence of membrane fragments is sufficient to promote transdifferentiation.

Figure 3.1 13: The average number of hepatocyte like cells per random field of view comparing B-13red cells alone, B-13red cells with dead myofibroblasts or cocultures with or without DEX treatment for 7 days. Data are expressed as mean of hepatocyte-like cells observed± SD ***P<0.001, and are typical of three separate experiments.

Figure 3.1.14 shows DAB staining for the pancreatic marker amylase and the liver marker CYP2E1 performed on cultures comparing the effects of B-13s alone, with myofibroblasts or with myofibroblast cell membrane fractions. Amylase staining was found to be positive in all B-13 cells however to a lesser degree after DEX treatment showing that a loss of some pancreatic function
Chapter 3: Results 3.1

had occurred. CYP2E1 expression was found in a small number of B-13 cells treated with DEX alone however in the presence of myofibroblasts (dead or alive) the number of CYP2E1 positive cells was much greater which supports data previously shown. Therefore a membrane bound factor present on myofibroblast cells is able to directly influence the rate of transdifferentiation of B-13 cells into B-13/H cells in the presence of DEX.
Figure 3.14: The expression of amylase and CYP2E1 in B-13 cells after cocultures shown by immunohistochemistry. Images show B-13 cells, and B-13 cells cultured with myofibroblast cells or myofibroblast cell membrane fragments with or without 10µM DEX treatment for 7 days. Pictures are typical of three separate experiments.
3.1.5 Chapter discussion

Data within this chapter has shown that in the presence of myofibroblasts the rate of DEX dependent transdifferentiation is enhanced such that the hepatic phenotype is observed in B-13 cells more rapidly compared to B-13s treated with DEX alone.

One simple explanation for the enhanced rate of transdifferentiation in the presence of myofibroblasts may be due to the scaffolding or matrix provided by the fibroblastic cells. Effective scaffolds, from either cellular or synthetic biomaterials, have been used successfully to promote liver functions of hepatocytes in vitro, and so this observation could be used to explain the enhanced hepatic differentiation of B-13 cells compared to their normal culture conditions on plastic. During liver regeneration the HSC population has been shown to be necessary for the HPC response; the HPCs are thought to reside within a niche in the liver where macrophages and HSCs act as companion cells; activated HSCs have been shown to be key to the niche structure by the production of a rich ECM. Observations have shown that the macrophages are responsible for the secretion of MMPs which results in the breakdown of the ECM and initiates the HPC response after injury. The importance of HSCs in liver regeneration has been further verified in vivo as their depletion led to a slowed or impaired regeneration. In coculture studies between HSCs and hepatocyte-like stem cells it has been shown that an increase of both cell proliferation and cell maturation was observed however it was not found whether these results were through direct cell contacts or via soluble factors in the medium. Together with the evidence from B-13 cocultures and cocultures from other groups it has been shown that the hepatic differentiation is enhanced by the presence of HSCs via a mutual synergism with the non parenchymal cells of the liver. Hepatocyte-like cells in the coculture might adhere firmly to the extracellular matrix, such as collagens, which are synthesized by activated HSCs.

To further understand the mechanisms involved in the coculture experiments it was necessary to clarify whether the myofibroblast-dependent promotion of B-13 transdifferentiation observed was due to a soluble factor within the media or
a result of direct cell contacts. Observations using both dead myofibroblast fragments and myofibroblast conditioned media suggest that the promotion of B-13 transdifferentiation is most likely due to direct cell contacts rather than a soluble factor being secreted by myofibroblasts as conditioned media from myofibroblasts had no effect on the rate of B-13 transdifferentiation. Findings from other studies have shown that soluble factors secreted from the HSCs were responsible for the maintenance of hepatocyte-like stem cells and that direct cell cocultures had less success\(^\text{[204]}\). Another group concluded that direct contact between hepatocytes and HSCs may play a negative role in the maintenance of hepatic differentiated functions but a positive role in cell proliferation, where increased DNA synthesis in hepatocytes was found\(^\text{[102]}\). From my observations it is most likely that direct cell contacts are necessary for the enhanced rate of transdifferentiation towards a hepatocyte phenotype. However once transdifferentiation had taken place the myofibroblast population became depleted suggesting that they may promote the hepatic phenotype in the B-13 cells but do not play a role in the maintenance of the functionality. It would therefore be interesting to investigate the coculture between B-13/H cells and myofibroblasts to elucidate whether myofibroblast cells are able to maintain the B-13/H cells for longer periods in culture and whether direct cell contacts or soluble factors play a role as seen by other in hepatocyte cocultures.

The depletion of myofibroblasts observed once B-13 cells had transdifferentiated towards a B-13/H phenotype is interesting, as if this phenomenon occurs \textit{in vivo} during regeneration, then the presence of new hepatocytes may lead to activated stellate cell death and thus aid in the reversal of the disease phenotype. The progressive death of myofibroblasts in cocultures was also observed by Carraro et al\(^\text{[102]}\), where after 7 days of cocultures with hepatocyte-like cells, the HSC population viability declined. From these studies it was concluded that HSCs initially are able to modulate the differentiation of human liver stem cells (HLSCs) before HSC cell death occurs and they are no longer able to support the hepatocyte cells. These observations could provide insights into the regeneration and disease reversal process as stellate cells could play an essential role in stimulating hepatocyte proliferation, or hepatic
progenitor cell differentiation, before they themselves are programmed for death to reduce the disease phenotype.

Wnt signalling is an essential pathway during liver development and specification and in addition to its role in liver development, the Wnt signalling pathway has been shown to be a key player in many aspects of postnatal liver homeostasis. It was reported that hepatocyte-specific knockout of β-catenin resulted in a decreased liver size and reduced hepatocyte proliferation\[^{176, 207}\]\ and complementary studies have shown the opposite effects when β-catenin is over expressed\[^{208}\]. As the transdifferentiation of B-13 cells towards a hepatic phenotype has been shown to be dependent on a transient repression of Wnt signalling the levels of Wnt activity in B-13 cells from the coculture was assessed. The level of Wnt activity was found to be lower in both coculture conditions independent of DEX when compared to B-13s alone however only transdifferentiation was evident in the conditions with DEX treatment. These data suggest that transdifferentiation is DEX dependent, however as the experiments were only cultured for 7 days it would be interesting to see whether a repression in Wnt signalling alone through coculture could result in B-13/H cells after a longer culture time. Several Wnt ligands have been found to be expressed by various liver cells\[^{172}\] suggesting a high degree of potential for crosstalk between different cells of the liver. In a study by Boulter et al\[^{203}\] the notch and Wnt signalling pathways were found to be implicated in lineage specification of cholangiocytes and hepatocytes respectively. It was found that interactions with activated stellate cells was shown to promote cholangiocyte specification through notch signalling and hepatocyte regeneration was shown to be dependent on an induction of Wnt signalling after macrophage engulfment of hepatocytes. Although it has been shown during regeneration, that liver Wnt signalling is required for hepatocyte specification; the opposite is required to induce a hepatic phenotype in B-13 cells. This difference may be due to the pancreatic origin of the B-13 cells where a repression of Wnt signalling is required to leave the pancreatic phenotype before it is restored to a high level in the B-13/H cells, and thus direct comparisons between HPCs and B-13 cells cannot be made.
Chapter 3: Results 3.1

The data presented in this chapter has shown a role of activated HSCs in promoting the hepatic phenotype of progenitor cells *in vitro* and they may be a good cell source for enhancing hepatic progenitor cell differentiation. Although the mechanisms by which the rate of transdifferentiation is enhanced are not completely understood it is evident that direct cell-cell contacts between the two cell populations is necessary. A repression of Wnt signalling was shown to occur in the presence of myofibroblasts independent of DEX treatment however as the rate of transdifferentiation was only enhanced in the presence of DEX then the mechanism cannot be solely explained through a repression of Wnt signalling and a crosstalk between multiple pathways is more likely.
3.2 Investigations into cytogenetics and tumour-like behaviour of B-13 cells
The transdifferentiation events leading from the B-13 phenotype towards the B-13/H phenotype have been extensively studied *in vitro* where mechanisms such as the Wnt signalling pathway have been shown to play key roles in the process\[^{167}\]. However, little is understood about the potential for B-13 transdifferentiation *in vivo* and what causes the cells to behave in the unique way that they do. Before investigations can be carried out *in vivo*, the cytogenetics and tumour forming potential of the cell line was assessed to establish whether any genetic abnormalities could be identified to explain the B-13 characteristics and to see whether there is a likelihood of tumour initiation if they are to be injected *in vivo*. 
3.2.1 Cytogenetic analysis of B-13 cells

Cytogenetic analysis of the B-13 chromosomes was carried out alongside rat myofibroblasts and HepG2 cells for normal diploid rat cells and transformed cell controls respectively. A number of metaphase spreads were analysed for each cell type. This analysis was performed to see if any genetic abnormalities such as chromosomal gains/losses could be observed which may explain the unique character of the cell line. Figure 3.2.1 shows a typical metaphase spread from the B-13 cells after Giesma banding (G-banding). It is evident that the B-13 cells do not possess a normal rat diploid chromosome count of 42 but instead appear near tetraploid with the majority of metaphase spreads analysed containing 84 chromosomes.

Figure 3.2 1: A typical G-banded metaphase spread of B-13 chromosomes. Magnification x100.

As expected the control rat myofibroblasts showed a diploid chromosome number of 42 in the majority of metaphase spreads analysed. The HepG2 cell line had an abnormal number of chromosomes, with a significant variation between different cells, with each cell showing highly inconsistent karyotypes. Figure 3.2.2 shows examples of G-banded metaphase spreads from each of the 3 cell types analysed and a summary of the results can be seen in table 3.2.1.
Figure 3.2 2: Metaphase spreads from B-13 cells, rat myofibroblasts and the human HepG2 cell line. B-13 cells show a near tetraploid (84) chromosome number whereas the rat myofibroblasts show the rat diploid chromosome number of 42. The HepG2 cell line shows a variable chromosome number ranging from 68-83.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Spreads analysed</th>
<th>Number of chromosomes (observed/cell-mean±SD)</th>
<th>Range (chromosomes/cell min-max)</th>
<th>Mode (chromosomes/cell)</th>
<th>Number of chromosomes/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-13</td>
<td>17</td>
<td>82.4±1.73</td>
<td>79-84</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>Myofibroblasts</td>
<td>11</td>
<td>41.6±0.67</td>
<td>40-42</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>HepG2</td>
<td>13</td>
<td>75.9±6.07</td>
<td>68-83</td>
<td>80</td>
<td>Variable</td>
</tr>
</tbody>
</table>

Table 3.2 1: The average number of chromosomes per metaphase spread analysed from each cell type. 17 spreads were analysed for B-13 cells, 11 for rat myofibroblasts and 13 spreads for HepG2 cells.

Once metaphase spreads had been G-banded, the chromosomes from the B-13 cells were sorted for karyotyping. Figure 3.2.3 shows a typical B-13 cell karyotype, illustrating its tetraploid karyotype of 84 chromosomes. It is evident that a number of structural abnormalities within some of the chromosomes were found resulting in G-bandng that prevented identification in confidence, and so
the identities of some chromosomes was ambiguous and were termed marker chromosomes.

![A typical G-banded karyotype of the B-13 cells.](image)

Figure 3.2 3: A typical G-banded karyotype of the B-13 cells. The karyogram shows a tetraploid karyotype with 8 marker chromosomes that could not be identified/placed.

Karyotyping clearly suggests that the B-13 cell line is derived from a male rat. To test this, the presence of the Y chromosome in the B-13 cell line was confirmed by PCR using male and female rat DNA as controls. PCR results verified the findings of the cytogenetic analysis (figure 3.2.4)

![PCR for the sex chromosomes in B-13 cells.](image)

Figure 3.2 4: PCR for the sex chromosomes in B-13 cells. The presence of the Y chromosome detected in cytogenetic analysis was confirmed. Male and female rats were used as positive controls. Data typical of three separate experiments.
### Table 3.2: Summary of the B-13 cell karyotype.

<table>
<thead>
<tr>
<th>Chromosome number</th>
<th>Copies positively identified/cell</th>
<th>Abnormalities</th>
<th>Unidentified (therefore presumed marker chromosome)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>n/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td></td>
<td>1</td>
<td>1 chromosome appears to have fragile site at 2q14</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>n/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>n/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>n/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>n/d</td>
<td></td>
<td></td>
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<td>4</td>
<td>n/d</td>
<td></td>
<td></td>
</tr>
<tr>
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3.2.2 Agar anchorage assay

As cytogenetic analysis of the B-13 cells revealed some chromosomal abnormalities, an agar anchorage assay was performed to investigate whether the cell line shows any tumour-like behaviour. The agar anchorage assay is an established method to monitor anchorage independent growth and is considered the most appropriate *in vitro* assay to detect the malignant transformation of cells\(^{[190]}\), where cells able to proliferate independent of anchorage to a substratum are likely to initiate tumours *in vivo*.

B-13 cells, rat myofibroblasts and HepG2 cells were all cultured in soft agar supplemented with cell media for 14 days, after which the cultures were stained with crystal violet to show any colonies formed. Figure 3.2.5 and 3.2.6 show examples of the stained 6-well plates and close up images of the cell colonies formed respectively. After 14 days of culture, both rat myofibroblast and B-13 cells failed to proliferate and no cell colonies were observed within any of the cultures. The HepG2 cell line showed clear evidence of colony formation suggesting that the cell line is independent of anchorage for cell proliferation and is likely to give rise to tumours *in vivo*. As shown in figure 3.2.7 the presence of colonies was only observed in HepG2 cultures which suggests that they are the only cell tested capable of anchorage independent growth.

![Figure 3.2.5: Crystal violet staining to show colony formation after culture in agar.](image)

An agar anchorage assay was carried out on B-13 cells, rat myofibroblasts and HepG2 cells and colonies were stained with crystal violet after 14 days. Cell colonies were observed in HepG2 wells only. Data is typical of three separate experiments.
Chapter 3: Results 3.2

Figure 3.2 6: The presence of cells or cell colonies after growth in soft agar for 14 days. B-13 cells and rat myofibroblasts failed to proliferate independent of anchorage and showed no colony formation. HepG2 cells showed large colonies throughout the agar. Data is typical of three separate experiments.

Figure 3.2 7: Colony formation after growth in soft agar. The average number of cell colonies observed per random field of view after 14 days culture in soft agar of B-13 cells, rat myofibroblasts, HepG2 cells and a no cell control. Data are expressed as mean of colonies observed ± SD. ***P<0.001, and are typical of three separate assays.

No cells

B-13

Myofibroblasts

HepG2

Cell colonies/random field of view

No cell  B-13  Myofibroblasts  HepG2

Cell type

112
3.2.3 In vivo assessment of tumour forming potential

To further assess their tumour-like behaviour, B-13 cells were injected subcutaneously into non-obese diabetic severe combined immunodeficient (NOD-SCID) mice, to investigate whether tumour initiation and/or metastasis was possible in vivo. HepG2 cells and PBS were used as positive and negative controls respectively. Mice were left for 6 weeks, with regular checks, before analysis of tumour formation was carried out. Contrary to the agar anchorage assay, the formation of tumours in the B-13 injected mice was observed. Tumours were only observed at the site of injection under the skin and no evidence of metastasis was found. Through RT-PCR and Western blot analysis the tumours were found to be composed of B-13 cells and showed no signs of transdifferentiation towards the B-13/H phenotype. Figure 3.2.8 shows that the expression of the pancreatic marker amylase was found only in the tumour and pancreas of the B-13 injected groups but no other organs. No evidence of tumour formation was observed in the PBS control and HepG2 injected mice at the time of culling.

Morphological analysis showed that the tumours were tightly packed clusters of B-13 cells and had a uniform patterning with no other cell types within the tumour. Figure 3.2.9 shows high expression of the pancreatic marker amylase, by IHC, confirming the presence of B-13 cells within the tumours, and no expression of the liver marker CYP2E1 which suggests no transdifferentiation towards B-13/H cells occurred.
Figure 3.2 8: RT-PCR and Western blot analysis of the expression for the pancreatic marker amylase in organs from NOD-SCID mice injected with B-13 cells, HepG2 cells and PBS. A) RT-PCR analysis comparing the expression of amylase at the mRNA level. Results shown are typical of three separate experiments. B) Western blot analysis comparing the expression of amylase at the protein level in coculture. Results shown are typical of three separate experiments.
Figure 3.2 9: The expression of amylase and CYP2E1 in tumours formed in B-13 injected NOD/SCID mice shown by immunohistochemistry. Liver and pancreas sections are used as positive controls for CYP2E1 and amylase expression respectively. Pictures are typical of 5 stained sections.
Chapter 3: Results 3.2

3.2.4 Chapter discussion

Cytogenetics analysis has demonstrated that the B-13 cell line has a tetraploid karyotype. Although an abnormal karyotype has been detected, this phenomenon is relatively common in cell lines such as embryonic stem cell lines that are propagated for some time and probably reflects the progressive adaptation of self-renewing cells to their culture conditions\textsuperscript{[209]}. The karyotypic instability of human embryonic stem cells was reported by Draper et al\textsuperscript{[210]} and Buzzard et al\textsuperscript{[211]} where they concluded that bulk passaging of cells resulted not only in abnormal karyotypes of cells but also led to quantitative differences in gene expression. Chromosomal abnormalities have been observed after as few as 23 passages in embryonic stem cells and chromosomal aneuploidy was found to be in conjunction with aberrant gene expression\textsuperscript{[212]}. The AR42JB-13 cell line was isolated in the mid-1990s and has been extensively expanded in culture for at least 18 years; in this time the cell line has retained both its proliferative capacity and the transdifferentiation response to dexamethasone. It is therefore likely that the tetraploid karyotype may be a result of a mis-division during its expansion and has not had any detrimental effects to the cells character, or alternatively the abnormal karyotype has always been present and may give the cells their unique characteristic. One explanation of the abnormal karyotypes often observed in embryonic stem cells is that it is a consequence of \textit{in vitro} selection, where hESCs with enhanced proliferative ability, due to increased expression of the relevant genes, may out grow other cells\textsuperscript{[212]}. It would be of interest to carry out cytogenetic analysis on the parent AR42J cell line, from which the B-13 cell line was derived, to clarify whether any karyotypic abnormalities can be observed. An understanding of the genetic changes which generate the B-13 phenotype may enable a human equivalent to be engineered which would have significant utility in basic and clinical science.

Due to the cytogenetic results, tumorigenicity tests were carried out on the B-13 cell line to assess whether they may give rise to tumours if they are to be used for \textit{in vivo} studies. The soft agar anchorage assay is a conventional method used to monitor anchorage independent growth and is considered the most appropriate \textit{in vitro} assay to detect malignant transformation of cells\textsuperscript{[190]}. B-13 cells were found to not grow in soft agar which indicates they retain a
requirement for anchorage-dependent growth and responsiveness to factors which present uncontrolled cell death. However many studies have reported that \textit{in vitro} methods of tumorigenicity testing do not always reliably reflect tumour initiation properties \textit{in vivo} and thus \textit{in vivo} testing is needed to demonstrate the cell behaviour reliably\cite{213}. \textit{In vivo} studies were therefore carried out with B-13 cells to see if tumour initiation could be detected. Tumours were formed at the site of inoculation after 4 weeks and appeared to consist of B-13 cells only with no signs of transdifferentiation. Analysis of all other tissues and organs showed no signs of B-13 metastasis. Due to the origin on the AR42J cell line being from pancreatic carcinomas, the ability of the B-13 cell to initiate tumours in not surprising. These data are in agreement with many findings that there is no direct relationship between anchorage independent growth and the ability to initiate tumour growth, thus care should be taken when using \textit{in vitro} tests for tumorigenicity testing. As tumour initiation is strongly influenced by both the immune system and the microenvironment, cell growth may in part reflect the ability of the cells to interact with the microenvironment in which they are placed and thus may be dependent on the site of inoculation. The term tumorigenicity can be defined by the capacity of a cell population inoculated into an animal model to produce a tumour by proliferation at the site of inoculation and/or at a distant site by metastasis\cite{214}. Although B-13s failed to grow in soft agar as tumour formation was observed \textit{in vivo}, a much more stringent tumorigenicity test, it can be concluded that B-13 cells are tumorigenic; however they are unlikely to metastasise and instead grow at the site of inoculation. Although B-13 cells are capable of tumour formation they still are a powerful cell model for the study of pancreatic to hepatic transdifferentiation and may provide insights that may facilitate in finding a human equivalent.
3.3 Investigations for the *in vivo* potential of the B-13 cell line as a liver progenitor cell
Since the first reports of pancreatic to hepatic transdifferentiation of the B-13 cell line, there has been much work into identifying the mechanisms involved and the functionality of the B-13/H cell. Much is now understood about the events that lead to the transdifferentiation process including a transient repression of the Wnt signalling pathway and an induction of SGK1 after glucocorticoid interactions with the glucocorticoid receptor. However the key goals for understanding these events are to assist us in identifying a hepatic progenitor cell from humans that could be an alternative or bridge to organ transplantation. It is therefore of interest to understand how the B-13 cell line behaves in vivo and whether it is able to engraft and transdifferentiate within the liver environment.
3.3.1 Determination of paracetamol and B-13<sup>rd</sup> cell dosage

Before investigating whether B-13 cells are able to engraft into the liver and are able to transdifferentiate in vivo, a pilot study was performed to find optimal doses of paracetamol treatment for mice. It has previously been reported that the mouse is much more sensitive than the rat to paracetamol<sup>215</sup>, and a study was carried out to find whether a mild injury dose to the rat (i.e. 20mg/kg (body weight)) could still induce liver damage in the mouse. By this method small amounts of liver damage could be induced in the mouse without affecting B-13 cell viability. Two groups of mice, (group 1 female and group 2 male) received 20mg/kg of paracetamol intraperitoneally (i.p.). Elevated levels of serum alanine transaminase (ALT) was observed in both groups 48 hours after paracetamol dosing, suggesting low levels of damage had been caused (figure 3.3.1). H&E staining of the liver sections confirmed the serum data by showing low levels of liver necrosis in all mice (figure 3.3.2); with inflammation and infiltration of macrophage cells observed.

![Figure 3.3.1: Levels of alkaline phosphatase and alanine transaminase in paracetamol treated mice.](image)

Serum levels show elevated levels of ALT in both male (Group 1) and female (Group 2) mice 48 hours after treatment with 20mg/kg paracetamol.
Figure 3.3: Morphology of livers from paracetamol treated mice and a non-treated control. Haematoxylin and Eosin staining after 20mg/kg paracetamol treatment. Small levels of liver necrosis can be observed with signs of inflammation and cell death. Images are typical of three mice per group.

As small amounts of damage were observed a pilot study investigating whether B-13 cells can engraft to the liver was carried out. The B-13\textsuperscript{red} cell line was chosen so that cells could be tracked \textit{in vivo} using the DsRed transgene. Before injection, B-13\textsuperscript{red} cells were visualised using an \textit{in vivo} imaging system (ivis) to determine how many B-13\textsuperscript{red} cells are required for detection both \textit{in vitro} and \textit{in vivo}. Under the DsRed wavelength as little as 0.1 million B-13\textsuperscript{red} cells could be detected \textit{in vitro}, however the biggest flux output was from 10 million cells as expected (figures 3.3.3 and 3.3.4). As B-13 cells are of rat origin, female non-obese diabetic/ severe combined immuno-deficient (NOD/SCID) mice were used to avoid rejection, and so that the Y chromosome of the B-13 cells could also be used for tracking, if required. Three 10-12 week old female NOD/SCID mice were pretreated with 20mg/kg of paracetamol three days prior to, and on the day of B-13\textsuperscript{red} cell injections to stimulate a moderate level of liver damage.
Chapter 3: Results 3.3

Figure 3.3 3: B-13ed cells were visualised under the DsRed wavelength using an *in vivo* imaging system (*ivis*). Cells could be detected in all tubes compared to PBS and B-13 cell controls. Cell numbers left to right: 0.1 million, 0.5 million, 1 million, 5 million and 10 million.

Figure 3.3 4: Total fluorescence detected in varying numbers of B-13ed cells under DsRed wavelengths using an *in vivo* imaging system (*ivis*). Cells could be detected in all B-13ed samples but not in PBS and B-13 cell controls.
Chapter 3: Results 3.3

To determine whether B-13$^{\text{red}}$ cells are able to engraft into the liver analysis of the livers of NOD/SCID mice was carried out by Western blot. As amylase expression has been shown to be retained in the B-13/H$^{\text{red}}$ cells \textit{in vitro}, the expression of amylase was employed as a marker to screen all tissues of the mice. Figure 3.3.5 shows that amylase expression was detectable in two of the three mice injected in the pilot study suggesting the presence of B-13/pancreatic cells within the livers. This was further confirmed by immunohistochemistry for amylase, which was performed in all tissues, where the presence of amylase positive cells was observed in the livers only and no other tissues. All tissues were observed under the DsRed wavelength to search for B-13$^{\text{red}}$ cells however no DsRed positive cells were found, including in the livers where amylase positive cells had been observed.

**Figure 3.3 5:** Western blot analysis and immunohistochemistry for the pancreatic marker amylase in pilot studies for injections of B-13$^{\text{red}}$ cells into NOD/SCID mice. A) Western blot analysis to show the protein expression of CYP2E1 and amylase in the livers and bone marrow of NOD/SCID mice after B-13$^{\text{red}}$ injection. B) Immunohistochemistry for amylase for two mice shown to have amylase expression in the liver by Western blot. Amylase positive cells were observed suggesting the B-13 cells successfully engrafted to the liver. No Amylase was detected in other tissues.
3.3.2 The B-13 cell is able to engraft to the liver independent of paracetamol treatment.

As B-13 cells engrafted to the periportal regions of the liver, and not the central lobular regions where paracetamol induced injury occurs, a larger study was performed where mice received B-13\textsuperscript{red} cell injections with or without paracetamol treatment to evaluate whether liver damage is necessary for B-13 cell engraftment. Immunohistochemistry for amylase was performed on the livers and a number of other tissues of NOD/SCID mice. Figures 3.3.6 and 3.3.7 show that clusters of amylase positive cells were found within the portal tracts of some of the livers indicating B-13 cell engraftment. Interestingly, amylase positive cells were observed in livers of both of the groups suggesting that engraftment of the B-13 cells to the liver is independent of paracetamol treatment prior to cell injection. No amylase positive cells were observed in any other tissues including the heart, lung, brain, kidneys, spleen, thymus, bone marrow and adipose tissue.

RT-PCR was performed to determine whether the presence of amylase within the livers could be confirmed at the mRNA level. Figure 3.3.8 shows that livers from all groups of the study displayed amylase expression at the mRNA level although only levels of amylase were detected in some livers suggesting less successful engraftment/injections of the cells. The expression of amylase was also found at the protein level in B-13\textsuperscript{red} injected NOD/SCIDs by Western blot analysis (figure 3.3.9), no amylase expression at the mRNA or protein levels were detected in the PBS-injected controls.
Figure 3.3 6: The expression of the pancreatic marker amylase in WT rat pancreas and liver and B-13^{red} injected NOD/SCID mouse pancreas, liver, heart and lung shown by immunohistochemistry. A number of amylase positive cells were observed in the livers of NOD/SCID mice around the portal tracts. No amylase expression was detected in other non-pancreatic organs such as the heart and lung. All images x20 magnification and are typical of animals from each group.
Chapter 3: Results 3.3

Figure 3.3 7: The expression of the pancreatic marker amylase detected within the livers of NOD/SCID mice after intravenous injections of B-13(red) cells shown by immunohistochemistry. Amylase positive cells were found around the portal tracts of the livers.

Figure 3.3 8: The expression for the pancreatic marker amylase in B-13(red) injected NOD/SCID mice shown by RT-PCR. All groups of injected NOD/SCID mouse showed the presence of amylase mRNA within their livers suggesting B-13(red) engraftment independent of paracetamol treatment. No amylase expression was detected in any other organs, in agreement with IHC performed.
Figure 3.3 9: Expression of amylase at the protein level in various organs of NOD/SCID mice after B-13 injections and non injected controls shown by Western blot. Data is in agreement with RT-PCR and IHC that amylase positive cells are present within the livers and pancreata but no other organs.
Although B-13<sup>red</sup> cells carried the DsRed transgene to allow tracking in vivo, DsRed positive cells could not be detected using the ivis imaging system following injection. Thus B-13 cells were tagged using the CellVue® NIR815 labelling agent to allow in vivo tracking of B-13 cells in the whole bodies and organs of injected mice. CellVue® NIR815 is a lipophilic near-infrared labelling reagent, optimally excited at 786 nm and has a peak emission of 814 nm. NOD/SCID mice were injected with Cellvue labelled B-13 cells or PBS for controls and left for 24 hours prior to imaging with the ivis. No Cellvue® NIR815 conjugated B-13 cells were detected in the whole bodies, however following dissection, fluorescence at the near-infrared wavelengths was detected in the liver of the B-13 injected animals only (figures 3.3.10 and 3.3.11). These observations confirm that the B-13 cells are present in the liver following injection, and no other tissues. No Cellvue® NIR815 conjugated B-13 cells were detected within the pancreata, although this could be due to the short 24 hour study which was restricted by the CellVue® NIR815 labelling agent.

Figure 3.3.10: Organs from NOD/SCID mice injected with Cellvue® NIR815 conjugated B-13 cells were visualised under the near infrared wavelength using an in vivo imaging system (ivis). Florescence could be detected in the liver B-13 of injected mice only and not in PBS injected controls. No fluorescence could be detected prior to dissection of the organs. Data is typical of two separate experiments.
Figure 3.3 11: Total radiance detected from the organs of Cellvue® NIR815 B-13 cell injected NOD/SCID mice and non-injected controls. Fluorescence was detected in the liver of injected mice only, suggesting the presence of B-13 cells. Data is typical of two separate experiments and ***P<0.001.
3.3.3 Amylase positive cells within the livers are of B-13 origin

To further confirm that the amylase positive cells observed within the livers of the injected NOD/SCID mice were derived from B-13<sup>rd</sup> cells, fluorescent in situ hybridisation (FISH) was undertaken. As the B-13 cells have been previously shown to be male and thus carry the Y chromosome, (chapter 3.2) a probe designed to specifically hybridise to the rat Y chromosome was used to track B-13 cells within mouse tissues. Figure 3.3.12 shows that the probe successfully hybridised specifically to the rat Y chromosome as positive cells were only visualised in male rat liver and not in female rat liver or male mouse liver. Successful hybridisation with the probe was also established in B-13 cells. FISH and amylase IHC were performed on serial sections of NOD/SCID livers, and in areas where amylase positive cells had been observed, cells positive for the Y chromosome were also detected (figure 3.3.13). Interestingly, more cells positive for the Y chromosome than amylase were within the same area suggesting that the expression of amylase may be fully repressed in vivo.
Figure 3.3 12: Fluorescent in situ hybridisation (FISH) staining performed on rat liver (male and female), mouse liver (male) and B-13 cells. Analysis confirmed that the probe was specific to the rat Y chromosome with positive staining observed in both the male rat and B-13 cells only. No cross reactivity with the mouse Y chromosome was seen.
Figure 3.3 13: FISH and amylase staining in serial sections from B-13 injected NOD/SCID mice livers. Sections where amylase positive cells were located also stained positively for the rat Y chromosome showing that amylase positive cells detected are most likely to be of B-13 origin rather than the hosts own pancreatic cells.
3.3.4 B-13 cells engraft to the liver and pancreas but show hepatocyte-like phenotype in the liver only.

No evidence was found for the engraftment of B-13 cells to other organs, however as only amylase has been used as a marker for B-13 cell engraftment it is not easy to determine whether B-13 cells engrafted to the pancreas. Thus the DsRed transgene expressed by the injected cells was used as a marker (FISH could not be performed in the pancreas due to the digestion steps). Sections from all organs were DAPI stained and observed under the DsRed wavelength. Interestingly DsRed positive cells were observed in both the pancreas and livers of B-13$^{\text{red}}$ injected NOD/SCID mice (figure 3.3.14), however the number of DsRed positive cells detected within the pancreata was limited. Within the liver, DsRed positive cells showed a clear hepatocyte-like morphology which was not evident in cells that engrafted within the pancreas.

Figure 3.3 14: Sections of liver and pancreas from B-13$^{\text{red}}$ injected NOD/SCID mice visualised under the DsRed wavelength show evidence of B-13 cell engraftment. Sections were DAPI stained and visualised under the DAPI and DsRed wavelengths. The expression of the DsRed transgene within pancreas and liver tissue from NOD/SCID was detected in cells within both organs suggesting B-13$^{\text{red}}$ engraftment. No DsRed positive cells were detected in other organs. Images are shown at x40 and x63 magnification.
Chapter 3: Results 3.3

Primers designed to amplify the rat specific transcripts for amylase and the hepatocyte marker CYP2E1 were used to confirm the engraftment of B-13\textsuperscript{red} cells into the pancreata as detected under the DsRed wavelength. Figure 3.3.15 shows that B-13 cells engrafted into both the livers and pancreata of NOD/SCID mice but not in any other tissues. Interestingly the rat specific CYP2E1 was detected within the livers where B-13 cells had engrafted, but not within the pancreata suggesting that \textit{in vivo} B-13 cells are able to transdifferentiate towards the B-13/H phenotype within the liver environment, but remain as pancreatic B-13 cells within the pancreas. These data are supported by the fact that the morphology of the engrafted cells appear hepatocyte-like suggesting a B-13/H phenotype. Furthermore immunocytochemistry for the liver marker albumin was performed and showed DsRed positive cells were also positive for albumin (figure 3.3.16).
Rat specific amylase and CYP2E1 mRNA was detected in the livers and pancreata of B-13<sup>red</sup> injected NOD/SCID mice. Primers designed to amplify rat specific transcripts of amylase and CYP2E1 were used for RT-PCR in organs of NOD/SCID B-13<sup>red</sup> injected mice. Rat amylase mRNA expression was detected within NOD/SCID livers and pancreata only. CYP2E1 was only detected in the livers and no other organs, and was only detected in livers that also showed positive amylase expression, suggesting both engraftment of the B-13 cells and transdifferentiation within the liver to B-13/H cells.

Figure 3.3 15: Rat specific amylase and CYP2E1 mRNA was detected in the livers and pancreata of B-13<sup>red</sup> injected NOD/SCID mice. Primers designed to amplify rat specific transcripts of amylase and CYP2E1 were used for RT-PCR in organs of NOD/SCID B-13<sup>red</sup> injected mice. Rat amylase mRNA expression was detected within NOD/SCID livers and pancreata only. CYP2E1 was only detected in the livers and no other organs, and was only detected in livers that also showed positive amylase expression, suggesting both engraftment of the B-13 cells and transdifferentiation within the liver to B-13/H cells.
Chapter 3: Results 3.3

Figure 3.3 16: DsRed and albumin expression in B-13<sup>red</sup> injected NOD/SCID mice. Immunocytochemistry for the liver marker albumin was performed on livers of B-13<sup>red</sup> injected and PBS-injected NOD/SCID mice. The expression of DsRed was shown to be found in cells also expressing albumin suggesting that B-13 cells had transdifferentiated towards the B-13/H phenotype in vivo.
3.3.5 Chapter discussion

Data presented in this chapter demonstrates for the first time that the B-13 progenitor is able to engraft specifically to the liver and pancreas of NOD/SCID mice. Hepatocyte transplantation has been widely investigated as the need for an alternative to organ transplantation is greatly needed. In humans it has been demonstrated that although possible, engraftment is not easy and producing direct evidence is difficult due to the invasive nature of liver biopsies\[^{214}\]. Studies have shown that transplanted cells do not always engraft directly to the liver structure but instead can survive and function in the portal vasculature where they lead to thrombi \[^{216}\]. In our study there was no evidence for engraftment within the portal vasculature and no evidence of tumour formation. Despite the fact that paracetamol injures the centrlobular region of the liver lobule, (zone 3 of the acinus) B-13 cells were consistently found to be present within the periportal region of the lobule in both control and paracetamol pre-treated mice. Furthermore hepatic engrafted cells were shown to differentiate into hepatocyte-like cells and appeared to completely down regulate amylase expression (which is downregulated but not lost completely in vitro) as they move further from the portal tracts. As B-13s appear to favour engraftment to the periportal region this may be in fact due to a supportive environment such as a stem cell niche, which has previously been reported to exist within the canals of Hering, close to the site of engraftment observed\[^{67}\]. In addition, the expression of amylase was shown to be lost in B-13 cells as they moved into the liver parenchyma, which is in support of the streaming hypothesis where progenitor cells migrate towards the central vein as progressively differentiated daughter hepatocytes\[^{81}\]. However more studies would be needed to clarify whether the local environment is supporting the engraftment and transdifferentiation of the B-13 cells towards functional hepatocyte-like cells.

Another interesting observation is that the B-13 cells were also found to engraft into the pancreas. However within the pancreas, no sign of transdifferentiation towards B-13/H cells was observed. These findings suggest that the in vivo environment in which the cells engraft has an important role in the maintenance of cell identity; within the hepatic environment the hepatocyte phenotype was promoted and within the pancreatic environment the pancreatic phenotype of
the B-13s was maintained. In a study by Jiang et al.\cite{217} it was shown that multipotent adult progenitor cells were able to engraft and differentiate into tissue-specific cells in response to local cues from the organ in which they engrafted. These data are in support with the findings of this chapter and if the mechanisms that drive these changes \textit{in vivo} could be identified then this could have great use in creating functional hepatocytes for transplantation therapies.

Although successful engraftment of the B-13 cells was observed into the liver, engraftment was also observed within the pancreas. In a study by Turner et al.\cite{218} restricted localisation to the liver was achieved with the use of hyaluronan grafts. The grafting strategies use matrix biomaterials that can be gelled into place, which are tailored to the microenvironment thus restricting the desired target tissue. These alternative methods offer possible strategies to reduce engraftment to the pancreas and may allow liver specific engraftment of B-13s only.
3.4 Culture of B-13 cells in 3D bio-artificial liver devices
Chapter 3: Results 3.4

There is currently no treatment for end stage liver disease, and at present the only option is organ transplantation. Recent advances in bioreactor technology bring new opportunities for assisting or replacing failing organs, where they can act as extracorporeal devices until transplantation can be performed\cite{74}. Bioreactors have been developed to provide a 3D environment for cells which aim to create conditions as close to the true cellular origin as possible, and thus support the cells to function as they would in vivo. The bioreactor systems used to support hepatocyte cells in Zeilinger’s lab are composed of a 3D network of three separate interwoven capillary membranes with integrated oxygenation and decentralised mass exchange, which are designed to represent an artificial equivalent of the hepatic vasculature at the level of the liver lobule\cite{192}. Bioartificial liver devices have been shown to support the recovery and maintenance of primary human hepatocytes with relatively stable metabolic activities for longer periods than achieved in 2D culture\cite{74}. After an initial adaptation phase following isolation, metabolic data has shown that hepatocytes can be functionally stable in the bioreactor systems for up to four weeks\cite{219}.

To date, a number of different bioartificial liver support systems have been developed for patients with liver disease and tested in phase I clinical trials\cite{220}. Various clinical treatments with bioreactor extracorporeal devices have been successful, where primary human hepatocytes from discarded transplant organs were used\cite{221}. As the potential of extracorporeal liver support for end stage liver disease is now being realised, it is the lack of available hepatocytes that is now limiting the advance of this new technology. All extracorporeal liver support systems that are currently under clinical investigation are based on the use of human or porcine hepatocytes\cite{222}, and thus an equivalent to human hepatocytes is required. A number of cell lines such as embryonic stem cell-derived hepatocytes, the human HepaRG, and xenogenic or tumour cell lines have been tested, however they carry many drawbacks such as low yields, poor functionality, and zoonotic or malignant potential\cite{223}.

Alternative cell lines are needed to provide a supply of functional hepatocytes, thus the B-13 cell line was cultured to test the hypothesis that ‘dexamethasone
induced generation of B-13/H cells is expected to be possible in 3D bioreactor cultures by adapting the treatment regimen established in 2D cultures’. If transdifferentiation of B-13s towards B-13/H cells can be conducted in 3D cultures then finding the human equivalent would provide a cell line for use in liver bioreactor technology.
3.4.1 Establishing optimal culture conditions for bioreactor cultures

Before seeding of the B-13 cells into the bioreactors could take place, a number of experiments were carried out to find optimal culture conditions. As the bioreactor system is composed of 40 layers of tightly packed capillaries there is a risk that the high levels of proteins in FCS could lead to frothing and/or blockage and therefore failure of the device. A lower FCS concentration of 2.5% rather than the normal 10% was therefore suggested for the bioreactors culture media. In addition to the new concentration of FCS, a higher DEX treatment of 10µM, compared to the standard 10nM, was chosen to promote a quicker transdifferentiation and to reduce the chance of DEX being absorbed by the capillaries before reaching the cellular compartment. To ensure that lower FCS concentrations are still able to support growth and transdifferentiation of the B-13s into B-13/H cells, 2D cultures were set up using the modified media. Cells cultured in 2.5% FCS with 10µM DEX showed a typical B-13/H morphology (figure 3.4.1) suggesting that transdifferentiation can still take place in low FCS containing media, though it was evident that the rate of proliferation was slowed (figure 3.4.2). The first bioreactor experiment was planned for 8 days and so after this period, samples were collected from each treatment for analysis at the mRNA and protein level. Results showed that B-13 cells treated with 10µM DEX expressed a number of hepatocyte-specific markers such as CYP2E1, CPS-1 and albumin confirming that the modified media is still able to support the B-13 transdifferentiation (figures 3.4.3). To confirm whether the CYP450 enzymes expressed were correctly folded and therefore functional a carbon monoxide binding assay was performed. CYP450 enzymes possess characteristic spectral properties due to a non-covalently bound protoporphyrin IX and this haem is able to bind carbon monoxide. When CYP450s are correctly folded and therefore functional, the binding of carbon monoxide yields a spectral peak at approximately 450nm, compared to a spectral peak of 420nm in the presence of a biologically inactive CYP450[^224]. Results from the assay demonstrated the presence of functional CYP450 enzymes with a peak at 450nm after exposure to carbon monoxide (figure 3.4.4). Together, these data show that the proposed media alterations for culture within the bioreactor devices does not affect the cell viability or inhibit the transdifferentiation towards functional B-13/H cells.
Chapter 3: Results 3.4

10% FCS and 10µM DEX

2.5% FCS and 10µM DEX

Figure 3.4 1: Typical cell morphology of B-13 cells treated with 10µM DEX in media containing 10% or 2.5% FCS. Both media types resulted in the appearance of B-13/H cells after 7 days of 10µM DEX.

Figure 3.4 2: Proliferation of B-13 cells in 10% or 2.5% FCS containing media with or without 10µM DEX treatment. Both DEX treatment and 2.5% FCS containing media result in a slower cell proliferation. All cell counts were made on 10 randomly selected fields of view at x20 magnification. Data are expressed as mean of cells per random field of view observed ± SD. **P<0.01, ***P<0.001, and are typical of three separate experiments.
Figure 3.4 3: RT-PCR and Western blot analysis for the expression of liver specific markers after culture with 10% or 2.5% FCS containing media ±10µM DEX. A) RT-PCR analysis comparing the expression of liver specific markers CPS-1 and CYP2E1 at the mRNA level in B-13 cells treated with high DEX (10µM) and low FCS (2.5%), compared to normal conditions of 10nM DEX and 10% FCS. Results shown are typical of three separate experiments. B) Western blot analysis comparing the expression of liver specific markers CYP2E1, albumin and CPS-1 at the protein level in B-13 cells treated with high DEX (10µM) and low FCS (2.5%), compared to normal conditions of 10nM DEX and 10% FCS. Results shown are typical of three separate experiments.
Figure 3.4: Cytochrome P450-carbon monoxide complexes in B-13/H cells and primary rat hepatocytes. A carbon monoxide binding assay indicating the CYP450 functionality in rat hepatocytes and B-13/H cells cultured in media containing 2.5% FCS and 10μM DEX. Peaks can be observed at 450nm suggesting the presence of correctly folded and functional CYP450 enzymes. Results are averages of four separate samples.
3.4.2 Pilot study in bioreactor (BR) 176

A pilot study was carried out to assess whether the B-13 cell line can be cultured and transdifferentiated in response to DEX treatment in the 3D bioreactor environment. A summary of the study can be seen in figure 3.4.5 where the timescale, the length of DEX treatment and the points at which CYP450 assays were performed are indicated. The cells were inoculated into the cell compartment before being left to recover overnight prior to the start of DEX treatment. In contrast to previous bioreactor studies that have used primary hepatocytes, the B-13 cell line is proliferative, and thus the standard cell number inoculated of 150 million, was reduced to 100 million, to avoid overcrowding within the cell compartment. During culture, daily measurements for a number of parameters were taken as described in methods section 2.11.4 and CYP450 assays were performed at the start and end of the bioreactor cultures. For comparison 2D cultures were set up in 6-well plates in parallel to the bioreactor system.

![Diagram showing the timescale of the pilot bioreactor study (BR 176) and the 2D parallel cultures. 100 million cells were seeded into the device 24 hours prior to DEX treatment was started.](image)

During culture a number of parameters were measured to assess the condition and performance of the cells. Figure 3.4.6 shows the lactate release and glucose consumption for both the 2D and bioreactor cultures. Both the lactate production and glucose consumption in the 2D culture was considerably higher than the bioreactor culture suggesting that the cells may have been proliferating at a much quicker rate in 2D. The parameters for metabolism in both cultures rose at a steady rate and therefore the differences in 2D and bioreactor cultures are likely due to the local environment and available space for the cells to grow.
Figure 3.4 6: Cell metabolism of B-13 cells cultured in bioreactor 176 and parallel 2D cultures. Cultures were over 8 days with 10μM DEX. Lactate and glucose levels were measured on a daily basis to calculate the levels of production and consumption respectively.
The amount of LDH, GLDH, AST and ALT released from the cell cultures was measured on a daily basis (figure 3.4.7). The levels detected were found to be at much lower and more stable levels in bioreactor cultures compared to the parallel 2D cultures. The pattern of release also matched that of the cell metabolism data where in bioreactor cultures day 6 showed a peak in all parameters. In 2D cultures, a higher and more varied level of enzyme release was observed, however this may be due to the higher metabolic activity observed in the 2D cultures or due to a quicker rate of transdifferentiation towards the hepatocyte phenotype. To assess whether there were any signs of transdifferentiation towards B-13/H cells the production of urea was measured (figure 3.4.8). Previously, urea has been shown to be an excellent parameter for hepatocyte cell performance as it corresponds to all other evaluated parameters and accordingly is ideal for describing general metabolic activity and function of hepatocytes\(^{[225]}\). No urea was detectable in 2D cultures over the 8 day study, however after just 2 days of DEX treatment urea production was observed in the bioreactor culture. This data suggests that transdifferentiation of the B-13 cell line can occur in the 3D bioreactor environment and form functional hepatocyte-like cells. More importantly, this is the first time that urea production has been shown in the B-13 cell line after transdifferentiation.
Figure 3.4 7: Enzyme release from B-13 cells cultured in bioreactor 176 and parallel 2D cultures. The enzyme release of lactate dehydrogenase (LDH), glutamate dehydrogenase (GLDH), alanine aminotransferase (ALT) and aspartate transaminase (AST), over 8 days with 10μM DEX. Enzyme levels were measured on a daily basis.
Figure 3.4.8: Urea production by B-13 cells cultured in bioreactor 176 and parallel 2D cultures. Cultures over 8 days with 10µM DEX, measured on a daily basis.

Samples of the cellular compartment were collected to assess the cell morphology. H&E staining showed that the cells still displayed a B-13 cell morphology rather than the expected B-13/H morphology after 7 days of 10µM DEX treatment (figure 3.4.9). The cells also appeared very tightly packed together and thus the apparent overcrowding may have restricted their proliferation and transdifferentiation.

DAB immunohistochemistry staining was performed to observe the expression of the liver markers albumin and CYP2E1, as well as the pancreatic marker amylase. Staining revealed that the expression of the liver markers albumin and CYP2E1 were detectable in bioreactor cultured cells, however at much lower levels compared to the parallel 2D cultures (figure 3.4.10). The differences in cell morphology between 2D and bioreactor cultures were also very apparent with clear hepatocyte-like cell morphology observed in 2D cultures only.
Figure 3.4 9: H&E stains of B-13 cells cultured in BR176 shown at x10 and x40 magnification. The morphology resembles that of B-13 cells rather than the expected B-13/H cells suggesting little transdifferentiation has occurred during culture.
Figure 3.4 10: Typical DAB staining for samples from BR176 and parallel 2D cultures. Staining for the hepatocyte markers albumin and CYP2E1 and the pancreatic marker amylase was performed. Rat liver and pancreas tissues were used as positive controls. All images are viewed at x40 magnification.
RT-PCR analysis was performed to look at the expression of liver markers at the mRNA level in samples from both the 2D and bioreactor cultures (figure 3.4.11). The RT-PCR data was somewhat mixed as the expression of some liver markers such as CYP4A1 and CYP2E1 were found to be only expressed in cells cultured in the bioreactor, in contrast other liver specific markers such as CYP2C11 were observed only in the 2D cultures. Interestingly the expression of CYP4A1 was detected in bioreactor cultured cells and not in B-13/H cells suggesting that the 3D environment may enhance expression of hepatocyte-specific enzymes not normally expressed in 2D cultures. However due to the conflicting results between 2D and bioreactor cultures caution should be taken in any review of this data.

Figure 3.4 11: RT-PCR analysis of mRNA samples from BR176 and parallel 2D cultures. Expression of liver specific markers and the pancreatic maker amylase were analysed at the mRNA level. B-13 cells were cultured in both bioreactor and 2D cultures for 8 days with 10μM DEX treatment.
Chapter 3: Results 3.4

In addition to RT-PCR and morphological data, functional data was obtained by carrying out CYP450 assays at the start and before the termination of the bioreactor and 2D cultures. Four different drugs were administered to the culture systems at the same time, before the levels of their respective metabolites were measured. A summary of the drug and the CYP450 activity being assessed can be seen in table 2.5 (material and methods). The data from the CYP450 assay showed that in the 2D cultures all of the drugs were metabolised into the products at 8 days, suggesting that functional B-13/H cells were present (figure 3.4.12). This data is in agreement with the morphological data where hepatocyte-like cells can clearly be seen. In contrast, no CYP450 activity was found in bioreactor cultures as no products were measured. Although the production of urea was detected in the bioreactor cultures and the expression of a number of liver specific markers were found by RT-PCR the morphological and metabolic data demonstrate that little transdifferentiation has occurred. Optimisation of bioreactor cultures for the B-13 cells is therefore needed to promote and enhance the B-13/H phenotype.

Figure 3.4.12: CYP450 metabolism assay of B-13 cells in BR176 and parallel 2D cultures at 8 days. The activity of four different CYP450 enzymes was measured by the conversion of the drugs into their respective metabolites by B-13 cells after 8 days with 10µM DEX treatment (see table 2.5).
3.4.3 Identifying factors affecting B-13 culture in BR176

Before further bioreactor studies can be carried out, reasons why the rate of transdifferentiation was restricted during the pilot run were investigated. From previous bioreactor studies it has been shown that the capillaries, which are made of polyethersulphone, can absorb some chemicals and thus disrupt their transport across the membrane into the cell compartment. The absorbance of DEX into the capillary membrane could perhaps provide an explanation for the low rate of transdifferentiation towards the B-13/H phenotype observed. Three different dexamethasone isoforms (H₂O soluble DEX, 21-Acetate DEX and 21 phosphate disodium DEX) were tested in 2D culture to see whether they can still promote the B-13/H phenotype in the hope that the different isoforms may not be absorbed by the capillaries. B-13 cells were treated with the three different DEX solutions for 7 and 14 days at a concentration of 10µM. RT-PCR analysis showed that all three of the alternative DEX solutions tested were able to drive transdifferentiation of B-13 cells towards a B-13/H cell phenotype, where the liver specific markers CYP2E1, CPS-1 and albumin were detected after both 7 and 14 days treatment (figure 3.4.13). These results show alternative forms of DEX may be used to allow transdifferentiation if the DEX is being absorbed by the capillary membranes within the bioreactor cultures.

To confirm whether DEX was being absorbed into the capillary membranes, media samples, which were collected from both the 2D and bioreactor cultures on days 2, 4 and 6, were sent to Pharmacelsus® for Liquid chromatography–mass spectrometry analysis to calculate the levels of DEX within the media. The media samples collected from the bioreactor were taken after passing through the bioreactor system and so if any DEX was absorbed by the capillaries an obvious difference between the cultures would be observed. Results showed similar concentrations of DEX present in the bioreactor culture and in parallel 2D cultures, indicating that there is no significant binding of DEX in the system (figure 3.4.14). As these data show that the DEX is not absorbed across the capillary membrane it should be acceptable to use the same DEX treatment used in the pilot study for future experiments.
Figure 3.4 13: RT-PCR analysis for the expression of liver specific markers CYP2E1, CPS-1, and albumin, and the pancreatic marker amylase, at the mRNA level in B-13 cells cultured with a range of different 10µM DEX solutions. Data is typical of three separate experiments.

Figure 3.4 14: Comparison of absolute levels of DEX (nM) measured in media samples from BR176 and parallel 2D cultures of B-13 cells on days 2, 4 and 6 of culture.
Another possible explanation for the limited transdifferentiation observed within the bioreactor system was the number of cells inoculated into the device. As previously discussed, the bioreactors are usually inoculated with non-proliferating cells and thus the proliferation of the B-13 cells may have led to overcrowding. To investigate whether the rate of transdifferentiation into B-13/H cells is density dependent, B-13 cells were cultured in T75 flasks with starting numbers of 5, 10 and 15 million. The B-13 cells were DEX treated for 96 hours with total cell counts and the number of B-13/H cell counts made every 24 hours. The rate of transdifferentiation was found to be significantly dependent on the confluency of the B-13 cells, as shown in figure 3.4.15, where lower seeding densities resulted in a much faster appearance of B-13/H cells. Any further bioreactor experiments will therefore need to use a much lower seeding cell number to allow optimal conditions for promoting the transdifferentiation towards the B-13/H phenotype.

Figure 3.4 15: The effect of seeding density on the rate of B-13 transdifferentiation. The percentage of B-13/H cells after 24, 48, 72 and 96 hours of DEX treatment in B-13 cell cultures at different seeding densities was calculated by performing cell counts in random fields of view. Data are expressed as mean of percentage of B-13/H cells observed ± SD. *P<0.05, **P<0.01, ***P<0.001, and are typical of three separate experiments.
3.4.4 Culturing of B-13 and B-13/H cells in a 3D environment

Once the problems from the pilot study had been addressed a number of new bioreactor runs were carried out. A repeat of the 8 day study was ran alongside another bioreactor with an increased culture time of 15 days; both of which had a reduced cell inoculation number of 40 million as opposed to 100 million cells seeded in the pilot study. As B-13 cells showed a reduced rate of transdifferentiation in the pilot study, B-13/H cells which had been DEX treated for 5 days were inoculated into another bioreactor for 15 days. This second approach will allow an assessment of whether transdifferentiation prior to inoculation leads to more functional B-13/H cell cultures or whether transdifferentiation within the device is preferable. Figure 3.4.16 shows a timescale for the bioreactor runs for B-13, B-13/H and rat hepatocyte cells. Primary rat hepatocytes were cultured in a bioreactor as a control bioreactor so that functionality could be directly compared to the B-13 bioreactors. For details on all the bioreactors cultures and their parameters see table 3.4.1.

Figure 3.4 16: The timescale of bioreactor cultures 196-199 and the 2D parallel cultures. Bioreactors were inoculated with either B-13, B-13/H or rat hepatocytes to find optimal bioreactor culture conditions for promoting the B-13/H phenotype.

Throughout the bioreactor runs a number of daily parameters were measured as for the pilot study. Cell metabolism was monitored by lactate production and glucose consumption, which showed similar patterns for each bioreactor (figure 3.4.17). The two bioreactors inoculated with B-13 cells (BR196 and BR197) showed the highest levels of lactate production and glucose consumption which can most likely be explained due to the proliferation of the cells. BR198, which was inoculated with B-13/H cells, showed a steady rate of lactate production and glucose consumption suggesting a stable culture performance. The rat hepatocyte (control) bioreactor showed significantly lower levels of lactate production and glucose consumption with a drop in glucose consumption after 5 days.
Figure 3.4 17: Cell metabolism of bioreactors 196, 197, 198 and 199 over 8/15 days. Bioreactors 196 and 197 were inoculated with B-13 cells, bioreactor 198 with B-13/H cells and bioreactor 199 with rat hepatocytes. Lactate and glucose levels were measured on a daily basis to calculate the levels of production and consumption respectively.
Chapter 3: Results 3.4

For all three bioreactors containing B-13 or B-13/H cells the levels of enzymes LDH, GLDH, ALT and AST released from the cultures were found to be at relatively low and stable levels throughout the whole cultures (figure 3.4.18). These data are in agreement with the pilot study where the bioreactor culture resulted in low enzyme release compared to the 2D cultures. A slight increase in enzyme release was observed in bioreactors 197 and 198 after prolonged culture suggesting cell death may be occurring. For the first 2-4 days of culture the levels of enzyme release in the rat hepatocyte control culture (BR199) was substantially higher most likely due to the stress of cell isolation prior to inoculation.

In bioreactors inoculated with B-13 cells (BR196 and BR197) urea was detected within 3-4 days of DEX treatment and was found to be at levels comparable to the control rat hepatocyte bioreactor (BR199) (figure 3.4.19). The urea production in BR198, which was inoculated with B-13/H cells, was much lower than the B-13 bioreactor cultures. Although no urea was detected on certain days, this may be due to the levels being below the limit of detection rather than the absence of urea production and thus a more sensitive assay would possibly provide more reliable results.
Chapter 3: Results 3.4

Graph 1: LDH activity over culture time for different cell types.

Graph 2: GLDH activity over culture time for different cell types.
Figure 3.4 18: The enzyme release in bioreactors 196, 197, 198 and 199 over 8/15 days. Bioreactors 196 and 197 were inoculated with B-13 cells, bioreactor 198 with B-13/H cells and bioreactor 199 with rat hepatocytes. Enzyme levels of Lactate dehydrogenase (LDH), Glutamate dehydrogenase (GLDH), Alanine Aminotransferase (ALT) and Aspartate transaminase (AST) were measured on a daily basis.
Chapter 3: Results 3.4

Figure 3.4 19: Urea production in bioreactors 196, 197, 198 and 199 over 8/15 days. Bioreactors 196 and 197 were inoculated with B-13 cells, bioreactor 198 with B-13/H cells and bioreactor 199 with rat hepatocytes. Urea levels were measured on a daily basis.

After termination of the bioreactor cultures the morphology of the cells was assessed by H&E staining (figure 3.4.20). A B-13/H morphology was observed in all B-13 bioreactor cultures even after 8 days suggesting that the reduced cell seeding number allowed a more successful transdifferentiation. B-13/H cells inoculated also showed a hepatocyte-like morphology, however some cells appeared enucleated with a rough membrane suggesting cell stress or death. The rat hepatocytes also showed evidence of enucleated cells; however this is not representative for the whole bioreactor as other data clearly shows the cells were still metabolically active. Due to the difficulty of preparing samples for morphological analysis from the bioreactors, the samples used may not be representative of the entire bioreactor culture.
Figure 3.4 20: H&E staining of bioreactors 199, 196, 197 and 198. Bioreactors 196 and 197 were inoculated with B-13 cells, bioreactor 198 with B-13/H cells and bioreactor 199 with rat hepatocytes. Images are shown at magnifications of x40 and x100. All images are representative of typical cell morphology found within the sections.

Further morphological analysis by DAB immunohistochemistry showed the expression of the liver markers albumin and CYP2E1 in both B-13 cell inoculated cultures (BR196 and BR197) and rat hepatocytes (BR 199), however no CYP2E1 was observed in B-13/H inoculated bioreactor (BR198) (figure 3.4.21). Although CYP2E1 and albumin were observed in both BR196 and 197 the intensity of staining for albumin was much stronger for BR197 and a loss of the pancreatic marker amylase was also observed suggesting that the longer culture time allowed a more accomplished transdifferentiation towards B-13/H cells.
### Figure 3.4 21: Typical images of DAB immunohistochemistry for bioreactors 196-199 after 8/15 days culture.

Staining is for the expression of liver markers albumin and CYP2E1 and the pancreatic marker amylase in B-13 cells after bioreactor cultures with no primary controls. Rat liver and pancreas act as positive controls for expression. All images are viewed at x40 magnification.
Chapter 3: Results 3.4

Analysis for expression of liver specific markers at the mRNA and protein level was carried out by RT-PCR and Western blotting respectively (figure 3.4.22). In contrast to the pilot bioreactor results, analysis showed expression of a number of liver markers from cells cultured in all bioreactor cultures. RT-PCR data showed a reduction in expression of liver markers such as CYP2E1 in bioreactor cultured rat hepatocytes compared to primary rat hepatocytes, however this was not confirmed by western blot analysis. To compare fold changes in gene expression real time PCR (qPCR) was performed for the liver specific markers, CYP2E1, albumin, CPS-1, CEBP/β and the pancreatic marker amylase compared to B-13 cells (figures 3.4.23-27). B-13/H cells cultured in 2D showed small fold changes in CYP2E1, CPS-1 and CEBP/β compared to non DEX treated B-13 cells. However the fold changes in liver marker expression from cells cultured in bioreactors were found to be significantly higher. Bioreactor 197 showed the highest up-regulation of liver specific markers with levels of expression higher than rat hepatocytes for all markers with the exception of albumin. These data show that the reduced cell number inoculated into the bioreactors along with the longer timescale has resulted in successful culture and transdifferentiation of B-13 cells in a 3D bioreactor system.
Figure 3.4 22: RT-PCR and Western blot analysis for the expression of liver specific markers in bioreactors 196-199. A) RT-PCR analysis comparing the expression of liver specific markers CYP2E1, CPS-1 CYP2C11 and albumin and the pancreatic marker amylase at the mRNA level in different BR culture approaches. Results shown are typical of three separate experiments. B) Western blot analysis comparing the expression of liver specific markers CYP2E1, CPS-1 albumin and CYP3A1 and the pancreatic marker amylase at the protein level in different BR approaches. Results shown are typical of three separate experiments.
Figure 3.4 23: Fold changes of CYP2E1 mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells. Freshly isolated rat hepatocytes are also shown for comparison. Data are expressed as mean of the ddCt values ± SD. *P<0.05, **P<0.01, ***P<0.001 = significantly different to control B-13 cells (Students t-test, two tailed). Data is typical of three separate PCR runs for each of the bioreactors samples.

Figure 3.4 24: Fold changes of albumin mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells. Freshly isolated rat hepatocytes are also shown for comparison. Data are expressed as mean of the ddCt values ± SD. *P<0.05, **P<0.01, ***P<0.001 = significantly different to control B-13 cells (Students t-test, two tailed). Data is typical of three separate PCR runs for each of the bioreactors samples.
Figure 3.4 25: Fold changes of CPS-1 mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells. Freshly isolated rat hepatocytes are also shown for comparison. Data are expressed as mean of the ddCt values ± SD. *P<0.05, **P<0.01, ***P<0.001= significantly different to control B-13 cells (Students t-test, two tailed). Data is typical of three separate PCR runs for each of the bioreactors samples.

Figure 3.4 26: Fold changes of C/EBPβ mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells. Freshly isolated rat hepatocytes are also shown for comparison. Data are expressed as mean of the ddCt values ± SD. *P<0.05, **P<0.01, ***P<0.001= significantly different to control B-13 cells (Students t-test, two tailed). Data is typical of three separate PCR runs for each of the bioreactors samples.
Chapter 3: Results 3.4

Figure 3.4 27: Fold changes of amylase mRNA detected by real time PCR in different bioreactor cultures 196, 197, and 198 compared to B-13 cells. Freshly isolated rat hepatocytes are also shown for comparison. Data are expressed as mean of the Delta-Delta-Ct (ddCt) ± SD. *P<0.05, **P<0.01, ***P<0.001= significantly different to control B-13 cells (Students t-test, two tailed). Data is typical of three separate PCR runs for each of the bioreactors samples.

Ethoxyresorufin-O-deethylase (EROD) assays were conducted during bioreactor runs to test the functionality of cells through the activity of CYP1A1 (figure 3.4.28). EROD activity describes the rate of the CYP1A1 mediated deethylation of the substrate 7-ethoxyresorufin to form the product resorufin and thus the rate is an indication of the amount of enzyme present. On day 1 no formation of the product was observed in all bioreactor cultures, however by day 8, the B-13/H inoculated bioreactor (BR198) had formation rates that were close to levels of the control rat hepatocytes. At the same time point no activity was found for BR196 and BR197 which may explain why metabolic activity was not observed in the pilot studies after 8 days. By day 15 the metabolic activity in bioreactors inoculated with B-13 cells was similar to levels of BR198 which suggests that longer periods are required for the cells to transdifferentiate and become metabolically functional in 3D bioreactor systems. As a result even longer culture periods may lead to even higher formation rates and hepatic functionality of B-13/H cells.
Figure 3.4 28: EROD CYP450 assay for bioreactors 196, 197, 198 and 199 performed on day 1, day 8 and day 15 of bioreactor cultures. The figure shows the formation rate of the product hydroxyresorufin from 7-ethoxyresorufin O-deethylase in cultures, representing the CYP450 activity within the culture systems. Bioreactors 196 and 197 were inoculated with B-13 cells, bioreactor 198 with B-13/H cells and bioreactor 199 with rat hepatocytes.
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<tr>
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<td>B-13 cells- 40 million /8 days</td>
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<td>BR 197</td>
<td>B-13 cells- 40 million /15 days</td>
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<tr>
<td>BR 198</td>
<td>B-13/H cells- 100 million /15 days</td>
<td></td>
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<tr>
<td>BR 199</td>
<td>Primary rat hepatocytes-150 million /8 days</td>
<td>Cells appeared to die around day 19 after variable pH</td>
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<td>BR 218</td>
<td>B-13 cells- 40 million /22 days</td>
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<td>BR 220</td>
<td>Primary rat hepatocytes-150 million /8 days</td>
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<td>BR 221</td>
<td>B-13 cells- 40 million /5 days</td>
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Table 3.4.1: A summary of the bioreactor cultures carried out with details of the length of culture, cell type and cell number.
3.4.5 Chapter discussion

Data presented in this chapter demonstrates for the first time that the B-13 progenitor cell is able to transdifferentiate in response to DEX within 3D bioreactor systems to form functional B-13/H hepatocyte-like cells. We have shown that low cell numbers and longer culture periods result in the most optimal culture conditions for transdifferentiation into functional B-13/H cells. The high cell numbers of cells cultured within the 3D systems allowed the detection or urea production for the first time, which was previously not detected in standard 2D cultured B-13/H cells. This finding has identified a further essential role of hepatocytes that B-13/H cells are able to perform, making the cell line a suitable candidate for metabolism studies and drug screening. In addition, compared to the control rat hepatocyte cells which lose functionality over time, the B-13 cell has demonstrated an increase in performance over time and it may be possible to culture these cells within bioreactors for extensive periods beyond that achievable in 2D. We have also shown that although B-13/H cells inoculated into the bioreactor are functional and produce urea, after 8 days, their culture performance declines, thus allowing transdifferentiation to occur within the bioreactor device is the best strategy.

Interestingly is has been shown that the longer bioreactor culture of 15 days for B-13 cells resulted in the most functional B-13/H cells with the highest expression of liver markers. Even though after 8 days of culture a number of liver specific markers are detectable, the EROD CYP450 assay only showed activity in cells cultured for 15 days. Therefore it may be possible that the transdifferentiation towards functional B-13/H cells takes longer in 3D, and cultures longer than 15 days may result in B-13/H cells with even higher liver functionality and expression of liver markers.

The bioreactor studies have allowed the functionality of B-13 cells to be further explored and B-13/H cells were shown to perform to similar or higher levels as primary rat hepatocytes in culture. As there is a lack of human hepatocytes available for use in extracorporeal bioreactor devices both in the clinical and laboratory setting, the use of alternative cell sources is the only option. The isolation or creation of a human equivalent to the B-13 cell line would provide an
unlimited supply of human hepatocyte-like cells that could be used both in the clinic to support the treatment of end stage liver disease and for toxicity screening for drugs using bioreactor cultures.
3.5 Culture of human pancreatic acinar cells for the generation of human hepatocyte-like cells
Chapter 3: Results 3.5

The B-13 cell line has shown great insight into the mechanisms of pancreatic to hepatic transdifferentiation in response to glucocorticoids in vitro. The progenitor cell appears to be hypersensitive to glucocorticoids with respect to hepatic differentiation, as the appearance of hepatocytes within the rodent pancreas has been shown to occur in response to physiologically abnormal high concentrations of glucocorticoids\[168\]. Previous work from our lab and other groups has shown that markers of liver gene expression can appear in rodent pancreata \[154, 226-228\] and human foetal acinar cells\[229\]. In addition we have shown that this mechanisms can take place in humans in vivo as tissues from a patient treated with glucocorticoids for at least 20 years was shown to express liver markers at levels comparable to human hepatocytes within the acinar regions of the pancreas\[183\]. We therefore sought to establish whether adult human pancreatic acinar cells are capable of transdifferentiating into hepatocyte-like cells in vitro in response to the glucocorticoid DEX. With the breakthrough of iPS technology in 2006\[4\], cell differentiation and lineage specification is now understood to be a reversible process allowing alternative approaches of generating differentiated adult cells to be explored\[230\]. Utilising the findings from work with the B-13 cell line and translating to human acinar cells will allow us to investigate whether the transdifferentiation of adult human acinar cells to hepatocyte-like cells is a feasible outcome in vitro.
3.5.1 The human pancreatic acinar cell (HPAC) line shows slowed proliferation in response to DEX.

The human pancreatic acinar cell (HPAC) line is a pancreatic adenocarcinoma epithelial cell line derived from a pancreatic adenocarcinoma after engraftment into nude mice. The cell line has been reported to express a functional glucocorticoid receptor (GR), the first reported human pancreatic adenocarcinoma cell line to do so, and in addition cell proliferation has been shown to be suppressed by dexamethasone and other glucocorticoids\textsuperscript{[187]}. B-13 cells also exhibit a slowed proliferation in response to glucocorticoids, and thus it was hypothesised that similarities may exist between B-13 and HPAC cell lines in response to DEX treatment.

HPAC cells were cultured with 10µM DEX or an ethanol vehicle control for 7 days to assess the effects of DEX treatment on cell proliferation. Figure 3.5.1 shows that after 7 days of culture there was a significant difference in cell numbers between HPACs treated with DEX compared to controls. These observations confirm those previously reported\textsuperscript{[187]}. In addition to an inhibited growth, HPACs showed an altered morphology after culture with DEX. Whilst in the control conditions HPACs reached confluency and possessed a fibroblast-like morphology when confluent, cells treated with DEX were tightly packed rounded cells with more distinct cell boundaries (figure 3.5.2). The changes in HPAC morphology observed following DEX treatment suggest that DEX influences more than cell proliferation alone. Analysis of gene expression with and without DEX may provide further insights into the alterations occurring at the transcriptional level.
Chapter 3: Results 3.5

Figure 3.5 1: HPAC cell proliferation with 10μM DEX treatment or ethanol vehicle controls. The average cell number per field of view was calculated using at least 4 separate observations for each treatment on each day.

Figure 3.5 2: Typical morphology of HPACs treated with ethanol vehicle and HPACs treated with 10μM DEX for 7 days. Control HPACs grown under standard cell culture conditions show typical HPAC morphology whereas after DEX treatment HPACs appear rounded and tightly packed with clear cell junctions.
3.5.2 Expression of liver-specific markers is induced in HPACS following DEX treatment and is dependent on activation of the GR.

As morphological changes were observed in HPACs following treatment with DEX, RT-PCR analysis was performed to determine any changes in gene expression. HPACs were treated with 10μM DEX for 7 or 14 days before samples were collected.

Analysis of gene expression at the mRNA level indicated that the liver-specific markers CYP2E1 and albumin were expressed following DEX treatment (figure 3.5.3A). These observations suggest that the HPAC cell line is able to transdifferentiate towards a hepatocyte-like cell in response to glucocorticoids as is observed in the B-13 cell line. To verify if the changes were also taking place at the protein level, Western blots were carried out. Figure 3.5.3B shows that after DEX treatment, no CYP2E1 protein was detected in HPAC cells, however low levels of albumin were observed after 7 and 21 days of culture. Although no protein expression of CYP2E1 was found in DEX treated HPACs by Western blotting, immunocytochemistry showed that CYP2E1 was induced in some cells after 7 days of DEX (figure 3.5.4). Although some cells were shown to express CYP2E1 by immunocytochemistry, only small areas were positive and therefore this may explain why no expression was detected at the protein level by Western blot. These results suggest that although changes in gene expression after DEX treatment occur, HPACs do not up-regulate the expression of hepatocyte-specific genes as convincingly as is observed in the B-13 cell line. The initial observations however are still exciting as they show the possibility of a human equivalent to the B-13 cell line, and may allow the mechanisms of pancreas to liver transdifferentiation to be further investigated with an in vitro model.
Figure 3.5 3: RT-PCR and Western blot analysis for the expression of liver specific markers in HPACs and HPACs treated with DEX. A) RT-PCR analysis comparing the expression of liver specific markers CYP2E1, albumin and CYP3A4 at the mRNA level in HPACs with or without culture with DEX for 7 or 14 days. Results are typical of three separate experiments. B) Western blot analysis comparing the expression of liver specific markers CYP2E1 and albumin at the protein level in control HPACs and DEX treated HPACs for 7, 14 or 21 days. Results are typical of three separate experiments.
As previously reported, the HPAC cell line is the first reported human pancreatic adenocarcinoma cell line to express a functional GR\cite{187}. RT-PCR and immunocytochemistry were performed to confirm the presence of the GR in HPACs in control cultured and DEX treated cells. As glucocorticoids are a member of the nuclear receptor family, RT-PCR was performed for other nuclear receptors known to be involved in regulating the expression of liver specific genes, including pregnane X receptor (PXR), farnesoid X receptor...
(FXR) and constitutive androstane receptor (CAR) to see if they are expressed in HPAC cells. Figure 3.5.5 and 3.5.6 show that expression of the GR was detected in both HPACs and DEX treated HPACs by both RT-PCR and immunocytochemistry respectively. Interestingly treatment with DEX resulted in a higher expression of the GR compared to WT HPAC cells. No other nuclear receptors were detected in the HPAC cultures, suggesting that the effects of DEX treatment observed are dependent on interactions with the GR.

Figure 3.5 5: RT-PCR analysis for the expression of nuclear receptors in HPAC cells. The expression of the nuclear receptors, glucocorticoid receptor (GR), pregnane X receptor (PXR), farnesoid X receptor (FXR) and constitutive androstane receptor (CAR) was carried out in control and DEX treated HPACs. Results are typical of three separate experiments.
Chapter 3: Results 3.5

Figure 3.5 6: Immunocytochemistry for the glucocorticoid receptor (GR) in control and DEX treated HPACs. All images are viewed at x40 magnification and are typical of 3 separate staining experiments.

To further confirm that the changes in morphology and gene expression observed in DEX-treated HPACs are through activation of the GR, HPACs were treated with a range of other nuclear receptor activators including: phenobarbital (PB), rifampicin (RIF) and metyrapone (MET) \[^{231-234}\]. HPACs were treated for 7 days with the various nuclear receptor activators before mRNA analysis was carried out by RT-PCR (figure 3.5.7). Interestingly the induction of CYP2E1 and albumin expression was only observed in DEX-treated HPACs and no other treatment conditions. These results support the hypothesis that induction of liver specific genes in HPAC cells by DEX are likely to be dependent on GR activation.
Figure 3.5 7: RT-PCR analysis for the expression of liver specific markers in HPACs treated with various nuclear receptor activators. HPACs were treated with either 10μM DEX, 1mM PB, 20μM RIF or 200μM MET for 7 days\textsuperscript{[235]}. The expression of liver specific genes, CYP2E1, albumin and CYP3A4 were analysed. Results are typical of three separate experiments.
3.5.3 **Expression of liver specific markers through DEX treatment is inhibited in cocultures with liver myofibroblasts.**

Induction of liver specific genes has been shown to occur upon DEX treatment in HPAC cells, however when comparing to the B-13 cell line the effects observed are minimal. It has previously been shown that the rate of transdifferentiation towards the B-13/H phenotype was enhanced through coculture with liver myofibroblasts. Coculture experiments were therefore set up for HPACs as carried out with B-13 cells to see if the induction of liver specific genes could be enhanced.

During culture the morphological changes in HPACs normally observed during DEX treatment were not seen. Instead in regions where myofibroblasts were growing, large areas could be observed where HPACs had detached (figure 3.5.8). This effect was seen for all cocultures independent of DEX treatment. These observations suggest that the presence of myofibroblasts leads to HPAC death rather than the supportive role hypothesised.

![Figure 3.5 8: Cocultures of control and DEX treated HPACs and liver myofibroblasts. Cells were DAPI stained before being visualised. Images are typical of two separate experiments.](image)

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186
Analysis of the liver-specific markers at the mRNA level was carried out by RT-PCR and revealed that in coculture conditions the treatment with DEX failed to induce the liver-specific marker CYP2E1, however albumin was still detectable (figure 3.5.9). These results show that the enhancing effects of coculture on transdifferentiation observed in B-13 cells is not found in HPACs. In contrast, coculture with myofibroblasts appears to not only have inhibited the induction of CYP2E1 expression but also promoted death of HPACs in proximity to myofibroblasts. These results were further confirmed by immunocytochemistry where CYP2E1 expression was only detected in HPACs treated with DEX not in the presence of myofibroblasts (figure 3.5.10). These results highlight clear differences between the HPAC and B-13 cell lines and thus the mechanisms occurring in the HPACs during liver gene induction need to be investigated further to identify optimal conditions for reprogramming HPACs towards a hepatocyte-like cell.

Figure 3.5 9: RT-PCR analysis for the expression of liver-specific markers in HPAC coculture experiments. HPACs, liver myofibroblasts or cocultures were treated with either 10μM DEX or ethanol controls for 7 days. The expression of liver specific genes CYP2E1 and albumin and GR, amylase and α-sma were analysed. Results are typical of two separate experiments.
Figure 3.5 10: Immunocytochemistry for CYP2E1 and albumin in HPACs and cocultures of myofibroblasts and HPACs ± 10μM DEX. All images are viewed at x40 magnification and are typical of two separate experiments.
Expression of pluripotency inducing factors in adult somatic cells to create iPS cells has transformed approaches in regenerative medicine\cite{4, 20}. Using non viral techniques, adult fibroblasts from both rodents and humans have been converted into iPS cells with ES like morphology using the pCAG2LMKOSimO vector which comprises the coding sequences of the four pluripotency factors c-Myc, KLF4, OCT4 and SOX2\cite{189}. Since B-13 cells express these four pluripotency factors, we used the same approach in HPAC cells to see if the progression towards a more pluripotent state would permit more complete induction of liver-specific genes following DEX treatment.

HPACs were transfected with pCAG2LMKOSimO and pCYL43 and stable transfectants were generated through antibiotic (G418) selection as judged by constitutive expression of IRES-mOrange.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Visualisation of HPAC and pCAG HPAC cells under excitation/emission 549 and 565nm respectively. HPACs and pCAG HPACS treated with or without DEX were cultured and visualised to show successful transfection with the pCAG2MKOSimO vector through detection of IRES orange expression.}
\end{figure}
Chapter 3: Results 3.5

Figure 3.5.12 shows that successful transfection of HPACs with the pCAG2MKOSimO construct resulted in an altered cell morphology. Cells were small and rounded compared to the normal epithelial morphology of WT HPACs. pCAG HPACS were cultured with DEX for 14 days to investigate whether hepatocyte-like cells would more readily appear after constitutive expression of the four pluripotent factors. No change in morphology was observed following DEX treatment, cells remained small and rounded and grew to confluency.

![Figure 3.5.12](image)

**Figure 3.5 12. Bright field images of pCAG HPACS in standard or DEX treated cultures.** Images are shown at x20 and x40 magnification and show typical representations of the cells morphology.

Figure 3.5.13 demonstrates that RT-PCR analysis confirmed pluripotent markers such as SOX2 were highly expressed in pCAG HPACS compared to normal HPAC cells. Treatment with DEX resulted in a loss of SOX2 expression in HPACs, however in pCAG HPACs no loss of SOX2 expression was observed and no induction of the liver specific gene CYP2E1 was found. This observation fits with those found in the B-13 cell line where KLF4, OCT4 and SOX2 expression falls as B-13 cells transdifferentiate towards B-13/H cells and the forced over expression was shown to block differentiation towards B-13/H cells in response to DEX. Thus over expression of the four pluripotency factors
through transfection with pCAG2MKOSimO has resulted in an inhibited expression of liver-specific markers such as CYP2E1 in pCAG HPAC cells following DEX treatment.

The results of the RT-PCR were confirmed by immunocytochemistry where no CYP2E1 induction was observed after treatment with DEX (figure 3.5.14). The expression of the pluripotency factor OCT4 was still highly expressed following DEX treatment. An induction of CYP2E1 and a loss of OCT4 expression can be seen in normal HPAC cells following DEX treatment, however the opposite observations were found in pCAG HPACS where no CYP2E1 was detectable after DEX treatment, and OCT4 was still highly expressed.
Chapter 3: Results 3.5

HPACS no DEX treatment

HPACS + 14 days 10µM DEX treatment
Figure 3.5 14: Immunocytochemistry in HPAC and pCAG HPAC cells with and without DEX treatment. Cells were stained for CYP2E1 and OCT4, no primers are shown. Images are all shown at x40 magnification and are typical of 2 separate experiments.
3.5.5 *Culture of human primary acinar cells.*

In addition to work on the HPACs, primary pancreatic acinar cells were cultured to establish whether adult human pancreatic acinar cells are capable of transdifferentiation into hepatocytes-like cells *in vitro.* Primary human exocrine cells from the fractions that are a by-product of the islet isolation procedure were cultured and the effects of DEX treatment on their morphology and gene expression profile were examined. The exocrine enriched fractions were placed in culture where the cells readily attached to the culture vessels and expanded into a monolayer of proliferative cells.

Over time cultured acinar cells adopted a fibroblastic morphology, an observation previously reported by Lima et al.[236] where cells are thought to undergo epithelial to mesenchymal transition (EMT) and dedifferentiate in culture. Reports have shown that the co-expression of amylase and vimentin at around day 10 confirmed that the cells were undergoing differentiation towards a mesenchymal phenotype[236]. Figure 3.5.15 shows the typical morphology of the acinar cells over 21 days following isolation. Interestingly the dedifferentiation of primary acinar cells was slowed in the presence of DEX, where an epithelial morphology was maintained for around 10 days before fibroblastic cells appeared, compared to around 5 days in control cultures.

![Figure 3.5.15: Typical morphology of human primary acinar cells cultured over 21 days.](image)

Cells cultured in basal media and media containing 10μM DEX were observed over 21 days of culture. EMT resulted in the dedifferentiation of acinar cells towards a fibroblastic morphology which was slowed in the presence of DEX. Images are at x20 magnification and are typical of 4 separate acinar isolations.
RT-PCR analysis revealed that following DEX treatment primary acinar cells were found to express a number of hepatic markers such as CYP2E1 and albumin which were not detectable in cells cultured in basal media (figure 3.5.16). Interestingly the highest levels of CYP2E1 and albumin were found after 7 days of DEX, and not 14, after which cells continued to adopt a fibroblastic morphology and hepatic gene expression was lost. Expression of the pancreatic marker amylase was also shown to be reduced in response to DEX treatment, whilst vimentin expression was gained overtime, suggesting that cells were losing expression of pancreatic markers as cells adopted either hepatic or fibroblastic gene profiles.

Figure 3.5.16: RT-PCR analysis for the expression of liver, pancreatic and fibroblast specific markers in primary acinar cells. Primary acinar cells were treated with either 10μM DEX or standard basal media for 7 and 14 days. The expression of liver specific genes CYP2E1 and albumin, the pancreatic marker amylase and the fibroblast marker vimentin were analysed. Results are typical of four separate cell isolations.
Although a loss of amylase expression was shown, the cultured cells retained some expression of amylase, thus DEX treatment resulted in the co-expression of the acinar marker and the liver specific marker CYP2E1. These findings were confirmed by dual immunohistochemistry staining for CYP2E1 and amylase (figure 3.5.17). Cells that were cultured in basal media showed no CYP2E1 expression whereas cells cultured with DEX for 3 or 7 days showed cells expressing both amylase and CYP2E1, suggesting cells are undergoing transdifferentiation.

Figure 3.5.17: Co-expression of amylase and CYP2E1 in primary acinar cells shown by immunohistochemistry. Primary acinar cells were treated with basal media or 10μM DEX for 3 or 7 days prior to staining. CYP2E1 expression was found in DEX treated cells only, and cells were shown to co-express the pancreatic marker amylase. Images are typical of four separate cell isolations and are shown at x20 magnification.


3.5.6 Chapter discussion

Results in this chapter have demonstrated that the transdifferentiation of pancreas to liver in response to the glucocorticoid DEX can be achieved in adult human pancreatic acinar cells. Although pancreatic to hepatic transdifferentiation has been observed multiple times in rodents both in vivo and in vitro, the observations shown in the HPAC cell line and primary acinar cells may prove a valuable model for studying the mechanisms for this process in human.

The mechanisms involved in the induction of hepatic gene expression in the HPAC cell line still need to be further explored, however initial findings suggest that the role of the GR may play a critical role as has been shown for the B-13 cell line[184]. Further experiments with the glucocorticoid antagonist RU486, which is effective at blocking dexamethasone binding to the GR[233], could be conducted to verify whether the induction of liver-specific genes in HPACs following DEX treatment is dependent on the GR.

DEX acts through the GR[237], which is expressed in the HPAC cell line[187]. However reports have shown that glucocorticoid sensitivity is not only dependent on GR expression but can be modulated by other factors such as type II bone morphogenic receptor (BMPRII) which is thought to influence glucocorticoid sensitivity[238]. Mechanisms of glucocorticoid sensitivity are largely unknown, however they may explain why certain cell lines such as the B-13s transdifferentiate in response to low concentrations of DEX, whereas the induction of transdifferentiation in vivo requires much higher doses. As a transient repression of Wnt signalling had been shown to play an early and critical role in the transdifferentiation of B-13 cells[167], it would be interesting to investigate the activity of Wnt signalling within the HPAC cell line. Elucidating any similarities in the mechanisms will allow a greater understanding of the pancreatic to hepatic transdifferentiation process and allow optimal conditions for promoting the hepatic phenotype to be found. Although similarities have been shown between HPACs and B-13 cells, some differences have also been observed, for example in coculture experiments myofibroblasts promoted transdifferentiation for B-13 cells but inhibited hepatic gene expression in
HPACs. The differences observed highlight the need for caution to be taken when translating findings from rodent to man.

Induction of pluripotent genes resulted in inhibition of hepatic gene expression in response to DEX, which has also been shown in the B-13 cell line\(^{\text{239}}\). It may be possible that following induction of pluripotency, other factors other than DEX may be needed to induce hepatocyte-specific gene expression. Two recent studies reported that ectopic expression of Gata4, HNF1α and foxa3\(^{\text{124}}\) or HNF4α plus one of Foxa1/Foxa2/Foxa3\(^{\text{125}}\) in adult mouse fibroblasts directly reprogrammed cells into hepatocyte-like cells. C/EBPβ has also been shown to be important in hepatic differentiation and was identified as an early marker in B-13 transdifferentiation\(^{\text{163}}\). Thus the ectopic expression of a number of genes involved with early hepatocyte development may enhance the hepatic gene expression in HPAC cells, instead of firstly driving cells towards a pluripotent state.

Although we have shown that induction of liver-specific genes in primary adult human pancreatic acinar cells in response to DEX treatment is possible in vitro, the maintenance of the cells at this point is problematic as they eventually dedifferentiate. The rapid dedifferentiation of acinar cells into a mesenchymal monolayer has been previously reported\(^{\text{236}}\) where genetic lineage studies have confirmed the occurrence of epithelial-mesenchymal transition (EMT). In spite of the delayed dedifferentiation through DEX treatment, hepatic expression could not be maintained; primary acinar cells still eventually go through EMT and lose both acinar and hepatic markers. EMT has previously been shown to play important roles in embryogenesis\(^{\text{240-242}}\) where multiple tissue types and organs are derived from the mesoderm. Recent work by Lima et al\(^{\text{236}}\) has shown that during reprogramming of exocrine pancreas towards β-like cells in vitro inhibition of EMT resulted in an enhanced reprogramming. By using inhibitors of TGF-β1 and Rho-kinase signalling pathways, TGF-β1 inhibitor and ROCK, EMT of the exocrine cells was suppressed and allowed enhanced reprogramming to functional β-cells. These findings suggest that the early inhibition of EMT in primary exocrine cultures, instead of allowing the culture to expand, enables the
maintenance of acinar cell gene expression and may result in enhanced reprogramming towards hepatic gene expression in future cultures.
Chapter 4: General Discussion
Work in this thesis has shown for the first time that adult human pancreatic acinar cells induce the expression of hepatocyte-specific genes in response to glucocorticoid exposure. Although much of the work carried out in this thesis was on the rat B-13 cell line, there seems to be a great deal of overlap between rodents and humans with regards to morphologic and transcriptional changes during pancreatic development \[243\]. We have shown that hepatocyte-like cells appeared in the adult human pancreas in response to glucocorticoids and thus the pathophysiological response to elevated glucocorticoid exposure exists in man\[^{183}\] as in rodents\[^{166}\]. In addition induction of hepatocyte-specific genes has been shown for the first time to be possible \textit{in vitro} in adult human acinar cells as well as the B-13 cell line. Therefore it is reasonable to predict that due to the similar mechanisms that have been shown between rodents and humans, with respect to pancreatic to hepatic transdifferentiation, that a human pancreatic acinar cell line could be identified that would have extensive utility for both bench and clinic.

Glucocorticoids play an important role in cellular differentiation as well as their role in metabolism; during late pregnancy a rise in glucocorticoid levels parallels organ maturation. An example of their central role is shown in lung development where respiratory distress in premature births can be reduced with glucocorticoid based therapies\[^{244}\]. The B-13 and HPAC cell lines both express high levels of GR and this may explain why relatively low concentrations of DEX are required for hepatocyte gene induction compared to \textit{in vivo} studies\[^{166}\]. Although the expression levels of GR may play a central part, other factors have been shown to affect GR activation and sensitivity. For example GR activity has been shown to be modulated by chaperone machinery composed of Hsp70 and Hsp90 chaperones, modulated by their respective co-chaperones, in a complex process which is likely to be in response to the cellular environment\[^{245}\]. In addition GR trans-activation is modulated by physical interactions with an adapter protein within the nucleus; modulator of non-genomic action of the estrogen receptor (MNAR), the nature of this complex is known to be dependent on cell type\[^{246}\]. Lastly tissue glucocorticoid sensitivity has been shown to be modulated by many factors such as BMPRII\[^{238}\], although the mechanisms are still largely unknown, therefore any of these elements could play a role in
inhibition of hepatocyte differentiation *in vivo*. The mechanisms that affect GR activation may be used to explain the observation of the *in vivo* studies in NOD/SCID mice. We have shown for the first time that the B-13 cell line is able to engraft and transdifferentiate into B-13/H cells *in vivo*; however the transdifferentiation was restricted to cells that engrafted to the liver, whereas cells in the pancreas maintained the acinar B-13 phenotype. Despite no glucocorticoid treatment being giving to cells prior to inoculation, transdifferentiation was still observed *in vivo* suggesting that the circulating levels of glucocorticoids were sufficient to promote the B-13/H phenotype within the liver environment, or that glucocorticoids are not required *in vivo*. The high levels of GR expressed in B-13 cells makes them sensitive to glucocorticoids and thus it is not surprising that normal physiological levels *in vivo* are adequate to promote transdifferentiation. However as there was no evidence for the B-13/H phenotype within the pancreas, this implies that other factors may be influencing GR sensitivity and inhibiting B-13/H identity. Since pancreatic tissue is a default state of differentiation for embryonic pancreatic cells capable of being directed towards a hepatic phenotype \(^\text{[247]}\) it is likely this mechanism may allow normal adult cells to transdifferentiate only when exposed to sufficiently high levels of glucocorticoids. In addition glucocorticoids have been shown to play a role on modulating the balance between endocrine and exocrine cell differentiation within the normal pancreas, where pancreatic buds treated with DEX have a decrease in insulin-producing cells and a doubled acinar population \(^\text{[248]}\). As a consequence it is critical that normal physiological levels *in vivo* do not promote the hepatic phenotype. It would be interesting to investigate whether abnormally high levels of circulating glucocorticoids would lead to B-13 transdifferentiation within the pancreatic environment, as hepatocytes have been observed in previous *in vivo* studies after high glucocorticoid exposure \(^\text{[166]}\).

Another interesting finding was the effects of cocultures with liver myofibroblasts on B-13 transdifferentiation *in vitro*. Although the mechanisms have not been fully elucidated the presence of myofibroblasts were found to increase the rate of transdifferentiation towards the B-13/H phenotype by further repression of the Wnt signalling pathway. Liver myofibroblasts, along with liver macrophages, have been identified as key components of the hepatic stem cell niche where
they can direct cell fate\textsuperscript{[249]}. It was reported by Boutler et al\textsuperscript{[203]} that macrophage cells drove hepatocyte regeneration through induction of Wnt3a expression, whilst myofibroblasts promoted biliary regeneration through notch signalling. Although these findings seem to oppose those from the coculture experiments it must be considered that promoting the hepatocyte phenotype from hepatic progenitor cells and inducing hepatic transdifferentiation of a pancreatic progenitor cell are very different events and thus may require different molecular signalling cues. Nonetheless it would be interesting to investigate the effects of coculture with liver macrophages or a combination of myofibroblasts and macrophages on B-13 transdifferentiation. The Wnt and notch signalling pathways play essential roles during acinar cell development and expansion, and sustained Wnt activity promotes proliferation of the cells\textsuperscript{[39, 250, 251]}, therefore a suppression of Wnt signalling may be essential to allow loss of the acinar phenotype before Wnt signalling is re-established to promote the hepatic phenotype.

No increased rate of transdifferentiation was observed in cocultures of HPACs and myofibroblasts, but instead myofibroblasts seemed to suppress induction of hepatic genes. It is important to investigate whether Wnt signalling plays a role in the HPAC cell line during induction of liver specific genes as the mechanisms have yet to be elucidated.

The hepatic stem cell niche is thought to be located within the terminal bile ductules known as the canals of Hering\textsuperscript{[67]} where hepatic progenitor cells are supported by liver macrophages, myofibroblasts and a rich laminin matrix\textsuperscript{[249]}. The laminin matrix is thought to maintain the progenitor cells in an undifferentiated phenotype and forms as a response to liver damage\textsuperscript{[249]}. Although paracetamol was used to induce liver damage, which occurs primarily around the central lobular area, during \textit{in vivo} studies the B-13 cells were found to engraft in portal tract areas, which is in proximity to the proposed hepatic niche. These findings suggest that signals originating from the niche may have influenced B-13 engraftment. Furthermore FISH analysis showed that cells further from the portal tracts lost amylase expression completely which indicates that cells in close proximity to the niche were maintained as progenitors whilst
those further from the portal tracts were more differentiated towards hepatocyte-like cells. These observations give further support to the ‘streaming hypothesis’ where progenitor cells proliferate within the smallest branches of the intrahepatic biliary tract before migrating towards the central vein as progressively differentiated daughter hepatocytes\cite{81}.

In recent years there have been advances in the field of regenerative medicine which have been fuelled by the first findings in iPS cell technology\cite{4}. The knowledge that adult somatic cells do not have a restricted differentiated state but instead are maintained through active gene expression, and thus can be reprogrammed, has allowed researchers to explore the developmental potential and provided new insights into creating cells of specific lineages. In addition to reducing the differentiation pathway needed to drive the cells towards the desired cell type, working with adult-derived cells avoids much of the ethical issues associated with embryonic stem cells. There are still many risks of contamination by undifferentiated pluripotent cells, which could lead to teratoma formation \textit{in vivo}, or the accumulation of genetic abnormalities after cellular reprogramming, which may result in excessive proliferation\cite{252}, and therefore translation to the clinic have been slow.

Stem cells, whether embryonic or adult, should not be considered the only potential source from which to generate hepatocytes; the strategy of cellular transdifferentiation which often does not involve extensive cell proliferation stands out as a feasible mechanism for generating specific cell types. Studies in recent years have shown many examples of somatic cells undergoing transdifferentiation into various cell types through transcription factors and/or a cytokine-dependent manner\cite{230}. iPS technology has inspired researchers to ectopically express combinations of transcription factors in differentiated adult cells, where cell fate can be overcome and the cells are driven towards other cell lineages. Two recent studies reported that ectopic expression of Gata4, HNF1\(\alpha\) and foxa3\cite{124} or HNF4\(\alpha\) plus one of Foxa1/Foxa2/Foxa3\cite{125} in adult mouse fibroblasts directly reprogrammed cells into hepatocyte-like cells. In addition Zhou et al showed that ectopic expression of the transcription factors
Pdx1, MafA and Neurog3 in acinar cells was able to drive cells to adopt a β-cell identity\textsuperscript{253}.

The process of transdifferentiation is generally seen from adjacent areas in the developing embryo such as pancreatic to hepatic\textsuperscript{159} and a greater understanding of this process will allow us to clarify what molecular mechanisms distinguish neighbouring regions. Elucidating the mechanisms of pancreatic to hepatic transdifferentiation at the molecular level will aid in identifying a human equivalent to the B-13 cell line. Many transcription factors have been associated with hepatocyte cells within the pancreas such as HNF-1, HNF-3α, HNF-3β, HNF-4, C/EBPα, C/EBPβ and C/EBPγ rats \textsuperscript{155, 156} which were termed liver-enriched transcription factors (LETFs), these may be involved in the mechanisms underlying the process of transdifferentiation. As C/EBPβ is not expressed in the normal pancreas this transcription factor may play a key role in promoting the hepatocyte phenotype in pancreatic cells, and this has been shown to be induced at an early stage of B-13 transdifferentiation\textsuperscript{157}. Therefore ectopic expression of C/EBPβ along with other liver enriched transcription factors or genes involved in early liver development may drive human pancreatic acinar cells successfully to more functional hepatocyte-like cells.

The ultimate goal of regenerative medicine is to produce healthy human cells for cell-based therapies\textsuperscript{230}. Utilising cellular transdifferentiation allows patients’ own tissues to be used, bypassing immune-rejection related problems\textsuperscript{254} and concerns with regards to ethical issues associated with embryo sourced cells. In addition, if an unlimited supply of hepatocyte-like cells could be produced, they would not only have great utility in the clinic but also in toxicity screening in pre-clinical drug trials.

The B-13 cell line is a powerful \textit{in vitro} model that permits the changes occurring at the individual cellular and molecular levels during pancreatic to hepatic transdifferentiation to be studied. Data presented as part of this thesis has shown that human acinar cells have the potential to transdifferentiate towards the hepatic phenotype in response to glucocorticoids \textit{in vitro}; as has been shown in the B-13 cell line. The challenge however still remains in
isolating and expanding the human equivalent capable of forming fully functional hepatocyte-like cells similar to the B-13/Hs. The HPAC cell line and primary acinar cells will be a great tool for elucidating the mechanisms in human, allowing us to understand the molecular and cellular changes, and support us to move closer to the ultimate goal of creating an unlimited supply of human hepatocyte-like cells.
References


References
References


77. Farber, E., *Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 3'-methyl-4-dimethylaminoazobenzene*. Cancer Res. 1956 Feb;16(2):142-8.
References


References


152. Rao, M.S., V. Subbarao, and D.G. Scarpelli, Development of hepatocytes in the pancreas of hamsters treated with 2,3,7,8-
References


References


References


214. W.H.O (2010) Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks.


References


References


Publications


Presented abstracts


Awards

- EASL registration bursary (2013)
- Newcastle University student travel bursary (2012-13)
- BTS annual meeting full bursary (2012)
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THE HEPATIC PROGENITOR B-13 CELL LINE ENGRAFTS INTO THE MOUSE LIVER AND TRANSDIFFERENTIATES IN VIVO

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**Background and Aims:** The AR42J-B-13 (B-13) cell line is a rat pancreatic acinar cell line capable of transdifferentiating into hepatocyte-like cells (B-13/H) in response to glucocorticoids. The transdifferentiation mechanisms have been shown to be dependent on a transient repression of WNT signalling upstream of an induction of liver enriched transcription factor C/EBPβ\([1,2]\). B-13s thus have great potential *in vitro*, as an unlimited supply of functional hepatocytes, for a wide range of uses such as toxicological screening. Our aims were to investigate whether the progenitor cells can engraft to the liver of severe-combined immuno-deficient (SCID) mice after paracetamol damage.

**Methods and Results:** Cytogenetic analysis confirmed that B-13s carry the Y chromosome thus female SCID mice were dosed with paracetamol to induce liver damage prior to intra-venous injections of B-13 progenitor cells. Immunohistochemistry, for the pancreatic marker amylase was performed in all tissues to determine if any B-13 cells were present. Clusters of amylase positive cells were found within the portal tracts of the liver but not in other tissues and showed hepatocyte morphology. To confirm the origin of amylase positive cells, fluorescent in situ hybridisation (FISH) was performed to probe for the rat Y chromosome and confirmed the presence of the B-13 cells within the mouse livers. Protein expression for the pancreatic marker confirmed the presence of amylase within the livers positive by immunohistochemistry. More interestingly RT-PCR for rat specific amylase and CYP2E1 showed that B-13 cells only engrafted to the liver and pancreas and transdifferentiation was only observed within the liver suggesting that within the liver environment their hepatic-phenotype is promoted.

**Conclusions:** These data show that the B-13 cell line is capable of engrafting to the damaged liver and differentiates into a hepatocyte-like morphology. This ability to transdifferentiate *in vivo* as well as *in vitro* brings new insights into the cell line and the isolation of a human equivalent would have great clinical potential for the treatment of liver disease.

**Reference(s)**

The hepatic progenitor B-13 cell engrafts into the Mouse liver.

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The AR42J-B-13 (B-13) cell line is a rat pancreatic acinar cell line capable of transdifferentiating into hepatocyte-like cells (B-13/H) in response to glucocorticoids. The transdifferentiation mechanisms have been shown to be dependent on a transient repression of the WNT signalling pathway upstream of an induction of the transcription factor C/EBP β[1,2]. Thus B-13 cells have great potential in vitro, as an unlimited supply of functional hepatocytes, for a wide range of uses such as toxicological screening.

We investigated whether the progenitor cells can engraft to the liver of severe-combined immuno-deficient (SCID) mice after paracetamol damage. Female SCID mice were dosed with paracetamol to induce liver damage prior to intra-venous injections of B-13 cells. Immuno-histochemistry, for the pancreatic marker amylase, was performed in all tissues to determine the presence of B-13 cells. Amylase positive cells with hepatocyte morphology were observed within the portal tracts of the liver but not in other tissues. Protein expression for the pancreatic marker confirmed the presence of amylase within the livers. To establish the origin of amylase positive cells, fluorescent in-situ hybridisation (FISH) was performed to probe for the rat Y chromosome and confirmed the presence of the B-13 cells within the liver.

These data show that the B-13 cell line is capable of engrafting to the damaged liver and transdifferentiates into hepatocyte-like cells. This ability to transdifferentiate in vivo brings new insights into the cell line. The isolation of a human equivalent would have great potential in screening for drug and chemical toxicity.

**Myofibroblasts modulate the transdifferentiation of hepatic progenitor cells in vitro**

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**Background**

Fibroblasts are known to regulate the pluripotency of embryonic stem cells (e.g. fibroblast feeder layers) (1). Since liver myofibroblasts secrete a variety of soluble factors (i.e cytokines, chemokines), we hypothesised that liver myofibroblasts would modulate progenitor cell differentiation to hepatocytes.

**Objectives**

To test this hypothesis, liver myofibroblasts stably expressing green fluorescent protein (GFP*) and an hepatic progenitor (B-13) cell (2) stably expressing a red fluorescent protein were generated. Fluorescently tagging cells ensured that the origin (from myofibroblast or progenitor) of any hepatocyte in cultures can be determined. The differentiation of the progenitor cell was then examined in culture alone or in coculture with liver myofibroblasts or in medium conditioned by liver myofibroblasts.

**Methods and Results**

Cocultures of liver myofibroblasts and B-13 cells were set up for 7 days and were treated ±10nM dexamethasone (DEX), a synthetic glucocorticoid which directs B-13 cells into hepatocytes via a modulation of Wnt signalling activity (2). After 7 days of treatment, the numbers of hepatocytes (which were all expressing the red fluorescent protein and were therefore derived from the progenitor cells) was significantly enhanced by the presence of myofibroblasts. An hepatocyte phenotype was observed in 96.43%± 2.14 of the cells derived from the progenitor cells whereas in the absence of myofibroblast cells, only 30.25%±4.3 of the progenitor cells differentiated into hepatocytes. Interestingly, a significant proportion of liver myofibroblasts had undergone apoptosis in the presence of differentiating progenitor cells.

Analysis of protein and mRNA levels confirmed phenotypic observations: myofibroblast marker expression was reduced in cocultures treated with DEX and hepatic markers such as cytochrome P4502E1, carbamoyl phosphate I (HepPar I) and albumin expression significantly enhanced.

The effects of liver myofibroblast coculture on progenitor cell differentiation were lost when coculture was replaced with medium conditioned by liver myofibroblasts. This indicates that cell-cell interactions, rather than soluble factors alone from liver myofibroblasts, play a role in enhancing the differentiation of liver progenitor cells into hepatocytes.

**Conclusions**

These data identify an important role for liver myofibroblasts in progenitor cell transdifferentiation into hepatocytes through cell-cell interactions.

**References**


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