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The differentiation of hepatic stem cells into pancreatic endocrine tissue: the influence of pancreatic mesoderm

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The differentiation of hepatic stem cells into pancreatic endocrine tissue: The influence of pancreatic mesoderm

Tanya Nadine Augustine

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science

Johannesburg, 2007

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I declare that this dissertation is my own, unaided work, submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Tanya Nadine Augustine

11th day of September 2007

ABSTRACT

The use of adult hepatic stem cells for the treatment of diabetes, based both on the close embryological association of the pancreas and liver, and on a putative shared tissue stem cell, has been proposed by a number of studies. This study investigated the capacity of hepatic oval cells to differentiate into pancreatic endocrine cells in the presence of pancreatic mesoderm. The GaIN model of hepatic injury was used to induce oval cell activation in Male Sprague-Dawley rats. A viable and significant oval cell population could not however, be isolated and propagated in culture. In order to continue experimentation, a PHeSC-A2 cell line, derived from normal adult porcine liver, was cultured with quail pancreatic mesoderm in the GFRM-Ham's F12.ITS culture system. Cells demonstrating positive immulocalization of the pancreatic markers, insulin and glucagon, were identified as PHeSC-A2-derived, by visual assessment of their nuclear morphology. Techniques used to confirm these results and preclude the derivation of the pancreatic endocrine cells from pancreatic endodermal contamination, proved ineffectual. The tentative results obtained in this study have lead to the following postulations: firstly, the PHeSC-A2 cell line may possess a higher level of potentiality than previously demonstrated; secondly, this potential may be due to the shared embryological origins of the pancreas and liver, and thirdly, permissive signaling from pancreatic mesoderm may have the capacity to induce the differentiation of hepatic oval cells into pancreatic endocrine cells. Further research is required to confirm the results obtained in this study and to substantiate the aforementioned propositions.

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Mariska van Heerden and Deborah Jones for their support,

And, my Virgil leading me through the Inferno, Beverley Kramer.

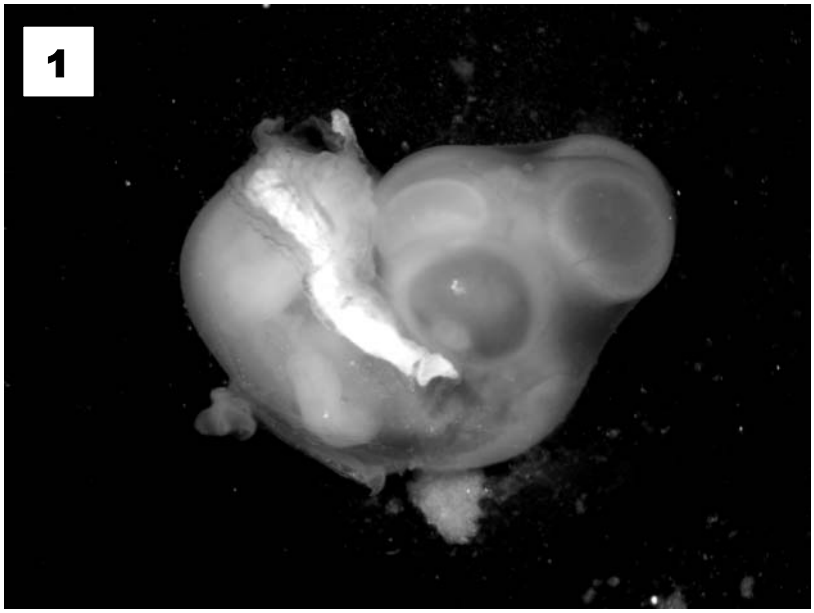
LIST OF ABBREVIATIONS

AAF	Acetylamino Fluorene
AFP	α-Fetoprotein
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenic Proteins
BSA	Bovine Serum Albumin
CCl_4	Carbon Tetrachloride
DAB	Diaminobenzodine
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
FACS	Fluorescent-Activated Cell Sorting
FBS	Foteal Bovine Serum
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
Flt-3 ligand	FMS-like Tyrosine Kinase-3 ligand
GaIN	d-Galactosamine
GFRM	Growth Factor-Reduced Matrigel
GGT	λ-glutamyl transpeptidase
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
HNF	Hepatocyte Nuclear Factors
ICC	Immunocytochemistry
IDX1	Islet Duodenum Homeobox -1
IMDM	Iscove's Modified Dulbecco's Medium
ITS	Insulin-Transferrin-Sodium selenite
LIF	Leukaemia Inhibitory Factor
PBS	Phosphate Buffered Saline
Pdx1	Pancreatic duodenum homeobox-1
PDL	Poly-D-Lysine
PE	Phycoerythrin
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SCF	Stem Cell Factor
TGF-β1	Transforming Growth Factor β1

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Figure 2. A photomicrograph illustrating a quail embryo at stage 25 (Hamburger and Hamilton, 1951), pinned down on to a black wax dish subsequent to the removal of the surrounding membranes. Stereomicroscopy. 60X


1

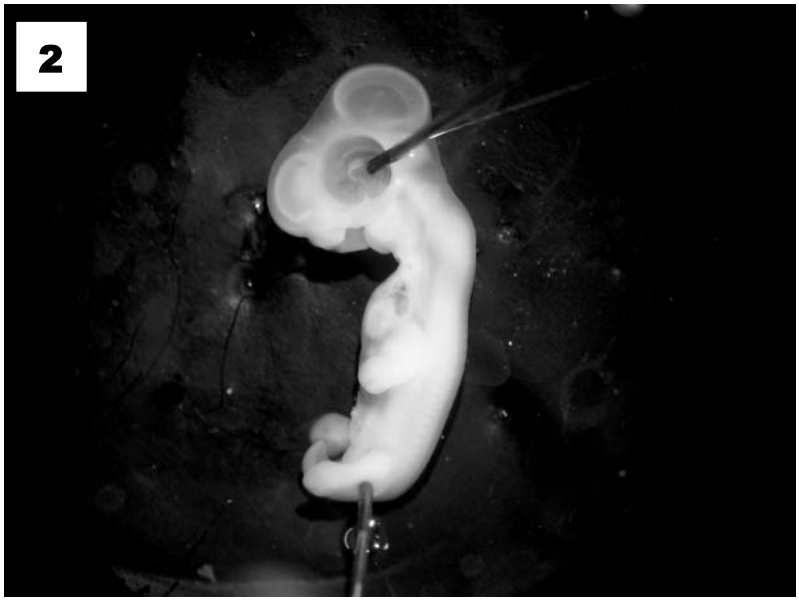
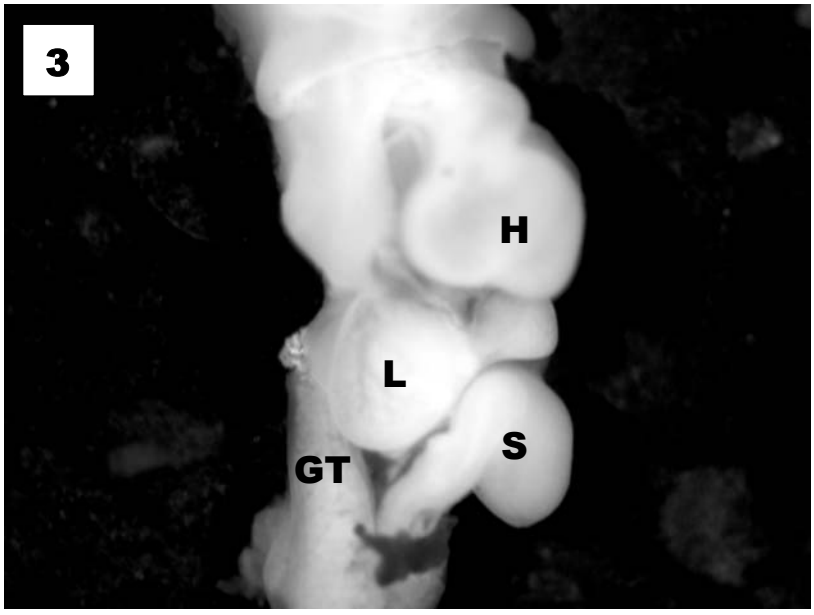

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3
H
L
S
GT

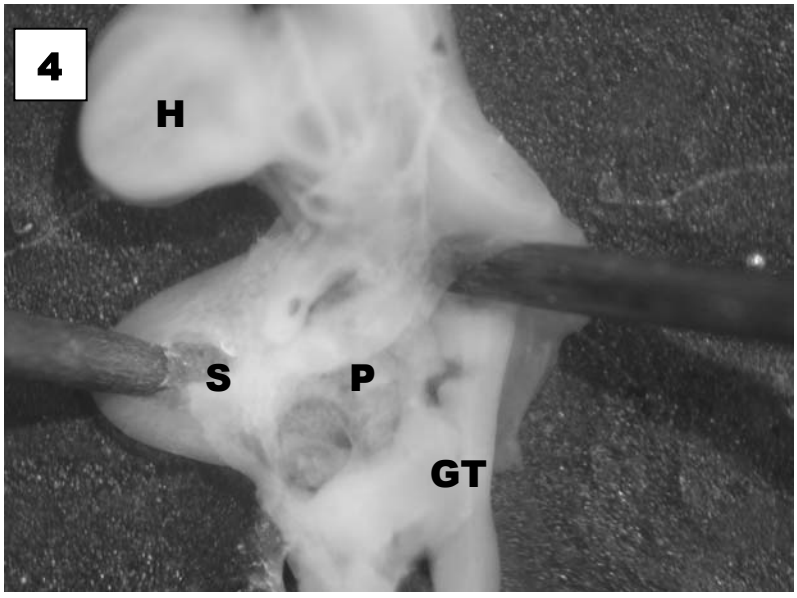
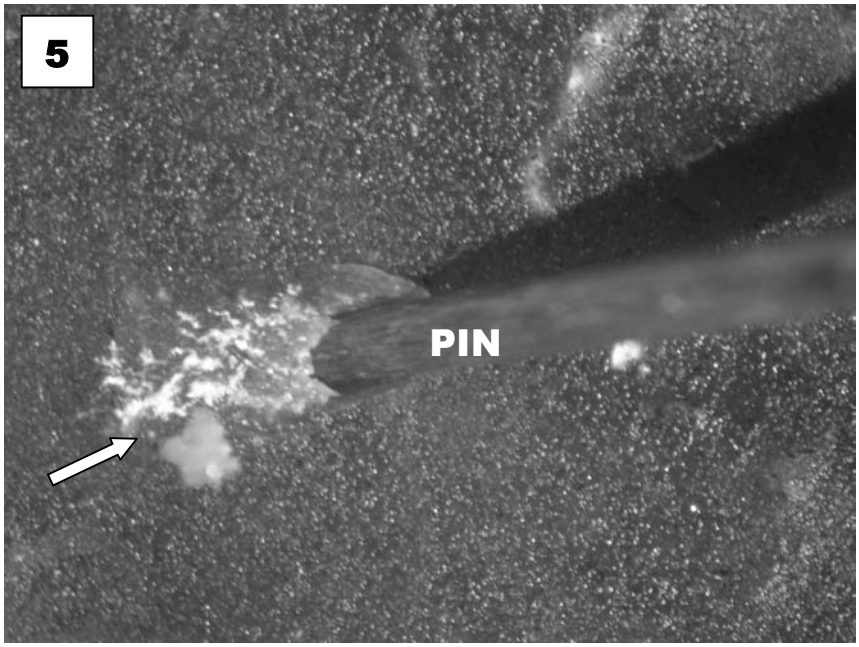

4
H
S
P
GT

Figure 5. A photomicrograph illustrating pancreatic mesoderm (white arrow), microdissected off the endoderm. Stereomicroscopy. 60X. (Courtesy of Roxanne Klopper)

Figure 6. A representative photomicrograph of normal rat liver illustrating the hepatic architecture as viewed using light microscopy. Cords of hepatocytes constitute the bulk of the tissue, radiating outward from the central vein toward the portal tracts (PT). Sections (8μm) were stained with haematoxylin and eosin. 100X.


5
PIN

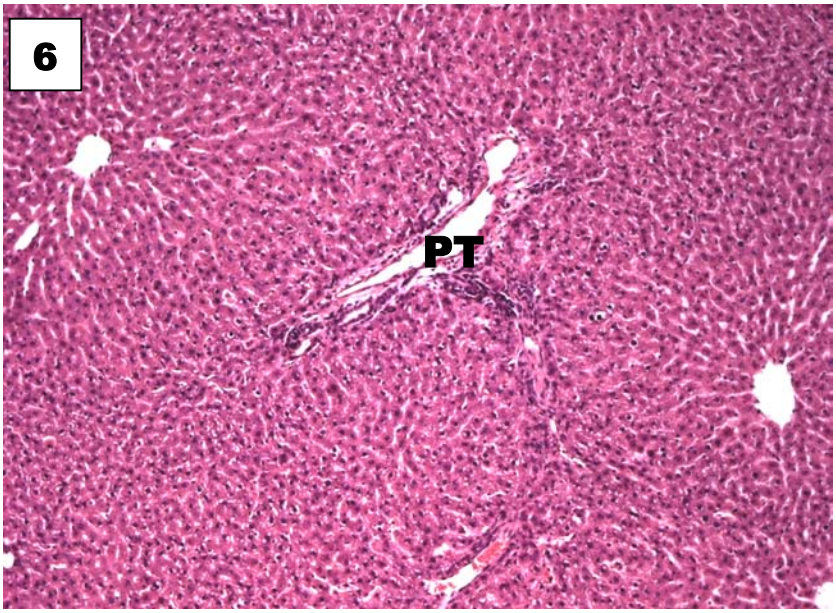
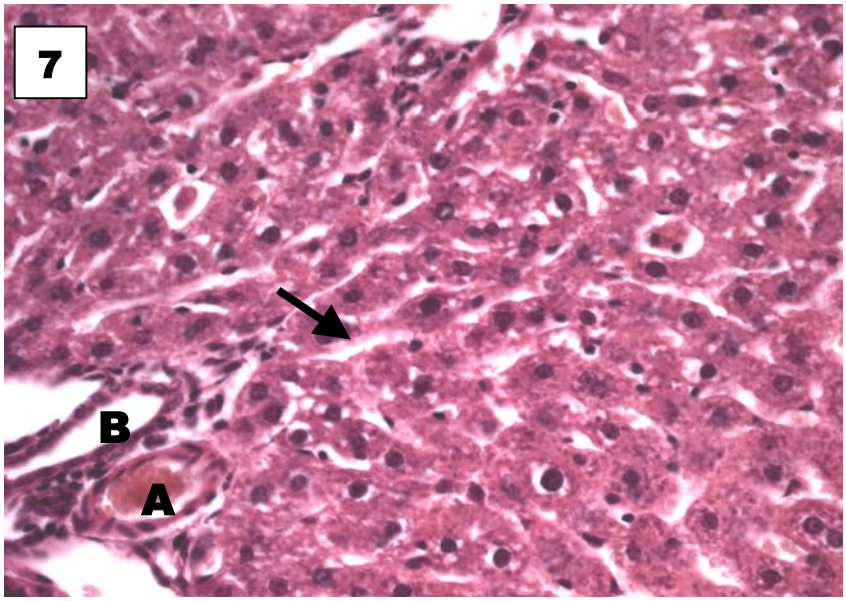

6
PT

Figure 7. A representative photomicrograph of normal rat liver illustrating hepatic architecture as viewed using light microscopy. The polygonal shape of hepatocytes can be noted as well as their arrangement into plates. These plates form the boundaries of the hepatic sinusoids (black arrow). Hepatocyte nuclei vary in size, exhibiting clumps of heterochromatin. A bile duct (B) and arteriole (A), constituents of the portal tract, are illustrated. Sections (8µm) were stained with haematoxylin and eosin. 400X.

Figure 8. A representative photomicrograph of rat liver treated with 40mg GaIN/100g body weight over a 4 day period. A portal tract (PT) is shown. The liver architecture does not differ markedly from that of normal liver illustrated in Figure 6. Hepatocyte cord structure does not appear to be adversely affected. Sections (8µm) were stained with haematoxylin and eosin. 100X.


7
B
A

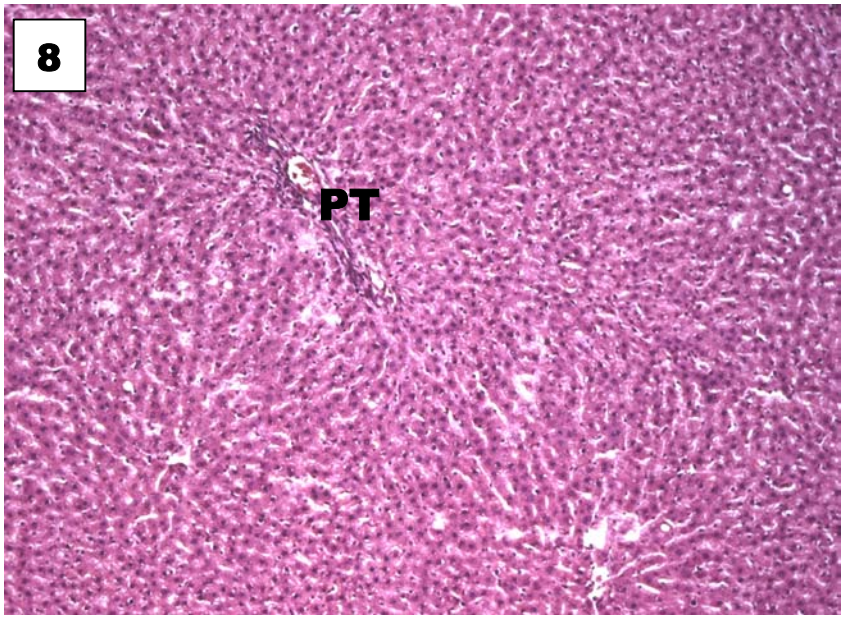
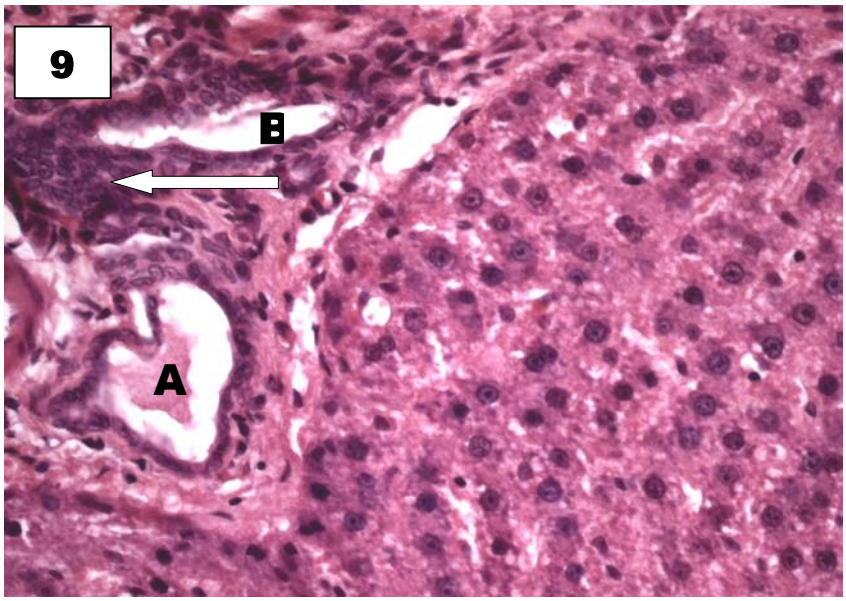

8
PT

Figure 9. A representative photomicrograph of rat liver treated with 40mg GaIN/100g body weight over a 4 day period, illustrating Figure 8 at higher magnification. A bile duct (B) and arteriole (A) have been included in the section. The arrangement of hepatocytes into plates can still be seen and no necrosis of the hepatic tissue is immediately evident. A few proliferating oval cells from areas closely associated with the bile duct can be seen. These cells, identified by the oval, palely basophilic nuclei (white arrow), have not, however, invaded the parenchyma. Sections (8μm) were stained with haematoxylin and eosin. 400X.

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.


9
B
A

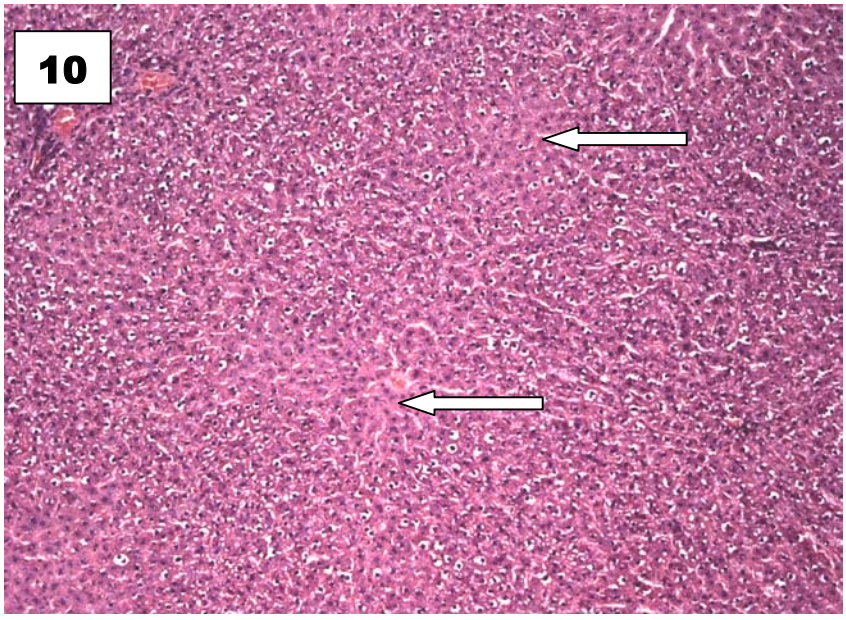
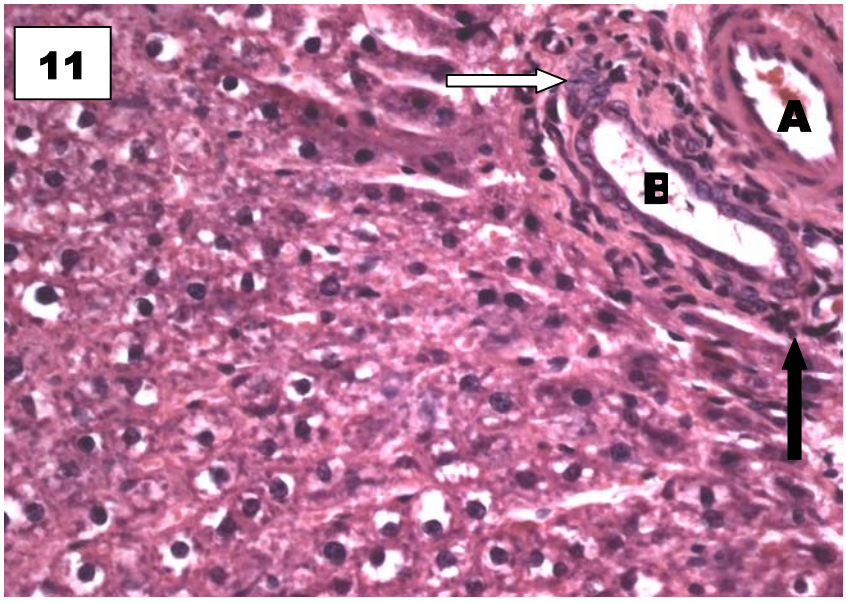

10

Figure 11. A representative photomicrograph of rat liver treated with 50mg GaIN/100g body weight with a duration of hepatic injury over 5 days. At an increased magnification, hepatocyte plate structure as well as cord structure is seen to be disorganized. A bile duct (B) and arteriole (A) are indicative of the position of the portal tract. As illustrated in liver treated with 40mg GaiN over a 4 day period (Figure 9), oval cells exhibiting palely basophilic, oval nuclei and scant basophilic cytoplasm (white arrow), remain closely associated with the bile duct. No oval cells are seen invading the surrounding parenchyma. An inflammatory response (black arrow) can also be seen. The leukocytes appear as small cells with darkly stained nuclei. Sections (8μm) were stained with haematoxylin and eosin. 400X.

Figure 12. A representative photomicrograph of rat liver treated with 70mg GaIN/100g body weight over a 4 day period. A portal tract containing a venule (V), arteriole (A) and bile duct (B), is indicated. The hepatic architecture shows severe disruption with loss of hepatocyte cord structure resulting in a disorganized pattern. Sinusoidal dilation and areas of increased eosinophlia (white arrows), signifying hepatocyte degeneration, were also evident. Sections (8μm) were stained with haematoxylin and eosin. 100X.


11
A
B

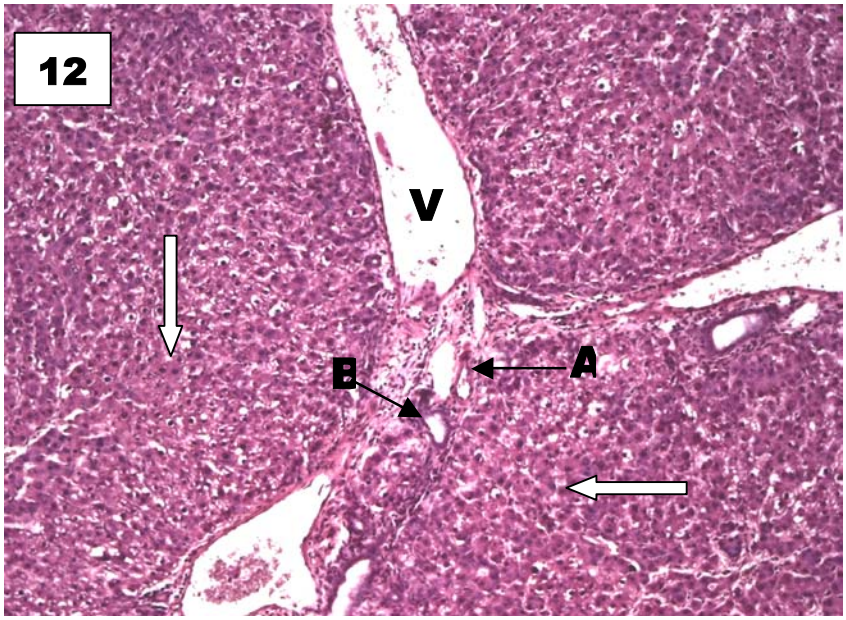
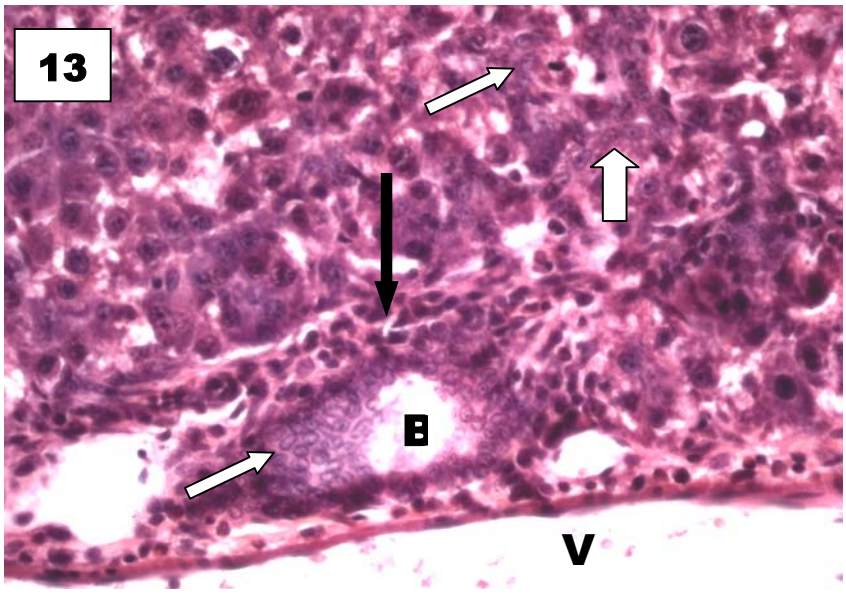

12
V
A
B

Figure 13. A representative photomicrograph of rat liver treated with 70mg GaIN/100g body weight over a 4 day period. A venule (V) and bile duct (B) are illustrated in this micrograph. Proliferation of oval cells (long white arrow), recognized by their small size, elongated shape and palely basophilic oval nuclei, can be seen. These cells have invaded the parenchyma (long white arrow) and have also formed atypical ducts (short white arrow). An intense inflammatory response, as evidenced by the presence of small cells exhibiting darkly stained nuclei (black arrow), is also indicated. Sections (8µm) were stained with haematoxylin and eosin. 400X.

Figure 14. A representative photomicrograph of rat liver treated with 70mg GaIN/100g body weight with a 4 day duration of hepatic injury. Rows of oval cells, exhibiting palely basophilic nuclei and scant basophilic cytoplasm, are seen to have invaded the parenchyma (white arrows). Hepatic sinusoids (short black arrows) appear dilated, while complete disruption of the hepatic cords has occurred. Hepatocyte arrangement has assumed an acinar form. Acidophilic bodies are demonstrated (long black arrows). These are indicative of hepatocyte degeneration, evidenced by the loss of nuclear structure and the increase in cytoplasmic eosinophilia exhibited by a number of cells. Sections (8µm) were stained with haematoxylin and eosin. 400X.


13
B
V

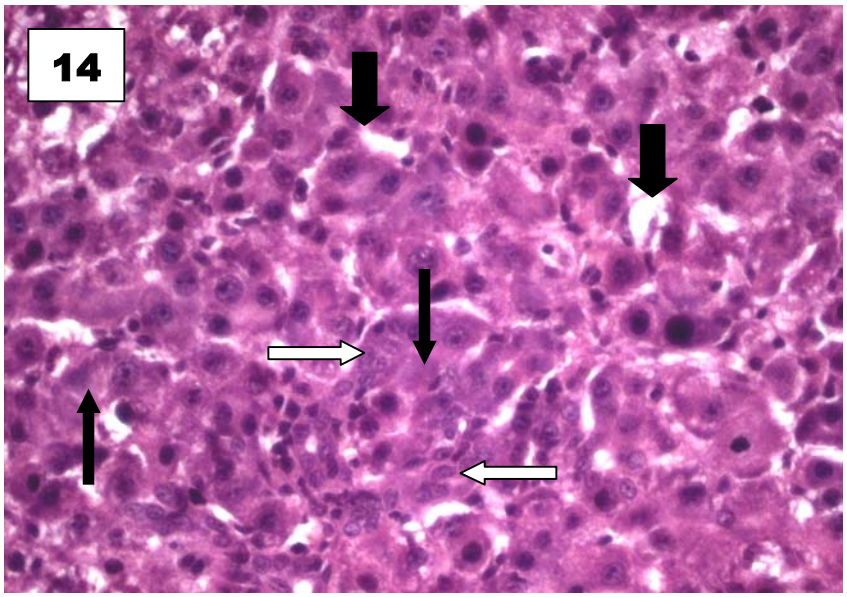
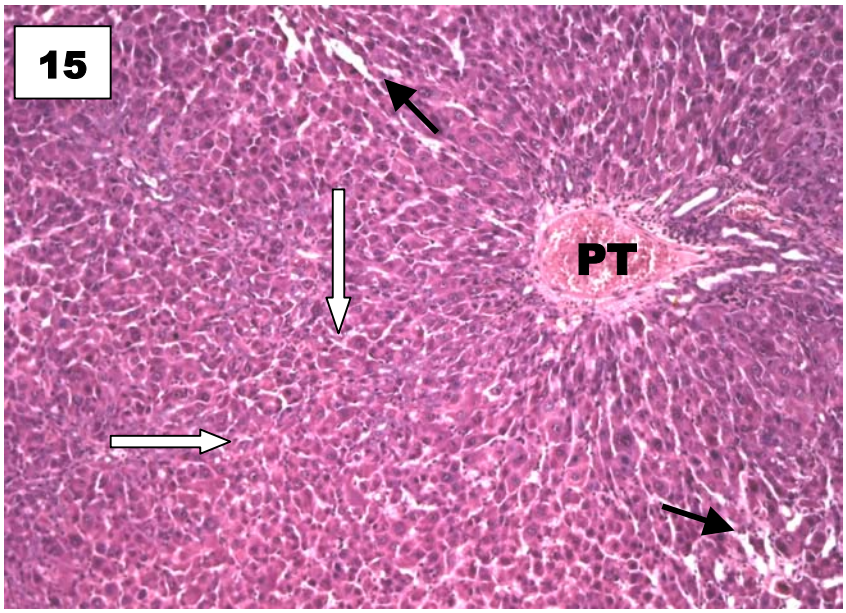

14

Figure 15. A representative photomicrograph of rat liver treated with 70mg GaIN/100g body weight with a 5 day duration of hepatic injury. Hepatocyte cord structure, as exhibited in normal liver (Figure 6), has been lost. The parenchyma exhibits scattered areas of intense eosinophilia, associated with hepatocyte degeneration (white arrows), as well as sinusoidal dilation (black arrows). A portal triad (PT) has been included in the section. Sections (8μm) were stained with haematoxylin and eosin. 100X.

Figure 16. A representative photomicrograph of rat liver treated with 70mg GaIN/100g body weight over a 5 day period. A venule (V), arteriole (A) and bile ducts (B) are indicated. Oval cells are identified by their palely basophilic small, oval nuclei and have come to line the bile ducts (white arrows). Acidophilic bodies (long black arrows) persist with a number of other hepatocytes also showing signs of degeneration. Leukocytes exhibiting small darkly stained nuclei, are prominent (short black arrows). Sections (8μm) were stained with haematoxylin and eosin. 400X.


15
PT

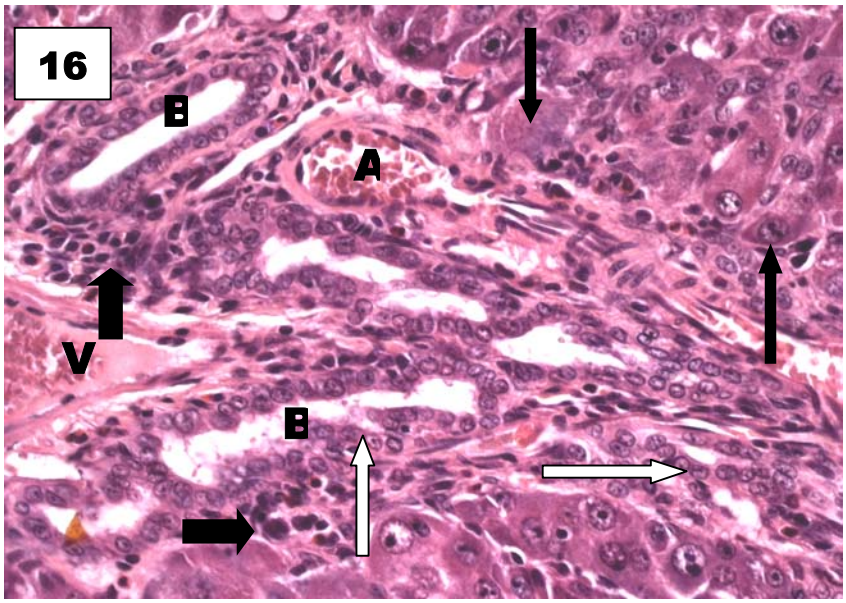
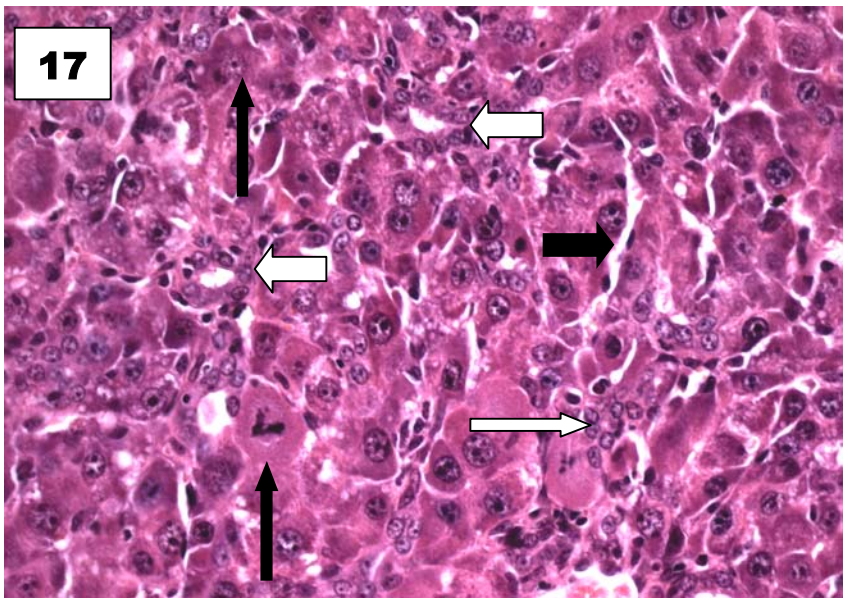

16
B
A
V
B

Figure 17. A representative photomicrograph of rat liver treated with 70mg GaIN/100g body weight with a 5 day duration of hepatic injury illustrating the liver parenchyma. Hepatocyte cord structure has been lost, with the hepatocytes appearing to form acini. Sinusoidal dilation (short black arrow) is evident. Degenerating hepatocytes exhibiting intensely eosinophilic cytoplasm and destruction of nuclear structure are demonstrated (long black arrows). Cords of oval cells (long white arrow) are noted as well as the formation of atypical ductular structures by these cells (short white arrows). Sections (8μm) were stained with haematoxylin and eosin. 400X.

Figure 18. A photomicrograph illustrating PHeSC-A2 cells in the PDL-NAIR-1 culture system, 1 day post-seeding. The cells have adhered to the substrate and assumed a stellate morphology. Phase contrast microscopy. 200X.


17

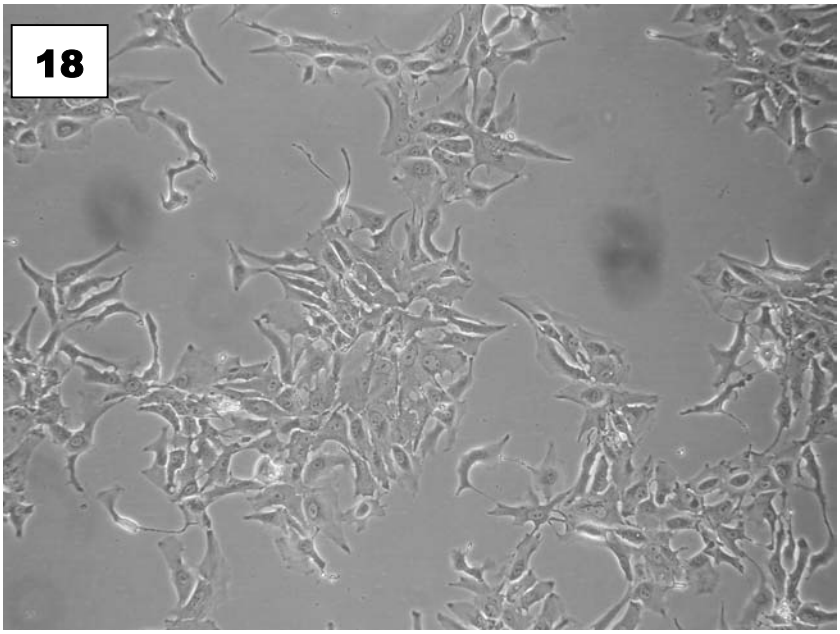
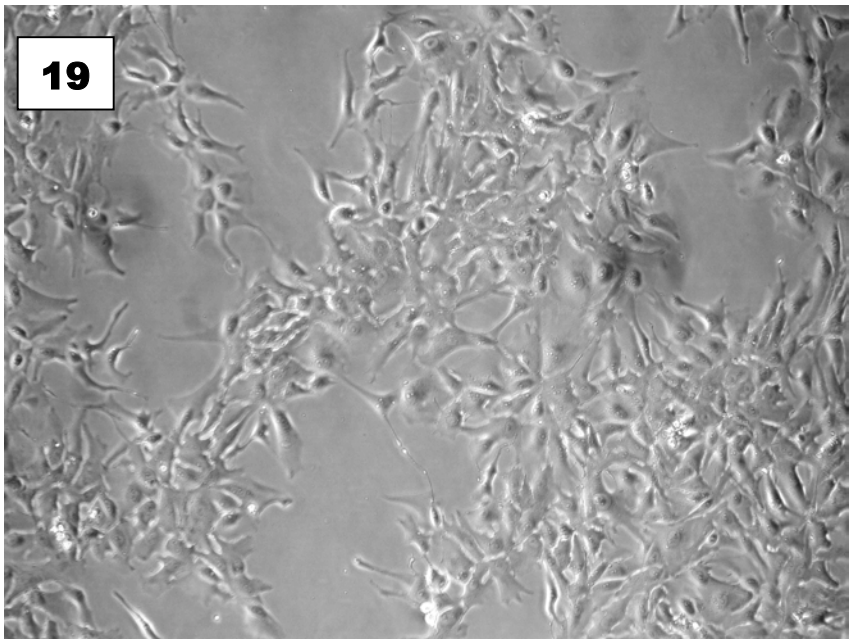

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Figure 19. A photomicrograph illustrating PHeSC-A2 cells in the PDL-NAIR-1 culture system, 3 days post-seeding. The cells have retained their stellate morphology and have proliferated, filling the spaces. Phase contrast microscopy. 200X.

Figure 20. A photomicrograph illustrating the co-culture of PHeSC-A2 cells with quail pancreatic mesoderm in the GFRM-Ham's F12.ITS system, on the second day of culture. The oval cells (C), were placed in close association with two pancreatic mesodermal explants (M). Proliferation of the mesodermal cells at the periphery of the explants is indicated (black arrows). Phase contrast microscopy. 40X.


19

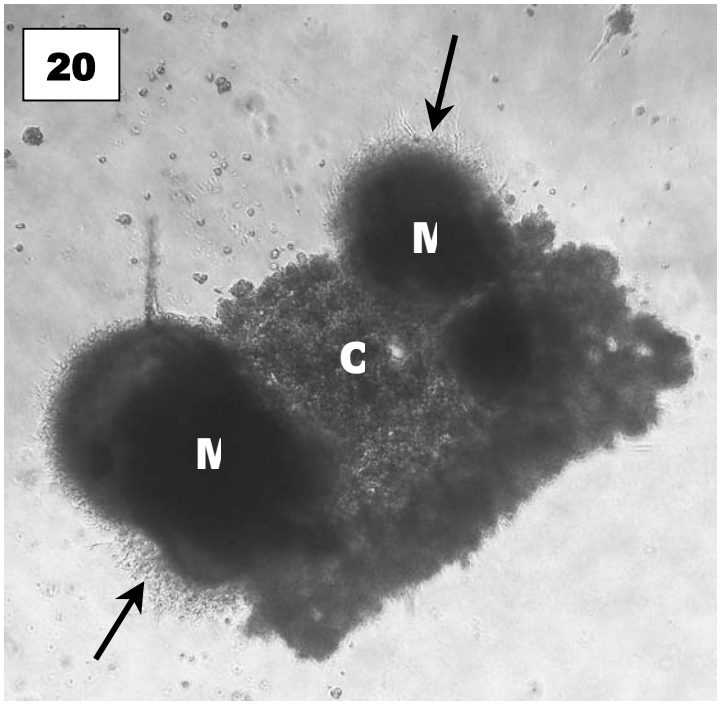
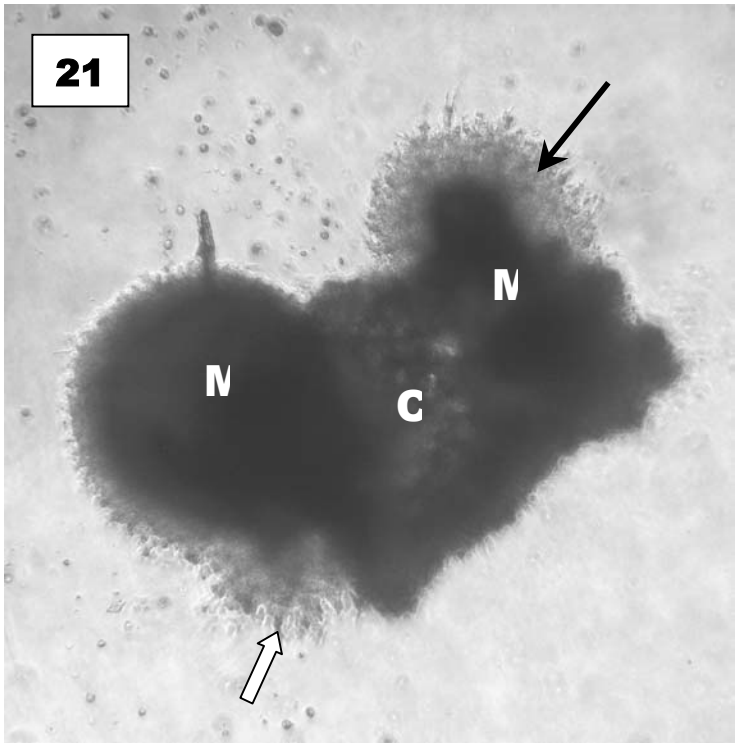

20
M
C
M

Figure 21. A photomicrograph illustrating PHeSC-A2 cells and quail pancreatic mesodermal explants in the GFRM-Ham's F12.ITS system, on day 4 of culture. Areas of proliferation of mesodermal cells can be seen (black arrow). Other areas of proliferation (white arrow) occur at the junction of mesoderm (M) and PHeSC-A2 cells (C) and it is unclear whether the former, latter or both components are undergoing proliferation. Furthermore, the oval cell area appears denser than that viewed on day 2 of culture (Figure 20). Phase contrast microscopy. 40X

Figure 22. A photomicrograph illustrating the culture of PHeSC-A2 cells in GFRM-Ham's F12.ITS system without pancreatic mesoderm (control), on day 2 of culture. Cells (C) remained grouped together following seeding on to the substrate. This clustering contrasts with PHeSC-A2 cells grown in the PDL-NAIR 1 system (Figure 19). Proliferation of cells was not evident in the GFRM-Ham's F12.ITS system. Phase contrast microscopy. 40X.


21
M
M
C

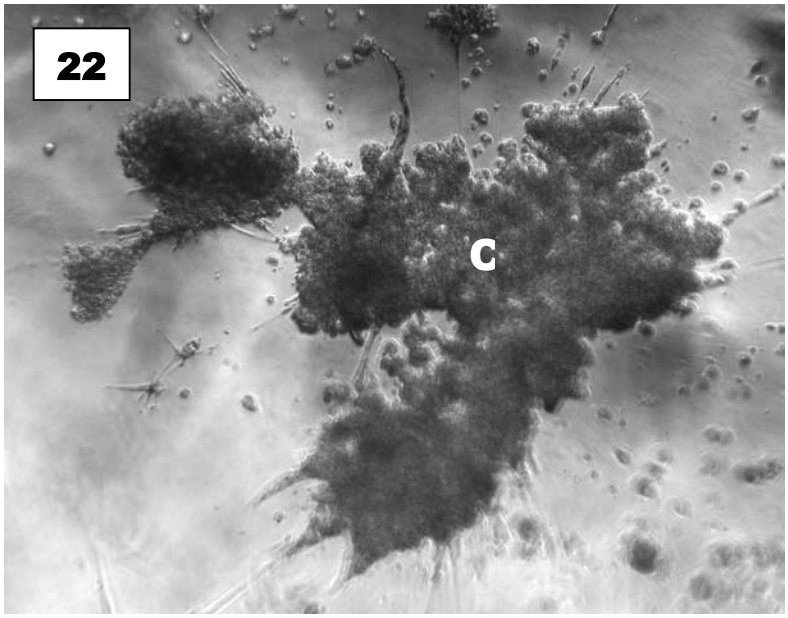
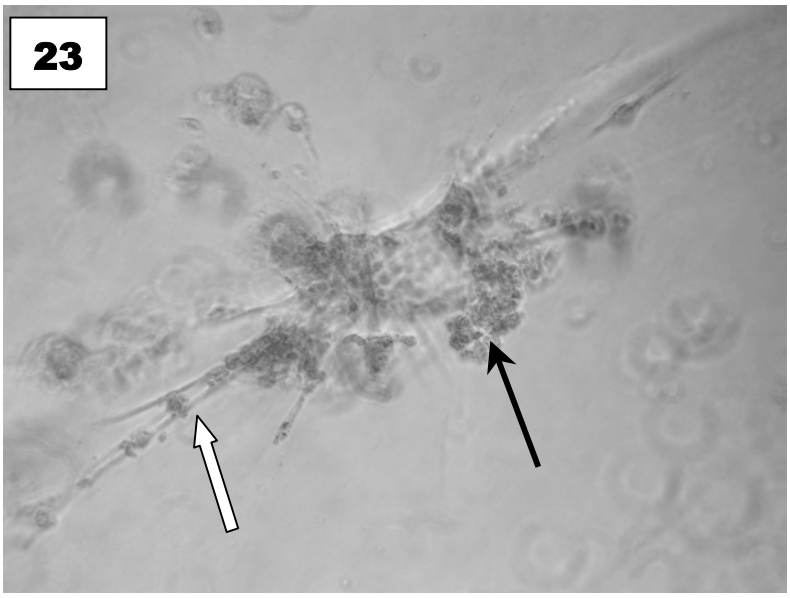

22
C

Figure 23. A photomicrograph illustrating the culture of PHeSC-A2 cells in GFRM-Ham's F12.ITS system without pancreatic mesoderm (control), on day 2 of culture. This illustrates Figure 22 at a higher magnification. A number of cells have clustered together (black arrow). Some cells have assumed a more spindle-shaped morphology (white arrow). This morphology is not comparable to PHeSC-A2 cells cultured in the PDL-NAIR-1 system (Figure 19). Phase contrast microscopy. 400X.

Figure 24. A photomicrograph illustrating PHeSC-A2 cells cultured on PDL-coated flasks using Ham's F12.ITS (control). The morphology of these cells is visually similar to cells propagated in NAIR-1 medium (Figures 18 and 19). Phase contrast microscopy. 40X.


23

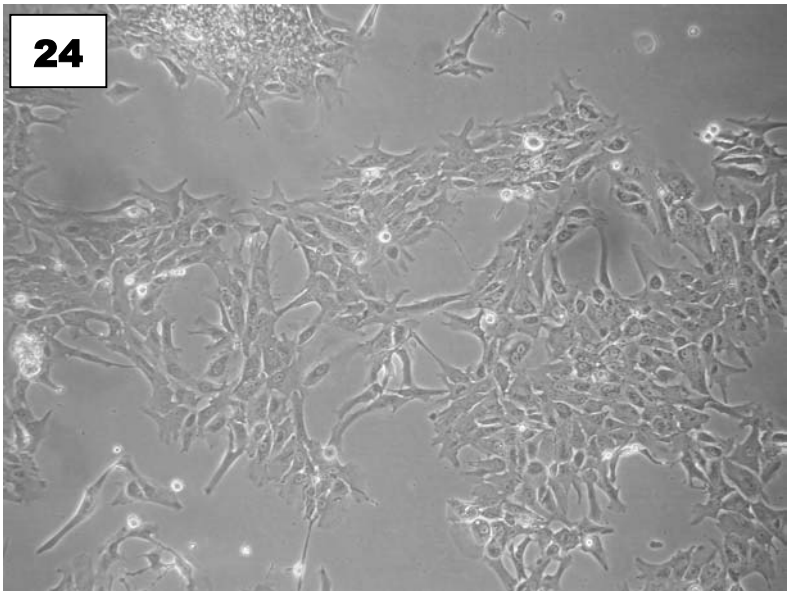
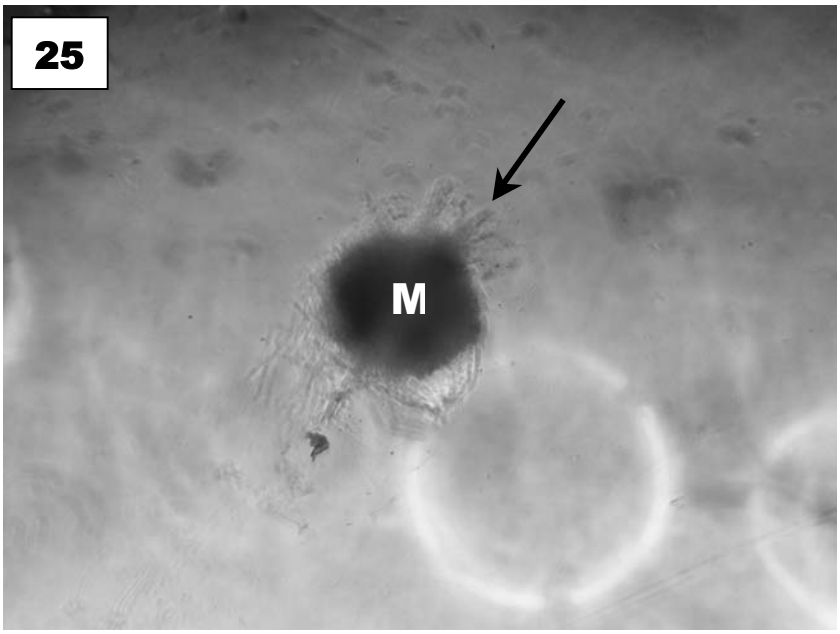

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Figure 25. A photomicrograph illustrating quail pancreatic mesoderm (M) cultured without oval cells in the GFRM-Ham's F12.ITS system (control), on day1. Putative proliferation of mesodermal cells can be noted at the edges of the explant (arrow). 40X.

Figure 26. A photomicrograph illustrating quail pancreatic mesoderm (M) cultured in the absence of oval cells in the GFRM-Ham's F12.ITS system (control), on day 2 of culture. Mesodermal cells can be seen invading the surrounding GFRM, extending laterally (arrows). This may be due to both proliferation and dispersal of cells from the explant. Phase contrast microscopy. 40X.


25
M

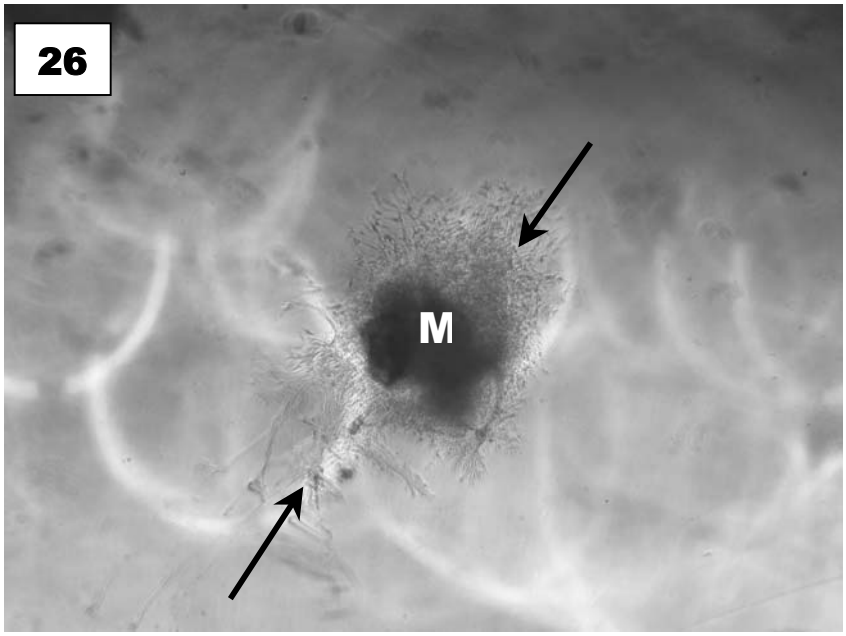

26
M

Figure 27. A representative photomicrograph of the immunolocalization of glucagon conducted on serial sections of chick pancreatic control tissue. Glucagon-positive cells, visualised brown with DAB, are located toward the centre of the pancreatic islet. Differential interference contrast microscopy. 400X.

Figure 28. This photomicrograph illustrates a negative control for glucagon localization conducted on serial sections of PHeSC-A2 and quail mesoderm co-cultures (See Figure 37 for immunolocalization of glucagon in adjacent section). No immunolocalization of glucagon is exhibited since the secondary antibody was omitted and replaced with Tris-saline buffer. Furthermore, no non-specific staining or background staining is evident. Differential interference contrast microscopy. 400X.


27

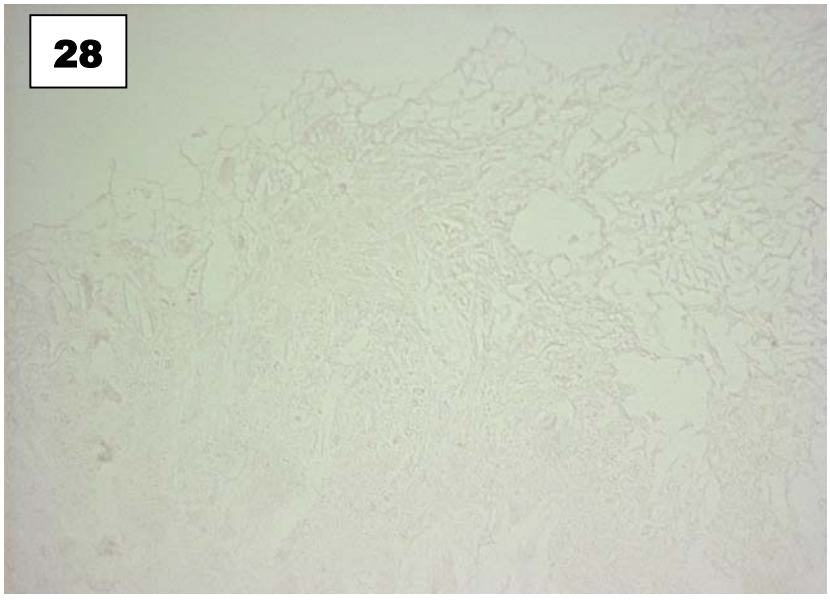

28

Figure 29. A photomicrograph illustrating an absorption control for glucagon localization in chick pancreatic tissue. No glucagon localization is evident since the glucagon antibody and its respective antigen were preabsorbed prior to the commencement of the ICC protocol. Differential interference contrast microscopy. 400X.

Figure 30. A representative photomicrograph demonstrating immunolocalization of insulin conducted on serial sections of chick pancreatic tissue. Insulin-positive cells, visualised brown with DAB, are confined to the pancreatic islets, more specifically, to the periphery of those islets. Differential interference contrast microscopy. 400X.


29

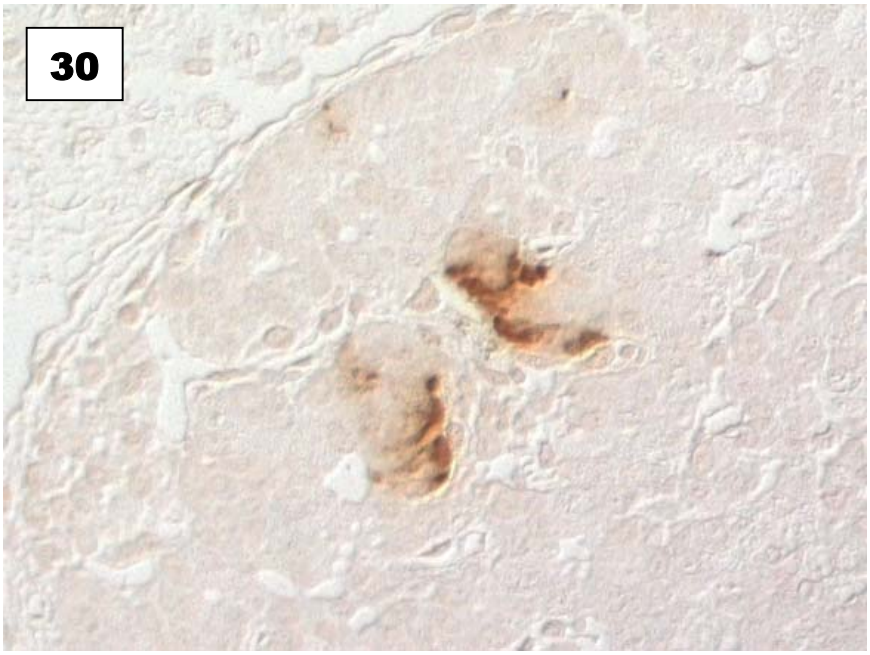
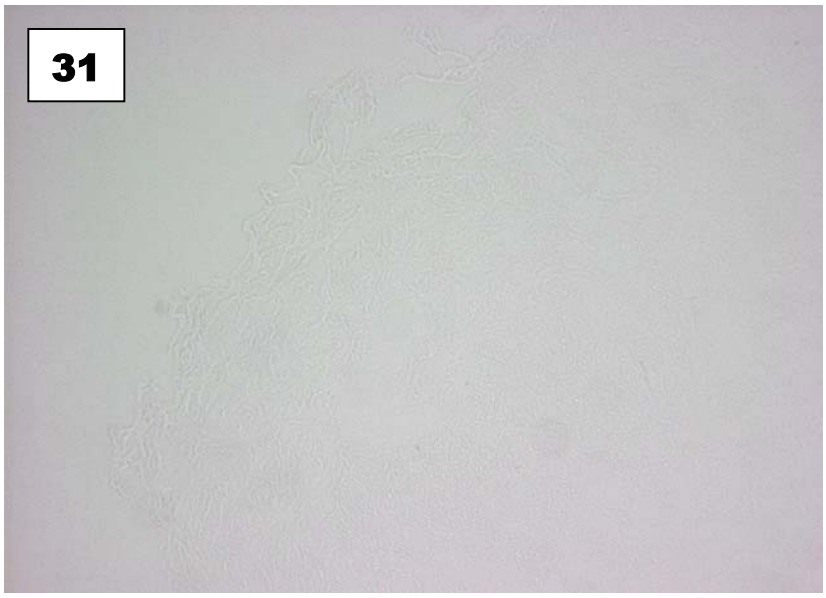

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Figure 31. This photomicrograph illustrates a negative control for insulin localization conducted on adjacent sections of PHeSC-A2 and quail pancreatic mesoderm co-cultures (See Figures 35 for immunolocalization of insulin in adjacent section). No localization of insulin is evident since the secondary antibody was omitted and replaced with Tris-saline buffer. Differential interference contrast microscopy. 400X.

Figure 32. A representative photomicrograph demonstrating an absorption control for the immunolocalization of insulin in chick pancreatic tissue. The insulin antibody was preabsorbed with its respective antigen overnight, prior to commencement of the ICC technique. No immunolocalization of insulin was thus evident. Differential interference contrast microscopy. 400X.


31

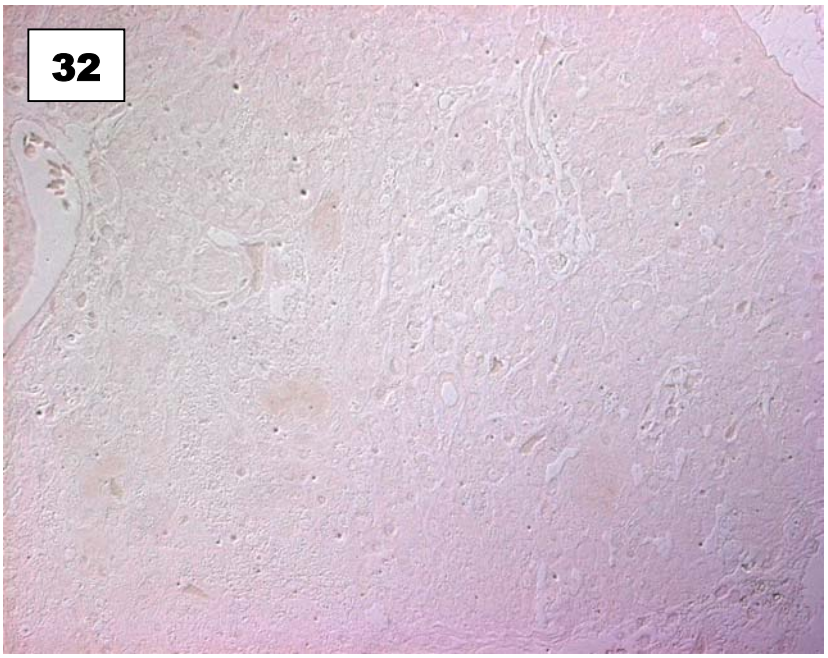
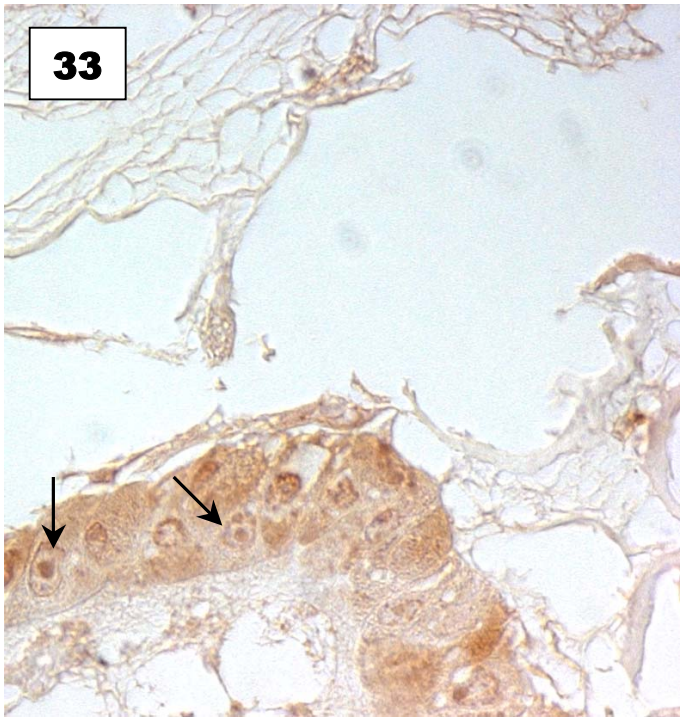

32

Figure 33. A photomicrograph illustrating quail mesodermal cells exhibiting a light background stain with DAB. Quail cells are easily distinguished by their large clear nuclei and prominent nucleoli (arrows). No insulin immunolocalization is exhibited, contrasting with insulin positive cells in chick pancreatic tissue as demonstrated in Figure 30. Differential interference contrast microscopy. 1000X.

Figure 34. A photomicrograph demonstrating the immunolocalization of insulin in PHeSC-A2 and quail pancreatic mesoderm co-cultures. Granules evident in the cytoplasm of insulin-positive cells are highlighted (arrows). These cells contrast dramatically with those of the quail mesodermal cells seen in Figure 33. It is thus proposed that the insulin-positive cells were derived from PHeSC-A2 cells. Differential interference contrast microscopy. 1000X.


33

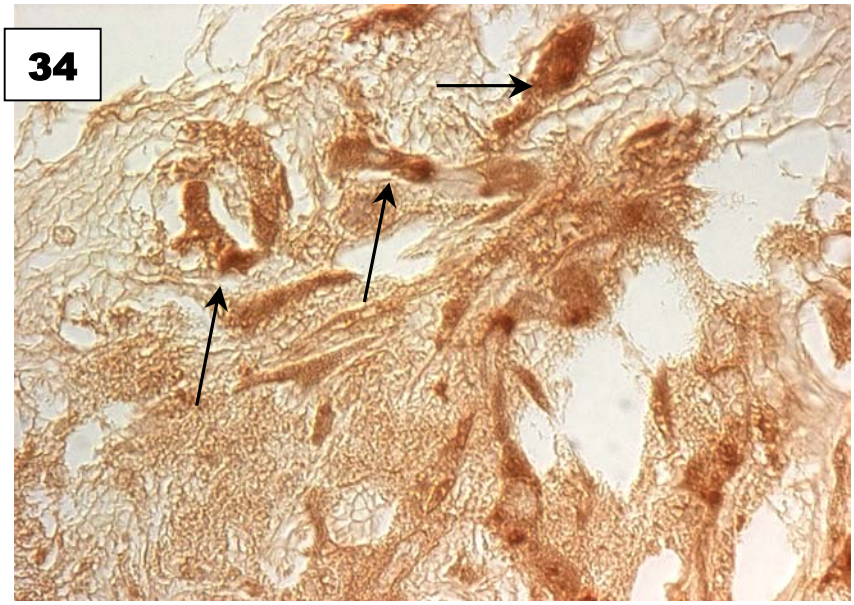

34

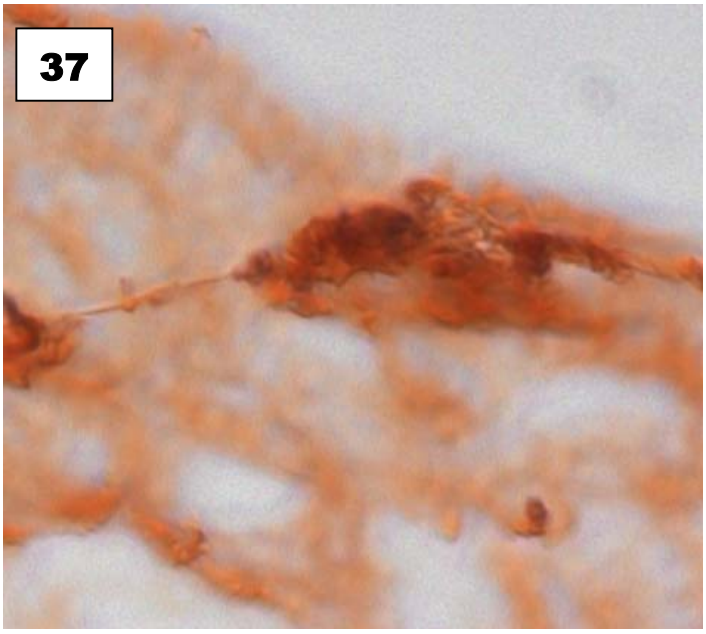
Figure 35. This photomicrograph illustrates a PHeSC-A2 and quail pancreatic mesoderm co-culture. Slight background staining is apparent in the GFRM (white arrow). Immunolocalization of two insulin-positive cells is evident (black arrows). Cell nuclei, seen as raised structures, are small and oval. The surrounding cytoplasm appears granular. This is indicative of insulin cells, moreover, their morphology when contrasted to quail mesodermal cells (Figure 33) implicates their origin from PHeSC-A2 cells. Differential interference contrast microscopy. 400X.

Figure 36. This photomicrograph illustrates insulin immunolocalization in a PHeSC-A2 and quail pancreatic mesoderm co-culture. Clusters of cells (black arrows) are visualised brown with DAB, indicating insulin-positive cells. Cell nuclei appear as small, oval, raised structures. The cytoplasm is granular. Since their morphology contrasts with that of quail mesodermal cells (Figure 33), it is proposed that these insulin-positive cells are derived from PHeSC-A2 cells. Differential interference contrast microscopy. 400X.


35


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Figure 37. A representative photomicrograph demonstrating immunolocalization of glucagon in a PHeSC-A2 and quail mesoderm co-culture. Two cells presenting with darkly staining oval nuclei and granular cytoplasm are demonstrated. This morphology indicates glucagon-positive cells as well as their derivation from PHeSC-A2 cells when compared with the quail cells seen in Figure 33. Differential interference contrast microscopy. 1000X.


37

1

INTRODUCTION

Diabetes mellitus is a metabolic disorder affecting more than 120 million people worldwide – a number expected to increase to 300 million over the ensuing two decades (Horb *et al.,* 2003). This illness is characterized by chronic hyperglycaemia, attributable to an insulin deficiency, insulin resistance or a combined effect. Insulin is a hormone secreted by β-cells within the pancreatic islets and is essential for glucose homeostasis (Horb *et al.,* 2003). The modulation of blood-glucose levels exists as an inverse relationship between the amount of glucose released by the liver and the amount of insulin produced by the pancreas. Blood-glucose levels, despite variations in food uptake, fasting and the utilization of glucose, are normally maintained within the range of 3.5-8.0mmol/L (Gale and Anderson, 2002). During periods of fasting, low levels of insulin act to modulate the amount of glucose released by the hepatic processes of glycogenolysis and gluconeogenesis. Postprandial, insulin levels increase, thereby suppressing the release of glucose from the liver and promoting the uptake of circulating glucose by fat and muscle (Gale and Anderson, 2002).

Diabetes mellitus presents itself in two forms: type I and type II. Although clinical symptoms may overlap, the epidemiology and aetiology of the two diseases differ. Type I diabetes is foremost a childhood disease, peaking in incidence at adolescence. It occurs primarily in Western countries, in individuals of European ancestry. Type I diabetes is not genetically predetermined; however, a definite polygenic susceptibility to the disease has been identified (Gale and Anderson, 2002). This susceptibility presents as specific variations of the polymorphic HLA genes that are responsible for the regulation of the immune system. Autoantibodies are directed against the insulin-producing cells of the pancreas. The presence of these autoantibodies in serum predates the clinical onset of the disease significantly. Type I diabetics thus suffer from progressive loss of β-cell structure and function, leading to an insulin-dependency (Stephenson, 2002).

Type II diabetes presents in all population groups with varying degrees of prevalence and is lowest in individuals of European ancestry. This disease is associated with the westernization of certain population groups, affluent living conditions and sedentary lifestyles. Incidence of type II diabetes increases markedly with age and obesity (Gale and Anderson, 2002). Although a clear pattern of inheritance has yet to be elucidated, a genetic component to this disease has been confirmed (Stephenson, 2002). However, unlike type I diabetes there is no involvement of the HLA genes. Type II diabetes is not an immune-mediated disease and only half of all sufferers progress to total insulin-dependency (Gale and Anderson, 2002). Factors in the pathogenesis of type II diabetes include insulin resistance as well as depletion of the β-cell population. Although insulin is able to bind to its cell surface receptor, genetic abnormalities disrupt the signaling pathway within the cell prohibiting insulin function, a phenomenon described as insulin resistance. Furthermore, the loss of β-cell mass, although not as significant as presented in type I diabetes, is sufficiently marked to disrupt glucose homeostasis (Gale and Anderson, 2002).

Without appropriate intervention, diabetes mellitus is a potentially fatal disease. Furthermore, despite that intervention, the cardiovascular, renal and nervous system may be affected, reducing life expectancy by almost a third (Montague, 1983). To date no cure for diabetes has been found, but a form of diabetes therapy has been postulated. Pancreatic islet transplantation has been reported to be successful (Lechner and Habenar, 2003), but presents problems both of immune rejection and donor supply (Horb *et al.,* 2003). An alternative proposition is the acquisition of adult stem cells from biopsy samples and the differentiation thereof into insulin-producing cells prior to transplantation. Such treatment would preclude problems of availability and rejection (Burns *et al.,* 2004). The use of adult hepatic stem cells for the treatment of diabetes, based both on the close embryological association of the pancreas and liver, and on the possibility of a shared tissue stem cell, has been proposed (Dabeva *et al.,* 1997).

Embryologically, the pancreas and liver develop from adjacent areas of the primitive gut (Yang *et al.,* 2002). During the process of gastrulation, the trilaminar embryonic disc

commences folding in the median and horizontal planes, creating a cylindrical embryo. The inclusion of the endodermal component of the dorsal aspect of the yolk sac during this folding forms the primitive gut which is divided into three parts: the foregut, midgut and hindgut (Allan and Kramer, 2002).

At this stage hepatic and ventral pancreatic cells are specified in the ventral foregut endoderm, while cells for the dorsal pancreas are specified in the middle and dorsal portions of the foregut endoderm (Yoshitoma and Zaret, 2004). Recent research has shown that both the dorsal and ventral regions of foregut endoderm are committed to a pancreatic fate as early as the 8-somite stage in the mouse model (Yoshitoma and Zaret, 2004). This contrasts dramatically with the competency of ventral foregut endoderm in hepatogenesis (Hogan and Zaret, 2002). Studies have shown that only the anterior portion of the ventral foregut endoderm is capable of producing completely competent hepatocytes just prior to the evagination of the hepatic diverticulum (Hogan and Zaret, 2002).

It has been postulated that despite the variable array of gene expression present within the endoderm, the differentiation capacity of this germ layer is predominantly controlled by signals from the overlying mesoderm (Yoshitoma and Zaret, 2004). The regions of foregut endoderm essential to hepatic and pancreatic development are exposed to a large mesodermal contingent including the septum transversum – an accumulation of mesoderm situated between the developing heart and midgut, and the notochord (Moore and Persaud, 1998; Allan and Kramer, 2002). Furthermore, it has been established that differentiation of the liver is dependant on interactions of the hepatic diverticulum with cardiogenic mesoderm via the septum transversum, without which the endoderm reverts to a pancreatic fate (Moore and Persaud, 1998; Kumar *et al.,* 2003; Yoshitoma and Zaret, 2004). This highlights the possibility that liver and pancreas progenitor cells specified in the endoderm maintain a level of plasticity enabling them to act as bipotential precursors with a 'pre-pattern' that allows for the induction of either hepatic or pancreatic fates, dependant on particular mesodermal influences.

The pancreas and liver also share phenotype-maintaining transcription factors, for example HNF transcription factors (Odom *et al.,* 2004), as well as roles in the regulation of glucose homeostasis (Burns *et al.,* 2004). In light of their common embryological origin, elucidated in the preceding paragraphs, it has been postulated that the two adult organs retain a common progenitor cell (Dabeva *et al.,* 1997). Progenitor cells, also known as transit-amplifying cells, are the progeny of stem cells residing within adult tissues and act as intermediaries between the tissue stem cell and terminally differentiated cells (Sell, 2004).

A stem cell is characterized by its potential to differentiate into a number of cell types (Preston *et al.,* 2003). The primordial stem cell, the fertilized ovum, is totipotent – capable of differentiating into all cell lineages of the organism in question. On cleavage, via a process of synchronous division, the egg gives rise to daughter cells that are identical in every aspect including totipotentiality (Sell, 2004). However, an inverse relationship exists between the potentiality of a stem cell and the acquisition of specialized function. As the zygote develops, cells undergo the process of determination where they become committed to certain cell fates and in doing so, limit their potential. The resulting multipotential stem cells of the trilaminar embryo in turn give rise to stem cells whose potential is postulated to be further limited. These stem cells, known as tissue stem cells, reside within the organs and are said to persist to adulthood (Sell, 2004).

Tissue stem cells give rise to progenitor cells, which act not only to produce committed cells capable of specific duties, but also to maintain their own populations. This is accomplished through a process of asymmetric division whereby the progenitor cell divides allowing one daughter cell to approach commitment and the other to retain its potential (Preston *et al.,* 2003; Sell, 2004). It can therefore be expected that a tissue may contain stem cells in differing states of potential – from multipotentiality to unipotentiality.

Hepatic stem cells were first identified in early cancer research (Petersen, 2001). However, the existence of such a population became the subject of much controversy due

to the liver's inherent ability to reconstitute its mass via the division of mature hepatocytes, independent of the involvement of putative stem cells (Sell, 2004). A number of studies have nonetheless demonstrated that when the liver is placed under stress considerable enough to impede the regenerative capacity of hepatocytes, the hepatic stem cell niche is activated. Differentiation of these stem cells into progenitor cells termed 'oval cells' allows for the restoration of the hepatic parenchyma (Sell, 2004).

Hepatocellular injury and subsequent oval cell activation has been achieved using a number of carcinogenic and non-carcinogenic models. These include the use of acetylamino fluorene (AAF) followed by partial hepatectomy (Evarts *et al.,* 1989), the administration of carbon tetrachloride (CCl_4) (Gil-Benso *et al.,* 2001) and d-galactosamine (GaIN) (Dabeva and Shafritz, 1993).

The source of oval cells remains a controversial subject. A number of studies postulate the derivation of oval cells from tissue stem cells residing within the canals of Hering (Germain *et al.,* 1988; Wang *et al.,* 2003). Other studies argue an extrahepatic source of oval cells – bone marrow – and propose the canals of Hering as a transitional compartment within which differentiation along a hepatic lineage occurs (Petersen, 2001). Such disparity is reflected in the characteristics of oval cells themselves. These progenitor cells express markers for both cell types of the hepatic lineage, CK19 for biliary epithelium and albumin for hepatocytes, as well as the immature liver cell markers α-fetoprotein (AFP) and λ-glutamyl transpeptidase (GGT) (Lemire *et al.,* 1991; Wang *et al.,* 2003). Interestingly they also express the haematopoietic stem cell markers CD34, CD90 (Thy1) and c-kit (Alison and Sarraf, 1998).

Although traditionally regarded as bipotential stem cells confined to the limits of the hepatic lineage, oval cells have been shown to differentiate into tissue other than liver, notably, pancreatic endocrine cells. Oval cells cultured in the absence of LIF (a factor capable of inhibiting stem cell differentiation) and in the presence of high glucose concentrations, are able to differentiate into insulin-producing β-cells (Yang *et al.,* 2002). Furthermore, the induction of PDX1 expression via gene transfection protocols in hepatic

progenitor cells has been shown to cause the differentiation of hepatic progenitor cells into insulin-producing cells (Nakajima-Nagata *et al.,* 2004).

Such research may merely propose a revisitation to the claimed bipotentiality of the oval cell, or may have deeper implications – that of highlighting the close association of the pancreas and liver and the affirmation of the existence of a hepatopancreatic stem cell. A number of studies have indicated that oval cells are not restricted to the liver, but astonishingly, have been identified in the pancreas of several mammalian models (Grompe, 2003).

Rats maintained on a copper-deficient diet for eight weeks presented with marked destruction of pancreatic acinar tissue with no involvement of either the pancreatic ducts or islets of Langherhans (Rao *et al.,* 1989). During this period, cells with characteristics of oval cells appeared within the pancreatic tissue. On the re-introduction of copper to the diet, these putative oval cells differentiated into hepatocytes (Rao *et al.,* 1989). Furthermore, hepatocytes derived from pancreatic oval cells are indistinguishable from those derived from hepatic oval cells, phenotypically and functionally (Dabeva *et al.,* 1997; Grompe, 2003). Pancreatic cell phenotypes have also been detected in the liver, specifically in cases of cholangiocarcinoma, in which hepatic oval cell involvement is also implicated (Petersen, 2001; Grompe, 2003). Several studies have proposed that pancreatic progenitor cells have a differentiation capacity restricted to that of the pancreatic lineage (Soria *et al.,* 2005). However, studies conducted by Suzuki *et al.* (2004) have identified the expression of hepatocyte growth factor (HGF) in pancreatic progenitor cells. Furthermore, pancreatic progenitor cells, expressing the neural-stem cell specific marker, nestin, have been shown to differentiate into cells expressing hepatic phenotypes (Zulewski *et al.,* 2001).

Although further characterization of both pancreatic and hepatic stem cells is needed, the highlighted studies validate the hypothesis of a hepatopancreatic stem cell. The role of mesodermal influences and the elucidation of the factors involved in the specification of the pancreatic and hepatic cell lineages are also in need of further exploration. This study

therefore aimed to investigate whether hepatic oval cells have the capacity to differentiate into pancreatic β-cells under the influence of *pancreatic* mesoderm in an *in vitro* system.

2
MATERIALS AND METHODS

All animals used in these experiments were obtained from the Central Animal Services, University of the Witwatersrand. Animal ethics clearance was obtained (AEC Number: 2004/39/3 and 2006/69/3) and is applicable to both Sprague Dawley rats and fertile quail eggs.

2.1 ISOLATION OF OVAL CELLS

2.1.1 Determination of appropriate GaIN dosage and duration of hepatic injury

Male Sprague Dawley rats within the weight range 240g-340g were initially injected, intraperitoneally, with various doses of GaIN (Sigma-Aldrich, SA), ranging between 40mg and 70mg GaIN/100g body weight in 1ml 0.14M NaCl, to determine whether the extent of hepatic injury was sufficient to induce oval cell proliferation. GaIN concentrates were prepared in sterile saline and filtered prior to use. Animals were killed either 4 or 5 days following GaIN administration to test whether the duration of hepatic injury impacted on the size of the oval cell population induced. Furthermore, animals injected with 1ml 0.14M NaCl only, served as normal controls with which to compare the GaIN-treated hepatic tissue.

Following hepatic injury animals were killed with an overdose of Euthanase (Centaur Labs, USA) via an intraperitoneal injection. Pieces of liver were removed, fixed in 10% buffered formalin and processed through a series of alcohols and chloroform overnight. Tissue was embedded in paraffin wax and 8μm sections were cut and stained using haematoxylin and eosin for histological analysis. Based on the results obtained as well as literature citations (Lemire *et al.,* 1991; Dabeva and Shafritz, 1993), the GaIN dosage was established at 70mg GaIN/100g body weight and the duration of hepatic injury, at 5 days post-GaIN administration.

2.1.2 Termination of animals and sorting of oval cells

Animals were anaesthetized with a weight-dependant dose of ketamine and rompin (4:1). Prior to the opening of the abdominal cavity, the abdomen was shaved and swabbed with 70% alcohol. Thereafter, experimental procedures were conducted under a laminar flow hood in sterile conditions. In order to perfuse the liver and cause dissociation of tissue, the aorta was tied off, the portal vein cut and approximately 20ml 0.05% TypeVIII collagenase (Sigma-Aldrich, SA) in IMDM (Sigma-Aldrich, SA) was injected into the inferior vena cava and allowed to circulate for 5 minutes.

Liver lobes were removed, rinsed in PBS and placed in 0.05% collagenase for 15 minutes. The tissue was then chopped with a fine blade and pressed through a fine wire mesh in order to obtain a single cell suspension. Three different methods of cell sorting were used to elucidate an oval cell fraction:

2.1.2.1 *Cell sorting with MiniMACS kit: Direct Labelling*

Briefly, the MiniMACS kit (Miltenyi Biotec, Germany) incorporates magnetic cell separation technology. Desired antigens may be either directly or indirectly labelled, as per normal immuncytochemistry principles, with magnetic microbeads. Labelled single cell suspensions are passed through a separation column which is placed within a powerful magnetic field. Thus, magnetically labeled cells from the suspension adhere to the column allowing the unlabelled fraction to pass through. Labelled cells are then collected by removing the separation column from the effects of the magnet and flushing out the cells. The procedure for direct labeling is as follows:

The cell suspension was washed in degassed phosphate buffered saline (PBS) (see Appendix I) supplemented with 2mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, SA) and 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, SA) at pH7.2, and centrifuged at 400*g* for 5 minutes. The cell pellet was resuspended in 90µl of buffer, to which 10µl of mouse CD90 magnetic microbeads (Miltenyi Biotec, Germany) were added. The solution was incubated

at 7ºC for 15 minutes. Thereafter, cells were washed with buffer and centrifuged at 300*g* for 10 minutes. The supernatant was removed and the pellet resuspended in 500µl of buffer.

In order to separate the unlabelled from the labeled fraction, the cell suspension was applied to a MACS separation column, attached to a powerful magnetic separator. The unlabelled fraction was allowed to pass through and discarded. The separation column was then removed from the separator and the labelled fraction flushed out into an eppendorf.

The labelled fraction was then seeded on to fibronectin-coated Nunc Wells (Nunc, Denmark) and grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10ng/ml stem cell factor (SCF) (Santa Cruz, USA), 10ng/ml leukaemia inhibitory factor (LIF) (Santa Cruz, USA), 10ng/ml Flt-3 ligand (Santa Cruz, USA) and 1µl/ml penicillin-streptomycin (Yang *et al.,* 2002). Cells were maintained in culture at 37 ºC with 5% CO_2 in air, in a humidified environment.

2.1.2.2 *Cell sorting with MiniMACS kit: Indirect Labelling*

Indirect labeling follows the same principle of magnetic cell sorting as elucidated in 2.1.2.1., the only difference being the use of a flurochrome-conjugated primary antibody. In doing so, this method precludes problems of magnetic microbead specificity by allowing the use of more specific antibodies. The antibody is directed against the antigen of interest whilst the magnetic microbeads are directed against the flurochrome conjugated to the antibody itself. Indirect labeling of cells proceeds as follows:

The cell suspension was centrifuged at 100*g* and the resulting pellet resuspended in 100µl degassed PBS buffer supplemented with 2mM EDTA and 0.5% fetal calf serum (FCS) (Highveld Biological, SA). Cells were labeled with 10µl CD90 phycoerythrin (PE)-conjugated antibody (US Biological, USA) and incubated in the dark at 7ºC for 10 minutes. Cells were washed in 1ml buffer and centrifuged at

300*g* for 10minutes. The supernatant was removed and the cell pellet resuspended in 80µl buffer. The suspension was incubated at 7 ºC for 15 minutes with 20µl Anti-PE Microbeads (Miltenyi Biotec, Germany).

Cells were washed further by adding 1ml buffer and centrifuging at 300*g* for 10minutes. The pellet was resuspended in 500µl buffer and applied to a MACS separation column. The unlabelled fraction was allowed to pass through. The column was then removed from the magnetic separator and the labeled fraction flushed out with 500µl buffer.

The labeled fraction was seeded onto poly-d-lysine (PDL) (Sigma-Aldrich, USA) coated flasks and grown in IMDM supplemented with SCF (10ng/ml), LIF (10ng/ml), Flt-3 ligand (10ng/ml) (Yang *et al.,* 2002) and penicillin-streptomycin (1µl/ml). Cells were cultured in a humidified environment at 37 ºC with 5% CO_2 in air.

2.1.2.3 *Cell sorting as adapted from Kano et al.(2003)*

The single cell suspension as obtained from methods elucidated in 2.1.2., was pipetted into centrifuge tubes and centrifuged at 60*g* for 2 minutes. The supernatant was removed, the cell pellet resuspended in PBS and centrifuged at 350*g* for 5 minutes. Two further centrifugation steps ensued, the cell suspension being centrifuged twice at 60*g* for 75 seconds.

The resulting cell pellet was seeded on to fibronectin (Sigma-Aldrich, USA) coated Ultra-Web Synthetic ECM (Donaldson, USA). These synthetic matrices, composed of nylon nanofibres, are designed to resemble the structure of basement membranes or extracellular matrix (ECM). Consequently, cells grown thereon assume phenotypes more similar to their *in vivo* morphology. The cell pellet once seeded, was left to adhere to the ECM for 30 minutes before the addition of IMDM supplemented with SCF (10ng/ml), LIF (10ng/ml), Flt-3 ligand (10ng/ml)

(Yang *et al.,* 2002) and penicillin-streptomycin (1µl/ml). Cells were cultured in a humidified environment at 37 ºC with 5% CO_2 in air.

2.2 CULTURE OF HEPATIC STEM-LIKE CELL LINE (PHESC-A2)

A porcine hepatic stem-like cell line (PHeSC-A2) was kindly donated by Prof. J. Kano (Department of Diagnostic Pathology, I.B.M.S., G.S.C.H.S., University of Tsukuba, Japan). PHeSC-A2 was propagated, under sterile conditions, on PDL-coated flasks in specifically formulated NAIR-1 medium (Highveld Biological, SA) (see Appendix I) with 1µl/ml penicillin-streptomycin (Kano *et al.,* 2003). The cell line was maintained in a humidified environment with 5%CO_2 in air at 37 ºC. When confluent, cells were treated with a solution of Trypsin-EDTA (Sigma-Aldrich, SA) to facilitate lifting and re-seeded in PDL-coated flasks.

2.3 CO-CULTURE OF PHESC-A2 CELLS AND QUAIL PANCREATIC MESODERM

All ensuing experimentation was conducted under sterile conditions within a laminar flow hood.

2.3.1 Microdissection of mesoderm from quail pancreatic buds

Fertile quail (*Coturnix coturnix japonica*) eggs were incubated in a humidified environment at 37ºC for 5 days to allow for development of the dorsal pancreatic bud. Embryos staged between 24-26 (Figure 1), according to Hamburger and Hamilton (1951), were pinned on to black wax dishes (Figure 2) in chick Ringer's solution (see Appendix I), prior to the removal of the surrounding membranes. The lateral body wall was removed exposing the underlying gut tube (Figure 3) which was dissected out and incubated in 0.04% TypeVIII collagenase (Sigma-Aldrich, SA) in Tyrode's solution (see Appendix I) at 20ºC for 20 minutes. Thereafter, utilizing transmitted and incident light, the pancreatic mesoderm was microdissected from the endoderm of the dorsal pancreatic bud (Figures 4 and 5) and left in Tyrode's solution at 37ºC until use.

2.3.2 Culturing of PHeSC-A2 cells and quail pancreatic mesoderm in the GFRM-Ham's F12.ITS system

A solution of Trypsin-EDTA was applied to a flask of near confluent PHeSC-A2 cells in order to lift them. The suspension was centrifuged at 100*g* for 5 minutes and the supernatant removed. 1µl of NAIR-1 medium was applied to the pellet. Thereafter, cells were pipetted directly on to polymerized growth factor-reduced Matrigel (GFRM) (BD Biosciences, USA), a biologically active matrix resembling *in vivo* basement membrane, and incubated at 37ºC for 30 minutes allowing the cells to adhere to the substrate. Subsequently, quail mesoderm was placed on to the cell aggregations and more cells pipetted on and around the explants. A final layer of GFRM was laid down over the PHeSC-A2 and mesoderm co-culture and left to polymerize at 37ºC for 30 minutes prior to the addition of Ham's F12.ITS medium containing additional insulin, transferrin and selenium (Highveld Biological, SA). The culture was maintained for 5 days at 37ºC in a humidified incubator in 5% CO_2 in air.

2.3.2.1 *Control: Culturing of PHeSC-A2 cells in the GFRM-Ham's F12.ITS system without pancreatic mesoderm*

Subsequent to the trypsinization and centrifugation steps as elucidated above, PHeSC-A2 cells were pipetted on to polymerized GFRM. The cells were covered with a further layer of GFRM which was allowed to polymerize prior to the addition of Ham's F12.ITS medium. These cells were cultured in the absence of quail pancreatic mesoderm for 5 days at 37ºC in a humidified incubator in 5%CO_2 in air. This experiment was carried out to visually assess the behaviour and morphology of PHeSC-A2 cells in the GFRM-Ham's F12.ITS system.

2.3.2.2 *Control: Culturing of quail pancreatic mesoderm in the GFRM-Ham's F12.ITS system without PHeSC-A2 cells*

Quail pancreatic mesoderm was laid down on to polymerized GFRM and subsequently covered with a layer of GFRM. The mesoderm explant was maintained in culture, in Ham's F12.ITS medium, for 5 days at 37ºC in a humidified incubator in 5% CO_2 in air. This experiment was conducted to visually

assess the proliferation of mesodermal cells and to provide a positive control for the immunolocalization of the quail nuclear marker (see 2.4.3.).

2.3.2.3 *Control: Culturing of PHeSC-A2 cells in the PDL-Ham's F12.ITS system*

A solution of Trypsin-EDTA was applied to a flask of PHeSC-A2 cells in order to detach the cells from the PDL substrate. The resultant suspension was centrifuged, washed in PBS and replated on to PDL-coated flasks. The cells were cultured in Ham's F12.ITS medium, in order to visually assess differences in structure when compared to those grown in the PDL-NAIR-1 system.

2.4 IMMUNOCYTOCHEMISTRY

2.4.1 Immunolocalization of AFP (an oval cell marker) in PHeSC-A2 cells

PHeSC-A2 cells were grown to near confluency prior to processing for immunocytochemistry. All processing procedures were carried out within the flask itself. Medium was poured off and cells washed in PBS (pH7.4). Cells were fixed in 2.5% glutaraldehyde in 1M PBS (pH7.4) at 4ºC for 1 hour followed by rinsing in PBS. Thereafter, they were dehydrated through a graded series of alcohols for 10 minutes at each percentage. The monolayer was thereafter infiltrated with Epon-Araldite resin (1:1) in ethanol and 2 changes of 100% for 10 minutes consecutively. Cells were then covered with a thin layer of Epon-Araldite resin (1:1).

The flask was left open and incubated at 60ºC to allow the resin to polymerize. The following day, the flask was broken open and the layer of resin removed. Strips were cut and placed into a rubber mould. Epon-Araldite resin (1:1) was poured into the moulds and left to polymerize again at 60ºC for 24 hours. Serial 1µm sections were cut for immunolocalization of AFP.

Sections were deplasticised in sodium ethoxide diluted 1:2 with absolute alcohol for 10 minutes. They were washed in absolute alcohol and hydrated through 95% alcohol to distilled water. Sections were washed in PBS and blocked in 10% rabbit serum in PBS

for 20 minutes. Primary antibody, goat polyclonal AFP (Santa Cruz, USA) diluted 1:500 in PBS supplemented with 1.5% BSA, was applied to sections and incubated at room temperature for an hour. Sections were washed in three changes of PBS for 5 minutes each. They were then incubated with a rhodamine-conjugated rabbit anti-goat secondary antibody (Santa Cruz, USA), diluted 1-5μg/ml, for 45 minutes. Following washing in PBS, sections were mounted with VectaShield Mounting Medium (Vectorlabs, USA).

2.4.1.1 *Controls for the immunolocalization of AFP in PHeSC-A2 cells*

Positive controls were used to determine the efficacy of the technique. A tissue sample in which AFP is a known component was used as a positive control for the oval cell marker AFP. Fourteen and a half day-old mouse fetal liver was quenched in chilled isopentane prior to being freeze-dried overnight at -40°C and 10^{-2} Torr. Tissue was then fixed in parabenzoquinone vapour for 3 hours at 60ºC, and infiltrated with Epon-Araldite resin (1:1) for 3 hours at 10^{-2} Torr. The resin was allowed to polymerize for 48 hours at 60°C. Serial 1μm sections were cut and deplasticized. ICC was conducted as per the experimental tissue procedure. Negative controls were conducted in which the primary and secondary antibodies were omitted and replaced with buffer.

2.4.2 Immunolocalization of insulin and glucagon in PHeSC-A2 and quail pancreatic mesoderm co-cultures

PHeSC-A2 and mesoderm co-cultures were dissected out of the GFRM and quenched in chilled isopentane. Tissue was freeze-dried overnight at -40°C and 10^{-2} Torr, fixed in parabenzoquinone vapour for 3 hours at 60ºC and infiltrated with Epon-Araldite resin (1:1) for 3 hours at 10^{-2} Torr. Tissue was embedded in resin which was allowed to polymerize for 48 hours at 60ºC. Serial 1μm sections were cut for routine histological examination with toluidine blue staining and immunolocalization of insulin and glucagon.

Sections were deplasticised in sodium ethoxide diluted 1:2 with absolute alcohol for 10minutes. They were washed in absolute alcohol, hydrated through 95% alcohol to distilled water and washed further in Tris/saline (see Appendix I). Slides were then

placed in 3% hydrogen peroxide in distilled water for 10 minutes and washed again in Tris/saline. They were then covered with 10% swine serum in Tris/saline for ten minutes in a damp chamber at room temperature. To detect insulin-producing cells, sections were incubated with guinea pig anti-insulin (Chemicon, USA) at 1:800 and for the detection of glucagon-producing cells sections were incubated with rabbit anti-glucagon (Euro Diagnostica, Sweden) at 1:1000, overnight. Both primary antibodies were diluted in Tris/saline.

The following day, sections were washed in three changes of 1% horse serum in Tris/saline for 5 minutes each. They were thereafter incubated with secondary antibody: rabbit anti-guinea pig peroxidase (Dako, Denmark) and swine anti-rabbit peroxidase (Dako, Denmark) for insulin and glucagon-labeled cells respectively, for 1 hour at room temperature in a damp chamber. Both antibodies were used at a 1:20 dilution with a specific diluent (see Appendix I). Sections were washed in three changes of Tris/saline for 5 minutes each. Thereafter they were incubated in DAB (Sigma-Aldrich, SA) solution (see Appendix I) for 5 minutes to allow for visualization of the antigen-antibody complex. Sections were then washed in Tris/saline followed by distilled water and were then washed further for 5 minutes in gently running tap water. Sections were dehydrated through a graded series of alcohols, cleared in xylene and mounted in Entellen.

2.4.2.1. *Controls for the immunolocalization of insulin and glucagon*

Positive controls were used to determine the efficacy of the technique. A tissue sample in which insulin and glucagon are known components, was used. Resin-embedded chick pancreas thus served as a control for both insulin and glucagon immunolocalization. ICC was conducted as above. Negative controls were conducted in which both primary and secondary antibodies for the insulin and glucagon immunocytochemical protocols, were omitted and replaced with buffer, thus prohibiting the formation of antigen-antibody complexes. Furthermore an absorption control was also carried out in which both primary antibodies were preabsorbed with their respective antigens overnight, prior to the execution of the ICC protocol, in order to ascertain the specificity of the antibodies in question.

2.4.3 Immunolocalization of the Quail Nuclear Marker

PHeSC-A2 and pancreatic mesoderm co-cultures were dissected out of GFRM and quenched in chilled isopentane. Tissue was freeze-dried overnight at -40°C and 10^{-2} Torr, fixed in parabenzoquinone vapour for 3 hours at 60ºC and infiltrated with Epon-Araldite resin (1:1) for 3 hours at 10^{-2} Torr. Tissue was embedded in resin which was allowed to polymerize for 48 hours at 60ºC. Serial 1µm sections were cut for immunolocalization of the quail nucleus.

Sections were deplasticised in sodium ethoxide diluted 1:2 with absolute alcohol for 10minutes. They were washed in absolute alcohol, hydrated through 95% alcohol to distilled water and washed in PBS (see Appendix I). Sections were placed in 10% rabbit serum in PBS for 45 minutes followed by incubation with the quail cell nuclear marker, QCPN mAB (Sigma-Aldrich, SA), overnight at 4°C. The primary antibody was used undiluted. Sections were rinsed in PBS and incubated with a rabbit anti-mouse peroxidase conjugated secondary antibody (Dako, Denmark) for 1 hour. The secondary antibody was diluted in a ratio of 1:50 with PBS. DAB was then applied to the sections for 5 minutes prior to rinsing, to allow for visualization of the antigen-antibody complex. Sections were thereafter dehydrated through a series of alcohols, cleared in xylene and mounted in Entellen.

2.4.3.1. *Controls for the immunolocalization of the quail nuclear marker*

Positive controls conducted on quail pancreatic mesoderm, served to test the effectiveness of the ICC protocol. Immunolocalization of quail nuclei was conducted as stated in the preceding paragraphs. Nuclei were expected to be visualized as brown with DAB. Negative controls, in which the primary and secondary antibody was omitted and replaced with PBS prior to the continuation of the normal ICC technique as highlighted above, were intended to investigate the specificity of the primary antibody.

2.5. Identification of the Quail Nucleus using the Feulgen Method (Bancroft and Cook, 1996)

PHeSC-A2 and mesoderm co-cultures were dissected out of the GFRM and quenched in chilled isopentane. Tissue was freeze-dried overnight at -40°C and 10^{-2} Torr, fixed in parabenzoquinone vapour for 3 hours at 60ºC and infiltrated with Epon-Araldite resin (1:1) for 3 hours at 10^{-2} Torr. Tissue was embedded in resin. The resin was allowed to polymerize for 48 hours at 60ºC. Serial 1µm sections were cut and deplasticised in sodium ethoxide diluted 1:2 with absolute alcohol for 10minutes. They were washed in absolute alcohol, hydrated through 95% alcohol to distilled water prior to execution of the Feulgen method.

Sections were rinsed in 1M HCl at room temperature followed by hydrolysis in 1M HCl at 60°C. In order to determine optimal hydrolysis time, required for the breaking of purine-deoxyribose bonds, sections were incubated in 1M HCl at 60°C for different periods ranging from 10 minutes to 1 hour. Sections were thereafter rinsed in 1M HCl at room temperature for 1 minute. In order to view the aldehydes resulting from HCl hydrolysis, sections were placed in Schiff's reagent (see Appendix I) for 45 minutes and rinsed 3 times in bisulphate solution (see Appendix I), followed by washing in distilled water. Sections were counterstained by placing them in 1% light green for 2 minutes. Sections were washed in distilled water, dehydrated through a graded series of alcohols, cleared in xylene and mounted in Entellen. Cell components containing DNA were expected to stain purple-red with the surrounding tissue staining a light green colour.

3
RESULTS

3.1 GAIN MODEL OF HEPATIC INJURY

Liver from untreated animals served as a control with which to compare the histology of the GaIN treated livers. The histological structure of the untreated liver revealed classic liver lobules with cords of hepatocytes radiating outward from the central vein toward the portal tracts (Figure 6). The parenchyma showed no signs of degeneration, presenting with both intact hepatic cord structure and sinusoidal spaces (Figure 7). Portal tracts, consisting of a bile duct, arteriole and venule were unaffected and demonstrated no evidence of oval cell proliferation (Figure 7).

The first animal to receive GaIN at a 70mg/100g body weight dose (Dabeva and Shafritz, 1993) died prematurely (refer to Table I). The dosage was thus reduced to 40mg GaIN/100g body weight with the duration of hepatic injury over 4 days. Histological analysis revealed no significant changes to the hepatic parenchyma (Figure 8) when compared to that of untreated liver (Figure 6). Hepatic cord structure remained intact with no obvious hepatocyte necrosis or sinusoidal dilation (Figure 9). However, a sparse oval cell response in the portal tract area was noted (Figure 9). These cells were identified by their palely basophilic oval nuclei, scanty basophilic cytoplasm and oval cell shape.

The dose was thus increased to 50mgGaIN/100g body weight. The duration of hepatic injury was also increased to 5 days. The results obtained from this experiment (Figure 10) contrast with those obtained from liver treated with 40mg GaIN/100g body weight (Figure 8). Disorganization of hepatic cord structure was apparent at both low and high magnifications (Figures 10 and 11, respectively). Although foci of hepatocyte degeneration, as evidenced by a scattered increase in eosinophilia, were evident (Figure 10), few putative oval cells were seen proliferating in the portal tract areas (Figure 11). These cells are seen in close association with the bile duct (Figure 11).

The dose was thus increased, once again, to 70mgGaIN/100g body weight based on the work of Dabeva and Shafritz (1993). The duration of hepatic injury was reduced to 4 days. The treated liver exhibited disruption of hepatic cord structure (Figure 12), when compared with that of the normal liver (Figure 6). An intense inflammatory response, as evidenced by the presence of leukocytes exhibiting small, darkly stained nuclei, was demonstrated associated with the portal tract (Figure 13). At a higher magnification, sinusoidal dilation and atrophy of hepatic plate organization was evident (Figure 14). This compares significantly with the structure of untreated liver (Figure 7) and GaIN-treated livers at 40mg GaIN/100g body weight (Figure 9) and 50mg GaIN/100g body weight (Figure 11). Degenerating hepatocytes (acidophilic bodies) exhibiting an increase in cytoplasmic eosinophilia and a loss of nuclear structure were evident (Figure 14). The appearance and severity of the aforementioned features was seen to increase concomitantly with an increase in GaIN concentration (Figures 9, 11 and 14). Furthermore, the proliferation of oval cells, identified by their pale-staining basophilic nuclei and scanty cytoplasm, was found in the vicinity of the bile duct (Figure 13). Oval cells are seen to invade the hepatic parenchyma in rows (Figure 13), forming atypical ducts (Figures 13 and 14).

The duration of hepatic injury was increased to 5 days in rats treated with 70mg GaIN/100g body weight, in order to increase the size of the oval cell population produced. Liver obtained from these experiments presented with similar pathology as seen in those animals treated with the same dose but over a 4 day period, as elucidated in the preceding paragraph. However, the severity of those pathologies was enhanced. Destruction of hepatocyte plate organization and cord structure, as well as sinusoidal dilation, were marked (Figure 15). An increase in scattered eosinophilia (Figure 15), indicative of acidophilic bodies was apparent (Figures 16). Putative oval cell proliferation, in the immediate surroundings of the portal triad was evident (Figures 16). These cells were seen to line existing biliary ducts (Figure 16), and to thus form atypical ducts (Figure 17). Oval cells were also seen to form cords extending into the hepatic parenchyma (Figure 17). It was thus established that a 70mg GaIN/100g body weight

dose and a duration of hepatic injury extending over a 5 day period, was sufficient to induce a significant oval cell population that could be isolated for primary cell cultures.

Table 1 illustrates the number of premature deaths of GaIN-treated rats experienced in this study. Prior to death, animals presented with severe weight loss and a tendency to bleed in the extremities. Autolysis of a number of the deceased precluded examination of body organs. Independent autopsies conducted on the others indicated severe necrosis of the liver parenchyma. Hepatocytes exhibited a loss of nuclear structure, eosinophilia of the cytoplasm and ultimate degeneration. Reports by the Central Animal Service veterinarian indicated that the evident pannecrosis, a severe lesion, is associated with toxin exposure.

Table 1.

Year	**Number Killed**	**Premature Deaths**	**% of Premature Deaths**
2004	6	1	16.67%
2005	9	2	22.22%
2006	9	5	55.55%
	Total Number Killed	**Total Number Premature Deaths**	**Total % Premature Deaths**
	24	8	33.33%

3.2 THE USE OF CELL-SORTING METHODOLOGY IN THE ESTABLISHMENT OF A PRIMARY CELL CULTURE FROM GAIN-TREATED ADULT RAT LIVER

The establishment of a primary cell culture is dependant on the acquisition of a significant number of viable cells from the organ in question. Furthermore, in cell sorting procedures based on the labelling of cell markers, these markers should not be adversely affected in the preparation of single cell suspensions (Carlsen *et al.,* 1981). As such, the technique involved in the production of a single cell suspension is of paramount

importance to the establishment of a primary cell culture. This will be expounded upon further in the following chapters.

All the cell sorting procedures used in this study proved ineffective – the resultant fraction exhibiting a low yield and an inability to proliferate in culture. The direct method of cell sorting incorporated the use of $CD90^{+}$ microbeads designed for use in a mouse model. The lack of specificity could be responsible for the low yield of cells derived from treated rat livers. However, the cell fraction obtained from the use of an indirect method of cell sorting using CD90 PE-conjugated primary antibody specific for rat tissue followed by labelling with Anti-PE microbeads, also produced a low yield of viable cells. This precludes reduced specificity as a cause for the ineffectiveness of the cell sorting procedure and rather implicates an ineffective isolation of single cell suspensions.

Furthermore, the use of a cell sorting method adapted from Kano *et al.* (2003) also proved ineffective. Although the yield appeared marginally higher, cells were static in culture. This yet again implicates the technique used to acquire an initial isolation of single cell suspensions, as inefficient. Specifically, the use of both enzymatic and mechanical dissociation procedures appear to have irreparably damaged the isolated cells. This will be discussed further in ensuing chapters.

Since a primary cell culture could not be established it was thus necessary to use an established cell line in order to continue experimentation.

3.3 PHeSC-A2 CELLS IN CULTURE

The PHeSC-A2 cell line, donated by Prof. Kano of the University of Tsukuba Japan, is a hepatic stem cell-like line derived from a normal porcine model. These cells have properties akin to those of oval cells and are regarded as such in this study. Their properties will be expounded upon in the ensuing chapters. Kano *et al.* (2003), established that the successful propagation of the PHeSC-A2 cell line was dependant firstly on the substrate on which the cells were to adhere, and secondly, on the medium in

which cells were grown. Kano *et al.* (2003) accordingly investigated a number of systems and found that the PDL-NAIR1 culture system was able to maintain both the proliferation and the stem-cell properties of the PHeSC-A2 cell line.

The PHeSC-A2 cell line, in this study, was thus cultured on PDL-coated flasks. Specifically formulated NAIR-1 medium was changed every two days. In these conditions, the cells appear stellate in shape (Figure 18). Clusters of cells were seen to proliferate towards each other (Figure 19). As confluency was approached, cells were passaged and reseeded in order to produce a sufficient population with which to continue experimentation.

3.4 IMMUNOLOCALIZATION OF AFP IN PHESC-A2 CELLS

Immunolocalization of AFP was conducted on 1μm sections of resin-embedded PHeSC-A2 cells in order to investigate whether they had maintained their oval cell properties in culture. The positive control, fourteen and a half day old fetal mouse liver, did not exhibit immunolocalization of AFP. The ineffectiveness of the technique was attributed to the use of an expired primary antibody (goat polyclonal AFP). As such, results obtained from the ICC protocol conducted on PHeSC-A2 cells, were null and void. Since no culture parameters were altered in this study from those elucidated in Kano *et al.* (2003) it was thus assumed that the PHeSC-A2 cell properties, as stated in the literature (Kano *et al.,* 2003), had been maintained in this study.

3.5 CO-CULTURE OF PHESC-A2 AND QUAIL PANCREATIC MESODERM

PHeSC-A2 cells and mesodermal explants were cultured in the GFRM-Ham's F12.ITS system for 5 days prior to processing for immunolocalization of insulin and glucagon. The culture system was arranged such that quail mesoderm lay between two layers of PHeSC-A2 cells, surrounded by GFRM (Figure 20). For the duration of the experiment, oval cells appeared to aggregate forming a cluster around the mesoderm explant. This appeared as an area that assumed a progressively denser appearance over time (Figures

20 and 21). The morphology and arrangement of PHeSC-A2 cells cultured in the GFRM-Ham's F12.ITS system differed to those grown in the PDL-NAIR-1 system. In the former culture system, most cells remained tightly clustered (Figure 22) while a few others assumed a more spindle-shaped morphology (Figure 23). Proliferation was not obviously evident (Figure 22). In the latter system (PDL-NAIR-1 system), oval cells did not cluster together, but proliferated radially, assuming a stellate structure (Figure 19). This morphological variance may be attributable to the different substrates on which they were grown. When cultured on PDL-coated flasks in Ham's F12.ITS medium (Figure 24), the structure of the oval cells is similar to those grown in NAIR-1 medium (Figures 18 and 19). Since immunocytochemical tests conducted on PHeSC-A2 cells in the PDL-NAIR-1 system were ineffectual (see 3.4.), no further tests on the cell marker, AFP, were conducted on the said cells in either the GFRM-Ham's F12.ITS culture system or PDL-Ham's F12.ITS system in this study. As such no inferences as to alterations in the nature of their properties, if any did in fact occur, can be made.

Proliferation of quail mesodermal cells varied in different experiments. This difference may be due to a reduction in cell viability following removal of the pancreatic mesoderm from the quail embryos. Mesoderm was cultured without PHeSC-A2 cells in GFRM as a control (Figure 25). The mesodermal cells proliferated extensively, invading the surrounding GFRM (Figure 26). Importantly, with respect to both mesodermal and oval cell contingencies, cell movement as seen using phase contrast microscopy, may not all be attributable to proliferation. Some cell movement may in fact be dissemination of cells from the aggregates in which they were seeded. However, no further experimentation was done to confirm these movements.

3.6 IMMUNOLOCALIZATION OF INSULIN AND GLUCAGON IN QUAIL PANCREATIC MESODERM AND PHESC-A2 CO-CULTURES

3.6.1. Controls

In order to determine the efficacy of the immunolocalization technique, a positive control in which a known sample of tissue (chick pancreatic tissue) possessing both insulin and

glucagon-containing cells, was subjected to the ICC process. Insulin and glucagon-positive cells were localized within the pancreatic islets of the chick pancreas. Glucagon-positive cells were located in the centre of islets (Figure 27) in the positive control. Neither the negative control (Figure 28) nor the absorption control exhibited immunolocalization of glucagon (Figure 29), indicating the specificity of the antibody in question and the efficacy of the ICC technique. Insulin-positive cells were confined to the periphery of the pancreatic islet (Figure 30). No immunolocalization of insulin was found in either the negative control (Figure 31) or the absorption control (Figure 32). Neither non-specific staining nor background staining was evident in any of the aforementioned controls for the immunolocalization of glucagon and insulin (Figures 27 – 29 and Figures 30 – 32, respectively), thus indicating the specificity and the effectiveness of the ICC technique.

3.6.2. Experimental tissue: PHeSC-A2 and quail mesoderm co-cultures

The histological structure of quail cells includes a large, pale-staining, vesicular nucleus and a prominent nucleolus (Figure 33). This feature allows for discrimination between quail cells and oval cells, the latter having a small, oval-shaped nucleus. As such, endodermal contamination of the co-cultures, which would result in the differentiation of quail endocrine cells, is immediately recognisable. Quail cells exhibited a light background stain with DAB. This is however, easily distinguishable from definitive immunolocalization of either glucagon (Figure 27) or insulin (Figure 30) which are identified by their dark brown, granular appearance in the positive control tissue.

Insulin-positive cells in the co-cultures were visualised using DAB. These cells exhibited a granular cytoplasm, indicative of insulin-producing cells, and condensed, darkly stained nuclei (Figures 34 - 36). These small, oval-shaped nuclei, as well as the appearance of the cytoplasm, contrasted significantly with those of quail cells (Figure 33). Insulin-positive cells appeared clustered together within the GFRM (Figures 34 - 36). This close association is characteristic of the nature in which PHeSC-A2 cells behaved in the GFRM-Ham's F12.ITS *in vitro* system (Figures 20 and 21).

Glucagon-positive cells, also visualised using DAB, were rare. The nuclei of these cells contrasted with those of the quail mesodermal cells (Figure 33), in that they were small, oval and did not display a prominent nucleolus (Figure 37). The cytoplasm of glucagon-positive cells again contrasted with those of quail cells, the former being darkly stained and granular in appearance (Figure 37), and the latter exhibiting a low background stain (Figure 33). Glucagon-positive cells were also found in clusters, reminiscent of the behaviour of oval cells in the *in vitro* culture system (Figures 20 and 21). Visual assessment of insulin and glucagon-positive cell nuclei suggested their origin from PHeSC-A2 cells. Although further tests were conducted to affirm this supposition, they proved inconclusive, as highlighted in the following paragraphs.

3.7 IMMUNOLOCALIZATION OF THE QUAIL NUCLEAR MARKER

Immunolocalization of the quail nuclear marker was conducted to allow for immunocytochemical discrimination between quail cells and PHeSC-A2 cells. However, the positive control, quail mesodermal tissue, exhibited no immunolocalization of the nuclear marker. The tissue exhibited no background staining or non-specific staining. This ICC technique was thus ineffective and could not be used to affirm visual identification of insulin and glucagon-positive cells as oval cell-derivations on sections adjacent to those exhibiting immunolocalization for the said pancreatic markers. The primary antibody used in this study had expired and is thus implicated in the inefficacy of the ICC technique.

3.8 IDENTIFICATION OF THE QUAIL NUCLEAR MARKER USING THE FEULGEN METHOD

The Feulgen method, which demonstrates DNA, has previously been used in chick-quail recombination studies to identify the quail nucleus. In this study it was intended to discriminate between quail and porcine cells and thereby confirm that insulin and glucagon were expressed by differentiated PHeSC-A2 cells, thus excluding the possibility of a quail pancreatic endodermal contaminant.

This technique, however, also proved ineffective. This is attributed to the manner in which the tissue was fixed in this study (freeze-drying, followed by fixation in parabenzoquinone vapour). The fixative used is critical to the efficacy of the Feulgen technique since it impacts directly on the hydrolysis of DNA by HCl (Bancroft and Cook, 1996). Although different hydrolysis times were investigated, none proved efficient and no DNA staining was evident.

The results obtained do indicate the presence of putative insulin-positive and glucagon-positive cells within the PHeSC-A2 and pancreatic mesoderm co-cultures, as determined by immunocytochemical techniques. By virtue of their cell and nuclear size, they have been identified as oval cell-derived cells. Quail cells as highlighted in preceding paragraphs, exhibit a large, round, vesicular nucleus with a prominent nucleolus. This contrasts with the nuclei of both the insulin-positive and glucagon-positive cells – being small, oval and lacking a prominent nucleolus. As such it is proposed that these cells were derived from PHeSC-A2 cells. Techniques designed to confirm the nature of insulin and glucagon-producing cells, were ineffective and could thus not be used to affirm the visual observations. Further studies are required to confirm the ability of pancreatic mesenchyme to induce the differentiation of oval cells to pancreatic endocrine cells. The results obtained in this study provide a basis with which to continue further experimentation both into mesodermal capabilities and the plasticity of oval cells.

4

DISCUSSION AND CONCLUSIONS

4.1 THE ROLE OF GaIN IN HEPATIC INJURY

The role of GaIN as a model for hepatic injury is based on its hepatotoxic effect. The action of this agent is associated with a rapid depletion of uracil nucleotides and consequently the inhibition of protein, glycoprotein, glycolipid, glycolipoprotein and phosphoprotein synthesis in hepatocytes (Dabeva and Shafritz, 1993; Kmieć *et al.,* 2000). The ensuing structural and functional damage to the liver, characteristically similar to acute hepatitis, is necessary for the activation of the oval cell compartment (Ferenčíková *et al.,* 2003). Oval cells are not targeted by the action of GaIN and thus act to restore the hepatic parenchyma by proliferation and differentiation along the hepatic lineage. This is a compensatory mechanism which is activated when the regenerative capacity of hepatocytes is impaired, as in the GaIN model of hepatic injury (Dabeva and Shafritz, 1993).

As evidenced in this study, degenerating hepatocytes exhibited an increase in cytoplasmic eosinophilia and loss of nuclear structure. Furthermore, hepatic cord structure was markedly disrupted with hepatocytes assuming an acinar arrangement. It was established in this study that the degeneration of the hepatic parenchyma increased in severity concomitantly with an increase in the dosage of GaIN. Furthermore, an increase in putative oval cell proliferation was also seen to be dependant on higher concentrations of GaIN, as well as on the duration of hepatic injury. Morphologically, oval cells are 10μm in size, with a large nuclear-cytoplasmic ratio and oval nucleus (Zhang *et al.,* 2003). This morphology was used to identify oval cells in this study. Moreover, oval cell nuclei were found to stain palely basophilic and were surrounded by scant basophilic cytoplasm, as reported by Dabeva *et al.* (1993). Oval cells were seen to proliferate in the periportal areas, in close association with the bile ducts. These cells were seen to invade the hepatic parenchyma as cords. The presence of both small hepatocytes and atypical duct structures are reminiscent of the ability of oval cells to differentiate into both hepatocytes and bile ductular cells (Dabeva *et al.*, 1993).

Dabeva and Shafritz (1993) have determined that oval cells are capable of restoring the hepatic parenchyma, re-establishing normal liver function and thus allowing GaIN-treated animals to resume their health. In this study, however, a number of animals died unexpectedly resulting in the termination of Animal Ethics Approval by the Animal Ethics Committee (AEC) of the University of the Witwatersrand for this part of experimentation.

The percentage of animals which died prematurely is illustrated in Table I. Of the 33.33% of untimely deaths, one animal succumbed at a 50mg GaIN/100g body weight dose and others at a 70mg GaIN/100g body weight dose. In Table I it can be noted that the number of premature deaths doubled year to year, from 2004. Animals exhibited severe weight loss, with a tendency to bleed at the extremities. Autopsy reports revealed severe necrosis of the liver parenchyma with degeneration of the hepatocytes. This included loss of nuclear structure and an increase in the eosinophilia of hepatocyte cytoplasm. Such pannecrosis is a severe lesion associated with toxin exposure. It was thus put forth by the AEC firstly, that the GaIN dose used was too high and secondly, that the toxicity of GaIN was directly responsible for the premature deaths of these animals.

Despite the association of hepatic necrosis with the effects of GaIN, the restoration of the liver parenchyma by putative progenitor cell proliferation has been documented. This forms the basis of the current understanding of oval cell behaviour and the subsequent reconstitution of the liver mass when hepatocyte replication is impeded. Although published work does not state the number of premature deaths that may have occurred in the duration of their research, personal correspondence with Dr. Dabeva (of Dabeva and Shafritz, 1993) from the Albert Einstein College of Medicine, New York, on whose work the dose was based, includes the following revelations.

Firstly, the 70mg GaIN/100g body weight dose is well tolerated by wild-type animals such as Sprague-Dawley rats. GaIN itself was purchased a number of times in the duration of this study, thereby precluding the possibility of batch contamination.

Moreover, GaIN is purchased from the same company used by Dabeva (personal communication). Furthermore, due to decreased levels of purity of GaIN, Dabeva (personal communication) has increased the dose to 110mg GaIN/100g body weight – almost twice the dose used in this study. It is therefore unlikely that the 70mg GaIN/100g body weight dose was too concentrated for toleration by the animals in this study.

Dabeva (personal communication) also highlighted the possibility of the exposure of these animals to an endotoxin, contact with which increases several-fold the effects of GaIN, notwithstanding that endotoxins are themselves capable of inducing hepatic injury. According to Galanos *et al.* (1979) variation occurs between and within animal species with regard to their reaction to the lethality of endotoxins. They postulated that GaIN increased the sensitivity of animals to this toxic effect. In their study, rabbits, Lewis rats and mice were used and kept under pathogen-free conditions. Lipopolysaccharide was purified from *Salmonella arbortus equi* and Lipid A was purified from *Salmonella minnessota* R345. The mice and rats received the endotoxins via IP injection, either combined with GaIN or immediately after GaIN administration (Galanos *et al.,* 1979). The GaIN dosage was 30mg/100g body weight – an amount sufficient to induce sensitization to endotoxin (1μg -10μg) in rats. An injection consisting of GaIN + 10μg endotoxin resulted in 100% death while GaIN + 1μg endotoxin resulted in a 50% death rate, 6-9 hours after administration (Galanos *et al.,* 1979). Also, in rabbit and mouse control groups (rat data not reported), receiving GaIN *sans* endotoxin, no deaths were recorded (Galanos *et al.,* 1979).

It was thus found by Galanos *et al.* (1979) that the administration of GaIN increased the susceptibility of animals to an endotoxin several thousandfold. Sensitivity was at its highest when the endotoxin was introduced together with GaIN or soon after GaIN administration. This sensitization has been linked to the early metabolic effect that GaIN has on the liver. It has thus been postulated that transient changes in metabolic activity can result in increased susceptibility of an animal to small amounts of endotoxin (Galanos *et al.,* 1979).

According to Dr. Herbst (personal communication) of the Albert Einstein College of Medicine's Animal Institute, New York reagent sterility and aseptic technique are of particular importance, in light of the influence of endotoxins. In this study GaIN concentrates were prepared with sterile saline and filtered prior to use. Trained personnel administered the majority of injections. The animals, however, were not disinfected prior to injection. It is thus possible that at this stage, bacteria from the surface were introduced into the peritoneal cavity. Following this communication, the site of injection was alcohol-sterilised. This did not however, prevent the ensuing premature deaths.

The effects of bacterial translocation during GaIN treatment need also be taken into account. Normally, the involvement of immunoglobulins, local and systemic macrophages and mucosal barriers prevent the translocation of enteric bacteria (Kasravi *et al.,* 1996). Such translocation may intensify the extent of damage caused by acute hepatic injury. In order to investigate such a phenomenon, Kasravi *et al.* (1996), administered 110mg GaIN/100g body weight to Sprague Dawley rats via an intraperitoneal injection. It was found that the intestinal mucosa was adversely affected, with permeability increasing concomitantly with decreasing arterial blood pressure. Furthermore, liver macrophages, activated by GaIN, release inflammatory mediators that not only enhance the hepatotoxic effect of GaIN, but also adversely affect the intestinal mucosa (Shiratori *et al.,* 1988). Furthermore, macrophages have also been implicated in the active translocation of enteric bacteria (Kasravi *et al.,* 1996). Despite a number of studies, bacterial translocation is a poorly understood phenomenon, with varying aetiologies and complications and thus remains a controversial subject. Nevertheless, such evidence may be used to explain the high rate of premature deaths experienced in this study. Further exploration in this area need be done before re-attempting to use the GaIN model of hepatic injury in the study of oval cell activation.

4.2. Establishment of Primary Cell Cultures from GaIN-treated Adult Rat Liver

The establishment of a primary cell culture proved problematic. Perfusion using a 0.05% collagenase solution followed by mechanical dissociation of the tissue did not yield a significant viable cell contingent. Carlsen *et al.* (1981) have investigated the properties of single cell suspensions of hepatocytes obtained both from enzymatic dissociation and mechanical dissociation of tissue. Both methods of isolation have adverse effects on cell-surface properties and consequently affect the propagation of cells in culture. Although a number of studies have used these methods in the isolation of single cell suspensions, minor alterations in methodology may have profound effects on cell properties and consequently, behaviour (Carlsen *et al.,* 1981). The cell suspensions obtained in this study did not propagate in culture. This could be attributed to a low cell yield or to excessive disruption of intercellular communication systems.

The use of magnetic microbeads for direct and indirect labeling of oval cells and subsequent sorting in a high gradient magnetic field proved unsuccessful. The efficacy of the use of magnetic microbeads in the sorting of cell populations in other studies has been documented (Servida *et al.,* 1996). However, this is dependant on the use of a single cell suspension with a significant yield of viable cells, as well as on the specificity of the microbeads used. Histological observations of the GaIN-treated liver in this study (70mg GaIN/100g body weight) revealed proliferation of a significant oval cell population however the single cell suspension obtained from these livers did not provide a significant yield of viable cells. Furthermore, the $CD90^{+}$ microbeads used in the direct labeling technique were not specific to rat and were intended for cell sorting in mouse samples. Indirect labeling however, incorporated the use of a CD90 PE-conjugated primary antibody specific for rat tissue, followed by labeling with Anti-PE microbeads. However, the resultant fraction did not proliferate in culture. A low cell yield was also obtained from the cell sorting method adapted from Kano *et al.* (2003) and was incapable of proliferating in culture. The establishment of a primary cell culture failed despite the use of three various techniques, a failure which is attributable to an ineffective isolation of a single cell suspension. This not only highlights the importance of appropriate and

effective tissue dissociation protocols but also that minor alterations in methodology may have profound effects on the resultant single cell suspension. Since a primary cell culture with which to continue experimentation was not obtained, the donation of a cell line (PHeSC-A2) by Prof. Kano of the University of Tsukuba, Japan, allowed for the progression of this study.

4.3. DERIVATION OF THE PHESC-A2 CELL LINE BY KANO *ET AL.* (2003)

Kano *et al.* (2000) investigated the differentiating capacity of the non-parenchymal epithelial cell fraction derived from porcine liver. Earlier work conducted by Kano *et al.* (2000) established that normal adult porcine liver contained a population of progenitor cells. The methodology used in the preparation of a non-parenchymal cell suspension thus precluded the use of hepatic injury models. The derivation of putative progenitor cells from untreated animals are of especial interest in lieu of the premature deaths of GaIN-treated rats experienced in this study.

Cells isolated by Kano *et al.* (2000) were plated on type-I collagen-coated culture dishes and propagated in specifically formulated NAIR-1 medium. Forty-eight hours after plating, clusters of cells began to proliferate and scatter, forming colonies (Kano *et al.,* 2000). Cells observed in the central region of these colonies were of a smaller size. It was found that 96% of scattered cell clusters developed duct-like structures, indicating a correlation between scattering and the emergence of such structures (Kano *et al.,* 2000). *In vivo*, proliferating oval cells, themselves non-parenchymal in nature, are known to invade the surrounding parenchyma, either differentiating into hepatocytes or organizing themselves into ductular structures (Alison and Sarraf, 1998). This remarkable phenomenon, was evidenced in this study, in haematoxylin and eosin-stained sections of GaIN-treated liver, and is reminiscent of the development of ductular structures identified in the *in vitro* studies conducted by Kano *et al.,* (2000).

Furthermore, the *in vitro* studies elucidated in the preceding paragraph by Kano *et al.* (2000) demonstrated that during the scattering stage, the cell clusters tested positive for the immature liver marker, AFP and hepatocyte marker, albumin, affirming their oval cell

nature as defined by other studies (Lemire *et al.,* 1991; Wang *et al.,* 2003). At the stage of duct-like structural development, these markers were expressed in subsets of cells surrounding the structures themselves and more strongly, at the periphery of the colonies (Kano *et al.,* 2000). Markers associated with biliary epithelial cells, CK19, CK14 and GGT, were expressed by cells forming duct-like structures but were not detected in the rest of the cells constituting the colonies. These markers are also associated with oval cells (Gordon *et al.,* 2000; Hixson *et al.,* 1997).

Kano *et al.* (2000) also investigated the expression of oval cell antigens OV.6 and OC.2. It was found that the scattered clusters of cells were OV6-postive but OC.2 negative. Cells constituting the duct-like structures expressed both these antigenic markers. Furthermore, these cells stained positive for vimentin, an intermediate mesenchymal filament (Kano *et al.,* 2000). Vimentin is also expressed by migrating oval cells. The co-expression of the mentioned cytokeratins and intermediate filaments, amongst others, are postulated to play a role in the migratory behaviour of cancerous cells (Alison and Sarraf, 1998). Oval cells have been implicated in the development of certain types of liver cancers, notably cholangiocarcinomas – adenocarcinomas of bile duct epithelium (Underwood, 2000; Grompe, 2003).

The biliary nature of oval cells is further highlighted in their proposed origins – the bile ductules, specifically, the canals of Hering, (Alison and Sarraf, 1998). It is postulated that oval cells are the progeny of intrahepatic stem cells residing either within the lining of the canals of Hering, or surrounding these ducts (Alison and Sarraf, 1998; Fausto, 2000). In this study, oval cell proliferation was seen to be closely associated with the bile ductules. Petersen *et al.* (1997), have demonstrated that damage to the biliary system, using methylene dianiline, prevents the proliferation of oval cells (Petersen *et al.,* 1997). Conversely, further studies conducted by Petersen *et al.* (1997) found that oval cell proliferation was not disrupted when using an acute dose of methylene dianiline followed by bile duct ligation (Petersen *et al.,* 1999). This implicated the derivation of oval cells from circulating haematopoietic stem cells. The demonstration by Kano *et al.* (2000), of the existence of hepatic progenitor cells in normal adult porcine liver could preclude the

invasion of haematopoietic stem cells as a compensatory mechanism for hepatocyte dysfunction and instead highlight the origin of oval cells as intra-hepatic. It is, however, plausible that haematopoietic stem cell involvement in liver regeneration is a compensatory mechanism for *oval cell* dysfunction, akin to the role oval cells themselves play when hepatocyte function is impeded. Haematopoietic stem cells are capable of differentiating into a myriad of cell types including skeletal and cardiac muscle, neurons, pneumocytes and hepatocytes (Alison, 2002). Petersen (2001) proposes that the oval cell compartment is a transitional compartment wherein haematopoietic stem cells differentiate into the hepatic lineages. That oval cells express a number of haematopoietic markers (Alison and Sarraf, 1998) is also of interest. Whether this feature could be linked to the embryological role of the liver in haemopoiesis is however, unknown. Despite such speculation it remains obvious that oval cells have yet to be fully characterized, their participation in hepatic regeneration yet to be fully comprehended and their capacity to differentiate yet to be fully investigated. Kano *et al.* (2003) have proposed that their establishment of a hepatic stem cell line is essential to the analysis of these cells and may assist in the understanding of oval cell character and behaviour in an *in vitro* environment.

A non-parenchymal population isolated from normal adult porcine liver was used by Kano *et al.* (2003), to establish a hepatic stem cell-like line (PHeSC-A2), which was donated for use in this study. As elucidated in the preceding paragraphs, this fraction was shown to contain hepatic progenitor cells with characteristics akin to that of oval cells (Kano *et al.,* 2000). It was found that cells cultured on PDL-coated dishes, in specifically formulated NAIR-1 medium were able to sustain proliferation through several passages (Kano *et al.,* 2000). In this study, it was demonstrated that the PDL-NAIR-1 system is essential to the maintenance of PHeSC-A2 cell proliferation, when compared to the GFRM-Ham's F12.ITS system. In the GFRM-Ham's F12.ITS system, PHeSC-A2 cells remained tightly aggregated. The cells neither disseminated, as in the case of mesodermal cells nor evidenced proliferation over a 5 day culture period. Furthermore, the morphology of the PHeSC-A2 cells in this culture system differed to those propagated in the PDL-NAIR-1 system. In the GFRM-Ham's F12.ITS culture system, most PHeSC-A2

cells remained clustered together with only a few cells assuming the spindle-shaped morphology exhibited by PHeSC-A2 cells grown in the PDL-NAIR-1 system.

Kano *et al.* (2003) found that after seven days in culture, cells from the non-parenchymal fraction of porcine liver formed aggregations expressing AFP mRNA, as determined by RT-PCR. The aggregations were then isolated and subjected to single cell sorting using FACS (Kano *et al.,* 2003). Ultimately two cloned cell lines – designated PHeSC-A1 and PHeSC-A2 were established. The characteristics of both cell lines were investigated using RT-PCR and immunocytochemical techniques. It was demonstrated that the cell lines exhibited immature hepatic phenotypes (Kano *et al.,* 2003). Although spontaneous differentiation of some cells into hepatocytes and biliary epithelium were noted, it was found that the expression of AFP mRNA increased significantly during each passage indicating the maintenance of both the stem-like phenotype and the proliferative capacity of the majority of the cells (Kano *et al.,* 2003).

In this study, localization of the AFP marker was attempted. Immunolocalization of AFP on positive controls (fourteen and a half day old fetal liver) yielded negative results. It thus followed that the efficacy of the primary antibody was significantly reduced and would therefore present itself as ineffective in the immunolocalization of AFP on the PHeSC-A2 cell line. Considering the battery of tests applied to the PHeSC-A2 cell line by Kano *et al.* (2003), with the results indicative of oval cell phenotypes, the failure of the ICC technique in this study was not sufficient to discontinue experimentation. The PHeSC-A2 cell line was thus deemed an appropriate cell line with which to continue experimentation in this study.

4.4. THE INFLUENCE OF PANCREATIC MESODERM ON PHESC-A2 CELLS

Pancreatic mesoderm, while essential for the differentiation of endocrine cells from the endodermal component of the dorsal and ventral pancreatic buds (Kim *et al.,* 1997), has also been shown to repress β-cell differentiation (Duvillié *et al.,* 2006). The inductive capability of pancreatic mesoderm is said to be dependant on the developmental stage at which it is utilised. Duvillié *et al.* (2006), have proposed that this phenomenon is

essential to the production of a significant pancreatic progenitor population before proceeding to the differentiation of specialized cells. The pancreatic mesoderm used in this study, obtained from quail embryos at stage 25 have been previously shown to have inductive properties, specifically of β-cell differentiation from pancreatic endoderm (Kramer *et al.,* 1987).

The influence of pancreatic mesoderm on the differentiation of oval cells (as the PHeSC-A2 cell line may be referred to in this study, in lieu of their characteristics as highlighted in 4.3.), was thus investigated in an *in vitro* system. A 'sandwich system' was created to ensure the close proximity of the PHeSC-A2 cells and the pancreatic mesoderm - necessary for the easy diffusion of mesodermal factors to their target. Mesoderm was thus placed between two layers of oval cells and covered in its entirety by GFRM prior to the addition of Ham's F12.ITS medium. Not all 1μm sections obtained from resin-embedded co-cultures contained mesodermal tissue, firstly due to the 'sandwich system' used in this study and secondly due to the dissemination of mesodermal cells from the explants and their ensuing proliferation.

Quail mesoderm was chosen specifically for use in this study, in order to preclude the possibility of endodermal contamination and the resultant differentiation of insulin-positive cells, by morphological discrimination. Quail nuclei are large and exhibit prominent nucleoli and are thus distinguishable from PHeSC-A2 cells. The insulin and glucagon-positive cells, immunolocalized in this experiment, exhibited small, oval, condensed nuclei. This nuclear morphology contrasts dramatically with that of quail cells. Insulin and glucagon-positive cells were thus deemed to be PHeSC-A2 cells which had been induced to differentiate into pancreatic endocrine cells. Although cells positive for the pancreatic markers in question, were few, the proportion of insulin-positive cells exceeded that of glucagon-positive cells. This may be attributable to the use of GFRM which has previously been shown to increase the proportion of insulin-positive cells relative to glucagon-positive cells (Rawdon and Andrew, 1998). This effect has been attributed to a reduced concentration of TGF-β1. TGF- β1 has an anti-proliferative action on insulin cells (Rawdon and Andrew, 1998) and has also been implicated in the

inhibition of hepatocyte proliferation and the induction of hepatocyte apoptosis in normal adult liver (Clark *et al.,* 2005).

A number of factors may have contributed to the low yield of the insulin-positive cells identified. The PDL-NAIR-1 culture system in which PHeSC-A2 cells are generally grown has been specifically formulated to maintain the viability and differentiating capacity of the cells in question (Kano *et al.,* 2003). The ability of the GFRM-Ham's F12.ITS culture system to maintain oval cell viability needs to be determined. Although this culture system has been previously shown to promote differentiation of pancreatic endocrine cells in cultures of the embryonic chick pancreas (Kramer and Penny, 2003), it is nonetheless probable that factors within the said system may have impeded the differentiating capacity of the oval cells. It is also possible that the oval cells and mesoderm were not in close enough proximity to allow the diffusion of signaling molecules to a significant population of oval cells.

In order to confirm visual observations suggesting the derivation of pancreatic endocrine cells from hepatic oval cells, immunocytochemical techniques, as well as the Feulgen method were conducted to distinguish the quail nuclear marker from PHeSC-A2 cell nuclei. However, positive controls in both techniques exhibited neither immmunolocalization of the quail nuclear marker nor the staining associated with the Feulgen method. The failure of the immunocytochemcal technique is attributed to the use of an expired quail nuclear marker, the primary antibody QCPN mAB. The ineffectiveness of the Feulgen technique, designed to demonstrate DNA is most likley due to the fixative used in this study (parabenzoquinone vapour). Fixatives used in the Feulgen technique are critical to the hydrolysis of DNA by HCl (Bancroft and Cook, 1996). This hydrolysis is used to break the purine-deoxyribose bond thereby exposing aldehydes. The resulting aldehydes are visualized using Schiff's reagent and appear red-purple (Bancroft and Cook, 1996). This was however, not demonstrated in this study. It is therefore put forward that the hydrolysis time was ineffective owing to the fixative, parabenzoquinone vapour, used in this study. Since neither the ICC technique nor the Feulgen method could identify known quail nuclei, no confirmation barring visual

observations of the insulin and glucagon-positive cells indicating their oval cell derivation could be made. Further studies are required to affirm these results and thus the ability of pancreatic mesenchyme to induce differentiation of oval cells into pancreatic endocrine cells. However, prior to such experimentation, it is firstly necessary to assess the proliferative capacity of PHeSC-A2 cells when cultured in GFRM, as well as their ability to retain their stem cell-like properties. The former is essential for obtaining a significant population of cells within the system in question, whilst the latter is essential to their capacity to accept influences from the mesodermal explants.

4.5. MESODERMAL SIGNALS

As previously mentioned, mesodermal signals are essential for the specification of the pancreas and for the differentiation of endocrine cells in the endodermal component of the dorsal and ventral pancreatic buds (Kim *et al.,* 1997; Kumar *et al.,* 2003). Rossi *et al.* (2001) have proposed that BMP signaling from the septum transversum mesenchyme, plays a role in hepatogenesis by priming foregut endoderm to respond to FGF signaling from cardiac mesenchyme, without which putative hepatic endoderm reverts to a default pancreatic fate. These mesodermal signals can either be 'permissive' or 'instructive'. Instructive signals can be defined as those capable of patterning the endoderm, or more specifically, establishing a pancreatic domain or pre-pattern within the endoderm. Permissive signals, however, are incapable of such patterning and act effectively only on endoderm for which a pancreatic fate is already specified (Kumar *et al.,* 2003). These signals allow for the maintenance of pancreatic gene expression and further cell differentiation.

Kim *et al.* (1997) have shown that signals from the notochord are also essential to pancreatic development, specifically for the formation of the dorsal pancreatic bud. Moreover, at stage 11 in chick embryos, the notochord is the only mesodermal component in direct contact with prospective pancreatic endoderm. Removal of the notochord prevents the expression of pancreatic genes in the dorsal endoderm but not in endoderm destined for ventral pancreatic bud fate. However, when combined with non-pancreatic endoderm, the notochord is incapable of inducing pancreatic gene expression

(Kumar *et al.,* 2003). As the only mesodermal contingent in contact with putative pancreatic endoderm at that specific developmental stage, this raises a number of possibilities. Firstly, that signals from the notochord, may be permissive not instructive; secondly, that direct contact with a mesodermal contingent may not be necessary and that thirdly, the endoderm in question may be patterned at an earlier stage. Although the mechanisms responsible for the patterning of the endoderm have yet to be elucidated, mesodermal influences, specifically from pancreatic mesoderm, are essential for the differentiation of pancreatic β-cells from pancreatic endoderm (Kim *et al.,* 1997).

According to Kumar *et al.* (2003), signals from the lateral plate mesoderm as early as stage 8 in chick embryos have been shown to be instructive. This occurs prior to pancreatic bud formation. These signals induce the expression of a number of pancreatic markers including insulin, glucagon and PDX1 (Kumar *et al.,* 2003). Kumar *et al.* (2003) attempted to identify the mesodermal signals responsible for inducing the expression of PDX1. Although the nature of these signals remain elusive, Kumar *et al.* (2003) have proposed that the growth factors, activin and a number of the family of BMP's, may play a role in the induction of pancreatic fate. PDX1 has also been proposed as the transcription factor most likely to differentiate pancreas from liver (Kumar and Melton, 2003). Although essential for pancreatic development, PDX1 is incapable of independently inducing cells toward a pancreatic fate without the assistance of co-factors (Kumar and Melton, 2003). Studies conducted by Ahlgren *et al.* (1996) found that pancreatic bud development was initiated in mice with a homozygous mutation for the PDX1 gene. The mesenchyme demonstrated normal morphology and function but signaling from this contingent was unable to further the development and differentiation of the endoderm (Ahlgren *et al.,* 1996).

Such data affirms the role of PDX1 as permissive rather than instructive in the patterning of the pancreatic domain. Moreover it highlights the importance of reciprocal communication between the mesoderm and endoderm, more specifically known as mesenchymal-epithelial interactions. It must also be noted that pancreatic development and differentiation is a multifaceted phenomenon involving an array of signals and

transcription factors. Although many may be identified, their roles in isolation may appear or may be permissive. It may therefore only be in complex relationships that a variety of factors acting in a cooperative, cohesive manner may assume an instructive role.

In the present study is it postulated that oval cells retain a higher level of potentiality than previous demonstrated (Kano *et al*., 2003) and are able to differentiate along either a hepatic or pancreatic lineage depending on the surrounding environmental influences. This proposition, based on the close embryological association of the pancreas and liver, has been validated by a number of studies (Deutsch *et al.,* (2001); Yang *et al.,* (2002). It is further postulated in this study, that the role of pancreatic mesoderm in the differentiation of endocrine cells from oval cells is permissive. Although it was found that oval cells are capable of differentiating into pancreatic endocrine cells under the influence of pancreatic mesoderm, further experimentation is needed to validate these results and thus the stated hypotheses.

4.6. LIVER-PANCREATIC SWITCHES

Deutsch *et al.,* (2001), have proposed the existence of a hepatopancreatic stem cell based on the developmental relationship between the liver and pancreas, more specifically between the liver and the ventral pancreatic bud. The dorsal pancreatic bud arises from the dorsal aspect of the foregut endoderm, contrasting with the development of the hepatic diverticulum and ventral pancreatic bud from ventral foregut endoderm. Deutsch *et al.* (2001), have thus proposed that the liver and ventral pancreatic bud, arising from adjacent areas of the same germ layer, share a common endodermal progenitor cell.

This however, does not preclude the existence of a hepatopancreatic stem cell within the dorsal pancreatic endoderm. It has already been established that signals from the lateral plate mesoderm play an instructive role in the patterning of the pancreatic domain prior to the emergence of either dorsal or ventral buds (Kumar *et al.,* 2003). As such, pancreatic stem cells within the endodermal component of both buds should only differ in action based on different mesenchymal-epithelial interactions – not on the initial patterning.

Moreover, considering that differentiation of endocrine cells occurs in the endodermal components of both buds, it follows that although these buds may initially interact with signals from different mesodermal sources, the signals themselves would not in fact differ. Furthermore, fusion of the dorsal and ventral pancreatic buds, midgestation, (Allan and Kramer, 2002) would expose both endodermal components to both mesenchymal components, and as such, to similar influences that would require reciprocal interaction with appropriate patterning in order to be effective.

A number of studies have investigated the plasticity of pancreatic and hepatic adult progenitor cells, more specifically the differentiation of these cells into hepatic and pancreatic lineages respectively. Yang *et al.* (2002) isolated hepatic oval cells from rat livers using the 2-AAF hepatic injury model. Flow cytometric cell sorting methodology was used to purify the cell sample, retaining cells positive for the CD90 marker. They were propagated in serum-free IMDM supplemented with 10ng/ml SCF, 10ng/ml LIF and 10ng/ml Flt-3 ligand. During continuous culture, the oval cells expressed markers for AFP, CD90, CD34 and c-kit. Cells were induced to differentiate into pancreatic endocrine cells by a change of medium to RPMI, supplemented with FBS and containing a total of 23.5mM glucose. Cells proliferated as spheroidal aggregates, similar morphologically to islet-cell clusters.

Yang *et al.* (2002) found that differentiation of hepatic progenitor cells into endocrine cells was dependant on the withdrawal of LIF from the culture conditions and the introduction of a high glucose medium. Differentiating cells expressed markers for PDX1, Pax4 and Pax6, as well as markers for the endocrine hormones, insulin and glucagon, as determined by RT-PCR. ICC and western blot assays demonstrated the presence of insulin and glucagon within differentiated cells. Furthermore, the cell clusters were shown to be competent in *in vivo* studies, actively reversing hyperglycaemia following transplantation in diabetic mice.

The differentiation capacity of oval cells has been further highlighted by Nakajima-Nagata *et al.* (2004). Hepatic progenitor cells isolated from rat liver were cultured in the

presence of 10ng/ml EGF, 20ng/ml HGF, 10mM nicotinamide and 10^{-7}M dexamethasone in 0.2% FBS-containing DMEM/F12K medium. It was found that these culture conditions were able to induce the differentiation of hepatic progenitor cells into insulin-producing cells. Furthermore, the forced expression of PDX1 via gene transfection protocols in differentiated cells was shown to significantly increase the amount of insulin produced, comparable to the amounts produced in an *in vivo* environment. Using early assays Nakajima-Nagata *et al.* (2004), also identified that the isolated hepatic progenitor cells, were positive for the neural stem cell marker, nestin. Zulewski *et al.* (2001) earlier identified multipotential stem cells within the pancreatic islets positive for the nestin marker. Cells were initially cultured in RPMI 1640 medium supplemented with 20ng/ml bFGF and 20ngEGF, 10% FBS, 10mmol HEPES buffer, 1mmol/L sodium pyruvate and 71.5μmol/L β-mercaptoethanol (Zulewski *et al.,* 2001). Although the nestin-positive cells exhibited none of the pancreatic markers initially, upon confluence and with the addition of HGF, betacellulinn, activin-A and nicotinamide to the media, they were shown to not only differentiate into both exocrine and endocrine components of the pancreas, but also into cells expressing hepatic phenotypes. Liver-specific markers exhibited included AFP, E-cadherin, c-MET and HGF, whilst pancreatic markers included insulin, glucagon and IDX1 (Zulewski *et al.,* 2001).

The PHeSC-A2 cell line used in this study had previously been shown to exhibit immature hepatic markers (Kano *et al.,* 2003). Whether these cells exhibit pancreatic markers is unknown. However, their putative differentiation into insulin and glucagon-producing cells, as determined using immunocytochemical techniques in this study, implicate a higher level of plasticity than demonstrated by Kano *et al.* (2003). The PHeSC-A2 cell line thus needs to be subjected to further experimentation. This experimentation could firstly, include further culture of the cell line in the presence of pancreatic mesoderm to confirm the results obtained in this study; secondly the plasticity of PHeSC-A2 cells could be investigated under the different culture conditions used by Yang *et al.* (2002) and Nakajima-Nagata *et al.* (2004).

The close association of liver and pancreas is further emphasized by studies establishing the existence of pancreatic oval cells (Grompe, 2003). Originating within the pancreatic ducts, these cells exhibit characteristics of hepatic oval cells. Rao *et al.* (1989) induced a progressive loss of pancreatic exocrine tissue with no involvement of either the pancreatic ducts or islets, by the administration of a copper-deficient diet in rats. Cells exhibiting the oval cell markers GGT and albumin were identified, increasing in number concomitantly with acinar degeneration. Following the withdrawal of the copper-deficient diet and a recovery period, it was found that islands of hepatocytes occupied the majority of the pancreatic tissue (60 –80%) (Rao *et al.,* 1989). Furthermore, Dabeva *et al.* (1997) established that pancreatic oval cells are able to differentiate into functional hepatocytes following transplantation into hepatic tissue. Moreover, pancreatic oval cell derived-hepatocytes are functionally and morphologically indistinguishable from hepatocytes derived from transplanted hepatic oval cells.

Although more evidence for the definitive existence of a shared hepatopancreatic stem cell needs to be obtained, the findings highlighted in the preceding paragraphs credits a higher level of potentiality to both hepatic and pancreatic progenitor cells, whose roles traditionally were thought to be restricted to differentiation along their own tissue-specific lineages. The plasticity of progenitor cells is presently under review by many scientific communities (Sell, 2004). Orthodox embryologists maintain that determination is unidirectional (Sell, 2004) i.e. that when cells are committed to a certain cell lineage they cannot dedifferentiate, a process whereby terminally differentiated cells are induced to lose their tissue specific phenotype (Stocum, 2004) or transdifferentiate, a process whereby a differentiated cell type is converted to a cell type of another lineage (Horb *et al.,* 2003). The transfer of a somatic cell nucleus to an enucleated oocyte followed by electrical or chemical stimulation to induce embryogenesis is an example of dedifferentiation (Sell, 2004), whilst transdifferentiation has been documented in studies of the conversion of liver to pancreas (Horb *et al.,* 2003).

According to Horb *et al.* (2003), the interconversion of liver and pancreas is facilitated by the nature of their organogenesis. Shen *et al.* (2003) have proposed that tissues that

develop adjacent to each other from a common cell sheet will differ from each other by the differential expression of either one or a few transcription factors. Horb *et al.* (2003) have suggested that only one developmental decision affecting a small number of genes is responsible for the divergence of somatic cells to either a liver or pancreatic fate. As such, if terminally differentiated hepatic cells can be directed along a pancreatic lineage, more so should it be expected from progenitor cells with a more accessible plasticity. Moreover, this brings to the fore the extent of influence exerted by microenvironmental factors, both chemical and mechanical, on cell differentiation (Huang and Ingber, 2004), regardless of the state of potential embodied by the cell.

4.7 CONCLUSIONS

Oval cell proliferation was successfully induced *in vivo* following GaIN administration. It was established that a 70mg GaIN/100g body weight dose and a 5 day duration of hepatic injury was sufficient to activate the proliferation of a significant oval cell population as determined visually. Further investigation of the action of GaIN is, however, required prior to continued use of this agent as a model of hepatic injury in lieu of the high incidence of premature deaths experienced in this study. Isolation of an oval cell population from non-treated liver, as conducted by Kano *et al*. (2003), should be applied across a species range, to ascertain firstly, whether hepatotoxic agents are in fact needed for oval cell activation, and secondly whether oval cells remain senescent or assume a more active role in the normal turnover of liver cells. Success in this regard would preclude the use of hepatotoxic agents and importantly, if applied to a human model, could be used for the isolation of oval cells from biopsy samples of liver. Thus, should the differentiation of pancreatic β-cells from oval cells and the transplantation thereof, prove successful, the initial isolation of oval cells would preclude problems of immune rejection.

Although the presence of oval cells in GaIN-treated liver was confirmed in this study, the establishment of primary cell cultures from the liver was found to be a precarious science whose success was affected by minor changes in technique. This illustrated the value of a number of parameters in the isolation of single cell suspensions. Furthermore, the

importance of culture systems and the factors within those systems on the maintenance of the viability, proliferation and differentiation capacity of cells, in lieu of the propagation of the PHeSC-A2 cell line, has also been highlighted. *In vitro* experimentation is in itself limited in application since cultured cells are not representative of the *in vivo* environment from which they are removed or intended to be part of. It is therefore only within the *in vivo* environment that results from *in vitro* studies can be functionally validated.

In order to understand the mechanisms underlying hepatic-pancreatic switches, it is necessary to identify the signals responsible firstly, for the patterning of the foregut endoderm into pancreas and liver and secondly, for the differentiation of pancreatic endocrine cells from pancreatic endoderm. Furthermore, the inherent plasticity of hepatic oval cells as well as their relationship to *pancreatic* oval cells requires thorough investigation. The theory of the unidirectionality of determination is in the process of being refuted; the implications being that somatic cells are capable of dedifferentiation and transdifferentiation and that their limitations may only be the terminology applied to them. The extent of influence exerted by microenvironmental factors – intracellular and extracellular, mechanical and molecular – are thus under investigation (Huang and Ingber, 2004). Signals are now thought not to act in a linear fashion, but rather to induce effects as part of a network. The signals themselves are thus seen to possess multiple functions dependant on the networks within which they act. Mechanical cues and topographical limitations need also be taken into account as able to effect certain selective pressures on cell states and fates (Huang and Ingber, 2004).

The tentative results obtained in this study have lead to the following postulations: firstly, that the PHeSC-A2 cell line may possess a higher level of potentiality than previously demonstrated by Kano *et al.* (2003), secondly that the potential may be due to the shared embryological origins of the pancreas and liver as proposed by Dabeva *et al.* (1997) and thirdly, that permissive signaling from the pancreatic mesoderm may be able to induce the differentiation of hepatic oval cells into pancreatic endocrine cells as demonstrated by

the results obtained in this study. Further research is however, required to confirm these results and to substantiate the aforementioned propositions.

Successful isolation of hepatic stem cells from untreated liver, and the differentiation of these cells into pancreatic endocrine tissue, under the influence of pancreatic mesoderm, could allow for the *in vitro* production of a significant β-cell population. The competency of this population within the *in vivo* environment could allow for the repopulation of the endocrine component of diabetic pancreata. The implications of success in this regard would be an improved quality of life for diabetics and consequently a significant reduction in morbidity and mortality rates.

5

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APPENDIX I

BISULPHITE SOLUTION

10%	Potassium metabisulphite
5ml	1M Hydrochloric acid
90ml	Distilled water

CALCIUM AND MAGNESIUM FREE TYRODE'S SOLUTION

Solution A

500ml	Distilled water
2.0g	Sodium chloride
0.05g	Potassium chloride
0.012g	Sodium dihydrogen phosphate

Solution B

480ml	Distilled water
0.025g	Sodium hydrogen carbonate

Solution C

20ml	Distilled water
1g	Glucose

Autoclave solutions A and B. Leave solution C overnight in refrigerator.
On the morning of the experiment add solution B to solution A. Filter solution C into A. Add 100µl pen/strep.

CHICK RINGER

8.5g	Sodium chloride
0.42g	Potassium chloride
0.25g	Calcium chloride
1L	Distilled water

Autoclave to sterilise and add 100µl pen/strep

DAB

Solution A

1mg	DAB
2ml	Tris/saline (pH 7.6)

Solution B

1ml	30% Perhydrol
29ml	Distilled water, chilled

Add 20µl of Solution B to Solution A. Mix well.

DILUENT FOR ANTISERA

100ml	Tris/saline
0.25g	Sodium azide
5mg	BSA
40mg	EDTA
1ml	Swine serum

NAIR-1 MEDIUM

1:1	DMEM/Ham's F12
10mmol/L	HEPES buffer
10ng/ml	Glucagon
10µg/ml	Insulin
60ng/ml	Hydrocortisone
25ng/ml	EGF
10µg/ml	Transferrin
50nmol/L	Triiodothyronin
5ng/ml	Sodium selenate
10mmol/L	Nicotinamide
0.1mmol/L	L-ascorbic acid phosphate magnesium salt
1µg/ml	[α]-tocopherole acetate
50ng/ml	Linoleic acid
5%	Fetal bovine serum

Add 50µl pen/strep per 100ml

PBS, pH 7.4

8.5g	Sodium chloride
1.07g	Disodium hydrogen orthophosphate
0.39g	Sodium dihydrogen orthophosphate dehydrate

Autoclave to sterilize

SCHIFF'S REAGENT

1g	Basic fuschin
200ml	Distilled water, boiling

Allow to cool to 50°C

2g	Potassium metabisulphite

Allow to cool to room temperature

2ml	1N Hydrochloric acid
2g	Activated charcoal

Leave overnight, at room temperature, in the dark
Filter and store in dark container at 4°C

TRIS/SALINE (pH 7.6)

Stock Solution: Tris/HCl (pH 7.6)

100ml	1M Tris
76.8ml	1N Hydrochloric acid

Make up to 2L with distilled water.

Tris/Saline

500ml	Tris/HCl
42.75g	Sodium chloride
4.5L	Distilled water