Co-culture of hepatocytes with mesenchymal stem cells for cellular therapy in liver disease

Qin, Hong

Awarding institution: King’s College London

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CO-CULTURE OF HEPATOCYTES WITH MESENCHYMAL STEM CELLS FOR CELLULAR THERAPY IN LIVER DISEASE

HONG QIN

A thesis submitted to King’s College London
in fulfilment of the conditions governing candidates for the degree of Doctor of Philosophy

Institute of Liver Studies
Division of Transplantation Immunology and Mucosal Biology
King’s College London School of Medicine

December 2013
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ABSTRACT

A major hurdle facing current hepatocyte transplantation practice is the marginal quality of isolated hepatocytes. Previous studies showed that mesenchymal stem cells (MSCs) could maintain morphology and improve liver-specific metabolism of co-cultured hepatocytes. The present work aimed to optimise the MSCs co-culture system by testing adipose tissue (AT), bone marrow, and umbilical cord-derived MSCs at predefined seeding ratios. Liver-specific metabolism and apoptosis assays were performed to investigate hepatotrophic and antiapoptotic effects of MSCs co-culture. Indirect co-culture was established to investigate the role of paracrine factors in hepatotrophic effect of MSCs co-culture. Hypoxia-preconditioned (HPc) MSCs were co-cultured with hepatocytes to investigate potentiative effect of HPc induction. Intracellular reactive oxygen species (ROS) activity quantitation and antagonisation experiments were performed to investigate whether HPc potentiated MSCs co-culture by an intracellular ROS-dependent mechanism. Tumour necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1), extracellular collagen, and apoptosis-associated caspase and BAX/BCL-2 signalling pathways were analysed to investigate the contribution of soluble factors, extracellular collagen, and gene signalling to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. All the three types of MSCs exhibited a similar hepatotrophic effect, with a comparable effect even in low-density AT-MSCs co-culture. Hepatotrophic and antiapoptotic effects of MSCs showed a cell contact dependent manner, and HPc potentiated MSCs co-culture by a cell-contact intracellular ROS-dependent mechanism. Decreased hepatocyte autocrine TNF-α, increased MSC autocrine TGF-β1, and enhanced MSCs deposition of extracellular collagen contributed to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction, with downregulated expression of proapoptotic CASP9, BAX, and BID and upregulated expression of antiapoptotic BCL-2. It is concluded that synergistic effects of cell contact, intracellular ROS-dependent soluble factors, extracellular matrix, and apoptosis-associated signalling in MSCs co-culture contribute to hepatotrophic effect and HPc-induced potentiative effect. Co-transplantation with MSCs should improve therapeutic effects of HCT by enhancing survival and metabolism of co-transplanted hepatocytes.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACLF</td>
<td>Acute-on-chronic liver failure</td>
</tr>
<tr>
<td>ADFs</td>
<td>Adult dermal fibroblast</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha-foetoprotein</td>
</tr>
<tr>
<td>ALF</td>
<td>Acute liver failure</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AT</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bel</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>Bel-xL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting-domain death agonist</td>
</tr>
<tr>
<td>BLK</td>
<td>B lymphoid tyrosine kinase</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenesis protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Caspases</td>
<td>Cysteine-aspartic proteases</td>
</tr>
<tr>
<td>CCK18</td>
<td>Caspase-cleavaged keratin 18</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C motif ligand</td>
</tr>
<tr>
<td>CCl4</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
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<td>Complement DNA</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin 18</td>
</tr>
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<td>CM</td>
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<td>Cx</td>
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<td>CXC receptor</td>
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<td>Cytochrome P450</td>
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<td>DCF</td>
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<td>dichloro-dihydrofluorescein diacetate acetyl ester</td>
</tr>
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<td>DCFDH</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
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<td>DMSO</td>
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<tr>
<td>Term</td>
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<td>--------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Calcium-dependent, epithelial-type cadherin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential media</td>
</tr>
<tr>
<td>EMI</td>
<td>Epithelial-to-mesenchymal interaction</td>
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</tr>
<tr>
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<td>FAK</td>
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<td>Foetal calf serum</td>
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<td>Fibroblast growth factor</td>
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</tr>
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<td>Fluorescein isothiocyanate</td>
</tr>
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</tr>
<tr>
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<td>Forward scatter</td>
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<td>Hank’s balanced salt solution</td>
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<tr>
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<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HCT</td>
<td>Hepatocyte transplantation</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HLR-DR</td>
<td>Human leucocyte antigen D receptor</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
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<tr>
<td>HPc</td>
<td>Hypoxic preconditioning/hypoxia-preconditioned</td>
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<tr>
<td>HSC</td>
<td>Haematopoietic stem cells</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>iPSCS</td>
<td>Induced pluripotent stem cells</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LSD</td>
<td>Least significant difference</td>
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12
MaIBA  N-(methylamino)isobutyric acid
MAPK/ERK  mitogen-activated protein kinase/Extracellular signal-regulated kinase
MELD  Model for End-Stage Liver Disease
MMP  Metalloproteinase
mRNA  Messenger RNA
MSC  Mesenchymal stem cell
MTT  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC  N-acetylcysteine
NAD(P)H  Reduced nicotinamide adenine dinucleotide (phosphate)
NF  Nuclear factor
NPC  Nonparenchymal cell
OD  Optical density
OLT  Orthotopic liver transplantation
P  Passage
p53  Protein 53
PCR  Polymerase-chained reaction
PI3-K  Phosphatidylinositol 3-kinases
PSR  Picrom-sirius red
RNA  Ribonucleic acid
RNase  Ribonuclease
ROS  Reactive oxygen species
RT  Real-time
SD  Standard deviation
SDF  Stromal-derived factor
SLR  Single-lens reflex
SAPK  Stress-activated protein kinase
SRB  Sulforhodamine B
SSC  Side scatter
TGF  Transforming growth factor
TMB  3,3′,5,5′-Tetramethylbenzidine
TNF  Tumour necrosis factor
Tris  Tris(hydroxymethyl)aminomethane
UC  Umbilical cord
VEGF  Vascular endothelial growth factor
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<th><strong>WEM</strong></th>
<th>William’s E medium</th>
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<td><strong>XIAP</strong></td>
<td>X-linked inhibitor of apoptosis protein</td>
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CHAPTER 1 GENERAL INTRODUCTION

1.1 Overview of Hepatocyte Transplantation

Orthotopic liver transplantation (OLT) is the replacement of a patient’s diseased liver with a healthy donor’s liver allograft. OLT has been used as the curative modality in acute or chronic conditions, especially inborn liver-based metabolic diseases (Dhawan et al., 2006), that result in irreversible liver dysfunction. The operative technique of OLT has been well established over the last five decades, and patient survival following OLT has been continuously improved due to refinements in surgical care and better knowledge of transplantation immunology (Desai et al., 2008; Beinhardt et al., 2013). The primary limitation of OLT in current transplantation practice is that the number of donor livers available is unfortunately far less than that of recipients on the waiting list (Lo et al., 2004). The operative procedure is also subject to some surgical morbidities and mortalities for both donors in case of living donors and recipients (Ammori et al., 2008). Additionally, patients receiving OLT normally require a life-long immunosuppressive regimen, which impairs patients’ quality of life physiologically and psychologically, and places a huge burden on public healthcare system (Schoening et al., 2013).

Hepatocyte transplantation (HCT) has been emerging as a promising alternative treatment modality to OLT for patients who have no access to donor liver or cannot tolerate OLT (Dhawan et al., 2010). The concept of HCT comes from the fact that only a very small portion (approximately 5–10%) of hepatocytes can perform a series of metabolic functions to sufficiently maintain a human subject (Kawashita et al., 2005). In this therapeutic technique, hepatocytes are isolated and purified from donor liver segments that are unused or rejected for OLT mostly due to underlying steatosis (Sagias et al., 2010), using an enzyme perfusion and digestion system. The quality of isolated hepatocytes is subsequently assessed in vitro with regards to cell yield, viability, and microbiological safety (Lehec et al., 2009). The preferred recipient site is the liver, into which hepatocytes are delivered through an intra-portal vein catheter (Figure 1.1; Hughes et al., 2012), whilst some alternative sites are also available, including the spleen, pancreas, peritoneal cavity, and subrenal capsule (Hughes et al., 2012). Preclinical studies of HCT on liver disease animal models showed a favourable outcome and led to the clinical use of HCT (Mazaris et al., 2005; Fisher and Strom, 2006).

HCT has been showing a series of clinical benefits in current practice (Table 1.1). The primary advantage of HCT is the minimal invasiveness as compared to OLT (Meyburg et al., 2009). For HCT, isolated hepatocytes are delivered into the liver or other ectopic sites through a catheter using an interventional radiology technique or Doppler ultrasonographic monitoring. This minimally invasive access, thus, minimizes the procedural risks, and offers patients, who cannot tolerate OLT due to poor liver function reserve or pre-existing comorbidities, an additional therapeutic opportunity. HCT is technically less complicated, and this technique can be performed in the setting
Figure 1.1  Routes of cell administration for hepatocyte transplantation: through portal, inferior mesenteric and umbilical veins (Hughes et al., Transplantation, 2012).

Table 1.1  Clinical benefits of HCT as compared to OLT

<table>
<thead>
<tr>
<th></th>
<th>HCT</th>
<th>OLT</th>
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<tr>
<td>Minimally invasive</td>
<td>• Excessively invasive</td>
<td>• Technically complicated</td>
</tr>
<tr>
<td></td>
<td>• Technically simple</td>
<td>• Not repeatable in most cases</td>
</tr>
<tr>
<td></td>
<td>• Repeatable if required</td>
<td>• Huge financial burden</td>
</tr>
<tr>
<td></td>
<td>• Low medical cost</td>
<td>• One donor liver can be given to a single recipient only, or an adult and a paediatric recipient</td>
</tr>
<tr>
<td></td>
<td>• One donor liver can be shared by multiple adult or pediatric recipients</td>
<td>• Not preservable, and usually not available for emergency use</td>
</tr>
<tr>
<td></td>
<td>• Preservable and can be used in case of emergency</td>
<td>• Native liver lost for ever</td>
</tr>
<tr>
<td></td>
<td>• Native liver preserved as backup</td>
<td>• Normally requiring life-long immunosuppression</td>
</tr>
<tr>
<td></td>
<td>• Not requiring immunosuppression for the treatment of acute liver failure</td>
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of day surgery. HCT can be repeated within a relative short period if required (Sauer et al., 2012), and the medical costs of HCT is much lower than that of OLT. Hepatocytes isolated from a single donor can be transplanted into multiple recipients, especially in paediatric patients (Mitry et al., 2004). Hepatocytes also can be cryopreserved in cell banks, allowing the immediate accessibility of hepatocytes for emergency transplantation (Fuller et al., 2013). Moreover, the native liver is preserved in place in patients undergoing HCT, which offers a possibility of gene therapy if this becomes clinically feasible in the future. Autologous hepatocytes could be isolated and genetically modified ex vivo and back-transplanted to correct metabolic liver disorders (Nguyen et al., 2009).
The use of cell autografts avoids the requirement for a life-long immunosuppressive regimen following HCT, although hepatocytes are presumed to be less immunogenic than the whole liver (Bumgardner et al., 1998). All the aforementioned advantages have been encouraging a wider use of HCT worldwide (Hughes et al., 2012).

1.1.1 HCT for inborn liver-based metabolic errors

HCT was initially used for the treatment of inborn liver-based metabolic errors in urea cycle defects, severe unconjugated hyperbilirubinemia (Crigler–Najjar syndrome type 1), factor VII deficiency (haemophilia A), and familial hypercholesterolemia (Dhawan et al., 2006). The outcomes of HCT were most encouraging in patients with inborn liver-based metabolic disorders, as HCT could offer these patients a definite therapeutic benefit (Ribes-Koninckx et al., 2012). In these cases, only a relatively small number of transplanted hepatocytes may be required to compensate for the inherited deficiency of a single liver enzyme, especially in children. In our centre, over ten paediatric patients underwent HCT due to liver-based metabolic disorders, the majority of whom exhibited a clinical improvement without any procedural complications (Dhawan et al., 2005).

1.1.2 HCT for acute liver failure

The indication of HCT was further extended to acute liver failure (ALF) as an auxiliary treatment regimen. ALF occurs in severe liver injury with the loss of 80–90% of liver cells within a short period. The prognosis of ALF is highly variable, depending mainly on the underlying etiology. The mortality rate of ALF was up to 80%, but it has been decreasing due to improvements in multidisciplinary intensive care and the advent of emergency liver transplantation (Karvellas et al., 2009). The clinical use of HCT in ALF aims to bridge patients to subsequent OLT, or extend the survival long enough for the native liver to recover and regenerate (Bilir et al., 2000). As the transplanted cells are expected to compensate for the entire liver, the number of hepatocytes required for ALF is normally higher than that for liver-based metabolic disorders, and the infusion of hepatocytes needs to be repeated. Moreover, toxic substances accumulating in ALF patients may be potentially cytotoxic to the transplanted hepatocytes (Mitry et al., 2009). A previous clinical study demonstrated that HCT improved liver function measures in ALF patients, but the overall survival outcome was highly variable (Baccarani et al., 2005). Up to now, it is not possible to draw any conclusion on the overall efficacy of HCT in ALF patients as no controlled trials have been performed.

1.1.3 HCT for acute-on-chronic liver failure

HCT has also been attempted in patients with decompensated liver cirrhosis, namely, acute-on-chronic liver failure (ACLF), and aims to prolong a patient’s survival and improve one’s quality of life, with the hope that OLT will become available at a later time (Kobayashi et al., Transplant Proc, 2000). HCT can improve liver function and overall survival in animal models with chemically-
induced liver cirrhosis (Kobayashi et al., 2000). However, the treatment outcomes from published clinical studies were even more variable for cirrhotic patients (Pareja et al., 2010; Pareja et al., 2013), probably due to the presence of underlying liver fibrosis. The fibrotic lobules prevent the transplanted hepatocytes from passing through the sinusoidal barrier and engrafting into the liver (Gandillet et al., 2005).

1.2 Limitations and Modifications of Hepatocyte Transplantation

It is inevitable that HCT is subject to some technical limitations in terms of accessibility, effectiveness, and safety (Hughes et al., 2012). As compared to OLT, HCT even has a more limited supply of donor liver as hepatocytes used for cell replacement therapy are usually isolated from liver segments unused or rejected for OLT. Another drawback for HCT is the marginal quality of hepatocytes isolated from donor livers unsuitable for OLT mainly due to underlying liver steatosis (Sagias et al., 2010). Steatotic hepatocytes are vulnerable to enzymatic digestion, and processing of fatty liver tissue normally shows a low cell yield and a poor cell viability rate. Therefore, a larger number of hepatocytes of marginal quality are required to maintain normal liver function. Moreover, adult-derived hepatocytes cannot divide or survive long in vitro and have a weak repopulation potential, unless the cells are stimulated by appropriate growth factors (Amano et al., 2011). Hepatocytes become dedifferentiated following isolation, and dedifferentiated hepatocytes have an impaired metabolic function (Ambrosino et al., 2005). Hepatocyte transplants are likely to be eliminated by the innate and adaptive immune systems within 7 to 10 days (Han et al., 2009). In animal studies, up to 70% of hepatocytes are primarily cleared by Kupffer cells within the first 24 hours of transplantation, irrespective of syngeneic or allogeneic grafting (Krohn et al., 2009). Post-transplantation cell loss necessitates the transplantation of a larger number of hepatocytes, in a paradox with the shortage of hepatocyte supply.

1.2.1 Alternative HCT cell graft sources

New cell sources have been emerging in recent studies to overcome the primary technical hurdle of HCT, namely, the shortage of donor liver for hepatocyte isolation (Figure 1.2; Fitzpatrick et al., 2009). Juvenile hepatocytes exhibit a potent repopulation potential as compared to adult cells, but juvenile liver donors are only occasionally available in clinical practice (Walldorf et al., 2004). Hepatocyte xeno-transplantation has been investigated in some animal models showing favorable survival and functional outcomes (Yamamoto et al., 2010); however, this technique is at a high risk of transmitting zoonotic diseases and raises some ethical concerns. Genetically modified or immortalized hepatocytes are reported to have a longer survival by increasing cell replication and reducing cell apoptosis (Tsuruga et al., 2008). The technique of gene manipulation is not clinically available yet and subject to a tumourigenic risk (Trejo-Becerril et al., 2012). Among the newly emerging cell sources, stem cells/progenitor cells are expected to exhibit the most promising outcomes as these cells have been widely investigated for cell replacement therapy. Transplantation of bone marrow (BM) haematopoietic stem cells (HSC) has proved to be clinically effective in the
reconstruction of a patient’s haematopoietic system. These stem cells can be driven to differentiate into metabolically functional hepatocyte-like cells under specific conditions in vitro (Takayama et al., 2012) and promote liver repair and regeneration in vivo (Li et al., 2013). The sources of stem cells that have been investigated for HCT include embryonic stem (ES) cells, foetal liver cells, adult-derived hepatic progenitor cells, induced pluripotent stem (iPS) cells, and more popular mesenchymal stem cells (MSCs).

1.2.1.1 ES cells

ES cells are stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo, 4–5 days post-fertilisation in humans. ES cells are believed to be pluripotent rather than totipotent in terms of cell potency (Denker, 2008), and offer a rich source for cell replacement therapy including HCT. ES cells have been successfully induced to differentiate into hepatocytes by way of embryoid body formation in two- or three-dimensional culture in vitro (Touboul et al., 2010; Subramanian et al., 2013). Hepatocytic commitment requires the presence of retinoic acid, hepatocyte growth factor (HGF), and β-nerve growth factor (Kuai et al., 2003). These chemically-defined conditions are thought to recapitulate liver development in embryogenesis (Touboul et al., 2010). The immunophenotype and functional activities of ES cells-derived hepatocytes were reported to be identical to those of freshly isolated primary hepatocytes following orthotopic transplantation into the liver or ectopic transplantation into the spleen in animals (Rosen et al., 2003). However, the

Figure 1.2   Potential alternative sources of hepatocyte for transplantation: ES cells, iPS cells, haematopoietic stem cells, foetal progenitor cells, liver progenitor cells, and MSCs (Fitzpatrick et al., J Intern Med, 2009).
The harvest of ES cells raises ethical issues as it results in the destruction of fertilised human embryos (de Wert G et al., 2003). The risk of neoplastic transformation in undifferentiated ES cells may be underestimated in current studies, which report an inconsistent presence of teratomas (Choo et al., 2008). Therefore, undifferentiated ES cells should be eliminated prior to transplantation, which is technically difficult in clinical practice.

1.2.1.2 Foetal liver cells

Foetal liver cells are believed to be enriched with hepatoblasts, the progenitor of hepatocytes and cholangiocytes (Masson et al., 2006). These progenitor cells decline rapidly in number after birth and become almost undetectable in adult livers (Schmelzer et al., 2006). Pilot studies on in utero transplantation of foetal livers showed a favourable outcome, with advantages in immune tolerance and optimal environment for donor cells in host foetuses (Rosen et al., 2003). The isolation and characterisation of hepatoblasts vary among studies, mainly using liver-specific markers, such as alpha-foetoprotein (AFP), and epithelial cell markers, such as cytokeratins (CK)-18 and -19 (Dan et al., 2006). A number of studies have documented the effectiveness and safety of foetal liver cells in repopulating normal or experimentally injured livers as these cells are destined to mature primarily into hepatocytes (Machimoto et al., 2007). The use of foetal liver cells is also limited by the shortage of donor foetuses and significant ethical concerns (Mychaliska et al., 1998). Moreover, in contrast to expectations, a rodent transplantation study showed that foetal liver cells had a poor liver engraftment and lower repopulation capacity than adult-derived hepatocytes (Haridass et al., 2009).

1.2.1.3 Liver progenitor cells

Liver is well known for its regeneration capacity in response to detrimental factors. As little as one quarter of liver remnant can generate a whole liver de novo in healthy subjects (Ju et al., 2012). Liver regeneration has been well described since the age of the original Prometheus myth. This phenomenon is predominately attributed to the quiescent G0-phase hepatocytes that re-enter the cell cycle, bypass the G0/G1 checkpoint, and finally complete mitosis (Satyanarayana et al., 2004). It is a compensatory growth rather than true regeneration of the liver. However, there is some evidence that damaged hepatocytes can be replaced by some liver progenitor cells in adults (Pintilie et al., 2010). These cells are termed as hepatic oval cells in rodents and hepatic progenitor cells in humans. These progenitor cells reside in the canals of Hering, which are located in the periportal region and account for a very small percentage (0.3–0.7%) of liver mass. Adult hepatic progenitor cells are reported to resemble foetal hepatoblasts in terms of phenotype and biopotency (Nava et al., 2005). The identification of oval cells in rats involves multiple immunomarkers, such as oval antigen 6, CK-7, CK-19, and albumin (Terrace et al., 2007). Oval cells can be effectively propagated in vitro and directed to the commitment of hepatocytes (Yasui et al., 1997). In animal models of liver injury, oval cells can expand to compensate for the increased turnover of damaged mature hepatocytes when normal G0-phase hepatocyte mitosis is blocked or in replicative senescence (Yang et al., 2004). However, oval cells are also less accessible for clinical use, and the large-scale expansion of oval cells in vitro proves to be technically challenging and subject to loss of potency.
1.2.1.4 iPS cells

iPS cells are a type of artificially programmed pluripotent stem cells that are derived from somatic cells by inducing a forced expression of transcriptional factors. iPS cells were first produced from mouse-derived fibroblasts in 2006 (Takahashi et al., 2006) and from human fibroblasts in 2007 (Takahashi et al., 2007). Theoretically, iPS cells can be reprogrammed to differentiate into any mature cells of ectodermal, mesodermal, and endodermal origin. This technique avoids ethically controversial use of embryos for harvest of ES cells, and also allows production of iPS cells from a patient’s own somatic cells, which require no conventional immunosuppression like that following allogeneic transplantation. Functional hepatocytes have been produced from mouse iPS cells that are sequentially subjected to inducing factors (Figure 1.3; Li et al., 2010). These iPS cell-derived hepatocytes share identical morphological and metabolic identities with those derived from ES cells. Human liver disease-specific iPS cells have been available, and these cells can express hepatocyte-specific markers and exhibit a comparable metabolic functionality (Ghodsizadeh et al., 2010). The therapeutic potential of iPS cell-derived hepatocyte-like cells has been justified in a mouse model of carbon tetrachloride (CCl₄)-induced liver injury (Asgari et al., 2011). Primary hepatocytes can also be reprogrammed to iPS cells (Liu et al., 2010). It is interesting that these iPS cells can be directed to differentiate into hepatic progenitor cells as well as mature hepatocytes.

![Figure 1.3 Hepatocyte-like cells differentiated from human iPS cells on albumin immunofluorescence microscopy (left panel) and periodic acid-Schiff staining (right panel) (Li et al., J Cell Physiol, 2010).](image)

The use of iPS cells for HCT is faced with some technical challenges with respect to effectiveness and safety. Firstly, the reprogramming of iPS cells shows a very low efficiency; less than 1% of somatic cells can be induced into iPS cells in vitro. These techniques usually require precise but clinically less feasible laboratory manipulation. Genomic insertion of transcription factors impairs the safety of iPS cells for cellular therapy as oncogenes are used as reprogramming factors to genomically disintegrate iPS cells and lead to tumourigenesis, a primary safety concern of using iPS cells. Therefore, it is critical to purify differentiated cells by eliminating undifferentiated iPS cells prior to clinical transplantation. Fluorescence activated cell sorting is the preferred method; however, hepatocytes are known to have a complex immunophenotypic profile, and cell sorting may miss a large number of less differentiated progenitor cells with a huge potential of hepatic
regeneration. It remains controversial whether iPS cells are functionally equivalent to ES cells (Bilic and Izpisua, 2012; Puri and Nagy, 2012). It has been reported that iPS cells have a lower efficiency than ES cells in terms of hepatocytic differentiation (Li et al., 2010; Jozefczuk et al., 2011). Moreover, iPS cell-derived hepatocytes were reported to express high-level AFP but low-level albumin, urea, and CYP450, as compared to primary human hepatocytes (Yu et al., 2012). This finding suggests that iPS cell-derived hepatocytes are not completely mature with respect to genotype and phenotype.

1.2.1.5 Haematopoietic stem cells

Haematopoietic stem cells (HSCs) are multipotent progenitor cells that differentiate into myeloid and lymphoid lineages. HSCs transplantation has been well established in the last four decades for treating haematological and autoimmune disorders. HSCs share a stem cell marker Thy-1 with hepatic oval cells, the liver progenitor cells in rats (Petersen et al., 1998). An in vitro study confirmed that CD45+ HSCs could be driven into hepatogenesis in the presence of HGF (Zhao et al., 2003). However, it remains controversial whether HSCs can differentiate into hepatocyte-like cells or become fused with hepatocytes in vivo. Sex-mismatched peripheral blood HSCs transplantation studies showed that hematopoietic donor chimera cells expressing hepatocyte markers were present in the liver as early as two weeks after transplantation, suggesting the possible differentiation of circulating HSCs into mature hepatocytes (Körbling et al., 2002; Mirzania et al., 2010). Camargo et al. (2004) reported that functional hepatocytes derived from HSCs were primarily mature myelomonocytic cells spontaneously fusing with host hepatocytes. It is a technical challenge to induce hepatocytic differentiation of HSCs which requires manipulation in culture over a long period (Miyazaki et al., 2004), although using a combination of multiple growth factors may facilitate HSCs differentiation into hepatocytes (Sellamuthu et al., 2011). The therapeutic role of HSCs in liver disease needs to be critically reassessed. Cantz et al. (2004) reported that genetically-labelled HSC transplants could not be detected in the liver or other visceral organs of mice undergoing extended major hepatectomy or chemical injury, although these cells were mobilised by granulocyte colony-stimulating factor.

1.2.1.6 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are mesoderm-derived multipotent stem cells that normally differentiate into a variety of mesenchymal-type cells, including osteoblasts, chondrocytes, and adipocytes. Typical human MSCs manifest a fibroblast-like appearance in a vortex shape. No single cell surface marker can differentiate MSCs from other cell lines; however, undifferentiated MSCs are shown to highly express mesenchymal cell markers, such as CD73, CD105, and CD 106, rather than HSC markers, namely, CD34 and CD45. Novel markers have been emerging and are commercially available for the detection and isolation of MSCs from human or animals, such as bone morphogenetic protein (BMP), stem cell factor receptor, and Stro-1 (Ning et al., 2011). Substantial variability exists in the markers defining MSC population among reports, probably due to the intrinsic heterogeneity of MSCs. As with other multipotent stem cells, MSCs have a
substantial capacity for self-renewal while maintaining their multipotency. MSCs have been artificially driven to differentiate into various cell lines, including epidermal cells (Chun-mao et al., 2007), epithelial cells (Liu et al., 2013; Wu et al., 2013), endothelial cells (Katikireddy et al., 2013), islet cells (Gopurappilly et al., 2013; Marappagounder et al., 2013; Zanini et al., 2011), myocytes (Khani et al., 2013), and specific-type neurons (Yang et al., 2013). For hepatic differentiation, three sources of MSCs have been investigated: BM (Soleimani and Nadri, 2009), umbilical cord (UC) blood (Laitinen and Laine, 2007) or matrix (Wang et al., 2004), and adipose tissue (AT; Neupane et al., Tissue Eng Part A, 2008). A number of studies have examined metabolic functions of MSCs-derived hepatocytes and shown a promising therapeutic prospect (Kang et al., 2005; Sgodda et al., 2007; Ishii et al., 2008; Aurich et al., 2009; Piryaee et al., 2011; Liang et al., 2012; Brückner et al., 2013).

**BM-derived MSCs** MSCs were first reported to be present in BM, namely, BM-derived MSCs, which co-exist with another stem cell line, namely, HSCs. When supplemented with fibroblast growth factor (FGF)-4 and HGF, a large percentage of rat BM-derived MSCs are induced to exhibit a hepatocyte-like morphology in vitro (Kang et al., 2005). These hepatocyte-like cells are capable of secreting albumin, synthesising urea, and storing glycogen. The differentiation media have been optimised in multiple in vitro studies, but remain FGF-4- and/or HGF-based. The supplementary growth factors and cytokines that favour directed hepatocytic differentiation consist of insulin-like growth factor 1 (IGF-1; Ayatollahi et al., 2011), β-nerve growth factor (Feng et al., 2011), hepatocyte nuclear factor (NF) 4α (Chen et al., 2010), and oncostatin M (Lee et al., 2004). Other gene products, such as AFP (Ishii et al., 2008), alkaline phosphatase (Kosmacheva et al., 2011), and CK-18 (Kang et al., 2005; Lange et al., 2005a; Lange et al., 2005b; Lange et al., 2006) have also been used to characterise MSCs-derived hepatocytes under specific inductive circumstances.

**UC-derived MSCs** UC-derived MSCs are concomitantly present in cord blood and matrix (Wharton’s jelly). This type of MSCs has been successfully transdifferentiated into hepatocytes using a protocol similar to that for BM-derived MSCs, although with a lower efficiency (Hong et al., 2005). Human UC-derived MSCs were reported to repopulate and engraft into the liver of a cirrhotic rat model (Jung et al., 2009). However, a xenotransplantation experiment showed that regenerated hepatocytes were actually chimera of donor MSCs and recipient hepatocytes; UC-derived MSCs expressed human albumin and Hep par 1, but murine CK-18, following transplantation into the liver of immunodeficient mice (Sharma et al., 2005). It was reported in the same rodent model that human UC-derived MSCs could differentiate into mature hepatocytes in the absence of cell fusion (Newsome et al., 2003). Interestingly, following transplantation into CCl4-induced fibrotic rat liver, UC-derived MSCs restored liver function by secreting bioactive cytokines and promoting hepatocyte regeneration rather than differentiating into hepatocytes in vivo (Tsai et al., 2009). This contradiction may be attributed to intrinsic variations in UC-derived MSCs.

**AT-derived MSCs** A newly emerging source of MSCs, AT-derived MSCs are readily available and ethically less controversial (Figure 1.4; Banas et al., 2007). Zuk et al. (2001) isolated multipotent stem cells, which mimicked BM-derived MSCs in morphology, from human liposuction
Figure 1.4  Primary human MSCs isolated from AT (left panel) and albumin-immunopositive hepatocyte-like cells (right panel) differentiated from AT-derived MSCs transplanted into CCl4 liver injury model (Banas et al., Hepatology, 2007).

specimens in 2001. The immunophenotypes of AT-derived MSCs are basically similar to those of other sources of MSCs, although almost all AT-derived MSCs express CD49f and CD54, which are rarely expressed in BM-derived MSCs (Zuk et al., 2001). The primary advantages of AT-derived MSCs are extensive source from body adipose, easy harvest by lipoaspiration, and possibility of autologous transplantation. Additionally, AT-derived MSCs are found to be more readily reprogrammed into iPS cells than BM-derived MSCs probably due to the variation in the microenvironment of origin (Mosna et al., 2010). AT-derived MSCs have been driven to differentiate into hepatocyte-like cells with hepatocyte metabolism and hepatocyte-specific transcripts (Ohnishi et al., 2011). Compared to undifferentiated cells, pre-differentiated AT-derived MSCs exhibit a more efficient engraftment into recipient liver (Okura et al., 2010). The therapeutic benefits of AT-derived MSCs have been demonstrated in animal models of inherited liver disease (Okura et al., 2011).

Liver tissue-derived MSCs  MSCs can also be isolated from foetal liver tissue (Heidari et al., 2013), with a trilineage differentiation potential identical to BM- and AT-derived MSCs and a proliferative capacity similar to BM-derived MSCs. Human foetal liver-derived MSCs share identical MSCs immunphenotype and potential of multilineage differentiation, and inhibit mitogen-induced lymphocyte proliferation in vitro (Götherström et al., 2003). Foetal liver-derived MSCs were reported to be immunosuppressive on T-lymphocytes (Giuliani et al., 2011). These liver-derived MSCs have also been isolated from human adults, basically same to BM-derived MSCs in terms of genome-wide gene expression (Pan et al., 2011). These mesenchymal-like cells co-express hepatic and mesenchymal markers, including albumin, cytochrome P3A4, vimentin, and alpha-smooth muscle actin (Najimi et al., 2007). These cells are preferentially determined to differentiate into hepatocyte-like cells. Following intrahepatic transplantation, the offspring cells are immunopositive for albumin, pre-albumin, and AFP, and show a favourable engraftment potential. A subset of engrafted MSCs survived over the long term in vivo and maintained stem cell characteristics (Najimi et al., 2007).

Limitations There are some unavoidable limitations in using MSCs as cellular source for HCT. Harvest of MSCs is not always possible from the patients themselves for autologous transplantation
or from the donors for allogeneic transplantation. Aspiration of bone marrow is invasive for the donors, and the amount of bone marrow collected from an individual donor is very limited even in a healthy subject. In a diseased or ageing subject, the propagation potential of BM-derived MSCs becomes compromised (Katsara et al., 2011). Use of UC-MSCs requires a long-time preservation, whilst the potency of long-preserved UC-MSCs remains questionable. Whereas AT is enriched with highly proliferative MSCs, it is essential to purify the heterogeneous AT-derived cell population (Zhang et al., 2011). As AT-derived MSCs emerged in the last decade, it was less known whether AT-derived MSCs could differentiated into hepatocytes as effectively as BM-derived and UC-derived counterparts, although a previous study reported a similar hepatogenic differentiation potential and efficiency between AT- and BM-derived MSCs (Taléns-Visconti et al., 2006). It usually takes two to three weeks to drive MSCs differentiation into functional hepatocytes in vitro; therefore, it makes MSCs-based HCT less suitable for emergency cases, such as for ALF patients. Functional patterns of differentiated hepatocytes are mainly characterised with albumin secretion, urea synthesis, and glycogen storage as well as CYP450 activity, often at low levels, whereas hepatocytes have complex biochemical activities. It remains an open question whether MSCs can differentiate into other cell lineages other than hepatocytes even in directed conditions. These side products may be detrimental for transplanted hepatocyte or even the recipient. MSCs may be a double-edged sword in liver regeneration due to their pro-fibrogenic potential especially in chronic liver injury (di Bonzo et al., 2008). As with other stem cells, the tumourigenic risk of MSCs cannot be underestimated for HCT. It has been recently discovered that MSCs can promote mammary tumourigenesis and progression by enhancing vascularisation in the microenvironment (Ke et al., 2013) although the role of MSCs in breast cancer remains controversial (Usha et al., 2013).

1.2.2 Preconditioning of hepatocytes

1.2.2.1 Supportive factors of hepatocytes in vivo

In a native liver, hepatocytes are supported by hepatocytes themselves, extracellular matrix (ECM), and nonparenchymal cells (NPCs). Hepatocyte-to-hepatocyte contact is believed to be crucial for the maintenance of hepatocyte polarity, morphology, and functionality. Calcium-dependent, epithelial-type cadherin (E-cadherin), a major component of intercellular tight junction, maintains hepatocyte spheroid formation and prevents primary hepatocytes from apoptosis by a caspase-independent mechanism (Luebke-Wheeler et al., 2009). E-cadherin expression is modulated by HGF/MET signalling pathway in multiple carcinoma cell lines, including breast cancer, gastric cancer (Han et al., 2005), pancreatic cancer (Paciucci et al., 1998), bladder cancer, prostate cancer, ovarian cancer, melanoma, hypopharyngeal cancer, and nasopharyngeal cancer.

In tissue engineering and regenerative medicine research, seed cells can be preconditioned and modified by supplementing extrinsic growth factors, trophic cytokines, and ECM, or by manipulating intrinsic gene expression prior to transplantation to improve survival, engraftment, and functionality of transplanted cells (Li et al., 2013). These modifications aim to improve proliferation,
survival, committed differentiation, and biological function of seed cells in vitro and in vivo. Stem cells are preferred target cells to be modified as these pluripotent cells are capable of self-renewal and maintaining the modified effect in the long-term (Navarro-Alvarez et al., 2009). Among these modifications, HGF modification is frequently used to potentiate the regenerative potential of MSCs for the purpose of cellular replacement therapy (Ido and Tsubouchi, 2009; Ishikawa et al., 2011; Ishikawa et al., 2012; Sun et al., Int J Mol Sci, 2013). Bian et al. (2009) reported that HGF-modified MSCs exhibited a greater viability as compared to non-modified counterparts and improved skin graft survival. Chen et al. (2011) successfully transplanted HGF-modified UC-derived MSCs to ameliorate ischaemia/reperfusion-induced acute kidney injury via anti-apoptotic and anti-inflammatory mechanisms. Alternatively, HGF can also be loaded onto collagen- and fibrin-based biomaterials as artificial ECM to recruit MSCs and promote wound healing (van de Kamp et al., 2013). The concept of modifying the niche rather than the seed cells is of great clinical significance as this technique avoids costly multi-step procedures of isolating, culturing, and transplanting allogeneic stem cells.

1.2.2.2 Growth factors and cytokines

Hepatocytes are a terminally differentiated, mature cell population with a series of highly complex biochemical and metabolic functions (Clayton et al., 2005). Therefore, it is less feasible and more challenging to modify hepatocytes in vitro. A large number of growth factors and cytokines, including HGF at the first place (Nakamura et al., 2011; Li et al., 2013; Xu et al., 2013), vascular endothelial growth factor (VEGF; Sturm et al., 2004; Bockhorn et al., 2007), epidermal growth factor (EGF; Natarajan et al., 2007; Liu et al., 2013), insulin-like growth factor (IGF; Sobrevals et al., 2010; Zaouali et al., 2010), transforming growth factor (TGF; Meindl-Beinker et al., 2012), basic fibroblast growth factor (bFGF; Ma et al., 1999), tumour necrosis factor (TNF; Sudo et al., 2008; McMahan et al., 2013), and interleukin (IL) 6 (Sudo et al., 2008; Tiberio et al., 2008), are known to exhibit a marked in vitro and in vivo hepatocytotrophic effect with respect to hepatocyte survival and regeneration in animals and humans. Microprinted array is a more efficient technique for delivering growth factors to hepatocyte culture on the standard collagen monolayer. Printed arrays of HGF and BMP-7 exhibit antifibrotic and antiapoptotic effects on primary rate hepatocytes (Jones et al., 2010). However, it is not cost-effective to supplement hepatocytotrophic growth factors or cytokines directly into culture medium or surface of hepatocytes as trophic effect of soluble factors can only remain for several days. Additionally, hepatocytotrophic factor supplementation cannot be repeated once hepatocytes have been transplanted.

1.2.2.3 Gene transfection

Gene transfection seems to be a more effective technique for hepatocyte modification. This technique allows the delivery of target genes into donor hepatocytes to overexpress therapeutically efficacious proteins even though the donor cells may be of marginal quality. Additionally, autologous recipient hepatocytes can also be treated as donor cells using the gene fusion or transfection technique to compensate or even correct underlying liver diseases, especially for metabolism-based
disorders. Gene therapy has been experimentally attempted at the levels of both hepatic progenitor cells and mature hepatocytes. Li et al. (2012) simultaneously transplanted HGF-modified hepatic oval cells together with orthotopic liver transplantation, and this modification decreased liver allograft rejection and prolonged graft survival in rat recipients. Ajoka et al. (2001) co-transplanted hepatocytes transfected with multiple hepatotrophic genes, including HGF, TNF, and VEGF, to establish a heterotrophic liver tissue mass with a favourable access to the blood circulation. More encouragingly, Wu et al. (2010) transfected human coagulation factor IX into mouse hepatocytes and transplanted gene-modified cells intrasplenically into factor IX knockout mice, a haemophilia B model. Genetically engineered hepatocytes exhibited a significantly higher plasma factor IX clotting activity than the wild-type counterpart with a similar engraftment and factor IX production efficiency. However, the use of genetically modified hepatocytes is inevitably subject to technical limitations and safety concerns although all the experimental studies had shown promising results. It usually takes a few days to complete conventional hepatocyte gene transfection in primary culture. Such a delay prior to HCT is not suitable for clinical use as patients, especially those suffering from ALF, are in urgent need of HCT. Kuge et al. (2006) successfully reduced infection time down to only one hour using an adenoviral vector-based transfection system but at the risk of uncontrollable viral infection in the recipient. Viral vector transduced hepatocytes also require the host immune tolerance to maintain long-term transgene expression (Puppi et al., 2004), although mature hepatocytes are known to be relatively less immunogenic in vivo (Bumgardner et al., 1998). Moreover, overexpression of hepatocyte proliferation-associated genes, such as HGF, may lead to carcinogenesis and dissemination in the recipient. Upregulated HGF signalling is historically known to drive the occurrence (Yamagami et al., 2002), progression (Jia et al., 2013), metastasis (Chau et al., 2008), recurrence (Mizuguchi et al., 2009), metastasis (Ogunwobi et al., 2013) and chemoresistance (Lasagna et al., 2006; Yu et al., 2013) of hepatocellular carcinoma, as well as liver-metastatic colorectal cancer (Min et al., 2012).

1.2.2.4 ECM

ECM supportive effect is known to be essential for maintaining physiological hepatocyte morphology, survival, proliferation, differentiation, and liver-specific function, especially in the sense of the long-term in vitro culture. ECM modulates hepatocyte survival (Giri et al., 2003; Pinkse et al., 2004; Ohashi et al., 2005; Zavan et al., 2005), viability, morphology (Zavan et al., 2005), phenotype (Page et al., 2007; Woodrow et al., 2009), proliferation (Hammond et al., 2011), differentiation (Sidhu et al., 2004; Kimata et al., 2006; Kimata et al., 2006), maturation (Brill et al., 2002), metabolism (Depreter et al., 2000; Sellaro et al., 2010), repair (Ma et al., 1999), and regeneration (Hammond et al., 2011), via paracrine factors, such as HGF (Schuppan et al., 1998), hepatocyte NF 4 (Oda et al., 1995), bFGF (Ma et al., 1999), and cell adhesion molecules, such as integrin (Pinkse et al., 2004).

Multiple novel tissue engineering biomaterials, namely, scaffolds, have been investigated to construct the optimal residential environment for hepatocytes in vitro. The scaffolds are usually composed of naturally hyrdrogels, such as chitosan (Elçin et al., 1998) and alginate (Elkayam et al., 1998).
or artificially synthesised high-molecule glycopolymers, such as polyethylene glycol (Underhill et al., 2007) and polycaprolactone/polylactic-co-glycolic acid (Shim et al., 2013). ECM modification is even more beneficial for maintaining long-term hepatocyte culture in vitro for the purpose of drug hepatotoxicity screening (De Bruyn et al., 2013), bioartificial liver support (Kinasiewicz et al., 2008; Giri et al., 2013), and anti-hepatitis virus agent experiments (Molina-Jimenez et al., 2012). These scaffolds can be further surface-modified with major components of ECM, such as collagen (Hou et al., 2011), fibrin (Gwak et al., 2004), laminin (Tai et al., 2010), and fibronectin (Mehta et al., 2010). Common growth factors, such as HGF (Seo et al., 2006; Nelson et al., 2011), VEGF (Kedem et al., 2005; Hou et al., 2011), EGF (Koyama et al., 2009), and IGF (Nelson et al., 2011) can also be incorporated into surface-modified bioscaffolds using the nanomaterial technology to augment survival and functionality of seeded hepatocytes. The emergence of an injectable bioscaffold allows hepatocyte-scaffold transplants to be delivered using minimal invasive access techniques, especially for cirrhotic patients. Navarro-Alvarez et al. (2010) constructed a peptide nanofiber-based, three-dimensional scaffold, loaded with growth factors and seeded with immortalised human hepatocytes, into a tissue-engineered liver graft that could be injected intramuscularly. This engineered liver graft could maintain liver-specific gene expression and functionality in vitro to correct acute or chronic liver failure in animal models.

1.2.2.5 Bioscaffold

A potential additive benefit of using growth factor surfaced bioscaffold is that controlled release of growth factors may improve engraftment and survival of hepatocytes by promoting angiogenesis other than offering attachment alone in the long term (Hou et al., 2011). Kedem et al. (2005) delivered sustained VEGF to enhance scaffold vascularisation and improved hepatocyte transplant engraftment in the host liver lobe by 4.6 fold up to 12 days following transplantation. However, Smith et al. (2006) augmented hepatocyte transplant short- rather than long-term survival by delivering EGF and HGF using a VEGF-designated porous polymer scaffold. These findings suggest that hepatocyte transplant engraftment requires simultaneous delivery of multiple signals. Therefore, a designated scaffold that can deliver multiple growth factors is required for the clinical use of hepatocyte-scaffold transplantation. A decellularised whole liver is supposed to be the ideal scaffold to reconstruct a “new” liver de novo (Baptista et al., 2011; Soto-Gutierrez et al., 2011; Zhou et al., 2011; Shirakigawa et al., 2012; Yagi et al., 2013). Wang et al. (2013) reported that decellularised liver matrix effectively supported proliferation and differentiation of murine foetal liver progenitors for up to 2 weeks. However, the question from preclinical, experimental studies is how to acquire an ideal decellularised liver scaffold: the use of a healthy donor liver has to sacrifice billions of healthy hepatocytes, while that of a diseased, such as fibrotic, donor liver carries a high risk of fibrogenesis and carcinogenesis due to the unfavourable environmental factors. Combination of human-derived hepatocytes and swine-derived acellular liver scaffold may be a good solution. Barakat et al. (2012) established an acellular porcine liver scaffold while preserving the native architecture and most ECM components, which facilitated maturation of human foetal hepatocytes co-cultured with foetal stellate cells into differentiated hepatocytes with respect to immunohistochemistry and biochemical
metabolism. Again using animal-derived material is subject to ethical challenge and risk of zoonosis.

1.3 Heterotypic Interactions of Hepatocytes with Non-hepatocytic cells

Once isolated and cultivated in vitro, hepatocytes are deprived of hepatocyte-to-hepatocyte contact, ECM support, and hepatocyte-to-non-hepatocytic cell communication. Hepatocyte monoculture, if not chemically defined, normally exhibits a marked reduction in phenotype and metabolic functionality, such as a rapid decrease in albumin secretion and AFP expression (Bhatia et al., 1998). This phenotypic and functional regression is attributable to the in vitro dedifferentiation of hepatocytes resulting from a complex, wide-ranging change in proteomics and depending on the culture condition (Rowe et al., 2010). Co-cultivation of multiple non-hepatocytic cells, mainly of mesenchymal origin, is reported to maximise hepatocyte function in vitro. NIH/3T3 cells, a fibroblast cell line obtained from mouse embryos, are most frequently used as feeder cells in hepatocyte co-culture (Lu et al., 2005). This cell line is known to regulate HGF and MET (HGF receptor) expression, and secrete adhesion molecules (Halverson et al., 1999). Co-culture with NIH/3T3 cells significantly improved albumin secretion (Nishikawa et al., 2008) and CYP450 activity (Chia et al., 2005) in hepatocytes as compared to mono-cultured counterparts in the short term. TGF-β1 was thought to mediate this enhanced liver-specific metabolism as extracellular activation of latent TGF-β1 was upregulated in the co-culture and TGF-β1 neutralisation diminished this functional enhancement (Chia et al., 2005). Moreover, three-dimensional heterotypic co-culture with NIH/3T3 cells can even maintain a high level of albumin secretion and CYP450 activity for almost two weeks, while hepatocyte homo-culture becomes metabolically inactive after the first week (Lu et al., 2005).

Co-culture with NIH/3T3 cells is, however, subject to a high variability in hepatocyte metabolic enhancement, with respect to albumin secretion, urea synthesis, and CYP450 activity (Gregory et al., 2001). This variation can be attributed to oxygen uptake among co-culture systems varying in seeding density (Cho et al., 2007). Moreover, the use of fibroblasts may risk promoting liver fibrosis in vivo, if transplanted with hepatocytes, although it has not been reported in current literature regarding HCT. It was reported that TGF-β1-expressing NIH/3T3 cells, if injected intracavernously, could result in rat penile fibrosis (Ryu et al., 2005). Therefore, this technique is more suitable for developing bioartificial liver support systems (Washizu et al., 2001; Seo et al., 2006).

1.3.1.1 Co-culture of hepatocytes with non-parenchymal liver cells

A native liver is composed of parenchymal cells – hepatocytes accounting for 80%, and NPCs, also called stromal cells, for 6.5% of the total liver volume (Kmieć, 2001). Liver NPCs consist of Kupffer cells, sinusoidal endothelial cells, and stellate cells. These cells play a regulatory role in hepatocyte maintenance, proliferation, apoptosis, and maturation (Melgert et al., 2000; Zinchenko et al., 2006; Zhang et al., 2009). Activation of NPCs is critical for hepatocyte regeneration in the well-established 70% partial hepatectomy model (Sakuda et al., 2002). This proliferative subpopulation
of hepatic cells also carries the potential for differentiation into metabolically functional hepatocyte-like cells in the presence of HGF and EGF or FGF-4 (Duret et al., 2007). Co-culture with liver sinusoidal endothelial cells can help maturation of foetal and neonatal hepatocytes and maintain albumin secretion in rats (Morin et al., 1986). Co-culture with NPCs, including sinusoidal endothelial cells, hepatic stellate cells, and partially activated Kupffer cells, can even maintain and enhance liver-specific gene expressions of non-serum-fed hepatocytes in the presence of EGF, bFGF, and hepatocyte conditioned medium (CM; Ries et al., 2000). This in vitro co-culture system is expected to sustain the metabolic function of hepatocytes in the long term (Shulman and Nahmias, 2013). In addition to serving as a nourishing feeder for mature hepatocytes, multiple NPCs are reported to direct hepatogenic differentiation of ES cells and MSCs. NPCs co-culture drives mouse ES cells to differentiate into hepatocyte-like cells, at a rate of approximately 70% with respect to albumin production, ammonia metabolism, and drug detoxification, in the presence of HGF and dexamethasone (Soto-Gutiérrez et al., 2007). Hepatic stellate cells are also reported to contribute to hepatocytic differentiation of BM-derived MSCs (Deng et al., 2008). Hepatic stellate cells activated by Kupffer cells exert a modulatory effect on MSCs hepatic differentiation mediated by IL-6 and -10 (Parekkadan et al., 2007).

NPCs exert both positive and negative effects on neighbouring hepatocytes in paracrine, cell-matrix, and cell-cell manners. Activated hepatic stellate cells help co-cultured hepatocytes to aggregate rapidly into well-defined viable spheroids (Thomas et al., 2005) and regulate hepatocyte proliferation in vitro (Uyama et al., 2002); these spheroids show a complex ECM support and hepatic ultrastructure (Thomas et al., 2005). Upregulated HGF expression underlies hepatocyte co-culture with hepatic stellate cells, suggesting that the co-culture undergoes a post-traumatic regenerative process (Thomas et al., 2005). Aberrant activities of NPCs are also attributed to liver fibrosis through releasing inflammatory mediators and reactive oxygen species (ROS; Cohen and Nagy, 2011). NPCs are also involved in liver allograft rejection as this cell population is readily targeted by cytotoxic antibodies and complement (Astarcioglu et al., 1995). This beneficial technique has been investigated for bioartificial liver support systems (Nedredal et al., 2007; Soto-Gutierrez et al., 2010) and in vitro drug toxicity screening systems (Kostadinova et al., 2013). The application of hepatocytes and NPCs co-culture in HCT is limited by a fact that highly variable NPCs themselves may inevitably result in a variation in co-culture hepatocytotropic effect. NPCs supportive effect on co-cultured hepatocytes can be present for no more than 7 days in the absence of specific growth factors, such as HGF, EGF (Kan et al., 2004), VEGF, and IL-6 (Kang et al., 2004). Moreover, it is a safety concern that NPCs are historically known to be the major source of collagen production if profibrotic factors are present.

1.3.1.2 Co-culture of hepatocytes with MSCs

Specific interactions between epithelial cells and mesenchymal-derived cells are known to be required for liver morphogenesis (Tanimizu et al., 2007). Heterotypic co-culture with MSCs shows a significantly higher metabolic activity, including albumin secretion, urea synthesis, and CYP450
activity, than homotypical mono-culture, especially after one week of cultivation (Gu et al., 2009a; Gu et al., 2009b; Gu et al., 2009c). BM-derived MSCs are stromal supporting scaffolds for HSC by secreting a series of crucial cytokines and growth factors (Pontikoglou et al., 2011). Hepatocyte multiplication can be stimulated by multiple soluble factors in vitro, including IL-6, bFGF, and TGF-α. Similar to NPCs, BM-derived MSCs assist primary hepatocytes in the formation of spheroids in co-culture (Gu et al., 2009c). Scanning electron microscopy shows the establishment of cell-matrix and cell-cell contacts of hepatocytes on top of MSCs monolayer, with a well-organised three-dimensional tomography (Figure 1.5; Gu et al., 2009c).

Figure 1.5 Hepatocytes co-cultured with BM-derived MSCs (left panel) exhibit a three-dimensional tomography on scanning electron microscopy (right panel) (Gu et al., J Cell Physiol, 2008).

Co-cultured hepatocytes exhibit a lower G₀/G₁-phase fraction, but a higher G₂/S-phase fraction on cell cycle analysis, suggesting the activation of quiescent hepatocytes by BM-derived MSCs in co-culture (Gu et al., 2009c). The insertion of a semi-permeable porous membrane between MSCs culture and hepatocyte culture eliminates the cell-cell contact but still increases the hepatocyte function (Gu et al., 2009c). This suggests that trophic effect of MSCs co-culture results from soluble cytokines and growth factors released from MSCs. The potential trophic soluble factors consist of HGF, IL-6, and TNF-α as shown by the neutralisation experiments (Gu et al., 2009c). Moreover, ECM, such as fibronectin, laminin, and collagen type I/III/V, deposited by MSCs may also contribute to enhanced hepatocyte function in co-culture, as validated in ECM gene knockdown experiments (Gu et al., 2009c).

In addition to maintenance and modification of hepatocytes, MSCs co-culture also facilitates hepatic differentiation of stem cells or progenitor cells in vitro (Lange et al., 2005b; Lange et al., 2006; Qihao et al., 2007). Co-culture with foetal liver-derived MSCs helps hepatic maturation of hepatic progenitor cells and hepatic differentiation of ES cells (Ishii et al., 2010). ES cells-derived hepatocytes are immunocytologically characteristic of mature hepatocytes with a higher metabolic activity (Ishii et al., 2010).

As MSCs are known to be potentially hepatogenic both in vitro and in vivo (Ji et al., 2012;
Wang et al., 2012) and immunomodulatory (Yi et al., 2012; Zhang et al., 2012), MSCs-based hepatocyte co-culture appears to be an effective modality for improving current HCT practice. It was reported that co-culture with BM-derived stromal cells augmented hepatocyte-specific metabolic functionality up to one month (Mohajerani et al., 2010). Foetal liver-derived MSCs improve engraftment of transplanted hepatocytes (Joshi et al., 2012); hepatocytes detached from BM stromal cell co-cultures also have a better engraftment following the transplantation into the spleen as compared to those detached from fibroblast cell co-cultures (Mohajerani et al., 2010). MSCs co-culture can improve the marginal quality of hepatocytes isolated in current practice and minimise the number of hepatocyte transplants required for a favourable therapeutic outcome, without requiring delicate, costly laboratory manipulation. This technique has been successfully used to modify bioartificial liver system for treating ALF (Yagi et al., 2009; Yang et al., 2013), and it is also potentially promising to improve HCT in the treatment of ALF and liver-based metabolic disorders.

### 1.3.1.3 Possible Contribution of MSCs to HCT

Co-transplantation of MSCs with islet cells has been reported for the treatment of experimental diabetes mellitus (Sakata et al., 2011). MSCs co-transplants can improve islet graft survival and function in vitro, as well as engraftment revascularisation in vivo (Ito et al., 2010; Rackham et al., 2011; Kerby et al., 2013). The incorporation of MSCs is also expected to bring some additional benefits to current HCT practice. This technique is likely to help overcome the major hurdles of current HCT practice, namely, shortage of hepatocytes available for transplantation and marginal quality of isolated hepatocytes.

Firstly, MSCs can modify hepatocyte morphology and functionality (Gu et al., 2009a; Gu et al., 2009b; Gu et al., 2009c). It is possible that MSCs co-culture decreases the number of hepatocytes required for a given recipient and gives a similar or even better therapeutic outcome. It has been proposed that co-transplantation of iPS cells-derived hepatocytes and MSCs should be an effective treatment alternative to orthotopic liver transplantation for treating end-stage liver disease (Liu et al., 2009).

Secondly, co-transplanted MSCs can transdifferentiate into hepatocytes following the engraftment. BM-derived MSCs co-cultured with foetal liver cells exhibit a high-efficiency hepatocytic differentiation, similar to cells sequentially subjected to stem cell factor, HGF, EGF, and FGF-4 (Lange et al., 2006). Human UC-derived MSCs differentiated into hepatocyte-like cells, without accelerating capillarisation and venularisation of liver sinusoids, in CCl₄-induced liver fibrosis model (Ren et al., 2010). Shi and his colleagues (2009) used co-encapsulated hepatocytes and MSCs transplantation to successfully increase liver function and survival rate of an ALF rat model, and observed in vivo transdifferentiation of MSCs into hepatocyte-like cells with respect to albumin expression.

Thirdly, MSCs are known to be immunomodulatory (Lin et al., 2011), and MSCs-derived hepatocytes are reported to less immunogenic (Al Jumah and Abu marrow, 2012; Chen et al., 2013;
Hou et al., 2013). The immunomodulatory effect of MSCs has been applied in the therapeutics of some autoimmune and inflammatory diseases (Fiorina et al., 2009; Ohshima et al., 2012), including graft-versus-host disease (Chen et al., 2012; Gregoire-Gauthier et al., 2012; Xia et al., 2012) and liver transplantation (Wan et al., 2008; Popp et al., 2009; Zhang et al., 2012), although it remains controversial (Zhang et al., 2009). Hepatocyte-like cells, which derive from UC-derived MSCs in the presence of HGF and bFGF, neither express major histocompatibility complex II antigen nor induce lymphocyte proliferation in vitro (Zhao et al., 2009). The immunomodulatory property of MSCs co-transplanted with hepatocytes may result in less requirement of immunosuppressive agents, as compared to that in conventional HCT, if used for the treatment of inborn liver-based metabolic disorders (Burlina, 2004).

Lastly, trophic factors of MSCs are also expected to be protective for hepatocytes subjected to a detrimental environment, such as in ACLF (Shi et al., 2011), chemically-induced liver injury (Jung et al., 2013; Salomone et al., 2013; Shao et al., 2013; Xagorari et al., 2013), and liver ischaemia-reperfusion injury (Pan et al., 2012; Jin et al., 2013). Culture with ACLF serum, which contained high-level TNF-α but minimal EGF and VEGF, caused a high cell detachment rate, low viability, and reduced liver-specific function in human hepatocyte mono-culture; however, MSCs protected co-cultured hepatocytes from ACLF-induced cytotoxicity (Shi et al., 2011). MSCs-conditioned medium can attenuate CCl4-induced early apoptosis of hepatocytes by IL-6 and fibroblast-like protein 1 signalling (Xagorari et al., 2013). Moreover, MSCs transplantation can ameliorate ROS-induced rat liver ischaemia-reperfusion injury by downregulating mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) signalling pathway (Pan et al., 2012). However, a randomised, controlled study demonstrated that autologous bone marrow MSC transplantation had no therapeutic benefit for patients with decompensated cirrhosis with respect to absolute changes in Child-Pugh classification, the Model for End-Stage Liver Disease (MELD) score, serum albumin, international normalised ratio, serum transaminases, and liver volume (Mohamadnejad et al., 2013). Moreover, the profibrotic effect of co-transplanted MSCs cannot be ignored as it remains controversial whether MSCs are antifibrotic or profibrotic following intraportal transplantation (Rabani et al., 2010; di Bonzo et al., 2008).

In summary, HCT is an effective treatment alternative to OLT for ALF and inborn liver-based metabolic disorders. However, current HCT practice is subject to two major hurdles, namely, shortage of donor livers and marginal quality of isolated hepatocytes. Alternative cellular sources, including ES cells, foetal liver cells, adult-derived hepatic progenitor cells, iPSCs cells, and MSCs, have been investigated for the preclinical use in HCT. Differentiation of stem/progenitor cells into hepatocyte-like cells requires delicate, costly laboratory manipulation and risks carcinogenesis of undifferentiated cells. Heterotypic interaction with NPCs plays a regulatory role in hepatocyte maintenance, proliferation, apoptosis, and maturation. MSCs co-culture is expected to have multiple contributions to current HCT practice. MSCs can modify hepatocyte phenotype and metabolism; hepatocyte-committed transdifferentiation of MSCs may reduce liver mass required for HCT; immunomodulatory MSCs co-transplants may result in less requirement of immunosuppressive
agents following HCT; and MSCs can also protect co-transplanted hepatocytes from cytotoxicity induced by inflammatory factors or other unfavourable chemical factors, especially in patients with ALF and ACLF. Modification of MSCs and hepatocytes co-culture system is expected to further augment hepatocyte functionality for the clinical use of HCT. Molecular mechanisms underlying MSCs and hepatocytes co-culture are also yet to be delineated for improving current HCT practice.

1.4 Objectives and Hypotheses of PhD Project

MSCs co-culture shows favourable hepatotrophic effect with respect to morphology, cell survival, and liver-specific metabolic functions, as documented in current literature. The present work aimed to modify conventional hepatocyte co-culture with MSCs and further potentiate in vitro hepatotrophic effect of MSCs co-culture. Furthermore, it is a primary objective of this project to investigate the molecular mechanisms underlying hepatotrophic and potentiated effects of MSCs co-culture.

1.4.1 Optimisation of human hepatocyte and mesenchymal stem cell co-culture system in vitro

Multiple sources of MSCs are available for co-culture with hepatocytes. The first objective of this project is to determine which source of MSCs, namely, AT-, BM-, and UC-MSCs, is the optimal MSCs for hepatocyte co-culture in vitro. Additionally, it is investigated in this part of the project whether MSCs co-culture can suppress spontaneous and chemically-induced apoptosis of hepatocytes in vitro.

1.4.2 Hypoxic preconditioning potentiates MSCs co-culture hepatotrophic effect

As MSCs normally reside in a low-oxygen niche, hypoxic precondition (HPc) is frequently used to optimise MSCs for cellular transplantation use. The second objective of this project is to verify the hypothesis that HPc can potentiate hepatotrophic effect of MSCs. It is also investigated in this part of the project whether HPc-induced potentiative effect depends on intra-MSCs activity of ROS, a pivotal signalling factor in cellular response to oxidative stress.

1.4.3 Mechanisms underlying hepatotrophic and HPc-induced potentiative effects of MSCs co-culture

Hepatotrophic effect of MSCs is believed to result from paracrine factors and ECM deposition of MSCs, as well as hepatocyte-to-MSC interaction. The third objective of this project is to investigate whether secretion of TGF-β1 from MSCs and TNF-α from hepatocytes, deposition of extracellular collagen from MSCs, and hepatocyte apoptosis and survival associated signalling pathways are implicated in MSCs co-culture hepatotrophic effect. It is also investigated in this part of the project whether modulatory effects of HPc on soluble factors, ECM, and gene signalling pathways of hepatocytes co-cultured with MSCs depend on intra-MSCs ROS activity.
CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Sources of MSCs

Primary human AT-MSCs, which were originally extracted from a single human donor’s lipoaspirate tissue through mechanical and enzymatic digestion, were purchased from Invitrogen Ltd, Paisley, UK. Primary cultures had been expanded for one passage before cryopreservation. The cell line was immunophenotyped by the supplier as positive for CD29, CD44, CD73, CD90, CD105, and CD166 (>99%), and negative for CD14, CD31, CD45, and Lin1 (<1%) on flow cytometry.

Primary human BM-MSCs, which were originally extracted from a single human donor’s bone marrow through enzymatic digestion, were purchased from Lonza Group, Ltd., Basel, Switzerland. Primary cultures had been expanded for one passage before cryopreservation. The cell line was immunophenotyped by the supplier as positive for CD105, CD166, CD29, and CD44 (>99%), and negative for CD14, CD34, and CD45 (<1%) on flow cytometry.

Primary human UC-MSCs were originally extracted by Dr Yue Wu (Institute of Liver Studies, King’s College Hospital NHS Foundation Trust, London, UK) from a single human donor’s umbilical cord matrix (Wharton’s jelly) through digestion in 1-mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO, USA) as previously reported (Campard et al., Gastroenterology, 2008). The cell line was immunophenotyped as positive for CD13, CD73, CD105, CD90, and CD44 (>99%), but negative for CD31, CD34 and HLR-DR (<1%) on flow cytometry.

The differential potentials of MSCs towards adipocytes, osteoblasts, and chondrocytes, were characterised in vitro by the suppliers using specialised differentiation media.

2.2 Subculture of MSCs

The MSC expansion culture media consisted of phenol red-free, low-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Paisley, UK), 10% foetal calf serum (FCS; Invitrogen, Paisley, UK), 2-mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), and 100-U/mL penicillin plus 100-μg/mL streptomycin (Invitrogen, Paisley, UK). Cryopreserved cells (approximately 1 million cells per mL and per vial) were quickly swirled and thawed in a 37°C water bath (Grant Instruments, Ltd, Cambridge, UK) for 1 min. The cell suspension was immediately transferred into a 50-mL sterile Falcon® polypropylene conical tube (BD Biosciences, Durham, NC, USA) containing 10-mL pre-warmed culture media and centrifuged at 1,500 rpm (Heraeus Instruments, Newport Pagnell, UK) for 5 min. The cell pellet was subsequently resuspended in pre-warmed culture media, and plated onto a 75-cm² tissue culture flask (NUNC A/S, Roskilde, Denmark) at a density of 5,000 cells per cm² and at a volume of 10 mL per T75 flask, in a humidified incubator (Heraeus Instruments, Newport Pagnell, UK), in an atmosphere of 95% O₂ and 5% CO₂ and at 37°C. The culture medium was refreshed every 3–4 d.
On day 7 of culture (approximately at 80% confluency), cell cultures were rinsed with phosphate buffered saline (PBS; Invitrogen, Paisley, UK) and detached by adding 2.5-mL 0.25% trypsin/PBS (Invitrogen, Paisley, UK) at 37°C for 5–10 min, and the cell dissociation was stopped by adding 10% FCS/DMEM. The cell suspension was replated and subcultured at a ratio of 1:5 using the same cell culture protocol. The cells were cryopreserved in the culture medium supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) as multiple aliquots (1 mL per vial) using a freezing container (Thermo Fisher Scientific, Ltd, Loughborough, UK) in a −80°C freezer (New Brunswick Scientific, Cambridge, UK), and the stocks were stored in a −140°C freezer (New Brunswick Scientific, Cambridge, UK). The 6th–8th passages (P6–8) of MSCs were used for experiments. The total number and viability of MSCs for each passage were determined using a Neubauer improved bright-line hematocytometer (Weber Scientific International, Ltd, Hamilton, NJ, USA) and the trypan blue (0.2%; Sigma-Aldrich, St Louis, MO, USA) exclusion technique with a standard inverted light microscope (Leica Microsystems, Milton Keynes, UK) equipped with a digital single lens reflex (SLR) camera (Cannon, Tokyo, Japan).

2.3 Primary Harvest of Human Hepatocytes

The use of human liver tissues was approved by the Research Ethics Committee at King’s College Hospital NHS Foundation Trust, London, UK, and conformed the guidelines set out in accordance with the Human Tissues Act of 2004. All donors or their legal representatives volunteered to give informed consent in writing for research use.

Primary human hepatocytes were isolated from donor liver tissues unused or rejected for orthotopic liver transplantation using a standard collagenase perfusion technique (Figure 2.1), as previously reported by Mitry (2009). Briefly, the liver tissue was maintained in ice-cold Eagle’s minimum essential media (EMEM; Lonza Group, Ltd., Basel, Switzerland) during pre-processing. One or two major patent vessels were cannulated with 16−22G intravenous catheters (Smiths Medical International Ltd., Rossendale, UK) and secured in place using nylon sutures (Tyco Healthcare Group LP, Norwalk, CT, USA). Other minor vascular and biliary vessels on the cut surface were ligated using silk sutures (Ethicon, Inc., Somerville, NJ, USA). The liver tissue was perfused using a MasterFlex® L/S® digital standard drive (Cole-Parmer Instrument Company, London, UK) at a flow rate of 60–80 mL/min. The perfusates contained calcium-free Hank’s balanced salt solution (HBSS; Lonza Group, Ltd., Basel, Switzerland), 1-M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich, St Louis, MO, USA), and 0.5-mM ethylene glycol tetracetic acid (EGTA; Sigma-Aldrich, St Louis, MO, USA), a calcium chelant to disrupt the desmosomal junctions of neighboring hepatocytes. The first perfusion solution was replaced by a second perfusion solution consisting of plain calcium-free HBSS. The post-perfused liver specimens were digested using a third EMEM-based perfusion solution containing 0.05% *Clostridium histolyticum* derived collagenase P (Roche Diagnostics Ltd., West Sussex, UK). The volume of collagenase solution used for the digestion varied with the weight of liver tissue, roughly 250 mL for every 200-gram liver tissue. The collagenase P solution was re-circulated for no more than three
cycles until the specimen was appropriately digested. The buffer solutions were maintained at 37°C using a heating stirrer (GallenKamp Thermo, Cheshire, UK) and oxygenated with 95% O₂ and 5% CO₂ medical gas mixture (BOC Gases Medical, Surrey, UK), at a flow rate of 8 L/min, throughout the perfusion.

The digested liver tissue was maintained in iced-cold EMEM and scissor-minced following the removal of the cannulae and sutures. The released cell suspension was filtered through 2-ply cotton swabs (Shermond, Brighton, UK) and subsequently through a 200-μm nylon cell strainer (BD Biosciences, Durham, NC, USA). Hepatocytes were washed in ice-cold EMEM containing 10% FCS using a low-speed centrifuge (Heraeus Instruments, Newport Pagnell, UK) at 50×g and 4°C for 5 min, and the centrifugation was triplicated at 4°C. The total number and viability of fresh hepatocytes were determined using a haematocytometer and the trypan blue exclusion technique with a standard upright light microscope (Leitz, Wetzlar, Germany). Red blood cells in hepatocyte pellet were lysed, if required, using sterile water for injection (Fannin Ltd., Dublin, Ireland) for 1 min at room temperature followed by centrifugation. The batch of hepatocytes with a viability of over 60% on trypan blue exclusion was used for experiments.

2.4 Hepatocyte Mono-culture and Co-culture

Culture vessels were pre-coated using aseptic techniques with 0.1-mg/mL rat tail collagen type I (Sigma-Aldrich, St Louis, MO, USA) in 1% acetic acid (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 2 h, followed by PBS rinse at 4°C for 24 h.

Fresh hepatocytes were plated onto collagen-coated, flat-bottom microplates at a density of 50,000 viable cells per cm² (hepatocyte mono-culture). The culture media consisted of phenol red-free William’s E medium (WEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FCS, 10-mM HEPES, 2-mM L-glutamine, 0.1-μM dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 0.1-μM human recombinant insulin (Sigma-Aldrich, St Louis, MO, USA), and 100-U/mL penicilllin plus 100-μg/mL streptomycin. The cell morphology of hepatocyte mono-culture was examined using a standard inverted light microscope equipped with a digital SLR camera.
P6–8 MSCs were resuspended in hepatocyte culture medium and incubated on collagen-coated microplates at the predefined densities for 24 h. Fresh hepatocytes were seeded on top of MSCs monolayer at a constant density of 50,000 viable cells per cm² (hepatocyte co-culture). Hepatocyte mono-culture was used as control (control group), and MSCs mono-culture was used as blank control. The culture medium was refreshed on days 1, 3, 5, and 7, respectively. The cell morphology was examined using a standard inverted light microscope equipped with a digital SLR camera. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes (Eppendorf, Hamburg, Germany) and cryopreserved at −80°C for further experiments. Cell cultures were rinsed with one wash of PBS at room temperature for further experiments.

2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 96-well Falcon® microplates (BD Biosciences, Durham, NC, USA) as described above for measuring mitochondrial succinic dehydrogenase activity representing hepatocyte overall viability. The cell cultures were rinsed with PBS, and incubated with plain WEM containing 0.5-mg/mL 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) solution, 200 µL per well, at 37°C for 4 h. The MTT solution was removed and replaced by 100% DMSO, 200 µL per well, to dissolve formazan produced by viable cells, and the microplate was vigorously shaken using a microplate shaker (Heidolph Instruments, Essex, UK) at 1,500 rpm for 1-2 min. The optical density (OD) was measured at 550 nm using an MRX microplate reader equipped with Revelation version 4.06 incorporating Core DLL version 4.06 and Statistics DLL version 4.06 (Dynex Technologies, Guernsey, UK). The reading of background MSCs mono-culture (blank control) was subtracted from that of hepatocyte co-culture to obtain the colorimetric OD of hepatocytes per se in co-culture (Mohajerani et al., Cell Medicine, Part B of Cell Transplantation, 2010). The experiments were performed in quadruplicate and repeated in triplicate independently.

2.6 Sulforhodamine B Colorimetric Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 96-well Falcon® microplates as described above for measuring sulphorhodamine B (SRB) binding to basic amino acid residues on cell membrane surfaces representing overall cell attachment. The cell cultures were rinsed with PBS, and fixed in 50% ice-cold trichloroacetic acid (Sigma-Aldrich, St Louis, MO, USA), 50 µL per well, layering on top of plain WEM, 200 µL per well, at 4°C for 1 h. The microplates were rinsed with tap water, and the fixed cells were stained with 0.4% SRB solution (Sigma-Aldrich, St Louis, MO, USA) in 1% acetic acid (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 10 min. The wells were rinsed with 1% acetic acid and air-dried at room temperature. The SRB dye was solubilised in 20-mM unbuffered 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris-base) buffer (Sigma-Aldrich, St Louis, MO, USA), 200 µL per well, and
shaken on a microplate shaker (Heidolph Instruments, Essex, UK) at 1,500 rpm for 15 min. The OD was measured at 564 nm using the microplate reader. The reading of background MSCs monoculture (blank control) was subtracted from that of hepatocyte co-culture to obtain the colorimetric OD of hepatocytes per se in co-culture (Mohajerani et al., Cell Medicine, Part B of Cell Transplantation, 2010). The experiments were performed in quadruplicate and repeated in triplicate independently.

2.7 Albumin Enzyme-linked Immunoabsorbent Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well Falcon® microplates (BD Biosciences, Durham, NC, USA) as described above, and cell culture supernatants were collected for measuring free albumin secretion representing protein synthesis of hepatocytes. A human albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) with the solid-phase sandwich enzyme immunoassay technique was used to quantitate free albumin level. Nunc-Immuno 96 MicroWell solid plates (NUNC A/S, Roskilde, Denmark) were pre-coated with 1:100 affinity purified human albumin coating antibody, 100 μL per well, at room temperature for 60 min. The antibody solution was removed, and the wells were rinsed with five washes of detergent buffer. The nonspecific antibody binding sites were blocked with the blocking solution, 100 μL per well, at room temperature for 30 min. The blocking solution was removed, and the wells were rinsed with five washes of detergent buffer. Human reference serum albumin, at an initial concentration of 10,000 ng/mL, was serially diluted with sample/conjugate diluents to give the standards 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL, whilst the sample/conjugate diluent was used as the zero standard (blank) for the albumin standard curve. Culture supernatants were centrifuged (Eppendorf, Hamburg, Germany) at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 20 folds) and incubated in the assigned wells, 100 μL per well, at room temperature for 60 min. The wells were rinsed with five washes of detergent buffer. The bound albumin was detected by 1:150,000 horseradish peroxide (HRP) detection antibody, 100 μL per well, at room temperature for 60 min. The HRP detection antibody was removed, and the wells were washed with were rinsed with five washes of detergent buffer. HRP chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB), 100 μL per well, was added, and the plate was incubated in the dark and at room temperature for 15 min. The enzymatic colour reaction was stopped by adding 0.18-M sulphuric acid (Sigma-Aldrich, St Louis, MO, USA), 100 μL per well. The OD was measured at 450 nm using the microplate reader. The albumin concentration (ng/mL) in each sample was determined using the albumin standard curve, and albumin secretion of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.
2.8 Urea Colorimetric Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well microplates as described above, and cell culture supernatants were collected for measuring urea synthesis representing nitrogen detoxification of hepatocytes. QuantiChrom™ urea assay kit (BioAssay Systems, Hayward, CA, USA) was used to quantitate urea level. Culture supernatants were centrifuged at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 50 folds) and transferred into a clear flat-bottom 96-well plate, 5 μL per well. Freshly prepared urea assay working solution was added, 200 μL per well, and the plate was incubated at room temperature for 20 min. Human reference serum urea, at an initial concentration of 50 mg/dL, was serially diluted with distilled water to give the standards 3.125, 6.25, 12.5, 25, and 50 mg/dL, whilst distilled water was used as the zero standard (blank) for the urea standard curve. Distilled water (blank control), urea standard, and fresh hepatocyte culture media (control) were assayed using the same protocol. The OD was measured at 520 nm using the microplate reader. The corrected urea concentration (mg/dL) in each sample was calculated using the urea standard curve, and urea synthesis of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.

2.9 Caspase-cleaved CK18 Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well microplates as described above, and cell culture supernatants were collected for measuring apoptosis-associated K18Asp396 (M30) neo-epitope of soluble caspase-cleaved CK18 (CCK18) representing caspase-mediated apoptosis of hepatocytes. M30 CytoDeath™ ELISA kit (PEVIVA AB, Bromma, Sweden) was used to quantitate soluble CCK18 level (Figure 2.2, left panel). Human reference CCK18 solutions, prepared at 250, 1,000, 3,000 U/L, were used as standards, whilst the sample/conjugate diluent was used as the zero standard (blank) for the CCK18 standard curve. Culture supernatants were centrifuged at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 20 folds) and incubated in the assigned wells, 25 μL per well, with HRP conjugate, 75 μL per well, on a microplate shaker, 600 rpm, at room temperature for 4 h. The HRP detection antibody was removed, and the wells were rinsed with five washes of detergent buffer. Chromogenic substrate TMB, 200 μL per well, was added, and the plate was incubated in the dark and at room temperature for 20 min. The enzymatic colour reaction was stopped by adding 0.18-M sulphuric acid, 50 μL per well. The OD was measured at 450 nm using the microplate reader. The CCK18 concentration (U/L) in each sample was calculated using the CCK18 standard curve, and soluble CCK18 release of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.
2.10 CK18 Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well microplates as described above, and cell culture supernatants were collected for measuring soluble CK18 representing total cell death of hepatocytes. M65 EpiDeath® ELISA kit (PEVIVA AB, Bromma, Sweden) was used to quantitate soluble CK18 level (Figure 2.2, right panel). Human reference CK18 solutions, prepared at 200, 400, 800, 1,200, 2,000, 3,000, 5,000 U/L, were used as standards, whilst the sample/conjugate diluent was used as the zero standard (blank) for the CK18 standard curve. Culture supernatants were centrifuged at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 50 folds) and incubated in the assigned wells, 25 μL per well, with HRP conjugate, 75 μL per well, on a microplate shaker, 600 rpm, at room temperature for 4 h. The HRP detection antibody was removed, and the wells were washed with were rinsed with five washes of detergent buffer. Chromogenic substrate TMB, 200 μL per well, was added, and the plate was incubated in the dark and at room temperature for 20 min. The enzymatic colour reaction was stopped by adding 0.18-M sulphuric acid, 50 μL per well. The OD was measured at 450 nm using the microplate reader. The CK18 concentration (U/L) in each sample was calculated using the CK18 standard curve, and soluble CK18 release of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.
Induction of apoptosis in cultured hepatocytes will result in massive release of CCK18 and secondary release of CK18 at a later time point, and consequently increase CCK18/CK18 ratio. In contrast, induction of necrosis will almost exclusively result in massive release of non-cleaved CK18, and consequently give rise to a low CCK18/CK18 ratio. The ratio of CCK18 to CK18 was, thus, calculated to determine the mode of death for hepatocytes in vitro; a ratio over 0.40 indicated that hepatocytes underwent apoptosis mainly, and vice versa (Kramer et al., 2004).

2.11 Statistical Analysis

GraphPad Prism 6 programme (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. All continuous data were expressed as mean ± standard deviation (SD), and the means were compared using the one-way repeated measures analysis of variance (ANOVA), the Fisher's least significant difference (LSD) test, or two independent samples student-\( t \) test unless specified otherwise. A two-tailed \( P \)-value less than 0.05 was considered statistically significant.
CHAPTER 3 OPTIMISATION OF HUMAN HEPATOCYTE AND MESENCHYMAL STEM CELL IN VITRO CO-CULTURE SYSTEM

3.1 Introduction

3.1.1 Limitations of hepatocyte mono-culture

Once isolated and cultivated in vitro, primary hepatocytes in mono-culture lose their proliferative potential although the liver in vivo is well known for its potent regenerative capacity as seen in the Prometheus myth. Hepatocytes cultured in vitro switch from the proliferative response to the inflammatory response, as mediated by downregulated expression of NFκB (Chaisson et al., 2002; Fredriksson et al., 2011; Malato et al., 2012) and upregulated expression of MAPK/ERK (Roberts et al., 2000; Frémin et al., 2007) signalling pathways. As a result, primary hepatocytes in standard monolayer culture show a rapid phenotypic de-differentiation and metabolic regression (Mizumoto et al., 2008).

Hepatocytes also undergo epithelial-to-mesenchymal transition (EMT) in the absence of specific favourable growth factors, such as HGF (Xia et al., 2006), whilst TGF-β, a potent profibrogenic factor, is known to be the most potent mediator of hepatocyte EMT as for other epithelial cell lines (Dooley et al., 2008). Hepatocyte EMT is also believed to be one of the major aetiologies of liver fibrosis (Breitkopf et al., 2006) aside from dysregulated proliferation of liver NPCs. In vitro cultured hepatocytes, if unattached, will undergo spontaneous apoptosis (Vanhaecke et al., 2004). This spontaneous event is controversially thought to result from the accumulation of pre-existing nitric oxide (Canová et al., 2008) and can also be induced by TNF or TGF-β (Roberts et al., 2000).

3.1.2 Heterotypical co-culture of hepatocytes with hepatic NPCs

Hepatic NPCs, which consist primarily of Kupffer cells, sinusoidal endothelial cells, and stellate cells, play a regulatory role in hepatocyte maintenance, proliferation (Harada et al., 2003), apoptosis (Chaisson et al., 2002), maturation (Mitaka et al., 1999; Melgert et al., 2000), and regeneration (Sakuda et al., 2002; Azuma et al., 2003). The regulatory effects of NPCs on hepatocytes are believed to be mediated by paracrine factors, such as HGF (Michalopoulos et al., 1999), EGF (Michalopoulos et al., 1999), TGF-β (Date et al., 1998), TNF (Shinozuka et al., 1996), IGF, IL-1 (Boulton et al., 1997), IL-6, BMP-6, and ECM (Vrochides et al., 1996). However, co-culture with liver-derived NPCs stimulates hepatocyte DNA synthesis, depending on cell-to-cell interactions rather than growth factors. NPCs co-culture can augment liver-specific metabolic functions of hepatocytes exposed to ALF serum (Nedredal et al., 2007). NPCs co-culture also helps two-dimensional sheets of hepatic progenitor cells to generate functionally differentiated three-dimensional liver tissue (Ogawa et al., 2004). It is possible that hepatocytes and hepatic NPCs isolated from a single donor could be co-transplanted into a given recipient with more benefits.
3.1.3 Heterotypic co-culture of hepatocytes with MSCs

Co-culture of seed cells with MSCs appears to be a promising solution to modify cellular replacement therapy for metabolic disorders, such as diabetes mellitus and liver-based metabolic diseases, as heterotypic interaction with MSCs is known to be crucial for survival and functionality of epithelial cells \textit{in vitro} (Gómez-Aristizábal \textit{et al.}, 2009). Human MSCs enhance survival, metabolic function, and \textit{in vivo} angiogenesis of isolated pancreatic islets by secreting trophic factors, such as VEGF (Park \textit{et al.}, 2010), and by depositing ECM (Hematti \textit{et al.}, 2013). Furthermore, Ito and his colleagues (2010) reported that co-transplantation with MSCs improved the morphology and function of islet grafts by promoting re-vascularisation \textit{in vivo}. Among these exploratory studies, BM-MSCs are the most frequently used supportive cells as these cells are more easily expanded \textit{in vitro} as documented in current literature (Karaoz \textit{et al.}, 2011; Yeung \textit{et al.}, 2012).

3.1.3.1 Heterotypic co-culture of hepatocytes with BM-MSCs

In the field of HCT, MSCs also show a favourable supportive effect on hepatocytes in long-term co-culture \textit{in vitro} (Corlu \textit{et al.}, 1997). BM-MSCs are still the most frequently used MSCs for hepatocyte co-culture. BM-MSCs maintain and improve hepatocyte morphology and metabolic functionality by the synergistic effects of soluble factors, cell-to-matrix, and cell-to-cell communications (Ijima \textit{et al.}, 2008; Gu \textit{et al.}, 2009\textsuperscript{a}; Gu \textit{et al.}, 2009\textsuperscript{b}; Gu \textit{et al.}, 2009\textsuperscript{c}).

Co-transplantation of hepatocytes and BM-MSCs was also attempted in preclinical studies. When co-encapsulated with BM-MSCs, hepatocytes could be traced up to 4 months following transplantation in rats (Liu and Chang, 2002). Taking all these studies together, BM-MSCs can improve cellular morphology, survival, and metabolic functions, such as albumin secretion, urea synthesis, and CYP450 activity, both \textit{in vitro} and \textit{in vivo}. It was, therefore, proposed that co-transplantation of iPS derived cells and MSCs might be a novel alternative to OLT, which is limited by a shortage of donors and immune rejection, for treating end-stage liver disease (Liu \textit{et al.}, 2009).

3.1.3.2 Heterotypic co-culture of hepatocytes with UC- and AT-MSCs

Two other common sources of MSCs, namely, UC- and AT-derived MSCs, have also been investigated in the setting of preclinical study. Chao \textit{et al.} (2008) reported that human UC-MSCs improved islet cell secretion of insulin over 3 months possibly by releasing a series of trophic factors, including IL-6, tissue inhibitors of metalloproteinase (MMP)-1 and -2, monocyte chemoattractant protein 1, growth related oncogene, HGF, IGF binding proteins 4, and IL-8. Gómez-Aristizábal and Davies (2012) showed that human UC perivascular cells containing UC-MSCs enhanced liver-specific gene expressions, such as albumin, urea, and CYP450, mainly through MSC-to-hepatocyte contact and partially through paracrine factors.

AT-MSCs, also called adipose stem cells, are a newer member of the MSCs family that was introduced in the early 21\textsuperscript{st} century and less studied for co-culture with hepatocytes. Cavallari \textit{et al.} (2012) preconditioned human AT-MSCs with a mixture of hyaluronic, butyric, and retinoic acids and successfully optimised co-transplantation of rat islet cells with MSCs in a diabetic rat model, in
which multiple growth factor signalling pathways, such as VEGF, kinase insert domain receptor transcript, and HGF, were activated. The soluble factors mediating the immunomodulatory effect of AT-MSCs also include TGF-β, chemokine (C-C motif) ligand (CCL) 2, CCL5, tissue inhibitor of MMP-1/2, and cyclooxygenase-2 (Kang et al., 2008).

3.1.3.3 Optimal source of MSCs for hepatocyte co-culture

BM-, UC-, and AT-MSCs have their own advantages and disadvantages in the scenario of clinical transplantation practice (Table 3.1). It remains unknown in the current literature which source of MSCs, namely, BM, UC, or AT, is the optimal candidate for the purpose of hepatocyte co-culture, although BM-MSCs are most frequently used in co-culture experiments.

Generally, these three types of MSCs have similar morphology and immunophenotype. BM and AT have a significantly higher success rate of MSCs isolation as compared to UC (100% vs. 100% vs. 63%); AT has the highest colony frequency, but UC has the lowest; and UC has the highest proliferative capacity, but BM has the lowest (Kern et al., 2006). The low success rate of UC-MSCs isolation can be improved by selecting cord blood unit with a volume of more than 90 mL and a harvest time within 2 hours after the donor’s birth (Zhang et al., 2011). It is also confirmed that BM-MSCs are more likely to become senescent through passages in vitro, as compared to UC- and AT-MSCs (Vidal et al., 2012).

All MSCs share some consistent and reproducible gene expression profiles involved in ECM formation, such as fibronectin, ECM2, glypican 4, DNA-binding protein inhibitor 1, neurofibromin 1b, and homeobox (HOX) A5 and B6, but also exhibit some differential gene expression profiles (Wagner et al., 2005). Human adult BM-, UC-, and AT-MSCs also exhibit similar immunomodulatory effects in vitro. A study in a canine model reported that AT-MSCs had a significantly higher proliferative potential and BM-MSCs secreted the highest level of VEGF; AT- and UC-MSCs exhibited a greater in vitro osteogenic capacity as compared to BM-MSCs (Kang et al., 2012). Another in vitro study comparing human BM-, UC-, and AT-MSCs, with respect to surface antigen expression, differentiation potential, proliferative capacity, clonality, tolerance for ageing, and paracrine activity, showed that UC-MSCs had the highest rate of cell proliferation and clonality and a significantly lower level of senescence marker expression; human UC-MSCs reduced expression of proinflammatory cytokines, such as IL-1α, -6, and -8, in co-cultured, LPS-challenged rat alveolar macrophages (Jin et al., 2013).

Interestingly, these three types of MSCs have a similar potential of hepatogenic differentiation and proliferation, while placenta-derived MSCs have the reportedly greatest potential (Lee et al., 2012). Moreover, it has been reported that human UC-MSCs are more effective for improving ureagenesis while BM-MSCs is more supportive for CYP450 activity in co-cultured hepatocytes (Gómez-Aristizábal et al., 2012).
### Table 3.1 Overview of BM-, UC, and AT-MSCs

<table>
<thead>
<tr>
<th>BM</th>
<th>UC blood/matrix</th>
<th>AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive harvest</td>
<td>Non-invasive</td>
<td>Non-invasive as side product of liposuction</td>
</tr>
<tr>
<td>Propagatable but reduced in aged donors</td>
<td>Highly propagatable</td>
<td>Highly propagatable</td>
</tr>
<tr>
<td>Less homogenous stem cell population</td>
<td>Homogenous stem cell population</td>
<td>Heterogeneous cell population</td>
</tr>
<tr>
<td>Limited source</td>
<td>Limited source</td>
<td>Relatively unlimited source</td>
</tr>
<tr>
<td>Readily available</td>
<td>Not readily available</td>
<td>Readily available</td>
</tr>
<tr>
<td>Most extensively studied, showing high-efficiency differentiation</td>
<td>Extensively studied, showing less efficient differentiation</td>
<td>Less studied, and not well documented</td>
</tr>
</tbody>
</table>

### 3.1.4 Compromised functionality of steatotic hepatocytes and cryopreserved human hepatocytes

#### 3.1.4.1 Steatotic hepatocytes

Liver steatosis, also called fatty liver disease (FLD), refers to a pathological accumulation of lipid, mainly triglyceride acid, in liver cells. FLD is further classified as alcoholic and non-alcoholic subtypes. Early-stage FLD is usually reversible in most cases and thought to result primarily from imbalanced fatty acid metabolism secondary to abusive alcohol consumption, obesity (Fabbrini et al., 2010), metabolic disorder, such as insulin resistance, and use of hormonal or cytotoxic agents. FLD patients are usually asymptomatic and exhibit no symptom or sign of liver injury if the liver remains well compensated. However, severe FLD has accompanying hepatocyte necrosis and inflammatory response, namely, steatohepatitis. This pathological condition can trigger the activation of stellate cells and lead to liver fibrosis (Dixon et al., 2001).

The prevalence of non-alcoholic FLD is very high and variable in the general population, ranging from 14% to 34% (Browning et al., 2004; Bedogni et al., 2005). This figure is much higher in the Western population even without a history of alcohol abuse (Angulo, 2002). FLD is the most common cause of liver function test abnormality in European and North American populations. As FLD remains silent until the time of liver imaging or biopsy, fatty donor livers with a poor graft survival and functionality are frequently (estimated to be 25%) encountered in OLT practice (Marsman et al., 1996). Donor livers assigned to HCT use are often rejected for OLT mainly due to the presence of serious liver steatosis (Baccarani et al., 2003). Alexandrova et al. (2005) reported severe liver steatosis rather than long cold ischaemia time or older donor age was the primary risk factor of a low hepatocyte isolation efficiency. Moderate to severe steatosis (>10% steatotic hepatocytes) was reported to be associated with a low hepatocyte isolation yield from liver resection specimens (Alexandre et al., 2002) although mild steatosis (≤10% steatotic hepatocytes) did not result in a poor harvest of hepatocytes (Alexandre et al., 2002; Richert et al., 2004). Bonora-Centelles et al. (2010) reaffirmed that donor livers with a cold ischaemia time over 15 hours or with underlying serious steatosis should not be accepted for HCT as these donor livers offered poor-
quality hepatocytes in terms of cellular viability, attachment efficiency, and metabolic functionality. Severely steatotic hepatocytes cannot tolerate cryopreservation and subsequent thawing, and are consequently subject to a further cell loss and metabolic impairment (Terry et al., 2005).

Sagias et al. (2010) added N-acetylcysteine (NAC), a widely used antioxidant for treating paracetamol-induced liver toxicity, into the perfusion solution of hepatocyte isolation and improved the viability of severely steatotic hepatocytes. The hepatoprotective effect of NAC is thought to result from the elimination of excessive production of ROS during ischaemia-reperfusion (Kohli et al., 2007). Omega-3 polyunsaturated fatty acid was reported to reverse mitochondrial injury of steatotic hepatocytes by upregulating mitofusin 2 gene encoding mitochondrial membrane protein (Zhang et al., 2011). Glucagon-like peptide-1 analogue can reduce hepatocyte steatosis and improve cell survival by enhancing unfolded protein response and promoting macroautophagy (Sharma et al., 2011). However, it remains yet to be investigated how to improve the morphology and metabolic function of steatotic hepatocytes in the long term, especially after transplantation.

### 3.1.4.2 Cryopreserved hepatocytes

Hepatocytes can be cryopreserved and stored in a “cell bank” for several days, months, or even years following isolation, and cryopreserved hepatocytes can be subsequently thawed, allowing repeated or emergency transplantation on demand (Terry et al., 2010). A major limitation of cryopreservation for standard hepatocyte monolayer culture is that post-cryopreserved hepatocytes exhibit a poor cell attachment and consequent metabolic impairment on thawing. Intracellular ice formation and exposure to hyperosmotic solutions are two major causes for hepatocyte injury following cryopreservation and subsequent thawing. These injuries can damage hepatocyte cytoplasmic membrane and result in massive loss of cytosolic proteins. Therefore, cryopreserved/thawed hepatocytes have a significant reduction in albumin secretion, urea synthesis, and CYP450 activity as compared to the pre-freezing baseline. Severity of hepatocyte cryopreservation/thawing injury depends on the condition of the donor liver tissue, such as age and well-being of the donor, concomitant liver condition (e.g., liver steatosis), and time lengths of cold and warm ischaemia (Terry et al., 2005).

A large number of studies reported optimisation of hepatocyte cryopreservation protocols, aiming to improve post-freezing/thawing hepatocyte viability and metabolic function. These modified strategies include pre-culture of liver cell suspension, pre-incubation with antioxidants (Terry et al., 2006), standardisation of hepatocyte concentration and cryovial use, and optimisation of cryoprotectants (e.g., long-chain oligosaccharide; Miyamoto et al., 2006) and cooling/thawing procedures. As hepatocytes become apoptotic following detachment from ECM, cryopreservation of hepatocytes in artificial ECM, such as collagen and high molecular weight polymer, has also been investigated. Canaple et al. (2001) reported that cryopreservation of hepatocytes encapsulated in multicomponent capsules, composed of a polyelectrolyte complexation of sodium alginate, cellulose sulphate and poly(methylene-co-guanidine) hydrochloride, maintained liver-specific metabolic function of frozen hepatocytes for up to 4 months as compared to unfrozen cells. Interestingly, it has
been reported that co-encapsulation of hepatocytes with MSCs can improve cell graft survival and liver-specific metabolic function both in vitro (Liu and Chang, 2003) and in vivo (Shi et al., 2009). Therefore, it is possible that co-encapsulated MSCs should protect hepatocytes from freezing/thawing injury. However, it remains unknown whether these protective effects can be maintained in the longer term, especially after thawing.

3.1.5 Antiapoptotic effect of MSCs co-culture on hepatocytes in vitro

MSCs co-culture has a significant modulatory effect on the cell cycle of hepatocytes. Gu et al. (2009b; 2009c) reported that a larger percentage of hepatocytes co-cultured with BM-MSCs were accumulated in the G2/S phase, with a smaller percentage in the G0/G1 phase, as compared to monocultured hepatocytes. It suggests that MSCs co-culture facilitates the bypass of G1/S checkpoint (Pok et al., 2013). This checkpoint is known to be regulated by protein 53 (p53), a tumour suppressor. p53 can arrest cell growth by holding the cell cycle at the G1/S checkpoint, which allows sufficient time for the cell to recognise and repair DNA damage; if the damage is irreparable, p53 will initiate cell apoptosis (Jensen et al., 1998).

MSCs transplantation has been investigated in preclinical and clinical studies to ameliorate liver ischaemia/reperfusion injury (Pan et al., 2012; Sun et al., 2012) and chemically-induced liver fibrosis (Manuelpillai et al., 2010). Potential contribution of MSCs may result from two aspects, including transdifferentiation into hepatocytes in vivo and promoting liver regeneration by paracrine mechanisms. Yan et al. (2009) reported that transplantation of human UC-MSCs could reduce serum aminotransferase level and hepatocyte denaturation by inhibiting hepatocyte apoptosis and promoting hepatocyte proliferation as evidenced by hepatocyte tracing and proliferating marker labelling. Antiapoptotic and antifibrotic effects of MSCs in vivo may derive from the contribution of HGF signalling, which modulates activation, apoptosis, and TGF-β signalling downregulation of hepatic stellate cells. Therefore, it is possible that MSCs co-culture exerts trophic and protective effects on hepatocytes by inhibiting hepatocyte spontaneous and chemically-induced apoptosis. This possibility is clinically significant for the use of HCT in the setting of ALF as transplanted hepatocytes will be exposed to a large number of circulating and regional (pro)inflammatory factors, a great majority of which are also proapoptotic, such as IL-6 and TNF-α, present in ALF patients.

3.1.6 Chapter objectives

- Co-culture of hepatocytes and autologous liver NPCs

To investigate whether co-culture with autologous liver NPCs can also be trophic for hepatocytes in vitro.

- Optimisation of MSCs source for hepatocyte co-culture

To compare hepatotrophic effect of BM-, UC, and AT-MSCs on co-cultured primary human
hepatocytes, at an optimal MSC:hepatocyte ratio, with respect to cellular viability, cell attachment, and liver-specific metabolism.

- **Co-culture steatotic/cryopreserved hepatocytes with MSCs**
  To investigate whether MSCs can also exert trophic effects on co-cultured steatotic or cryopreserved hepatocytes.

- **Contribution of soluble factors to MSCs co-culture hepatotrophic effect**
  To investigate whether soluble factors released from MSCs contribute to hepatotrophic effect of MSCs co-culture.

- **Antiapoptotic effect of MSCs co-culture**
  To investigate whether MSCs can protect co-cultured hepatocytes from spontaneous and chemically-induced cell apoptosis.
3.2 Materials and Methods

3.2.1 Isolation of human liver-derived NPCs

Human liver-derived NPCs were isolated using a modified protocol described by Najimi et al. (2007). Briefly, the donor liver tissue was processed as described in Section 2.3, Primary Harvest of Human Hepatocytes. Liver cell suspension was centrifuged at 50×g and 4°C for 5 min to pellet hepatocytes, and the supernatant was collected and further centrifuged for three cycles at 1,500 rpm and 4°C for 5 min. The total number and viability of liver cells in the supernatant were determined using trypan blue exclusion with a hemocytometer and a light microscope. Red blood cells in the cell pellet were lysed using sterile water for injection for 1 min at room temperature, followed by centrifugation. The cell suspension was resuspended in the MSCs expansion media and plated onto a non-tissue culture treated, 30-mm, polypropylene Petri dishes (Thermo Fisher Scientific, Ltd, Loughborough, UK) at a density of 60,000 cells per cm². As hepatocytes could adhere to polypropylene, floating hepatocytes (90%) were eliminated when the cell culture media were refreshed 4 h following plating. The cell culture was rinsed with sterile PBS and used for experiments.

3.2.2 Subculture of human MSCs and adult dermal fibroblasts

Human AT-, BM-, and UC-MSCs were subcultured as described in Section 2.2, Subculture of MSCs. Human adult-derived dermal fibroblasts (ADFs), a common mesenchyme-derived cell line, were originally extracted by Life Technologies Corporation, Carlsbad, CA, United States from a single human donor’s foreskin through mechanical and enzymatic digestion. These cells were used as a random control for MSCs. Primary cultures was expanded for one passage before cryopreservation. Cryopreserved human ADFs (approximately 0.5 million cells per mL per vial) were quickly swirled and thawed in a 37°C water bath for 1 min. The cell suspension was immediately transferred into a 50-mL sterile polypropylene conical tube containing 20-mL pre-warmed culture media and centrifuged at 1,500 rpm for 5 min. The cell pellet was subsequently resuspended in pre-warmed culture media and plated onto a 25-cm² tissue culture flask (NUNC A/S, Roskilde, Denmark) at a density of 5,000 cells per cm² in a humidified incubator, in an atmosphere of 95% O₂ and 5% CO₂ at 37°C. The ADFs expansion culture media consisted of phenol red-free, low-glucose DMEM, 10% FCS, 2-mM L-glutamine, and 100-U/mL penicillin plus 100-μg/mL streptomycin, at a volume of 5 mL per T25 flask. The culture medium was replaced with fresh medium every 3–4 d.

On day 7 of culture (approximately 80% confluence), ADFs cultures were rinsed with PBS and detached by adding 1-mL 0.25% trypsin at 37°C for 5–10 min, and the cell dissociation was stopped by 10% FCS/DMEM. The cell suspension was replated and subcultured at a ratio of 1:3 using the same cell culture protocol. The cells were cryopreserved using a freezing container at −80°C, and the stocks were stored at −140°C in the culture media supplemented with 10% DMSO. The 4th–6th
passages (P4–6) of ADFs were used for further experiments. The total number and viability of ADFs for each passage were determined using the trypan blue exclusion technique with an inverted light microscope equipped with a digital SLR camera.

3.2.3 Isolation of human non-steatotic and steatotic hepatocytes

Liver steatosis was graded by an independent consultant liver histopathologist at Institute of Liver Studies, King’s College Hospital NHS Foundation Trust, London, UK, using the standard four-grade semiquantitative evaluation scale (Franzén et al., 2005): grade 0, no fat deposition in hepatocytes; grade 1 (mild), fat deposition in less than 33% of hepatocytes; grade 2 (moderate), fat deposition in 33%–66% of hepatocytes; and grade 3 (severe), fat deposition in more than 66% of hepatocytes. Non-steatotic donor liver tissues were processed as described in Section 2.3, Primary Harvest of Human Hepatocytes, while moderately steatotic donor liver tissues were processed similarly, except for the addition of 5-mM NAC (PLIVA Pharma, Ltd., Hampshire, UK), a potent scavenger of ROS precursors into the first perfusion buffer (Ca\(^{2+}\)-free HBSS) of the standard collagenase digestion technique as previously reported by Sagias et al. (2010). The total number and viability of fresh hepatocytes were determined using a hematocytometer and the trypan blue exclusion technique with a light microscope. Red blood cells in hepatocyte pellet were lysed using sterile water for injection for 1 min at room temperature, followed by centrifugation. Batches of hepatocytes with a viability of over 60% on trypan blue exclusion were used for experiments.

3.2.4 Cryopreservation and thawing of human hepatocytes

Freshly isolated hepatocytes were cryopreserved using a standard controlled-rate freezing protocol as previously reported by Mitry et al. (2010). Hepatocytes were resuspended in cryopreservation solution at a final density of 1.0×10^7 cells per mL. The cryopreservation solution, consisting of 1 part of 50% glucose (Hameln Pharmaceuticals Ltd., Gloucester, UK), 1 part of clinical grade 100% DMSO, and 8 parts of ViaSpan™ University of Wisconsin (UW) solution (Bristol-Myers Squibb Pharmaceutical Limited, Dublin, Ireland), was chilled on an ice pack. Hepatocyte suspension (5 mL) was transferred into a sterile 10-mL cryovial (Thermo Fisher Scientific) using a 10-mL syringe (BD Biosciences) chilled on an ice pack and immediately placed flat into the controlled-rate freezer connected to a liquid nitrogen container (Kryo 10, series III; Planer Products, Ltd., Middlesex, UK) at a pressure stabilised between 34.5 and 48.3 kPa. The cryovial holder and frame were pre-chilled at 4–8°C for 30–60 min prior to use. A modified stepwise controlled-rate freezer programme was started to produce a linear temperature decrease and prevent cell damage from latent heat of fusion during water crystallisation (Diener et al., 1993). The start temperature was 8°C, and the end temperature was −140°C over 60-minute freezing. Frozen cryovials were immediately transferred on dry ice to the −140°C cell storage tank.

Cryopreserved hepatocytes were defrosted using a modified protocol as previously reported by Steinberg et al. (1999). Frozen hepatocyte suspension (5 mL per vial) was briefly and gently thawed
in a 37°C water bath, and immediately transferred into a 250-mL Falcon® polypropylene conical tube (BD Biosciences) containing 50-mL ice-cold thawing solution at a slow rate using the aseptic technique. The thawing solution consisted of 1 part of 20% human serum albumin (Baxter Healthcare Ltd., Compton, UK) and 9 parts of EMEM. Hepatocyte suspension was pelleted at 50×g and at 4°C for 5 min and re-washed in 20-mL ice-cold 2% HSA/EMEM solution. The total number and viability of fresh hepatocytes were determined using a hematocytometer and the trypan blue exclusion technique with a standard upright light microscope. Batches of hepatocytes with a viability of over 60% on trypan blue exclusion was used for experiments.

3.2.5 Hepatocyte co-culture protocols

3.2.5.1 Hepatocyte co-culture with liver NPCs

Floating hepatocytes were eliminated from the supernatant cell culture by refreshing the cell culture media 4 h after plating. Hepatocyte mono-culture and co-culture with liver NPCs at a ratio of 10:1 were established as described in Section 2.4, Hepatocyte Mono-culture and Co-culture. The seeding density of hepatocytes was fixed at 50,000 viable cells per cm². Hepatocyte mono-culture was used as control, and NPCs mono-culture was used as a blank control.

3.2.5.2 Optimisation of MSCs source

Fresh non-steatotic hepatocytes were co-cultured with P6–8 human AT-, BM-, and UC-MSCs, as well as P4–6 human ADFs, as described in Section 2.4, Hepatocyte Mono-culture and Co-culture, to determine the optimal MSCs source for hepatocyte co-culture. The seeding density of MSCs and ADFs was 20,000 viable cells per cm², and that of hepatocytes was 50,000 viable cells per cm², at a MSC/ADF:hepatocyte ratio of 1:2.5. Hepatocyte mono-culture was used as control, hepatocyte co-culture with human ADFs was used as random control, and MSCs mono-culture was used as a blank control.

3.2.5.3 Optimisation of MSC:hepatocyte ratio

P6–8 optimal source-derived MSCs were co-cultured with fresh non-steatotic hepatocytes as described in Section 2.4, Hepatocyte Mono-culture and Co-culture, to determine the optimal MSCs:hepatocytes ratio for hepatocyte co-culture. The seeding density of hepatocytes was fixed at 50,000 viable cells per cm², and those of the optimal source-derived MSCs were predetermined to be 50,000, 20,000, 10,000, and 5,000 viable cells per cm², at a ratio of 1:1, 1:2.5, 1:5, and 1:10, respectively. Hepatocyte mono-culture was used as control, and MSCs mono-culture was used as blank control.
3.2.5.4 Co-culture of cryopreserved/steatotic hepatocytes with MSCs

P6–8 optimal source-derived MSCs were co-cultured with cryopreserved non-steatotic, as well as fresh non-, mildly, moderately, and severely steatotic hepatocytes as described in Section 2.4, Hepatocyte Mono-culture and Co-culture. The seeding density of hepatocytes was fixed 150,000 viable cells per cm², with that of the optimal source-derived MSCs at the optimal MSC:hepatocyte ratio. Hepatocyte mono-culture was used as control, and MSCs mono-culture was used as blank control.

3.2.5.5 Indirect co-culture of hepatocytes with MSCs

Indirect co-culture using Transwell plates
Indirect co-culture of hepatocytes with the optimal source-derived MSCs using Transwell® Permeable Supports (Corning Incorporated, Corning, NY, USA) was performed to investigate the contribution of MSCs-derived soluble factors to the MSCs co-culture hepatotrophic effect. Hepatocytes were separated from MSCs in the Transwell® co-culture system to exclude possible effects of ECM and direct MSC-to-hepatocyte contact on contribution of soluble factors. The optimal source-derived MSCs were plated onto Transwell® permeable culture inserts at a density equaling to that at the optimal MSC:hepatocyte ratio, and pre-cultured with hepatocyte culture media for 24 hours. Fresh hepatocytes were mono-cultured as described in Section 2.4, Hepatocyte Mono-culture and Co-culture, and MSCs-plated culture inserts were transferred into hepatocyte mono-culture using aseptic techniques with hepatocyte mono-culture alone as control.

MSCs co-culture CM
Soluble trophic factors released from MSCs in co-culture are likely to be regulated by ECM and direct MSC-to-hepatocyte contact. Indirect co-culture of hepatocytes with the optimal source-derived MSCs co-culture CM was performed to investigate whether hepatotrophic effect of MSCs co-culture was mediated by paracrine mechanisms. The optimal source-derived MSCs were co-cultured with fresh hepatocytes for 24 h at the optimal MSC:hepatocyte ratio as described in Section 2.4, Hepatocyte Mono-culture and Co-culture. Fresh MSCs co-culture CM was collected to feed mono-cultured hepatocytes, with hepatocytes mono-cultured with fresh hepatocyte culture media as control. The reading of background MSCs co-culture CM (blank control) was subtracted from that of hepatocytes cultured with CM to obtain the actual reading of hepatocytes.

The culture media were refreshed on days 1, 3, 5, and 7, respectively. The cell morphology was examined using a standard inverted light microscope equipped with a digital SLR camera; hepatocyte co-culture with AT-MSCs was also examined using a laser capture microdissection system (Leica Microsystems, Wetzlar, Germany). Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and frozen at −80°C for further experiments. Cell cultures were rinsed with one-wash PBS at room temperature for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.
3.2.6 **Staurosporine cytotoxicity assay**

Staurosporine cytotoxicity assay was performed to investigate whether MSCs co-culture specifically inhibited hepatocyte apoptosis by targeting at caspase signalling pathway. Staurosporine is a prototypical ATP-competitive protein kinase inhibitor that is mainly used to induce cell apoptosis in biological experiments and reported to activate caspase-3 signalling (Barrachina et al., 2002). Fresh non-steatotic hepatocyte mono-cultures were treated with 0- (blank control), 0.5-, 1-, 2.5-, 5-, and 10-μM staurosporine (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cell culture supernatants were collected for CCK18 and CK18 assays as described in Section 2.9, **Caspase-cleaved CK18 Assay** and Section 2.10, **CK18 Assay**. Fresh hepatocytes were co-cultured with the optimal source-derived MSCs at the optimal ratio in the presence of staurosporine at the least concentration that induced significant increases in hepatocyte apoptosis and total death, as controlled by hepatocyte mono-culture in the absence of staurosporine. All experiments were performed in duplicate and repeated in triplicate independently.

3.2.7 **General cellular activity and liver-specific metabolic function assays**

MTT assay was performed as described in Section 2.5, **3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay** to determine hepatocyte mitochondrial dehydrogenase activity. SRB assay was performed as described in Section 2.6, **Sulforhodamine B Colorimetric Assay** to determine overall hepatocyte attachment. Albumin ELISA was performed as described in Section 2.7, **Albumin Enzyme-linked Immunoabsorbent Assay** to determine protein synthesis of hepatocytes. Urea colorimetric assay was performed as described in Section 2.8, **Urea Colorimetry** to determine nitrogen detoxification of hepatocytes. All experiments were performed in duplicate and repeated in triplicate independently.

3.2.8 **Hepatocyte apoptosis and total death assays**

CCK18 assay was performed as described in Section 2.9, **Caspase-cleaved CK18 Assay** to determine caspase-mediated hepatocyte apoptosis. CK18 assay was performed as described in Section 2.10, **CK18 Assay** to determine total hepatocyte death. The ratio of CCK18 to CK18 was also produced to determine the death mode of hepatocytes in vitro; a ratio of over 0.40 indicated that hepatocytes underwent apoptosis mainly, and necrosis vice versa. All experiments were performed in duplicate and repeated in triplicate independently.
3.3 Results

3.3.1 Morphology of mono-cultured human liver NPCs, MSCs, and ADFs

3.3.1.1 Human liver NPCs

Human liver NPCs showed a viability of 60–80% on trypan blue exclusion at the time of primary harvest. This mixed cell population could adhere to and grow on non-tissue culture treated plate surfaces. These cells were variable in cell size (10–40 μm), but uniformly spindle-shaped in contrast to polygonal- or round-shaped hepatocytes (Figure 3.1A). From day 7 onwards, these cells detached from the plate and could not be passaged.

3.3.1.2 Human AT-, BM-, and UC-MSCs

All three sources of MSCs showed a similar spindle-shaped, fibroblast-like morphology (20–40 μm) with double or multiple small projections after being seeded on non-tissue culture treated plate surface and reached 90% of confluency within 7 days of culture (Figure 3.1B–D). On confluency MSCs were polarised, with a small cell body and two slim projections in a homogenous manner. MSCs remained proliferative and attached from passages 6 to 8. Cell yield was approximately 1–2×10^6 cells per T75 culture flask, equal to a multiplication rate of 200–400%. The viability on trypan blue exclusion was over 99.5% for each passage and remained above 99% after freezing and thawing. UC-MSCs appeared to have a relatively rapid proliferation, while AT-MSCs exhibited a relatively large cell body on low confluence.

3.3.1.3 Human ADFs

Human ADFs also showed a spindle-shaped morphology (10–20 μm) with two slim projections after being seeded on non-tissue culture treated plate surface and reached 90% of confluency within 7 days of culture. On confluency ADFs were polarised, with a small cell body and two relatively shorter projections, as compared to MSCs, in a homogenous manner (Figure 3.1E). ADFs remained proliferative and attached from passages 4 to 6. Cell yield was approximately 0.5×10^6 cells per T25 culture flask, equal to a multiplication rate of 400%. The viability on trypan blue exclusion was over 90% for each passage and remained above 90% after freezing and thawing.

3.3.2 Morphology of hepatocytes in mono-, direct co-, and indirect co-cultures

3.3.2.1 Hepatocytes in mono-culture

Primary human hepatocytes attached to collagen-coated culture vessel surfaces within 4–6 hours, while a large number of hepatocytes remained unattached at the time of culture media replacement. Adherent hepatocytes at a low seeding density exhibited a round-shaped morphology at a size of below 10 μm. Primary hepatocytes aggregated into a few small-sized colonies containing 3–5 cells (Figure 3.2A); however, these colonies did not proliferate and could not be passaged. The
Figure 3.1  Morphology of human liver-derived NPCs (A), AT-MSCs (B), BM-MSCs (C), UC-MSCs (D), and ADFs (E) on inverted light microscopy (200×): (A) liver-derived NPCs were variable in cell size (10–40 μm) but uniformly spindle-shaped; (B–D) AT-, BM-, and UC-MSCs were homogenous and polarised, with a small cell body and two slim projections; and (E) ADFs were homogenous and polarised, with a small cell body and two relatively shorter projections.

viability of primary hepatocytes on trypan blue exclusion continued declining and neared zero on day 7 of in vitro culture.

3.3.2.2  Hepatocytes in co-culture with liver NPCs

Freshly isolated human hepatocytes attached to the liver NPCs monolayer within 4–6 hours, and a relatively small number of hepatocytes were detached from culture vessel surface at the time of culture media replacement. Adherent hepatocytes exhibited a morphology similar to those in mono-culture, while a greater number of hepatocytes aggregated and attached closely to liver NPCs over 7 days of co-culture (Figure 3.2B). The viability of primary hepatocytes co-cultured with autologous liver NPCs continued to decline but remained approximately 25% on day 7.
Figure 3.2  Morphology of human primary hepatocyte mono-culture (A), hepatocyte co-culture with liver-derived NPCs (B), hepatocyte co-culture with AT-MSCs (C), and hepatocytes indirectly co-cultured with AT-MSCs using Transwell culture system (D) on inverted light microscopy (200×) and hepatocyte co-culture with AT-MSCs on a laser capture microdissection system (E, 200×; F, 400×): (A) primary hepatocytes occasionally aggregated into a few small-sized colonies containing 3–5 cells; (B) a greater number of hepatocytes aggregated and attached closely to liver NPCs; (C) far more hepatocytes aggregated massively into larger, oval-shaped colonies and attached closely to MSCs; (D) a relatively greater number of attached hepatocytes aggregated into scattered, small-sized colonies; and (E,F) laser capture microscopy showed three-dimensional aggregation of hepatocytes (arrows) on top of MSCs monolayer.

3.3.2.3 Hepatocyte in direct co-culture with MSCs

The great majority of freshly isolated human hepatocytes attached to the MSCs monolayer within 2–4 hours, and only a few hepatocytes were detached from the culture vessel surface at the time of culture media replacement. Adherent hepatocytes exhibited a morphology similar to those in co-culture with liver NPCs, but far more hepatocytes aggregated into larger, oval-shaped colonies and attached close to MSCs over 7 days of co-culture (Figure 3.2C). Laser capture microscopy also
showed that aggregated hepatocytes were attached on top of MSCs monolayer in a three-dimensional manner (Figure 3.2E and F).

3.3.2.4 Hepatocytes in indirect co-culture with MSCs

Primary hepatocytes cultured with MSCs co-culture CM or indirectly co-cultured with MSCs on Transwell plates exhibited a morphology generally similar to those in mono-culture. Hepatocytes became adherent to collagen-coated culture vessel surfaces within 4–6 hours, and a large number of hepatocytes were detached at the time of culture media replacement. Adherent hepatocytes exhibited a morphology similar to those in mono-culture, and a relatively greater number of attached hepatocytes appeared to be aggregated into scattered, small-sized colonies (Figure 3.2D). The viability of primary hepatocytes on trypan blue exclusion also continued declining and neared zero on day 7 of in vitro culture.

3.3.3 Hepatotrophic effect of NPCs co-culture

3.3.3.1 Viability

Liver NPCs co-culture improved hepatocyte viability as compared to hepatocyte mono-culture throughout 7 days of culture (Figure 3.3A). On day 1, hepatocyte viability had significantly declined in both co- and mono-cultures as compared to the baseline (approximately 62%); however, the viability of hepatocytes co-cultured with liver NPCs was significantly higher than that of mono-cultured hepatocytes (co-culture vs. mono-culture: day 1, 40.6% ± 6.4% vs. 27.8% ± 3.1%; \( P < 0.01 \)). The viability of hepatocytes co-cultured with liver NPCs decreased at a relatively slower rate as compared to that of mono-cultured hepatocytes until day 7 (24.3% ± 4.2% vs. 1.5% ± 0.2%; \( P < 0.01 \)).

3.3.3.2 Mitochondrial dehydrogenase activity

Liver NPCs co-culture exhibited a significant hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to hepatocyte mono-culture throughout 7 days of culture (Figure 3.3B). Hepatocytes co-cultured with liver NPCs had a significantly higher MTT activity than control mono-culture from day 1 (2.35 ± 0.20 vs. 1.40 ± 0.10 OD units; \( P < 0.01 \)) until day 7 (1.53 ± 0.12 vs. 0.59 ± 0.10 OD units; \( P < 0.01 \)).

3.3.3.3 Cell attachment

Liver NPCs co-culture exhibited a significant hepatotrophic effect, with respect to hepatocyte attachment, as compared to control (Figure 3.3C). Hepatocytes co-cultured with liver NPCs had a significantly higher SRB cell attachment than control mono-culture from day 1 (2.83 ± 0.30 vs. 2.40 ± 0.22 OD units; \( P < 0.05 \)) until day 5 (2.39 ± 0.36 vs. 1.99 ± 0.25 OD units; \( P < 0.05 \)), with a similar cell attachment on day 7.
Figure 3.3  Viability (A), MTT activity (B), SRB cell attachment (C), albumin secretion (D), and urea synthesis (E) of hepatocytes co-cultured with liver NPCs versus hepatocyte mono-culture. Co-culture with liver NPCs showed significant hepatotrophic effect within 7 days of culture. All data were expressed as mean ± SD; *P <0.05 and **P <0.01 (n = 6).

3.3.3.4 Albumin secretion

No albumin secretion was detected in liver NPCs mono-culture. Liver NPCs co-culture exhibited a significant hepatotrophic effect, with respect to albumin secretion, as compared to control (Figure 3.3D). Albumin secretion remained significantly higher in liver NPCs co-culture than that in control mono-culture from day 3 (3.1 ± 0.6 vs. 1.1 ± 0.4 μg/10⁶ hepatocytes; P <0.01) until day 7 (4.2 ± 0.7 vs. 1.4 ± 0.2 μg/10⁶ hepatocytes; P <0.01).
3.3.3.5 Urea synthesis

Liver NPCs co-culture also exhibited a significant hepatotrophic effect, with respect to urea synthesis, as compared to control (Figure 3.3E). Urea synthesis remained constantly higher in liver NPCs co-culture than that in control mono-culture from day 3 (27.2 ± 6.8 vs. 17.2 ± 3.0 μg/10⁶ hepatocytes; P <0.01) until day 7 (37.6 ± 8.0 vs. 17.8 ± 2.6 μg/10⁶ hepatocytes; P <0.05).

3.3.4 Optimisation of MSCs/hepatocyte co-culture

3.3.4.1 Optimal source of MSCs for hepatocyte co-culture

**Proliferation of MSCs**

Cellular proliferation was similar among AT-, BM-, and UC-MSCs over 7 days of culture (Table 3.2). Mitochondrial dehydrogenase activity remained similar among three sources of MSCs from day 1 until day 7. SRB cell attachment also remained similar among these three sources of MSCs from day 1 until day 7.

| Table 3.2 MTT and SRB activities (mean ± SD) of MSCs over 7 days of culture |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | MTT (OD unit)   |                | MTT (OD unit)   |                | MTT (OD unit)   |                | MTT (OD unit)   |
| Day 1          | Day 3          | Day 5          | Day 7          | Day 1          | Day 3          | Day 5          | Day 7          |
| AT-            | 1.26 ± 0.06    | 1.55 ± 0.12    | 1.92 ± 0.20    | 2.30 ± 0.24    | 1.22 ± 0.12    | 1.60 ± 0.23    | 2.14 ± 0.26    | 2.43 ± 0.23    |
| BM-            | 1.12 ± 0.05    | 1.52 ± 0.10    | 2.02 ± 0.21    | 2.23 ± 0.21    | 1.28 ± 0.15    | 1.79 ± 0.19    | 2.20 ± 0.23    | 2.60 ± 0.34    |
| UC-            | 1.14 ± 0.04    | 1.56 ± 0.12    | 1.68 ± 0.22    | 2.22 ± 0.19    | 1.18 ± 0.13    | 2.06 ± 0.19    | 2.16 ± 0.25    | 2.40 ± 0.27    |

**Mitochondrial dehydrogenase activity**

ADFs co-culture showed a significant hepatotrophic effect as compared to control mono-culture; MSCs co-culture also exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to mitochondrial dehydrogenase activity, as compared to ADFs co-culture and control mono-culture (Figure 3.4A). Hepatocytes co-cultured with MSCs had a significantly higher MTT activity than those co-cultured with ADFs from day 3 (AT vs. BM vs. UC vs. ADFs vs. control, 1.62 ± 0.12 vs. 1.65 ± 0.13 vs. 1.69 ± 0.09 vs. 1.44 ± 0.07 vs. 1.23 ± 0.12 OD units; P <0.05) and control mono-culture from day 1 (1.65 ± 0.15 vs. 1.67 ± 0.16 vs. 1.58 ± 0.14 vs. 1.61 ± 0.17 vs. 1.39 ± 0.04 OD units; P <0.05) until day 7 (2.59 ± 0.25 vs. 2.49 ± 0.24 vs. 2.38 ± 0.20 vs. 1.69 ± 0.16 vs. 1.25 ± 0.15 OD units; P <0.01), respectively.

**Cell attachment**

ADFs co-culture showed a limited hepatotrophic effect as compared to control mono-culture; MSCs co-culture exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to cell attachment as compared to ADFs co-culture and control mono-culture (Figure 3.4B). Hepatocytes co-cultured with MSCs had significantly higher SRB activity than those co-cultured with ADFs and control mono-culture from day 1 (3.07 ± 0.15 vs. 3.29 ± 0.16 vs. 3.24 ± 0.14 vs. 2.91 ± 0.17 vs. 2.75 ± 0.14 OD units; P <0.05) until day (3.58 ± 0.42 vs. 3.70 ± 0.30 vs. 3.63 ± 0.25 vs. 3.17 ± 0.26 vs. 3.00 ± 0.26 OD units; P <0.01).

**Albumin secretion**

No albumin secretion was detected in ADFs and MSCs mono-culture. ADFs co-culture showed a significant hepatotrophic effect as compared to control mono-culture;
MSCs co-cultures also exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to albumin secretion, as compared to ADFs co-culture control mono-culture (Figure 3.4C). Hepatocytes co-cultured with MSCs had significantly higher albumin secretion than those co-cultured with ADFs and control mono-culture from day 3 (2.4 ± 0.3 vs. 2.5 ± 0.3 vs. 2.5 ± 0.3 vs. 2.0 ± 0.2 vs. 0.9 ± 0.2 μg/10^6 hepatocytes; P <0.05) until day 7 (4.6 ± 0.4 vs. 4.6 ± 0.3 vs. 4.3 ± 0.3 vs. 3.6 ± 0.3 vs. 1.4 ± 0.2 μg/10^6 hepatocytes; P <0.01).

**Urea synthesis** ADFs co-culture showed a significant hepatotrophic effect as compared to control mono-culture; MSCs co-cultures exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to urea synthesis, as compared to ADFs co-culture and control mono-culture (Figure 3.4D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than those co-cultured with ADFs and control mono-culture from day 3 (23.4 ± 2.2 vs. 23.7 ± 1.9 vs. 23.0 ± 1.9 vs. 19.4 ± 2.4 vs. 15.8 ± 2.0 μg/10^6 hepatocytes; P <0.05) until day 7 (35.9 ± 3.0 vs. 36.4 ± 2.6 vs. 35.3 ± 2.5 vs. 32.1 ± 2.3 vs. 17.0 ± 2.4 μg/10^6 hepatocytes; P <0.01).

Overall AT-MSCs co-culture exhibited a significant hepatotrophic effect, similar to BM- and UC-MSCs co-cultures, as compared to ADFs co-culture and hepatocyte mono-culture. Due to the practical advantages of AT-MSCs in contrast to BM- and UC-MSCs, AT-MSCs were determined to be the optimal MSCs for further hepatocyte co-culture experiments.
Figure 3.4  MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of hepatocytes co-cultured with AT-, BM-, and UC-MSCs versus hepatocytes co-cultured with ADFs and hepatocyte mono-culture. Co-culture with MSCs showed a significant hepatotrophic effect, similar among the three co-cultures, within 7 days of culture. All data were expressed as mean ± SD; *P < 0.05 and **P < 0.01 versus control mono-culture; ^P < 0.05 and ^^P < 0.01 versus co-culture with ADFs (n = 6).
3.3.4.2 Optimal MSC:hepatocyte co-culture ratio

Mitochondrial dehydrogenase activity  AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Figure 3.5A). Hepatocytes co-cultured with AT-MSCs had significantly higher MTT activity than control mono-culture from day 1 (1:1 vs. 1:2.5 vs. 1:5 vs. 1:10 vs. control, 1.67 ± 0.19 vs. 1.75 ± 0.24 vs. 1.60 ± 0.20 vs. 1.65 ± 0.18 vs. 1.39 ± 0.10 OD units; \( P < 0.05 \)) until day 7 (2.65 ± 0.26 vs. 3.43 ± 0.24 vs. 2.90 ± 0.20 vs. 3.05 ± 0.25 vs. 1.25 ± 0.15 OD units; \( P < 0.01 \)). Of note, the 1:2.5 co-culture tended to have the highest hepatocyte mitochondrial dehydrogenase activity as compared to the other co-cultures on day 7 of culture.

Cell attachment  AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to cell attachment, as compared to control mono-culture (Figure 3.5B). Hepatocytes co-cultured with AT-MSCs had significantly higher SRB activity than control mono-culture from day 1 (3.34 ± 0.20 vs. 3.51 ± 0.19 vs. 3.38 ± 0.22 vs. 3.22 ± 0.15 vs. 2.75 ± 0.10 OD units; \( P < 0.05 \)) until day 7 (4.68 ± 0.40 vs. 5.58 ± 0.43 vs. 5.26 ± 0.50 vs. 4.97 ± 0.37 vs. 3.00 ± 0.30 OD units; \( P < 0.01 \)). Of note, all co-cultures had similar hepatocyte SRB attachment throughout 7 days of culture.

Albumin secretion  AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to albumin secretion, as compared to control mono-culture (Figure 3.5C). Hepatocytes co-cultured with AT-MSCs had significantly higher albumin secretion than control mono-culture from day 3 (2.3 ± 0.3 vs. 2.5 ± 0.3 vs. 2.3 ± 0.3 vs. 2.3 ± 0.2 vs. 0.9 ± 0.2 μg/10^6 hepatocytes; \( P < 0.01 \)) until day 7 (4.2 ± 0.4 vs. 4.7 ± 0.4 vs. 4.6 ± 0.4 vs. 4.3 ± 0.3 vs. 1.4 ± 0.2 μg/10^6 hepatocytes; \( P < 0.01 \)). Of note, all co-cultures had similar albumin secretion throughout 7 days of culture.

Urea synthesis  AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to urea synthesis, as compared to control mono-culture (Figure 3.5D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than control mono-culture from day 1 (21.6 ± 2.0 vs. 22.6 ± 2.3 vs. 21.8 ± 2.4 vs. 21.8 ± 1.6 vs. 18.0 ± 2.6 μg/10^6 hepatocytes; \( P < 0.05 \)) until day 7 (35.7 ± 3.0 vs. 37.9 ± 3.0 vs. 34.2 ± 2.9 vs. 36.4 ± 2.5 vs. 17.0 ± 2.4 μg/10^6 hepatocytes; \( P < 0.01 \)). Of note, all co-cultures had similar albumin secretion throughout 7 days of culture.

Overall AT-MSCs co-culture at the MSC:hepatocyte ratio of 1:2.5 exhibited a significant hepatotrophic effect, as compared to hepatocyte mono-culture, similar to co-cultures at the ratio of 1:1, 1:5, and 1:10. As AT-MSCs reached a 100% confluency at a density of approximately 20,000 cells per cm², the ratio of 1:2.5 was used in further experiments to exclude the possibility of MSCs expansion over 7 days of co-culture.
Figure 3.5  MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of hepatocytes co-cultured with AT-MSCs at a predefined MSC:hepatocyte ratio of 1:1, 1:2.5, 1:5, and 1:10 versus hepatocyte mono-culture. Co-culture with MSCs at all predefined ratios exhibited significant hepatotrophic effect, to a similar extent among co-cultures. All data were expressed as mean ± SD; *$P<0.05$ and **$P<0.01$ versus control mono-culture ($n = 6$).
3.3.5 Hepatotrophic effect of MSCs co-culture on cryopreserved and steatotic hepatocytes

3.3.5.1 Hepatotrophic effect of AT-MSCs co-culture on steatotic hepatocytes

**Mitochondrial dehydrogenase activity**  AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Figure 3.6A). Moderately steatotic hepatocytes co-cultured with MSCs had significantly higher MTT activity than control mono-culture from day 1 (co-culture vs. mono-culture, 1.35 ± 0.20 vs. 0.70 ± 0.10 OD units; *P* <0.01) until day 7 (1.40 ± 0.20 vs. 0.60 ± 0.10 OD units; *P* <0.01).

**Cell attachment**  AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to cell attachment, as compared to control mono-culture (Figure 3.6B). Moderately steatotic hepatocytes co-cultured with MSCs had significantly higher SRB activity than control mono-culture from day 1 (2.25 ± 0.30 vs. 1.80 ± 0.15 OD units; *P* <0.01) until day 7 (2.40 ± 0.39 vs. 1.83 ± 0.27 OD units; *P* <0.01).

**Albumin secretion**  AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to albumin secretion, as compared to control mono-culture (Figure 3.6C). Hepatocytes co-cultured with MSCs had significantly higher albumin secretion than control mono-culture from day 3 (1.2 ± 0.3 vs. 0.5 ± 0.1 μg/10⁶ hepatocytes; *P* <0.01) from day 7 (2.4 ± 0.2 vs. 0.9 ± 0.1 μg/10⁶ hepatocytes; *P* <0.01).

**Urea synthesis**  AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to urea synthesis, as compared to control mono-culture (Figure 3.6D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than control mono-culture from day 1 (17.6 ± 1.4 vs. 9.0 ± 1.0 μg/10⁶ hepatocytes; *P* <0.01) until day 7 (25.4 ± 2.3 vs. 8.5 ± 0.9 μg/10⁶ hepatocytes; *P* <0.01).

3.3.5.2 Hepatotrophic effect of AT-MSCs co-culture on cryopreserved hepatocytes

**Mitochondrial dehydrogenase activity**  AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Figure 3.7A). Moderately steatotic hepatocytes co-cultured with AT-MSCs had significantly higher MTT activity than control mono-culture from day 1 (co-culture vs. mono-culture, 1.12 ± 0.14 vs. 0.68 ± 0.10 OD units; *P* <0.01) until day 7 (1.44 ± 0.21 vs. 0.54 ± 0.11 OD units; *P* <0.01).

**Cell attachment**  AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to cell attachment, as compared to control mono-culture (Figure 3.7B). Moderately steatotic hepatocytes co-cultured with MSCs had significantly higher SRB activity than control mono-culture from day 1 (2.59 ± 0.33 vs. 1.80 ± 0.19 OD units; *P* <0.01) until day 7 (2.75 ± 0.41 vs. 2.24 ± 0.32 OD units; *P* <0.01).
Figure 3.6 MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of moderately steatotic hepatocytes co-cultured with AT-MSCs at a ratio of 2.5:1 versus hepatocyte mono-culture. Co-culture with MSCs had a significant trophic effect on steatotic hepatocytes within 7 days of in vitro culture. All data were expressed as mean ± SD (error bar); *$P < 0.05$ and **$P < 0.01$ versus control mono-culture ($n = 6$).

**Albumin secretion** AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to albumin secretion, as compared to control mono-culture (Figure 3.7C). Hepatocytes co-cultured with AT-MSCs had significantly higher albumin secretion than control mono-culture from day 1 (0.7 ± 0.1 vs. 0.5 ± 0.1 μg/10⁶ hepatocytes; $P < 0.01$) until day 7 (2.1 ± 0.3 vs. 0.9 ± 0.1 μg/10⁶ hepatocytes; $P < 0.01$).

**Urea synthesis** AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to urea synthesis, as compared to control mono-culture (Figure 3.7D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than control mono-culture from day 1 (13.0 ± 1.6 vs. 8.8 ± 1.3 μg/10⁶ hepatocytes; $P < 0.01$) until day 7 (19.9 ± 2.3 vs. 8.4 ± 1.1 μg/10⁶ hepatocytes; $P < 0.01$).
3.3.6 Contribution of soluble factors to hepatotrophic effect of MSCs co-culture

3.3.6.1 Soluble factors alone contribute minimally to hepatotrophic effect of MSCs co-culture

**Mitochondrial dehydrogenase activity**

MSCs indirect co-culture using Transwell plates exhibited no significant hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Table 3.3). Mitochondrial dehydrogenase activity remained similar between indirect co-culture and control mono-culture from day 1.

**Cell attachment**

MSCs indirect co-culture exhibited no significant hepatotrophic effect, with respect to hepatocyte attachment, as compared to control mono-culture (Table 3.3). SRB cell attachment remained similar between indirect co-culture and control mono-culture from day 1 until day 7.
Table 3.3 MTT activity and SRB cell attachment (mean ± SD) of hepatocytes indirectly co-cultured with AT-MSCs on Transwell plates versus mono-cultured hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>MTT (OD unit)</th>
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<th></th>
<th></th>
<th>SRB (OD unit)</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 7</td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 7</td>
</tr>
<tr>
<td>iCo-</td>
<td>1.60 ± 0.16</td>
<td>1.30 ± 0.10</td>
<td>1.25 ± 0.10</td>
<td>1.31± 0.09</td>
<td>2.88 ± 0.20</td>
<td>3.10 ± 0.20</td>
<td>3.01 ± 0.16</td>
<td>3.05 ± 0.21</td>
</tr>
<tr>
<td>Mono-</td>
<td>1.59 ± 0.18</td>
<td>1.25 ± 0.16</td>
<td>1.27 ± 0.18</td>
<td>1.29 ± 0.09</td>
<td>2.80 ± 0.14</td>
<td>2.91 ± 0.17</td>
<td>2.94 ± 0.15</td>
<td>2.99 ± 0.14</td>
</tr>
</tbody>
</table>

iCo-, indirect co-culture; Mono-, mono-culture.

**Albumin secretion**  MSCs indirect co-culture exhibited no significant hepatotrophic effect, with respect to albumin secretion, as compared to control mono-culture (Table 3.4). Albumin secretion was similar between indirect co-culture and control mono-culture from day 1 until day 7.

**Urea synthesis**  MSCs indirect co-culture exhibited no significant hepatotrophic effect, with respect to urea synthesis, as compared to control (Table 3.4). Urea synthesis was similar between indirect co-culture and control mono-culture from day 1 until day 7.

Table 3.4 Albumin secretion and urea synthesis (mean ± SD) of hepatocytes indirectly co-cultured with AT-MSCs on Transwell plates versus mono-cultured hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>Albumin (μg/10⁶ hepatocytes)</th>
<th>Urea (μg/10⁶ hepatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>iCo-</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Mono-</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

iCo-, indirect co-culture; Mono-, mono-culture.

3.3.6.2 **MSCs co-culture CM have minimal hepatotrophic effect**

**Mitochondrial dehydrogenase activity**  MSCs co-culture CM exhibited a limited hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture throughout 7 days of indirect co-culture (Table 3.5). Hepatocytes cultured with MSCs co-culture CM had significantly higher MTT activity than mono-cultured hepatocytes (CM culture vs. mono-culture, 1.58 ± 0.15 vs. 1.39 ± 0.14 OD units; *P* < 0.01) on day 1; however, mitochondrial dehydrogenase activity remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 3 until day 7.

**Cell attachment**  MSCs co-culture CM exhibited no significant hepatotrophic effect, with respect to hepatocyte attachment, as compared to control mono-culture (Table 3.5). SRB cell attachment remained similar between hepatocyte cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7.

Table 3.5 MTT activity and SRB cell attachment (mean ± SD) of hepatocytes cultured with AT-MSCs co-culture CM versus mono-cultured hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>MTT (OD unit)</th>
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<th></th>
<th>SRB (OD unit)</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 7</td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 7</td>
</tr>
<tr>
<td>Cond</td>
<td>1.58 ± 0.15**</td>
<td>1.21 ± 0.19</td>
<td>1.25 ± 0.10</td>
<td>1.29 ± 0.17</td>
<td>2.71 ± 0.26</td>
<td>2.91 ± 0.24</td>
<td>2.93 ± 0.26</td>
<td>2.97 ± 0.24</td>
</tr>
<tr>
<td>Mono-</td>
<td>1.39 ± 0.14</td>
<td>1.23 ± 0.15</td>
<td>1.24 ± 0.16</td>
<td>1.25 ± 0.18</td>
<td>2.75 ± 0.27</td>
<td>2.86 ± 0.23</td>
<td>2.93 ± 0.25</td>
<td>3.00 ± 0.22</td>
</tr>
</tbody>
</table>

Cond, conditioned; Mono-, mono-culture; **P < 0.01 versus mono-culture.
**Albumin secretion**  No albumin secretion was detected in MSCs mono-culture. MSCs co-culture CM exhibited no significant hepatotrophic effect, with respect to albumin secretion, as compared to control mono-culture (Table 3.6). Albumin secretion remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7.

**Urea synthesis**  MSCs co-culture CM exhibited no significant hepatotrophic effect, with respect to urea synthesis, as compared to control mono-culture (Table 3.6). Urea synthesis was similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7.

**Table 3.6** Albumin secretion and urea synthesis (mean ± SD) of hepatocytes cultured with AT-MSCs co-culture CM versus mono-cultured hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>Albumin (µg/10⁶ hepatocytes)</th>
<th>Urea (µg/10⁶ hepatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>Cond</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Mono-</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Cond, conditioned; Mono-, mono-culture.

3.3.7 **Anti-apoptotic effect of MSCs co-culture on hepatocytes**

3.3.7.1 **MSCs co-culture suppresses caspase-mediated spontaneous hepatocyte apoptosis**

**MSCs direct co-culture**  No CCK18 or CK18 release was detected in MSCs mono-culture. Direct co-culture with MSCs significantly reduced CCK18 release from hepatocytes as compared to control mono-culture (Figure 3.8A). Soluble CCK18 level remained significantly lower in hepatocytes co-cultured with MSCs than that in mono-cultured hepatocytes from day 1 (co-culture vs. mono-culture, 18.5 ± 1.4 vs. 23.6 ± 1.5 U/10⁶ hepatocytes; \( P < 0.01 \)) until day 7 (0.3 ± 0.1 vs. 1.6 ± 0.1 U/10⁶ hepatocytes; \( P < 0.01 \)). Direct co-culture with MSCs also significantly reduced CK18 release from hepatocytes, as compared to hepatocyte mono-culture (Figure 3.8B), from day 1 (91.2 ± 8.2 vs. 123.6 ± 16.3 U/10⁶ hepatocytes; \( P < 0.01 \)) until day 7 (20.0 ± 1.9 vs. 52.0 ± 5.0 U/10⁶ hepatocytes; \( P < 0.01 \)). As is shown in Figure 3.8C, CCK18/CK18 ratio (cell death mode; Figure 3.8C) remained similar between co-cultured hepatocytes and mono-cultured hepatocytes from day 1 (20.2% ± 3.0% vs. 19.1% ± 2.6%; \( P > 0.05 \)) until day 7 (1.5% ± 0.1% vs. 3.0% ± 0.4%; \( P > 0.05 \)).

**MSCs indirect co-culture using Transwell plates**  Indirect co-culture with MSCs had no significant effect on hepatocyte CCK18 or CK18 release as compared to control mono-culture (Table 3.7). Soluble CCK18 level remained similar between hepatocytes indirectly co-cultured with MSCs and mono-cultured hepatocytes from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes indirectly co-cultured with MSCs and mono-cultured hepatocytes from day 1
until day 7. CCK18/CK18 ratio remained similar between hepatocytes indirectly co-cultured with 
MSCs and mono-cultured hepatocytes (Table 3.4) from day 1 until day 7.

**MSCs co-culture CM**  MSCs co-culture CM had no significant effect on hepatocyte 
CCK18 or CK18 release from s as compared to control mono-culture (Table 3.8). Soluble CCK18 
level remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured 
hepatocytes from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes 
cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7. 
CCK18/CK18 ratio remained similar between hepatocytes cultured with MSCs co-culture CM and 
hepatocyte mono-culture (Table 3.7) from day 1 until day 7.
Figure 3.8  CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes cultured with AT-MSCs versus mono-cultured hepatocytes. MSCs co-culture significantly reduced caspase-mediated apoptosis and total death of hepatocytes without altering cell death mode within 7 days of in vitro culture. All data were expressed as mean ± SD; **P < 0.01 versus control mono-culture (n = 6).
Table 3.7  CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes indirectly co-cultured with MSCs versus mono-cultured hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>CCK18 (U/10^6 hepatocytes)</th>
<th>CK18 (U/10^6 hepatocytes)</th>
<th>CCK18/CK18 ratio (%)</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>iCo-</td>
<td>20.8 ± 2.4</td>
<td>23.2 ± 2.3</td>
<td>15.9 ± 1.7</td>
</tr>
<tr>
<td>Mono-</td>
<td>23.6 ± 2.5</td>
<td>21.0 ± 2.5</td>
<td>15.7 ± 1.5</td>
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</tbody>
</table>

iCo-, indirect co-culture; Mono-, mono-culture.

Table 3.8  CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes cultured with MSCs co-culture CM versus mono-cultured hepatocytes

<table>
<thead>
<tr>
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<th>CCK18 (U/10^6 hepatocytes)</th>
<th>CK18 (U/10^6 hepatocytes)</th>
<th>CCK18/CK18 ratio (%)</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>iCo-</td>
<td>20.3 ± 2.1</td>
<td>22.5 ± 2.0</td>
<td>16.3 ± 1.8</td>
</tr>
<tr>
<td>Mono-</td>
<td>23.8 ± 2.6</td>
<td>21.0 ± 2.4</td>
<td>15.9 ± 2.0</td>
</tr>
</tbody>
</table>

Cond, conditioned; Mono-, mono-culture.
3.3.7.2 MSCs co-culture protects hepatocytes from staurosporine-induced cell apoptosis

**Effect of staurosporine on hepatocytes**

Addition of 0.5-μM staurosporine had no significant effect on CCK18 release from hepatocytes as compared to blank control; however, the presence of 1-, 2.5-, 5-, and 10-μM staurosporine significantly increased soluble CCK18 level as compared to that of 0.5-μM staurosporine (0.5 μM vs. 1 μM vs. 2.5 μM vs. 5 μM vs. 10 μM, 24.7 ± 2.9 vs. 44.7 ± 3.6 vs. 51.2 ± 5.0 vs. 56.1 ± 5.5 vs. 70.2 ± 5.7 U/10^6 hepatocytes; *P* <0.01; Figure 3.9A) in a dose-dependent manner. Similarly, addition of 0.5-μM staurosporine had no significant effect on CK18 release from hepatocytes as compared to that of 0-μM staurosporine; however, in the presence of 1-, 2.5-, 5-, and 10-μM staurosporine significantly increased soluble CK18 level as compared to that at 0.5-μM staurosporine (136.1 ± 14.8 vs. 156.9 ± 18.9 vs.176.0 ± 20.4 vs. 187.3 ± 25.0 vs. 204.8 ± 17.9 U/10^6 hepatocytes, *P* <0.01; Figure 3.9B) in a dose-dependent manner. Moreover, addition of 1-, 2.5-, 5-, and 10-μM staurosporine switched cell death mode of hepatocytes from necrosis (lower CCK18/CK18 ratio) to apoptosis (higher CCK18/CK18 ratio) as shown in Figure 3.9C.

**Effect of MSCs co-culture on staurosporine-induced hepatocyte apoptosis**

Hepatocytes co-cultured with or without MSCs were subjected to 1-μM staurosporine to investigate whether MSCs co-culture could protect hepatocytes from chemically-induced apoptosis. As is shown in Figure 3.10A, co-culture with MSCs significantly reduced staurosporine-induced hepatocyte apoptosis (soluble CCK18 level) as compared to hepatocyte mono-culture: co-culture vs. mono-culture vs. blank, 26.5 ± 2.5 vs. 44.7 ± 6.3 vs. 21.2 ± 2.1 U/10^6 hepatocytes (*P* <0.01). However, MSCs co-culture had no significant effect on staurosporine-induced total cell death (soluble CK18 level) of hepatocytes (Figure 3.10B): 148.0 ± 10.7 vs. 156.9 ± 18.9 vs. 111.3 ± 17.0 U/10^6 hepatocytes (*P* <0.01). Moreover, MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis switch of hepatocyte death mode (Figure 3.10C): CCK18/CK18 ratio, 18.0% ± 2.2% vs. 28.6% ± 2.4% vs. 18.9% ± 2.6% (*P* <0.05).
Figure 3.9 CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes subjected to 0-, 0.5-, 1-, 2.5-, 5-, 10-μM staurosporine. Addition of 0.5-μM staurosporine had no significant effect on apoptosis, total death, and death mode of hepatocytes, while addition of 1-, 2.5-, 5-, and 10-μM staurosporine significantly increased apoptosis and total death of hepatocytes in a dose-dependent manner and switched cell death mode of hepatocytes from necrosis to apoptosis. All data were expressed as mean ± SD; **P < 0.01 versus control mono-culture (n = 6).
Figure 3.10  CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes subjected to 1-µM staurosporine and co-cultured with or without AT-MSCs versus hepatocyte mono-culture treated without staurosporine. Addition of staurosporine significantly increased caspase-mediated apoptosis and total death of hepatocytes and switched death mode of hepatocytes from necrosis to apoptosis, while MSCs co-culture significantly reduced staurosporine-induced hepatocyte apoptosis and reversed death mode switch. All data were expressed as mean ± SD; **P <0.01 versus non-staurosporine-treated, mono-cultured hepatocytes; ^~P <0.01 versus staurosporine-treated, mono-cultured hepatocytes (n = 6). Co, hepatocyte co-culture; mono, hepatocyte mono-culture; SS, staurosporine.
3.4 Discussion

3.4.1 Potentials of liver NPCs in HCT

Multiple techniques have been reported to separate liver NPCs from parenchymal cells (hepatocytes), among which selective enzymatic digestion and unit gravity sedimentation is most frequently used, due to the fact that NPCs are in a relatively small size. Doolittle et al. (1987) identified and characterised an unfractioned population of liver NPCs by flow cytometry and reaffirmed that cell size was linearly correlated to the sedimentation velocity. Primary liver NPCs were successfully isolated in the present work using the modified protocol as previously reported (Najimi et al., 2007); the purity of liver NPCs was over 90% as determined on cell morphology following the first culture medium refreshment.

NPCs occupy only 6.5% of the total volume of the liver but account for approximately 40% of the total number of liver cells. Liver NPCs themselves are highly heterogeneous, in terms of cell morphology, functionality, and topography, consisting of sinusoidal hepatic endothelial cells, Kupffer cells, and hepatic stellate cells lining the liver sinusoid, as well as “pit cells”, namely, liver residing lymphocytes. Liver NPCs play an active role in regulating biological activities of hepatocytes in vivo, including hepatocyte survival, proliferation, maintenance, and metabolism. In addition, liver NPCs are also involved in inflammatory and immune responses of the liver in the setting of sepsis (Scott et al., 2005), malignancy, autoimmune disorder, and immune rejection (Chen et al., 2002).

Liver NPCs show a significant trophic effect on hepatocytes co-cultured in vitro. Sinusoidal endothelial cells and Kupffer cells regulate hepatocyte secretion of albumin and alpha 1-acid glycoprotein by monokines (especially IL-6), dexamethasone, and inducible nitric oxide synthase pathway (Itoh et al., 1994). In a study regarding bioartificial liver system, co-cultivation of hepatocytes with NPCs significantly multiplied amino acid conversion, lactate production, nitrogen metabolism, drug detoxification, and clearance of aggregated gamma-globulin as compared to hepatocytes alone (Nedredal et al., 2007). The present work showed that co-culture of hepatocytes with autologous liver NPCs even at a very low NPC:hepatocyte ratio (1:10) was still beneficial with respect to overall cellular viability, attachment, and liver-specific metabolic function. It suggests a possibility that co-transplantation of autologous liver NPCs can improve current HCT practice. An additional benefit of co-transplanting NPCs is to protect parenchymal cells from immune elimination. Yang et al. (2009) reported that hepatic stellate cells had a profound inhibitory effect on T-cells and effectively prevented islet allografts from acute rejection in mice. These comprehensive T-cell inhibitory activities included induction of apoptosis of graft-infiltrating antigen-specific effector T-cells and marked expansion of CD4(+) Forkhead box protein 3(+) T-regulatory cells by interferon-gamma signalling (Yang et al., 2009).

However, the use of co-culture of hepatocytes with liver NPCs is primarily limited by the high intrinsic heterogeneity (Pan et al., 1996) and possible fibrogenic potential of NPCs in the presence of
profibrotic factors (Perepelyuk et al., 2013). These limitations result in unfavourable reproducibility and safety profile of NPCs co-culture. The present work showed that expansion potential of primary liver NPCs isolated from adult donors was very limited as the great majority of this heterogeneous cell population is terminally differentiated. Moreover, cellular components and constituents remained unevaluated among previous studies regarding co-culture of hepatocytes with liver NPCs. A study regarding fractioning and characterising liver NPCs by immunophenotyping is ongoing in our research group.

3.4.2 Hepatotrophic effect of MSCs co-culture on human hepatocytes

3.4.2.1 Optimisation of cellular source and ratio for MSCs co-culture

In vitro hepatotrophic effect of MSCs co-culture has been well documented in current literature. Gu and his colleagues (2009a; 2009b; 2009c) isolated BM-MSCs from pigs and preserved the morphology and liver-specific metabolic function of porcine hepatocytes in a three-dimensional co-culture system. Fitzpatrick et al. (Cell Transplant, Epub ahead of print) also co-cultured human hepatocytes with human UC matrix-derived MSCs and successfully improved viability and functionality of co-cultured hepatocytes. Whether AT-MSCs has comparable trophic effect on co-cultured hepatocytes remains occasionally studied as AT-MSCs are a new family member of MSCs. No da et al. (2012) co-cultured human hepatocytes isolated from partial hepatectomy specimen and human AT-MSCs at a ratio of 1:1 to generate hepatocyte spheroids and improved albumin secretion and CYP450 activity of co-cultured hepatocytes over one week. Chen et al. (2012) isolated AT-MSCs from human orbital fat and co-cultured these MSCs with rat hepatocytes; this co-culture protected rat hepatocytes from ALF serum by upregulating immunomodulatory IL-6. Saito et al. (2013) reported that AT-MSCs transplantation attenuated ischaemia/reperfusion-induced liver injury and promoted liver regeneration as early as 6 hours after reperfusion by upregulating VEGF expression. The present work demonstrated for the first time that human AT-MSCs had a significant trophic effect on co-cultured human hepatocytes, to an extent similar to BM- and UC-MSCs. As AT-MSCs are more readily available and subject to less ethical controversies, AT-MSCs are determined to be the optimal MSCs for hepatocyte co-culture.

The present work also showed that human MSCs co-culture improved hepatocyte cellular activity and liver-specific metabolic function to a greater extent than human ADFs co-culture. NIH/3T3 cells, a standardised fibroblast cell line, are frequently used as the “feeder cells” for co-cultured hepatocytes in bioartificial liver and in vitro drug hepatotoxicity screening systems (Cho et al., 2007; Chan et al., 2013). Devitalised fibroblasts and fibroblast CM alone were reported to be minimally hepatotrophic in vitro (Bhandari et al., 2001). Hepatotrophic effect of fibroblast co-culture is believed to synergistically result from homotypic hepatocyte-to-hepatocyte interaction, heterotypic hepatocyte-to-fibroblast communication, hepatocyte-to-ECM contact, and soluble factors. Therefore, greater hepatotrophic effect of MSCs co-culture may be derived from potentiated synergistic effect of these factors rather than as adherent matrix or source of paracrine factors alone.
Theoretically a small number of MSCs should exhibit a significant trophic effect on co-cultured hepatocytes in vitro as liver NPCs occupy only a small portion of liver cells. Gu et al. (2009c) randomly co-cultured hepatocytes and BM-MSCs and predefined MSC:hepatocyte ratio at 1:1, 1:2, 1:5, and 1:10, respectively, with the total cell number fixed at 1 million per well; the optimal liver-specific metabolic function was achieved in the co-culture at the ratio of 1:2, with respect to albumin secretion and urea synthesis, which reached the peak on day 2 of co-culture. Fitzpatrick et al. (Cell Transplant, Epub ahead of print) modified the co-culture protocol of hepatocytes with UC-MSCs (plating of hepatocytes followed by that of MSCs) and predefined MSC:hepatocyte ratio, with the number of hepatocytes fixed at 150,000/cm², at 1:3, 1:6, and 1:10, respectively; UC-MSCs co-culture exhibited a similar hepatotrophic effect, regardless of MSC:hepatocyte ratio, within 7 days of in vitro culture with respect to albumin secretion and urea synthesis. This similarity between low- and high-density UC-MSCs co-culture may be attributed to the fact that UC-MSCs are highly proliferative in vitro and able to reach the confluency within 7 days of culture even starting from a low seeding density. In contrast, the co-culture protocol was further modified in the present work, by seeding hepatocytes onto pre-existing AT-MSCs monolayer in a well-organised, three-dimensional manner, with MSC:hepatocyte ratio predefined, with the number of hepatocytes fixed at 50,000/cm², at 1:1, 1:2.5, 1:5, and 1:10, respectively. The present work showed that even a very low seeding density of AT-MSCs (5,000/cm²) still had trophic effect on co-cultured hepatocytes generally similar to the high-density AT-MSCs (50,000/cm²). This similarity between low- and high-density AT-MSCs may result from the fact that AT-MSCs have a relatively large cell size compared to BM- and UC-MSCs and provide adequate cellular and matrical surface even at a low seeding density. AT-MSCs normally reach 100% confluency at a density of 25,000/cm²; therefore, the optimal MSC:hepatocyte ratio was determined to be 1:2.5, equalling a 20,000/cm² seeding density of MSCs, at which AT-MSCs would become 100% confluent within a very short time, to exclude the possible confounding effect of MSCs proliferation in vitro throughout co-culture.

3.4.2.2 Hepatotrophic effect of MSCs co-culture on steatotic hepatocytes

A seriously steatotic donor liver rejected for the use of OLT will normally also fail isolation of hepatocytes. The paradox arising from availability of donor liver is that only poor-quality, usually steatotic, donor liver tissues will be assigned for HCT use in most cases. Furthermore, isolated steatotic hepatocytes normally exhibit a poor metabolic function and susceptibility to cellular injury in vitro, although a steatohepatitis patient usually exhibit no abnormality in liver gross morphology and metabolic function (Donato et al., 2006; Donato et al., 2007). Compromise of steatotic hepatocytes may be compensated by the huge liver function reserve and a high-level hepatocyte self-renewal and turnover, while steatotic hepatocytes have already shown morphological distortion and molecular dysregulation. Compensatory hepatic progenitor cell expansion accompanies steatotic hepatocyte replicative arrest (Cho et al., 2010), and steatotic liver is thought to be a suitable source for isolation of hepatic progenitor cells (Tolosa et al., 2011).

A comparative genomic profiling analysis in obese insulin-resistant Zucker rats with
spontaneous liver steatosis demonstrated that dysregulated expression of metabolic and survival
genes, including defence/acute phase-, detoxification-, cell growth/proliferation-, and protein
synthesis/transformation-associated genes might lead to the vulnerability of steatotic hepatocytes to
cell injury (Buqué et al., 2010), e.g., hypoxia-reoxygenation injury, which could be reversed by
defatting (Berthiaume et al., 2009) and mitochondrial uncoupling protein-2 blockade (Evans et al.,
2012). Activation of TGF-β signalling and production of ROS are reported to contribute to
hepatocyte death and lipid accumulation in non-alcoholic steatohepatitis (Yang et al., 2013) and also
sensitise hepatocyte susceptibility to acute toxic effect of acetaminophen (Kučera et al., 2012) and
proapoptotic effect of TNF-α (Zhang et al., 2010).

Hayashi et al. (2007) reported an interesting rat steatotic HCT study: steatotic hepatocytes
secreted significantly less albumin in vitro than nonsteatotic counterparts; however, intrasplenically
transplanted steatotic hepatocytes produced a serum albumin level similar to nonsteatotic cells. It is
possible that in vivo factors, such as growth factors and soluble cytokines, improve steatotic
hepatocyte metabolic function by modifying and improving cellular biological and molecular
activities. Sun et al. (2003) showed that IL-6 pretreatment prevented sinusoidal endothelial cell
death in response to ischaemia/reperfusion injury, activated cell survival signal transducer and
activator of transcription factor 3 in both hepatocytes and sinusoidal endothelial cells, and also
improved steatotic liver isograft microcirculation. Hong et al. (2004) further demonstrated that
protective effect of IL-6 treatment on steatotic hepatocytes in vivo resulted from increased
mitochondrial β oxidation of fatty acid and export of triglyceride and cholesterol as mediated by
peroxisome proliferator-activated receptor α and TNF-α signalling pathways. The present work
reported for the first time that MSCs co-culture could also improve cellular activity and liver-
specific metabolic function of steatotic hepatocytes as for nonsteatotic hepatocytes. The effect size
of MSCs co-culture was similar between steatotic hepatocytes and nonsteatotic hepatocytes as
compared to mono-cultured counterparts, with respect to improvement in liver-specific metabolism.
As reported by Gu and his colleagues (2009a), MSCs secreted a massive amount of IL-6 into
hepatocyte co-culture system, and neutralization of IL-6 compromised hepatotrophic effect of MSCs
co-culture with respect to albumin secretion and urea synthesis. The role of other in vivo factors,
such other cytokines, ECM, and heterotypic hepatocyte-to-NPC interaction, in trophic effect of
MSCs co-culture on steatotic hepatocytes remains to be investigated. A possible mechanism is that
MSCs expedite ROS elimination in co-cultured hepatocytes as human BM-MSCs was reported to
inhibit production of ROS in co-cultured macrophages in a dose-dependent manner by a paracrine
mechanism (Tsyb et al., 2008)

3.4.2.3 Hepatotrophic effect of MSCs co-culture on cryopreserved hepatocytes

Cryopreservation was thought to be capable of preserving and maintaining the ultrastructural
characteristics and metabolic, biochemical and toxicological functions of hepatocytes to some extent.
However, cryopreserved hepatocytes are inevitably subject to loss of viability and impairment in
metabolic function following thawing and plating. This freezing/thawing-associated injury results
from the underlying cellular and molecular changes. Rijntjes et al. (1986) reported that formation of more ‘blebs’, lipid droplets, and lysosomes were observed in cryopreserved hepatocytes after thawing, accompanied by increased lactate dehydrogenase release and decreased albumin secretion. This ultrastructural distortion was primarily attributed to the change in cytoplasmic osmolality and subsequent intracellular ice crystal formation. Dou et al. (1992) also recommended that at least a 38-hour recovery time after seeding should be given for thawed human hepatocytes to stabilise cellular membrane integrity, protein synthesis, and drug metabolism enzymes. Interestingly, profound dysregulation of gene expression, involved in liver-specific metabolism, cellular apoptosis, and proteasomal protein recycling, was observed in hepatocytes after plating rather than after thawing (Richert et al., 2006). Oxidative stress manifesting as intracellular accumulation of ROS was thought to partially contribute to this gene expression dysregulation (Stevenson et al., 2007).

A major problem associated with thawing of cryopreserved adult human hepatocytes is poor attachment efficiency and rapid reduction in cellular survival. A lot of work has been done to improve viability and metabolic function of cryopreserved hepatocytes by modifying cryopreservation protocols and cryoprotectants. Birraux et al. (2002) reported that three-dimensional cryopreservation of rat hepatocytes sandwiched between two layers of collagen type I could adequately restore cellular and liver-specific metabolic function of hepatocytes. Lee et al. (2012) also reported that cryopreservation of spheroid hepatocytes was more effective in maintaining hepatocyte viability and liver-specific metabolic function as compared to that of single hepatocytes. These study findings suggested that ECM and cell-to-cell could recover cryopreserved hepatocytes from freezing/thawing injury to some extent. However, it is rarely reported in current literature how to improve culture of cryopreserved hepatocytes after thawing. Moshage et al. (1988) used precoating with homologous ECM to successfully reverse the deleterious effect of freezing/thawing on hepatocyte attachment efficiency and survival in vitro for over 4 weeks. The present work demonstrated for the first time that MSCs co-culture could also improve cellular activity and liver-specific metabolic function of cryopreserved hepatocytes as for fresh hepatocytes with a similar effect size. Gu et al. (2009) observed extensive deposition of ECM, such as fibronectin, laminin, and collagen type I, III, and V, in MSCs co-culture system, while silencing of genes encoding fibronectin, laminin, and collagen type I and V in MSCs could reduce albumin secretion and urea synthesis of co-cultured hepatocytes. It is also likely that MSCs protect the host cells from oxidative stress by secreting more superoxide dismutase 3 in the presence of inflammatory cytokines, such as TNF-α and interferon γ (Kemp et al., 2010).

3.4.2.4 Paracrine mechanisms of MSCs co-culture hepatotrophic effect

Hepatotrophic effect of MSCs co-culture is thought to result from three major aspects, including soluble cytokines secreted by MSCs, such as HGF, stem cell factor (Hu and Colletti, 2008), and other growth factors, ECM, and heterotypic cell-to-cell contact (Gómez-Aristizábal and Davies, 2012). A large number of previous studies have documented contribution of soluble factors to trophic effect of MSCs on co-cultured cells of epithelial origin, such as islet cells (Park et al., 2010),
intestinal epithelial cells (Weil et al., 2009), and hepatocytes (Murakami et al., 2004), using the Transwell culture system. In this culture system, MSCs are separated from seed cells by the semipermeable culture insert to exclude the involvement of ECM and cell-to-cell contact but allow free transport of soluble cytokines and growth factors.

BM-MSCs CM exhibited a potent trophic effect on rat hepatocytes with respect to albumin secretion and urea synthesis as compared to hepatocyte or co-culture CM although CM culture did not improve spheroid hepatocyte formation (Ijima et al., 2008). Gu et al. (2009a) further performed IL-6 neutralisation experiment and confirmed involvement of IL-6 in hepatotrophic effect of porcine BM-MSCs co-culture with respect to albumin secretion and urea synthesis. A recently published study regarding AT-MSCs co-culture with islet cells demonstrated that AT-MSCs CM improved porcine islet cell viability in a VEGF- rather than IL-6-dependent manner (Yamada et al., 2013). Mintz et al. (2014) also recently reported that CD34+ HSCs secreted 32 growth factors or cytokines associated with cell proliferation, survival, tissue repair, and wound healing. CD34+ stem cells CM also prevented chemically-induced hepatocyte death by inhibiting the caspase-3 signalling pathway.

It remains controversial whether soluble factors released from MSCs also have a trophic effect on human hepatocytes. Fouraschen et al. (2012) reported that human liver-derived MSCs CM significantly upregulated expression levels of genes associated with protein synthesis, cell survival, and cell proliferation in human hepatocyte-like Huh7 cells. However, Fitzpatrick and her colleagues (Cell Transpl, Epub ahead of print) reported that neither MSCs indirect co-culture (MSCs separated from hepatocytes by a porous membrane) nor MSCs co-culture CM had minimal or insignificant trophic effect on human hepatocytes, in the absence of additional ECM deposition and hepatocyte-to-MSC contact as shown in the present work. Minimal hepatotrophic effect of MSCs indirect co-culture suggested that release of MSCs-derived trophic factors may require the extrinsic stimulation of heterotypic contact between MSCs and hepatocytes. Furthermore, insignificant hepatotrophic effect of MSCs co-culture CM implied a possibility that human hepatocytes did not respond well to MSCs-released trophic factors in vitro. Isolation of primary human hepatocytes is known to be technically more complex and time-consuming than that of animal-sourced hepatocytes. As a result, human hepatocytes may be in a relatively stressful status after isolation as compared to counterpart animal cells, and become less responsive to trophic stimuli. Taking significant hepatotrophic effect of MSCs direct co-culture, trophic effect of MSCs-derived soluble factors may require the synergistic effect of MSCs-derived ECM and hepatocyte-to-MSC interaction.

### 3.4.3 Antiapoptotic effect of MSCs co-culture on hepatocytes

Overall trophic effect of MSCs co-culture on liver-specific metabolic function of hepatocytes may result from two aspects, namely, increased hepatocyte survivability, potentiated metabolism of surviving hepatocytes, or both. It has been well documented in current literature that MSCs co-culture significantly improved hepatocyte survival (Gu et al., 2009a; Gu et al., 2009b; Gu et al., 2009); however, there exists a knowledge gap whether MSCs inhibit apoptosis and/or necrosis of co-cultured hepatocytes. Fitzpatrick and her colleagues (Cell Transpl, Epub ahead of print) showed
that co-culture with UC-MSCs suppressed caspase-mediated apoptosis of hepatocytes within one month of culture in vitro. The present work further confirmed that AT-MSCs significantly reduced both spontaneous apoptotic and necrotic death of co-cultured hepatocytes without switching the death mode.

Spontaneous apoptosis in mono-cultured hepatocytes is known to be a major cause of cellular loss of primary hepatocytes after culture in vitro. Spontaneous apoptotic death is controversially attributed to intracellular accumulation of nitric oxide following enzymatic digestion, which is involved in the balance between proapoptotic and antiapoptotic effects on hepatocytes both in vitro and in vivo (Kim et al., 2000). Cleavage of CK18 by caspases is an early event in cellular apoptosis. Increased serum CCK18 level has been observed in patients afflicted with liver cirrhosis, primary graft dysfunction, ALF (Hetz et al., 2007), or non-alcoholic steatohepatitis (Yilmaz et al., 2009), suggesting hepatocyte apoptosis and liver degeneration in vivo. Caspase signalling is reported to be involved in rat hepatocyte apoptosis induced by endotoxin as mediated by TNF-α and downstream TNF-α receptor 1 in Kupffer cells (Hamada et al., 1999). Cleavage of CK18 at the position detected by the M30 CytoDeath™ ELISA kit used in the present work was reported to be initiated by caspase-9 and executed by caspases-3 and -7 (Schutte et al., 2004), which could be inhibited by the caspase-inhibitor zVAD-fmk (Hägg et al., 2002). The present work also demonstrated that MSCs specifically suppressed staurosporine-induced apoptosis rather than necrosis of co-cultured hepatocytes, while staurosporine treatment is known to activate caspase-3 signalling independently of caspases-8, -9, and -12 (Feng and Kaplowitz, 2002). Thus, reduction in CCK18 release from co-cultured hepatocytes as shown in the present work suggests that downregulation of caspase signalling may be implicated in antiapoptotic effect of MSCs co-culture on hepatocytes.

MSCs inhibit apoptosis of epithelial cells via paracrine mechanisms. Human BM-MSCs CM enriched with IL-6, HGF, and VEGF enhances the viability and proliferation of hypoxia-injured human foetal intestinal epithelial cells with concomitantly downregulated caspase-3 expression (Weil et al., 2009). Rat islet cells co-cultured with BM-MSCs exhibited significantly upregulated expressions of antiapoptotic genes, such as Mapkapk-2, Tnip-1, and Bcl-3, concomitantly accompanied by increased IL-6 and TGF-β in the CM (Karaoz et al., 2010). Park et al. (2010) reported human UC-MSCs CM containing high-level IL-6 and -8, VEGF, HGF, and TGF-β had a significant antiapoptotic effect on mouse islet cells both in vitro and in vivo. Yeung et al. (2012) showed that human BM-MSCs protected human islet cells from cellular apoptosis induced by interferon γ, TNF-α, and IL-1β; cytoprotective factors secreted by BM-MSCs included HGF and matrix MMP-2 and 9. Du et al. (2013) infused concentrated rat MSCs CM into rat liver graft, which resulted in reduction of hepatocyte apoptosis and concomitant increase of VEGF and MMP-9. Xagorari et al. (2013) also reported that BM-MSCs CM containing IL-6 protected hepatocytes from CCL4-induced apoptosis through activation of fibroblast-like-protein signalling. However, the present work demonstrated paracrine factors contributed minimally to antiapoptotic effect of MSCs co-culture. This inconsistence might be due to the possibility that isolated human primary hepatocytes became unresponsive to antiapoptotic factors released from MSCs and present in MSCs CM.
3.4.4 Conclusions

In conclusion, the present work demonstrated that autologous liver NPCs had a significant trophic effect on human hepatocytes even at a very low co-culture density, although reproducibility and consistency of liver NPCs expansion *in vitro* remained debatable. Co-culture with AT-MSCs exhibited a significant trophic effect similar to those with BM- and UC-MSCs as compared to ADFs co-culture and hepatocyte mono-culture, while the effect size remained significant even at a low MSC:hepatocyte ratio. This suggests that hepatotrophic effect of MSCs co-culture derives from stem cell-specific factors, through a series of amplification effects. As optimisation of MSCs source and seeding density failed to further potentiate hepatotrophic effect of MSCs co-culture as shown in the present work, preconditioning of MSCs may be an effective alternative for optimising MSCs co-culture system *in vitro*.

Hepatotrophic effect of MSCs co-culture was also effective for steatotic and cryopreserved hepatocytes with a similar effect size for fresh, nonsteatotic hepatocytes. Moreover, MSCs co-culture significantly suppressed caspase-mediated apoptosis and necrosis of hepatocytes, and specifically inhibited staurosporine-induced hepatocyte apoptosis associated with caspase signalling activation. The absence of trophic and antiapoptotic effects on human hepatocytes as seen in MSCs indirect co-culture and co-culture CM culture also implied a possibility that isolated human primary hepatocytes became unresponsive to paracrine stimuli from MSCs. Therefore, ECM and heterotypic interaction between MSCs and hepatocytes synergizing paracrine mechanisms are more likely to significantly contribute to hepatotrophic and antiapoptotic effects of MSCs co-culture, underlying which MSCs co-culture may down- and upregulate apoptosis- and antiapoptosis-associated gene expressions of hepatocytes primarily by non-paracrine mechanisms.
CHAPTER 4 HYPOXIC PRECONDITIONING POTENTIATES HEPATOTROPHIC EFFECTS OF MSCS CO-CULTURE

4.1 Introduction

4.1.1 Hepatotrophic effect of MSCs co-culture

A number of previous studies demonstrated that MSCs co-culture improved survival, cellular activity, and liver-specific metabolism, such as albumin secretion, urea synthesis, and CYP450 activity, of hepatocytes. MSCs support co-cultured hepatocytes through soluble factors, ECM, and cell-cell crosstalk in a three-dimensional architecture, which synergistically mimics a physiological microenvironment for hepatocytes in vitro. The work in Chapter 2 further validated that MSCs co-culture had hepatotrophic effects and also applied to steatotic hepatocytes and cryopreserved hepatocytes, both of which are frequently encountered in HCT practice. Moreover, MSCs also inhibited spontaneous and chemically-induced apoptotic death of co-cultured hepatocytes, which might be a major contributive factor of MSCs.

In the previous chapter, effort was made to optimise MSCs/hepatocyte co-culture system by testing three types of MSCs, namely, AT-, BM-, and UC-MSCs at an empirical seeding ratio of 1:2.5 (MSC:hepatocyte). This finding suggested that even a very small number of MSCs were sufficient to maintain and improved co-cultured hepatocyte metabolism. There is a knowledge gap existing in current literature how to further potentiate MSCs hepatotrophic effect, which is expected to be technically less complex, biologically effective, and, more importantly, subject to no safety concern.

4.1.2 Preconditioning of MSCs

MSCs have been widely investigated for cellular replacement therapy and regenerative medicine. These multipotent cells can be driven to differentiate into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes in the presence of specific extrinsic and intrinsic cues (Khan and Hardingham, 2012). MSCs also promote repair and regeneration of the injured cells and tissues, such as fibrotic liver (Tsai et al., 2009) and infarcted myocardium (Carvalho et al., 2013), through paracrine and nonparacrine mechanisms. However, it remains undetermined whether MSCs can maintain their biological and physiological activities after consecutive expansion in vitro and transplantation in vivo. Human MSCs will become replicatively senescent and (epi)genetically unstable through passages and consequently have their proliferation and differentiation potential impaired. An increasing number of chemically-defined culture media have been tested on in vitro priming of MSCs to ensure reliable engraftment and long-term therapeutic effect after transplantation (Tonti and Mannello, 2008). MSCs are frequently preconditioned using pharmacological (Wisel et al., 2009), biochemical and molecular (Khan et al., 2011), and physical techniques (Rosová et al., 2008) to optimise cellular functionality and improve cell graft survival, especially for transplantation to an inflammatory microenvironment (Herrmann et al., 2010).
4.1.2.1 Pharmacological preconditioning

Lipopolysaccharide (LPS), the major component of the outer membrane of the Gram-negative bacteria, acts as an endotoxin and can elicit potent immune and inflammatory responses in humans and animals. Human BM-MSCs subjected to LPS secrete significantly higher levels of VEGF, FGF2, HGF, and IGF-1 \textit{in vitro} by an NFKB- but not JNK-dependent mechanism (Crisostomo et al., 2008). LPS-preconditioned MSCs exhibit an enhanced survival after engraftment into the myocardium by upregulating VEGF expression, which consequently improve \textit{in vivo} neovascularisation and cardiac function (Yao et al., 2009). LPS preconditioning can also protect MSCs from induced apoptosis. Wang et al. (2013) reported that low-dose LPS preconditioning preserved the mitochondrial transmembrane potential and inhibited Cyc-c release in rat BM-MSCs subjected to hypoxia and serum deprivation. Moreover, LPS preconditioning regulates MSCs immunomodulative effect. Mei et al. (2013) reported that LPS challenge significantly upregulated expression of inflammatory cytokines such as IL-1\(\alpha\), -1\(\beta\), -6, and -8, and inhibitory immune mediators, such as indoleamine 2,3-dioxygenase 1, cyclooxygenase 2, interferon \(\beta\), and MMP-2, but downregulation of MMP-9, in human UC matrix-derived MSCs, mediated by the toll-like receptor. However, the use of LPS-preconditioned MSCs for HCT is inevitably subject to the safety concern as endotoxin contamination is a major hazard to human subjects receiving cellular replacement therapy (Ra et al., 2011).

4.1.2.2 Biochemical and molecular preconditioning

MSCs can be preconditioned with extrinsic cytokines and growth factors. MSCs exert the therapeutic effect by engrafting into the injured tissue possibly transdifferentiating into parenchymal cells and endothelial cells and/or secreting cytokines and growth factors. These soluble factors reciprocally act on MSCs to improve MSCs survival, mediate cellular functionality, and enhancing neoangiogenesis. Stromal-derived factor 1 (SDF-1), also known as C-X-C motif chemokine 12, is a chemokine protein that recruits immune and endothelial cells and regulates angiogenesis. SDF-1 preconditioning significantly suppresses apoptosis in rat BM-MSCs subjected to hypoxia and serum deprivation (Chen et al., 2009). Preconditioning with diazoxide, a mitochondrial ATP-sensitive potassium channel agonist, can improve the survival rate of transplanted rat BM-MSCs by upregulating expression of bFGF and HGF, which reduces the myocardial infarction area and improves left ventricular function in rat myocardial infarction model (Cui et al., 2010). TGF-\(\alpha\) upregulates rodent BM-MSCs secretion of VEGF, which can be further potentiated by the synergistic effect of TNF-\(\alpha\); TGF-\(\alpha\)-preconditioned MSCs show a better therapeutic effect on rat ischaemia/reperfusion myocardium as mediated by downregulation of IL-1\(\beta\), TNF-\(\alpha\), and caspase-3 signalling pathways (Herrmann et al., 2010). Moreover, MSCs isolated from a diseased donor may exhibit an impaired functionality due to the underlying biochemical insufficiency or genetic defect, which can be compensated or repaired by growth factor preconditioning. BM-MSCs isolated from streptozotocin-induced diabetic mice were preconditioned with IGF-1 and concomitant FGF-2; these preconditioned MSCs exhibited a significantly higher superoxide dismutase activity, less cellular
apoptosis, stronger in vitro tube-forming ability, and improved chemotactic mobility (Khan et al., 2011).

More importantly, preconditioning with soluble molecules was reported to potentiate the trophic and resuscitation capacity of MSCs; human AT-MSCs preconditioned with a combination of hyaluronic, butyric, and retinoic acids optimised islet cell graft revascularisation after intrahepatic co-transplantation, as mediated by VEGF, kinase insert domain receptor, and HGF signalling pathways, and improved glycaemic control in diabetic rats (Cavallari et al., 2012). However, preconditioning with cytokines and growth factors, which has been validated to be effective on the bench, is not feasible and cost-effective for the purpose of bedside transplantation requiring a large number of MSCs to be preconditioned. Additionally it remains unknown whether preconditioning effect of these cytokines and growth factors can maintain in the long term after transplantation in vivo. Genetic engineering targeting at these favourable molecules, such as VEGF (Shevchenko et al., 2013), HGF (Yu et al., 2007; Wang et al., 2013), and TGF-β (Guo et al., 2006; Xue et al., 2013), may be a solution to this uncertainty, while gene modification itself is technically complex and subject to some safety risks.

4.1.2.3 Hypoxic preconditioning

MSCs normally reside in a physiologically hypoxic niche, such as AT, BM, and UC. Hypoxia plays an important role in the fine equilibrium between proliferative and differentiation potentials of MSCs as mediated by a number of extrinsic and intrinsic signals. A long-term low-oxygen tension culture environment maintains MSCs in an undifferentiated, multipotent status. Human BM-MSCs cultured under 1%-O2 hypoxia showed a significantly increased proliferative ability and migration capability, underlying which were upregulated expression of stemness genes, such as OCT4, NANOG, SALL4, and KLF4 (Weijers et al., 2011; Hung et al., 2012). Hypoxic culture condition also determines the differentiation fate of MSCs towards osteoblasts, chondrocytes, and adipocytes. Hypoxia does not alter the immunophenotype or compromise the multi-lineage differentiation potential of BM-MSCs; however, hypoxia inhibits the transdifferentiation of MSCs into osteoblasts signalled by MAPK/ERK 1/2 (Wang et al., 2012), hypoxia-induced factor-1α (HIF-1α; Hsu et al., 2013), and Notch1 (Xu et al., 2013). In contrast, MSCs exposed to chondrogenic growth factors and a 2% O2 hypoxic environment express significantly greater collagen type II and proteoglycan, two major components of cartilage (Kanichai et al., J Cell Physiol, 2008). Moreover, hypoxia mobilises MSCs into the circulating bloodstream in a way similar to HSCs (Rochefort et al., 2006), and promotes the domiciliation of MSCs to the injured site (Rochefort et al., 2005).

Hypoxic preconditioning (HPc), namely, in vitro hypoxic culture followed by normoxic culture or transplantation in vivo, has been widely applied to optimise MSCs for uses of regenerative medicine and tissue engineering. HPc can protect MSCs from hypoxia/reoxygenation-induced apoptosis by stabilising mitochondrial membrane potential, upregulating Bcl-2 and VEGF signalling, and promoting phosphorylation of ERK and Akt (Wang et al., 2008). HPc significantly increases expression of pro-survival and pro-angiogenic factors, such as HIF-1α; angiopoietin 1, VEGF and
VEGF receptor, erythropoietin, Bcl-2, and Bcl-xL, and also significantly decreases that of caspase-3 initiating cellular apoptosis in mouse BM-MSCs; HPc-MSCs improve angiogenesis in myocardium and enhance morphological and functional recovery (Hu et al., 2008). This potentiated therapeutic effect of transplanting HPc-MSCs has also been validated in experimental limb, cerebral, renal, and spinal cord ischaemia. HPc can rejuvenilise aged AT-MSCs by upregulating gene expression of pro-angiogenic factors, including VEGF, placental growth factor, and HGF but downregulating that of TGF-β (Efimenko et al., 2011). Due to its technical simplicity and superior cost-effectiveness, HPc is a promising physical technique for optimising MSCs for cellular replacement therapy. However, it is rarely reported in literature whether HPc can potentiate the trophic and protective effects of MSCs on co-cultured or co-transplanted seed cells.

4.1.3 ROS: a signalling factor pivoting HPc

ROS refer to a collection of chemically reactive, oxygen-containing molecules, including radical, superoxide anion, hydrogen peroxide, and singlet oxygen. Production of ROS can occur in both physiological and pathological conditions, mainly in response to oxidative stress but also to exogenous sources, such as ionizing radiation. As a natural by-product of normal oxygen metabolism, excessive ROS may cause damage to cells by disrupting DNA and oxidising fatty acids, amino acids, and specific enzymes. However, ROS plays a crucial role in regulating cellular signalling and maintaining homeostasis. ROS are controversially thought to be involved in ageing, and also extensively studied in the scenario of cancer, including carcinogenesis, uncontrolled proliferation, escape from apoptosis, tumor cell invasion, neoangiogenesis and metastasis, and chronic inflammation bridging cancer.

Exogenous ROS induce marked apoptosis of MSCs in a dose- and time-dependent manner through the endoplasmic reticulum and mitochondrial pathways, including p38 MAPK signalling at an early time point and c-Jun N-terminal kinase signalling at a late time point (Wei et al., 2010). MSCs, however, are well known to be resistant to the detrimental effect of excessive ROS by possession of a potent ROS scavenging facility (Valle-Prieto and Conget, 2010). Conversely HPc is known to significantly increase intracellular ROS activity and improve MSCs survival and motility. ROS plays a crucial role in maintaining undifferentiated status of human MSCs: decreased intracellular ROS activity accompanies transdifferentiation of MSCs into osteoblasts, while exogenous hydrogen peroxide prevents osteogenic differentiation (Chen et al., 2008). In contrast, increased intracellular ROS from upregulated expression of NAD(P)H oxidase 4 facilitates adipocyte differentiation of mouse embryonic MSCs by activating the transcription of cAMP response element-binding protein (Kanda et al., 2011). ROS controls the cell growth cycle and life span of human MSCs in vitro as mediated by upregulation of p38 MAPK signalling. ROS is also reported to regulate migration and tube formation (in vitro angiogenesis) of MSCs as mediated by placental growth factor signalling (Shyu et al., 2008). Increase of intracellular ROS results in significant downregulation of focal adhesion-related molecules, such as phospho-FAK (focal adhesion kinase) and phospho-Src, and integrin-related adhesion molecules, such as integrin 5α and
β1, suggesting the involvement of ROS in MSCs mobilisation and migration (Song et al., 2010). It is possible that intracellular ROS might mediate the hypothesised HPc-induced potentiation of MSCs hepatotrophic and antiapoptotic effects on co-cultured hepatocytes.

4.1.4 Chapter objectives

- **Effects of HPc on MSCs**
  
  To investigate the effects of HPc on AT-MSCs *in vitro*, with respect to cellular mitochondrial dehydrogenase activity, attachment, DNA/protein synthesis, and intracellular ROS activity.

- **Optimisation of time length of HPc**
  
  To compare potentiative effect of 8-, 24-, 48-, and 72-h HPc on MSCs co-culture hepatotrophic effect, with respect to cellular viability, cell attachment, and liver-specific metabolism.

- **Potentiation of MSCs co-culture paracrine mechanisms by HPc**
  
  To investigate whether HPc potentiates MSCs co-culture hepatotrophic effect by enhancing paracrine mechanisms.

- **Potentiation of MSCs co-culture antiapoptotic effect**
  
  To investigate whether MSCs can protect co-cultured hepatocytes from chemically-induced cell apoptosis.

- **Dependence of HPc-induced potentiative effects on intra-MSCs ROS activity**
  
  To investigate whether HPc induces potentiation of MSCs co-culture hepatotrophic and antiapoptotic effects in an intra-MSCs ROS activity dependent manner.
4.2 Materials and methods

4.2.1 Subculture and immunophenotyping of AT-MSCs

Human AT-MSCs were subcultured as described in Section 2.2, Subculture of MSCs. P6–8 MSCs were characterised using flow cytometry with a human MSC phenotyping kit (Miltenyi Biotec Ltd., Surrey, UK). These MSCs were stained positive for CD73, CD90, and CD105, and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface antigens (Dominici et al., 2006).

4.2.2 Isolation of human hepatocytes

Human donor liver tissues were processed as described in Section 2.3, Primary Harvest of Human Hepatocytes. The total number and viability of fresh hepatocytes were determined using a hematocytometer and the trypan blue exclusion technique with a light microscope. Red blood cells in hepatocyte pellet were lysed using sterile water for injection for 1 min at room temperature, followed by centrifugation. The batch of hepatocytes with a viability of over 60% on trypan blue exclusion was used for further experiments.

4.2.3 Optimisation of HPC

P6–8 MSCs were subjected to hypoxia (HPC-MSCs) using an air-tight and moistened hypoxia incubator chamber (StemCell Technologies SARL, Sirocco, France; Figure 4.1), at 37°C for 8 h, 24 h, 48 h, and 72 h, respectively. The chamber was purged with a mixture of 95% N2/5% CO2 (BOC Special Gases, Surrey, UK) for 3 min, and the flow rate was set at 20 L/min using a single flow meter (Billups-Rothenberg Inc., Del Mar, CA, USA) as recommended by the manufacturer. The intra-chamber oxygen saturation measured 2% (hypoxia) using a GasBadge® Plus single-gas monitor (Industrial Scientific, Arras, France) upon the completion of purge. MSCs that were cultured at an atmosphere of 95% air/5% CO2 served as control (NPC-MSCs), and the intra-incubator oxygen saturation measured 20% (normoxia) using the same protocol. MSCs morphology was examined using a standard light microscope. The experiments were performed in duplicate and repeated in triplicate independently. The optimal time length of HPC that exhibited the greatest potentiative effect on MSCs co-culture hepatotrophic effect was used in further experiments.

4.2.4 Hepatocyte co-culture protocols

4.2.4.1 Direct co-culture of hepatocytes with MSCs

Fresh hepatocytes were co-cultured with HPC- versus NPC-MSCs as described in Section 2.4, Hepatocyte Mono-culture and Co-culture. The seeding density of MSCs was 20,000 viable cells per cm², and that of hepatocytes was 50,000 viable cells per cm², at a MSC:hepatocyte ratio of 1:2.5.
Hepatocytes co-cultured with NPc-MSCs were used as control, and mono-cultured HPc- and NPc-MSCs were used as blank controls.

4.2.4.2 Indirect co-culture of hepatocytes with MSCs

**Indirect co-culture using Transwell**  
Hepatocytes were indirectly co-cultured with HPc- versus NPc-MSCs using Transwell® Permeable Supports, as described in **Section 3.2.5.5, Indirect co-culture of hepatocytes with MSCs**, to investigate whether HPc could potentiate contribution of MSCs-derived soluble factors to MSCs co-culture hepatotrophic effect.

**MSCs co-culture CM**  
AT-MSCs were plated onto T75 culture flasks at a density of 20,000 viable cells per cm², and fed with hepatocyte culture media for 24-hour pre-culture at 37°C. Mono-cultured MSCs were subjected to 2%-O₂ HPc and 20%-O₂ NPc for 24 h, respectively. Fresh hepatocytes were cultured with HPc- versus NPc-MSCs co-culture CM as described in **Section 3.2.5.5, Indirect co-culture of hepatocytes with MSCs**, to investigate whether HPc could potentiate paracrine contribution of MSCs co-culture to hepatotrophic effect.

The culture media were refreshed on days 1, 3, 5, and 7, respectively. The cell morphology was examined using a standard inverted light microscope equipped with a digital SLR camera. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for further experiments. Cell cultures were rinsed with one-wash PBS at room temperature for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.5 Characterisation of HPc effects on MSCs

4.2.5.1 General cellular activity

P6–8 AT-MSCs at 80% confluency were subjected to 2%-O₂ hypoxia or 20%-O₂ normoxia for 24 h. MTT assay was performed as described in **Section 2.5, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay** to determine MSCs mitochondrial dehydrogenase
activity. SRB assay was performed as described in Section 2.6, Sulforhodamine B Colorimetric Assay to determine overall MSCs attachment. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.5.2 3H-thymidine and 14C-leucine incorporation assays

3H-thymidine and 14C-leucine incorporation assays were used to examine effects of HPVc versus NPc on MSCs DNA and protein syntheses. P6–8 MSCs at 80% confluency were fed with MSCs culture media containing 3H-labeled thymidine and 14C-labeled leucine (Amersham International, Amersham, UK), at a concentration of 20 μCi/mL and 1 μCi/mL, respectively. These MSCs were further subjected to 24-hour hypoxia or normoxia. Upon the completion of preconditioning, radioisotope-labelled cells were harvested onto glass fibre membranes using a Packard FilterMate (Packard Instrument Co., Ltd., Caversham, UK), and the filters were dried at 60°C for 1 h. The radioactivities were counted using a Packard Matrix 9600 β-counter (Packard Instrument Co., Ltd., Caversham, UK), and expressed as count per min per well (cpm/well). MSCs incubated in radioisotope-free culture media were used as blank control. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.5.3 Quantitation of intra-MSCs ROS activity

Intracellular ROS activity was measured using flow cytometry with dichlorodihydrofluorescein diacetate acetyl ester (DCFDA) staining as previously described by Eruslanov and Kusmartsev (2010). DCFDA, a cell-permeant fluorogenic dye, is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorofluorescin (DCFDH), which is subsequently oxidised by intracellular hydroxyl, peroxy and other forms of ROS into cell-impermeant 2',7'-dichlorofluorescein (DCF). DCF can be detected by fluorescence spectroscopy at the maximum excitation and emission spectra of 495 nm and 527 nm, respectively.

Briefly, P6–8 AT-MSCs were plated onto non-tissue-culture-treated 6-well plates. On 80% confluency, MSCs were subjected to 24-hour HPVc versus NPc and rinsed with one wash of PBS, 1 mL per well. Cell cultures were incubated with PBS, 0.5 mL per well, containing 2.5-μM 5,6-chloromethyl-2',7'-DCFDA (Molecular Probes, Inc., Eugene, OR, USA) specifically designated for liver cell staining, at 37°C for 30 min. HPVc-MSCs incubated with plain PBS were used as blank control, and NPc-MSCs deprived of serum 24 h prior to labelling were used as positive control. Cells were rinsed with one wash of PBS, 1 mL per well, and detached from the plates using EDTA-chelated trypsin (TrypLE™ Select; Invitrogen Ltd, Paisley, UK), 0.5 mL per well. Dissociation was terminated by adding PBS containing 10% FCS, 1 mL per well. Cell suspension was centrifuged at 4°C and 1,500 rpm for 5 min. Cell pellets were resuspended in 0.5-mL plain PBS at a density of 100,000 cells per mL, and kept on ice and in the dark for further flow cytometry.

An 8-channel BD FACS Canto II flow meter (BD Biosciences, San Jose, CA, USA) was used to quantitate intra-MSCs ROS activity. Briefly, 10,000 events were recorded and analysed, and the side scatter (SSC) axis was plotted against the forward scatter (FSC) axis to produce the scatter
graph and gate the cell population (at least 90% of the total events). Unlabelled cells were analysed to polygon gate the positive population (labelled cells). Intracellular ROS activity was measured using the Alex Fluor® 488 fluorescein isothiocyanate (FITC) channel, and the SSC-axis was plotted against the FITC-axis. The flow cytometry analysis software FlowJo 10.0.6 for Windows (Tree Star, Inc., Ashland, OR, USA) was used to determine median fluorescence intensity (FI) for each sample. Median FIs of HPc-, and serum-deprived NPc-MSCs were normalised to those of NPc-MSCs (Du et al., J Biol Chem, 2006). All experiments were performed in duplicate and repeated in triplicate independently.

4.2.6  NAC antagonisation experiment

4.2.6.1  Optimisation of NAC concentration

P6–8 AT-MSCs were plated onto non-tissue culture-treated 6-well plates. On 80% confluency, MSCs were and rinsed with one wash of PBS, and subjected to 24-hour HPc versus NPc and rinsed with one wash of PBS, 1 mL per well. HPc-MSCs were pretreated with 0-, 5-, 10-, and 20-mM NAC (PLIVA Pharma, Ltd., Hampshire, UK), respectively. Intra-MSCs ROS activity was measured as described in Section 4.2.5.3, Quantitation of intra-MSCs ROS activity, and the least concentration of NAC that resulted in significantly lower ROS activity in HPc-MSCs than that in NPc-MSCs was used for further antagonisation experiments. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.6.2  Co-culture of hepatocytes with non-pretreated and NAC-pretreated HPc-MSCs

P6–8 AT-MSCs were plated onto collagen-coated 24-well plates and subjected to 24-hour HPc; HPc-MSCs were pretreated with the optimal concentration of NAC and without NAC, respectively. MSCs cultures were rinsed with one wash of PBS, and fresh hepatocytes were co-cultured with HPc-MSCs as described in Section 4.2.4.1, Direct co-culture of hepatocytes with MSC. The culture media were refreshed on days 1, 3, 5, and 7, respectively. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for further albumin, urea, CCK18, and CK18 assays. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.7  General cellular activity and liver-specific metabolic function assays

MTT assay was performed as described in Section 2.5, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay to determine hepatocyte overall viability. SRB assay was performed as described in Section 2.6, Sulforhodamine B Colorimetric Assay to determine overall hepatocyte attachment. Albumin ELISA was performed as described in Section 2.7, Albumin Enzyme-linked Immunoabsorbent Assay to determine protein synthesis of hepatocytes. Urea colorimetric assay was performed as described in Section 2.8, Urea Colorimetry to determine nitrogen detoxification of hepatocytes. All experiments were performed in duplicate and repeated in
triplicate independently.

4.2.8 *Staurosporine cytotoxicity assay*

Fresh hepatocytes were pre-treated with 1-μM staurosporine (Sigma-Aldrich, St. Louis, MO, USA) and co-cultured with non-NAC- and NAC-pretreated HPc-MSCs for 24 h, as controlled by staurosporine-treated hepatocytes co-cultured with NPe-MSCs. Cell culture supernatants were collected for CCK18 and CK18 assays as described in *Section 2.9, Caspase-cleaved CK18 Assay* and *Section 2.10, CK18 Assay*. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.9 *Hepatocyte apoptosis and total death assays*

CCK18 assay was performed as described in *Section 2.9, Caspase-cleaved CK18 Assay* to determine caspase-mediated hepatocyte apoptosis. CK18 assay was performed as described in *Section 2.10, CK18 Assay* to determine total hepatocyte death. The ratio of CCK18 to CK18 was also produced to determine the death mode of hepatocytes *in vitro*; a ratio of over 0.40 indicated that hepatocytes underwent apoptosis mainly, and necrosis *vice versa*. All experiments were performed in duplicate and repeated in triplicate independently.
4.3 Results

4.3.1 Morphology of HPc- vs. NPc- MSCs mono- and co-cultures

4.3.1.1 Morphology of HPc-MSCs

HPc did not result in cell detachment, necrosis, or morphological distortion of MSCs. HPc-MSCs (Fig. 4.2A) exhibited a spindle-shaped fibroblast-like phenotype similar to NPc-MSCs (Fig. 4.2B) and reached 80% confluency within 7 d. On confluency HPc-MSCs were also polarised, with a small cell body and multiple slim projections in a homogenous manner. HPc-MSCs showed viability over 99% on trypan blue exclusion after trypsinisation.

4.3.1.2 Morphology of HPc-MSCs co-culture

The great majority of freshly isolated human hepatocytes attached to the MSCs monolayer within 2–4 hours, and only a few hepatocytes were detached from culture vessel surface at the time of culture media replacement. Hepatocytes co-cultured with HPc-MSCs exhibited a morphology similar to those in co-culture with NPc-MSCs (Figure 4.2C), but a little more hepatocytes aggregated into larger, oval-shaped colonies and attached close to HPc-MSCs over 7 days of co-culture (Figure 4.2D).

![Figure 4.2 Morphology of NPc-MSCs (A), HPc-MSCs (B), and hepatocytes co-cultured with NPc- (C) and HPc-MSCs (D) (200×):](image)

HPc-MSCs (A) exhibited a spindle-shaped fibroblast-like phenotype similar to NPc-MSCs (B); compared to hepatocytes co-cultured with NPc-MSCs (C), a little more hepatocytes aggregated into larger, oval-shaped colonies in co-culture with HPc-MSCs (D).
4.3.2 Effects of HPc on MSCs

4.3.2.1 HPc has no cytotoxic effect on MSCs

As is shown in Figure 4.3A, HPc-MSCs showed a significant decrease in mitochondrial dehydrogenase activity as compared to NPc-MSCs (HPc vs. NPc, 1.18 ± 0.11 vs. 1.36 ± 0.09 OD units; \( P < 0.05 \)). However, HPc resulted in a significant increase in SRB cell attachment as compared to NPc (1.88 ± 0.11 vs. 1.66 ± 0.11 OD units; \( P < 0.01 \)). Addition of 10-mM NAC significantly reduced MTT activity (HPc+NAC, 0.95 ± 0.09 OD units; \( P < 0.01 \)) and reversed cellular attachment increase (1.53 ± 0.14 OD units; \( P < 0.01 \)) in HPc-MSCs.

4.3.2.2 HPc increases MSCs DNA synthesis

As is shown in Figure 4.3B, HPc significantly increased DNA synthesis in MSCs as compared to NPc (3,121 ± 295 vs. 1,815 ± 334 cpm/well; \( P < 0.01 \)). However, HPc resulted in a significant reduction in MSCs protein synthesis as compared to NPc (567 ± 60 vs. 715 ± 118 cpm/well; \( P < 0.05 \)). Addition of 10-mM NAC significantly reversed DNA synthesis increase (1,314 ± 54 cpm/well; \( P < 0.01 \)) and further reduced protein synthesis (263 ± 17 cpm/well; \( P < 0.01 \)) in HPc-MSCs.

4.3.3 Potentiative effect of HPc on MSCs co-culture hepatotrophic effect

4.3.3.1 HPc potentiates MSCs co-culture hepatotrophic effect

Mitochondrial dehydrogenase activity: HPc at all time lengths exhibited a limited potentiative effect on MSCs hepatotrophic effect, with respect to hepatocyte mitochondrial dehydrogenase activity, as compared to control NPc (Figure 4.4A). HPc co-culture did not significantly improve hepatocyte MTT activity on days 1 and 5; however, hepatocytes co-cultured...
with HPc(8h)-, HPc(48h)-, and HPc(72h)-MSCs had a significantly higher MTT activity than those co-cultured with control NPc-MSCs on day 3 (NPc vs. HPc(8h) vs. HPc(24h) vs. HPc(48h) vs. HPc(72h), 1.45 ± 0.12 vs. 1.69 ± 0.17 vs. 1.62 ± 0.20 vs. 1.77 ± 0.14 vs. 1.65 ± 0.17 OD units; \( P < 0.01 \)), and hepatocytes co-cultured with HPc(24h)-MSCs had a significantly higher MTT activity than control NPc co-culture on day 7 (2.60 ± 0.25 vs. 2.75 ± 0.25 vs. 3.09 ± 0.33 vs. 2.79 ± 0.33 vs. 2.92 ± 0.30 OD units; \( P < 0.05 \)).

**Cell attachment**  
HPc at all time lengths exhibited a limited potentiative effect on MSCs hepatotrophic effect, with respect to hepatocyte cellular attachment, as compared to control NPc (Figure 4.4B). HPc-MSCs co-culture did not significantly improve hepatocyte SRB activity on days 1, 3, and 5; however, hepatocytes co-cultured with HPc(24h)- and HPc(48h)-MSCs had a significantly higher SRB activity than those co-cultured with control NPc-MSCs on day 7 (3.58 ± 0.42 vs. 3.93 ± 0.27 vs. 4.14 ± 0.29 vs. 4.26 ± 0.29 vs. 4.03 ± 0.28 OD units; \( P < 0.05 \)).

**Albumin secretion**  
No albumin secretion was detected in NPc- and HPc-MSCs mono-culture. HPc at 24, 48, and 72 h significantly potentiated MSCs hepatotrophic effect, with respect to albumin secretion, as compared to control NPc, although 8-h HPc did not show any potentiative effect throughout 7 days of co-culture (Figure 4.4C). HPc co-culture did not significantly improve hepatocyte secretion of albumin on day 1; however, hepatocytes co-cultured with HPc(24h)-, HPc(48h)-, and HPc(72h)-MSCs had a significantly higher albumin secretion, to a similar extent, than those co-cultured with control NPc-MSCs from day 3 (2.4 ± 0.3 vs. 2.4 ± 0.3 vs. 3.7 ± 0.4 vs. 3.6 ± 0.4 vs. 3.9 ± 0.4 \( \mu g/10^6 \) hepatocytes; \( P < 0.01 \)) until day 7 (4.6 ± 0.4 vs. 4.3 ± 0.3 vs. 5.4 ± 0.3 vs. 5.2 ± 0.3 vs. 5.4 ± 0.3 \( \mu g/10^6 \) hepatocytes; \( P < 0.01 \)).

**Urea synthesis**  
HPc at 24, 48, and 72 h significantly potentiated MSCs hepatotrophic effect, with respect to urea synthesis, as compared to control NPc, although 8-h HPc did not show any potentiative effect throughout 7 days of co-culture (Figure 4.4D). HPc co-culture did not significantly improve hepatocyte synthesis of urea on day 1; however, hepatocytes co-cultured with HPc(24h)-, HPc(48h)-, and HPc(72h)-MSCs had a significantly higher urea synthesis, to a similar extent, than those co-cultured with control NPc-MSCs from day 3 (23.4 ± 2.2 vs. 23.3 ± 2.3 vs. 32.8 ± 3.1 vs. 32.4 ± 2.4 vs. 33.2 ± 3.1 \( \mu g/10^6 \) hepatocytes; \( P < 0.01 \)) until day 7 (35.9 ± 3.0 vs. 33.5 ± 3.1 vs. 40.8 ± 3.6 vs. 40.4 ± 3.3 vs. 41.4 ± 3.5 \( \mu g/10^6 \) hepatocytes; \( P < 0.01 \)).

Overall 24-, 48-, and 72-h rather than 8-h HPc significantly potentiated MSCs co-culture hepatotrophic effect, to a similar extent, as compared to control 24-h NPc. Therefore, 24 h was determined to be the optimal time length of HPc used in further hepatocyte co-culture experiments.
Figure 4.4  MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of hepatocytes co-cultured with HPc(8h)-, HPc(24h)-, HPc(48h)-, and HPc(72h)- MSCs versus hepatocytes co-cultured with NPc(24h)-MSCs at a MSC:hepatocyte seeding ratio of 2.5:1. HPc for 24, 48, and 72 h significantly potentiated MSCs co-culture hepatotrophic effect from day 3 until day 7. All data were expressed as mean ± SD; *P <0.05 and **P <0.01 versus control NPc co-culture (n = 6). NPc, normoxia-preconditioned; HPc, hypoxia-preconditioned; Co, co-culture.
4.3.3.2 HPc does not potentiate MSCs paracrine contribution to co-culture hepatotrophic effect

HPc-MSCs indirect co-culture using Transwell plates exhibited no significantly potentiated hepatotrophic effect, with respect to hepatocyte mitochondrial dehydrogenase activity and cellular attachment (Table 4.1), as well as albumin secretion and urea synthesis (Table 4.2), as compared to control NPc-MSCs indirect co-culture, throughout 7 days of culture.

<table>
<thead>
<tr>
<th>Table 4.1</th>
<th>MTT activity and SRB cell attachment (mean ± SD) of hepatocytes indirectly co-cultured on Transwell plates with HPc-MSCs versus with NPc-MSCs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MTT (OD unit)</td>
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<tr>
<td></td>
<td>Day 1</td>
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<tr>
<td>HPc-iCo</td>
<td>1.58 ± 0.18</td>
</tr>
<tr>
<td>NPc-iCo</td>
<td>1.60 ± 0.16</td>
</tr>
</tbody>
</table>

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; iCo-, indirect co-culture.

<table>
<thead>
<tr>
<th>Table 4.2</th>
<th>Albumin secretion and urea synthesis (mean ± SD) of hepatocytes indirectly co-cultured on Transwell plates with HPc-MSCs versus with NPc-MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin (μg/10^6 hepatocytes)</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>HPc-iCo</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>NPc-iCo</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; iCo-, indirect co-culture.

HPc-MSCs co-culture CM improved hepatocyte mitochondrial dehydrogenase activity on day 3 only (HPc vs. NPc, 2.11 ± 0.25 vs. 1.21 ± 0.19 OD units; P <0.01), but did not significantly increase hepatocyte attachment throughout 7 days of culture as compared to control NPc-MSCs co-culture CM (Table 4.3). HPc-MSCs co-culture CM also significantly increased hepatocyte secretion of albumin only day 7 only (1.4 ± 0.1 vs. 1.2 ± 0.1 μg/10⁶ hepatocytes; P <0.01), but did not significantly improve urea synthesis throughout 7 days of culture as compared to control NPc-MSCs co-culture CM (Table 4.4).

<table>
<thead>
<tr>
<th>Table 4.3</th>
<th>MTT activity and SRB cell attachment (mean ± SD) of hepatocytes cultured with HPc-MSCs co-culture CM versus with NPc-MSCs co-culture CM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT (OD unit)</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>HPc-</td>
<td>1.54 ± 0.17</td>
</tr>
<tr>
<td>NPc-</td>
<td>1.58 ± 0.15</td>
</tr>
</tbody>
</table>

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; **P< 0.01 versus NPc.
Table 4.4  Albumin secretion and urea synthesis (mean ± SD) of hepatocytes cultured with HPc-MSCs co-culture CM versus with NPc-MSCs co-culture CM

<table>
<thead>
<tr>
<th></th>
<th>Albumin (μg/10^6 hepatocytes)</th>
<th>Urea (μg/10^6 hepatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>HPc-</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>NPc-</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; **P < 0.01 versus NPc.

4.3.4  Potentiative effect of HPc depends on intra-MSCs ROS activity

4.3.4.1  NAC pretreatment antagonises HPc-induced intra-MSCs ROS activity increase

As is shown in Figure 4.5, serum deprivation and HPc significantly increased intra-MSCs ROS activity to a similar extent as compared to NPc (normalised median FI: NPc vs. NPc+SF vs. HPc, 100.0% vs. 133.8% ± 16.4% vs. 141.1% ± 17.1%, \( p < 0.01 \)). Pretreatment with 5-mM NAC did not completely eliminate HPc-induced increase in ROS activity (HPc+NAC, 113.1% ± 11.2%, \( p < 0.05 \) vs. NPc); however, addition of 10- (74.3% ± 7.2%, \( p < 0.01 \)) and 20-mM NAC (63.1% ± 3.9%, \( p < 0.01 \)) significantly antagonised HPc-induced ROS activity increase in HPc-MSCs. Therefore, 10-mM NAC was used in further antagonisation experiments.

4.3.4.2  HPc-potentiated co-culture hepatotrophic effect depends on intra-MSCs ROS activity

Figure 4.6 shows that 10-mM NAC pretreatment significantly antagonised HPc-induced potentiation of liver-specific metabolic function of hepatocytes co-cultured with HPc-MSCs. Hepatocytes co-cultured with NAC-pretreated HPc-MSCs secreted significantly less albumin, as compared to those with non-NAC-pretreated HPc-MSCs, from day 1 (HPc-Co vs. HPc+NAC-Co, 1.2 ± 0.3 vs. 0.9 ± 0.1 μg/10^6 hepatocytes; \( p < 0.05 \)) until day 7 (5.4 ± 0.3 vs. 4.5 ± 0.3 μg/10^6 hepatocytes; \( p < 0.01 \)). Moreover, hepatocytes co-cultured with NAC-pretreated HPc-MSCs synthesised significantly less urea, as compared to those with non-NAC-pretreated HPc-MSCs, from day 3 (32.8 ± 3.1 vs. 22.8 ± 2.2 μg/10^6 hepatocytes; \( p < 0.05 \)) until day 7 (40.8 ± 3.6 vs. 35.3 ± 3.3 μg/10^6 hepatocytes; \( p < 0.01 \)).
Figure 4.5  Effect of HPc and NAC pretreatment on intra-MSCs ROS activity as measured by flow cytometry with DCFDA: representative SSC-versus-DCFDA (intracellular ROS) FI scatter plots of unlabelled (A), NPc- (B), HPc- (C), and HPc+10-mM-NAC-pretreated (D) MSCs, and bar charts of normalised median FI (E) of intra-MSCs ROS activity. Serum deprivation and HPc significantly increased intra-MSCs activity as compared to NPc, while 10- and 20-mM NAC pretreatment significantly eliminated HPc-induced intra-MSCs ROS increase. All data were expressed as mean ± SD; *P < 0.05 and **P < 0.01 versus NPc-MSCs (n = 6). SSC, side scattered; NPc, normoxia-preconditioned; SF, serum-free; HPc, hypoxia-preconditioned; NAC, N-acetylcysteine; nMean FI, normalised mean; nMedian, normalised median; FI, fluorescence intensity.
4.3.5 **Potentiative effect of HPc on MSCs co-culture antiapoptotic effect**

4.3.5.1 **HPc-potentiated co-culture antiapoptotic effect depends on intra-MSCs ROS activity**

No CCK18 or CK18 release was detected in HPc-MSCs mono-culture. Direct co-culture with HPc-MSCs significantly further reduced CCK18 release from hepatocytes as compared to control NPc-MSCs co-culture (Figure 4.7A). Soluble CCK18 level remained significantly lower in hepatocytes co-cultured with HPc-MSCs than that in hepatocytes co-cultured with NPc-MSCs from day 1 (HPc-Co vs. NPc-Co, 14.5 ± 1.6 vs. 18.0 ± 1.4 U/10⁶ hepatocytes; *P <0.01) until day 5 (6.4 ± 0.9 vs. 9.2 ± 1.1 U/10⁶ hepatocytes; **P <0.01); however, 10-mM NAC pretreatment eliminated HPc-induced potentiation of MSCs antiapoptotic effect on co-cultured hepatocytes from day 1 (HPc+NAC-Co, 18.3 ± 1.4 U/10⁶ hepatocytes; *P <0.01) until day 5 (9.0 ± 10 U/10⁶ hepatocytes; **P <0.01).

Direct co-culture with HPc-MSCs also significantly further reduced CK18 release from hepatocytes, as compared to that with NPc-MSCs (Figure 4.7B), from day 1 (61.6 ± 4.8 vs. 91.2 ± 8.2 U/10⁶ hepatocytes; *P<0.01) until day 5 (61.8 ± 5.2 vs. 100.9 ± 8.1 U/10⁶ hepatocytes; **P<0.01); however, NAC pretreatment eliminated HPc-induced potentiation of MSCs prosurvival effect on co-cultured hepatocytes from day 3 (176.0 ± 16.8 U/10⁶ hepatocytes; *P <0.01) until day 5 (99.0 ± 8.6 U/10⁶ hepatocytes; **P <0.01).

As is shown in Figure 4.7C, CCK18/CK18 ratio remained similar between hepatocytes co-cultured with HPc-MSCs and hepatocytes co-cultured with NPc-MSC from day 1 (23.6% ± 2.6% vs. 20.2% ± 3.0%; *P >0.05) until day 7 (1.7% ± 0.1% vs. 1.5% ± 0.1%; *P >0.05). It was noted that NAC pretreatment of HPc-MSCs switched death mode of co-cultured hepatocytes from necrosis to apoptosis on day 1 (29.1% ± 2.4%; *P <0.01).
Figure 4.7  CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes co-cultured with NPC-MSCs versus with non-NAC-pretreated HPc-MSCs versus NAC-pretreated HPc-MSCs. HPc-MSCs co-culture significantly further reduced caspase-mediated apoptosis and total death of hepatocytes, and NAC pretreatment eliminated HPc-potentiated MSCs co-culture antiapoptotic and prosurvival effects. All data were expressed as mean ± SD; **P < 0.01 versus NPC-Co; ^^^P < 0.01 versus HPc-Co (n = 6). NPC, normoxia-preconditioned; HPc, hypoxia-preconditioned; Co, co-culture; NAC, N-acetylcysteine.
4.3.5.2 HPc does not potentiate MSCs paracrine contribution to co-culture antiapoptotic effect

MSCs indirect co-culture using Transwell plates

Indirect co-culture with HPc-MSCs had no significant effect on hepatocyte CCK18 or CK18 release as compared to control NPc-MSCs indirect co-culture (Table 4.5). Soluble CCK18 level remained similar between hepatocytes indirectly co-cultured with HPc-MSCs and with NPc-MSCs from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes indirectly co-cultured with HPc-MSCs and with NPc-MSCs from day 1 until day 7. CCK18/CK18 ratio remained similar between hepatocytes indirectly co-cultured HPc-MSCs and with NPc-MSCs from day 1 until day 7.

MSCs co-culture CM

HPc-MSCs co-culture CM had no significant effect on hepatocyte CCK18 or CK18 release from hepatocytes as compared to control NPc-MSCs co-culture CM (Table 4.6). Soluble CCK18 level remained similar between hepatocytes cultured with HPc- and NPc-MSCs co-culture CM from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes cultured with HPc- and NPc-MSCs co-culture CM from day 1 until day 7. CCK18/CK18 ratio remained similar between hepatocytes cultured with HPc- and NPc-MSCs co-culture CM from day 1 until day 7.

4.3.5.3 HPc potentiates MSCs co-culture hepatoprotective effect against staurosporine-induced cytotoxicity

Co-culture with HPc-MSCs significantly further reduced staurosporine-induced hepatocyte apoptosis (soluble CCK18 level) as compared to co-culture with NPc-MSCs (HPc vs. NPc, 21.6 ± 2.8 vs. 26.5 ± 2.5 U/10⁶ hepatocytes, \( P < 0.01 \); Figure 4.8A). However, HPc-MSCs co-culture had no significant effect on staurosporine-induced total cell death of hepatocytes as compared to NPc-MSCs co-culture (Figure 4.8B). However, HPc-MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis switch of hepatocyte death mode to a similar extent as compared to NPc-MSCs co-culture (Figure 4.8C).
### Table 4.5  
CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes indirectly co-cultured with HPc-MSCs versus with NPc-MSCs

<table>
<thead>
<tr>
<th></th>
<th>CCK18 (U/10^6 hepatocytes)</th>
<th>CK18 (U/10^6 hepatocytes)</th>
<th>CCK18/CK18 ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 Day 3 Day 5 Day 7</td>
<td>Day 1 Day 3 Day 5 Day 7</td>
<td>Day 1 Day 3 Day 5 Day 7</td>
</tr>
<tr>
<td>HPc-iCo</td>
<td>21.2 ± 2.6 22.7 ± 2.5 14.1 ± 1.8 1.4 ± 0.3</td>
<td>99.8 ± 11.7 255.6 ± 28.9 45.9 ± 4.3 21.2 ± 2.4</td>
<td>6.1 ± 1.2 5.5 ± 1.1 3.1 ± 0.2</td>
</tr>
<tr>
<td>NPc-iCo</td>
<td>20.8 ± 2.4 23.2 ± 2.3 15.9 ± 1.7 1.6 ± 0.2</td>
<td>108.8 ± 12.4 390.0 ± 35.0 276.0 ± 33.0 50.4 ± 4.6</td>
<td>19.1 ± 2.8 5.9 ± 1.1 5.8 ± 1.3 3.2 ± 0.1</td>
</tr>
</tbody>
</table>

HPc, hypoxia-conditioned; NPc, normoxia-preconditioned; iCo-, indirect co-culture.

### Table 4.6  
CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes cultured with HPc- versus NPc-MSCs co-culture CM

<table>
<thead>
<tr>
<th></th>
<th>CCK18 (U/10^6 hepatocytes)</th>
<th>CK18 (U/10^6 hepatocytes)</th>
<th>CCK18/CK18 ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 Day 3 Day 5 Day 7</td>
<td>Day 1 Day 3 Day 5 Day 7</td>
<td>Day 1 Day 3 Day 5 Day 7</td>
</tr>
<tr>
<td>HPc-</td>
<td>22.1 ± 2.2 23.2 ± 2.1 17.1 ± 1.9 2.1 ± 0.3</td>
<td>112.2 ± 12.2 358.0 ± 31.1 290.0 ± 35.5</td>
<td>52.5 ± 4.5 19.7 ± 2.4 6.5 ± 1.2 5.9 ± 1.1 4.0 ± 0.3</td>
</tr>
<tr>
<td>NPc-</td>
<td>20.3 ± 2.1 22.5 ± 2.0 16.3 ± 1.8 2.0 ± 0.4</td>
<td>110.5 ± 12.5 360.0 ± 33.3 286.0 ± 31.7</td>
<td>54.1 ± 4.0 18.4 ± 2.0 6.3 ± 1.1 5.7 ± 1.0 3.7 ± 0.2</td>
</tr>
</tbody>
</table>

HPc, hypoxia-conditioned; NPc, normoxia-preconditioned.
Figure 4.8  CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes subjected to 1-μM staurosporine and co-cultured with HPc-MSCs versus NPc-MSCs. HPc-MSCs co-culture significantly further reduced staurosporine-induced hepatocyte apoptosis and had no significant effect on total death of hepatocytes; HPc-MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis death mode switch to a similar extent as compared to NPc-MSCs co-culture. All data were expressed as mean ± SD; **P <0.01 versus control NPc-MSCs co-culture (n = 6). HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; Co, MSCs co-culture; SS, staurosporine.
4.4 Discussion

4.4.1 Effects of HPc on MSCs

MSCs are known to be highly sensitive to oxygen tension in the culture environment. Previous reports were controversial regarding hypoxic effects on MSCs, probably due to the variations in MSCs sources as well as hypoxic conditioning protocols. The variation in hypoxic effect on MSCs is most striking in cellular apoptosis: hypoxic conditioning was reported to be pro-apoptotic (Rasmussen et al., 2011), anti-apoptotic (Zhang et al., 2009), or without effect (Efimenko et al., 2011). Severe hypoxia will distort the morphology and ultrastructure of MSCs, especially the mitochondria and nucleus; however, moderate hypoxic atmosphere favours human MSCs expansion in vitro. Hypoxia normally results in a smaller size of MSCs containing a less number of mitochondria, but hypoxia alone does not induce apoptosis in MSCs, but serum or glucose deprivation will cause marked apoptotic death in MSCs. In the present work, 24-hour 2% O2 hypoxia had no cytotoxic effect on human AT-MSCs as shown by the morphological results consistent with a previous report (Fu et al., 2011). It is generally accepted that HPc-MSCs exhibit a higher survival rate when subsequently subjected to hypoxia or hypoxia-reoxygenation, mainly by attenuating apoptosis in MSCs. Low-dose oxidative preconditioning can protect MSCs from cellular apoptosis induced by high-dose oxidative stress (Li et al., 2009).

Hypoxia, as a major cause of mitochondrial oxidative stress, has a negative effect on mitochondrial metabolism. Hypoxia (1% O2) decreases human BM-MSCs mitochondrial activity by inhibiting expression of respiratory enzymes and oxygen consumption; under a hypoxic condition, MSCs depends mainly on anaerobic glycolysis for energy supply, accompanied by upregulated expression of glycolytic enzymes and increased production of lactate acid. Hypoxia also activates cell-matrix contact of MSCs. Hypoxia under 2% O2 upregulates expression of integrin subunits, including α1, α3, α5, α6, α11, β1, and β3, in human BM-MSCs (Saller et al., 2012). The HPc protocol used in the present work did inhibit mitochondrial dehydrogenase activity as evidenced by decreased MTT activity but improved cellular attachment as shown by the increased SRB activity.

HPc is reported to positively affect the colony-forming potential and proliferation of BM- and UC-MSCs (Martin-Rendon et al., 2007; Pilgaard et al., 2009), while hypoxia-mimetic agents can inhibit the hypoxic effect on MSCs proliferation by inducing a G1-phase cell cycle arrest (Zeng et al., 2011). BM-MSCs cultured under 2% O2 exhibit an advanced exponential growth phase and greater cell division kinetics (Dos Santos et al., 2010). Physiological hypoxia (1%–3% O2) enhances rat BM-MSCs proliferation, which manifests as increases in the ratio of S-phase cells, bromodeoxyuridine incorporation, and proliferating cell nuclear antigen expression, by upregulating phosphorylation of p38 MAPK and nuclear translocation of HIF-1α (Wang et al., 2013). Hypoxic condition also enhances the propagation ability of human BM-MSCs possibly by maintaining telomere length (Tsai et al., 2011). The 3H-thymidine incorporation assay in the present work demonstrated that 24-hour of 2%-O2 hypoxia promoted DNA synthesis in MSCs. The effect of hypoxia on overall protein synthesis was less reported in previous studies, although expression of
specific proteins was inhibited or activated in hypoxic MSCs (Abdollahi et al., 2011). The $^{14}$C-leucine incorporation assay in the present work showed that hypoxia slightly decreased overall protein synthesis. It is expected that MSCs synthesis of protein is inhibited under a hypoxic micro-environment as hypoxia switches the energy metabolism from oxidation phosphorylation to glycolysis; however, it is known that translation of protein generally requires a large amount of energy supply. It is likely that the stimulatory effect of hypoxia on MSCs protein synthesis will become evident after a longer time as reported in previous studies (Sengupta et al., 2010).

4.4.2 HPc potentiates trophic and protective effects of MSCs co-culture on hepatocytes by non-paracrine, ROS-dependent mechanisms

Enhanced angiogenesis of HPc-MSCs has been well documented in literature, especially in the setting of myocardial, limb and cerebral ischaemia. HPc upregulates expression of various pro-survival and pro-angiogenic factors, especially angiopoietin-1, erythropoietin, and VEGF in MSCs, transplantation of which consequently enhances angiogenesis as well as morphological and functional restoration of the infarcted myocardium (Hu et al., 2008). HPc also helps BM-MSCs to more effectively restore the blood flow in an experimental rat hind limb ischaemia model by improving revascularisation through activating HGF signalling (Rosová et al., 2008). Moreover, HPc AT-MSCs also significantly increased the viability and decreased apoptotic death of co-cultured or co-transplanted neural stem cells by downregulating Bax signalling in an experimental rat spinal cord ischaemia model (Oh et al., 2010). The present work demonstrated for the first time that HPc potentiated the hepatotrophic effect of MSCs on co-cultured hepatocytes with respect to liver-specific metabolic function in vitro.

Putative paracrine mechanisms are thought to primarily contribute to the potentiative effect of HPc on MSCs. HPc was reported to significantly upregulate expression of a large number of soluble cytokines and growth factors, such as HGF, VEGF, FGF2, IGF-1, and IL-6 in MSCs (Lam et al., 2010). HGF and VEGF are two candidate factors that have been most frequently studied. HPc significantly increases the secretion of both HGF and VEGF from human BM-MSCs into the culture medium; concentrated HPc-MSCs conditioned medium favours the regeneration of neurons in vivo by limiting neuronal apoptosis resulting from experimental traumatic brain injury in rats, and further improves motor and cognitive function of the rat model (Chang et al., 2013). Hsiao et al. (2013) reported that HPc enhanced paracrine angiogenic activity of human AT-MSCs through VEGF-A and angiogenin signalling pathways. HPc also increases human AT-MSCs secretion of VEGF and bFGF to the culture medium, and HPc-MSCs conditioned medium improves vitality and reduces apoptosis in human umbilical vein endothelial cells with enhanced tube formation in vitro (Liu et al., 2013). Yu et al. (2013) recently reported an interesting study regarding BM-MSCs therapy for an experimental rat extensive hepatectomy model. NPc-MSCs did improve hepatocyte proliferation in vivo but not liver regeneration or animal survival, while HPc-MSCs significantly enhanced liver regeneration and animal survival. Activation of VEGF signalling in HPc-MSCs was thought to be
the underlying mechanism as expression of VEGF signalling was significantly upregulated and the improved therapeutic effects could be diminished by VEGF neutralisation antibody. However, the present work showed that HPc minimally potentiate paracrine contribution to trophic and antiapoptotic effects of MSCs on co-cultured human hepatocytes. It was possible that isolated human hepatocytes became unresponsive to HPc-potentiated MSCs release of soluble trophic and antiapoptotic factors. Therefore, HPc-induced potentiation of MSCs co-culture hepatotrophic and antiapoptotic effects may result primary from enhanced ECM construction and heterotypic MSC-hepatocyte communication.

Intracellular ROS is known to finely modulate biological activities of MSCs. Exposure to moderate hypoxia is known to immediately increase intracellular ROS production in human BM-MSCs (Busletta et al., 2011), while pretreatment with NAC significantly inhibits increased ROS production and ameliorates oxidative stress associated cellular damages in hypoxic MSCs (Fan et al., 2011). NAC pretreatment improves the antioxidant capacity of human MSCs to restore the reduction-oxidation balance by eliminating intracellular ROS, elevating intracellular glutathione level, and enhancing cellular adhesion when exposed to in vitro oxidative stress (Wang et al., 2013). Oxidative stress resulting from low-level broadband visible light illumination was also found to stimulate the proliferative potential of BM-MSCs, in which production of ROS increased (Lipovsky et al., 2013). These findings demonstrate that ROS is a major factor mediating the effects of oxidative stress, such as hypoxia and ion radiation, on cellular proliferation, survival/apoptosis, and attachment of MSCs in vitro.

The present work showed that scavenging of increased ROS reversed HPc-induced increases in synthesis of DNA and membrane proteins of human AT-MSCs, implying that ROS are involved in regulating DNA synthesis and cellular attachment. However, the paradox that addition of NAC further significantly reduced mitochondrial dehydrogenase activity and protein synthesis suggested the possibility that a pre-existing, relatively higher intracellular ROS level facilitates MSCs recovery from moderate hypoxia and resistance to subsequent hypoxia/reoxygenation-induced inhibitive effect on mitochondrial activity and protein synthesis of MSCs (Wang et al., 2008). This also indicated the involvement of non-ROS-dependent mechanisms, such as HIF-1α, in HPc of MSCs. Upregulation of HIF-1α expression occurs at a late phase of HPc and mediates the cascades of hypoxic response (Busletta et al., 2011). Overexpression of HIF-1α enhances the survivability, attachment, migration, ECM synthesis, osteogenic differentiation and energy metabolism of MSCs (Palomäki et al., 2013).

The present work showed that HPc potentiated hepatotrophic and antiapoptotic effects of MSCs on co-cultured hepatocytes and NAC pretreatment diminished HPc-induced potentiative effects as evidenced by liver-specific metabolism and caspase-mediated hepatocyte apoptosis assays. Induction of ROS production accompanies phosphorylation of EGF receptor in human BM-MSCs, which can be antagonised by NAC treatment (Park et al., 2013). Aged AT-MSCs show a relatively limited angiogenic capacity associated with significant downregulation of VEGF, placental growth factor, and HGF, while HPc significantly upregulated expression of these pro-angiogenic factors and
restored angiogenesis of aged AT-MSCs (Efimenko et al., 2011). De Barros et al. (2013) also reported that aged human AT-MSCs had a relatively lower ROS level, while HPc could improve angiogenic capacity of aged MSCs; conversely NAC treatment eliminated HPc-induced potentiation of MSCs angiogenesis both in vitro and in vivo. It remains less investigated whether ROS also regulate ECM activity involved in trophic and antiapoptotic effects of MSCs on co-cultured hepatocytes. Chemically-induced oxidative stress was reported to upregulate expression of bone morphogenetic protein 2 and FGF2 in human AT-MSCs in an intra-MSCs ROS dependent manner (Moriyama et al., 2012). These two cytokines are well known to actively participate in ECM formation and modification of bone and cartilages (Krawczak et al., 2009).

4.4.3 Conclusions

In conclusion, as compared to NPc, HPc potentiated trophic effect of MSCs on co-cultured hepatocytes with respect to liver-specific metabolic function. HPc also enhanced antiapoptotic and protective effects of MSCs co-culture in an ROS-dependent manner as pretreatment with antioxidative NAC diminished HPc-induced potentiative effects. However, This potentiative effect might not result primarily from enhanced paracrine activities of MSCs regardless of the presence or absence of MSC-hepatocyte direct contact. It remains to be investigated whether HPc-induced potentiative effect on MSCs co-culture results from nonparacrine contribution of soluble factors and ECM secreted by MSCs by an ROS-dependent mechanism. Signalling pathways involved in apoptosis and survival of hepatocytes, such as caspases and BAX/BCL-2, will also be addressed in the next chapter.
CHAPTER 5 MECHANISMS OF MSCs CO-CULTURE HEPATOTROPHIC EFFECT AND HPC-INDUCED POTENTIATIVE EFFECT

5.1 Introduction

5.1.1 Contributive factors to hepatotrophic effect of MSCs co-culture

Trophic and protective effects of MSCs on co-cultured hepatocytes in vitro have been well documented in the present work and in previous studies. MSCs also have pronounced effects on hepatocyte proliferation, repair, and regeneration in physiological and pathological conditions in vivo (Esrefoglu, 2013). MSCs transplantation (Meier et al., 2013) and co-transplantation with hepatocytes (Joshi et al., 2012) are expected to be therapeutically effective for acute and chronic liver diseases. Putative mechanistic factors contributing to the hepatotrophic effects of MSCs co-culture in vitro mimic supportive factors for hepatocytes in vivo. These factors consist mainly of paracrine factors (soluble cytokines and growth factors), ECM, and heterotypic MSC-to-hepatocyte interaction (Figure 5.1; Gómez-Aristizábal et al., 2009).

Figure 5.1 Putative mechanisms of trophic effect of MSCs on co-cultured hepatocytes, including soluble cytokines, ECM, and direct cell contact (Gómez-Aristizábal et al., Mol Ther, 2009).

5.1.1.1 Soluble factors in MSCs co-culture

Soluble factors contributing to the trophic effect of MSCs on co-cultured epithelial cells and other cell lines, such as cardiomyocytes, hepatocytes, and islet cells, have been relatively well studied in the current literature. Injection with CM from Akt-overexpressing MSCs enriched with VEGF, FGF-2, HGF, IGF-I, and TB4 significantly shrank infarction size and improved ventricular function (Gnecchi et al., 2006). Gu and his colleagues (2009) reported that MSCs co-culture with hepatocytes significantly increased secretion of IL-6 rather than TGF-α or TNF-α; conversely IL-6 neutralisation significantly diminished the trophic effects of MSCs co-culture CM on liver-specific metabolism. Moreover, MSCs-derived soluble factors have supportive effect on transplanted cells
after transplantation in vivo. Park et al. (2010) demonstrated that HGF, IL-6, TGF-β, and VEGF-A were the trophic molecules of human BM- and UC-MSCs enhancing survival, function, and angiogenesis of isolated islets after transplantation. Overexpression of VEGF and HGF in MSCs significantly increased cardiomyocytes survival and peri-infarct vessel density in the border zone of acute myocardial infarction (Deuse et al., 2009). MSCs CM also protects hepatocytes from CCl₄-induced apoptosis as mediated by IL-6 signalling and consequent activation of fibroblast-like-protein 1 (Xagorari et al., 2013). Preliminary analysis of MSCs secretome using the proteomics technique showed out of all chemokines only heparin-bound eluent was effective for treating fulminant hepatic failure (Parekkadan et al., 2007). Interestingly, trophic factors may have an autocrine regulative effect on MSCs. Ex vivo treatment of human BM-MSCs with ischaemic rat brain extract containing high levels of brain-derived neurotrophic factor, VEGF, and HGF results in a further increase in production of these growth factors (Choi et al., 2010). The physiological activities of lipid microvesicles released from MSCs have been recently discovered but not well understood. Generally these microvesicles play a sophisticated role in transporting proteins, lipids, and RNAs to neighbouring cells (Baglio et al., 2012).

5.1.1.2 ECM in MSCs co-culture

ECM is historically known to play an essential role in maintaining and regulating hepatocyte survival, proliferation, maturation, regeneration, and metabolism (Depreter et al., 2000). ECM supports hepatocyte directly through cell-to-ECM interaction or indirectly through modulating release of growth factors, such as HGF (Schuppan et al., 1998) and bFGF (Sakakura et al., 1999). Minimal self-deposition of ECM may result in deterioration of cell survival and metabolic function in hepatocyte mono-culture due to the absence of cell-to-matrix interaction (Thomas et al., 2005); interruption of cellular attachment to ECM, such as laminin, fibronectin, and collagen type I and V by neutralising β1-integrin results in marked apoptosis of hepatocytes (Pinkse et al., 2004). Engineered bioscaffolds containing synthetic ECM, such as xyloglucan (Seo et al., 2005) have been developed to maintain and support hepatocytes both in vitro and in vivo. Co-culture of rabbit chondrocytes with allogeneic BM-MSCs at an optimal ratio (chondrocytes:MSC, 2:1) improves expression of type II collagen and aggrecan, two major components of cartilaginous ECM (Qing et al., 2011). MSCs co-encapsulation was also reported to synergistically enhance insulin secretion of pancreatic islets with ECM by upregulating expression of insulin I/II, glucagon, somatostatin, and pancreatic and duodenal HOX 1, and downregulating expression of CK19 and vimentin (Davis et al., 2012). Gu and his colleague (2009c) reported that mono-cultured hepatocytes deposited minimal ECM, such as fibronectin, laminin, and collagen type I, III, and V, while MSCs co-culture secreted fibronectin and collagen type I, as well as some laminin and collagen type V; further siRNA knockdown experiments validated that inhibition of fibronectin, laminin, and collagen type I and V compromised the hepatotrophic effect of MSCs on co-cultured hepatocytes with respect to liver-specific metabolic function. Interaction between MSCs and ECM also modulates capillary morphogenesis of vascular endothelial cells both in vitro and in vivo, suggesting the role of MSCs in
neoangiogenesis in addition to paracrine activity (Kniazeva et al., 2011). It is noted that MSCs have a biphasic modulatory effect on ECM formation of the liver (Zhao et al., 2012). Li et al. (2013) reported that BM-MSCs transplantation significantly decreased hydroxyproline content and collagen accumulation in an experimental liver fibrosis model, accompanied by upregulated expression of HGF, IL-10, VEGF, and MMP-9.

5.1.1.3 Cell-to-cell contact in MSCs co-culture

Epithelial-to-mesenchymal interaction (EMI) plays a paramount role in reciprocal regulation of biological activities involved in development and regeneration of the liver. Moreover, liver MSCs also promotes differentiation and maturation of hepatic progenitor cells by direct cell-to-cell contact (Hoppo et al., 2004; Ito et al., 2013). The contribution of cell-to-cell contact to trophic effect of MSCs co-culture is evidenced by the fact that direct contact co-culture is superior to indirect non-contact co-culture and MSCs-derived CM in the present work and previous studies. Jung et al. (2011) reported that contact between MSCs and islet cells was a major factor favouring survival, morphology maintenance, and insulin release of pancreatic islets in the presence of synergic regulation of inflammatory cytokine production. Gómez-Aristizábal and Davies (2012) also reported that human UC perivascular cells contact co-culture significantly improved ureagenesis of human hepatocytes as compared to non-contact co-culture. EMI is modulated by multiple growth factors, such as EGF, TGF-α, HGF, TGF-β, bFGF, platelet-derived growth factor β, and IGF-1; out of these factors IGF-1 is the key to EMI during gastric mucosal healing (Watanabe et al., 2000). This interaction is also modulated by membrane-associated ligand/receptor. The Notch signalling pathway is a highly conservative cellular signalling system regulating cell-to-cell contact and involved in remodelling liver progenitor cell niche in the setting of liver fibrosis (Spee et al., 2010). Human BM-MSCs expresses a high level of Notch ligand on contact with rat neural stem cells, which highly express the receptor NOTCH1 (Robinson et al., 2011). Moreover, human UC perivascular cells in co-culture with rat hepatocytes exhibited marked upregulation of HGF and Jagged1 (a ligand of NOTCH1 receptor) expression, suggesting the role of Notch-Jagged1 interaction in maintaining functional polarity of hepatocytes (Gómez-Aristizábal and Davies, 2013). Liver-regulating protein, which is widely expressed by BM-MSCs, hepatocytes, and liver NPCs, is thought to partially contribute to hepatotrophic effect of MSCs co-culture; blocking antibody against LRP will diminish the trophic effect of MSCs on co-cultured hepatocytes (Corlu et al., 1997). Conversely direct contact with distinct differentiated cells also plays a critical role in determining MSCs fate. Wang et al. (2006) reported that direct contact co-culture with rat cardiomyocytes rather than indirect non-contact co-culture or culture with CM drove rat BM-MSCs differentiation into cardiomyocytes or smooth muscle cells.
5.1.2 HPc-induced potentiative effect

5.1.2.1 HPc potentiates paracrine activity

MSCs exhibit a potentiated paracrine activity in response to hypoxia (Das et al., 2010), and HPc-potentiated paracrine effect of MSCs can improve the therapeutic benefit of MSCs transplantation (Cheng and Yau, 2008). Hypoxia triggers activation of Akt signalling pathway and consequently upregulates expression of genes encoding VEGF, FGF-2, HGF, IGF-1, and thymosin β4, enriched with which MSCs CM results in marked reduction of cardiomyocytes apoptosis (Gnecchi et al., 2006). Hypoxic challenge was reported to upregulate expression of MSC VEGF, FGF2, HGF, and IGF-1 by an ERK- and JNK-independent, NFκB-dependent, mechanism (Crisostomo et al., 2008). Hypoxic exposure also significantly increased secretion of VEGF, HGF, and bFGF, which depends on HIF, a pivotal signalling factor in hypoxic response (Tamama et al., 2011). After HPc, rat BM-MSCs exhibit significantly upregulated expression of pro-survival and pro-angiogenic factors, such as HIF-1, angiopoietin-1, VEGF and its receptor, Flk-1, and erythropoietin (Hu et al., 2008). HPc-MSCs CM shows a significantly greater protective effect on cardiomyocytes deprived of serum under 1%-O2 hypoxia as compared to NPc counterpart (Fidelis-de-Oliveira et al., 2012). HPc-MSCs enhanced skeletal muscle regeneration, to a greater extent as compared to NPc-MSCs, by improving blood flow and vascular formation in an experimental rat hind limb ischaemia model; the putative mechanism might be activation of Wnt4 gene (Leroux et al., 2010), encoding a secreted protein regulating myogenic proliferation. HPc also potentiates neurotrophic effects of rat BM-MSCs by upregulating expression of a series of trophic and growth factors, including brain- and glial cell-derived neurotrophic factor, VEGF and its receptor, erythropoietin and its receptor, SDF-1, and CXC chemokine receptor 4 (Wei et al., 2012). These HPc-potentiated paracrine mechanisms are likely to offer additional benefits with respect to neovascularisation and progenitor cell recruitment if transplanted in vivo. HPc increases secretion of angiogenic VEGF and bFGF from AT-MSCs, by which HPc-MSCs CM improves survival and tube formation of human umbilical vein endothelial cells (Liu et al., 2013). HPc stimulates secretion of proangiogenic and mitogenic factors and improved MSCs chemotaxis; upregulation of SFDF-1α expression in the ischaemic tissue selectively recruited HPc- rather than NPc-MSCs by a CXC receptor (CXCR) 7-independent, CXCR4-dependent manner (Liu et al., 2012).

5.1.2.2 HPc enhances formation and organisation of ECM

Microenvironmental oxygen tension finely modulates chondrogenesis and osteogenesis of MSCs by switching on and off MSCs formation of ECM. Hypoxic conditions stimulate MSCs to deposit multiple components of ECM and adhesion molecules. A previous genomic study demonstrated that HPc had a late-phase upregulative effect on expression of genes involved in ECM by up to 60 folds (Basciano et al., 2011). Hypoxia (5% O2) rather than normoxia (21% O2) in combination with TGF-β3 can induce chondrogenic differentiation of foetal synovium-derived MSCs in a serum free culture (Li et al., 2011). Expression of MMP-2, collagen type II and XI, aggrecan, and integrins α2 and β3 is significantly upregulated in rat MSCs subjected to TGF-β1 and
2%-O₂ hypoxia along with phosphorylation of MAPK/ERK1/2 signalling pathway (Risbud et al., 2004). IL-1β has an inhibitory effect on chondrogenesis, while hypoxic condition can reverse the inhibitory effects of IL-1β on BM-MSCs deposition of ECM (Felka et al., 2009). Stimulatory effect of hypoxia on MSCs deposition of ECM can also be maintained after transplantation in vivo. Feng et al. (2011) reported that HPc in combination with TGF-β1 drove rabbit MSCs differentiation towards a nucleus pulposus-like phenotype in vitro, as evidenced by significantly upregulated expression of aggregan, collagen type II, Sox-9, glycosaminoglycan, and HIF-1α; subcutaneous implantation experiment showed that HPc facilitated MSCs-scaffold construct to maintain chondrial morphology and prevent secondary calcification. Oxygen tension tunes MMPs and tissue inhibitor of MMPs in a differential manner; low oxygen tension inhibits expression of MMP-13 and tissue inhibitor of MMP-1 involved in ECM remodelling and vascular invasion, but has significant effects on expression of MMP-2, an enzyme involved in cell migration, in human MSCs (Raheja et al., 2010).

More interestingly, MSCs have a beneficial regulative effect on ECM remodelling in response to hypoxia. NPc- or HPc-MSCs co-culture downregulates expression of MMP and upregulates expression of tissue inhibitor of MMP-1 in cardiac fibroblasts subject to severe hypoxia (0.5% O₂) mediated by erythropoietin and its receptor as well as ERK1/2 signalling pathway (Wang et al., 2011).

5.1.2.3 HPc potentially augments cell-to-cell contact

Effect of hypoxia on interaction inbetween MSCs and between MSCs and other cell lines is rarely reported in literature. Connexins are a family of structurally related transmembrane proteins that assemble the gap junction and serve as the electrical coupling in the neuromuscular tissue. Long-term hypoxic culture of human MSCs significantly upregulates expression of connexin 43 (Cx43), a connexin family member mainly found in myocardium, along with enhanced ECM formation. The biological role of Cx43 is not well understood; knockdown of Cx43 will reduce cardiomyocyte survival and diminish protective effect of IGF-1 on HSCs (Lu et al., 2009). Overexpression of Cx43 significantly upregulates expression of Bcl-2, downregulates expression of Bax, increases phosphorylation of Akt signalling pathway in MSCs; Cx43-overexpressing MSCs shows an enhanced survival in infarcted myocardium (Wang et al., 2010). Enhanced expression of Cx43 in response to hypoxia accompanies expression of prosurvival and proangiogenic factors in MSCs (Chacko et al., 2010). Integrins are another collection of transmembrane receptors mediating adhesion between a cell and the neighbouring cells or ECM and transducing signals that regulate a number of biological activities, such as cellular survival, division, growth, differentiation, and apoptosis (Docheva et al., 2007). Overexpression of integrin-linked kinase enhances attachment of hypoxic MSCs to ischaemic myocardium (Song et al., 2009). It remains to be investigated whether hypoxia augments heterotypical interaction of MSCs with epithelial and other cells and how this augmented interaction improves supportive effect of MSCs.
5.1.3 Chapter objectives

- Contribution of TNF-α and TGF-β1

To investigate whether autocrine TNF-α activity of hepatocytes and autocrine TGF-β1 activity of MSCs mediate hepatotrophic effect of MSCs and potentiative effect of HPc induction.

- Contribution of collagen

To investigate whether extracellular collagen mediates hepatotrophic effect of MSCs and potentiative effect of HPc induction.

- Pro- and antiapoptosis-associated gene expression analysis

To characterise expression profiles of pro- and antiapoptosis-associated genes, such as caspase and BAX/BCL-2 signalling pathways, in MSCs co-culture and HPc co-culture as compared to those in mono-cultured hepatocytes.
5.2 Materials and methods

5.2.1 Cell culture protocols

5.2.1.1 Subculture and HPc of AT-MSCs and isolation of hepatocytes

Human AT-MSCs were subcultured as described in Section 2.2, Subculture of MSCs. P6–8 MSCs were subjected to 2%O₂ hypoxia (HPc-MSCs) or 20%O₂ normoxia (NPc-MSCs) for 24 h as described in Section 4.2.3, Optimisation of HPc. Non-steatotic donor liver tissues were processed as described in Section 2.3, Primary Harvest of Human Hepatocytes. Batches of hepatocytes with a viability of over 60% on trypan blue exclusion were used for experiments.

5.2.1.2 Direct co-culture of hepatocytes with MSCs

Fresh hepatocytes were co-cultured with HPc- versus NPc-MSCs as described in Section 4.2.4.1, Direct co-culture of hepatocytes with MSC. The seeding density of MSCs was 20,000 viable cells per cm², and that of hepatocytes was 50,000 viable cells per cm², at a MSC:hepatocyte ratio of 1:2.5. Mono-cultured hepatocytes were used as control, and mono-cultured HPc-/NPc-MSCs were used as blank controls.

5.2.1.3 Indirect co-culture of hepatocytes with MSCs

Hepatocytes were indirectly co-cultured with HPc- versus NPc-MSCs using Transwell® Permeable Supports, as described in Section 4.2.4.2, Indirect co-culture of hepatocytes with MSC, to investigate soluble factors underlying the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction.

The culture media were collected 24 h after culture. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for assays. Cell cultures were rinsed with one-wash PBS and cryopreserved at −80°C for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

5.2.2 Pretreatment experiments

5.2.2.1 NAC antagonisation experiment

HPc-MSCs were pretreated with 10-mM NAC as described in Section 4.2.6.1, Optimisation of N-acetylcysteine concentration. MSCs cultures were rinsed with one wash of PBS, and fresh hepatocytes were co-cultured with NAC-treated versus non-treated HPc-MSCs as described in Section 4.2.4.1, Direct co-culture of hepatocytes with MSC.

5.2.2.2 Staurosporine cytotoxicity experiment

Fresh hepatocytes were pre-treated with 1-μM staurosporine and co-cultured with NPc-, HPc-, and NAC-pretreated HPc-MSCs for 24 h, as controlled by staurosporine-treated mono-cultured hepatocytes.
5.2.2.3 TNF-α ELISA and neutralisation experiment

**TNF-α ELISA**  The Quantikine® human TNF-α ELISA immunoassay kit (R&D Systems Europe, Ltd., Abingdon, UK) with the solid-phase sandwich enzyme immunoassay technique was used to quantitate soluble TNF-α level in cell cultures. The 96-well (12 strips of 8 wells) polystyrene microplate is pre-coated with a mouse monoclonal antibody against human TNF-α. All reagents were brought to room temperature 20 min prior to use. TNF-α standard was reconstituted with deionised water to prepare a stock solution of 10,000 pg/mL 15 min prior to dilution, and was serially diluted with the diluted calibrator diluent RD6-35 (animal serum) to give the standards 1,000, 500, 250, 125, 62.5, 31.2, 15.6 pg/mL, whilst the calibrator diluent was used as the zero standard (0 pg/mL). Cell culture supernates were thawed at room temperature and centrifuged at 1,500 rpm for 1 min to pellet any cell debris or particulates. The assay diluent RD1F (buffered protein base), 50 μL per well, was added, and the standards, samples, and controls were incubated in the assigned wells, 200 μL per well, at room temperature for 2 h. The wells were rinsed with four washes of detergent buffer. The bound TNF-α was detected by TNF-α conjugate (polyclonal antibody against TNF-α conjugated to HRP), 100 μL per well, at room temperature for 60 min. The HRP detection antibody was removed, and the wells were rinsed with four washes of detergent buffer. The substrate solution containing stabilised hydrogen peroxide and stabilised chromogen TMB in an equal volume, 200 μL per well, was added, and the plate was incubated in the dark and at room temperature for 20 min. The enzymatic colour reaction was stopped by adding 2 N sulphuric acid, 50 μL per well. The OD was measured at 450 nm using the microplate reader. The TNF-α concentration (pg/mL) in each sample was determined using the TNF-α standard curve, and the reading of background hepatocyte culture medium (blank control) was subtracted from that of mono- or co-cultured hepatocytes or MSCs to obtain the colorimetric OD of hepatocyte or MSCs culture per se.

**TNF-α neutralisation experiment**  TNF-α neutralisation experiment was performed to investigate proapoptotic effect of TNF-α on mono-cultured hepatocytes. Human TNF-α affinity purified polyclonal antibody was purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom) and reconstituted at 1 mg/mL in sterile PBS. Freshly isolated primary human hepatocytes were seeded onto collagen-precoated 96-well plates at a density of 50,000 viable cells per cm² for a 24-h pre-incubation. The culture media were replaced by fresh hepatocyte culture media containing 0× (control), 1× (10 μg/mL), 2.5×, 5×, 10×, and 20× TNF-α neutralisation antibody for further 24-h mono-culture.

5.2.2.4 TGF-β ELISA and neutralisation experiment

**TGF-β1 ELISA**  The Quantikine® human TGF-β1 ELISA immunoassay kit (R&D Systems Europe, Ltd., Abingdon, UK) with the solid-phase sandwich enzyme immunoassay technique was used to quantitate soluble TGF-β1 level in cell cultures. The 96-well (12 strips of 8 wells) polystyrene microplate is pre-coated with a mouse monoclonal antibody against human TGF-β1. All reagents were brought to room temperature 20 min prior to use. TGF-β1 standard was reconstituted with deionised water to prepare a stock solution of 2,000 pg/mL 15 min prior to dilution, and was Ironically, the original focus on TNF-α was briefly lost during this section, which may have caused confusion. The parallel discussion of TGF-β1 should ideally have been presented with equal emphasis to maintain conceptual coherence. Further, the experimental design and procedural details for both TNF-α and TGF-β1 ELISAs should have been consistent to ensure a balanced approach to understanding their respective biological functions and how they modulate cell culture outcomes. This would have involved a careful examination of concentration ranges, dilution protocols, and the choice of control samples to accurately assess the impact of these cytokines on various cell cultures.
serially diluted with the diluted calibrator diluent RD5-53 (buffered protein base) to give the standards 1,000, 500, 250, 125, 62.5, 31.2, 15.6 pg/mL, whilst the calibrator diluent was used as the zero standard (0 pg/mL). Cell culture supernates were thawed at room temperature and centrifuged at 1,500 rpm for 1 min to pellet any cell debris or particulates. TGF-β1 is generally secreted as a latent form to the cell culture medium and becomes immunoreactive following acid activation and neutralisation. Briefly, the cell culture supernate (100 μL) was incubated with 1-N hydrochloride acid (20 μL; Sigma-Aldrich, St Louis, MO, USA) at room temperature for 10 min, and the acidified sample was neutralised by adding 1.2-N hydroxyl peroxide (20 μL; Sigma-Aldrich, St Louis, MO, USA) containing 0.5-M HEPES to a pH value of 7.2–7.6. The assay diluent RD1-21 (buffered protein solution), 50 μL per well, was added, and the standards, activated samples (diluted in 1.4 folds), and controls were incubated in the assigned wells, 50 μL per well, at room temperature for 2 h. The wells were rinsed with four washes of detergent buffer. The bound TGF-β1 was detected by TGF-β1 conjugate (polyclonal antibody against TGF-β1 conjugated to HRP), 100 μL per well, at room temperature for 2 h. The HRP detection antibody was removed, and the wells were washed with were rinsed with four washes of detergent buffer. The substrate solution containing stabilised hydrogen peroxide and stabilised chromogen TMB in an equal volume, 100 μL per well, was added, and the plate was incubated in the dark and at room temperature for 30 min. The enzymatic colour reaction was stopped by adding hydrochloride acid solution, 100 μL per well. The OD was measured at 450 nm using the microplate reader. The TGF-β1 concentration (pg/mL) in each sample was determined using the TGF-β1 standard curve, and the reading of background hepatocyte culture medium (blank control) was subtracted from that of mono- or co-cultured hepatocytes or MSCs to obtain the colorimetric OD of hepatocyte or MSCs culture per se.

**TGF-β neutralisation experiment**

TGF-β1 neutralisation experiment was performed to investigate whether soluble TGF-β1 secreted by MSCs contributed to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. Human TGF-β1 affinity purified polyclonal antibody was purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom) and reconstituted at 1 mg/mL in sterile PBS. P4–6 human AT-MSCs were seeded onto collagen-precoated 96-well plates at a density of 20,000 viable cells per cm² for a 24-h pre-incubation. The culture media were replaced by fresh hepatocyte culture media containing 0× (control), 1× (1 μg/mL), 2.5×, 5×, 10×, and 20× TGF-β1 neutralisation antibody. Mono-cultured MSCs were further subjected to HPc versus NPc for 24 h, and fresh hepatocytes were co-cultured with HPc- and NPc-MSCs for additional 24 h as described in Section 4.2.4.1, Direct co-culture of hepatocytes with MSC.

**5.2.2.5 Cellular collagen assay and inhibition experiment**

Cellular collagen was semi-quantitated using the colorimetric assay with picro-sirius red (PSR) staining. PSR specifically binds to hydroxyproline residuals enriched in collagen (up to 14% by weight). PSR was gifted by Dr Qihe Xu (Department of Renal Medicine, King’s College London) and reconstituted at 0.1% in saturated aqueous solution of picric acid (Sigma-Aldrich, St Louis, MO, USA). Cell cultures in chamber slides (Thermo Scientific Nunc,
Loughborough, UK) or 96-well plates were fixed in methanol (Sigma-Aldrich, St Louis, MO, USA) at -20°C overnight, and rinsed with two washes of PBS for 5 min. Cell cultures were stained in 0.1% PSR solution at room temperature for 4 h. The staining solution was discarded, and cell cultures were rinsed with three washes of 0.1% acetic acid for 5 min. PSR-stained cell cultures in chamber slides were dehydrated in three washes of absolute ethanol for 5 min, permeabilised in three washes of xylene for 10 min, and mounted with coverslips for light photomicrography. PSR-stained cell cultures in 96-well plates were eluted in 0.1 N sodium hydroxide (Sigma-Aldrich, St Louis, MO, USA), 200 μL per well, for 1 h for spectrophotometry. The OD was measured at 540 nm using the microplate reader.

**Soluble and extracellular collagen assays** Soluble and extracellular collagen was quantitated using the colorimetric assay with the Sircol™ soluble collagen assay kit (Biocolor, Carrickfergus, UK). Fresh (blank control) and thawed culture media, 1 mL per vial, were transferred into 1.5-mL low-protein-binding Eppendorf tubes (Eppendorf UK Limited, Stevenage, UK). The ice-cold isolation and concentration reagent containing Tris-HCl buffered polyethylene glycol (pH = 7.6) was added, 200 μL per vial, for an overnight incubation at 4°C. The tubes were centrifuged to pellet hydrated collagen at 12,000 rpm for 10 min, and the supernatant was removed using a 1-mL micropipette. The Sircol dye reagent containing PSR was added, 1 mL per vial, and the tubes were gently shaken for 30 min. The tubes were centrifuged to pellet the collagen-dye complex at 12,000 rpm for 10 min, and the supernatant was removed using a 1-mL micropipette. The ice-cold acid-salt wash reagent containing acetic acid, sodium chloride, and surfactants was gently layered on the collagen-dye pellet, 750 μL per vial, to remove unbound dye. The tubes were centrifuged to pellet the bound collagen-dye complex at 12,000 rpm for 10 min, and the supernatant was removed using a 1-mL micropipette. The alkali reagent containing 0.5-M sodium hydroxide was added, 250 μL per vial, to release the bound collagen-dye into the solution for 5 min. Samples were transferred to assigned wells of a clear-bottom 96-well plate, 200 μL per well. Extracellular collagen was solubilised by overnight incubation with 0.1 mg/mL pepsin (Sigma-Aldrich, St Louis, MO, USA) in 0.5-M acetic acid at 4°C. The acid extracts were neutralised by adding the acid neutralising reagent, 1 mL per vial, containing Tris-HCl buffered sodium hydroxide. Extracellular collagen was further stained using the same protocol, and collagen standards 0, 5, 10, and 15 μg reconstituted in 100-μL, 0.5-M acetic acid were also stained using the same protocol to produce the standard curve. The OD was measured at 540 nm using the microplate reader. The collagen content in each sample was determined using the collagen standard curve, and the reading of background hepatocyte culture medium (blank control) was subtracted from that of mono- or co-cultured hepatocytes or MSCs to obtain the colorimetric OD of hepatocyte or MSCs culture per se. The collagen content was normalised to that (mg) of 1 million hepatocytes or MSCs.

**Collagen inhibition experiment** N-(methylamino)isobutyric acid (MaIBA) is a competitive inhibitor of the neutral amino acid transport A system in collagen synthesis. HPc- and NPc-MSCs were subjected to 0-, 0.1-, 0.5-, 1-, 2.5-, 5-, 10-, 20-mM MaIBA (Sigma-Aldrich, St Louis, MO, USA) for 24 h. Cytotoxic effect of MaIBA on MSCs was evaluated using MTT and SRB
attachment assays as described in MTT assay was performed as described in Section 2.5, *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay* and Section 2.6, *Sulforhodamine B Colorimetric Assay*. Extracellular collagen deposit of MalBA-treated HPc- and NPc-MSCs was determined using the Sircol™ soluble collagen assay kit as described above. The least concentration of MalBA that resulted in significant reduction in extracellular collagen deposit of HPc- and NPc-MSCs was used for further inhibition experiment. MalBA-treated versus non-treated HPc- and NPc-MSCs were co-cultured with hepatocytes for 24 h as described in Section 5.2.1.2, *Direct co-culture of hepatocytes with MSCs*.

The culture media were collected 24 h after culture. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for further assays. Cell cultures were rinsed with one-wash PBS and cryopreserved at −80°C for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

### 5.2.3 Hepatocyte apoptosis and total death assays

CCK18 assay was performed as described in Section 2.9, *Caspase-cleaved CK18 Assay* to determine caspase-mediated hepatocyte apoptosis. CK18 assay was performed as described in Section 2.10, *CK18 Assay* to determine total hepatocyte death. The ratio of CCK18 to CK18 was also produced to determine the death mode of hepatocytes *in vitro*; a ratio of over 0.40 indicated that hepatocytes underwent apoptosis mainly, and necrosis *vice versa*. All experiments were performed in duplicate and repeated in triplicate independently.

### 5.2.4 Two-step, semi-quantitative, real-time polymerase-chained reaction assay

#### 5.2.4.1 Total RNA extraction

The Direct-zol™ RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA, USA) was used to isolate and purify RNA samples. Fresh cell cultures in 6-well plate were lysed by adding the TRIzol® reagent (Life Technologies Ltd., Paisley, UK), 1 mL per well. Cell sample homogenates were centrifuged at 12,000 × g for 1 min to remove any particulates and transferred into RNase-free Eppendorf tubes. One volume of absolute ethanol, molecular biology grade (Sigma-Aldrich, St Louis, MO, USA), was added into one volume of sample homogenate (1:1). The mixture was loaded into a Zymo-Spin™ IIC Column² and centrifuged in a collection tube at 12,000 × g for 1 min. The flow-through was discarded, and the Zymo-Spin™ column was transferred into a new collection tube. The concentrated (5 ×) RNA wash buffer was diluted with absolute ethanol and loaded into the Zymo-Spin™ column. The column was centrifuged 12,000 × g for 1 min, and the flow-through was discarded. The in-column DNase I digestion was performed using the DNase I cocktail (Promega, Madison, WI, USA) prepared as below (Table 5.1).
Table 5.1  In-column DNase I digestion cocktail recipe

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume per column (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilised DNase I (1 U/μL)</td>
<td>5</td>
</tr>
<tr>
<td>10× RNase-free DNase I reaction buffer</td>
<td>8</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>3</td>
</tr>
<tr>
<td>RNA wash buffer</td>
<td>64</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
</tr>
</tbody>
</table>

The cocktail was well mixed in an RNase-free Eppendorf tube by gentle inversion. The cocktail was directly loaded into the column matrix, 80 μL per column, and incubated on a heat block at 37°C for 15 min. The column was centrifuged at 12,000 × g for 30 sec. The Direct-zol™ RNA PreWash (5 ×, diluted in absolute ethanol) was loaded onto the column, 400 μL per column. The column was centrifuged at 12,000 × g for 1 min, and the flow-through was discarded. The step of prewash was repeated once. The RNA wash buffer was loaded onto the column, 700 μL per column, and the column was centrifuged at 12,000 × g for 1 min. The flow-through was discarded, and the column was centrifuged for additional 2 min. The column was transferred to an RNase-free Eppendorf tube. DNase/RNase-free water, 25 μL per column, was directly loaded into the column matrix, and the column was centrifuged at the maximum speed for 1 min. Additional 25-μL DNase/RNase-free water was added to further elute RNA sample. The quantity and purity of the eluted RNA sample were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA samples were reconstituted, at 1 μg for mono- or co-cultured hepatocytes and 0.5 per μg for mono-cultured MSCs per 26-μL final stock solution, in 0.2-mL PCR-clean Eppendorf tubes. An RNA sample with a ratio of the absorbance at 260 and 280 nm (A$_{260/280}$) above 2.0 was determined to be pure RNA (Okamoto and Okabe, Int J Mol Med, 2000). The reconstituted stock solutions were stored at -20°C for further experiments.

5.2.4.2 cDNA synthesis

RNA samples were thawed on ice and reversely transcribed into complement DNA (cDNA) using the Omniscript® Reverse Transcriptase kit (Qiagen, West Sussex, UK). The reverse transcription (RT) cocktail was prepared using the following recipe (Table 5.2).

Table 5.2  Reverse transcription cocktail recipe

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume per sample (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× RT buffer</td>
<td>4</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>4</td>
</tr>
<tr>
<td>Oligo dT primer</td>
<td>2</td>
</tr>
<tr>
<td>RNase out</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>2</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>1.5</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
</tr>
</tbody>
</table>
RNA samples were preheated at 65°C using a PCR thermal cycler (Thermo Scientific, Wilmington, DE, USA) for 5 min, and snap chilled on ice. The RT cocktail, 14 μL per vial, was added into the RNA sample, and the mixture was incubated at 37°C using the PCR thermal cycler for 60 min. The resultant cDNA samples were stored at -20°C for further experiments.

5.2.4.3 Semi-quantitative, real-time polymerase-chained reaction assay (qRT-PCR)

cDNA samples were thawed on ice and amplified using the TaqMan® PreAmp master mix kit (Applied Biosystems, Foster City, CA, USA). The qPCR Master Mix was prepared using the following recipe (Table 5.3).

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume per column (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® gene expression MasterMix</td>
<td>10</td>
</tr>
<tr>
<td>Primers</td>
<td>1</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
</tr>
</tbody>
</table>

The qPCR Master Mix was added into a chilled MicroAmp® fast optical 96-well reaction plate (Applied Biosystems, Foster City, CA, USA), 18 μL per well. The following proprietary primers (Table 5.4) for human gene expression assays were used and synthesised by Invitrogen (Paisley, UK). cDNA samples were added to the assigned wells, 2 μL per well, and well mixed by gentle pipetting.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Assay ID</th>
<th>Dye Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M (reference gene)</td>
<td>Hs00984230_m1</td>
<td>VIC</td>
</tr>
<tr>
<td>CASP3</td>
<td>Hs00234385_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>CASP8</td>
<td>Hs01018151_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>CASP9</td>
<td>Hs00154261_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>CASP14</td>
<td>Hs00201637_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>BAX</td>
<td>Hs00180269_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>BCL-2</td>
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<td>FAM</td>
</tr>
<tr>
<td>BID</td>
<td>Hs00609632_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>BLK</td>
<td>Hs00176441_m1</td>
<td>FAM</td>
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Semi qRT-PCR assay was performed using the ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: uracil-DNA glycosylase was activated at 50°C for 2 min; AmpliTaq® gold enzyme was activated at 50°C for 2 min; and cDNA template was denatured at 95°C for 15 sec and annealed/extended at 60°C for 1 min, for a total of 40 cycles. Cycle threshold (Ct) was produced with an automatic threshold using the Sequence Detection Software version 1.2.3 with 7000 System SDS Software RQ Study Application (Applied Biosystems, Foster City, CA, USA).
The qPCR results were analysed using the semi-quantitative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, Methods, 2001) with the following formulae $\Delta\Delta Ct = \Delta Ct_{(treated)} - \Delta Ct_{(control)}$, where $\Delta Ct_{(treated/control)} = Ct_{(target\ gene)} - Ct_{(reference\ gene)}$, and expressed as mRNA expression level (fold) relative to the control (mono-cultured hepatocytes). A $2^{-\Delta\Delta Ct}$ value higher than 1 fold indicates upregulated expression of the target gene, and vice versa. All experiments were performed in duplicate and repeated in triplicate independently.
5.3 Results

5.3.1 Contribution of TNF-α and TGF-β to MSCs co-culture and HPc-induced potentiation

5.3.1.1 TNF-α secretion and neutralisation

Mono-cultured hepatocytes secreted a high level of TNF-α, while NPc-, HPc-, and NAC-pretreated HPc-MSCs secreted no detectable (below the lower assay limit) TNF-α (Figure 5.2A). Direct co-culture with NPc-MSCs significantly decreased hepatocyte secretion of TNF-α as compared to hepatocyte mono-culture, while indirect co-culture decreased TNF-α secretion to a significantly lesser extent (mono- vs. direct co- vs. indirect co-culture, 154.5 ± 12.6 vs. 10.6 ± 1.3 vs. 134.1 ± 12.6 pg/mL, P <0.01). Co-culture with HPc-MSCs significantly further reduced hepatocyte secretion of TNF-α as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly increased TNF-α secretion (NPc- vs. HPc- vs. NAC-treated HPc-MSCs co-culture, 10.6 ± 1.3 vs. undetectable vs. 10.3 ± 1.6 pg/mL, P <0.01).

TNF-α neutralisation even at the lowest dose (1×, 10 μg/mL) significantly inhibited CCK18 release from mono-cultured hepatocytes (0× vs. 1×, 64.6 ± 6.4 vs. 40.4 ± 6.2 U/10^6 hepatocytes, P <0.01), while TNF-α neutralisation at higher doses did not significantly further inhibit apoptosis of mono-cultured hepatocytes (Figure 5.2B). Similarly, TNF-α neutralisation at 2.5× significantly suppressed total death of hepatocytes, although that at 1× showed no significant effect (0× vs. 1× vs. 2.5×, 114.5 ± 7.2 vs. 105.9 ± 8.1 vs. 99.2 ± 7.3 U/10^6 hepatocytes, P <0.01; Figure 5.2C). TNF-α neutralisation switched the death mode of mono-cultured hepatocytes from apoptosis to necrosis (0× vs. 1× vs. 2.5×, 56.4% ± 4.5% vs. 38.1% ± 5.9% vs. 38.7% ± 4.8%, P <0.01; Figure 5.2D).

5.3.1.2 TGF-β1 secretion and neutralisation

Mono-cultured hepatocytes and MSCs secreted high levels of TGF-β1. HPc significantly increased MSCs secretion of TGF-β1, while NAC pretreatment significantly decreased TGF-β1 secretion (NPc vs. HPc vs. HPc+NAC, 172.5 ± 15.1 vs. 784.0 ± 35.0 vs. 596.9 ± 46.0 pg/mL, P <0.01; Figure 5.3A). Direct co-culture with NPc-MSCs significantly increased TGF-β1 secretion as compared to hepatocyte or MSCs mono-culture, while indirect co-culture increased TGF-β1 secretion to a significantly lesser extent (mono- vs. direct co- vs. indirect co-culture, 369.5 ± 19.6 vs. 871.0± 44.6 vs. 370.3 ± 22.4 pg/mL, P <0.01). Co-culture with HPc-MSCs further increased TGF-β1 secretion as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly decreased TGF-β1 secretion (NPc- vs. HPc- vs. NAC-treated HPc-MSCs co-culture, 871.0± 44.6 vs. 1,370.8 ± 101.0 vs. 1,017.7 ± 64.1 pg/mL, P <0.01).

TGF-β1 neutralisation starting from 5× (1× = 1 μg/mL) significantly diminished antiapoptotic effect of NPc- and HPc-MSCs co-culture (0× vs. 5×, NPc: 35.2 ± 2.4 vs. 45.0 ± 1.8 U/10^6 hepatocytes, P <0.01; HPc: 32.0 ± 2.2 vs. 40.4 ± 2.8 U/10^6 hepatocytes, P <0.01; Figure 5.3B). Similarly, TGF-β1 neutralisation at 5× significantly diminished prosurvival effect of NPc- and HPc-
Figure 5.2  TNF-α ELISA and neutralisation experiments. TNF-α secretion of mono-/co-cultured hepatocytes (A); effects of autocrine TNF-α neutralisation on apoptosis (B), total death (C), and death mode (D) of mono-cultured hepatocytes. All data were expressed as mean ± SD; **P <0.01 versus control mono-/co-culture; ^^P <0.01 versus non-NAC treated; ***P <0.01 versus control mono-culture; +++P <0.01 versus direct co-culture. Hx, mono-cultured hepatocytes; UD, undetectable; iCo, indirect co-culture; α-TNF, anti-TNF.
Figure 5.3  TGF-β1 ELISA and neutralisation experiments. TGF-β1 secretion of mono-/co-cultured hepatocytes and NPc-/HPc-MSCs (A); effects of MSCs TGF-β1 neutralisation on apoptosis (B), total death (C), and death mode (D) of co-cultured hepatocytes. All data were expressed as mean ± SD; *P <0.05 and **P <0.01 versus control mono- or co-culture; ^^P <0.01 versus non-NAC treated; ^P <0.01 versus control mono-culture; ^^P <0.01 versus direct co-culture. Hx, mono-cultured hepatocytes; UD, undetectable; iCo, indirect co-culture; α-TGF, anti-TGF.
MSCs co-culture (0× vs. 5×, NPC: 89.5 ± 8.2 vs. 96.3 ± 9.9 U/10⁶ hepatocytes, P <0.05; HPC: 80.5 ± 7.1 vs. 91.2 ± 7.4 U/10⁶ hepatocytes, P <0.05; Figure 5.3C). TGF-β1 neutralisation resulted in a necrosis-to-apoptosis switch in death mode of hepatocytes co-cultured with NPC-MSCs starting from 1× (0× vs. 1×, 39.3% ± 3.3% vs. 44.7% ± 3.6%, P <0.05) and with HPC-MSCs from 10× (0× vs. 10×, 39.8% ± 4.1% vs. 46.6% ± 3.4%, P <0.05; Figure 5.3D).

5.3.2 Contribution of cellular and extracellular collagen

5.3.2.1 Cellular and extracellular collagen content

Hepatocytes expressed some cellular rather than extracellular collagen in mono- (data not shown) and co-culture (Figure 5.4A), while MSCs secreted massive cellular and extracellular collagen in both mono- (Figure 5.4B) and co-culture (Figure 5.4A).

HPc significantly increased MSCs expression of cellular collagen, while NAC pretreatment significantly decreased cellular collagen expression (NPC vs. HPC vs. HPC+NAC, 0.19 ± 0.04 vs. 0.31 ± 0.06 vs. 0.24 ± 0.04 OD units, P <0.01; Figure 5.5A). Direct co-culture with NPC-MSCs significantly increased cellular collagen expression as compared to hepatocyte or MSCs mono-culture, while indirect co-culture increased cellular collagen expression to a significantly lesser extent (mono- vs. direct co- vs. indirect co-culture, 0.07 ± 0.01 vs. 0.30 ± 0.06 vs. 0.22 ± 0.03 OD units, P <0.01). Co-culture with HPC-MSCs further increased cellular collagen expression as compared to that with NPC-MSCs, while co-culture with NAC-pretreated HPC-MSCs significantly decreased cellular collagen expression (NPC- vs. HPC- vs. NAC-treated HPC-MSCs co-culture, 0.30 ± 0.06 vs. 0.55 ± 0.04 vs. 0.33 ± 0.04 OD units, P <0.01).

Mono-cultured hepatocytes secreted minimal extracellular collagen (below the lower assay limit), while MSCs deposited extracellular collagen (Figure 5.5B). HPC significantly increased MSCs deposition of extracellular collagen, while NAC pretreatment significantly decreased
extracellular collagen deposition (NPc vs. HPc vs. HPc+NAC, 0.39 ± 0.04 vs. 0.56 ± 0.06 vs. 0.40 ± 0.02 mg/10^6 cells, P < 0.01). Direct co-culture with NPc-MSCs significantly increased extracellular collagen deposition as compared to hepatocyte or MSCs mono-culture, while indirect co-culture increased extracellular collagen deposition to a significantly lesser extent (mono- vs. direct co- vs. indirect co-culture, 0.39 ± 0.04 vs. 2.89 ± 0.26 vs. 0.41 ± 0.02 mg/10^6 cells, P < 0.01). Co-culture with HPc-MSCs further increased cellular collagen expression as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly decreased cellular collagen expression (NPc- vs. HPc- vs. NAC-treated HPc-MSCs co-culture, 2.89 ± 0.26 vs. 5.97 ± 0.53 vs. 3.30 ± 0.30 mg/10^6 cells, P < 0.01).

5.3.2.2 Effects of MaIBA on NPc- and HPc-MSCs co-culture

MaIBA at a dose range from 0.1 to 20 mM had no toxic effect on MSCs with respect to mitochondrial dehydrogenase activity and cellular attachment (Table 5.5). MaIBA pretreatment at 5, 10, and 20 mM significantly inhibited extracellular collagen deposition of NPc- and HPc-MSCs (0 mM vs. 5 mM vs. 10 mM vs. 20 mM, NPc: 0.39 ± 0.04 vs. 0.33 ± 0.03 vs. 0.32 ± 0.03 vs. 0.30 ± 0.02 mg/10^6 cells, P < 0.01, Figure 5.5C; HPc: 0.56 ± 0.06 vs. 0.45 ± 0.05 vs. 0.44 ± 0.04 vs. 0.42 ± 0.04 mg/10^6 cells, P < 0.01; Figure 5.5D); therefore, 5-mM MaIBA was used for further collagen inhibition experiment.

<table>
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<th>SRB (OD unit)</th>
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<tr>
<td>0</td>
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<tr>
<td>0.1</td>
<td>0.33 ± 0.02</td>
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<td>0.5</td>
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<td>1</td>
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<td>0.50 ± 0.03</td>
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<td>5</td>
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<td>20</td>
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Pretreatment of 5-mM MaIBA significantly inhibited extracellular collagen deposition of hepatocytes co-cultured with NPc- and HPc-MSCs (0 mM vs. 5 mM, NPc: 2.89 ± 0.26 vs. 2.03 ± 0.28 mg/10^6 cells, P < 0.01; HPc: 5.97 ± 0.23 vs. 4.47 ± 0.29 mg/10^6 cells, P < 0.01; Figure 5.6A). MaIBA pretreatment significantly diminished antiapoptotic effect of MSCs co-culture (0 mM vs. 5 mM, 18.5 ± 2.4 vs. 25.5 ± 2.5 U/10^6 hepatocytes, P < 0.01) and potentiative effect of HPc induction (14.5 ± 2.3 vs. 20.8 ± 2.1 U/10^6 hepatocytes, P < 0.01; Figure 5.6B). However, MaIBA pretreatment had no significant prosurvival effect of MSCs co-culture and potentiative effect of HPc induction (Figure 5.6C). MaIBA pretreatment resulted in a necrosis-to-apoptosis switch in hepatocytes co-cultured with NPc-MSCs (30.2% ± 3.2% vs. 38.9% ± 4.5%, P < 0.01) and with HPc-MSCs (28.1% ± 3.0% vs. 35.4% ± 4.5%, P < 0.01; Figure 5.6D).
Figure 5.5  **Cellular and extracellular collagen assays and collagen inhibition experiment.** Cellular (A) and extracellular collagen (B) content of mono-/co-cultured hepatocytes and NPc-/HPc-MSCs; inhibitory effect of 0- to 20-mM MaIBA on extracellular collagen deposit of NPc- (C) and HPc-MSCs (D). All data were expressed as mean ± SD; *P <0.05 and **P <0.01 versus control mono- or co-culture; ^^P <0.01 versus non-NAC treated; ^^P <0.01 versus control mono-culture; ++P <0.01 versus direct co-culture. Hx, mono-cultured hepatocytes; UD, undetectable; Co, co-culture; iCo, indirect co-culture.
Figure 5.6 Effects of 5-mM MalBA pretreatment on extracellular collagen deposit (A), cellular apoptosis (B), total death (C), and death mode (D) of hepatocytes co-cultured with NPC-versus HPc-MSCs. All data were expressed as mean ± SD; **P <0.01 versus NPC-Co. Co, co-culture.

5.3.3 Pro- and antiapoptosis-associated gene expressions

5.3.3.1 CASP9

Expression of CASP3, CASP8, and CASP14 mRNA was undetectable in mono- or co-cultured hepatocytes. MSCs co-culture significantly downregulated expression of CASP9 mRNA (0.72 ± 0.07 fold, P <0.01), while indirect co-culture slightly downregulated CASP9 mRNA expression (0.90 ± 0.07 fold, P >0.0; Figure 5.7A). HPc further downregulated CASP9 mRNA expression in MSCs co-culture (0.55 ± 0.05 fold, P <0.01), while NAC pretreatment diminished HPc-induced further downregulation (0.71 ± 0.07 fold, P <0.01). Additionally, staurosporine pretreatment significantly upregulated expression of CASP9 mRNA in mono-cultured hepatocytes (1.44 ± 0.12 folds, P <0.01), while MSCs co-culture significantly decreased staurosporine-induced upregulation of CASP9 mRNA expression (0.90 ± 0.07 fold, P <0.01).

5.3.3.2 BAX, BCL-2, BAX/BCL-2 ratio, and BID

MSCs co-culture significantly downregulated expression of BAX mRNA (0.39 ± 0.11 fold, P <0.01), while indirect co-culture slightly downregulated BAX mRNA expression (0.83 ± 0.17 fold, P >0.05; Figure 5.7B). HPc further downregulated BAX mRNA expression in MSCs co-culture (0.12 ± 0.03 fold, P <0.01), while NAC pretreatment diminished HPc-induced further downregulation (0.26 ± 0.10 fold, P <0.01). Additionally, staurosporine pretreatment significantly upregulated
expression of BAX mRNA in mono-cultured hepatocytes (1.37 ± 0.24 folds, *P* <0.01), while MSCs co-culture significantly decreased staurosporine-induced upregulation of BAX mRNA expression (0.65 ± 0.17 fold, *P* <0.01).

MSCs co-culture significantly upregulated expression of BCL-2 mRNA (1.28 ± 0.17 fold, *P* <0.01), while indirect co-culture slightly downregulated BCL-2 mRNA expression (0.95 ± 0.10 fold, *P* >0.05; Figure 5.7C). HPc further upregulated BCL-2 mRNA expression in MSCs co-culture (1.86 ± 0.26 folds, *P* <0.01), while NAC pretreatment diminished HPc-induced further upregulation (1.34 ± 0.09 folds, *P* <0.01). Additionally, staurosporine pretreatment significantly downregulated expression of BCL-2 mRNA in mono-cultured hepatocytes (0.58 ± 0.17 fold, *P* <0.01), while MSCs co-culture significantly increased staurosporine-induced downregulation of BCL-2 mRNA expression (0.86 ± 0.10 fold, *P* <0.01).

MSCs co-culture significantly decreased BAX/BCL-2 ratio (0.30 ± 0.07 fold, *P* <0.01), while indirect co-culture slightly decreased BAX/BCL-2 ratio (0.88 ± 0.12 fold, *P* >0.05; Figure 5.7D). HPc further decreased BAX/BCL-2 ratio in MSCs co-culture (0.06 ± 0.01 fold, *P* <0.01), while NAC pretreatment diminished HPc-induced further decrease (0.20 ± 0.07 fold, *P* <0.01). Additionally, staurosporine pretreatment significantly increased BAX/BCL-2 ratio in mono-cultured hepatocytes (2.36 ± 0.22 folds, *P* <0.01), while MSCs co-culture significantly decreased staurosporine-induced BAX/BCL-2 ratio increase (0.76 ± 0.12 fold, *P* <0.01).
Figure 5.7  Relative mRNA expression levels of pro- and antiapoptosis-associated genes in mono- and co-cultured hepatocytes: *CASP9* (A), *BAX* (B), *BCL-2* (C), BAX/BCL-2 ratio (D), *BID* (E), and *BLK* (F). All data were expressed as mean ± SD; **P < 0.01 versus control hepatocyte mono-culture; §§P < 0.01 versus SS-treated, mono-cultured hepatocytes; §§§P < 0.01 versus SS-treated, mono-cultured hepatocytes; ++P < 0.01 versus NPc co-culture; +P < 0.05 and ++P < 0.01 versus non-NAC-treated HPc co-culture. Hx, mono-cultured hepatocytes; SS, staurosporine; Co, co-culture; iCo, indirect co-culture.
MSCs co-culture significantly downregulated expression of \textit{BID} mRNA (0.25 ± 0.13 fold, \(P < 0.01\)), and indirect co-culture also significantly downregulated \textit{BID} mRNA expression to a significantly lesser extent (0.65 ± 0.12 fold, \(P < 0.01\); Figure 5.7E). HPc further downregulated \textit{BID} mRNA expression in MSCs co-culture (0.10 ± 0.04 fold, \(P < 0.01\)), while NAC pretreatment diminished HPc-induced further downregulation (0.15 ± 0.02 fold, \(P < 0.01\)). Additionally, staurosporine pretreatment significantly upregulated expression of \textit{BID} mRNA in mono-cultured hepatocytes (1.46 ± 0.17 folds, \(P < 0.01\)), while MSCs co-culture significantly decreased staurosporine-induced upregulation of \textit{BID} mRNA expression (0.53 ± 0.12 fold, \(P < 0.01\)).

5.3.3.3 \textit{BLK}

MSCs co-culture significantly downregulated expression of \textit{BLK} mRNA (0.48 ± 0.05 fold, \(P < 0.01\)), and indirect co-culture also significantly downregulated \textit{BLK} mRNA expression to a significantly lesser extent (0.63 ± 0.05 fold, \(P < 0.01\); Figure 5.7F). HPc further downregulated \textit{BLK} mRNA expression in MSCs co-culture (0.32 ± 0.06 fold, \(P < 0.01\)), while NAC pretreatment diminished HPc-induced further downregulation (0.44 ± 0.12 fold, \(P < 0.01\)). Additionally, staurosporine pretreatment significantly upregulated expression of \textit{BLK} mRNA in mono-cultured hepatocytes (1.69 ± 0.24 folds, \(P < 0.01\)), while MSCs co-culture significantly decreased staurosporine-induced upregulation of \textit{BLK} mRNA expression (0.61 ± 0.05 fold, \(P < 0.01\)).

5.3.3.4 \textit{Summary of gene expression assays}

Direct MSCs co-culture significantly downregulated expression of proapoptotic \textit{CASP9}, \textit{BAX}, and \textit{BID} mRNA and significantly upregulated expression of antiapoptotic \textit{BCL-2} mRNA. In contrast, indirect MSCs co-culture significantly downregulated expression of \textit{BID} and \textit{BLK} mRNA to a significantly lesser extent only. HPc further significantly downregulated expression of \textit{CASP9}, \textit{BAX}, \textit{BID}, and \textit{BLK} mRNA and significantly upregulated expression of \textit{BCL-2} mRNA in MSCs co-culture; however, NAC pretreatment diminished HPc-induced down- and upregulation of the respective genes. Additionally, staurosporine pretreatment culture significantly upregulated expression of \textit{CASP9}, \textit{BAX}, \textit{BID}, and \textit{BLK} mRNA and significantly downregulated expression of \textit{BCL-2} mRNA, while MSCs co-culture diminished staurosporine-induced down- and upregulation of the respective genes.
5.4 Discussion

5.4.1 MSCs inhibit autocrine TNF-α activity of co-cultured hepatocytes

TNF-α is a pleiotropic cytokine chiefly secreted by activated immune cells, and plays a central role in inflammation and apoptosis. TNF-α is known to mediate LPS-induced hepatocyte apoptosis, manifesting as DNA fragmentation and cytoplasmic translocation of alanine aminotransferase, which can be antagonised by pretreatment with IL-1β (Leist et al., 1995). TNF-α induced hepatocyte apoptosis depends on caspase signalling pathway, including activation of caspases-2 (Guicciardi et al., 2005), -8 (Imao et al., 2006), and -9 (Imao et al., 2006). Hepatocytes secrete a high level of TNF-α (600 pg/mL) within 4 h if challenged with LPS (Saad et al., 1995). However, TNF-α inhibitors suppress hepatocyte proliferation in response to mitogen (Kubo et al., 1996), while stimulatory effect of TNF-α on hepatocytes may result from liver NPCs response to TNF-α (Shinozuka et al., 1996). It was also reported that hepatocytes resisted TNF-α induced apoptosis by a mechanism dependent on pre-existing intracellular glutathione, a major effector reducing ROS (Xu et al., 1998). This bidirectional regulation of TNF-α on epithelial cells may be modulated by other extrinsic and/or intrinsic factors, such as redox (Kim et al., 2000), NF-κB (Nagaki et al., 2000), EGFR (Argast et al., 2004), and HGF (Grant-Tschudy and Wira, 2005).

The present work showed that mono-cultured hepatocytes secreted a baseline level of autocrine TNF-α (approximately 640 pg/10^6 hepatocytes/24 h), and TNF-α neutralisation significantly decreased spontaneous apoptosis and total death of mono-cultured hepatocytes. MSCs expressed no TNF-α and inhibited autocrine activity of TNF-α in co-cultured hepatocytes. This finding was contradictory to that reported by Gu et al. (2009), in which TNF-α was not expressed in mono- or co-cultured hepatocytes. This inconsistency might derive from the differences in source of hepatocytes (human versus porcine) and co-culture protocol (layered versus mixed). Shi et al. (2011) reported that MSCs co-culture protected hepatocytes from apoptosis induced by exposure to acute-on-chronic liver failure serum containing a high level of TNF-α. It was also reported that TNF-α treatment significantly upregulated expression of VEGF, FGF2, HGF, and IGF-1 in human MSCs by an NF-κB-independent, JNK-independent mechanism (Crisostomo et al., 2008). The present work demonstrated for the first time that trophic and protective effects of MSCs on co-cultured hepatocytes might result from inhibition of proapoptotic TNF-α in addition to expression of trophic factors by MSCs. However, this finding should be cautiously explained as MSCs co-culture CM containing a very low level of TNF-α could not improve liver-specific metabolism or protect monocol cultured hepatocytes from spontaneous apoptosis, suggesting the role of synergistic factors aside from TNF-α. The present work showed that inhibitory effect of MSCs co-culture on autocrine TNF-α activity of hepatocytes depended on MSC-to-hepatocyte contact as evidenced by the minimal effect of indirect non-contact co-culture. This inhibitory effect could also be potentiated by HPc in an intra-MSCs ROS activity dependent manner as NAC pretreatment diminished the potentiative effect of HPc induction.
5.4.2 Hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction depend on autocrine TGF-β activity of MSCs

TGF-β is a polypeptide secreted by multiple cell lines, including platelets, macrophages, fibroblasts, and mesenchymal stem cells, and involved in a large number of biological activities, such as cell growth, cell proliferation, cell differentiation, and apoptosis (Ng et al., 2008). TGF-β plays a crucial role in regulating MSCs differentiation and promotes chondrogenic differentiation of human MSCs by upregulating expression of SOX9, type II collagen, and aggrecan (Miyanishi et al., 2006). Synergistic treatment of IL-1β and TGF-β1 can also increase MSCs secretion of VEGF and improve cardioprotective effect of MSCs transplantation (Luo et al., 2012). TGF-β1 is also believed to be a major trophic factor released from MSCs for the host cells. MSCs maintain phenotype and pluripotency of human ES cells through TGF-β and FGF receptor in response to bFGF (Montes et al., 2009).

Generally, TGF-β has a negative effect on biological activities of hepatocytes. TGF-β inhibits hepatocyte DNA synthesis, proliferation, differentiation, and liver-specific metabolism. TGF-β is well known as a potent stimulant that induces EMT. TGF-β treatment in vitro can induce EMT in human foetal hepatocytes accompanied by upregulation of Snail signalling (mesenchymal cell marker) and downregulation of E-cadherin (epithelial cell marker) expression (Caja et al., 2011). Abnormal TGF-β expression is believed to be a major etiology of liver fibrosis (Dooley et al., 2008; Ciucu et al., 2010). However, TGF-β1 was reported to be required for functional enhancement in hepatocytes co-cultured with NIH/3T3 cells as this functional enhancement could be eliminated by TGF-β1 depletion and restored by TGF-β1 reconstitution (Chia et al., 2005). This paradox might result from the complex interplays between soluble factors and MSCs/hepatocytes.

The present work showed that both hepatocytes and MSCs secreted a baseline level of TGF-β1, while co-culture of hepatocytes with MSCs exhibited an additive or synergistic effect with respect to TGF-β1 production. This additive or synergistic effect depended on MSC-to-hepatocyte contact as indirect non-contact co-culture only secreted a similar level of TGF-β1 to direct co-culture. This finding was consistent with the previous report (Chia et al., 2005). HPc potentiated MSCs secretion of TGF-β1 in both mono- and co-culture by an intracellular ROS-dependent manner as evidenced by the reversal effect of NAC pretreatment. It has recently been reported that hypoxia increases MSCs secretion of TGF-β1 and promotes breast cancer cell progression, and the major hypoxia-regulated element is determined to be HIF-1 binding to the hypoxia response element of TGF-β1 promoter (Hung et al., 2013). The present work also demonstrated that autocrine TGF-β1 activity of MSCs was required for the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction as evidenced by neutralisation of MSCs-derived TGF-β1. As MSCs co-culture CM containing high-level TGF-β1 had no significant hepatotrophic and antiapoptotic effect, autocrine TGF-β1 of NPc- and HPc-MSCs might not act on hepatocytes through ECM and/or cell-to-cell contact rather than a paracrine mechanism. TGF-β activation is reported to mediate the crosstalk between hepatocytes and the stromal niche in the setting of HCV infection (Benzoubir et al., 2013).
5.4.3 Hepatotropic effect of MSCs co-culture and potentiative effect of HPc induction depend on MSCs deposition of extracellular collagen

Collagen is the major component and the most abundant protein in ECM. The main biological function of collagen, especially extracellular form, is to support resident cells in the form of fibrillar protein. In physiological conditions, liver collagen is mainly produced by hepatic NPCs rather than hepatocytes, while pathological hepatocytes deposit collagen in response to profibrotic factors, such as TGF-β (Vadasz et al., 2005). The present work confirmed that hepatocytes secreted little extracellular collagen; however, hepatocytes were reported to produce some collagenous components, such as collagen type I, II, III, and V (Diegelmann et al., 1983). Collagen attachment is known to support hepatocyte with respect to metabolic function; as a result, monolayer collagen coating and modified collagen gel “sandwich” culture are widely used for hepatocyte culture and bioartificial liver system (Wang et al., 2004). Maintenance effect of collagen was thought to result from persistent phosphorylation of HGF and EGF receptors (Engl et al., 2004). Extracellular collagen also regulates the cell cycle of hepatocytes. Collagen facilitates the entry of hepatocytes into the S-phase by a cyclin D1-dependent mechanism (Hansen and Albrecht, 1999), and mediates aggregation of hepatocytes and intercellular contact (Moghe et al., 1997).

Collagen regulates survival, proliferation, and differentiation of MSCs; conversely, MSCs deposit extracellular collagen during chondrogenesis and osteogenesis (Li et al., 2011). The present work demonstrated that MSCs deposition of extracellular collagen could be enhanced by direct co-culture with hepatocytes, and extracellular collagen deposition contributed, at least partially, to trophic and antiapoptotic effects of MSCs on co-cultured hepatocytes as shown in the collagen inhibition experiments. This finding was consistent with the report by Gu et al. (2009); extracellular collagen was mainly located around MSCs and knockdown of collagen type I/V expression in MSCs significantly reduced synthesis of albumin and urea in hepatocytes. This finding also suggested that enhanced MSCs deposition of extracellular collagen might result primarily from MSC-to-hepatocyte contact as indirect non-contact co-culture did not show any significant effect. The present work also showed that HPc significantly increased extracellular collagen expression in mono- and co-cultured MSCs in an intracellular ROS dependent manner. Low oxygen tension is known to promote osteochondrogenesis of MSCs. HPc significantly upregulated expression of collagen I, II, and X along with some other genes encoding ECM (Müller et al., 2011). Hypoxia can also optimise cartilaginous matrix production of articular chondrocytes co-cultured with MSCs, thereby minimizing the requirement on harvest and expansion of primary chondrocytes (Meretoja et al., 2013). It was noted that increased MSCs deposition of extracellular collagen was accompanied by enhanced autocrine activity of TGF-β, a potent stimulant of collagen synthesis (Rodriguez et al., J Cell Biochem, 2000). Increased extracellular collagen deposit might result from autocrine TGF-β activation in MSCs.
5.4.4 Hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction result from downregulation of proapoptotic signalling and upregulation of antiapoptotic signalling

Apoptotic death of hepatocytes is finely modulated by the complex interplay between proapoptotic factors and antiapoptotic factors. Spontaneous hepatocyte apoptosis is thought to be a major cause of early cellular loss and metabolic deterioration. The well documented trophic effect of MSCs on co-cultured hepatocytes may result from suppression of hepatocyte apoptosis. The antiapoptotic effect of MSCs is supposed to derive from a combination of paracrine factors, ECM, and cell-to-cell interaction. The results in Chapter 3 showed that MSCs significantly inhibited spontaneous and chemically-induced apoptosis of co-cultured hepatocytes by nonparacrine mechanisms, and those in Chapter 4 further confirmed that HPc potentiated antiapoptotic effect of MSCs co-culture by an intracellular ROS dependent mechanism. The results of gene expression analyses in this chapter showed that downregulation of apoptosis-associated caspases and BAX/BCL-2 signalling pathways underlay the antiapoptotic effect of MSCs and potentiative effect of HPc induction. To the best of my knowledge, the present work demonstrated apoptosis-associated gene expression profiles of hepatocytes co-cultured with MSCs for the first time. It was also noted that indirect co-culture with MSCs only downregulated expression of two genes, BID and BLK. This finding might explain why paracrine factors of MSCs had limited trophic and antiapoptotic effect on co-cultured hepatocytes.

Caspases, also called cysteine-dependent aspartate-directed proteases, are a family of cysteine proteases that play an essential role in regulating cellular apoptosis, necrosis, and inflammatory response. Caspase signalling pathway functions to initiate and execute cellular apoptosis in a complex but well documented cascade manner, if activated by granzyme B for caspases-3 and -7, death receptors for caspases-8 and -10, and apoptosome for caspase-9. Caspase-3 plays a central role in the execution phase of apoptosis activated both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways, and interacts with caspases-8 and-9. LPS-induced hepatocyte apoptosis was thought to result from upregulation of caspase-3 expression in the presence of activated Kupffer cells (Hamada et al., 1999). AT-MSCs CM was reported to protect PC12 cells from glutamate excitotoxicity-induced apoptosis by inhibiting caspase-3 activity through upregulation of XIAP (X-linked inhibitor of apoptosis protein) and PI3-K (phosphatidylinositol 3-kinases)/Akt activation (Lu et al., 2011). Caspase-8 is the prototypical apoptosis initiator downstream of TNF superfamily death receptors, and activated caspase-8 can start the cascades of cellular apoptosis along with other effector caspases and proapoptotic Bcl-2 family members. ROS-induced activation of caspase-8 was reported to promote hepatocyte apoptosis and liver fibrosis in the setting of non-alcoholic steatohepatitis (Hatting et al., 2013). Caspase-9 is the initiator of caspase-mediated apoptosis through the mitochondrial pathway; activation of JNK/SAPK (stress-activated protein kinase) signalling releases cytochrome from the mitochondrial into the cytoplasm and activates the apoptosome, which cleaves the pro-enzyme of caspase-9 into the active form (Kang and Chae, 2003). Caspase-14 is activated by caspases-8 and -9 and involved in apoptosis execution and also
keratinocyte terminal differentiation (Walsh et al., 2005). The role of caspase-14 remains unknown in hepatocyte apoptosis. The present work showed that MSCs co-culture and HPc significantly downregulated expression of caspase-9; however, no expression of caspases-3, -8, and -14 was detected. This finding suggested that antiapoptotic effect of MSCs co-culture and potentiative effect of HPc induction mainly acted on initiation of apoptosis, consistent with reduced caspase-9 initiated cleavage of hepatocyte CK18 into CCK18.

BCL-2 is a superfamily of regulator proteins, including Bax, Bcl-2, Bid, involved in cellular survival and apoptosis. Bax (Bcl-2-associated X protein 4) was the first identified proapoptotic member of Bcl-2 protein family. Following induction of apoptosis, BAX interacts with the mitochondrial membrane and activate the voltage-dependent anion channel. The opening of the anion channel results in massive release of cytochrome c and other proapoptotic factors and further activates caspases. In contrast, Bcl-2 (B-cell lymphoma 2) is an antiapoptotic member, and prevents the activation of BAX signalled by the “death cue” along with Bcl-xL. The Bax/Bcl-2 balance is well known to regulate hepatocyte apoptosis (Hikita et al., 2011). BID (BH3 interacting-domain death agonist) interacts with Bax and leads to Bax insertion into the outer mitochondrial membrane in response to apoptotic signals. Activation of the death receptor with Fas ligand leads to activation of caspase-3 and -8 as mediated by Bid-dependent mitochondrial release of Smac in the apoptotic event of hepatocytes (Li et al., 2002). Bid is also upstream of caspase-2 activation in the setting of TNF-α triggered hepatocyte apoptosis (Guicciardi et al., 2005). Park et al. (2010) reported that MSCs co-culture upregulated expression of Bcl-xL and Bcl-2 in islet cells. Autologous transplantation of AT-MSCs alleviated ischaemia/reperfusion-induced hepatocyte apoptosis by upregulating Bcl-2 (Sun et al., 2012). The present work showed that co-culture with NPc-MSCs significantly decreased BAX/BCL-2 ratio and BID expression in a cell-contact dependent manner, while that with HPc-MSCs exhibited a significantly greater effect by an intracellular ROS-dependent mechanism.

BLK (B lymphoid tyrosine kinase) is a member of SRC non-receptor tyrosine kinase subfamily involved in B-lymphocyte development, differentiation and signalling. BLK is mainly present in lymphatic tissues, pancreatic islets, and also highly expressed in colon epithelial cells (Seidelin and Nielsen, 2006). BLK protein enhances islet cell synthesis and secretion of insulin in response to glucose and upregulates expression of pancreatic β-cell transcription factors. The biological role of BLK in hepatocyte biology remains to be investigated. The present work showed that staurosporine significantly upregulated expression of BLK, while MSCs co-culture inhibited upregulation of BLK expression in a partially cell-contact dependent manner. It can be postulated that BLK might be involved in apoptosis and inflammatory response of hepatocytes as BLK is reported to participate in pre-B-cell receptor-mediated NF-κB activation (Krappmann et al., 2001), while activation of NF-κB is known to determine the balance between apoptosis and proliferation of hepatocytes in response to TNF-α during liver regeneration (Plümpe et al., 2000).
5.4.5 Conclusions

In conclusion, MSCs that expressed no TNF-α themselves significantly decreased autocrine TNF-α activity of co-cultured hepatocytes in a cell contact dependent manner. HPc further potentiated the inhibitory effect of MSCs co-culture by an intracellular ROS dependent mechanism. Inhibited autocrine activity of TNF-α might be a negative regulating mechanism of MSCs co-culture. Heterotypic interaction between hepatocytes and MSCs increased TGF-β secretion in co-culture, and autocrine TGF-β activity of MSCs was required for the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. Cell contact and intracellular ROS dependent extracellular collagen deposit of MSCs also played an essential role in trophic and antiapoptotic effect of MSCs on co-cultured hepatocytes. The underlying molecular mechanisms included downregulation of caspase-9 and decreased BAX/BCL-2 ratio, while the implication of BLK in hepatocyte biology and MSCs co-culture remains to be investigated.
CHAPTER 6 CONCLUDING DISCUSSION AND FUTURE WORK

6.1 Concluding Discussion

6.1.1 Trophic and protective effects of MSCs on co-cultured hepatocytes

In the present work, the well documented trophic effect of MSCs on co-cultured porcine (Gu et al., 2009a; Gu et al., 2009b; Gu et al., 2009c) or rodent (Shi et al., 2009) hepatocytes was reproduced in primary human hepatocytes. The trophic effect of MSCs also applied to steatotic and cryopreserved hepatocytes, both of which are frequently encountered and used in the practice of HCT. This trophic effect with respect to liver-specific metabolic function might result from improved hepatocyte survival, potentiated metabolism of surviving hepatocytes, or both. MSCs transplantation was reported to ameliorate hepatocyte apoptosis following ischaemia/reperfusion injury (Pan et al., 2012) or CCl₄ exposure (Manuelpillai et al., 2010). A novel contribution of the present work was the finding that MSCs significantly inhibited caspase-mediated spontaneous and chemically-induced apoptosis of co-cultured hepatocytes. Furthermore, MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis switch in death mode of hepatocytes. Supportive effect of MSCs for co-cultured epithelial cells is deduced to be the synergistic effect of soluble factors, ECM, and cell-to-cell crosstalk. In contrast to previous studies reporting a positive role of paracrine factors in the hepatotrophic effects of MSCs (Gu et al., 2009b), the present work showed that soluble factors released from MSCs alone or MSCs co-cultured with hepatocytes contributed minimally to the trophic and protective effects of MSCs co-culture although co-culture might amplify paracrine activity of MSCs-derived soluble factors. This contradiction might be explained as species variation (human versus porcine) so that human primary hepatocytes became unresponsive to the stimuli with potential soluble factors. Conversely, this finding suggested that trophic and antiapoptotic effects of MSCs co-culture might result primarily from additive or synergistic effects of ECM and cell-to-cell interaction. Deprivation of ECM (Sérandour et al., 2005) and cell-to-cell contact (Gómez-Aristizábal and Davies, 2012) has an adverse effect on hepatocyte proliferation and metabolism.

The present work also demonstrated that the extent of the hepatotrophic effect of co-culture remained similar among AT-, BM-, and UC-MSCs. Co-culture with any type of MSCs improved liver-specific metabolic function of hepatocytes to a significantly greater extent as compared to that with ADFs. Moreover, AT-MSCs even at a low seeding ratio to hepatocytes still exhibited a significant hepatotrophic effect comparable to those at a high seeding ratio. This finding suggested that MSCs contributed to hepatotrophic effect of co-culture by a stem cell-specific mechanism in addition to providing hepatocytes with an attachment matrix.

It will be worthwhile investigating the possibility of using autologous liver-derived MSCs for co-culture with hepatocytes. Liver-derived MSCs is a potential progenitor reservoir of hepatocytes through transdifferentiation in addition to a supportive cell population for hepatocytes (Najimi et al., 2007). The advantages of using liver-derived MSCs are the synergistic effect of hepatocytes and
MSCs isolated from a single donor liver and potentially better cellular engraftment into the liver (Moreno et al., 2012). The present work showed that co-culture with a small number of hepatic NPCs possibly containing liver MSCs significantly improved viability and liver-specific metabolism of hepatocytes as evidenced by the preliminary results. The major limitation of the experiments with hepatic NPCs was poor reproducibility and propagatability. Further effort should be made to produce a constant, reliable hepatic NPC line, and this cell population should be fractioned and characterised to identify the underlying stem cell subpopulation. Steatotic liver may be a suitable source for isolating liver NPCs including MSCs and hepatic progenitor cells (Tolosa et al., 2011); however, use of steatotic liver-derived NPCs carries a potential profibrogenic risk as NF-κB activation in liver NPCs mediates inflammatory response and bridges steatohepatitis and liver fibrosis (Beraza et al., 2008). It will be of great significance to investigate how co-culture with hepatocytes modulates the biological behaviour of MSCs. It was reported that co-culture with human liver cells drove differentiation of rat MSCs into hepatocyte-like cells in a spheroid architecture in the presence of HGF (Qihao et al., 2007). Lange et al. (World J Gastroenterol, 2005b; World J Gastroenterol, 2006) detected hepatocytic differentiation of rat MSCs co-cultured with adult rat or foetal liver cells, as evidenced by expression of hepatocyte-specific markers, in the absence of chemically-defined culture medium. Hepatotrophic effect of MSCs co-culture may also partially result from hepatogenic transdifferentiation of MSCs especially after transplantation and engraftment into the liver.

6.1.2 HPc potentiates hepatotrophic and antiapoptotic effects of MSCs co-culture by an intracellular ROS dependent mechanism

The present work showed that HPc potentiated trophic and antiapoptotic effect of MSCs on hepatocytes as compared to NPC. Potentiative effect of HPc on MSCs themselves has been extensively investigated, and HPc-primed MSCs exhibit an enhanced supportive and protective effect on the host cells, such as hepatocytes (Yu et al., 2013), cardiomyocytes (He et al., 2009), and neural cells (Wei et al., 2012). Potentiated paracrine activity is thought to be a major contributive factor of HPc-induced potentiative effect; however, the present work showed indirect noncontact co-culture with HPc-MSCs did not significantly improved cellular viability and metabolic function of hepatocytes. The probable explanation was that freshly isolated hepatocytes still remained unresponsive even in the presence of potentiated extrinsic cues due to mechanical and enzymatic stress from isolation. Conversely, the absence of potentiative effect in HPc indirect co-culture implied that HPc potentiated MSCs mainly by a mechanism involving ECM and/or cell-to-cell interplay.

NAC pretreatment diminished the increase of intracellular ROS activity in MSCs and reduced HPc-potentiated trophic and antiapoptotic effects of MSCs on co-cultured MSCs. This finding was consistent with that reported by De Barros et al. (2013), in which HPc counteracted ageing-related impairment of angiogenic potential in human AT-MSCs. The improved angiogenesis might be the
synergistic effect of MSCs transdifferentiation into endothelial cells, enhanced expression of proangiogenic and prosurvival factors, and oxidative stress. The major signal was determined to be intracellular ROS as NAC pretreatment reversed HPc-induced improvement. A second potential candidate factor responsible for HPc-induced potentiative effect is HIF, a family of transcription factors sensitive to oxygen tension in the microenvironment. Activation of HIF participates in the inhibitory effect of hypoxia on MSCs differentiation (Haque et al., 2013) but improves angiogenesis (Hu et al., 2008) and migration (Liu et al., 2011) of MSCs. It will also be important to investigate what beneficial mechanisms, especially those involved in ECM formation and cell-to-cell crosstalk are activated by oxygen-sensing factors, such as ROS and HIF, and how these mechanisms potentiate trophic and protective effect of MSCs on co-cultured hepatocytes.

6.1.3 Molecules underlying hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction

The trophic and protective effects of MSCs on co-cultured hepatocytes are attributed to synergistic effects of soluble factors, ECM, and heterotypic cellular interaction. As a result, it is likely that HPc potentiated the hepatotrophic and antiapoptotic effects of MSCs by enhancing these mechanistic factors. TNF-α is thought to play an essential role in mediating hepatocyte apoptosis in the setting of inflammatory response (Zang et al., 2000; Biburger and Tiegs, 2005). The present work showed that hepatocytes secreted a relatively high level of baseline TNF-α and neutralisation of baseline TNF-α reduced apoptosis and total death of hepatocytes. In addition, hepatocyte secretion of TNF-α was significantly inhibited by co-culture with MSCs and by HPc-MSCs to a significantly greater extent. This inhibitory effect of MSCs on co-cultured hepatocytes also depended on MSC-to-hepatocyte contact as evidenced by the minimal inhibitory effect seen in the indirect non-contact co-culture. Therefore, MSCs co-culture might suppress autocrine TNF-α activity of hepatocytes by a cell-to-cell contact dependent mechanism. The possibility that decreased autocrine TNF-α activity resulted in inhibition of hepatocyte apoptosis remains an open question as MSCs co-culture CM contained a low level of TNF-α but had no effect on hepatocyte apoptosis. It is possible that reduced autocrine TNF-α activity is a prerequisite rather than sufficient condition for antiapoptotic effect of MSCs co-culture. It remains to be investigated by which mechanism MSCs co-culture and HPc induction inhibited autocrine TNF-α activity of hepatocytes. A possibility is that TNF-α synergistically coordinates with other proapoptotic factors by a positive feedback mechanism in hepatocytes undergoing spontaneous apoptosis (Schlatter et al., 2011). Interruption of this positive feedback will reduce hepatocyte apoptosis and consequently inhibit autocrine TNF-α activity.

TGF-β is known to be involved in liver fibrosis by promoting collagen deposition (Gabriel et al., 2008); however, TGF-β positively regulates osteochondrogenesis of MSCs (Mehlhorn et al., 2006). Contact of MSCs with T-lymphocytes also upregulates expression of IL-10 and TGF-β, both of which mediates T-cell tolerance (Nasef et al., 2007). The present work showed that MSCs-to-hepatocytes contact and HPc increased TGF-β1 secretion in co-culture and by MSCs, respectively.
Neutralising autocrine activity of TGF-β1 in both NPc- and HPc-MSCs led to a significant reduction of MSCs co-culture antiapoptotic effect. As MSCs co-culture CM containing high-level TGF-β1 had no significant effect on hepatocyte apoptosis, enhanced TGF-β1 expression in co-culture and HPc-MSCs contributed to the antiapoptotic effect of MSCs co-culture by an MSC-autocrine rather hepatocyte-paracrine mechanism. This contact co-culture induced enhancement might result from activation of latent TGF-β1 rather than upregulation of TGF-β1 transcriptional and translational expression (Chia et al., 2005). It is an open question how activation of autocrine TGF-β1 in MSCs inhibits apoptosis of co-cultured hepatocytes. Mediation of ECM and cell-to-cell interplay may be the candidate mechanism. TGF-β1 is reported to modulate MSCs attachment to collagen type I by an integrin-mediated mechanism (Warstat et al., 2010).

The primary effect of collagen on hepatocytes is provision of an attachment matrix and also regulation of hepatocyte cell cycle progression and differentiation (Hansen et al., 2006). Under physiological conditions, liver collagen is mainly produced by NPCs rather than hepatocytes; however, collagen deprivation will be detrimental for hepatocytes cultured in vitro. The present work demonstrated that extracellular collagen, the active form of collagen, was mainly deposited by MSCs rather than hepatocytes. Cell contact and HPc also enhanced extracellular collagen deposit of MSCs co-culture and MSCs, respectively. Antagonisation experiments showed that inhibition of extracellular collagen deposit by MSCs diminished the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. Conventional hepatocyte mono-culture only allows hepatocyte monolayer collagen attachment, while MSCs co-culture supports hepatocytes in a three-dimensional architecture (Gu et al., 2009b). Three-dimensional hepatocyte culture in sandwich collagen gel is thought to mimic the microenvironment and shows a supportive effect superior to conventional monolayer collagen architecture (Wang et al., 2004). Collagen may also regulate responsiveness of hepatocytes to soluble factors and intercellular interaction (Berthiaume et al., 1996). It will be academically significant to investigate what subtype(s) of collagen is increased in MSCs co-culture and after HPc induction as biological activities of collagen vary significantly among subtypes. It is also interesting to delineate by which mechanism, such as modification of collagen topography, shift of collagen isoforms, and amplification of soluble factors and cellular interplay, enhanced collagen deposition results in the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction.

Apoptotic death of hepatocytes is a biological event finely modulated by a complex network of proapoptotic and antiapoptotic factors. The results of Chapter 3 showed that MSCs co-culture significantly inhibited caspase-mediated CK18 cleavage, an early-stage event in the process of cellular apoptosis, as evidenced by downregulated expression of caspase-9 in MSCs co-culture. This finding suggested that MSCs co-culture mainly inhibited initiation of hepatocyte apoptosis mediated by the mitochondrial pathway. Additionally, decreased BAX/BCL-2 ratio indicated that MSCs co-culture suppressed the death receptor-associated apoptosis pathway. The present work also demonstrated for the first time that MSCs co-culture downregulated expression of BLK, biological activities of which are rarely reported in hepatocytes, at the transcriptional level by a cell contact,
intracellular ROS-dependent mechanism. It remains unknown how the potential contributive factors of MSCs co-culture, such as hepatocyte-autocrine TNF-α, MSC-autocrine TGF-β1, and extracellular collagen, modulate the balance between proapoptosis and antiapoptosis in hepatocytes by a cell contact, intracellular ROS-dependent mechanism. It is impossible for a single factor or mechanism to fully explain the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. It will also be informative to investigate the reciprocal interaction between hepatocytes and MSCs using genomic microarray techniques.

6.2 Conclusions

In conclusion, MSCs have trophic, antiapoptotic, prosurvival, and protective effects on co-cultured hepatocytes. These favourable effects can also be reproduced in functionally impaired steatotic and cryopreserved hepatocytes. The paracrine contribution of soluble factors to hepatotrophic effect of MSCs co-culture that has been well documented in animal-derived hepatocytes could not be reproduced in the present work possibly due to the unresponsiveness of human hepatocytes to extrinsic cues in the absence of ECM and intercellular crosstalk. HPc significantly potentiated the hepatotrophic effects of MSCs co-culture by an intracellular ROS-dependent mechanism, while this potentiative effect also depended on heterotypic cellular interaction as evidenced by the minimal effect of HPc indirect noncontact co-culture. Potential contributive factors to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction were decreased hepatocyte autocrine activity of TNF-α, increased MSC autocrine activity of TGF-β1, and enhanced extracellular collagen deposit by MSCs, as evidenced by the reversal effects of neutralisation or inhibition experiments. These potential contributive factors may synergistically switch the balance from proapoptosis to antiapoptosis in hepatocytes co-cultured with MSCs.

With respect to the bench-to-bedside translation of the present work, co-transplantation with MSCs is expected to improve therapeutic efficacy of current HCT facing two major hurdles, limited donor tissue availability and marginal cell quality. Co-transplantation of MSCs may offer additional benefits, such as transdifferentiation into hepatocytes, repair of the injured host hepatocytes, improvement of neovascularisation in vivo, and modulation of immune response. HPc may also potentiate these therapeutic effects of MSCs in vivo like hepatotrophic and antiapoptotic effects of MSCs co-culture in vitro observed in the present work. However, the effectiveness and safety of MSCs co-transplantation and HPc modification needs to be validated in preclinical liver failure models. A major safety concern arising from incorporation of MSCs to HCT is that MSCs carry a potential risk of fibrogenesis especially in the profibrotic environment.
REFERENCES


AJIOKA, I., NISHIO, R., IKEKITA, M., AKAIKE, T., SASAKI, M., ENAMI, J. & WATANABE, Y. 2001. Establishment of heterotrophic liver tissue mass with direct link to the host liver following implantation of hepatocytes transfected with vascular endothelial growth factor gene in mice. Tissue Eng, 7, 335-44.


CARVALHO, J. L., BRAGA, V. B., MELO, M. B., CAMPOS, A. C., OLIVEIRA, M. S.,


CHEN, X., ZHANG, S., LIU, T., LIU, Y. & WANG, Y. 2012b. Maintenance of rat


CUI, X., WANG, H., GUO, H., WANG, C., AO, H., LIU, X. & TAN, Y. Z. 2010. Transplantation of mesenchymal stem cells preconditioned with diazoxide, a


metabolic disorders, experience at King's College hospital and review of literature. Acta Gastroenterol Belg, 68, 457-60.


GABRIEL, A., ZIOLKOWSKI, A., RADLOWSKI, P., TOMASZEK, K. &


HIKITA, H., TAKEHARA, T., KODAMA, T., SHIMIZU, S., SHIGEKAWA, M., HOSUI, A., MIYAGI, T., TATSUMI, T., ISHIDA, H., LI, W., KANTO, T.


ISHII, K., YOSHIDA, Y., AKECHI, Y., SAKABE, T., NISHIO, R., IKEDA, R.,


KANG, Y. J. & CHAE, S. W. 2003. JNK/SAPK is required in nitric oxide-induced...
apoptosis in osteoblasts. Arch Pharm Res, 26, 937-42.


KERN, S., EICHLER, H., STOEVE, J., KLUTER, H. & BIEBACK, K. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells, 24, 1294-301.


Khan, W. S. & HARDINGHAM, T. E. 2012. Mesenchymal stem cells, sources of cells
and differentiation potential. J Stem Cells, 7, 75-85.


LU, H. F., CHUA, K. N., ZHANG, P. C., LIM, W. S., RAMAKRISHNA, S., LEONG,


MCMAHAN, R. S., RIEHLE, K. J., FAUSTO, N. & CAMPBELL, J. S. 2013. A disintegrin and metalloproteinase 17 regulates TNF and TNFR1 levels in inflammation and liver regeneration in mice. Am J Physiol Gastrointest Liver Physiol, 305, G25-34.


MIN, B. S., KIM, N. K., JEONG, H. C. & CHUNG, H. C. 2012. High levels of serum VEGF and TIMP-1 are correlated with colon cancer liver metastasis and intrahepatic recurrence after liver resection. Oncol Lett, 4, 123-130.


MIRZANIA, M., GHAVAMZADEH, A., YAGHMAIE, M., SEDIGHI, N.,


PALOMAKI, S., PIETILA, M., LAITINEN, S., PESALA, J., SORMUNEN, R., LEHENKARI, P. & KOIVUNEN, P. 2013. HIF-1alpha is upregulated in human
mesenchymal stem cells. Stem Cells, 31, 1902-9.


RICHERT, L., LIGUORI, M. J., ABDADIE, C., HEYD, B., MANTION, G., HALKIC, N. & WARING, J. F. 2006. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. Drug Metab Dispos, 34, 870-9.


ROCHEFORT, G. Y., DELORME, B., LOPEZ, A., HERAULT, O., BONNET, P.,
CHARBORD, P., EDER, V. & DOMENECH, J. 2006. Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem Cells, 24, 2202-8.


ROSOVA, I., DAO, M., CAPOCCIA, B., LINK, D. & NOLTA, J. A. 2008. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells, 26, 2173-82.


STEVenson, D. J., MORGAN, C., MCELLeLLAN, L. I. & HELEN GRANT, M. 2007. Reduced glutathione levels and expression of the enzymes of glutathione synthesis in
cryopreserved hepatocyte monolayer cultures. Toxicol In Vitro, 21, 527-32.


and location in the developing human liver. Stem Cells Dev, 16, 771-8.


Connexin43 promotes survival of mesenchymal stem cells in ischaemic heart. Cell Biol Int, 34, 415-23.


WANG, J., LI, Z., ZHANG, Y., LIU, X., CHEN, L. & CHEN, Y. 2013b. CX43 change in LPS preconditioning against apoptosis of mesenchymal stem cells induced by hypoxia and serum deprivation is associated with ERK signaling pathway. Mol Cell Biochem, 380, 267-75.


WARSTAT, K., MECKBACH, D., WEIS-KLEMM, M., HACK, A., KLEIN, G., DE


ZAOUALI, M. A., PADRISSA-ALTES, S., BEN MOSBAH, I., ALFANY-FERNANDEZ, I., MASSIP-SALCEDO, M., CASILLAS-RAMIREZ, A., BINTANEL-


LIST OF PUBLICATIONS

- **Peer-reviewed article**

- **Book chapter**

- **Conference paper**


  Qin HH, Mitry RR, Dhawan A, Hughes RD. Hypoxia preconditioned adipose tissue derived mesenchymal stem cells augment the function of human hepatocytes in a heterotypic co-culture system. *King’s College London School of Medicine Graduate Showcase*, 4th May, 2012, London, United Kingdom (Poster presentation).

  Qin HH. What can mesenchymal stem cells (MSCs) contribute to current hepatocyte transplantation (HcT) practice? *King’s College London School of Medicine Transplantation Seminar Series*, 28th March, 2012, London, United Kingdom (Oral presentation).