REVIEWS

Factors influencing stem cell differentiation into the hepatic lineage in vitro

BOON CHIN HENG,* HANRY YU,† YIJUN YIN,‡ SENG GEE LIM§ AND TONG CAO*

*Stem Cell Laboratory, Faculty of Dentistry, National University of Singapore, †Institute of Bioengineering and Nanotechnology, Singapore Science Park II, Singapore, ‡The Stem Cell Institute, Department of Medicine, University of Minnesota, Minneapolis, MN, USA and §Division of Gastroenterology, Faculty of Medicine, National University of Singapore, Singapore

Abstract A major area of research in transplantation medicine is the potential application of stem cells in liver regeneration. This would require well-defined and efficient protocols for directing the differentiation of stem cells into the hepatic lineage, followed by their selective purification and proliferation in vitro. The development of such protocols would reduce the likelihood of spontaneous differentiation of stem cells into divergent lineages upon transplantation, as well as reduce the risk of teratoma formation in the case of embryonic stem cells. Additionally, such protocols could provide useful in vitro models for studying hepatogenesis and liver metabolism. The development of pharmokinetic and cytotoxicity/genotoxicity screening tests for newly developed biomaterials and drugs, could also utilize protocols developed for the hepatic differentiation of stem cells. Hence, this review critically examines the various strategies that could be employed to direct the differentiation of stem cells into the hepatic lineage in vitro.

INTRODUCTION

In recent years, there has been growing interest in utilizing stem cells for treatment of chronic and acute liver diseases. The extensive self-renewal capacity of both embryonic and adult stem cells makes them particularly useful in cell-transplantation therapy for liver regeneration, as in theory this could provide an unlimited supply of donor material.1 Besides cell-transplantation, differentiated hepatic derivatives of stem cells has also been suggested for use in bioartificial liver support devices,2 which can either be used for curative treatment of acute liver failure, or as a ‘bridge’ to liver transplantation.

The clinical application of stem cells in liver diseases would require well-defined and efficient protocols for directing stem cells into the hepatic lineage in vitro. This is because the transplantation of differentiated hepatic derivatives is likely to result in higher engraftment efficiency and better integration within the failing liver, as compared to undifferentiated stem cells. Additionally, undifferentiated stem cells possess the capacity for spontaneous differentiation into multiple divergent lineages at the transplantation site,3 other than the hepatic lineage. This could very well reduce the efficacy of cell-transplantation therapy for liver regeneration. Moreover, in the case of embryonic stem cells, some degree of differentiation would be required to reduce the risk of teratoma formation within the transplant recipient.

Besides clinical therapy, there is also a dire need for in vitro models of hepatogenesis and liver metabolism. It is extremely difficult to elucidate the molecular mechanisms and signaling pathways that regulate hepatogenesis in vivo within live animal models. Efficient protocols for directing the hepatic differentiation of stem cells in vitro will therefore provide a model that is more amenable to molecular characterization and genetic manipulation. An added advantage is that these protocols may also facilitate the genetic manipulation of stem cells for the delivery of recombinant genes/proteins in liver regeneration therapy.3

The development of pharmokinetic and cytotoxicity/genotoxicity screening tests for newly developed biomaterials and drugs may also utilize protocols for the hepatic differentiation of human stem cells in vitro.

Correspondence: Dr Tong Cao, Stem Cell Laboratory, Faculty of Dentistry, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074. Email: dencaot@nus.edu.sg
Accepted for publication 12 August 2004.
Because the liver is the major organ of detoxification in the human body, cultures of human stem cell-derived hepatocytes would be particularly useful for assaying drug metabolism, as well as for toxicological research and testing. Compared to laboratory testing on animal cell/tissue cultures or on live animals, screening tests based on human stem cell-derived hepatocytes will be much more clinically relevant, timely, and accurate, as well as more cost-effective. Moreover, ethical issues surrounding the use of live animals for pharmokinetic and cytotoxicity/genotoxicity testing would also be circumvented.

Hence, the purpose of this review is to critically examine the various strategies that could be employed to direct the differentiation of stem cells into the hepatic lineage in vitro.

ADULT STEM CELLS VERSUS EMBRYONIC STEM CELLS FOR LIVER REGENERATION

Stem cells for liver regeneration can be derived from two major sources: (i) embryonic stem (ES) cells derived from the inner cell mass of blastocyst stage embryos; and (ii) adult stem cells isolated from postnatal tissues. The latter category encompasses a diverse array of cell-types that have been shown to be capable of giving rise to the hepatic lineage. Within the liver itself, there are oval cells and small hepatocyte-like progenitor cells, while within the bone marrow there are mesenchymal stem cells, hematopoietic stem cells, as well as a putative independent subpopulation of bone marrow-derived liver stem cells (BDLSC). Adult stem cells of the peripheral blood circulation are of particular interest, since these are readily accessible without the need for any invasive surgical procedure. Indeed, the hepatic lineage has been demonstrated to arise from a number of cellular subpopulations within peripheral blood. These include the putative monocyteic stem cells and the so-called side population (SP) stem cells, which have also been identified in multiple tissue types besides peripheral blood.

Because the immunological barrier is undoubtedly the most formidable challenge in stem cell transplantation therapy, it would be preferable to utilize an autologous source of adult stem cells, rather than an allogenic source of either adult or embryonic stem cells. Hence, if ES cells are to be successfully utilized for liver regeneration, it would first of all be necessary to overcome immunological rejection from the transplant recipient; unless an isogenic source of ES cells is derived from therapeutic cloning. Although this has recently been achieved in the human model, this is still a remote option, given the technical difficulties of such an approach. A more viable alternative would be to create a bank of ES cells with different major histocompatibility complex (MHC) genotypes, for matching with the transplant recipient. It could also be possible to down-regulate the antigenicity of ES cells through suppression of MHC gene expression. Another major problem is the risk of teratoma formation by ES cells upon transplantation in situ, which is not the case for adult stem cells.

The most obvious advantage of utilizing ES cells, instead of adult stem cells for liver regeneration is that ES cells are immortal, and could potentially provide an unlimited supply of differentiated hepatocytes for transplantation. By contrast, the self-renewal and proliferative capacity of adult stem cells is very much limited, and may decrease with age. This would obviously limit their usefulness in autologous cell-transplantation therapy for the treatment of liver failure in older patients. Putative adult stem cells isolated from postnatal tissues are in fact highly heterogenous populations with only a limited proportion of cells being capable of differentiation into the hepatic lineage. If the capacity for continuous self-renewal is taken strictly as the defining criteria for stem cells, then it is likely that an extremely low proportion of putative adult stem cell populations could be considered as true stem cells. This would pose a major challenge in the development of isolation and purification protocols. Additionally, adult stem cells may also contain more genetic abnormalities than ES cells, caused by exposure to free radicals, metabolic toxins, and errors in DNA replication accumulated during the course of a lifetime.

Currently, it is ambiguous as to whether the future in liver transplantation would hold more promise for adult or embryonic stem cells. This will ultimately depend on how the various technical challenges associated with each can be successfully overcome in the near future.

DEVELOPMENT OF DEFINED CULTURE MILIEU FOR DIRECTING THE HEPATIC DIFFERENTIATION OF STEM CELLS IN VITRO

For clinical applications of stem cell transplantation therapy, it is imperative that in vitro culture protocols should be devoid of animal or human products, to avoid potential contamination with pathogens. The avoidance of products of animal or human origin would also reduce variability within the culture milieu, and provide a more stringent level of quality control. Moreover, supplemented animal or human proteins may adhere onto the surface of cultured stem cells, which could possibly enhance their antigenicity upon transplantation. Hence, the ideal culture milieu for promoting the hepatic differentiation of stem cells in vitro should be chemically defined, and either be serum-free or utilize synthetic serum replacements with the possible supplementation of specific recombinant cytokines and growth factors (Table 1) if so required.

Numerous cytokines and growth factors have been shown to have potent effects on hepatic growth and differentiation under in vitro culture conditions (Table 1). These include hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF), acid fibroblast growth factor (aFGF), insulin, insulin-like growth factor (IGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stromal derived factor-1 alpha (SDF-1α), stem cell factor...
Hepatic differentiation of stem cells

Of these various cytokines and growth factors, HGF, EGF, TGF, and aFGF are the most commonly used in the majority of studies, to promote hepatic differentiation in vitro.8,32,33 It is important to note that for any one particular hepatogenic-promoting cytokine or growth factor, there probably exist subtle differences in its effects at different developmental stages. Hence, for optimal stimulation of hepatic differentiation, the dosage, timing and combination of cytokines/growth factors should be fine-tuned according to the origin and type of stem cell (i.e. ES cells, bone marrow-derived mesenchymal stem cells or hepatic stem cells). These would, in turn, be expected to differ significantly in their expression of specific surface receptors for hepatogenic-promoting cytokines and growth factors, at different developmental stages. For example, HGF and aFGF are the only cytokines that have so far been reported to promote the hepatic differentiation of ES cells in vitro;6,34 while TGF have been shown to be particularly useful for maintaining the differentiated phenotype of primary hepatocytes within in vitro culture.35 Currently, the formulation of ‘optimal’ combinations of cytokines/growth factors to promote the hepatic differentiation of different types of stem cells is still at a very immature stage of development.

Another major consideration is the dichotomy between hepatocyte proliferation and expression of differentiated function.36 Culture conditions that are advantageous for promoting proliferation of hepatogenic progenitors would not necessarily be optimal for expression of differentiated function, and vice versa. Hence, the dosage and combination of cytokines and growth factors that is supplemented within the in vitro culture milieu should be fine-tuned, according to this dichotomy between hepatocyte proliferation and differentiation.

In addition to protein-based cytokines and growth factors, a number of non-proteinaceous chemical compounds have also been shown to promote hepatic differentiation in vitro (Table 2). Such chemicals tend to be less labile, with a longer active half-life in solution, compared to protein-based cytokines and growth fac-

### Table 1 Cytokines and growth factors which promote hepatic differentiation

<table>
<thead>
<tr>
<th>Cytokines/growth factors</th>
<th>ES cells</th>
<th>Putative hepatic stem cells/oval cells</th>
<th>Primary fetal/adult hepatoblasts or hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte growth factor (HGF)</td>
<td>Hu et al.8</td>
<td>—</td>
<td>Michalopoulos et al.33</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>—</td>
<td>He et al.163</td>
<td>Michalopoulos et al.33</td>
</tr>
<tr>
<td>Transforming growth factor (TGF)</td>
<td>—</td>
<td>—</td>
<td>Lilja et al.35; Block et al.32</td>
</tr>
<tr>
<td>acid Fibroblast growth factor (aFGF)</td>
<td>Hu et al.8</td>
<td>Evarts et al.164</td>
<td>—</td>
</tr>
<tr>
<td>Insulin</td>
<td>—</td>
<td></td>
<td>Katsura et al.165</td>
</tr>
<tr>
<td>Insulin-like growth factor (IGF)</td>
<td>—</td>
<td></td>
<td>Streck and Pintar166; Brill et al.167</td>
</tr>
<tr>
<td>Granulocyte macrophage colony-stimulating factor (GM-CSF)</td>
<td>—</td>
<td></td>
<td>Brill et al.167</td>
</tr>
<tr>
<td>Stromal derived factor-1α (SDF-1α)</td>
<td>—</td>
<td>Hatch et al.168</td>
<td>—</td>
</tr>
<tr>
<td>Stem cell factor (SCF)</td>
<td>—</td>
<td>Fujio et al.169</td>
<td>—</td>
</tr>
<tr>
<td>Oncostatin M (OSM)</td>
<td>—</td>
<td></td>
<td>Lazaro et al.170</td>
</tr>
<tr>
<td>Serum-derived hepatocyte growth stimulating factor (HGSF)</td>
<td>—</td>
<td></td>
<td>Barone et al.171</td>
</tr>
</tbody>
</table>

### Table 2 Non-proteinaceous chemical factors which promote hepatic differentiation

<table>
<thead>
<tr>
<th>Chemical factors</th>
<th>ES cells</th>
<th>Putative hepatic stem cells/oval cells</th>
<th>Primary fetal/adult hepatoblasts or hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethsone</td>
<td>—</td>
<td></td>
<td>Michalopoulos et al.33</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>—</td>
<td></td>
<td>Alisi et al.38</td>
</tr>
<tr>
<td>Sodium butyrate</td>
<td>Rambhatla et al.7</td>
<td>—</td>
<td>Rogler39</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>—</td>
<td></td>
<td>Sato et al.40</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>—</td>
<td></td>
<td>de Juan et al.41</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>—</td>
<td></td>
<td>De La Vega and Mendoza-Figueroa42</td>
</tr>
</tbody>
</table>
tors. This would be advantageous for prolonged in vitro cell-culture over several days or even weeks. Moreover, unlike proteins that have to be synthesized in living cells and subjected to complex post-translational modifications (i.e., glycosylation, peptide-processing, conformational folding), non-protein based chemical compounds can be manufactured by chemical reactions in the laboratory, and hence are more structurally and chemically defined compared to proteins.

Among the non-proteinaceous chemical compounds that are known to promote hepatic differentiation in vitro (Table 2), are dexamethasone, retinoic acid, sodium butyrate, nicotinamide, norepinephrine, and dimethylsulfoxide. Dexamethasone is a synthetic corticosteroid that is routinely supplemented in primary hepatocyte cultures. It is thought to promote expression of differentiated hepatocyte phenotype through suppression of cell division. Retinoic acid, a derivative of vitamin A, has also been reported to promote expression of differentiated hepatocyte function through growth inhibition. Sodium butyrate is a histone deacetylase inhibitor that was reported to be a potent inducer of hepatic differentiation in ES cells, as well as with primary fetal hepatoblasts. Nicotinamide (vitamin B3) and its derivatives have been reported to stimulate the clonal growth of hepatic progenitors and subsequent formation of small hepatocyte colonies in vitro. Norepinephrine is a member of the catecholamine family of neurotransmitters. It has been reported to act synergistically with EGF to promote clonal growth of hepatic progenitors during liver regeneration. Dimethyl sulfoxide (DMSO) is commonly used as a cryoprotectant, but has also been reported to promote expression of differentiated hepatocyte phenotype under in vitro culture conditions. As with the case of cytokines and growth factors, the dosage and combination of non-proteinaceous chemical factors have not yet been optimized for the hepatic differentiation of stem cells in vitro. Probably, this would also require some degree of fine-tuning with different types of stem cells.

**NATURALLY OCCURRING AND ARTIFICIALLY SYNTHESIZED EXTRACELLULAR MATRIX SUBSTRATUM FOR PROMOTING HEPATIC DIFFERENTIATION**

*In situ*, functional liver tissue consists of parenchymal (i.e., hepatocytes) and non-parenchymal cells embedded within an acellular extracellular matrix (ECM) that serves to maintain its structural integrity. This is composed of five distinct types of collagens (types I, III, IV, V, and VI), seven classes of non-collagenous proteins (fibronectin, laminin, entactin/nidogen, tenasin, thrombospondin, SPARC and undulin) and a diverse array of proteoglycans, and glycosaminoglycans. In addition to its structural role, liver ECM plays a physiological role, by influencing the immediate microenvironment of the hepatocytes and other cell types embedded within it. Histological studies have reported extensive remodelling of liver ECM during growth and development as well as under various pathological conditions. Hence, the introduction of appropriate ECM substratum within in vitro culture would certainly enhance the directed differentiation of stem cells into the hepatic lineage. These can either be based on naturally occurring components of liver ECM, or may utilize synthetic materials. Additionally, composite matrix scaffolds of both natural and synthetic materials have also been fabricated.

It is important to note the various properties of ECM substratum that would be favorable for hepatogenesis. For tissue engineering applications, it is imperative that the synthesized matrix substratum is biocompatible, and has no cytotoxic properties. Biodegradability is also another preferred characteristic, although this is not absolutely critical. Culture on 3-D matrix scaffolds or embedment within sandwich culture, have both been reported to be superior to conventional 2-D monolayer culture for maintaining differentiated hepatocyte phenotype. Studies with synthetic substratum demonstrated that hepatocyte adhesion and biological function are enhanced with increasing surface ‘roughness’, hydrophilicity, and porosity. Additionally, porosity of the ECM substratum is advantageous for 3-D tissue growth and vascularization, and this would be particularly useful if the scaffold matrix is to be implanted in vivo.

ECM substratum used for promoting the differentiation of stem cells into the hepatic lineage should preferably incorporate naturally occurring constituents of liver ECM, so as to provide a more physiological environment for hepatic differentiation in vitro. Because collagen is an integral component of liver ECM, it is commonly supplemented in primary hepatocyte culture, and has been reported to be beneficial for maintaining differentiated hepatocyte phenotype under in vitro culture conditions. Additionally, the presence of collagen has also been reported to have a stimulatory effect on the hepatic differentiation of both adult and ES cells in the presence of appropriate cytokines and growth factors. To enhance its structural properties for tissue-engineering applications, collagen-based substratum are often fabricated as composites that may incorporate a variety of synthetic and natural occurring materials, such as polypropylene, TER polymer, alginate-poly L-lysine, chondroitin sulfate, and chitosan. Besides collagen, fibronectin and laminin are also major constituents of liver ECM. Indeed, these have been reported to enhance hepatic differentiation in the presence of the appropriate cytokines and growth factors.

The non-proteinaceous component of liver ECM is primarily composed of glycosaminoglycans (GAG), which are essentially long-chain sugar molecules. With the exception of hyaluronan, all of these (heparan sulfate, chondroitin sulfate, keratan sulfate, and dermatan sulfate) are sulfated branch-chained molecules that are conjugated to proteins, in the form of proteoglycans. Both chondroitin sulfate and hyaluronan have been reported to be beneficial for maintaining the differentiated phenotype of primary hepatocytes cultured in vitro.
However, their specific effects on the hepatic differentiation of stem cells remain uncharacterized.

Besides purified ECM molecules, heterogenous mixtures of ECM components can also be used to promote hepatic differentiation in vitro. Of particular interest is the basement membrane extract of Engelbreth–Swarms mouse sarcoma, better known by its commercial product name: Matrigel® (Becton-Dickinson Inc., USA). This has been reported to be beneficial for maintaining differentiated hepatocyte phenotype, as well as for promoting hepatic differentiation under in vitro culture conditions. Other naturally occurring materials that are not found in liver ECM, have also been utilized as substratum for hepatocyte culture. These include gelatin, chitosan, and alginate or composites of these materials. Additionally, matrix scaffolds based completely on synthetic materials such as polyglycolic acid, polylactic acid, polylactic-coglycolic acid, polyethylene oxide, polyurethane, polyethylene glycol, and dextran sulfate have also been fabricated. Nevertheless, it would be preferable to utilize these as composites with naturally occurring ECM components.

It must be noted that none of the naturally occurring or artificial ECM substrata that have been described so far, actually possess a specific effect on hepatic differentiation. Instead, their use should be combined together with appropriate cytokines/growth factors (Table 1), so as to achieve a synergistic effect on hepatic differentiation in vitro. Also, the overwhelming majority of studies on the effects of ECM substrate on hepatic differentiation were based on primary explanted hepatocytes or hepatoblasts, with few if any studies being conducted on purified populations of adult stem cells or even ES cells; hence, this field of research is still relatively immature. Nevertheless at present, the development of ECM substratum for application in liver tissue engineering is progressing very rapidly. It is anticipated that more novel types of composite matrix scaffolds incorporating a variety of natural and synthetic materials would be developed in the near future, for the controlled differentiation of stem cells into the hepatic lineage.

**COCULTURE AND CELL-CONDITIONED MEDIA**

Another strategy to induce hepatic differentiation of stem cells would be to coculture the stem cells with a different cell population. Primary cultures of mature hepatocytes rapidly lose their differentiated phenotype under in vitro culture conditions. Coculture with a variety of different cell types have been reported to be beneficial for maintenance of differentiated hepatocyte function. These include a variety of non-parenchymal liver cell types, such as the fat-storing stellate cells, biliary epithelial cells, and sinusoidal endothelial cells. More recently, an immortalized hepatic stellate cell line was specifically developed for long-term maintenance of hepatocyte primary cultures. Additionally, coculture with non-liver cell types such as pancreatic islet cells, 3T3 fibroblast, and STO cell lines have also been reported to be beneficial for maintenance of differentiated hepatocyte function.

Liver non-parenchymal cells were reported to stimulate transdifferentiation of bone marrow-derived hematopoietic stem cells into the hepatic lineage. Surprisingly, coculture with embryonic cardiac mesoderm induced hepatic differentiation of ES cells. With hepatic stem-like cells, coculture with fat-storing liver stellate cells was shown to have a stimulatory effect on hepatic differentiation. Further investigation revealed that one of the surface proteins found on stellate cells, epimorphin, could play a crucial role in the stimulation of hepatic differentiation. In an interesting study, it was reported that embryonic chick lung mesenchyme could promote further differentiation of mouse hepatic primordium cultured in vitro.

The main advantage of coculture systems is that this allows intimate contact between different cell types, which may lead to a more efficient transduction of molecular signals that induce hepatic differentiation. The surface receptors of cocultured cells come into direct physical contact, and the autocrine and paracrine factors secreted by one cell type readily interact with the other cell type. More recently, there is evidence that intimate physical contact may lead to fusion of different cell types, resulting in the formation of heterokaryons. In fact, cell-fusion phenomenon has been used to explain the ability of adult stem cells to trans-differentiate into cell types that are radically different from their tissue of origin, when transplanted in vivo. Nevertheless, there is as yet no evidence that the stimulatory effect of coculture on hepatic differentiation is the result of cell-fusion.

Coculture of two or more distinct cell populations (from different sources) also carries a strong risk of transmission of pathogens, in particular viruses. This would constitute a major obstacle to the clinical application of coculture for hepatic differentiation. In the clinical situation, it would simply not be practical to stimulate hepatic differentiation of stem cells through coculture with either an autogenic or donated cell source. Obviously, a donated source of primary explanted cells is difficult to come by and not always readily available; while the pathological state of autogenic cells from the diseased liver would clearly make these unsuitable for coculture with stem cells. An alternative would be to utilize established cell lines for coculture with stem cells, but there would be overwhelming safety concerns if the cocultured stem cells are to be utilized in clinical transplantation.

Another major shortcoming of coculture is the difficulty in the separation of cocultured cell populations. The highest degree of purity upon separation could be achieved by fluorescence activated cell sorting (FACS). However, FACS is skill-intensive and requires expensive instrumentation. Magnetic-affinity cell sorting (MACS) is much cheaper compared to FACS, but the degree of purity upon separation is much lower. The problem of separating distinct cell populations, as well as the potential problem of cell-fusion, may be overcome by keeping cocultured cell popula-
tions physically separated through the use of commercially available Transwell inserts. The other alternative would be to utilize filtered cell-conditioned media instead.

Indeed, culture media conditioned by non-parenchymal liver cells, biliary epithelial cells, and 3T3 fibroblasts have been shown to be beneficial for maintaining differentiated hepatocyte function within in vitro culture. Suzuki et al. reported that embryonic fibroblast-conditioned media promoted differentiation and subsequent colony formation of hepatic stem cells. However, culture media conditioned by liver stellate cells had no beneficial effect on the maintenance of differentiated hepatocyte function within in vitro culture. It is important to note that the use of filtered conditioned media does not alleviate the risk of viral transmission. Also, secreted factors within conditioned media may be labile and hence may not be suitable for prolonged durations of in vitro culture.

**INDUCTION OF INTERCELLULAR COUPLING THROUGH GAP JUNCTION FORMATION**

A common means of intercellular communication in vivo is through gap junctions, which form small connecting channels (pore size = 1.5 nm) between the cytoplasm of adjacent cells. Gap junctions are primarily composed of transmembrane proteins that are generically referred to as connexins, of which 20 different isoforms have currently been identified. The most abundantly expressed isoform in mature hepatocytes is connexin 32. At early stages of hepatic differentiation, connexin 26 and 43 are also strongly expressed, but these are gradually down-regulated at the later stages of hepatic differentiation. Adjacent cells coupled by gap junctions can share small molecules (<1500 Da) such as inorganic ions and metabolites, but not macromolecules (proteins, nucleic acid, and polysaccharides). Even so, the electro-chemical and metabolic coupling achieved through gap junctions, as a result of the passage of small molecules, plays an important role in the regulation of tissue physiology.

Gap junction-mediated intercellular coupling plays an integral role in liver function. Differentiation and expression of mature hepatocyte phenotype is associated with increased gap junction formation. Hence, a possible strategy to enhance hepatic differentiation, would be to induce intercellular coupling by gap junction formation between stem cells that are differentiating into the hepatic lineage in vitro.

With primary hepatocytes, gap-junction mediated intercellular coupling was reported to be enhanced by a variety of protein factors such as insulin and glucagon, polysaccharides (i.e. glycosaminoglycans), and other non-proteinaceous chemical compounds. Among the most potent stimulators of gap-junction formation are the glycosaminoglycans and their protein conjugates, the proteoglycans, in particular chondroitin and dermatan sulfate proteoglycans. Purified glycosaminoglycans were reported to have only a weak stimulatory effect on gap junction mediated intercellular coupling, with the exception of liver-derived heparin. Other polysaccharides (i.e. glycosaminoglycans) also have weak stimulatory effects, with the exception of lambda-carrageenan. Other non-proteinaceous chemicals that have been reported to enhance gap junction formation in hepatocytes, includes retinoic acid, diallyl sulfide, dimethyl sulfoxide, melatonin, glucocorticoids such as dexamethasone and hydrocortisone, and dibutryl-cAMP. It is important to note that the overwhelming majority of these studies were based on mature primary hepatocytes, with little investigation being carried out on hepatic progenitors and stem cells differentiating into the hepatic lineage.

With different cell types, other than the hepatic lineage, a variety of cytokines and non-proteinaceous chemical factors have also been reported to enhance gap junction mediated intercellular coupling. These include parathyroid hormone, Interleukin-1-beta, prostaglandin E2, low-density lipoprotein, dopamine, inhibitors of glycosylation, and potassium conductance blockers such as tetraethylammonium and 4-aminopyridine. It would be interesting to test the effects of these cytokines and chemical factors on either primary hepatocytes, or on stem cells that are differentiating into the hepatic lineage in vitro.

The major limitation of gap junction communication is that only inorganic ions and small molecular weight metabolites (<1500 Da) can transverse through the cytoplasm of coupled cells, while much larger cytosolic signaling proteins and mRNA transcripts cannot pass through gap junctions. Hence, there might not be an effective transduction of differentiation signals through gap junctions alone.

**POSSIBLE USE OF CYTOPLASMIC EXTRACTS AND CYBRIDIZATION**

A novel technique that could possibly be used to direct the hepatic differentiation of stem cells in vitro, would be to expose permeabilized stem cells (i.e. with Streptolysin-O) to concentrated cytoplasmic extracts derived from mature hepatocytes or hepatic progenitors. Using a similar technique, Håkelien and colleagues managed to reprogram 293T fibroblasts to express T-cell and neuronal function, by exposure to cytoplasmic extracts of T-cells, and neuronal progenitors, respectively. However to date, there are as yet no reported studies on the use of cytoplasmic extracts of hepatocytes or hepatic progenitors, to direct the differentiation of either fibroblasts or stem cells into the hepatic lineage.

A major limitation of this approach is the risk of viral transmission. For clinical therapy, it is simply not practical to obtain cytoplasmic extracts of primary hepatocytes or hepatic progenitors from diseased patients, to reprogram autogenic adult stem cells. Perhaps, if there are efficient protocols for generating hepatocytes or hepatic progenitors from ES cells, then this could potentially provide a limitless supply of donor cytoplasm to reprogram autogenic cells.
Another possible strategy to direct hepatic differentiation would be to fuse autologous adult stem cells with enucleated cytoplasts derived from hepatocytes or hepatic progenitors, to form cytoplasmic hybrids or cybrids. This approach could possibly utilize ES cell-derived hepatocytes or hepatic progenitors, to form cytoplastic hybrids or cybrids. Again, this approach could possibly utilize ES cell-derived hepatocytes or hepatic progenitors, to form cytoplasmic hybrids or cybrids. The enucleated cytoplasts can then be fused with nucleated cells through a variety of different techniques utilizing electrical pulse, polyethylene glycol, or with Sendai virus.

The advantage of cybridization is the intracellular exposure of the nuclei to cytosolic proteins and mRNA transcripts of another cell type, which could in turn lead to a more effective transduction of differentiation signals. Intracellular signaling molecules are likely to be extremely labile, and hence easily destroyed by the relatively harsh procedure used to make a cytoplasmic extract, whereas the stability of such labile molecules is likely to be preserved within intact cytoplasts. To date, there are no studies that have yet been reported on the use of cybridization to direct hepatic differentiation. Nevertheless, previous studies have shown that cybridization could be used to induce teratocarcinoma cells to express myoblast function, as well as direct erythroid and myeloid differentiation.

As with the use of cytoplasmic extracts, cybridization entails the risk of viral transmission. Moreover, the enucleation procedure by high speed centrifugation may lead to some cytoplasts retaining fragments of chromosomal DNA, which could result in aneuploidy upon fusion with nucleated cells. There could also be structural disruptions to the cytoskeleton and organelles within the enucleated cytoplasts, as a result of high-speed centrifugation. This could possibly lead to aberrant cellular function upon fusion with nucleated cells. Another major problem is the relatively slow efficiency of cell fusion, and relatively poor viability of the resulting cybrids. This would pose a formidable challenge with relatively scarce adult stem cells.

DIRECTING HEPATIC DIFFERENETIATION THROUGH GENETIC MODULATION

A novel strategy for directing and controlling the hepatic differentiation of stem cells in vitro is through genetic modulation. This could be achieved by transfecting stem cells with recombinant DNA constructs encoding for the expression of certain proteins that promote hepatogenesis. Of particular interest are liver-enriched transcription factors such as the hepatic nuclear factor (HNF) family of proteins (HNF 1, 3, 4, & 6), CCAAT/enhancer binding protein (C/EBP), and D binding protein (DBP), which are implicated in the pathway of hepatic differentiation. These can be thought of as ‘master switches’ that control the expression of the entire array of proteins specific to the hepatic lineage. Indeed, recombinant overexpression of members of the HNF family has all been reported to promote hepatic differentiation. Besides transcription factors, the recombinant expression of the gap junction protein connexin 32, has also been shown to stimulate hepatic differentiation, since gap junction mediated intercellular coupling plays an integral role in hepatocyte function.

The disadvantage of directing hepatic differentiation through genetic modulation is one of the potential risks associated with utilizing recombinant DNA technology in human clinical therapy. For example, the constitutive over-expression of any one particular protein or transcription factor within transplanted stem cells would certainly have unpredictable physiological effects upon transplantation in vivo. This problem may be overcome by placing the recombinant expression of the particular protein under the control of ‘switchable’ promoters, several of which have been developed for expression in eukaryotic systems. Such ‘switchable’ promoters could be responsive to exogenous chemicals, heat shock, or even light. Of particular interest are light-responsive promoters, since these would avoid the potentially toxic or pleiotropic effects of exogenous chemicals and heat treatment. At present, there are as yet no reported studies on the coupling of hepatocyte-specific genes to light-responsive promoters. Indeed, the creation of such recombinant constructs and the subsequent transfection within stem cells would certainly make an interesting study, with potentially useful clinical applications.

The cellular signaling pathway for hepatic differentiation could be given a ‘kick-start’ through temporary expression of liver-enriched transcription factors coupled to light-inducible promoters. After that, it is possible that the pathway for hepatic differentiation could carry on independently of the recombinant expression of these transcription factors, since the entire array of hepatocyte-specific genes would have already been activated. The advantage of this approach is that there is no constitutive over-expression of any one particular transcription factor. Also, the natural cellular pathway for hepatic differentiation could carry on physiologically upon switching off the recombinant expression of these transcription factors, through removal of light stimulus. Upon transplantation in vivo, it is extremely unlikely that light-inducible promoters would again be activated, since light stimulus would be completely absent in situ.

Genetically modified stem cells may also run the risk of becoming malignant within the transplanted recipient. Moreover, there are overriding safety concerns with regards to the use of recombinant viral-based vectors in the genetic manipulation of stem cells. It remains uncertain as to whether legislation would ultimately permit the use of genetically modified stem cells for human clinical therapy. At present, the potential detrimental effects of transplanting genetically modified stem cells in vivo are not well-studied. More research needs to be carried out on animal models to address the safety aspects of such an approach.
More recently, there is emerging evidence that some transcription factors (which are commonly thought of as cytosolic proteins) have the ability to function as paracrine cell to cell signaling molecules. This is based on intercellular transfer of transcription factors through atypical secretion and internalization processes.

Hence, there is an exciting possibility that liver-enriched transcription factors implicated in hepatic differentiation, may in the future be genetically engineered to incorporate domains that enable them to participate in novel paracrine signaling mechanisms. This in turn would have tremendous potential for directing the differentiation of stem cells into the hepatic lineage.

**CONCLUDING REMARKS**

Despite the large number of studies that have recently been carried out on the hepatic differentiation of stem cells *in vitro*, this particular area of research is still in its relative infancy. Hepatic differentiation may be further enhanced, if the various techniques that have so far been discussed are used in combination, rather than exclusively by themselves. In the natural milieu, the hepatic differentiation of stem cells probably involves multiple signaling pathways. This may be mimicked *in vitro* by using a combination of these various techniques, to achieve a synergistic effect on the differentiation of stem cells into the hepatic lineage.

It must, however, be kept in mind that for clinical applications it is imperative to develop well-defined and efficient *in vitro* protocols for the hepatic differentiation of stem cells that would utilize chemically defined culture media supplemented with recombinant cytokines and growth-factors. This will then provide the stringent levels of safety and quality control that would make the clinical applications of stem cell transplantation therapy realizable. Hopefully, this will be achieved in the near future.

**REFERENCES**

18. Wulf GG, Luo KL, Jackson KA, Brenner MK, Goodell MA. Cells of the hepatic side population contribute to liver regeneration and can be replenished with bone marrow stem cells. *Haematologica* 2003; 88: 368–78.
26. Wagemaker G, Neelis KJ, Wognum AW. Surface markers and growth factor receptors of immature hemopoie-
Hepatic differentiation of stem cells


59 Ranucci CS, Kumar A, Batra SP, Moghe PV. Control of hepatocyte function on collagen foams: sizing matrix


Hepatic differentiation of stem cells


124 Huard C, Druesne N, Guyonnet D. Cyclic AMP enhances gap-junctional intercellular communication by both direct and indirect mechanisms in rat liver cells. Carcinogenesis 2004; 25: 91–8.

125 Yoshizawa T, Watanabe S, Hirose M, Miyazaki A, Sato N. Dimethylsulfoxide maintains intercellular communication by preserving the gap junctional protein...


Hepatic differentiation of stem cells


