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Signalling pathways involved in the process of mesenchymal stem cells differentiating into hepatocytes

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Abstract

End-stage liver disease can be the termination of acute or chronic liver diseases, with manifestations of liver failure; transplantation is currently an effective treatment for these. However, transplantation is severely limited due to the serious lack of donors, expense, graft rejection and requirement of long-term immunosuppression. Mesenchymal stem cells (MSCs) have attracted considerable attention as therapeutic tools as they can be obtained with relative ease and expanded in culture, along with features of self-renewal and multidirectional differentiation. Many scientific groups have sought to use MSCs differentiating into functional hepatocytes to be used in cell transplantation with liver tissue engineering to repair diseased organs. In most of the literature, hepatocyte differentiation refers to use of various additional growth factors and cytokines, such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), oncostatin M (OSM) and more, and most are involved in signalling pathway regulation and cell–cell/cell–matrix interactions. Signalling pathways have been shown to play critical roles in embryonic development, tumourigenesis, tumour progression, apoptosis and cell-fate determination. However, mechanisms of MSCs differentiating into hepatocytes, particularly signalling pathways involved, have not as yet been completely illustrated. In this review, we have focused on progress of signalling pathways associated with mesenchymal stem cells differentiating into hepatocytes along with the stepwise differentiation procedure.

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Introduction

Numbers of aetiologies are able to cause liver dysfunction resulting in chronic liver disease and acute liver failure; many people annually, worldwide, die of liver disease. The therapy of choice for end-stage liver diseases, liver metabolic diseases and (perhaps) hepatocellular carcinoma is liver transplantation. Although achievements of liver transplantation have improved enormously and despite increasing numbers for whom this surgical procedure is performed, solutions to address the growing numbers of patients eligible for transplantation and decreasing numbers of available donors. Considering the prohibitive cost of liver transplantation, major complications (1), long-term requirement for immunosuppressive drugs (2,3) and necessity of an intensive care, pursuit of alternative therapies for end-stage liver diseases are particularly important.

Over the past decade, cell-based therapies and hepatic tissue engineering have started to be considered as promising alternatives for treatment of liver diseases (4–6). Mesenchymal stem cells (MSCs) have attracted considerable attention as potential therapeutic tools as they can be obtained with relative ease and can be expanded in culture. MSCs should be efficiently proliferating and adherent fibroblastic-type cells with features of the unique expression profile of cell surface molecules and cell–cell interaction proteins. MSCs have self-renewal and multilineage differentiation shown by mesodermal (7) and ectodermal differentiation (8), to such as adipose tissue, bone, cartilage, tendon and muscle. In addition, numerous pieces of research indicate that MSCs have low inherent immunogenicity (9) and suppress immunological responses through interaction with immune cells (10,11). This is exceptionally useful in clinical applications to therapy-resistant severe acute graft-versus-host liver diseases, liver allograft rejection and liver tissue engineering. Also, consideration of their ease of harvest and various tissue sources, such as from

bone marrow (12), adipose tissue(13), umbilical cord (14), placenta (15) and menstrual blood (16), propose MSCs to be seed cell candidates for therapeutic purposes in clinical application to liver diseases and liver cell-tissue engineering.

MSCs obtained from various sources have been demonstrated to possess endodermal differentiation potential which allows them to differentiate into hepatocyte-like cells (12–14,16) *in vitro* under appropriate culture conditions. Most differentiation procedures include various additional growth factors and cytokines, such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), oncostatin M (OSM) and more. HGF is a mesenchymally derived, pleiotropic, multifunctional growth factor that acts as an important regulator of diverse processes, including mitogenesis, motogenesis, morphogenesis and apoptosis, in a variety of cell types (17,18). It exerts its biological effects through the HGF receptor (19), c-Met, which is expressed by normal hepatocytes, gastric and intestinal epithelium, ovarian and endometrial endothelia. FGF, on the other hand, is a fundamental member of heparin-binding proteins and interacts with cell surface-associated heparan sulphate proteoglycans shown to be essential for FGF signal transduction (20,21). FGF is a key player in processes of proliferation and differentiation of a wide variety of cells and tissues (21,22). EGF is the founding member of the EGF-family of proteins and is a growth factor that stimulates proliferation and differentiation by binding to its receptor EGFR (21,23,24). OSM is a pleiotropic cytokine that belongs to the interleukin 6 (IL-6) group of cytokines (25) which closely resembles leukaemia inhibitory factor (LIF) in both structure and function, and has been proven to be important in liver development (26–28). These growth factors and the cytokine are involved in hepatocyte differentiation together with other differentiation regents, such as dexamethasone (29,30), insulin–transferrin–selenium (ITS) (31,32) and L-ascorbic acid (33) and are involved in enormous varieties of signalling transduction pathways.

Signal transduction and communication involve binding of extracellular signalling molecules and ligands to cell surface receptors to trigger intracellular events. Signalling pathways have been shown to play critical roles in embryonic development, tumourigenesis, tumour progression, apoptosis and cell-fate determination through pathway crosstalk-communication and cell–cell/cell–matrix interactions (34–36). In the complex process of mesenchymal stem cells differentiating into hepatocytes, investigators define the procedure into three stages: initiation, differentiation and maturation, consistent with sequences of liver developmental processes of specification and differentiation into mature hepatocytes. In this review, we have focused on signalling pathways associated with mesenchymal stem cells differentiating into hepatocytes *in vitro*, with the stepwise differentiational procedure.

Passage of hepatogenesis from mesenchymal stem cells

To understand hepatocyte differentiation protocols utilizing various growth factors and cytokines related to cell development and cell communicative signalling pathways, it is essential to overview the passage of hepatogenesis from mesendoderm, *in vivo*.

Specification of mesendoderm to endoderm definition

This begins with establishment of the endodermal germ layer during gastrulation. Fate mapping studies in the mouse embryo at embryonic gestation day 8, indicate that embryonic liver originates from ventral foregut endoderm (37). Nodal signaling is a signal transduction pathway that is important in pattern formation and differentiation during embryo development. Nodal signalling initiates both endodermal and mesodermal formation in a concentration-dependant manner, low Nodal doses inducing mesoderm while higher doses inducing endoderm (38). Huelsken *et al.* (39) revealed that lacking Nodal, or β-catenin, mouse embryos fail to form appropriate primitive development, indicating that the canonical Wnt pathway and Nodal act in synergy to specify definitive endoderm. Nodal signalling stimulates expression of a critical set of endoderm transcription factors including HMG domain DNA-binding factor Sox17 and fork head domain proteins Foxa1–3(HNF3α/β/γ) (40) which in turn regulate a cascade of genes committing cells to the endoderm lineage. Recent research has also indicted that treatment with high Activin A and Wnt3a enabled ED-cells to differentiate into endodermal lineages (41).

Specification of the hepatoblast

The hepatoblast structure begins with an invagination in the ventral domain of the foregut (42). By embryonic day (ED) 8.5, specification of the hepatoblast is detfined to a portion of the ventral foregut endoderm adjacent to the heart, and with the embryo developing the endoderm, forms a gut tube. The liver domain moves to the midgut (43). Establishment of foregut progenitors is an important step in hepatogenesis as *in vivo* only, the foregut endoderm (but not midgut nor hindgut) is competent to develop into the liver (37,42,44). As dependent on appropriate Activin/Nodal signalling in the definitive endoderm at early stages

of differentiation, the hepatoblast responds to cAMP and is able to differentiate further into hepatic progenitors, using fibroblast growth factor 10, retinoic acid and an inhibitor of Activin/Nodal receptor *in vitro* (45). Wnt/β-catenin signalling is not only a critical effect during hepatoblast specification (42), but also plays important roles in hepatoblast cell proliferation and differentiation (46). Further investigation (47) has suggested that hepatoblasts derived from direct conversion of non-hepatic endodermal cells, can form in the endoderm posterior to the liver by activation of Wnt/β-catenin signalling. This manifests that the Wnt/β-catenin pathway exerts autonomy in endodermal cells to induce hepatic conversion. In summary, these results demonstrate that Wnt/β-catenin signalling can induce fate change of non-hepatic endodermal cells to hepatoblast fate, during specification of the hepatoblast.

Determination of hepatocytes

Differentiation of bi-potential hepatoblasts into hepatocytes or biliary epithelial cells (BECs) begins around ED13 of mouse development when the cell population is considered to be the foetal source of hepatic progenitor cells (48,49). Bi-potential hepatoblasts express foetal liver genes (e.g. *alpha foetal protein*, *AFP*) as well as markers of both hepatocytes albumin (ALB) and BEC cytokeratin 19 (CK19). Evidence suggests that TGFβ and Wnt signals restrain expression of hepatogenic transcription factors from the periportal mesenchyme (50), such as HNF4 and C/EBP, but strengthen expression of BEC promoting transcription factors, such as onecut1 (OC1), onecut2 (OC2) and hepatocyte nuclear factor1β (HNF1β) in adjacent hepatoblasts. Continued Notch signalling pathway from the periportal mesnechyme promotes ductal plate remodelling (51,52) along the cholangiocytic lineage, while cytokine oncostatin M (OSM) in combination with HGF promotes hepatocyte differentiation (27,53,54) and complements glucocorticoid hormones and HGF to trigger complete hepatic maturation (55). Interestingly, although evidence (47) supports the role of β-catenin in proliferation and differentiation of hepatoblasts during liver bud expansion, Lade *et al.* (56) utilized truncated 75 kDa β-catenin, which appears in developing liver; its localization and appearance in hepatocytes coincides with hepatocyte maturation. Lade *et al.* further demonstrate that this form of β-catenin is produced post-translationally *via* proteolytic cleavage of its N-terminal 95 amino acids by calpain, and that it localizes to membranes and nuclei of hepatocytes in late foetal liver. This indicates that calpain-mediated cleavage of β-catenin plays a role in inducing hepatocyte maturation in mouse and human liver.

Together, various signalling pathways and molecular cross-talk events are patterned to occur appropriately and temporally and accomplish homoeostatic regulation of hepatogenesis (Table 1).

Protocols for mesenchymal stem cells (MSCs) differentiating into hepatocytes

MSCs obtained from various sources have been demonstrated to have endodermal potential enabling them to differentiate into hepatocyte-like cells (12–14,16) *in vitro* under appropriate culture conditions. It is essential to include adequate stimulus for maintenance of cell function, such as growth factors, hormones and cytokines during induction of hepatocyte differentiation *in vitro*. Various protocols are well established, while they may differ considerably in detail; this causes difficulty in standardization of hepatocyte acquirement. To recapitulate liver development, we address *in vitro* hepatocyte differentiation in three stages: initiation, differentiation and maturation, and focus on the signalling pathways involved in each.

Initiation

Mesenchymal stem cells were initially described as being from the bone marrow and termed as mesodermally-derived non-haematopoietic bone marrow cells, while hepatocytes are the principal cell type of the liver, derived from the embryonic endoderm. Though MSCs have the capacity to differentiate into mesodermal and non-mesodermal-derived tissues according to product-orientation induction medium, definition and specification of 'endoderm' is essential and considered to be the initiation stage in the process of hepatocyte differentiation *in vitro* (40,45,57). Activin/Nodal family members initiate mesendodermal/endodermal transition when Activin A expression elevates 3-fold compared to baseline. Activin/Nodal family members release inhibitory signals generated by phosphoinositide 3-kinase (PI3K) through insulin/IGF (58). Although, Toivoen *et al.* have shown that definitive endodermal cells derived from human pluripotent stem cells with high Activin A and Wnt3a treatment have been able to differentiate further into both hepatic and pancreatic lineages (41), Filby *et al.* demonstrated that stimulation of the Nodal signalling pathway with Activin A was insufficient to induce definitive endoderm formation from unrestricted somatic stem cells (59). So *et al.* used mosaic analysis with a transgenic cell line expressing Axin1 and showed when Axin1, a central component of the β-catenin destruction complex, was greatly overexpressed *via* heat shock, 17 h post-fertilization, liver size was greatly reduced in Axin1-overexpressing embryos compared to

Table 1. summary track of hepatogenesis *in vivo*. various signalling pathways are involved in the right place at the right time and accomplish homoeostatic regulation of hepatogenesis. To clarify the functions of related signalling pathways in different hepatogenesis stages, the hypothetical development hepatogenesis demarcations are presented here

Stage of Hepatogenesis *in vivo*	Principal activities of signalling pathways	Functions of signalling pathways regulations	References
Specification of mesendoderm to endoderm definition	Nodal signalling pathway initiates both endoderm and mesoderm formation	Low Nodal doses induces mesoderm while higher doses induces endoderm	(38)
	Canonical Wnt pathway and Nodal act in synergy to specify definitive endoderm	Mouse embryos fail to form a primitive development in lacking of Nodal or β-catenin	(39)
	Nodal signalling stimulates the expression of transcription factors, such as HMG domain DNA-binding factor Sox17 and the fork head domain proteins Foxa1–3(HNF3α/β/γ)	Regulate a cascade of genes committing cells to the endoderm lineage	(40)
	Treatment of high Activin A and Wnt3a	Differentiate into endodermal lineages	(41)
Specification of hepatoblast	Appropriate Activin/Nodal signalling enhance endoderm definition at early stages of differentiation	Hepatoblast responds to cAMP and is able to differentiate further into hepatic progenitors	(45)
	Wnt/beta-catenin signalling	Performs a critical effect during the hepatoblast specification and plays important roles in hepatoblast proliferation and differentiation	(42,46)
	Activation of Wnt/β-catenin signalling	Exerts cell autonomously in endodermal cells to induce hepatic conversion	(47)
Determination of hepatocytes	TGFβ and Wnts signals	Restrain the expression of hepatogenic transcription factors from the periportal mesenchyme, but strengthen the expression of BEC-promoting transcription factors in the adjacent hepatoblasts	(50)
	Continued Notch signalling pathway from the periportal mesenchyme	Promotes ductal plate remodelling along cholangiocytic lineage	(51,52)
	Oncostatin M (OSM) in combination with HGF	Promotes hepatocyte differentiation	(27,53,54)
	OSM works together with glucocorticoid hormones and HGF	Trigger for complete hepatic maturation	(55)
	Utilizes a truncated 75 kDa β-catenin species	The localization and appearance of t runcated β-catenin in hepatocytes coincides with hepatocyte maturation	(56)

controls. This suggested that Wnt/β-catenin signalling autonomously induced non-hepatic endodermal cells to the liver fate (47). Characteristic time-dependent expression of transcriptional factors HNF4α, C/EBPα and C/EBPβ during hepatocyte differentiation by bone marrow stem cells demonstrates that expression of these transcription factors is closely related to initiation and maintenance of hepatocyte differentiation (50,60). Hence, in the initiation stage of hepatocyte differentiation, MSCs can be induced to become a homogenous population of endodermal cells using a combination of activin, fibroblast growth factor 2 and bone morphogenetic protein 4 together with phosphoinositide 3-kinase (PI3K) inhibition (45,58).

Differentiation

Cells of the hepatoblast are considered to be somatic stem/progenitor cells in foetal livers as they have high proliferative potential and ability to differentiate into both hepatocytes and cholangiocytes, during middle to late embryonic stages (42,45,61). Proliferation and differentiation of hepatoblast cells are regulated by a number of soluble factors. By addition of growth factors such as FGF4, HGF and EGF cultured for 5 days *in vitro*, MSCs can be directed into proliferation of a certain cell population, of which in the order of 50% of cells co-express hepatocyte marker AFP and cholangiocyte marker of cytokeratin 19. This result suggests that that cell population corresponds to the hepatoblast population (62). Of various growth factors, HGF has particularly attracted the attention of most investigators concerned with induction of differentiation from MSCs. For example, Forte *et al.* (63) demonstrated that HGF played an essential role in development and regeneration of the liver by comparative observations of short-term exposure of MSCs to HGF, being able to induce activation of its cognate Met receptor and downstream effectors extracellular signal-regulated kinase1/2 (ERK1/2), p38,

MAPK and PI3K/Akt. Long-term exposure to HGF, however, resulted in cytoskeletal rearrangement, cell migration and marked inhibition of proliferation by arrest at the G1–S checkpoint. Ghaedi *et al.* (17) cultured adipose stem cells on HGF/collagenI(Col) spots for 2 weeks and found that mRNA levels for albumin, α-foetoprotein and α1 antitrypsin were 10–20-fold higher in stem cells cultured on HGF/Col arrays compared to stem cells on Col only spots, indicating that HGF induced expression of the hepatic phenotype in mesenchymal stem cells *in vitro*. Neuss *et al.* (19) also demonstrated that HGF and c-Met are constitutively expressed by hMSC and that expression of HGF is down-regulated by transforming growth factor-β (TGF-β). Furthermore, HGF exerts a strong chemotactic stimulus to hMSC, which may be further enhanced by autocrine signalling through the HGF c-Met pathway. While Zhou *et al.* (64) demonstrated HGF supported a mid/late hepatic phenotype such as ALB and dipeptidyl peptidase IV expression, but failed to induce functional hepatocyte maturation. Together, the above investigations suggest that HGF stimulates rapid hepatoblast proliferation *via* Wnt/β-catenin signalling and the HGF c-Met pathway and is crucial for hepatogenesis *in vivo* and is considered to be a critical and preliminary growth factor in the process of hepatocyte differentiation *in vitro*.

A further family of FGFs, is also effective in mediating early hepatic differentiation, even though optimal choice of the most suitable FGF type depends on the species involved and differentiation orientation (14,16,65). Sekson *et al.* (66) showed FGF1- and FGF4-enriched bipotential hepatic progenitors, whereas FGF8 further promoted embryonic liver enrichment for unipotential hepatocyte progenitors. FGFs expressed by cardial mesoderm play a role in induction of ventral foregut endoderm to initiate early liver development. Many other growth factors are associated with hepatocyte differentiation, many researchers having demonstrated that differentiation of MSCs might be triggered and induced into hepatocytes by cooperation of HGF and FGF-4 *via* the HGF/c-Met signalling pathway and interaction with Wnt signalling by analysis of substantially increasing expressions of ALB, CK18 and CK19, but of strongly reduced AFP (12,14,17,67,68).

Maturation

In the late stage of hepatic differentiation *in vitro*, many investigations have referred to OSM, insulin–transferrin–selenium (ITS), dexamethasone and nicotinamide as major factors. OSM is an interleukin-6 (IL-6) subfamily member produced by haematopoietic cells during the early stages of embryogenesis (26). Kamiya *et al.* (55) demonstrated that OSM play an important role in progression of hepatocyte development to liver maturation through the signalling pathway of signal transducer and activator of transcription 3 (STAT3), despite it failing to induce the differentiated hepatocyte phenotype alone. Sakai *et al.* (69) also indicated that OSM alone had only very weak effects on hepatocyte function, while albumin secretion was greatly enhanced when OSM was combined with nicotinamide. NAD^+ and $NADP^+$ play important roles in maintaining energy for cell functions including DNA repair and genomic stability. Nicotinamide, serves as primary precursor of NAD^+ and the phosphorylated-derivative $NADP^+$ synthesis, has been shown to enhance proliferation of primary hepatocytes and formation of small hepatocyte colonies (70). By over-expression of an activated form of C/EBPβ in dexamethasone/EGF-treated cells, Al-Adsani *et al.* (29) have shown that expression of hepatocyte markers increased and expression of ductal markers reduced indicating that hepatocytes and ductal cells could be induced from pancreatic exocrine cells following treatment with dexamethasone; conversion was dependent on expression of C/EBPβ and *via* regulation of phosphoinoside 3-kinase (PI3K) signalling pathway. On the other hand, Teramoto *et al.* also showed suppression of IL-6 expression by dexamethasone depended on inhibition of OSM-mediated activation of PI3K signalling during the formation of vacuoles in human foetal liver cells (27). Vollmer *et al.* (71) indicated that treatment of hepatocytes and hepatoma cells with OSM lead to increased protein level of HIF-1alpha under normoxic and hypoxic conditions *via* Janus kinase (JAK)/STAT3 and mitogen-activated protein kinase kinase (MAPKK)/ERK1/2 pathways, and demonstrated that OSM-mediated HIF-1alpha up-regulation did not result from increase in HIF-1alpha protein stability but from increased transcription of the Hypoxia inducible factor-1alpha (HIF-1alpha) gene, suggesting that OSM treatment promoted activation of HIF-1alpha, and HIF-1alpha in reverse contributed to OSM downstream signalling events, pointing to cross-talk between cytokine and hypoxia signalling in liver development and regeneration. Researches have also reported that dexamethasone is required for maintaining expression of liver-enriched transcription factors essential for stimulating liver-specific gene transcription and promotion of expression of the hepatocyte phenotype by suppressing cell division correlated with activation of several stress signalling pathways including MAPK, SAPK/JNK and c-Jun (29,72). ITS is known to be a chemically defined supplement for supportive *in vitro* proliferation of various mammalian cells. For hepatogenic induction, ITS has been shown to be effective in promoting proliferation and survival of primary hepatocytes (73). In sum-

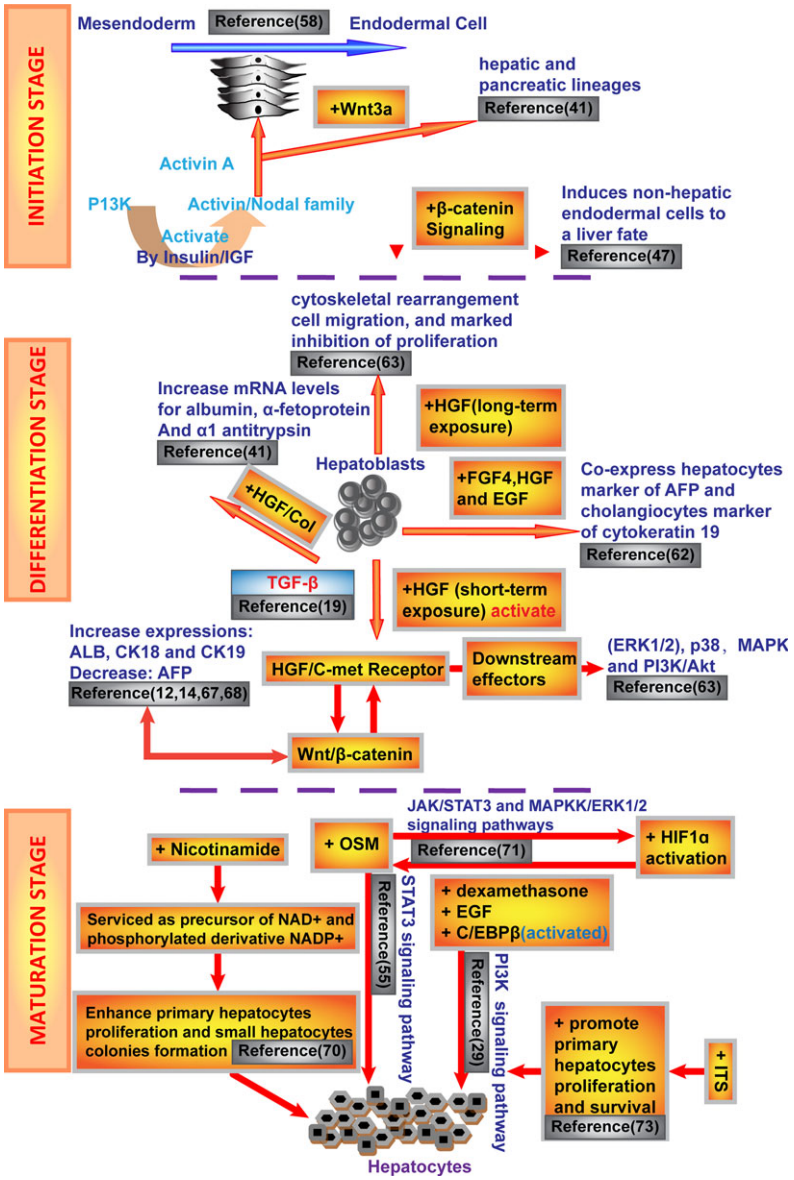

Figure 1. Schematic signalling pathways of MSCs differentiating into hepatocytes *in vitro*.

mary, different cytokines and growth factors involved in different stages of hepatocyte differentiation from MSCs are primarily related to the signalling pathways, such as the Activin/Nodal pathway, PI3K/Akt pathway, HGF/c-Met pathway, ERK1/2, MAPK, JAK/STAT3, hypoxia pathway, although further precise mechanisms remain to be further illustrated (Fig. 1).

Conclusion

Given the ease of isolation, expansion and their multilineage differentiation potential, additionally, superiority of low inherent immunogenicity and capability of regulating immunological responses by interaction with immune cells, MSCs are considered to be an ideal source for clinical applications for a variety of diseases and regeneration with tissue engineering. Signalling pathways have been illustrated to participate in regulation of numerous life events through cell–cell/cell–matrix interactions and crosstalk-communication between diversified signals, such as embryonic development, self-renewal and differentiation, tumourigenesis, tumour progression, apoptosis and cell-fate determination. In the research field of stem cells, most important of all, signalling pathways provide a valuable structure and method for understanding cell–cell/cell–matrix regulation, concomitantly with providing

information of how the stem cell microenvironment couples and integrates extrinsic with intrinsic stem-cell fate determinants and maintain the capability of self-renewal. Also, comprehensive understanding of regulatory mechanisms underlying liver development and hepatocyte differentiation has influenced diagnosis of liver diseases and further progress will be critical to future advances in liver disease therapy.

MSCs derived from various sources have been demonstrated to possess endodermal differentiation potential and can differentiate into hepatocyte-like cells *in vitro* under appropriate culture conditions. However, current understanding of mechanisms that facilitate hepatocyte proliferation and differentiation from mesenchymal stem cells is limited, which has hampered generation of therapeutically effective cells. Thus, it is necessary to clarify the mechanisms of MSC differentiation to build and standardise production of functional hepatocytes from MSCs. Here, we have proposed a number of directions to help guide future research into liver cell therapy and tissue engineering based on MSCs. (i) Knowledge of liver development and regeneration can best provide exhaustive information of MSC differentiation into hepatocytes *in vitro*. It is good choice to thoroughly investigate signalling bases and molecular mechanisms of liver development over the period from foetal to adult liver. (ii) MSCs can be induced into differently functional mature cells in terms of differently stimulative conditions. Thus, dosage and combinations of induced factors need to be accurately adjusted according to the differentiated stages of hepatogenesis. (iii) Molecular mechanisms of each step of hepatocyte differentiation from MSCs is essential for cell therapy. Both positive and negative factors responsible for initiation, differentiation and maturation of hepatocyte differentiation need to be specially considered. (iv) Although we have attempttd to divide the process of hepatocyte differentiation into three stages, it is actually a continuous evolving process in which many critical factors may not only take effect at some stage of hepatocyte differentiation from MSCs. (v) In addition, attention needs to be paid to the various signalling pathways involved in stages of hepatocyte differentiation and also in cross-talk and interaction between signalling pathways, throughout the whole intricate network of signals.

In summary, more scrupulous understanding of instructive signalling pathways emanating from liver development *in vivo*, to MSC interaction with the microenvironment, together with a deeper analysis of cell-intrinsic mechanisms governing proliferation versus differentiation-inducing signalling pathways, is necessary to better understand the mechanisms of hepatocyte differentiation with high efficiency *in vitro*.

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