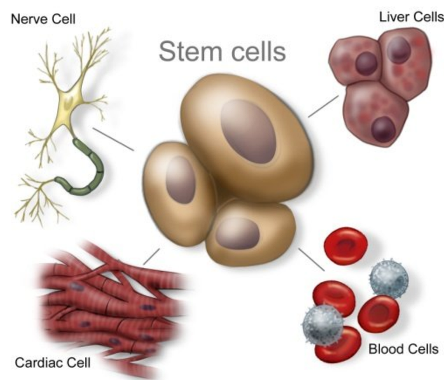
Essay

New approaches in the differentiation of stem cells towards hepatocytes and endothelial cells



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Abstract

Currently, orthotopic liver transplantation is the only effective treatment for end-stage liver diseases. However, the procedure is highly limited by the shortage of liver donors. Cell-based liver therapy could be employed as an alternative approach to whole liver transplantation, thus reducing the mortality rate of patience on the waiting list. Stem cells are currently considered a potentially infinite source for generation of various cell types. Hepatocyte- and endothelial-like cells have been derived from different sources, such as embryonic stem cells (ESCs), human induced pluripotent stem cells (iPSCs), bone marrow stem cells, adipose tissue etc., each of which has its advantages and limitations. Accordingly, tumorogenicity is still one of the major obstacles in clinical application of ESCs and iPSCs. However, a progress in developing efficient non-viral methods of reprogramming somatic cells into pluripotent cell lines and availability of new methods to remove oncogenes after the induction of pluripotencyalleviates some tumorigenic concerns around iPSCs and holds promise for their clinical application in the coming years. Also, adult stem cells were suggested to be non-oncogenic and were extensively studied with respect to their ability to differentiate into hepatocyte- and endothelial-like cells. Mesenchymal stem cells (MSCs) derived from adipose tissue appear to be the most abundant source of adult stem cells that can be used to generate autologous therapies, minimizing or even eliminating problems associated with immune rejection after orthotropic transplantation. Previously used differentiation protocols for hepatocytes and endothelial cells generation were shown to be inefficient and cell differentiation appeared to be inadequate. Newly applied techniques, such as co-cultivation, manipulations of the genome to induce certain genes, considerably improved generation of vascular and hepatic cells from stem cells. Development of functional vascularized *in vitro* hepatic tissue constructs would make it possible to mimic *in vivo* environment and ultimately create bioartificial liver for clinical use.

Keywords: stem cells, hepatocytes, endothelial cells, transplantation, pluripotency.

List of abbreviations

ADSCs	adipose-derived stem cells
AFP	α-fetoprotein
BDSCs	blood derived stem cells
BM-MSCs	bone marrow mesenchymal stem cells
BMP	bone morphogenetic protein
CBSCs	cord-blood stem cells
EB	embryoid bodies
ECM	extracellular matrix
ECs	endothelial cells
EGF	epidermal growth factor
eNOS	endothelial NO synthase
ESCs	embryonic stem cells
FGF	fibroblast growth factor
GF	growth factors
HIF1-α	hypoxia-inducible factor 1-α
HGF	hepatocyte growth factor
HNF	hepatocyte nuclear factor
HSCs	hepatic stellate cells
ICG	indocyanine Green
iPSCs	induced pluripotent stem cells
LDL	low density lipoprotein
MSCs	mesenchymal stem cells
SMC	smooth muscle cells
VEGF	vascular endothelial growth factor

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I. Introduction

Acute liver failure and end-stage liver disease are severe clinical syndromes leading to hepatic encephalopathy, systemic inflammation and multi-organ failure, resulting in a mortality rate of 80% (1, 2). To date, liver transplantation is the only effective treatment for end-stage liver disease considerably decreasing the mortality rate of patients. However, orthotopictransplantation is limited by the shortage of liver donors (3-6). Therefore, cell-based liver therapy including creation of bioartificial liver, could be employed as an alternative approach to whole liver transplantation, reducing the mortality rate of patients on the waiting list: in the Western world about 20-25% of patients die while waiting for a donor liver (1,5,7).

As yet, it is not clear which cell sources would be more efficient in generating liver cell lines for transplantation.Though usage of primary hepatocytes has been recognized as a promising treatment approach for patients with some liver diseases, as well as a ‘’temporal bridge’’ to liver transplantation for people on a waiting list, their low *in vitro*viability, loss of function and limited availability are still the major limitations for their clinical application (3-4,8). Therefore, alternative sources of hepatocytes are requiredto meet the existing clinical needs.

In this essay I tried to summarize up-to-date information regarding new approaches and possibilities in differentiation of stem cells into functional hepatocytes and endothelial cells. The understanding of opportunities and prospects available in generating different cell lineswill bring us closer to the desirable development of bioartificial liver.

II. Hepatocyte-like cells

Various human cells sources have been reviewed regarding their therapeutic functionality and potential of clinical implementation. Accordingly, hepatocyte-like cells have been derived from hepatoma-derived cell lines, embryonic stem cells (ESCs), human induced pluripotent stem cells (iPSCs), bone marrow stem cells, adipose tissue, multipotent progenitor cells in the human umbilical cord, etc (1,3,9) The aim of this essay is to discuss recent data with respect to capability of different proliferative human cell types to differentiate toward hepatocytes.

2.1. ESCs and iPSCs-derived hepatocyte-like cells

Each cell population has its own differentiation potential. Unipotent cells have an ability to sustain only one cell type (e.g. spermatogenial stem cells). Pluripotency, on the other hand, refers to the capacity of cells to give rise to any human cell type,while multipotency is an ability of cells to differentiate into different cell types within the same cell lineage (31,32). Accordingly, ESCs are a pluripotent stem cell linederived from the inner cell mass of blastocyst which possesses an ability of self-renewal and differentiation into virtually all human cell types (3,10). Although each cell type has its differentiation potential, it is possible to revert differentiated cells into a less differentiated using the reprogramming(31). Thus, iPSCs are pluripotent stem cells derived from reprogrammed somatic cells (usually fibroblasts) by introducingOct3/4, Sox 2, Klf4

and c-Myc (3, 11, 32). Currently, different methodologies can be applied to reprogram adult somatic cells to iPSCs, such as integrating or non-integrating viral vectors, plasmids, transposons and recombinant proteins (30). To date, iPSCs are the most attractive cell source for clinical purposes, considering their pluripotency and availability (32). Moreover, the discovery of iPSCs opened a possibility of autologous hepatocyte transplantation, minimizing the risk of transplant rejection (3).

2.1.1. Early liver development

To date, numerous varieties of protocols have been suggested to generate hepatocyte-like cells from ESCs and human iPSCs. Many of them tried to mimic a sequential embryonic liver development (3,8)

Liver progenitor cells are found in the endoderm and express albumin, transthyretin and α-fetoprotein (AFP): the first markers of liver development. Subsequently, these cells start to differentiate and form hepatic endoderm. Wntsignaling factors, fibroblast growth factor (FGF) 4 and bone morphogenetic protein (BMP)-2 and (BMP)-4 are indispensable at this stage of development and are responsible for the hepatic induction and stimulation of hepatic gene expression (Fig.1). Moreover, it was suggested, that hepatic specification is enhanced by stimulation of retinoic acid and insulin. Newly formed hepatoblastsexpress albumin and AFP, which are recognized markers of hepatic differentiation. Proliferation of these hepatoblasts and their migration is regulated by a variety of factors, such as hepatocyte nuclear factor 6 (HNF-6), HNF-4, prosperohomebox protein 1(Prox1), T-box transcriptional factor, GATA-6, etc. Hepatocyte growth factor (HGF), epidermal growth factor (EGF) and FGF4 enhance following hepatocytes proliferation and maturation (3,8).

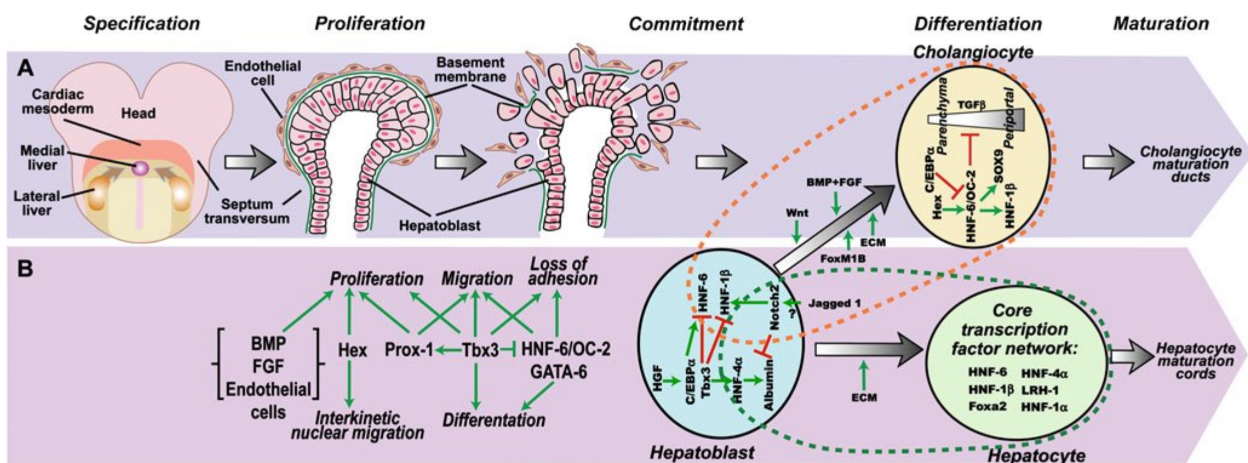

Figure1. A. Early liver development; B. Transcription factors network involving in liver development. (3)

The complexity of this gradual process makes it difficult to set up a unique step-wise differentiation protocol. Therefore, numerous protocols were tried in different studies and their effectiveness tested *in vitro*and *in vivo*.

2.1.2. Hepatic differentiation protocols

At the beginning, ESC differentiation via embryoid bodies (EB) under the influence of different growth factors (GFs) has been applied. However, due to the phenotype diversity of the hepatocyte-like cell population, this method was found to be inefficient (3). Definite endoderm formation (from which hepatocytes differentiate later on) is under the nodal signaling pathway regulation (3). It has been shown that *Activin a* initiates this nodal pathway by binding to the type II receptor that results in a cascade response with the activation of gene-specific transcription factors. Following this discovery the majority of present-day protocols include *Activin a* application, together with FGF2 or bFGF, as a first step of endoderm differentiation of ESCs and iPSCs(3,10) Furthermore, it was suggested that combination of *Activin a* with Wnt3a inducesarapid increase in the expression of definitive endoderm markers, such as SOX17 and FOXA2 (12). SOX17 regulates endoderm differentiation in the early embryonic development and FOXA2 contributes to the hepatic precursors formation (15). Hepatic induction usually involves a combined treatment of ESCs or iPSCs with different combinations of FGF and BMP: BMP4+FGF10, BMP2+FGF4, BMP4+FGF4 etc (3,10).DMSO seems to induce hepatocytes specification through modification of histone acetylation (3).During the last step hepatocyte-like cells undergo functional maturation under the influence of HGF and EGF and/or oncostatin M (OSM), which play an important role in liver development (3, 10).Short summary of the ESCs and iPSCs differentiation towards hepatocytes and crucial factors involved in this process is shown on the Fig.2. Detailed description of different protocols is specified in appendix A.

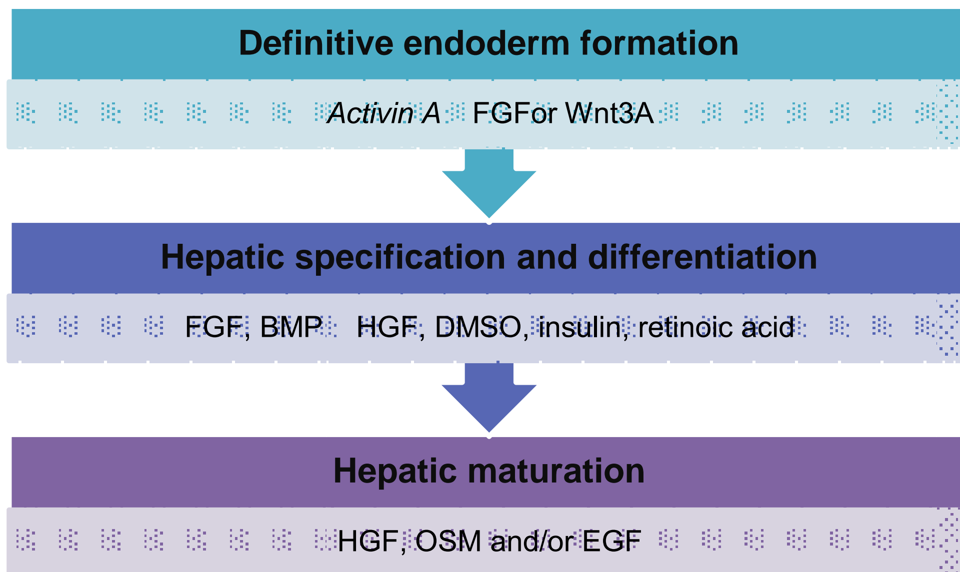

Figure 2. ESC or iPSC differentiation process towards hepatocytes.

2.1.3. Characterization and functional evaluation of differentiated hepatocytes

Generally, characterization and functional evaluation of differentiated hepatocyte-like cells is performed in the following way: by their morphology, liver-specific mRNAs, proteins and functional characteristics (Table 1). Polygonal shaped cells with distinct round multiple

nuclei,well-developed bile canaliculi and tight junctions are characteristics of mature hepatocytes. Analysis of specific hepatic markers differs from study to study, depending on a differentiation phase of development. Commonly, hepatocytes are defined by such markers as CYPs, albumin, HNF1α, HNF4α, tryptophan-oxygenase (TO), tyrosine amino-transferase (TAT), C/EBPα, asialoglycoproteinreceptor 1 (AGPR1), CK18 and α-1-antitripsin (α-1-AT). Glycogen accumulation, low density lipoprotein (LDL) uptake, indocyanine Green (ICG) uptake and secretion, albumin and urea secretion are recognized characteristics of mature hepatocytes (3, 6, 8, 10, 13).

	Criteria	Description
1	Morphological features	Polygonal shape, multiple nuclei, developed bile canaliculi, tight cell-cell junctions
2	Gene/protein expression levels	CYPs, ALB, HNF1α, HNF4α, TO, TAT, C/EBPα, AGPR1, CK18, α-1-AT
3	Liver function tests	Glycogen accumulation, albumin synthesis, LDL uptake, urea secretion, ICG uptake and secretion

Table 1. Criteria used for characterization and evaluation of differentiated hepatocytes.

2.1.4. New approaches in hepatic differentiation of ESCs and iPSCs

Although, the use of GF-cocktails was shown to be efficient for a stimulation of hepatic differentiation in many previous studies, generation of a heterogeneous and immature hepatocyte population remained to be the main problem (13). Recent studies suggested that transduction of specific transcription factors enhance the hepatic differentiation from ESCs and iPSCs. For example, hepatocyte nuclear factor (HNF)4α is known to play a crucial role in liver development: it is responsible for anexpression of variety of liver enzymes and formation of normal liver morphology (11, 13, 15). Accordingly, sequential transduction of SOX17, HEX and HNF4α using adenoviral vectors was demonstrated to promote hepatic differentiation and maturation of ESCs and iPSCs-derived hepatoblasts: higher expression levels of albumin, α-1-AT, CYPs, hepatic conjugating enzymes, hepatic transporters as well as hepatic transcription factors were detected in comparison with HNF4α non-transduced cells.Applying this method, it was possible to generate nearly homologous population: 80% of met proto-oncogen-positive cells, CYPs-, ALB-positive cells and AGPR1-positive cells. Moreover, LDL uptake was detected to be 87% in SOX17/HEX/HNF4α transduced cells versus 44% SOX17/HEX-only transduced cells; ICG uptake and excretion, and storage of glycogen were also higher in a former group. It has been speculated that this sequential three-factors transduction mimics the early embryogenesis, resulting in better hepatocytes differentiation and maturation (13).

In the following studies, the impact of another hepatocyte nuclear factor, HNF6, on hepatic differentiation was investigated. Accordingly, transcriptional activation of CYP3A4 was observed following HNF6 introduction. Since HNF6 did not affect CYP3A4 expression levels in already matured hepatocytes, it was hypothesized that HNF6 plays an important role in hepatocytes maturation (11).

It has been reported that other factors, such as the timing, concentration of GFs and oxygen supply can considerably influence hepatic differentiation. For example, a concentration of FGF2 in the medium, as well as an optimal stage-specific HNF4α transduction determines the hepatic or pancreatic differentiation.Furthermore, it was shown that definitive endoderm formed at a normal oxygen pressure, and hepatic specification was conducted in a hypoxic condition. However, the advantage of low oxygen pressure on hepatic differentiation in particular is still disputed. Therefore, a lot of different factors should be taken into account in order to effectively generate homogenous population of hepatocyte-like cells (3,13).

Several *in vivo*studiesshowed thatiPSC- and ESC-derived hepatocytes could successfully engraft in a recipient liver (3). However, only recent studies revealed the nature of the interaction between host and donor tissues. Thus, it was suggested that ESCs-derived hepatocytes not only engraft to the host tissue, but also exhibit paracrine effect by stimulating endogenous host liver regeneration and neovascularization. The exact mechanism of secretome action is still under investigation, however vascular endothelial growth factor A (VEGF-A), milk fat globule-EGF factor 8 (MFGE-8) and growth arrest-specific 6 (GAS-6) might contribute to the endogenous liver regeneration (14).

2.2. MSCs-derived hepatocyte-like cells

Though ESCs are a potentially powerful means for regenerative medicine, the ethical issues surrounding their use hampered their chances of future clinical application (3,15). Moreover, ESC- and iPSC-derived hepatocyte-like cells were shown to have a potential to form teratomas in several *in vivo* studies (3,16). However, generation of integration-free iPSCs (without genetic alterations), availability of new methods to remove oncogenes from differentiated cells and improved differentiation protocols alleviates some tumorigenic concerns and holds promise for the clinical application of iPSCs in the coming years (30,34,35). More attention has been recently given to the search of alternative sources for hepatocyte-like cell generation.Thus, it has been suggested that adult stem cells are not tumorigenic. These cells arepresent in different organs or tissues and have an ability to differentiate into various tissue lineages (4,15). Accordingly, it was demonstrated that mesenchymal stem cells (MSCs) possess characteristics such as self-renewal, multipotency, proliferation, and they can differentiate towards hepatocytes (4-6, 15). Bone marrow-derived MSCs (BM-MSCs) have been use to generate hepatocyte-like cell populations, however thelow number of MSCs derived with this method and the distress of a traditional bone marrow procurement procedure limit the use of thissource of MSCs.Adipose tissue, on the other hand, represents an abundant source of MSCs, commonly obtained from routine liposuction procedure(4,6). Previously, abdominal adipose tissue was mostly used for MSCs procurement. However, recent studies demonstrated

that thigh adipose tissue can also be used as a source for adipose-derived stem cells (ADSCs) (6).

2.2.1. Hepatic differentiation protocols

Similar to protocols for ESCs and iPSCs, ADSCs acquire features of hepatocytes after several steps of hepatic differentiation. Common protocols include treatment of ADSCs with *Activin a* and FGF4, following by addition of DMSO, HGF, EGF and OSM (4,6,15). Additional components of supplementation medium differed from study to study. Differentiated cells exhibit features of hepatocytes: they store glycogen, synthesize urea, produce albumin and take up LDL (4,15). Also ADSC-derived hepatocyte-like cells displayed CYP activity and were capable to metabolize drugs. Their morphology resembles that of the hepatocyte polygonal shape with tight cell-to-cell junctions and bile canaliculi structures (4,6). Moreover, gene expression pattern of ADSCs-derived hepatocytes was similar to that of human hepatocytes (4).

Though ADSC differentiation towards the hepatocyte lineage was achieved, it was shown to be incomplete: expression levels of some hepatocyte-specific transcriptional factors, such as FOXA1, FOXA2, SOX17 GATA4 and HNF4a, were low. Therefore, the viral transduction has beenapplied and has been proven to be efficient. Accordingly, lentiviral transduction of above-mentioned factors increased albumin expression compared with uninfected cells (15).

2.2.2. Useful properties of ADSCs

It is known that ADSCs contribute to organ regeneration and wound healing, possibly due to the ability to stimulate the activity of antioxidant chemicals, free radical scavengers and heat-shock proteins, as well as to secrete various bioactive molecules, such as IL-6, cytokines, HGF, EGF, nerve growth factor etc (4,5). Moreover, it was reported that ADSCs exhibit immunosuppressive propertiesthrough releasing HGF, leukemia inhibitory factor and prostaglandin E2 (4). Therefore, aside its hepatocyte-like properties, ADSCs possess intrinsic anti-inflammatory and regenerative activity (4,5,15).

Transplantation of ADSC-derived hepatocytes resulted in engraftment of differentiated cells to the host liver, promotion of liver regeneration and decline in markers of liver injury (4, 15).Although tumor formation was not detected with ADSCs, more studies need to be done to prove the safety of these cells. Also, since ADSCs might participate in wound healing and scar formation, concern may arise whether these cells might enhance the fibrotic process in the liver (4).

In conclusion, abundance and ability ADSCs to differentiate into functional hepatocytes make this source of stem cells attractive to regenerative medicine. Moreover, it became possible to use autologous-ADSCs for transplantation that will significantly reduce cases of donor cells/tissue rejection (6, 15).

2.2.3. New approaches in culturing of primary hepatocytes and ADSC-derived hepatocytes

Previously, it has been shown that culturing cells as spheroids increased their viability and function. Accordingly, formation of hepatospheres of primary hepatocytes alone or together

with hepatic stellate cells (HSCs) resulted in better maintenance of liver-specific functions, such as albumin secretion, urea production and metabolism, compared to thecell monolayer (5,17). Possibly, culturing hepatocytes together with HSCs is more beneficial due to similarity of these structures with *in vivo* microenvironment. HSCs were shown to improve aggregation of hepatocytes and cell viability (to 95%).In addition, a well-developed hepatic structure was observed, such as tight junctions, bile canaliculi and mitochondria in presence of HSCs.Moreover, albumin secretion increased on 30% compared with primary hepatocytes mono-spheres and the expression of cytochrome P450 was detected to be higher as well (17).

Concave microwells were proved to be more effective than cylindrical microwells or planer surface (17). Spheroids detached easily from the wells and had uniform size and form (5,17). Because of the concave-morphology effect and non-adherent surface, the treatment with trypsin was not needed by this method that in turn maintained cell viability (5,29). Also, the structure of formed spheroids was demonstrated to be porous, so gas and nutrients could easily reach the inner part of spheroids preventing necrosis formation (5). Therefore, cell spheres produced by concave microstructures reproduce *in vitro* environment that resembles the liver *in vivo* and could be used for the construction of bioartificial liver.

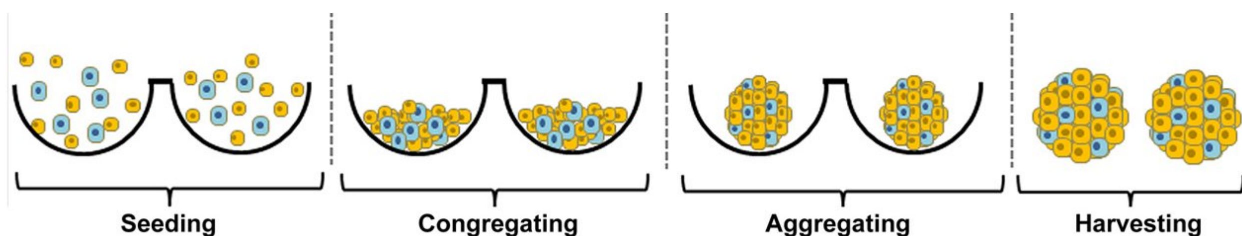

Figure. Co-culture 3D model of heterospheres using concave microwells.

Additionally, arecent study has demonstrated that co-culturing ADSCs with primary hepatocytes 1:1 in concave microwell arraysyieldsformation of a highly functional three-dimensional (3D) hepatic tissue construct. ADSCs were shown to improve viability and function of primary hepatocytes: cell viability after 3 days of culturing was 47% versus 99% in mono- and hetero-cultures accordingly. This phenomenon might be explained by paracrine healing effects of ADSCs mentioned before. Additionally, albumin secretion was 10 % higher and cytochrome P450 activity 28% higher compared with primary hepatocytes mono-culture, though in mono-culture the number of hepatocytes was two times that of co-culture. Urea production was similar in hetero- and mono-culture. It was shown that co-cultured spheroids had smoother surface and aggregated more tightly and rapidly: the size of spheres decreased more promptly after the cell-seeding in co-cultured spheroids in comparison with mono-cultured.Remarkably, ADSCs in co-culture with hepatocytes differentiated into hepatocyte-like cells exhibiting hepatocyte-like features without any additional processes and addition of GFs,which is possibly due to direct cell-cell interactions (5). This together with paracrine effects of ADSCs most likely contributed to the high performance of co-cultured spheroids.

Co-culturing of primary hepatocyte with ADSCs makes it possible to overcome low viability and shortage of primary hepatocytes. Moreover, transdifferentiation of ADSCs towards hepatocyte-like cells increases the number of hepatic functional cells in co-cultured spheroids.

2.3. Other sources for hepatocyte-like cells generation

In addition to ESCs, iPSCs and ADSCs, alternative sources of stem cells are under investigation. Accordingly, it was shown that human blood-derived stem cells (BDSCs) express markers of pluripotencyand have the ability to differentiate into various lineages, including hepatic cells. Treatment of BDSCs with HGF and FDF-4 resulted in generation of hepatocyte-like cells which exhibited hepatic morphology and function. Accordingly, cells acquired polygonal shape and expressed AFP. Also, urea production and glycogen storage were confirmed. The advantage of BDSCs-use is a short length of differentiation time: transformation of stem cells into hepatocyte-like cells took 7 days only, whereas other protocols took approximately 15-20 days to generate functional hepatocytes. Comparable to the adipose tissue, peripheral blood is a readily available source of adult stem cells that can be used in anautologouscell-based therapy. Although it was demonstrated that BDSCs-derived hepatocyte-like cells exhibited some of liver features and function, more studies need to be done to prove ability of these cells to perform other liver functions, such as metabolism and albumin secretion (9).

2.4. Optimization of differentiation conditions

Despite successfullyapplied protocols to generate hepatocytes from different sources, optimization of differentiation conditions is still in progress. Thus, application of soluble GFs coupled with high expenses due to the daily change of media should be resolved. Interestingly, it was demonstrated that the use of printed arrays of GFs is more efficient compared to supplementation of culture media with soluble GFs. Printed arrays contained fibronectin and collagen to enhance stem cell attachment and GF binding respectively. Accordingly, it was shown that, a decrease of markers of pluripotency in stem cells residing on top of printed ECM/HGF/BMP4/bFGF was faster compared to stem cells cultured on ECM and exposed to the soluble combination of these three GFs: Oct4 gene (marker of pluripotency) was 5 times lower in the former group. Furthermore, albumin level was detected to be higher in cells residing on top of printed GFs compared to the cells exposed to the media containing soluble GFs. These results indicate that the function of printed GFs wasmaintained and hepatic differentiation of stem cells was achieved.Besides, co-cultivation of ESCs with HSCs on printed GFs was even more effective regarding ESCs differentiation towards hepatocyte-like cells (7).

Moreover, it was suggested that printing GFs on a substrate representsa more physiological environment: *in vivo* GFs associate with extracellular matrix (ECM) and are released by degradation of the matrix.Also, GFs bound to ECM proteins seem to possesshigher stability and function compared to the soluble GFs. Therefore, the use of printed GFs represents more effective and economical way to achieve desirable results (7).

2.5. Summary

Summarizing, the differentiation of stem cells into hepatocyte-like cells is a complex multi-step process. The GFs and other signaling molecules that are crucial in the liver development are numerous and their combinations vary between different protocols. To date,

scientists successfully generated hepatocytes from stem cells of various origins that brought us closer to the desired goal of the development of functional liversystem. Although good results were achieved in generating functional hepatocytes, a lot of issues still need to be addressed. Thus, the use of viral transductionof stem cells should be applied with caution, and non-viral genetic manipulations should preferably be employed. Furthermore, the possibility of tumor formation cannot be ruled out even by using adult stem cells as a source for hepatocytes generation.Finally, more liver function tests should be performed to prove that newly generated hepatocytes are metabolically competent and represent all vital liver functions.

III. Endothelial cells (ECs)

In order to generate tissues for regenerative medicine, vascularization of engineered constructs should be successfully achieved. Although mature endothelial cells can be procured from patient blood vessels, poor proliferation *in vitro*and limited availability limit their possible clinical use considerably (26,28). To date, vascular and mural (smooth muscle) cells have been rathersuccessfully derived fromdifferent sources, including ESCs and iPSCs.

3.1. ESC and iPSC-derived endothelial cells

Several regulators have been identified to play a crucial role in the generation of endothelial cells, including Indian hedgehog (Ihh), VEGF, bFGF, BMP and hypoxia-inducible factor (HIF) 1-α. VEGF is a crucial factor during the differentiation of ECs. Wnt, BMP2 and HIF1-α seem to play a role in vascular development through VEGF activation. Additionally, an important role of histone acetylation in the promotion of endothelial phenotype has been suggested (18,30).

3.1.1. Endothelial differentiation protocols and characterization of differentiated endothelial cells

Therefore, current protocols of endothelial differentiation of ESCs and iPSCsusuallyinclude application of three above-mentioned GFs: VEGF, bFGFand/orBMP(18,19). To determine whether generated endothelial cells are comparable to *in vivo* counterparts, gene expression studies and functional tests*in vitro* and *in vivo*, such as LDL uptake, *in vitro* Matrigel assays and Matrigel plug models for the*in vivo* testing of blood vessel formation should be evaluated. For the *in vitro* Matrigel test, cells are seeded on plates with Matrigel and tubular network formation is assessed after 24h incubation. For the *in vivo* test, Matrigel is mixed with bFGFand ECs and injected into mice. After defined period of time (usually 2 weeks), plugs are removed and ECsare stained for specific endothelial markers (19, 20, 22).

	Criteria	Description
1	Morphological features	Elongated shape, tube structure, tight cell-cell junctions, confluent cell monolayer with little or no cellular overlaps
2	Gene/protein expression levels	VEGF, bFGF, SDF-1, CD31, CD31, eNOS, vWF, VEGFC, VEGFA, VCAM-1, Flk1, PECAM-1, VEGFR-2, F8, PDGF,

		ANG-1
3	Functional tests	LDL uptake, *in vitro*Matrigel test, *in vivo*Matrigel plug model, NO production, ECs activation by TNF-α

Table 2. Criteria used for characterization and evaluation of differentiated endothelial cells.

Recent evidence indicates that thegene expression profile and somatic epigenetic memory differ between ESCs and iPSCs (19,21). On the other hand, several other studies have showed that the global gene expression and histone modification pattern were similar between human ESCs and iPSCs and observed gene expression differences between them is not higher than that between different human ESCs lines (30, 34). Therefore, a study was performed aimed to identify the gene expression variance between ECs population derived from different lines of ESCs and iPSCs that might potentially have an impact on their future application in cell-based therapy. Interestingly, the gene expression profile was very similar in newly generated ECs. These results suggest that gene expression heterogeneity in these two pluripotent cell types is much higher than in individual lineages derived from these cells (21).

Accordingly, it was shown that ECs derived from ESCs and iPSCsexpress endothelial markers, such as platelet-endothelial cell adhesion molecule-1 (CD31), VE-cadherin (CD144), endothelial NO synthase (eNOS), von Willebrand factor (vWF) and fetal liver kinase-1 (Flk1 or VEGF2), as well as genes related to EC proliferation and migration, such as VEGFC, VEGFA andVCAM-1. eNOS is an enzyme that generates NO in ECs, that regulates many physiological processes in the cardiovascular system and promotes endothelialization (26). Furthermore, generated ECs were capable of LDL uptake, NO production andECs activation by TNF-α that leads to the upregulation of cell adhesion molecules. ECs were shown to have an ability to form tube-like networks *in vitro* that represents their vasculogenic and angiogenic potential. Additionally, it was shown that ESCs- and iPSCs-derived ECs formed functional vessels *in vivo* in immunodeficient mice (19-22).

3.1.2. Optimization of differentiation conditions

*In vivo*ECs are heterogeneous, which is related to the maintenance of specialized tissue functions. Accordingly, ECsexhibit different structure and function depending on their subtype: arterial, venous or lymphatic. Remarkably, it was shown that ECs that are derived from iPSCs are heterogeneous: all three above-mentioned cell subtypes were present in the culture (20).

Differentiation conditions determine the fate of generated ECs. Accordingly, high concentrations of VEGF promote arterial subtype formation, whereas lower concentrations of VEFG induce venous subtype formation. Also, cell density plays an important role in differentiation: higher density facilitates cell-cell interaction and as a result cell differentiation. Extracellular matrix plays a substantial supportive role in vasculogenesis. It was demonstrated that matrix, such as fibronectin, gelatin, collagen type I and IV contribute to the endothelial

differentiation. Co-culture of murine ESCs with OP9 stromal cells yield similar results: better endothelial differentiation was demonstrated (20).

Also, the important role of mural cells for vessel formation and maturation has been well characterized. Accordingly, enhanced engraftment and vascularization was shown *in vivo* by using ECs together with mural cells compared with the use of ECs alone (18).

3.1.3. In vivo studies

Vascular regeneration properties of iPSCs-derived ECs have been demonstrated in several *in vivo*animalmodels. iPSC-derived ECs transplanted into the mouse ischemic limb were shown to increase miscrovessel regeneration in the ischemic tissue. Moreover, it was suggested that aside direct incorporation of transplanted cells into existing vasculature, iPSCs-ECs display paracrine effects due to the secretion of angiogenic cytokines and GFs, that promoteshost angiogenesis(22,23). Additionally, injection of ESC-derived ECs into the ischemic murine myocardium resulted in improvement of the systolic function and restoration of myocardial capillary density (20).

3.2. MSCs-derived endothelial-like cells

The ability of MSCs to secrete a variety of angiogenic growth factors and cytokines has been well studied and the ability of these cells to promote vascularizationhas been suggested (24,25). Because of the limited availability of BM-MSCs and possibility of donor-site morbidity, as has been mentioned before, adipose tissue has been considered as an alternative abundant source of MSCs for EC generation. ADSCs can differentiate into ECsfollowing exposure to VEGF and FGF(26-28).

3.2.1. Endothelial differentiation of ADSCs

Accordingly, several studies have shown that ADSCs express angiogenic factors, such as VEGF, bFGF, SDF-1 and IL-8; and exhibit angiogenic activity *in vitro* and *in vivo*: ADSCs integrated into the host tissue and contributed to thenew vessel formation through paracrine pathways (24). Moreover, ADSC-derived endothelial-like cells express endothelial markers and factors involved in theblood vessel formation, such as CD31, eNOS, VCAM-1, PECAM-1,VEGFR-2, F8, PDGF, ANG-1 and vWF (26, 27). Regarding endothelial factors, PECAM-1 plays an important role in vascular permeability and coagulation; VCAM-1 mediates adhesion of lymphocytes to the vascular endothelium and F8 is necessary for adhesion of platelets to the endothelium. Angiogenic factors, such as PDGF and ANG-1 are important for the vessel stability (27).

Several studies have shown that ADSCsdo not perform angiogenesis themselves, but do exhibit paracrine effects and promote vascularization without incorporation into the vessel walls.This fact might limit the therapeutic efficacy of *ADSCs*for clinical application or use in

tissue engineering (24,28).Therefore, new methods were sought to promote endothelial differentiation of ADSCs.

3.2.2. Optimization of differentiation conditions

As noted, 3D cell culture methods significantly increase cell viability and promote cell stabilization and differentiation. Accordingly, 3D culture method using a substratedisplaying immobilized FGF2 was developed and tested *in vitro* and *in vivo*. It was demonstrated that cells within the spheroids differentiated into ECs and smooth muscle tissue cells (SMCs), expressing endothelial markers, such as CD31 and CD34, and SMCs markers, such as α-SMA, respectively.

Also, spheroid formation contributed to the endothelial differentiation through hypoxic environment, that is established within spheroidsdue to the insufficient oxygen supply to the inner cells. Hypoxic conditions lead to theupregulation of hypoxia adaptive signaling, such as HIF 1-α and CXCL12, and to the increase in secretion of angiogenic factors, such as HGF, VEGF and FGF2. Accordingly, the level of VEGF in spheroids was 5 fold higher compared to the monolayer culture. It was suggested that these factors are stored within the spheroids promoting endothelial differentiation of ADSCs in autocrineor paracrine manner. Remarkably, transplanted 3D spheres of differentiated cells intothe rat ischemic heart integrated with host vessels and contributed to the neovascularization. Therefore, 3D culture methods show advantages compared to the mono-layers culture both *in vitro* and *in vivo*: higher cell viability, retention and angiogenesis have been demonstrated in 3D spheroids (28).

Many other attempts have been taken to improve the endothelial differentiation potential of stem cells. The use of viral transfection to overexpress genes for pluripotencyis limited due to safety concerns (22, 27).Possibly, changes in epigenetic makeup using non-viral methods are a promising tool to influence differentiation potential of cells. Accordingly, the methyltransferaseinhibitorBIX-01294 (BIX) has been shown to increase the ability of ADSCs to differentiate into ECs.DNA and histone methylation play an important role in cell differentiation during embryonic development. BIX inhibits histone methyltransferase, responsible for methylation of Histone 3 Lysine 9 (H3K9), that leads to chromatin unfolding and increased expression of pluripotency genes(27). Obtained results suggest that enhanced pluripotency of ADSCs by this method and following exposure to an endothelial medium, leads to the increase in expression of endothelial and angiogenic markers (27).

Moreover, recent studies have shown that surface features can have a great impact on stem cells differentiation into ECs. Accordingly, a nanostructured surface showed advantage compared to amicrostructured surface: ADSCs demonstrated better endothelial differentiation when seeded on a nanostructured surface (26).

3.3. Other sources for ECs generation

In addition to ESCs, iPSCs and ADSCs alternative sources of stem cells have been sought. Accordingly, cord-blood stem cells (CBSCs) were shown to have a potential to

differentiate into ECs. Newly generated ECs were capable of FGF-2, VEGF and Ang-1 production, as well as LDL uptake and tubule formation. Moreover, it was demonstrated that CBSC-derived ECs exhibit paracrine effects on surrounding cells due to the bioactive molecules secretion, that induces angiogenesis and vasculogenesis. However, superiority of ESC-derived ECs has been demonstrated compared to morphology, proliferation, secreted cytokines, GFs and wound-healing efficacy of CBSC-derived ECs (23).

To date, only few clinical studies with adult stem cell-derived ECs have been done involving patients with myocardial ischemia or peripheral arterial disease. Preliminary data indicates that ECs cell-based therapy can provide some benefit in the recovery of ischemic tissue (22).

3.4. Summary

In summary, endothelial-like cells have been isolated from several human sources such as ESCs, iPSCs, BM-MSCs, ADSCs and CBSCs. Additionally, it has been shown that stem cells are capable not only to differentiate into endothelial cells, but also to promote paracrine effects and to stimulate vascularization through the secretion of different bioactive molecules (22-24,28).To date, protocols describing endothelial cells generation from different types of stem cells are not standardized. Also, the diversity of endothelial markers and functional tests used to confirm successful endothelial differentiation makes the direct comparison of different studies difficult (20). Despite all these limitations, progress in this field is prominent. The application of new techniques such as nano-modeling, 3D culture and epigenetic manipulations, open new possibilities to derive functional vascular cells from stem cells, that would beultimately used in regenerative medicine. Also, angiogenic activity of stem cells may prove beneficial for vasculature formation in engineeredtissue constructs (24).

IV. Conclusions

Stem cells are currently considered a potential, infinite source for generation of various cell types. Hepatocyte- and endothelial-like cells have been derived from different stem cell sources, each of which has its advantages and limitations. ESCs have a favorable ability of self-renewal and differentiate into different lineages, including hepatocytes and endothelial cells. However, ethical considerations and immunogenic barrier associated with these cells encouraged scientists to search for alternative sources of human stem cells. iPSCs share many similar characteristics with ESCs, avoiding ethical issues surrounding the ESCs use. On the other hand, tumorogenicity is still considered obstacles in clinical application of these cell types. However, continuous advancement in technology has substantially reduced the risk of iPSCs cells and clinical application of iPSC-derived somatic cells is expected in the coming years. Adult (multipotent) stem cells were suggested to be non-oncogenicand were extensively studied in respect of their ability to differentiate into hepatocyte- and endothelial-like cells. MSCs derived from adipose tissue appear to be the most accessible source of adult stem cells. iPSCs and adult MSCs can be used to generate autologous therapies, minimizing or even eliminating problems associated with immune rejections after orthotropic transplantation.

Currently, there are numerous protocols available describing generation of hepatic and endothelial cells from ESCs, iPSCs, BM-MSCs and ADSCs. However, some of these protocols were shown to be inefficient and cell differentiation appeared to be inadequate: cells were not sufficiently mature for future therapeutic application. Therefore, new techniques aimed to improve generation ofvascular and hepatic cells from stem cells were sought and applied, such as co-cultivation, non-viral manipulations with genome, use of 3D hetero- and mono- cell structures and printed arrays.

Hepatocyte-like cells, which are differentiated from stem cells, would be useful for basic research, drug discovery and regenerative medicine. Furthermore, vascularization of *in vitro* hepatic tissue constructs would make it possible to mimic *in vivo* environment and ultimately create bioartificial liver.

In conclusion, achievements in generation of hepatic- and endothelial-like cells from stem cells brought us one step closer to the construction of functionalliver tissue. However, the fidelity and safety of cell-based therapy needs to be confirmed in more *in vitro* and *in vivo*studies before clinical application can be considered.

V. Appendix

Cell line	Before definitive endoderm	Definitive endoderm induction	Hepatic specification	Hepatic differentiation	Hepatic maturation	Special evaluation	Reference
H1, H9	–	Activin A 3 days	FGF4 BMP2 5 days	HGF 5 day	OSM, Dex 5 days	Evaluation of entry of HIV-HCV Pseudotype viruses into hESC-derived hepatic cells. In vivo study in SCID mice	Jun Cai et al. 2007 [77]
H1, H9	–	Activin A 100 ng/ml Wnt3a 50 ng/ml, RPMI 1640, B27 3 days	1% DMSO, KO DMEM, 20% SR, 4 days	–	HGF, OSM, L-15, 10% FBS, 7 days	Intra splenic injection of cells in NOD-SCID mice	Hay D et al. 2008 [76]
H1, H9	–	Activin A 0.5% FBS, RPMI 3 days 2% KOSR instead of FBS in Days 3–5	FGF-4, HGF, 2% KOSR, RPMI media on Collagen I days 5–8	MM media, BSA, FGF-4, HGF, OSM, Dex Days 8–10	HCM Media, FGF-4, HGF, OSM, Dex day 11–20	Portal injection of definitive endoderm cells in NOD-SCID mice	Agarwal S et al. 2008 [81]
H1	Embryoid body formation 2 days	Activin A bFGF 3 days	HGF DMSO 8 days	–	Dex 3 days	Splenic injection of ASGPR 1 positive cells to uPA-SCID mice, Nagase-analbuminemic-rat, NOD-SCID mice	Basma H et al. 2009 [5]
SA001, SA002, SA002.5, SA167	–	Activin A FGF-2 1–5 days	BMP 2/4 FGF 1/2/4 6–17 days	–	EGF, Insulin, Transferri, Hydrocortion, Ascorbic acid 18–45 days	Differentiation in three cell lines was successful	Brolén G et al. 2010 [75]
H9	–	No Serum-Activin A 2 days Low serum, Activin A, SB, in RPMI 1640 3–6 days	FGF4, HGF, Dex, BMP2/4, Insulin, DMSO, IMDM 10–14 days	–	FGF4, HGF, Dex, DMSO, OSM, HCM Until use	Drug metabolism evaluation by LC/MS/MS	Duan Y et al. 2010 [4]
H9	Activin A10 ng/ml FGF2 12 ng/ml 2 days	100 ng/mL Activin A, 20 ng/mL FGF2, 10 ng/mL BMP4, 10 µM LY294003 3 days	50 ng/mL FGF10, 10^{-7} M retinoic acid, 10 µM SB431542 3+2 days	–	30 ng/mLFGF4, 50 ng/mL HGF, 50 ng/mL EGF. 10 days	uPAxrag2gammac$^{-/-}$ mice injection	Touboul T et al. 2010 [6]

Origin	iPS induction	iPS conformation	Before definitive endoderm	Definitive endoderm	Hepatic specification	Hepatic proliferation	Hepatic maturation	
Moue GFP expressing fibroblast	Oct3/4, Sox2, Klf4, c-Myc	Teratoma formation	Embryonic body formation with 50 ng Activin A, 50 ng Wnt, 6 days	10 ng/ml bFGF 1% DMSO 3 days	10 ng/ml HGF 1% DMSO days 9–18	–	10 ng/ml HGF, 10 ng/ml OSM, Dex, days 18–25	Gai H et al. 2010 [97]
Mouse embryonic fibroblast	Oct3/4, Sox2, KLF4 c-Myc	Teratoma formation neural differentiation mesodermal differentiation	–	–	1% DMSO day 1–4	–	sodium butyrate day 5–11	Li W et al. 2010 [98]
Human fibroblast	OCT4, SOX2, NANOG, LIN28	Teratoma formation	–	Activin A, RPMI media B27 in 20% O2 5 days	RPMI, B27, BMP4, FGF-2 in 4% O2 5 days	RPMI, B27, HGF in % 4 O2, 5 day	HCM + OSM 20% O2	Si-Tayeb K et al. 2010 [99]
Fibroblasts of normal caucasian male	Oct3/4, Sox2, Klf4, c-Myc	Teratoma formation	–	Activin A, Wnt 3a, RPMI, B27 days 1–3	Activin A, RPMI, B27 days 4–5	KO/DMEM 1% DMSO 20% SR days 5–8	L15/10% FCS HGF OSM days 9–14	Sullivan GJ et al. 2010 [101]
Human fibroblast	Oct-4, Sox2, Klf4, Utf1	Testing expression of OCT4, NANOG, SOX)2, SSEA4, TRA1-60 a, TRA1-81, by RT-PCR or immunofluorescence assay,	–	Activin A, 3 days	FGF4, BMP-2 4 days	HGF, KGF, 6 days	OSM, Dex 5 days then OSM, Dex, N2B27 3 days	Song Z et al. 2010 [100]

VI.References:

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