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# Differentiation of Hepatocytes from Mice Embryonic Stem Cells in Three-Dimensional Culture System Imitating in vivo Environment

Tetsuya Imamura
*Shinshu University School of Medicine*
*Japan*

## 1. Introduction

Liver transplantation is accepted as the standard treatment for saving patients affected with serious liver disease. However the demand for donated livers for transplantation far exceeds the supply. One potential way to compensate for the chronic shortage is the development of bioartificial (Aoki et al., 2008, Mizumoto et al., 2004, Qian et al., 2003) and secondary livers (Kosone et al., 2008, Nguyen et al., 2009, Strom & Fisher, 2003). A necessity for development of these liver systems is the availability of sufficient high quality hepatocytes (Dan & Yeoh, 2008, Haridass et al., 2009, Mizumoto et al., 2008, Umehara et al., 2008). In addition, the high quality hepatocytes have great potentials to be source for pharmaceutical models to assess toxicity of new drugs, a critical step in drug discovery and development. In this review, we describe recent progress towards using embryonic stem (ES) cells differentiated in three-dimensional (3D) culture systems that imitate *in vivo* environment of hepatic histogenesis and liver regeneration. We suggest that the combination of the ES cells and 3D culture systems have the potential to provide high quality hepatocytes.

Pluripotent ES cells, which are derived from the inner cell mass of blastocysts, are able to replicate and differentiate into various cell types composed of whole body. Thus, ES cells have great potentials to serve as sources of hepatocytes to construct liver systems, bioartificial or secondary livers. Hepatocyte-like cells differentiated from ES cells by various methods have already been reported (Hamazaki et al., 2001, Matsumoto et al., 2008, Rambhatla et al., 2003). This review introduces our culture systems for differentiation into the hepatocyte-like cells and describes the characteristics of the ES cell-derived hepatocyte-like cells induced by the culture systems.

## 2. Produce of three-dimensional (3D) culture systems

We maintain undifferentiated mice ES cells of the cell line 129/sv strain. The cells are cultured in Dulbecco's modified eagle medium supplemented with 20% Knockout Serum Replacement, 100 mM non-essential amino acids, 100 μM sodium pyruvate, 100 mM 2-mercaptoethanol, 50 units/ml penicillin and 50 μg/ml streptomycin, 1000 units/ml leukemia inhibitory factor on 0.1% gelatin-coated dishes with STO fibroblasts treated with mitomycin C. The cultures are maintained at 37°C in humidified air with 5% $CO_2$.

Following, we form embryoid bodies (EBs) including germ layers with our original method, conical tube (CT) method (Kurosawa et al., 2003). The ES cells are dissociated using 0.1% trypsin–EDTA, and resuspended at $2x10^4$ cells/ml with EB-formation medium composed of Iscove's modified Dulbecco's medium containing 20% fetal bovine serum, 100 mM non-essential amino acids, 100 μM sodium pyruvate, and 100 mM 2-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin. One milliliter of the cell suspension is placed into polypropylene 1.5-ml conical tubes with round bottoms and the screw caps loosely closed for gas exchange. The cells are cultured for 5 days in 5% $CO_2$ at 37°C.

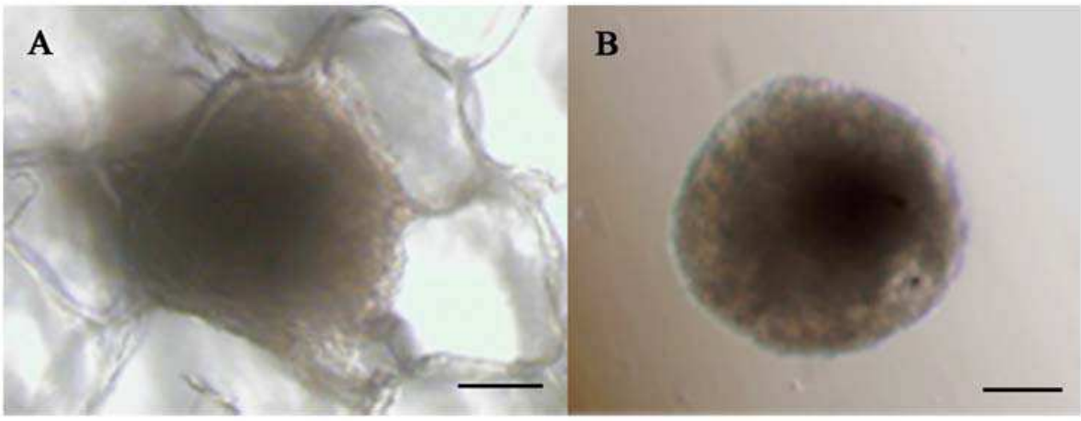



Fig. 1. (A) Day 0 EB derived from $2.0x10^4$ ES cells formed by the CT method inserted into collagen scaffold, 3D culture system. Bar=100 μm. (B) EB is put on the culture dish, 2D culture sysytem. Bar=100 μm

We construct 3D culture system by inserting a single EB prepared by the CT method into the three-dimensional space of collagen scaffolds (Fig. 1A). The scaffold is composed of atelocollagen type I derived from cow skin, and the diameters of the scaffold pores are approximately 200-400 μm. To estimate the 3D culture system, we put a single EB on the collagen-coated dishes (two-dimensional (2D) culture system, Fig. 1B). The EBs inserted into each scaffold, or put on each dish are cultured for 24 days with originally cocktailed EB-differentiation medium. The medium are composed of EB-formation medium supplemented with 100 ng/ml acidic fibroblast growth factor, 20 ng/ml hepatocyte growth factor, 10 ng/ml mouse oncostatin M, $10^{-7}$ M dexamethasone, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite. Every 3 days, one half of the old medium is removed and an equal volume of fresh medium is added to replace it.

## 3. Hepatocyte-like cells induced from ES cells in the 3D culture system

To induce hepatocyte from the EBs, we imitate environments of hepatic histogenesis by using acidic fibroblast growth factor, hepatocyte growth factor, oncostatin M, dexamethasone, insulin, transferrin and sodium selenite. These exogenous growth factors and hormones are known to relate with liver development (Hamazaki et al, 2001). In the 3D culture system, the cells derived from the EB fill many of the scaffold pores, and proliferating cell nuclear antigen (PCNA)-positive cells are present inside the collagen

scaffolds. The cells in the 2D culture system also proliferate and spread. Both culture systems induce formation of various cell types including spontaneously beating cardiac muscle cells.

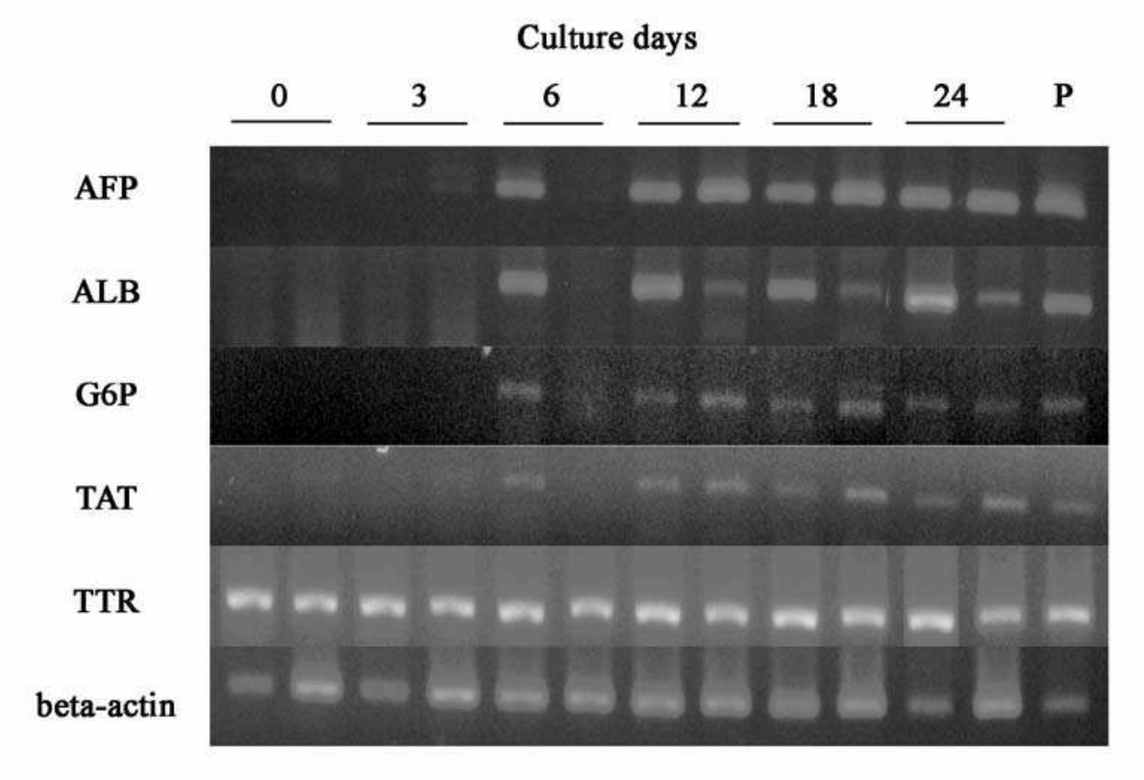



Fig. 2. In the 3D culture system, the cells cultured from 6 to 24 days express AFP, ALB, G6P and TAT genes. In contrast, these genes in 2D culture systems are expressed only after Day 12. P, positive control from fetal liver of pregnant ICR mouse at Day 12

The cells derived from EB are analyzed to determine if hepatocyte-like cells are induced in both culture systems. Reverse transcription polymerase chain reaction (RT-PCR) data show that the cultured cells express hepatocyte-specific mRNAs alpha-fetoprotein (AFP), albumin (ALB), glucose-6-phsphatase (G6P), tyrosine aminotransferase (TAT) and the endodermal transthyretin (TTR) mRNA (Fig. 2). Because EBs include endoderm, TTR expression is apparent at Day 0 and is maintained for the period of culture in both 3D and 2D culture systems. The 3D culture system shows that cells express all of the hepatocyte marker mRNAs on Day 6, and these are maintained throughout the culture to Day 24. In the 2D culture system, these mRNAs appear at Day 12 and each is maintained to Day 24.

Some of the cells derived from EBs in both systems were positively stained with anti-albumin antibody. These albumin-positive cells have large nuclei and polyhedral contours. In 3D culture systems, the albumin-positive hepatocyte-like cells formed cord-like structures (Fig. 3A). In 2D culture systems, the albumin-positive cells are usually distributed singly, but also occasionally form small clusters (Fig. 3B). However, the cells do not show cord-like structures.

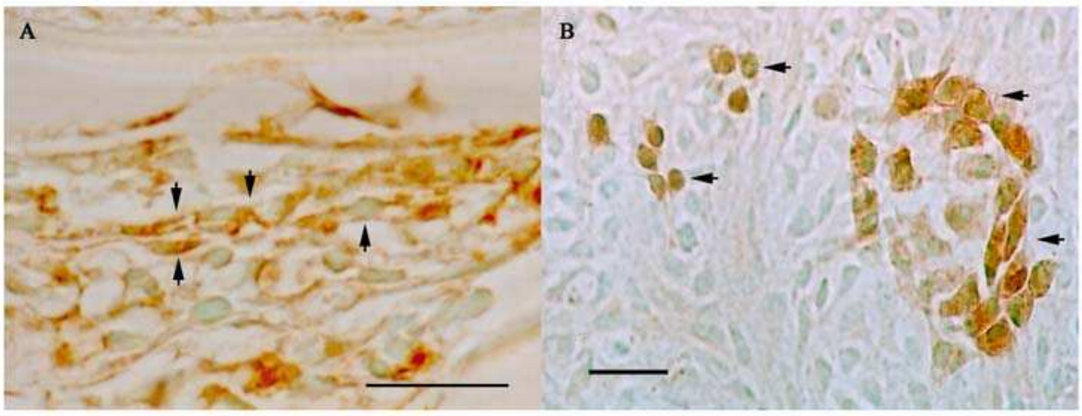



Fig. 3. (A) At 24 days, cells cultured in a 3D culture system contain albumin-positive cells. The albumin-positive cells form cord-like structures (arrows). Bar=20 μm. (B) Outgrowths derived from EBs in monolayers of 2D cultures systems also include albumin-positive cells. The albumin-positive cells distribute singly or in small clusters (arrows). Bar=50 μm

These results show that the both culture systems induce hepatocyte-like cells from EBs. However, there are two differences between 3D and 2D culture systems. One is that on the sixth day of culture, all hepatocyte-specific genes are detected in cells of the 3D culture system, but not in the 2D culture system. Another is that the albumin-positive cells induced in the 3D culture system form cord-like structures that are not present in the 2D culture system. These differences suggest that the 3D culture system promotes differentiation of ES cells and formation of tissue-like structures better than the 2D culture system. This may be due to the presence of cell-to-cell and/or cell-to-matrix interactions in the 3D culture system, which is one of the advantages, similar to those likely to occur *in vivo*.

## 4. Hepatocyte-like cells induced from ES cells in the 3D culture system

We have investigated the embryologic characteristics of the hepatocyte-like cells that are differentiated from ES cells within the 3D culture system *in vitro*. Firstly, RT-PCR shows that the cells derived from EBs express the following genes: AFP, an endodermal marker of early fetal hepatocyte differentiation; ALB, an early fetal and mature hepatocyte differentiation marker; G6P, predominantly expressed in the hepatocytes in late gestational or perinatal stages; and TAT, a marker for perinatal or postnatal hepatocyte-specific differentiation (Hamazaki et al., 2001). Although AFP and ALB are expressed in other cells, G6P and TAT are selectively expressed in developmental hepatocytes (Harada et al., 2003).
Secondly, immunohistochemistry shows that the ALB-positive EB-derived hepatocyte-like cells are positive for AFP, a marker of hepatoblasts, and cytokeratin 19, a marker of hepatic-progenitor cells, but not for cytokeratin 18, a marker of mature hepatocytes. On embryonic day 9.5 in mice, hepatoblasts, immature liver epithelial cells expressing both albumin and alpha-fetoprotein, appear in the hepatic bud. The hepatoblasts then develop into hepatic-progenitor cells, bipotential cells that are capable of differentiating into hepatocytes or bile duct epithelial cells, both of which express albumin and cytokeratin 19 (Dabeva et al, 2000). The cells develop into mature hepatocytes expressing both albumin and cytokeratin 18 (Rambhatla et al., 2003).

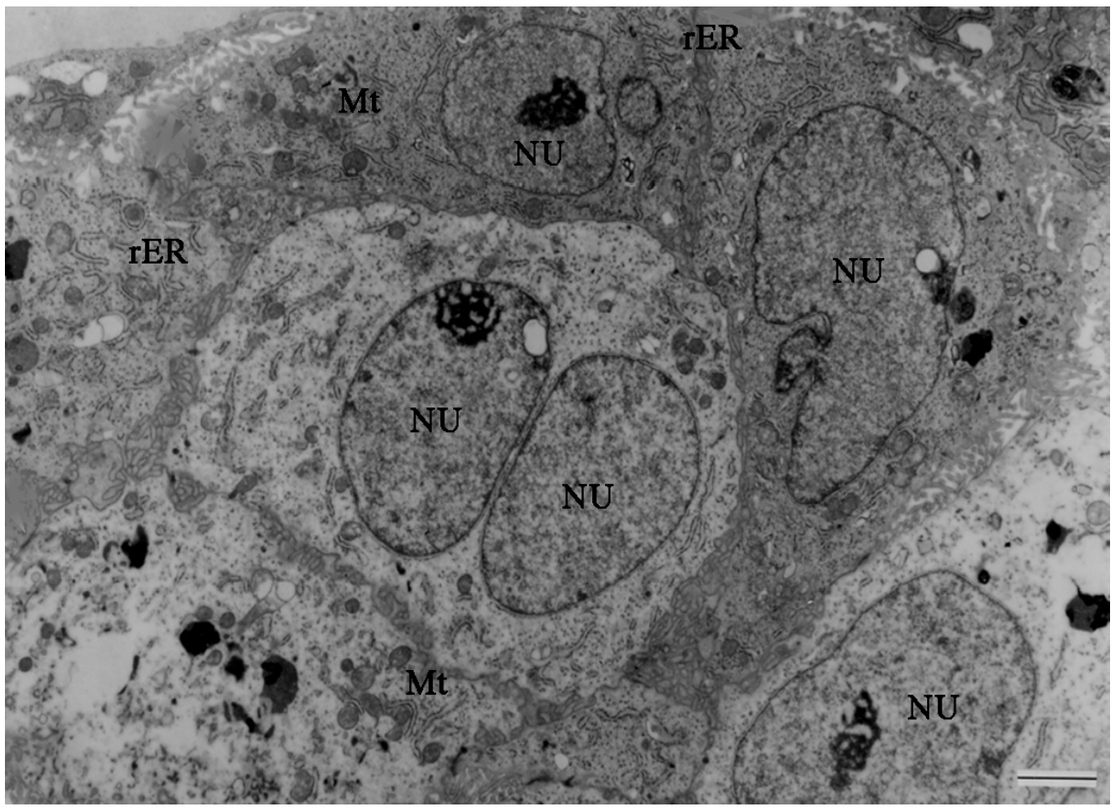



Fig. 4. Electron microscopy shows that hepatocyte-like cells in the collagen scaffold have numerous free ribosomes, moderate numbers of mitochondria and rough surfaced endoplasmic reticulum. The nuclear-cytoplasm ratio is high, and some nuclei are slightly pleiomorphic. Bar=2 μm. NU: nuclear; Mt: mitochondria; rER: rough surfaced endoplasmic reticulum

Finally, electron microscopy shows that the hepatocyte-like cells have typically immature intracellular organelles including numerous free ribosomes, mitochondria and rough-surfaced endoplasmic reticulum (rER) (Fig. 4). However they do not have glycogen particles, smooth-surfaced endoplasmic reticulum (sER), lipid droplets, peroxisomes, or iron deposits that are typically found in mature hepatocytes. In addition, the nuclei are occasionally irregular in shape, with a high nuclear-cytoplasmic ratio. Developmentally, cytoplasmic structures associated with hepatocyte synthetic and secretory function, i.e., rER and Golgi apparatus, are the first to differentiate (Bielanska-Osuchowska, 1996, Kanamura et al., 1990, Luzzatto, 1981), and glycogen particles, sER and peroxisomes appear later. Therefore, the hepatocyte-like cells induced from EBs in the 3D culture system *in vitro* resemble an immature type, which are developmentally intermediate between embryonal and fetal types (Nonoyama et al., 1988).

## 5. Hepatocyte-like cells derived from ES cells in the liver regeneration environment

We have attempted to use the *in vivo* environment of regenerating liver to induce the formation of mature hepatocyte-like cells. To provide an *in vivo* environment of liver

regeneration, we use partially hepatectomized livers of BALB/c nu/nu male nude mice at postnatal week 5. The nude mice are anesthetized with an intraperitoneal injection of pentobarbital sodium solution (0.05 mg/g-body weight), and then approximately 60% of the liver is removed. At that time, we transplant a single EB-collagen scaffold cultured for 24 days into the median lobe. We have analyzed the cells within the scaffolds implanted for 7 and 14 days. The transplanted cells are clearly demarcated with a capsule composed of collagen fibers. The scaffolds have many translucent structures that contained red blood cells, and PCNA-positive cells are present inside the transplanted scaffolds. In the recipient liver, the cells derived from EB do not form teratomas, and then these cells or host tissues do not expand and invade each other.

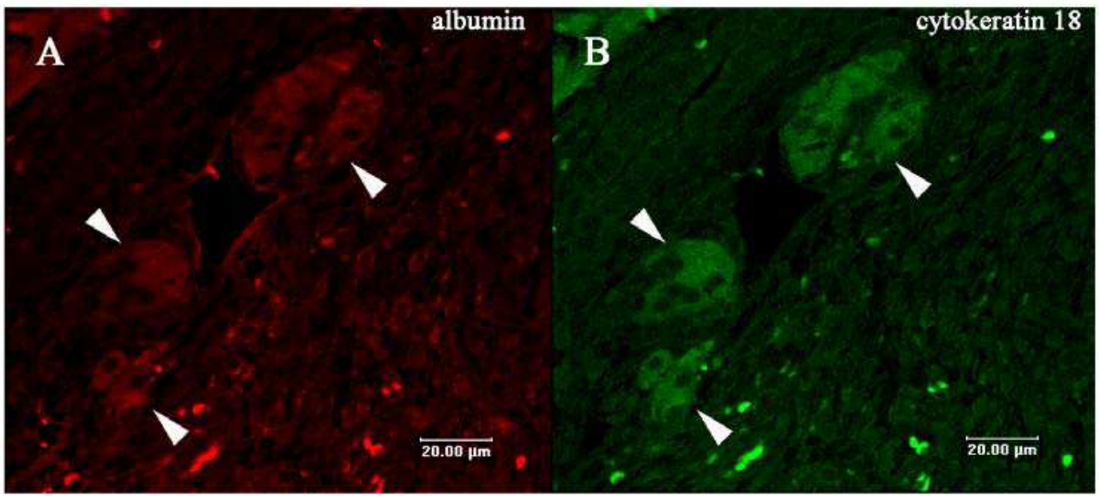



Fig. 5. At 14 days after implantation, the EB-derived hepatocyte-like cells expressing both albumin (A, red) and cytokeratin 18 (B, green) form hepatic lobule-like aggregates (Bar=20 µm)

We are investigating whether the hepatocyte-like cells derived during *in vitro* culture developed into mature cells or, alternatively, if other transplanted cells differentiated into them. At 7 days after transplantation, albumin positive cells are routinely present but alpha-fetoprotein positive hepatocyte-like cells disappear, even though cytokeratin 19 positive cells remain in the scaffolds. Importantly, some hepatocyte-like cells are clearly stained with both anti-albumin and anti- cytokeratin 18 antibodies. These are not recognizable in the cells after 24 days of *in vitro* culture. At 14 days, the hepatocyte-like cells positive for both albumin and cytokeratin 18 progressively develop from the cord-like structures into aggregates similar to hepatic lobules (Fig. 5). These results clearly indicate that immunohistochemically mature hepatocyte-like cells are induced from ES cells, and these cells construct hepatic lobule-like structures in the *in vivo* environment of liver regeneration.
The differentiation of hepatocyte-like cells from ES cells is complex. It requires not only an appropriate supporting matrix, but also an abundance of chemical mediators. During liver regeneration, nonparenchymal hepatocytes and parenchymal hepatocytes secrete various factors including tumor necrosis factor-alpha, interleukin-6, heparin bridge-epidermal growth factor, tumor growth factor, hepatocyte growth factor, and vascular endothelial growth factor (Duncan et al., 2009, Ishii et al., 1995). These factors might be responsible the

induction of immunohistochemically mature hepatocyte-like cells in the ES cell-implanted livers. Therefore, culture systems that imitate the *in vivo* environment and include co-cultured freshly isolated hepatocytes supplemented with growth factors and sufficiently high oxygen tension (Kimura et al., 1998), have the potential to promote development of new hepatocytes from the ES cells. Culture systems using scaffolds make this possible because they can provide an optimal structure that imitates *in vivo* histoarchitecture (Kamiya et al., 2002, Takimoto et al., 2003).

## 6. Advantage of the 3D culture systems and imitation of in vivo environment

This review shows two advantages of 3D culture systems. One is that 3D culture systems using scaffolds can provide cell-to-cell and/or cell-to-matrix interactions. These interactions promote differentiation of ES cells and construction of tissue-like structures (Carlberg et al., 2009, Elisseeff et al., 2006, Gao et al., 2010, Tian et al., 2008, Yim et al., 2006). Another benefit of the 3D culture system is that it can be manipulated to retrieve the cells or tissues derived from EBs without injuring or destroying them. Although hepatocyte-like cells can be induced from EBs in monolayer culture systems, these cells must be harshly treated to dislodge them from the culture dish before use. This operation can injure cells and destroy tissue structures. In the case of the scaffold culture system, we show that hepatocytes and hepatic structures derived from EBs can be used without any prior treatment.
Furthermore, this review shows that two points are worth highlighting regarding the development of bioartificial and secondary livers. The first is that ES cells have the potential to differentiate into hepatocytes and hepatic structures in suitable environments. The second point is that the best of these environments are ones that closely imitate the *in vivo* environment.

## 7. Conclusion

This review has shown that EBs formed from ES cells in collagen scaffolds when cultured *in vitro* for 24 days with exogenous growth factors and hormones associated with liver development. The 3D culture system induces hepatocyte-like cells from ES cells, and these cells form cord-like structures that are not present in monolayer cultures. However, the hepatocyte-like cells produced under these conditions are immature. Seven days after the cultured cells in scaffolds are transplanted into mice livers after partial hepatectomy, immunohistochemically mature cells, expressing both albumin and cytokeratin 18 are induced. At 14 days after transplantation, the cells are configured into hepatic lobule-like aggregates. Threfore, the combination of ES cells and culture systems imitating the *in vivo* environment warrant further development as a potential source of hepatocytes and liver structures for *in vivo* use.

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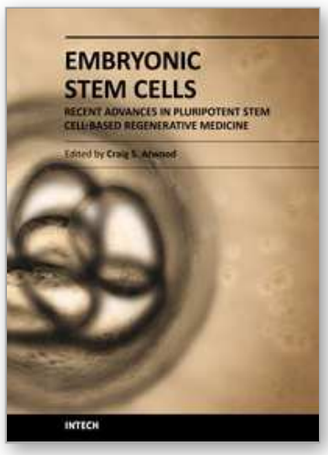
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**Abbreviations:** ES cell: embryonic stem cell; EBs: embryoid bodies; 3D: three-dimensional; 2D: two-dimensional; CT method: conical tubes method; ALB: albumin; AFP: alpha fetoprotein; TTR: transthyretin; G6P: glucose-6-phsphatase; TAT: tyrosine aminotransferase; CK19: cytokeratin19; CK18: cytokeratin18; PCNA: Proliferating Cell Nuclear Antigen; rER: rough surfaced endoplasmic reticulum; sER: smooth surfaced endoplasmic reticulum




**Embryonic Stem Cells - Recent Advances in Pluripotent Stem Cell-Based Regenerative Medicine**

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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