Hepatic maturation in differentiating embryonic stem cells in vitro

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Received 11 January 2001; accepted 20 April 2001

First published online 3 May 2001

Edited by Veli-Pekka Lehto

Abstract We investigated the potential of mouse embryonic stem (ES) cells to differentiate into hepatocytes in vitro. Differentiating ES cells expressed endodermal-specific genes, such as α-fetoprotein, transthyretin, α 1-anti-trypsin and albumin, when cultured without additional growth factors and late differential markers of hepatic development, such as tyrosine aminotransferase (TAT) and glucose-6-phosphatase (G6P), when cultured in the presence of growth factors critical for late embryonic liver development. Further, induction of TAT and G6P expression was induced regardless of expression of the functional SEK1 gene, which is thought to provide a survival signal for hepatocytes during an early stage of liver morphogenesis. The data indicate that the in vitro ES differentiation system has a potential to generate mature hepatocytes. The system has also been found useful in analyzing the role of growth factors and intracellular signaling molecules in hepatic development.

Key words: Embryonic stem cell; Hepatocyte; In vitro differentiation; SEK1; Tyrosine aminotransferase

1. Introduction

Embryonic stem (ES) cells are continuously growing stem cell lines of embryonic origin first isolated from the inner cell mass of blastocysts [1,2]. The distinguishing features of ES cells in mice are their capacity to be indefinitely maintained in an undifferentiated state in culture and their potential to develop into every cell type. ES cells can proliferate in vitro in an undifferentiated state on a feeder layer of mouse embryonic fibroblast cells (MEF) or in a medium containing leukemia inhibitory factor (LIF). The most rigorous test of the developmental potential of mouse ES cells is their ability to contribute to all cell lineages of chimeric animals, including the germ line [3]. Alternatively, when ES cells are injected into immunocompromised animals, they form teratomas composed of multiple lineages [2].

In addition to their pluripotent ability to differentiate in vivo, ES cells can differentiate into multiple cell lineages in vitro as well. The in vitro differentiation of ES cells is induced by removing the ES cells from the feeder layer of MEF or by removing LIF from the culture medium, and then allowing them to form aggregates in suspension. ES cells aggregate into structures termed embryoid bodies (EBs), in which all three germ layers develop and interact with each other. Well-differentiated EBs are composed of multiple differentiated cell types including neuronal, cardiac muscle, hematopoietic, and chondrocytic cells. EBs recapitulate many processes that take place during development [4]. Certain aspects of the kinetics of lineage development observed within EBs show remarkable similarities to that observed in the developing embryo [5]. Such development, however, is spontaneous and influenced by accidental cell-to-cell interactions. In case of hematopoietic commitment, exogenous growth factors efficiently and reproducibly induce lineage-specific precursors [6].

While previous studies have shown that ES cells demonstrate early endodermal differentiation in vitro [7–10], it remains unclear whether they have the ability to differentiate into mature endodermal phenotypes such as hepatocytes in vitro. To this end, we investigated the ability of ES cells to differentiate into mature hepatocytes in vitro. Further, we examined the effects of growth factors, cell culture matrix, and gene modulation on the hepatic maturation in this in vitro system.

2. Materials and methods

2.1. Cell culture

The ES cell lines R1 (129Sv strain), W9.5 (129Sv), and SEK1 null (established from W9.5) [11] were maintained undifferentiated in gelatin-coated dishes in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) containing 15% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES (Gibco BRL), 300 μM monothioglycerol (Sigma, St. Louis, MO, USA), and 250 U/ml recombinant mouse LIF (Esgro, Chemicon, Temecula, CA, USA). To induce differentiation, ES cells were suspended in Iscove's modified Dulbecco's medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL), 20% fetal bovine serum (Atlanta Biologicals) and 300 μM monothioglycerol (Sigma). Cells were cultured for 2 days by the hanging drop method (1×10^3 ES cells per 30 μl in each drop) [12]. EBs in hanging drops were transferred to suspension culture in 100 mm petri dishes and cultured for an additional 3 days. The resulting EBs were plated onto six-well tissue culture dishes coated with or without Vitrogen (collagen type I) (Cohesion, Palo Alto, CA, USA). In some experiments, the growth factors were added into culture medium (100 ng/ml acidic fibroblast growth factors (aFGF), 20 ng/ml hepatocyte growth factor (HGF), 10 ng/ml oncostatin M (OSM), with 10^{-7} M dexamethasone (Dex; Sigma), and ITS (5 mg/ml insulin, 5 mg/ml trans-

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PII: S0014-5793(01)02423-1

ferrin, 5 μg/ml selenious acid, Collaborative Biomedical Products, Benford, MA, USA)).

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by using an RNA aqueous kit (Ambion, Austin, TX, USA). cDNA was synthesized from 2 μg total RNA by using SuperScript II first-strand synthesis system with oligo (dT) (Gibco BRL). PCR was performed by using Taq DNA polymerase (Eppendorf, Westbury, NY, USA) (94°C, 1 min; specific annealing temperature below, 1 min; 72°C, 1 min). Primers were synthesized for the following mouse genes (oligonucleotide sequences are given in brackets in the order of anti-sense, sense primer followed by the annealing temperature, cycles used for PCR and length of the amplified fragment): transthyretin (TTR) (5′-CTCACCACAGATGAGAAG, 5′-GGCTGAGTCTCTCAATTC; 55°C; 25 cycles; 225 bp), α-fetoprotein (AFP) (5′-TCGTATTCCAACAGGAGG, 5′-AGGCTTTTGCTTCACCAG; 55°C; 25 cycles; 173 bp), α 1-anti-trypsin (AAT) (5′-AATGGAAGAAGCCATTCGAT, 5′-AAGACTGTAGCTGCTGCAGC; 55°C; 30 cycles; 484 bp), albumin (ALB) (5′-GCTACGGCACAGTGCTTG, 5′-CAGGATTGCAGACAGATAGTC; 55°C; 25 cycles; 260 bp), glucose-6-phosphatase (G6P) (5′-CAGGACTGGTTCATCCTT, 5′-GTTGCTGTAGTAGTCGGT; 55°C; 30 cycles; 210 bp), tyrosine aminotransferase (TAT) (5′-ACCTTCAATCCCATCCGA, 5′-TCCCGACTGGATAGGTAG; 50°C; 30 cycles; 206 bp), β-actin (5′-TTCCTTCTTGGGTATGGAAT, 5′-GAGCAATGATCTTGATCTTC; 55°C; 20 cycles; 200 bp), SEK1 (5′-TGTATGGAGCTCATGTCTACC, 5′-GTCTATTCTTTCAGGTGCCA; 50°C; 30 cycles; 300 bp).

For each gene, the DNA primers were originated from different exons to ensure that the PCR product represents the specific mRNA species and not genomic DNA. Relative quantitation of ALB gene was performed by ABIPRISM5700 sequence detection system and SYBR green PCR master mix (PE Biosystems, Foster City, CA, USA). β-Actin was used as the endogenous control.

2.3. JNK activity

JNK activity in cell lysates was measured by immunocomplex protein kinase assays using the substrate glutathione *S*-transferase (GST)-c-Jun (1–79) fusion protein [13,14]. Cell lysates were incubated 30 min with GST-c-Jun (1–79) fusion proteins immobilized on glutathione–Sepharose beads to precipitate JNKs. These beads were resuspended in 50 μl of kinase buffer (20 mM HEPES, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 50 μM Na_3VO_4, and 10 mM $MgCl_2$, 10 μCi [γ-^{32}P]ATP). The kinase reaction was performed at 30°C for 20 min, and stopped by adding SDS sample buffer. The samples were resolved in a SDS gel. The gel was stained with Coomassie brilliant blue solution for 5 min and destained, then air dried. Phosphorylated GST-c-Jun was visualized by autoradiography.

3. Results

3.1. In vitro ES differentiation to hepatic lineage

To assess the level of endodermal and hepatic differentiation, we examined the mRNA expression of endodermal- and liver-specific genes. TTR and AAT represent endodermal or yolk-sac-like differentiation and are expressed throughout liver maturation [15,16]. AFP is a marker of the endodermal differentiation as well as an early fetal hepatic marker, and its expression decreases as the liver develops into adult phenotype. Expression of ALB, the most abundant protein synthesized by mature hepatocytes, starts in early fetal hepatocytes (E12) and reaches the maximal level in adult hepatocytes [17]. Although ALB is known to be a hepatocyte differentiation marker, it is also expressed weakly in yolk sac. At a late gestational or perinatal stage, G6P is predominantly expressed in the liver [18]. TAT represents an excellent enzymatic marker for peri- or postnatal hepatocyte-specific differentiation. These enzymes are not synthesized in significant quantities prior to birth but are rapidly activated early in the neonatal developmental period [19]. Since hormone-regulated TAT activity is strictly limited to the parenchymal cells of the adult liver, it has been used extensively for monitoring cellular differentiation in experimental models for liver development/maturation in vitro [20,21].

Undifferentiated ES cells did not express these endodermal or hepatocyte lineage genes. Fig. 1 depicts the in vitro ES differentiation procedure used in this study. Fig. 2 illustrates the pattern of endodermal-specific gene expression in differentiating EBs without additional growth factors. TTR was expressed within 6 days after removal of the LIF. AFP and AAT were expressed within day 9. ALB mRNA expression first appeared within day 12. Late differential markers of hepatocyte, TAT and G6P were not detectable throughout the time course (up to day 18). These data indicate that ES cells spontaneously differentiate toward hepatic or yolk sac lineage cells, but they do not differentiate into mature hepatocytes.

3.2. Induced hepatic maturation in vitro

During embryonic development of mice, the initial event of liver ontogeny occurs on embryonic day 9 (E9). In this early stage, FGFs, derived from adjacent cardiac mesoderm, commit the foregut endoderm to forming the liver primodium [22]. Over the next 2 days, the liver bud proliferates and migrates into surrounding septum transversum, which consists of loose connective tissue containing collagen. Hepatic precursors are in direct contact with connective tissue matrix [23]. During and after the mid-stage of hepatogenesis, surrounding mesenchymal cells secrete HGF and support fetal hepatocytes. In-

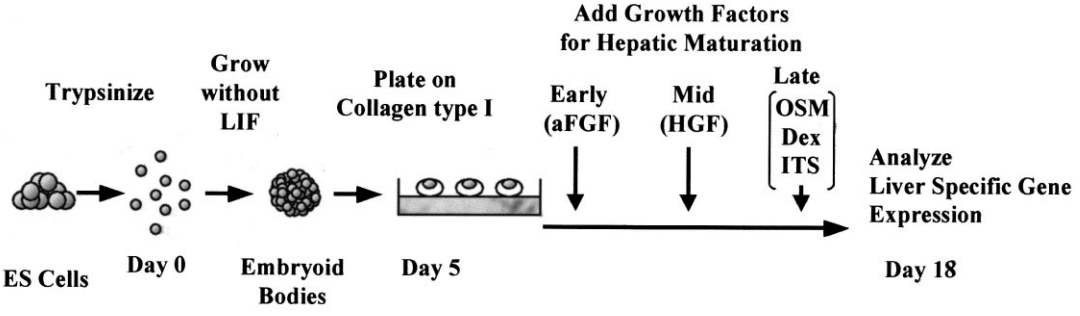

Fig. 1. In vitro hepatic lineage differentiation of ES cells. The in vitro differentiation protocol used in the study is illustrated. In some experiments, growth factors were added based on previous studies in embryonic liver development. Acidic FGF (100 ng/ml) was added between days 9 and 12 as an early stage factor for hepatic maturation. HGF (20 ng/ml) was added between days 12 and 18 as a mid-stage factor. OSM (10 ng/ml), Dex (10^{-7} M), and ITS mixture (5 mg/ml insulin, 5 mg/ml transferrin, 5 μg/ml selenious acid) were added as late stage factors between days 15 and 18.

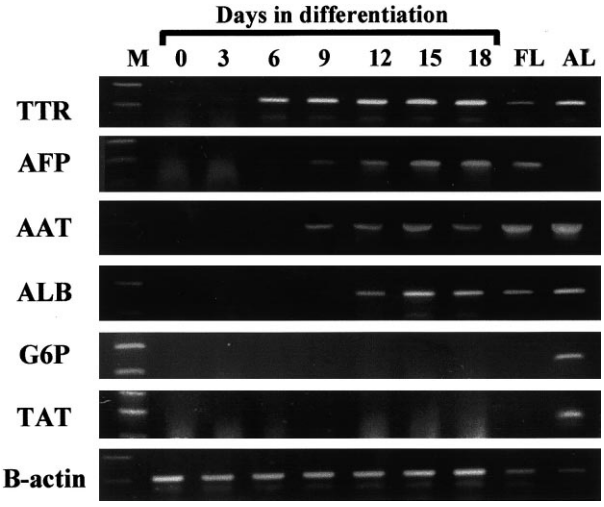

Fig. 2. Expression of endodermal and hepatic differentiation marker genes in differentiating ES cells. ES cells were cultured under the protocol shown in Fig. 1 without using collagen-coated dish nor additional growth factors for hepatic maturation. Cells were harvested at the indicated time (days). mRNA expression of TTR, AFP, AAT, ALB, G6P, TAT and β-actin was examined by RT-PCR as described in Section 2. Molecular size marker for DNA (M), fetal liver at embryonic day 12 (FL), adult liver at 3 weeks old (AL). Results shown here and below are representative of three to five independent experiments.

deed, in mice genetically lacking HGF, the embryonic liver is reduced in size and shows extensive loss of parenchymal cells [24,25]. From E12 through E16, the fetal liver becomes the major site for hematopoiesis [26]. During this late stage, hematopoietic cells produce OSM that induces maturation of murine fetal hepatocytes [27].

Based on these previous reports for embryonic liver development, we applied growth factors and cell culture matrix to induce hepatic maturation of EBs in vitro (Fig. 1). Initially we attached EBs on collagen-coated culture plates at day 5 in vitro differentiation. As an early stage factor potentially inducing hepatic differentiation, aFGF was added from day 9 to day 12. From day 12 to day 18, HGF was added as a mid-stage factor. OSM, Dex and a mixture of insulin, transferrin and selenious acid (ITS) were added as late stage factors from day 15 to day 18. We analyzed the patterns of hepatic lineage gene expression at day 18. As shown in Fig. 3, a combination of these growth factors enhanced the expression of ALB mRNA, which is an indicator of hepatocyte maturation. The expression of ALB was increased 9.5-fold and 7.4-fold (real-time PCR) by the growth factors on collagen-uncoated culture and collagen-coated culture, respectively. Moreover, G6P and TAT genes, indicators of hepatocyte maturation, were now expressed in EBs in the presence of the growth factors. It appeared that collagen coating further enhanced the expression of G6P and TAT.

We then examined the effects of growth factors at individual stages on hepatic development using EBs plated on collagen-coated dishes. As demonstrated in Fig. 4a, the mid-stage factor (HGF) or late stage factors (OSM, Dex, ITS) were critical for G6P expression. The late stage factors exclusively enhanced TAT gene expression. Although Dex, by itself, slightly induced TAT expression, the mixture of the late stage factors mostly enhanced the TAT expression (Fig. 4b).

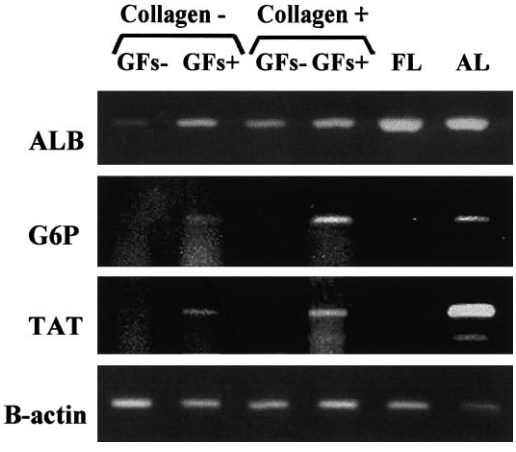

Fig. 3. Effects of collagen-coated dish and addition of growth factors on expression of late hepatic differentiation markers. ES cells were cultured under the protocol shown in Fig. 1 with or without the use of a collagen-coated dish, in the absence or presence of growth factors (GFs) for hepatic maturation. Cells were harvested at day 18, and mRNA expression of ALB, G6P, TAT and β-actin was examined by RT-PCR as described in Section 2. Fetal liver at embryonic day 12 (FL), adult liver at 3 weeks old (AL).

3.3. *Maturation of SEK1 null ES cells into hepatic lineage in vitro*

SEK1 (also known as MKK4 and JNKK) is a member of the mitogen-activated protein kinase activator family [28,29]. SEK1 deficiency leads to an embryonic lethality between E10.5 and E12.5 and is associated with abnormal liver devel-

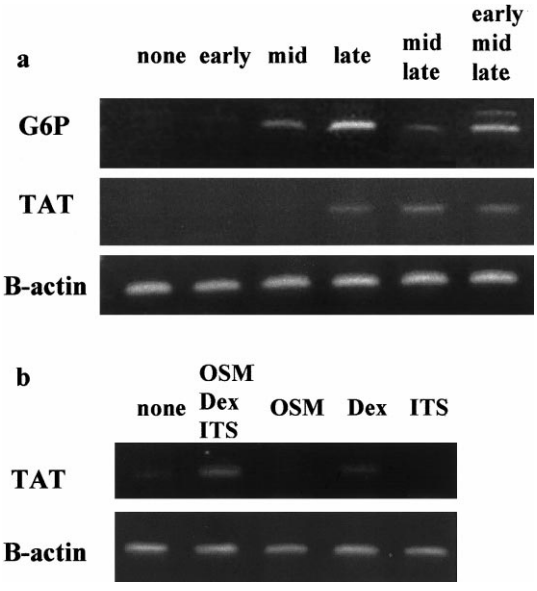

Fig. 4. Effects of early, mid- and late stage factors on expression of late hepatic differentiation markers. ES cells were cultured under the protocol shown in Fig. 1. a: No growth factor (none), early stage factor alone (early), mid-stage factor alone (mid), late stage factors alone (late), mid- and late stage factors (mid/late), or early, mid- and late stage factors (early/mid/late) were added as growth factors for hepatic maturation. b: No growth factor (none), OSM, Dex and ITS mixture (OSM/Dex/ITS), OSM, Dex, or ITS mixture (ITS) were added as late growth factors for hepatic maturation. No early or mid factors were added. Cells were harvested at day 18, and mRNA expression of G6P, TAT, and β-actin was examined by RT-PCR as described in Section 2.

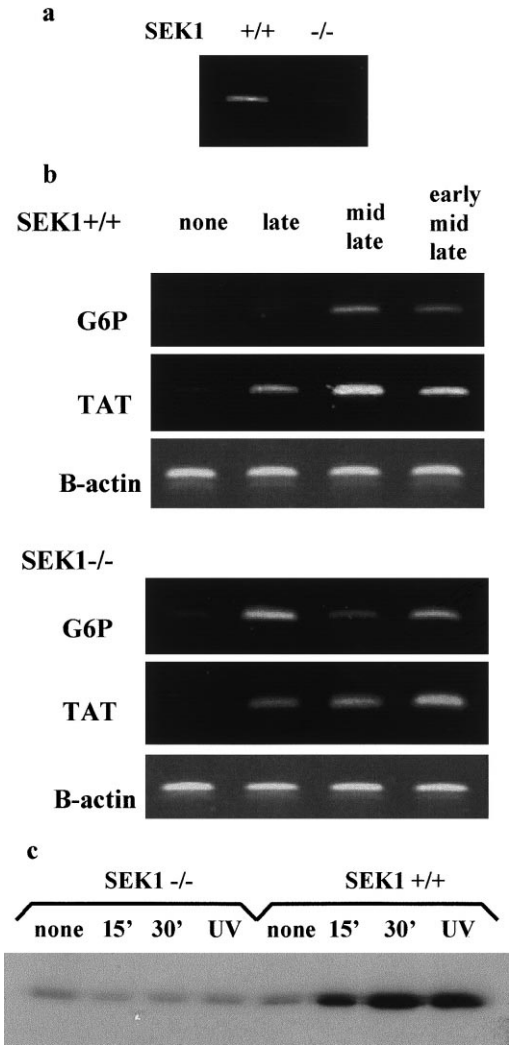

Fig. 5. Effects of SEK1 knockout on expression of late hepatic differentiation markers. SEK1 null ES cells (SEK1−/−) and their parental ES cells (SEK1+/+) were cultured under the protocol shown in Fig. 1. a: SEK1 expression. Cells were harvested at day 18, and expression of SEK1 mRNA was examined using RT-PCR as described in Section 2. b: Induced expression of G6P and TAT. No growth factor (none), late stage factors alone (late), mid- and late stage factors (mid/late), or early, mid- and late stage factors (early/mid/late) were added as growth factors for hepatic maturation. Cells were harvested at day 18, and mRNA expression of G6P, TAT, and β-actin was examined by RT-PCR as described in Section 2. c: JNK activity. Late stage factors were added into differentiating ES cells at day 15. Cells were harvested at the indicated time after addition of the factors (0, 15 or 30 min later). As a control, cells were irradiated by ultra-violet for 15 min. JNK activity in the cell lysates was measured as described in Section 2.

opment [30]. SEK1 null fetal mice show that the visceral endoderm normally develops into primordial liver, but parenchymal hepatocytes undergo massive apoptosis. This phenomenon indicates that the SEK1 signaling pathway is exclusively required after a certain period of early hepatogenesis [31,11]. Because of its embryonic lethality, it is hard to further assess the role of SEK1 in the late stage hepatogenesis in vitro.

Using the in vitro ES cell differentiation system described above, we investigated the potential of SEK1 null ES cells to differentiate into mature hepatocytes. Fig. 5a showed the expression of SEK1 mRNA in differentiated EBs (day 18). We confirmed that SEK1 mRNA was not detected in SEK1 null EBs. The expression of TAT and G6P mRNA was induced in SEK1 null EBs by late stage growth factors as well as in control wild type EBs (Fig. 5b). The late stage growth factors induced JNK activity, a downstream kinase of SEK1, in wild type EBs but not in SEK1 null EBs (Fig. 5c). These data indicated that the SEK1 signaling pathway is not indispensable in late stage maturation of hepatocytes.

4. Discussion

The ability of ES cells to develop into a wide range of cell types in vitro has drawn much attention to ES cells as a basic research tool and as a novel source for potential cell therapy [32], particularly after human ES cells were established [33,34]. Despite intensive studies of in vitro differentiation of ES cells in other lineages, hepatic differentiation of ES cells has not yet been reported with the exception of early to mid-endodermal differentiation of ES cells up to the stage of ALB production, which, by itself, could not distinguish hepatic differentiation from yolk sac lineage differentiation. Here we demonstrated that ES cell differentiation in vitro would not surpass this stage spontaneously without additional growth factors. However, addition of hepatocyte maturation factors did induce expression of mature hepatocyte-specific genes in differentiating ES cells, suggesting that ES cells have a potential to become mature hepatocytes in vitro. This finding indicates that it is possible to generate functional hepatocytes in vitro from differentiating ES cells. Since cell replacement therapy using primary hepatocytes has been shown to be effective in animal models of hepatic failure and liver-based metabolic diseases [35–38], an alternative source of functional hepatocytes would be beneficial.

Since differentiating EBs recapitulate many processes taking place in normal embryonic development, in vitro ES differentiation serves as an excellent system to study lineage-specific development. In the current study, endodermal and hepatocyte lineage genes were sequentially expressed in a fashion similar to that found in the normal embryo. In addition, late hepatocyte maturation factors, including OSM, indeed induced expression of mature hepatocyte-specific genes in differentiating ES cells. These data indicate that the in vitro ES differentiation system is a useful tool to study hepatic development.

Another advantage of this in vitro ES-to-hepatocyte differentiation system is the ability to rapidly determine the role of a specific gene in specific tissues. In contrast, most studies in gene function require generation of transgenic and/or gene knockout animals, which are considered to be long-term assays. We previously demonstrated the usefulness of this in vitro ES differentiation system combined with targeted gene disruption to define complex regulatory events in a cardiac disease model [39]. In particular, this in vitro system is useful when targeting of a gene leads to an early embryonic death as seen with the SEK1 knockout mice. It has been suggested that the SEK1 signaling cascade is related with cell survival under a variety of cellular stresses or under stimulation with growth factors [29,40]. SEK1 appears to induce anti-apoptotic genes in developing hepatocytes and/or directly affect signaling pathways required for cell survival [31,11]. In these in vivo studies, it was shown that hepatic differentiation itself was not disturbed by SEK1 deletion, at least to the stage of ALB

expression. By using the in vitro ES differentiation system, we demonstrated that SEK1 is not indispensable for hepatic maturation to a later stage of liver development.

In summary, we have demonstrated the potential of mouse ES cells to differentiate into mature hepatocytes in vitro. Further, we demonstrated the ES differentiation system in vitro is a useful model for analyzing the role of specific growth factors and intracellular signaling molecules in hepatic development.

Acknowledgements: We thank James Crawford and Bryon Petersen (Gainesville, FL, USA) for critical reading of the manuscripts, and Stephen Duncan (Milwaukee, WI, USA) and Hirobumi Teraoka (Tokyo, Japan) for helpful discussion. This work was supported in part by a grant from the National Institutes of Health (DK-59699) to N.T.

References

[1] Evans, M.J. and Kaufman, M.H. (1981) Nature 292, 154–156.
[2] Martin, G.R. (1981) Proc. Natl. Acad. Sci. USA 78, 7634–7638.
[3] Bradley, A., Evans, M., Kaufman, M.H. and Robertson, E. (1984) Nature 309, 255–256.
[4] Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985) J. Embryol. Exp. Morphol. 87, 27–45.
[5] Leahy, A., Xiong, J.W., Kuhnert, F. and Stuhlmann, H. (1999) J. Exp. Zool. 284, 67–81.
[6] Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M.V. (1993) Mol. Cell. Biol. 13, 473–486.
[7] Abe, K., Niwa, H., Iwase, K., Takiguchi, M., Mori, M., Abe, S.I., Abe, K. and Yamamura, K.I. (1996) Exp. Cell. Res. 229, 27–34.
[8] Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S. and Parmacek, M.S. (1998) Genes Dev. 12, 3579–3590.
[9] Coffinier, C., Thepot, D., Babinet, C., Yaniv, M. and Barra, J. (1999) Development 126, 4785–4794.
[10] Barbacci, E., Reber, M., Ott, M.O., Breillat, C., Huetz, F. and Cereghini, S. (1999) Development 126, 4795–4805.
[11] Ganiatsas, S., Kwee, L., Fujiwara, Y., Perkins, A., Ikeda, T., Labow, M.A. and Zon, L.I. (1998) Proc. Natl. Acad. Sci. USA 95, 6881–6886.
[12] Metzger, J.M., Lin, W.I. and Samuelson, L.C. (1994) J. Cell Biol. 126, 701–711.
[13] Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R.J., Johnson, G.L. and Karin, M. (1994) Science 266, 1719–1723.
[14] Ishizuka, T., Chayama, K., Takeda, K., Hamelmann, E., Terada, N., Keller, G.M., Johnson, G.L. and Gelfand, E.W. (1999) J. Immunol. 162, 2087–2094.
[15] Makover, A., Soprano, D.R., Wyatt, M.L. and Goodman, D.S. (1989) Differentiation 40, 17–25.
[16] Thomas, T., Southwell, B.R., Schreier, G. and Jaworwski, A. (1990) Placenta 11, 413–430.
[17] Sellem, C.H., Frain, M., Erdos, T. and Sala-Trepat, J.M. (1984) Dev. Biol. 102, 51–60.
[18] Pan, C.J., Lei, K.J., Chen, H., Ward, J.M. and Chou, J.Y. (1998) Arch. Biochem. Biophys. 358, 17–24.
[19] Greengard, O. (1969) Science 163, 891–895.
[20] Chou, J.Y. (1988) Arch. Biochem. Biophys. 263, 378–386.
[21] Shelly, L.L., Tynan, W., Schmid, W., Schutz, G. and Yeoh, G.C. (1989) J. Cell Biol. 109, 3403–3410.
[22] Jung, J., Zheng, M., Goldfarb, M. and Zaret, K.S. (1999) Science 284, 1998–2003.
[23] Cascio, S. and Zaret, K.S. (1991) Development 113, 217–225.
[24] Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E. and Birchmeier, C. (1995) Nature 373, 699–702.
[25] Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T. and Kitamura, N. (1995) Nature 373, 702–705.
[26] Kinoshita, T., Sekiguchi, T., Xu, M.J., Ito, Y., Kamiya, A., Tsuji, K., Nakahata, T. and Miyajima, A. (1999) Proc. Natl. Acad. Sci. USA 96, 7265–7270.
[27] Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T. and Miyajima, A. (1999) EMBO J. 18, 2127–2136.
[28] Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R. and Templeton, D.J. (1994) Nature 372, 798–800.
[29] Nishina, H., Fischer, K.D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E.A., Bernstein, A., Mak, T.W., Woodgett, J.R. and Penninger, J.M. (1997) Nature 385, 350–353.
[30] Yang, D., Tournier, C., Wysk, M., Lu, H.T., Xu, J., Davis, R.J. and Flavell, R.A. (1997) Proc. Natl. Acad. Sci. USA 94, 3004–3009.
[31] Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., De la Pompa, J.L., Furlonger, K., Paige, C., Hui, C., Fischer, K.D., Kishimoto, H., Iwatsubo, T., Katada, T., Woodgett, J.R. and Penninger, J.M. (1999) Development 126, 505–516.
[32] Keller, G. and Snodgrass, H.R. (1999) Nat. Med. 5, 151–152.
[33] Thomson, J.A., Itskovitz Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998) Science 282, 1145–1147.
[34] Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. and Bongso, A. (2000) Nat. Biotechnol. 18, 399–404.
[35] Demetriou, A.A., Reisner, A., Sanchez, J., Levenson, S.M., Moscioni, A.D. and Chowdhury, J.R. (1988) Hepatology 8, 1006–1009.
[36] Roger, V., Balladur, P., Honiger, J., Baudrimont, M., Delelo, R., Robert, A., Calmus, Y., Capeau, J. and Nordlinger, B. (1998) Ann. Surg. 228, 1–7.
[37] Gupta, S., Gorla, G.R. and Irani, A.N. (1999) J. Hepatol. 30, 162–170.
[38] Yoshida, Y., Tokusashi, Y., Lee, G.H. and Ogawa, K. (1996) Gastroenterology 111, 1654–1660.
[39] Minamino, T., Yujiri, T., Papst, P.J., Chan, E.D., Johnson, G.L. and Terada, N. (1999) Proc. Natl. Acad. Sci. USA 96, 15127–15132.
[40] Liu, Y.F. (1998) J. Biol. Chem. 273, 28873–28877.