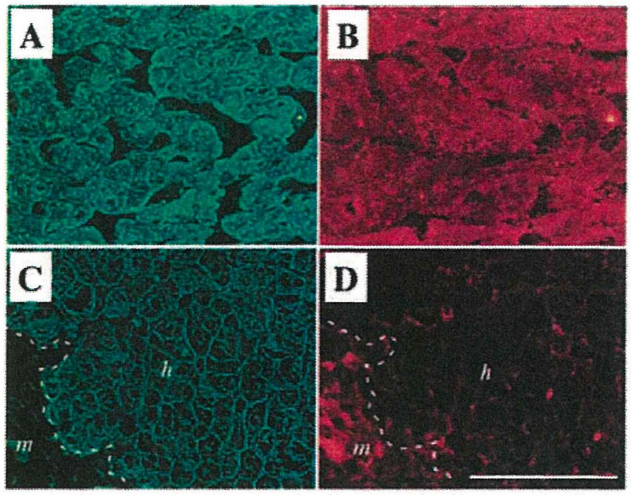

Fig. (8). Expression of TGFBR2 in normal and *h*-chimeric mouse livers. A 49-year-old male liver tissues were used as control normal human liver (**A**, **B**). uPA/SCID mice were transplanted with *h*-hepatocytes from 9-month-old male and sacrificed at 11 weeks (T-phase) after transplantation. The liver tissues were isolated from the mice (**C**, **D**). Liver sections were double stained for hCK8/18 for identifying *h*-hepatocytes (green; **A** and **C**) and TGFBR2 (red; **B** and **D**). The dashed lines in **C** and **D** indicate the boundary between *h*-hepatocyte (*h*) and *m*-hepatocyte regions (*m*). Scale bar represents 100 μm.

soon (within 24 h) for cell division to replace the damaged cells, but correctly terminates DNA synthesis/cell division when the mass ratio of the liver to the body weight reaches to a defined value determined by intercellular signaling between parenchymal and nonparenchymal cells [17], although underlying mechanisms have not been still completely understood. Studies with cultured hepatocytes suggested that TGF-β and its type II receptor TGFBR2 (TGF-β/TGFBR2) are antimitogenic for hepatocytes [18]. Two research groups independently generated hepatocyte-targeted TGFBR2-knockout mice and examined the role of TGF-β/TGFBR2 signaling in the termination of partial (70%) hepatectomy-induced liver regeneration, one by Romero-Gallo *et al.* claiming that TGF-β/TGFBR2 is a terminating factor [19] and the other by Oe *et al.* suggesting that activin A may be a principal terminator [20].

Based upon these previous studies, we asked whether TGF-β/TGFBR2 or activin A is involved in the observed hepatic hyperplasia in *h*-chimeric mouse [9]. The livers from *h*- and *r*-hepatocytes chimeric mice generated as above were examined for the expression levels of TGFBR1, TGFBR2, and Activin A type IIA receptor (ACVR2A) genes during repopulation of xenogeneic hepatocytes. The obtained expression levels were compared to those of the respective resting normal livers determined in the parallel experiments. Expressions of all these genes in *r*-hepatocyte chimeric mouse livers were suppressed to half those of normal rat livers in P-phase (at 2 weeks after transplantation, RI = 57%) and rapidly returned to normal levels in T-phase (at 3 weeks, RI = 97%) and were saturated thereafter (at 4 weeks, RI = 99%), suggesting roles of these genes as replication termination signals. In *h*-hepatocyte chimeric mouse livers, h-TGFBR1 showed a similar expression pattern as rTGFBR1 in *r*-hepatocyte chimeric mouse livers except that changes progressed quite slowly taking 11 weeks after transplantation to regain normal levels. The expression levels of TGFBR2 and ACVR2A genes were also lower than the resting livers, the extent of suppression being quite prominent, less than one-third on the normal level. However, importantly *h*-hepatocyte chimeric livers kept the reduced levels thereafter up to 11 weeks after transplantation. The expression levels of TGFBR2 strictly down-regulated during the experimental period and those of ACVR2A showed a tendency to increase, but still much lower than the normal levels even at 11 weeks after transplantation.

We then immunohistologically tested our explanation of *h*-chimeric liver enlargement that *h*-hepatocytes fail to regain expression of TGFBR2 at a level seen in the normal resting liver in T-phase [9]. Liver sections at P- and T-phase from *h*- and *r*-hepatocyte chimeric livers were doubly stained with antibodies against "hCK8/18 and h/rTGFBR2 (cross-reactive for both humans and rats)" and "rRT1A (rat major histocompatibility complex class 1, for staining *r*-hepatocytes) and h/rTGFBR2", respectively, together with sections from normal resting human and rat livers. *r*-Hepatocytes in both normal rats and *r*-chimeric mice at T-phase were heavily positive to TGFBR2. Similarly *h*-hepatocytes in resting normal livers were heavily positive to TGBR2 (Fig. **8A** and **B**), but not only those in P-phase at 5 weeks after transplantation, but also those in T-phase (11 weeks) were negative (Fig. **8C** and **D** for *h*-hepatocytes in T-phase) as we thought. There were some positive cells, but these cells were identified as *m*-nonparenchyma cells by their morphology and distribution patterns. These results indicate that the hyperplasia caused by *h*-hepatocyte repopulation might be a result of persistent low sensitivity of *h*-hepatocytes in *h*-hepatocyte chimeric mouse toward TGF-β/activin, especially TGF-β. As mentioned above, TGFR1 mRNA was increased to the normal level at T-phase, which, however, appears not to affect the sensitivity of *h*-hepatocyte to the ligand, because TGF-β initially binds to TGFBR2 homodimers, which recruits TGFBR1 dimers forming a hetero-tetrameric complex, and TGF-β signals are transferred intracellularly [21].

The next question was which type of liver cells produce TGF-β as an anti-mitogenic factor for hepatocytes and induce them to get into T-phase when the host regains the correct $R_{L/B}$. TGF-β-expressing cells were identified by double immunostaining for desmin and TGF-β, which reveals the following facts: (1) When cells are positive for TGF-β, they are mostly desmin-positive; (2) Hepatic stellate cells of normal rats, normal humans, and SCID mice do not express TGF-β protein. Hepatic stellate cells are markedly activated and, as a result, increase their numbers and all express TGF-β protein at high levels in uPA/SCID mice (Fig. **9A**, **B**); (3) In *r*-chimeric mice, the number of stellate cells markedly decreases and these cells do not express TGF-β protein in P-phase (Fig. **9C**, **D**). Their number increases >4-fold as compared to P-phase and these cells all express TGF-β protein at considerably high levels in T-phase (Fig. **9G**, **H**); (4) In *h*-chimeric mice, the number of stellate cells greatly decreases and these cells do not express TGF-β protein in P-phase as in *r*-chimeric mice (Fig. **9E**, **F**). Their number increases >2-

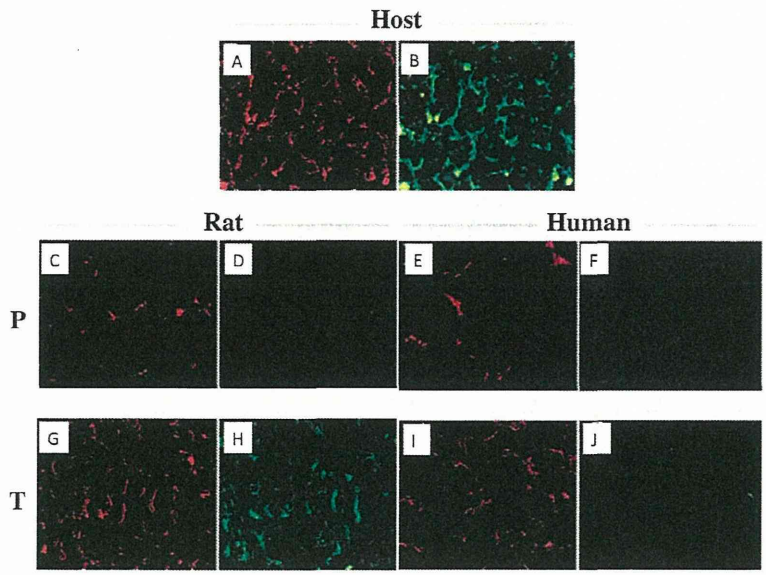

Fig. (9). Expression and distribution of TGF-β in normal and chimeric mouse livers. Livers were removed from a host mouse (1-month-old uPA/SCID mouse) (A, B), from *r*-chimeric mice with Fisher 344 rat hepatocytes (C, D, G, H) at P-phase (2 weeks post transplantation) (P: C, D) and at T-phase (three weeks post transplantation) (T: G, H) and from *h*-chimeric mice with 9-month-old male hepatocytes (E, F, I, J) at P-phase (five weeks post transplantation) (P: E, F) and at T-phase (11 weeks post transplantation) (T: I, J). These livers were cryosectioned and double-immunostained for desmin (A, C, G, E, I, red) and TGF-β (B, D, H, F, J, green). Serial sections from *r*- and *h*-hep-mouse livers were immunostained for rRT1A and hCK8/18 to identify *r*- and *h*-hepatocytes, respectively (data not shown). Scale bar represents 100 μm.

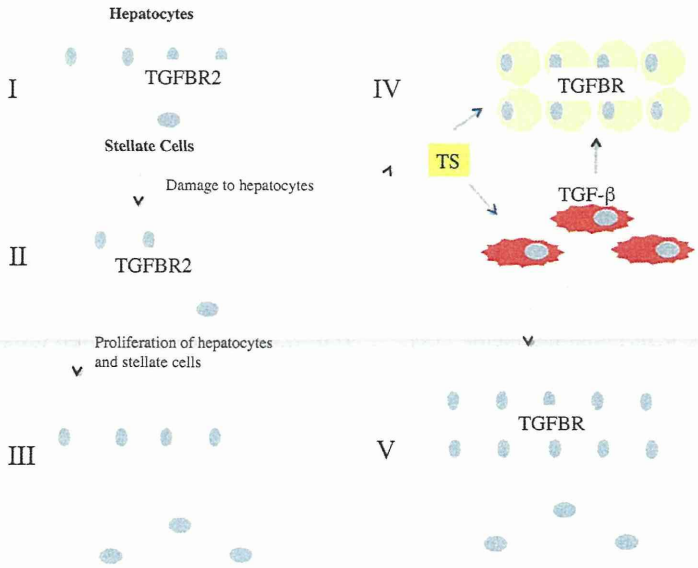

Fig. (10). Plausible interactions between hepatocytes and stellate cells to terminate the proliferation of hepatocytes during liver growth. Hepatocytes and stellate cells are in a normal resting state (Step I). Hepatocytes express TGFBR2. The liver is injured and the number of hepatocytes is reduced (Step II). The hepatocyts start to proliferate and get into Step III in which the TGFBR2 expression is reduced. When hepatocytes increase the number, which is adequate to sufficiently respond to metabolic demands of body. Such situations generate a termination signal(s) (TS), which makes hepatocyts to express TGFBR2 and activate stellate cells to express TGF-β (Step IV). The establishment of TGF-β signaling leads hepatocytes to terminate proliferation and stellate cells to terminate expression of TGF-β, which brings these cells into the resting state (Step V).

fold as compared to P-phase, but these cells all still do not express TGF-β protein in T-phase (Fig. **9I, J**), which is quite different from the case observed in *r*-chimeric mice.

These facts suggest that stellate cells are generally less distributed around proliferating hepatocytes in liver at P-phase and they do not produce TGF-β protein. However,

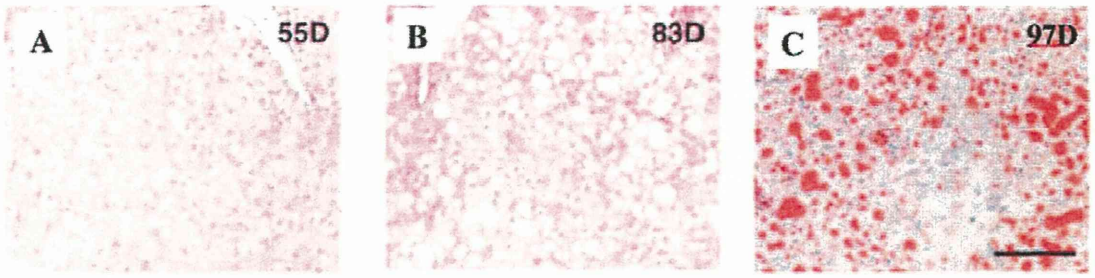

Fig. (11). Lipid accumulation in chimeric mouse *h*-hepatocytes. Chimeric mice with 6-year-old female were killed at 55 days (55D) (**A**) and 83 days (83 D) after transplantation for histological examinations of *h*-hepatocyte regions by H&E. No visible cytoplasmic vacuolation in the *h*-hepatocytes at 55 days, but extensive and intensive vacuolation at 83 days. *h*-Hepatocyte regions in the mice killed at 97 days (97D) were stained with Oil Red O together with nucler staining (blue). Bar represents100 μm. Photos are cited from the literature No. 10.

when the number of hepatocytes becomes close to the physiologically adequate number, stellate cells become abundantly distributed around the hepatocytes and these cells express TGF-β. These stellate cells cease to express TGF-β when the liver actually gets the adequate number of hepatocytes and become physiologically stable. *h*-Chimeric mouse liver are able to provide *h*-hepatocytes and *m*-stellate cells with environments which permit the progression to P-phase as in *r*-chimeric mouse liver, but these environments do not permit the progression to T-phase. The failure of TGF-β/TGFBR signaling in *h*-hepatocytes of *h*-chimeric mouse was supported by experiments in which we showed the absence of detectable levels of Smad2/3, major intracellular effectors in both TGFBR and ACVR, in *h*-hepatocyte nuclei in *h*-chimeric mouse livers at T-phase, contrasting to *r*-chimeric mouse livers whose hepatocyte nuclei contained high levels of the effector proteins [9].

The results we have obtained suggest the presence of plausible interactions between hepatocytes and stellate cells to terminate the proliferation of hepatocytes during liver growth (Fig. **10**). Hepatocytes in a normal resting state (Step I) express TGF-β type II receptor (TGFBR2). When the liver is injured (Step II), hepatocytes start to proliferate to recover the original number and get into Step III in which the TGFBR2 expression is reduced. When hepatocytes increase the number, which is adequate to normally respond to and treat metabolic demands of body (Step IV), the liver generates a termination signal(s) (TS), which makes hepatocytes to express TGFBR2 and activate stellate cells to express TGF-β. The establishment of TGF-β signaling leads hepatocytes to terminate proliferation and stellate cells to terminate expression of TGF-β, which brings these cells into the resting state (Step V). These hypothetical mechanisms could be applicable to interactions between *r*-hepatocytes and *m*-stellate cells due to phylogenetic closeness. Apparently, there are some failures in these mechanisms in the interactions between *h*-hepatocytes and *m*-stellate cells. One is that *m*-stellate cells are not able to proliferate or increase their number around *h*-hepatocytes (failure in Step III). Another is that hypothetical TS is not generated in Step IV and, thus, *h*-hepatocytes do not up-regulate TGFBR2 and *m*-stellate cells do not express TGF-β. Molecular entities of TS remain to be challenged for identifications.

INSENSITIVITY OF CHIMERIC MOUSE *H*-HEPATOCYTES TO MOUSE GH

Growth hormone (GH) is a key regulator of postnatal growth and metabolism [22] and exerts its effects by binding to its specific cell surface receptor, GH receptor (GHR), which triggers various intracellular signaling cascades [23]. GHR is a single transmembrane protein of class 1 cytokine receptor family [23, 24]. Exceptionally among peptide and protein hormones in mammals, although primate GH is effective to not only primates, but also to other mammals, non-primate mammalian GH is effective among non-primate mammals, but not to primates, which is called "species specificity" of GHR [25]. Arg^{43} in hGHR is responsible for the species specificity as GH/GHR binding, because replacement of Arg^{43} to Leu as in non-primate bovine GHR, for example bovine, greatly reduced its affinity to hGH [25].

Supporting the notion of GHR-species specificity, *h*-hepatocytes in *h*-chimeric mouse does not respond to mGH, because hIGF-1, the principal GH mediator [26], was undetectable even *h*-chimeric mice with RI >90%, which became detectable when the mice were treated with recombinant hGH [27]. GH was reported to stimulate liver regeneration induced by partial hepatectomy [28], which is especially noteworthy in relation to ageing. Hepatic regenerative capacity is generally decreased with ageing [29], which is associated with a progressive decline in GH secretion [30]. It was experimentally demonstrated that GH stimulated proliferation of old-aged regenerating liver through forkhead box m1b [31]. Involvement of GH in liver regeneration as a physiological pro-regeneration agent was also shown utilizing GH antagonist transgenic mice, in which the action of GH is blocked [32].

From these our and other researchers' studies we thought that *h*-chimeric mice are quite useful as an *in vivo* model to study effects of GH on human liver, because first the *h*-hepatocytes in the mice are in the hGH-deficient state and second GH shows physiological and pathological actions on liver including regeneration in an age-dependent manner. Meantime, we noticed the deposition of lipid droplets in *h*-hepatocytes of chimeric mice when the animals are reared for a relatively longer period of time (> ~70 days after transplantation). It is generally known that GH plays a major role in the regulation of lipid metabolism, and impairment in the GH axis elicits major changes in glucose and lipid metabolism [33].

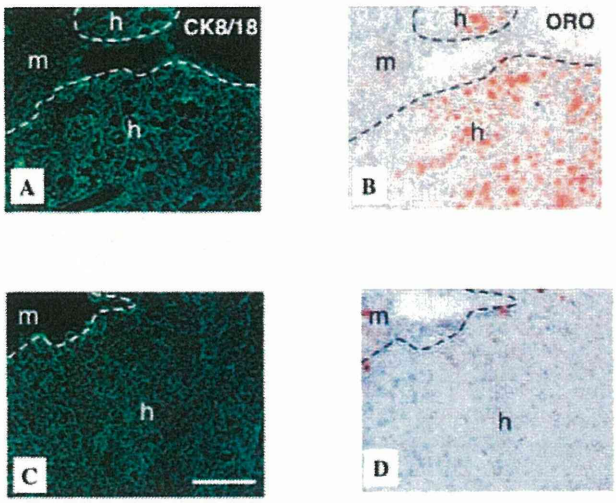

Fig. (12). Effects of hGH on liver steatosis. Two groups of chimeric mice with a 6-year-old girl's hepatocytes were used to test the effect of hGH on liver steatosis, one without treatment with hGH (A, B) and the other with the hormone treatment (C, D). The animals of the experimental group were given hGH in the last 2 wk before being killed 70–90 days after transplantation. Liver tissues were stained for hCK8/18 immunohistochemistry to identify *h*-hepatocytes (A, C). Primary antibodies were visualized with Alexa 594-conjugated anti-mouse IgG goat sera. Their serial sections were stained with Oil Red (ORD) (B, D). Small to large droplets are diffusely distributed in *h*-hepatocytes from control chimeric livers, but are absent in hGH-treated animals. m and h indicate regions of *m*- and *h*-hepatocytes, respectively. Dotted lines show the boundary between the two regions. Scale bar is shown in C and represents 100 μm. Photos are cited from the literature No. 10.

With these backgrounds we investigated hepatic lipogenesis in chimeric mouse liver in relation to GH-deficiency [10]. *h*-Chimeric mice transplanted with 6-year-old girl started to present vacuoles in the cytoplasm of donor hepatocytes around 70 days post-transplantation, which gradually increased in numbers and size thereafter (Fig. **11A** and **B**). These vacuoles were stainable with Oil Red O (ORO) (Fig. **11C**), thus, concluded to be lipid deposits. More than 90% of the tested chimeric mice whose RIs were >70% spontaneously became fatty 90 days post-transplantation. It was most plausible that the observed hepatic steatosis is induced by the lack of available hGH in the hosts and this possibility was soon verified by the following studies. Chimeric mice with RI >70% (hepatocytes from a 6-year-old girl) were infused with hGH at 2.5 mg/kg/day for the last 2 weeks of the transplantation experiments. As expected, GH administration raised the hIGF-1 serum concentration from undetectable levels to levels (~73 ng/ml) comparable to normal human serum levels and prominently abolished lipid granules (Fig. **12**). Thus, we conclude that first *h*-hepatocytes in *h*-chimeric mice are deprived of hGH, gradually accumulate lipid droplets, and finally become steatotic, and second hGH plays a principal role in the etiology of human liver steatosis [10].

Comparison of gene expression profiles between *h*-hepatocytes from hGH-untreated and -treated mice by microarray and real-time quantitative RT-PCR enabled us to identify the following 14 GH-up-regulated genes in the descending sequence from the highest ratio to the lowest, ASCL1 (achaete-scute complex-like 1), IGF-I, SOCS2 (suppressor of cytokine signaling 2), nicotinamide N-methyltransfease, KL (klotho), P4AH1 (procollagen-proline, 2-oxoglutarate 4-dioxygenase, a-polypeptide 1), DSCR1L1 (down syndrome critical region gene 1-like1), IGFLS (IGF-binding protein, acid labile subunit), CREM (camp-responsive element modulator), MBL2 (mannose-binding lectin 2, soluble), AMIGO2 (adhesion molecule with Ig-like domain 2), C50RF13 (chromosome 5 open reading frame 13), SLC16A1 (solute carrier family 16, member 1), and SRD5A1 (steroid-5-a-reductase, a-polypeptide 1), together with 4 GH-down-regulated genes such as FADS1 (fatty acid desaturase 1), PCOLCE22 (procollagen C-endopeptidase enhancer 2), AKR1B10 (aldo-keto reductase family 1, member B10), and SERPINE1 (serine proteinase inhibitor, clade E, member 1). Actually, when the expression levels of these genes were compared to those in normal human livers, most of the former and latter genes were down- and up-regulated in *h*-chimeric mouse livers, respectively, except that KL was not down-regulated in chimeric mice [10].

Among them, ASCL1, or also termed ASH1 (achaete-scute homolog 1), is a basic helix-loop-helix transcription factor, and might be worthy to be considered in relation to hepatic steatosis observed in *h*-chimeric mice, because Hes6 is included as the target genes of ASCL1 [34]. Recently, Talianidis and coworkes identified Hes6 as a novel HNF4a target and reported that these two transcription factors regulate the gene expressions responsible for hepatic fatty acid metabolism such as CPT1, Acot1, FGF21, HMGCS2, and CD36, coordinating with PPARα and γ [35].

There are studies that show reciprocal regulations of GH and SOCS-2 expressions. GH increases the expression of SOCS-2 in cultured rat hepatocytes [36] and in mouse liver *in vivo* [37], which supports our results. In contrast, SOCS-2 appears to negatively regulate the expression of GH, because deletion of SCCS-2 genes results in increase of GH signaling including lipogenesis [38, 39]. STAT5β is a transcription factor that associates with intracellular domain of the GHR. Upon GH stimulation, STAT5β is phosphorylated by Janus kinase (JAK), dimerizes, translocated to nucleus, and promotes the transcription of some GH-regulated genes [40]. SOCS2 is one of the STAT5β target genes and its promoter regions contain an E-box followed by STA5β binding sites, both of which are shown to be required for full GH responsiveness [40]. Activities of intracellular GH signaling through Stat5a and Stat5b seem to be high in GH-treated *h*-chimeric mouse hepatocytes, which might induce SOCS-2 gene expression and its gene products might contribute to homeostasis of GH signaling and lipid metabolism.

Regarding the upregulation of P4HA1 in h-GH treated *h*-hepatocytes, it is relevant to state three points: firstly proly 4-hydroxylase (P4H), a α2β2 heterodimer, plays a critical role(s) in biosynthesis and formation of all known types of collagen, the representative extracellular matrix necessary for growth and morphology of tissues, and the abundance of the α–subunit restricts the enzymatic activity [41]; second, its gene transcription is mediated through binding of upstream stimulatory factors with its E-box-containing pro-

moter regions [41], suggesting GH-signaling pathway could be involved in this regulation as in SOCS-2; third, however, biological significance of up-regulation of P4HA1 gene by h-GH remains unclear, because it is generally known that hepatocytes do not express collagen genes in both biological and pathological conditions [42]. The upregulation of P4Ha1 gene by hGH appears not to be related with lipolysis observed in hGH-treated chimeric livers.

At least two possibilities are considered for the role of DSCR1-L1 (or termed rcan2), which is known to inhibit calcineurin signaling. It was reported that cyclosporine A, an inhibitor of calcineulin, inhibits apolipoprotein A1 (apAI) gene expression [43]. Thus, DSCR1-L1 plays a role in regulating amounts of lipid through balancing high density lipoprotein (HDL) metabolism. Or this gene expression does not reflect the lipid metabolism, but reflect hyperplastic growth of *h*-hepatocytes in *h*-chimeric mouse, because a study showed that bax inhibitor-1 (BI-1) regulates liver regeneration through dephosphorylation and nuclear translocation of nuclear factor of activated T cells (NFATI) by calcineurin [44]. CREM is a transcription factor that binds to the cAMP response element (CRE) found in a number of gene promoters. To our knowledge, there have no reports whether lipid metabolism-associated genes are included in the target genes of CREM. Interestingly, CREM gene was reported as hGH-upregulated genes in which gene expression in peripheral blood mononuclear cells was compared between children with GH deficient (GHD) and those hGH-treated children with GHD [45].

Significance of upregulation of MBL2, a protein of the humoral innate immune system, is not clear in relation to GH signaling and lipid metabolism. However, MBL2 might play a role in activating lipid metabolisms, because MBL2 gene expression was reported to be significantly up regulated in *h*-hepatocytes by Wy14643, an agonist of peroxisome proliferator-activated receptor alpha (PPARα) which is known to be activated by endogenous agonists such as fatty acids and fatty acid derivatives [46]. C5ORF13 gene, also known as P113 gene, is a gene originally discovered as abundant transcript at site of active embryonic and postnatal nerurogenesis [47]. Recently, the possibility was proposed that C5ORF13 upregulates several classes of genes associated with lipid synthesis in a retinoic acid dependent manner [48]. It is plausible that the retinoic acid/C5ORF13 signaling functions in choresterol and lipid homeostasis in hGH-treated chimeric mouse hepatocytes.

SRD5A1 converts testosterone into 5-alpha-dihydrotestosterone, a potent androgen hormone, and progesterone or corticosterone into their corresponding 5-alpha-3-oxosteroids. This enzyme could be involved in hGH-dependent lipid metabolism, because it was reported that first GH up-regulated SRD5A1 and GHR in cultured haematopoietic cells [49], and second, a study with mice lacking hepatic androgen receptor (AR) suggested that AR may play a vital role in preventing the development of hepatic steatosis [50]. We currently consider that hGH-induced upregulation of SL16A1 gene is not related to lipid metabolism, but to hyperplastic growth of *h*-hepatocytes in *h*-chimeric liver, because it was reported that miR-124 whose target gene was identified as SL16A1 gene is down-regulated in medulloblastoma [51].

FADS1 encodes the membrane bound delta-6 desaturase, which is a rate-limiting enzyme for the formation of long-chain polyunsaturated fatty acids and plays a key role in determining plasma and tissue fatty acid profiles [52]. PCOLCE2, now known as PCPE2, is related to the lipid metabolism by its regulatory function on pro-apolipoprotein A-I (pro-apoA-I) processing by BMP-1 to apoA-1 that is exported to circulation and associated with HDL [53]. HDL binds cholesterols produced from peripheral tissue cells and transports them to hepatocytes, which remove cholesteros from HDL particles and eliminate them into bile, the process known as reverse cholesterol transport (RCT) responsible for cholesterol clearance [54]. ApoA-I is the principal protein present in the outer hydrophilic phase of the HDL particle whose inner hydrophobic phase contains phospholipids and cholesterol esters, and its structure modulates HDL function [55]. It is thought that in pro-apoAI and BMP-1 or BMP-1 and PCOLCE1/PCPE2 first are associated and then a ternary complex of pro-apoAI/BMP-1/PCPE2 is formed in the cytoplasm, in which PCPE2 might play a chaperon-like function or enhance BMP-1's processing of pro-apoAI [53]. Based upon a series of experiments to investigate the mechanism of interactions among PCPE2, apoA-I, and BMP2, Zhu et al. (2009) have proposed that although currently the functional significance of the apoA-1 processing is unclear, its prosegment might as an activator or repressor of the processing of apoA-I depending its concentration [53], by which we speculate, PCPE2 levels could regulate HDL-C–dependent lipid metabolisms.

AKR1B10, also designated aldose reuctase-like-1 (ARL-1), is known to participate in the pathway of lipid synthesis through stabilizing acetyl-CoA carboxylase as an rate-limiting enzyme of *de nove* synthesis of long chain fatty acids [56]. Serpine1, commonly called plasminogen activator inhibitor-1 (PAI-1), might be related to hepatic lipid metabolism, because hepatocytes are reported as PAI-secreting cells [57]. Its plasma levels are elevated in nonalcoholic steatohepatitis (NASH) patients [58]. The promoter region of its gene shows several response elements related with either metabolic or inflammatory pathways including very-low-density lipoprotein [59], and its gene is dramatically upregulated in steatohepatitis [60]. There is also a study that showed the correlation of liver fat content with serum level of serpine1 [61].

RESPONSES OF CHIMERIC MOUSE LIVER TO HUMAN HEPATITIS VIRUSES

Chimpanzees had been used for efficacy studies of antiviral drugs as HBV- and HCV-susceptible animals [62]. However, chimpanzees have been becoming less accessible for experimental uses because of expensiveness and animal welfare regulations. Since the advent of *h*-hepatocyte chimeric mouse [3, 4], researchers have recognized its usefulness as an appropriate animal model for study on HBV and HCV. Although studies on HBV/HCV using chimeric mice are still progressively increasing worldwide, in this review we just summarize major studies performed in our group or

in association with our group. These mice are susceptible to HBV and HCV and maintain viremia with high titers for long term [5]. They were successfully infected with HBV-containing serum or genetically engineered HBV, and their viremia levels were effectively reduced by administration of Lamivudin, which indicates that the chimeric mouse model is also usable for R&D of anti-HCV/HBV drugs [5]. Lamivudine-resistant mutated HBV were insensitive to Lamivudin compared to non-mutated HBV [63]. It has been assumed that the non-structural X protein of HBV, HBx, plays an important role in HBV replication, which was supported by a study using the chimeric mice demonstrating that HBx protein is indispensable for development of viremia in the chimeric mice [64]. A study with chimeric mice also contributed to elucidating poorly understood cytopathic effects of HBV and its association with particular viral genotypes or genetic mutations, which demonstrated that different HBV genotypes resulted in different virologic and histopathologic outcome of infection, and particular genetic variants of HBV may be directly cytopathic in immunosuppressive conditions [65].

There have been studies showing efficacy of candidates of anti-HCV drugs using HCV-infected chimeric mice: Myriocin (Serine palmitoyltransferase inhibitor) [66], 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (HSP90 inhibitor) [67], DEBIO-025 (cyclophilin Inhibitor) [68], NS3-4A and NS5B protease inhibitor [69, 70], and an inhibitor of 24-dehydrocholesterol reductase (DHCR24) [71]. These accumulating data all show that the chimeric mouse model of HBV or HCV infection is a quite useful tool for the study of HBV or HCV virology and evaluation of anti-viral drugs.

RESPONSES OF CHIMERIC MOUSE LIVER TO DRUGS

Compared to studies on HCV/HBV, chimeric mice have been less utilized for studies to predict human metabolisms of drugs, but their usefulness has been increasingly appreciated among researchers in pharmaceutical areas [7, 8]. In this review we briefly introduce recent progresses of studies of drug metabolisms using chimeric mice. It is generally known that cytochrome P450 enzymes (CYPs) play an important role in the metabolism of medicines and other xenobiotics [7, 8]. The presence of animal species differences in the metabolism of many drugs is the key factor in adopting chimeric mouse model for R&D of medical drugs. In the chimeric mouse liver, expressions of human enzymes that play critical roles in drug metabolisms have been reported including cytochrome P450 enzymes such as CYPs, CYP1A1, 1A2, 2A6, 3A4, 3A5, 2C9, 2C8, 2C19, and 2D6), phase II enzymes (UGT, SULT, CST, TPST, NAT, PEMT, and AMST), and also aldehyde oxigenase [6, 72-77]. Recently, it was reported that S-warfarin is metabolized to S-7-hydroxywarfarin, catalyzed by CYP2C9, and is primarily recovered in urine in humans [78]. Mass balance and metabolic disposition of S-warfarin in chimeric mice is similar to the reported human data [79, 80]. These currently available data, although still not so abundant, strongly suggest that chimeric mice are a useful and powerful model for the examination of absorption, distribution, metabolism, and excretion (ADME) and drug interactions via enzyme induction and inhibition *In vivo* [6-8, 74-79].

Regarding utilization of chimeric mouse in drug development, it is relevant here to refer to *h*-hepatocytes isolated from chimeric mouse livers. Cryopreserved *h*-hepatocytes are currently regarded as the best *in vitro* model for predicting human intrinsic clearance of xenobiotics in spite of the fact that fresh *h*-hepatocytes have greater plating efficiency on dishes and greater metabolic activities than cryopreserved cells, because experimentation for reproducible studies using fresh hepatocytes from the same donor is not practical and an "on demand" supply of fresh hepatocytes is not realistic. Recently, we sought for the possibility that the chimeric mouse could serve as a novel source of fresh *h*-hepatocytes for *in vitro* studies [81]. *h*-Hepatocytes were isolated from chimeric mice, and CYP activities were determined. Expressions of CYP1A2, 2C9, 2C19, 2D6, 2E1, 3A and glucuronidation of fresh hepatocytes derived from chimeric mice were equal or superior to cryopreserved cells. Thus, we propose that chimeric mice could be precious sources of fresh *h*-hepatocytes on demand that retain high and stable phase I enzymes and glucuronidation activities.

PERSPECTIVES

In the previous reviews we described a short research history on the advent of a *h*-chimeric mouse and its utilization as a convenient animal model that is small-sized, relatively easy in maintaining in laboratory, and easy in experimental handlings for drug testing and HBV/HCV-infection studies [7, 8]. As stated therein and in the present review, *h*-chimeric mice have been increasingly utilized among researchers as such models, because hitherto accumulated experiences with *h*-chimeric mice have showed that the liver tissues composed of human parenchymal cells and mouse nonparenchymal cells in mouse body appreciably represent the phenotypes of liver tissues of human body, although not perfectly, and are practically quite useful if we utilize the model recognizing its limitations.

One of the major limitations inherent to the current *h*-chimeric mouse model is the incomplete replacement of *m*-hepatocytes with *h*-hepatocytes. Although it is possible to generate chimeric mice with RI = ~100% ("complete" *h*-chimeric mouse) under appropriate conditions with adequate animal cares for the sake of a purely research purpose, chimeric mice with RI = 70 - 80% are practical for industrial drug R&D activities and sufficient for usual studies for HBV/HCV infection mechanisms. However, a problematic case was reported in which chimeric mice had been administered with a test drug that is well metabolized by *m*-hepatocytes, but to a limited extent by *h*-hepatocytes and its mouse metabolites include all its human metabolites [82]. In this case the serum metabolic profile was closely similar to that of the control mice, and no significant increase in the peaks of human metabolites were found. To avoid this type of problems coming from metabolic activities by the residual *m*-hepatocytes, we have to develop a technology by the residual host hepatocytes can be specifically eliminated from the currently available chimeric mice with with RI = 70 - 80%.

Unlike previous reviews, we emphasize herein the dissimilarity in biological features between artificial *h*-hepatocyte chimeric livers and natural human livers based upon the facts obtained in our recent studies that chimeric livers are under hyperplasia and under GH-deficiency, hoping to provide researches with our current opinions on *h*-chimeric mouse model. First, it should be noted that in spite of the presence of these dissimilarities *h*-chimeric mice have been shown to be practically quite useful at least for pharmaceutical researches and virological studies on HCB/HBV if researchers correctly understand merits and demerits in utilizing the animals in relation to the aims of their studies. Secondly, it is apparent that knowing the presence of dissimilarity will give us ideas and hints to generate a *h*-chimeric mouse whose liver shows biological features much closer to natural human livers than currently available *h*-chimeric mice. For example, livers of *h*-chimeric mice given appropriate amounts of hGH depending on the purposes of researches could reproduce the situations more resembling human livers and, thus, such a modified chimeric mouse will be an appropriate model for studying age-dependent changes in drug metabolisms and virological infections.

Although not referred to in this review, lack of human systems of inflammations and immunological recognitions in the current chimeric mice largely limit the usability of the *h*-chimeric mouse model in the study of pathological processes induced by drugs and viruses. Introduction of these systems into the *h*-chimeric mice will be a big and attractive challenge to generate a more useful humanized mouse liver model.

CONFLICT OF INTEREST

Declared none.

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Suppression of hepatic stellate cell activation by microRNA-29b

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ABSTRACT

MicroRNAs (miRNAs) participate in the regulation of cellular functions including proliferation, apoptosis, and migration. It has been previously shown that the miR-29 family is involved in regulating type I collagen expression by interacting with the 3′UTR of its mRNA. Here, we investigated the roles of miR-29b in the activation of mouse primary-cultured hepatic stellate cells (HSCs), a principal collagen-producing cell in the liver. Expression of miR-29b was found to be down-regulated during HSC activation in primary culture. Transfection of a miR-29b precursor markedly attenuated the expression of Col1a1 and Col1a2 mRNAs and additionally blunted the increased expression of α-SMA, DDR2, FN1, ITGB1, and PDGFR-β, which are key genes involved in the activation of HSCs. Further, overexpression of miR-29b led HSCs to remain in a quiescent state, as evidenced by their quiescent star-like cell morphology. Although phosphorylation of FAK, ERK, and Akt, and the mRNA expression of c-jun was unaffected, miR-29b overexpression suppressed the expression of c-fos mRNA. These results suggested that miR-29b is involved in the activation of HSCs and could be a candidate molecule for suppressing their activation and consequent liver fibrosis.

1. Introduction

Liver fibrosis is characterized by excessive accumulation of extracellular matrices (ECMs) and is a common feature of chronic liver injury. Hepatic stellate cells (HSCs) are considered to be the primary population that contributes to fibrogenic reactions by producing ECM in response to liver trauma. HSCs, which reside in the space of Disse outside the liver sinusoids, maintain a quiescent phenotype and store vitamin A under physiological conditions. When liver injury occurs, they become activated and trans-differentiate into myofibroblast-like cells, which are proliferative cells that lose their vitamin A droplets, express α-smooth muscle actin (α-SMA), and secrete profibrogenic mediators and ECM proteins [1,2]. Therefore, controlling the activation of the HSC population is considered a potential therapeutic target for liver fibrosis.

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs that work as post-transcriptional regulators of gene expression through their interaction with the 3′ untranslated region (3′UTR) of target mRNAs [3]. They participate in various biological phenomena, such as cell proliferation, development, differentiation, and metabolism [3]. Regarding HSCs, it was reported that miR-15b and miR-16 are down-regulated upon HSC activation and that their overexpression induces apoptosis and a delay in the cell cycle progression of HSCs [4,5]. Knockdown of miR-27a and miR-27b in activated HSCs reportedly allowed their reversion to a quiescent phenotype and decreased their rate of cell proliferation [6]. MiR-150 and miR-194 were reported to suppress proliferation, activation, and ECM production by HSCs [7]. We also reported the involvement of miR-195 in the proliferation of HSCs when treated with interferon [8].

Previously, we showed that miR-29b was induced by interferon treatment and that it suppressed type I collagen production in the human HSC line LX-2 [9]. Moreover, Roderburg et al. reported that miRNAs in the miR-29 family were significantly decreased in the

Abbreviations: BSA, bovine serum albumin; Col1a1, alpha 1 (I) collagen; Col1a2, alpha 2 (I) collagen; DDR, discoidin domain receptor; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; ITGB1, integrin β1; miRNA, microRNA; PBS, phosphate buffered saline; PDGFR-β, platelet-derived growth factor receptor-β; PI3K, phosphatidylinositol-3 kinase; SDS, sodium dodecyl sulfate; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; 3′UTR, 3′ untranslated region.

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