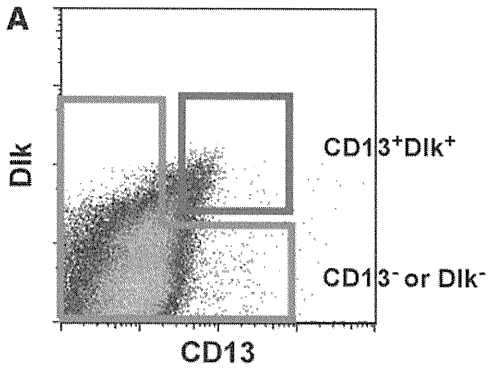
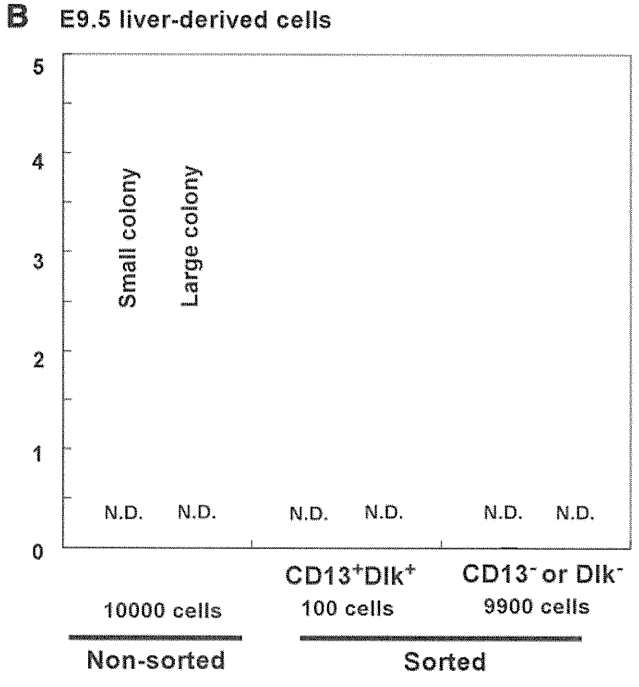
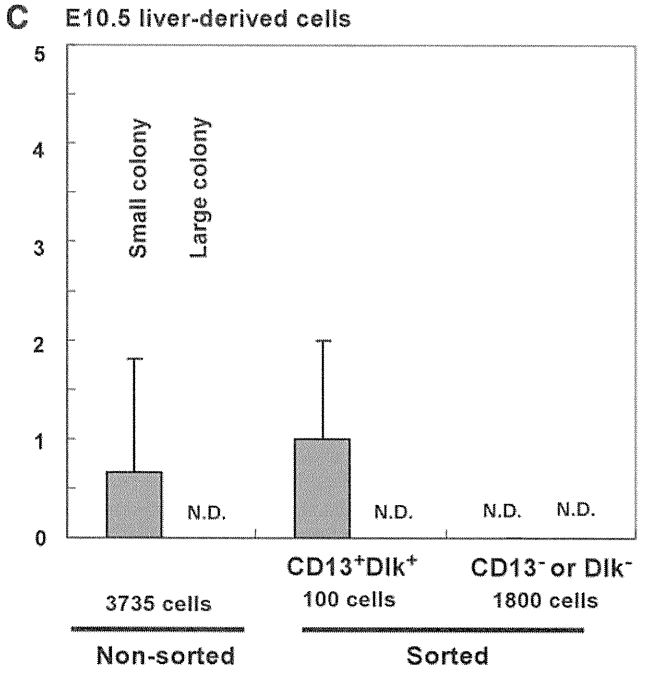
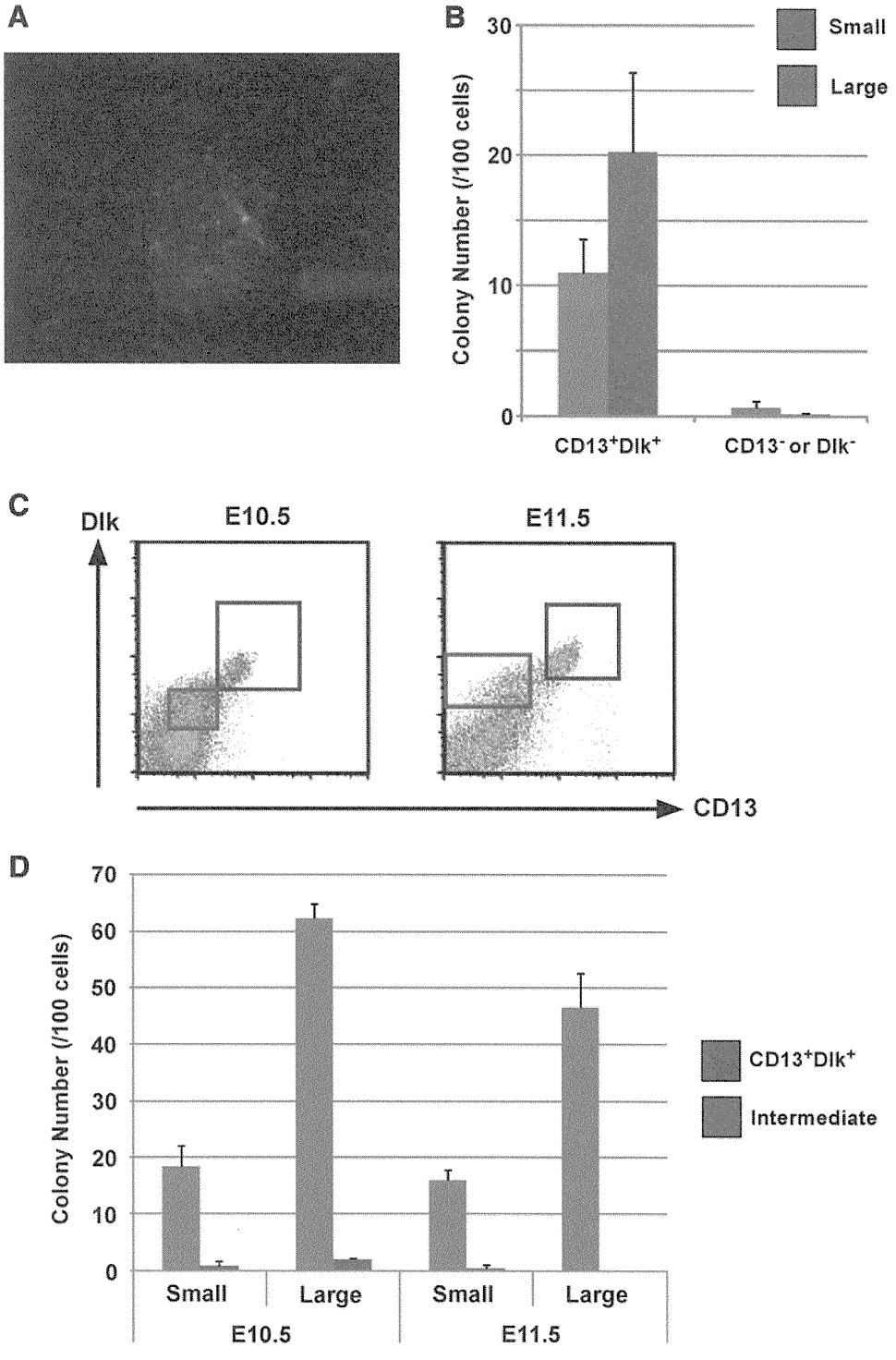






SUPPLEMENTARY FIG. 3. $CD45^-Ter119^-CD13^+Dlk^+$ cells derived from E9.5 and E10.5 livers could not form large colonies under conventional culture condition. **(A)** Flow-cytometric analysis of fetal liver cells in E9.5 mouse embryos. Sorting gates were set for the $CD13^+Dlk^+$ fraction (CD13, Dlk-double positive) and the $CD13^-$ or Dlk^- fraction (CD13, Dlk-not double positive) in nonhematopoietic cells. **(B, C)** E9.5 and E10.5 liver cells were directly inoculated into collagen-coated dishes. About 10,000 nonsorted cells, 100 CD13, Dlk-double-positive cells, and 9,900 CD13, Dlk-not double-positive cells were cultured for 6 days. Small colonies (*blue bars*) consisting of 50–100 cells and large colonies consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples). N.D., not detected.



SUPPLEMENTARY FIG. 4. Expansion of early fetal $CD13^+Dlk^+$ cells in our culture system. **(A, B)** Colony formation of early fetal $CD13^+Dlk^+$ cells derived from C3H mice. **(A)** A representative view of a colony formed from a single E10.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells. *Blue*, nuclei; *red*, albumin; *green*, CK19. **(B)** E10.5 fetal liver cells were sorted and cocultured with MEF for 6 days. Small colonies (*blue bars*) consisting of 50–100 cells and large colonies (*red bars*) consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples). **(C)** Cells stained with anti-mouse CD13 and Dlk antibodies were analyzed using flow cytometer. Blue gates, the $CD13^+Dlk^+$ cells; red gates, $CD13^{mid}Dlk^{mid}$ cells. **(D)** The intermediate fractions ($CD13^{mid}Dlk^{mid}$ cells) were cocultured with MEF. In contrast to $CD13^+Dlk^+$ cells (*blue bars*), $CD13^{mid}Dlk^{mid}$ cells (*red bars*) could barely form large (>100 cells) and small (50–100 cells) colonies. Results are represented as mean colony count ± SD (triplicate samples). MEF, mouse embryonic fibroblast.

SUPPLEMENTARY TABLE 1. PRIMERS FOR QUANTITATIVE RT-PCR

Albumin	Fw	agtgttgtgcagaggctgac
	Rv	ttctccttcacaccatcaagc
	Probe number	27
AFP	Fw	catgctgcaaagctgacaa
	Rv	ctttgcaatggatgctctctt
	Probe number	63
CK19	Fw	tgacctggagatgcagattg
	Rv	cctcagggcagtaatttcctc
	Probe number	17
c-Met	Fw	caccaccaagtcagatgtgtg
	Rv	aggggctcctctcgtcat
	Probe number	82
E-cadherin	Fw	atcctcgccctgctgatt
	Rv	accaccgttctcctccgta
	Probe number	18
Sox17	Fw	cacaacgcagagctaagcaa
	Rv	cgcttctctgccaaggtc
	Probe number	97

AFP, α-fetoprotein; CK19, cytokeratin 19; Fw, forward primer; Rv, reverse primer.

SUPPLEMENTARY TABLE 2. EXPRESSION PROFILES OF HEPATIC GENES IN CULTURED $CD13^+Dlk^+$ CELLS

Genes	*E10.5 $CD13^+Dlk^+$ cells (3 days cultured)*	*E13.5 $CD13^+Dlk^+$ cells (3 days cultured)*
Cyp1a1	−	+
Cyp1a2	−	+
Cyp3a11	+	+ +
Cyp3a13	+ +	+ +
Cyp7a1	+ +	+
G6Pase	+	+ +
TAT	+	+ +
Albumin	+ +	+ +
CK19	+	+ +

Cyp, cytochome P450; G6Pase, glucose-6-phosphatase; TAT, tyrosine aminotransferase; CK, cytokeratin.
+ +, high expression; +, low expression; −, no expression.

Mice with Artificial Human Liver

Chen A, Thomas D, Ong L, Schwartz R, Golub T, Bhatia S. Humanized mice with ectopic artificial liver tissues. Proc Natl Acad Sci U S A 2011;108:11842-11847. (Reprinted with permission.)

Abstract

"Humanized" mice offer a window into aspects of human physiology that are otherwise inaccessible. The best available methods for liver humanization rely on cell transplantation into immunodeficient mice with liver injury but these methods have not gained widespread use due to the duration and variability of hepatocyte repopulation. In light of the significant progress that has been achieved in clinical cell transplantation through tissue engineering, we sought to develop a humanized mouse model based on the facile and ectopic implantation of a tissue-engineered human liver. These human ectopic artificial livers (HEALs) stabilize the function of cryopreserved primary human hepatocytes through juxtacrine and paracrine signals in polymeric scaffolds. In contrast to current methods, HEALs can be efficiently established in immunocompetent mice with normal liver function. Mice transplanted with HEALs exhibit humanized liver functions persistent for weeks, including synthesis of human proteins, human drug metabolism, drug–drug interaction, and drug-induced liver injury. Here, mice with HEALs are used to predict the disproportionate metabolism and toxicity of "major" human metabolites using multiple routes of administration and monitoring. These advances may enable manufacturing of reproducible in vivo models for diverse drug development and research applications.

Comment

Analysis of a complex biological system often requires *in vivo* experimental systems, and numerous mouse models to study functions of genes, cells, and tissues *in vivo* have been developed by using genetic manipulations. However, the study of human biology *in vivo* is limited by ethical and technical constraints, and animal models that recapitulate human biological systems are needed. Various immunodeficient mouse strains have been developed to engraft human cells and tissues. The engraftment of human hematopoietic stem cells in immunodeficient mice has been used extensively and successfully to study the development and pathogenesis of blood cells.[1]

Hepatocytes express various enzymes and proteins involved in drug and xenobiotic metabolism and transport. Because of the difference in drug metabolism between human and experimental animals,[2,3] the generation of functional human hepatocytes has been a major focus for the application of hepatocytes to drug development.[4] However, because cultured hepatocytes usually retain very limited functions, attempts have been made to use chimeric mice with human hepatocytes.[5,6] When human hepatocytes are transplanted into immunodeficient mice in which hepatocytes have been genetically engineered to die under specific conditions, they gradually replace the endogenous mouse hepatocytes. After several weeks or months under specific conditions, as many as 90% of hepatocytes can be replaced with human hepatocytes. Such chimeric mice have been used to study human drug metabolism and infections by hepatitis viruses and parasites.[7-11] There are, however, some drawbacks, in that immunodeficient mice must be used, the preparation of chimeric mice requires weeks or months, and the engraftment is variable.

The article by Chen et al. describes the development of a novel humanized mouse model based on human ectopic artificial livers (HEALs). The investigators previously showed that the viability[12] and functions of primary rat hepatocytes *in vitro* are modulated by cell-cell interactions with fibroblasts in tunable, photopolymerizable polyethylene glycol (PEG) hydrogels.[13] They now show that the addition of the fibronectin-derived monomeric peptide, RGDS,[14] to the polymer scaffold improves the synthetic and secretory functions of encapsulated primary human hepatocytes with mouse fibroblasts (HEP/FIB). The encapsulated coculture systems (i.e., HEALs), prepared from fresh and cryopreserved hepatocytes, show comparable levels of function and viability. They examined whether nonparenchymal liver cells improve the functions of hepatocytes. Interestingly, coencapsulation of the human liver endothelial cell line, TMNK-1, improved rat hepatocellular functions, whereas the human hepatic stellate cell line, TWNT-1, had no effect (Fig. 1).

Gene-expression studies revealed enzymes involved in drug metabolism to be expressed at similar or higher levels in three-dimensional (3D) HEP/FIB cultures than 2D HEP/FIB control cultures. Importantly, cytochrome P450 (CYP)3A4, 1A2, 2D6, 2E1, and the 2C isoforms, which metabolize more than 90% of clinical drugs, were expressed in HEALs from two donors. In response to omeprazole or rifampin, HEALs (HEP/FIB+TMNK1) produced CYP1A2 or CYP3A4, respectively, which metabolized the respective substrates. HEALs also recapitulated clinically relevant drug-drug interactions involving CYP2A6- and CYP2D6-inhibiting compounds. These results indicate that HEALs express functional drug-metabolizing enzymes, suggesting that HEALs provide a potential means to screen inducers and inhibitors for CYPs.

The investigators then generated reporter HEALs by transducing lentiviruses expressing firefly luciferase under

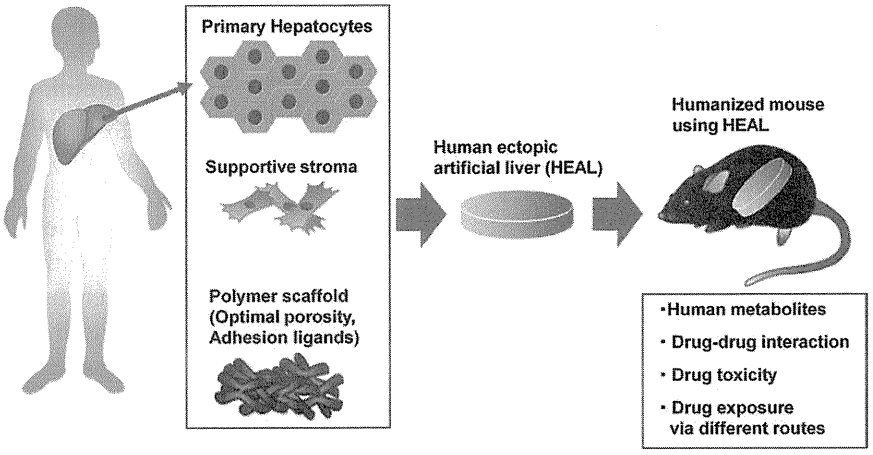


Fig. 1. Generation of humanized mice using HEAL. Primary hepatocytes are co-cultured with fibroblasts for 7-10 days and encapsulated with liver endothelial cells in scaffolds. HEALs are then transplanted into mice. Humanized mice using HELAs exhibit various human liver functions.

the control of the albumin promoter into HEP/FIB+TMNK1 cultures, and the encapsulated HEALs were implanted at an intraperitoneal (IP) site in athymic nude mice. Bioluminescence imaging showed that the IP site could support the HEALs. Albumin promoter activity was maintained for several weeks, and human albumin and alpha-1-antitrypsin were secreted in the mouse sera. HEALs were reproducibly engrafted (91.6% of 131 mice transplanted) and microcomputed tomography angiography of extracted HEALs confirmed host vessel recruitment to, around, and inside the implant. HEAL-humanized mice could be rapidly and reproducibly generated from fresh and also cryopreserved hepatocytes. Moreover, unlike humanized chimeric mouse models, HELAs were maintained in immunocompetent and non-liver-injury mice for up to 8 days.

HEAL-humanized mice expressed various human CYPs. Because major drug metabolites can be missed in standard animal models because of differences in drug-metabolism pathways among species, the investigators assessed whether the mice could be used for the identification of major metabolites. Debrisoquine (DB) is a CYP2D6 substrate converted to 4-hydroxydebrisoquined (4-OHDB) in humans, but not in mice.[2] In fact, HEAL-humanized mice metabolized DB to 4-OHDB. More important, CYP2D6 is responsible for the metabolism of 25% of known drugs and its highly polymorphic nature contributes to significant interindividual variability.[15] The investigators prepared HEALs from two donors with different levels of CYP2D6 and compared the ability of mice harboring the HEALs to metabolize DB. Mice with HEALs from the donor with lower levels of CYP2D6 metabolized DB less efficiently than those with HEALS from the donor with higher expression levels. Thus, HEAL-humanized mice can be used to detect breakdown products of drugs, which are missed in standard mouse models.

Drug-drug interactions are critical determinants of drug efficacy and safety because of the potential for drugs to alter the therapeutic or toxic effect of concomitantly administered compounds. Finally, the investigators explored the utility of the humanized mice for modeling toxic drug-drug interactions *in vivo*. Mice were first given rifampin (RIF) and then a therapeutic dose of acetoaminophen (APAP), a hepatotoxin at a high dose because of the CYP-mediated formation of *N*-acetyl-*p*-benzoquinone (NAPQI).[16] Although mice were resistant to RIF+APAP as a result of species-specific drug-drug interaction and HEAL-humanized mice given either RIF and APAP alone showed no sign of liver injury, HEAL-humanized mice given RIF+APAP showed evidence of human hepatocellular injury. Thus, HEAL-humanized mice can be used for screening hepatotoxic drug-drug combinations and doses *in vivo*.

The article describes basically two new additions to previous works.[17] First, the investigators have improved their tissue-engineering protocol (i.e., the addition of liver endothelial cells in addition to mouse fibroblasts in cocultures and the addition of the RGDS peptide to the PEG scaffold). These modifications significantly increased the metabolic and synthetic functions of hepatocytes. Second, they demonstrated that the implantation of HEALs in not only immunodeficient mice, but also in immunocompetent mice allows the expression of human liver functions rapidly and reproducibly, allowing them to mimic human drug metabolism and drug-drug interactions in mice. However, because HEAL-humanized mice have an intact mouse liver, drug metabolism can be affected by mouse enzymes, and the interpretation of results may be difficult in some cases. This is a common problem in various humanized models.

HEAL-humanized mice have several advantages over currently available chimeric mouse models. The

transplantation of human hepatocytes in mice, in which hepatocytes are conditionally injured, allows human hepatocytes to replace mouse hepatocytes, and the chimeric mice can be used to study drug metabolism and viral infections. However, this procedure requires immunodeficient mice and special conditions. Furthermore, the chimera vary, and it takes many weeks to prepare chimeric mice for testing human liver functions. By contrast, HEALs are relatively easy to prepare, and human liver functions can be assayed just days after implantation. Neither immunodeficient mice nor a special injury model is required.

Some questions remain unanswered. The investigators show that the addition of the human liver endothelial cell line, TMNK-1, to cocultures of human hepatocytes with mouse fibroblasts improved the expression of human functions, but the hepatic stellate cell (HSC) line, TWNK-1, had no effect. However, it is not clear from the article whether this effect is specific to liver sinusoidal endothelial cells (LSECs) or not. It would be interesting to test the effect of more liver cell lines or fresh nonparenchymal liver cells. In contrast to chimeric mice that carry only human hepatocytes, HEALs can be added by other human cells, such as LSECs or HSCs, and their contribution to liver functions may be assessed. A combination of different liver cells may improve their functions. Also, it is not stated in the article whether the implantation of HEALs in mice changes their functions. Because HEALs in mice are well vascularized, a rich blood supply may further improve the functions of HEALs *in vivo*. It is also worth testing whether HBV or HCV can replicate in these mice. Furthermore, if HEAL-humanized mice are prepared using immunodeficient mice in which human hematopoietic stem cells have been engrafted, the interaction between human liver cells and the immune system can be assessed in mice. Thus, HEAL-humanized mice provide a novel system to study human liver functions and physiology in mice.

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