Humanized mice with ectopic artificial liver tissues

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Humanized mice with ectopic artificial liver tissues

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“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.

Preclinical models provide a critical window into human physiology and are important for determining drug safety and efficacy. Bioengineering advances have produced in vivo platforms with improved disease modeling (1, 2), absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) (3, 4), and circulation-mimicking capabilities (4). “Human-on-a-chip” systems, however, show limited recapitulation of pharmacokinetics and cannot support clinically relevant administration routes. Elsewhere, humanized mice, based on genetic manipulation or transplantation of human cells, have also been highly enabling (5). Mice with chimeric livers show encouraging drug responsiveness (6–9) and pathogen susceptibility (10–12). However, these models are confined to liver-injury, immunodeficient recipients and depend on the coordinated injection of high-quality human hepatocytes, which must home to the liver from the injection site, engraft, and expand over weeks to months within the injured host (13, 14). Given the limitations of current preclinical models, human metabolites and their downstream effects often go undetected until clinical trials, the most costly and dangerous phase of drug development (15).

Here we develop an improved humanized mouse model by implantation of tissue-engineered human ectopic artificial livers (HEALs). In the field of tissue engineering, polymeric scaffolds are widely used for cell and drug delivery in vivo (16, 17). Primary hepatocytes, however, are challenging to maintain and implant via biomaterials due to their unstable phenotype, variable engraftment efficiencies, and high metabolic needs (18). Use of partial hepatectomy with portal-caval shunt has been shown to improve hepatocyte engraftment and function in allogenic rat models (19, 20), suggesting that transplanted hepatocytes depend on signals (hepatotropic factors) circulating from the portal vein. Despite these findings, support of implanted hepatocytes by sequential delivery of seeded cells, growth factors, hormones, and/or proangiogenic molecules has had limited success (21–24), and recent evidence showing promise for the survival of hepatocytes transplanted in the ectopic lymph node remains dependent on mouse liver injury (25). We hypothesized that an integrated system, based on hepatocyte encapsulation and the incorporation of juxtacrine and paracrine signals to stabilize the cell phenotype before, rather than during/after transplantation, could mitigate the requirement for portal venous flow or liver injury in vivo. To pursue our hypothesis, we engineered HEALs, functionalized polymer scaffolds that encapsulate primary human hepatocytes within a supportive microenvironment, and tested their ability to become integrated with recipient mouse circulation and perform human hepatic functions (Fig. S1).

Results and Discussion

Engineering HEALs. We previously reported that the viability and functions of primary rat hepatocytes are critically modulated by cell–cell interactions with nonparenchymal fibroblasts within 3D biomaterials (26). Therefore, to engineer HEALs, we sought to determine whether these findings could be extended to primary human hepatocyte encapsulation. Cocultivation of primary human hepatocytes with 32-3T3 mouse fibroblasts (HEP/FIB) for 1 wk, followed by encapsulation in PEG diacrylate (PEG-DA) polymer scaffolds (∼0.5 × 10⁶ total encapsulated hepatocytes), led to sustained albumin secretion and urea synthesis, whereas hepatocytes encapsulated alone declined over 2–4 d of culture (Fig. 1 B and C). Next, to study the impact of cell-matrix interactions on encapsulated primary human hepatocytes, we used the tunability of the PEG scaffold chemistry to incorporate the functional fibronectin-derived monomeric peptide sequence “RGDS” or negative control peptide “RGES” into the scaffold (Fig. L4). The presence of RGDS improved the synthetic and secretory functions of the encapsulated HEP/FIB by two- to threefold, as compared to RGES controls (Fig. 1 B and C). These data, taken with studies from our group, which profiled the integron expression of primary hepatocytes in vitro (27), suggest a dependence on juxtacrine cell interactions in 3D and responsiveness to the RGDS ligand via the hepatocyte cell surface integrin, αvβ3.

Notably, in exploring alternative primary cell sources for HEAL fabrication, we compared fresh and cryopreserved hepatocyte HEP/FIB and found that they showed comparable levels of func-


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tional markers and viability staining after coculture and encapsulation (Fig. S2). Due to their availability and potential for improved reproducibility, subsequent HEALs were built from cryopreserved primary hepatocytes. Human liver-derived nonparenchymal cells provide soluble signals important for hepatocyte development and maintenance (28). To investigate the effects of paracrine signaling on HEAL stability, we mixed human liver nonparenchymal cell lines with HEP/FIB clusters prior to encapsulation, entrapping HEP/FIB near, but not clustered with, the nonparenchymal cells (Fig. 1D). Coencapsulation of the human liver endothelial cell (LEC) line TMNK-1 (28, 29) was the most beneficial to rat hepatocellular function (Fig. 1 E and F), whereas additional mouse FIB were moderately and transiently supportive, and the human hepatic stellate cell (HSC) line TWNT-1 (30) had no effect (Fig. S3). The stabilizing influence of coembedded LECs was conserved between human and rat HEP/FIB cocultures, required initial cocultivation of HEP with FIB, and could not be reproduced with conditioned medium from cultured LECs (Fig. S4). Collectively, these data suggest that FIB provide critical spatiotemporal adhesive cues to help stabilize primary hepatocytes after isolation (31), whereas coembedded LEC may improve encapsulated cell function through secretion of short-range or rapidly turned over soluble factors. Ultimately, the optimization of cellular and chemical factors to provide juxtacrine and paracrine signaling within HEALs resulted in over 3 wk of human hepatic tissue function in vitro.

Characterization of HEALs for Drug Metabolism Gene Expression and Functions. In order to assess the utility of tissue-engineered hepatocyte cultures for drug metabolism studies, we characterized HEALs for the expression and function of human drug-metabolizing enzymes, comparing 3D-encapsulated HEP/FIB HEALs to same-donor 2D HEP/FIB cultures on day 10 of culture. The 2D condition acts as reference for a stable hepatocyte coculture model, previously shown to express a number of genes pertinent to ADME/Tox in vitro (32). However, to date, assessment of hepatocyte models has hinged on the measurement of only a small handful of drug-metabolizing enzymes. Noting that drug-metabolizing enzymes are regulated primarily at the level of transcription, we hypothesized that we could comprehensively assess drug-metabolizing enzyme expression levels in a low-cost, high-throughput assay based on the multiplexed ligation mediated amplification (LMA) of transcripts coupled to detection on Luminex beads (33). Accordingly, we designed probes for 83 human drug metabolism-encoding transcripts including phase I detoxification enzymes, phase II conjugating enzymes, phase III transporters, several key transcription factors, and albumin (Fig. 2A and Dataset S1). Comparing the relative gene expression between 2D and 3D cultures for two donors in independent experiments, we found that on average 7/7 nuclear receptors, 34/36 phase I [including cytochrome P450 superfamily enzymes (CYP450s)], 11/22 phase II, and 16/17 phase III genes showed similar or higher levels of expression in 3D HEP/FIB HEALs, compared to the 2D HEP/FIB control (Fig. 2B). Importantly, CYP3A4, 1A2, 2D6, 2E1, and the 2C isoforms, which collectively metabolize >90% clinical drugs, were expressed in HEALs established from both donors. These results indicate that relevant human enzymes are expressed in 2D and 3D hepatocyte cocultures and lay groundwork for future comprehensive and patient-specific profiling of drug-metabolizing enzyme expression and induction. To functionally validate the gene expression studies and to test the utility of HEALs for predicting clinical drug metabolism and interactions, we focused on one donor cell type and treated optimized (HEP/FIB + LEC) HEALs with omeprazole (OME) or rifampin (RIF), inducers of CYP1A2 and CYP3A4, respectively, and assessed CYP450 enzymatic activity upon exposure to known substrates (Fig. 2C). Compared to the vehicle control, OME induced CYP1A2-mediated metabolism of ethoxyresorufin (ER) by 11.5 ± 2.0-fold, but had only a minimal effect on the CYP3A4-mediated metabolism of testosterone (TEST). Conversely, RIF induced the metabolism of TEST by 13.7 ± 2.9-fold, but had no effect on the metabolism of ER. HEALs also recapitulated...
clinically relevant drug–drug interactions involving CYP2A6- and CYP2D6-inhibiting compounds (Fig. S5). Thus, HEALs express functional human drug-metabolizing enzymes and show potential for screening compounds for CYP450 inducer, inhibitor, or effector responses.

**Humanized Mice via Implantation of HEALs.** We next sought to test whether stabilized HEALs could be implanted and functionally maintained in mice. We encapsulated HEP/FIB + LEC expressing luciferase under the human albumin promoter (Fig. S6) and transplanted the resulting reporter HEALs in the subcutaneous or intraperitoneal site of athymic nude mice (Fig. 3A). Whole-animal bioluminescence imaging and quantification of luciferase production indicated that the intraperitoneal site could support hepatic survival and albumin promoter activity for several weeks (Fig. 3A and B), and up to 3 mo in one cohort of five mice (Fig. 3A). Serum analysis from HEAL-humanized mice confirmed the production and secretion of human proteins, albumin, and alpha-1-antitrypsin (Fig. 3C and Fig. S7). Based on qualitative inspection of vessels supplying the implants at day 6 or greater, engraftment of HEALs in vivo was highly efficient (91.6% of n = 131 mice transplanted). Vascular casting at day 35, followed by microcomputed tomography (micro-CT) angiography of extracted HEALs, confirmed host vessel recruitment to, around, and penetrating the implant (Fig. 3D–F and Movie S1). Together, these results demonstrate that stabilizing hepatocytes prior to implantation protects cells from death due to anoikis, loss of cellular signaling, and/or compromised oxygen transport during engraftment, and also decreases dependence on hepatotrophic factors from the portal vein. These advances enable rapid (<1 wk) and reproducible generation of HEAL-humanized mice using cryopreserved hepatocytes from different primary donors (Fig. 4A and B and Fig. S8). In contrast, humanizing mice by current cell transplantation methods requires 2–6 mo and results in unpredictable and highly variable repopulation yields (ranging from single-digit to nearly complete percent humanization). Furthermore, current humanized mice are limited to liver-injury strains, whereas humanization with HEALs could be achieved in multiple strains (Swiss–Webster, C57/BL6) of immunocompetent, non-liver-injury mice for up to 8 d (Fig. 4C).

**Prediction of Human Metabolites.** The drug-metabolism profile of HEALs and the relatively facile generation of humanized mice through their implantation suggested the potential for in vivo preclinical studies. Major metabolites can pass undetected in standard animal models due to differences in drug metabolism pathways among species. Upon discovery in man, major metabolites require new preclinical phase evaluation and contribute to an alarming rate of prelaunch failures (15). To assess whether mice with HEALs could be used for early identification of major metabolites, we challenged wild-type and humanized athymic nude or immunocompetent C57/BL6 mice with drugs known to be metabolized differently by mice and humans. Coumarin, a CYP2A6 substrate, is primarily metabolized by humans to 7-hydroxycoumarin (7-HC), but preferentially metabolized in mice to coumarin-3,4-exopoxide by cyp1a1/2 and 2e1 isoforms (34). Debl-
risoquine, a CYP2D6 probe, is metabolized to 4-hydroxyrisoquine (4-OHDDB) in humans and is not metabolized in mice (35). Importantly, human CYP2D6 is responsible for metabolism of 25% of known drugs and, due to its high number of polymorphisms, contributes to pronounced interindividual variability (up to 30–40-fold metabolic differences) (36). Indeed, debrisoquine hydroxylation genotyping and phenotyping can be used to classify patients as poor, intermediate, extensive, or ultrarapid metabolizers (36). We assessed CYP2D6 mRNA expression levels and debrisoquine hydroxylation activity of two human hepatocyte donors in vitro (Figs. 2A and 4B) and implanted HEALs derived from the poorer metabolizer into C57/BL6 mice. Following coumarin or debrisoquine exposure, serum and urine were analyzed by liquid chromatography (LC)/MS/MS to quantify parent drug and metabolite concentrations over time. Based on pharmacokinetic profiles, HEAL-humanized mice metabolized the parent compounds more efficiently than wild-type mice, as shown by higher maximum metabolic ratio (metabolite AUC over parent AUC) (15) (Fig. 4A) and implanted HEALs derived from the poorer metabolizer into C57/BL6 mice. Following coumarin or debrisoquine exposure, serum and urine were analyzed by liquid chromatography (LC)/MS/MS to quantify parent drug and metabolite concentrations over time. Based on pharmacokinetic profiles, HEAL-humanized mice metabolized the parent compounds more efficiently than wild-type mice, as shown by higher maximum metabolic ratio (metabolite AUC over parent AUC) (15) (Fig. 4A).

**Prediction of Drug-drug Interactions.** We next characterized the ability of HEAL-humanized mouse to probe clinical drug–drug interactions. Drug–drug interactions are critical determinants of drug efficacy and safety due to the potential for CYP450-inducing or CYP450-inhibiting drugs to alter the therapeutic or toxic effect of concomitantly administered compounds. We systematically exposed HEAL-implanted animals to the CYP3A4 inducer, RIF, and assessed the downstream effects by analyzing explanted HEALs. In response to intraperitoneal RIF, the enzymatic activity associated with explanted HEALs exposed to a second drug—CYP3A4 substrate 7-benzyloxy-4-trifluoromethylcoumarin (BFC) or TEST—was greater than fivefold induced as compared to DMSO-treated HEALs (Fig. 4H and Fig. S10). CYP1A2 activity, as probed by metabolism of the drug ER, was not altered by RIF, confirming the specificity of the inducer (Fig. 4H). These results indicate that HEALs can recapitulate the interaction of drug pairs and highlight the utility of HEAL explant following mouse exposure as a facile assay for drug–drug interactions.

To explore the utility of humanized mice for modeling toxic drug–drug interactions in vivo, we dosed RIF-induced or noninduced (DMSO-treated) mice with therapeutic levels of acetaminophen (APAP) and monitored human hepatotoxicity by assay of the mouse serum. APAP is a common analgesic that is severely hepatotoxic at high doses due to the CYP450-mediated formation of the reactive metabolite N-acetyl-p-benzoquinone (NAPQI); it is innocuous at therapeutic doses due to detoxification of NAPQI by cellular glutathione (39). Four hours after oral administration of APAP, mice that had been preinduced with RIF–AP or RIF alone exhibited similar levels of NAPQI to untreated controls (Fig. 4F and Fig. S11). Mouse livers exposed to RIF, APAP, or RIF + APAP appeared uninjured based on serum liver enzyme tests and histopathological analysis (Fig. S12), consistent with recent findings on the species specificity of this drug interaction (40). These results indicate that HEAL-humanized mice can be useful for screening hepatotoxic drug–drug combinations and doses by multiple administration routes in vivo. By leveraging tissue-engineering strategies to stabilize the functions of primary human hepatocytes within a biomaterial scaffold, we have established a unique humanized mouse model and demonstrated its utility for predicting human drug responses, pharmacokinetics upon multiple routes of administration, and metabolite formation in vivo. Unlike current transgenic and transplantation approaches, engineered HEAL-humanized mice...
Materials and Methods

Process by which new therapeutics reach patients. The potential to reduce clinical trial attrition rates and accelerate the integration of this model into drug development pipelines has the potential to reduce clinical trial attrition rates and accelerate the integration of this model into drug development pipelines.

HEAL-implanted mice may prove useful for the study of immune-dominant toxicity, idiosyncratic toxicity, and gut-liver interactions. HEALs can be rapidly generated (<2 wk), reproducibly, at high yields, and in the context of immunocompetent, non-liver-injury murine backgrounds. These advantages point to applications beyond drug safety, including investigations of human liver disease and hepatotropic infections, and potential integration with studies of normal mouse immunity or humanized immunity. We demonstrate the feasibility of implanting HEALs in immunocompetent mice for drug metabolism studies, and consider that the encapsulating polymer scaffold unique to this method of humanization may serve as not only a supportive microenvironment for hepatocytes but a drug delivery vehicle and potential rejection-delaying barrier.

Donor A

Recipient

Fig. 4. Humanized mice using different donors or recipients. Drug dosing via multiple routes of administration and prediction of major human metabolites, drug-drug interactions, and toxicity. (A) Representative micrographs of cryopreserved primary hepatocytes from donors A and B, cocultivated with stromal fibroblasts for 7 d prior to 3D encapsulation. (Scale bar: 75 μm.) (B) Characterization of donors A- or B-derived HEALs for CYP2D6 activity by exposure to CYP2D6 substrate debrisoquine and quantification of debrisoquine hydroxylation. (C) In vivo bioluminescence imaging and quantification of reporter albumin-firefly luciferase HEALs from donor A, implanted in the intraperitoneal cavity of athymic nude, immunocompetent C57/B6, or Swiss–Webster mice. Representative images 3 d after implantation are shown. (D) Pharmacokinetic analysis of serum metabolite 7-HC formation in humanized nude mice exposed to coumarin via i.p. injection (Upper), and serum metabolite 4-OHDB formation in humanized C57/B6 mice exposed to debrisoquine via oral gavage (Lower). (E) Detection of urinary 7-HC (Upper) or 4-OHDB (Lower) metabolite excretion over 4 h in vivo. (F) Identification of major human metabolites based on the metabolic ratio (MR; parent AUC over metabolite AUC). Red dashed line represents the lower threshold for classification as a major metabolite (0.1 MR). (G) Timeline of drug–drug interaction (DDI) study. Humanized mice (n = 6 per group) were administered RIF (25 mg/kg) daily for 3 d before (H) extraction of HEAL and incubation with CYP1A2 substrate ER or CYP3A4 substrate TEST ex vivo, or (I) in vivo oral exposure to APAP and serum assessment of human albumin production. Fold-induction of CYP450 activity was determined by normalization to DMSO. *p < 0.05, **p < 0.01, ***p < 0.001 for n as indicated and SEM. Error bars are SEM for n = 3 or greater.

DNA Isolation and LMA-Luminex Analysis. Total RNA from three HEALs per donor was purified using Trizol (Invitrogen) and Mini-RNeasy kit (Qiagen) and pooled for analysis. LMA-Luminex procedures and probes are described in SI Materials and Methods. Data for replicate loadings, expressed in mean fluorescent intensity of at least 100 beads per sample, were scaled to the human transferrin gene and row-normalized for heat map representation using Gene Pattern open software (Broad Institute).

Implantation and Assessment of HEALs. HEALs were placed in the subcutaneous or periportal cavity of anesthetized nude mice (Taconic) or Webster mice. Representative images of the subcutaneous or periportal cavity of anesthetized nude mice (Taconic) or Webster mice.
Mice were administered 250 μL of 15 mg/mL D-Luciferin/PBS solution (Caliper Life Sciences) by intraperitoneal injection and imaged using the IVIS Spectrum (Xenogen) system and Living Image software (Caliper Life Sciences). The Committee for Animal Care in the Department of Comparative Medicine at Massachusetts Institute of Technology approved all animal procedures.

Vascular Perfusion and Micro-CT Imaging. Mice under isoflurane anesthesia were treated with intracardiac perfusions of 10 units/mL of heparin, 4 mg/L papaverin, and 1 g/L adenosine vasodilator agents, 2% (vol/vol) paraformaldehyde in PBS fixative, and Microsil contrast reagent (yellow; FlowTech). Micro-CT scans were obtained using the explore Locus MicroCT platform (GE Healthcare; 0.021-mm voxel size, 400 views, 2,000-ms exposure, 80-kV photon energy, and 450-μM current) and analyzed using GE Microview and Qirtix rendering (Pixmeo Sarl) software.

Biochemical Assays. Human albumin or human alpha-1-antitrypsin was quantified by enzyme-linked immunosorbent assay using goat antihuman albumin or alpha-1-antitrypsin antibodies (Bethyl Labs). Urea was measured by acid- and heat-catalyzed detection of diacetylmonoxime conversion to a colormetric product (StanBio Labs). Alanine aminotransferase and aspartate aminotransferase levels were detected with clinical kits (Teco Diagnostics).

Cytocrome-P450 Studies. All chemicals were purchased from Sigma. For induction studies, in vitro HEALs were treated daily for 3 d with CYP450 inducers 20 μM Rif or 50 μM OME in media (stocks <0.1% DMSO). Mice were injected intraperitoneally for daily 3 d with 3 mg/kg Rif saline solution, or oral-gavaged daily for 3 d with 10 mg/kg OME saline water. For inhibition study, developing embryos were treated with b-estradiol (0–0.4 μM), or quinidine (0–1 μM) were incubated with HEALs in vitro at indicated concentrations for 1 h prior to addition of CYP450 substrates: BFC (50 μM), ER (5 μM) with 10 μM dicumarol, TEST (200 μM), coumarin (100 μM), or debrisoquin (DB, 100 μM), for 2 h at 37 °C with 5% CO2. Glucuronidase/sulfatase-mediated phase II metabolites from coumarin or debrisoquin reactions were hydrolyzed by incubating with b-glucuronidase/arylsulfatase (Roche) for 2 h at 37 °C. The metabolites of BFC, ER, and coumarin were quantified at wavelengths 410/510, 530/590, 355/560 ex/em, respectively. Substrates and metabolites of TEST, coumarin, and DB were quantified by LC/MS/MS (Aperlica).

Pharmacokinetic Analysis. Mice administered 80 mg/kg of coumarin injected intraperitoneally or 2 mg/kg DB oral-gavaged were subjected to retroorbital droplet collection, 10, 15, 30, 60, 90, and 120 min, and terminal urine collection at 8 h. Glucuronidase/sulfatase-mediated phase II metabolites were hydrolyzed with b-glucuronidase/arylsulfatase (Roche) for 2 h at 37 °C. Metabolites 7-HC and 4-OHD were quantified using LC/MS/MS (Aperlica). The area under the curve from time 0 until the last measurable plasma concentration (AUC0–t) was calculated using the linear trapezoidal rule. Peak concentration (Cmax) and time to reach maximum concentration (tmax) were obtained from the plasma concentration-time profile. Metabolic ratios were determined by dividing AUC0–t of metabolite by AUC0–t of the parent drug.

Statistical analysis. Experiments were independently repeated 2–3 times with replicate samples as indicated in figure captions. Statistical analysis was performed using one-way ANOVA and Tukey’s post hoc test for group comparisons. Errors bars represent SEM.

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