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Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling

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Human embryonic stem cells (hESCs) are a valuable source of pluripotent primary cells. To date, however, their homogeneous cellular differentiation to specific cell types in vitro has proven difficult. Wnt signaling has been shown to play important roles in coordinating development, and we demonstrate that Wnt3a is differentially expressed at critical stages of human liver development in vivo. The essential role of Wnt3a in hepatic cyte differentiation from hESCs is paralleled by our in vitro model, demonstrating the importance of a physiologic approach to cellular differentiation. Our studies provide compelling evidence that Wnt3a signaling is important for coordinated hepatocellular function in vitro and in vivo. In addition, we demonstrate that Wnt3a facilitates clonal plating of hESCs exhibiting functional hepatic differentiation. These studies represent an important step toward the use of hESC-derived hepatocytes in high-throughput metabolic analysis of human liver function.

Hepatic endoderm | function | hepatocyte | drug metabolism | high throughput

Results

Wnt3a Plays an Important Role in Developing Human Liver. During development the liver is formed from DE. The coordination of anterior region of the PS to mesoderm or endoderm is likely to depend on the duration and magnitude of Nodal signaling (9, 10). Following its commitment the definitive endoderm (DE) lines the ventral region of the developing embryo and is patterned by adjacent mesenchyme. Liver formation depends on 2 mesenchymal structures, the cardiac mesoderm and septum transversum, which provide convergent instructive signals [reviewed by Zaret (11)].

Additional Wnt3a promotes hepatocyte-like cell (HLC) function and facilitates clonal plating of hESCs capable of hepatic differentiation. These results represent an important step toward the use of hESC-derived HLCs in biomedical applications and high-throughput drug toxicity studies.


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cell signaling during this process is critical for normal liver development. Wnt signaling plays an essential role in embryonic development and cellular differentiation, and we were interested in studying the instructive role played by Wnt during human liver development. Wnt3a expression was examined in first (T1) and second (T2) trimester human liver sections by immunostaining. Wnt3a expression was maximal at day 1 in AW cells (Fig. 2A, AW1–3) than in AB-treated cells (Fig. S2B, AB1–3). The decrease in Brachyury and faster induction of SOX17 and HNF3β levels was induced earlier in AW-treated cells (Fig. S2B, AW1–3) than in AB-treated cells (Fig. S2B, AB1–3). Similarly, WNT11 was induced in both AW and AB cultures over the 3-day time course (Fig. 2C, AW1–3 and AB1–3), consistent with its role in regulating gastrulation. Of note, the high levels of WNT11 expression coincided with the expression of endodermal markers SOX17, HNF3β, NODAL, and CRIPTO in both models.

**AW-Driven hESC Differentiation Induces Rapid and Homogeneous Hepatic Endoderm Differentiation.** In addition to a role for Wnt3a in facilitating endoderm differentiation, we also studied the capability of those endodermal cell populations to form hepatic
endoderm. Cells treated with AW or AB were transferred to other types of media known to specify hepatic endoderm (23). Cells primed with AW exhibited hepatocyte morphology by day 10 (Fig. 3A, AW H1 and AW H9), in contrast with cells differentiated with AB, which required 14 days’ differentiation before they developed characteristic hepatic morphology (Fig. 3A, AB H1 and AB H9). In addition to the relative delay in hepatocyte appearance, we also detected many areas of overgrowth in AB cultures (Fig. 3A, AB H1 and AB H9). The number of hESC-derived hepatocytes in culture was assessed morphologically, by their polygonal shape and prominent nucleoli, and confirmed by albumin staining. The differentiation of H1 hESCs using AW yielded 90% ± 3% albumin-positive hepatocytes (Fig. 3B, AW H1) on day 17, compared with 72% ± 8% of albumin-positive cells seen in response to AB (Fig. 3B, AB H1) on day 17. We also tested an independent hESC line, H9, and demonstrated that AW and AB treatment elicited similar amounts of human hepatocyte differentiation (Fig. 3B, AW H9 and AB H9). Specificity of immunostaining was confirmed using an IgG-negative control (Fig. 3B, IgG). Cells were maintained in culture to day 18, and their gene expression was analyzed by RT-PCR (Fig. 3C). Our results show that H1 or H9 cells differentiated by either method expressed CYP7A1, seen in liver but not yolk sac, and a repertoire of hepatocyte markers: ALB, AFP, HNF4α, TAT, TO, APOF, and CYP3A4.

AW-Driven hESC Differentiation Produces HLCs That Express a Wide Variety of Hepatocyte-Specific Functions. In addition to marker expression, we studied hESC-derived hepatocyte functionality. hESC-derived AW and AB HLCs (AWHLCs and ABHLCs, respectively) were matured using either L-15 (26) or Eagle’s Minimal Essential Medium (MEME) in the presence or absence of a mixture known to induce hepatocyte function in vitro (27). After incubation, hepatocyte metabolism was measured by two assays, ureagenesis and gluconeogenesis. AWHLCs matured in either L-15 or MEME plus lactate, pyruvate, octanoate, and ammonium chloride (LPON) exhibited greater urea production than their ABHLC counterparts (Fig. 4A). hESC-derived hepatocytes from either protocol exhibited a similar pattern of gluconeogenesis, which was enhanced in the presence of LPON (Fig. 4B). Liver synthetic function was measured using ELISA. In addition to α fetoprotein (AFP), other proteins included fibrinogen, fibronectin, thyroxin-binding prealbumin (TBPA), and haptoglobin. AWHLCs secreted greater amounts of AFP (Fig. 4C), fibrinogen (Fig. 4D), and TBPA (Fig. 4E) than ABHLCs. The production of fibronectin (Fig. 4F), haptoglobin, and albumin (Fig. 4G) was similar in both treatment groups.

AW-Differentiated HLCs Engraft in Vivo and Are More Functional than ABHLCs. A critical ability of hESC-derived hepatocytes to engraft into the spleen was examined in vivo using NODscid mice.
hESC-derived HLCs ($10^6$) were injected intrasplenically into adult mice (aged 8–10 weeks). Three days after injection the animals were killed, and the presence of hESC-derived hepatocytes was detected using human-specific CK18, CK19, human FISH, and albumin staining. Clusters of AW-derived human CK18- and CK19-positive HLCs were detected in the spleen of recipient animals (Fig. 5A–C). Hepatocytes present in the spleen also stained positive for human DNA by FISH, which colocalized with albumin staining, demonstrating hepatocellular function in vivo (Fig. 5D–F). In addition to AWHLCs, clusters of ABHLCs exhibiting the above attributes were also detected in the spleen of recipient animals (Fig. 5G–L). Human albumin production in mouse serum was measured using ELISA. ELISA serum absorbance values for mice with AW HLCs engrafted were 7.5-fold greater than those for control mice, whereas for AB HLCs values were 3.3-fold greater than those of control mice. These results indicate either that AW HLCs are a more functional cell population or that a greater number of cells proliferated or engrafted in vivo.

Wnt3a Treatment Facilitates Single-Cell Cloning of hESCs Capable of HLC Differentiation and Function in Vitro. Because AW HLCs exhibited a broader range of hepatic function than AB HLCs, AW HLCs were used in p450 metabolic studies. Cytochrome p450s metabolize both exogenous and endogenous compounds, accounting for their central importance in human health. hESC-derived HLCs displayed CYP1A2 (0.012 pmol/min/mg protein) metabolic activity that could be assayed in 12-well format (Fig. 6A). The ability to move models of liver function, in particular p450 metabolism, to a high-throughput format is attractive to drug toxicity testing programs. In an attempt to provide high-throughput format models, we studied the ability of hESC to plate as small colonies that could be seeded into 96-well plates. Wnt3a pretreatment of hESCs (whESC) promoted hESCs self-renewal, and after passaging, hESCs displayed looser and smaller colony structure than non-treated hESCs. We reasoned that if hESCs could self-renew in vitro, AWHLCs display increased human liver function in vitro. (A) Urea synthesis, J Urea ($\mu$mol·g$^{-1}$·gTP$^{-1}$), was measured from cells derived from both AW and AB protocols. hESC-derived hepatocytes differentiated with AW exhibited greater urea production than cells differentiated with AB. (B) Gluconeogenesis was comparable in both treatment groups. Gluconeogenesis, [Glc] (umol·h$^{-1}$·gTP$^{-1}$), was greater in cells treated with LPON (L). (C–G) Serum protein production was measured by ELISA on days 15, 16, and 17, and units are quoted as $\mu$g/24 h. AW-differentiated cells exhibited greater production of AFP, fibrinogen, and TBPA than AB cells. *, $P < 0.01$ by Student’s t test. HLCs from both protocols exhibited similar levels of fibronectin, haptoglobin, and albumin, (n = 6). (H) Albumin protein production was measured by ELISA on day 17, and units are quoted as $\mu$g/24 h.

Fig. 5. AW and AB HLCs engraft in the spleen and liver in vivo. (A and B) Clusters of AW-derived hepatocytes expressing CK18 (A) and CK19 (B) in the spleen. (C) IgG control. (D) Clusters of AW-derived hepatocytes detected by FISH. (E) Human cells express albumin in the spleen. (F) FISH/immunostaining overlay. (G and H) Clusters of AB-derived hepatocytes expressing (G) CK18 and (H) CK19 in the spleen. (I) IgG controls. (J) Clusters of AW-derived hepatocytes detected by FISH. (K) Human cells express albumin in the spleen. (L) FISH/immunostaining overlay. (M) Transplanted AW and AB HLCs express albumin in vivo as detected by ELISA. The results presented are a mean of four animals ± SE. *, $P < 0.01$ by Student’s t test. Phase images were recorded using a Zeiss Axioplan fluorescent microscope. (Scale bar, 100 $\mu$m.) Immunostaining was recorded using a Zeiss Axioplan fluorescent microscope.
smaller colonies, they may also tolerate single-cell cloning. To test our hypothesis we seeded 96-well plates with single WhESCs and grew them to confluence. Five WhESC clones, selected from 50, were expanded and passaged 10 times before hESC identity and differentiation potential were assessed using FACS (Fig. 6B). Clonally derived hESC expressed the correct repertoire of hESC cell-surface markers (SSEA4, Tra1–60, and Tra1–81), expressed Oct-4, and were capable of forming embryo bodies exhibiting cell types from all three germ layers. In addition to hESC self-renewal and pluripotentiality, we tested clonally derived lines’ ability to differentiate toward hepatic endoderm. AW-driven hepatocyte differentiation was carried out as before, and hepatic identity was assessed by morphology, albumin expression, and CYP1A2 metabolic activity (0.071 ± 0.026 pmol/min/mg protein) indicative of scalable hepatic differentiation and high-throughput function in vitro. The HLCs were compared with freshly isolated human hepatocytes. Populations of HLCs derived in 12-well plates exhibited CYP1A2 activity equivalent to 4% of that observed from adult hepatocytes. In contrast, HLCs derived from clonal lines exhibited CYP1A2 activity at 24% of adult hepatocytes, demonstrating significantly improved drug metabolism.

Discussion

The results presented here demonstrate efficient and scalable human HLC generation in vitro. The exposure of hESCs, in a manner designed to mimic events in the developing embryo, resulted in their homogeneous hepatocellular differentiation and displayed increased hepatocellular function in vitro and in vivo. Wnt treatment, in addition to promoting cellular functionality, also facilitated the high-throughput study of human liver function in vitro.

The liver bud is formed from foregut endoderm during development. The generation of this structure begins with establishment of the PS and its derivative germ layers. Both Wnt and Activin signaling pathways have been shown to play important roles in mesoderm and endoderm specification (28–30). In addition, Wnt3a’s expression in developing human liver made it a candidate factor for directing efficient hESC hepatocyte differentiation. Our second model, AB, was chosen for its ability to form relatively high levels of hepatic endoderm, and we reasoned that this would serve as an established comparative (31). Monolayers of hESCs were differentiated using the two models producing HLCs that express epithelial and biliary markers in vitro and in vivo, which mimics the phenotype of oval/progenitor cell in vivo. hESCs treated with AW promoted a more rapid transition through the PS than cells differentiated with AB. Of note, the dramatic increase in WNT3A and BRACHYURY at day 2 in AB cells could be responsible for the late induction of endoderm markers SOX17 and HNF3β. The subsequent differentiation of DE to hepatic endoderm, using AW, was more rapid and promoted hepatic function in vitro and in vivo.

In addition to its role in cellular differentiation, Wnt3a has also been studied in hESC single-cell plating. In a previous study Wnt3a promoted both cellular replating and differentiation and as a result was consequently less effective than hESC clonal passaging (32). In contrast, our results demonstrate that Wnt3a treatment of hESCs promoted their single-cell dissociation and self-renewal, promoting undifferentiated hESC clonal passaging.

There are a number of signaling pathways involved in the complex cascade of events that lead to DE formation. In both our models, cells initially expressed genes indicative of PS formation and went on to express endodermal genes in a manner akin to human development. The induction of Brachyury by Wnt3a (24) and Sox17 by Nodal (33) are well documented and consistent with our observations. In addition, both Brachyury and Sox17 are known to be essential components in driving WNT3A expression and, in agreement with previous experiments, we demonstrate that increased WNT11 expression is paralleled by a decrease in WNT3A and BRACHYURY expression (35). In effect, Wnt11 could be acting as a molecular switch responsible for the commitment of differentiating cells to DE. The mesendodermal-to-endodermal transition requires a fundamental change in cellular gene expression programs. Although we observe down-regulation of BRACHYURY and WNT3A, WNT3 expression was still detectable, indicating that β-Catenin signaling, responsible for Cripto induction (36), is still active in differentiating cells. It may be the case that Wnt11-mediated shutdown of Wnt3a-driven β-Catenin/TCF signaling dampens down mesoderm formation and that Wnt3-driven β-Catenin signaling is respecified to an endodermal program of gene expression. With this in mind, it is interesting to note that Sox17 and β-catenin heterodimers have been shown to cooperate in the regulation of endodermal gene expression (37) and therefore may represent one of the Wnt11 effector complexes in endoderm specification.

In conclusion, we demonstrate the efficient generation of hESC-derived HLCs that differentiate through appropriate in vivo intermediates. This has led to the generation of a scalable liver differentiation model that bypasses the problems associated with sourcing and culturing primary human hepatocytes. Additionally, we predict that our high-throughput technology will not only be
important in the prediction of hepatotoxicity but will be extremely useful in the generation of other developmental testing models.

Materials and Methods

Cell Culture and Differentiation. hESCs (H1 and H9) were cultured and propagated as described (23, 38). Differentiation was initiated at 60–70% confluency, by replacing mouse embryonic fibroblast conditioned medium (MEF-M) with differentiation medium (RPMI1640 containing 1× BG [both from Invitrogen], 1 mM sodium butyrate (Sigma), and 100 ng/ml ActinA (Peprotech) or 50 ng/ml Wnt3a (R&D Systems) and 100 ng/ml ActinA. After 72 h (changing medium every day), differentiating cells were cultured in subsequent differentiation media as described in ref. 31.

RNA Isolation and RT-PCR. Total RNA was harvested from cells and underwent RT-PCR as described (23, 38). Total RNA from mouse tissue was isolated using RNA Isolation and RT-PCR. cDNA was generated using TaqMan reverse transcription reagent (Applied Biosystems NB008234). Quantitative PCR was carried out as described (39) and measured on an ABI Prism 7900 (Applied Biosystems). Optimized primer conditions for PCRs are shown in Tables S1 and S2.

Immunofluorescence and Immunohistochemistry. Monolayer cultures were fixed and stained as described (23). Human and mouse tissues were embedded in paraffin and 3-μm serial sections were cut. Sections were microwaved in the following heat retrieval methods: 10 mM Tris, DAKO target retrieval high pH (pH 9.5), or 10 mM citrate and calcium signaling. Beta-catenin-independent pathways. Cell Calcium 38:439–446.

Western blotting. Western blotting was performed as described (42). Quantitative Western blots were analyzed using the Image J program (Wayne Rasband, National Institutes of Health).

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Flow Cytometry. Optimized conditions for flow cytometry were used as described (42).

19. Johnson V, Johnson K (2000) Using 2 μM 7-ethoxresoruﬁn in culture medium for 24 h at 37°C. Liberated resoruﬁn was ﬂuorescently analyzed (excitation, 550 nm; emission, 585 nm) in a Safire spectrophotometer (Tecan) and reported as speciﬁc activity (pmol/min/mg protein) = SEM.
20. Animals. Adult NOD.CB17-Prkdcr(RecI)/mice were purchased from the Jackson Laboratory and housed in individual ventilated cages. Experimental protocols were carried out in accordance with permits and guidelines issued by the University of Edinburgh Ethical Review Committee and the United Kingdom Home Office. Project Licence no. 30/1134. Cells were differentiating in the absence of the AA0 on the AB protocol for 10 or 14 days, respectively. Cells were harvested using trypsin-EDTA and ﬁltered through a 100-mum ﬁlter. NODscid mice (aged 8–10 weeks) were injected intrasplenically with a 10×106 cells per laparotomy.
21. Fish. Formalin-ﬁxed, parafﬁn-embedded sections were dewaxed and rehydrated. The sections were then microwaved in 10 mM citrate buffer for 20 min and denatured in 70% formamide in 2× SSC for 3 min, dehydrated through a series of 90–100% ETOH, and air dried digoxigenine (DIG)-labeled human genomic DNA (150 ng) was hybridized overnight at 37°C. Slides were washed 4× 3 min in 0.1× SSC at 45°C, followed by 4× 3 min in 0.1× SSC at 60°C. Slides were then incubated in FITC-labeled sheep anti-DIG for 30 min, washed 3× 2 min in 4× SSC/0.1% Tween, and then incubated in FITC-labeled anti-anti antibody for 30 min, washed 3× 2 min in 4× SSC/0.1% Tween, mounted in Vectashield DAPI (Vector Laboratories), and visualized using a Zeiss Axiopt fluorescent microscope.