A small molecule screen reveals that HSP90β promotes the conversion of iPSC-derived endoderm to a hepatic fate and regulates HNF4A turnover.

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Summary statement

Our results reveal cellular processes with unappreciated roles in controlling cell differentiation and highlight the utility of using chemical screens during iPSC differentiation to uncover novel developmental mechanisms.

Abstract

We have previously shown that the transcription factor HNF4A is required for the formation of hepatic progenitor cells from endoderm that has been derived from human induced pluripotent stem cells. We reasoned that we could uncover regulatory pathways with novel roles in hepatocyte differentiation by identifying cellular processes that regulate HNF4A. We, therefore, performed a screen of 1120 small molecules with well-characterized mechanisms of action to detect those that affect the abundance of HNF4A in iPSC–derived hepatic progenitor cells. This approach uncovered several small molecules that depleted HNF4A. Of those, we chose to focus on an inhibitor of Heat Shock Protein 90-beta (HSP90β). We show that mutation of the gene encoding HSP90β represses hepatocyte differentiation during the formation of hepatocytes from iPSCs. We reveal that HSP90β, although dispensable for expression of HNF4A mRNA, directly interacts with HNF4A protein to regulate its half-life. Our results demonstrate that HSP90β has an unappreciated role in controlling hepatic progenitor cell formation and highlight the efficiency of using small-molecule screens during the differentiation of iPSCs to reveal novel molecular mechanisms that control hepatocyte formation.

Keywords: liver development, small molecule screening, iPSC-derived hepatocytes
Introduction

Much effort has been made to elucidate the molecular mechanisms that underlie liver development. Success in identifying proteins that control hepatic specification and hepatocyte differentiation has facilitated the generation of protocols that can be used to produce hepatocyte-like cells from human pluripotent stem cells (Cai et al., 2007; Hay et al., 2008; Basma et al., 2009; Si-Tayeb et al., 2010b; Sullivan et al., 2010; Touboul et al., 2010). Several reviews have discussed the molecular basis of liver development in depth (Lemaigre, 2009; Si-Tayeb et al., 2010a; Iwafuchi-Doi and Zaret, 2016). Briefly, the parenchymal cells of the liver originate from the ventral foregut endoderm. Competence of the foregut endoderm to adopt a hepatic fate is influenced by pioneer transcription factors such as Forkhead Box A (FOXA) and GATA binding proteins (GATAs) (Gualdi et al., 1996; Bossard and Zaret, 1998). Fibroblast Growth Factors (FGFs) from the developing heart, Bone Morphogenetic Proteins (BMPs) from the septum transversum mesenchyme, and dynamic regulation by wingless-type MMTV integration site (WNT) proteins, induce endoderm cells to differentiate into hepatic progenitors expressing transcription factors that drive hepatocyte differentiation (Jung et al., 1999; Rossi et al., 2001; Ober et al., 2006; McLin et al., 2007).

Hepatocyte Nuclear Factor 4 Alpha (HNF4A) is a transcription factor in the nuclear hormone family that is essential for hepatocyte formation. Previous work has shown that HNF4A is expressed at the beginning of hepatic progenitor cell formation and regulates the onset of hepatic gene expression (Duncan et al., 1994). Therefore, depletion of HNF4A in human pluripotent stem cells prevents the endoderm from adopting a hepatic fate (Delaforest et al., 2011). Although several studies emphasize the importance of HNF4A as a central regulator of hepatocyte differentiation, little is known about the control of HNF4A expression during early development of the liver.

We rationalized that identifying cellular processes that control HNF4A protein levels could provide new insight into cellular mechanisms that govern hepatocyte formation during hepatogenesis. To find pathways that are required to maintain HNF4A protein during the transition of human iPSC–derived endoderm to a hepatic fate, we performed a screen of 1120 small molecules that have known mechanisms of action. We report that disruption of the molecular chaperone heat shock protein 90 kDa alpha, class B, member 1 (HSP90AB1, referred to here as HSP90β) causes a dramatic reduction in the levels of HNF4A protein.
and, as a consequence, negatively affects the conversion of the endoderm to a hepatic fate. We also reveal that HSP90β directly interacts with HNF4A to control its half-life. These findings indicate that heat shock proteins can facilitate differentiation by fine-tuning the levels of transcriptional regulators that determine cell fate.

Results

The differentiation of iPSCs into hepatocyte-like cells in 96-well plates is compatible with screening for inhibitors of HNF4A

We have previously described a protocol to generate human hepatocyte-like cells from iPSCs (Si-Tayeb et al., 2010b). We initially optimized this protocol for the differentiation of cells in 6–well plates. Unfortunately, such a culture format was incompatible with our proposal to screen large numbers of compounds. To establish a platform that could facilitate medium throughput analyses of small molecules, we, therefore, sought to scale down the differentiation to 96–well plates. After initial trials, we noted that following the formation of endoderm, the cells had a tendency to peel off from the surface of tissue culture plates coated with Matrigel. We speculated that cell peeling was a consequence of transport of fluids to the basal surface resulting in an increase in basal pressure that could drive epithelial detachment. However, we found that transiently exposing iPSC–derived endoderm cells to 0.02% EDTA (PH7.2) at day 5 of differentiation substantially reduced the peeling. This treatment had no effect on cell viability or differentiation because the differentiated cells robustly expressed HNF4A and Albumin (Fig. S1).

To measure the impact of small molecule treatment on the formation of hepatic progenitors, we next developed an assay suitable for quantification of HNF4A protein in a medium throughput screen. We chose to focus on measuring HNF4A protein levels rather than generating cells with a reporter gene regulated by the HNF4A promoter because we believed that such an approach could identify cell processes that reached beyond transcriptional regulation. We performed immunostaining using an antibody to detect endogenous HNF4A protein. To confirm that this method was compatible with screening, we used an HNF4A-depleted iPSC line that we had generated previously (Delaforest et al., 2011). Immunostaining detected HNF4A in hepatic progenitors generated from wild type iPSCs but was undetectable in the HNF4A-depleted cells (Fig. 1A). A statistical measurement called a z–factor is commonly used to evaluate the suitability of using an assay for high throughput screening (Zhang et al., 1999). A z–factor of 1 indicates the test is perfect, between 0.5 to 1.0 is excellent, while a score below 0.5 suggests that the assay is
marginal and incompatible with screening. We, therefore, calculated the z-factor of HNF4A immunostaining to determine with a small molecule screen. Wild-type cells (n=30 wells) and HNF4A-depleted cells (n=30 wells) were differentiated to hepatic progenitor cells and then HNF4A immunostaining performed. Images were processed using ImageJ software to calculate the average pixel intensity in each well. The level of HNF4A in each well was highly reproducible; however, we identified a clear distinction between the positive and negative (HNF4A-depleted) groups, which computed to a z-factor of 0.9 (Fig. 1B).

To confirm that the assay was capable of identifying a small molecule that inhibited hepatic specification, we treated differentiating iPSCs with 5 µM Dorsomorphin. Dorsomorphin inhibits BMP signaling, which is required for the onset of hepatic development (Rossi et al., 2001). HNF4A immunostaining was performed on both untreated (n = 24 wells) and treated cells (n = 24 wells). As before, the images were processed by ImageJ software to calculate pixel intensity. The immunostaining revealed that the cells treated with Dorsomorphin had a reduced level of HNF4A compared to control cells. A comparison of the pixel intensity between control and treated cells revealed that HNF4A levels were significantly lower compared to untreated cells (p=2.4E–26, n=24) (Fig. 1C). Based on these results we concluded that the immunostaining assay was compatible with a medium throughput small molecule screen and could quantitatively and reproducibly detect differences in HNF4A protein levels.

A screen of small molecules reveals several pathways that regulate HNF4A protein levels

To identify pathways that affect the formation of hepatic progenitors, we examined the effect of 1120 small molecules on HNF4A protein during the differentiation of iPSCs toward a hepatic fate (Fig. 2A). Human K3 iPSCs were plated on 96-well plates (day 0) and then induced to differentiate into hepatic progenitor cells. Following the formation of the definitive endoderm at day 6, individual small molecules were added to each well at a concentration of 7 µM. For each 96-well plate, one column of wells was treated with DMSO (vehicle) and one was untreated. Thus for the full screening, 14 X 96-well plates were used comprising 1120 experimental wells, 112 untreated wells, and 112 wells treated with DMSO (vehicle). By the end of day 8, all cells were fixed and stained for HNF4A protein by immunocytochemistry and counterstained with DAPI to reveal cell number. We captured a representative fluorescent microscopic image for each well (Fig. 2B). HNF4A protein levels appeared similar in DMSO and untreated wells. In contrast to the control wells, the micrographs
revealed that several wells treated with small molecules showed a substantial change in the level of HNF4A staining.

Next, we processed all 1344 images with ImageJ software to calculate the pixel intensity. The quantitative values were plotted and used for statistical analysis. We set as a threshold plus and minus three standard deviations from the average level of HNF4A expression. The treatments that met these criteria were considered to be primary ‘hits’ (Fig. 2C). In the screening, ~85% of the small molecules had no effect on the expression of HNF4A, while ~15% (189 of 1120) were found to either cause a change in HNF4A level or affect cell viability. To verify these data, we repeated the treatment of all 189 hits in 24-well plates of differentiations and found that 132/189 (~70%) were consistent with the initial screen (Fig. S2). A complete list of small molecules tested, in addition to those affecting HNF4A is presented in Table S1.

We next attempted to identify biological processes that could impact the formation of hepatic progenitors by performing bioinformatic analyses. A software tool called STITCH (‘Search Tool for Interacting Chemicals’) has recently been developed that allows investigators to explore interactions between chemicals and proteins (Szklarczyk et al., 2016). The STITCH 4.0 database describes interactions between 300,000 small molecules and 2.6 million proteins from diverse organisms. Using STITCH software, we identified 396 proteins that interacted with the small molecules identified as hits in our screen (Table S1). Gene ontology analyses were then performed on the target proteins to identify cellular processes that were affected by the small molecules using PANTHER (Protein ANalysis THrough Evolutionary Relationships) (Mi et al., 2013) (Fig. S3). As expected PANTHER revealed that several of the small molecules affected pathways targeting biological processes that are indispensable for cell survival, such as protein synthesis (Anisomycin), cell cycle progression (Aminopurvalanol A), and cell survival (AT101). In our screen, treatment with such small molecules usually resulted in a detrimental effect on cell viability as evidenced by loss of DAPI staining. We also identified small molecules targeting signaling pathways that are necessary for hepatic progenitor cell formation from pluripotent stem cells, including the FGF (PD161570) and WNT (XAV939) pathways (Twaroski et al., 2015). In addition to the known pathways, several of the small molecules that affected HNF4A protein levels were agonists or antagonists of kinases or signaling receptors. These included the SRC family of kinases (1-Naphthyl PP1), Spleen Tyrosine Kinase (SYK) (ER27319 maleate), and Transient Receptor Potential Vanilloid 1 (TRPV1) (AMG9810). Network analyses using STITCH revealed that these pathways also form a complex interacting network of cellular functions.
that influence HNF4A levels (Fig. S4). Such pathways may, therefore, make unappreciated contributions to regulating the early stages of hepatocyte differentiation.

**HSP90β plays a role in hepatocyte differentiation by regulating HNF4A levels**

Although gene ontology analyses had identified several provocative pathways, we chose to focus on the effect of CCT-018159. This small molecule targets HSP90β (Sharp et al., 2007), which is an ATP-dependent chaperone. HSP90β stabilizes and promotes folding of a broad repertoire of client proteins (Karagöz and Rüdiger, 2015). Moreover, our STITCH analyses of the small molecule targets had identified HSP90β as being part of a network of cell functions that impact HNF4A levels (Fig. S4). Several transcription factors rely on HSP90β including nuclear hormone receptors (Sanchez, 2012); however, regulation of HNF4A has not been previously described. We, therefore, sought to determine whether HSP90β contributes to hepatic progenitor cell formation by regulating HNF4A protein levels. First, we repeated the CCT-018159 treatment in multiple wells of iPSCs from day 6 to day 8 of differentiation, which allowed us to exclude experimental artifacts and assess reproducibility (Figs 3A, B). The result confirmed that CCT-018159 treatment significantly reduces HNF4A protein levels by ≥ 3-fold (p≤0.0001). We verified the reduction of HNF4A protein in response to CCT-018159 treatment by immunoblot analysis (Fig. 3C).

The IC50 value for inhibition of HSP90β by CCT-018159 is 3.2 µM; however, at 50 µM CCT-018159 has been found to inhibit the activity of some kinases including GSK3β, LCK, and PDGFRα (Sharp et al., 2007). We, therefore, examined the effect of different doses of CCT-018159 on HNF4A protein levels by immunostaining (Fig. 3D). Doses as low as 1.25 µM visibly reduced HNF4A protein in the iPSC-derived hepatic progenitor cells and the calculated IC50 was 0.79 µM (Fig. 3E).

Although the dose-response data suggested that the reduction in HNF4A levels following CCT-018159 treatment was likely due to inhibition of HSP90β, we recognized that off-target effects could potentially confound interpretation. We believed that if CCT-018159 acted by specifically inhibiting HSP90β then mutating the HSP90AB1 gene (encoding HSP90β) using CRISPR-Cas9 (Ran et al., 2013) should recapitulate the effect of CCT-018159. A CRISPR guide RNA was, therefore, designed to target exon 2 of the HSP90AB1 gene (Fig. 4A). We detected INDELS by PCR amplification of genomic DNA and confirmed the nature of the mutations by nucleotide sequencing (Fig. 4A, B). Through this approach, we identified a cell line that contained deletions of 10 bp in one allele and 11 bp in the other allele of
HSP90AB1. These mutations introduce frameshifts (p.[Glu42LeufsTer56];[Glu42Ter]) that are predicted to disrupt HSP90β function. For simplicity we referred to this iPSC cell line as \( HSP90\beta^{--} \).

It has been reported that HSP90β is expressed ubiquitously during embryogenesis in multiple species (Krone and Sass, 1994; Voss et al., 2000; Dugyala et al., 2002; Vanmuylder et al., 2002). We used RT-PCR to confirm the presence of \( HSP90\beta \) mRNA in the developing liver bud throughout hepatic development in mouse embryos ranging from embryonic day (E)10.5 to E18.5 (Fig. S5A). We also confirmed that HSP90β protein was present in undifferentiated human iPSCs as well as iPSC–derived endoderm (day 5), hepatic progenitors (day 8), and hepatocytes (day 20) (Fig. S5B). To determine whether the introduction of deletions within the HSP90AB1 gene caused a loss of function, we compared the expression of HSP90β protein by immunostaining and immunoblot analyses between control and \( HSP90\beta^{--} \) iPSC–derived endoderm (Fig. 4C, D). While we observed HSP90β in the control endoderm, it was undetectable in endoderm derived from \( HSP90\beta^{--} \) iPSCs. Similarly, RT-qPCR revealed that HSP90AB1 mRNA was reduced by approximately 5–fold in \( HSP90\beta^{--} \) endoderm compared to control cells (Fig. 4E). The reduction in HSP90AB1 mRNA in \( HSP90\beta^{--} \) endoderm likely reflects nonsense-mediated decay of the transcript. Based on these data we conclude that the frameshift deletions in exon 2 of HSP90AB1 result in loss of HSP90β protein.

We next examined the impact of the loss of HSP90β on hepatic progenitor cell formation. We measured the levels of characteristic endoderm and hepatic progenitor cell markers by RT-qPCR, immunoblot analyses, and immunostaining (Fig. 5). We observed uniform expression of HNF4A protein in hepatic progenitors derived from \( HSP90\beta^{++} \) iPSCs, while the protein was barely detectable in \( HSP90\beta^{--} \) cells at an equivalent stage of differentiation (Fig. 5A). Quantification by immunoblot analyses revealed that the amount of HNF4A was approximately 5–fold lower in \( HSP90\beta^{--} \) cells compared to control cells (Fig. 5B, C). No morphological change was observed in the \( HSP90\beta^{--} \) endoderm cells (Fig. S6A), and the endoderm markers GATA4, FOXA2, CXCR4, and SOX17 were expressed at similar levels in both \( HSP90\beta^{++} \) and \( HSP90\beta^{--} \) differentiated cells, demonstrating that loss of HSP90β did not significantly impact endoderm formation (Fig. 5A, B, Fig. S6B).

Given the essential role of HNF4A in regulating hepatic progenitor cell formation (Delaforest et al., 2011), we predicted that an HSP90β–mediated reduction of HNF4A protein would impact hepatocyte differentiation from iPSCs by affecting the expression of hepatic...
progenitor cell mRNAs. We have previously defined a series of genes that are direct targets of HNF4A (Odom et al., 2004; Bolotin et al., 2010; Delaforest et al., 2011). We, therefore, performed RT-qPCR to determine the mRNA level of these genes in hepatic progenitor cells generated from wild type and HSP90β−/− iPSCs. We observed a substantial reduction in the steady-state mRNA levels encoded by these genes in the HSP90β−/− hepatic progenitor cells compared to control cells (Fig. 5D). When we extended the differentiations to day 20, the majority of HSP90β+/+ cells expressed Albumin, HNF4A and other markers that are characteristic of relatively mature hepatocytes (Fig. 5E, F). In contrast to the HSP90β+/+ cells, day 20 hepatocyte–like cells derived from HSP90β−/− iPSCs exhibited a marked reduction in Albumin and HNF4A protein levels. The levels of mRNA encoding CLRN3, ASGR1, AADAC, and SLC10A1, all of which are characteristic of late stages of hepatocyte differentiation, were also severely reduced in the absence of HSP90β. Although the impact on expression of markers such as Albumin was reduced to 30% of controls, we noted that the impact of losing HSP90β was less dramatic than in HNF4A−/− iPSC–derived hepatocytes where Albumin was undetectable (Fig. S7). The retention of some marker expression presumably reflects the impact of residual levels HNF4A that are retained in the HSP90β−/− cells. Cumulatively, these data confirm that, while HSP90β is dispensable for differentiation of iPSCs into endoderm, it is essential for maintenance of normal HNF4A protein levels and is required for the efficient differentiation of hepatocytes from iPSCs.

**HSP90β maintains HNF4A levels by regulating HNF4A protein turnover**

Finally, we sought to determine the mechanism through which HSP90β controls the level of HNF4A. We first considered the possibility that HSP90β interfered with HNF4A mRNA expression by repressing the FGF signaling pathway. FGF is critical for hepatic specification and tissue growth through the activation of the Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K) pathways (Jung et al., 1999; Calmont et al., 2006; Twaroski et al., 2015). It has been reported that HSP90β inhibition blocks MAPK1/3 (ERK1/2) and V-Akt Murine Thymoma Viral Oncogene Homolog 1 (AKT) activation, which are downstream effectors of the FGFR (Hackl et al., 2010). However, immunoblot analyses revealed that the level of phosphorylated–ERK and phosphorylated–AKT was indistinguishable in hepatic progenitor cells derived from either HSP90β+/+ or HSP90β−/− iPSCs (Fig. S8). Moreover, despite our finding that the absence of HSP90β significantly reduced the levels of HNF4A protein, RT-qPCR revealed that the steady-state level of HNF4A mRNA was comparable between control and HSP90β−/− cells (Fig. 6A).
These data imply that the regulation of HNF4A by HSP90β is post-transcriptional and independent of FGFR activity.

As an ATP–dependent chaperone, HSP90β protein aids in the folding and stability of its clients (Karagöz and Rüdiger, 2015). We, therefore, considered the possibility that HSP90β directly regulates HNF4A protein turnover. To test this hypothesis, we determined the half-life of HNF4A protein in hepatic progenitor cells in the presence and absence of HSP90β. Control cells and HSP90β−/− cells were differentiated until they formed hepatic progenitor cells at day 8. The cells were then cultured with cycloheximide (CHX, 100µM), to prevent the synthesis of nascent protein, and we measured HNF4A protein levels over time by immunoblot (Fig. 6B). Because the level of HNF4A was lower in HSP90β−/− cells (Fig. 5B), we normalized the loading of lysates to ensure equivalent levels of HNF4A at time 0 hours (Fig. 6B). In contrast to HSP90β+/+ cells, where HNF4A was easily detected after 24 hours of cycloheximide treatment, HNF4A was barely identifiable in HSP90β−/− cells. Quantification of the immunoblots after normalization to total protein revealed that the t½ of HNF4A protein in HSP90β+/+ hepatic progenitor cells was approximately 6 hours, whereas in HSP90β−/− cells HNF4A half-life was closer to 3 hours (Fig. 6C). HNF4A is a member of the nuclear hormone receptor class of transcription factors. Several other transcription factors within this family have been described as interacting with HSP90β, raising the possibility that HNF4A is an HSP90β client (Sanchez, 2012). We, therefore, performed co-immunoprecipitation experiments to determine whether endogenous HNF4A and HSP90β physically interact in iPSC–derived hepatic progenitor cells. Immunoprecipitations were performed on cells differentiated from either HSP90β+/+ or HSP90β−/− hepatic progenitor cells using anti–HSP90β or IgG antibodies. The presence of HNF4A and HSP90β in the immune–precipitate was detected by immunoblot. HNF4A was co–precipitated from HSP90β+/+ cell extracts using an anti-HSP90β antibody, but was not precipitated from HSP90β−/− cells or when we performed precipitations with IgG (Fig. 6D). Based on these data we conclude that HSP90β interacts with HNF4A and controls its steady–state levels and as a consequence is required for the efficient differentiation of hepatocyte–like cells from human iPSCs.

**Discussion**

Developmental biologists have focused on identifying growth factors, signaling pathways and transcription factors with the belief that gene expression controls cell fate. Such studies have been very successful with significant practical implications. For example, by recapitulating
the molecular events that drive cell differentiation during embryonic development, investigators have been able to design approaches that allow the generation of cell lineages from pluripotent stem cells. Despite such success, the focus on regulation of gene expression, either through growth or transcription factors, often overlooks the complexities of cell biology that govern cell behavior. Such biological processes may indirectly affect cell fate by regulating, for example, cell metabolism, protein function, intracellular protein transport, or subcellular structure. Given that intracellular functions are intricately linked, it seems logical to assume that cellular processes exist that have an unappreciated impact on cell fate decisions. Support for such a view comes from genetic studies demonstrating that cilia and intraflagellar transport control signal transduction through the Hedgehog pathway (Huangfu et al., 2003). Similarly, cell junctions recruit and process specific miRNAs that impact multiple cell properties (Kourtidis et al., 2015).

One challenge that limits our ability to identify cellular mechanisms that control cell differentiation is the availability of suitable models that can mimic the developmental process. Genetic approaches are powerful but in screening for cellular processes that affect development they are in general limited to low-throughput studies. Pluripotent stem cells offer a cell culture model that can dynamically recapitulate cell differentiation. Such a platform opens the possibility of using chemical screens to reveal novel mechanisms that affect cell fate. Here we successfully used the differentiation of iPSCs to hepatocytes to identify cellular pathways that regulate the conversion of the endoderm to a hepatic fate.

The mechanisms underlying the development of the liver have been studied extensively (Lemaigre, 2009; Si-Tayeb et al., 2010a; Iwafuchi-Doi and Zaret, 2016). Such studies have identified several growth factors and transcription factors that contribute to hepatocyte formation. HNF4A is a particularly appealing marker of hepatic progenitor cells because it is expressed at the onset of hepatic progenitor cell formation. Moreover, previous work from our group demonstrated that HNF4A is essential for the generation of the hepatic lineage from iPSCs (Delaforest et al., 2011). We, therefore, reasoned that cellular pathways that impact HNF4A protein levels would have important roles in controlling hepatocyte differentiation and formation. Although we could have used a reporter gene targeted to the HNF4A locus as a read-out of expression, we felt that this approach would restrict us to identifying processes that affected transcriptional regulation of the HNF4A gene. Instead, we elected to measure the impact of small molecules with known mechanisms of action on the endogenous HNF4A protein levels, which we believed would capture a broader class of regulatory mechanisms.
Our screen of a library of 1120 small molecules identified 132 that could reproducibly impact HNF4A protein levels. The hits included chemicals that affect signaling pathways involved in the development of hepatic cells, including FGF (PD 161570) and WNT (XAV939, endo-IWR 1, BIO) signaling. The successful identification of proteins known to control hepatic fate provided confidence in the fidelity of the screen. Although our studies focused on small molecules that reduced the level of HNF4A without affecting cell viability, several of the hits resulted in a loss of cells. Many of the small molecules that repressed processes vital for cell survival, such as protein and mRNA synthesis, were not considered further. However, it is important to note that cell death per se should not be considered a criterion for exclusion because disruption of many developmentally important pathways can manifest in a cell death phenotype. For example, acute inhibition of FGF signaling blocks specification, but when the FGFR is chronically repressed it diminishes cell viability (Twaroski et al., 2015). With this in mind, if we avoid exclusion based solely on viability, several additional small molecules could be considered provocative. This group would include those that target pathways that control an array of liver functions but have not so far been implicated in the conversion of the endoderm to a hepatic fate. For example, PHA 665752 is an inhibitor of MET Proto-Oncogene, Receptor Tyrosine Kinase (C-MET), which is a receptor for Hepatocyte Growth Factor (HGF). Both Hgf−/− and Met−/− mice die during embryogenesis between embryonic days 13.5 and 16.5 due to defects in the development of multiple tissues including liver (Schmidt et al., 1995). For this reason, HGF is included in many protocols that generate hepatocyte–like cells from iPSCs (Cai et al., 2007; Hay et al., 2008; Basma et al., 2009; Si-Tayeb et al., 2010b; Sullivan et al., 2010; Touboul et al., 2010). Whether signaling through MET or related receptors affected by PHA 665752 are required for hepatic specification has not been determined and we believe that the use of an IPSC-based differentiation model will clarify its role.

In addition to processes known to regulate hepatocyte function, our screen surprisingly identified many agonists and antagonists of receptors that have predominantly been studied in the nervous system. Dopamine, 5-hydroxytryptamine (serotonin), adrenergic, and ryanodine receptors accounted for approximately 30% of all hits. While we recognize that the effect exhibited on hepatic progenitor cell formation by these small molecules may in some cases reflect off–target effects, in many instances multiple drugs purported to target a given receptor all repressed HNF4A levels. For example, the Sigma-1 receptor that modulates calcium signaling is targeted by five individual small molecules with distinct chemical structures (Bd 1047, astemizole, Gbr 12935, sertraline, vanoxerine). Similarly, six molecules in the library target the dopamine receptors and eight are 5-HT receptor antagonists. All of these receptor classes have subtypes that are expressed in the liver (Nassar et al., 1986;
Klouz et al., 2002). Whether they affect liver development has not been described, but our data suggests that such a role should be investigated.

At the completion of the screen, we chose to focus on HSP90β. HSP90β is a protein chaperone abundantly expressed in all eukaryotic cell types (Johnson, 2012). It binds to client proteins and regulates their maturation (Wayne et al., 2011), localization, (Kazlauskas et al., 2001) and activation (Vaughan et al., 2008). The clients of HSP90β include transcription factors (Sato et al., 2003), kinases (Xu et al., 1999), and receptors (Morishima et al., 2000), that affect diverse functions of cells (Taipale et al., 2010; Karagöz and Rüdiger, 2015). HSP90β has been primarily studied as a target for cancer therapy because many HSP90β clients are oncoproteins and the expression of HSP90β is up-regulated in several types of malignancies (Trepel et al., 2010). Although the perturbation of expression leads to developmental abnormalities (Voss et al., 2000), the role of HSP90β in the context of organogenesis and development of the liver is not understood.

We show that pharmacological inhibition or mutation of the gene encoding HSP90β, substantially reduced HNF4A protein levels during the formation of hepatic progenitor cells from iPSCs without causing cell death. In addition to its role in hepatocyte differentiation in the fetus, HNF4A also has significant roles in adults. For example, a haploinsufficiency of HNF4A causes maturity-onset diabetes of the young (MODY) (Yamagata et al., 1996). Moreover, HNF4A regulates expression of wide variety of genes in both fetal and adult hepatocytes and is intimately associated with control of many liver functions including cholesterol homeostasis, carbohydrate metabolism, secretion of serum factors and xenobiotic responses (Odom et al., 2004; Battle et al., 2006; Bolotin et al., 2010). It seems likely, therefore, that HSP90β through its regulation of HNF4A protein levels, will also contribute to the control of the adult liver function.

Consistent with the fact that signaling pathways that control HNF4A expression were not affected, we found that HNF4A mRNA levels were normal HSP90β−/− cells. Given that loss of HSP90β reduces the half-life of HNF4A protein and that HSP90β directly interacts with HNF4A, we believe it most likely that HNF4A is a client of the HSP90β chaperone. HSP90β regulates ligand binding, inactivation, protein transport and degradation of various nuclear receptors (Pratt and Toft, 1997). However, no one has reported a role for HSP90β in controlling HNF4A. Based on our understanding of HSP90β mechanism of action in the context of other nuclear receptors, we favor a model whereby disruption to HSP90β results in a failure to control maturation of HNF4A protein structure. As a consequence of improper
folding, HNF4A is degraded by the proteasome in HSP90β−/− cells. Other studies have documented that turnover of HNF4A can occur through ubiquitin–mediated proteasomal degradation (Zhou et al., 2012). Unfortunately, treatment of iPSC–derived hepatic progenitors with the proteasome inhibitor MG 132 resulted in extensive and rapid cell death (not shown). This toxicity, therefore, prevented us from directly testing whether loss of HNF4A in HSP90β−/− hepatic progenitors was mediated by the proteasome. While we have demonstrated that HSP90β regulates HNF4A protein levels, it is also important to acknowledge that the impact of HSP90β on hepatocyte differentiation could be multifaceted. As we have discussed, the list of HSP90β clients is broad and so it seems likely that other client proteins could contribute and experiments to address this possibility are currently ongoing.

In summary, we have combined the use of a small molecule screen with the differentiation of human iPSCs to identify novel cellular pathways that impact the conversion of the endoderm to a hepatic fate. One caveat of using a chemical for screen is that small molecules can have off-target effects and so require significant follow-up analyses. Nevertheless, small molecules have several advantages compared to genetic approaches. For example, chemicals can be applied at a particular stage of the differentiation procedure, thereby circumventing any early requirement for a target pathway that would limit traditional mutagenesis screens. In addition, the availability of small molecules that affect diverse targets provides an unbiased opportunity to identify contributions made by cellular processes that could not have been predicted. While our study directly establishes the role of HSP90β in controlling the fate of the endoderm, we are also confident that further work on other targets that influence HNF4A protein levels will advance our general understanding of hepatic development.

Materials and Methods

Culture and Differentiation of Human iPSC Cells
Human K3 iPSCs (Si-Tayeb et al., 2010c) were regularly tested for contamination and cultured in mTeSR medium (Ludwig et al., 2006) with 4 ng/ml zbFGF on an E-cadherin-IgG Fc fusion protein matrix (Nagaoka and Duncan, 2010) in 4% O₂/5% CO₂. K3 cells were seeded on Matrigel (2 mg/ml)–coated tissue culture plates 24 hours prior to differentiation. Cells were induced to form hepatocyte-like cells as described in a stepwise protocol published previously (Mallanna and Duncan, 2013).
Small molecule screening

Human K3 iPSCs were seeded on thirteen 96-well plates and induced to form endoderm. Small molecules from the Tocriscreen Mini library (Tocris, MN, cat #2890) were individually applied between day 6 to day 8 of differentiation. In each 96-well plate, 8 wells were untreated, 8 wells were treated with DMSO, and the remaining wells were treated with small molecules at a concentration of 7 μM. At the end of day 8, cells were fixed for immunostaining to detect HNF4A and cell number was determined by DAPI staining.

Immunostaining

Cultured cells were fixed with 4% PFA for 30 minutes and made permeable using 0.5% Triton X-100 in PBS for 15 minutes. Cells were treated with with 3% BSA in PBS for 30 minutes followed by overnight incubation with primary antibody at 4°C. Antibodies used were HNF4A (Santa Cruz, CA, #sc-1556, 1:250), GATA4 (Santa Cruz, CA, #sc-1237, 1:250), and HSP90β (Abcam, MA, #ab32568, 1:500). Cells were rinsed with PBS 3 X 5 minutes and incubated with DAPI (1 µg/ml) and secondary antibody for 1 hour at room temperature. Alexa fluor antibodies (594 nm anti-goat, 488 nm anti-rabbit, 488 nm anti-goat) were used at 1:1000 dilution. Images for quantitative analysis were captured using identical microscopy and image settings for each sample. ImageJ software was used to measure pixel intensity of the whole image under a linear range (Schneider et al., 2012). After analyses, the images were processed using Adobe photoshop to optimize brightness/contrast. Control and experiment wells were processed identically.

Immunoblot and Immunoprecipitation

Whole cell lysates were collected using NP-40 buffer with protease inhibitor cocktail (ThermoFisher Scientific, NY, #78443). 30μg total protein was separated by SDS–PAGE using Any kD™ Mini-protean TGX stain-free™ precast gels (BioRad, CA, #4568123), and transferred to PVDF membranes using the Trans-Blot Turbo™ Transfer System (BioRad, CA, #1704155). Membranes were incubated overnight with antibodies against HNF4A (Santa Cruz, CA, #sc-1556, 1:1000), HSP90β (Abcam, MA, #ab32568, 1:100000), phospho-ERK (Cell Signaling Technology, MA, #9101, 1:2000), Phospho-AKT (Cell Signaling Technologies, MA, #4060, 1:2000), pan-AKT (Cell Signaling Technology, MA, #4691, 1:2000), pan-ERK (Cell Signaling Technology, MA, #4695, 1:2000) or GAPDH (Novus Biologicals, CO, #NB600-502, 1:6000) at 4°C. HRP-conjugated secondary antibodies were used at a dilution of 1:2000. Protein levels were calculated using BioRad stain-free Imaging System and were normalized to total protein using Image Lab software from BioRad. To determine HNF4A half-life, cells were cultured in the presence of 100 μg/ml CHX and lysed at different time points. Collected samples were used for Western Blot. To detect HNF4A
and HSP90 interaction, co-immunoprecipitation was performed using Catch and Release® V2.0 kit (EMD Millipore, CA, #17-500) following the manufacturer’s directions.

**Quantitative Real-Time PCR analysis**

RNA was isolated from K3 cells or iPSC–derived hepatocyte–like cells using the RNeasy mini Kit (Qiagen, #74106). Genomic DNA was removed using the TURBO DNA-free™ Kit (ThermoFisher/Ambion, NY, #AM1907). First strand cDNA was synthesized using M-MLV Reverse Transcriptase (ThermoFisher/Invitrogen, NY, #28025-013). Quantitative real-time PCR was performed on a BioRad CFX384 real-time PCR machine using TaqMan® Gene Expression assay (ThermoFisher/Applied Biosystems, NY, #4369016) or Power SYBR Green PCR assays (ThermoFisher/Applied Biosystems, NY, #4367659) following the manufacturer’s directions. SYBR green primers and Taqman assays are listed in Table S2.

**CRISPR/Cas9 Genome Editing**

CRISPR guide RNAs targeting Exon 2 of the HSP90AB1 gene were designed following the protocol established by Zhang and colleagues (Ran et al., 2013). A guide sequence (GTAATCTCTAGTTGAGGGCT) was cloned into PX459 pSPCas9(BB)-2A-Puro vector (Ran et al., 2013). The plasmid was introduced into K3 cells by electroporation using a BTX electroporator. Electroporated iPSCs were cultured on Matrigel for 24 hours in the presence of ROCK inhibitor Y27632 (StemRD, CA, #146986-50-7) and then treated with 1μg/ml Puromycin for two days. Cells that survived the selection were expanded until clones could be collected. Genomic DNA was extracted from the clones using QuickExtract™ DNA extraction solution (Epicentre, WI, #QE09050). The targeted region of HSP90AB1 gene was amplified using Herculase Fusion Polymerase (Agilent, CA, #600675) and run on Novex® 4-20% TBE gels (ThermoFisher/Invitrogen, CA, #EC6225BOX) to detect INDELs (For:AGGAGGAGGTGGAGACTTT, Rev:AGGCCAAACCACTCCTTTT). Amplicons were cloned into plasmid and subjected to nucleotide sequencing to confirm the identity of the INDELs.

**Statistical Analysis**

Z-factor was defined based on the means and standard deviations of both the positive and negative control values and calculated using online Z-factor calculator. (http://www.screeningunit-fmp.net/tools/z-prime.php). Student’s T-test (unpaired) was used to determine the significance of difference between control and experimental results generated by immunostaining pixel intensity measurement, immunoblot densitometry and RT-qPCR assays.
Competing interests: The authors declare that they have no competing interests.

Author contribution: R.J. was responsible for experimental design, analysis of data, and drafting the manuscript, C.B.D. contributed technical support for the collection of images, S.A.D. helped write the manuscript, contributed to experimental design and interpretation of results.

Funding: This work was supported by gifts from the Marcus Family, the Phoebe R. and John D. Lewis Foundation, the South Carolina Smart State Endowed Chair in Regenerative Medicine, and by National Institutes of Health grants DK102716, HG006398, and HD082570.
Abbreviations:

- **HSP90β**  heat shock protein 90 beta
- **HNF4A**  hepatocyte nuclear factor 4 alpha
- **iPSC**  induced pluripotent stem cell
- **mRNA**  messenger RNA
- **FOXA**  forkhead box A
- **GATA s**  GATA binding proteins
- **FGF**  fibroblast growth factor
- **BMP**  bone morphogenetic protein
- **WNT**  wingless-type MMTV integration site protein
- **STITCH**  search tool for interacting chemicals
- **PANTHER**  protein analysis through evolutionary relationships
- **SYK**  spleen tyrosine kinase
- **TRPV1**  transient receptor potential vanilloid 1
- **IC50**  half maximal inhibitory concentration
- **HGF**  hepatocyte growth factor
- **MODY**  maturity-onset diabetes of the young
References:


Figure 1. Differential expression of HNF4A protein can be quantified by immunocytochemistry. A) Micrographs showing the results of immunostaining to detect HNF4A in hepatic progenitor cells after 8–days of differentiation from control K3 iPSCs (Si-Tayeb et al., 2010c) and HNF4A-depleted iPSCs (Delaforest et al., 2011). B) Graphs showing the result of quantification of HNF4A immunostaining by measuring average pixel intensity using ImageJ software in each of 30 independent wells of differentiated control (blue diamonds) or HNF4A–depleted cells (red circles). Results were plotted and used to calculate the Z-factor ($z_{robust} = 0.919$). C) Graphs showing the ability to detect differences in the level of HNF4A protein by immunostaining after inhibition of BMP signaling by Dorsomorphin. K3 iPSCs were differentiated and treated with DMSO (blue diamonds) or Dorsomorphin (5μM) (red circles) from day 6 to day 8. Immunostaining was performed to detect HNF4A on day 8 of differentiation (n=24). The average pixel intensity in each well was quantified using ImageJ and significance calculated by Student’s t-test (p=2.4E−26).
Figure 2. Identification of small molecules that reduce HNF4A in iPSC-derived hepatic progenitor cells. A) A schematic representation of the small molecule screening approach. B) Micrographs showing the result of immunostaining for HNF4A in hepatic progenitor cells following treatment with 1120 small molecules. The first column in each plate was treated with vehicle (DMSO) and the last column (column 12) was untreated. C) Graphs showing quantification of HNF4A immunostaining following the screen. The average pixel intensity in each well was quantified using ImageJ. On each plate, drugs that affected HNF4A levels by ±3 standard deviations (inset: red bars) compared to the average reading per plate (inset: blue bar) were considered hits. AVE, average (mean); SD, standard deviation.
Figure 3. An inhibitor of HSP90β reduces the level of HNF4A protein in hepatic progenitor cells. A) Micrographs showing the result of immunostaining for HNF4A in iPSC–derived hepatic progenitor cells treated between days 6 and 8 of differentiation with either DMSO (n= 6 wells) or the HSP90β inhibitor CCT-018159 (n= 6 wells). Scale bar = 100 µM. B) Bar graph showing the quantification of HNF4A levels by measuring the average pixel intensity using ImageJ. Significance was determined by Student’s t-test (mean±s.e.m., p ≤
0.001, n = 6). C) Representative western blot used to measure the steady state levels of intracellular HNF4A in iPSC–derived hepatic progenitor cells treated with either DMSO (control) or CCT-018159 between days 6 and 8 of differentiation. GAPDH was used as a loading control. D) Micrographs showing immunostaining for HNF4A in hepatic progenitor cells treated with DMSO or 20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mM CCT018159. DAPI staining was used as an indication of cell viability. Scale bar = 100 µM. E) Graph showing the CCT018159 dose response curve, determined by quantification of HNF4A immunostaining by ImageJ, that was used to calculate the IC_{50} (http://ic50.tk) (n = 3).
Figure 4. Generation of \textit{HSP90\beta^{+/-}} iPSCs. A) Schematic illustration of the \textit{HSP90AB1} gene structure showing the position of the CRISPR-Cas9 guide nucleotide sequence (red arrow) and PAM sequence (blue line) used to target exon 2. The nucleotide sequences of the \textit{HSP90AB1} wild type allele (\textit{HSP90\beta^{+/-}}), and both alleles (\textit{HSP90\beta} allele 1 and 2) that were mutated in \textit{HSP90\beta^{+/-}} iPSCs are shown. Black arrows indicate the relative position of PCR primers used to identify INDELs. B) Image of showing the result of PAGE to identify PCR amplicons of the PAM region in control and \textit{HSP90\beta^{+/-}} cells. INDELs result in an electrobility shift in the amplicon derived from \textit{HSP90\beta^{+/-}} cells compared to \textit{HSP90\beta^{+/-}} cells and the DNA standards (M). C) Immunostaining reveals depletion of HSP90\beta protein (green) in endoderm derived from \textit{HSP90\beta^{+/-}} iPSCs. Total cell number was revealed by DAPI staining (blue). Scale bar = 100 μM. D) Western blot of HSP90\beta protein in endoderm derived from
HSP90β+/+ and HSP90β−/− iPSCs. GAPDH was used as loading control. E) Bar graph showing the relative level of HSP90AB1 mRNA detected by qRT-PCR in HSP90β+/+ and HSP90β−/− derived endoderm. Significance was determined by Student’s t-test (mean±s.e.m., * p ≤ 0.0001).
Figure 5. Loss of HSP90β inhibits the differentiation of hepatocyte–like cells from iPSCs.

A) Micrographs showing immunostaining for HNF4A and GATA4 in HSP90β^+/+ and HSP90β^−/− hepatic progenitor cells on day 8 of differentiation. Scale bar = 100 µM. B) Western blot of HSP90β, FOXA2, and HNF4A in HSP90β^+/+ and HSP90β^−/− cells on day 8 of differentiation. GAPDH was used as a loading control. C) Quantification of Western blot analyses of HNF4A by densitometry. HNF4A protein level was normalized to total protein and significance was determined by Student’s t-test (mean±s.e.m., p ≤ 0.05, n=3). D) Bar graph showing fold change in the steady-state level of mRNA encoding HNF4A target genes in HSP90β^+/+ and
HSP90β−/− cells at day 8 of differentiation. Significance was determined by Student’s t-test (mean±s.e.m., * p ≤ 0.05, ** p ≤ 0.01, n=3). E) Micrographs showing immunostaining of Albumin (ALB) and HNF4A in hepatocyte–like cells derived from HSP90β+/+ and HSP90β−/− iPSCs at day 20 of differentiation. Scale bar = 100 µM. F) Bar graph shows relative steady-state level of characteristic hepatocyte mRNAs in hepatocyte–like cells derived from HSP90β+/+ and HSP90β−/− iPSCs at day 20 of differentiation. Significance was determined by Student’s t-test (mean±s.e.m., * p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, n = 3).
Figure 6. HSP90β interacts with HNF4A to regulate protein half-life. A) Bar graph showing relative levels of HNF4A mRNA in HSP90β+/+ and HSP90β−/− cells at day 8 of differentiation (mean±s.e.m.). B) Western blots of cycloheximide (CHX) chase analyses of HNF4A protein in day 8 hepatic progenitor cells derived from HSP90β+/+ and HSP90β−/− iPSCs. Samples were collected at 0, 3, 6, 12, 24, 36, 48, and 60 hrs after incubation of cells with 100 µM CHX to inhibit protein synthesis. C) Quantification of HNF4A protein levels by densitometry of western blots performed on CHX-chase samples. The level of HNF4A was calculated at each time point and expressed relative to the level of HNF4A in untreated cells (0 hour). D) Western blots to detect HNF4A and HSP90β following immunoprecipitation of HSP90β+/+ and HSP90β−/− day 8 hepatic progenitor cell lysates with an HSP90β antibody. Wild type iPSC-derived hepatic progenitor cell lysate was used to measure total HNF4A and HSP90β, and immunoprecipitation of the same lysate using IgG was used as a negative control.
Supplemental Figure S1 Transient treatment of cells with EDTA on day 5 of differentiation. K3 iPSCs were induced to differentiate toward a hepatic fate. On day 5, cells were untreated or transiently treated with Versene EDTA (30s). Immunostaining of HNF4a on day 8 and Albumin on day 20 showed no difference in control and treated cells. Scale bar = 100 μm.
**Supplemental Figure S2.** Confirmation of primary hits. Immunostaining of HNF4A on ten (1–10) 24-well plates iPSC–derived hepatic progenitor cells at day 8 of differentiation following treatment between days 6–8 with 189 small molecules identified in the primary screen.
**Figure S3. Pathways targeted by small molecules that reduce HNF4A levels.**

Bar graph showing representation of gene ontology pathways. Protein targets of small molecules that reduced HNF4A were defined by STITCH analyses. A list of 396 target proteins were categorized by PANTHER using Gene Ontology (GO) terms. Overrepresentation of a pathway was defined by comparison of target proteins to a human reference list which contained 20814 proteins. GO terms that were statistically overrepresented (p\(\leq\)0.05) in the target list compared to the reference list are presented.
**Supplemental Figure S4. Network of proteins targeted by small molecules affecting HNF4A levels.** Network analysis by STITCH of small molecules identified as affecting HNF4A levels in iPSC-derived hepatic progenitor cells revealed pathways of chemical-protein and protein-protein interactions. Only liver related interactions are shown. Nodes represent either chemicals (pill) or proteins (circle). Links represent experimentally determined interactions. HSP90AB1 (HSP90B) is indicated by a dashed circle.
Supplemental Figure S5. Expression of HSP90β. A. Steady-state mRNA level of HSP90β in liver buds from E10.5 to E18.5 mouse embryos and liver from an adult mouse were identified by RT-PCR. HPRT was used as loading control. B) Immunoblot showing HSP90β protein in wild type iPSCs (Day 0), iPSC–derived definitive endoderm (Day 5), hepatocyte progenitors (Day 8), and mature hepatocytes (Day 20). GAPDH was used as loading control.
Supplemental Figure S6. Endoderm formation by HSP90β+/+ and HSP90β−/− iPSCs.
A. HSP90β+/+ and HSP90β−/− iPSCs were differentiated to day 5 and cell morphology was examined by phase contrast microscopy. Scale bar = 100 μM. Bar graph shows relative steady-state level of characteristic endoderm mRNAs in definitive endoderm cells derived from HSP90β+/+ and HSP90β−/− iPSCs at day 5 of differentiation.
Supplemental Figure S7. Expression of Albumin in hepatocytes derived from control, HSP90B−/− and HNF4A−/− iPSCs. Bar graph shows relative steady-state level of ALB mRNAs at day 20 of differentiation that were determined by qRT-PCR. Error bars represent SEM from 3 biological replicates.
Supplemental Figure S8. Activation of AKT and ERK is unaffected by loss of HSP90β during hepatic progenitor cell formation. A) Immunoblot analyses comparing the level of phosphorylated AKT, total AKT, phosphorylated ERK, and total ERK in HSP90β+/+ and HSP90β−/− cells following their differentiation to hepatic progenitor cells (day8). B) Immunoblots were quantified by densitometry. Phospho-AKT and phospho-ERK levels were normalized to total protein.
Supplemental Table 1 Screen of Small Molecules to Identify Pathways Affecting HNF4A. A) All Molecules Screened: Complete list of small molecules present in the TOCRIS library that was used in the initial screen. B) Primary Hits: Small molecules found to impact HNF4A protein levels. C) Functional Partners: Proteins defined to interact with small molecules that affect HNF4A levels.

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Supplemental Table S2. Primers used for PCR amplifications

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