DESIGN AND DEVELOPMENT OF METABOLICALLY COMPETENT CELL LINES FOR HIGH THROUGHPUT, CHEMICAL COMPOUND, TOXICITY TESTING

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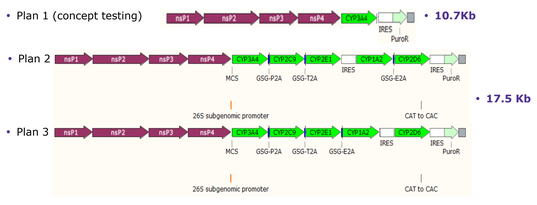
Introduction

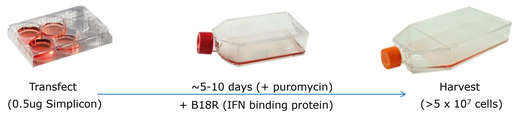
MilliporeSigma Simplicon™ Technology can be used to introduce multiple genes into cells with transfection via a single, synthetic, polycistronic, self-replicating RNA vector. The transfected cell line can be placed under selection to maintain expression of the proteins of interest for a limited number of cell divisions, while leaving no genetic footprint in the modified cell line. Recently, this technology was successfully utilized to introduce four, integration-free, reprogramming factors into human iPSC fibroblasts[1]. For this work, we created three Simplicon™ vectors expressing Phase I, human, xenobiotic metabolizing enzymes. The first vector expressed just a single cytochrome P450 enzyme (CYP3A4), to determine optimal expression conditions and activity profile in HepG2 cells and HEK293 cell lines which stably expressed human liver transporters. To perform this optimization, testosterone was used a substrate to measure 6-beta-hydroxytestosterone produced, via mass spectrometry. Subsequently, we replaced the iPSC reprogramming factors with five cytochrome P450 enzymes (e.g. CYP3A4, CYP2C9, CYP2E1, CYP1A2 and CYP2D6) in two differently designed vectors and tested each separately in a 96-well plate assay using the same cell lines. Activity of each enzyme was quantified by mass spectrometry of the metabolites produced from the substrates testosterone, tolbutamide, chlorzoxazone, phenacetin and buproprion. Relative expression of each enzyme was determined by qPCR measurement. Unlike the HepG2 cell line, to achieve substrate uptake, HEK293 cells were transfected to stably express liver transporters (i.e. OATP1B1 or OATP1B3). While cytochrome P450 metabolism varied with the substrate used, both cell lines show a 10-fold increase of testosterone metabolism over background in a 96-well plate format, after two hours of substrate incubation. Cell lines transfected with a Simplicon™ vector, scaled, frozen and subsequently assayed (6 days, total), demonstrated a similar metabolism profile to those assayed 48hrs post transfection. Currently, most commercial high-throughput cell based assay systems used for chemical toxicity screening utilize cells which lack metabolic competence. Here, the MilliporeSigma Simplicon™ Technology is used to create a cell line which expresses and shows metabolic activity of five human xenobiotic metabolizing enzymes, and can be scaled to screen thousands of chemical compounds in a 96-well plate format.

Methods



Simplicon Design: Two larger RNA constructs were created, each containing five CYP450 enzymes (Plan 2 and Plan 3) separated by either a 2A peptide cleavage site, or an IRES sequence. Additionally, a smaller construct containing only the CYP3A4 enzyme was designed to begin experiments while the larger constructs were being assembled. A puromycin resistance gene was included on all constructs.

HEK293 cells stably expressing OATP1B1 or OATP1B3 or HepG2 cells were plated into 6-well plates at 3 X 10^5 cells/well. After overnight incubation, the cells were transfected with 0.5ug – 2ug Simplicon/well in the presence of 200ng/ml B18R IFN binding protein. The following day, cells were removed, counted and plated into 96-well plates at 1 X 10^5 cells/well in 200ul DMEM/F12 growth media containing 0.1ug/ml puromycin and 200ng/ml – 600ng/ml B18R protein. The next day, the cells were treated with substrate compounds (10uM, with the exception of phenacetin which was 40uM) for 2-3 hours in serum-free medium and assayed for metabolite production by mass spectrophotometry.
Cells that were incubated for 144 hours were passaged and scaled into the same growth media supplemented with 0.1ug/ml puromycin and 200ng/ml B18R IFN binding protein.



Results

Figure 1 graphically shows the relative amount of mRNA of each of the CYP450 enzymes in cells transfected with the Plan 3 SimpliconTM, 48hrs post transfection compared to a liver cell line, HepaRG, which had been differentiated in 1.7% DMSO for 2 weeks. A similar amount of mRNA, if not more, is expressed in HEK293 cells.

HEK293 cells required the expression of a liver transporter, as well as, exogenously added P450 enzymes to efficiently metabolize substrate compounds (Figure 2). Both OATP1B1 and OATP1B3 were capable of substantially improving substrate metabolism and stable HEK293 cell lines stably expressing these transporters were created (data not shown).

While the first experiments were performed using 0.5ug SimpliconTM transfected per well, the optimal amount of SimpliconTM required was ~2ug/well (Figure 3). Higher concentrations proved toxic. The reason for this toxicity was not investigated.

To create cell banks of Simplicon™ expressing cells, HEK293 cells stably expressing a liver transporter was transfected and placed under puromycin selection (0.1ug/ml) for up to 5 days. After expansion, cells could be plated and assayed, or frozen for later use without loss of activity. Selection in the presence of puromycin was required to retain CYP3A4 activity (Figure 4).

Simplicon™ vectors containing multiple CYP450 enzymes (i.e. Plan 2) show activity of all CYP450 enzymes six days post transfection into HepG2 cells or HEK293 cells stably expressing OATP1B1 while in the presence of 0.1ug/ml puromycin (Figure 5). HepG2 cells do not require the addition of a liver transporter to show substrate compound metabolism. Difficulty in assaying for CYP2C9 activity prevented this data from being shown.

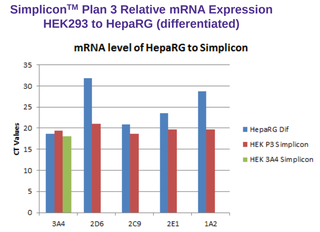


Figure 1. mRNA Expression of Five CYP450 Enzymes in HepaRG cells and Plan 3 Transfected HEK293 cells. qPCR was performed on HEK293 cells stably expressing hOATP1B1 48 hours after transfection with the Plan 3 Simplicon™ and HepaRG cells differentiated in 1.7% DMSO for 2 weeks. The five CYP450 enzymes present on the Simplicon™ were evaluated.

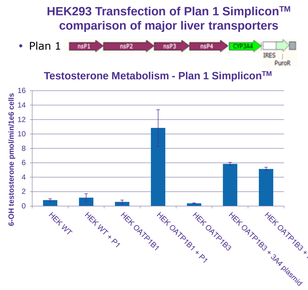


Figure 2. Requirement of Human Liver Transporters for Xenobiotic Activity in HEK293 Cells. HEK293 cells stably expressing either hOATP1B1 or hOATP1B3 were transfected with the Plan 1 Simplicon™ and incubated in the presence of 10mM testosterone for 2 hours, approximately 48hrs post transfection. For comparison, a pool of HEK293 cells stable expressing OATP1B3 and CYP3A4 under a CMV promoter was included. 6B-OH-testosterone in medium was measured by mass spectrometry.

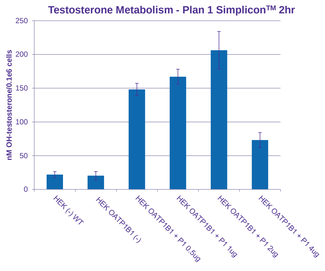


Figure 3. Optimizing the Concentration of Simplicon™ to be Transfected into HEK293 cells. HEK293 cells stably expressing OATP1B1 were transfected with increasing concentrations of Plan 1 Simplicon™/well . **Testosterone metabolism was assayed 48hrs post transfection.**

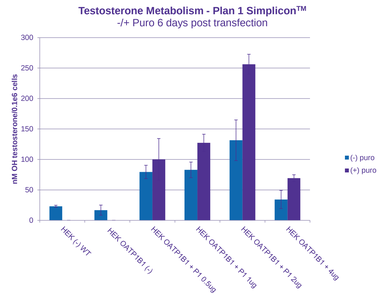


Figure 4. Metabolism of Testosterone Six Days Post Transfection. HEK293 OATP1B1 cells transfected with Plan 1 SimpliconTM was expanded in the presence of 0.1ug/ml puromycin for 5 days, harvested , plated into 96-well plate for assay, and subsequently assayed for testosterone metabolism the following day.

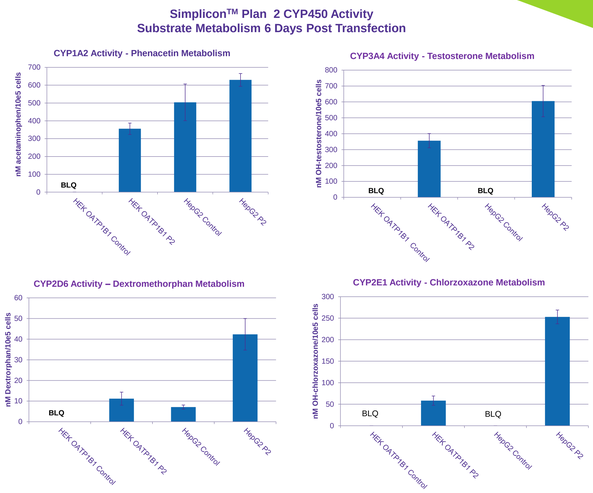


Figure 5. Activity of Four CYP450 Enzymes in Plan 2 Transfected HepG2 and HEK293 cells. HEK293 cells stably expressing OATP1B1 or HepG2 cells were transfected with 2ug/well of Plan 2 Simplicon™ and expanded in growth media containing 0.1ug/ml puromycin and 600ng/ml B18R. Metabolites were analyzed by mass spectrometry after 3 hour incubation with substrate.

Summary

Previously, MilliporeSigma Simplicon™ Technology was utilized to generate human iPSC cultures from human foreskin fibroblasts via the introduction of four reprogramming factors[1]. Here, we demonstrate the use of the technology to transiently introduce CYP450 enzymes into HEK293 cells stably expressing a liver transporter, and HepG2 cells for high throughput, compound toxicity screening. In this study, both cell types were transfected with a single polycistronic, self-replicating RNA vector containing five CYP450 enzymes, scaled under puromycin selection, and assayed for metabolic activity of those enzymes in a 96-well plate assay, 48 hour and 6-days post transfection.

Three Simplicon™ vectors were created to test the concept. Plan 1 Simplicon™, containing only CYP3A4, was created to determine the transfection and subsequent cell scale-up conditions. The two larger Simplicon™ vectors, containing all five CYP450 enzymes, differing only by the location of the IRES site, were used to test for enzyme activity after cell scale-up.

Here, we show that expression of each of the enzymes was similar to a liver cell line (DMSO treated HepaRG) typically used for toxicity screening, by qPCR of the mRNA from transfected HEK293 cells. Utilizing Plan1 Simplicon™, we establish that 2ug RNA is required to obtain best CYP3A4 activity via metabolism of testosterone, and puromycin supplemented growth medium is required to maintain CYP450 activity during HEK cell scale-up. While Plan 2 and Plan 3 Simplicon™ yielded similar results in compound metabolism screening (data not shown), the use of Plan 2 Simplicon™ demonstrates that both transfected HEK293 and HepG2 cells could be scaled in culture for five days and still retain metabolic activity of each enzyme.

While transfected HepG2 cells did yield higher metabolic activity of the enzymes tested compared to transfected HEK293 cells, they showed a higher background of activity for some enzymes (i.e. CYP2D6 and CYP1A2).

[1]Yoshioka N, Gros E, Li HR, Kumar S, Deacon DC, Maron C, Muotri AR, Chi NC, Fu XD, Yu BD, Dowdy SF. Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell.* 2013 Aug 1;13 (2):246-54.