Engineering Spatial-Organized Cardiac Organoids for Developmental Toxicity Testing

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Summary

Emerging technologies in stem cell engineering have produced sophisticated organoid platforms by controlling stem cell fate via biomaterial instructive cues. By micropatterning and differentiating human induced pluripotent stem cells (hiPSCs), we have engineered spatially organized cardiac organoids with contracting cardiomyocytes in the center surrounded by stromal cells distributed along the pattern perimeter. We investigated how geometric confinement directed the structural morphology and contractile functions of the cardiac organoids and tailored the pattern geometry to optimize organoid production. Using modern data mining techniques, we found that pattern sizes significantly affected contraction functions, particularly in the parameters related to contraction duration and diastolic functions. We applied cardiac organoids generated from 600 μm-diameter circles as a developmental toxicity screening assay and quantified the embryotoxic potential of nine pharmaceutical compounds. These cardiac organoids have potential use as an *in vitro* platform for studying organoid structure-function relationships, developmental processes, and drug-induced cardiac developmental toxicity.

Introduction

Recent progress in stem cell-based organoid technology offers unique opportunities to *in vitro* recapitulate biological processes of organogenesis into spatially organized tissue structures that resemble the architecture and functions of specific tissues (Fatehullah et al., 2016). Integration of organoid technology and microfabrication has provided promising ways to guide self-organization and spatial pattern formation of developing biological tissues (Brassard and Lutolf, 2019). ~~For example, microwell technology has been widely applied to control the aggregate sizes of human pluripotent stem cells (hPSCs) for generating brain (Lancaster et al., 2017), kidney (Czerniecki et al., 2018), and blastocyst (Rivron et al., 2018) organoids of a desired architecture.~~ Microfluidic systems could precisely control the localization of morphogen source and gradient to guide the spatial hPSC differentiation and organization into *in vitro* synthetic embryonic tissues, such as in germ layer patterning (Manfrin et al., 2019), as well as amniotic and epiblast layer separation (Zheng et al., 2019). Surface micropatterning techniques were also able to provide geometric confinement for modeling gastrulation process, where PSCs were patterned and differentiated to form concentric rings indicative of specific germ layers (Morgani et al., 2018; Warmflash et al., 2014). These examples illustrate the critical need for spatiotemporal engineering of cell microenvironments to guide the structure and function of stem cell organoids to specific biological tissues.

Despite extensive efforts in controlling stem cell lineage specification via biophysical inputs, there are few studies focusing on how 2D patterned cell colonies could give rise to organoids with defined structure-function relationships. Understanding these relationships require comprehensive analysis of multiple variables simultaneously, which increases the data dimensionality for analysis and visualization. As methods to detect cardiac functions evolve and become more sophisticated, large-scale multidimensional data requires more advanced analytics to effectively comprehend the functional outcomes. Currently, data dimensionality reduction techniques, which are the basis of bioinformatics analysis, are still underexplored for analyzing tissue level structural and functional properties. The combination of tissue engineering, organoid technology and advanced data mining techniques would potentially provide the versatility and

capability to discover trends and relationships to guide new engineering designs with a spectrum of biological structures and functions.

hPSC-derived organoids exhibit characteristics of specific tissue lineages at their early developmental stages, thus providing great potential as *in vitro* assays of developmental drug toxicity. *In vivo* animal models and *in vitro* mouse embryonic stem cell tests (mEST) are widely implemented by pharmaceutical companies as biological assays for embryotoxicity screening (Seiler and Spielmann, 2011). To overcome species barriers that impose limitations in traditional drug screening, hPSC technology has been proposed to replace the mEST for better predictions of human-specific developmental toxicity (Kumar et al., 2012). However, most 2D stem cell-based assays lack the capability of morphological scoring of 3D tissue morphogenesis. This undermines the predictability of drug-induced teratogenicity, which potentially leads to structural malformations manifested in late prenatal fetus development. Moreover, traditional organoid technology exhibits relatively random positioning of tissue regions of specific cell types, and these regions are not reliably spatial-organized relative to one another. ~~Lack of spatial information in organoid formation does not faithfully recapitulate the developmental process to distinct spatial tissue patterns.~~ Heterogeneity in organoid formation also makes it difficult for embryotoxicity drug testing purposes with high consistency and reproducibility. Advances in microtechnologies have produced organoids with consistent 3D morphology and spatial structure, enabling robust morphological scoring of *in vitro* tissue formation, which is crucial for screening drug-induced developmental toxicity.

In this work, we optimized cardiac organoid production from 2D micropatterned human induced PSCs (hiPSCs) by generating organoid arrays with different geometries (Hoang et al., 2018). Organoids generated from genome engineered GCaMP6f hiPSCs were used for integrated functional analysis of calcium transient and contraction motion. Using data mining tools, we established relationships amongst a multitude of organoid metrics of tissue structure and contractile functions. We also explored the inter-dependency amongst these parameters associated with the geometric sizes, which highlighted that biophysical

microenvironment could modulate tissue structure and cardiac function. Next, we implemented this model for evaluating cardiac developmental toxicity by quantifying the drug effects based on cardiac differentiation, contractile behaviors and 3D tissue morphology. We also compared drug-induced cardiac developmental toxicity on hiPSC-based cardiac organoids to effects on *in vivo* cardiac development using whole embryo culture (WEC) of living *danio rerio* (zebrafish) embryos. We envision the human cardiac organoid model as a versatile platform to assess cardiac organogenesis and developmental toxicity, which can be adopted for pharmaceutical development and fetal safety assessments.

Results

Cardiac differentiation of patterned hiPSCs produces spatially organized cardiac organoids

Controlling biophysical microenvironments through cell patterning techniques has been used for regulating stem cell differentiation and modeling mammalian embryonic development (Hwang et al., 2008; Ma et al., 2015; Shao et al., 2015; Warmflash et al., 2014). Geometric confinement to hiPSCs was provided by a poly(ethylene glycol) (PEG)-based micropatterned substrate created by oxygen plasma etching (Fig. 1a). hiPSCs seeded on the micropatterned substrates (Fig. 1b). Upon confluency, hiPSCs were differentiated via small molecule modulation of the Wnt/β-catenin pathway (Supplemental Fig. 1a, b) (Lian et al., 2012). Patterned hiPSCs were able to proliferate, conform to the pattern geometry and retain pluripotency, as indicated by positive immunofluorescence of OCT4, NANOG, E-cadherin, SOX2 and SSEA4 (Supplemental Fig. 1c). This approach generated robust contracting cardiac organoids arrays of 50-100 organoids within 20 days.

At early differentiation stages, we verified positive expression of mesoderm marker BRA (Day 1) (Supplemental Fig. 1d), and cardiac progenitor markers ISL1 (Day 8), NKX2.5 and GATA4 (Day 10) (Supplemental Fig. 1e-g). Expression of early cardiac progenitor makers was distributed across the entire pattern before the cardiac organoids began contracting around Day 12. As the contraction became robust over time, the cardiac tissue compacted towards the center, revealing the underneath stromal cells. At Day

20 of differentiation, cardiomyocytes were primarily differentiated on the center top of the organoids, demonstrated by multiple cardiac-specific markers (Fig. 1c, d): cardiac troponin T, myosin heavy chain β, sarcomeric α-actinin and cardiac troponin I, while smooth muscle-like stromal cells along the pattern perimeter, indicated by positive expression of α-smooth muscle actin, calponin, α-SM22 and vimentin.

Next, we compared the gene expression profile between cardiac organoids from three different pattern sizes (200 μm, 600 μm and 1000 μm in diameter) and traditional 2D monolayer differentiation. In general, gene expression showed upregulation of cardiac-specific genes and downregulation of WNT signaling at Day 20 differentiation, though the gene expression profile had closer similarity amongst different organoids than 2D differentiation (Fig. 1e). The organoids also showed significantly higher gene expression related to TGFβ signaling (TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR1). This might relate to a high content of stromal cells in the organoids under geometric confinement on the patterned substrate (Supplemental Fig. 1h). To compare the organoids generated from different pattern sizes, we found that organoids of 600 μm and 1000 μm showed upregulation of cardiac-specific genes (MYL4, MYH7, NKX2.5), while smaller 200 μm organoids had higher expression of stromal cell genes (ACTA2, TAGLN, VIM). These results illustrated that high geometric confinement from smaller patterns promoted the differentiation into supportive cell types.

Pattern geometry dictates structural morphology and contractile physiology of cardiac organoids

Using immunofluorescence staining, we measured the cardiac tissue distribution by calculating the *area ratio* between the areas of cardiomyocyte differentiation and entire pattern area (Supplemental Fig. 2a, b). Using confocal microscopy and 3D image reconstruction (Supplemental Movies 1-6), we assessed the three-dimensionality of cardiac organoids by measuring the *height* and *full-width at half-maximum* (FWHM) (Supplemental Fig. 2c). To study the contractile functions of cardiac organoids, we generated the cardiac organoids from genome-edited hiPSC line with GCaMP6f reporter (Chen et al., 2013; Huebsch et

al., 2016; Mandegar et al., 2016), which provided us the capability to assess the cardiac contractions using brightfield motion tracking analysis (Huebsch et al., 2015) (Supplemental Fig. 2d, Supplemental Movies 7-9). Motion data was integrated with fluorescent calcium flux (Supplemental Fig. 3a, b, Supplemental Movies 10-12) for a comprehensive characterization of contraction functions. t-SNE plots (Fig. 2e-i), together with a heatmap (Fig. 2k) were generated using quantified contractile function parameters from individual organoids with different pattern sizes: *area ratio* between GCaMP6 fluorescence and pattern area, *beat rate*, *maximum calcium flux*, *pulse duration* of calcium flux cycle (τ_0, τ_{50}, τ_{75}), *peak-to-peak interval*, *maximum contraction velocities* and *relaxation velocities* (Supplemental Fig. 3c).

To examine the effects of pattern size on cardiac organoid development, we generated organoids from circular patterns ranging 200–1000 μm in diameter (Fig. 2a, Supplemental Fig. 4). The 200 μm patterns did not reliably produce the organoids with a relatively low organoid production efficiency (~ 20%), in comparison to all other sizes (~ 80%). Albeit with the largest variability, 200 μm circle patterns produced the cardiac organoids with the largest area ratio of cardiac muscles amongst all the sizes (Fig. 2b). Regarding large sizes (800 μm and 1000 μm in diameter), these patterns produced the organoids with a greater area ratio, but lower organoid height than the ones from 400 μm and 600 μm circles (Fig. 2c). Additionally, the FWHM steadily increased with increasing pattern size, peaked at 600 μm patterns and decreased at larger sizes (Fig. 2d). This indicated that 600 μm patterns produced the cardiac organoids with high consistency and large 3D morphology.

From the t-SNE plot (Fig. 2e) generated from data mining of contraction function parameters (Supplemental Fig. 5), organoids of larger sizes (600 μm, 800 μm and 1000 μm) were better clustered with high consistency. Organoids from 200 μm and 400 μm patterns showed significant organoid divergence with separated clusters, indicating less consistency in organoid properties. We also used t-SNE plots to illustrate the gradients of each parameter across the organoid sample distribution (Fig. 2f-I, Supplemental Fig. 6a-f). Organoids of larger patterns exhibited higher beat rate (Fig. 2f), while smaller patterns exhibited higher

contraction and relaxation velocities (Fig. 2g, Supplemental Fig. 6a). Consistent with the confocal structural analysis, the 200 μm organoids produced the largest area ratio of cardiac tissue relative to pattern size (Fig. 2h). In these t-SNE plots, the divergence of small organoids from 200 μm and 400 μm pattern primarily resulted from the significant differences on parameters of contraction duration (peak-to-peak intervals, pulse duration, τ_{50}, τ_{75}) (Fig. 2i and Supplemental Fig. 6b-d). The metrices of τ_0 and max calcium flux exhibited no apparent trends amongst different organoid sizes (Supplemental Fig. 6e, f).

These parameters for the cardiac organoids of different sizes were then compared in the heat map (Fig. 2j). Organoids larger than 200 μm exhibited comparable trends of beat rate and maximum calcium flux, indicating efficient calcium handling and consistent beat frequency. Meanwhile, the contraction duration parameters of peak-to-peak intervals, pulse duration, τ_{50}, and τ_{75} showed high level of size dependency. The prolongation of contraction duration, especially regarding calcium decay in the cardiac organoids from small patterns, is prone to arrhythmia related to the abnormal diastolic functions (Gaasch & Zile, 2004; Kass et al., 2004; Periasamy & Janssen, 2008; Weber et al., 2006). From the correlation matrix (Fig. 2k), strongest correlations were observed between parameters of calcium analysis (τ_0, τ_{50}, τ_{75}, pulse duration), which had negative correlation with beat rate (Supplemental Fig. 6g, h). Furthermore, we were able to establish the correlation across different functional analysis. There was a strong positive correlation between calcium decay from calcium flux analysis and peak-to-peak interval from contractile motion analysis. More importantly, area ratio from morphological analysis is positively correlated with calcium decay and contractile motion velocities from different functional analysis, indicating a structure-function relationship between the relative tissue size and cardiac contraction cycles.

Additionally, we compared circle, square, and triangle organoids with the same area (Supplemental Fig. 7a). Cardiac organoids from circular patterns exhibited significantly higher contraction velocity, lower beat rate and longer beat duration (Supplemental Fig. 7b-d). Furthermore, circle patterns produced the organoids with larger area ratio, height and FWHM than squares and triangles (Supplemental Fig. 7e-g). Overall, the

circular pattern geometry promoted more robust formation of cardiac organoids with large size and high contractile functions. These observations illustrated that the development of cardiac organoids was affected by pattern shape, in which the angular geometries presented high physical constraint to the cells and promoted a higher degree of differentiation into the stromal cell types, rather than cardiomyocytes. By plotting the structure-function correlations and structure-structure, we observed a decline in the beat rate that corresponded to the increasing area ratio (Supplemental Fig. 7h, i) as a negative correlation between the cardiac tissue size and the beat rate.

Cardiac organoids as an *in vitro* assay for cardiac developmental toxicity

The heart is the first functional organ to form, thus cardiac differentiation is often used as a key evaluation for developmental toxicity (Tandon and Jyoti, 2012). Since 600 μm-diameter circular patterns gave robust organoid production, large 3D morphology, consistent contractile functions and high level of cardiac-specific differentiation, arrays of cardiac organoids from this geometry were used to test the capabilities of this platform to be implemented as a cardiac developmental toxicity assay. Using flow cytometry, we quantified the consistency of cardiac differentiation efficiency of organoids and 2D monolayer differentiation from 5 different batches ($n = 5$) across 10 passages of hiPSCs from two different lines. Both organoids of 600 μm and 2D differentiation produced over 50% cells that were positive for cardiac troponin T (Supplemental Fig. 8a, b). These results illustrated that cardiac organoids can be generated consistently across multiple cell batches and multiple hiPSC lines. Furthermore, the result of ~ 50% cTnT+ cells from flow cytometry was consistent with the area ratio calculation, where 600-μm organoids exhibited an average area ratio of approximately 0.5 (Fig. 2b). Since the percentage of cTnT+ cells that composed organoids was comparable to the area ratio metric, we inferred that the area ratio is an appropriate metric to approximate cardiac differentiation efficiency for the drug screening purposes.

Nine drugs (Supplemental Table 1) used in this study covered the entire spectrum of the past FDA pregnancy category system that ranked drugs from A (safe) to X (toxic) based on predicted teratogenic risk.

The drugs were individually introduced on Day 1 of differentiation, 24 hours after treatment of GSK3 inhibitor CHIR99021 (Fig. 3a). This initial timepoint was chosen because it specifically targets cardiac differentiation after mesoderm induction. First, we compared two drugs between category A (doxylamine succinate) and category X (thalidomide). and found that doxylamine succinate (Category A, Fig. 3b) treatment had no negative effects on cardiac differentiation, whereas thalidomide (Category X, Fig. 3c) had produced the abnormal cardiac organoids with less cardiac tissue coverage relative to the untreated controls (Fig. 3d, e). A similar trend was seen when quantifying cardiac differentiation efficiency via flow cytometry analysis (Supplemental Fig. 8c). Doxylamine succinate had prominent effects on the contractile functions with lower contraction velocity and slower beat rate (Fig. 3f, h) due to its anticholinergic effects as an H1 receptor antagonist. Generally used as a sleeping aid due to its relaxant effects, doxylamine succinate is also prescribed as an analgesic to reduce muscle tension. It is possible that the reduction of muscle contraction due to doxylamine treatment led to less tissue compaction during organoid development, which resulted in an increase of area ratio. In contrast, there is no significant effect from thalidomide on the contraction velocity (Fig. 3g), though high concentration of drug dosage led to high variability in the beat rate (Fig. 3i). More importantly, high concentration of thalidomide (100 μM) impaired the 3D morphology of the cardiac organoids with significantly lower height and FWHM relative to organoids treated with doxylamine succinate (Fig. 3j) and to the controls (Fig. 3k-m). The impairment of cardiac tissue is potentially caused by FGF antagonism of this drug, resulting in decreased muscle development. These results indicated that exposure of a well-known teratogen resulted in severe impairment to hiPSC differentiation and organization into 3D cardiac organoids, which confirmed that this organoid model was sensitive to the morphological defects as a result of drug exposure.

Next, we tested cardiac developmental toxicity of drugs from the other pregnancy categories. Three antibiotic drugs tested on the cardiac organoids showed increased developmental toxicity with the increase of their risk classification in the pregnancy category. Amoxicillin (category B antibiotics) in mammalian cells has been shown to induce DNA lesions as a result of amoxicillin-induced oxidative stress (Li et al.,

2007). In the organoids, amoxicillin showed no clear toxic effect on either structure or functions of the cardiac organoids at all three tested concentrations (Supplemental Fig. 9). Rifampicin (category C antibiotics) targeting bacterial RNA and DNA for its effectiveness, has been shown to inhibit protein synthesis in mammalian cells (Buss et al., 1978). Rifampicin showed severe developmental toxicity at a high concentration (100 μM) with no organoid formation (Supplemental Fig. 10). Doxycycline (category D antibiotics) inhibits the synthesis of bacterial proteins by binding to the 30S ribosomal subunit, but also showed adverse effects on mitochondrial ribosomes within mammalian cells (Ahler et al., 2013). Doxycycline treatment resulted in severe impairment on cardiac differentiation and organoid formation even at a moderate concentration (10 μM) (Supplemental Fig. 11a).

We then tested other category D dugs with various therapeutic applications. Lithium carbonate (category D antidepressant), which inhibits the PKC signaling for its psychiatric medication purpose, appeared to inhibit phosphatidylinositol cycle and Wnt pathway activation, based on mEST assays (Shaikh Qureshi et al., 2014). Exposure of lithium did not affect the contractile functions of the cardiac organoids but exhibited mild toxicity to the organoid formation measured by area ratio and FWHM (Supplemental Fig. 11b). Phenytoin (category D anticonvulsant) protects against seizures via voltage-dependent antagonism of voltage-gated sodium channels. Phenytoin exposure produced smaller cardiac organoids than controls, but still maintained structural integrity (Supplemental Fig. 11c). Due to its inhibition on sodium channels, the cardiac organoids stopped beating at a high concentration (100 μM), until the drug was removed to allow the organoids to recover the contractile functions. Tretinoin (all-trans-retinoic acid, category D retinoid) treatment completely abolished the cardiac differentiation at the concentration as low as 10 μM, but still produced the organoids with comparable height and overall size as controls (Supplemental Fig. 12a).

Overexposure to retinoids was shown to result in birth defects from retinoic acid deficiency with decreased levels of retinoic acid-producing enzymes (Lee et al., 2012). Hence, we also tested isotretinoin (13-cis-retinoic acid, category X retinoid) (Supplemental Fig. 12b) on the cardiac organoids. Similar to tretinoin,

isotretinoin produced large organoids at all tested concentrations, but totally abolished the cardiac differentiation at even lower concentration at 1 μM. This implied that retinoids caused severe impairment to the cardiac differentiation, but not tissue growth. Overall, these results verified that this cardiac organoid model was sensitive to the drug-induced cardiac developmental toxicity and offered the capability of morphological scoring based on the 3D tissue formation, which is often not available from other stem cell-based *in vitro* assays.

Last, we compared the developmental toxicity of these drugs between cardiac organoid model and zebrafish whole embryo culture (zWEC), a well-established toxicity assay with promising potential to screen for teratogenicity (Lantz-McPeak et al., 2015). We used transgenic *Tg(myl7:GFP)* (Huang et al., 2003) with GFP only in cardiomyocytes, which allowed us to readily score myocardial development and heart tube looping at 48 hours post-fertilization (hpf) (Sarmah and Marrs, 2016) (Fig. 3n, Supplemental Fig. 13). Live zebrafish embryos were collected and exposed to the identical drugs at identical concentrations used in the cardiac organoid assay. Consistent with the organoid model, exposure with doxylamine succinate (Category A) had negligible effect on the zebrafish embryonic heart development, as there was a considerable proportion of embryos exhibiting normal D-looped heart across all concentrations. However, the effects of thalidomide on zebrafish embryonic heart development were not as significant as what we observed in the organoid model. We found that the proportion of normal heart looping did not notably decrease at higher concentrations of thalidomide (Fig. 3n). Rifampicin (Supplemental Fig. 13b) and phenytoin (Supplemental Fig. 13d) showed mild embryotoxic effects to the zebrafish embryos at the highest concentration, while amoxicillin (Supplemental Fig. 13a), lithium carbonate (Supplemental Fig. 13c) and doxycycline (Supplemental Fig. 13e) showed moderate toxicity. Retinoic acid derivatives, tretinoin (Supplemental Fig. 13f) and isotretinoin (Supplemental Fig. 13g), caused severe defects to the embryo heart development. At low concentration (0.1 μM), embryos had severe heart defects, including smaller size and abnormal morphology. At higher concentrations, the hearts failed to develop, as indicated by 0% of embryos expressing the GFP transgene. Upon comparing the organoid model with the zWEC model, we saw

developmental toxicity that was comparable for between these two systems for most of drugs (doxylamine succinate, amoxicillin, lithium carbonate, phenytoin, tretinoin, and isotretinoin). However, rifampicin, doxycycline and thalidomide showed distinct mismatch between these two model systems. We infer that this might be due to a number of factors, including species differences, method of drug exposure, and differences in the range of effective treatment concentrations.

Discussion

The majority of *in vitro* cardiac tissue models focus on accurate recapitulation of physiologically relevant tissue structures of adult human heart, which are generally achieved by populating pre-fabricated 3D biomaterial scaffolds with pre-differentiated hiPSC-derived cardiomyocytes (hiPSC-CMs) (Ogle et al., 2016; Rodrigues et al., 2018; Turnbull et al., 2018). These adult-mimicking model systems are designed to enhance the maturity of hiPSC-CMs for the purpose of drug screening and disease modeling, but not designed for studying dynamic cellular self-organization occurring along with the cardiac differentiation process. In contrast, stem cell-derived organoids are designed to resemble the early developing organs through self-organization of differentiating cells into spatial-distinct tissue-specific structures. However, cardiac organoids are still largely generated by aggregating pre-differentiated hiPSC-CMs with other stromal cells (Hookway et al., 2019; Richards et al., 2017, 2020), instead of originating from directed stem cell differentiation. Our technique to generate cardiac organoids started with 2D micropatterned hiPSC colonies, allowing for cell self-organization into 3D tissue structures during the differentiation process under geometric confinement. Our new approach opens the possibility to create cardiac organoids that could resemble the similarity to a certain extent of biological process of tissue self-assembly and morphogenesis during early heart formation.

Commonly reported birth defects are heart related, and the potential for generating cardiac defects is a primary concern in determining drug developmental toxicity (Pamies et al., 2011). The cardiac organoid model allowed us to evaluate human-specific drug-induced developmental toxicity based on its' disruption

of forming correct 3D organoid structures and developing normal cardiac contractile functions. By exposing the cardiac organoids to a range of drugs with different risk, we found an overall increase of teratogenic severity on cardiac organoid formation, corresponding to an increase of test concentrations, and an increase of risk category from A to X. Especially, category D drugs (phenytoin, lithium, doxycycline and tretinoin) showed diverse effects on developmental toxicity. Surprisingly, exposure of lithium only showed mild developmental toxicity of slight reduction in cardiac differentiation and organoid formation, though there has been a long debate of this drugs' developmental toxicity, especially resulting in congenital heart defects (Hoberman et al., 1990; Shaikh Qureshi et al., 2014).

~~For the highly toxic drugs, doxycycline failed to create 3D organoids due to massive cell apoptosis at high concentrations, whereas exposure of tretinoin created supersized organoids, but abolished all cardiac differentiation.~~ Our drug response results of thalidomide were generally consistent with published works of embryotoxicity using both whole embryo and *in vitro* stem cell models. Previous work showed reduced cardiac differentiation efficiency in a hiPSC embryotoxicity test due to thalidomide treatment (Aikawa et al., 2014). A recent study demonstrated that thalidomide inhibited early mesoderm differentiation in chick embryos (Belair et al., 2020), which is the transitional stage during cardiac differentiation. From an EB-based cardiac differentiation assay, thalidomide treatment showed decreased EB volume, similar to the decreased organoid height and FWHM from our cardiac organoids. Retinoids actually act as morphogens in embryogenesis, guiding gastrulation and body axis formation (Piersma et al., 2017). Overdose of retinoids has been linked to cardiovascular and skeletal developmental malformations (Collins and Mao, 1999). ~~From iPSC models, retinoids impair metabolic responses involved in cell proliferation and differentiation (Palmer et al., 2017).~~ In our study, retinoids impaired the cardiac differentiation but promoted the formation of giant tissues. It is possible that progenitor cells in our retinoid-treated organoids retained a high proliferative capacity to give rise large tissue growth, but inhibited the terminal differentiation of cardiomyocytes (Drowley et al., 2020). Another possibility is that the cells were directed

to the endoderm lineages, as exposure to these compounds was shown to severely disrupt mesoderm formation (Liu et al., 2018).

Embryotoxicity assays based on WEC have been invaluable in drug toxicology for decades, because they can study drug effects on whole systematic biological processes (Augustine-rauch et al., 2010; Flick and Klug, 2006). Generally, WEC assays focus on drug toxicity on structural and morphological features, such as limb and appendage malformations, but suffer from species differences that can lead to inaccurate predictions in humans (He et al., 2014). In contrast, stem cell-based assays, including mEST and newly developed *in vitro* platforms using human pluripotent stem cells, offer a cheaper and less invasive method to measure drug toxicity on mammalian and human cell differentiation (Seiler and Spielmann, 2011). However, they cannot characterize tissue morphogenesis and organ formation. ~~Meaningful comparisons between these systems are difficult to draw due to significant variations in characterization and measurement readouts from each model.~~ In a comparison of triazole exposure to rat WEC, zebrafish WEC and mEST, the zebrafish tests showed the best correlation, followed by mEST tests, regarding their toxicity levels relative to *in vivo* studies conducted in industry (de Jong et al., 2011). Rat WEC had the lowest correlation scores, which was likely caused by differences in drug exposure times calculated for each system, illustrating the challenges in embryotoxicity model comparisons. Other studies on embryotoxicity indicated a comparable result between WEC and mEST models, but poor correlation with *in vivo* reports (Dimopoulou et al., 2018; Inoue et al., 2016; Strikwold et al., 2012). Furthermore, these works suggest that a combination of different testing systems can provide better predictivity of embryotoxic potential. One study integrated mEST and zebrafish WEC to understand biological mechanisms of triclosan on early development (Chen et al., 2015), and found that triclosan causes developmental defects via disruption of pluripotent markers. In relation to the apparent heterogeneity of the cardiac organoids, we envision that the cardiac organoids can serve as complementary tests to current well-established assays to assess

teratogenicity in both cell differentiation and tissue morphogenesis, which can provide a comprehensive risk-assessment toolkit to better predict drug toxicity on fetal health.

Recently, fully 3D heart organoids have been reported that form from Matrigel embedded hPSC aggregates and develop into early heart and foregut endoderm tissues (Drakhlis et al., 2021). Though our method to micropattern hiPSCs and generate organoids is versatile and reproducible for various applications, one primary limitation is that the organoids are artificially attached to the bottom substrate, instead of embodying a fully 3D culture system. ~~Stimuli-responsive biomaterials can be explored in future to facilitate the on-demand detachment of organoids from surface with proper triggering (*e.g.* temperature changes).~~ Furthermore, the maturity of cardiac organoids has not been assessed, though we think the cardiomyocytes within our organoids are less mature compared to other engineered tissue model systems that applied different external stimulations to promote the maturation (Kolanowski et al., 2020; Nunes et al., 2013; Ronaldson-Bouchard et al., 2018). Additionally, the presence of stromal cells in cardiac microtissues has been shown to promote maturity as well (Hookway et al., 2019). Currently, our model is purposed to studying early developmental events and drug effects on embryonic cardiogenesis, instead of mimicking adult-like physiology and drug responses. Future work to expand this study, such as lineage tracking, fate mapping, and single cell genomic sequencing of organoids at different developmental stages, will reveal parallelism between human cardiac development and cardiac organoid formation through comprehensive molecular evidence. In the perspective of assay development for drug embryotoxicity screening, our cardiac-based model system presents a major limitation that it only focuses on cardiac differentiation as key assessment variables but cannot evaluate the developmental toxicity to a wide range of organ types (Seiler and Spielmann, 2011). Developmental toxicology can encompass a wide range of organ targets, depending on the molecular target of the specific drug, but may not particularly result in cardiac defects (Xu et al., 2020). Lastly, many drug compounds are safe in their native chemical makeups, but undergo

metabolism to produce toxic metabolites, which could lead to misclassifications of drug safety unless the screening can be achieved in a multi-organ system.

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Author contributions: Z.M. and P.H. conceived the study and designed the experiments. P.H. S.S. and A.A. performed surface patterning and organoid generation-related experiments. S.S. acquired the data of GCaMP6f organoids and performed the calcium flux analysis. A.K. performed data mining on contractile function data from cardiac organoids. T.W. performed flow cytometry analysis. A.G.E.C., A.G., W.L. and M.I.K generated the Yale WT hiPSC line and kindly provided to us for cell line comparison. P.H. performed all the organoid-based drug toxicity studies. P.H. and J.A. performed zebrafish whole embryo experiments. P.H. and S.L. collected and analyzed bulk motion tracking and image analysis data sets. P.H. and Z.M. wrote the manuscript with discussion and improvements from all authors. Z.M. supervised the project development and funded the study.

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Figure Legends

Figure 1. Human iPSC micropatterning and differentiation into cardiac organoids. (a) The procedure schematic for micropatterning hiPSCs on standard tissue culture plate using a selective PEG-based etching method. (b)Bright-field microscopy images of arrays of micropatterned hiPSCs with different sizes and shapes. Scale bar, 400 μm. (c) Spatial-organized cardiac organoids showed cardiac muscle on the top center with cardiomyocyte-specific proteins (cardiac troponin T (cTnT), β myosin heavy chain (MHC-β) sarcomeric α-actinin and cardiac troponin I (cTnI)) and smooth muscle-like cells on the perimeter of the organoids with stromal cell markers (smooth muscle actin (α-SMA), calponin, transgelin (α-SM22), and vimentin from 600 μm organoids. Scale bar, 200 μm. (d) Maximum intensity confocal projections of cardiomyocytes on the cardiac organoids. Scale bar, 50 μm. (e) Gene expression profile of cardiogenesis was compared between organoids of varying sizes with 2D monolayer differentiation. ΔCt values used in this gene map were calculated relative to the average Ct of GAPDH. HPRT1 and GUSB housekeeping controls.

Figure 2. Pattern size effects on cardiac organoid characteristics. (a) Cardiac organoids generated from the circular patterns with different sizes (200 μm – 1000 μm in diameter) showed spatial-organization of cardiomyocytes and stromal cells and 3D dome-shape in the X-Z plane. Scale bar, 200 μm. (b) The area ratio between cardiomyocyte staining and entire pattern size showed larger coverage but higher variation in cardiac muscle differentiation on the cardiac organoids of 200 μm, 800 μm and 1000 μm patterns ($n = 9$, $*p \leq 0.0001$ between 200 μm and 400/600 μm; $*p \leq 0.0001$ between 400/600 and 800/1000 μm). The cardiac organoids of 600 μm patterns exhibited better 3D morphology with largest values in (c) height ($n = 8$, $*p = 0.0126$ between 200 μm and 600 μm; $*p = 0.0126$ between 600 and 800/1000 μm) and (d) FWHM ($n = 8$, $*p \leq 0.0001$ between 600 μm and 200/400 μm; $*p \leq 0.0001$ between 600 and 800/1000 μm). (e) Contractile function parameters were used to cluster individual organoids with different pattern sizes and generate a t-SNE plot to evaluate organoid-to-organoid correlations. t-SNE gradients were plotted for (f)

beat rate, (g) maximum contraction velocity, (h) τ_{75}, and (i) area ratio. (j) Heat map illustrating all contractile functions parameters relative to pattern size showed strong correlation between size and prolonged contraction duration, especially for the organoids of small patterns. (k) Correlation plot illustrated proportional relationships between contraction duration parameters, but inverse correlations between contraction duration and contraction rate. All box plots show the minimum, maximum, median, and 25th and 75th percentiles, and statistical analysis was performed based on analysis of variance (ANOVA) with Tukey's multiple comparison test.

Figure 3. Cardiac organoids as a developmental toxicity screening assay. (a) The timeline of cardiac organoid generation and continuous drug exposure. Epi-fluorescent microscopy images of the cardiac organoids for comparison between untreated controls with (b) doxylamine succinate (category A drug) and (c) thalidomide (category X drug). The area ratio of cardiac muscle coverage showed (d) an increase with doxylamine succinate ($n = 29$, $*p \leq 0.0001$), but a decrease with thalidomide at high concentration ($n \geq 28$, $*p \leq 0.0001$). The contraction velocity of cardiac contractile motion showed no drug effect from (f) either doxylamine succinate (g) or thalidomide. The beat rate showed (h) a decrease with doxylamine succinate ($n \geq 9$, $*p \leq 0.0001$), and an increase with thalidomide at 1 μM and 10 μM concentrations ($n \geq 12$, $*p \leq 0.0001$). (j) The cardiac organoids under doxylamine succinate exposure showed no drug effects on the 3D organoid morphology at all the tested concentrations. (k) Thalidomide exposure induced significant structural impairment on 3D organoid formation, showed as lower height ($n \geq 9$, $*p \leq 0.0001$) and FWHM ($n \geq 14$, $*p \leq 0.0001$) at high concentrations. Representative confocal projections of cardiac organoids at (k) untreated control and (m) 100 μM thalidomide exposure showed severe abnormal organoid formation for quantitative morphological scoring. (n) Cardiac looping scored as a cardiac developmental toxicity evaluation in the zebrafish whole embryo culture assay (zWEC) showed no significant drug effect from doxylamine succinate exposure, and a very mild toxicity level from high dosage exposure of thalidomide. A sample size of $n>40$ embryos were analyzed for these treatment groups. In all panels, box plots show the

minimum, maximum, median, and 25th and 75th percentiles, and statistical analysis was performed based on analysis of variance (ANOVA) with Dunnett's multiple comparison test against controls.