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# Modeling trastuzumab-related cardiotoxicity *in vitro* using human stem cell-derived cardiomyocytes

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ABSTRACT

Trastuzumab (Herceptin®), a monoclonal antibody against the ErbB2 (HER2) receptor, has significantly improved clinical outcomes for HER2$^+$ breast cancer patients. However, the drug also has known cardiotoxic side effects through mechanisms that are not fully understood. Here we utilized human induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs) to model trastuzumab-related cardiotoxicity *in vitro*. We demonstrate that cardiotoxic effects of ErbB2 inhibition by trastuzumab can be recapitulated only when the cardioprotective effects of ErbB2/4 signaling is observed. We observed no cardioprotective effects of ErbB2/4 signaling without cellular stress (doxorubicin exposure in this study). In addition to neuregulin-1 (NRG-1), we show that heparin-binding epidermal growth factor-like growth factor (HB-EGF) also provides cardioprotective effects for iPS-CMs. Finally, we demonstrate a simple, high-throughput co-culture platform utilizing iPS-CMs and endothelial cells that is capable of detecting trastuzumab-related cardiotoxicity. We conclude that iPS-CMs can recapitulate trastuzumab-related cardiotoxicity, and may be used to elucidate additional modes of toxicity of trastuzumab and related compounds.

## 1. Introduction

Cardiotoxicity screening using human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) has rapidly evolved over the past decade. Initial efforts, including the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative, have focused on using hPSC-CMs to determine the arrhythmogenic potential of drugs, as several FDA-approved drugs have been pulled off the market due to proarrhythmogenic side effects (Cavero and Holzgrefe, 2014; Fermini et al., 2016; Gintant et al., 2016). More recently, researchers have explored the use of hPSC-CMs to better understand drug-induced structural cardiotoxicity, defined as compounds that lead to decreased cardiomyocyte viability (Clements et al., 2015; Doherty et al., 2015; Pointon et al., 2013; Sharma et al., 2017). Given the wide use of anticancer drugs with known clinical cardiotoxicity through loss of cardiomyocytes (*e.g.* anthracyclines such as doxorubicin) (Ewer and Ewer, 2015), it is especially important to have a human *in vitro* platform that can rapidly screen new compounds for potential structural cardiotoxicity. Several recent studies have leveraged hPSC-CMs to develop a mechanistic understanding of doxorubicin-induced cardiotoxicity (Burridge et al., 2016; Holmgren et al., 2015; Maillet et al., 2016; Zhao and Zhang, 2017).

Separate from anthracyclines, the use of targeted drugs that inhibit specific pathways critical for cancer progression have also gained broad clinical use (Brown, 2016). These compounds have led to improved clinical outcomes in certain cancer types; however, incidents of clinical cardiotoxicity associated with these drugs have been reported (Cross et al., 2015; Force and Kolaja, 2011). One such compound is trastuzumab (Herceptin®), a monoclonal antibody against ErbB2 (HER2) used to treat patients with HER2$^+$ breast cancer (Slamon et al., 2001). Trastuzumab, administered with an anthracycline or on its own, significantly increases the incidence of left ventricular dysfunction (Cardinale et al., 2010; Feldman et al., 2000; Narayan et al., 2017; Seidman et al., 2002; Slamon et al., 2001). Animal studies have shown that ErbB2, together with its co-receptor ErbB4 and its activating ligand neuregulin-1 (NRG-1) are critical for normal cardiac development and homeostasis (Crone et al., 2002; Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). Studies using isolated rat cardiomyocytes have shown that activation of the ErbB2/4 pathway with NRG-1 ameliorates anthracycline-induced cardiotoxicity, suggesting a mechanism for trastuzumab's cardiotoxic effects (Fukazawa et al., 2003; Sawyer et al., 2002).

The objective of our study is to utilize human iPS-CMs to model trastuzumab-related cardiotoxicity. We hypothesize that the ErbB2/4 pathway must be active in order to detect trastuzumab-related

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cardiotoxicity and that the toxicity is mediated by the inhibition of the cardioprotective effects of ErbB2/4 signaling. We first explore the effects of trastuzumab on iPS-CMs with or without ligands activating the ErbB2/4 pathway. We observe that the cardioprotective effects of ErbB2/4 signaling is observed only in the presence of a cardiotoxic compound (doxorubicin in this study). We demonstrate that under conditions where we observe cardioprotective effects of ErbB2/4 signaling, we can observe the cardiotoxic effects of trastuzumab *via* ErbB2 inhibition. In addition to NRG-1, we show that heparin-binding EGF-like growth factor (HB-EGF) similarly activates the ErbB2/4 pathway and provides cardioprotective effects. We also demonstrate that a CM-EC co-culture platform enables the detection of trastuzumab-related cardiotoxicity through the activation of the ErbB2/4 pathway *via* EC-secreted NRG-1. Our results demonstrate the potential of using hPSC-CMs to detect pathway-specific cardiomyocyte toxicities that impact viability.

## 2. Materials and methods

### 2.1. Cell culture

Wild-type human iPSCs reprogrammed from dermal fibroblasts of a healthy male volunteer (cell line WTC-11) were cultured as previously described (gifted by Dr. Bruce Conklin, Gladstone Institutes) (Kurokawa et al., 2017). The iPSCs express the calcium indicator GCaMP6f, which increases in fluorescent intensity in response to increasing concentrations of $Ca^{2+}$ in the cytosol (Chen et al., 2013; Huebsch et al., 2015). Routine checks for mycoplasma were performed every 6 months using MycoAlert mycoplasma detection kit (Lonza) following manufacturer's protocol. The iPSCs were used between passages 40–60.

Endothelial colony forming cell-derived endothelial cells (ECFC-ECs) were isolated and cultured in endothelial growth medium-2 (EGM-2, Lonza) as described previously (Moya et al., 2013). The ECFC-ECs were used between passages 5–8.

### 2.2. Cardiomyocyte differentiation

A small molecule Wnt modulatory protocol was used as previously described, with modifications (Fig. S1A) (Lee et al., 2015; Lian et al., 2012). Briefly, iPSCs were grown to ~85% confluence on 6-well plates coated with growth factor reduced Matrigel (Corning) in Essential 8 (E8) medium (Gibco). On Day 0, the medium was changed to RPMI 1640 (Gibco) with B-27 supplement without insulin (RPMI/B-27 −Ins, Gibco) containing 6 μM CHIR99021 (LC Laboratories). On Day 2, the medium was changed to RPMI/B-27 −Ins. On Day 3, the medium was changed to RPMI/B-27 −Ins containing 5 μM IWP2 (Tocris). On Day 5, the medium was changed to RPMI/B-27 −Ins. On Day 7 and 10, the medium was changed to RPMI/B-27 (with insulin), with spontaneously contracting cardiomyocytes appearing on Day 8. Non-cardiomyocytes were removed using lactate selection (Tohyama et al., 2013), changing the medium every two days between Day 13 and 21 with RPMI 1640 without glucose, with 4 mM lactic acid (Sigma-Aldrich), and 25 mM HEPES (Gibco). On Day 24, the iPS-CMs were passaged by incubating in 200 U/ml collagenase II (GIbco) for 1 h followed by TrypLE Express (Gibco) for 4 min. The cells were either cryopreserved using CryoStor10 (STEMCELL Technologies) or replated on Matrigel-coated 6-well plates.

### 2.3. Flow cytometry

The iPS-CMs were passaged between Day 27–30 and fixed in Fixation Buffer (BioLegend) for 20 min. The cells were permeabilized in PBS + 0.1% Triton-X (Sigma-Aldrich) and stained using APC-pre-conjugated anti-cardiac troponin T (cTnT) antibody (Miltenyi Biotec) or a APC-preconjugated isotype control following manufacturer's protocol. The samples were read using the Guava easyCyte flow cytometer (Millipore) and analyzed using FlowJo.

### 2.4. Drug exposure

For all drug exposure studies, the iPS-CMs were passaged and replated on Matrigel-coated 96-well plates at 50,000 cells/well in RPMI/B-27. The cells were cultured for 4 additional days with a medium change 2 days before drug exposure. The targeted concentrations of doxorubicin (LC Laboratories), NRG-1β (R&D Systems), HB-EGF (R&D Systems), trastuzumab (Genentech), and/or corresponding volumes of vehicle control (Milli-Q ultrapure water) was added to the media as specified. For the single-dose analysis, the following concentrations were used: 10 μM doxorubicin, 1 μM trastuzumab, 1 ng/ml NRG-1, and 100 ng/ml HB-EGF. Analysis was performed 72 h after exposure to drugs. The drug exposure studies were performed using iPS-CMs between Day 30–40 of differentiation.

### 2.5. Lactate dehydrogenase (LDH) measurement

After 72 h of drug exposure, the LDH release was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) following manufacturer's protocol. Absorbance was measured using the Epoch microplate spectrophotometer (BioTek). For dose-dependent analysis, measured absorbance values were calculated relative to the average of the no-drug control condition ($A_{control}$). For column analysis, the measured values were first baseline-subtracted using the average of the untreated control group, then normalized to the average of the doxorubicin group ($A_{DOX}$) using the formula: normalized LDH release = $(A_{measured} - A_{control})/(A_{DOX} - A_{control})$. In these cases, the untreated control group is not shown (mean = 0).

### 2.6. CM-EC co-culture

Both iPS-CMs and ECFC-ECs were passaged and mixed at the density to seed 40,000 and 2500 iPS-CMs and ECFC-ECs per well, respectively, in a Matrigel-coated 96-well plate. The cells were fed with EGM-2 for 3 days before drug exposure. The drug exposure was performed and analyzed as described for the iPS-CM mono-culture experiments.

### 2.7. Quantitative real-time PCR

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following manufacturer's protocols, and cDNA was produced using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Gene expression was measured using Taqman Gene Expression Assays (*NRG1*: Hs01101538_m1, *18S*: Hs99999901_s1) using the CFX96 Real-Time PCR Detection System (Bio-Rad). Relative expression was calculated using the comparative $C_t$ method (Schmittgen and Livak, 2008), normalizing the expression level to the iPSCs.

### 2.8. Statistical analysis

All experiments were performed using at least 3 biological replicates per condition, and all results are reported as mean ± SD. Statistical analysis was performed using GraphPad Prism 6. For column analysis, significance was calculated using one-way analysis of variance (ANOVA) in conjunction with Tukey's multiple comparison test. For dose-response analysis, significance was calculated using two-way ANOVA in conjunction with Dunnett's (comparing dose-response to its control) or Holm-Sidak's (comparing dose-response across different curves) multiple comparison test, with p-values less than 0.05 considered statistically significant. Data were compiled, analyzed, and graphed using Microsoft Excel and GraphPad Prism 6.