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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.

3. Results

3.1. Differentiation and isolation of iPS-CMs

We differentiated the iPS-CMs using an established Wnt modulatory protocol, which was followed by a metabolic selection whereby cardiomyocytes were enriched through their ability to utilize lactate as an energy source. The resulting cell population stained positively for cTnT at 95.08 ± 2.06% as measured by flow cytometry (Fig. S1B). A monolayer of iPS-CMs replated in a 96-well plate and stained for cTnT demonstrated semi-organized troponin structures (Fig. S1C), indicative of the immature phenotype of iPS-CMs (Robertson et al., 2013; Yang et al., 2014).

3.2. Trastuzumab-related cardiotoxicity at baseline conditions

We first exposed iPS-CMs to trastuzumab at a baseline condition where the medium contained no ligands activating the ErbB2/4 pathway. Unsurprisingly, we did not detect a significant cardiotoxic effect of trastuzumab as measured by LDH release at any concentration (Fig. 1). We also tested for trastuzumab-related cardiotoxicity using iPS-CMs with NRG-1 supplemented into the culture medium. The addition of trastuzumab under this condition also led to no cardiotoxic effects as measured by LDH release (Fig. 1). We also observed that the addition of NRG-1 did not decrease LDH release compared to the untreated control (decrease of 5.4 ± 10.7%, p = 0.51 between control and +NRG-1 without trastuzumab) (Fig. 1). The data suggest that the activation and/or inhibition of ErbB2/4 does not affect iPS-CM viability at baseline conditions.

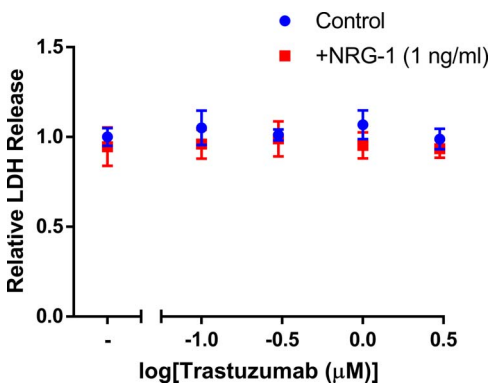

Fig. 1. Trastuzumab-related cardiotoxicity at baseline conditions. We observe no increase in relative LDH release with increasing concentrations of trastuzumab with or without NRG-1. We also observe no cardioprotective effects of NRG-1 compared to control. (−) indicates no trastuzumab.

3.3. Cardioprotective effects of NRG-1

As previous studies have shown NRG-1 to provide cardioprotective effects against cardiac stress, we studied whether we could detect the cardioprotective effects of NRG-1 using iPS-CMs exposed to cardiotoxic compounds. For this purpose, we utilized doxorubicin, an anthracycline commonly used in conjunction with trastuzumab to treat breast cancer patients, which has known cardiotoxic side effects. We initially examined the dose-dependent response of iPS-CMs to doxorubicin with or without NRG-1 (Fig. 2A). The addition of NRG-1 provided cardioprotective effects, significantly reducing the LDH release by iPS-CMs and shifting the half-maximal effective concentration (EC_{50}) from 163.9 μM to 263.6 μM (95% confidence interval of 126.1–213.1 μM to 181.8–382.2 μM, $p < 0.05$). The cardioprotective effects were most evident at the higher concentrations of doxorubicin, with statistically significant differences between each point of the curves above 10 μM doxorubicin concentration. Therefore, all

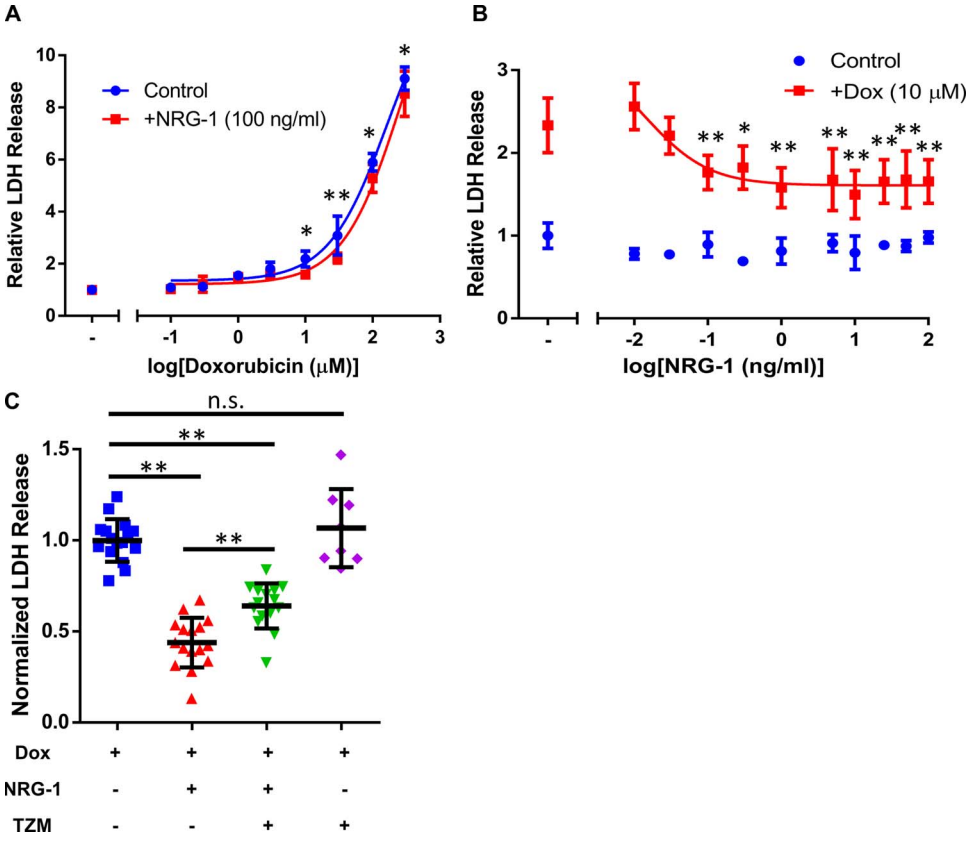

Fig. 2. Trastuzumab inhibits cardioprotective effects of NRG-1. (A) A dose-response curve of doxorubicin with or without the presence of NRG-1 as measured by relative LDH release. The addition of NRG-1 results in a statistically significant change in the EC_{50} from 163.9 μM to 263.6 μM (95% confidence interval of 126.1-213.1 μM to 181.8-382.2 μM, $p < 0.05$). (−) indicates no doxorubicin. *$p < 0.05$, **$p < 0.005$ between the control and +NRG-1 for the given doxorubicin concentration. (B) Dose-response curve of NRG-1 with or without the presence of doxorubicin as measured by relative LDH release. (−) indicates no NRG-1. *$p < 0.05$, **$p < 0.005$ compared to control condition with doxorubicin (no NRG-1). (C) Trastuzumab (1 μM), combined with doxorubicin (10 μM) and NRG-1 (1 ng/ml), increases the normalized LDH release compared to having doxorubicin and NRG-1 without trastuzumab. Adding trastuzumab and doxorubicin without the addition of NRG-1 does not increase LDH release compared to having doxorubicin alone. **$p < 0.005$. Dox = doxorubicin, TZM = trastuzumab.

subsequent studies utilized doxorubicin concentration of 10 μM, which falls within the range of *in vivo* peak plasma concentration of doxorubicin (Burridge et al., 2016).

Next, we observed that the cardioprotective effects of NRG-1 was dose-dependent, with concentrations above 0.1 ng/ml causing a statistically significant reduction in LDH release in response to doxorubicin (Fig. 2B). We also observed no significant changes in LDH release following the addition of NRG-1 at any concentration without doxorubicin exposure, suggesting that the cardioprotective effects of NRG-1 is only detectable under cellular stress. Subsequent experiments utilized NRG-1 concentration of 1 ng/ml, which is in the range of average NRG-1 plasma concentration (Geisberg et al., 2011).

3.4. Trastuzumab inhibits cardioprotective effects of NRG-1

After observing the cardioprotective effects of NRG-1, we tested to see if trastuzumab-related cardiotoxicity can be recapitulated using the same culture condition. In the presence of both NRG-1 and doxorubicin, we observed cardiotoxic effects of trastuzumab (Fig. 2C). While the addition of NRG-1 resulted in a 56 ± 14% decrease in normalized LDH release compared to doxorubicin alone, the addition of both NRG-1 and trastuzumab led to a smaller decrease (36 ± 12%); in other words, comparing the two conditions with both doxorubicin and NRG-1, the addition of trastuzumab led to a 58 ± 38% increase in normalized LDH release. No difference was observed by the addition of trastuzumab and doxorubicin (without NRG-1) compared to doxorubicin alone (increase of 6.8 ± 21.4%, $p = 0.69$), suggesting that trastuzumab is specifically inhibiting the cardioprotective effects of ErbB2/4 signaling activated by NRG-1.

3.5. Cardioprotective effect of HB-EGF

Next, we explored whether we can observe a similar cardioprotective effect using HB-EGF, another ligand that binds to ErbB4 and activates the ErbB2/4 pathway. Similar to NRG-1, we observed a dose-dependent cardioprotective effect of HB-EGF against doxorubicin-induced cardiotoxicity (Fig. 3A). We also did not observe a change in LDH release by the addition of HB-EGF without the presence of doxorubicin. In the presence of HB-EGF and doxorubicin, we also detected the cardiotoxic effects of trastuzumab (Fig. 3B). The addition of HB-EGF and doxorubicin resulted in a 26 ± 19% reduction in the normalized LDH release compared to doxorubicin alone, but the addition of both HB-EGF and trastuzumab removed any statistically significant cardioprotective effects compared to doxorubicin alone (decrease of 7.8 ± 23%, $p = 0.63$). Comparing the two conditions with both doxorubicin and HB-EGF, the addition of trastuzumab resulted in a 27 ± 24% increase in normalized LDH release.

Comparing the degree of cardioprotection provided by NRG-1 and HB-EGF, we observed that NRG-1 significantly reduced the normalized LDH release compared to HB-EGF (Fig. 3C). Importantly, the addition of both NRG-1 and HB-EGF did not provide any additional cardioprotective effects compared to NRG-1 alone (56 ± 14% *vs.* 63 ± 5%, $p = 0.55$).

3.6. CM-EC co-culture platform

Since NRG-1 is mainly secreted by endothelial cells in the cardiac microenvironment (Brutsaert, 2003), we aimed to recapitulate this phenomenon using a co-culture of iPS-CMs and ECFC-ECs. First, we measured that NRG-1 is expressed by the ECFC-ECs using qPCR and ELISA. Compared to iPSCs and iPS-CMs, the ECFC-ECs have

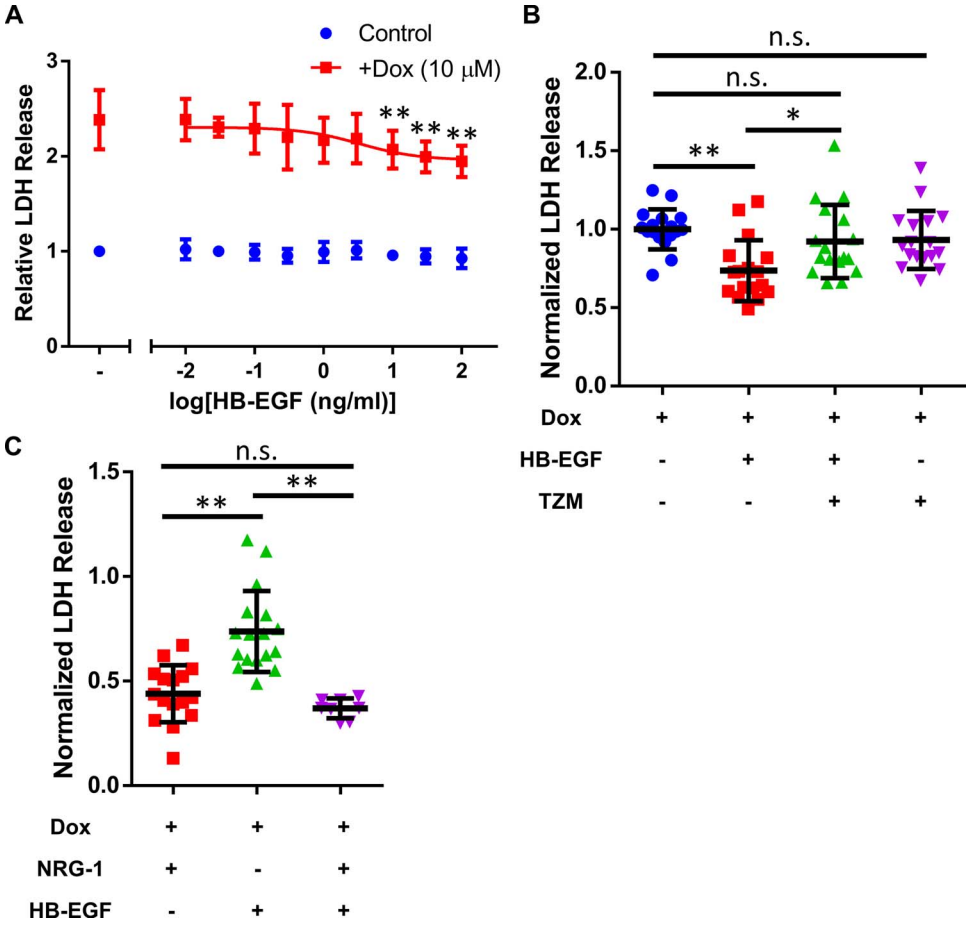

Fig. 3. Cardioprotective effects of HB-EGF. (A) A dose-response curve of HB-EGF with or without the presence of HB-EGF as measured by relative LDH release. (−) indicates no HB-EGF. **p < 0.005 compared to control condition with doxorubicin (no HB-EGF). (B) The addition of HB-EGF (100 ng/ml) with doxorubicin enables the detection of trastuzumab-related cardiotoxicity as measured by increased normalized LDH release. *p < 0.05, **p < 0.005. (C) No added cardioprotective effects were detected by adding both NRG-1 (1 ng/ml) and HB-EGF (100 ng/ml) compared to NRG-1 alone as measured by normalized LDH release. **p < 0.005.

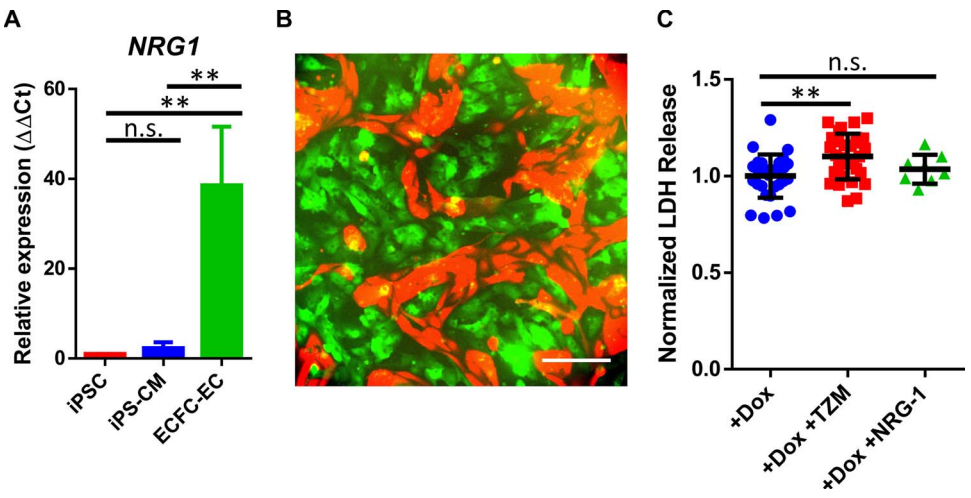

Fig. 4. Co-culture of iPS-CM and ECFC-EC. (A) Relative expression of *NRG1* between the iPSCs, iPS-CMs, and ECFC-ECs. **p < 0.005. (B) Representative image of the CM-EC co-culture. Green = iPS-CM (GCaMP6), red = ECFC-EC (transduced). Scale bar = 250 μm. (C) The addition of trastuzumab with doxorubicin increases normalized LDH release compared to doxorubicin alone. **p < 0.005.

significantly higher *NRG1* expression (Fig. 4A). The secretion of NRG-1 into the growth medium was confirmed using ELISA, with protein concentrations reaching 604 ± 142 pg/ml in 48 h. This level of NRG-1 is sufficient to observe a significant cardioprotective effect based on the NRG-1 dose-response experiment (Fig. 1B).

In order to produce a simple and reproducible co-culture platform that is amenable to high-throughput analysis, we utilized a monolayer co-culture in 96-well plates. The iPS-CMs and ECFC-ECs were seeded at a ratio of 16:1 (40,000:2500) in order to promote even distribution of the two cell types (Fig. 4B), whereas using higher concentrations of ECFC-ECs resulted in the overcrowding of ECFC-ECs (Fig. S2A). Using this co-culture platform, we were able to detect the cardiotoxic effects of trastuzumab. The addition of both doxorubicin and trastuzumab increased the normalized LDH release by 10 ± 12% compared to doxorubicin alone, which was statistically significant. The addition of exogenous NRG-1 did not result in a reduced LDH release, suggesting that the cardioprotective effects of ErbB2/4 signaling is already active *via* EC-secreted NRG-1. The addition of trastuzumab to ECFC-ECs (without iPS-CMs) led to no increase in normalized LDH release (Fig. S2B).

4. Discussion

Since the advent of hPSC-CMs, their potential use in drug screening applications has rapidly expanded (Gintant et al., 2017). In our study, we demonstrate that iPS-CMs are capable of detecting trastuzumab-related cardiotoxicity when ErbB2/4 signaling is active, suggesting that trastuzumab is blocking the cardioprotective effects of the ErbB2/4 pathway. While NRG-1 is recognized as the main activator of the ErbB2/4 pathway in the cardiac microenvironment, we demonstrate that HB-EGF can also activate the same cardioprotective pathway. Additionally, we demonstrate that a co-culture platform of iPS-CMs and ECs recapitulates the physiological effects of EC-secreted NRG-1 activating the ErbB2/4 pathway.

ErbB2/4 signaling has critical functions in cardiac development, including trabeculation and formation of the conduction system (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Rentschler et al., 2002). While previous studies have explored the role of ErbB2/4 signaling in hPSC-CM differentiation (Ramachandra et al., 2016; Zhu et al., 2010), it remained unclear whether hPSC-CMs can recapitulate the cardiotoxic effects of ErbB2 inhibition *via* trastuzumab exposure. Here we demonstrate that, while functionally immature, iPS-CMs can recapitulate the cardioprotective effects of ErbB2/4 signaling and the cardiotoxic effects of ErbB2 inhibition. Without the addition of NRG-1 or HB-EGF to activate the ErbB2/4 pathway, we were not able to detect trastuzumab-related cardiotoxicity. This result differs from two recent studies that observed changes in cell impedasnce and gene expression when hPSC-CMs were exposed to trastuzumab without the addition of NRG-1 or HB-EGF (Eldridge et al., 2014; Necela et al., 2017). Interestingly, both studies used a commercially available source of iPS-CMs and a proprietary maintenance medium, which may contain growth factors that activate the ErbB2/4 pathway. The proprietary medium also contains animal-derived serum, which has inherent variability between lots and can mask the effects of drugs (Dambrot et al., 2014; Tiburcy et al., 2017). While we utilized a serum-free growth medium, it will be important to develop a chemically-defined medium that contains growth factors appropriate for the cardiac microenvironment in order to improve the relevance of iPS-CMs for drug screening applications.

Trastuzumab has clinically been shown to induce cardiotoxic side effects including reduction in the left ventricular ejection fraction (LVEF) and increases in the serum cardiac troponin I (cTnI) concentration, suggesting ErbB2 inhibition directly affects cardiomyocyte viability (Cardinale et al., 2010; Seidman et al., 2002). Loss of cardiomyocytes is largely irreversible in the adult heart, and a recent study showed that treatments using trastuzumab (with or without doxorubicin) resulted in reduced LVEF that does not recover back to baseline after 5 years (Narayan et al., 2017). On the other hand, we observed that the addition of doxorubicin was necessary to detect trastuzumab-related cardiotoxicity using iPS-CMs. The finding supports the existing theory that trastuzumab potentiates pre-existing cardiac stress (drug-induced or otherwise) by inhibiting the cardioprotective effects of ErbB2/4 signaling (Chien, 2006; Plana et al., 2014; Tocchetti et al., 2012). Mice with cardiac deletion of *ErbB2* led to increased susceptibility to anthracycline-induced cardiomyocyte death, suggesting that the physiological functions of ErbB2/4 signaling are critical in preventing the progression of cardiac injury (Crone et al., 2002; Ozcelik et al., 2002). No overt phenotype initially appears in these mice, consistent with our finding that ErbB2 inhibition on its own does not induce acute cardiotoxic effects. Other potential mechanisms of trastuzumab-related cardiotoxicity have been proposed (Barth et al., 2012; Collins et al., 2012; Kirabo et al., 2017; Wilkinson et al., 2016), which warrant further exploration.

While NRG-1 is widely known as the activating ligand of the ErbB2/4 pathway, we identify HB-EGF as having a similar pattern of activation that results in cardioprotective effects against doxorubicin. The addition of both NRG-1 and HB-EGF did not confer additive cardioprotective effects, suggesting that both ligands activate ErbB2/4 signaling that is saturated by the addition of NRG-1 alone at the tested concentrations. Therapeutic use of recombinant NRG-1 has been extensively explored in animal models, showing improved cardiac function in disease models such as myocardial infarction and doxorubicin-induced cardiomyopathy (Vermeulen et al., 2016). Clinical trials have also shown promising data on treating chronic heart failure patients using recombinant

NRG-1 (Gao et al., 2010; Lenihan et al., 2016). HB-EGF has been shown to be critical in cardiac development and homeostasis in mice through ErbB2/4 activation (Iwamoto et al., 2003), but its potential therapeutic use in treating cardiovascular disease has remained largely unexplored. While the cardioprotective effects of HB-EGF was less pronounced compared to NRG-1 in our study, the clinical potential of HB-EGF warrants further investigation.

One limitation in our study is that we utilized a single iPSC line. Recent studies have demonstrated that clinically-relevant cardiotoxicity of doxorubicin and sotalol can be predicted using iPS-CMs differentiated from patient-derived iPSCs (Burridge et al., 2016; Stillitano et al., 2017). Much like doxorubicin, not every patient treated using trastuzumab shows clinical symptoms of cardiac dysfunction (Onitilo et al., 2014). It remains to be seen if trastuzumab-related cardiotoxicity is a phenotype that is similarly dependent on the genetic background of the patient and can be recapitulated using patient-derived iPS-CMs. Such patient-specific models can be utilized to further our mechanistic understanding of trastuzumab-related cardiotoxicity and aid in the development of therapeutic targets to more effectively combat trastuzumab-related cardiotoxicity. While primary ECs were used in this study due to their defined phenotype, it will also be critical to develop a co-culture platform that incorporates iPSC-derived ECs in order to perform patient-specific drug screening.

Another limitation in our study is the use of LDH release as the readout of cardiotoxicity. LDH release is a clinically-relevant readout for cardiac injury but lacks specificity (Mair, 1997). We observed that in the CM-EC co-culture platform, the ECs contributed a non-negligible amount of LDH, which resulted in decreased sensitivity in detecting LDH release by the cardiomyocytes. While more costly and time-consuming, cardiomyocyte-specific markers of toxicity, such as cTnI and cTnT release, can be utilized in the future for improved sensitivity and specificity in cardiotoxicity screening. For screening drugs of unknown mechanism of action, such methods will be required in order to distinguish between cardiac and endothelial toxicities.

In conclusion, we demonstrate that iPS-CMs can recapitulate the cardiotoxic effects of ErbB2 inhibition by trastuzumab. We also establish a simple and high-throughput CM-EC co-culture platform that recapitulates physiological crosstalk between the two cell types including activation of ErbB2/4 signaling *via* EC-secreted NRG-1. Further advancements in our ability to model the human cardiac microenvironment *in vitro* will be critical in broadening our understanding of trastuzumab-related cardiotoxicity.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxlet.2018.01.001.

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