Calreticulin Is Required for TGF-β-Induced Epithelial-to-Mesenchymal Transition during Cardiogenesis in Mouse Embryonic Stem Cells

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SUMMARY

Calreticulin, a multifunctional endoplasmic reticulum resident protein, is required for TGF-β-induced epithelial-to-mesenchymal transition (EMT) and subsequent cardiomyogenesis. Using embryoid bodies (EBs) derived from calreticulin-null and wild-type (WT) embryonic stem cells (ESCs), we show that expression of EMT and cardiac differentiation markers is induced during differentiation of WT EBs. This induction is inhibited in the absence of calreticulin and can be mimicked by inhibiting TGF-β signaling in WT cells. The presence of calreticulin in WT cells permits TGF-β-mediated signaling via AKT/GSK3β and promotes repression of E-cadherin by SNAIL2/SLUG. This is paralleled by induction of N-cadherin in a process known as the cadherin switch. We show that regulated Ca²⁺ signaling between calreticulum (ER) (Michalak et al., 2009), is highly expressed in the early embryonic heart. It is silenced in the adult heart, highlighting its importance as a key regulator of cardiac differentiation progresses (Imanaka-Yoshida et al., 1996; Park et al., 1998; Liu et al., 2002; Franzini-Armstrong et al., 2005). Among the Ca²⁺-handling proteins, calreticulin, an evolutionarily conserved, major Ca²⁺ storage protein of the endoplasmic reticulum (ER) (Michalak et al., 2009), is highly expressed in the early embryonic heart. It is silenced in the adult heart, highlighting its importance as a key regulator of cardiac differentiation (Michalak et al., 2002a, 2004; Papp et al., 2009b; Faustino et al., 2016). The crucial role of calreticulin during cardiogenesis has been demonstrated by the lethal phenotype of calreticulin-deficient mice due to cardiac defects (Mesaelli et al., 1999; Rauch et al., 2000) and disorganized cardiomyogenesis in calreticulin-deficient ESCs (Li et al., 2002; Papp et al., 2009a, 2009b; Faustino et al., 2010). On the other hand, overexpression of calreticulin is also lethal due to conductivity problems (Nakamura et al., 2001; Mery et al., 2005; Hattori et al., 2007), underscoring the importance of calreticulin in both cardiac development and function (Papp et al., 2008; Wang et al., 2012).

The molecular mechanisms underlying calreticulin’s effects on cardiac development continue to be elucidated. It is known that intranuclear translocation of cardiogenic transcription factors is affected by calcineurin, a Ca²⁺/calmodulin-regulated cytosolic phosphatase (Crabtree, 1999; Frey et al., 2000; Crabtree and Schreiber, 2009). The Ca²⁺ influx required to activate calcineurin depends on the sustained release of Ca²⁺ from ER stores (Crabtree, 2001), which is dependent on calreticulin abundance (Michalak et al., 2002b). Importantly, overexpression of constitutively activated calcineurin specifically targeted to the heart rescues the lethal cardiac phenotype in calreticulin-null (CRT-KO) mice (Guo et al., 2002). Lynch and Michalak (2003) elegantly demonstrated that calreticulin is an upstream regulator of calcineurin, thus providing a mechanism for the control of early cardiogenesis.

Gene regulation, cellular migration, and cell-cell and cell-substratum communication during cardiogenesis involve a variety of intrinsic and extrinsic factors (Maltev et al., 1993; Hescheler et al., 1997; Sachinidis et al., 2003). Epithelial-to-mesenchymal transition (EMT), has been identified as one of the first steps of cardiac differentiation (Thiery and Sleeman, 2006; Thiery et al., 2009; Kovacic et al., 2012). Expression of N-cadherin and the transcription factors SNAIL1 (SNAIL), SNAIL2 (SLUG), TWIST1, delta-EF1 (ΔEF1), and SIP1/ZEβ2 are increased during EMT, while E-cadherin expression is decreased (Lee et al., 2006; Zeisberg and Neilson, 2009). A multitude of transcription factors play a role in cardiomyocyte specification, including NKX2-5, MEF2c, GATA 4,5,6, MESP1, MYOCARDIN, TBX5, and TBX20, which work in a combinatorial manner to turn on the transcriptional activity of cardiac gene promoters (Droother and Nemer, 1998; Brand, 2003; Olson, 2006). Also, pleiotropic growth factors, most notably transforming growth factor β (TGF-β), induce cardiac
Calreticulin promotes cardiac differentiation

**Figure 1. Calreticulin Absence Impairs Cardiac Differentiation in Mouse ES Cells In Vitro**

(A) Functional analysis of EBs during cardiac differentiation in WT and CRT-KO and CN cells. WT and CN EBs begin to beat earlier and the percentage of beating is higher in WT and CN EBs throughout the differentiation compared with the CRT-KO. Beating EBs were counted at day 3 (D3), day 5 (D5), day 10 (D10), and day 14 (D14) of differentiation.

(B) Quantification of cardiac marker protein expression by intracellular flow cytometry using anti-cardiac troponin I (Alexa Fluor 488) antibody on D14 WT, CRT-KO, and CN EBs, with rabbit monoclonal IgG (Alexa Fluor 488) used at the same concentration and conditions as primary antibody in WT EBs as control. Representative plot analysis corresponds to the fluorescence intensity at 488 nm on the x axis and the y axis corresponds to live cell counts. The graph shows mean florescent intensity (MFI), and the bars represent the SEM of the values from three individual experiments.

(C) Western blot analysis shows the protein level of cardiac MHC increases in WT and CRT-KO cells during differentiation.

(D) Bar graph shows quantification of cardiac MHC band density versus GAPDH from three independent experiments.

(legend continued on next page)
development in general (Schneider and Parker, 1991; MacLellan et al., 1993; Schneider et al., 1994) and cardiac differentiation from ESCs in particular (Behfar et al., 2002; Sachinidis et al., 2003; Lim et al., 2007; Faustino et al., 2008), reviewed in (Puceat, 2007; Kovacic et al., 2012). It has also been established that TGF-β induces EMT (Zavadil and Böttinger, 2005; Xu et al., 2009; Lamouille et al., 2014), thereby promoting exit from pluripotency in ESCs (Acloque et al., 2009; Lamouille et al., 2014; Kim et al., 2014). Importantly, Joanne Murphy-Ullrich’s lab has demonstrated that calreticulin-regulated Ca²⁺ signaling controls TGF-β-induced deposition of extracellular matrix by mouse embryonic fibroblasts (Zimmerman et al., 2013) and of collagen I by vascular smooth muscle cells both in vitro and in vivo (Zimmerman et al., 2015). This has been extended to pathological settings such as fibrosis (Kypreou et al., 2014) and in vivo (Zimmerman et al., 2015). The aim of the present paper was to elucidate interconnections between calreticulin’s role as a Ca²⁺ homeostasis regulator and TGF-β signaling in the context of cardiomyogenesis of mouse ESCs.

RESULTS

Calreticulin Promotes Cardiac Differentiation

To examine if the absence of calreticulin impairs cardiac differentiation of mouse ESCs, we counted contracting (“beating”) EBs during differentiation from day 3 to day 14 (D3-D14) in wild-type (WT), CRT-KO, and constitutively activated calcineurin-overexpressing CRT-KO (CN) cultures. The percentage of beating EBs was higher in WT versus CRT-KO EBs; the difference was already substantial at D7, became 2-fold at D10, and 3-fold by D14. In addition, the percentage of beating in CN EBs was significantly higher than in CRT-KO EBs (Figure 1A). Flow cytometry analysis on D14 WT, CRT-KO, and CN EBs using anti-cardiac troponin I antibody Alexa Fluor 488 conjugated showed higher cardiac troponin I in WT and CN EBs compared with CRT-KO EBs (Figure 1B). WT EBs showed increasing levels of cardiac myosin heavy chain (MHC) as differentiation progressed, while in CRT-KO cells MHC levels remained low (Figures 1C and 1D). During cardiomyogenesis of mouse ESCs, expression of the mesodermal and cardiac markers Mesp1, Gata4, and Nkx2-5 was induced in the WT cells but not in the CRT-KO cells, in which all markers remained at very low levels (Figure 1E).

Calreticulin Is Required for the Cadherin Switch and EMT during Cardiac Differentiation

Immunoblot analysis was carried out to examine the expression levels of E- and N-cadherins in WT, CRT-KO, and CN cells throughout the differentiation period (Figures 2A and 2B). The expression of E-cadherin in WT EBs progressively decreased as differentiation progressed, while remaining abundant in CRT-KO EBs. In contrast, the N-cadherin expression increased significantly starting at D7 in WT EBs and continued to increase up to D14. N-Cadherin expression remained very low in CRT-KO EBs. CN cells showed a similar trend as the WT cells for both cadherins.

We also analyzed the mRNA expression level of E-Cadherin, N-Cadherin and their upstream transcription factors Snail1, Snail2/Slug, and Twist1 (Figure 2C). Similar to the protein expression pattern, E-Cadherin mRNA was higher overall in CRT-KO cells, peaking at D10. Snail2 expression, a repressor of E-Cadherin, remained very low throughout differentiation in these CRT-KO cells. On the other hand, N-Cadherin mRNA expression was very low in CRT-KO cells, while it was high and increased with time in WT cells. Twist1, an upstream regulator of N-Cadherin, also increased during differentiation in WT cells. Snail1 expression did not show any significant difference between CRT-KO and WT cells, and was thus not further examined.

Calreticulin Regulates TGF-β-Induced EMT during Cardiac Differentiation

TGF-β regulates GSK3β activity and SNAIL2/SLUG nuclear translocation, thereby affecting E-Cadherin expression (Zhou et al., 2004; Kim et al., 2012; Wakefield and Hill, 2013). We thus examined the expression of TGF-β receptor genes in WT and CRT-KO EBs throughout cardiac differentiation. The expression of all three TGF-β receptors was suppressed in CRT-KO EBs (Figure 3A). We then evaluated E- and N-cadherin expression, phosphorylation status of AKT on serine 473 (S473), indicative of AKT activation, and GSK3β phosphorylation on serine 9 (S9), indicative of GSK3β inactivation (Fang et al., 2000). Figure 3B shows that, in contrast to WT cells, N-cadherin was significantly lower in CRT-KO cells, while E-cadherin was elevated. The CN cells followed a similar trend to that of the WT cells (Figure 3B). Phosphorylation of AKT on S473 and GSK3β on S9 was higher in WT and CN cells at D14 compared with...
Calreticulin is responsible for Cadherin switch and EMT during cardiac differentiation

**Figure 2. Calreticulin Absence Impairs E-Cadherin/N-Cadherin Switch during EMT**

(A) Western blot analysis shows the expression of E-cadherin and N-cadherin in WT and CRT-KO cells during differentiation at D0, D3, D5, D7, D10, and D14. In WT cells E-cadherin is decreasing, while N-cadherin is increasing during differentiation. E-cadherin expression is high in CRT-KO cells and it remains high throughout the differentiation, while N-cadherin expression is very low. The graphs are the quantification of band density for N- and E-cadherin normalized versus GAPDH of these particular western blots.

(B) Western blot analysis the E-Cadherin and N-Cadherin expression in CN cells.

(C) qPCR showed that N-Cadherin, Snail2/Slug, and Twist1 mRNA expression increased in WT cells during differentiation and they were low in CRT-KO cells, while the E-Cadherin mRNA level is higher in CRT-KO cells.

The qPCR data are the average of three individual experiments; *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001.

CRT-KO cells. Total AKT and GSK3β levels were similar in all three cell lines. Since S9 phosphorylation renders GSK3β inactive, this indicates that the enzyme is more active in the absence of calreticulin. A detailed phosphorylation pattern for AKT and GSK3β in earlier days of cardiac differentiation is provided in Figure S1. We also assessed the nuclear localization of SNAIL2/SLUG in WT and CRT-KO cells by subcellular fractionation. Figure 3C shows that SNAIL2/SLUG expression is high in the nucleus of WT cells throughout cardiomyocyte differentiation, while the protein is almost undetectable in the nucleus of the CRT-KO cells.

Next, we examined the expression levels of E-cadherin, N-cadherin, and GSK3β (S9) phosphorylation in the presence or absence of the GSK3β inhibitor SB415286 and the TGF-β inhibitor SB431542 by western blot analysis (Figures 4A and 4B). GSK3β inhibition reduced E-cadherin levels in both WT and KO cells, with no significant effect on N-cadherin in WT cells. This is in stark contrast to CRT-KO cells, where N-cadherin abundance increased by 2-fold following GSK3β inhibition. TGF-β inhibition caused significant effects only in WT cells and was without effect on the CRT-KO cells. Specifically, TGF-β inhibition caused an increase in E-cadherin expression and
Calreticulin stimulates TGFβ induced cadherin switch via GSK3β inhibition

Figure 3. Calreticulin Absence Reduces S9 Phosphorylation of GSK3β and Affects SNAIL2/SLUG Nuclear Translocation

(A) qPCR analysis shows the mRNA expression of TGF-β receptor markers Tgfbr1, Tgfbr2, and Tgfbr3 in WT and CRT-KO cells during differentiation.

(B) N-cadherin, E-cadherin, pAKT (S473), and pGSK3b (S9) versus total AKT and total GSK3β were examined in WT, CRT-KO, and CN cells at D10 of cardiac differentiation from total cell lysates by western blot analysis. Compared with calreticulin-containing WT cells, in KO cells E-cadherin is more abundant, while in contrast N-cadherin is more abundant in WT cells than in calreticulin-deficient KO cells. CN cells express less E-cadherin and more N-cadherin. AKT phosphorylation at serine 473 and GSK3β phosphorylation at S9 is low in CRT-KO cells. The bar graphs show the quantification of E-cad/N-cad, pAKT, and pGSK3b band density versus GAPDH from three independent experiments; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

(C) Western blot detection of SNAIL2/SLUG in nuclear fractions of in WT, CRT-KO, and CN cells during cardiac differentiation in D10 and D14.

A decrease in N-cadherin expression in WT cells. GSK3β inhibition did not appreciably affect the level of S9 phosphorylation of GSK3β in WT cells, while it increased S9 phosphorylation of GSK3β in CRT-KO cells finally. TGF-β inhibition effectively abolished S9 phosphorylation of GSK3β in the calreticulin-containing WT cells, while it did not affect the already low level of S9 phosphorylation of GSK3β in CRT-KO cells.

Double-label confocal immunocolocalization of EBs (Figure 4C) shows that in control WT EBs (DMSO only), the E-cadherin signal was weak compared with the N-cadherin signal. In the untreated CRT-KO EBs (DMSO only), the E-cadherin signal was much stronger than the N-cadherin signal and it was predominantly cytoplasmic with a weak junctional component. GSK3β inhibition (GSK3β inh.) in WT EBs further lowers the weak E-cadherin signal. In contrast, GSK3β inhibition in WT EBs had a similar effect on the intensity of N-cadherin fluorescence as WT DMSO EBs. In CRT-KO EBs treated with GSK3β inhibitor (GSK3β inh.), the E-cadherin signal became very weak while there was a visible increase the N-cadherin signal. TGF-β inhibition in WT EBs increased the intensity of E-cadherin labeling in comparison with the control EBs; however, it substantially reduced N-cadherin fluorescence labeling, again in comparison with the untreated EBs. In CRT-KO EBs, TGF-β inhibition did not appear to bring about any substantial changes in labeling with either antibody in comparison with the control CRT-KO EBs.

Finally, we examined GATA4 protein level and its nuclear localization. GATA4 was more abundant in WT and CN cells compared with CRT-KO cells (Figure 5A). Cell fractionation showed that GATA4 was substantially enriched in the nucleus of WT and CN cells at D10 and D14 of differentiation, while it was absent from the nuclei of the CRT-KO
TGFβ inhibition impairs cadherin switch via GSK3β activation

Figure 4. Inhibition of TGF-β Impairs E-Cadherin/N-Cadherin Switch during Mouse ESC Cardiac Differentiation
(A) The effects of GSK3β inhibitor (SB415286) and TGF-β inhibitor (SB431542) on the expression of E- and N-cadherin in WT and CRT-KO cells at D14 of differentiation following treatments at D3–D5.
(B) The graph shows the quantification of band density normalized to GAPDH for the blot shown at left. Further details in the text. Results are average of at least three independent experiments for each condition; error bars, SD. *p ≤ 0.05, **p ≤ 0.01.
(C) Double-label confocal microscopy of EBs at D14 of differentiation labeled with anti-E-cadherin and anti-N-cadherin antibodies. Microscope settings were kept identical for all conditions during imaging. Scale bar, 10 μm.

cells (Figure 5B). qPCR showed that, in WT cells, GSK3β inhibition did not affect Gata4 mRNA expression but TGF-β inhibition reduced Gata4 mRNA expression significantly (Figure 5C). In contrast, GSK3β inhibition significantly increased mRNA expression of Gata4 in CRT-KO cells, and TGF-β inhibition significantly decreased Gata4 mRNA expression (Figure 5D). The effect of TGF-β and GSK3β inhibition on mRNA expression of Gata4 in earlier days of differentiation is shown in Figure S2.

DISCUSSION

Ca2+ has been identified as a major second messenger in directing stem cells toward cardiomyocyte differentiation (Puceat and Jaconi, 2005). In vitro cardiomyocyte differentiation of EBs revealed that CRT-KO cells failed to differentiate and beat properly (Mesalet al., 1999; Papp et al., 2009a, 2009b; Faustino et al., 2010, 2016), but its mechanism has remained elusive. Depleting Ca2+ using BAPTA
Cardiac transcription factor GATA4 is regulated via TGFβ/GSK3β pathway

(A) Western blot analysis shows that the expression of GATA4 is low in the absence of calreticulin (CRT-KO) compared with calreticulin-containing cells (WT) and calcineurin-overexpressing cells (CN) at D14 of differentiation. (B) Western blot detection of GATA4 in nuclear fractions of WT, CRT-KO, and CN cells during cardiac differentiation at D10 and D14. (C) show qPCR of Gata4 mRNA at D14. (D) In WT cells, GSK3β inhibition does not significantly affect Gata4 mRNA, while TGF-β inhibition significantly decreases its abundance. (D) In CRT-KO cells, GSK3β inhibition significantly increases the abundance of Gata4 mRNA, while TGF-β inhibition significantly decreases it. Results are average of three independent experiments. 

**p ≤ 0.001, ****p ≤ 0.0001.

In the presence of calreticulin, a cadherin switch occurs between E- and N-cadherins during the course of cardiomyocyte differentiation. This is exhibited by a decrease in E-cadherin abundance with a progressive increase in (and replacement by) N-cadherin. The presence of N-cadherin at the cell surface is necessary for the proper formation and function, as demonstrated by contraction, of cardiomyocytes (Radice et al., 1997; Ong et al., 1998; Luo and Radice, 2003). Accordingly, we show an induction of TWIST1, a transcriptional regulator of N-cadherin (Derycke and Bracke, 2004), and increases in both N-Cadherin mRNA and protein, as the calreticulin-containing ESCs begin to express cardiac MHC and EBs start to beat. The expression of E-cadherin is downregulated by the SNAIL2/Slug transcription factor, which suppresses the E-cadherin promoter (Batlle et al., 2000; Kim et al., 2012). GSK3β phosphorylates SNAIL2/Slug exposing its nuclear export sequence (Domínguez et al., 2003; Kim et al., 2012). In calreticulin-containing WT cells, GSK3β is inactivated by phosphorylation at S9, whereby it is unable to facilitate export of SNAIL2/Slug from the nucleus to the cytoplasm. SNAIL2/Slug remains in the nucleus where it suppresses E-cadherin expression (Cano et al., 2000; Domínguez et al., 2003; Zhou et al., 2004; Ko et al., 2007); our current findings are in full accordance with these reports. Thus, in the absence of calreticulin, the expression of SNAIL2/Slug is low and its nuclear translocation is impaired as well. This explains the absence of a cadherin switch in CRT-KO cells and the abundant expression of E-cadherin throughout their dysregulated cardiomyocyte differentiation period.

TGF-β is a potent inducer of EMT during differentiation (Derynck et al., 2014). We thus asked if the effect of...
calreticulin on EMT may be due to impaired TGF-β signaling in the absence of calreticulin. We show that the expression of TGF-β receptor mRNA is downregulated in the absence of calreticulin. Moreover, we analyzed AKT activation by phosphorylation on S473 downstream of TGF-β receptor activation (Alessi et al., 1996; Shin et al., 2001) and we show that AKT activation is impaired in the absence of calreticulin. Normally, activated, S473-phosphorylated AKT inhibits GSK3β activity by phosphorylating it on S9 (Fang et al., 2000). In the absence of calreticulin, GSK3β phosphorylation on S9 is diminished, and thus activity of this kinase is increased. In brief, we show here that inhibition of signaling from AKT/GSK3β causes downstream inhibition of nuclear accumulation of SNAIL2/SLUG and, in the end result, E-cadherin overexpression. Furthermore, we showed that overexpression of calcineurin in CRT-KO cells rescues AKT activation. It is important to note here that calcineurin is an effector of calreticulin, in that Ca²⁺ influx required to activate calcineurin depends on Ca²⁺ stored in the ER by calreticulin (Michalak et al., 2002b). Conversely, it has been shown that inhibition of calcineurin impacts negatively, signaling downstream of AKT/GSK3β (De Windt et al., 2000; Soleimanpour et al., 2010). Collectively, in the absence of calreticulin, phosphorylation of AKT on S473 and GSK3β on S9 are diminished, thus rendering the TGF-β signaling pathway dysregulated.

Our results show that inhibition of TGF-β during mesodermal formation in WT EBs from D3 to D5 with SB431542-compound, which interferes with Tgfr1-mediated TGF-β signaling, significantly decreases the abundance of N-cadherin and increases E-cadherin levels to that seen in CRT-KO EBs. Inhibition of TGF-β does not have any significant effect on either E-cadherin or N-cadherin levels in CRT-KO EBs, as TGF-β signaling is already impaired in the absence of calcineurin. Consequently, we infer that the impairment in TGF-β signaling is the cause of the impaired EMT in the absence of calcineurin and, as a result, impaired cardiomyogenesis. TGF-β is one of the main regulators of GSK3β, whereby TGF-β decreases activity of this kinase (Yoshino et al., 2007; Millet et al., 2009; Byun et al., 2014; Cheng et al., 2014; Lal et al., 2014; see Guo and Wang, 2009; Xu et al., 2015 for reviews). This is in agreement with our findings that GSK3β S9 phosphorylation is high (i.e., activity is low) in the calreticulin-containing WT EBs, while GSK3β S9 phosphorylation is lower in the absence of calreticulin. SNAIL2/SLUG expression is higher in WT EBs and it is enriched in the nucleus, which may account for the lower levels of E-cadherin in WT EBs compared with CRT-KO EBs. Furthermore, GSK3β is phosphorylated on S9 in calreticulin-containing WT EBs. Hence, in these EBs, inhibition of GSK3β lacks substantial effect on either E- or N-cadherin expression as GSK3β is already inactive in the presence of calreticulin. Conversely, in CRT-KO cells, inhibition of GSK3β induces EMT as demonstrated by reduced levels of E-cadherin and increased levels of N-cadherin. TGF-β signaling is impaired in CRT-KO EBs where GSK3β remains active; hence GSK3β inhibition can rescue EMT in CRT-KO EBs. Finally, low mRNA expression levels of the cardiac transcription factor Gata4 in CRT-KO EBs can be rescued by inhibition of GSK3β during mesodermal formation. TGF-β inhibition completely impairs Gata4 mRNA expression in both WT and CRT-KO EBs. Thus, we conclude that the absence of calcineurin impairs TGF-β-mediated EMT and also impairs expression and nuclear translocation of cardiac transcription factors such as GATA4.

In summary, we show that calreticulin and TGF-β signaling intersect downstream of TGF-β receptors I and II. The presence of calreticulin permits TGF-β-mediated inactivation of GSK3β and thus promotes SNAIL2/SLUG-regulated repression of E-cadherin. This condition is sine qua non for the expression and activation of EMT components and, further downstream, cardiac factors during cardiomyocyte differentiation. We demonstrate here, that regulated Ca²⁺ signaling from calreticulin to calcineurin is critical for unabated TGF-β signaling necessary for the exit from pluripotency and cadherin switch during EMT, and commencement of cardiomyogenesis from mouse ESCs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cardiomyocyte Differentiation from Mouse ESCs**

Calreticulin-null R1 ESC lines (Li et al., 2002) and their WT counterparts were used to obtain cardiomyocytes via the hanging drop method (Ilescheler et al., 1997). The WT CGR8 cell line (Nichols et al., 1990) was used as an additional control. Calcium-independent constitutively activated calcineurin-overexpressing CRT-KO ESCs (CN) were a kind gift from Dr. Marek Michalak (University of Alberta). ESCs were grown on a mitomycin C-treated (10 μg/mL; Sigma) mouse embryo fibroblast feeder layer to passage two in propagation medium consisting of high glucose DMEM (Multicell, Wisent), 10% fetal bovine serum (FBS; Multicell Wisent), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM minimum essential medium non-essential amino acids, 0.007 μL/mL β-mercaptoethanol, and 1,000 U/mL of mouse leukemia inhibitory factor (LIF; Millipore). The differentiation medium was identical to the propagation medium, except that it contained 15% FBS without LIF. A total of 500 ESCs per 25 μL drops was placed on the lids of tissue culture dishes in differentiation medium for 2 days. The aggregated ESCs, now termed EBs, were floated in bacteriological dishes in differentiation medium for 3 days, after which they were plated onto tissue culture dishes (coated with 0.1% gelatin in PBS for 1 hr at 37°C). Spontaneously beating areas could be detected in the CRT-KO EBs as early as D7 (D0 = creation of hanging drops).
Quantification of Beating Activity of EBs

Individual EBs were plated on D5 into separate wells of a 24-well tissue culture plate (Falcon) in 1 mL of differentiation medium. At the same time on the indicated days, the EBs were viewed under a Nikon TMS inverted light microscope and the number of spontaneously beating EBs were recorded. The size of the area of beating was not taken into consideration. Of the EBs, 48 were counted for each cell line, and the number of beating EBs were graphed as a percentage of the total number of EBs plated.

Immunolabeling and Microscopy

For immunolocalization, cells were cultured on 0.1% gelatin in 1× PBS covered four-chamber polystyrene culture slides (BD Falcon) and fixed in 3.7% formaldehyde in PBS for 10 min. After washing (three times for 5 min) in PBS, the cells were permeabilized with 0.1% Triton X-100 in buffer containing 100 mmol/L of PIPES, 1 mmol/L EGTA, and 4% (w/v) of polyethylene glycol 8000 (pH 6.9) for 20 min, and the cells were washed again (three times for 5 min in PBS). Staining with primary antibodies E- and N-cadherin (Abcam) diluted 1:100 in 1× PBS was done overnight in 4°C. After the final wash (three times for 5 min) in PBS, the slides were mounted in prolong Gold Antifade with DAPI (Cell Signaling Technology). A Zeiss spinning disk confocal was used for fluorescence imaging and images were processed with ZEN software.

Flow Cytometry

EBs from D14 cultures were harvested and washed in ice-cold PBS. Cells were trypsinized for 2 min. Single-cell suspensions were created and adjusted to a concentration of 1 × 10⁶ cells/mL in ice-cold PBS with 1% FBS. Cells were stained with fixable viability dye eFluor 780 (1:1,000; Life Technologies) for 15 min at 4°C. Then washing three times with ice-cold PBS, cells were stained with fixable viability dye eFluor 780 (1:1,000) for 15 min at 4°C. After washing three times with ice-cold PBS, cells were fixed in 3.7% formaldehyde in PBS for 15 min, followed by blocking with 1% FBS in PBS 1× for 30 min at 4°C. Anti-cardiac troponin I antibody (Alexa Fluor 488) (Abcam catalog no. 196384) was diluted 1:50 in 1% FBS in PBS and incubated with cells for 30 min at 4°C following washing three times with PBS. Isotype control antibody was rabbit monoclonal IgG Alexa Fluor 488 (Abcam catalog no. 199091) used at the same concentration and conditions as the primary antibody. Non-viable cells were excluded from analysis. An LSR II flow cytometer was used for cell sorting, where at least 10,000 events of viable cells were collected. FlowJo V10 (Treestar) was used for data analysis and gating of positive populations was performed based on appropriate isotype-matched antibody control. Different repeats were analyzed using the same settings for gating.

SDS-PAGE and Western Blotting

Cells were lysed in 1× RIPA buffer containing 10 μL/mL of phosphatase inhibitor cocktail (Sigma) and 10 μL/mL of protease inhibitor cocktail (Sigma). Protein samples were quantified by using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer’s instructions. Finally, protein samples were subjected to SDS-PAGE and western blotting as described previously (Mery et al., 1996). The primary antibodies were used at the following dilutions in Tris-buffered saline supplemented with 0.1% Tween 20: mouse anti-calreticulin (Abcam catalog no. 92516), 1:1,000; rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling catalog no. 14C10), 1:2,000; mouse anti-cardiac MHC from (Abcam catalog no. S0967), 1:1,000; rat anti-E-cadherin (Abcam catalog no. 11512), 1:1,000; rabbit anti-N-cadherin (Abcam catalog no. 12221), 1:1,000; rabbit anti-pGSK3β (S9) (Abcam catalog no. 131092), 1:1,000; rabbit anti-GSK3β (Cell Signaling catalog no. 27C10), 1:1,000; rabbit anti-pAKT (S473) (Cell Signaling catalog no. 9271S), 1:1,000; mouse anti-AKT (Cell Signaling catalog no. 9272S), 1:1,000; rabbit anti-SNAIL2/SLUG (Thermo Fisher Scientific catalog no. PA1-86737), 1:1,000; rabbit anti-Histone 3 (Cell Signaling catalog no. S192), 1:100; rabbit anti-GATA4 (Abcam catalog no. 134057), 1:100. All secondary antibodies were conjugated to horseradish peroxidase and used at a dilution of 1:10,000 (Abcam). The protein bands were visualized by using the ECL-Plus Detection System (Amersham).

Subcellular Fractionation

Nuclear fractions were obtained by a modification of the method described previously (Dimas et al., 2012). Briefly, cells were collected and washed with ice cold 1× PBS, lysed on ice for 30 min in cold cytosolic extraction buffer (20 mM sucrose, 50 mM Tris-HCl [pH 7.4], 5 mM MgCl₂, and protease and phosphatase inhibitors). The cytosolic protein was collected by centrifugation at 800 × g for 15 min at 4°C. The pelleted nuclei were treated with nuclear extraction buffer (NEB) (20 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 0.5 mM EDTA, 20% glycerol, 1% Triton X-100, and protease and phosphatase inhibitors). The nuclei were incubated with NEB on ice for 30 min followed by centrifugation at 9,000 × g for 30 min at 4°C to remove cellular debris from the nuclear extract. Protein concentration was measured for both the nuclear and cytosolic fractions using the Bio-Rad DC Protein Assay followed by denaturing and boiling with 2× SDS-PAGE sample buffer.

Quantitative RT-PCR

Total RNA was extracted from EBs using the QIAGEN RNaseasy Mini Kit according to the manufacturer’s instructions: 4 μg of RNA was reverse transcribed to cDNA using Superscript II (Invitrogen) in a total reaction volume of 48 μL. To examine the mRNA expression of cardiac and EMT markers, cDNA was amplified using real-time PCR. Real-time PCR analysis was performed in a Bio-Rad CFX384 Touch detection system. The PCR products were monitored by measuring the fluorescence caused by the binding of SYBR green (Applied Biosystems) to double-stranded DNA. The cDNA levels were normalized using L32 (a housekeeping gene). Reaction conditions were as follows: 10 min at 95°C, 10 s at 95°C, and 30 s at 60°C for 40 cycles. The primer sequences used are detailed in Table S1.

Statistical Analysis of the Data

All data are presented as mean ± SD. Differences between mean values for different treatments were calculated by using the two-way ANOVA with a Bonferroni correction for multiple comparisons. Differences between two groups were assessed with the Student’s t test for normally distributed data. Data were processed in GraphPad Prism version 6.0 (GraphPad Software). p Values less than 0.05 were considered to be significant (**p ≤ 0.05, ***p ≤ 0.01, ****p ≤ 0.001).
Treatments
TGF-β inhibitor, SB431542 (Sigma) (Callahan et al., 2002), was added to the differentiation medium at 10 μM from D3 to D5, and GSK3β inhibitor, SB415286 (Sigma) (Coghlan et al., 2000) was added at 1 μg/mL from D3 to D5. Both inhibitors were diluted in DMSO and DMSO used as negative control.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.03.018.

AUTHOR CONTRIBUTIONS
Conception and Design, F.K. and M.O.; Collection and Assembly of Data, E.K.; Data Analysis and Interpretation, F.K. and M.O.; Manuscript Writing, F.K. and M.O.; Final Approval, M.O.

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