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CstF-64 is necessary for endoderm differentiation resulting in cardiomyocyte defects

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Abstract Although adult cardiomyocytes have the capacity for cellular regeneration, they are unable to fully repair severely injured hearts. The use of embryonic stem cell (ESC)-derived cardiomyocytes as transplantable heart muscle cells has been proposed as a solution, but is limited by the lack of understanding of the developmental pathways leading to specification of cardiac progenitors. Identification of these pathways will enhance the ability to differentiate cardiomyocytes into a clinical source of transplantable cells. Here, we show that the mRNA 3′ end processing protein, CstF-64, is essential for cardiomyocyte differentiation in mouse ESCs. Loss of CstF-64 in mouse ESCs results in loss of differentiation potential toward the endodermal lineage. However, CstF-64 knockout ($Cstf2^{E6}$) cells were able to differentiate into neuronal progenitors, demonstrating that some differentiation pathways were still intact. Markers for mesodermal differentiation were also present, although $Cstf2^{E6}$ cells were defective in forming beating cardiomyocytes and expressing cardiac specific markers. Since the extraembryonic endoderm is needed for cardiomyocyte differentiation and endodermal markers were decreased, we hypothesized that endodermal factors were required for efficient cardiomyocyte formation in the $Cstf2^{E6}$ cells. Using conditioned medium from the extraembryonic endodermal (XEN) stem cell line we were able to restore cardiomyocyte differentiation in $Cstf2^{E6}$ cells, suggesting that CstF-64 has a role in regulating endoderm differentiation that is necessary for cardiac specification and that extraembryonic endoderm signaling is essential for cardiomyocyte development.

Introduction

Embryonic stem cell (ESC) derived cardiomyocytes offer a source of transplantable heart cells (Passier et al., 2008; Segers and Lee, 2008; Chong et al., 2014), but the developmental and molecular pathways leading to specification of cardiac progenitors remain unclear. In vertebrates, the heart is the first organ to become functional after gastrulation. For this process, the primitive endoderm must be present to promote specification of the nascent mesoderm into cardiomyocytes (Murry and Keller, 2008; Pauklin et al., 2011). This process can be mimicked *in vitro* either by co-culture of ESCs with endodermal cell lines (Mummery et al., 1991, 2002, 2003) or by addition of conditioned media from various primitive

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endodermal cell lines (Brown et al., 2010). Recent experiments have elucidated the role of the endoderm in cardiomyocyte differentiation (Cai et al., 2013). Our interests here are to understand the role of RNA processing in controlling the cardiogenic factors required to enable differentiation of ESC-derived cardiomyocytes.

CstF-64 is the RNA-binding component of the cleavage stimulation factor (CstF) that is necessary for efficient and accurate polyadenylation of most mRNAs (Takagaki et al., 1990; Salisbury et al., 2006; Hockert et al., 2010; Darmon and Lutz, 2012; Tian and Manley, 2013). As such, CstF-64 is involved in the expression of many cellular mRNAs (Takagaki et al., 1996; Martin et al., 2012; Yao et al., 2012) including mRNAs encoding replication-dependent histones (Kolev and Steitz, 2005; Sullivan et al., 2009; Sabath et al., 2013; Yang et al., 2013). Previously, we showed that CstF-64 was necessary for correct histone mRNA 3′ end processing as well as maintenance of pluripotency in mouse ESCs (Youngblood et al., 2014). CstF-64 knockout ESCs (*Cstf2*^{E6} cells) displayed decreased expression of pluripotency markers and partial differentiation toward ectodermal and endodermal lineages. Wild type ESCs and *Cstf2*^{E6} cells also express the mammalian paralog of CstF-64, τCstF-64, which is necessary for spermatogenesis (Dass et al., 2001, 2002, 2007; Li et al., 2012). Increased expression of τCstF-64 in *Cstf2*^{E6} cells probably accounted for their viability in the absence of CstF-64 (Youngblood et al., 2014).

In the *Cstf2*^{E6} cells, loss of CstF-64 reduced pluripotency and led to partial differentiation in ESCs (Youngblood et al., 2014). Therefore, we wondered whether CstF-64 was also required for differentiation of ESCs to other cell lineages including endoderm, ectoderm, and mesoderm (Chambers and Smith, 2004; Chambers and Tomlinson, 2009). Here we demonstrate that CstF-64 is needed for proper differentiation of mouse ESCs into the endodermal lineage, but not into ectodermal or mesodermal endpoints; and that endoderm is required for further differentiation of mesoderm into cardiomyocyte cells. *Cstf2*^{E6} cells, when differentiated into embryoid bodies (EBs), displayed a defect in cavitation and a decrease in both primitive and definitive endoderm markers, suggesting disruption of endoderm differentiation. However, both mesoderm and ectoderm markers were expressed normally. In agreement with the EB data, *Cstf2*^{E6} cells were capable of differentiating into neuronal progenitors (an ectodermal lineage). However, in contrast to their expression of mesodermal markers, *Cstf2*^{E6} cells displayed a profound defect in cardiomyocyte differentiation, showing a significant decrease in spontaneous beating and expression of cardiac markers. To account for this, we determined that endoderm differentiation was severely disrupted in the *Cstf2*^{E6} cardiomyocytes, although mesoderm markers were increased. However, we were able to rescue the spontaneous beating and expression of cardiac markers in the *Cstf2*^{E6} cardiomyocytes through the addition of conditioned medium from extraembryonic endodermal (XEN) stem cells, demonstrating that mesodermal and post-mesodermal potential of the cells was normal. These data support a necessary role for the primitive endoderm in ESC-derived cardiomyocyte differentiation and suggest that CstF-64 is needed for cardiomyocyte differentiation through the expression of paracrine factors that regulate endoderm differentiation.

Materials and methods

Cell culture

The *Cstf2*$^{Gt(IST10905E6)Tigm}$ cell line, herein called Cstf2^{E6} cells, was obtained from Texas A&M Institute for Genomic Medicine (TIGM) and derived from mouse C57BL/6N-derived Lex3.13 ESC lines in which a gene-trap cassette was inserted between the first and second exons (*Cstf2*$^{Gt(IST10905E6)Tigm}$, Youngblood et al., 2014). Mouse embryonic stem cells (ESCs) were maintained on 0.1% gelatin-coated 10 cm dishes without feeder cells in Embryo Max DMEM (Millipore) supplemented with 15% ESC-qualified fetal bovine serum (Hyclone/Thermo), 2 mM L-glutamine (Gibco), 0.1 mM-mercaptoethanol (Sigma), 0.1 mM MEM non-essential amino acid stock (Gibco), and 10 ng/mL human leukemia inhibitory factor (LIF, InVitria). ESCs were grown at 37 °C in a humidified incubator in 5% CO_2 and passaged every two days (~70–80% confluency).

XEN cells (Brown et al., 2010) were a gift from Ann C. Foley (Clemson University), and were cultured in 10 cm dishes in ESC complete media without LIF at 37 °C in a humidified incubator in 5% CO_2. For cardiomyocyte differentiation, XEN complete media were obtained from 70% confluent cells and 0.2 micron-filtered.

Embryoid body differentiation

ESCs were deprived of LIF in complete media for 96 h in 10 cm dishes. After 96 h, 2×10^6 cells were plated in ultra low-attachment 10 cm dishes (Corning) for 5, 10, or 15 days in complete ESC media without LIF.

Neuronal differentiation

N2B27 protocol

Wild type and *Cstf2*^{E6} cells were plated on 0.1% gelatin-coated 6-well plates in N2B27 media (19 mL DMEM/F12 Glutamax medium (Invitrogen), 19 mL neurobasal medium (Invitrogen), 0.4 mL 100× N2 (Invitrogen), and 0.8 mL 50× B27 (Invitrogen) (Ying et al., 2003)) at 10,000 cells/cm^2. Cells were cultured in N2B27 media for 6 days, followed by RNA extraction.

4−/4+ protocol

Wild type and *Cstf2*^{E6} cells were plated as hanging drop embryoid bodies in 10 cm dishes in complete ESC media without LIF (Bain et al., 1995). Briefly, approximately 200 cells/drop were plated for 24 h, followed by plating in ultra low-attachment 10 cm dishes (Corning) for 8 days. On the first 4 days, EBs were cultured in complete ESC media without LIF, followed by the addition of 5×10^{-7} M all-*trans* retinoic acid (Sigma) for the last 4 days. After 8 days, the EBs were plated on laminin (Sigma)-coated dishes for 6 days in ESC complete media without LIF, followed by photography and RNA extraction.

Cardiomyocyte differentiation

ESCs were plated as hanging drop EBs in complete ESC media without LIF for 5 days. After 5 days, EBs were plated in 0.1% gelatin coated dishes for indicated times. Incidence of

beating was determined by counting at least 50 EB foci for spontaneous beating. Statistical significance of beating frequency was determined by counting 3 biological replicates. Cardiomyocyte differentiation with the XEN conditioned media was performed according to Brown et al. (2010). Briefly, hanging drop EBs were formed and incubated for 24 h, followed by plating on 0.1% gelatin-coated dishes in complete ESC media without LIF. XEN media was added on days 4–6, followed by the addition of complete ESC media without LIF for the duration of differentiation.

RNA extraction

RNA was extracted from ESCs using the Qiagen RNAeasy kit following the manufacturer's instructions. Genomic DNA was eliminated using gDNA spin columns provided in the Qiagen RNAeasy kit. The quantity of RNA was determined using a NanoDrop device.

RT-PCR

Complementary DNA was prepared from total mouse ESC RNA by reverse transcription with Super Script II RT (Life Technologies) following the protocol recommended by the manufacturer. cDNA was amplified using 2× EmeraldAMP PCR Master Mix (Clonetech) with 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension of 10 min at 72 °C. For negative −RT control, Super Script II was not added to cDNA synthesis reaction. PCR products were displayed on 1.5% agarose (Invitrogen) gels stained with ethidium bromide (0.5 μg/mL). Primers used in this study are listed in Supplemental Table 1.

Real-time RT-PCR

For real-time PCR, 20 ng of cDNA was amplified in triplicates using 2× SYBR Green Master Mix (Invitrogen). The PCR program consisted of 95 °C for 10 min, 40 cycles of 95 °C for 15 s followed by 55 °C for 1 min. The ribosomal protein S2 (*Rps2*) served as a loading control and reference gene. Relative expression was calculated using the comparative C_t method as described previously (Youngblood et al., 2014). Statistical significance was determined using a two-tailed *t* test comparing WT and *Cstf2*^{E6} cells.

Western blots

For Western blots, protein extracts were prepared as previously described (Youngblood et al., 2014). Briefly, protein was extracted using RIPA buffer (50 mM Tris–HCl pH 8.8, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 0.5% NP-40) and the concentrations quantified using BCA assay (Thermo). Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF (Millipore) for immunoblot detection. Primary antibodies for all the polyadenylation proteins were purchased from Bethyl Laboratories (Montgomery, TX) with the exception of anti-CstF-64 (3A7) and anti-τCstF-64 (6A9), which were used as previously described (Wallace et al., 1999).

Results

CstF-64 and τCstF-64 are downregulated during *in vitro* differentiation

Cstf2^{E6} cells are C57BL/6N-derived Lex3.13 mouse ESCs that have a gene trap cassette inserted between the first and second exons of *Cstf2* (Fig. 1A), and thus do not express detectable CstF-64 (Youngblood et al., 2014). *Cstf2*^{E6} cells have lost pluripotency markers and display characteristics of partially differentiated cells (*ibid.*). Thus, we were curious how these cells responded to differentiation signals. We induced wild type ESCs and *Cstf2*^{E6} cells to differentiate into embryoid bodies (EBs, see Materials and methods) and examined the expression of CstF-64 and other polyadenylation factors. In wild type EBs, CstF-64 and τCstF-64 expression seemed to decrease after 15 days of differentiation (Fig. 1B, lanes 1, 3, 5, and 7). In the *Cstf2*^{E6} EBs, τCstF-64 expression was consistently increased compared to wild type (lanes 2, 4, 6, and 8), possibly due to a compensatory mechanism that is activated upon CstF-64 depletion (Youngblood et al., 2014; Ruepp et al., 2011; Yao et al., 2013). We also examined CstF-77 and CPSF-100 protein expression, which was unchanged between WT and *Cstf2*^{E6} cells at all time points (Fig. 1B). These data suggest that both CstF-64 and τCstF-64 respond independently to differentiation signals, while other polyadenylation factors are unaffected; the other polyadenylation factors also do not respond to loss of CstF-64. This is consistent with our previous observation that these proteins did not change in *Cstf*^{E6} cells (Youngblood et al., 2014).

Cstf2^{E6} embryoid bodies do not cavitate

EBs are capable of differentiating into derivatives of all three germ layers (Murry and Keller, 2008; Pauklin et al., 2011). Upon removal of leukemia inhibitory factor (LIF) and growth as either hanging drop or in suspension culture, ESCs form aggregates that mimic mammalian pregastrulation development and early gastrulation stages (Murray and Edgar, 2001). The primitive endoderm, specifically the visceral endoderm gives rise to a fluid-filled cavity that is essential for proper gastrulation. To examine the differentiation potential of the *Cstf2*^{E6} cells, we performed embryoid body *in vitro* differentiation experiments. Interestingly, the *Cstf2*^{E6} EBs did not form cavities (Fig. 1D), as did wild type EBs (Fig. 1C). Lack of cavitation suggested a defect in primitive endoderm differentiation (Coucouvanis and Martin, 1999). To further verify endoderm disruption in the *Cstf2*^{E6} EBs, we performed qRT-PCR on markers for all three germ layers and markers representing pluripotency. Consistent with the lack of cavitation observed, the *Cstf2*^{E6} EBs displayed significant reduction of endodermal markers compared to wild type EBs, including the primitive endoderm marker, *Afp*, and the endoderm markers *Foxa2* and *Sox17* (Figs. 1E–G). In contrast, the mesodermal markers, *Hand1*, *Mixl1*, *Msx1* and the primitive streak marker, *Eomes* were significantly increased in the *Cstf2*^{E6} EBs (Figs. 1H–J). However, the hemato-cardiovascular marker, *Flk1* was significantly increased in the wild type EBs compared to the *Cstf2*^{E6} EBs (Supplemental Table 2). In addition, expression of some of these markers, *e.g.*, *Mixl1*

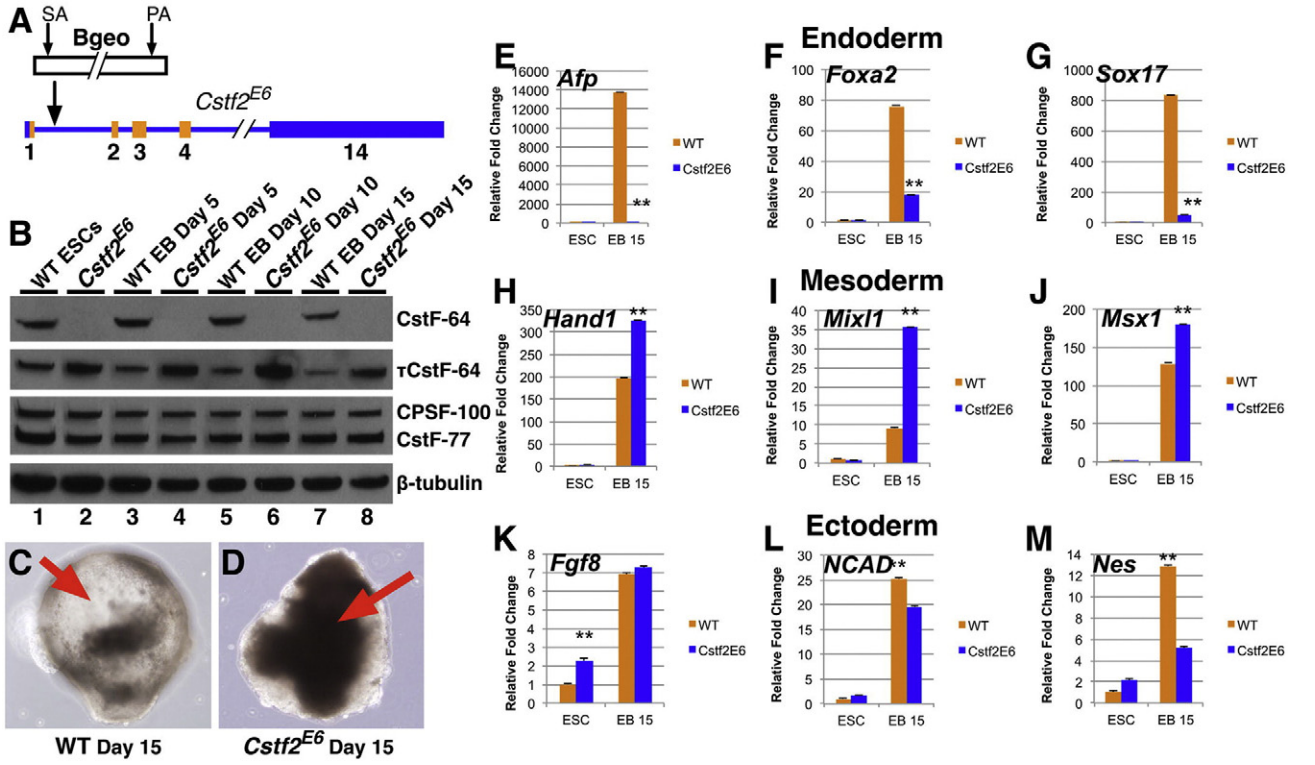

Figure 1 Loss of CstF-64 alters ESC differentiation patterns. (A) Schematic representation of insertion of the gene-trap β-galactosidase-neomycin (Bgeo) fusion protein in the first (*Cstf2*^{E6}) intron of the *Cstf2* gene in the ESC line (Youngblood et al., 2014). The gene-trap consists of a splice acceptor (SA) site and polyadenylation (PA) signal. Yellow bars represent the open reading frame of *Cstf2* mRNA. (B) Western blot analysis shows that *Cstf2*^{E6} cells do not express detectable CstF-64 protein either when grown in the presence of LIF (lane 2) or in the absence of LIF for up to 15 days (lanes 4, 6, and 8). Lanes 1, 3, 5, and 7 are identically treated wild type cells. Also shown are protein expression levels of τCstF-64, CPSF-100, CstF-77, and β-tubulin. Photomicrographs (100× magnification) of wild type embryoid bodies (C) or *Cstf2*^{E6} cells treated by the same protocol (D). Note the cavity formed in wild type embryoid bodies (arrow in C) that is absent in *Cstf2*^{E6} cells (arrow in D). (E–M) Relative mRNA expression (qRT-PCR) of markers for endoderm (*Afp*, *Foxa2*, and *Sox17*, E, F, and G), mesoderm (*Hand1*, Mixl1 and *Msx1* H, I, and J), or ectoderm (*Fgf8*, *NCAD* and *Nes*, K, L, and M) in wild type ESCs or EBs (orange bars) and *Cstf2*^{E6} cells (blue bars). ** denotes $p < 0.01$. Bars indicate standard deviation of at least three biological replicates.

and *Mesp1*, seemed delayed compared to other studies (Ng et al., 2005; Lim et al., 2009). This may reflect impaired differentiation or delay in mesoderm formation. Ectoderm markers did not show a consistent expression pattern between the *Cstf2*^{E6} and wild type EBs (Figs. 1K–M).

The pluripotency markers *Nanog* and *Klf4* displayed decreased expression in the wild type EBs, consistent with the activation of differentiation transcriptional programs (Supplemental Table 2). Interestingly, *Nanog* displayed increased expression in the *Cstf2*^{E6} EBs on day 15, whereas *Klf4* followed a similar pattern to wild type EBs. This increased expression of *Nanog* is consistent with the lack of endoderm differentiation (Hamazaki et al., 2004). These data suggest that the *Cstf2*^{E6} EBs have a defect in endodermal lineage expression, consistent with their lack of cavitation, but have higher mesodermal characteristics. Ectoderm lineage markers are only partially affected.

CstF-64 is not necessary for *in vitro* neuronal differentiation

To further test the differentiation potential, we subjected wild type ESCs and *Cstf2*^{E6} cells to either the N2B27 or the 4−/4+ protocol to differentiate them into neuronal progenitors. N2B27 is a chemically defined serum free media that only allows neuronal progenitors to proliferate in a monolayer (Ying et al., 2003), whereas the 4−/4+ protocol requires embryoid body formation and retinoic acid supplementation followed by laminin attachment (Bain et al., 1995). Both wild type ESCs and *Cstf2*^{E6} cells seemed competent to differentiate into morphologically neuronal cell types (Figs. 2A, B). We further performed RT-PCR and qRT-PCR on neuronal markers *Blbp* and *Mash1* (Fig. 2C and Supplemental Table 3). Wild type ESC- and *Cstf2*^{E6} cell-derived neuronal progenitors demonstrated increased expression of both *Blbp* and *Mash1* mRNAs using both protocols, with the 4−/4+ protocol inducing a greater increase (Fig. 2C). These data suggest that CstF-64 is not necessary for *in vitro* differentiation of ESCs into the neuronal lineage, possibly due to the functional redundancy of τCstF-64.

Cstf2^{E6} cells are unable to form beating cardiomyocytes

Cardiomyocytes, while of mesodermal origin, require paracrine factors from endoderm for early specification (Brown et al., 2010). Although mesodermal markers were increased

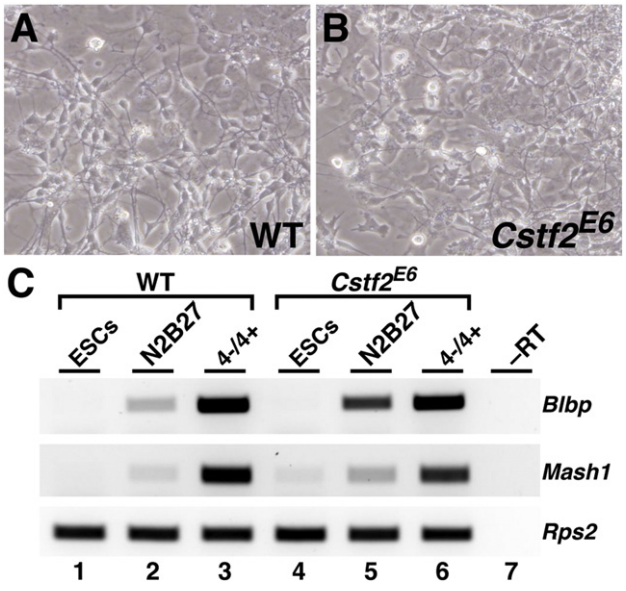

Figure 2 *Cstf2*^{E6} cells are able to differentiate into neuronal cells *in vitro*. Wild type ESCs (A) and *Cstf2*^{E6} cells (B) were differentiated using the 4−/4+ protocol for 14 days. Both cell types form indistinguishable neurite structures (A and B). (C) Agarose gel electrophoresis of RT-PCR amplimers made using RNA from wild type (lanes 1–3) or *Cstf2*^{E6} cells (lanes 4–6). Cells were either grown in the presence of LIF (lanes 1 and 4), or subjected to differentiation using N2B27 medium (lanes 2 and 5) or using the 4−/4+ protocol (lanes 3 and 6). Primers were directed against mouse neuronal markers *Blbp* or *Mash1*, or *Rps2* as a loading control.

in the *Cstf2*^{E6} cells (Figs. 1H–J), endodermal markers were significantly decreased relative to wild type (Figs. 1E–G). Therefore, we wondered whether the *Cstf2*^{E6} cells were capable of differentiation to cardiomyocytes, a mesoderm-derived cell type. We subjected both wild type ESCs and *Cstf2*^{E6} cells to a cardiomyocyte differentiation protocol (Maltsev et al., 1994) and counted the number of EBs that demonstrated spontaneous beating on days 8, 10, and 12. Wild type ESC-derived cardiomyocytes demonstrated 65–75% beating EBs for all three days, indicating efficient cardiomyocyte differentiation (Fig. 3A). In contrast, the *Cstf2*^{E6} ESC derived cardiomyocytes demonstrated very little spontaneous beating, with only 2–5% EBs displaying beating (Fig. 3A). In wild type ESCs, CstF-64 levels decreased at day 10 of the cardiomyocyte differentiation protocol, then increased slightly at day 15 (Supplemental Fig. 1, lanes 1, 3, and 5). τCstF-64 decreased at both 10 and 15 days of the protocol (lanes 1, 3, and 5), but increased at day 10 in the *Cstf2*^{E6} cells (lanes 2, 4, and 6). CstF-77 did not appear to change throughout the protocol.

Consistent with the lack of rhythmic beating, the *Cstf2*^{E6} cells displayed significantly reduced cardiac markers (*Myl2*, *Myh6*, *Myh7*, *Myl7*, and *Actc1*), significantly higher mesodermal markers (*Msx1*, *Hand1*, and *Mixl1*), and significantly decreased primitive and definitive endodermal markers (*Afp*, *Ttr*, *Foxa2*, *Sox17*, and *Gata4*) compared to wild type cells (Figs. 3B, C, and Supplemental Fig. 2). In addition, expression of the transcription factor *Eomes* mRNA in *Cstf2*^{E6} cardiomyocytes was delayed compared to wild type cardiomyocytes (Fig. 3B). Consistent with *Eomes* role in activating *Mesp1* expression to specify the cardiac mesoderm (Costello et al., 2011), *Mesp1* expression also demonstrated a delayed pattern in *Cstf2*^{E6} cardiomyocytes (Fig. 3B). These data demonstrate that *Cstf2*^{E6} cells are impaired in cardiomyocyte differentiation, though not in mesoderm differentiation. They further suggest that the impairment is due to a disruption in endoderm signals and not due to defective mesoderm differentiation.

Cstf2^{E6} cardiomyocyte potential can be rescued using XEN cell-conditioned media

Extraembryonic endoderm XEN stem cells are derived from late blastocyst stage embryos and serve as a developmentally relevant source of primitive endoderm factors (Brown et al., 2010). To examine whether defective primitive endoderm differentiation led to cardiomyocyte disruption in *Cstf2*^{E6} cells, we used medium that had been conditioned by XEN stem cells to promote cardiomyocyte differentiation.

Wild type and *Cstf2*^{E6} cells were subjected to the cardiomyocyte differentiation protocol using either ESC medium without LIF or XEN cell-conditioned medium. As before, *Cstf2*^{E6} cells formed few beating cardiomyocytes compared to wild type ESCs (Fig. 4A). Addition of XEN media to wild type cardiomyocytes did not significantly change the beating efficiency. However, the addition of XEN cell-conditioned media on days 4–6 of differentiation almost completely rescued the ability of the *Cstf2*^{E6} cells to form beating cardiomyocytes, with approximately 65% of EBs displaying beating (Fig. 4A). Consistent with the increase in beating incidence, there was an increase in the expression of mRNAs encoding the cardiac markers, *Myh7*, *Myl2*, and *Actc1* in the *Cstf2*^{E6} XEN medium treated cells compared to the non-treated cells (Fig. 4B). Addition of XEN cell-conditioned medium to the *Cstf2*^{E6} cardiomyocytes decreased the expression of the mesoderm marker *Mixl1* relative to non-treated *Cstf2*^{E6} cells. In addition, there was an increase in the expression of the primitive endoderm marker *Afp* in the *Cstf2*^{E6} XEN-treated cells compared to the non-treated cells. These data demonstrate that the cardiomyocyte differentiation potential in *Cstf2*^{E6} cells can be rescued using conditioned media from the XEN cells, suggesting that endoderm-derived paracrine factors are sufficient to promote cardiomyogenesis in these cells. Further, they demonstrate that CstF-64 is necessary for proper endoderm differentiation that is necessary to specify cardiac progenitors for efficient cardiomyocyte differentiation, but that CstF-64 is not otherwise necessary for differentiation to the mesoderm lineage or cardiomyocytes.

Discussion

The use of stem cell-derived cardiomyocytes is an appealing approach for treating heart disease (Passier et al., 2008; Segers and Lee, 2008), but it has not yet demonstrated clinical success in humans (Chong et al., 2014). However, before it can be used in regenerative medicine, an efficient and reproducible cardiomyocyte differentiation protocol must be established, requiring a molecular investigation

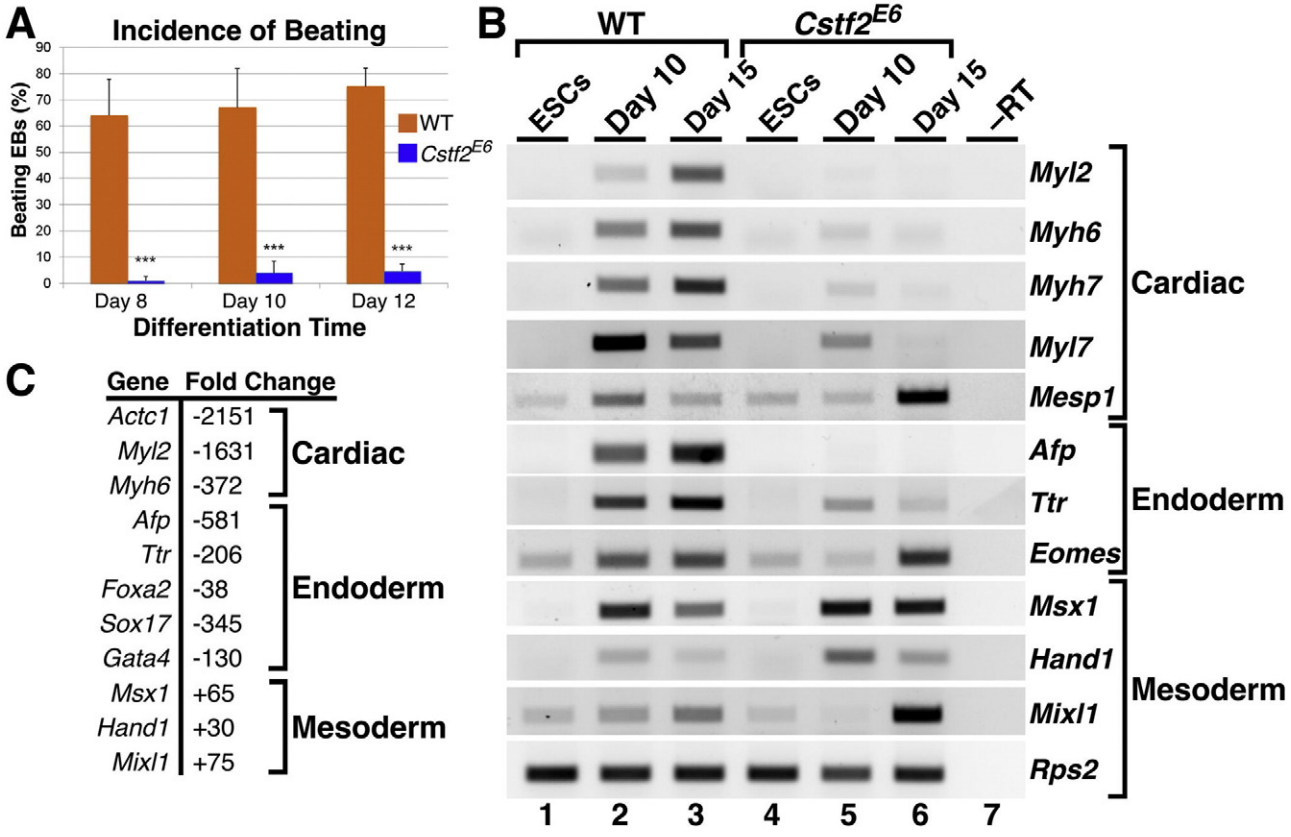

Figure 3 Loss of CstF-64 impairs cardiomyocyte differentiation. (A) Incidence of beating (percent) of wild type (orange) and *Cstf2*^{E6} (blue) cardiomyocytes on days 8, 10, and 12 of differentiation. *** denotes $p < 0.001$. Bars indicate standard deviation of at least three biological replicates. (B) Agarose gel electrophoresis of RT-PCR amplimers made with RNA from wild type (lanes 1–3) or *Cstf2*^{E6} cells (lanes 4–6). Cells were either grown in the presence of LIF (lanes 1 and 4) or differentiated into cardiomyocytes (lanes 2–3 and 5–6). Primers were directed against cardiac (*Myl2*, *Myh6*, *Myh7*, *Myl7*, and *Mesp1*), endoderm (*Afp*, *Ttr*, and *Eomes*), and mesoderm (*Msx1*, *Hand1*, and *Mixl1*) markers. (C) Table displaying the relative mRNA fold change of cardiac, endoderm, and mesoderm genes acquired from qRT-PCR in wild type and *Cstf2*^{E6} day 15 cardiomyocytes. Fold change is relative to wild type cardiomyocytes on day 15.

of cardiomyocyte development. Cardiac progenitor cells require inductive signals from the primitive endoderm to specify the nascent mesoderm into the cardiac fate. However these cardiogenic signals have not all been identified. Here we demonstrate that the mRNA 3′ end processing factor, CstF-64 is required for differentiation of mouse ESCs into endoderm, which in turn produces signals necessary to support cardiomyocyte formation. EB-differentiated *Cstf2*^{E6} cells display significant defects in both primitive and definitive endoderm expression compared to EB-differentiated wild type ESCs (Murray and Edgar, 2001), for example, failing to form interior cavities as do wild type ESCs (Fig. 1). Interestingly, the endodermal differentiation defect was specific, as neither mesodermal nor ectodermal lineage expression markers were significantly reduced in *Cstf2*^{E6} cells. Using two different differentiation protocols, we were able to induce neuronal differentiation in *Cstf2*^{E6} cells (Fig. 2), demonstrating that specification of mouse ESCs to the neuronal pathway can progress without CstF-64. While it is somewhat surprising that this differentiation pathway continues to function in the absence of CstF-64 (Youngblood et al., 2014), it is likely that many functions of CstF-64 are subsumed by τCstF-64 in mammals (Youngblood et al., 2014; Dass et al., 2007; Yao et al., 2013). Therefore, we presume that retinoic acid- and sonic hedgehog-dependent signaling pathways continue to function in *Cstf2*^{E6} cells (Wichterle et al., 2002).

On the other hand, *Cstf2*^{E6} cells displayed significant defects in cardiomyocyte differentiation demonstrated by the decrease in spontaneous beating and the decrease in cardiac and endoderm markers compared to wild type ESCs (Fig. 3). However, the majority of mesoderm markers were significantly increased, suggesting that mesoderm precursors to cardiomyocytes were not dependent on CstF-64. In contrast to the other mesodermal markers, *Flk1* expression was increased in wild type EBs (Supplemental Table 2). *Flk1* positive cells give rise to cardiovascular cell lineages and may explain the decreased expression displayed by the *Cstf2*^{E6} EBs compared to wild type (Ishitobi et al., 2011). To implicate endoderm differentiation disruption in the block to cardiomyocyte differentiation in the *Cstf2*^{E6} cells, we used conditioned media from the XEN endodermal stem cell line (Brown et al., 2010) that almost completely rescued cardiomyocyte beating potential in the *Cstf2*^{E6} cells and resulted in a significant increase in cardiac and endoderm markers (Fig. 4). This suggests that CstF-64 regulates the expression of paracrine factors inducing endoderm differentiation required for cardiomyocyte differentiation.

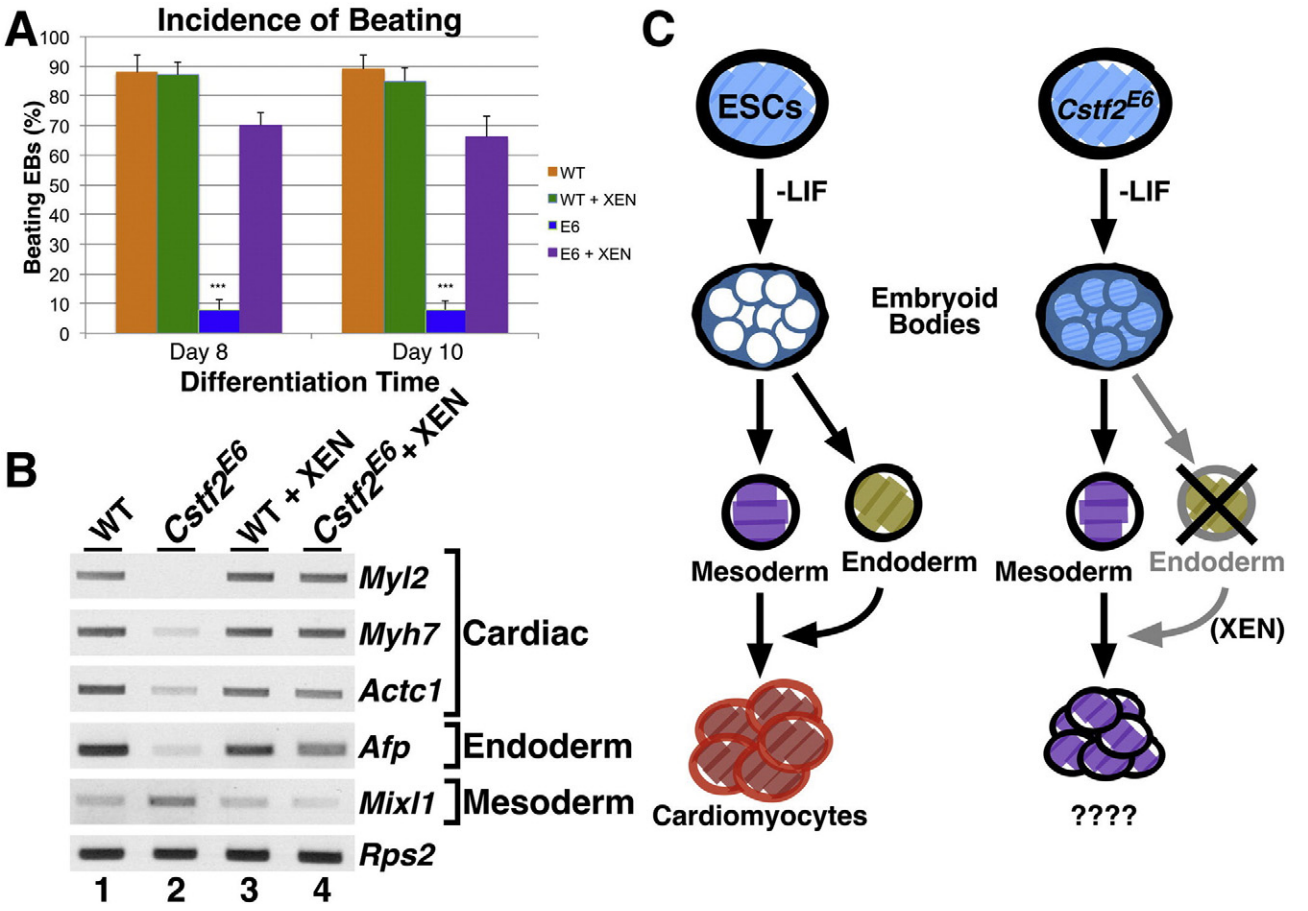

Figure 4 XEN cell-conditioned medium can rescue the cardiac differentiation defect in *Cstf2*^{E6} cells. (A) Graph displaying the beating incidences of wild type cardiomyocytes (WT), wild type cardiomyocytes grown in the presence of XEN cell-conditioned medium (WT + XEN), *Cstf2*^{E6} cardiomyocytes (E6), and *Cstf2*^{E6} cardiomyocytes grown in the presence of XEN cell-conditioned medium (E6 + XEN) on days 8 and 10 of differentiation. *** denotes $p < 0.001$. Bars indicate standard deviation of three biological replicates. (B) Agarose gel electrophoresis of RT-PCR amplimers made with RNA from wild type ESCs (lanes 1), *Cstf2*^{E6} cells (lanes 2), wild type ESCs treated with XEN cell-conditioned medium (lanes 3), or *Cstf2*^{E6} cells treated with XEN cell-conditioned medium on day 10 of cardiomyocyte differentiation. Primers were directed against cardiac (*Myl2*, *Myh7*, and *Actc1*), endoderm (*Afp*), or mesoderm (*Mixl1*) markers. (C) Cartoon depiction of cell lineages and the role of CstF-64 in cardiomyocyte differentiation. In wild type ESCs (left), embryoid bodies give rise to both the mesoderm cell lineage that can further differentiate to cardiomyocytes and the endoderm cell lineage that is needed to provide paracrine factors necessary for cardiomyocyte differentiation. Like wild type ESCs, *Cstf2*^{E6} cells lacking CstF-64 (right) differentiate into embryoid bodies that give rise to mesoderm cell lineages. However, CstF-64 is required for differentiation of ESCs to endoderm. Thus, lacking endoderm-supplied paracrine factors, *Cstf2*^{E6} cells fail to differentiate completely to cardiomyocytes. Addition of XEN cell-conditioned medium can rescue cardiomyocyte differentiation of *Cstf2*^{E6} cells by activating endoderm signaling required for proper cardiomyocyte differentiation.

Candidates include but are not limited to *Afp*, *Ttr*, *Foxa2*, and other downregulated genes (Figs. 3B, C), and other CstF-64-regulated genes such as bone morphogenic protein 2 (*BMP2*, Liu et al., 2008), which has been previously been implicated in cardiac fate specification (Monzen et al., 1999; van Wijk et al., 2007). We propose that CstF-64 regulates the expression of several of these genes directly through polyadenylation, resulting in regulation of paracrine pathways in endoderm cells necessary to support differentiation of ESCs into cardiomyocytes.

Previously, CstF-64 expression was shown to increase in embryonic development as well as in the reprogramming of somatic cells into iPS cells (Ji et al., 2009; Ji and Tian, 2009). In contrast, we show here that both CstF-64 and τCstF-64 decrease upon differentiation of ESCs into EBs, while other polyadenylation factors do not (Fig. 1). The CstF-64 mRNA 3′ end-processing factor is involved in both polyadenylation (Takagaki et al., 1990; Hockert et al., 2010) and replication-dependent histone mRNA processing (Kolev and Steitz, 2005; Sabath et al., 2013; Yang et al., 2013; Youngblood et al., 2014). Changes in both these processes result in changes to pluripotency, cell cycle and growth characteristics of mouse ESCs (Youngblood et al., 2014 and this manuscript). Similarly, loss of τCstF-64 resulted in global changes in gene expression in mouse spermatogenic cells (Li et al., 2012) resulting in male infertility (Dass et al., 2007; Hockert et al., 2011; Tardif et al., 2010). These data suggest that loss of CstF-64, such as that of τCstF-64, will act at one of two levels: (1) it will act directly to

affect the expression of key genes through improper 3′ end processing, or (2) it will act indirectly to affect the expression of a larger number of genes as consequences of changes in the first set of genes. In other experiments, we will determine whether genes involved in the endoderm differentiation pathway are due to direct or indirect effects, or both, and what roles CstF-64 and τCstF-64 play in their regulation. From the data presented here, we know that τCstF-64 can only partially compensate for the multiple functions of CstF-64, since the *Cstf2*^{E6} cells display a phenotype relative to wild type ESCs (Youngblood et al., 2014). This suggests that specific gene targets of CstF-64 and τCstF-64 differ in differentiating ESCs. Investigation of these targets will help better understand the pluripotency and differentiation potentials of ESCs, and advance their therapeutic utility.

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