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Functional consequences of overexpressing the gap junction Cx43 in the cardiogenic potential of pluripotent human embryonic stem cells

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ABSTRACT

Gap junctions, encoded by the connexin (Cx) multi-gene family, couple adjacent cells and underlie cell–cell communications. Previous mouse studies suggest that Cxs play an important role in development but their role in *human* cardiogenesis is undefined. Human embryonic stem cells (hESC) provide a unique model for studying human differentiation. Lentivirus-mediated stable overexpression of Cx43 in hESC (Cx43-hESC) did not affect colony morphology, karyotype and expression of pluripotency genes such as Oct4 but completely suppressed the formation of spontaneously beating, cardiomyocyte-containing clusters in embryoid bodies (EBs). Unlike control hEBs, the transcripts of several mesodermal markers (kallikrein, δ-globin, and CMP), ventricular myosin light chain and cardiac troponin I were absent or delayed. Transcriptomic and pathway analyses showed that 194 genes crucial for movement, growth, differentiation and maintenance were differentially expressed in Cx43-hESC. We conclude that Cx43 mediates the expression of an array of genes involved in human cardiogenesis, in addition to intercellular communication.

Gap junctions, the membrane channels that link adjacent cells by spanning two neighboring plasma membranes, function in a range of biological processes from electrical signaling to transportation of metabolites. In the heart, gap junctions are important for effective propagation of electrical signals to induce coordinated contraction of the cardiac muscles for blood pumping [1]. Experiments with transgenic and knockout mice have underscored the importance of gap junctions in embryonic development, including cardiac differentiation [2–4]; their ability to allow the transfer of important signaling molecules between cells contribute to the *in vivo* cardiac tissue homeostasis [5].

Gap junctions consist of two connexons (a.k.a. hemichannels) that are composed of six connexins (Cx) and to date, over 20 connexin genes have been identified (reviewed in [6]). Of the different isoforms, Cx43 is the most abundantly expressed form in the mammalian heart [7,8]. Although homozygous Cx43 knockout (KO) mice die at birth due to pulmonary outflow tract obstruction and conotruncal heart malformation [2], heart defects associated with malformations in the conotruncus were observed in transgenic mice that *globally* overexpress Cx43 [6], hinting that the precise temporal expression level of Cx43 plays an important role in mammalian cardiac development. However, the role of Cx43 in early human cardiac development has not been studied due to a lack of readily accessible experimental models. Since human embryonic stem cells (hESC), derived from the inner cell mass of blastocysts, can propagate indefinitely in culture, maintain pluripotency and differentiate into cardiomyocytes (CMs), they can be regarded as a simplified *in vitro* model of early human development [9]. Indeed, CMs derived from hESC have structural, molecular and electrophysiological properties that closely resembled those of early human CMs [9]. As a first step, here we employed genetically engineered hESC and their cardiac derivatives for exploring the role of Cx43 in early human cardiac development.

Materials and methods

Maintenance, differentiation and lentiviral transduction of hESC. The hESC line H1 (WiCell, Madison, WI) was maintained, propagated and differentiated into embryoid bodies (EBs) as we previously described [10]. Both continuously and episodically beating EBs were considered when assessing the percentage of spontaneously-contracting EBs. For transgene delivery, we employed the same lentiviral vector (LV) that we already described [10–12]. The rat (r) Cx43 transgene was employed to generate

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pLV-CAG-rCx43-IRES-GFP (Supplementary Fig. 1). Standard procedures for RT-PCR, Western blot analyses, etc. are given in Supplementary methods.

Scrape loading/dye transfer assay. The degree of gap junction communication between hESC was determined by the scrape loading/dye transfer assay as described previously [13]. Briefly, the cells were rinsed three times with PBS buffer (Invitrogen) and Lucifer Yellow (LY) (1 mg/mL, Sigma) diluted in PBS was added to the colonies. The colonies were then immediately scraped with a scalpel blade and after 1 min of incubation were washed several times with PBS. Normal cell culture medium was added to the cells and they were left undisturbed in the dark. The dye transfer was observed by fluorescence microscope and the resulting dye spreading was recorded digitally using the same gain and exposure for each sample.

Transcriptomic and pathway analyses. Samples of mRNAs were analyzed with Affymetrix GeneChip Human Genome U133 Plus2 Arrays. Data analysis was conducted on the chips' CEL file probe signal values at the Affymetrix probe pair (perfect match (PM) probe and mismatch (MM) probe) level, using statistical techniques and GC Robust Multiarray Analysis (GCRMA) [14,15]. With two biological replicates in the experiment, we chose to use fold change to filter differentially expressed genes. A change of 2-fold was chosen as the cutoff. For Ingenuity Pathway Analysis (www.ingenuity.com), biological functions were assigned to the overall analysis based on findings in the scientific literature and those stored in the Ingenuity Pathways Knowledge Base. The calculations of significant score for networks in IPA are based on Fischer's exact test. The *p*-values for Functions and Pathways are calculated using the right-tailed Fisher's exact test.

Results

Firstly, a hESC line that stably overexpresses rat (r) Cx43 (Cx43-hESC) was generated (Fig. 1). One week after transduction by LV-CAG-rCx43-IRES-GFP, the cells were passaged manually and green portions of the hESC colonies were microsurgically segregated. In the following weeks the Cx43-hESC line was passaged enzymatically. Six weeks after the initial transduction, cells that expressed GFP, which accounted for ~91% and 52% of the gated population for control (GFP-) and Cx43-hESC, were FACS-sorted. When recovered, stably LV-CAG-rCx43-IRES-GFP-transduced hESC colonies displayed a morphology not different from WT (untransduced) or GFP-hESC (Fig. 1B) and remained green for >1.5 years.

To confirm rCx43 overexpression, real-time qPCR was performed with primers to the rat (r) and human (h) isoforms. Fig. 1C (right bars) shows that the rCx43 transcript was highly expressed in the Cx43 but not the GFP-hESC lines. However, the endogenous hCx43 expression remained unchanged (Fig. 1C, left bars). Consistent with this result, Western blot analysis using a polyclonal antibody against the C-terminus of Cx43 (which recognizes both r and hCx43) generated detectable bands at 43 kDa (Fig. 1D, left panel) confirming that the total Cx43 proteins were overexpressed in Cx43-hESC. However, the protein expression of Oct4 was unchanged (Fig. 1D, right panel).

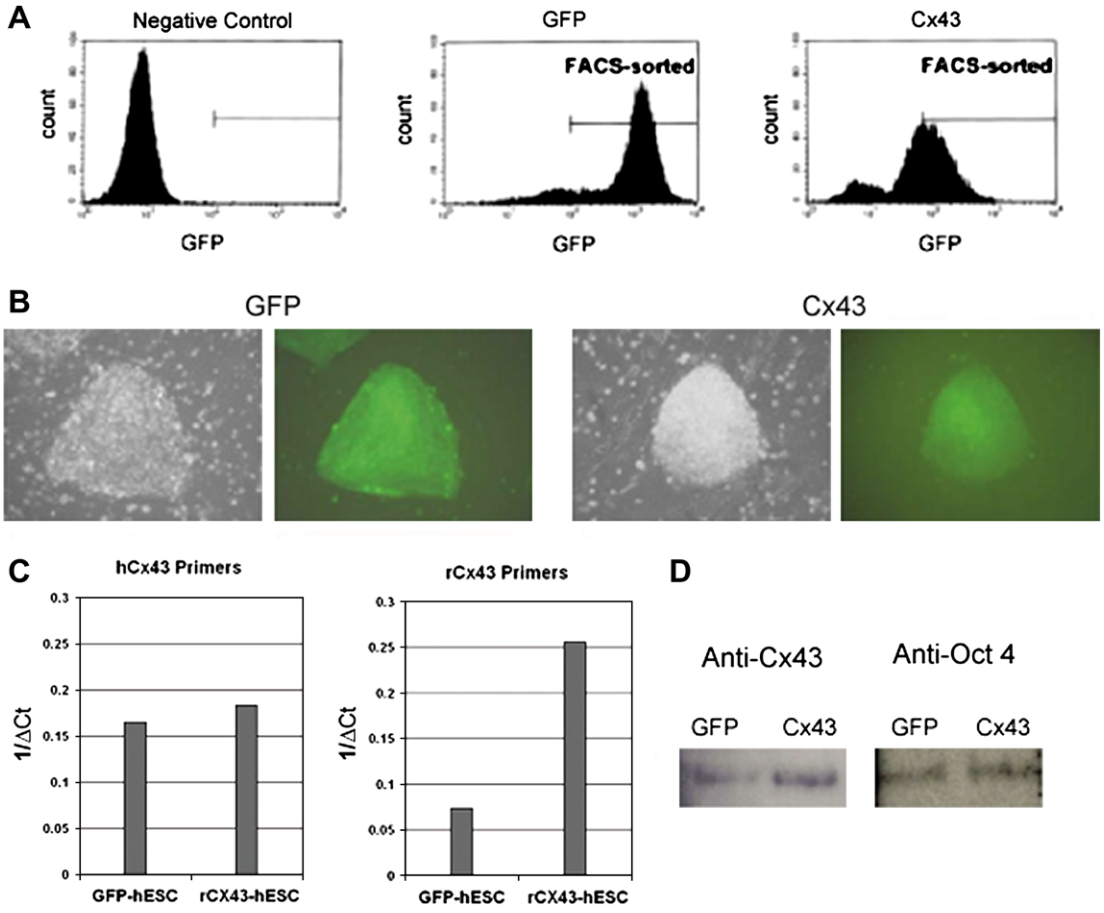

Fig. 1. (A) FACS of LV-CAG-GFP- (middle panel) and LV-CAG-Cx43-IRES-GFP- (right panel) transduced hESC. (B) Representative images (left—phase contrast; right—green fluorescence) of LV-CAG-GFP-transduced (left) and LV-CAG-Cx43-IRES-GFP-transduced (right) hESC after recovery from sorting. (C) Quantitative RT-PCR of both human Cx43 (hCx43, endogenous) and rat Cx43 (rCx43) mRNA transcripts. (D) Western blots of total Cx43 and Oct4.

To explore the sub-cellular distribution of Cx43, we performed immunofluorescence experiments (Supplementary Fig. 2). Undifferentiated Cx43-hESC consistently displayed Cx43 expression at the plasma membranes between adjacent cells. By contrast, the expression pattern of GFP-hESC was much more mosaic, with a number of cell–cell contact interfaces that displayed little or even no Cx43 expression (also see Supplementary Fig. 3). Of note, Cx43-hESC also expressed cytoplasmic Cx43 proteins that could be detected as punctate spots. Lucifer Yellow (LY) dye spreading assays were employed to functionally determine if Cx43 overexpression resulted in enhanced gap junction-mediated transportation of molecules [13]. As shown in Fig. 2A, gap junction-mediated communication was enhanced in Cx43-hESC as indicated by the further spread of LY in their colonies as compared to WT. The dye spreading in the GFP-hESC was consistent with previous findings showing that Cx43 is endogenously expressed in pluripotent hESC [13,16,17].

Human ESCs can be induced to form three-dimensional aggregates, termed human embryoid bodies (hEBs). Upon attachment, these hEBs contain spontaneously contracting CMs [9,16]. Fig. 2B shows the formation of hEBs during the suspension phase and after attachment. Human EBs derived from both GFP- and Cx43-hESC remained green upon differentiation, consistent with the constitutive promoter activity of CAG [10]. For GFP-hEBs, rhythmically contracting areas appeared after days 9 and 10. At day 29, 14% of hEBs contained beating areas (Fig. 2C). Interestingly, Cx43 overexpression abolished *all* beating activity although hEB formation was not affected as gauged by their ability to aggregate, sizes, and morphologies (cf. Fig. 2B; five independent, differentiation reactions). While electrical field stimulations of GFP-hEBs could readily overdrive their beating activities, it did not rescue the loss of function of Cx43-hEBs, suggesting that the lack of spontaneously contracting Cx43-EBs was not due to the simple lack of pacemaker cells but contractile dysfunction. Human EBs made of a mixture of Cx43- and GFP-hESC partially rescued this loss-of-function and resulted in the re-appearance of beating areas, although only ~1/3 of that found in hEBs derived from GFP-hESCs alone. Both GFP- [10] and Cx43-hESC displayed normal karyotypes (Fig. 2D), indicating that the lack of contracting hEBs from Cx43-hESCs was not secondary to karyotypic abnormalities.

To further investigate the negative cardiogenic effect of Cx43 overexpression, the expression levels of several germ layer-specific genes in hEBs derived from GFP- and Cx43-hESC were assessed at days 14 and 32 post-differentiation. As anticipated, GFP-hESC could readily differentiate into all three germ layers as assayed by RT-PCR for various germ layer markers (Fig. 3A). By contrast, the mesodermal markers kallikrein, δ-globin and CMP were absent in hEBs differentiated from Cx43-hESC. This observation hints that the ability of Cx43-hESC to differentiate into mesoderm (which in turn gives rise to the cardiac lineages) has been compromised.

To determine whether and how cardiac differentiation was affected by Cx43 overexpression, we next compared the transcript expression of several heart-specific genes (Fig. 3B). EBs derived from GFP-hESC, which contained spontaneously beating CMs, expressed all the cardiac-specific genes tested. Similarly, Cx43-hESC also expressed atrial natriuretic factor (ANF), α-actinin, α-myosin heavy chain (α-MHC), cardiac actin (c-actin), cardiac troponin T (cTnT), atrial myosin light chain (MLC-2A) on days 14 and 32 post-differentiation. However, the ventricular myosin light chain (MLC-2V) transcript, which has previously been shown to express only in beating EBs [18], was completely absent in Cx43-hEBs at day 14. Additionally, cTnI, a major contractile element in cardiac muscle, was also not detectable at day 14. Thus, Cx43 overexpression compromised the expression of sarcomeric proteins important for contractile function and beating activity. Interestingly, co-incubation with 0.1% or 0.5% DMSO, a known cardiogenic agent, partially rescued the developmental defects caused by Cx43 overexpression (from 0 to ~7.0 and 2.0% of spontaneously beating hEBs, respectively), although higher concentrations (>0.5%) inhibited EB formation. RT-PCR showed that there was a concomitant re-appearance of MLC-2V and cTnI gene expression.

To explore the pathways and networks that have been affected by Cx43 overexpression, we performed transcriptional analysis on GFP- and Cx43-hESC. Of more than 47,000 cDNA clones examined, 86 transcripts were up-regulated by >2-fold while 108 transcripts were down-regulated by >2-fold in Cx43 hESC (vs. GFP hESC). These transcripts are given in Supplementary Table 2. In Fig. 4, genes with significant up- or down-regulation were grouped into functional groups by Ingenuity Pathway Analysis software. Groups

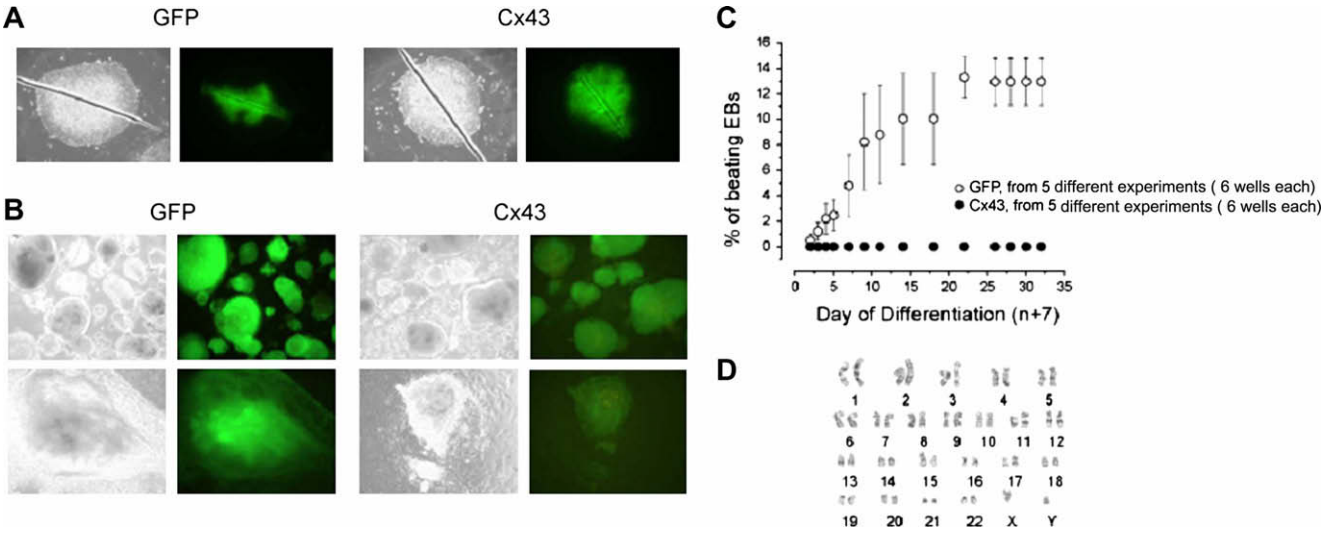

Fig. 2. (A) Gap junctional intercellular communication using Lucifer Yellow as an indicator of dye transfer. Representative images (left, phase contrast; right, yellow fluorescence) of GFP (left) and Cx43 (right) hESC. The dye spreading was greater in the Cx43 overexpressing cell line. (B) Representative images (left—phase contrast; right—green fluorescence) of GFP and Cx43 hEBs in suspension (top panel) and after attachment (and bottom panel). (C) Cumulative percentage of EBs containing spontaneous beating areas as a function of the number of days after EB plating. (D) The Cx43-hESC line displayed a normal karyotype.

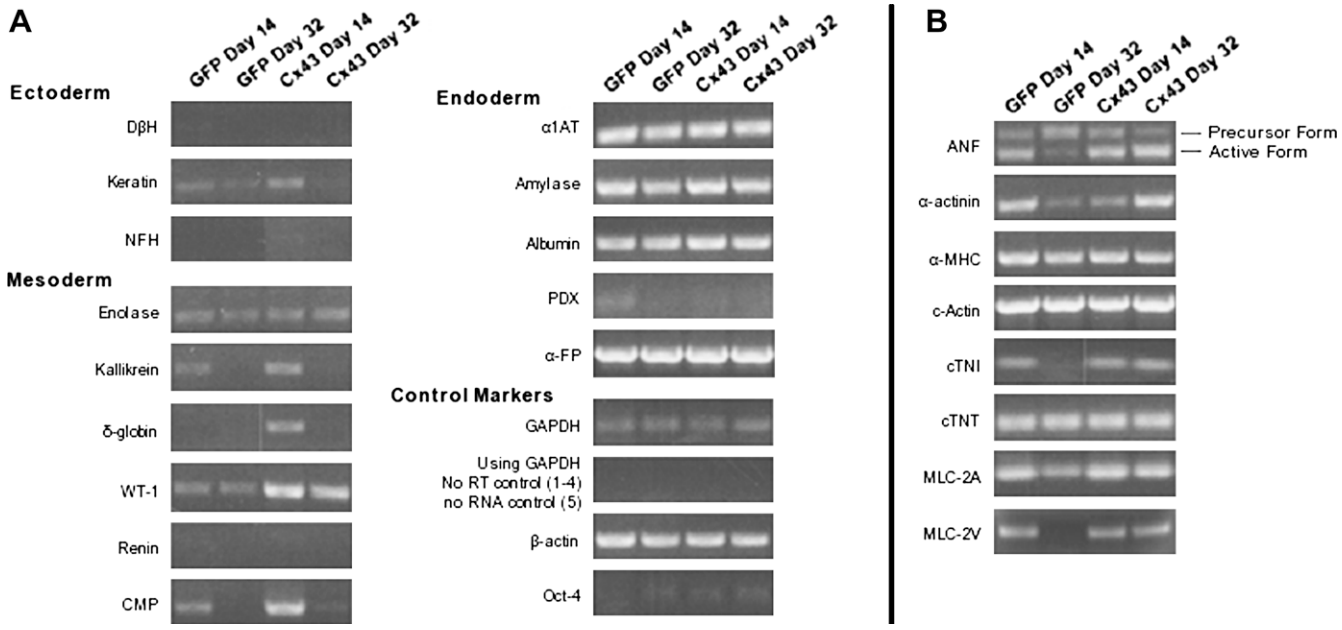

Fig. 3. (A) RT-PCR of different germ layer markers in differentiated GFP and Cx43 on days 14 and 32 after differentiation. (B) RT-PCR on days 14 and 32 after differentiation. Gene expression of the sacromeric proteins MLC-2V and cTnI were completely absent on day 14 in the differentiated Cx43 line while both markers were present in differentiated GFP line under the same conditions.

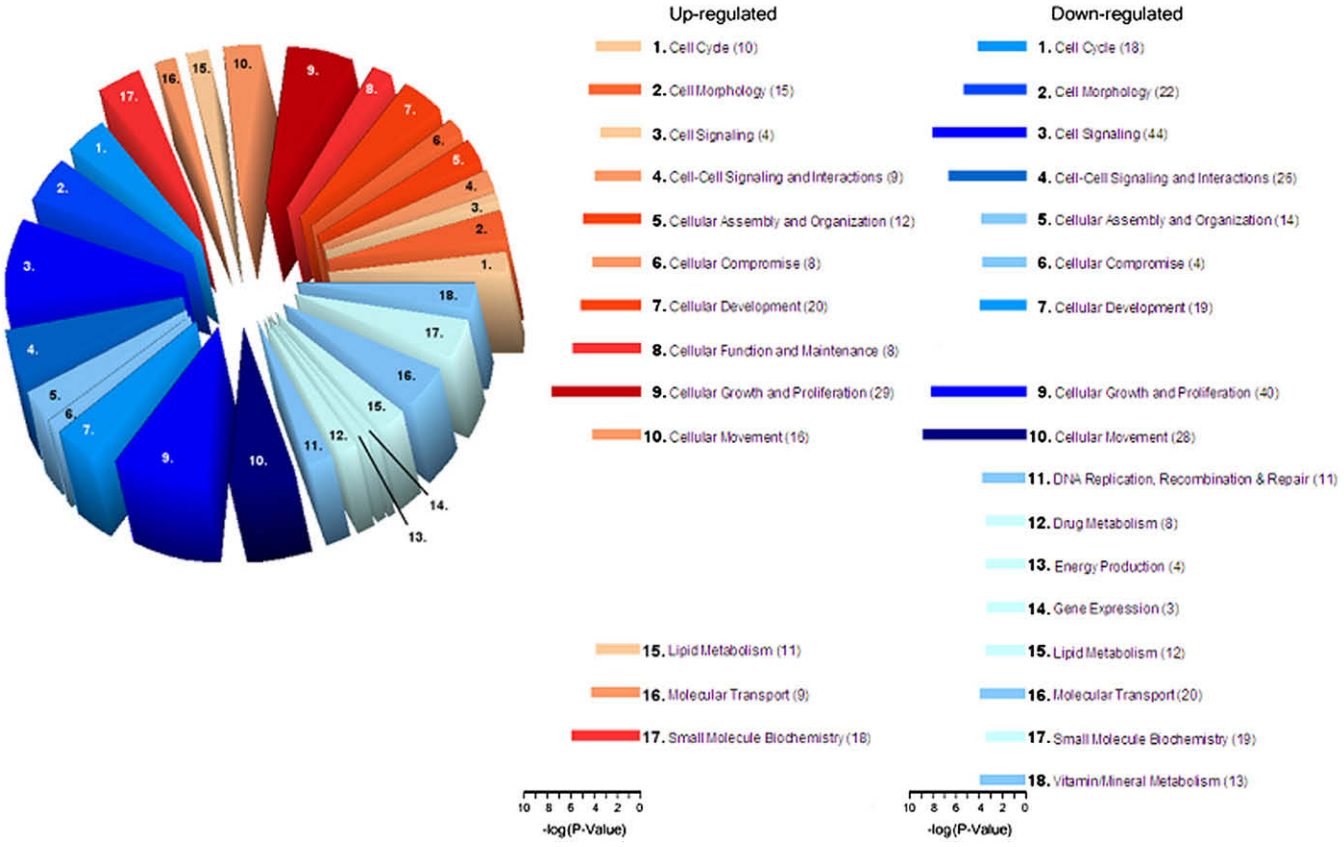

Fig. 4. Pie chart summarizing differentially expressed transcripts according to their functions.

with no statistically significant representation (p-value > 1e−3) were not analyzed further. The remaining 18 are illustrated in this pie chart, with the number of genes in parenthesis. Some genes may participate in multiple functions, therefore some redundancy exists in gene number. The maximum −log(p-value) is represented by a bar to the left of each functional group on a scale of 0.1 − log(p-value) per pixel. The color gradient is also consistent with −log(p-value), a higher value is indicated by an increasing

intensity in color—either red/orange for up-regulated genes or blue for down-regulated genes.

For the up-regulated genes, the most affected networks include (score > 20): 1. Cancer, cellular growth and proliferation, cell death; 2. Cellular growth and proliferation, cellular development, hematological system development and function; and 3. Cellular growth and proliferation, connective tissue development and function, cancer. From these networks, the significant molecular and cellular functions are ($p > 10^{-6}$): 1. Cellular growth and proliferation; 2. Cellular function and maintenance; 3. Small molecular biochemistry; and 4. Cellular development. Significant canonical pathways affected are ($p < 10^{-3}$): 1. Axonal guidance signaling; 2. Ephrin receptor signaling; and 3. The transforming growth factor-β (TGF-β)-signaling.

For the down-regulated genes, the most affected networks include (score > 20): 1. Cardiovascular and nervous system development and function; 2. Cardiac hemorrhaging, embryonic development, hematological disease; and 3. Lipid metabolism and small molecule biochemistry. Significantly affected molecular and cellular functions include ($p > 10^{-6}$): 1. Cellular movement; 2. Cellular Growth and Proliferation; 3. Cellular signaling; 4. Cell-to-cell signaling and interaction; and 5. Cellular morphology. Significant canonical pathways affected are ($p < 10^{-3}$): 1. Complement and coagulation cascades; 2. The transforming growth factor-β (TGF-β)-signaling; 3. G-Protein coupled receptor signaling; and 4. cAMP-mediated signaling. A more complete list of all the affected networks is summarized in Supplementary Table 3.

Discussion

The present study serves as a first step to elucidate the role of Cx43 in human cardiogenesis. Indeed, Cx43 overexpression abolished the formation of spontaneously beating EBs normally observed upon differentiation of hESC. Several possibilities could underlie this outcome. For instance, non-contracting but electrically-active cardiomyocytes could be derived from Cx43-hESC. Perhaps their lack of fully functional contractile apparatus resulted in the absence of contractions. Alternatively, it is possible that cardiomyocytes derived from Cx43-hESC were functional and electrically excitable but quiescent (i.e., a phenotype similar to adult ventricular myocytes). However, this possibility can be ruled out because hEBs derived from Cx43-hESC remained non-contracting when subjected to electrical field stimulations. A final explanation is that Cx43 overexpression led to non-functional heart cells with defects in their contractile apparatus (e.g., altered expression pattern of proteins that are necessary for contractile function). Our present results are in favor of the last possibility. The transcripts of the sarcomeric proteins MLC-2V and cTnI were completely absent 14 days after differentiation, unlike control GFP-hESC. Interestingly, at 32 days post-differentiation these transcripts were present, but there were still no spontaneously contracting CMs. Further immunohistochemical experiments will be needed to investigate to what degree structural and electrophysiological differentiation, in terms of myofibril organization and excitability, has been altered. However, the identification of hESC-CMs has been conventionally based on their ability to spontaneously beat [9,10,12,16] and since Cx43-hESC did not give rise to contracting heart cells, these experiments will require genetic labeling of cardiac cells [19].

Previous reports have demonstrated the presence of gap junctions in hESC [13,20]. Changes in cell–cell coupling and compartmentalization that are mediated by the temporal and spatial expression of gap junction are important in the regulation of embryonic development; alterations of these communications may negatively impact on cardiogenesis. Indeed, transgenic mice that (1) overexpress Cx43 (under the control of CMV), (2) have been knocked out (KO) for Cx43, and (3) overexpress a dominant-negative inactive form of Cx43 all display cardiac malformations due to the increase or decrease in Cx43 function [3,21]. Migration changes during cardiogenesis occur in step with connexin gene or transgene dosage in the homozygous vs. hemizygous Cx43-overexpressed and KO embryos, respectively. Dye coupling analysis of the cardiac neural crest cells in the outgrowth cultures and also in the living embryos shows an elevated gap junction communication in the Cx43-overexpressed mice but reduction in both the dominant-negative and KO groups. Overall, these findings suggest that Cx43-mediated communication plays an important role in cardiac neural crest migration. Furthermore, they support that cardiac neural crest perturbation is the likely underlying cause for heart defects in mice with the gain or loss of Cx43 function.

Cx43 has also been implicated in other physiological processes independent of gap junction-mediated cell–cell communications, such as cell growth, proliferation, differentiation, apoptosis, and tumorigenicity [21–26]. Recent experiments comparing the transcriptomes of Cx43 null and wild type mice have also shown significant gene regulation differences when Cx43 is absent, particularly in the heart and brain [27–29]. Evidently, genes that are down-regulated in Cx43 null mice are co-expressed temporally and spatially with Cx43, lending further supports to the role of Cx43 in transcription mediation [27]. Our transcriptomic experiments and pathway network analysis of hESC were therefore consistent with and complementary to these previous murine experiments. The lack of spontaneously beating cardiomyocytes from Cx43-hESC may be due to the altered expression pattern of a number of genes and their related pathways. Thus, Cx43 also seems to play a role in transcriptional mediation of human differentiation.

Finally, cell replacement therapy using skeletal myoblasts for repairing cardiac tissues after myocardial infarction is now in phase 2 clinical trials. Although improved contractile function has been reported, a significant number of patients have been reported to suffer from lethal ventricular tachycardia [30,31]. One possible cause of these arrhythmias is the lack of connecting gap junctions, leading to ineffective electrical communication between the transplanted skeletal myoblasts and the host cardiomyocytes. Overexpression of Cx43 has been employed as a strategy to improve electrical conduction between the two cell types [32–34] and to attenuate arrhythmia *in vitro* [33]. Although hESC-derived cardiomyocytes express Cx43-encoded gap junctions [9,17] and Cx43 was shown to express at the interface between the hESC-derived and host heart cells [10], significantly slower conduction velocity was recorded within the hEBs [16] in comparison to intact human heart. While the possibility of improving the effectiveness of electrical conduction by overexpressing Cx43 remains to be determined, the present study suggests that the temporal expression level of Cx43 is an important determinant of human cardiac differentiation. The results implicate that conditional Cx43 overexpression (e.g., post-differentiation) may be needed for future genetically engineered hESC-derived cardiac grafts for improved electrical conduction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.076.

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