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TGFβ-Dependent Epithelial-to-Mesenchymal Transition Is Required to Generate Cardiospheres from Human Adult Heart Biopsies

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Autologous cardiac progenitor cells (CPCs) isolated as cardiospheres (CSps) represent a promising candidate for cardiac regenerative therapy. A better understanding of the origin and mechanisms underlying human CSps formation and maturation is undoubtedly required to enhance their cardiomyogenic potential. Epithelial-to-mesenchymal transition (EMT) is a key morphogenetic process that is implicated in the acquisition of stem cell-like properties in different adult tissues, and it is activated in the epicardium after ischemic injury to the heart. We investigated whether EMT is involved in the formation and differentiation of human CSps, revealing that an up-regulation of the expression of EMT-related genes accompanies CSps formation that is relative to primary explant-derived cells and CSp-derived cells grown in a monolayer. EMT and CSps formation is enhanced in the presence of transforming growth factor β1 (TGFβ1) and drastically blocked by the type I TGFβ-receptor inhibitor SB431452, indicating that TGFβ-dependent EMT is essential for the formation of these niche-like 3D-multicellular clusters. Since TGFβ is activated in the myocardium in response to injury, our data suggest that CSps formation mimics an adaptive mechanism that could potentially be enhanced to increase in vivo or ex vivo regenerative potential of adult CPCs.

Introduction

Cardiovascular disease remains the leading cause of mortality and morbidity in Western countries. The vast majority of clinically relevant cardiovascular disease results from the death of cardiac cells that are replaced by non-contractile fibrotic tissue, thus leading to pathological ventricular remodeling and heart failure [1,2]. Therefore, an intense effort during the last decade has been focused on identifying endogenous cardiac progenitor cells (CPCs) that can be expanded ex vivo and reintroduced as an autologous regenerative therapy [3,4]. A promising candidate population of resident CPCs can be readily obtained from cells that spontaneously migrate out of primary cardiac explants (explant-derived cells [EDCs]) and form cardiospheres (CSps) which recreate in vitro a niche-like microtissue [5]. CSp-derived cells (CDCs) can be expanded in monolayers [6] and retain the ability to form secondary cardiospheres (IICSps) when cultured under appropriate conditions. These cells can contribute in vivo to all the 3 main cell lineages of the heart (endothelial, smooth muscle cells, and cardiomyocytes), [7] and can provide a sufficient number of adult autologous CPCs for clinical applications. CDCs have been successfully employed for a randomized phase I clinical trial (CADUCEUS), showing the safety of these cells and an unprecedented increase in viable myocardium, which is consistent with therapeutic regeneration [8]. Furthermore, in a recent head-to-head comparative study, CDCs injected into infarcted mice hearts resulted in superior improvement of cardiac function, the highest cell engraftment and myogenic differentiation rates, and the least-abnormal cardiac remodeling 3 weeks after treatment, compared with other populations of human adult stem cells of diverse origin and to a sorted c-kit$^+$ subpopulation [9]. In addition, preclinical studies conducted on both small [10] and large animal models [11] show that CSps are superior to CDCs in improving hemodynamics and regional function, and in attenuating ventricular remodeling [12], thus paving the way for a future clinical trial (RECONSTRUCT, Study NCT01496209). The regenerative capability of CSps is reportedly linked to their 3D niche-like structure, which favours the maintenance of "stemness" features, while conferring higher resistance to oxidative stress, thus enhancing in vivo engraftment [10]. Despite these advances, modulation of the balance between differentiation

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and paracrine signalling of CSps and CDCs [13] as reparative mechanisms remains unclear and needs to be resolved in order to interpret the emerging clinical data. In particular, the signals that govern CSps formation and cardiogenic, as distinct from a fibrogenic, differentiation remain unclear, especially when cells are recovered from or transplanted into the inhospitable ischemic environment of an infarcted heart [14].

Here, we analyzed the role of epithelial-to-mesenchymal transition (EMT) and its reverse process, mesenchymal-to-epithelial transition (MET), during generation of CSps from human auricular biopsies by means of gene expression analysis at different culture stages and in vitro treatments with transforming growth factor β (TGFβ) [15,16], which is a key EMT inducer, and its antagonist SB431452 [17]. EMT and MET are well known to play pivotal roles in embryogenesis [18], with 4 distinct waves of EMT occurring at different stages of heart morphogenesis [19]. EMT has also been associated with the acquisition of stem cell properties in both adult tissues and cancer [19–21] and also with cardiac post-ischemic remodeling [14]. We found that EMT controlled by TGFβ signaling is essential for the formation of CSps. Moreover, the gene profiles of CSps formation in vitro resembles the in vivo response of epicardial cells to ischemic cardiac injury, which also involves EMT, suggesting that TGFβ and EMT during CSps formation recapitulate aspects of the subepicardial niche and support the proliferation of adult CPCs.

Materials and Methods

Cell cultures

Human auricolar biopsies (10 patients) were cultivated as explants, and CPCs were isolated with the CSp protocol, as previously described [5,22]. Briefly, EDCs were collected weekly up to 3 times from each explant, and seeded on poly-D-lysine (BD-Biosciences) coated wells (9,000 cells/cm^2) to obtain CSps. CSps were then collected by gentle pipetting and expanded on fibronectin (BD-Biosciences) coating as CDCs [6]; IICSps were obtained by replating CDCs in CSp-forming conditions.

In vitro treatments

EDCs or CDCs from 4 different patients were treated with TGFβ (2.5–5 ng/mL) (PeproTech, Inc.) or its inhibitor SB431542 (1, 10, or 25 μg/mL) (Sigma-Aldrich) for 1, 2, 3, 4, or 7 days. Preformed CSps (3 days after EDCs seeding) from 3 different patients were treated 3 or 4 days with TGFβ (5 ng/mL) or its inhibitor SB431542 (10 μg/mL). Proliferation was measured by WST-8 assay (Alexis Bioch.) according to the manufacturer's instructions. For each condition, we plated 2,000 cells/well in triplicate for 4 time points in 96-multiwell plates. CSps were imaged, then counted, and measured at different time points using ImageJ software. Five random fields per well acquired at 4× magnification were used for a quantitative analysis. Cells were collected after 7 days of treatments for RNA extraction.

Immunostainings

Immunostainings were performed on EDCs, EDCs plated to obtain CSps in presence of 10 μM SB431542 or 5 nM TGFβ, CSps, and CDCs. All samples were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 4°C for 10 min, washed thrice with phosphate-buffered saline (PBS) and twice with washing buffer: 1% bovine serum albumin (Sigma-Aldrich), 0.02% Triton (Panreac Química S.L.U.) in PBS with Ca^{+2}/Mg^{+2}, and 50 mM Glycine (Sigma-Aldrich). Nonspecific antibody binding sites were blocked with 10% goat serum (Sigma-Aldrich) before incubation with primary antibodies: Snai1, α smooth muscle actin, Wilms tumor 1 (Abcam), Notch3, NOTCH1 (Santa Cruz Biotechnology, Inc.), β-catenin, and E-cadherin (BD-Biosciences). After washing 4 times in washing buffer, slides were incubated with the appropriate secondary antibody (Alexa 488- or 568-conjugated antibody IgG; Life Technologies Corporation), then counterstained with Hoechst 33342 (5 μg/mL in PBS; Life Technologies Corporation), and mounted with Vectashield medium (Vector Laboratories). Fluorescence micrographs were acquired using an Olympus FV-1000 spectral confocal microscope with a 20× and 40× (oil immersion) objectives and Olympus Fluoview software (Olympus).

RNA isolation and real-time quantitative PCR

EDCs ($n=7$), CSps ($n=9$), CDCs ($n=4$), IICSps ($n=4$), EDCs, or CDCs plated to form CSps/IICSps in the presence of TGFβ ($n=7$) or SB431542 ($n=6$) were collected, and RNA was extracted using the column-based purification kit (Genaid). 500 ng of RNA were retro-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). The obtained cDNA was used to load 2 lanes of the Taqman Customed MicroArray Cards (Applied-Life Technologies). Each card allows the analysis of 48 genes for 4 samples in duplicate. The preloaded taqman probes have been selected from the Applied Biosystem database and include genes involved in cellular reprogramming of induced-pluripotent stem cells (iPSC) [23,24], induced-cardiomyocyte generation [25,26], EMT [18], MET, endothelial-to-mesenchymal transition (EndMT), hypoxia [27], migration, stemness, and markers of cardiac, and vascular and mesenchymal cells (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). TaqMan Univ PCR MasterMix no UNG was used on an ABI PRISM 7900 HT sequence detector (Applied Biosystems). Quantification analysis was performed using the ΔΔCq method, with *GAPDH* as the reference gene (selected as the best and most stable among 3 analyzed), and EDCs or untreated CSps as calibrator.

Statistical analysis

Data are presented as mean value ± standard error of the mean. Two-sided Student's *t*-test was used to evaluate the statistical significance in gene expression, CSps number and diameter, and metabolic activity among the different culture stages. Significance threshold was set at $P \leq 0.05$.

Results

EMT is involved in CSps formation

Quantitative gene expression analyses conducted on human adult EDCs, CSps, CDCs, and IICSps (Fig. 1A, B) revealed several key trends (Fig.1C, D). First, the EMT associated genes [18] *SNAI1*, *TGFBR2*, and *SNAI2* were significantly up-regulated in 3D structures (CSps/IICSps) compared with the cells in monolayers (EDCs/CDCs)

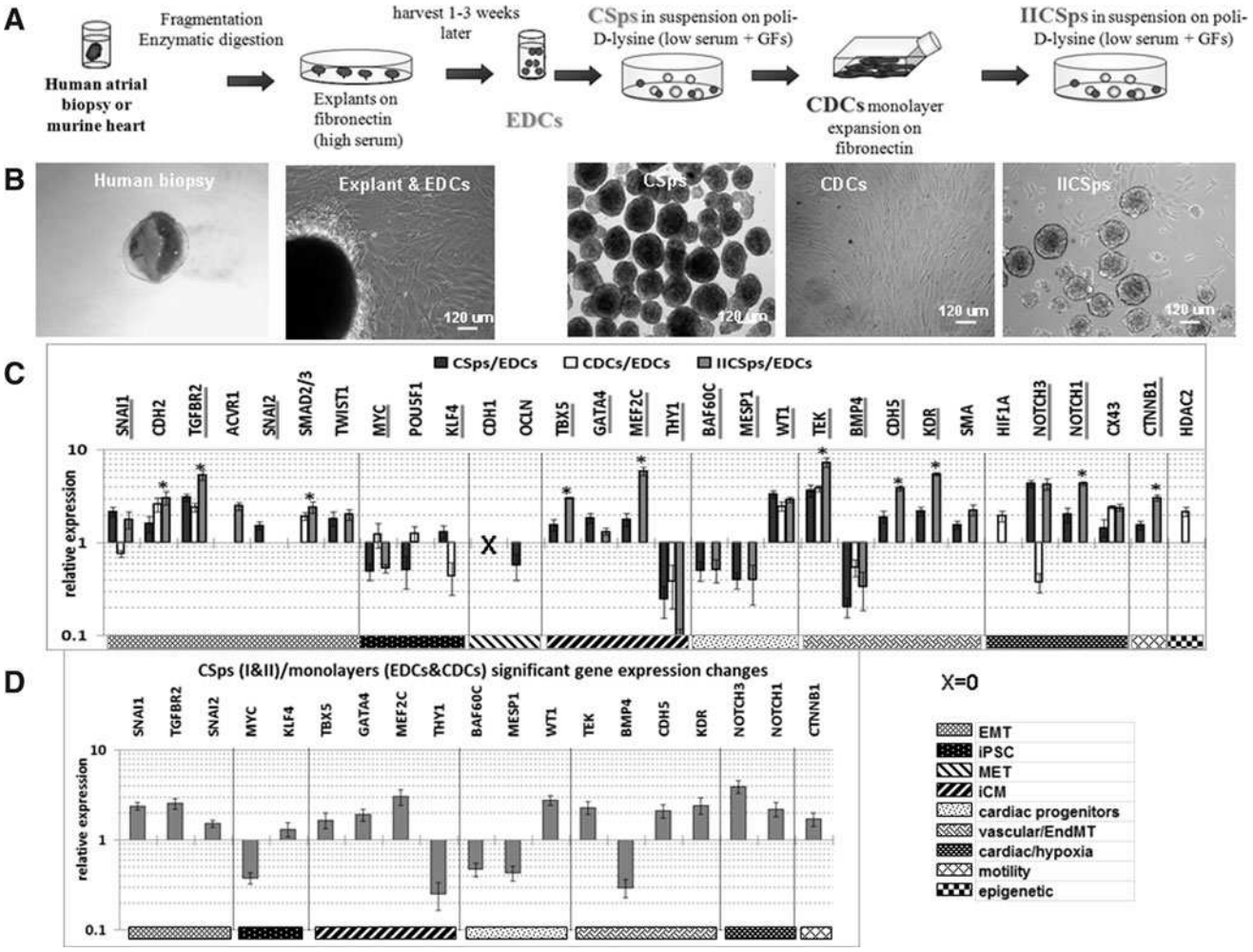

FIG. 1. EMT is involved in CSps formation. **(A)** Schematic representation of cell culture procedures. **(B)** Representative images of a human auricular biopsy, a cardiac explant surrounded by migrating EDCs, CSps, CDCs, and IICSps. **(C)** Significantly modulated ($P < 0.05$) gene expression levels in CSps ($n = 9$), CDCs ($n = 4$), and IICSps ($n = 4$), normalized to EDCs ($n = 7$), assessed by Q-RT-PCR ($\Delta\Delta$Ct method, *GAPDH* as reference gene for all experiments), and presented on a logarithmic scale. *$P < 0.05$ IICSps versus CSps. **(D)** Significantly modulated ($P < 0.05$) gene expression levels between 3D structures (CSps and IICSps, $n = 13$) and monolayers (EDCs and CDCs, $n = 11$) assessed by Q-RT-PCR and presented on a logarithmic scale. Scale bars = 120μm. EDCs, explant-derived cells; CDCs, cardiosphere-derived cells; IICSps, secondary cardiospheres; EMT, epithelial-to-mesenchymal transition; iPSC, induced pluripotent stem cells; MET, mesenchymal-to-epithelial transition; iCM, induced cardiomyocyte; EndMT, endothelial-to-mesenchymal transition; BAF60c, SMARCD3.

(Fig. 1D). *CDH2*, *SMAD2/3*, and *TGFBR2* expression increased at all culture stages compared with EDCs, even in CDCs (Fig. 1C), suggesting the possible occurrence of intermediate states of EMT [28]. Another mediator of EMT that is directly activated by both HIF1α and the BMP/TGF pathway [27,29] is *NOTCH1*, which sustains CPC self-renewal [30]. *NOTCH1* was up-regulated in CSps/IICSps compared with EDCs/CDCs. *NOTCH3* was similarly up-regulated in 3D cultures and down-regulated in CDCs, suggesting that NOTCH3 and NOTCH1 might act in a similar manner (NOTCH3 controls self-renewal and hypoxia survival in human stem/progenitor cells isolated as mammospheres [31]). Second, we investigated stemness genes used in reprogramming to obtain iPSC [23,24] that might reflect a spontaneous loss or acquisition of a stem state by EDCs at the CSp stage. We observed that *MYC* was down-regulated in 3D structures compared with monolayers, and *POU5F1* was down-regulated in CSps versus EDCs/CDCs, which is consistent with their role in promoting MET through the down-regulation of *TGFβ/TGFβR* and *SNAI1*, respectively [32]. An exception is *KLF4*, which can potentiate the TGFβ pathway [33], and was up-regulated in CSps and down-regulated in CDCs compared with EDCs. *BMP7*, a MET inducer and a TGFβ antagonist [34], was never detectable; *CDH1* was significantly down-regulated at all culture stages compared with EDCs, in accordance with previous studies [10], while *CTNNB1* was significantly up-regulated in 3D structures versus monolayers, consistently with a greater mobility. Third, mesenchymal markers varied according to cell type. *ACTA2* was significantly up-regulated in I/IICSps, and *GJA1* [35] was up-regulated in all culture stages compared with EDCs. In contrast, *THY1*, as a marker of cardiac fibroblasts [25], was significantly down-regulated at all culture stages, in particular CSps and IICSps. Markers of

embryonic mesendoderm, including uncommitted cardiac progenitors, were down-regulated in I/IICSps compared with monolayers, including *MESP1* [36,37] and *SMARCD3* (encoding the BAF60c subunit of the Swi/Snf-like chromatin remodeling complex) [38]. However, later markers of CPCs, such as *GATA4*, *TBX5*, and *MEF2C* (which were recently reported as being capable of inducing cardiac fibroblast transdifferentiation into cardiomyocyte-like cells [25,26]), were up-regulated in 3D structures compared with monolayers, suggesting that CSps comprise atypical cardiac mesoderm [39]. Further, *TBX5* and *MEF2C* were up-regulated in IICSps compared with CSps. Endothelial progenitor markers (*KDR*, *CDH5*, and *TEK*) were similarly up-regulated at all culture stages compared with EDC, and more so in IICSps. *BMP4*, mostly involved in EndMT [40] and late stages of cardiomyocyte differentiation, was down-regulated in 3D structures. Lastly, investigating epicardial markers, we found *WT1* to be up-regulated in all 3D structures. Hypoxia in the CSp core [41] might induce *WT1*, a crucial mediator of epicardial EMT, similar to *WT1* activation in vivo by ischemia in the adult epicardium [42–44].

Inhibition of TGFβ pathway blocks CSps formation

To establish a functional relationship between EMT and CSps formation or differentiation, cultures were treated with TGFβ, a potent promoter of EMT in numerous normal and pathological contexts [15,16]. TGFβ reproducibly enhanced primary or IICSps formation (Fig. 2A, C), whereas the divergent TGFβ superfamily member BMP4 and Activin had

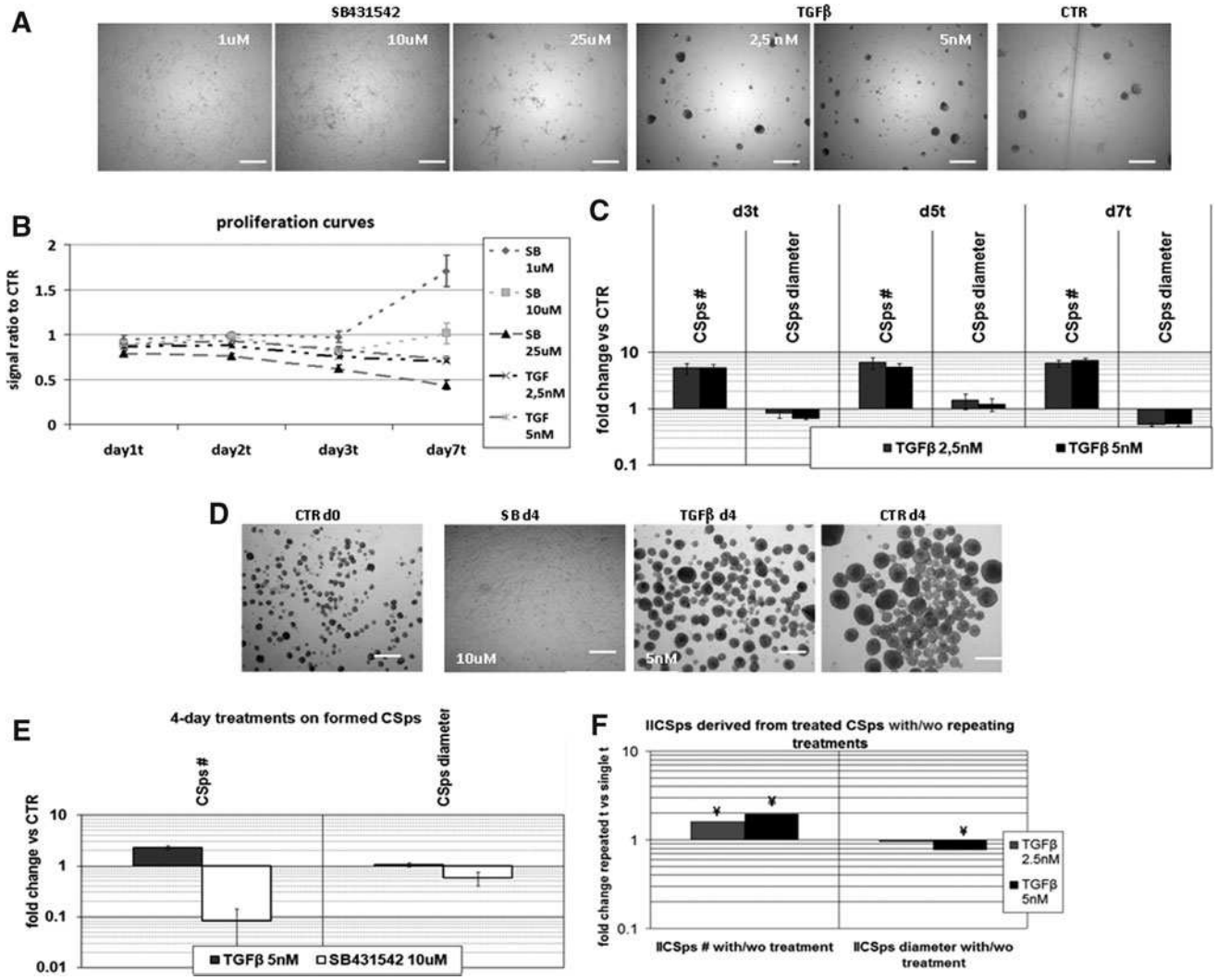

FIG. 2. SB431542 inhibits and TGFβ enhance CSps formation. **(A)** Representative images of EDCs plated to form CSps with or without (CTR) 7 day treatments with SB431542 (1, 10 or 25μM) or TGFβ (2.5 or 5 nM). **(B)** Proliferation curve by WST-8 assay of different TGFβ and SB431542 treatments, normalized to standard culture conditions ($n=4$). **(C)** CSps yield and size in TGFβ-treated samples versus controls ($n=4$). No CSps were detected in the presence of SB431542. **(D)** Representative images of preformed CSps at day 0 and after 4 days of TGFβ or SB431542 treatments. **(E)** CSps number and diameter in TGFβ 5 nM or SB431542 10 μM treatments for 4 days on preformed CSps ($n=3$). **(F)** IICSps derived from CSps in the presence of TGFβ do not maintain memory of the treatment. *Bars* show the average fold change in the number and diameter of IICSps (from a cell line of already treated primary CSps) obtained by repeating the TGFβ treatment, normalized to IICSps obtained without the second TGFβ treatment. Scale bar = 280 μm. SB, SB431542; d, days; t, treatment; TGFβ, transforming growth factor β; ¥, $p<0.05$ versus untreated.

no effect (data not shown). TGFβ caused CSps to form 2 days earlier than control and increased the overall yield, producing more compact structures (Fig.2A, C). Cell proliferation, measured indirectly in terms of metabolic activity of viable cells [45], was moderately reduced (Fig. 2B), consistently with previous studies showing that the proliferation rate is lower in CSps culture compared with adherent cells [10]. These effects were not observed in IICSps derived from previously TGFβ-treated primary CSps in the absence of TGFβ; only repeated treatments with the growth factor produced significantly higher numbers of IICSps compared with controls (Fig. 2F).

We repeated the experiments with SB431542, which blocks the type I receptors (Alk4/5/7) for TGFβ and Activin [17,46]. TGFBR1 mediates most TGFβ1 functions, including EMT [47–49]. No I/IICSps were obtained in the presence of this inhibitor, neither from EDCs nor from CDCs, (Fig. 2A, C), while it induced the spreading and dissolving of previously formed CSps after 3 (Supplementary Fig. S1) or 4 days (Fig. 2D, E).

Treatment of EDCs/CDCs with SB431542 or TGFβ elicited opposite effects on the analyzed genes (Fig. 3). In particular, genes characteristically up-regulated in 3D culture (CSps/IICSps), including *SNAI1*, *ACVR1*, *TGFBR1*, *FGFRI*, *SMAD2/*

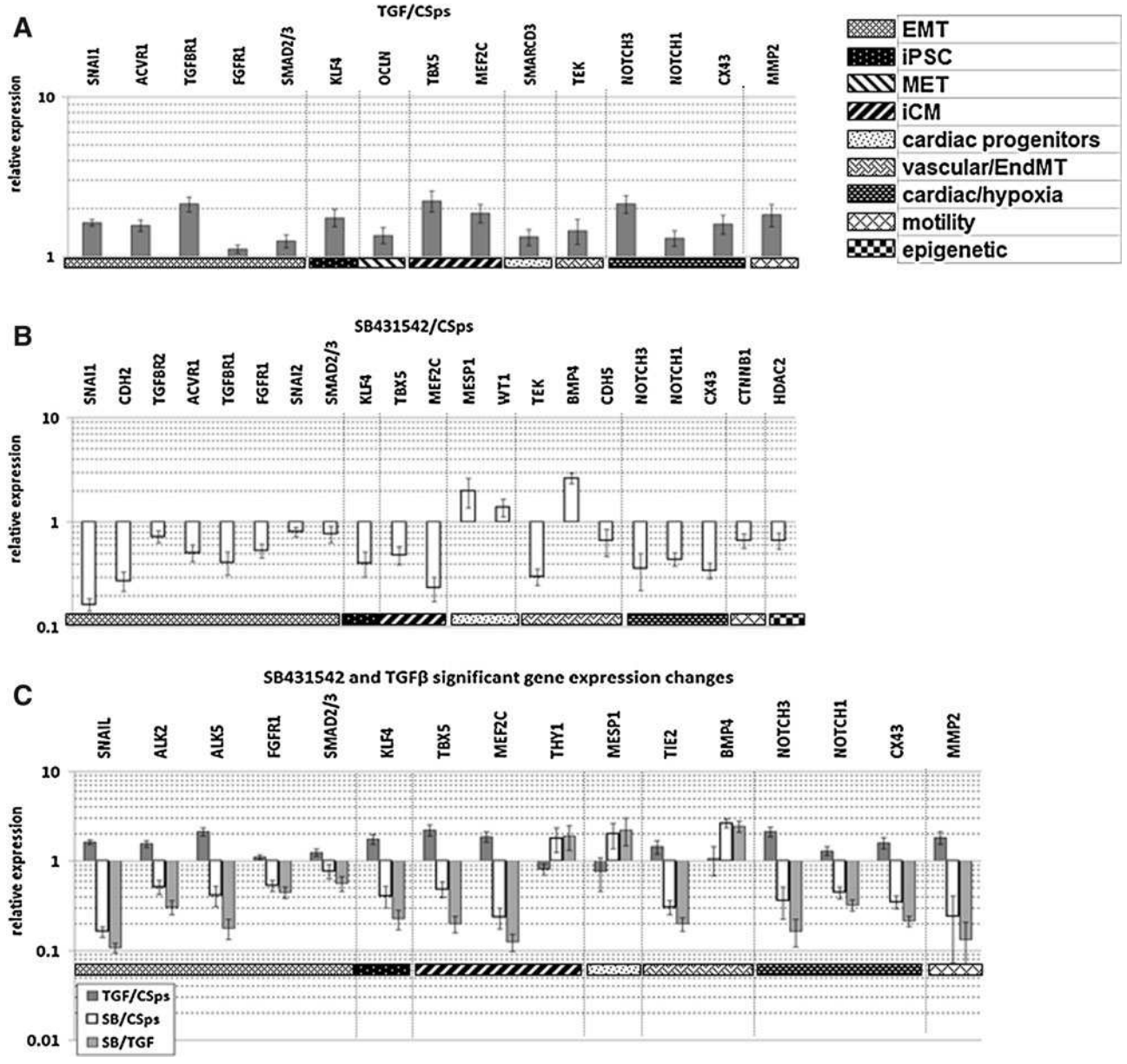

FIG. 3. SB431542 treatment down-regulates, while TGFβ further up-regulates the expression of genes that are up-regulated in 3D structures versus monolayers. **(A)** Significantly modulated ($P < 0.05$) gene expression levels in CSps/IICSps obtained in presence of TGFβ ($n = 7$) versus untreated controls ($n = 13$) assessed by Q-RT-PCR and presented on a logarithmic scale. **(B)** Significantly modulated ($P < 0.05$) gene expression levels in EDCs/CDCs plated to form CSps/IICSps, respectively, in the presence of SB431542 10 μM for 7 days ($n = 6$) versus untreated CSps/IICSps as control ($n = 13$). **(C)** Normalized gene expression levels that were significantly modulated both in TGFβ ($n = 7$) and SB431542 ($n = 6$) treated samples versus untreated CSps ($n = 13$). SB, SB431542; SB/TGF, SB431542 treated CSps versus TGFβ treated CSps, both normalized versus untreated CSps.

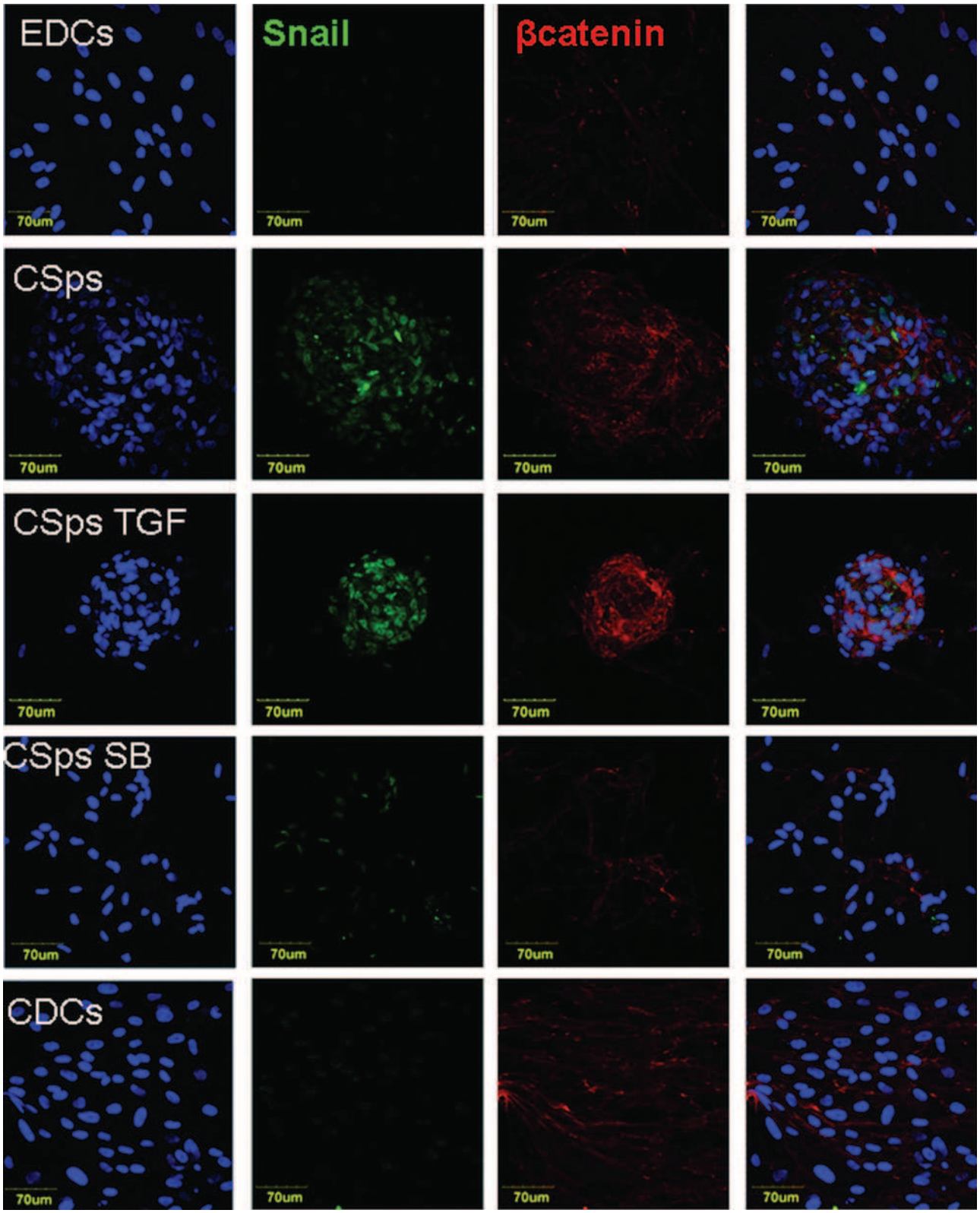

FIG. 4. SNAI1 expression and CTNNB1 localization at different culture stages. Immunofluorescence staining of EDCs, CSps, and EDCs plated to form CSps in the presence of TGFβ (5 nM) or SB431542 (10 μM), and CDCs for SNAI1 (SNAIL, *green*), CTNNB1 (βcatenin, *red*), and Hoechst (*blue*). Confocal images were acquired with a 40× oil immersion objective, with the same settings and from the same set of stainings. For CSps, 1 representative slice has been selected out of a stack of 10–15 slices (1.5 μm step). Color images available online at www.liebertpub.com/scd

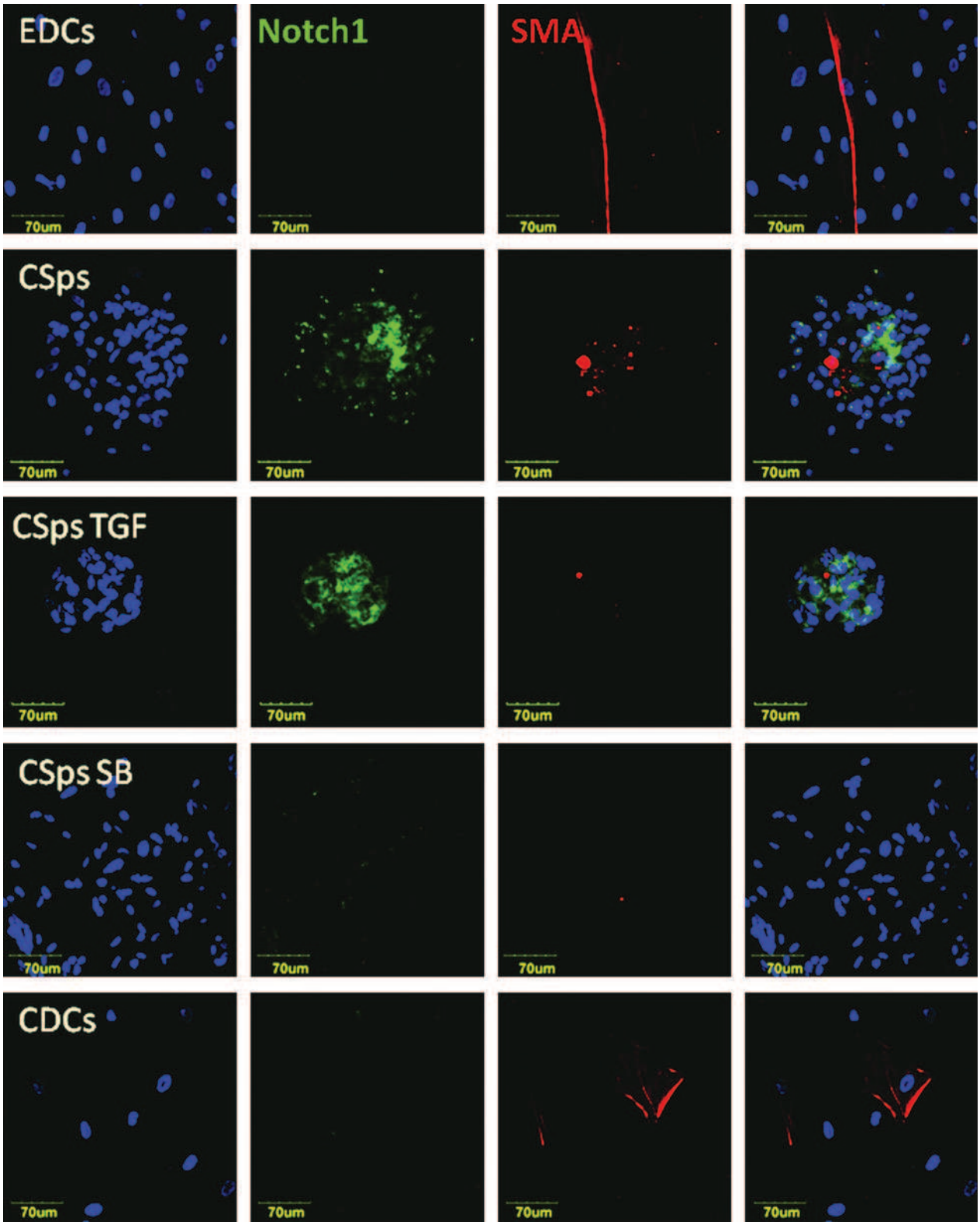

FIG. 5. NOTCH1 is up-regulated in 3D structures. Immunofluorescence staining of EDCs, CSps, and EDCs plated to form CSps in the presence of TGFβ (5 nM) or SB431542 (10 μM), and CDCs for NOTCH1 (*green*), ACTA2 (SMA, *red*), and Hoechst (*blue*). Confocal images were acquired with a 40× oil immersion objective, with the same settings and from the same set of stainings. For CSps, 1 representative slice has been selected out of a stack of 10–15 slices (1.5 μm step). Color images available online at www.liebertpub.com/scd

3, *KLF4*, *TBX5*, *MEF2C*, *NOTCH3*, *NOTCH1*, *GJA1*, and *TEK*, were significantly up-regulated in the presence of TGFβ and down-regulated by its inhibitor. On the other hand, *BMP4*, *MESP1*, and *THY1* (down-regulated in 3D structures compared with monolayers) were significantly up-regulated in the presence of SB431542, and unchanged or slightly down-regulated in the presence of TGFβ compared with control CSps. Immunostainings confirmed the differential regulation at the protein level. Comparing confocal images, acquired with the same settings, brighter and more widespread fluorescence signals were related to SNAI1 (Fig. 4); NOTCH1 (Fig. 5) and Notch3 (Supplementary Fig. S2) in 3D structures were detectable compared with monolayers (EDCs, CDCs, and SB-treated EDCs). Similarly, WT1 (Supplementary Fig. S3) also appeared more abundant in CSps and TGFβ/SB-treated CSps, than in EDCs and CDCs. Taken together, these results are consistent with the idea that TGFβ-dependent EMT promotes and controls CSps formation.

Discussion

CSps are a promising candidate for autologous cardiac cell therapy. The aim of this study was to gain an insight into both the origin and the mechanism underlying the generation of these niche-like 3D multicellular clusters. Profiling the morphological and gene expression changes that occur during formation of the 3D structures (CSps/IICSps), relative to monolayers (EDCs/CDCs), suggested that EMT underlies the formation of CSps and the subsequent differentiation of later-stage cardiogenic progenitors. Thus, CSps and IICSps formation involved the up-regulation of markers of EMT (e.g., *SNAI1*, *TGFBR2*, *TGFBR1*, and *TWIST*) and cardiac progenitors (e.g., *TBX5*, *MEF2C*, *CDH5*, *KDR*, *NOTCH1*, and *CTNNB1*) (Fig. 1). Interestingly, some of the same genes also changed significantly in CDCs versus EDCs, but in the opposite way compared with 3D structures (*SNAI1*, *KLF4*, and *NOTCH3*), indicating divergence of lineage specification between 3D and monolayer cultures. In some cases, however, gene profiles in CDCs were more similar to CSps than to EDCs (e.g., ↑*TGFBR2*, ↓*THY1*, ↑*TEK*, and ↓*BMP4*), suggesting that CDCs represent an intermediate stage that retains memory of passing through the CSp stage, which seems to function as a selective/inductive stage [50]). Together, these data indicate that the formation of the 3D structures involves EMT and supports the differentiation of cardiac progenitors.

EMT is a critical morphogenetic process for heart development [19]. In particular, cells from the epicardial layer of the developing heart delaminate during early gestation and migrate into the underlying myocardium to form fibroblasts, certain cells of the coronary vasculature, and possibly a minor proportion of cardiomyocytes [51,52]. Recent studies suggest that this process might be recapitulated in the adult heart after myocardial infarction, such that epicardial-derived progenitor cells, or EPDCs, may provide a source of cells for myocardial regeneration [53]. A key finding of our study is that WT-1, a zinc-finger transcription factor that marks the activated epicardium [52], is significantly up-regulated on CSps formation. WT-1 binds to promoters of *SNAI1* and *CDH1* to induce or inhibit their activity, respectively, and promotes EMT [54]. Together with the EMT gene profile, the significant up-regulation of *WT1* on CSps formation suggests a re-activation of the developmental and injury-induced epicardial program that might constitute an in vitro recapitulation of the formation of cardiogenic progenitors during development and in the adult [53,55–57].

Mechanistically, we provide evidence that TGFβ directs EMT during CSps formation. Accordingly, genes that characterize 3D structures (relative to monolayer cultures) were up-regulated by TGFβ and down-regulated by SB431542. Significantly, TGFβ enhanced CSps yield, whereas SB431542 drastically inhibited it while inducing the spreading of pre-existing CSps. The significant up-regulation of NOTCH in the 3D structures is in agreement with previous studies showing the integration of TGFβ and NOTCH signaling during EMT [29,58,59], and is possibly related to the stem-cell promoting properties of TGFβ in other adult tissues [20,21].

In conclusion, our study implicates TGFβ-dependent EMT as an essential pathway for CSps formation and the acquisition of cardiogenic properties by progenitor cells, mimicking the niche aspects proposed for the subepicardium after injury in the adult heart. Significantly, these studies define a transcriptomic signature for human CSps and their subsequent in vitro culture stages that will aid future studies to enhance their cardiogenic potential.

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Authors Disclosure Statement

No competing financial interests exist.

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