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Original article

Human epicardium-derived cells reinforce cardiac sympathetic innervation

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ABSTRACT

Rationale: After cardiac damage, excessive neurite outgrowth (sympathetic hyperinnervation) can occur, which is related to ventricular arrhythmias/sudden cardiac death. Post-damage reactivation of epicardium causes epicardium-derived cells (EPDCs) to acquire a mesenchymal character, contributing to cardiac regeneration. Whether EPDCs also contribute to cardiac re/hyperinnervation, is unknown.

Aim: To investigate whether mesenchymal EPDCs influence cardiac sympathetic innervation.

Methods and results: Sympathetic ganglia were co-cultured with mesenchymal EPDCs and/or myocardium, and neurite outgrowth and sprouting density were assessed. Results showed a significant increase in neurite density and directional (i.e. towards myocardium) outgrowth when ganglia were co-cultured with a combination of EPDCs and myocardium, as compared to cultures with EPDCs or myocardium alone. In absence of myocardium, this outgrowth was not directional. Neurite differentiation of PC12 cells in conditioned medium confirmed these results via a paracrine effect, in accordance with expression of neurotrophic factors in myocardial explants co-cultured with EPDCs. Of interest, EPDCs increased the expression of nerve growth factor (NGF) in cultured, but not in fresh myocardium, possibly due to an "ischemic state" of cultured myocardium, supported by TUNEL and Hif1α expression. Cardiac tissues after myocardial infarction showed robust NGF expression in the infarcted, but not remote area.

Conclusion: Neurite outgrowth and density increases significantly in the presence of EPDCs by a paracrine effect, indicating a new role for EPDCs in the occurrence of sympathetic re/hyperinnervation after cardiac damage.

1. Introduction

Cardiac function is influenced by the cardiac autonomic nervous system tone. Cardiac sympathetic stimulation increases heart rate, contraction force and atrioventricular conduction velocity, whereas parasympathetic stimulation largely results in opposite effects. Balanced cardiac sympathetic and parasympathetic activity is critical for maintaining normal cardiac function. Excessive sympathetic activation can be pro-arrhythmogenic, whereas parasympathetic activation is considered to be cardioprotective [1,2]. An accumulating amount of reports indicate a relation between ventricular arrhythmias, sudden cardiac death and activity of the sympathetic autonomic nervous system [3,4]. Although sudden cardiac death after myocardial infarction (MI) is in general attributed to heterogeneous conduction in the infarct border zone [5], autonomic hyperinnervation after MI is also related to sudden cardiac death in numerous reports [6,7]. In addition, sympathetic hyperinnervation has been described in other states of cardiac damage/overload, such as hypertension, RV overload and pulmonary hypertension [8,9].

The definition of post-MI sympathetic hyperinnervation is most often linked to an increase of sympathetic nerve sprouting which can be observed between 3h to several months after the cardiac event [10]. The increased sympathetic nerve density is considered to result from sprouting from cardiac sympathetic ganglia, clusters of neuron cell bodies located bilaterally to the spinal cord at cervical and upper thoracic levels [11].

During development, ventricular innervation develops in close relationship with ventricular vascularization and axons use the developing coronary veins as guides. Neurotrophic factors secreted by coronary veins/epicardium-derived smooth muscle cells guide this process

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[12,13].

The epicardium is a monolayer of tissue covering the heart. Although it is predominantly quiescent in the healthy adult heart, in the fetal phase it is a multipotent population with various functions. Cells derived from the epicardium, so called epicardium-derived cells (EPDCs), function as a powerful population of cardiac progenitor cells [14–16]. EPDCs expressing e.g.Tbx18, Tcf21 and WT1 can undergo a process known as epithelial-to-mesenchymal transition (EMT) [17,18]. These post-EMT EPDCs acquire mesenchymal cell characteristics and the ability to migrate and populate the subepicardial space by differentiating into different cell types [16]. Of interest, the fetal epicardium also transiently expresses the β2-adrenergic receptor (β2-AR), and was shown to be essential for the early autonomic response [19].

In the adult, epicardium can get reactivated after cardiac damage such as MI. After reactivation, EPDCs regain an embryonic phenotype with an enrichment of EPDCs in the infarcted area [15,20,21]. Exogenous administrated adult-derived mesenchymal EPDCs have also been shown to migrate to the infarcted area where they contribute to neovascularization and amelioration of left ventricular function [22]. Whether EPDCs also contribute to cardiac re-innervation as is shown after MI, is currently not known.

The current study is aimed at exploring the effect of adult-derived mesenchymal EPDCs on cardiac sympathetic innervation and demonstrates that these potent cells promote sympathetic nerve sprouting towards “damaged” cultured adult myocardium in vitro.

2. Materials and methods

A detailed overview of methodology for all sections described below, is provided in the *Supplemental Materials*.

2.1. Experimental animals

C57BL/6 J (Jackson Laboratory) adult mice (n = 42) and mice embryos of embryonic day (E) 18.5 (n = 81) were used. All animal experiments were carried out according to *the Guide for the Care and Use of Laboratory Animals* published by NIH and approved by the Animal Ethics Committee of the Leiden University (License number AVD1160020185325), Leiden, The Netherlands).

2.2. MI induction

8-week male C57BL/6 J mice (n = 4) were anesthetized with isoflurane 5% for induction and 1.5–2% for maintenance. MI was induced by permanent ligation of left anterior descending coronary artery for 7 days [22].

2.3. Isolation and culture of human EPDCs

EPDCs were isolated from adult human cardiac tissue and EMT was induced as previously described [23,24]. Briefly, the epicardium was carefully peeled off and cells were dissociated, to exclude contamination with other cells. Isolated EPDCs of epithelial state have a typical cuboidal morphology, therefore EPDCs can readily be distinguished from other cell types. Only cultures that displayed a clear epithelial morphology were used for further experiments to ensure their derivation from the epicardium. Mesenchymal EPDCs were obtained after several days of TGFβ stimulation and were cultured for several passages in an mesenchymal state. Mesenchymal post-EMT EPDC are characterized by their morphology and gene expression pattern (Supplemental fig. S1). Human mesenchymal EPDCs of passage 6 to 9 were prepared into aggregates and cultured for 4 days until ready for further co-culture. All experiments were performed according to the guidelines of the Leiden University Medical Centre (Leiden, The Netherlands) and according to the Dutch regulations on the use of human tissues. The study was conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and national and institutional guidelines, regulations and acts.

2.4. Dissection and culture of adult murine myocardium

Pregnant mice were euthanized in a CO_2 chamber. Left ventricular myocardium was isolated from mother mice and processed into pieces of similar size of 0.1mm^3. To induce myocardial damage, processed myocardium pieces were cultured in a cell incubator (at 37 ˚C and 5% CO_2) for 7 days before subsequent culturing.

2.5. Isolation of murine embryonic sympathetic ganglia

After resection of the uteri of euthanized pregnant mice, embryos were collected and euthanized in cold PBS. Superior cervical ganglia (SCG) were isolated from E18.5 embryos and kept in cold PBS. Each SCG was processed into 3–4 pieces for subsequent culturing.

2.6. Sympathetic ganglion culture and neurite outgrowth assay

Collagen gel was used to provide a 3-dimentional environment for neurite projection from ganglia explants. Freshly isolated SCG explants were randomly cultured under 5 different conditions in complete medium for 6 days before fixation and further staining.

2.7. Immunostaining of ganglion explants in gel

All SCG cultured in gel were whole-mount immuno-stained with anti-β-Tubulin III (Tubb3) to visualize outgrowth. Expression of the enzyme Tyrosine hydroxylase (TH), that is part of the catecholamine biosynthesis pathway necessary for stimulating myocardial contraction, was used to demonstrate the sympathetic phenotype of these ganglia [25]. To verify that growing sympathetic SCG were of a similar phenotype as those freshly isolated, whole-mount anti-TH immunostaining was performed in cultured growing SCG as well as in freshly isolated SCG. Growing SCG show a similar TH expression profile as compared to freshly isolated SCG (Supplemental Fig. S2A,B). In addition, post-ganglionic varicosities, the site in the axon were the neurotransmitters are contained and released, forming the site of the synapses [26,27] were detected in our co-cultured ganglionic neurites (Supplemental Fig. S2C).

2.8. Quantification of neurite outgrowth

The work flow of outgrowth quantification is illustrated in supplemental Fig. S3A. Tubb3+ images of cultured SCG were used to quantify the neurite outgrowth according to the presence of neurite outgrowth and its directional preference. For directional outgrowth, a method to quantify the length and density of directional neurites was developed based on different existing plugins in ImageJ: briefly, Quadrant picking was used to identify the quadrant showing directional outgrowth towards myocardium/EPDCs; NeuriteJ was utilized to count the directional neurites length and density in this quadrant. To correct for potential differences in amount of myocardium used in each experiment, all data were normalized for myocardial volume. The myocardial volume was measured and calculated by applying z-stack in confocal imaging. The normalized directional neurite outgrowth was calculated as: Normalized directional neurite number = Counted neurite number/Relative myocardium volume.

2.9. PC12 neurite outgrowth assay

Serum-free conditioned media were collected after 48-h culturing. PC12 cells were cultured in low-serum media (with/without the addition of NGF) or conditioned media supplemented with 1% FBS. After culturing, the PC12 cells were immuno-stained with anti-β-Tubulin III

(tubb3) and neurite differentiation of PC12 in each condition was quantified.

2.10. Real time PCR and immuno-blotting

Left-ventricular myocardium explants (freshly isolated or cultured for 7 days in vitro) were cultured exclusively or co-cultured with EPDCs for 2 days. Fresh and cultured myocardial explants were collected for gene expression analysis by real time PCR and protein expression analysis by dot blotting and WES (automated Western Blots).

2.11. TUNEL assay and immuno-staining in myocardium sections

Sections of freshly isolated, in-vitro cultured (for 7 days) left-ventricular myocardium explants and 7-day post-MI hearts were stained (see Supplement for further details) with TUNEL, anti-NGF and anti-Wilms' tumor 1 (WT-1) antibodies. NGF expression in myocardium explants and post-MI hearts was quantified with ImageJ.

3. Results

3.1. Adult mesenchymal EPDCs enhance neurite outgrowth of sympathetic ganglia

To investigate the relationship of EPDCs with the establishment and remodeling of cardiac sympathetic innervation, murine sympathetic superior cervical ganglia (SCG) of late embryonic stages (E18.5) were isolated and cultured with human-derived mesenchymal EPDCs in a 3D-culture system. To mimic the in-vivo absence of direct neuron-EPDC communication (cell-cell contact) and determine the paracrine potential, cellular contact between EPDCs and SCG was prevented by preparing the mesenchymal EPDCs as aggregates in collagen gel. In this way, EPDC aggregates were formed, 4 days prior to co-culturing them with E18.5 ganglia in vitro. Each of these EPDC-ganglion co-cultures was allowed to sprout for 6 days, followed by staining to detect the effects of EPDCs on nerve sprouting of SCG in vitro (Fig. 1A).

The SCG explants cultured in the vehicle condition (Fig. 1B) showed very limited neurite outgrowth, with either absent or very short neurites. SCG explants in +EPDCs condition (Fig. 1C) exhibited robust neurite outgrowth with regard to both density and length of neurites. Comparison of the occurrence of neurite outgrowth (regardless of the length and density of neurites) between SCG in vehicle condition and in +EPDCs condition demonstrated that EPDCs significantly increase the neurite outgrowth of sympathetic ganglia (Fig. 1F).

3.2. Directional sympathetic nerve sprouting is induced by myocardium and this effect is amplified by EPDCs

Communication between target organs and nerve cells contributes to the innervation of target organs [28]. To study whether adult mesenchymal EPDCs participate in facilitating sympathetic neurite sprouting to target tissue (i.e. the heart), ventricular myocardium was included into the co-culture. Adult-derived murine ventricular myocardium explants were prepared and were cultured ex-vivo for 7 days prior to co-culture with SCG explants, after which similar procedures were followed as described (Fig. 1A). The SCG explants cultured in the +EPDCs+M condition showed not only abundant but also directionally sprouted neurites towards ventricular myocardium explants (Fig. 1D) compared to the abundant but non-directional neurite outgrowth of SCG in the +EPDCs condition (Fig. 1C).

To clarify the separate roles as well as potential interactions of mesenchymal EPDCs and myocardium affecting ganglionic outgrowth, SCG explants isolated from the same embryos were randomly divided and cultured in either +EPDCs+M or +M conditions. After a matched co-culture period of 6 days, anti-Tubb3 staining showed directionally sprouted neurites towards myocardium explants in both co-culture conditions (Fig. 1D, E). However, the sprouting density of neurites appeared higher in the +M+EPDCs compared to the +M group.

When comparing the +M+EPDCs group and the +EPDCs group (Fig. 1F), no difference in the total occurrence of outgrowth from the ganglia was detected but neurite outgrowth showed a significant increase in occurrence of *directional outgrowth* in the +M+EPDCs condition compared to +EPDCs condition (Fig. 2A).

3.3. Quantification of neurite density shows significant increase in growth and branching of directional neurites in presence of EPDCs

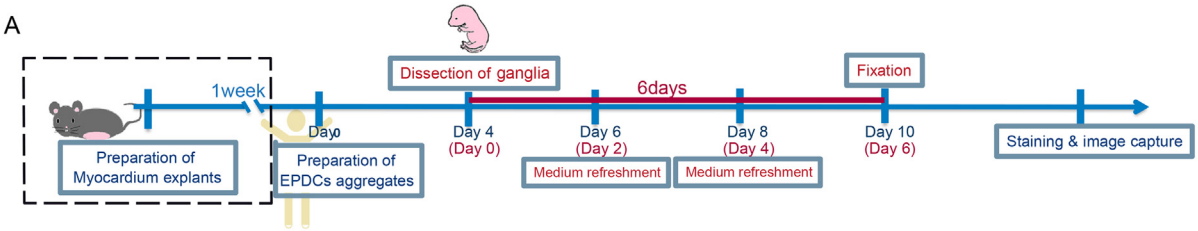
As a next step the density of *directional sprouting* of neurites towards myocardium was quantified in both groups (i.e. +M versus +EPDCs +M).

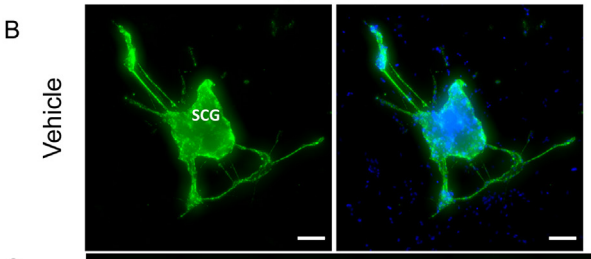
Quadrants in anti-Tubb3 images were determined and #1 was assigned to the quadrant showing neurites outgrowth towards myocardium/EPDCs (supplemental Fig. S3A, Fig. 2B). Outgrowth was designated as "*directional outgrowth*" when denser and longer neurites were observed in quadrant 1 (as is depicted in the graphs of Supplemental Fig. S3B as red lines). Likewise, neurites sprouting in quadrant 1 were named "*directional neurites*". When each quadrant showed a similar neurite density and length, as was the case when SCG were co-cultured with EPDCs only, the outgrowth was designated as "*non-directional*". In this case, the colored lines in Supplemental Fig. S3C depicting the different quadrants overlap, as is shown.

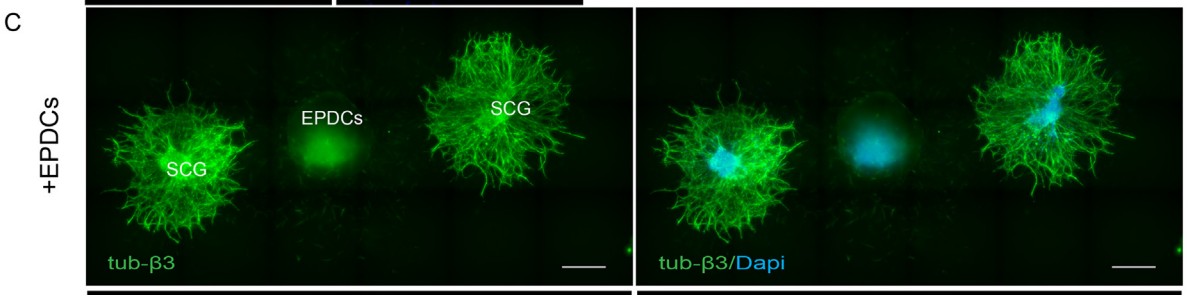
Subsequently, once SCG explants in +M+EPDCs and +M conditions showed directional outgrowth, the directional neurites were semi-automatically quantified and normalized for myocardium volume in each corresponding co-culture. The normalized number of directional neurites at each length (from 100 μm to1600 μm) projecting from the SCG explants is shown in a heatmap (Fig. 2C, relative myocardium volume is shown in supplemental Fig. S3D). As is demonstrated in the heat map, directional neurites with high density (red areas) are predominantly encountered in +M+EPDCs condition. Addition of EPDCs also resulted in a significant increase in growth and branching of directional neurites (Fig. 2D).

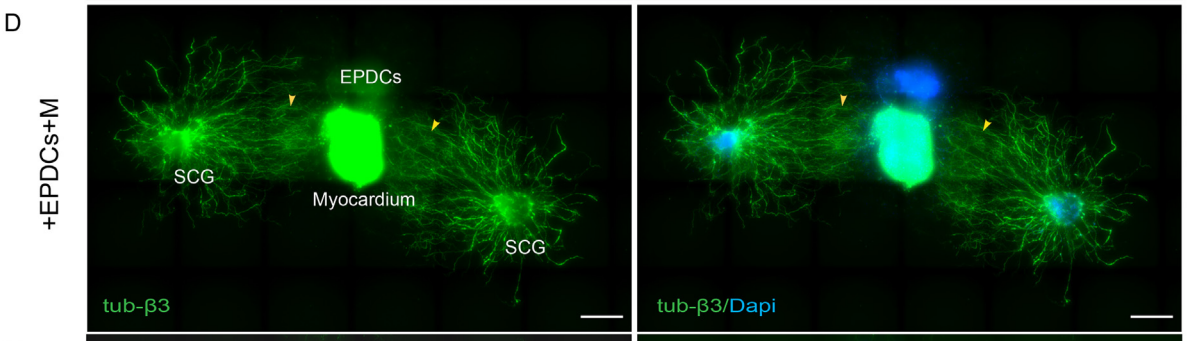
3.4. EPDCs and myocardium augment neurite outgrowth via a paracrine effect

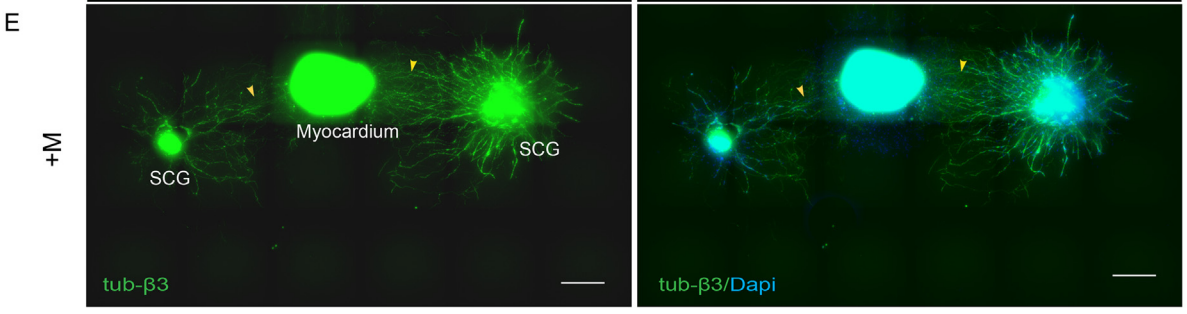
In the SCG/myocardium/EPDC co-cultures, the most dense and elongated neurites were observed in quadrant 1, while outgrowth/density was less in quadrant 2 and 4 and least in quadrant 3 (supplemental Fig. S3B), suggesting a potential paracrine effect. Therefore, the next experiments used conditioned medium of the different culture groups. PC12 cells, derived from rat adrenal medullary tumor, have been shown as an adequate model to study neurite outgrowth in vitro [29,30]. After exposure to NGF, PC12 cells are induced to switch from a proliferating phenotype to a neuron-like phenotype, i.e. neurite differentiation [31]. Conditioned medium of mesenchymal EPDCs (EPDCs med), ventricular myocardium (M med) and co-cultured myocardium with EPDCs (M+EPDCs med) was collected to study the effect on neurite differentiation of PC12 cells. As PC12 cells need a "trigger" to switch to a differentiating phenotype, the cells were first triggered by exposure to 50 ng/ml NGF followed by culturing in different conditioned mediums to study their outgrowth (Supplemental table 2). All conditioned media could induce clear neurite differentiation of PC12 cells after 2-day triggering with NGF (Fig. 3A). PC12 cells triggered by NGF and subsequently cultured in vehicle medium served as a negative control (NGF+/−) and showed limited neurite differentiation. In contrast, PC12 cells cultured in different conditioned mediums after pre-treatment with NGF showed different degrees of augmentation of neurite differentiation. This was significantly different from the PC12 cells in the negative control (NGF+/−) condition, but comparable to the cells in a positive control (NGF+/+) condition (Fig. 3A,B). Quantification showed a significantly higher neurite differentiation in











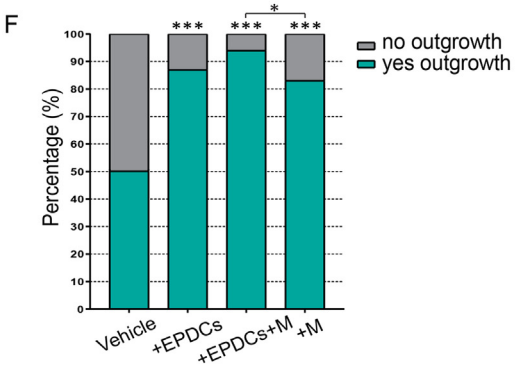


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Fig. 1. Mesenchymal EPDCs enhance the outgrowth of sympathetic ganglia.
A. Schematic timeline of events in co-culturing superior cervical ganglia (SCG) with mesenchymal EPDCs or with mesenchymal EPDCs and ventricular myocardium (indicated in the dashed square).
B-E. Outgrowth of SCG after 6-days of culturing in indicated conditions. Both EPDCs and cultured ventricular myocardium increase the outgrowth of SCG. Directionally organized neurite outgrowth of SCG (indicated by arrowheads in D and E) are detected in +EPDCs+M (panel D) and +M (panel E) co-culturing conditions. Scale bar represents 100 μm in B and 500 μm in C-E.
F. Quantification of the percentage of SCG showing neurite outgrowth shows significant increase of neurite outgrowth in ganglia co-cultured with mesenchymal EPDCs and/or ventricular myocardium as compared to vehicle $n = 48$ for vehicle condition; $n = 38$ for +EPDCs condition; $n = 96$ for +EPDCs+M condition; $n = 95$ for +M condition. Chi-square test was applied to detect the difference among groups, $^{*}P < .05$, $^{***}P < .001$.

combined M+EPDCs medium as compared to cells cultured exclusively with either M medium or EPDCs medium (Fig. 3B), in concordance with findings in the SCG co-cultures (Fig. 2D). Culturing PC12 cells with conditioned media without pre-treatment of NGF showed no significant neurite differentiation (Supplemental table 1, Supplemental Fig. S4A).

3.5. EPDCs increase the expression of NGF in cultured myocardium compared to fresh myocardium

In contrast to the robust neurite sprouting of SCG co-cultured with pre-cultured myocardium, co-culturing SCG with freshly isolated myocardium (+fresh M), showed strikingly less directional outgrowth and only limited density of directional neurites (Fig. 4A). TUNEL assay revealed apoptosis in cultured myocardium but not in freshly isolated myocardium. (Fig. 5A, B). Hif1a (hypoxia-inducible factor 1-alpha), known to regulate the cellular and systemic homeostatic response to hypoxia and activate genes involving energy metabolism and apoptosis, plays a critical role in adaption of ischemic disease [32,33]. Its expression was increased in the ex-vivo cultured myocardium explants, especially in the myocardium co-cultured with EPDCs (Supplemental Fig. S4B). This led to the hypothesis that cultured myocardium explants might show signs of ischemia related cell death leading to the release of factors stimulating neuronal outgrowth. Therefore as a next step several neurotrophic factors known to induce innervation, including Gdnf, Ntf3 and Igfbp6 were examined by RT-PCR, but were not significantly changed in cultured myocardium. The expression of these genes was not significantly altered by the presence of EPDCs either (Supplemental Fig. S4B). However, expression of other genes, like Bmp2, Fgf2, Tgfb1 and Tgfb3 was significantly increased in cultured myocardium (Supplemental Fig. S4B). These genes are known to be involved in various events including cardiac development (i.e. epicardial EMT and differentiation), angiogenesis, neuron differentiation/growth and peripheral nerve regeneration.

In addition to the neurotrophic factors mentioned above, expression of NGF was studied, as it is one of the most critical factors for neuronal survival and nerve growth and has been shown to increase under hypoxic conditions and is released acutely from ischemic myocardium [34,35]. Its induction is regulated by ET-1 via the endothelin receptor A (ETA), which participates in cardiac sympathetic nerve growth during development [36]. As we previously found an increase of ET-1 in re-activated mesenchymal human EPDCs (Supplemental Fig. S4C), we first examined the expression of gene Endothelin receptor A (EdnrA) in myocardium. Increased EdnrA expression was observed in cultured myocardium as compared to fresh isolated myocardium (Fig. 4B). As a next step, NGF expression in cultured versus fresh myocardium was examined by real time PCR. Results showed that NGF in cultured myocardium was significantly upregulated compared to fresh isolated myocardium (Fig. 4B). SEMA3A, a chemorepellent of axonal outgrowth critical for the formation of normal cardiac innervation, was also examined and a decline of SEMA3A expression was detected in the cultured myocardial tissue at mRNA level (Fig. 4B). Both NGF and SEMA3A were further confirmed at protein level by WES (automated Western Blotting) and showed more outspoken alterations in cultured myocardium at protein level (Fig. 4C, D). After co-culturing of myocardium explants with EPDCs, NGF protein was further upregulated in myocardium explants (Fig. 4C).

To confirm the finding of NGF at the tissue level, fresh and cultured myocardial explants as well as explants co-cultured with EPDCs from the same mouse were sectioned and stained with anti-NGF antibody. In fresh myocardium, only a very limited amount of anti-NGF staining was detected (Fig. 5C). In cultured myocardium however, robust staining of NGF was detected, mainly observed in non-cardiomyocyte cells, like cells in vessels with slightly increased NGF detection in cardiomyocytes (Fig. 5D). The presence of EPDCs, as is shown in Fig. 5E, further increased the expression of NGF, which could be widely detected in both cardiomyocytes and non-cardiomyocytes of cultured myocardium. To compare with the in-vivo situation, sections of cardiac tissue after myocardial infarction were studied (Fig. 6). As expected, activation of epicardium was observed with expression of WT-1 in the epicardium (Fig. 6A,B) as reported previously [20]. In the ischemic zone, NGF expression was observed in cardiomyocytes and non-cardiomyocytes including the activated epicardial cells, similar to the findings in myocardium explants co-cultured with EPDCs in vitro (Fig. 6C). In contrast, in the area remote from the infarction, only a low amount of NGF expression was observed similar to the pattern in fresh isolated healthy myocardium explants (Fig. 6D,E), supporting a role of EPDCs on up-regulation of NGF in “damaged” myocardium. Quantification of NGF protein confirmed an significant increase in both the myocardial explants co-cultured with EPDCs and in the ischemic area of post-MI hearts (Fig. 6F, G).

4. Discussion

Ventricular tachyarrhythmias secondary to myocardial infarction (MI) are a major risk factor for sudden cardiac death and have been increasingly associated with ventricular sympathetic hyperinnervation [7,37]. For ventricular (hyper)innervation to occur, axons must grow out of sympathetic chain ganglia towards the heart [11]. Likely, factors from the heart in response to ischemia/hypoxia cause retrograde stimulation of ganglia to induce axonal outgrowth [38]. In the current study, we aimed to explore a potential role of EPDCs in this process.

The epicardium is composed of multifunctional, multipotent progenitor cells with important roles during fetal development [14,15,39]. Of interest, the fetal epicardium transiently expresses the autonomic β2-adrenergic receptor, and presence of this mono-layer on the surface of the myocardium was shown to be essential for the early autonomic response [19]. However, whether and how epicardium or EPDCs participate in establishment of cardiac sympathetic innervation, especially in the remodeling of sympathetic innervation in states of cardiac damage is largely unknown. Although mesenchymal EPDCs are able to differentiate into several cell types, including cardiac fibroblasts, which have been suggested to induce cardiac sympathetic hyperinnervation after MI [40], there is no direct evidence linking EPDCs to sympathetic innervation, as yet.

During development, cardiac autonomic ganglia and nerve fibers have their origin in the neural crest [41], multipotent cells that derive from the embryonic neural plate. Ventricular autonomic innervation develops in parallel with ventricular vascularization, guided by neurotrophic factors [12]. In the current study, in which we co-cultured mouse sympathetic chain ganglia with myocardium and human EPDCs in a collagen based 3D co-culture system, we found evidence for a role of EPDCs in stimulating neuronal outgrowth. This observation opens up

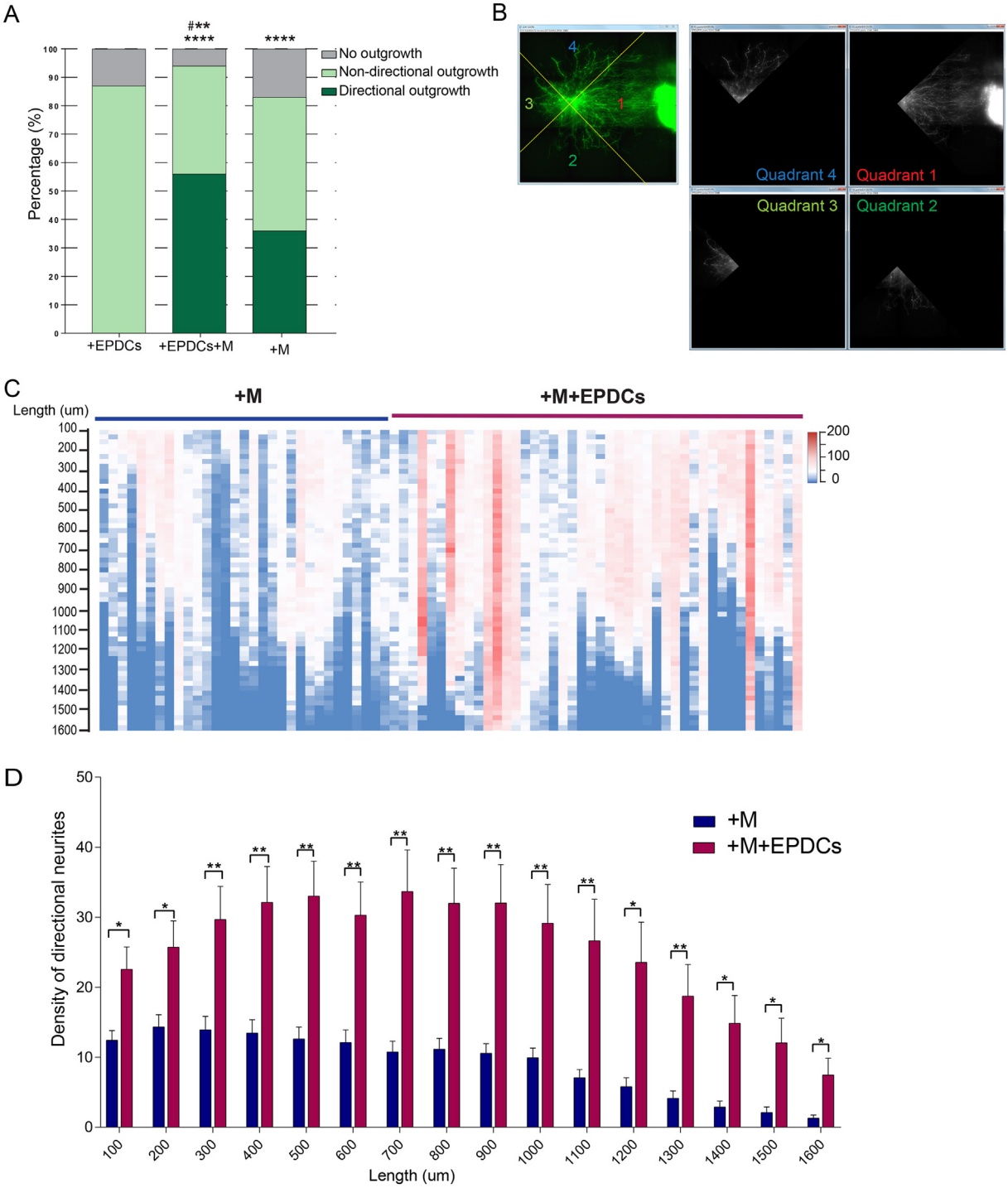


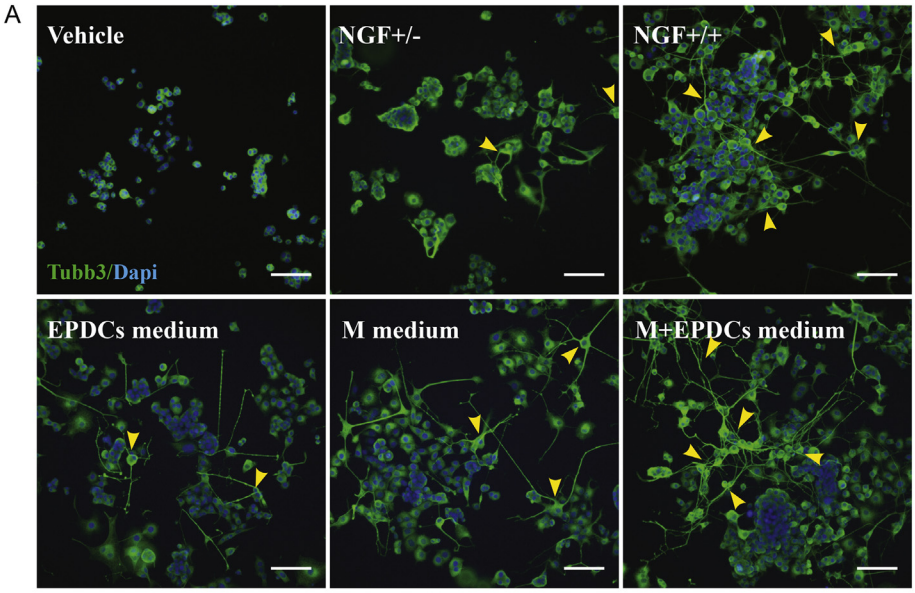
Fig. 2. Mesenchymal EPDCs enhance directional neurite growth of SCG towards ventricular myocardium.

A. Cultured myocardium triggers directional outgrowth (compare +M condition with +EPDCs condition). EPDCs augment this occurrence of directional outgrowth (compare +M condition with +EPDCs+M condition). +EPDC+M condition n = 96, +M condition $n = 95$. Chi-square test was applied to detect the difference among conditions, **** $p < .001$ compared to +EPDCs group; #** $p < .01$ compared to +M group.

B. To define directional outgrowth, Tubb3+ images with detectable neurite outgrowth were first processed by Quadrant picking in ImageJ, which divided the image in 4 quadrants according to the ganglion-myocardium position (SCG explants shown in Fig. 1D are shown as an example). The quadrants were numbered 1 to 4, where quadrant 1 is always the one facing the myocardium/EPDC aggregate, followed clockwise by quadrant 2, 3 and 4.

C. The normalized density of directional neurites (i.e. the neurites in quadrant 1) at the indicated length is shown as a heatmap. Neurite density increases from blue (lowest density) to dark red (hig h density).

D. Statistical analysis indicates a significant difference of neurite density (y-axis) between directional neurite outgrowth in the +M condition and in the +M+EPDCs condition at each distance (x-axis) ($n = 32$ for +M group, $n = 45$ for +M+EPDCs group). Multiple *t*-test was applied to detect the difference between +M and +M+EPDCs, * $p < .05$, ** $p < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



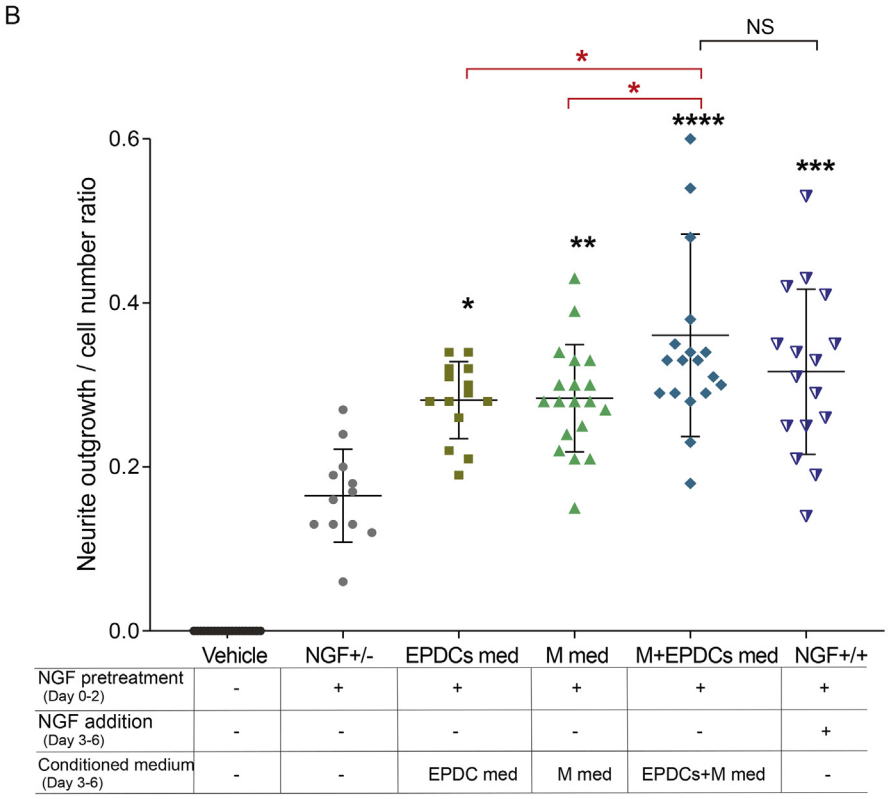


Fig. 3. EPDCs and myocardium augment neurite outgrowth via a paracrine effect.
A. Tubb3 staining of PC12 cells cultured under different conditions as indicated (also see supplemental Table 2). Differentiated PC12 cells and neurites are indicated by arrow heads. Scale bar = 100 μm.
B. Neurite outgrowth of PC12 cells cultured in the indicated conditions (shown in the table below the graph) is quantified and displayed as mean ± SD. One-way ANOVA was applied to detect differences in neurite outgrowth among conditions, and Post Hoc Test (Tukey's multiple comparisons test) in one-way ANOVA was performed to detect the specific difference between groups, * $p < .05$, ** $p < .01$, *** $p < .001$ **** $p < .0001$ compared to NGF+/− condition.

a novel approach to understanding of the regulation of the autonomic innervation of the heart. In all circumstances, adding EPDCs to the ganglion cultures induced a synergistic effect as compared to culturing ganglia with either EPDCs or myocardium alone. The mutual interaction between myocardium and EPDCs has been demonstrated by our group as well in other circumstances, where EPDCs influenced for instance the proper maturation and alignment of myocardium [18,42].

The question remains why healthy murine ganglia, started to show an increase in outgrowth in our in vitro culturing model. We postulated that ischemia/hypoxia induced conditions mimicked a condition of cardiac damage. This was supported by the ganglia-EPDC co-cultures with fresh myocardium, that showed remarkably less outgrowth. These finding are in line with observations in other cell culture systems, showing that even concentrations of oxygen in the physiological range may exert a significant negative impact on cells in culture [43]. Our data on the possible role of induction of “cardiac damage” was further supported by the increase of levels of Hypoxia-inducible factor 1-alpha (HIF1a) in cultured myocardial cells, already starting after 1 day of culture. Hif1a is known to regulate the cellular and systemic homeostatic response to hypoxia and activate genes involving energy metabolism and apoptosis, and plays a critical role in adaption to ischemic disease [32,33].

Paracrine effect of mesenchymal EPDCs on neurite outgrowth. The current culturing experiments using PC12 cells cultured with conditioned

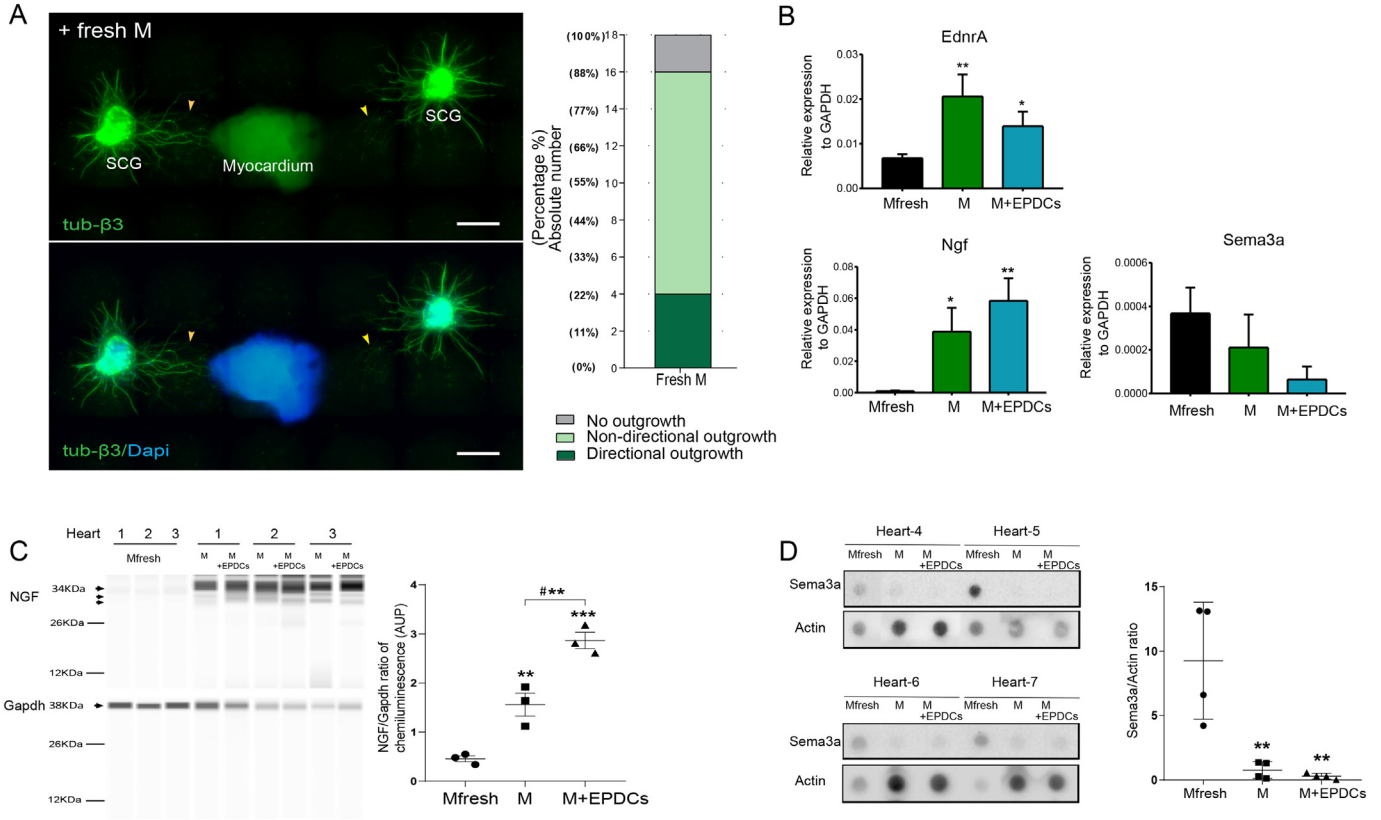


Fig. 4. EPDCs increase the expression of neurotrophic factors in cultured myocardium.
A. SCG co-cultured with freshly isolated myocardium explants (left panel) and the quantification of outgrowth (right panel). Directional neurite outgrowth is indicated with arrowheads. Scale bar = 500 μm. B. Real time PCR results of Ngf, Sema3a and EdnrA expression in freshly isolated myocardium, cultured myocardium and myocardium co-cultured with EPDCs (n = 6). Mfresh = freshly isolated myocardium; Mculture = cultured myocardium ** p < .01 compared to Mfresh. C. The expression of NGF protein in cultured myocardium and in myocardium co-cultured with EPDCs (n = 3), determined by WES (automated western blots). Mfresh = freshly isolated myocardium; M = cultured myocardium; M + EPDCs = the myocardium co-cultured with EPDCs. *p < .05, ** p < .01 compare to Mfresh, #** p < .01 compared to M + EPDCs with M. D. SEMA3A protein in myocardium explants as determined by dot-blotting (n = 4). * p < .05, ** p < .01 compared to Mfresh. Mfresh = freshly isolated myocardium; M = cultured myocardium; M + EPDCs = the myocardium co-cultured with EPDCs. One-way ANOVA and Post Hoc Test (Tukey's multiple comparison test) were performed to detect the differences.

media of myocardium, EPDCs and both, confirmed a paracrine effect of EPDCs on the differential potential of neurons. PC12 cells are derived from rat adrenal medullary tumor cells and have been shown an adequate model to study neurite outgrowth in vitro [29,30]. In line with the in vitro results of ganglion cultures, a synergistic effect of the addition of EPDC medium to the PC12 cell culture was observed, with an increase in neurite sprouting as compared to cultures with either myocardium or EPDCs medium alone. It is well established that epicardium and its EPDCs exert effects on the heart through paracrine signaling via secreted factors [44], which is considered crucial in the regenerative role of EPDCs after MI [16], although a potential effect on neuronal outgrowth has not been described to date. Interestingly, comparable like the in vivo situation after MI, where neurons only start growing fast after an “ischemic hit”, PC12 cells are in a “default” dormant proliferating state and only start differentiating and sprouting when triggered by addition of NGF. NGF is a critical neurotrophic factor supporting neuron survival and axonal growth. Levels of myocardial NGF increased substantially in the presence of EPDCs, demonstrated at both mRNA and protein level. NGF has two main isotypes in vivo; it is initially synthesized as a precursor, proNGF (a complex of alpha, beta and gamma unit) and can be cleaved into a mature functional beta NGF [45]. We only found a low amount of mature NGF staining in fresh myocardium, consistent with a previous report describing the absence of mature NGF in most healthy tissues in both mouse and human [46]. In vivo, anti-mature NGF staining in epicardium-activated hearts (7-day post-MI) supported our findings that EPDCs increase the NGF expression in both non-cardiomyocyte cells as well as in cardiomyocytes in the myocardium.

Similar results in our study were found for other factors involved in regulation of cardiac innervation. Endothelin 1, increasing cardiac innervation via NGF in animal models [36], was upregulated in myocardium in the presence of EPDCs. In contrast, axonal repellent factor, SEMA3A, that guides the establishment of normal epicardium-to-endocardium cardiac sympathetic innervation [47], was decreased in cultured myocardium, the effect of which was again augmented by the presence mesenchymal EPDCs. This decrease in SEMA3 expression could underlie an increased potential for neuronal growth, as SEMA3A has a suppressive function on neuronal growth [48]. Conversely, overexpression of SEMA3A in either the ischemic border zone of myocardium or in sympathetic ganglia was able to diminish cardiac sympathetic hyperinnervation in the damaged heart [47,49].

Besides a modulation of myocardial NGF and SEMA3A expression by EPDCs, an upregulation of transcriptional expression of Bmp2, Fgf2, Tgfb1 and Tgfb3 was also detected. These genes have various functions including their role as growth factors for multiple cell types, participating in cardiac development, directing cells differentiation, as well as promoting peripheral nerve regeneration [50–53].

5. Study limitations

Murine ganglia and myocardium were cultured with human adult EPDCs. In an ideal setting tissues of the same species (preferably

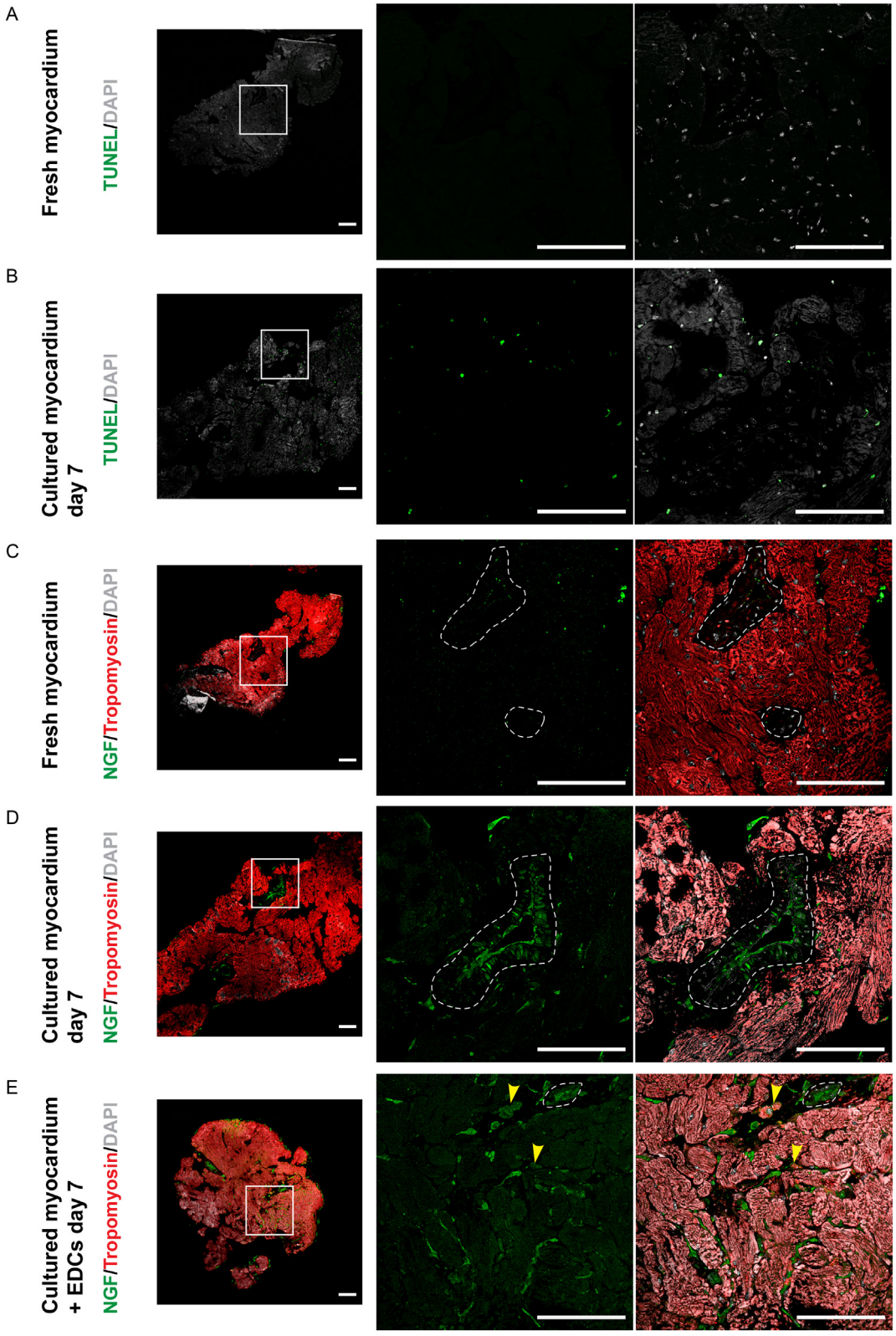


Fig. 5. Cultured myocardium explants shows apoptosis and expression of NGF.
A-B. Detection of apoptosis in fresh and 7-day cultured myocardium explants with TUNEL assay. Scale bar = 100 μm. C-E. Anti-NGF staining in fresh myocardium explants, in cultured myocardium explants and in myocardium explants co-cultured with EPDCs. Anti-Tropomyosin was performed to label cardiomyocytes. Arrowheads indicate cardiomyocytes with NGF staining of high intensity. Dash lined-circles indicate vessels. Scale bar = 100 μm.

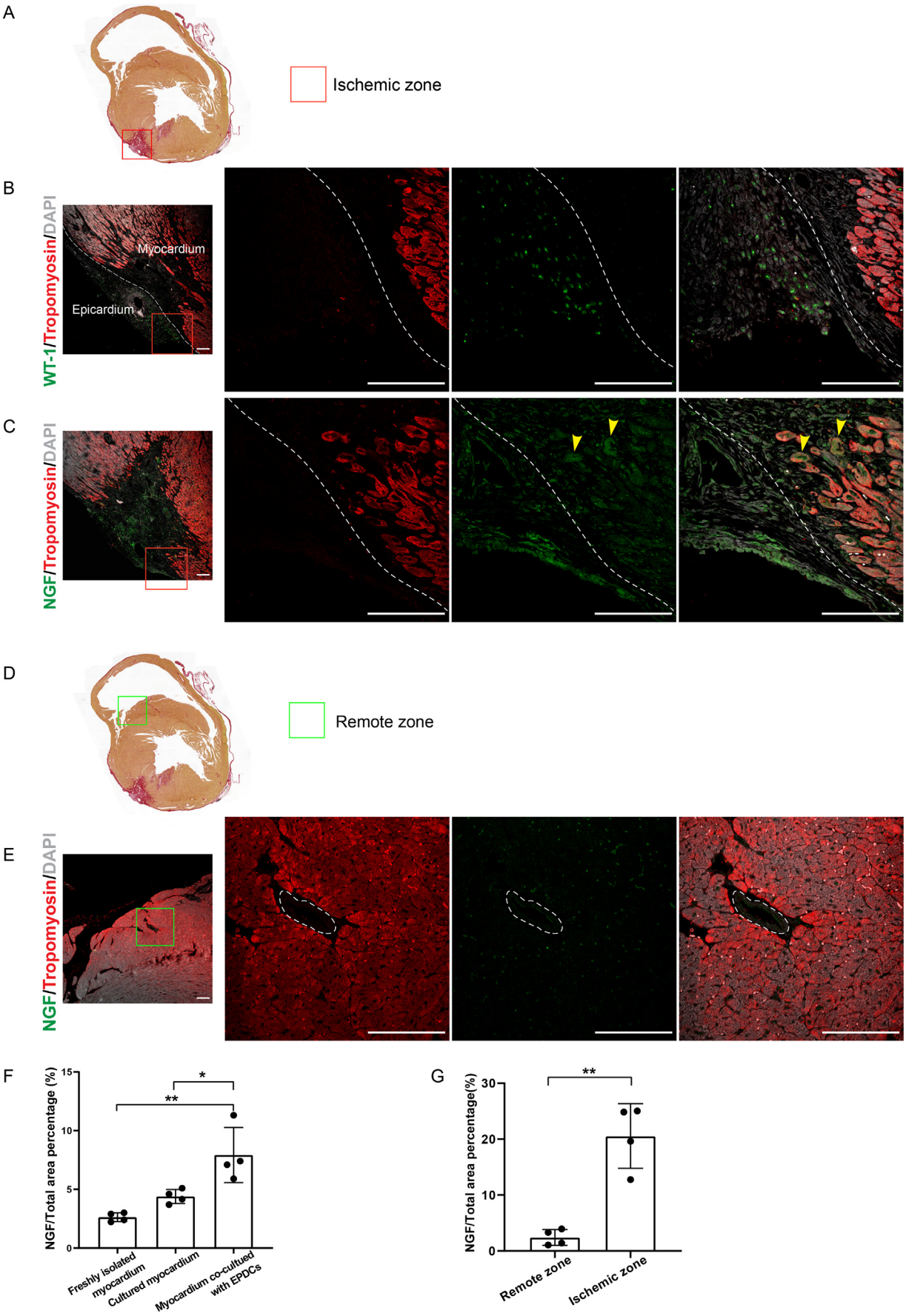


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Fig. 6. Expression of NGF at Ischemic zone of infarcted heart.
A,D Sirius red staining of infarcted murine heart (7 days after ligation of the left anterior descending coronary artery). The red square indicates the ischemic zone shown in B and C. B. Activation of epicardium in the infarcted murine heart with the re-expression of WT-1 in epicardial cells. The dashed line indicates the borderline of epicardium at ischemic zone. Scale bar = 100 μm. C. Anti-NGF and anti-tropomyosin staining of the ischemic zone. Arrowheads indicate cardiomyocytes with NGF staining of high intensity. The dashed line indicates the borderline of epicardium at the ischemic zone. Scale bar = 100 μm.D, E. Anti-NGF and anti-tropomyosin staining of remote zone. The dashlined-circle indicates the vessel. Scale bar = 100 μm. D. Sirius red staining of the infarcted murine heart (7 days after ligation of the left anterior descending coronary artery). The green square indicates the remote (un-infarcted) zone shown in E. E. Anti-NGF and anti-tropomyosin staining of the remote (un-infarcted zone). The dash-lined-circle indicates the vessel. F. Quantification of NGF expression in freshly isolated myocardium, cultured myocardium and the myocardium co-cutured with EPDCs in vitro for 7 days (n = 4). G. Quantification of NGF expression at ischemic zone and remote zone of ischemic murine hearts (n = 4). * $P < .05$, ** $P < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human) would be used. The use of our setup was partly directed by the difficulties encountered of culturing murine EPDCs in vitro. In addition, acquiring fresh ventricular human tissues and ganglia suitable for prolonged culture is challenging. On the other hand, the use of human cells is beneficial from a translational point of view. In other studies we also successfully cultured murine and human tissues [22,54], as was also reported by other researchers [55].

As EPDCs are derived from human adult cells, obtained as anonymous surplus operation tissues, we were blinded to any patient information. This may explain some of the variations we encountered in our cell cultures. In the future, we strive to obtained immortalized/clonal cell lines, that would provide a solution for these interindividual variations. On the other hand, the current study more closely mimics the situation encountered in clinical settings, were individuals factor will always play a role in outcome.

6. Conclusions and clinical perspective

In conclusion, in the current paper we propose a new role for EPDCs extending their potential role in cardiac regeneration in states of ischemia to include an effect on cardiac (re)innervation. Because of its complexity, the autonomic nervous system is in many respects still enigmatic and the pathways regulating neural growth and differentiation, especially in disease states are complex and largely unresolved. We show that the promotional effect of mesenchymal EPDCs on sympathetic neurite sprouting is via paracrine signaling, and indicate a role of NGF, Endothelin-1 and SEMA3A in the process. This response may be in essence beneficial in vivo, restoring loss of functional nerves after damage, but given the occurrence of hyperinnervation-related lethal arrhythmias, an “overshoot” may occur, with adverse effect on prognosis after cardiac damage. Which of those factors released by the ventricular myocardium and/or EPDCs are crucial in promoting nerve outgrowth, requires further exploration including metabolomics/proteomics. Our findings warrant more extensive molecular studies, aimed at exploring the neural-myocardial-epicardial axis such as RNA sequencing of ganglionic, myocardial and epicardial tissues in normal and disease states, exploring other signaling pathways involved.

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Author contributions

All authors contributed to the study conception and design. Material preparation were performed by Yang Ge and Conny van Munsteren. Data collection and analysis were performed by Yang Ge, supervised by Monique Jongbloed and Anke Smits. The first draft of the manuscript was written by Yang Ge, Anke Smits and Monique Jongbloed and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Comprting Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2020.04.006.

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