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Original Article

Cardiac fibroblast paracrine factors modulate mouse embryonic stem cells

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Abstract: The study aims to investigate the effects of cardiac fibroblast (CF) paracrine factors on murine embryonic stem cells (ESCs). Conditioned mediums from either neonatal cardiac fibroblasts (ConM-NCF) or adult cardiac fibroblasts (ConM-ACF) were diluted by 1:50 and 1:5, respectively, to investigate whether these conditioned mediums impact murine ESCs distinctly with RT-real time PCR techniques, cell proliferation essay, ELISA and by counting percentage of beating embryoid bodies (EBs) during ESCs differentiation. The data showed that the paracrine ability of CFs changed dramatically during development, in which interleukin 6 (IL6) increased with maturation. ConM-NCF 1:50 and ConM-NCF 1:5 had opposite effects on the pluripotent markers, although they both reduced mouse ESC proliferation. ConM-ACF 1:50 promoted ESCs pluripotent markers and proliferation, while ConM-ACF 1:5 exerted negative effects. All CF-derived conditioned mediums inhibited cardiac differentiation, but with distinguishable features: ConM-NCF 1:50 slightly decreased the early cardiac differentiation without altering the maturation tendency or cardiac specific markers in EBs at differentiation of day 17; ConM-ACF 1:50 had more significant inhibitory effects on early cardiac differentiation than ConM-NCF 1:50 and impeded cardiac maturation with upregulation of cardiac specific markers. In addition, IL6 neutralization antibody attenuated positive effect of ConM-ACF 1:50 on ESCs proliferation, but had no effects on ConM-NCF 1:50. Long-term IL6 neutralization reduced the percentage of beating EBs at early developmental stage, but did not alter the late cardiac differentiation. Taken together, both the quality and quantity of factors and cytokines secreted by CFs are critical for the ESC fate. IL6 could be a favorable cytokine for ESC pluripotency and the early cardiac differentiation.

Key words: murine embryonic stem cells; microenvironment; paracrine factors; cell therapy; interleukin 6

心脏成纤维细胞旁分泌能力影响小鼠胚胎干细胞的命运

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摘 要：本研究旨在探讨心脏成纤维细胞旁分泌能力对小鼠胚胎干细胞的影响。新生小鼠和成年小鼠成纤维细胞的条件培养基(ConM-NCF、ConM-ACF)分别稀释50倍(1:50)或5倍(1:5)，通过RT-real time PCR技术、ELISA、细胞增殖检测以及计数心肌分化过程中跳动拟胚体(embryoid body, EB)的比例，观察这些条件培养基对小鼠胚胎干细胞的影响。结果显示，成纤维细胞细胞因子的分泌能力在发育过程中发生显著变化，其中白细胞介素6 (interleukin 6, IL6)随发育成熟显著增加。ConM-NCF抑制干细胞的增殖，但高、低浓度对干性基因表达的作用不同。ConM-ACF 1:50促进干细胞增殖并上调干性基因，但ConM-ACF

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1:5作用相反。ConM-NCF和ConM-ACF都能抑制干细胞的心肌细胞分化能力，但又各有特点：ConM-NCF 1:50抑制早期心肌分化但不影响晚期心肌细胞标记物的表达；ConM-ACF 1:50抑制早期心肌分化比ConM-NCF 1:50更显著，阻碍心肌成熟并上调分化晚期的心肌细胞标记物表达水平。此外，IL6中和抗体抑制ConM-ACF 1:50对干细胞的促增殖作用，但不影响ConM-NCF 1:50的作用。干细胞分化过程中长期应用IL6中和抗体，抑制早期心肌细胞分化，但对晚期分化不产生影响。以上结果提示，心脏成纤维细胞分泌的细胞因子种类和浓度都对小鼠胚胎干细胞的增殖、干性基因表达和心肌分化有重要意义，其中ConM-ACF分泌的IL6是干细胞干性维持和心肌细胞分化的有利因素。

关键词：小鼠胚胎干细胞；微环境；旁分泌；细胞移植；白细胞介素6
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Regenerative medicine using exogenous sources [1, 2] to generate new cardiomyocytes and direct cardiac reprogramming [3] serves as a game changer to improve cardiac function. However, promoting cell proliferation or cardiac differentiation remains the cornerstone although many candidates and possibilities have been proposed [4–6]. The non-cardiomyocytes in the heart function as the irreplaceable microenvironment in the heart [7–14]. Cardiac fibroblasts (CFs) are the largest non-myocyte cell population in the heart [15, 16], and could possibly influence the fate of candidates for cell therapies [17–21]. However, the relative proportion of cardiac cell types changes during development and can differ during heart diseases [7, 15, 20, 22]. It is of importance to well address whether such developmental changes could also have distinct impact on cell therapy.

Embryonic stem cells (ESCs) have been taken as a promising cell source for cell therapy [23, 24]. Modifying the microenvironment which affects the ESCs proliferation and their differentiation [12, 27, 28] to improve the efficacy of ESC-based cell therapy has been focused for decades [25, 26]. Especially, some studies have focused on the potential function of fibroblasts on ESC cell fate [29–33], however, mostly based on 3D-cardiac tissue engineering. By producing conditioned medium derived from neonatal CFs (NCF) or adult CFs (ACF) and diluting them as 1:50 or 1:5 in the culture medium, we investigated the effects of CF paracrine factors on murine ESCs in order to provide additional information on developmental changes in CFs and their effects on mouse ESCs, which could be critical for cardiac regeneration in different models.

1 MATERIALS AND METHODS

1.1 *CFs isolation and culture*

Ventricles from neonatal (postnatal day 3) or adult (6–8 weeks old) mice (C57, 2019-s1842) were cut into small pieces and put into 0.25% trypsin solution for 8 min at 37 °C. The cell suspension containing enzyme was collected into Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) fetal bovine serum (FBS) to end the digestion. Such digestion was run for 3–5 cycles until the tissue debris was almost invisible. Thereafter cells were filtered with 40 μm plastic mesh and counted on a haemocytometer, diluted to a density of about 250 000 cells per cm^2 and plated into 35 mm petri dishes with 2 mL culture medium. After 2 h culture, the attached cells were supplemented with fresh DMEM containing 15% FBS for further incubation for 2 days until confluent to collect the original conditioned medium. ConM-NCF and ConM-ACF indicated the conditioned medium from NCF and ACF. 1:50 and 1:5 meant that the original conditioned medium was diluted with stem cell culture medium by 50-fold and 5-fold, respectively.

1.2 *Mouse ESCs culture and cardiomyocyte differentiation*

The D3 mouse ESC line was purchased from ATCC (Manassas, USA). As previously described [34, 35], the cells were cultured on a feeder layer of inactivated mouse embryonic fibroblasts (MEFs) with 1 000 U/mL recombinant mouse leukaemia inhibitory factor (LIF, Chemicon, Temecula, USA) to maintain the cells in an undifferentiated state. ESC proliferation and pluripotent markers were studied to reveal the stemness properties. After removal of LIF and feeder layers, the mouse ESCs could initiate the differentiation as embryoid bodies (EBs) in DMEM containing 20% FBS. Percentage of beating EBs and cardiac specific markers were investigated to evaluate the cardiac differentiation. EBs at differentiation of day 17 (EB17) were collected for reverse transcription (RT)-real time PCR analysis of cardiac specific markers.

Different conditioned mediums were applied on the mouse ESCs for two days to investigate their effects on mouse ESC proliferation and stemness markers. The

stem cell culture medium was refreshed every two days with each condition medium to study their influences on cardiac differentiation.

1.3 *Cell proliferation assay*

The proliferation of ESCs was evaluated with Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). According to the manufacturer's instructions, cells were seeded in 96-well plates at 1 000 cells/well and cultured for 48 h. Thereafter 10 μL CCK-8 solution was added to each well for microplate reader to measure the optical density at a wavelength of 450 nm. Triplicate wells were used in each group.

1.4 *RT-real time PCR*

Total RNA was prepared using Trizol method and RT was carried out with oligo (dT) primers in aid of superscript first-strand cDNA synthesis kit (Invitrogen, USA) [36]. Gene-specific primers (Table 1) were designed to perform real time quantitative PCR using the SYBR-Green PCR MasterMix (Toyoboco., LTD., Japan). The reaction volume was 20 μL composed of 1.6 μL first strand cDNA, 0.4 μL 10 mmol/L forward and reverse primers, and 10 μL SYBR-Green PCR MasterMix. After an initial denaturation at 95 °C for 10 min, each reaction was run for 40 cycles at 95 °C for 10 s, 60 °C for 6 s, and 72 °C for 6 s. Signal detection was measured by Mx3000P real-time PCR system (Agilent Stratagene, USA). The CT values of each gene were converted into absolute copy numbers using a standard curve for GAPDH. All reactions were run in triplicate.

1.5 *Enzyme linked immunosorbent assay (ELISA)*

Culture medium from CFs was collected and the concentration of IL6 and TGFβ1 (R&D Systems, USA) was determined according to the manufacturer's protocol. 50 μL medium was assayed and the amount of growth factors/cytokines was determined via a standard curve.

1.6 *Statistics*

Data were expressed as the mean ± SEM, and paired or unpaired *t* test was used to test significance of differences when applicable. The difference was statistically significant when $P < 0.05$.

2 RESULTS

2.1 ConM-NCF and ConM-ACF functioned distinctly in mouse ESC proliferation and pluripotency markers expression

As shown in Fig. 1, ConM-NCF consistently inhibited mouse ESC proliferation in a concentration dependent manner (Fig. 1*A*). Without feeder/LIF interference, ConM-NCF exhibited stronger effects. ESC pluripotent markers, Oct4 and Sox2, were downregulated in ConM-NCF 1:5, and unexpectedly increased in ConM-NCF 1:50 (Fig. 1*B*). ConM-ACF played a double-edged role in ESC proliferation and the expression of stemness markers (Fig. 1*C*, *D*): ConM-ACF 1:50 promoted murine ESC proliferation and stemness marker Oct4/Sox2 expression, and on the contrary

Table 1. Primers for real time PCR analysis

Gene	Primer sequence
Oct4	F: CAGCCAGACCACCATCTGTC; R: GTCTCCGATTTGCATATCTCCTG
Sox2	F: TGGACTGCGAACTGGAGAAG; R: ATTTGGATGGGATTGGTGGT
c-myc	F: CTGTGGAGAAGAGGCAAACC; R: TTGTGCTGGTGAGTGGAGAC
α-MHC	F: ATGACAGACAGATCCCTCCTATCTCC; R: CTCATCACTCGTTGCATCA TCGAC
MLC2v	F: TGTGGGTCACCTGAGGCTGTGGTTCAG; R: GAAGGCTGACTATGTCCGGGAGATGC
Gata4	F: TCTCACTATGGGCACAGCAG; R: GCGATGTCTGAGTGACAGGA
NKX2.5	F: CAAGTGCTCTCCTGCTTTCC; R: GGCTTTGTCCAGCTCCACT
FLK1	F: GCGGGCTCCTGACTACACTA; R: CCAAATGCTCCACCAACTCT
TGFβ1	F: GCAACATGTGGAACTCTACCAGAA; R: GACGTCAAAAGACAGCCACTCA
TGFβ2	F: GCAGATCCTGAGCAAGCTG; R: GTAGGGTCTGTAGAAAGTGG
IL6	F: TTCCATCCAGTTGCCTTCTT; R: CATTTCCACGATTTCCCAGA
IGF1	F: CTCCTCGCATCTCTTCTACC; R: GCAGCACTCATCCACTATTC
FGF1	F: AGCCCGTCGGTGTCCATGG; R: GATGGCACAGTGGATGGGAC
TNFα	F: GAACTGGCAGAAGAGGCACT; R: AGGGTCTGGGCCATAGAACT
EDN1	F: CCACAGGAAGAGATGCCA; R: GGAACGCTTGGACCTGGAAGAAC
GAPDH	F: AACTTTGGCATTGTGGAAGG; R: GGATGCAGGGATGATGTTCT

F: forward; R: reverse.

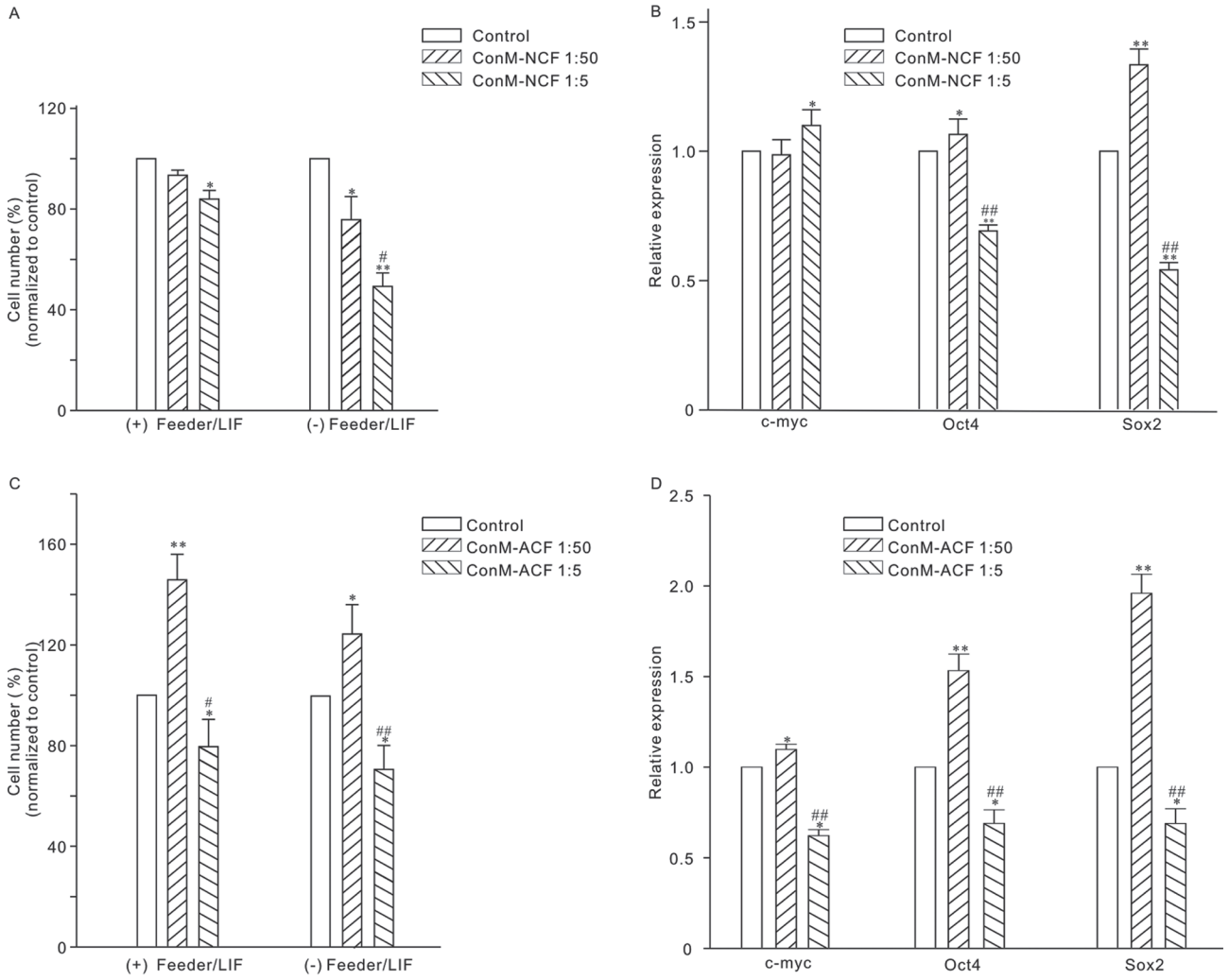

Fig. 1. Cardiac fibroblast modulated mouse embryonic stem cells (ESCs) proliferation and stemness maintenance distinctly in dependence on quality and quantity. *A*: Exposure to ConM-NCF markedly led to decreased cell proliferation in a concentration dependent manner. *B*: Oct4 and Sox2 expression was upregulated in ConM-NCF 1:50, whereas downregulated in ConM-NCF 1:5. *C*, *D*: ConM-ACF 1:50 remarkably increased cell proliferation with upregulation of pluripotent genes c-myc, Oct4, and Sox2. Increasing ConM-ACF (1:5) contrarily decreased cell proliferation and the stemness markers expression. ConM-NCF and ConM-ACF indicated the conditioned medium from neonatal cardiac fibroblasts and adult cardiac fibroblasts. 1:50 and 1:5 meant that the original conditioned medium was diluted with stem cell culture medium by 50-fold and 5-fold, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* ConM-NCF1:50 or ConM-ACF 1:50. Mean ± SEM, $n = 4$ for both ConM-NCF and ConM-ACF.

ConM-ACF 1:5 exhibited negative effects.

2.2 ConM-NCF and ConM-ACF distinguishably changed the cardiomyocyte differentiation of mouse ESCs

During differentiation, we monitored the cardiac differentiation by monitoring the beating EBs, markers of cardiac precursors and cardiomyocytes [25, 37–39]. The maximum percentage of beating EBs in control was taken as the internal control for all values. ConM-NCF 1:50 slightly decreased the percentage of beating EBs at the early stage (EB9–13) of cardiac differentiation ($P < 0.05$ in EB13) (Fig. 2*A*) without affecting the maturation tendency (beating EBs decreased from EB11 to EB17 as in control panel). ConM-ACF 1:50 acted on early cardiac differentiation more potently ($P < 0.05$ or $P < 0.01$ in EB9–11) and impeded cardiac maturation (similar amount of beating EBs from EB13 to EB17) (Fig. 2*B*). In ConM-NCF 1:5, the murine ESCs lacked cardiac differentiation (Fig. 2*A*). Nevertheless, in ConM-ACF 1:5, the murine ESCs had stable percentage of beating EBs (Fig. 2*B*). RT-real time PCR were then performed to investigate the cardiac specific marker

genes expression in EB17. ConM-NCF 1:50 changed neither the cardiac progenitor markers Gata4/NKX2.5/FLK1 nor the cardiac markers α-MHC/MLC2v, whereas these cardiac specific markers were incredibly low in ConM-NCF 1:5 (Fig. 2*C*). Surprisingly, both ConM-ACF 1:50 and ConM-ACF 1:5 upregulated expression of these marker genes (Fig. 2*D*). The molecular level of these genes fit well with the observations in the amount of beating EBs.

2.3 CFs-secreted growth factors and cytokines changed during development

The above difference in ConM-NCF and ConM-ACF might lie in the variation of these CFs during development. The RT-real time PCR data (Fig. 3*A*) revealed that ACF expressed much less endothelin 1 (EDN1), insulin growth like factor 1 (IGF1), fibroblast growth factor 1 (FGF1), and tumor necrosis α (TNFα) than NCF. IL6 and tumor growth factor β2 (TGFβ2) expressed at higher level in ACF than in NCF. TGFβ1 expression in CF remained constant during differentiation from neonatal to adult. IL6 and TGFβ1 were then taken as the candidates to test their concentrations with ELISA in the conditioned medium (Fig. 3*B*). The finding was coincided with the mRNA expression.

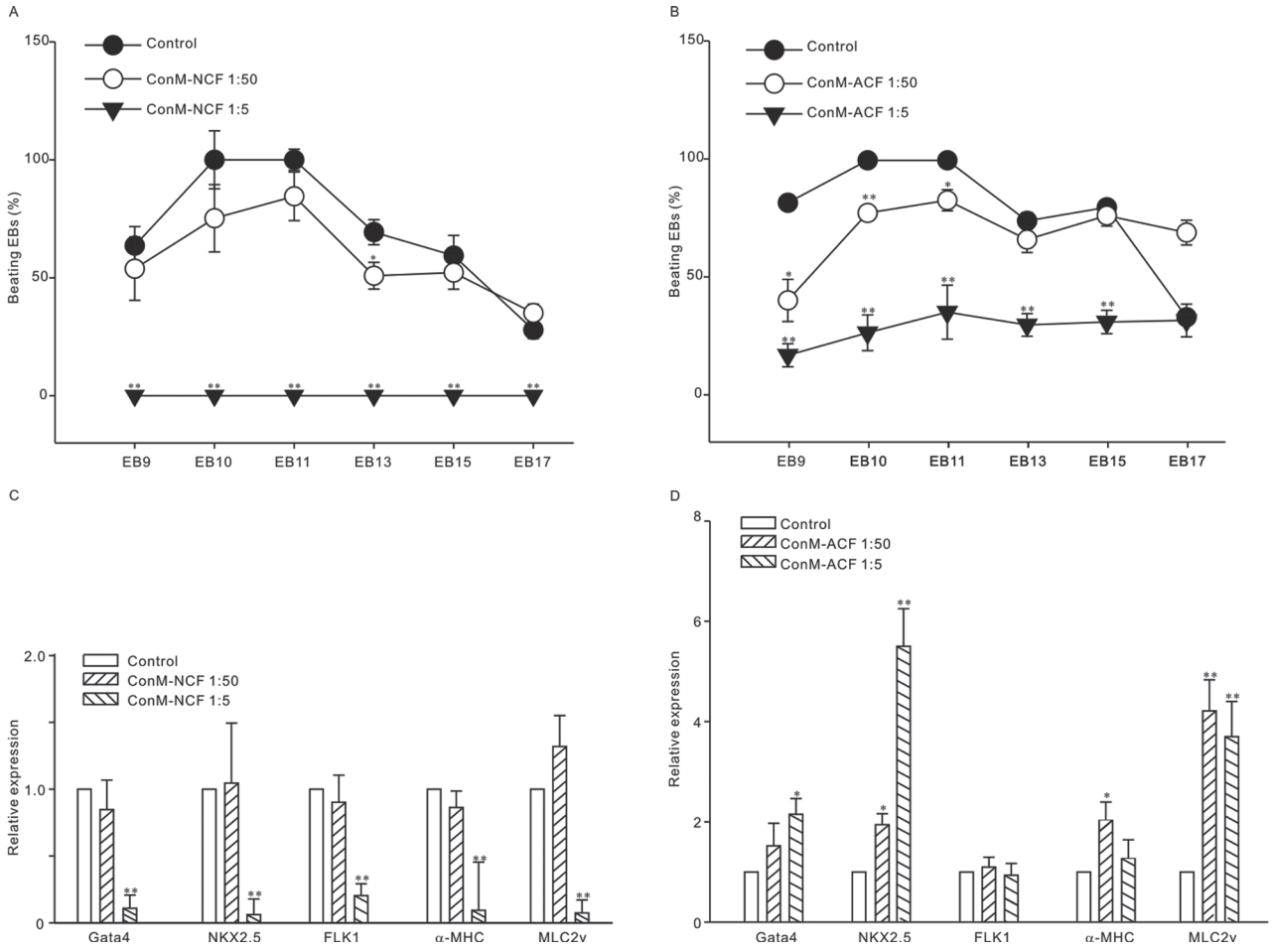

Fig. 2. Cardiac fibroblast diversely interfered cardiac differentiation from murine ESCs due to the variations in quality and quantity. *A*: ConM-NCF 1:50 inhibited the percentage of beating embryoid bodies (EBs) without impacting the cardiac maturation. ConM-NCF 1:5 depleted the cardiac differentiation potential of mouse ESCs. *B*: ConM-ACF significantly reduced the beating EBs during differentiation from EB9 to EB11, and the percentage of beating EBs did not decline compared with the control from EB13 on. *C*: ConM-NCF 1:50 did not influence the mRNA expression of cardiac progenitor markers Gata4/NKX2.5 /FLK1 or cardiac markers α-MHC/MLC2v in EB17. The mRNA expressions of these cardiac progenitor and cardiomyocyte markers were greatly low in ConM-NCF 1:5 in EB17. *D*: Both ConM-ACF 1:50 and ConM-ACF 1:5 raised the mRNA expression of these cardiac progenitor and cardiomyocyte markers in EB17. ConM-NCF and ConM-ACF indicated the conditioned medium from neonatal cardiac fibroblasts and adult cardiac fibroblasts. 1:50 and 1:5 meant that the original conditioned medium was diluted with stem cell culture medium by 50-fold and 5-fold, respectively. EB9–EB17: EBs at differentiation of day 9–17. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control. Mean ± SEM, $n = 4$.

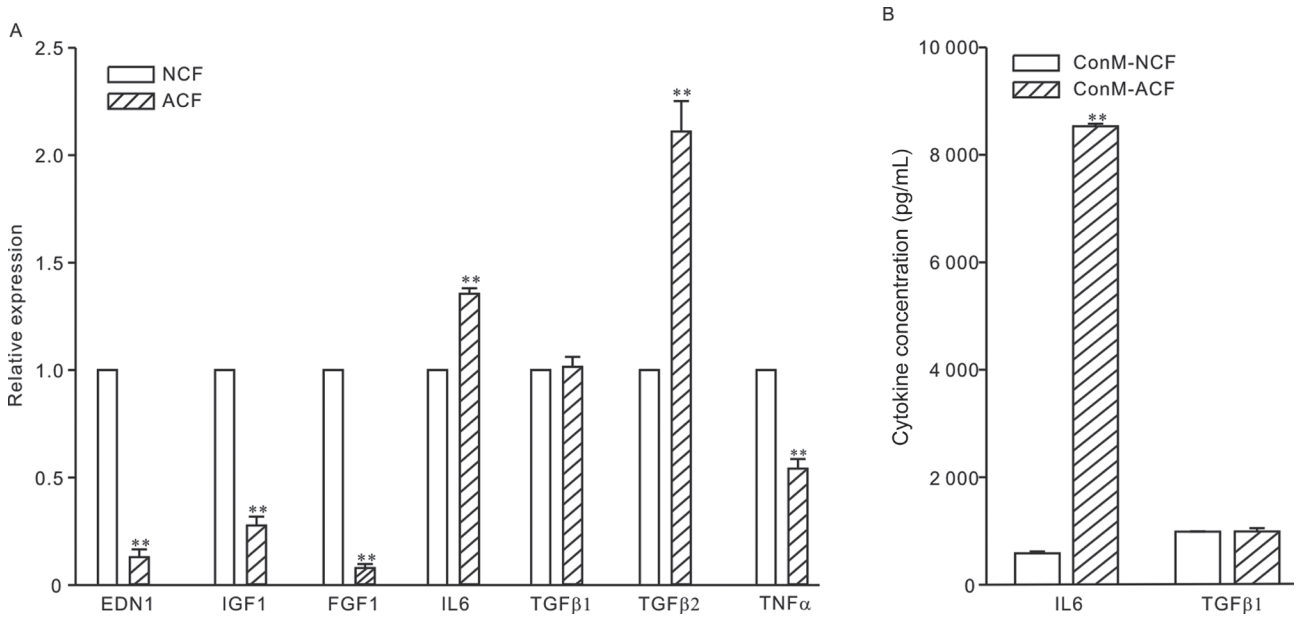

Fig. 3. Factors and cytokines secreted by cardiac fibroblasts experienced developmental changes. *A*: As compared to NCF, ACF expressed less EDN1/IGF1/FGF1/TNFα and more IL6/TGFβ2. *B*: IL6 concentration in ConM-ACF stayed much higher than that in ConM-NCF. NCF: neonatal cardiac fibroblasts; ACF: adult cardiac fibroblasts. ConM-NCF and ConM-ACF indicated the conditioned medium from neonatal cardiac fibroblasts and adult cardiac fibroblasts. $^{**}P < 0.01$ *vs* NCF or ConM-NCF. Mean ± SEM, $n = 3$.

2.4 IL6 dominated partial functions of the ConMs derived from CFs in mouse ESCs

IL6 favors the cardiac repair by improving cardiomyocyte regeneration in different models [40–42]. We then took IL6 as a candidate to explore whether it was critically involved in the developmental changes of CFs' function on ESCs. IL6 antibody (2 ng/mL) attenuated the positive effects of ConM-ACF 1:50 on mouse ESC proliferation (Fig. 4*A*). However, IL6 antibody failed to change the influence of ConM-NCF 1:50. Likely we applied IL6 neutralization antibody to block the endogenous IL6 during ESC cardiac differentiation. Long term blocking intrinsic IL6 (Fig. 4*B*) remarkably inhibited the amount of beating EBs during differentiation from EB9 to EB11. Such function progressively disappeared during late differentiation toward EB17.

3 DISCUSSION

Previous studies have revealed that NCF expresses more growth factors Hbeg and Ptn, while ACF secretes more growth related cytokines IL1 and IL6 [43]. The present study showed similar developmental changes in majority of the growth factors and cytokines, but with some exceptions like cytokine EDN1, and growth factor TGFβ2 was illustrated to change in an opposite manner, whereas TGFβ1 had no developmental changes. These findings provided more vital information on developmental changes in CFs, which could be critical for cardiac regeneration in different models.

In our study, ConM-NCF consistently inhibited mouse ESC proliferation, whereas less ConM-NCF (1:50) increased the expression of the pluripotency markers Oct4/Sox2, and more ConM-NCF (1:5) decreased the pluripotent markers. More ConM-ACF (1:5) inhibited both the ESCs proliferation and pluripotent markers. In these markers, Oct4 expression increased by more than 50% in ConM-ACF 1:50, which could possibly trigger the differentiation into primitive endoderm or mesoderm [44]. This raised the doubt on the effects on pluripotent maintenance. However, undoubtedly difference between ConM-NCF and ConM-ACF might lead to distinct influence on mouse ESCs. Additionally, studies of ESCs proliferation and pluripotency should attach importance not only to the quality but also to the quantity (concentration) of these CF released growth factors and cytokines, because different amount of the same conditioned medium exerted opposite effects on ESCs.

Studies focusing on 3D-cardiac tissue engineering have shown that fibroblast could promote ESC derived-cardiomyocytes to form a functional 3-D tissue [29] and

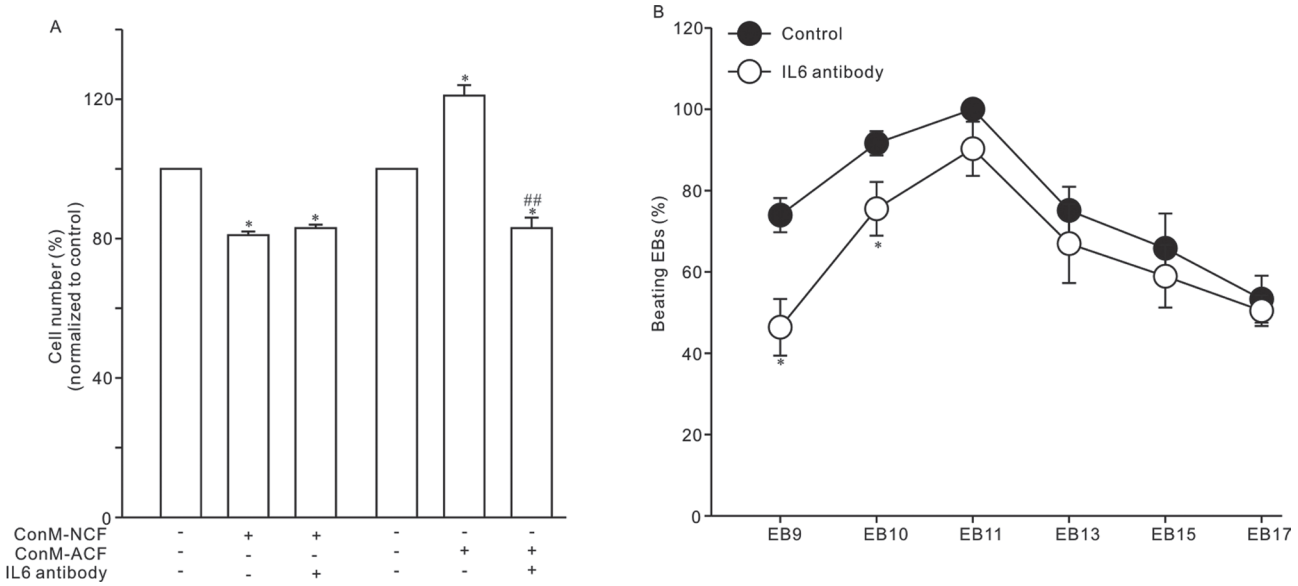

Fig. 4. IL6 was involved in the function of ConM-CF on mouse ESCs. *A*: IL6 antibody played distinguishable roles in the effects of ConM-NCF and ConM-ACF on mouse ESC proliferation. ConM-NCF and ConM-ACF indicated the conditioned medium from neonatal cardiac fibroblasts and adult cardiac fibroblasts. $^{*}P < 0.05$ *vs* ConM-NCF (-) or ConM-ACF (-); $^{\#\#}P < 0.01$ *vs* ConM-ACF. *B*: Long term IL6 antibody administration moderately decreased the percentage of beating embryoid bodies (EBs) during early cardiac differentiation from EB9 to EB11. EB9–EB17: EBs at differentiation of day 9–17. $^{*}P < 0.05$ *vs* control. Mean ± SEM, $n = 4$.

enhance the electrical maturation of ESC-cardiomyocyte microtissues [31]. We found that ConM-NCF 1:50 slightly inhibited the early cardiac differentiation (from EB9–EB13), but did not alter the cardiac specific markers in EB17, and higher concentration of ConM-NCF (1:5) completely disabled the cardiac differentiation. This was in good agreement with the previous finding that the MEFs were not friendly in cardiac differentiation [45]. More profoundly and significantly, ConM-ACF 1:50 decreased the cardiac differentiation from EB9–EB13, and the percentage of beating EBs remained stable during cardiac differentiation from EB13–EB17 with upregulation of cardiac specific markers in comparison to control. This suggested a relative immature status of cardiomyocytes during cardiac development [25, 38] because working cardiomyocytes would progressively lose the autorhythmicity with maturation. Upregulation of cardiac specific markers during cardiac development mostly implies more cardiomyocytes [25, 38], yet the decreased beating EBs during early cardiac development (from EB9 to EB13) indicated a hindered cardiac generation. We proposed that this contradictory observation in ConM-ACF lies in the inconsistent relationship between the cardiac maturation and cardiac proliferation. This mattered greatly for the immature cardiomyocytes to integrate with the host cardiomyocytes when being implanted for cell therapy [10, 11, 20, 22, 46] in case that the failed electrical integration leads to arrhythmia and thereafter the heart dysfunction.

We found that IL6 neutralization antibody successfully attenuated stimulatory effect of ConM-ACF 1:50 on ESCs proliferation, and did not produce any effect under ConM-NCF 1:50. This definitely attributed to the high concentration of IL6 in ConM-ACF and we proposed that IL6 at appropriate concentration favors the murine ESC proliferation. This suggested that IL6 indeed played an essential role in ACF to influence ESC cell fate. Long term application of IL6 antibody reduced the percentage of beating EBs at early developmental stage (EB9–EB10) and did not alter the maturation tendency. Combining with the fact that ConM-ACF inhibited the early cardiac differentiation, we proposed that IL6 may be a favorable cytokine for early cardiac differentiation, while such function lasts shortly. This result is contrary to the previous study which showed that IL6 benefited the explant-derived stem cells (EDC)-mediated cardiac regeneration and favored the cardiac repair [47]. This additionally indicated the diverse function of IL6 in different pluripotent stem cells.

Multiple stem cells locating in the heart or circulating into the heart might be responsible for the cardiac

regeneration [48, 49]. We took murine ESCs as a model and found that in cardiac microenvironment both the quality (different types) and quantity (amount) of factors and cytokines secreted by CFs were critical for the pluripotent stem cell fate. Our studies provided additional knowledge on the essential roles of CFs in cell therapy.

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