Carbon nanotube-composite hydrogels promote intercalated disc assembly in engineered cardiac tissues through β1-integrin mediated FAK and RhoA pathway

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Abstract

Carbon nanotube (CNT)-based hydrogels have been shown to support cardiomyocyte growth and function. However, their role in cellular integrity among cardiomyocytes has not been studied in detail and the mechanisms underlying this process remain unclear. Here, single walled CNTs incorporated into gelatin with methacrylate anhydride (CNT/GelMA) hydrogels were utilized to construct cardiac tissues, which enhanced cardiomyocyte adhesion and maturation. Furthermore, through the use of immunohistochemical staining, transmission electron microscopy and intracellular calcium transient measurement, the incorporation of CNTs into the scaffolds was observed to markedly enhance the assembly and formation in the cardiac constructs. Importantly, we further explored the underlying mechanism behind these effects through the use of immunohistochemical staining and western blotting. The β1-integrin-mediated FAK and RhoA signaling pathways were found to be responsible for CNT-induced upregulation of electrical and mechanical junction proteins respectively. Together, our study provides new insights into the facilitative effects of CNTs on ID formation, which has important significance for improving the quality of engineered cardiac tissue and applying them to cardiac regenerative therapies.

Statement of Significance

Currently, the bottleneck to engineering cardiac tissues (ECTs) for cardiac regeneration is the lack of efficient cellular integrity among adjacent cells, especially the insufficient remodeling of intercalated discs (IDs) in ECTs. Recently, carbon nanotube (CNT) hydrogels provide an advantageous supporting microenvironment and therefore benefit greatly the functional performance of ECTs. Although their beneficial effect in modulating ECT performance is evident, the influence of CNTs on structural integrity of ECTs has not been studied in detail, and the mechanisms underlying the process remain to be determined. Here, we utilized carbon nanotube incorporated into gelatin with methacrylate anhydride (CNT/GelMA) hydrogels to construct cardiac tissues, determined the influence of CNTs on intercalated discs (IDs) assembly and formation and explored the underlying mechanisms.

1. Introduction

Cardiac tissue engineering aims to fabricate functional cardiac tissues in vitro, that later will be transplanted into the damaged myocardium for replacement therapy [1–5]. Although significant improvement has been made in this respect, there are still many challenges that remain to be addressed. A major roadblock/challenge in the development of functional engineered cardiac tissues (ECTs) is the lack of efficient cellular integrity among adjacent cells [6–8], which ensures the structural integrity and functionality of the native myocardium.

The structural integrity of the heart is maintained by the end-to-end connections between cardiomyocytes known as the
intercalated disc (ID). The ID, a highly specialized cell-cell contact structure, is comprised of different junctions, including adherens-, desmosomal-, and gap junctions, which electromechanically couple cardiomyocytes (CMs) into a syncytium and are essential for the morphogenesis, differentiation, and maintenance of tissues [9,10]. Furthermore, studies have demonstrated that the mutation of even one ID protein can cause inherited heart diseases, such as arrhythmogenic right ventricular cardiomyopathy (ARVC) [11,12]. Therefore, in order to induce proper cardiac tissue assembly into functional ECTs, it is essential to investigate the development mode of IDJs in the microenvironment provided by the scaffold biomaterial.

Among various biomaterials, nanocomposite hydrogels have received significant and growing interest for biomedical applications, such as drug delivery, tissue engineering, and regenerative medicine [13,14]. In this regard, carbon nanotubes (CNTs) based hydrogels present important advantages for cardiac tissue engineering since they mimic the native extracellular matrix (ECM) [15–22]. These CNT hydrogels provide topographical, electrical and mechanical cues for proper organization and tissue formation. To date, CNTs have been incorporated to various synthetic and natural polymers to fabricate various conductive nanocomposite hydrogels, which exhibit excellent electrical and mechanical properties. In a series of studies, we and other groups have previously shown that CNTs embedded within natural hydrogels, such as gelatin, alginate and chitosan, support or direct the attachment, electrical coupling and function of CMs [18–20]. Most notably, Shin et al. cultured cardiomyocytes on CNTs incorporated into gelatin methacrylate (GelMA) hydrogels and found the enhanced electro-physiological activities of CMs and ultimately developed functional ECTs [21,22]. These works highlight that CNT hydrogels provide an advantageous supportive microenvironment and improving the functional performance of constructed cardiac tissue. Although their beneficial effect in modulating ECT performance is evident, the influence of CNT hydrogels on structural integrity of ECTs has not been studied in detail, and the mechanisms underlying the process remain to be determined.

In our recent work, we have shown that CNT-collagen substrates enhanced ID assembly in cultured cardiomyocytes [23]. Here, we utilized CNT/GelMA hydrogels to construct cardiac tissues and determined the influence of CNTs on ID assembly and formation on the tissue level. Furthermore, we explored the underlying mechanisms by which signaling pathway CNTs regulate this process.

2. Materials and methods

2.1. Preparation of CNT/GelMA hydrogels

All chemicals were purchased from Sigma-Aldrich. Single wall carbon nanotubes (SWCNTs; 0.7–1.3 nm in diameter and 5–20 μm in length) were purchased from US Nanomaterials Research Inc. CNT/GelMA hydrogels were fabricated and characterized as previously described [21]. In brief, GelMA precursors were first obtained by adding methacrylate anhydride (MA) into 10% (w/v) of gelatin solution at 50 °C. CNTs with various concentrations of 0, 0.5, 1 and 2 mg/mL were prepared by dispersing in 2% Pluronic copolymer solution. Subsequently, the CNT solution was added to GelMA solution containing photoinitiator (2-hydroxy-1-(4-hydroxyethoxy) phenyl)-2-methyl-1-propanone, 0.5% w/v). The mixtures were ultra-sonicated for 1 min to allow the cross-linking reaction to occur.

2.2. Characterization of CNT/GelMA hydrogels

Scanning electron microscopy (SEM) was utilized to observe the surface topography of CNT/GelMA and GelMA hydrogels. The samples were dried through a freeze-drying procedure and sputter-coated with gold (Autoconductavac IV, See-Vac). The microscope was operated under a working voltage of 10 kV. For the porosity analysis, six SEM images were obtained and the data was analyzed using Imagej software.

For atomic force microscope (AFM) analysis, the microscope (Nanoscope IIIa, Digital Instruments Inc., USA) was registered in tapping mode with a scanning rate of 1 Hz and scanning line of 512. A scan field of 1 × 1 μm was used for measurements and the data was analyzed using NanoScope imaging software.

For mechanical testing, the compressive modulus of the hydrogels was obtained from a TA.XT2i Texture Analyzer (Texture Technologies Corp.). The samples were measured from the slope in the linear region corresponding to 5–15% strain at 0.1 mm/s.

For degradation analysis in vitro, the hydrogels were immersed in PBS (pH = 7.4) at 37 °C for 3 and 7 days respectively. The detailed method was characterized and determined as previously described [24].

2.3. Construction and evaluation of ECTs

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-day-old Sprague-Dawley (SD) rats as previously described [23]. All SD rats were purchased from Chengdu Da Shuo Biotech Co., Ltd. (Chengdu, China). All experiments in the study were performed in compliance with the Committee on the Ethics of Animal Experiments of Chengdu Military General Hospital (Chengdu, China). Following isolation and enrichment through 2 h preplating, NRVMs (>90% purity) were immediately seeded into either CNT/GelMA or GelMA hydrogels to construct ECTs (7 × 10^5 cells/cm^2). In some experiments, the gelatin-coated dish was used as control. All samples were cultivated in DMEM (Invitrogen, Carlsbad, CA, USA) culture medium containing 15% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO2. The culture medium was changed daily.

2.4. Cell viability assays

A Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, Grand Island, NY) was utilized to assess the viability of cardiac constructs based on CNT/GelMA and GelMA hydrogels. At day 1, cardiac constructs were incubated with 2 μM calcein AM and 4 μM EthD-1 in phosphate-buffered saline (PBS) for 30 min. Ten randomly selected fields of each group were visualized under a fluorescence microscope ((Nikon AZ-100 multipurpose microscope) using a 10× objective lens. The obtained images were processed by ImageJ software. Additionally, cytotoxicity evaluation was performed by AlamarBlue based colorimetric assays. Experiments were conducted in triplicate per each group.

2.5. Immunofluorescence staining and confocal microscopy

Immunofluorescence was performed as previously described [27]. Cardiac constructs based on CNT/GelMA and GelMA hydrogels were fixed in 4% formaldehyde for 10 min and permeabilized in 0.3% Triton X-100 for 10 min, and then blocked in 2% BSA in PBS for 1 h at room temperature. The samples were then incubated with primary antibodies overnight at 4 °C, including mouse monoclonal anti-alpha-actinin (sarcomeric) (EA-53, a-SA) (1:100, AbCam, Cambridge, MA), rabbit monoclonal anti-Troponin I (Tnl) (1:100, AbCam, Cambridge, MA), and rabbit polyclonal anti connexin-43 (1:1000, AbCam, Cambridge, MA), anti-N-cadherin (1:200, Abcam Cambridge, MA), rabbit monoclonal anti-plakophilin2
(1:800, Abcam, Cambridge, MA) and anti-plakoglobin (1:1000, Sigma, Italy). After washing three times with PBS, the samples were incubated with the appropriate secondary antibodies, Alexa Fluor 488- and Alexa Fluor 548-conjugated secondary antibodies (1:500; Invitrogen, Carlsbad, CA, USA). For nuclei detection, the cardiac constructs were incubated with Hoechst 33258 (1:100; Sigma-Aldrich, Italy). The images were visualized using a Zeiss confocal microscope (Volocity Demo 6.1.1).

2.6. Assay of sarcomere length and Z-line width

The sarcomere length was presented as the distance between α-SA-stained Z-line structures from α-SA⁺ cells with visible sarcomere structures as previously reported [25]. Using ImageJ, different striation regions of 10 NRVMs were measured. Z-line width was also determined in 10 NRVMs by ImageJ according to a previous study [26].

2.7. Analysis of contraction behavior of the NRVMs

The spontaneous contraction of NRVMs was determined and monitored at day 7. The beating video was acquired using a video capture. For spontaneous contraction analysis, the sample number was six cardiac constructs each group.

2.8. TEM Analysis of NRVMs grown on GelMA or CNT/GelMA hydrogels

The cardiac constructs were fixed in a 2.5% solution of glutaraldehyde, post fixed in 1% phosphate-buffered OsO₄, and embedded in epoxy resin. Semi-thin sections were prepared by a LKB NOVA ultra-microtome. After staining with toluidine blue, the areas of interest were sliced into ultra-thin sections and imaged by a transmission electron microscope (Technai10, Philip, Eindhoven, Netherlands).

2.9. Intracellular calcium transient measurements

For calcium Transient analysis, cardiac constructs based on CNT/GelMA and GelMA hydrogels were placed in Tyrode’s containing 10 mM fluo-4 AM (Invitrogen, Carlsbad, CA, USA) and 0.1% Pluronic F-127 (Sigma-Aldrich, Italy) for 30 min at 37 °C. Then, the samples were perfused for 15 min to de-esterify the fluo-4 AM in normal Tyrode’s solution. The fluorescence imaging was performed with a confocal laser scanning system (Nikon Eclipse Ti-E, Japan) equipped with a Perkin-Elmer Ultra view spinning-disc scanner unit. Fluo-4 AM was monitored at excitation wavelengths of 488 nm, emitted fluorescence (F) was collected using a 505 nm band-pass filter and fluorescent signals were normalized to the basal cell fluorescence after dye loading (F₀). The obtained images were analyzed using Volocity software. Intracellular Ca²⁺ was determined by line scan mode, presented as [Ca²⁺] = K_d(F/F₀) / (K_d[Ca]₀ + 1 - F/F₀) with K_d = 1100 nmol/L and [Ca²⁺]₀ = 100 nmol/L.

2.10. Protein isolation and western blotting

For protein analysis, the cardiac constructs were lysed in lysis buffer (Tiangz, Beijing, China) to prepare the total protein. 100 μg total protein was separated by SDS-PAGE electrophoresis and blotted onto PVDF membrane (Millipore, MA). The membranes were blocked in 5% nonfat milk and incubated with primary antibodies overnight at 4 °C, including anti-β1-integrin (Abcam, Cambridge, MA), β-catenin (Cell Signaling Technology, Beverly, MA), anti-connexin-43 (Abcam, Cambridge, MA), ranti-N-cadherin (Abcam, Cambridge, MA), anti-phospho-FAK and anti-FAK antibodies (Cell Signaling Technology, Beverly, MA), and anti-RhoA antibodies (Abcam, Cambridge, MA). After washing, the proper secondary antibodies were incubated with the membranes for 1 h. The labeled proteins were detected using a ECL chemiluminescence reagent (Amersham, Sweden). In some experiments, serum-free medium containing anti-β1integrin antibody (clone Ha2/5, BD Pharmingen, USA) was utilized to incubate the cardiac constructs for 1 h in order to block signaling mediated by β1-integrin. Then the treated constructs were cultured in normal medium for 7 days. In some other experiments, the constructs were incubated with FAK specific inhibitors, F-573228 (10 μM, Pfizer, USA) or RhoA specific inhibitors, Y27632 (10 μM, Calbiochem, USA) for 7 days. The band intensities were quantified by ImageJ software.

2.11. Statistical analysis

Data was presented as mean ± standard error of the mean. Statistical analysis was performed either with one-way ANOVA with Tukey’s post hoc test or an unpaired two-tailed Student’s t-test. P values < 0.05 were regarded as significant (‘p < 0.05, **p < 0.01, ***p < 0.001). Analyses were performed with SAS statistical software version 9.1 (Cary, NC).

3. Results

3.1. Physical characteristics of CNT/GelMA hydrogels

The effects of CNT incorporation into the scaffolds on morphological and structural properties of GelMA hydrogels can be seen in Fig. 1. Hydrogels with higher CNT concentration were observed to exhibit darker samples (Fig. 1a). Scanning electron microscopy (SEM) observation showed that CNT incorporation did not notably influence the porosity percentage of the GelMA hydrogels (Fig. 1b and c). Importantly, CNT/GelMA hydrogels showed a well-developed CNT network distributed on the surface of the pore walls. Additionally, the incorporation of CNTs into the GelMA hydrogels displayed an increase in the tensile and compressive Young’s modulus (Fig. 1d and f). Moreover, hydrogels with the CNT concentration ranging from 50 to 200 ppm showed an increasing trend with higher mechanical strength. The conductivity of CNT/GelMA hydrogels was observed to be significantly greater compared to the GelMA hydrogels (Table. S1). The in vitro degradation analysis demonstrated that CNT incorporation significantly decreased the degradation rate of GelMA hydrogels (Fig. 1f). These results indicated that the incorporation of CNTs improved the morphological, mechanical and electrical properties of the GelMA hydrogels.

3.2. Construction and evaluation of engineered cardiac tissues

To determine the optimal concentration of CNTs for the construction of ECTs, neonatal rat ventricular myocytes (NRVMs) were cultured within CNT/GelMA hydrogels with varying concentration (0, 50, 100, 150 and 200 ppm) and cell viability was systematically evaluated. NRVMs cultured within hydrogels with CNT concentrations of 100 ppm or lower had >80% viability and showed no significant difference in cell viability compared to those within GelMA hydrogels (Fig. 2a–c). However, at 200 ppm, samples showed significantly reduced cell viability (Fig. 2b and c), suggesting that the potential cytotoxicity of CNTs was closely related to their concentration. Given that the electrical and mechanical properties of CNTs also depend on their dose, we chose GelMA hydrogels containing 100 ppm CNTs for further study.

To evaluate the effects of CNTs on cardiomyocyte function, the cardiac constructs were immunostained for α-SA on day 3 and 7. Confocal images revealed that NRVMs grown within the GelMA
hydrogels developed shorter cell spreading and no typical bundle was observed (Fig. 3a). In contrast, NRVMs grown within the CNT/GelMA hydrogels exhibited an aligned and elongated morphology with massive actin striation (Fig. 3a). These NRVMs organized into elongated and aligned cardiac-cell bundles, resembling the natural morphology of cell bundles in the myocardium [10]. Furthermore, the cardiac constructs based on CNT/GelMA hydrogels exhibited significant increase in both sarcomere length and Z-line width than those on GelMA hydrogels (Fig. 3b, c). Importantly, an apparent increase in the contraction rate was observed in the cardiac constructs based on CNT/GelMA hydrogels in comparison to the control group (Fig. 3d). These results demonstrate that the incorporation of CNTs facilitated cardiomyocyte assembly into an organized and dense tissue with a strong and anisotropic contraction potential.

3.3. CNT/GelMA hydrogels promoted ID formation in the ECTs

To determine the roles of CNTs on ID formation, we first examined the spatiotemporal expression profile of ID-related proteins by immunofluorescent staining in cardiac constructs based on CNT/GelMA and GelMA hydrogels on 3, 7, and 14 days, including gap junctions (connexin43, Cx43), adherens junctions (N-cadherin, NC) and desmosomes (plakophilin 2, PKP2).

Representative confocal images are shown in Fig. 4. On day 3, Cx43, N-cadherin and PKP2 were discrete spots and distributed extensively on the surface of cardiomyocytes. However, there is an increased accumulation of these ID-related proteins in the cardiac constructs based on CNT/GelMA hydrogels when compared to the control groups (Fig. 4a). It is remarkable that Cx43 exhibited a strong staining in the cardiac constructs based on CNT/GelMA hydrogels. In contrast, fluorescent signal in Cx43 was rarely detected in the control group (Fig. 4b).

On day 7, these ID-related proteins exhibited a progressive increase and gradually localized towards the lateral regions of NRVMs with increasing time in culture (Fig. 4). On the lateral surface of NRCMs, NC and PKP2 were observed to be larger puncta in a linear distribution when compared to those in the control group. Meanwhile, Cx43 showed strongly stained plaques in the cardiac constructs based on CNT/GelMA hydrogels while few plaques were observed in the intercellular regions in the control group. These results demonstrate that the addition of CNTs promoted the assembly of mechanical junctions and gap junctions.

On day 14, large amounts of localized structures were seen in the intercellular regions, including GJ plaques, strand-like adherens junction and stair-like desmosomes (Fig. 4). Compared to the control group, larger clusters of mechanical junctions were found on the termini of the cardiac constructs based on CNT/GelMA hydrogels where IDs were formed. Meanwhile, Cx43 exhibited a marked increase in length and area of GJ plaques in the CNT/GelMA group compared to the GelMA hydrogels. These results indicate that the addition of CNTs into the hydrogels promotes ID formation in the ECTs.

To further determine whether the CNT/GelMA hydrogel is superior to standard 2D substrates, NRVMs were cultivated on the gelatin-coated dish used as control. As shown in Fig. 5, Cx43 and...
NC proteins were also noted in the whole cell membrane at day 3. After 7 days of culture, some cell-cell contacts were seen in intercellular region and increased slightly on day 14. However, compared to those in the CNT/GelMA hydrogel, fluorescent signals of ID-related proteins were much less in the gelatin group. The data suggests that CNT/GelMA hydrogel is superior to standard 2D substrates for the formation and assembly of IDs among cardiomyocytes.

3.4. CNT/GelMA hydrogels enhanced ID formation on the ultrastructural level

To further assess the effect of CNTs on ID development and formation at the ultrastructural level, transmission electron microscopy was performed on the cardiac constructs based on CNT/GelMA and GelMA hydrogels.

ID structures were clearly visible in the cardiac constructs based on CNT/GelMA hydrogels, with adherens junctions, desmosomes and gap junctions represented by submembranous electron dense plaques adjacent to intercellular space among the CMs (Fig. 6a). In contrast, classic ID structures were absent and only some developing intercellular junctions were observed in the cardiac constructs based on the GelMA hydrogels (Fig. 6b). Furthermore, the lack of intercellular space between the myocytes made it difficult to discern where the myocytes made contacts with their neighbors. At higher magnification, the sarcomeres exhibited dramatically increased sarcomere length, wider and more dense Z-lines in the cardiac constructs based on CNT/GelMA hydrogels than on GelMA hydrogels (Fig. 6a). These results further indicate that the incorporation of CNTs enhanced ID development and formation in the cardiac constructs, thereby resulting in efficient structure integrity in the constructed tissues.

3.5. The enhanced functionality of IDs in the cardiac constructs based on CNT/GelMA hydrogels

Given that the well-organized structure integrity among CMs is a prerequisite for the functionality in the cardiac constructs, we asked whether the incorporation of CNTs into the scaffolds would enhance the contracted and electrophysiological performance of cardiac constructs. Therefore, we performed calcium transient measurements using a fluorescent calcium indicator (Fluo-4 AM, 10 mM) to measure the intracellular Ca²⁺ concentration. We noticed that the cardiac constructs based on CNT/GelMA hydrogels contracted synchronously at day 7 and showed apparent spontaneous electrical activity in calcium images (Fig. 7a). In contrast, the cardiac constructs based on GelMA hydrogels displayed no visual contraction and little spontaneous electrical activity.
Fig. 3. Evaluation of the engineered cardiac tissues. a, Through immunostaining of sarcomeric α-actinin, cardiac constructs based on CNT/GelMA hydrogels exhibited an aligned and elongated morphology with massive actinin striation while those on GelMA hydrogels displayed shorter cell spreading and no typical bundle. b, Sarcomere length had significant increase in cardiac constructs based on CNT/GelMA hydrogels in comparison to those on GelMA hydrogels; n = 10 cells per group. c, Z-line width had significant improvement in cardiac constructs based on CNT/GelMA hydrogels than those on GelMA hydrogels, n = 10 cells per group. d, The contraction rate of the cardiac constructs based on CNT/GelMA hydrogels and GelMA hydrogels at day 7. Data are means ± s.e.m. *p < 0.05, **p < 0.01. All experiments were performed in triplicate.

Fig. 4. Immunofluorescent staining of ID-related proteins in cardiac constructs for 3, 7 and 14 days. a, The expression and distribution of Cx43, NC and PKP2 in cardiac constructs based on GelMA hydrogels. b, The expression and distribution of ID-related proteins in cardiac constructs based on CNT/GelMA hydrogels on 3, 7, and 14 days, including gap junction protein (connexin43, Cx43), adherens junction protein (N-cadherin, NC) and desmosome protein (plakophilin 2, PKP2). All experiments were performed in triplicate.
Fig. 7b. Furthermore, the cardiac constructs based on CNT/GelMA hydrogels exhibited relatively rhythmic Ca\textsuperscript{2+} transients and increased Ca\textsuperscript{2+} fluctuation, whereas those on GelMA hydrogels exhibited arrhythmic transients and lower Ca\textsuperscript{2+} fluctuation (Fig. 7a and b). Average intracellular Ca\textsuperscript{2+} transients in NRVMs (n = 20, 276 ± 36 nmol/L) grown in CNT/GelMA hydrogels significantly increased when compared to those (n = 20, 199 ± 24 nmol/L) in GelMA hydrogels (Fig. 7c). These results suggest the incorporation of CNTs into the scaffolds enhanced the functionality of the cardiac constructs.

Fig. 5. The expression and distribution of Cx43 and NC in NRVMs grown on the gelatin-coated dish for 3, 7 and 14 days by immunofluorescent staining. Scar bars = 20 µm. All experiments were performed in triplicate.

Fig. 6. Electron microscopy observation of the ultra-microstructure and of IDs in cardiac constructs for 7 days. a, ID structures were clearly visible in the cardiac constructs based on CNT/GelMA hydrogels, with adherens junctions (white star), desmosomes (white arrow) and gap junctions (white triangle) represented by submembranous electron dense plaques adjacent to intercellular space among the cardiomyocytes. b, Classic ID structures were absent and only some developing intercellular junctions were observed in the cardiac constructs based on the GelMA hydrogels. Myo: myofibril; N: nucleus; Z: Z-line; CNT: carbon nanotubes.
3.6. β1 integrin signaling plays a crucial role in CNT-induced ID formation

Since that various signal pathways such as integrin, wnt and p38 mitogen-activated protein kinase have been shown to regulate ID formation [27–29], we wondered whether these signaling molecules would have a different expression in the cardiac constructs based on CNT/GelMA hydrogels than those on GelMA hydrogels during the period of ID formation. Through quantitative western blot experiments, the expression levels of integrin and β-catenin were determined. The data showed that β1 integrin was expressed at about three times in NRVMs grown within the CNT/GelMA hydrogels relative to their level in GelMA hydrogels while no apparent changes in β-catenin were noted in the two groups (Fig. 8a–c). These results indicate that the β1-integrin may be involved in the regulation of ID formation.

To further determine their roles in the process of ID formation, the cardiac constructs based on CNT/GelMA hydrogels were incubated with anti-β1-integrin blocking antibodies and were compared to those on GelMA hydrogels after 7 days of culture. As shown in Fig. 8d, e and c, anti-β1-integrin antibody significantly suppressed the increase of Cx43 and NC expression in the CNT/GelMA group compared to those in the control group. These observations suggest that the β1-integrin pathway plays a critical role in the modulation of CNT-triggered electrical and mechanical junction proteins.

3.7. Activation of FAK and RhoA regulates the formation of gap junction and mechanical junction respectively

β1 integrin signaling intracellularly can activate focal adhesion kinase (FAK) as well as small G protein like RhoA, which has been reported to regulate cell-cell junctions [30,31]. To determine whether these downstream signaling molecules play a role in the stimulation of CNT-induced ID formation, we investigated the phosphorylation levels of FAK and the expression level of RhoA in cardiac constructs based on CNT/GelMA hydrogels at day 7. Significant increases in the expression of p-FAK and RhoA were observed in cardiac constructs based on CNT/GelMA hydrogels at day 7 when compared to those on GelMA hydrogels (Fig. 9a–c). Furthermore, FAK and RhoA were inhibited via the addition of specific inhibitors PF-573228 and Y27632 in cardiac constructs based on CNT/GelMA and GelMA hydrogels for 7 days, respectively. When FAK was inhibited, gap junction protein (Cx43) was impaired in cardiac constructs on CNT/GelMA hydrogels whereas no apparent differences in mechanical junction protein (NC) were observed than those on GelMA hydrogels (Fig. 9d). These results suggest that the FAK pathway plays a critical role for the formation of gap junction.

In contrast, RhoA inhibitor resulted in a significant decrease in mechanical junction in the cardiac constructs based on CNT/GelMA hydrogels while it had no obvious effect on gap junction in the control groups (Fig. 9e). The results indicate that RhoA plays a major role in the regulation of the CNT-induced mechanical junction protein.

On the basis of the above findings, we proposed the mechanism responsible for CNT-induced ID formation in cardiac constructs. CNTs activate the β1-integrin signaling at the cell membrane and trigger the downstream signaling kinases FAK and Rho, thereby regulating the formation of gap junctions and mechanical junctions respectively.

4. Discussion

Enhancing the efficient assembly and formation of IDs among cardiac cells is essential to create high-quality cardiac tissues for cardiac regeneration. Here, we provide evidence of the facilitative effects of CNTs in the assembly and formation of IDs, indicating
that the incorporation of CNTs into GelMA hydrogels is an efficient method to improve cellular integrity in the engineered cardiac tissues, and reveal a novel CNT-promoting mechanism. The important findings are that (i) the incorporation of CNTs enhances ID assembly and formation in engineered cardiac tissues; (ii) β1-integrin mediated FAK signaling pathway plays a critical role in the CNT-induced gap junction formation; (iii) β1-integrin mediated Rho pathway is responsible for the CNT-induced mechanical junction formation.

Recently, various types of electroconductive nanomaterials such as CNTs, graphene oxide and gold nanowires have been incorporated into polymers to obtain nanocomposite hydrogels with superior properties [15–22,32,33]. These hydrogels have been shown to provide proper microarchitecture and electrically conductive networks for cardiac myocytes, thereby improving the performance of constructed cardiac tissues [15–19,22,32,33]. In this regard, the incorporation of CNTs into GelMA hydrogel exhibits superior mechanical and electrical properties, which significantly improves cardiomyocyte adhesion, organization, and electrophysiological functions. Given the beneficial effects of CNT/GelMA hydrogels on cardiac constructs, we thus utilized the CNT hydrogel based tissue-engineering platform to study the regularity of ID assembly and formation during cardiac construct reconstitution and to explore the underlying mechanism.

Several lines of evidence demonstrate that conductive nanomaterials dynamically increase the expression of gap junction protein and improve the performance of cardiac constructs [19,22,32,33], indicating its superiority to the electrically insulating matrices in cardiac tissue engineering. Given that IDs are essential structures that enable the myocardium to function as an electromechanical syncytium [9,10], it is vital for us to understand their assembly and formation on the overall level during cardiac tissue reconstitution. In our recent study [23], we found that the addition of CNTs into collagen substrates enhanced the overall assembly of IDs in cultured cardiomyocytes on the cellular level. In the present study, we extended these findings by showing that the incorporation of CNTs into GelMA hydrogels enhanced the formation of mechanical junctions and gap junctions in constructed cardiac tissues. For the first time, we determined the roles of CNTs in ID formation from the cellular level to the tissue level, which has important significance for understanding the facilitative effects of electroconductive nanomaterials on cardiac constructs.

Matrix stiffness plays a key role in regulating cardiomyocyte structure and function [22,33,34–37]. Specially, the elastic modulus of the extracellular matrix has great effect on the sarcomere organization and contractility of cardiomyocytes. Thus, the elastic modulus of material has been extensively utilized to be a key indicator to evaluate its ability to accommodate compressive strain from cardiac contraction in the field of cardiac tissue engineering [22,33,34–37]. Current reports indicate that cardiomyocytes possess good structure and function when were grown on matrixes that mimic the elastic modulus of native myocardium [22,33–35]. In this study, we mainly investigated the effects of CNT/GelMA hydrogels with the CNT concentration of 100 ppm (around a 20 kPa

![Fig. 8.](image)

β1 integrin signaling plays a crucial role in CNT-induced ID formation. a–c, Western blot showed the markedly increased expression of β1-integrin in cardiac constructs based on CNT/GelMA hydrogels compared to those on GelMA hydrogels on day 7 while no apparent changes in β-catenin were noted in the two groups. d–f, Western blot showed that anti-β1-integrin antibody (Ab) significantly suppressed the increase of Cx43 and NC expression in the CNT/GelMA hydrogels whereas no remarkable changes were noted on the control group. Data are means ± s.e.m. **p < 0.01, ***p < 0.001. All experiments were performed in triplicate.
However, the optimal modulus of CNT/GelMA hydrogels would need to be modified. Therefore, future research should focus on introducing human cardiac cells to CNT/GelMA hydrogels and defining the optimal modulus of the hydrogels on ID formation. The study will be crucial for cardiac regenerative medicine for myocardial infarction.

In terms of understanding the interrelationships between mechanical and gap junctions, disparate perspectives hold. Some groups have reported that mechanical junctions (adherens junctions and desmosomes) are the prerequisites for the formation of gap junctions [38]. By contrast, loss of gap junctions in the peri-infarct region is observed to be accompanied by downregulation of adhesion junctions in experimental animal models of myocardial infarction [39]. Additionally, coordinate upregulation of adherens junction and gap junction proteins has been shown in cultured myocytes as a response to pulsatile stretch [40]. In agreement with the latter, our study found that the incorporation of CNTs into GelMA hydrogels remarkably enhanced the expression of mechanical and gap junction proteins in the cardiac constructs. However, it has yet to be investigated further whether gap junction can directly influence the expression pattern of adherens junctions and desmosomes in response to conductive nanomaterials.

As is known, adherens junction establishment is a critical aspect during the ID formation [41,42]. In this study, we found that the addition of CNTs increased the size and area of adherens junction in the cardiac constructs. Moreover, we provide evidence that CNTs enhance the assembly and formation of adherens junctions by β1-integrin-dependent activation of RhoA signaling. Rho family small GTPases have been shown to regulate adherens junction formation. For example, RhoA signaling is required to drive initiation and expansion of adherens junction in epithelial cells [43]. Our data indicated that RhoA in cardiomyocytes was activated in response to CNT/GelMA hydrogels. In line with this, inhibition of RhoA by C2I-C3 resulted in a significant decrease of N-cadherin clusters. This mechanistic insight into the enhanced effects of CNTs on adherens junction establishment has important significance for advancing current quality of engineered cardiac tissues for cardiac regeneration.

Cardiac tissues based on CNT/GelMA hydrogels were observed to have improved functionality. This is supported by the fact that intracellular Ca2+ transients in cardiac constructs based on CNT/GelMA hydrogels significantly increased when compared to those on GelMA hydrogels, which is consistent with the previous reports [47,48]. In our opinion, two factors may account for this effect. Firstly, the improved mechanical property of CNT/GelMA hydrogels affected cell phenotype and improved cell adhesion. Secondly, the improved conductivity of composite hydrogels provides beneficial microenvironment for the intercellular junction and cell-cell coupling, which enhanced cardiomyocyte maturation.

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**Fig. 9.** Activation of FAK and RhoA regulates the formation of gap junction and mechanical junction respectively. a–c, Western blot showed significant increases in the expression of p-FAK and RhoA in cardiac constructs based on CNT/GelMA hydrogels at day 7 when compared to those on GelMA hydrogels. d, Immunofluorescence microscopy showed FAK inhibitor, PF-573228, apparently impaired the expression of gap junction protein, Cx43 in cardiac constructs on CNT/GelMA hydrogels than those on GelMA hydrogels whereas no apparent differences in mechanical junction protein (NC) were observed. e, RhoA inhibitor, Y27632, significantly decreased CNT-induced upregulation of mechanical junction protein expression whereas it did not prevent upregulation of Cx43 compared to the control group. Data are means ± s.e.m. *p < 0.01. All experiments were performed in triplicate.
5. Conclusion

In this study, we report that the incorporation of CNTs into GelMA hydrogel is an effective strategy to regulate the structural integrity of engineered cardiac tissues. The incorporation of CNTs into the scaffolds accelerated the assembly and formation of IDs in the engineered cardiac tissues. Furthermore, β1-integrin-mediated FAK and RhoA signaling pathways play a crucial role during this process. Our study has important significance for improving the quality of engineered cardiac tissue and applying them to cardiac regenerative therapies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.10.025.

References


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