The Electrical Stimulation of Carbon Nanotubes to Provide a Cardiomimetic Cue to MSCs

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Abstract

Once damaged, cardiac muscle has little intrinsic repair capability due to the poor regeneration potential of remaining cardiomyocytes. One method of overcoming this issue is to deliver functional cells to the injured myocardium to promote repair. To address this limitation we sought to test the hypothesis that electroactive carbon nanotubes (CNT) could be employed to direct mesenchymal stem cell (MSC) differentiation towards a cardiomyocyte lineage. Using a two-pronged approach, MSCs exposed to medium containing CNT and MSCs seeded on CNT based polylactic acid scaffolds were electrically stimulated in an electrophysiological bioreactor. After electrical stimulation the cells reoriented perpendicular to the direction of the current and adopted an elongated morphology. Using qPCR, an upregulation in a range of cardiac markers was detected, the greatest of which was observed for cardiac myosin heavy chain (CMHC), where a 40-fold increase was observed for the electrically stimulated cells after 14 days, and a 12-fold increase was observed for the electrically stimulated cells seeded on the PLA scaffolds after 10 days. Differentiation towards a cardioprogenitor cell was more evident from the western blot analysis, where upregulation of Nkx2.5, GATA-4, cardiac troponin t (CTT) and connexin43 (C43) was seen to occur. This was echoed in immunofluorescent staining, where increased levels of CTT, CMHC and C43 protein expression were observed after electrical stimulation for both cells and cell-seeded scaffolds. More interestingly, there was evidence of increased cross talk between the cells as shown by the pattern of C43 staining after electrical stimulation. These results establish a paradigm for nanoscale biomimetic cues that can be readily translated to other electroactive tissue repair applications.

1.0 Introduction

In recent years, CNT have attracted great interest in the field of biomedical engineering for a range of applications including biosensors, cell delivery agents and as supporting structures for tissue engineering scaffolds [1–3]. Although potential cytotoxicity of CNT remains a controversial issue, we have previously demonstrated no adverse effects on MSC behaviour at low concentrations of both single (SWNT) and multiwall nanotubes (MWNT) [4]. Furthermore, cellular uptake of these electroactive nanoparticles [4,5] provides a platform for the manipulation of MSC differentiation pathways using electrical stimulation. Previous studies have shown that electrical stimulation promotes a range of cell responses including reorientation and angiogenesis [6], muscle cell regeneration [7–9], myogenesis of fibroblasts [10,11], cardiomyogenesis of embryonic stem cells [12–14] and enhanced cardiomyocyte phenotype [15–17].

Some of the initial attempts at promoting cardiomyogenesis using mesenchymal stem cells (MSC) involved the use of the controversial demethylating agent 5-azacytidine [18], which has been shown to induce apoptosis *in vivo* [19]. Since then a variety of different approaches have been attempted [20,21] many of which have been the subjects of clinical trials in the past number of years. However, evidence of MSC differentiation to a cardiomyogenic phenotype *in vivo* has been controversial [22,23] leading to the concept that functional benefits of MSCs are largely due to paracrine mechanisms [24,25]. Moreover, recent advances in cell-based therapies have suggested that cell fate can be manipulated by internalising micron-sized particles with phenotype altering capabilities, without the use of genetic alteration or growth factor manipulation [26–28]. Directional neurite growth has been observed using

carbon nanotube patterned structures as a biomimetic cue in other applications (29). Indeed, cardiac muscle itself has a complex structure with microscale to nanoscale organisation and previous studies have suggested that small topographical cues can affect cardiomyocyte attachment and tissue remodeling (17,30,31). The exploitation of the synergy between electrical properties of CNT and the differentiation potential of stem cells presents an opportunity to promote cardiomyogenesis *in vitro*. To this end, we introduce a platform for creating cells of a cardioprogenitor phenotype that combines an electroactive nanoparticle as a biomimetic cue with electrical stimulation of human MSCs. This study aimed to test the hypothesis by applying an electrical stimulus using two approaches, 1) utilisation of CNT as an internal conduit of the stimulus to elicit a biological response in MSCs and 2) provision of an external promoter in the form of a CNT-based randomly oriented nanofibre scaffold to induce MSC differentiation towards a cardiomyocyte lineage *in vitro*.

2.0 Materials and Methods

2.1 Screening CNT concentration for optimal electrical conductivity

As a first step, it was necessary to determine the optimum concentration of CNT for electrical stimulation. As a follow up to a previous study (4), a range of CNT concentrations from 0-0.16mg/ml were screened in terms of electrical conductance. In brief, human MSCs were isolated, characterized and their phenotype confirmed as previously described (4). Subsequently, 3,000 MSCs/cm^2 were seeded per well of a 6-well plate. After 24 hours, the cells were exposed to aseptically prepared CNT suspensions of 0.00128, 0.0064, 0.032, 0.16 and 0.8mg/ml of COOH-functionalized SWNT in MSC medium (DMEM-low glucose containing 10% foetal bovine serum and 1% antibiotic-antimycotic). The electrical resistance was measured continuously

for 24 hours using a UT70B data acquisition card connected to a computer as described previously (4). The resistance of beating neonatal rat cardiomyocytes in cell culture medium was measured as a positive control. Neonatal cardiomyocytes were isolated from 1-4 day old rats; rat hearts were removed, homogenized and digested with trypsin overnight. Collagenase was added to digest the extracellular matrix and cardiomyocytes were isolated by differential centrifugation through a discontinuous Percoll gradient (32). As described previously, it is noted that above concentrations of 0.032mg/ml CNT, the conductance of the cell culture medium reached its percolation threshold (Figure 1). Moreover, concentrations of CNT above 0.032mg/ml CNT were shown to adversely affect cell viability (4). As a result, a concentration of 0.032mg/ml CNT was selected as the optimal concentration for electrical stimulation in this study.

2.2 CNT/PLA Nanofibre Scaffold Preparation

A 30-wt % solution of poly-l-lactide acid (PLA) (Sigma, UK) in a 70:30 mixture of dichloromethane and dimethylformamide was created. As previously described (33), 2-wt % COOH functionalized SWNT (Nanocyl, Belgium) were added to the PLA solution and used to create the electrospun randomly oriented nanofibre scaffolds using a voltage of 15 kV and a feed rate of 0.05 ml/min, with the collector screen 15 cm from the syringe needle. As a method of control electrospun randomly oriented PLA nanofibre scaffolds were also produced without CNT.

2.3 Electrical Stimulation of MSCs in the presence of CNT

As a first step, human ss were seeded in T75 tissue culture flasks at a density of 3000 cells/cm^2 and cultured in MSC growth medium (αMEM, 10%FBS, 1% penicillin/streptomycin). After 24 hours, the medium was replaced with MSC growth medium containing 0.032mg/ml CNT for a further 24 hours as described previously (4). CNT containing medium was removed and cultures washed twice with growth medium. Thereafter, cultures were allowed to grow for 4-5 days to approx. 80% confluence in medium without CNT. Control cultures were treated equivalently but not exposed to CNT. Confluent cultures were trypsinised and replated at 5,000 cells/cm^2 in 4-well tissue culture plates (Nunc Multidishes Nunclon™Δ). Each plate, with two wells seeded with control MSCs and two wells with MSCs previously exposed to CNT containing medium, were placed in a custom built chamber of an electrophysiological bioreactor. Once approx. 60% confluency was reached to enable cells to withstand the initial shock of the current, two wells with MSCs and two wells with MSCs previously exposed to CNT on each plate were exposed to an electrical current of 0.15 V/cm for 2 ms duration at a frequency of 1 Hz for a 14-day period with MSC growth medium changed every 3 days. Additional plates were set up in the same manner as unstimulated controls.

The CNT based scaffolds were seeded at a density of 20,000 cells/cm^2 as previously described (33), placed in the same tissue culture vessels, cultured in MSC growth medium and exposed to the same electrical signal for 10 days; this time was found to be optimal as cultures were over confluent on the scaffolds by 14 days. As controls, MSCs were cultured in the absence of carbon nanotubes with and without electrical stimulation.

2.4 Quantification of cell alignment

In order to quantify the orientation of cells after electrical stimulation, images were taken from 4 independent experiments for 2 donors for the 4 experimental conditions (32 images in total, with >1000 cells). Using ImageJ analysis as previously described (34), images of the cells were thresholded and in some cases manually edited to highlight cell boundaries. The particle analysis tool was used to give a best-fit ellipse for each cell. 0° alignment was set perpendicular to the direction of the current i.e., parallel to the electrodes. The degree of alignment was measured and results were represented as a percentage of the total number of cells captured in the field of view.

2.5 Gene expression analysis using quantitative real time polymerase chain reaction (qPCR)

Changes in gene expression were investigated using semi-quantitative real time polymerase chain reaction (qPCR) for a range of cardiac markers including the early cardiac muscle marker myocyte-specific enhancer factor 2C (MEF2C), cardiac troponin t (CTT) and the later marker cardiac myosin heavy chain (CMHC), as they are known to be involved in morphogenesis, myogenesis, the assembly of muscle proteins and the coordination of contractile response in the developing myocardium after mid-foetal development (35–37). Therefore, the presence of these markers would provide evidence of differentiation towards a cardiac genotype (16,20). Moreover, to determine whether there was direct communication between the cells connexin43 (C43), a gap junction protein found in myocardium, was also investigated. RNA was isolated from MSCs directly after electrical stimulation using TRIzol reagent and transcribed to cDNA using reverse transcriptase. GAPDH was

used as a housekeeping control and all cultures were normalized to unstimulated MSCs.

2.6.1 Protein expression using Western blot analysis

Changes in MSC phenotype were examined by Western blot analysis and immunofluorescent staining for a range of cardiac-associated proteins, including, $NKx_{2.5}$, GATA-4, CTT, CMHC and connexin43. $NKx_{2.5}$ is one of the earliest markers of cardiogenesis and is thought to work in combination with GATA-4, an early marker shown to regulate some of the genes involved in cardiac muscle differentiation and function during embryonic development (38); hence the expression of these cardiac transcription factors would indicate early cardiomyogenesis. For western blot analysis 50μg of protein was separated by SDS-PAGE and transferred to PVDF-membrane for detection of the cardiac markers $NKx_{2.5}$, GATA-4, cardiac troponin T, and connexin43. CMHC was not used, as the size of the protein was too large for western blot analysis. Densitometry was performed using the Fluor Chem analysis tool with the background value subtracted from individual values and bands normalized to housekeeping CuZn SOD (Copper Zinc Superoxide Dismutase) values. Fold change was calculated with respect to protein from control MSCs cultured in MSC growth medium.

2.6.2 Immunofluorescence staining

As it was not possible to collect enough protein from the MSCs seeded on the CNT scaffolds, protein expression was examined using immunofluorescence staining for both the cell cultures and the cell-seeded scaffolds. The medium was removed from

the 4-well plate and the cultures were washed twice in D-PBS. Cells were fixed in 4% paraformaldehyde for 20 min. and the cell membrane permeabilized with 0.5% Triton-X100 for 15 min. After intensive washing with D-PBS, the cultures were blocked with 10% normal goat serum and 0.5% bovine serum albumin (BSA) for 1 hour to prevent non-specific binding of antibodies. Cells were incubated overnight at 4°C with the following mouse monoclonal primary antibodies at a 1:100 dilution in 1% normal goat serum and 0.5% BSA; GATA-4 (Santa Cruz), cardiac troponin T (Abcam), cardiac myosin heavy chain (Abcam) or connexin43 (Santa Cruz). Following 4 x 5 min. washes with D-PBS; cells were incubated with Alex Fluor 488-conjugated Goat Anti-Mouse secondary antibody (Molecular Probes) at a dilution of 1 in 500 for 1 hour in the dark at room temperature. Cell nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI) at a dilution of 1:1000 in D-PBS. Following further intensive washing, cells were covered with D-PBS and imaged using the fluorescent Olympus 1X71 microscope.

2.7 Statistical Analysis

Differences between the test groups for the electrical conductance were assessed for significance using a one-way ANOVA and Tukey post hoc analysis using the software programme GraphPad Prism. A p value of < 0.05 was considered statistically significant.

3.0 Results

3.1 Electrical Conductance

As seen in Figure 1, there appeared to be a correlation between the concentration of CNT in the medium containing MSCs and the electrical conductivity. Moreover, the

electrical conductivity of cell culture medium containing MSCs exposed to a concentration of 0.032mg/ml CNT was approximately twice that of medium without CNT (Figure 1), while there was no significant difference between the electrical conductivity of medium alone when compared to medium plus MSCs.

3.2 Cell Morphology and Orientation

After electrical stimulation the unstimulated MSCs either exposed to the medium containing CNT or seeded on the CNT/PLA scaffolds maintained a typical fibroblast-like morphology, while the electrically stimulated MSCs, MSCs exposed to medium containing CNT and MSCs seeded on the CNT based scaffolds appeared to elongate and became more spindle-like (Figure 2A and Figure 2B). With respect to cell alignment, it can be seen that the electrically stimulated cells reorient perpendicular to the direction of the current, while the unstimulated cells retain a random orientation. This is especially evident for the electrically stimulated MSCs exposed to medium containing CNT. Using ImageJ analysis, this observation was quantified, where it was revealed that 49% of the electrically stimulated MSCs exposed to medium containing CNT realigned between 0-10°. This trend was also observed for the MSCs seeded on the CNT/PLA scaffolds and electrically stimulated for 10 days, where 30% of the cells reoriented between 0-10°.

3.3 MSC Gene Expression

As shown in Figure 4, an upregulation in a range of cardiac markers was observed for MSCs exposed to medium containing CNT and electrically stimulated MSCs. Although gene expression was examined 14 days after treatment, there was still evidence of an upregulation in the early marker MEF2C, with a 4.8 fold increase

observed for MSCs exposed to medium containing CNT and a 5 fold increase seen for electrically stimulated MSCs. When the electrical stimulus and the CNT were combined the affect was diminished with a 2.1 fold increase in expression observed. In terms of cardiac function, there was no trend revealed between the test groups for SMA expression, however, CTT was upregulated by 2.8 fold and 3.1 fold for the electrically stimulated MSCs and electrically stimulated MSCs exposed to medium containing CNT, respectively. The greatest increase in cardiac marker expression was observed for CMHC, a later cardiac marker, where a 36 fold increase in gene expression was observed after 14 days electrical stimulation consistent with a change in MSC genotype. In terms of cell-to-cell communication, an increase in C43 was observed for MSCs exposed to medium containing CNT and after electrical stimulation.

With respect to the cell-seeded scaffolds, although the presence of the early markers was detected, there was no dramatic change in expression as a result of electrical stimulation, either with or without the presence of CNT (Figure 4B). As in the case of the cells, the greatest increase in cardiac marker gene expression was observed for CMHC, whereby a 5.6 fold increase was observed when cells were seeded on the CNT based scaffold, a factor of 12 when the MSCs were electrically stimulated on the PLA and a factor of 2.7 when electrically stimulated on the CNT/PLA scaffold, suggesting that the presence of the CNT or electrical stimulation are important regulators of gene expression. Interestingly, when CNT and electrical stimulation were combined, the resulting change in gene expression was reduced, suggesting that maximal differentiation has been reached and a more mature cardioprogenitor cell is observed.

3.4 Protein Expression of MSCs

After 14 days electrical stimulation, protein was isolated from the MSCs exposed to medium containing CNT for western blot analysis. Electrically stimulated MSCs exposed to medium containing CNT showed positive bands for $NKx_{2.5}$, GATA-4, CTT and connexin43 (Figure 5Ai), with over 4 times more $NKx_{2.5}$, twice more GATA-4, and 2.4 times more CTT present compared to unstimulated MSCs (Figure 5Aii). Electrically stimulated MSCs also revealed evidence of cardiomyogenesis with a 4 fold increase in $NKx_{2.5}$, a 1.4 fold increase in GATA-4 and a 1.2 fold increase in CTT (Figure 5Aii). With respect to the gap junction protein C43, there was an increase observed for both electrically stimulated cultures with over 3.5 times more protein present for the stimulated MSCs and 1.4 times more protein present in the MSCs exposed to medium containing CNT when compared to the unstimulated MSC control.

Electrically stimulated MSCs exposed to medium containing CNT stained positive for CTT, CMHC and connexin43 (Figure 5Bi). MSCs and MSCs exposed to medium containing CNT controls also stained positive for connexin43. However, staining in these cultures had a punctuate appearance with little or no evidence of cell-to-cell cross talk. Electrical stimulation resulted in significantly induced levels of connexin43 and, more importantly, evidence of cross talk with neighbouring cells, as highlighted in Figure 5Bi. This cell-to-cell communication appeared to be more pronounced after electrical stimulation of MSCs exposed to medium containing CNT. A similar observation was made for the scaffolds, where increased levels of CTT and CMHC fluorescent staining were observed for the electrically stimulated MSCs (Figure 5Bii).

4.0 Discussion

Cardiac muscle is an electroactive tissue capable of transferring electrical signals and allowing the heart to beat. In an effort to develop a repair modality for damaged cardiac muscle, electroactive carbon nanotubes were employed as an electrical stimulus to provide a pathway to promote MSC differentiation towards a cardioprogenitor phenotype. At present, there are few reports assessing the conductivity of cells containing CNT, however, a recent study by Ateh revealed that the conductivity of CNT was stable in biological microenvironments (39). We examined the affect of CNT concentration on MSC conductance in cell culture medium and found a positive correlation in electrical properties without adversely affecting the biological properties of the MSCs, thereby confirming the findings of Ateh (39).

Electroactive CNT based scaffolds with following materials properties; T_g of 63°C, T_m of 172°C, percentage crystallinity of 44%, tensile strength of 2.3 MPa, an elastic modulus of 159 MPa, a percentage elongation of 110% and an electrical resistance of 7×10^{-6} Ω were also employed (33) to provide an electrical stimulus for MSC manipulation. Although, CNT based scaffolds have been employed for bone, cartilage and neural tissue repair (2,40–46), this is the first study to show the potential of CNT based scaffolds for MSC differentiation towards a cardioprogenitor cell. Nonetheless, the results compare favorably with these other studies, in that they highlight the importance of electrical properties in the design of biomaterials for electroactive tissue repair.

As mentioned previously, it is well known that mechanical and electrical stimulation alters cell morphology and cell alignment (7,17,47–50); herein the MSCs are elongated in shape after electrical stimulation both in the presence or absence of CNT. However, it is interesting to note that the majority of cells exposed to medium containing CNT or seeded on the CNT based scaffolds reorient perpendicular to the electrical current at an angle between 0 and 10°. This correlates with previous studies by Robinson (51) suggesting that cells align perpendicular to the direction of the current to minimize the voltage drop across the cells. With respect to electrical stimulation of MSCs for cardiac muscle applications, these changes in cell alignment compare well with previous studies by Guan (47) and Genovese (54) where changes in cell alignment were observed after electrical stimulation.

The effect of electrical stimulation on cardiac marker gene expression was examined for cells exposed to medium containing CNT and cells seeded on electrospun randomly oriented nanofibre CNT based PLA scaffolds. Although the presence of range of cardiac markers was detected for all conditions, the levels of expression remained largely unaffected for smooth muscle alpha actin or cardiac troponin t. However, changes were observed for MEF2C and CMHC mRNA levels. These genes were upregulated approx. 5 fold in MSCs previously exposed to medium containing CNT at 14 days. Electrical stimulation also resulted in increased levels of MEF2C (5 fold) and CMHC (40 fold) mRNA at this time point. Paradoxically, the combination of CNT and electrical stimulation had no impact on expression of these genes, a pattern that was repeated for expression of CMHC on cell-seeded scaffolds. To resolve this issue and the higher levels of CMHC protein levels detected in both systems after exposure to a combination of CNT and electrical stimulation we briefly

examined gene expression at 7 days. Preliminary data in one donor indicated that expression of MEF2C and CMHC in MSCs was not increased in response to electrical stimulation at 7 days, however, exposure to CNT alone result in increased expression of MEF2C (8 fold) and CMHC (11 fold). Electrical stimulation synergised with exposure to CNT resulting in a slight increase of MEF2C mRNA levels to 10 fold over that in MSCs alone and a significant increase in CMHC mRNA levels (>8,000 fold; results not shown). This data correlates with other studies (54, 55, 56) and in particular with the findings of Guan and co-workers who reported that cells achieving a higher degree of cell alignment had a greater expression of the cardiac markers MEF2C, Nkx2.5 and GATA-4 (47).

Of more interest, protein expression of cardiac associated markers was increased in the presence of CNT after electrical stimulation. In particular, the immunofluorescent staining for CTT and CMHC expression showed a synergistic effect, suggesting that the CNT are providing a biomimetic stimulus for MSC differentiation. Initially, this may seem at odds, but can be explained, by suggesting that the combination of CNT and electrical stimulation may lead to a more rapid differentiation to a cardioprogenitor phenotype with maximal effect achieved at the times used, resulting in downregulation of gene expression. These findings compare with other studies where micron sized particles, biomaterials and carbon nanotubes have been shown to provide cellular cues for promoting MSC differentiation or altering cell fate (26,28–31,52,53). These data suggest that the synergy between electrical stimulation and carbon nanotubes offers a different approach for the pre-differentiation of MSCs to create cardioprogenitor cells. In addition to agreeing with previous studies where mechanical stimulation (47) and growth factors (20) were employed to pre-

differentiate MSCs for cardiac muscle tissue repair, it opens up the opportunity to use undifferentiated MSCs and electrically stimulate them *in situ* using pacemaker technology.

Conclusions

Using a two-pronged carbon nanotube approach, these data show that by providing a biomimetic electroactive cue, manipulation of the MSC differentiation pathway can be achieved by harnessing the electrical properties of a carbon nanotube based medium or scaffold. Since proof of principle has been established herein, the biomimetic properties of such a platform can be now exploited even further and tailored for other electroactive environments in the heart, the brain or the spinal cord. Ultimately, this strategy provides an opportunity for future studies in the quest to use CNT and MSCs to promote electroactive tissue repair.

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Figure Captions

Figure 1 The effect of CNT concentration on electrical conductance of MSC containing CNT in culture medium. Control was neonatal rat cardiomyocytes. Electrical conductance of cell culture medium with MSC containing CNT was twice that of cell culture medium with MSC alone, *P ≤ 0.05 between 0 and 0.0064mg/ml, 0 and 0.032mg/ml and 0 and 0.16mg/ml. *** P≤0.0001 between test groups and control neonatal rat cardiomyocytes. There was no significant difference (ns) in electrical conductance between the 0.032mg/ml and the 0.16mg/ml. Error bars represent standard error of the mean (n=3).

Figure 2 Effect of CNT and/or electrical stimulation on MSC morphology in (A) monolayer of MSC containing CNT after 14 days electrical stimulation (Magnification 10x). Stimulated MSC appeared elongated in shape after 14 days compared to the fibroblastic morphology of the unstimulated control cultures and (B) MSC seeded on CNT scaffolds after 10 days electrical stimulation. Electrically stimulated MSC appeared to have an elongated morphology similar to that of the electrically stimulated MSC containing CNT.

Figure 3 Cell reorientation after electrical stimulation. Quantification of cell orientation using ImageJ analysis for (A) MSC exposed to medium containing CNT and (B) MSC seeded on CNT scaffolds. Electrical stimulation of MSC exposed to medium containing CNT or MSC seeded on CNT scaffolds resulted in cell reorientation perpendicular to the direction of the current between 0-10°. Results are representative of 4 independent experiments for 2 donors.

Figure 4 (A) Detection of cardiac genes for MSC exposed to medium containing CNT after electrical stimulation for 14 days in culture using qPCR. Graph represents expression of cardiac genes in samples normalized to MSC cultured in MSC growth medium. **(B)** Detection of cardiac genes for MSC seeded on CNT scaffolds after electrical stimulation for 10 days in culture using qPCR. In both cases there was an upregulation in gene expression of cardiac myosin heavy chain in the presence of CNT and after electrical stimulation. Results are representative of 2 independent experiments for 2 donors.

Figure 5 (A) Examination of protein expression for a range of cardiac markers using **(i)** Western blot analysis and quantification using **(ii)** densitometric analysis. Cardiac marker expression was normalized to CuZn SOD levels with MSC alone as the control cultures (Results are representative of 2 independent donors). **(B)** Immunofluorescence staining for detection of cardiac troponin T, cardiac myosin heavy chain and connexin43 for **(i)** MSC exposed to medium containing CNT and **(ii)** MSC seeded on CNT scaffolds. Scale bar 130μm. In both cases there was an upregulation in protein expression, more noticeably with CNT present after electrical stimulation.

Figure 1 Electrical Conductance

Figure 1

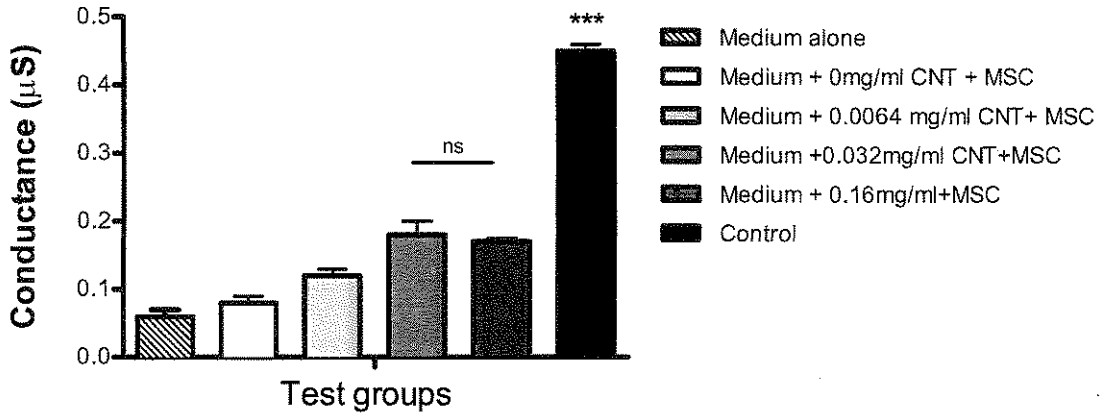

Figure 2A Cell morphology
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Figure 2A

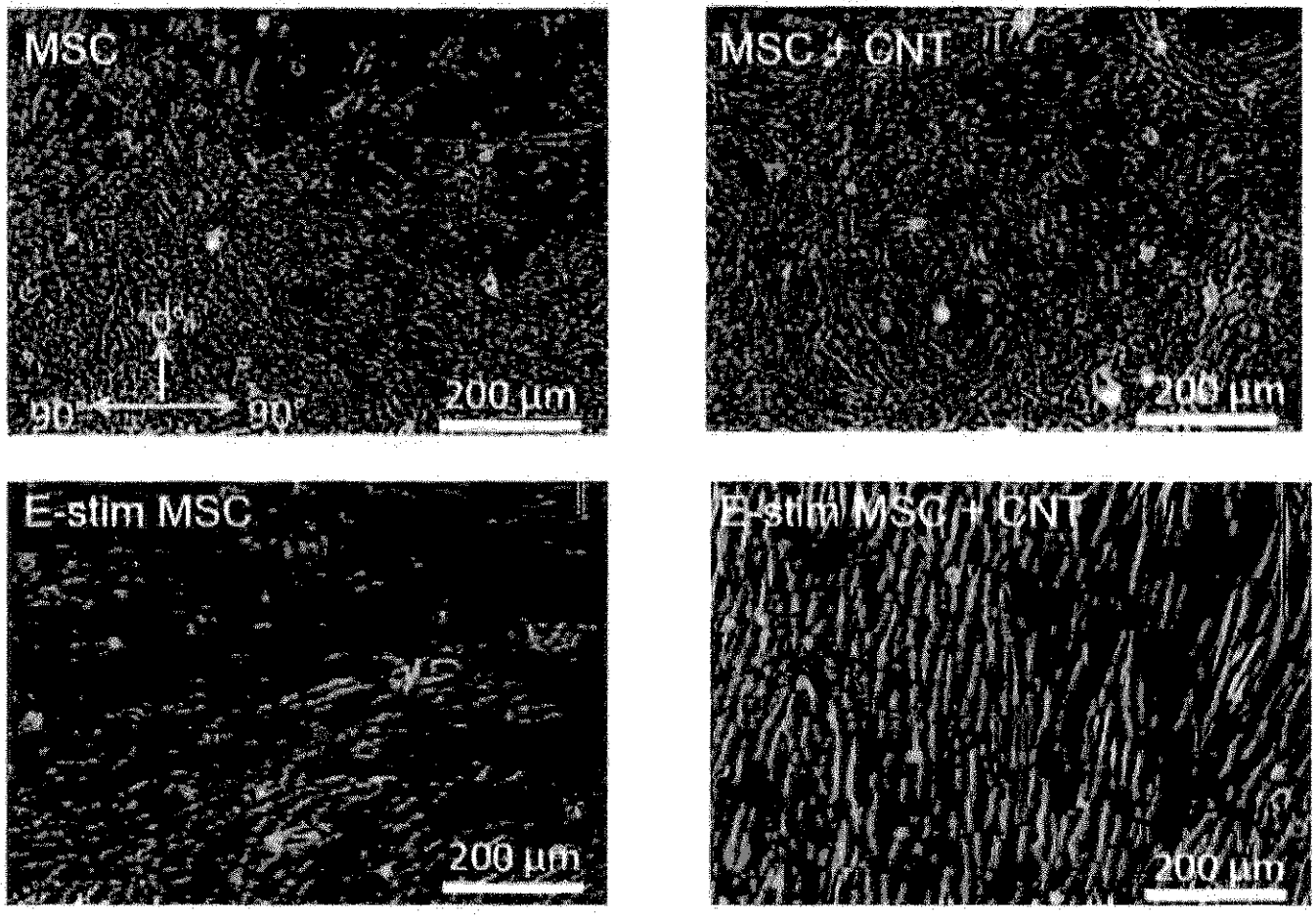

Figure 2B Morphology of cells on scaffold
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Figure 2B

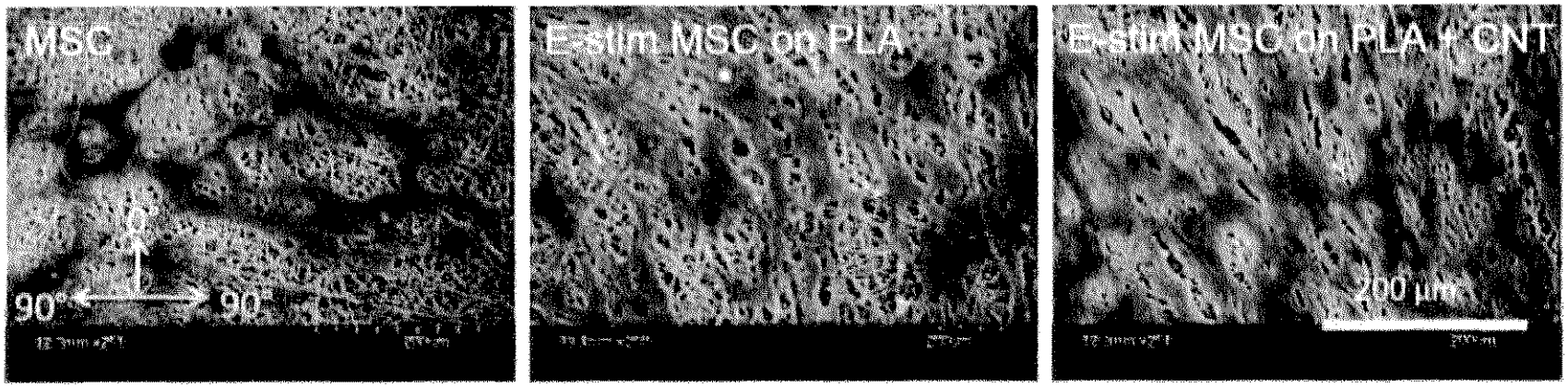

Figure 3A Alignment of cells

Figure 3A

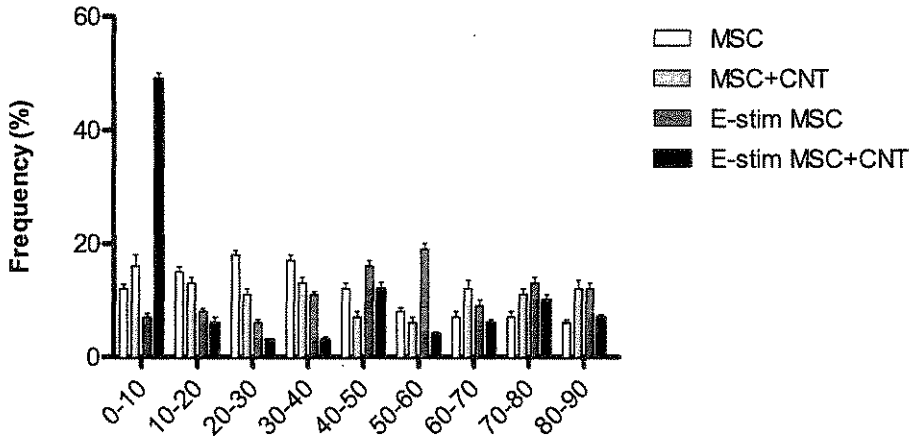

Figure 3B Alignment of cells on scaffold

Figure 3B

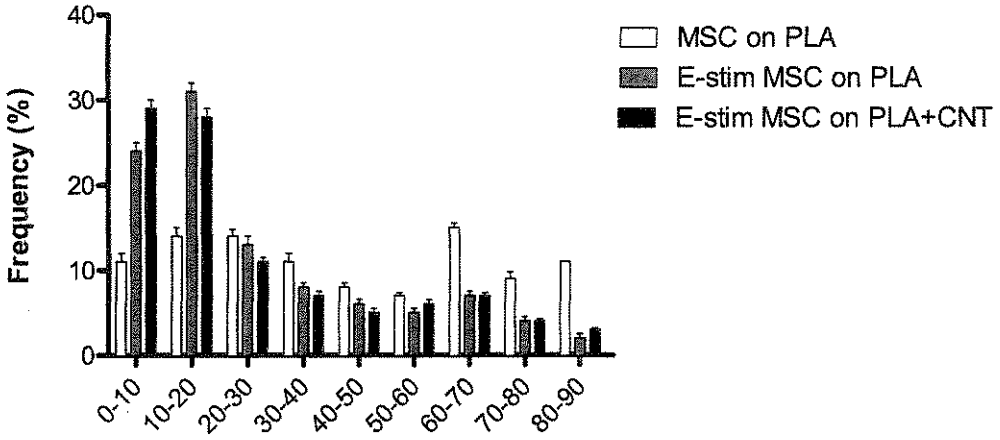

Figure 4A Gene expression of cells

Figure 4A

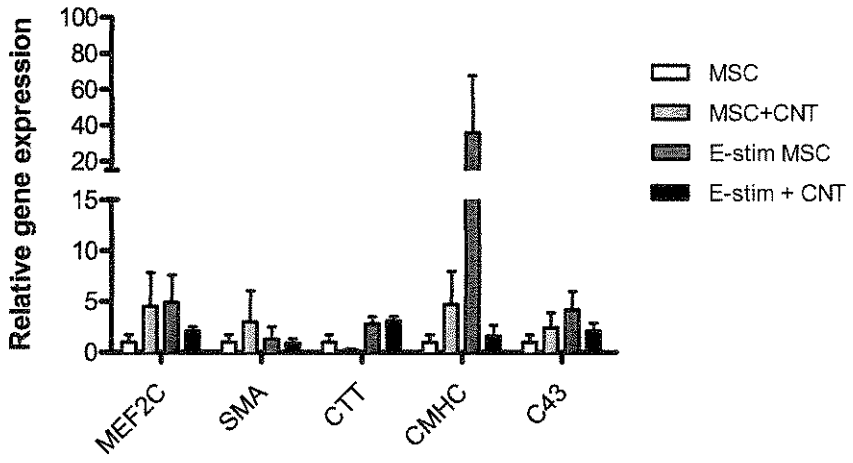

Figure 4B Gene expression of cells on scaffold

Figure 4B

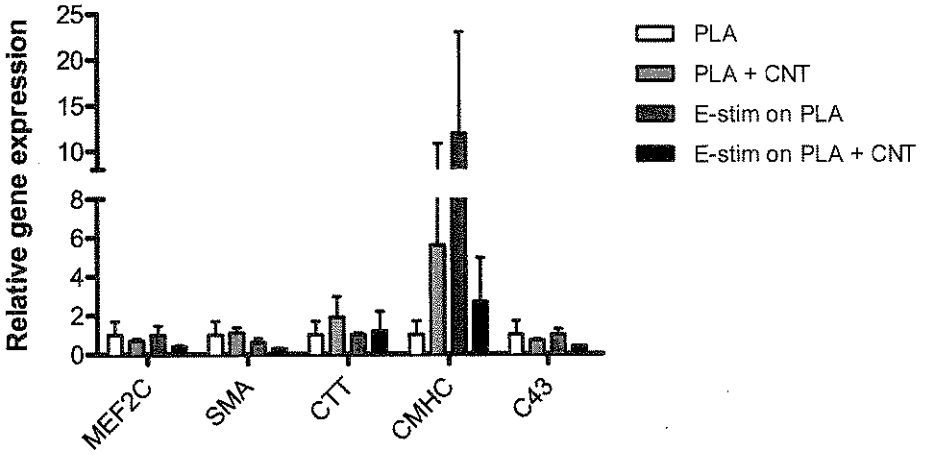

Figure 5Ai Western blot
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Figure 5 Ai

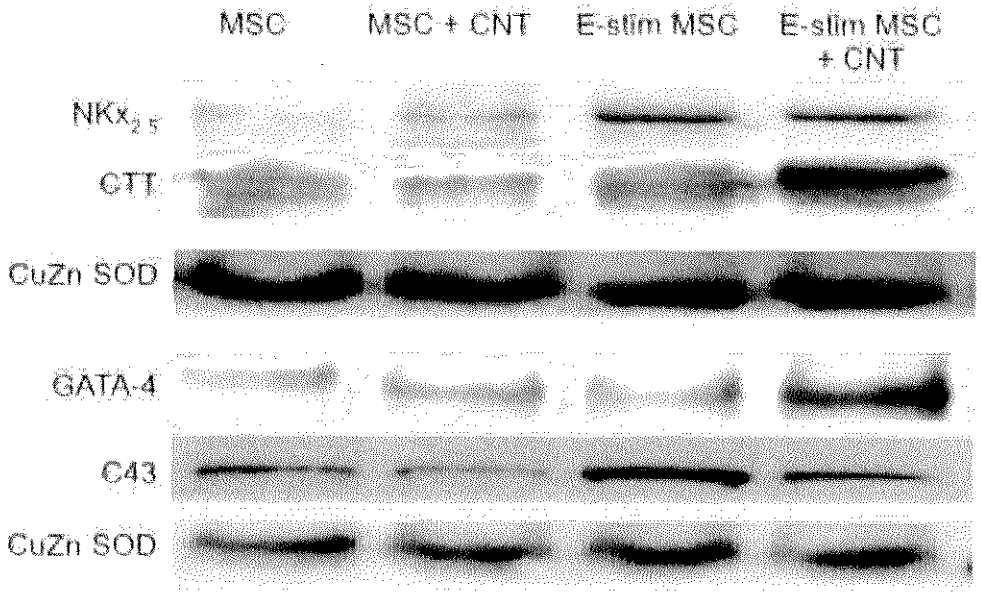

Figure 5Aii Densitometry

Figure 5Aii

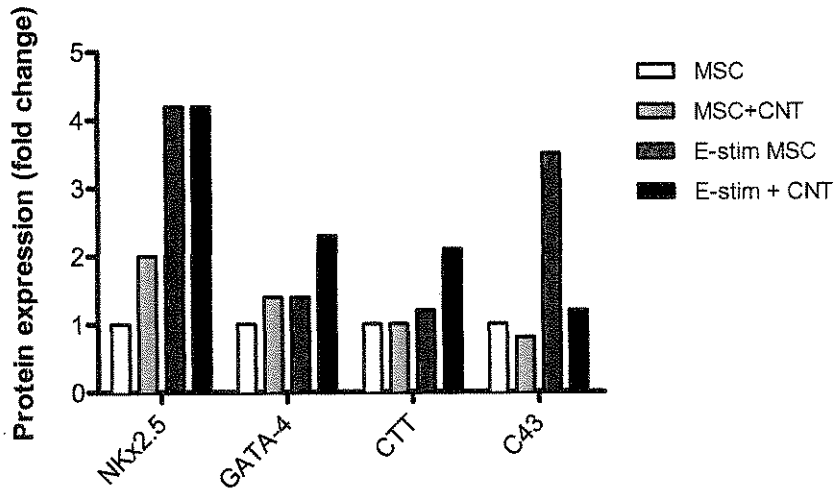

Figure 5Bi Immunostaining of cells
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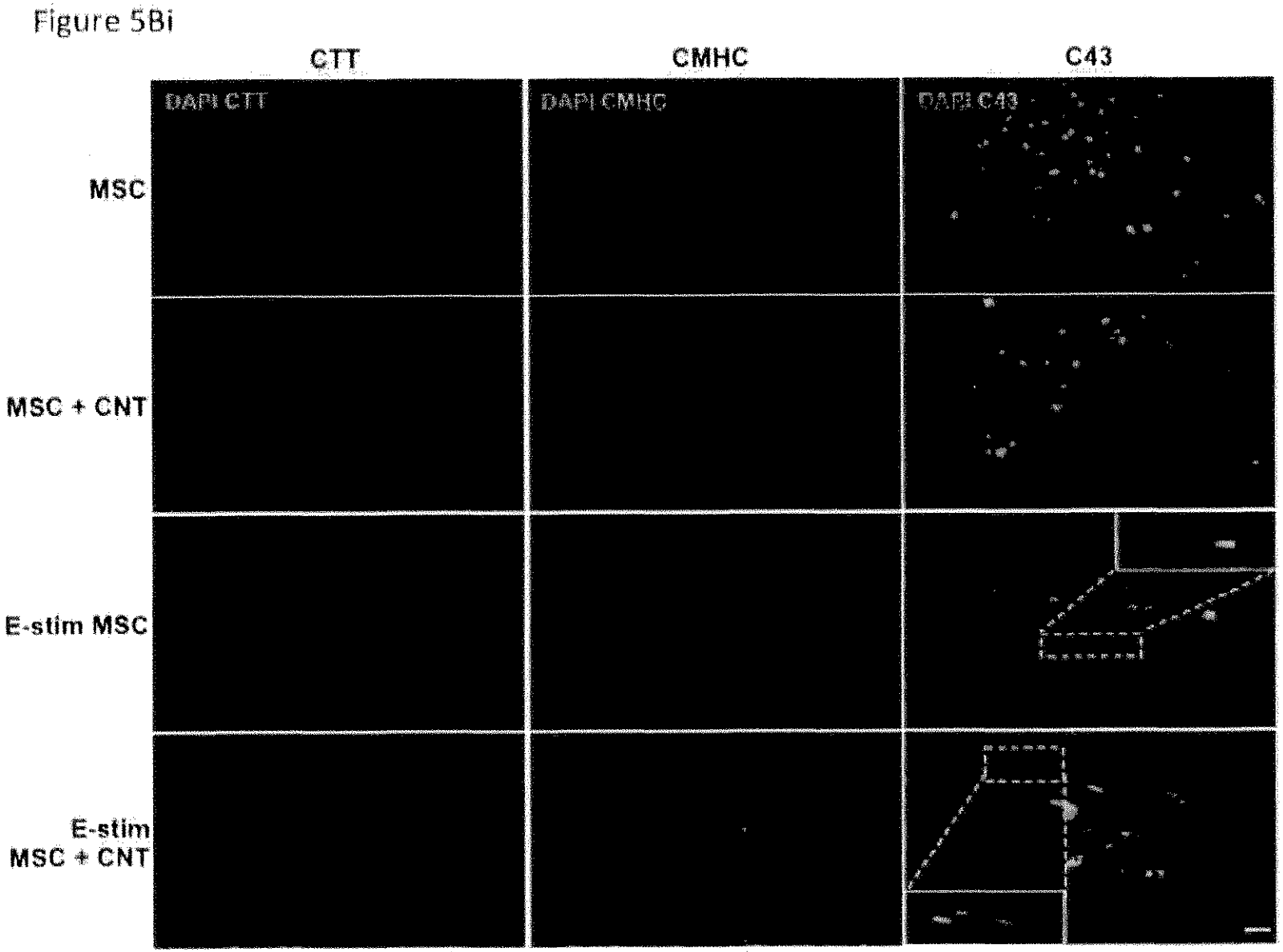

Figure 5Bii Immunostaining of cells on scaffold
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