Modulation of chondrocyte motility by tetrahedral DNA nanostructures

Sirong Shi* | Shiyu Lin* | Xiaoru Shao | Qianshun Li | Zhang Tao | Yunfeng Lin

State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China

Correspondence

Yunfeng Lin, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China.

Email: yunfenglin@scu.edu.cn

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Abstract

Objectives: Contemporarily, a highly increasing attention was paid to nanoconstructs, particularly DNA nanostructures possessing precise organization, functional manipulation, biocompatibility and biodegradability. Amongst these DNA nanomaterials, tetrahedral DNA nanostructures (TDNs) are a significantly ideal bionanomaterials with focusing on the property that can be internalized into cytoplasm in the absence of transfection. Therefore, the focus of this study was on investigating the influence of TDNs on the chondrocytes locomotion.

Materials and methods: Tetrahedral DNA nanostructures was confirmed by 6% polyacrylamide gel electrophoresis (PAGE) and dynamic light scattering (DLS). Subsequently, the effect of TDNs on chondrocyte locomotion was investigated by real-time cell analysis (RTCA) and wound healing assay. The variation of relevant genes and proteins was detected by quantitative polymerase chain reaction (qPCR), western blotting and immunofluorescence respectively.

Results: We demonstrated that tetrahedral DNA nanostructures have positive influence on chondrocytes locomotion and promoted the expression of *RhoA*, *ROCK2* and *vinculin*. Additionally, upon exposure to TDNs with the concentration of 250 nmol L^{-1} , the chondrocytes were showed the highest motility via both RTCA and wound healing assay. Meanwhile, the mRNA and protein expression of *RhoA*, *ROCK2* and *vinculin* were also significantly enhanced with the same concentration.

Conclusions: It can be concluded that the TDNs with the optimal concentration of 250 nmol L^{-1} could extremely promoted the chondrocytes locomotion through facilitating the expression of *RhoA*, *ROCK2* and *vinculin*. These results seemed to reveal that this special three-dimensional DNA tetrahedral nanostructures may be applied to cartilage repair and treatment in the future.

1 | INTRODUCTION

DNA, one of the most well-utilized therapeutic gene, is used for gene therapy for years, with significantly specific Watson-Crick base pairing and a well characterized double-helical structure.^{1,2} To date, as a

kind of nano-biomaterials, DNA nanostructures has attracted a highly increasing attention on account of its multiple functions and extensive application.^{3,4} DNA nanostructures are promising carriers for drug and gene delivery vehicles due to following advantages¹: their intrinsic biocompatibility and biodegradability,² they are easily controllable in

Abbreviations: TDNs, tetrahedral DNA nanostructures; RTCA, real-time cell analysis; siRNA, short interfering RNA; DMEM, Dulbecco's Modified Eagle Media; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; DLS, dynamic light scattering; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; qPCR, quantitative polymerase chain reaction; *RhoA*, Ras homolog gene family, member A; *ROCK*, Rho-associated coiled-coil protein kinase.

*Sirong Shi and Shiyu Lin contributed equally to this work.

size and shape at a tailor-made level,³ they can be internalized in mammalian cells in the absence of transfection agent.⁵⁻⁸ Recently, DNA nanostructures have been exploited to deliver bioactive molecules such as anti-senses,⁹ aptamers,¹⁰ siRNA,^{5,11} anti-cancer drugs^{12,13} and immunogenic molecules.¹⁴⁻¹⁶ Among the many potential DNA nanostructures, tetrahedral DNA nanostructures (TDNs) originally developed by Turberfield et al.¹⁷ are remarkably attractive because of their brilliant mechanical rigidity, prominent structural stability, rapidly and easily synthesis and simple functionalization. More significantly, it has been proved that tetrahedral DNA nanostructures can be permeable the cell membrane even without the help of transfection reagents and remains substantially intact within the cytoplasm.⁷ Subsequently, Le liang et al. has confirmed that TDNs rapidly entered into the cell by a caveolin-dependent pathway and with the aid of nuclear location signals, TDNs can escape from the lysosome and entry into the cellular nuclei.¹⁸ In fact, TDNs have been studied and functionalized with CpGs,¹⁴ siRNA,⁵ microRNA,¹⁹ metal complexes.²⁰ Nevertheless, how about the TDNs itself influencing on the cell bioactivity such as motility, especially for chondrocytes motility?

Motility and migration of chondrocytes are significantly essential in the period of growth, development, wound healing and in the manufacturing of scaffolded cartilage implants.^{21,22} As is well known to everyone, cartilage repair is a challenge in both therapeutics and research since cartilage has limited self-regeneration capabilities.²³ It was hypothesized that motility of chondrocytes in the cartilage matrix occurs during the formation of chondrocyte clusters in osteoarthritis, however.²⁴⁻²⁷

Moreover, chondrocyte clusters adjacent to severe cartilage degeneration have specific progenitor, proliferative potential and locomotory activity.²⁸ Based on these results, could the TDNs promote the locomotion of the chondrocytes for treatment of cartilage injuries in the future? In our study, we report our observations on the role of TDNs in rat chondrocyte migration and molecular mechanism in vitro.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

Animal materials used for this study were obtained according to governing ethical principles and our protocol was reviewed and approved by our Institutional Review Board (IRB).

Chondrocytes were obtained from knee-joint of 3-day old SD rats. Briefly, under sterile conditions, collected cartilage tissues was cut into small pieces, pretreated with 0.25% trypsin for 30 minutes, washed with phosphate-buffered saline (PBS) for three times, and digested with type II collagenase (0.1%) for 1-3 hours in 37°C shaking bath. Enzyme activity was neutralized by the 1:1 (v/v) addition of fresh Dulbecco's Modified Eagle Media (DMEM) containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin solution. After centrifuged at 200×g for 5 minutes, removing the supernatant, DMEM containing 10% FBS was added to the tubes, re-suspending the chondrocytes. Subsequently, the chondrocytes in suspension were seeded on T25 culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO₂ atmosphere till passage II for usage.^{29,30}

TABLE 1 The sequence of four designed specific ssDNA

DNA	Sequence
S1	5'-ATTATACACCCGCCATAGTAGACGTATCACC AGGCAGTTGAGACGAACATTCCTAAGTCTGAA-3'
S2	5'-ACATGCGAGGGTCCAATACCGACGATTACA GCTTGCTACACGATTGAGACTTAGGAATGTTTCG-3'
S3	5'-ACTACTATGGCGGGTGATAAAACGTGTAGCA AGCTGTAATCGACGGGAAGAGCATGCCCATCC-3'
S4	5'-ACGGTATTGGACCCTCGCATGACTCAACTGC CTGGTGATACGAGGATGGGCATGCTCTTCCCG-3'

2.2 | TDNs synthesis and characterization with dynamic light scattering (DLS) and polyacrylamide gel electrophoresis (PAGE)

Tetrahedral DNA nanostructures were prepared as previously described.^{31,32} Briefly, we designed four single-stranded DNAs (Table 1) that were synthesized by Takara Bio (Otsu, Japan) and combined in an equimolar ratio in buffer composed of 10 mmol L⁻¹ Tris-HCl (pH 8.0) and 50 mmol L⁻¹ MgCl₂. The solution was heated to 95°C for 10 minutes and then rapidly cooled to 4°C for 20 minutes. The successful synthesis of TDNs was confirmed by 8% PAGE; the TDNs were diluted in ddH₂O at a concentration of 250 nmol L⁻¹. Dynamic light scattering was performed using a ZetaPALS analyzer (Brookhaven Instruments, Holtsville, NY, USA).

2.3 | Real-time cell analysis (RTCA) migration/invasion assays

Real-time cell motility was monitored using an xCELLigence® DP RTCA instrument (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's standard protocol. In brief, Prior to cell seeding, 165 µL fresh culture medium containing 1% FBS was added to lower chamber and 30 µL same medium was added to the upper chamber, and then the solutions were left to equilibrate in the incubator at 37°C and 5% CO₂ for 1 hour. After this incubation step, a background reading was taken for each well. Following, 6×10⁵ cells/well suspended in culture medium containing 1% FBS were seeded in the upper chamber according to the manufacturer's manual (Roche Diagnostics). Subsequently, TDNs were added into the upper chamber at various concentrations (62.5-250 nmol L⁻¹). The cell invasion/migration plates were placed onto the RTCA station and the cell indices were measured every 15 minutes for up to 48 hours with the RTCA software.

2.4 | Wound healing assay

wound healing assay was obtained to detect migration of chondrocytes. Cells were seeded in 12-well plates, cultured in incubator for 24 hours. Next, a sterile pipette tip was used to scratch and form a bidirectional wound. Each hole washed for three times with PBS.

Cells were exposed to medium containing 1% FBS only (control) and the same medium containing TDNs (concentration from 62.5 to 250 nmol L⁻¹). Wound closure was photographed 0, 8, 12, and 24 hours after incubation.

2.5 | Immunofluorescence

For immunofluorescence analysis, chondrocytes were cultured in a confocal microscopy culture dish. After treatment with TDNs, cells were washed three times with PBS, fixed with 4% (w/v) paraformaldehyde for 15 minutes, permeabilized with 0.1% (v/v) Triton X-100 for 15 minutes, blocked by adding 1% (v/v) goat serum in PBS for 1.5 hours at room temperature, and incubated at 4°C overnight in primary antibody solution. The following day, DyLight 594 goat anti-rabbit secondary antibodies were applied to the cells for 1 hour at 37°C. Fluorescein isothiocyanate-labelled phalloidin and 4',6-diamidino-2-phenylindole were used to label the cytoskeleton and nuclei respectively. Cells were imaged by confocal laser scanning microscopy.

2.6 | Western blotting

After treatment with TDNs or ddH₂O (control), cells were washed, harvested, and lysed in lysis buffer. Protein concentration was determined with the bicinchoninic acid assay, and proteins were denatured by boiling in sodium dodecyl sulphate (SDS) buffer and separated by SDS-PAGE. They were then transferred to a polyvinylidene difluoride membrane that was blocked for 1 hour and then incubated overnight at 4°C with primary antibodies. After washing and incubation with secondary antibody for 1 hour at room temperature followed by additional washes with Tris-buffered saline with 0.1% Tween-20, immunoreactivity was visualized by enhanced chemiluminescence.

2.7 | Quantitative (q)PCR analysis

For qPCR, Total RNA was extracted from chondrocytes using an RNeasy® Plus Mini Kit (Qiagen, CA, USA) with a genomic DNA eliminator. RNase-free water was used to dissolve extracted RNA samples and RNA concentration was quantified by the absorbance at 260 nm. 0.5 µg of each total RNA sample was reverse transcribed using a first strand cDNA synthesis kit (Fermentas, Burlington, Canada) after treatment with DNase I. evaluating the expression of target mRNAs (Table 2), qPCR was performed (denaturation for 3 minutes at 94°C, followed by

40 cycles of 5 seconds at 94°C and 34 seconds at 60°C. For each reaction, a melting curve was generated to test for primer dimer formation and false priming,) using SYBR® Green I PCR master mix and an ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA, USA).

2.8 | Statistical analysis

In each experiment, samples were prepared in triplicate and the assay was repeated at least three times. Data are shown as the mean±SD. The Student's *t* test or one-way analysis of variance was used to evaluate the statistical significance of differences between group means (*P*<.05). Analyses were performed using SPSS v.19.0 software (IBM Corp., Armonk, NY, USA).

3 | RESULTS

3.1 | Preparation and characterization of TDNs

Tetrahedral DNA nanostructures were readily self-assembled from four 55-mer specific designed single strands (Table 1) with a simple annealing process. The schematic plot is shown in Figure 1A. Successfully constructed TDNs were verified in 6% PAGE (Figure 1B), suggesting that one prominent band on the right for functional nanostructures. The nanostructure was also characterized by the dynamic light scattering (DLS), demonstrating that the hydrodynamic size of TDNs was ~10 nm (Figure 1C).

3.2 | Effects of various concentration of TDNs on cell migration

To explore the effect of TDNs on chondrocytes in migration and invasion, RTCA and wound healing assay were performed. As shown in Figure 2A, TDNs were confirmed to remarkably enhanced chondrocytes migration and invasion at a concentration dependent manner (62.5, 125, 250 nmol L⁻¹), suggesting that TDNs displayed a potential to facilitate chondrocytes cell migration. Statistical analysis reveal that a significant increase in the migratory cell index following treatment with TDNs at 6, 12 and 24 hours in Figure 2B. In addition to the results of migration analysis conducted with RTCA DP, similar results were also obtained by wound healing assay. It was shown that chondrocytes migration was highly up-regulated with raising concentration and prolonged treatment of TDNs in Figure 2C,D. Interestingly, 8, 12 and 24 hours after scratching, the chondrocytes with TDNs were found to migrate markedly

TABLE 2 The primers sequences of selected genes designed for qPCR

mRNA	Product length	Primer pairs	
β-actin	140bp	Forward	CCTAGACTTCGAGCAAGAGA
		Reverse	GGAAGGAAGGCTGGAAGA
RhoA	132 bp	Forward	AACAGGATTGGCGCTTTTGG
		Reverse	GATGAGGCACCCCGACTTTT
Rock2	114 bp	Forward	GACATTGAACAGCTTCGGTCG
		Reverse	TCGTGATTCTGGAAATCCGTCA
VCL	108 bp	Forward	AAGGAGGCAAAAGGAAAC
		Reverse	GAAAGAAGAGGCAGAAAAACA

FIGURE 1 Synthesis and characterization of tetrahedral DNA nanostructures (TDNs). (A), Schematic illustration of TDNs self-assembly from four single-stranded DNAs. (B), Native polyacrylamide gel electrophoresis (PAGE) analysis confirming the successful formation of TDNs. (C), Hydrodynamic size of TDNs, as determined by dynamic light scattering (DLS)

FIGURE 2 (A, B) Chondrocyte migration at different tetrahedral DNA nanostructures (TDNs) concentrations, as detected by real-time cell analysis (RTCA). (C, D) Effect of TDNs concentration on chondrocyte migration at 12, 24, 36, and 48 h, as determined by the wound healing assay. Data are presented as mean $\pm$ SD ($n=3$). * $P < .05$, ** $P < .01$

faster than untreated cells in a concentration-dependent manner in the range of 62.5–250 nmol L⁻¹. In contrast, the TDNs concentration of 250 nmol L⁻¹ importantly led more cells migrated into wounding zone.

Combining the results of RTCA and wound healing assay, it suggests that TDNs can be capable of promoting chondrocytes motility, the optimum concentration of TDNs for 250 nmol L⁻¹.

3.3 | The expression of RhoA highly up-regulated under the influence of TDNs

We further investigated the molecular mechanism involved. *RhoA* which is closely related to cell locomotion was detected by real-time q-PCR, immunofluorescence and western blotting. As shown in Figure 3B, the expression level of *RhoA* was detectable and remarkable different between treatment with TDNs and control groups, except the concentration of 62.5 nmol L^{-1} . It is worth mentioning, noteworthy differences in TDNs concentration of 250 nmol L^{-1} were detected in contrast with control groups, TDNs highly enhancing the *RhoA* expression up to 2.025-fold. Hence, 250 nmol L^{-1} may be the most suitable concentration for enhancing the motility of chondrocytes. Subsequently, the concentration of 250 nmol L^{-1} was used to treat the chondrocytes in the immunofluorescence and western blotting. Not coincidentally, the results of immunofluorescence about RhoA indicated that TDNs with the concentration of 250 nmol L^{-1} extremely promoted the production of RhoA (Figure 3A). In general, RhoA production under the treatment of TDNs is much higher in comparison to control groups (** $P < .001$) in chondrocytes (Figure 3E). Similarly, there

is a remarkable difference (** $P < .001$) between treatment groups and control groups in the result of western blotting in Figure 3C,D.

3.4 | TDNs significantly modulated the expression of Rho-associated coiled-coilprotein kinase 2 (ROCK2) and vinculin about chondrocytes

Same as to *RhoA*, *ROCK2* and *vinculin* are also closely related to cell motility. Therefore, the mRNA expression of *ROCK2* and *vinculin* were detected by real-time qPCR about treatment groups and control groups. As shown in Figure 4B, TDNs (the concentration of $62.5\text{--}250 \text{ nmol L}^{-1}$) facilitated expression of *ROCK2* than control groups, detailedly, under the influence of TDNs concentration of 62.5 nmol L^{-1} , *ROCK2* expression in the treatment groups was higher than in the control groups, increasing to 1.618-fold. Likewise, there is a remarkable difference (* $P < .05$) between experimental groups in the concentration of 250 nmol L^{-1} and control groups. In contrast to *RhoA*, there is no concentration dependent manner about TDNs in the expression of *ROCK2*. As for the *vinculin*, it increased in a concentration-dependent manner from 62.5 to 250 nmol L^{-1} treated with TDNs, especially

FIGURE 3 (A), Confocal micrographs of chondrocytes incubated without (control) or with tetrahedral DNA nanostructures (TDNs) (250 nmol L^{-1}) for 24 h, showing RhoA (red), cytoskeleton (phalloidin, green), and cell nuclei (blue). Scale bars: $25 \mu\text{m}$. (B), Quantitative polymerase chain reaction (qPCR) analysis of *RhoA* expression in chondrocytes cultured without (control) or with TDNs (250 nmol L^{-1}) for 12 h. Results were normalized to the levels of the reference gene β -actin. (C), *RhoA* protein expression, as determined by western blotting. Lane 1, control; lane 2, TDNs (250 nmol L^{-1}). (D), Histogram representation of *RhoA* protein expression from the western blot shown in (C). (E), Histogram representation of *RhoA* protein expression, as determined by immunocytochemistry. Data are presented as mean $\pm$ SD ($n=3$). * $P < .05$, ** $P < .01$

FIGURE 4 (A), Confocal micrographs of chondrocytes incubated without (control) or with tetrahedral DNA nanostructures (TDNs) (250 nmol L⁻¹) for 24 h, showing ROCK2 (red), cytoskeleton (phalloidin, green), and cell nuclei (blue). Scale bars: 25 μm. (B), Quantitative polymerase chain reaction (qPCR) analysis of ROCK2 expression in chondrocytes cultured without (control) or with TDNs (250 nmol L⁻¹) for 12 h. Results were normalized to the levels of the reference gene β-actin. (C), ROCK2 protein expression, as determined by western blotting. Lane 1, control; lane 2, TDNs (250 nmol L⁻¹). (D), Histogram representation of ROCK2 protein expression from the western blot shown in (C). (E), Histogram representation of ROCK2 protein expression, as determined by immunocytochemistry. Data are presented as mean±SD (n=3). **P*<.05, ***P*<.01, ****P*<.001

250 nmol L⁻¹ (***P*<.01) (Figure 5B). Subsequently, the protein expression of ROCK2 and vinculin were determined by immunofluorescence and western blotting treated with the concentration of 250 nmol L⁻¹ of TDNs. Shown in the Figures 4A and 5A, the ROCK2 and vinculin protein expression in treatment group were markedly higher than control group respectively. Meanwhile, statistical analysis showed that both ROCK2 and vinculin were significantly enhanced in the Figures 4E (***P*<.01) and 5E (***P*<.01) respectively. The results of western blotting revealed that no matter ROCK2 or vinculin were enhanced when 250 nmol L⁻¹ TDNs added into the culture medium of chondrocytes (Figures 4C and 5C). In addition to, as shown in Figures 4D and 5D, statistical analysis showed that there was a significant difference between treatment groups and control groups in ROCK2 (***P*<.01) as well as vinculin (***P*<.01) separately.

4 | DISCUSSION

Nowadays, it has been proposed and determined that DNA nanoconstructs could be used for molecular delivery carriers in diagnostics and

therapeutics as one of most promising candidates considering their versatility.¹ Among them, there was a significantly practical DNA nanoconstruct named tetrahedral DNA nanostructure possessing the characteristic of excellent mechanical rigidity and brilliant structural stability.¹⁷ As reported previously,³¹ TDNs can be readily and rapidly formed via a simple annealing process with four equimolar single strand DNA (Figure 1A). Tetrahedral DNA nanostructures were determined mainly through 8% PAGE and DLS (Figure 1B,C), both of which demonstrating that TDNs successfully self-assembled and their hydrodynamic size was ~10 nm on average. More significantly, it has been reported that TDNs can easily be taken into the mammalian cells even lack of transfection reagents and remains substantially intact in the cytoplasm.⁷ Meanwhile, TDNs entered into the cell primarily via a caveolin-dependent pathway and can access to the cellular nuclei with the aid of nuclear location signals.¹⁸ Although TDNs have been used for delivery all kinds of compound, such as CpGs, siRNA, microRNA, metal complexes etc.^{5,14,19,20} There was little research about TDNs itself influencing on the cell bioactivity. Hence, we want to study and analyse how TDNs affected the locomotion of chondrocytes, isolated from the primary rat articular cartilage.

FIGURE 5 (A), Confocal micrographs of chondrocytes incubated without (control) or with tetrahedral DNA nanostructures (TDNs) (250 nmol L^{-1}) for 24 h, showing vinculin (VCL; red), cytoskeleton (phalloidin, green), and cell nuclei (blue). Scale bars: $25 \mu\text{m}$. (B), Quantitative polymerase chain reaction (qPCR) analysis of VCL expression in chondrocytes cultured without (control) or with TDNs (250 nmol L^{-1}) for 12 h. Results were normalized to the levels of the reference gene β -actin. (C), VCL protein expression, as determined by western blotting. Lane 1, control; lane 2, TDNs (250 nmol L^{-1}). (D), Histogram representation of VCL protein expression from the western blot shown in (C). (E), Histogram representation of VCL protein expression, as determined by immunocytochemistry. Data are presented as mean $\pm$ SD ($n=3$). * $P<.05$, ** $P<.01$

In the physiological condition, articular cartilage is an avascular tissue, deriving both its nutrition and oxygen via diffusion from the synovial fluid and the subchondral bone.³³ Additionally, articular cartilage has an awfully limited capacity to heal itself once injured by trauma or pathology.³⁴ Meanwhile, in our previous study, we have demonstrated that TDNs could remarkably promote cell proliferation via Wnt/ β -catenin pathway.³¹ It is generally known that cartilage regeneration is highly dependent on chondrocytes proliferation and migration.³⁵ It has been demonstrated that TDNs can facilitate the proliferation of cells including chondrocytes. Therefore, TDNs may also enhance the motility of chondrocytes and we have confirmed it subsequently.

Chondrocytes motility is one of the most important during the process of cartilage regeneration.³⁵ The locomotion of chondrocytes treated with TDNs in different concentration from 62.5 to 250 nmol L^{-1} and vehicle was detected by RTCA and wound healing assay. Real-time cell analysis is a non-invasive, impedance-based bio-sensor system that can monitor cell processes including cell proliferation, viability, growth, spreading and migration with highly sensitive and accurate.³⁶ From the results of RTCA, it can be clearly determined

that chondrocytes migration and invasion were extremely enhanced by TDNs at a concentration-dependent manner (62.5 - 250 nmol L^{-1}), suggesting that TDNs have the potential to promote chondrocytes motility. Additionally, the same results were also obtained from wound healing assay. Chondrocytes motility were greatly up-regulated with raised concentration and prolonged treatment of TDNs. It's significantly noteworthy that the concentration of 250 nmol L^{-1} of TDNs is the most capable concentration to facilitate chondrocytes locomotion showing in both RTCA and wound healing assay. Therefore, TDNs can be capable of promoting chondrocytes motility with the optimum concentration of 250 nmol L^{-1} .

It is universally known that rearrangement the actin cytoskeleton induces the cell migration basically through four steps¹: constituting new lamellipodia and filopodia,² adhering to the substratum at the front of the cell,³ detaching from the substratum at the tail of the cell,⁴ retracting their tails. More importantly, there are two essential steps during cell locomotion: formation of adhesive structures and cellular contraction.³⁷ Meanwhile, members of the Rho family of small GTPases are key regulators of the actin cytoskeleton, especially

associated with the cell shape changes and the adhesion dynamic that drive cell migration.³⁸ In these members, *RhoA* plays a particularly vital role in charge of stimulation of cell locomotion, mainly through regulation of the actin cytoskeleton in the formation of stress fibres and focal adhesion assembly.³⁹ Therefore, we detected the mRNA expression of *RhoA* of chondrocytes treatment with different concentration of TDNs (62.5, 125 and 250 nmol L⁻¹) via real-time PCR (RT-PCR). It was apparently shown that the *RhoA* expression of chondrocytes highly increased when the concentration of TDNs increased from 62.5 to 250 nmol L⁻¹, and the *RhoA* has the highest expression in TDNs concentration of 250 nmol L⁻¹. Subsequently, the protein expression of *RhoA* treatment with the TDNs concentration of 250 nmol L⁻¹ was also detected using immunofluorescence and western blotting. The results of both immunofluorescence and western blotting demonstrated that TDNs significantly up-regulated the *RhoA* protein expression. These results strongly suggested that *RhoA* activity is essential for TDNs-enhanced chondrocytes migration. Likewise, Ding Ma et al., reported that the increased expression of *RhoA* in human umbilical vein endothelial (HUVE) cells significantly enhanced the morphogenetic changes and cytoskeletal reorganization of the transfected cells, and also enhanced cell migration.⁴⁰

Because the *ROCK2* is the major downstream effector of *RhoA* exerting a control on actin stress fibre assembly through the action of *ROCK*,^{41,42} we next investigated its role in TDNs-effective motility of chondrocytes. Similarly, we found that *ROCK2* was greatly up-regulated when treated with TDNs from the concentration 62.5 to 250 nmol L⁻¹ via RT-PCR. We also detected the protein level of *ROCK2* using the TDNs concentration of 250 nmol L⁻¹, suggesting that the protein expression of *ROCK2* was extremely enhanced. These results are consistent with previous finding indicating that activation of the small GTPase *RhoA* and its effector *ROCK* have been involved with the amoeboid movement.⁴³ Concomitantly, it has been demonstrated that $\alpha\beta1$ was closely involved in the regulation of migration via a predominant *RhoA/ROCK*-dependent mechanism.⁴⁴ Additionally, *vinculin* plays a vital role in cell motility. It can promote lamellipodial extension mainly through interacting directly with the Arp2/3 complex and providing a link between integrin and the adhesion complex to the machinery.⁴⁵⁻⁴⁸ Indeed, Mierke et al., suggested that *vinculin* could significantly promote the wildtype MEFs invade deeper and highly migrate into dense and relatively stiff 3D collagen gels.⁴⁹ Similarly, in our study, the mRNA expression of *vinculin* was up-regulated in chondrocytes treatment with TDNs including three different concentration 62.5, 125, 250 nmol L⁻¹, showing the concentration-dependent manner. Treated with TDNs of 250 nmol L⁻¹, the *vinculin* protein expression significantly high than control group both in immunofluorescence and western blotting. Collectively, our data provide important advances in our understanding of TDNs influencing on the chondrocytes motility. The locomotion of chondrocytes was extremely enhanced treatment with the TDNs self-assembled from four specific designed single stranded DNA, showing a concentration-dependent manner in the range of 62.5-250 nmol L⁻¹. It is remarkable that the TDNs with the concentration of 250 nmol L⁻¹ strongly promoted chondrocytes motility and enhanced the expression of migration related genes

including *RhoA*, *ROCK2* and *vinculin*. It can be demonstrated that TDNs particularly facilitated the motility of chondrocytes mainly through the up-regulated the *RhoA/ROCK2* and *vinculin*. Tetrahedral DNA nanostructures might be promising DNA nanostructures applying to cartilage tissue engineering.

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REFERENCES

- Seeman NC. DNA in a material world. *Nature*. 2003;421:427-431.
- Mangraviti A, Tzeng SY, Kozielski KL, et al. Polymeric nanoparticles for nonviral gene therapy extend brain tumor survival in vivo. *ACS Nano*. 2015;9:1236-1249.
- Gong T, Xie J, Liao J, Zhang T, Lin S, Lin Y. Nanomaterials and bone regeneration. *Bone Res*. 2015;3:15029.
- Pinheiro AV, Han D, Shih WM, Yan H. Challenges and opportunities for structural DNA nanotechnology. *Nat Nanotechnol*. 2011;6:763-772.
- Lee H, Lytton-Jean AK, Chen Y, et al. Molecularly self-assembled nucleic acid nanoparticles for targeted in vivo siRNA delivery. *Nat Nanotechnol*. 2012;7:389-393.
- Smith D, Schüller V, Engst C, Rädler J, Liedl T. Nucleic acid nanostructures for biomedical applications. *Nanomedicine (Lond)*. 2013;8:105-121.
- Walsh AS, Yin H, Erben CM, Wood MJ, Turberfield AJ. DNA cage delivery to mammalian cells. *ACS Nano*. 2011;5:5427-5432.
- Hamblin GD, Carneiro KM, Fakhoury JF, Bujold KE, Sleiman HF. Rolling circle amplification-templated DNA nanotubes show increased stability and cell penetration ability. *J Am Chem Soc*. 2012;134:2888-2891.
- Keum JW, Ahn JH, Bermudez H. Design, assembly, and activity of antisense DNA nanostructures. *Small*. 2011;7:3529-3535.
- Chang M, Yang CS, Huang DM. Aptamer-conjugated DNA icosahedral nanoparticles as a carrier of doxorubicin for cancer therapy. *ACS Nano*. 2011;5:6156-6163.
- Lee JB, Hong J, Bonner DK, Poon Z, Hammond PT. Self-assembled RNA interference microsponges for efficient siRNA delivery. *Nat Mater*. 2012;11:316-322.
- Kim KR, Kim DR, Lee T, et al. Drug delivery by a self-assembled DNA tetrahedron for overcoming drug resistance in breast cancer cells. *Chem Commun (Camb)*. 2013;49:2010-2012.
- Jiang Q, Song C, Nangreave J, et al. DNA origami as a carrier for circumvention of drug resistance. *J Am Chem Soc*. 2012;134:13396-13403.
- Li J, Pei H, Zhu B, et al. Self-assembled multivalent DNA nanostructures for noninvasive intracellular delivery of immunostimulatory CpG oligonucleotides. *ACS Nano*. 2011;5:8783-8789.
- Mohri K, Nishikawa M, Takahashi N, et al. Design and development of nanosized DNA assemblies in polypod-like structures as efficient vehicles for immunostimulatory CpG motifs to immune cells. *ACS Nano*. 2012;6:5931-5940.
- Liu X, Xu Y, Yu T, et al. A DNA nanostructure platform for directed assembly of synthetic vaccines. *Nano Lett*. 2012;12:4254-4259.
- Goodman RP, Schaap IA, Tardin CF, et al. Rapid chiral assembly of rigid DNA building blocks for molecular nanofabrication. *Science*. 2005;310:1661-1665.
- Liang L, Li J, Li Q, et al. Single-particle tracking and modulation of cell entry pathways of a tetrahedral DNA nanostructure in live cells. *Angew Chem Int Ed Engl*. 2014;53:7745-7750.
- Miao P, Wang B, Chen X, Li X, Tang Y. Tetrahedral DNA nanostructure-based microRNA biosensor coupled with catalytic recycling of the analyte. *ACS Appl Mater Interfaces*. 2015;7:6238-6243.

20. Huang Y, Huang W, Chan L, Zhou B, Chen T. A multifunctional DNA origami as carrier of metal complexes to achieve enhanced tumoral delivery and nullified systemic toxicity. *Biomaterials*. 2016;103:183-196.
21. Schüttler KF, Struwer J, Rominger MB, Rexin P. Repair of a chondral defect using a cell free scaffold in a young patient—a case report of successful scaffold transformation and colonisation. *BMC Surg*. 2013;16:11.
22. Marmotti A, Bonasia DE, Bruzzone M, et al. Human cartilage fragments in a composite scaffold for single-stage cartilage repair: an in vitro study of the chondrocyte migration and the influence of TGF- β 1 and G-CSF. *Knee Surg Sports Traumatol Arthrosc*. 2013;21:1819-1833.
23. Bedi A, Feeley BT, Williams RJ. Management of articular cartilage defects of the knee. *J Bone Joint Surg Am*. 2010;92:994-1009.
24. Kouri JB, Jiménez SA, Quintero M, Chico A. Ultrastructural study of chondrocytes from fibrillated and non-fibrillated human osteoarthritic cartilage. *Osteoarthr Cartil*. 1996;4:111-125.
25. Morales TI. Chondrocyte moves: clever strategies? *Osteoarthr Cartil*. 2007;15:861-171.
26. Fu N, Liao J, Lin S, et al. PCL-PEG-PCL film promotes cartilage regeneration in vivo. *Cell Prolif*. 2016;49:729-739.
27. Liao J, Cai X, Tian T, et al. The fabrication of biomimetic biphasic CAN-PAC hydrogel with a seamless interfacial layer applied in osteochondral defect repair. *Bone Res*. 2017;5:17018.
28. Hoshiyama Y, Otsuki S, Oda S, et al. Chondrocyte clusters adjacent to sites of cartilage degeneration have characteristics of progenitor cells. *J Orthop Res*. 2015;33:548-555.
29. Zhong J, Guo B, Xie J, et al. Crosstalk between adipose-derived stem cells and chondrocytes: when growth factors matter. *Bone Res*. 2016;4:15036.
30. Shi S, Xie J, Zhong J, et al. Effects of low oxygen tension on gene profile of soluble growth factors in co-cultured adipose-derived stromal cells and chondrocytes. *Cell Prolif*. 2016;49:341-351.
31. Peng Q, Shao XR, Xie J, et al. Understanding the biomedical effects of the self-assembled tetrahedral DNA nanostructure on living cells. *ACS Appl Mater Interfaces*. 2016;8:12733-12739.
32. Shi S, Peng Q, Shao X, et al. Self-assembled tetrahedral DNA nanostructures promote adipose-derived stem cell migration via lncRNA XLOC 010623 and RHOA/ROCK2 signal pathway. *ACS Appl Mater Interfaces*. 2016;8:19353-19363.
33. Falchuk KH, Goetzl EJ, Kulka JP. Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. *Am J Med*. 1970;49:223-231.
34. Wakitani S, Goto T, Young RG, Mansour JM, Goldberg VM, Caplan AI. Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel. *Tissue Eng*. 1998;4:429-444.
35. Uddin SM, Richbrough B, Ding Y, et al. Chondro-protective effects of low intensity pulsed ultrasound. *Osteoarthr Cartil*. 2016;24:1989-1998.
36. Stefanowicz-Hajduk J, Adamska A, Bartoszewski R, Ochocka JR. Reuse of E-plate cell sensor arrays in the xCELLigence real-time cell analyzer. *Biotechniques*. 2016;61:117-122.
37. Kiosses WB, Daniels RH, Otey C, Bokoch GM, Schwartz MA. A role for p21-activated kinase in endothelial cell migration. *J Cell Biol*. 1999;147:831-844.
38. Tkach V, Bock E, Berezin V. The role of RhoA in the regulation of cell morphology and motility. *Cell Motil Cytoskeleton*. 2005;61:21-33.
39. Vega FM, Fruhwirth G, Ng T, Ridley AJ. RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. *J Cell Biol*. 2011;193:655-665.
40. Zhao L, Xu G, Zhou J, et al. The effect of RhoA on human umbilical vein endothelial cell migration and angiogenesis in vitro. *Oncol Rep*. 2006;15:1147-1152.
41. Zohrabian VM, Forzani B, Chau Z, Murali R, Jhanwar-Uniyal M. Rho/ROCK and MAPK signaling pathways are involved in glioblastoma cell migration and proliferation. *Anticancer Res*. 2009;29:119-123.
42. Zhang T, Gong T, Xie J, et al. Softening substrates promote chondrocytes phenotype via RhoA/ROCK pathway. *ACS Appl Mater Interfaces*. 2016;8:22884-22891.
43. Lämmermann T, Sixt M. Mechanical modes of 'amoeboid' cell migration. *Curr Opin Cell Biol*. 2009;21:636-644.
44. Benoit YD, Lussier C, Ducharme PA, et al. Integrin α 8 β 1 regulates adhesion, migration and proliferation of human intestinal crypt cells via a predominant RhoA/ROCK-dependent mechanism. *Biol Cell*. 2009;101:695-708.
45. Craig SW, Chen H. Lamellipodia protrusion: moving interactions of vinculin and Arp2/3. *Curr Biol*. 2003;13:236-238.
46. DeMali KA, Barlow CA, Burridge K. Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. *J Cell Biol*. 2002;159:881-891.
47. Zhang T, Xie J, Sun K, et al. Physiological oxygen tension modulates the soluble growth factor profile after crosstalk between chondrocytes and osteoblasts. *Cell Prolif*. 2016;49:122-33.
48. Shao X, Lin S, Peng Q, et al. Tetrahedral DNA nanostructure: a potential promoter for cartilage tissue regeneration via regulating chondrocyte phenotype and proliferation. *Small*. 2017;13:1602770.
49. Mierke CT, Kollmannsberger P, Zitterbart DP, et al. Vinculin facilitates cell invasion into three-dimensional collagen matrices. *J Biol Chem*. 2010;285:13121-13130.

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