Chaperonin containing T-complex polypeptide subunit eta is a potential marker of joint contracture: an experimental study in the rat

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Abstract Joint contracture is a fibroproliferative disorder that restricts joint mobility, resulting in tissue degeneration and deformity. However, the etiology of joint contracture is still unknown. Chaperonin containing T-complex polypeptide subunit eta (CCT-eta) is reported to increase in fibrotic diseases. The purpose of this study was to investigate whether CCT-eta is implicated in joint contracture and to determine the role of CCT-eta in the progression of joint contracture by analyzing a rat model. We immobilized the left knee joint of rat by internal fixation for 8 weeks. The non-immobilized right leg served as a control. The range of motion (ROM) of the knee was investigated. Fibroblasts were obtained from the posterior joint capsule of the joints. The outcome was followed by quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, fibroblast migration assay, and collagen assay. The effect of CCT-eta on the functions of fibroblasts was observed by utilizing a short inhibitory RNA (siRNA) targeting CCT-eta. The ROM of the immobilized joints was significantly limited compared to the contralateral joints ($p < 0.05$). Fibroblasts derived from the contractive joints showed higher mRNA and protein expressions of CCT-eta in parallel with alpha-smooth muscle actin (α -SMA)

compared to the cells from the contralateral knees ($p < 0.05$). siRNA-mediated downregulation of CCT-eta inhibited the expressions of both CCT-eta and α -SMA. Moreover, the reduction of CCT-eta also significantly decreased fibroblast functions such as cell mobility and collagen synthesis (all $p < 0.05$). Our findings indicate that CCT-eta appears to be a potential marker of joint contracture disease.

Keywords Immobilization · CCT-eta · Joint capsule · Fibrosis formation · Joint contracture

Introduction

Joint contracture is a fibroproliferative disorder that limits the range of motion (ROM) of a joint, and it can cause irreversible disability (Trudel et al. 2003). The contracture of a joint is frequently the result of longtime immobilization, which is the regular treatment of joint trauma including joint fracture or dislocation (Trudel and Uthoff 2000). Loss of joint motion diminishes activities of daily living and increases the burden of nursing care (Akeson et al. 1987). Although joint contracture can induce disability, hardly any appropriate strategies have been developed for the prevention or treatment of joint contracture due to its obscure biomechanism. The main treatment for joint contracture is excision of the fibrous tissue by arthroscopy or open surgery, but only 36 % of the patients achieve full functional recovery (Nwachukwu et al. 2011). Additionally, damage to the blood vessels and nerves is a serious complication of the surgery (Ghani et al. 2012). Thus, there remains an urgent need for a novel therapeutic target to address joint contracture.

Recently, the chaperonin containing T-complex polypeptide subunit eta (CCT-eta) was reported to be implicated in the progression of fibrous diseases. CCT is a cytosolic

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chaperonin composed of two stacked rings, and each ring comprises eight discrete polypeptide subunits (Kubota et al. 1995). Most studies on CCT function have focused on the holoenzyme, but there is increasing evidence that the different subunits of CCT have distinct functions. CCT-gamma mutation causes neurological defects in zebrafish (Matsuda and Mishina 2004). CCT-epsilon was found to bind the EBNA-3 nuclear protein from the Epstein-Barr virus (Hanafy et al. 2004). CCT-eta is a molecule believed to assist in the folding of cellular proteins, including cytoskeletal proteins such as actin and tubulin (Kubota et al. 1995; Hanafy et al. 2004). According to Satish et al., increased CCT-eta is observed in fibrous diseases such as Dupuytren's contracture (Satish et al. 2013) and skin contractive scars (Satish et al. 2010a), while the inhibition of CCT-eta results in decreased contractility in skin-derived fibroblasts (Satish et al. 2010a). The high expression level of CCT-eta from fibroblasts increases the stiffness of connective tissue, and this function is not correlated to any other CCT subunit (Satish et al. 2008, 2010a, b). These changes in contractive diseases caused by CCT-eta, but not other subunits of CCT, may result in structural changes that are thought to influence stiffness of the joints.

Since fibroblast proliferation and myofibroblast differentiation are the principal effectors for the progression of fibrous diseases, reduced contracture of joints may be achieved via inhibiting the functions of fibroblasts (Hildebrand et al. 2004; Verjee et al. 2010; Desmouliere et al. 2005). Myofibroblasts are differentiated from fibroblasts, and they are typically recognized by their characteristic expression of α -smooth muscle actin (α -SMA), which has been implicated in both cell motility and its ability to exert a deforming contractile force on surrounding tissues (Hinz 2007). Targeting molecules that enable myofibroblast function is therefore an attractive option to potentially mitigate the progression or recurrence of joint contracture at a cellular level.

The goal of this study was to investigate the effect of CCT-eta expression in fibroblasts from joint contracture in a rat model. In our study, the left knees of rats were immobilized by internal fixation for 8 weeks and were compared with the non-immobilized contralateral knees. Outcomes were followed by analyzing fibroblasts obtained from the posterior joint capsule of the joints. The effect of CCT-eta inhibition on the behaviors of fibroblasts from fibrotic joints was investigated by using a short inhibitory RNA (siRNA) versus CCT-eta. Cell migration, collagen synthesis, genes, and proteins expressions were then investigated.

Materials and methods

Animal experiments

This animal study was conducted by permission of the Institutional Animal Research Committee Approval of Sun Yat-sen

University. Skeletally mature Sprague-Dawley male rats aged 12 weeks, weighing approximately 180 ± 30 g, were obtained from the Animal Center for Medical Experiment of Guangdong. Ten rats were prepared for joint angle measurement and cell culture, respectively. Under 50 mg/kg sodium pentobarbital anesthesia, the left knee joints of rats were rigidly immobilized at 135° flexion with a polyformaldehyde plate (South China University of Technology) and two metal screws for 8 weeks (Hagiwara et al. 2009). The plastic plate was fixed with one screw to the distal tibia and another to the proximal femur (Fig. 1a). The fixed left knees could not move while the non-immobilized right knee joints were able to move freely (Fig. 1b). Trudel et al. reported that the contralateral non-immobilized knee did not have a restricted ROM (Trudel et al. 1999). Thus, the contralateral knee served as the control group. The animals were administered sodium salicylate (150 mg/kg) for pain control.

Joint angle measurements

At the end of the 8-week immobilization period, the rats were euthanized. Then, the plastic plate, screws, and skeletal muscles were removed from the joints (Fig. 1c, d). Joint ROM of the immobilized and contralateral knee was analyzed with an arthrometer adapted to the rat knee described by Trudel et al. (2000b). The arthrometer measured the joint

Fig. 1 **a** The left hind limb was immobilized by a polyformaldehyde plate, and the femur (F) and tibia (T) were fixed at an angle of approximately 45° . **b** Eight weeks after the immobilized surgery. The fixed knee could not be moved (red arrow), and the right knee joints served as the non-immobilized joints and were able to move freely. **c** Image of the contralateral joint after the skin and skeletal muscles were removed. **d** The plastic plate, screws, and skeletal muscles were removed from the immobilized joint after 8-week immobilization

angles reached at two torque values, 667 g/cm (torque 1) and 1060 g/cm (torque 2).

Cell culture

The posterior capsule tissues of ten rat knees were obtained as the method described by Lee et al. (Online Resource Fig. S1) (Lee et al. 2010), since the posterior capsule of the knee was the main anatomic factor for knee contracture (Trudel et al. 2008). Posterior capsule tissues were washed with minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA). Then, sub-sectioned tissues were placed in tissue culture plates containing a growth medium of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10 % fetal bovine serum (FBS; Invitrogen) to obtain fibroblasts (Buss et al. 2010). Fibroblasts were then incubated in a 5 % CO₂ incubator at 37 °C. In about 3–5 days, fibroblasts started to migrate from the sub-sections. Once the culture reached approximately 90 % confluence, the tissues were removed. The medium was changed every couple of days. Fibroblasts were then divided into four groups: cells derived from the contralateral knees (CF), cells from the immobilized knees (IF), IF cells treated with CCT-eta siRNA (IF-CS), and scramble siRNA (IF-SS).

Construction of siRNAs and transfections

Short interfering RNA (siRNA) was synthesized and purified by Forevergen Inc. (Guangzhou, China). The siRNA sequence targeting rat CCT-eta and a scrambled siRNA that served as a control are shown in Online Resource Table S1. Transfection of siRNAs was performed according to the manufacturer's protocol utilizing Lipofectamine 2000. Briefly, 1 µl of Lipofectamine 2000 was diluted into 50 µl of Opti-MEM; 50 µl of 100 mM siRNA was diluted with 1.25 µl of Opti-MEM and incubated for 5 min at room temperature. After the incubation, the diluted Lipofectamine 2000 was combined with the diluted siRNAs. After 20-min incubation, the siRNA-Lipofectamine 2000 complex was added to each well of cultured fibroblasts at 90 % confluence. After 24-h incubation at 37 °C, the fibroblasts were switched to growth medium for 48 h. After transfection, cell metabolic activity was determined using the thiazolyl blue tetrazolium bromide (MTT) assay (Shi et al. 2006). To investigate the specificity of CCT-eta downregulation in vitro, the fibroblasts were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot after transfection.

Transwell migration assay

The migratory ability of fibroblasts from CF, IF, IF-CS, and IF-SS groups was investigated by the transwell migration assay. Fibroblasts were cultured in DMEM media without FBS

for 12 h; then, cells were detached by trypsin and counted using a Scepter (based on the Coulter method) automated cell counter (Millipore, USA). For the transwell assay, 50,000 cells per 200 µl were seeded in the upper chamber of a transwell containing DMEM media without FBS. The lower chamber contained DMEM media with 10 % FBS served as an attractant. After incubation for 24 h at 37 °C with 5 % CO₂, the cells on the lower chamber were fixed with methanol and stained with crystal violet (Sigma, USA). The number of fibroblasts that migrated across the membrane was counted for five microscopic fields at ×100 magnification (Shelef et al. 2014).

Quantitative real-time polymerase chain reaction

The gene expressions of CCT-eta and α-SMA of fibroblasts from CF, IF, IF-CS, and IF-SS groups were characterized by qRT-PCR as described in a previous work from our group (He et al. 2015). After fibroblasts were cultured at 90 % confluence, mRNA extractions were conducted by an RNeasy Kit with QIAshredder (QIAGEN, Netherlands) according to the manufacturer's instructions. A NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) was used to investigate the mRNA concentration. cDNA synthesis was conducted with a Superscript Vilo cDNA Synthesis Kit (Invitrogen, USA) on an iCycler thermal cycler (Bio-Rad, USA). qRT-PCR was then performed utilizing an Express GreenER QPCR AB Kit (Invitrogen, USA) on a StepOne™ Plus real-time PCR system (Applied Biosystems). The relative expressions of the target genes were normalized to the reference gene GAPDH. The sequences of the primers used are summarized in Online Resource Table S2.

Western blots

Fibroblasts from CF, IF, IF-CS, and IF-SS groups were lysed with M-PER mammalian protein extraction reagent (ThermoScientific, USA), and the total protein concentration was determined by a Micro BCA™ Protein Assay kit (ThermoScientific). Equal amounts of protein from the different groups were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies specific for CCT-eta, CCT-beta and α-SMA. In particular, we employed CCT-beta as a control to investigate whether CCT-eta has the only marked change in the treated fibroblasts. GAPDH was used as a loading control. The signals were analyzed utilizing SuperSignal West Femto Trial Kit Prod (ThermoScientific).

Collagen assay and collagen type I staining

Fibroblasts from CF, IF, IF-CS, and IF-SS groups (50,000 cells per group) were cultured in growth medium for 5 days. The medium of each group was removed, and the extracellular

collagen produced by fibroblasts was extracted via incubation in pepsin solution (0.1 mg/mL in 0.5 M acetic acid) overnight at 4 °C (He et al. 2015). A SircolTM Collagen Assay Kit (Biocolor, UK) was used to quantify the extracted collagen. The collagen type I was also imaged after staining with a collagen type I antibody (Hinz et al. 2002): Briefly, fibroblasts were fixed by 4 % PFA and permeabilized with 0.1 % Triton X-100 solution. Then, cells were incubated in 2 % bovine serum albumin (BSA) at room temperature for 2 h. The rabbit anti-mouse collagen type I primary antibody (Millipore, USA) was employed, followed by the Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, USA) as a secondary antibody. The cells were stained with DAPI after the collagen staining process (Sigma, USA), then visualized utilizing confocal laser scanning microscope (CLSM, Olympus, Japan).

Statistical analysis

At least five samples for each experimental condition were analyzed. A one-way analysis of variance (ANOVA) was utilized to analyze the data. Significant difference was accepted at $p < 0.05$.

Results

Restricted total ROM of rat knee joints after immobilization

Ten rats were included in the measurement of total knee ROM, and neither infection nor severe edema was found in any animal. Importantly, the knee of normal Sprague Dawley rat does not reach full extension due to the knee flexion posture (Table 1). As shown in Table 1, the contracture was greater ($p < 0.05$) in the immobilized knees than the non-immobilized contralateral knees at both torques after 8-week immobilization. These results indicated that the joint contracture model in our study was successfully established.

Table 1 Range of motion in extension of rat knees^a

Groups	Number of Joints	Mean lack of knee extension $\pm$ SD (°)	
		Torque 1	Torque 2
Immobilized	10	55.8 $\pm$ 1.9*	44.3 $\pm$ 2.7*
Contralateral	10	12.5 $\pm$ 2.3	4.3 $\pm$ 1.9

SD standard deviation

*Indicates significant difference ($p < 0.05$) compared with the contralateral knee

^a Range of motion measurement correspond to the angle missing to reach complete extension (0°) at the knee; torque 1=667 g/cm, torque 2=1060 g/cm.

mRNA and protein expressions of CCT-eta and α -SMA were significantly increased in fibroblasts from the IF group when compared to the CF group

We examined both mRNA and protein levels of CCT-eta and α -SMA in fibroblasts of IF and CF groups. qRT-PCR indicated that CCT-eta and α -SMA mRNA expressions were significantly elevated by about twofold ($p < 0.05$) in the IF group when compared to the CF group (Fig. 2a, b.). Western blot also demonstrated that CCT-eta and α -SMA protein expressions were similarly increased in the IF group when compared to the CF group, while no difference was observed in CCT-beta protein expression (Fig. 2c).

siRNA targeting CCT-eta effectively inhibited the mRNA and protein expressions of α -SMA in fibroblasts from the IF group

We firstly employed siRNA interference to inhibit the mRNA and protein expression of CCT-eta in fibroblasts of the IF group. The mRNA expression of CCT-eta in fibroblasts from the IF-CS group decreased to 40 $\pm$ 2 % ($p < 0.05$, Fig. 3a), and the protein level decreased to 32.3 $\pm$ 3 % ($p < 0.05$, Fig. 3c) while there was no detectable effect on CCT-beta protein. These results indicated that the siRNA targeting CCT-eta effectively inhibited the expression of CCT-eta in fibroblasts from the IF group. Analysis of mRNA and protein expressions of α -SMA in fibroblasts from the IF-CS group revealed a statistically significant ($p < 0.05$) reduction to 37.5 $\pm$ 4 % (Fig. 3b) and 39.3 $\pm$ 2 % (Fig. 3c), respectively. Moreover, fibroblasts from the IF-SS group treated with scrambled siRNA had no detectable effect on the mRNA and protein expressions of both CCT-eta and α -SMA ($p > 0.05$, Fig. 3).

Fibroblasts from the IF group have significantly higher migratory ability than those from the CF group, while siRNA-mediated depletion of CCT-eta decreased the mobility of fibroblasts from the IF group

As shown in Fig. 4, fibroblasts from the IF group had a higher ($p < 0.05$) migratory ability compared to those from the CF group (Fig. 4a and c). Since we have confirmed that CCT-eta was specifically decreased in the IF-CS group, we then investigated whether selective inhibition of the CCT-eta might alter the migratory ability. In the transwell assay, fibroblast mobility in the IF-CS group decreased to 30 $\pm$ 2 % (Fig. 4, $p < 0.05$) when compared to the IF group, whereas the scrambled siRNA had no effect on cellular migration. In addition, MTT assay results showed that no significant inhibition of cell proliferation was observed for the cells in the IF, IF-SS, and IF-CS groups 24 h after siRNA transfection (Fig. S1).

Fig. 2 Relative gene expressions of **a** CCT-eta and **b** α -SMA by fibroblasts from CF and IF groups ($n=5$). **c** Expression of CCT-eta, CCT-beta, and α -SMA proteins in fibroblasts from CF and IF groups. Western blot analyses using antibodies specific for CCT-eta, CCT-beta, and α -SMA. GAPDH antibody was used as a loading control. A representative immunoblot is shown. The asterisk indicates $p<0.05$ compared with the CF group. *CF* cells derived from the contralateral knees, *IF* cells from the immobilized knees

Collagen synthesis of fibroblasts from the IF group was significantly increased when compared to cells from the CF group. siRNA-mediated reduction of CCT-eta decreased the collagen synthesis of fibroblasts from the IF group

Figure 5 qualitatively showed that the amount of collagen produced by fibroblasts of the CF and IF-CS groups was significantly reduced compared to the IF group. Quantitative results show that the amount of collagen produced by fibroblasts

of the CF and IF-CS groups was ~60 % lower than that produced by the IF group ($p<0.05$) (Fig. 5e). The scrambled siRNA did not have any effect on cellular migration when compared to the IF group.

Discussion

Although joint contracture after immobilization is a common complication, the mechanisms that regulate the formation of fibrous tissue around the joint are still obscure. Since

Fig. 3 Relative gene expressions of **a** CCT-eta and **b** α -SMA by fibroblasts from IF, IF-SS, and IF-CS groups ($n=5$). **c** Expression of CCT-eta, CCT-beta, and α -SMA proteins in fibroblasts from IF, IF-SS, and IF-CS groups ($n=5$). Western blot analyses using antibodies specific for CCT-eta, CCT-beta, and α -SMA. GAPDH antibody was used as a loading control. A representative immunoblot is shown. The asterisk indicates $p<0.05$ compared with the IF group. *CF* cells derived from the contralateral knees, *IF* cells from the immobilized knees, *IF-SS* IF cells treated with scramble siRNA, *IF-CS* IF cells treated with CCT-eta siRNA

Fig. 4 Fibroblasts derived from **a** CF, **b** IF, **c** IF-SS, and **(d)** IF-CS groups were allowed to migrate for 24 h across a transwell. Scale bar=500 μ m. **e** Quantification of the migrated cells per microscopic field at $\times 100$ magnification ($n=5$). The asterisk indicates $p < 0.05$ compared with the IF group. CF cells derived from the contralateral knees, IF cells from the immobilized knees, IF-SS IF cells treated with scramble siRNA, IF-CS IF cells treated with CCT-eta siRNA

fibroblasts are critical for fibrous disease, ultimately fibroblast physiology must be involved in the mechanisms of joint contracture. This is the first study to demonstrate how the fibroblast behaviors implicated in joint contracture are affected by the regulation of CCT-eta. In our study, CCT-eta mRNA and protein expression significantly increased in parallel with α -SMA in the fibroblasts from the immobilized joints. Other studies comparing cells derived from fibrous tissue and normal tissue showed consistent results. Increased CCT-eta was observed in fibrous diseases such as Dupuytren's contracture (Satish et al. 2010b) and skin wound scars (Hildebrand et al. 2004), and a similar coincident elevation in CCT-eta and α -SMA is found in corneal fibrosis (Koulikovska et al. 2005). For joint contracture, the

biomechanisms are quite different from other fibrous diseases. The pathological changes of joint contracture are characterized by joint capsule stiffness induced by decreasing water and hyaluronic acid, which are the main components of the joint capsule (Akeson et al. 1973, 1980). Since the posterior joint capsule is the main contributor in joint contracture (Trudel et al. 2008), we analyzed the fibroblasts to myofibroblasts differentiation by observing the level of α -SMA in the posterior joint capsule. α -SMA is the maker of myofibroblasts (He et al. 2015), which are differentiated from fibroblasts and responsible for fibrous diseases. The transformation of fibroblasts into myofibroblasts is a key point in the progression of joint contracture (Verjee et al. 2010; Serini et al. 1998). Therefore, the progression of joint contracture can be observed by the increased expression of α -SMA, and in our study, the increased CCT-eta may be an attractive target for molecular intervention in joint contracture. In addition, we employed CCT-beta as the appropriate control subunit for CCT-eta since CCT-beta is the subunit most closely evolutionarily related to CCT-eta (Satish et al. 2010a). The striking finding in our study indicates that CCT-eta, not CCT-beta, is the unique potential marker for active fibroblasts that is contributing to joint contracture.

We also observed that the siRNA-downregulation of CCT-eta simultaneously knocked down α -SMA expression, indicating that CCT-eta, not CCT-beta, is essential for α -SMA accumulation. α -SMA is an actin subtype, and it has to be folded to a correct and functional structure after protein translation (Koulikovska et al. 2005). This process may be regulated by CCT-eta, which can mediate the folding of unfolded actins to their functional structures (Kubota et al. 1995). On the other hand, according to Satish et al., siRNA-mediated CCT-eta inhibition suppressed the expression of α -SMA in wound scars and Dupuytren contracture by inhibiting the cellular response to growth factor stimulation (Satish et al. 2010a, b, 2013). However, the exact mechanism of the modulation of CCT-eta to α -SMA is still unclear and remains to be further investigated. Our results suggest that CCT-eta may be a potential marker of α -SMA in fibroblasts in joint contracture. Therefore, interference with CCT-eta, and thus α -SMA, would potentially prevent differentiation of fibroblasts into myofibroblasts and ultimately may prevent progression of clinically fibrotic disease.

Cell migration plays a critical role in fibrotic disease, metastasis, tissue morphogenesis, and angiogenesis (Satish et al. 2010a, b; Webb et al. 2005). Hildebrand et al. indicated that myofibroblasts migrated and

Fig. 5 CLSM images of stained collagen type I (green) generated by fibroblasts derived from **a** CF, **b** IF, **c** IF-SS, and **d** IF-CS groups after 5-day culture. Scale bar=500 μ m. **e** Quantification of collagen type I generated by fibroblasts ($n=5$). The asterisk indicates $p<0.05$ compared with the IF group. CF cells derived from the contralateral knees, IF cells from the immobilized knees, IF-SS IF cells treated with scrambled siRNA, IF-CS IF cells treated with CCT-eta siRNA

accumulated around the contractive joint capsule in patients with elbow contracture (Vi et al. 2010). During the procession of joint contracture, the migration of fibroblasts to the contractive capsule is indispensable. Utilizing a widely accepted cell transwell assay, we first investigated the migratory capability of fibroblasts from immobilized joints and in response to the reduction of CCT-eta. As shown in Fig. 4, fibroblasts with higher levels of both CCT-eta and α -SMA were clearly better able to migrate than the relatively CCT-eta- and α -SMA-deficient fibroblasts. Moreover, MTT assay results showed that no significant inhibition of cell proliferation was observed for the cells in the IF, IF-SS, and IF-CS groups 24 h after siRNA transfection (Fig. S1). These results indicated that the reason for the reduced cell migration in the IF-CS group was not the inhibition of cell proliferation. In fact, cellular motility has previously been well connected to α -SMA actin expression. Tsai et al. indicated that the increased α -SMA in tendon-derived fibroblasts resulted in a corresponding increase in fibroblast motility (Tsai et al. 2008). Conversely, treatment of fibroblasts reduced α -SMA expression with a correlated decrease in cell motility (Tsai et al. 2003). Our own findings indicated that fibroblast motility was decreased with an induced reduction of α -SMA by siRNA-mediated downregulation of CCT-eta.

Collagen type I is the critical component in fibrous tissue (Ivarsson et al. 1998), and the deposition of collagen by fibroblasts is responsible for the development of joint contracture (Lee et al. 2010). Figure 5 shows that the amount of collagen produced by fibroblasts

treated with CCT-eta siRNA was significantly reduced ($p<0.05$). Therefore, the collagen assay results in this study indicate that the reduction of CCT-eta may be a promising solution to combat joint contracture. As fibroblasts are the principle cells for collagen synthesis, the reason for the reduction of collagen deposition in IF-CS group may be due to the prevention of fibroblast differentiation to myofibroblasts caused by reduction of α -SMA. However, further work should be carried out to understand the exact mechanism of CCT-eta and collagen synthesis in joint contracture.

There are some limitations related to our study. The joint contracture model we investigated in this study mainly focused on joint immobilization. However, according to Kodde et al., joint contracture can be induced by the effect of a traumatic or surgical wound healing response in addition to joint immobilization (Kodde et al. 2013). Future work regarding in vivo inhibition of CCT-eta gene expression during forced immobilization or surgery would address this problem.

Conclusions

In a rat model of joint contracture, the expression of CCT-eta was significantly increased in parallel with α -SMA in fibroblasts derived from immobilized joints. siRNA-mediated depletion of CCT-eta effectively inhibited the expression of α -SMA as well as fibroblast functions such as migratory ability and collagen synthesis. Therefore, CCT-eta may be a potential marker of the progression of joint contracture.

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Conflict of interest The authors declare that they have no competing interests.

References

- Akeson WH, Woo SL, Amiel D, Coutts RD, Daniel D (1973) The connective tissue response to immobility: biochemical changes in periarticular connective tissue of the immobilized rabbit knee. *Clin Orthop* 93:356–362
- Akeson WH, Amiel D, Woo SL (1980) Immobility effects on synovial joints the pathomechanics of joint contracture. *Biorheology* 17:95–110
- Akeson WH, Amiel MF, Garfin SR, Woo SL (1987) Effects of immobilization on joints. *Clin Orthop Relat Res* 219:28–37
- Buss DG, Giuliano EA, Sharma A, Mohan RR (2010) Isolation and cultivation of equine corneal keratocytes, fibroblasts and myofibroblasts. *Vet Ophthalmol* 13(1):37–42
- Desmouliere A, Chaponnier C, Gabbiani G (2005) Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 13:7–12
- Ghani H, Maffulli N, Khanduja V (2012) Management of stiffness following total knee arthroplasty: a systematic review. *Knee* 19(6):751–759
- Hagiwara Y, Ando A, Chimoto E, Saijo Y, Ohmori-Matsuda K, Itoi E (2009) Changes of articular cartilage after immobilization in a rat knee contracture model. *J Orthop Res* 27(2):236–242
- Hanafy KA, Martin E, Murad F (2004) CCT-eta, a novel soluble guanylyl cyclase-interacting protein. *J Biol Chem* 279(45):46946–46953
- He RH, Hu XF, Tan HC, Feng J, Steffi C, Wang K, Wang W (2015) Surface modification of titanium with curcumin: a promising strategy to combat fibrous encapsulation. *J Mater Chem B* 3:2137–2146
- Hildebrand KA, Sutherland C, Zhang M (2004) Rabbit knee model of post-traumatic joint contractures: the long-term natural history of motion loss and myofibroblasts. *J Orthop Res* 22:313–320
- Hinz B (2007) Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127:526–537
- Hinz B, Gabbiani G, Chaponnier C (2002) The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. *J Cell Biol* 157:657–663
- Ivarsson M, McWhirter A, Borg TK, Rubin K (1998) Type I collagen synthesis in cultured human fibroblasts: regulation by cell spreading, platelet-derived growth factor and interactions with collagen fibers. *Matrix Biol* 16:409–425
- Kodde IF, van Rijn J, van den Bekerom MP, Eygendaal D (2013) Surgical treatment of post-traumatic elbow stiffness: a systematic review. *J Shoulder Elb Surg* 22(4):574–580
- Koulikovska M, Podskochoy A, Fagerholm P (2005) The expression pattern of the subunit of chaperonin containing T-complex polypeptide 1 and its substrate, alpha-smooth muscle actin, during corneal wound healing. *Acta Ophthalmol Scand* 83:543–548
- Kubota H, Hynes G, Willison K (1995) The chaperonin containing T-complex polypeptide 1 (TCP-1). Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. *Eur J Biochem* 230:3–16, **Review**
- Lee S, Sakurai T, Ohsako M, Saura R, Hatta H, Atomi Y (2010) Tissue stiffness induced by prolonged immobilization of the rat knee joint and relevance of AGEs (pentosidine). *Connect Tissue Res* 51(6):467–477
- Matsuda N, Mishina M (2004) Identification of chaperonin CCT gamma subunit as a determinant of retinotectal development by whole-genome subtraction cloning from zebrafish no tectal neuron mutant. *Development* 131:1913–1925
- Nwachukwu BU et al (2011) Arthrofibrosis after anterior cruciate ligament reconstruction in children and adolescents. *J Pediatr Orthop* 31(8):811–817
- Satish L, Abdulally A, Oswald D, Johnson S, Hu FZ, Post JC, Ehrlich GD, Kathju S (2008) Differential expression of chaperonin containing T-complex polypeptide (CCT) subunits during fetal and adult skin wound healing. *Cell Stress Chaperones* 13:527–533
- Satish L, Johnson S, Wang JH, Post JC, Ehrlich GD, Kathju S (2010a) Chaperonin containing T-complex polypeptide subunit eta (CCT-eta) is a specific regulator of fibroblast motility and contractility. *PLoS One* 5(4), e10063
- Satish L, Johnson S, Abdulally A, Post JC, Ehrlich GD, Kathju S (2010b) Cloning and expression of rabbit CCT subunits eta and beta in healing cutaneous wounds. *Cell Stress Chaperones* 15(6):819–826
- Satish L, O’Gorman DB, Johnson S, Raykha C, Gan BS, Wang JH, Kathju S (2013) Increased CCT-eta expression is a marker of latent and active disease and a modulator of fibroblast contractility in Dupuytren’s contracture. *Cell Stress Chaperones* 18(4):397–404
- Serini G et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J Cell Biol* 142:873–881
- Shelef MA et al (2014) Focal adhesion kinase is required for synovial fibroblast invasion, but not murine inflammatory arthritis. *Arthritis Res Ther* 16(5):464
- Shi Z, Neoh KG, Zhong SP, Yung LY, Kang ET, Wang W (2006) In vitro antibacterial and cytotoxicity assay of multilayered polyelectrolyte-functionalized stainless steel. *J Biomed Mater Res A* 76(4):826–834
- Trudel G, Uthoff HK (2000) Contractures secondary to immobility: is the restriction articular or muscular? An experimental longitudinal study in the rat knee. *Arch Phys Med Rehabil* 81:6–13
- Trudel G, Uthoff HK, Brown M (1999) Extent and direction of joint motion limitation after prolonged immobility: an experimental study in the rat. *Arch Phys Med Rehabil* 80:1542–1547
- Trudel G, O’Neill PA, Goudreau LA (2000) A mechanical arthrometer to measure knee joint contracture in rats. *IEEE Trans Rehabil Eng* 8:149–155
- Trudel G, Jabi M, Uthoff HK (2003) Localized and adaptive synovial cell proliferation characteristics in rat knee joint contractures secondary to immobility. *Arch Phys Med Rehabil* 84:1350–1356
- Trudel G, Zhou J, Uthoff HK, Laneuville O (2008) Four weeks of mobility after 8 weeks of immobility fails to restore normal motion: a preliminary study. *Clin Orthop Relat Res* 466:1239–1244
- Tsai WC, Tang FT, Wong MK, Pang JH (2003) Inhibition of tendon cell migration by dexamethasone is correlated with reduced alpha-smooth muscle actin gene expression: a potential mechanism of delayed tendon healing. *J Orthop Res* 21:265–271
- Tsai WC et al (2008) Therapeutic ultrasound stimulation of tendon cell migration. *Connect Tissue Res* 49:367–373
- Verjee LS, Midwood K, Davidson D, Eastwood M, Nanchahal J (2010) Post-transcriptional regulation of alpha-smooth muscle actin determines the contractile phenotype of Dupuytren’s nodular cells. *J Cell Physiol* 224:681–690
- Vi L, Gan BS, O’Gorman DB (2010) The potential roles of cell migration and extra-cellular matrix interactions in Dupuytren’s disease progression and recurrence. *Med Hypotheses* 74(3):510–512
- Webb DJ, Zhang H, Horwitz AF (2005) Cell migration: an overview. *Methods Mol Biol* 294:3–11