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Trend of telomerase activity change during human iPSC self-renewal and differentiation revealed by a quartz crystal microbalance based assay

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Telomerase plays an important role in governing the life span of cells for its capacity to extend telomeres. As high activity of telomerase has been found in stem cells and cancer cells specifically, various methods have been developed for the evaluation of telomerase activity. To overcome the time-consuming procedures and complicated manipulations of existing methods, we developed a novel method named Telomeric Repeat Elongation Assay based on Quartz crystal microbalance (TREAQ) to monitor telomerase activity during the self-renewal and differentiation of human induced pluripotent stem cells (hiPSCs). TREAQ results indicated hiPSCs possess invariable telomerase activity for 11 passages on Matrigel and a steady decline of telomerase activity when differentiated for different periods, which is confirmed with existing golden standard method. The pluripotency of hiPSCs during differentiation could be estimated through monitoring telomerase activity and compared with the expression levels of markers of pluripotency gene via quantitative real time PCR. Regular assessment for factors associated with pluripotency or stemness was expensive and requires excessive sample consuming, thus TREAQ could be a promising alternative technology for routine monitoring of telomerase activity and estimate the pluripotency of stem cells.

Human telomeres are specialized tandem repeats of a TTAGGG sequence locating at the terminal of chromosomes and maintain the stability of genome. The progressive shortening of telomere during cell replication leads to cell senescence[1,2]. Telomerase is a ribonucleoprotein complex that mainly consists of telomerase reverse transcriptase (TERT) and telomerase RNA (TERC)[3–5]. It adds repeats sequence to the end of the chromosome by using TERC as a template, so as to keep the telomere length stable and alleviate cell senescence[6].

Previous evidence has shown that telomerase activity in cancer cells and stem cells is higher than that in somatic cells[7]. Human induced pluripotent stem cells (hiPSCs) with a high differentiation potential provides a new source for regenerative medicine and it shares comparative high-level telomerase activity with human embryonic stem cells (hESCs)[8]. Recently, hiPSCs is emerging as an important new tool to be applied in drug screening and treatment of human diseases[9–11]. Together, these results prompt the critical importance of telomerase activity. Further, correlational analyses have been conducted between telomerase activity and cell differentiation of ESCs, mesenchymal stem cells (MSCs) and cancer cells, but not iPSCs[12–14]. This situation leads us to monitor the telomerase activity during self-renewal and differentiation of hiPSCs.

Conventional telomerase activity detection method was originally established by Kim et al. in 1994, which called telomerase repeat amplification protocol (TRAP)[7]. As this polymerase chain reaction (PCR)-based method was frequently used, some novel alternative methods have been developed to overcome certain shortcomings of TRAP[15–27]. Several of them had been widely used and commercially available. These approaches provided us various methods for the detection of telomerase activity. Nevertheless, most of them had its shortcomings including time-consuming procedures, complicated manipulations and the need for labels and elaborate instruments (see Supplementary Table S1 online). Also, the features of high-throughput ability and ease of automation

were always been ignored. Telomerase researches on basic life science and biomedical research need a more simple and reliable method for measuring telomerase, which is expected to create new opportunities in this research field.

Quartz crystal microbalance (QCM), a well-studied instrument, is ideally suited for in situ characterization study and has been applied to monitor bimolecular interactions at the solid-liquid interface, such as antigen-antibody recognition, DNA-protein interaction and DNA hybridization[28–30]. Highly sensitive QCM is also used for the study of DNA-related enzyme reactions[31,32]. Our group have engaged in QCM study for a long time and proposed a "solidified liquid layer" (SLL) model that made it feasible for quantitative analyzing of frequency change in liquid[33,34]. We also developed a DNA methylation detection method base on QCM and applied to evaluate the effect of 5-aza-2′-deoxycytidine (decitabine), a methyltransferase inhibitor used in clinical treatment[35].

In this study, we introduce TREAQ detection, a rapid and PCR-free method, easily to detect telomerase activity. By mimicking telomere elongation in vivo, we can monitor the behavior of telomerase extending the primer in real time on different surfaces (gold chips and polymer-coated chips) by F_T (the absolute value of QCM resonance frequency shift (Δf) caused by extension of primers), which mirrors the activity of telomerase (Fig. 1A). Our results suggest that TREAQ is a helpful tool to rapidly carry out high-throughput detections of telomerase activity (using off-line detection method introduced in Results section) and has wide application prospects in life science and biomedical research.

Results

Immobilization of Oligonucleotides Primers. Biotin-avidin detection system was applied to ensure the immobilization of oligonucleotides primer on polymer-coated QCM chips, as schematically shown in Fig. 2A. The first step was the binding of 3′ biotin modified primer on the polymer surface by amide linkage, consistent with the immobilization of Primer (1)/(2) as indicated in Methods section. The second step was the binding of avidin-HRP to the biotin modified primer. With the coverage of Luminol, the final images were presented, as shown in Fig. 2C for chemiluminescence. Evidently, image with the binding of biotin modified primer (left) owned high rate of luminous intensity, compared with the control group (right), which confirmed the immobilization of oligonucleotides primer (Fig. 2C). SYBR Green II stain was used as another approach to detect single-stranded primer immobilized on polymer-coated QCM chips (Fig. 2B). Amido modified primers were first immobilized on the polymer surface and SYBR Green II then bind to the primers and fluoresces. The results showed the significant differences between chips with primers (left panel, Fig. 2C) and chips without primers (right panel, Fig. 2C). Thus, the effectiveness of the immobilization strategy was confirmed.

TREAQ measurement of HeLa Cells. We first used common cancer cell line HeLa as model to estimate a linear dynamic range between the telomerase-containing cell lysate concentration and the Δf value of TREAQ (F_T). As shown in Figure. 3A, F_T of HeLa lysates (ranging from 0.1 g/L to 0.6 g/L) was steadily changing with the concentration increased. An equal volume of 1 × CHAPS Lysis buffer was also measured by TREAQ as a control. We found a linear relationship between F_T and the cell lysate concentration indicating that QCM was able to quantitatively measure telomerase activity (Fig. 3B). For cell lysate (active telomerase) treated by heat inactivation (90°C for 30 min), a F_T tending to zero indicated eliminated telomerase activity (Fig. 3C). The same samples from HeLa cell lines are measured with a standard TRAP assay for verification (see Supplementary Fig. S1 online). Also, the relationship between different cell lysate concentrations and Total Product Generated (TPG) was investigated for the comparison with traditional methods (see Supplementary Fig. S2 online).

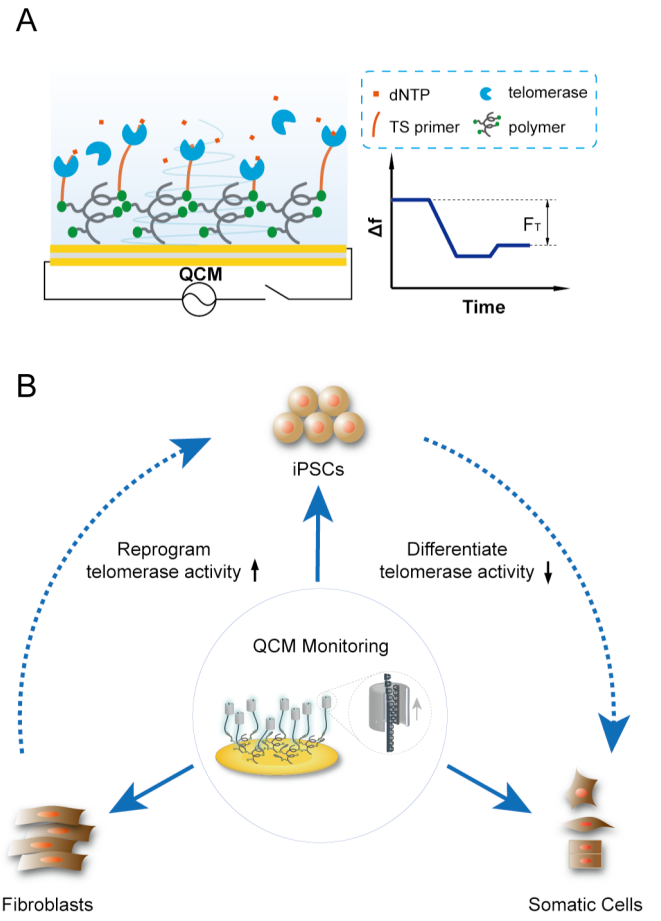

Figure 1 | Illustration of TREAQ on polymer-coated chips. (A) Amido modified primers were immobilized on the polymer surface and extended by telomerase in reaction buffer. (B) The schematic of monitoring of telomerase activity during the self-renewal and differentiation of hiPSCs by QCM.

Monitoring telomerase activity during hiPSCs self-renewal and differentiation. An off-line telomerase activity detection is a method that recording frequency value only at the beginning and the end of QCM process. It means real-time consecutive QCM curve for displaying primers extension is not required. Thus, high-throughput TREAQ is feasible based on this off-line system. With suitable instruments, high-throughput TREAQ will provide us a more convenient way not only for the detection of telomerase activity but also for more applications like drug screening. With the polymer-coated QCM chip immobilized by oligonucleotides primers on its surface and placed in an open-top reaction chamber, we use the off-line TREAQ, as indicated above, to detect the human fibroblasts, HNF1-4C11 hiPSCs and differentiated HNF1-4C11 hiPSCs. As shown in Figure 4, about 36 Hz frequency shift triggered by extension of primers was detected by QCM, which indicated low telomerase activity of human fibroblasts. Evidently increasing frequency of hiPSCs indicated up-regulation of telomerase activity during reprogramming, in accordance with telomere elongation mediated by telomerase in the meantime[36]. High-level telomerase activity of hNF1-4C11 hiPSCs (F_T between 70 Hz and 80 Hz) was maintained for 11 passages according to TREAQ. However, progressively decrease of frequency after differentiated for various periods (F_T: 1 day for about 38 Hz, 3 days for about 29 Hz and 5 days for about 16 Hz) indicated progressively down-regulation of

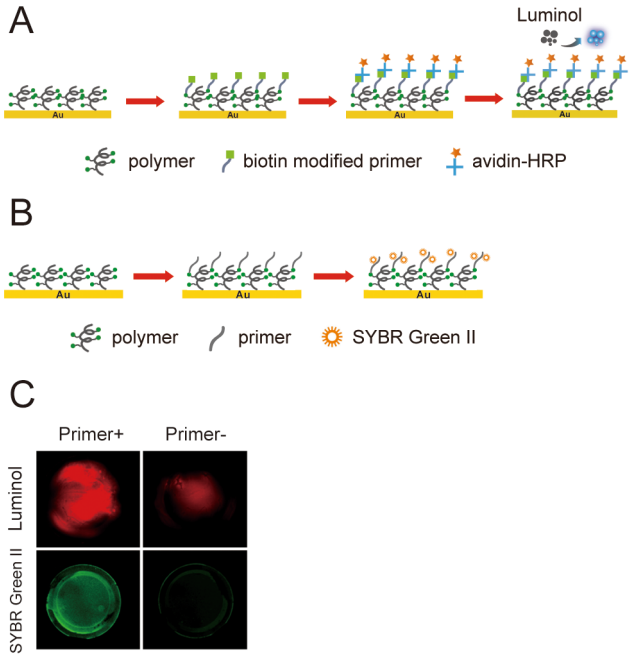

Figure 2 | Oligonucleotides Primers Immobilization and Verification. (A) Three steps immobilization of oligonucleotides primers on polymer-coated QCM chips. Step 1: immobilization of biotin modified primers, step 2: immobilization of avidin-HRP, step 3: chemiluminescence. (B) Two steps immobilization of oligonucleotides primers on polymer-coated QCM chips. Step 1: immobilization of amido modified primer, step 2: immobilization of SYBR Green II. (C) Coloration results for (A) and (B).

telomerase activity (Fig. 4). Morphological characteristics of hNF1-4C11 hiPSCs differentiation were shown in Figure 5.

Demonstration of compatibility of TREAQ to other cell lines. As human Mesenchymal Stem Cells (MSCs) and human Osteosarcoma cells MG63 were both cell types existing in human bone, we detected their telomerase activity by TREAQ. F_T of both measurements in Figure 6, MSC for about 53 Hz and MG63 for about 117 Hz, indicated the higher telomerase activity of human Osteosarcoma cells MG63 compared with human MSCs, which verified the higher telomerase activity of cancer cells compared with MSCs[37].

Expression analysis of pluripotency factors. Our quantitative RT-PCR data quantified the Oct4, Nanog, Sox2 and Rex-1 transcripts relative to the ACTB housekeeping gene in differentiated hiPSCs. As shown in Figure 7, Oct-4 and Sox2 expression significantly decreased during differentiation. On the other hand, flow cytometric results indicated invariable Oct4 expression during the self-renewal of hiPSCs (Fig. 8).

Discussion

In this paper, we have established an easy handling method for the detection of telomerase activity based on QCM. As mentioned above, telomerase activity is calculated by the frequency decrease of QCM signals, labeling or PCR-related artifacts and complicated manipulation are not required. Thus, TREAQ can be considered as another form of telomeric repeat elongation (TRE) assay[19]. Some control experiments were conducted on gold chips for the evidence of telomerase binding (see Supplementary table S2 online). We also demonstrated that TREAQ based on polymer-coated chips is superior to that on gold chips (see Supplementary Fig. S3 online).

Significantly, this method can give the information of the length of elongated-primer molecule, which most current methodologies could not measure. We can apply SLL model to calculate the DNA

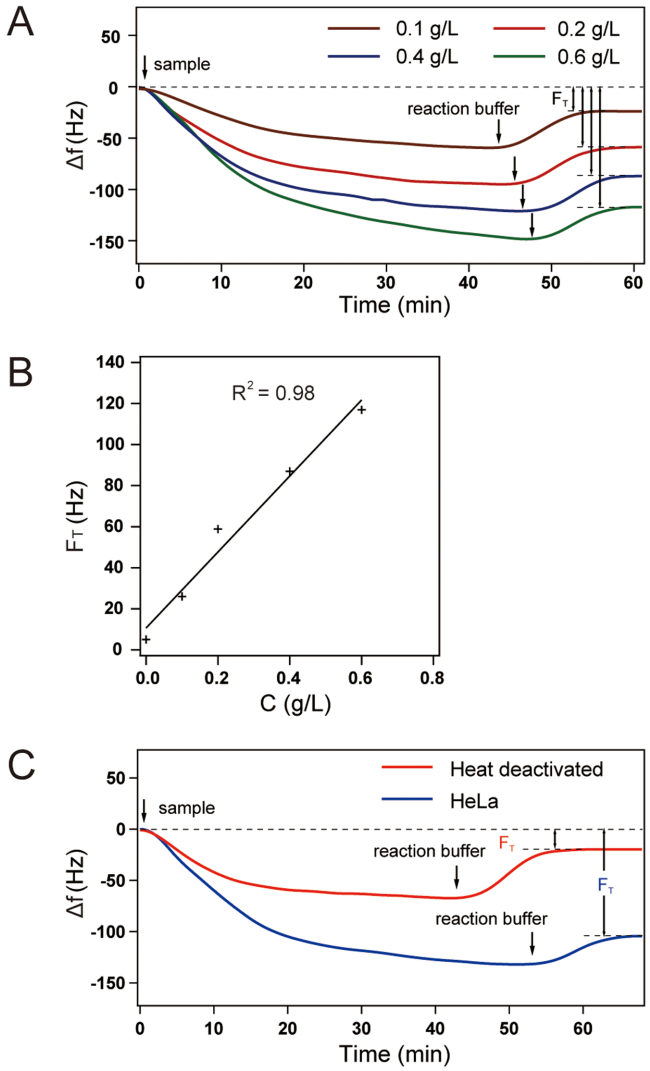

Figure 3 | TREAQ measurement of HeLa Cells. (A) F_T for HeLa cells at four different total Protein Concentrations. (B) A linear relationship ($y = 185.22x + 10.6$) between F_T and the cell lysate concentration. (C) F_T for HeLa cells on polymer surface. Samples which were heated in 90°C for 30 min were used as negative control.

extended primers layer thickness (DNA length) in the telomerase reaction buffer. If the vertical distance between adjacent bases planes is 0.34 nm, we can later gain how many DNA bases the telomerase has extended at the end of DNA primers (see Supplementary for the detailed calculation process). However, there are several reasons that make these calculations less accurate: (1) Structure difference between ssDNA and dsDNA exists, distance of the vertical base plane would not be exactly 0.34 nm. (2) The flexibility of ssDNA chain would be much stronger than the dsDNA, the persistence length of ssDNA is about 3 nm, much shorter than the latter[38]. Tinland et al. found that for single-stranded DNA in high salt concentrations, the persistent distance of ssDNA–the rigid length of polymer segments, lies between 8 and 13 Å[39]. Smith et al. studied ssDNA mechanical property with laser tweezers and found the persistence distance of ssDNA is 7.5 Å[40]. In our experiment, the DNA on the surface is in some extent coiled and twisted states, not rigid in the right vertical direction, so the calculated result of length of extended-DNA bases is a rough assessment.

This application of QCM provides us a novel approach to study the telomerase activity of various types of cells, including cancer cells and hiPSCs. Since the discovery of how chromosomes were protected

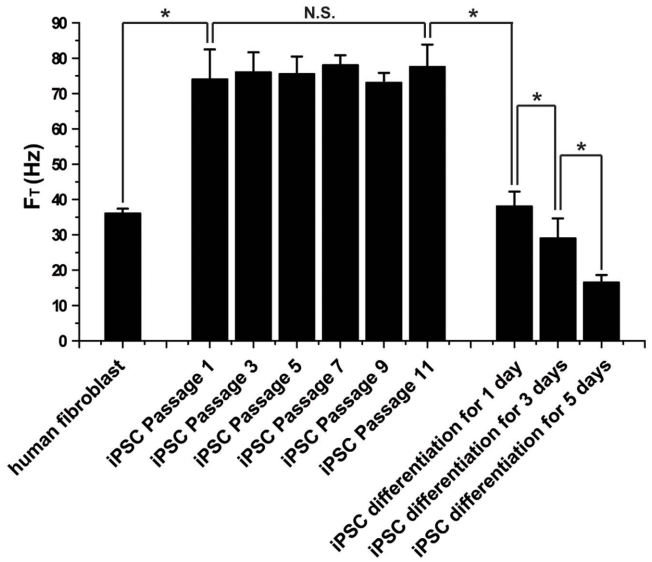

Figure 4 | F_T for human fibroblasts, 6 self-renewal passages and differentiation of hNF1-4C11 hiPSCs. The data are presented as mean ± SD. (n = 4/human fibroblast; 3/different passages; 3/differentiation). *P < 0.05 as determined by a Student's t-test.

by telomerase, critical importance should be self-evident as it has opened a promising avenue in early diagnosis and anti-cancer therapy[4,7,10]. But the clinical implementation of telomerase-based therapeutic strategies is proceeding at a pace slower than was probably expected. Questions like "Can we select patients with a higher likelihood of responding to this novel anticancer (telomerase-targeted) approach?" are always been asked, which stressed the importance of relationship between the type of carcinoma and telomerase activity[4].

Furthermore, stem cell pluripotency steps down during the cell differentiation prompts us to find the relationship between pluripotency and telomerase activity during iPSC differentiation, coupling with the recent report which summarized that telomerase was believed to act as a "stemness" enzyme[41]. In the present study, high telomerase activity and pluripotency of hiPSCs are gradually decreasing steadily and simultaneously in the process of differentiation. Through TREAQ, it was observed that telomerase activity was positively correlated with pluripotency during the self-renewal and differentiation of hiPSCs. Indeed, telomerase activity of somatic cell experienced a process from increase to decrease during iPSC induction and differentiation, along with pluripotency changing (Fig. 1B). As the consistent high expression of pluripotency factors and telomerase activity had been reported in human stem cell lines[42,43], as well as the same trends were discovered between factors like Oct-4 and telomerase activity in long-term cultures of hESCs[44,45], the quantitative RT-PCR and flow cytometric results of pluripotency factors (Oct-4, etc) also supported our presumption (Fig. 7, Fig. 8). That means we can roughly estimate the extent of pluripotency at a certain stage during differentiation through monitoring telomerase activity of hiPSCs. Regular assessment for those factors associated with pluripotency or stemness is expensive and requires excess sample consumption, thus TREAQ could be a promising alternative technology.

In summary, we have demonstrated that TREAQ, a label-free method which is superior to the existing detection method based on QCM[46], can be applied in monitoring telomerase activity during the self-renewal and differentiation of hiPSCs. Furthermore, as telomerase is also over-expressed in more than 85% of cancer cells, which makes it a universal cancer marker[4,7,47], the TREAQ could be applied for the detection of telomerase activity in tumor therapy and regenerative medicine as a novel tool, because of its simplicity and rapidity.

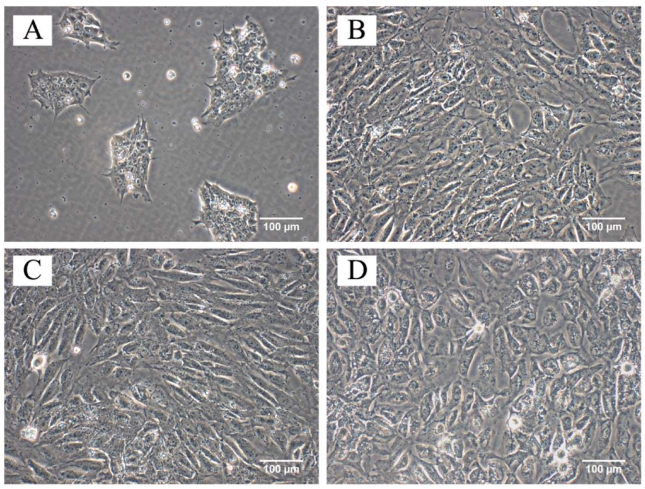

Figure 5 | Morphological characteristics of hNF1-4C11 hiPSCs differentiation. (A) Undifferentiated hNF1-4C11 hiPSCs. (B) Morphological characteristics of hNF1-4C11 hiPSCs after differentiation for 1 day. hiPSCs became scattered and irregular. (C) Morphological characteristics of hNF1-4C11 hiPSCs after differentiation for 3 days. The cellular outlines were becoming vague. (D) Morphological characteristics of hNF1-4C11 hiPSCs after differentiation for 5 days. Significant changes in morphology and integrality of hiPSCs indicated high degree of cellular differentiation.

Methods

Materials. SYBR GREEN II, DNA marker, dNTP mixture were obtained from Fermentas (Massachusetts, USA). The oligonucleotides primer functionalized with a thiol group was synthesized by Invitrogen Biotechnology (Shanghai, China).

Preparation of QCM chips. The gold and polymer-coated QCM chips (AT cut, 5 MHz) were purchased from Dongwei Biotech (Hangzhou, China) after the parameter were determined from previous studies[48]. UV/Ozone cleaner (Uvotech Systems, CA, USA) was used to clean the gold surface for 30 minutes. Then the gold QCM chips were washed by Milli-Q water, ethanol and dried under nitrogen gas flow. For the polymer-coated QCM chips, an aqueous mixture of N-ethyl-N′-[3-dimethylaminopropy]carbodiimide hydrochloride (EDC, 0.1 M) and hydroxy-2,5-dioxopyrolidine-3-sulfonicacid sodium salt (NHSS, 0.2 M) was used to activate the carboxyl groups, which were modified at the end of polymers so as to immobilize the primer through the amino-carboxyl reaction. Afterwards, the chips were washed by ultrapure water (resistivity > 18 MΩ cm^{-1}, Milli-Q) and dried under nitrogen gas flow.

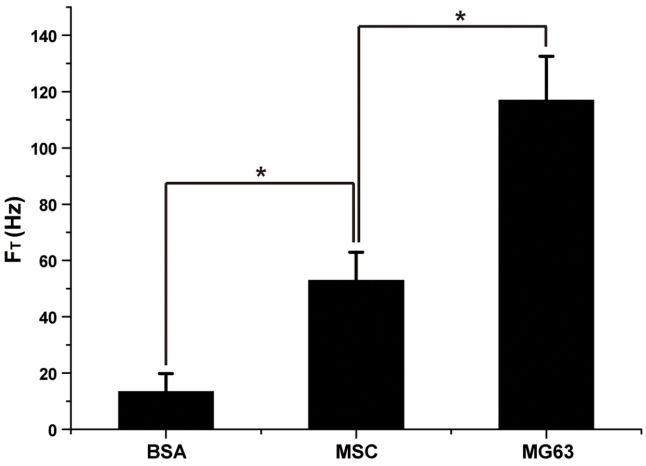

Figure 6 | F_T for MSCs and human Osteosarcoma cells MG63. Albumin from Bovine Serum (BSA, 0.5 g/L) was used as a control. The data are presented as mean ± SD. (n = 5/BSA; 3/MSC; 3/MG63). *P < 0.05 as determined by a Student's t-test.

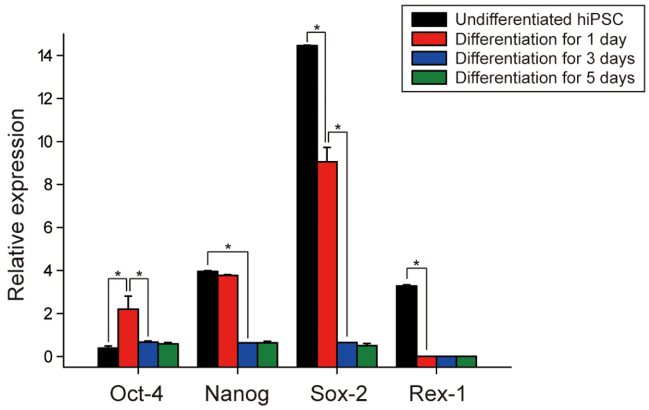

Figure 7 | Quantitative Real-time RT-PCR analysis of pluripotency factors. Oct4, Nanog, Sox2 and Rex-1 transcripts of hNF1-4C11 hiPSCs were detected by quantitative real-time RT-PCR. Transcripts of ACTB were used as internal control. The data are presented as mean ± SD. (n = 3/undifferentiated hiPSC; 4/differentiation for 1, 3, 5 days). *P < 0.05 as determined by a Student's t-test.

Immobilization of oligonucleotides primer. Primers were immobilized on gold substrate or polymer substrate through Au-S bond or amido bond respectively. The sequences of the oligonucleotides primers we used are as follows: 5′-$HS(CH_2)_6$TTTTTTAATCCGTCGAGCAGAGTT-3′ (1), 5′-$NH_2(CH_2)_6$TTTTTTTTTTAATCCGTCGAGCAGAGTT-3′ (2). 8 μL 100 μM Primer (1) and Primer (2) were respectively introduced into two copies of the same 200 μL mixture of 0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 0.5 M NaCl, pH 7.4. These two kinds of reaction buffer were placed on the surface of gold and polymer-coated chips respectively in single face reactor for 3 hours at room temperature. Afterwards, the chips were washed by Milli-Q water and dried under nitrogen gas flow. Milli-Q water with a resistivity of 18.2 MΩ cm^{-1} was used for the solution preparation.

Cultures of HeLa, MG63 and human fibroblast cells. The human cervical cancer cell line HeLa, human Osteosarcoma cells MG63 and human fibroblast cell line were provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). HeLa, MG63 and human fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin. Those reagents all were purchased from Hyclone (Logan, USA). Cells were incubated at 37°C in a humidified 5% (v/v) CO_2 incubator (HERAcell 150i, Germany).

Cultures of hiPSCs. hNF1-4C11 hiPSCs generated from human skin fibroblasts by introducing four classical factors, Oct-4, Sox-2, c-Myc and Klf-4 (provided from Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, China). Cells were cultured on Matrigel™ (BD Biosciences, New Jersey, USA) coated 6-well plates using chemically defined mTeSR™1 media (StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions. The culture medium was changed with fresh every day. hNF1-4C11 hiPSCs were passaged to the next generation at 1 : 3 splitting ratio every 3–4 days by exposure to 0.5 mM EDTA for 4–5 min at 37°C. The cells were passaged to the 11th generation. Every two generations for hiPSCs, the telomerase activity was measured by TREAQ detection and compared with the expression of Oct-4.

hiPSCs differentiation. hNF1-4C11 hiPSCs were cultured on Matrigel™ coated 6-well plates for two days, then mTeSR™1 media was changed with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin for differentiation. The medium was changed with fresh every day. After differentiated for various periods (1 days, 3 days and 5 days), the TREAQ detection and quantitative RT-PCR for hiPSCs were performed.

Preparation of cell extracts. Resuspend the cell pellet in 150 μL of 1 × CHAPS Lysis Buffer (Millipore) with RNase inhibitor (100–200 units/mL for the final concentration)/10^6 cells and incubate the suspension on ice for 30 minutes. Spin the sample in a micro-centrifuge at 12000 × g for 20 minutes at 4°C. Transfer the supernatant into a fresh tube and prepare for the QCM measurements. Protein concentration of all the cell extracts samples were determined by Nanodrop 2000c from Thermo and were demonstrated to be consistent (0.5 g/L).

TREAQ detection of telomerase activity. In order to avoid the nonspecific protein adsorption (NPA) on gold QCM chips, we used EG_3 (2,5,8,11-tetraoxadocosane-22-thiol) as blocking agent, which contains sulfydryl and can be anchored on the

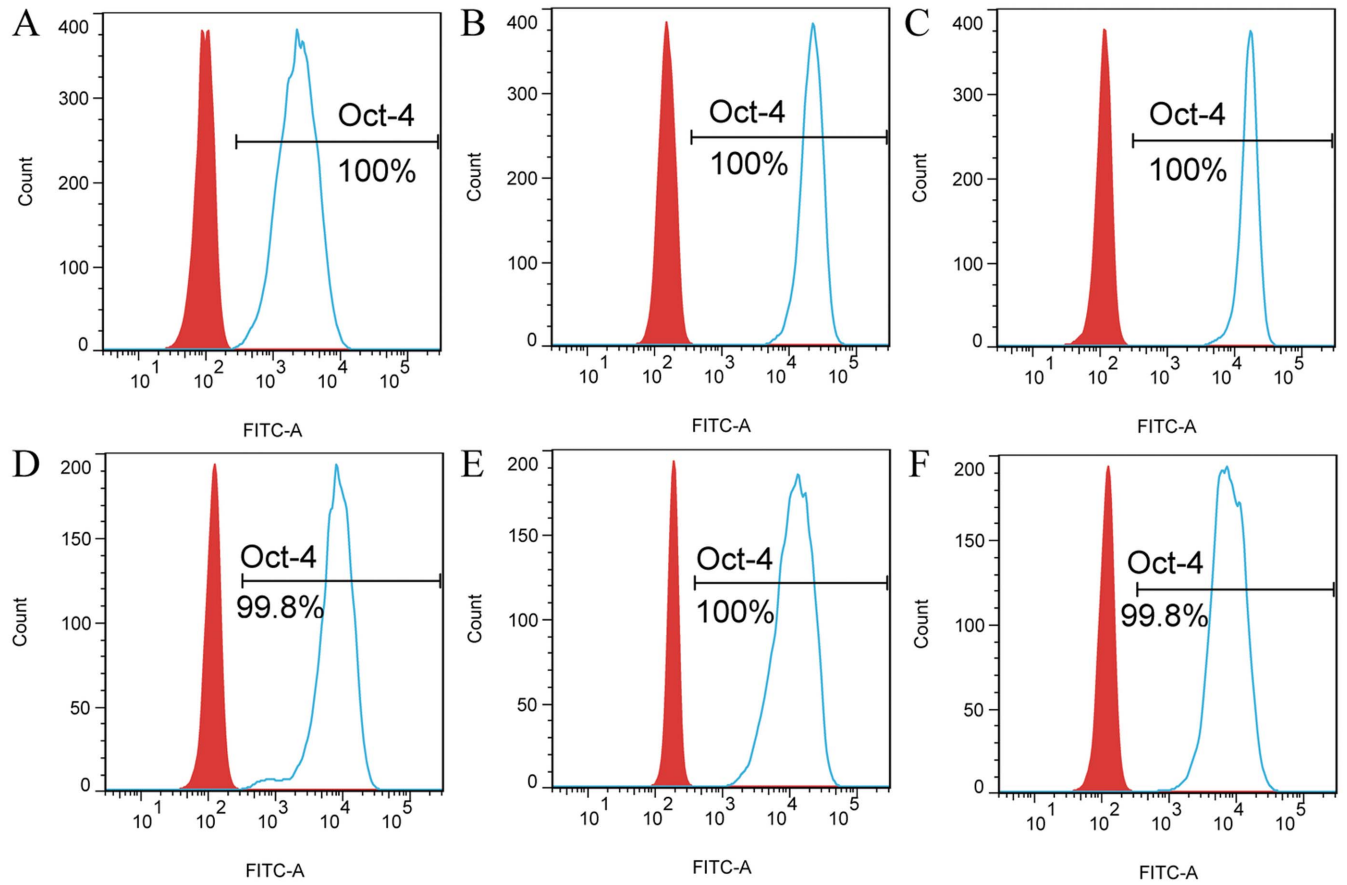

Figure 8 | FACS analysis of hNF1-4C11 hiPSCs after 1 (A), 3 (B), 5 (C), 7 (D), 9 (E), 11(F) passages on Matrigel™ for Oct-4. The percentage of positive cells is listed in parentheses, which indicate the stable pluripotency of hiPSCs.

Table 1 | Primer Sequences for Quantitative RT-PCR

Gene	Forward primer	Reverse primer
Oct-4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT
Nanog	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG
Sox-2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
Rex-1	TCGCTGAGCTGAAACAAATG	CCCTTCTTGAAGGTTTACAC
ACTB	CCCAGAGCAAGAGAGG	GTCCAGACGCAGGATG

remaining sites of gold surface[49]. After immobilization of oligonucleotides primer (1), EG_3 (5 mM) was placed on the surface of gold chips in single face reactor for 2 hours incubation. Then the chips were washed by Milli-Q water and dried under nitrogen gas flow.

All of QCM telomerase activity detections were conducted with a relative humidity controlled below 25%. The operation temperature was set at 37°C. The gold and polymer-coated QCM chips were placed in a home-built QCM reaction chamber with control software purchased from Resonant Probes GmbH (Goslar, Germany). For the real time measurements, QCM was operated in a flow-through mode at a speed of 80 mL min^{-1}, reaction buffer (composed of DEPC-treated water that included 20 mM Tris buffer (pH 8.3), 1.5 mM $MgCl_2$, 0.63 mM KCl and 1 mM EGTA) was introduced into the reaction chamber to establish a baseline first. Then the cell extracts (30 μL) and 10 mM dNTP mix (8 μL) were added to 362 μL reaction buffer and this solution was introduced into the QCM chamber and reacted with QCM chips. After incubation for 50 minutes, we introduced the reaction buffer into the chamber again to get a steady line (corresponding to the baseline).

We define off-line QCM telomerase activity detections as follow: Place the QCM chip in an open-top QCM reaction chamber (purchased from Dongwei Biotech, Hangzhou, China) first. Introduce 400 μL reaction buffer and establish a baseline and then stop the online monitoring, replace the reaction buffer by the mixture of cell extracts (30 μL), dNTP mix (8 μL) and reaction buffer (362 μL) and incubate in 37°C. Take out the QCM chip from the open-top chamber after 50 minutes, wash it by reaction buffer and Milli-Q water respectively and dry it under nitrogen gas flow. Reload the QCM chip in open-top QCM reaction chamber, introduce 400 μL reaction buffer again and monitor a terminal steady line after the extension reaction by telomerase.

TRAP assay. Telomerase activity was measured by telomere repeat amplification protocol (TRAP) for the verification of QCM results with the TRAPeze Telomerase Detection Kit (S7700; Chemicon, Temecula, CA) according to the manufacturer's protocol. Briefly, 10^5–10^6 cultured cells were lysed in 0.2 mL of CHAPS Lysis buffer, and 0.2–1 g of cell extract protein was used in TRAP assay. The extended and amplified TRAP products were resolved on 12.5% nondenaturing polyacrylamide gel. The relative telomerase activity was determined by SA-1000 (red) from ProteinSimple, USA.

RNA Isolation and Quantitative RT-PCR. The total RNA was isolated from cells using TRIzol (Invitrogen, USA). Reverse transcription was carried out by means of RevertAid™ First Stand cDNA Synthesis Kit (Toyobo, Japan). Quantitative RT-PCR analysis was performed using SYBR Green I (Takara, Japan) and an ABI 7500 RT-PCR machine (Applied Biosystems, USA). All experiments were performed in triplicate, and values were normalized on the basic of ACTB value. Primers used in this study were (5′–3′) as indicated in Table 1.

Fluorescence-Activated Cell Sorting Analysis. For flow cytometry, hNF1-4C11 hiPSCs were trypsinized when grown to 90% confluence. Cells were washed and resuspended in FACS buffer (PBS buffer without Ca^{2+} and Mg^{2+} with 2% FBS). After treatment with 200 μL 90% formaldehyde solution on ice for 30 min, hiPSCs were incubated with Oct-3/4 primary antibodies (StemCell Technologies, Canada) and FITC 488-conjugated goat anti-mouse IgG1 (Invitrogen, USA) for 30 min at 37°C respectively. The mouse IgG1 (StemCell Technologies, Canada) was conducted as the isotype. Cells were analyzed by BD FACS Calibur System and the data were analyzed using Flowjo Software.

Statistical analysis. All values were expressed as mean ± SD. A one-tailed Student's t-test was used to determine the statistical significance. A P value of < 0.05 was considered significant.

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Author contributions

J.W., Z.Z., S.W. and H.M. conceived and designed the experiments. Y.X., P.Z. and J.H. performed the cell experiments, Y.Z. conducted the QCM study. Y.Z., P.Z. and Y.X. analyzed the data and Y.Z., Y.X. and H.M. wrote the paper.

Additional information

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