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Overexpression of hTERT Extends Replicative Capacity of Human Nucleus Pulposus Cells, and Protects Against Serum Starvation-Induced Apoptosis and Cell Cycle Arrest

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ABSTRACT

The nucleus pulposus (NP) cells are chondrocyte-like cells that are required for the resistance of compressive loads through the synthesis of collagen fibrils and proteoglycan aggrecans, and the generation of a hydrostatic swelling pressure, and thus play an important role in the intervertebral disc. Here, we report the production and characterization of an immortalized human NP cell line from normal NP cells using stable transfection of recombinant human telomerase reverse transcriptase (hTERT) gene. The hTERT-transfected NP cells exhibited morphological characteristics typical of native cells. When compared with the first generation of normal NP cells, the hTERT-transfected NP cells grew faster and had an increased level of IGF-1 and TGF-β gene expression. They were successfully passaged over 20 generations without significant change in the levels of type II collagen and proteoglycan aggrecan expression. In addition, they showed resistance to serum starvation-induced apoptosis, G1 cell cycle arrest, and gene expression of p53, CCNE1, Fas, and Caspase 3. Moreover, histology revealed that no tumorigenicity of NP cells over expressing hTERT was observed after they were implanted in nude mice. Taken together, an immortalized human NP cell line was established, which has an extended lifespan, retains phenotypic features similar to primary parent NP cells, and should provide a suitable model for studying the biology of NP cells. J. Cell. Biochem. 113: 2112–2121, 2012.

KEY WORDS: hTERT; NUCLEUS PULPOSUS CELLS; IMMORTALIZED; CELL APOPTOSIS; CELL CYCLE

The intervertebral disc (IVD) plays an essential role in motion, flexibility, and support of loading of the spine. Degeneration of the IVD is commonly associated with back pain, a major chronic healthcare problem [Williams and Sambrook, 2011]. Current clinical treatments focus on alleviating pain as well as restoring the structure and function of the disc [Popovich et al., 2011]. Understanding the pathogenesis of IVD disorders will help provide a better approach to facilitate restoring the structure and function of the IVD.

The IVD consists of the gelatinous nucleus pulposus (NP), the collagenous and lamellar annulus fibrous [Roberts et al., 2006]. Among these, the NP consist of chondrocyte-like NP cells, which are required for providing resistance of compressive loads through the generation of a hydrostatic swelling pressure [Uchiyama et al., 2007]. NP cells synthesize proteoglycan (i.e., aggrecan) and type II collagen, and reside in an environment with limited vascular supply [Roberts et al., 2006]. In pathological conditions, increased degradation of aggrecan molecules and changes in extracellular matrix (ECM) content are frequently observed in NP tissues which, accompanied with increased fibrous composition and necrotic cell death, leading to increased disc degeneration, stiffness and back pain [Tsai et al., 2006].

One major limitation of studying NP cells has been a lack of good sources of available cells. Replicative senescence occurs in somatic cells due to telomere shortening when cells divide, and primary cells

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usually undergo a limited number of cell divisions during in vitro culture [Gadalla and Savage, 2011]. Likewise, primary human NP cells exhibit poor proliferation and growth retardation [Schek et al., 2011]. To facilitate our investigation of the physiology and pathogenesis of NP cells, primary cells with extended replicative capacity or immortalized cells are required.

Telomeres are repetitive DNA sequences present at the termini of chromosomes, and approximately 50–200 base pairs of DNA are lost from the telomere ends of chromosomes during each cell division [Cui et al., 2003]. Telomerase is required for the restoration of the DNA base pairs lost from the telomeres, and for preventing cells from replicative senescence [Erusalimsky and Skene, 2009]. Through this biological process, chromosomal integrity is maintained, and cells are able to continue to divide [Bolzan, 2011]. Telomerase activity is very low in most normal cells, thus permitting cells to undergo replicative senescence [Wikgren et al., 2011]. Telomerase is a holoenzyme that contains a catalytic component (called hTERT) and overexpression of hTERT can lead to preservation of the telomere end-regions of chromosomes, which in turn suppresses replicative senescence, resulting in cell immortalization [Chen et al., 2009; Zhang et al., 2011]. Unlike cancer cell lines which often consist of genetic mutations and display an unstable genotype, hTERT immortalized cell lines have been shown to exhibit a stable genotype, retain normal growth responses to serum and mitogens, and lack tumorigenicity [Klinger et al., 2006]. hTERT-mediated immortalized cells have a normal karyotype and contact inhibition, similar to reproducing stem cells [Yeager and Reddel, 1999].

Primary NP cells display a short life span and can only undergo limited numbers of divisions under normal culture conditions, which hampers their ability to be used in our research. As an initial step towards understanding the pathogenesis of NP cells, we aimed to generate an immortalized NP cell line via overexpressed hTERT in primary NP cells. These cells have an extended lifespan, retain phenotypic features similar to primary parent NP cells, and should make a suitable model for studying the function of NP cells.

MATERIALS AND METHODS

MATERIALS

RNA isolation kits were purchased from TaKaRa (Tokyo, Japan), and plasmid DNA preparation kits, restriction enzymes, T4 DNA ligase, and Taq polymerase were obtained from MBI Company (Shanghai, China). Western blot kits were purchased from GE Healthcare (Piscataway, NJ). Antibody to hTERT was supplied by Abcam (Cambridge, MA), and the anti-mouse IgG-HRP by Santa Cruz (CA). Anti-collagen-II antibody was purchased from Calbiochem (Darmstadt, Germany). The CY3 secondary antibody was obtained from Boster Biological Engineering Co., Ltd. (Wuhan, China).

PRIMERS USED TO GENERATE hTERT CONSTRUCTS

The following primers were used to generate the hTERT constructs; primer RT-1: 5′-tggaaggagttcgatgccgcgcgctccccgctg-3′ engineered with *Xmn*1 restriction enzyme cutting site; primer RT-2: 5′-caccctcgaggtgagacgctcgg-3′ engineered with *Xho*1 restriction enzyme cutting site; primer RT-3: 5′-acctcgagggtgaaggcactgttca-3′ engineered with *Xho*1 restriction enzyme cutting site; and primer RT-4: 5′-cgctcgagctagtccaggatggtcttgaagtct-3′ engineered with Xho1 restriction enzyme cutting site.

CONSTRUCTION OF hTERT EXPRESSION CLONE USING EUKARYOTIC EXPRESSION PLASMID

To generate the hTERT expression construct, total RNA was extracted from a human liver biopsy using an RNA extraction kit according to the manufacturer's instructions (TaKaRa). Reverse transcription was performed with M-MULV reverse transcriptase to obtain cDNA using oligo (dT) primers and total RNA as templates. RT-1/RT-2 and RT-3/RT-4 primers were used in PCR to amplify hTERT gene. PCR products were isolated from 1% agarose gel after electrophoresis. PCR products were purified using rapid gel extraction kit, and cloned into the T-Easy vector (Promega, Beijing, China). The identity of T-hTERT was verified by DNA sequencing using bi-directional DNA sequencing (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China). T-Easy-hTERT was completely digested using *Xmn*1 and *Xho*1, vector pAAV-MCS plasmid DNA was completely digested using *Hin*c2 and *Xho*1, respectively. DNA fragments were isolated using 1% agarose gel electrophoresis, purified, and then ligated. Following the transformation into DH5α competent *Escherichia coli*, the pAAV-hTERT plasmid was identified using restriction enzyme analysis. To generate a pGFP/hTERT construct, plasmid pAAV-hTERT and pGFP/Neo were completely digested with *Not*I, the pGFP/Neo was ligated with hTERT expression cassette to generate pGFP/hTERT/Neo, and the construct was verified by restriction enzyme analysis and DNA sequencing.

ISOLATION AND PASSAGE OF NORMAL NUCLEUS PULPOSUS CELLS

Normal NP cells were obtained from three cases of scoliosis that underwent orthopedic surgery (two cases aged 13 years, one case aged 15 years) and a pooled population of these cells was employed for all the experiments. The intervertebral disk was removed and soaked in saline containing penicillin-streptomycin antibiotics for 10 min, and the NP tissues were gently separated from the disc using a curette, followed by three to four washes with saline until no blood was visible. NP tissues which showed normal appearance of a peripheral white fibrous ring surrounding the central jelly-like NP structure were then cut into $1 \times 1 \times 1\,mm^3$ pieces with ophthalmic scissors, and placed in a 100 ml beaker filled with 10 ml of 2% collagenase II and stirred for 60 min. The completely digested tissues were centrifuged at 1,000 rpm for 10 min. The supernatant was aspirated and the cells were dispersed using 1 ml DMEM containing 10% fetal calf serum, and cells were then cultured in a T25 tissue culture flask with 6 ml DMEM containing 10% fetal bovine serum at 37°C, saturated humidity, and 5% CO_2 for 3 days.

DETECTION OF THE TRANSFECTED hTERT EXPRESSION

pGFP/hTERT/Neo was prepared according to the instruction of BIOMIGA plasmid extraction kit, and cell transfection was performed according to the Lipofectamine 2000 instructions. pGFP/hTERT/Neo was transfected into the NP cells for 24 h, and

the culture medium was supplemented with G418 (250 μg/ml) and changed every 2 days for the selection. The empty vector pGFP/Neo was also transfected and used as a control. Fluorescence microscopy of GFP expression in NP cells was employed to determine transfection efficiency. To confirm the expression of hTERT, Western blot was performed by 10% SDS–PAGE gel sample electrophoresis (90 V, constant voltage) for 120 min, followed by transfer to 0.45 μM PVDF membrane. Membranes were incubated with anti-hTERT (1:500) and anti-mouse-HRP (1:3,000) antibodies. The Western blot signals were detected by ECL reagents and imaged for analysis.

GROWTH CURVE OF NUCLEUS PULPOSUS CELLS AS DETECTED BY XTT

Cell growth curves were examined using XTT cell proliferation assays. In brief, cells were trypsinized and plated at 2×10^4/ml into 96-well culture plates. After 24 h, 5 mg/L XTT solution (20 μl) was added, and incubated for an additional 4 h. The absorbance at 490 nm was used to detect signals on a microplate reader. Growth curves of the NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo control (1st generation) were tested for 7 days. Six duplicate wells were measured, and their average values with standard deviation were presented.

REAL-TIME PCR ANALYSIS OF GROWTH FACTORS AND APOPTOSIS FACTORS IN HUMAN NUCLEUS PULPOSUS CELLS

Fluorescence quantitative PCR (SYBR Green) was performed to detect the expression of type II collagen, aggrecan, BMP 2, IGF-1, TGF-β, BMP 4, PDGF, and FGF mRNA between the NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo control (1st generation). RT-PCR analysis was also performed for P53, CCNE1, Fas, and Caspase 3 gene expression between the NP cells transfected with pGFP/hTERT/Neo(20th generation) or pGFP/Neo control (1st generation) at 0, 6, 12, 18, 24, and 48 h after serum starvation. Primer express 2.0 software was used for primers design (Table I).

DETECTION OF TYPE II COLLAGEN AND AGGRECAN CONTENT IN EXTRACELLULAR FLUID BY ELISA ASSAY

The expression of type II collagen and aggrecan in extracellular fluid was determined by ELISA assays using an ELISA kit purchased from USCNLIFE (Wuhan, China). We compared type II collagen expression or aggrecan in NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo control (1st generation). The sample groups consisted of blank wells, standard wells, and detected sample wells.

TYPE II COLLAGEN IMMUNOFLUORESCENCE STAINING IN NUCLEUS PULPOSUS CELLS

For type II collagen immunofluorescence staining analysis, the first and fourth passage of the empty vector pGFP/Neo transfected NP cells, and the first and twentieth passage of pGFP/hTERT/Neo transfected NP cells, were grown on poly-L-lysine-coated coverslips at 1×10^4 cells/ml (2 ml per well) at 37°C, 5% CO_2, 95% air, and saturated humidity for 24 h. The cells received their various experimental paradigms, and the coverslips were then collected and rinsed with 0.02 mM phosphate buffer solution three times (5 min each), fixed with 4% paraformaldehyde for 30 min, and washed with distilled water four times (5 min each). Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 10 min, and the cell membrane was disrupted by incubation in 0.3% Triton X-100 for 15 min and blocked using non-immune goat serum. Cells were incubated with appropriate rat anti-human type II collagen (1:200 in 1% bovine serum albumin) in a wet box at 4°C overnight. Cells were rewarmed at 35°C for 30 min, rinsed with 0.02 mM PBS, and then incubated with 1:50 CY3-labeled goat anti-mouse IgG or goat anti-rabbit IgG at 35°C in a humidity box in the dark for 30 min. Cells were washed with 0.02 mM PBS, followed by DAPI mounting. Images were collected using a fluorescence microscope system, digital color CCD camera, and microscope image analysis system (Siemens Company, Munich, Germany).

INDUCTION OF CELL APOPTOSIS BY SERUM STARVATION

Cell apoptosis was performed in NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo control (1st generation). Apoptosis rate was quantified by detecting surface exposure of phosphatidylserine in apoptotic cells using an Annexin V-FITC/PI (propidium iodide) apoptosis detection kit (BD Biosciences Clontech) according to the manufacturer's instruction. The cell culture medium was replaced with serum-free medium and the cells were collected following 0, 6, 12, 18, 24, and 48 h of serum starvation and washed twice with cold phosphate-buffered saline (PBS). 5×10^5 cells were resuspended in 0.5 ml binding buffer, containing Annexin-V (1:50) and 40 ng/sample of propidium iodide (PI), and

TABLE I. Primer Sequences for PCR

Primers	Forward	Reverse	Length (bp)
TGFβ	5′-CGAGCCTGAGGCCGACTAC-3′	5′-AGATTTCGTTGTGGGTTTCCA-3′	71
IGF-1	5′-TGATCTAAGGAGGCTGGAGATGTA-3′	5′-GCGTTCTTCAAATGTACTTCCTTCT-3′	123
FGF	5′-AGCGACCCTCACATCAAGCT-3′	5′-CGGTTAGCACACACTCCTTTGATA-3′	72
BMP2	5′-AAAACGTCAAGCCAAACACAAA-3′	5′-TCCACGTACAAAGGGTGTCTCTT-3′	72
BMP4	5′-TTTCCACTGGCTGACCACCT-3′	5′-TTCAGTGGGCACACAACAGG-3′	105
PDGF	5′-CTGTCTGCAAGACCAGGACG-3′	5′-TGCAGCGTTTCACCTCCAC-3′	108
Type II collagen	5′-TGGTGGCTTCCATTTCAGCT-3′	5′-TGTTCTGGGAGCCTTCCGT-3′	104
AGGRECAN	5′-AGCCTGCGCTCCAATGACT-3′	5′-GGAACACGATGCCTTTCACC-3′	103
P53	5′-CCGAGTGGAAGGAAATTTGC-3′	5′-AGTCAGAGCCAACCTCAGGC-3′	101
CCNE1	5′-GGAAGGCAAACGTGACCGT-3′	5′-TTATTGTCCCAAGGCTGGCT-3′	103
FAS	5′-GGGCATCTGGACCCTCCTA-3′	5′-GGCATTAACACTTTTGGACGATAA-3′	70
Caspase 3	5′-TACCAGTGGAGGCCGACTTC-3′	5′-CAAAGCGACTGGATGAACCA-3′	99
β-actin	5′-GCATGGGTCAGAAGGATTCCT-3′	5′-TCGTCCCAGTTGGTGACGAT-3′	106

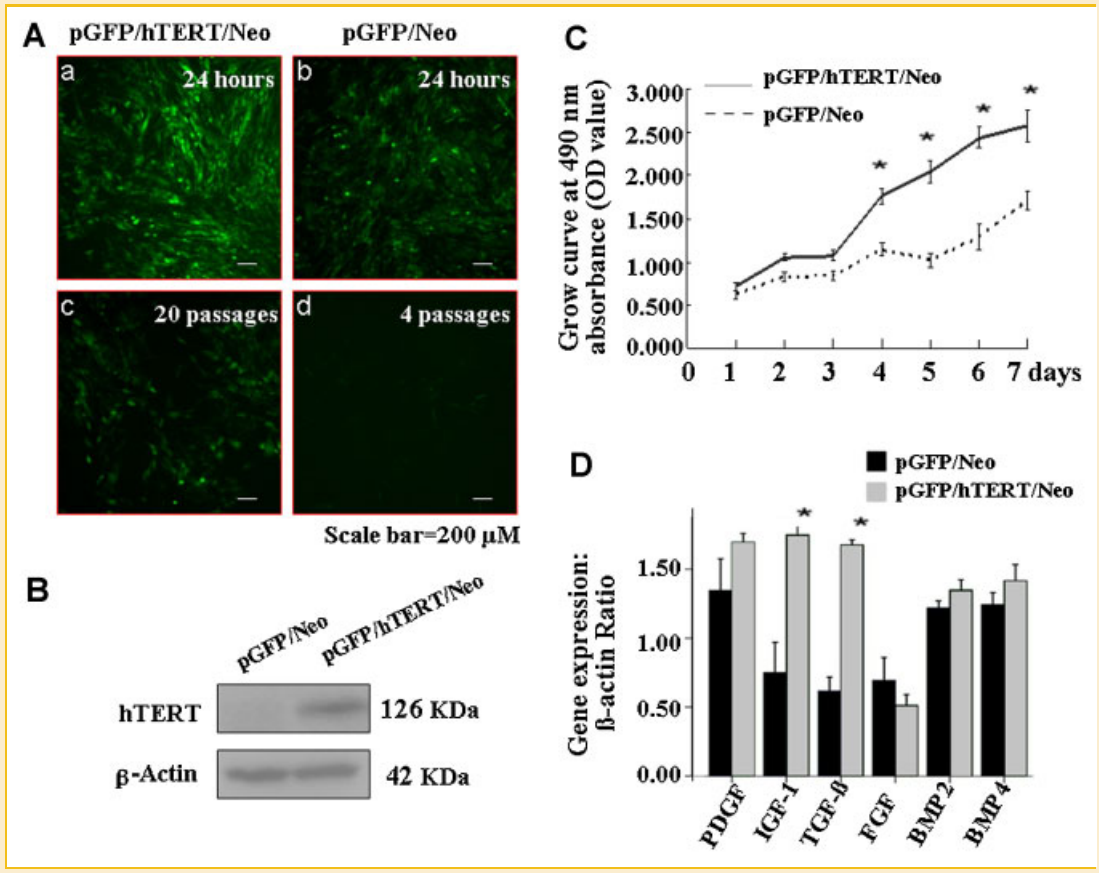

Fig. 1. A: GFP protein expression was detected in NP cells after 24 h transfection with either pGFP/hTERT/Neo or pGFP/Neo construct (a–b). The GFP signals were still present at the 20th generation of NP cells transfected with pGFP/hTERT/Neo but appeared to be diminishing in NP cells transfected with the pGFP/Neo at the 4th generation (c–d). B: Western blot analysis showing that hTERT was detected in the NP cells transfected with pGFP/hTERT/Neo, but rarely detectable in NP cells transfected with the pGFP/Neo control. C: The NP cells overexpressing hTERT (20th generation) showed a small but insignificant increase in proliferation rate than NP cells transfected with pGFP/Neo (1st generation) from days 1 to 3, but increased significantly from days 4 to 7. D: The levels of IGF-1 and TGF-β in pGFP/hTERT/Neo NP cells were significantly higher than those of pGFP/Neo NP cells, while other growth factors including PDGF, bFGF, BMP 2, and BMP 4 were at a similar level in both cell types ($^*P$-value < 0.05). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

incubated for 30 min at 37°C in the dark. The number of viable, apoptotic, and necrotic cells was quantified by flow cytometer (Becton Dickinson) and analysis was carried out using Cell Quest software. At least 10,000 cells were analyzed for each sample. The apoptosis rate (%) = (the number of apoptotic cells/the number of total cells observed) × 100% was recorded. Experiments were repeated three times.

CELL CYCLE ANALYSIS

Cell cycle analysis was conducted in NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo control (1st generation). The cell culture medium was replaced with serum-free medium and the cells were collected following 0, 6, 12, 18, 24, and 48 h of serum starvation and washed twice with cold PBS. Then cells were fixed in 70% ice-cold ethanol overnight. After washing twice with PBS, 5×10^5 cells were resuspended in 0.5 ml PBS containing ribonuclease A (100 mg/ml) and PI (100 mg/ml) for 30 min at 37°C in the dark. The DNA content of cells was then analyzed by flow cytometer (Becton Dickinson).

ANIMAL EXPERIMENTS

Ten nude mice were randomly divided into two groups (experimental and control; n = 5 per group). In the experimental group, 2×10^6 pGFP/hTERT/Neo (20th generation) NP cells in 200 μl of PBS were subcutaneously seeded on the right dorsal scapular back skin by a single injection in nude mice. In the control group, 2×10^6 pGFP/Neo (1st generation) NP cells in 200 μl of PBS were subcutaneously seeded on the right dorsal scapular back skin by a single injection in nude mice. At 2, 3, and 4 weeks after implantation, the injection site in nude mice was assessed for growth size. The animal experiments were conducted with an animal ethics approval from the Experimental Animal Management Committee of Zhongshan University in china. The approval number is 0069802.

HISTOPATHOLOGICAL EXAMINATION

The samples were fixed with 4% formaldehyde solution, decalcified using 100 g/L formic acid, followed by gradient ethanol dehydration, paraffin-embedding, sectioning, and conventional hematoxy-

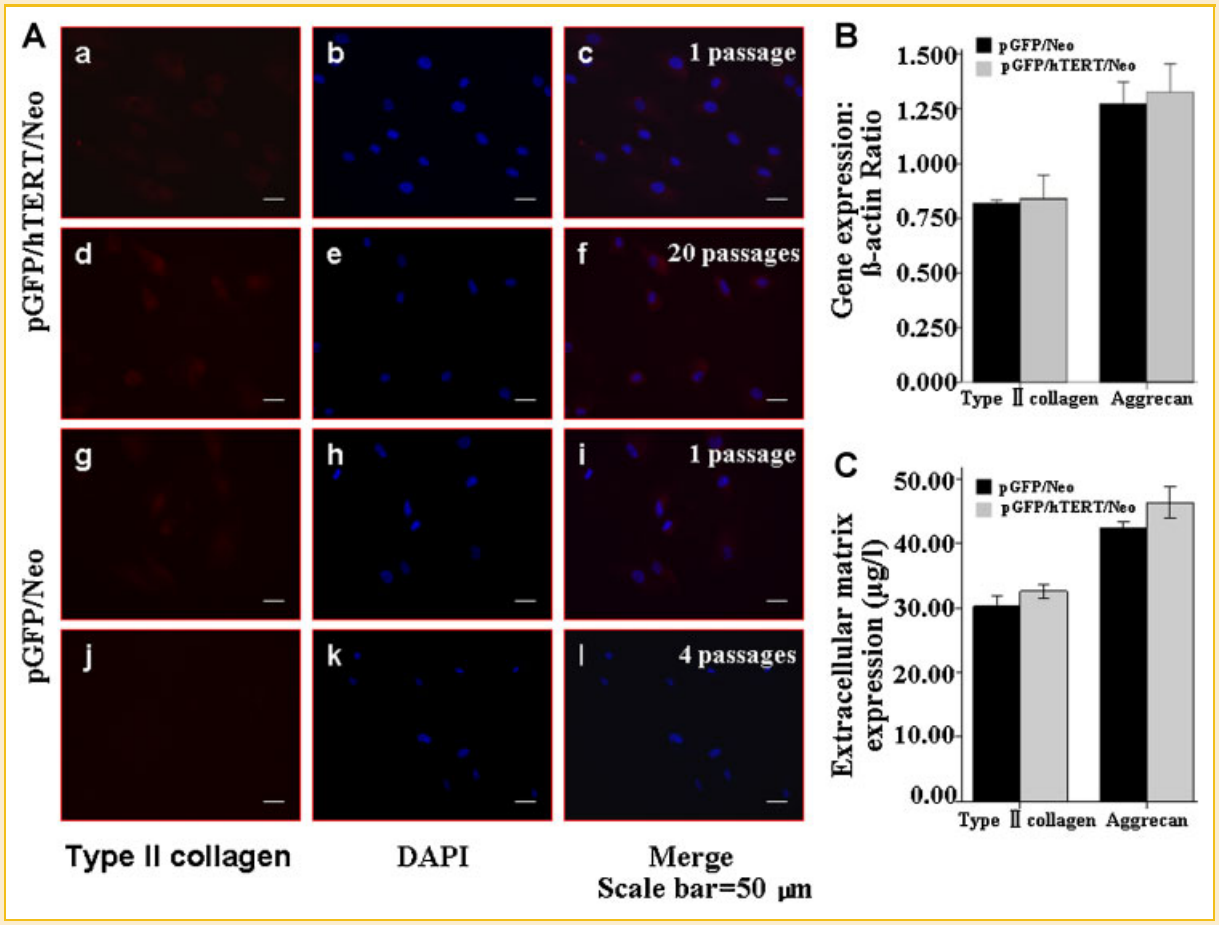

Fig. 2. A: Type II collagen expression was examined in NP cells transfected with pGFP/hTERT/Neo (1st, 20th generation) or pGFP/Neo (1st, 4th generation) by immunofluorescence staining. The immunofluorescence staining on NP cells transfected with pGFP/hTERT/Neo (1st generation, 20th generation) was positive (a–f). The immunofluorescence staining in NP cells transfected with pGFP/Neo (1st generation) was positive but it was negative in 4th generation cells (g–l). B: RT-PCR analysis revealed that type II collagen and aggrecan gene expression in NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) show no apparent difference. C: Protein expression analysis of type II collagen and aggrecan in extracellular fluid by ELISA assays revealed that there was no significant difference in the synthesis of type II collagen and aggrecan between the NP cells transfected with pGFP/hTERT/Neo or pGFP/Neo.

lin–eosin staining. Samples were then observed by light microscopy and photographed to observe their characteristics.

RESULTS

GENERATION AND CHARACTERIZATION OF HUMAN NUCLEUS PULPOSUS CELLS OVEREXPRESSING hTERT

Following the transfection of pGFP/hTERT/Neo or pGFP/Neo constructs, we examined GFP protein expression in NP cells (NP) cells using fluorescent microscopy. As shown in Figure 1A, GFP protein expression was detected in approximately 80% of NP cells after 24 h transfection with either pGFP/hTERT/Neo or pGFP/Neo construct (Fig. 1A, a and b). The GFP signals were still present at the 20th generation of NP cells transfected with pGFP/hTERT/Neo after G418 selection, but appeared to be diminishing in NP cells transfected with the pGFP/Neo at the 4th generation (Fig. 1A, c and d). To confirm the protein expression of hTERT, Western blot analysis of NP cells was performed. hTERT was detected in the NP cells transfected with pGFP/hTERT/Neo, but rarely detectable in NP cells transfected with the pGFP/Neo control (Fig. 1B). We observed that the NP cells overexpressing hTERT proliferated more than 20 generations and retained similar morphology to the primary parent cells, whereas the NP cells transfected with pGFP/Neo control stopped proliferating at 4–5 generations.

We further examined the proliferation rate of immortalized human NP cells using the XTT colorimetric assay. As shown in Figure 1C, the NP cells overexpressing hTERT (20th generation) showed a small but insignificant increase in proliferation rate when compared to NP cells transfected with pGFP/Neo control (1st generation) from days 1 to 3, but increased significantly from days 4 to 7 (Fig. 1C). It was observed that the NP cells overexpressing hTERT continued to proliferate and ceased proliferation after 30–50 generations. We next tested the gene expression of common growth factors and cytokines between the immortalized NP cells over-expressing hTERT (20th generation) and the NP cells transfected with pGFP/Neo control (1st generation). We found that the levels of

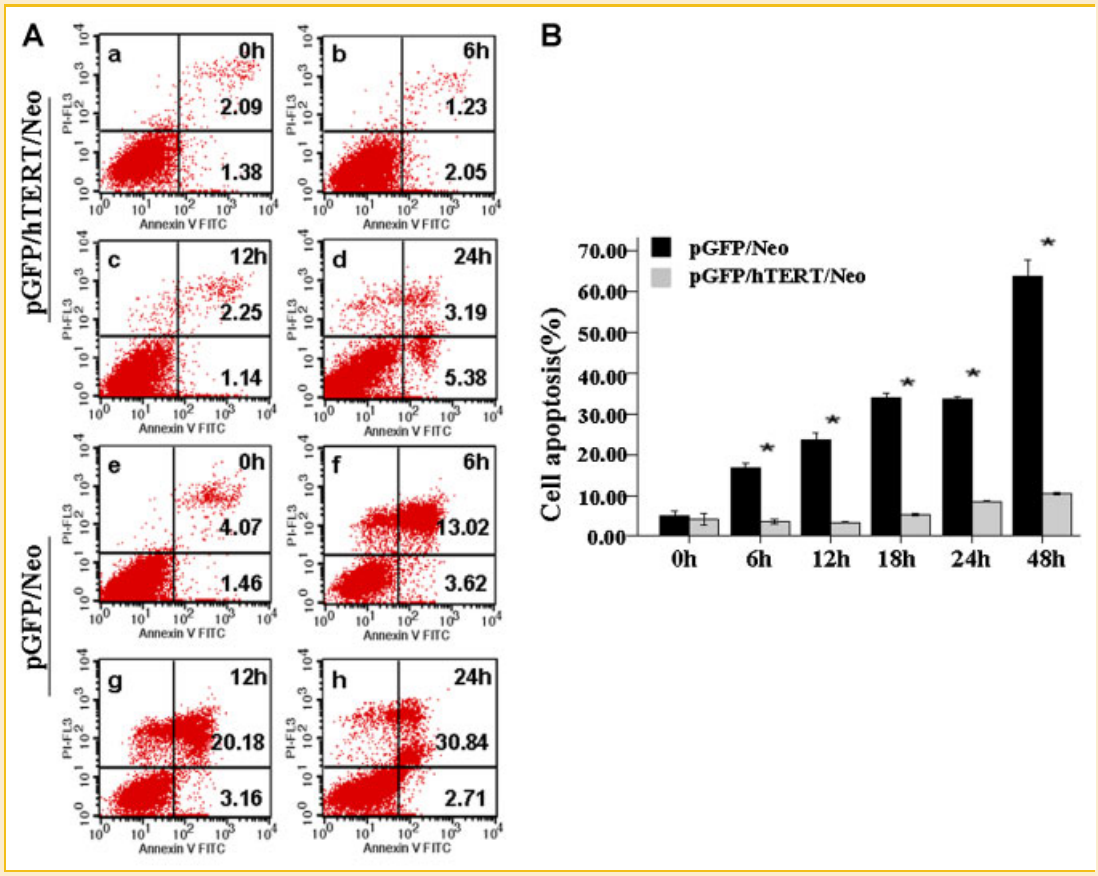

Fig. 3. A: Serum starvation-induced apoptosis analyzed in NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation). Representative graphs showing cell apoptosis following serum starvation (0, 6, 12, and 24 h) in the pGFP/hTERT/Neo (20th generation) group (a–d) or in the pGFP/Neo (1st generation) group: (e–h). B: Comparison of the apoptosis rate between NP cells transfected with he pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) following serum starvation at 0, 6, 12, 18, 24, and 48 h ($^*P$-value < 0.05). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

IGF-1 and TGF-ß in pGFP/hTERT/Neo NP cells were significantly higher than those pGFP/Neo transfected NP cells, while other growth factors including PDGF, bFGF, BMP 2, and BMP 4 were at a similar level in both cell types (Fig. 1D).

We next evaluated the protein expression of specific makers of NP cells. Type II collagen expression was examined in NP cells transfected with pGFP/hTERT/Neo (1st, 20th generation) or pGFP/Neo (1st, 4th generation) as a control using immunofluorescence staining. Cells were co-stained with DAPI (nuclear staining) and a type II collagen antibody. The immunofluorescence staining on pGFP/hTERT/Neo NP cells was positive at both 1st generation and 20th generation (Fig. 2A, a–f). The control pGFP/Neo NP cells were positive for type II collagen in 1st generation cells, but negative in the 4th generation (Fig. 2A, g–l). Next we compared the gene expression of type II collagen and aggrecan in pGFP/hTERT/Neo (20th generation) and pGFP/Neo (1st generation) using real-time PCR and ELISA assays. As shown in Figure 2B, type II collagen and aggrecan gene expression showed no differences between pGFP/hTERT/Neo (20th generation) and pGFP/Neo (1st generation). Protein expression analysis of type II collagen and aggrecan in extracellular fluid by ELISA revealed that there was no significant difference in the synthesis of type II collagen and aggrecan between the pGFP/hTERT/Neo NP cells and pGFP/Neo NP cells (Fig. 2C). These results indicate that the levels of type II collagen and aggrecan present in the extracellular fluid in the immortalized human NP cells at 20 passages were similar to that in the first generation of NP cells transfected with pGFP/Neo control. Taken together, NP cells overexpressing hTERT retain expression of NP-specific markers and morphologically resemble their primary parent cells.

OVEREXPRESSION OF hTERT IN NP CELLS PROTECT AGAINST SERUM STARVATION-INDUCED APOPTOSIS

Serum withdrawal represents an effective trigger to induce apoptosis in cell cultures. Here, we examined if overexpression of hTERT could protect against serum starvation-induced apoptosis. Apoptosis was analyzed to compare the apoptotic rates between NP cells transfected with pGFP/hTERT/Neo (20th generation) and NP cells transfected with pGFP/Neo (1st generation). Following the withdrawal of bovine serum (0, 6, 12, 18, 24, and 48 h), NP cells transfected with pGFP/hTERT/Neo (20th generation), had an average apoptosis rate of 3.47%, 3.28%, 3.39%, 5.20%, 8.57%, and 10.23% of cells respectively (Fig. 3A, a–d and B). In comparison, in NP cells transfected with pGFP/Neo (1st generation), following the withdrawal of bovine serum (0, 6, 12, 18, 24, and 48 h), the apoptosis rate

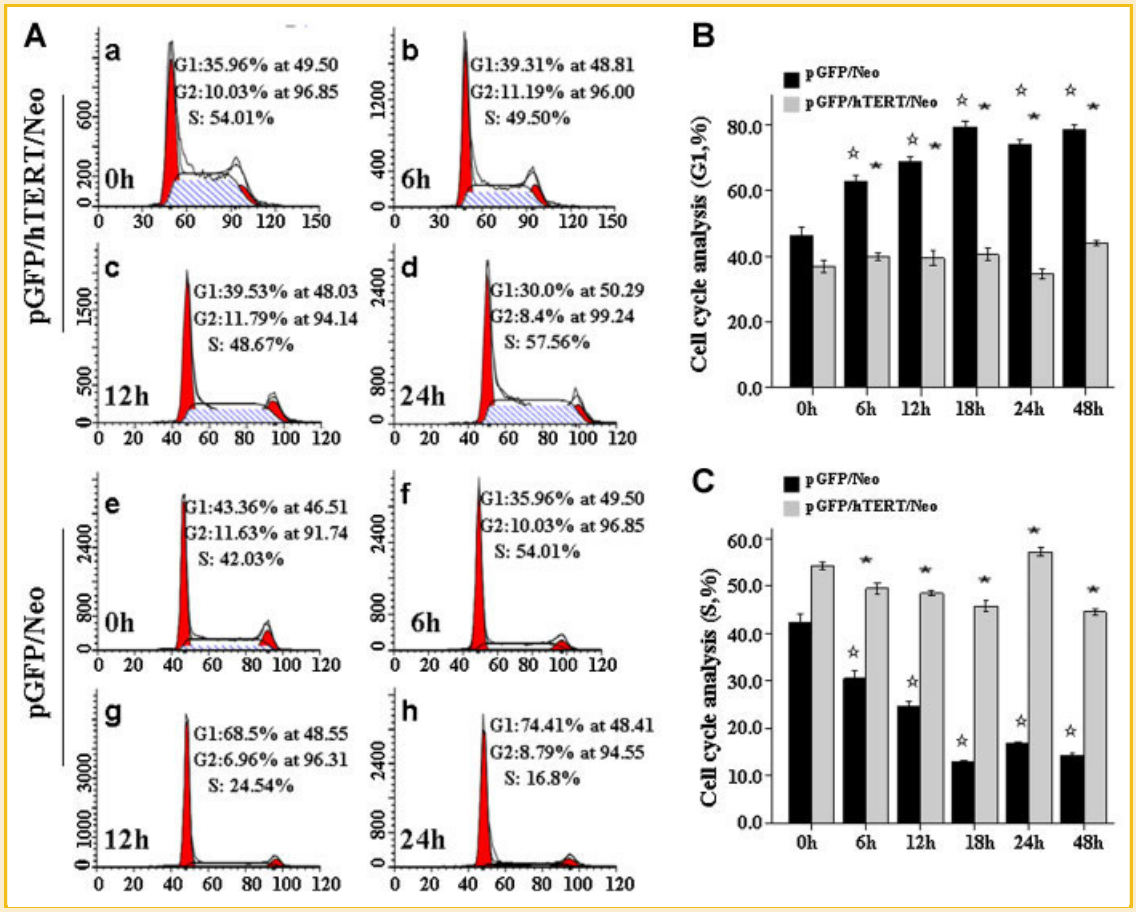

Fig. 4. A: Serum starvation-induced cell cycle analyzed in NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation). Representative graphs showing the cell cycle following serum starvation (0, 6, 12, and 24 h) in the pGFP/hTERT/Neo (20th generation) group (a–d) or in the pGFP/Neo (1st generation) group: (e–h). B: Comparison of the cell cycle in the G1 phase between NP cells transfected with he pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) following serum starvation at 0, 6, 12, 18, 24, and 48 h ($^*P$-value < 0.05). $^*P$-value when comparing with cells at 0 h. C: Comparison of the cell cycle in the S phase between NP cells transfected with he pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) following serum starvation at 0, 6, 12, 18, 24, and 48 h ($^*P$-value < 0.05). $^*P$-value when comparing with cells at 0 h. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

averaged 5.53%, 16.64%, 23.34%, 33.31%, 33.55%, and 62.12% of cells, respectively (Fig. 3A, e–h and B). As expected, there was an increase in apoptosis rates in NP cells transfected with pGFP/Neo control upon serum starvation in a time-dependent manner. In comparison, the NP cells overexpressing hTERT were resistant to serum starvation-induced apoptosis over the time course tested up to 48 h.

OVEREXPRESSION OF hTERT IN NP CELLS PROTECTS AGAINST SERUM STARVATION-INDUCED G1 ARREST

Cells are arrested at the G1 phase after serum withdrawal. We examined if overexpression of hTERT could protect against serum starvation-induced G1 arrest. Cell cycle was analyzed to compare G1 arrest between NP cells transfected with pGFP/hTERT/Neo (20th generation) and NP cells transfected with pGFP/Neo (1st generation) at 0, 6, 12, 18, 24, and 48 h after serum starvation (Fig. 4A). We observed that the NP cells transfected with pGFP/Neo (1st generation) were arrested at the G1 phase of the cell cycle, whereas the NP cells transfected with pGFP/hTERT/Neo (20th generation) were not (Fig. 4B,C). These results indicate that immortalized human NP cells are protected against serum starvation-induced G1 arrest via overexpression of pGFP/hTERT/Neo (Fig. 5).

SERUM STARVATION-INDUCED P53, CCNE1, FAS, AND CASPASE 3 GENE EXPRESSION WERE ABSENT IN NP CELLS OVEREXPRESSION OF hTERT

Induction of p53, CCNE1, Fas, and Caspase 3 gene expression regulates the progress of apoptosis and the cell cycle. We hypothesized that serum starvation-induced apoptosis and cell cycle G1 arrest is regulated via the gene expression of p53, CCNE1, Fas, and Caspase 3 in the NP cells overexpressing hTERT. RT-PCR analysis of p53, CCNE1, Fas, and Caspase 3 gene expression was performed and compared between NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) at 0, 6, 12, 18, 24, and 48 h after serum starvation. It was observed that p53, CCNE1, FAS, and Caspase 3 gene expression was up-regulated upon serum starvation in NP cells transfected with pGFP/Neo control (1st generation), whereas in the immortalized NP cells, the gene expression of p53, CCNE1, FAS, and Caspase 3 were largely unaffected. These results indicate that serum starvation induces the gene expression of p53, CCNE1, Fas, and

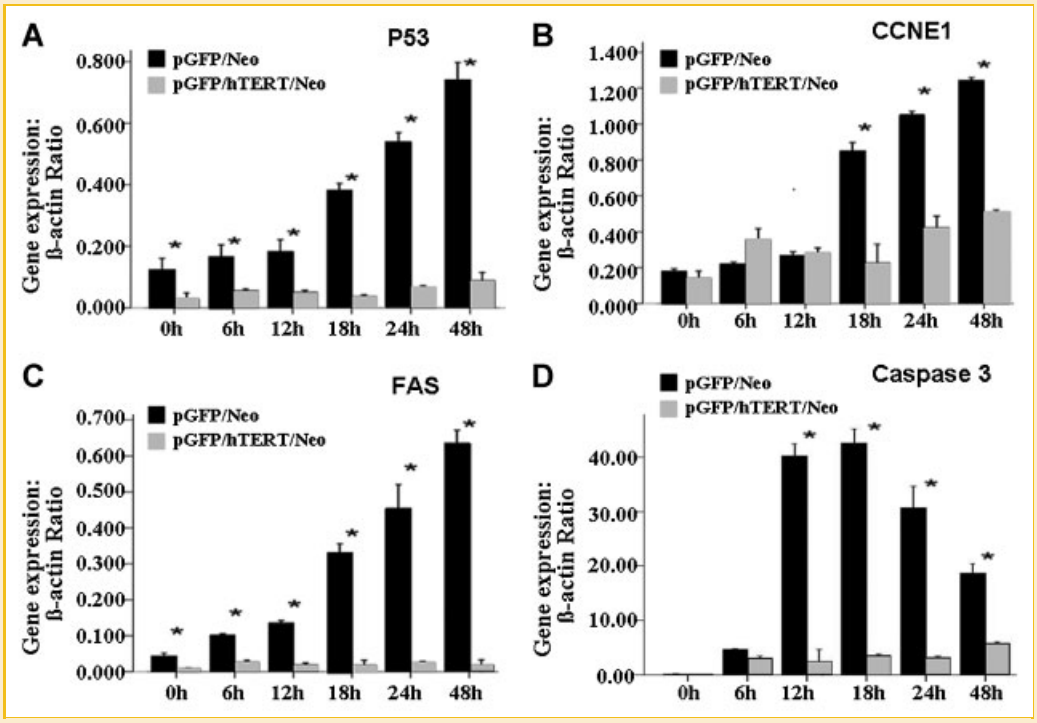

Fig. 5. P53, CCNE1, Fas, and Caspase 3 gene expression analyzed by real-time RT-PCR. A: Comparison of the gene expression of p53 (A), CCN1 (B), FAS (C), and Caspase 3 (D) between NP cells transfected with he pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) following serum starvation at 0, 6, 12, 18, 24, and 48 h (*P-value < 0.05).

Caspase 3 in normal cells, whereas overexpression of hTERT protects from these effects.

IN VIVO CELL GROWTH CHARACTERISTICS OF THE IMMORTALIZED NUCLEUS PULPOSUS CELLS

hTERT immortalized cell lines usually display a stable genotype and do not cause cell transformation. To confirm if hTERT immortalized NP cell lines do not show changes associated with tumorigenicity, the transfected NP cells were implanted into the right scapular region of nude mice. We observed a cell mass in NP cells transfected with pGFP/hTERT/Neo (20th generation) at 2, 3, and 4 weeks which appeared to be hard and moveable. The cell mass was $1 \times 1\,cm^2$ at week 2 and appeared to subside to $0.7 \times 0.7\,cm^2$ at week 3 and $0.3 \times 0.3\,cm^2$ at week 4 (Fig. 6). In the NP cells transfected with pGFP/Neo control (1st generation), there was a smaller mass of $0.3 \times 0.3\,cm^2$ in size, which was only observed at week 4 (Fig. 6). Histology of the NP cells transfected with pGFP/hTERT/Neo (20th generation) showed that the mass consisted of cell clusters, necrotic tissue, and inflammatory cells (Fig. 6I,J). Similar histology features were observed in the NP cells control group (Fig. 6L,M). No tumor growth was observed in both groups.

DISCUSSION

Using overexpression of hTERT, we successfully established immortalized NP cells that exhibit a longer lifespan than primary NP cells, and grow faster than normal cells. These high proliferative characteristics may be explained by the gene expression of IGF-1, TGF-β, and other growth factors [Ross Richards et al., 2010]. The immortalized NP cells were able to be passaged over 20 generations. hTERT expressing cells produce high levels of TGF-β, and IGF-1 (Fig. 1D), and the production of these factors does not change the production of ECM components; including type II collagens and aggrecan (Fig. 2B,C) between immortalized cells and normal NP cells. These results indicate that overexpression of hTERT retains phenotypic features resembling primary parent NP cells.

Our study demonstrated that transfection with hTERT alone is fully capable of immortalizing NP cells. Bodnar et al. [1998] first introduced exogenous hTERT into human retinal pigment epithelial cells and foreskin fibroblasts, which increased in vitro culture time by 20 doublings. hTERT has since been used to successfully immortalize human skin keratinocytes, dermal fibroblasts, muscle satellite (stem), and vascular endothelial, myometrial, retinal-pigmented, and breast epithelial cells [Shay and Wright, 2005]. We have now added NP cells into this growing list. The availability of a human NP cell line is critical to advancing the field and will drive innovation in drug discovery and regenerative medicine in the degeneration of the IVD.

The probability of cell spontaneous immortalization is very low in both rodent (10^{-5} to 10^{-6}) and human (10^{-12}) cells [Katakura et al., 1998]. As such, immortalization is often achieved through gene transfection. For instance, DNA tumor viruses have been used for developing immortalized cell strains including the SV40 virus, HPV, adenovirus E1A gene, and EB virus [Schwab et al., 2000]. The SV40

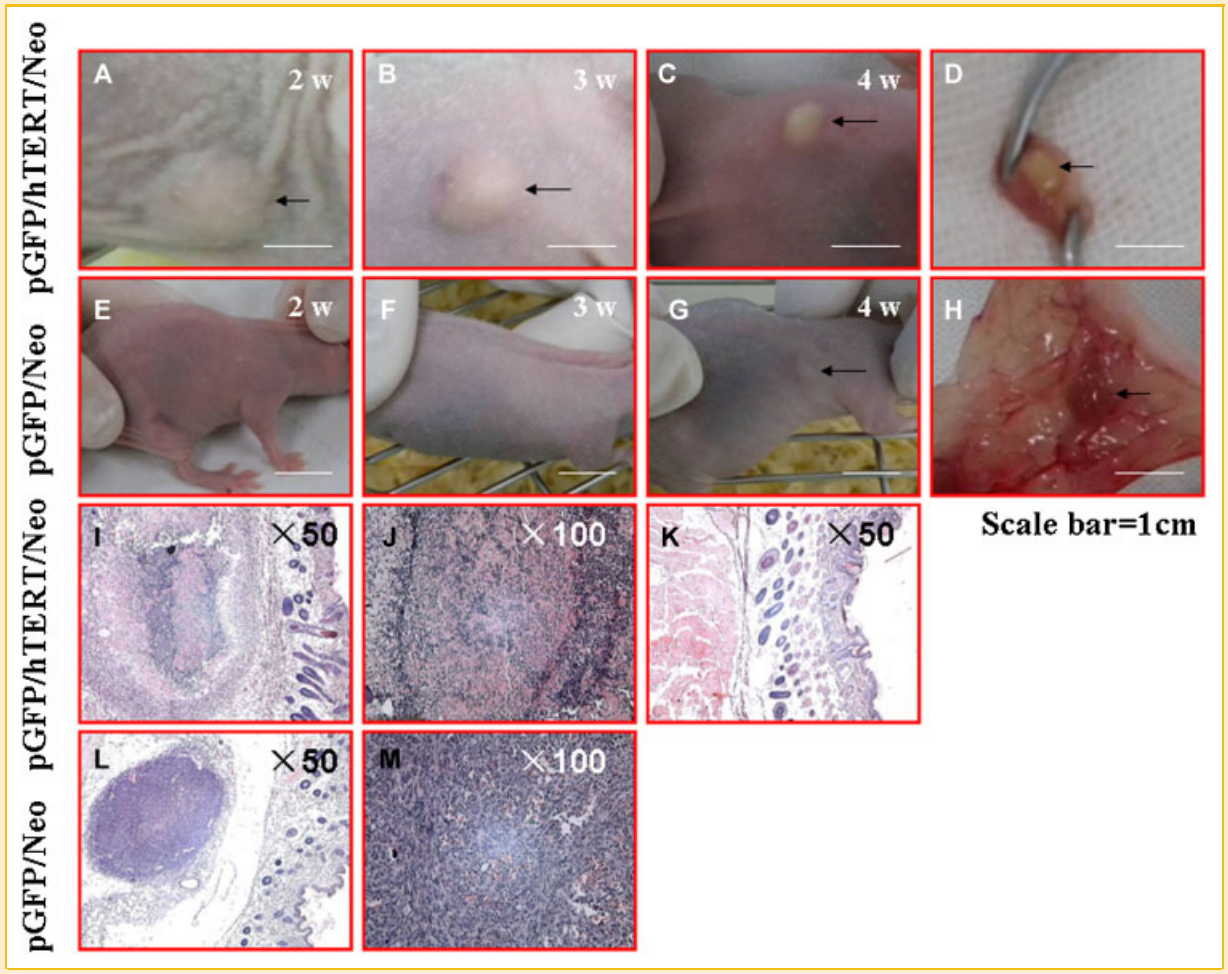

Fig. 6. Examination of tumorigenicity of implanted NP cells in nude mice. NP cells transfected with pGFP/hTERT/Neo (20th generation) or pGFP/Neo (1st generation) were implanted into the right scapular region of nude mice, the injection site was monitored at weeks 2, 3, and 4 (A–G). Histology sections are presented which show cell clustering, necrotic tissue, and inflammatory cells (I, J, L, M) in the cell injection sites. Histology of a normal skin control is also shown (K). No tumor characteristics are detected.

virus was originally separated from monkey kidney cells, and is the most common virus used to promote cell immortalization [Liu et al., 2005]. The virus-encoded transformation proteins in the cytoplasmic membrane and cell nucleus also inactivate pRB, p53, SEN6, and other growth inhibitory factors by regulating DNA replication and gene expression, thus maintaining telomere stability [Argiris et al., 2011]. In addition, several oncogenes or proto-oncogenes, including Myc, Jun, Ras, and Ab5 can also be employed to induce cell immortalization [Vandel et al., 1996]. However, both oncogene and proto-oncogene can be randomly integrated into DNA resulting in unexpected changes in cellular physiological pathways [Song et al., 2006]. By comparison, hTERT transfection helps to restore the DNA base pairs lost from the telomeres during cell division and to maintain chromosomal integrity without random integration. Because telomerase length determines the capacity of cell proliferation [Rodriguez-Brenes and Peskin, 2010], exogenous hTERT can be used to effectively activate telomerase in target cells, maintain telomere length, and stabilize the chromosome, thus resulting in cell immortalization [Haber, 1995]. The hTERT-mediated immortalized cells are normal cells that maintain more differentiated functions and less chromosomal damage, and are therefore more stable [Toouli et al., 2002]. Thus, the use of hTERT overexpressing NP cells in this study has advantages over other approaches such as virus and oncogene-mediated cell immortalization [Sakai et al., 2004].

Several checkpoints are in place to ensure that damaged or incomplete DNA is not passed on to daughter cells. Two main checkpoints exist: the G1/S checkpoint and the G2/M checkpoint. G1/S transition is a rate-limiting step in the cell cycle and is also known as the restriction point [Deckbar et al., 2011]. Serum starvation is able to halt the cell in the G1 phase [Huang et al., 1996]. Generation of immortalized human NP cells via overexpression of hTERT can overcome serum starvation induced G1-S phase arrest, which could be explained by the low levels of expression of p53, CCNE1, FAS, and Caspase 3 gene. p53 is a tumor suppressor protein that regulates the cell cycle, and plays an important role in triggering the control mechanisms at both G1/S and G2/M checkpoints [Yamaki et al., 2011]. CCNE1 (cyclin E1) protein accumulates at the G1/S phase boundary and is degraded as cells progress through S phase. FAS has been shown to play a central role

in the physiological regulation of programmed cell death [Jantscher et al., 2011]. Caspase 3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [Liu et al., 2009]. Collectively, serum-induced G1/S phase arrest was inhibited by overexpression of hTERT via the regulation of p53, CCNE1, FAS, and Caspase 3 gene expression.

Enhanced tumorigenicity of immortalized NP cells is a potential risk associated with extended lifespan. However, we found no evidence of tumor formation when NP cells overexpressing hTERT were implanted into nude mice. The development of an immortalized NP cell line may help to elucidate the biological function of NP cells and provide a standard cell line for investigating the mechanisms underlying degenerative disc disease. These cells may also be useful for tissue engineering regeneration methods on the treatment of degenerative disc disease and should make a suitable model for studying the biology of NP cells.

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