Establishment and Functional Characterization of Immortalized Rabbit Dermal Papilla Cell Lines

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Abstract

Background: Hair follicle (HF) undergo periodic growth and development in mammals, which regulated by dermal papilla cells (DPCs) are reported to play an important role in the HF morphogenesis and development. However, primary DPCs have low proliferative activity, age quickly, and fresh cell isolation is both time-consuming and laborious.

Method: In this study, we introduced the SV40 large T antigen (SV40T) into dissociated early passage rabbit vibrissae DPCs with lentiviral vectors and established seven immortalized DP cell lines (R-1, R-2, R-3, R-4, R-5, R-6 and R-7).

Result: These cell lines displayed early passage morphology and displayed high alkaline phosphatase activity. RT-PCR and immunofluorescence staining showed that all the immortalized cell lines expressed the DPC markers (*α-SMA ,IGF1*, *ALPL*, *FGF2*, *BMP2* and *TGFβ2*; α-SMA and VIM protein), but *α-SMA* was only expressed well in R-3, R-4, and R-7. Furthermore, it was found that R-7 was the only line to survive beyond 50 passages. Compared to melanoma cells, R-7 did not undergo malignant transformation. Karyotyping and cell growth viability analysis illustrated that the R-7 cell line preserved the basic characteristics of primary DPCs.

Conclusion: The R-7 DPCs established have potential application for future hair research. The study provides the theoretical basis in the cell research of HF growth and development.

Introduction

The HF is a distinguishing organ in mammals, consisting of both dermal and epidermal components[1].It is highly sensitive and cyclic transformed from phases of rapid growth (anagen), via apoptosis-driven regression (catagen) to relative quiescence (telogen)[2-5]. There are numerous factors that can affect these three periods.HFs are composed of concentric epithelial sheaths (hair shafts, inner root sheaths and outer root sheaths) which are surrounded by dermal sheaths connected by a dermal papillae (DP) at the bottom of the HF. Epidermal stem cells converge in the middle of an outer root sheath called a bulge. These cells interact with DPs at the bottom of the HF and produce several types of HF cells[6,7].

The DP is considered necessary for the hair follicles growth and cyclic development[8-10]. Such as, dermal papilla cell number specifies hair size, shape and cycling and its reduction causes follicular decline[11]. Human DPCs was first isolated via the microdissection techniques[12,13]. Subsequently, the DPCs of the murine sources were also isolated[14-16]. Although DPCs can be cultivated and passaged several times *in vitro,* there were stillmany problems exist in DPC culture[16-19]. Firstly, a primary DPC culture can take up to 4 weeks[20]. Then, cultured DPCs undergo dramatic changes in morphology, and have low proliferative activity and a limited lifespan. In addition, following cell passage, the cells lose their inductive capacity in culture[21-24]. The primary DPCs that passages less than 13 present triangular or short fusiform, retaining

their unique aggregative growth characteristics[25].Therefore, it is necessary to establish immortalized DP cell lines which could help researchers obtain DPCs in the absence of complex culture conditions.

Cell immortalization is a characteristic of cells gaining continuous proliferation ability. The *SV40T* can overcome the blockage of cell proliferation[26] and is widely used in experiments[27]. SV-40 is a closed-loop double-stranded DNA tumor virus that has been widely used in studies of cell transformation. The T antigen fragment of SV40 is the most commonly used which integrates into the host genome leading to immortalized proliferative[28].In 2010, human DPCs were transduced with a combination of viral vectors containing SV40T and cmyc[20]. After that, human immortalized DP cell line were also established with SV40T and hTERT[29,30]. Based on the optimized culture strategy, mouse immortalized DP cell strains were established with SV40T[31].These cells showed high proliferation rates and comparable genotypic and phenotypic properties to primary cells.

As a model animal, rabbit plays important roles in the animal products and medical research, but rabbit DPCs have not been established which severely restricts HF growth and development related studies. In this study, we introduced SV40T into dissociated early passage rabbit vibrissae DP cells and report the establishment of seven immortalized DP cell lines. Especially, R-7 survived over 50 passages. We characterized the cell lines for ALPL activity, DPC marker expression, malignant transformation and cell growth viability.

Materials And Methods

Primary culture of rabbit vibrissae DPCs and the establishment of immortalized cell lines

All work with rabbits and mice were approved by the Animal Care and Use Committee of Yangzhou University (Yangzhou, China, 14 March 2019, No. 201903005). Three four-month-old white rex-rabbits (female) were purchased from the Yuyao Xinnong Rabbit Industry. Rabbits were injected intravenously with zoletil-50 (6mg/kg) and waited to collect vibrissae skin after death. We tried to isolate DPCs three times in total. DPCs were isolated as previously described[15]. Cells were passaged in 0.25% trypsin and sub-cultured at a ratio of 1:3 when sub-confluent. DPCs are mesenchymal-derived follicular stem cells[32,33] so we tried to culture them in MSCM (Mesenchymal Stem Cell Medium) complete medium (ScienCell, USA). primary DPC (passage 3) were transfected with pLVX-IRES-Puro-SV40LT lentiviral expression vector[34] (Yingrun Biotechnology, Changsha, China). The puromycin resistance gene (Puro) was used for cell screening[35]. After 72 hours of transfection, DPCs were inoculated with MSCM containing 2.0 μg/mL puromycin for 7 days, and replaced with MSCM containing 1.0 μg/mL puromycin for 2 weeks. Cells were harvested in 0.25% trypsin, diluted with medium, and single cells were seeded into 96-well plates. Seven clones identified by immunofluorescence were selected and propagated. SV40T antigen expression was evaluated by immunofluorescence and RT-PCR analysis. Immortalized DPCs were cultured in MSCM medium. Immortalized DPC lines of passage 50 were used in this study.

Alkaline Phosphatase Activity

Primary DPCs (passage 2) and seven cell line(passage 10) were seeded into 24-well plates and cultured for incubated for 24 h. Cell alkaline phosphatase stain kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to fix and stain the cells. Positive reactions were observed through gray-black particles or massive strip-like precipitates in the cytoplasm.

Immunofluorescence assay

DPCs (passage 15) were seeded into 24 well plates and cultured for 24 hours. Cells were fixed in 4% paraformaldehyde for 30 min and washed with PBS. Cells were permeabilzed in 0.5% Triton X-100 for 1 hour, washed in PBS, and blocked for 30 min in 1% BSA (Boster, AR0004). Cells were then probed with primary antibodies (4 μg/mL) in blocking solution overnight. Primary antibodies against α-SMA (Boster, BM0002), VIM (Boster, BM0135), SV40LT (Santa Cruz (Pab 101): sc-147) were used. Cells were then washed and labeled with secondary fluorochrome-conjugated antibodies (Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488), abcam, ab150077, Cy3–conjugated Affinipure Goat Anti-Mouse IgG(H+L), proteintech, SA00009-1) for 2 hours at room temperature (25˚C). Finally, cells were stained with DAPI (Beyotime, C1006) for 10 min and imaged under a fluorescence microscope.

RT-PCR analysis

Total RNA was isolated using the RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China) and used as a template for cDNA synthesis with HiScript Q Select RT Super Mix for qPCR Kit (Vazyme Biotech, Nanjing, China). The synthesized cDNA was used as a template for gene amplification using primers and 2×Rapid Taq Master Mix(Vazyme Biotech, Nanjing, China). Primer sequences are summarized in Table S1. PCR conditions are summarized in Table S2-S3. PCR products were separated by 1% agarose gel electrophoresis and observed on a gel doc ez imager (BIO-Rad, shanghai, China).

Soft agar assays

Pre-heated 1.2% thawed agar was mixed with DMEM medium containing 20% fetal bovine serum as the basal layer[36]. B16F10 and DPCs (1×10^3 cells/mL)were mixed with the pre-heated 0.7% agarose ensuring no air bubbles were generated. Cells were spread onto agarose plates and 200 ul of complete medium was added every two days. Colony formation was observed via microscopy for up to 2 weeks.

Karyotype analysis

1.5μl colchicine (1 mg/ml) to was added to DPCs in 25 cm^2 cell culture flasks for 4 h at 37˚C, 5% CO_2. Cells were collected and low-permeated in 0.075 M KCl at 37 ˚C for 32 min. Cells were collected, fixed in 3:1 methanol: acetic acid three times for 30 min, 15 min and 15 min. Finally, cell suspensions were dripped onto pre-cooled slides and stained with working fluid prepared by Giemsa (Amresco, Solon, OH, USA).

Tumorigenesis assay

Four-week-old female BALB/c nude mice were purchased from the College of veterinary medicine (Yangzhou University, China) (http://syxy.yzu.edu.cn/art/2017/6/6/art_40971_559648.html). Cells ($5x10^6$) were resuspended in 200 μl PBS. Subcutaneous injection with PBS was performed into the right armpit of mice (n=33)[29]. Mice were observed weekly for 3 weeks and sacrificed with carbon dioxide. Mouse melanoma cells (B16F10) were used as a positive control.

Cell cycle analysis

To analyze cell cycle progression and apoptosis in primary DPCs (passage 2) and R-7 (passage 50), the Cell Cycle and Apoptosis Analysis Kit (Beyotime, Jiangsu, China) was used. Collected cells were incubated overnight in 70% ethanol and treated for 5 min with RNase A. After 30 min of incubation with propidium iodide (PI) in the dark at 37˚C, DNA content was analyzed on a Flow Cytometer through fluorescent analysis (LSRFortessa, BD Company, American).

Cell growth curves

Primary DPC (passage 2), R-7 (passage 5, passage 19 and passage 50) were harvested and seeded into 24-well plates at a density of ~10^3 cells/mL (n=3 per passage). Then Cells were counted every 24 h using an Automatic cell counter (BIO-Rad, shanghai, China) continuously for 8 days. Uncounted cells were replaced with fresh MSCM medium every 2 days.

Cell proliferation assessments

Cell Counting Kit-8 (CCK-8) (Vazayme, Nanjing, China) assays were used to detect cell apoptosis. Cells (10^5 per well) were seeded into 96-well plates and cell viability assessments were performed through the addition of CCK-8 (10μL) for 2-4 hours. The optical density of each well was determined at 0, 24, 48, and 72 hours at 450 nm with Infinite M200 Pro (Tecan, Männedorf, Switzerland).

Western blot

Cell lysates were obtained using RIPA Lysis Buffer (PPLYGEN, Beijing, China). Protein concentrations were determined with the Enhanced BCA Protein Kit (Beyotime)[37]. These proteins were detected and analyzed using the Wes automated Western Blot Analysis System[38]. The following antibodies were used: 1:200 Anti-SV40LT (Santa Cruz (Pab 101): sc-147), 1:200 Anti-GAPDH mouse monoclonal antibody (Abcam, ab8245), 1:100 Anti-Vimentin (VIM) monoclonal antibody (Boster, BM0135), 1:100 Anti-α smooth muscle actin(α-SMA) antibody monoclonal antibody (Boster, BM0002). Expected protein sizes, SV40LT:92 kDa, VIM:55 kDa, α-SMA:43 kDa, GAPDH: 39 kDa.

Statistical analysis

SPSS 22.0 was used for data analysis. Each analysis has three biological replicates, and all error bars in the results represent the mean ± SD.*p <0.05 were considered significantly different, and **p<0.01 considered extremely significantly different.

Results

Establishment of the immortalized rabbit DP cell lines

Primary rabbit vibrissae DPCs spread slowly and showed various shapes (Fig.1a). Primary DPCs (passage 2) were divided into two groups, pLVX-IRES-Puro-SV40LT and pLVX-IRES-Puro-GFP lentiviral expression vector.The fluorescence observation showed the successful transfection of pLVX-IRES-Puro-GFP transfection (Fig.1b) showed. Then Seven clones were selected from the pLVX-IRES-Puro-SV40LT group, termed R-1, R-2, R-3, R-4, R-5, R-6 and R-7 (Fig.1c). Various cell morphologies were observed with R-3 and R-7 both of fusiform shapes[29]. R-5 and R-6 showed an oval shape or egg-like appearance, consistent to the previous study [39]. The morphologies of R-1 were similar to primary DPCs, while R-3 and R-7 showed obvious aggregative behavior. In addition, alkaline phosphatase can be used as marker for the induction of DPCs[40,41]. The results of alkaline phosphatase tests showed that all DPC lines expressed ALPL, which means rabbit DPC lines have been successfully obtained (Fig. 1d).

Expression of DPC markers in the immortalized DPC lines

We investigated the ability of the cell lines to maintain the characteristics of primary DPCs at both the mRNA and protein levels. We designed a series of primers to detect expression in seven DPC lines. The results of RT-PCR showed that all cell lines expressed DPC specific marker genes, such as lpha smooth muscle actin (*α-SMA*) [42], insulin-like growth factor 1 (*IGF1*), alkaline phosphatase (*ALPL*), fibroblast growth factor 2 (*FGF2*), bone morphogenetic protein (*BMP2*) and *TGFβ1*. Instead, Cytokeratin 8 (*KRT8*) an epithelial cell marker didn't expressed in the DPCs, suggesting high cell homogeneity (Fig. 3). Immunofluorescence staining confirmed that the seven DPC lines (passage 7) expressed SV40T and VIM protein (Fig. S2 and S4), but α-SMA protein was only expressed well in R-3, R-4, and R-7 (Fig.2, Fig. S3). Especially, DPC signature genes were significantly expressed in R-7 cell line.

R-7 cell line maintains the characteristics of primary DPCs

For further analysis of the R-7 cell line over passage 50, we investigated malignant transformation and cell growth activity. The colony-forming ability of R-7 was assessed in soft agar colony formation assays, there were no enlarged colonies were observed in DPCs after two weeks, suggesting they underwent no transformation and proliferated without tumorigenic properties (Fig.4a). Furthermore, R-7 was used for karyotype analysis by colchicine (Figure.S5) and the visual field was randomly selected to show that the karyotypes were normal (22 pairs of chromosomes), which was consistent with the normal rabbit chromosome (Fig. 4b). R-7 was examined for tumor formation in BALB/c nude mice. Excluding B1F10, none of the cells promoted tumor formation (Fig.4c).

The growth curves and cell proliferation assessments of primary DPC and R-7 were comparable with all showing similar growth curves as observed for primary DPC, with high proliferative activity (Fig.5a). Immortalized DPCs and viability assessments were performed via CCK8 assays. Immortalized DPCs showed similar proliferation rates to primary DPCs (Fig.5b). Western blotting analysis showed the R-7 cell

line expressed the DPC proteins (α-SMA, VIM) (Fig.5c). There is no significant difference between the primary DPC and the R-7 cell line in cell apoptosis (Fig.5d). To investigate the long-term culture of DP cell lines, we used flow cytometry to analyze cell cycle [43]. The distribution of the cells is shown in Fig.5e, as a percentage of cells containing 2n DNA (G1 phase), 4n > S phase > 2n of DNA, and 4n DNA (G2 and M phases) levels assessed via PI staining. R-7 was consistent with the cell cycle analysis of primary DPC (Fig.5e). These above results demonstrated that the R-7 cell line maintained the characteristics of primary DPC and showing a lack of malignant transformation.

Discussion

For most animals, hair has many useful biologic functions[4]. It can control heat dissipation in the body, maintain body temperature, protect skin, also be concerned with social and sexual communication[44]. In HFs, DPCs play a role in inducing, controlling and regulating the morphogenesis of hair follicles and maintaining the growth and development and periodic circulation of hair follicles. DPC depletion in mice causes the changes in hair follicle structure and cycling that characterize progressive alopecia in humans, including reduction in both hair and follicle size, a prolonged telogen phase and an ultimate failure to produce new terminal hairs[45]. Currently, DPC lines have been established via SV40T in human[29] and mouse[31]. Therefore, we used SV40LT to establish DPC line in rabbit.

In the previous studies, many genes have effects in the biological processes in the HF growth and development.*IGF-1* affects follicular proliferation, tissue remodelling and the hair growth cycle, as well as follicular differentiation[46].BMP signaling in dermal papilla cells is required for their hair follicle-inductive properties[47].*TGF-β_2* is specifically expressed in human dermal papilla cells and modulates hair folliculogenesis[48].Human DPC line expressed *Wnt5a*, *ALP*, and *FGF7*[29]. Mouse DPC line expressed*FGF7* and*α-SMA*, and had activity of alkaline phosphatase[31].The *KRT8* mRNA expression levels indicated that the cell lines had not originated from any contaminated epithelial cells. The primary DP cells contained some epithelial cells, which reflected the benefits of monoclonal selection.*α-SMA,IGF1, ALPL,FGF2, BMP2*, and *TGFß* have been shown to be expressed in rabbit DPC line, and HF expression plays a key role in the regulation of cell signaling[49-52].

In this study, we further introduced SV40T into rabbit Vibrissae DPCs and passed the cells to more than 50 passages. The growth of the DPCs was unchanged and the cells maintained a normal DP signature. The percentage of cells in S phase cell can act as an indicator for tumor proliferation status[53]. The number of R-7 in the S phase cell showed similarity compared to primary DPCs. The chromosome number and karyotype of R-7 remained normal. It proved that the introduction of the SV40LT did not cause chromosomal mutations in R-7. B16F10 is a murine melanoma cell line from a C57BL/6J mouse and it is tumorigenic[54,55].Soft agar assays showed that R-7 kept single cell in long-term soft agar culture, but B16F10 could form obvious clones. The transplantation tumor experiment in nude mice also showed that R-7 malignant transformation. Cell proliferation and cell death are essential yet opposing cellular processes[56]. The cell proliferation assessments of primary DPCs and R-7 were no big difference. The

results of cell apoptosis were the same. The growth curves of primary DPCs and R-7 were comparable with all showing similar growth curves as observed for primary DPCs. As stated before, R-7 performed best as a DP cell line and did not undergo transformation even at passage 50.

Conclusions

In conclusion, we successfully establish the immortalized rabbit DPC line, which could be as a tool for the studies of hair growth, hair morphogenesis and hair cycle. These findings will ultimately contribute to the treatment and prevention of hair loss in future studies.

Declarations

Ethics approval and consent to participate

The experimental procedures were approved by the Animal Care and Use Committee of Yangzhou University (Yangzhou, China, 14 March 2019, No. 201903005). The animals were used in accordance with Laboratory Animal Requirements of Environment and Housing Facilities (GB14925-2001). The study was carried out in compliance with the ARRIVE guidelines.

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

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Acknowledgements

Not applicable.

Consent for publication

Not applicable.

Data Availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors' contributions

Jiali Li wrote the main manuscript text. Bohao Zhao provided methodological guidance. Bohao Zhao and Yang chen reviewed the first draft and provided comments. Chen Zhang, Xiyu Zhang, Yingying Dai helped

during the experiment. Shuaishuai Hu, Naisu Yang helped with data processing.

Xinsheng Wu provided resources. All authors reviewed the manuscript.

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Figures

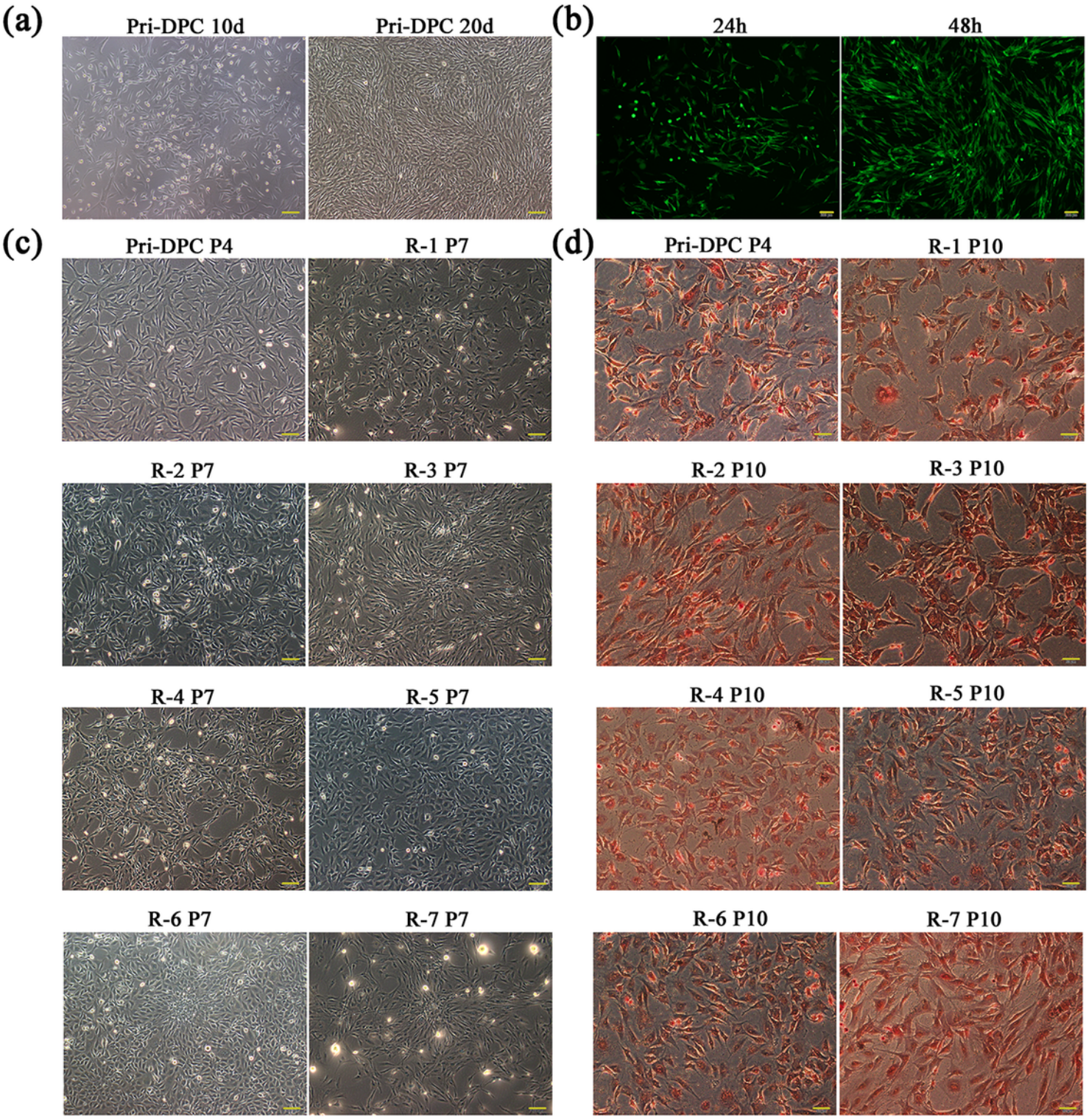

Figure 1

Morphology of the seven DPC lines and primary DPCs. (a) Isolation and morphological observations of primary DPCs (Scale Bar: 100 μm). (b) Fluorescence analysis of pLVX-IRES-Puro-GFP transfection after 24h, 48h. (Scale Bar: 100 μm). (c) Cell morphologies (Scale Bar: 100 μm). (d) Alkaline phosphatase analysis. Primary DPCs and immortalized cell lines were used for ALPL detection (Scale Bar: 50 μm).

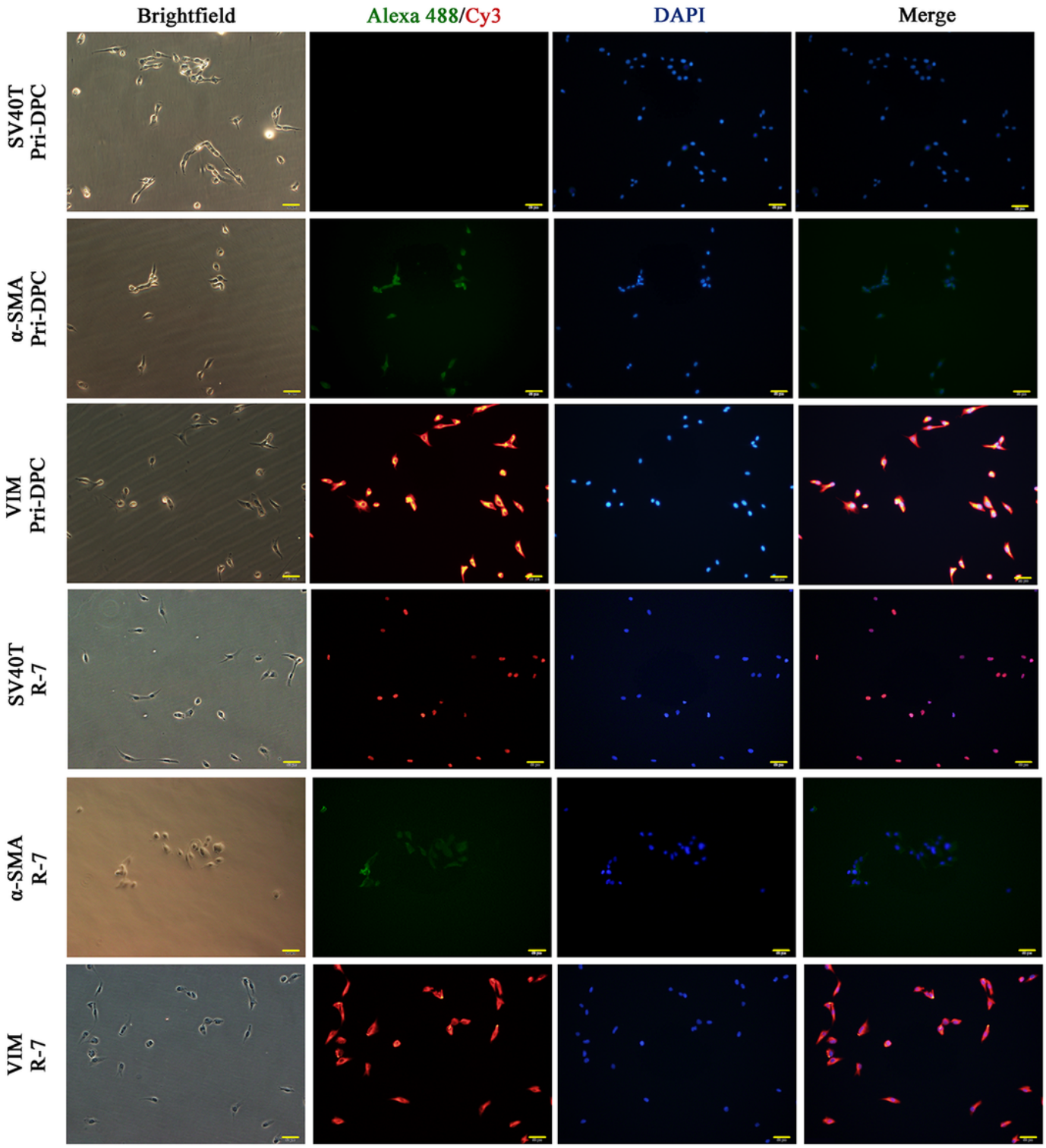

Figure 2

Indirect immunofluorescence staining of primary DPCs and R-7. All the DPCs were incubated with antibodies against α-SMA, SV40T and VIM (Scale Bar: 50 μm). Passage 5 of DPCs were used.

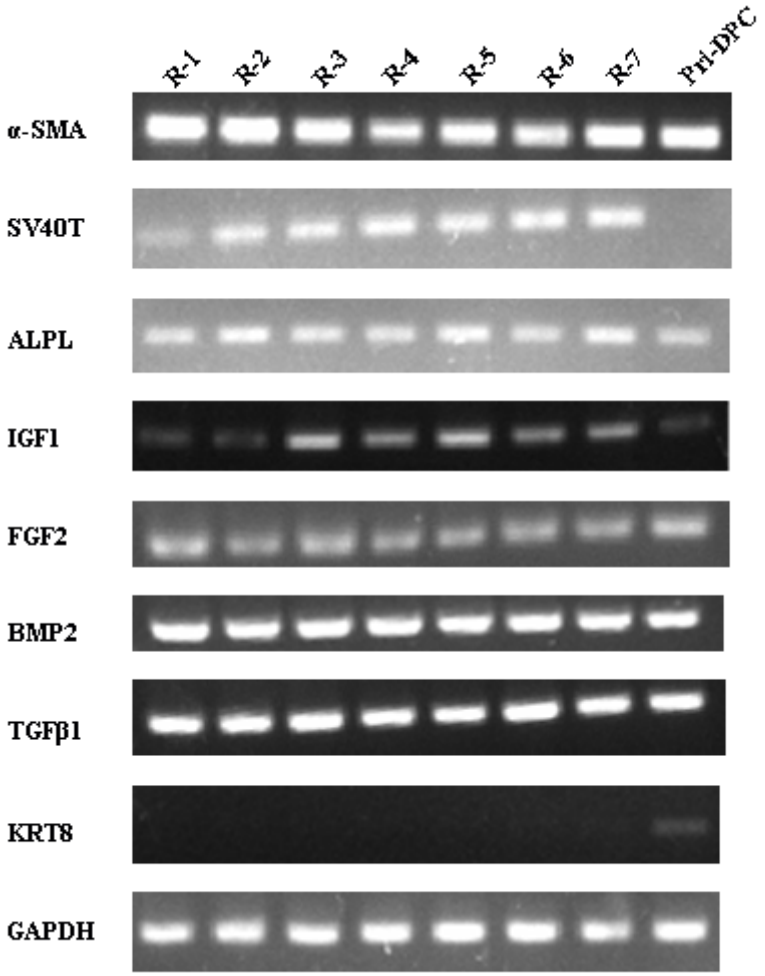

Figure 3

RT-PCR analysis. Equivalent amounts of total RNA from the immortalized DPCs (passage 7) (lanes 1-7), primary DPCs (passage 2) (lane 8), were used for RT-PCR analysis. GAPDH was used as a loading control. Pri-DPC: primary DPC.

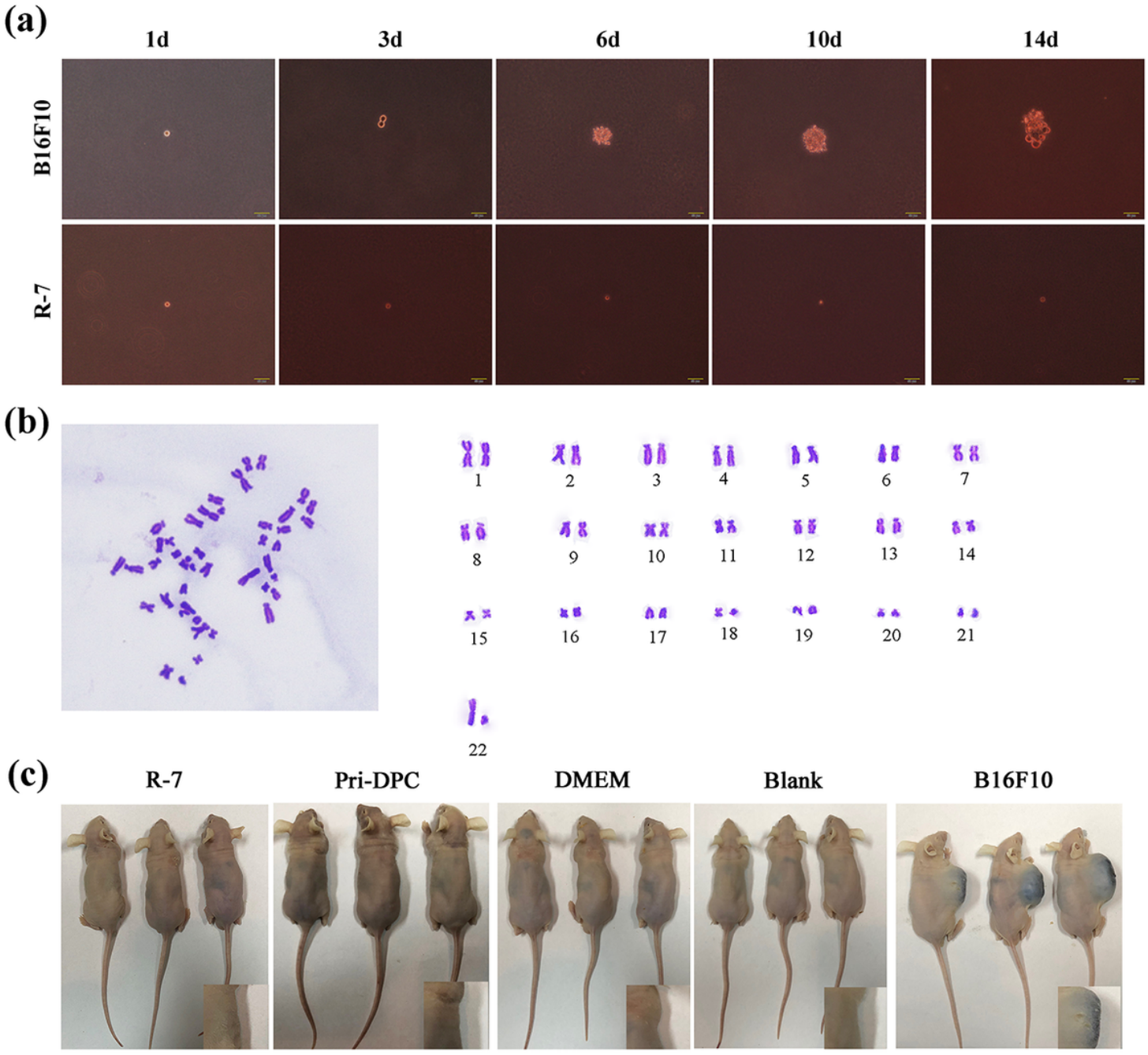

Figure 4

Malignant transformation of R-7 (passage 50) cells. (a) Colony-forming ability of R-7(passage 50) cells. Colony-forming ability of B16F10 cells (positive control) (above) and DPCs (below) at days 1, 3, 6, 10 and 14 were assessed by a soft agar assays (Scale Bar: 50 μm). (b) Metaphase chromosome (left) and karyotypes (right) of rabbit R-7(passage 50) cells ( ) XY type. (c) Tumorigenicity in nude mice. B16F10 mouse melanoma cells (positive control) formed tumors 2-weeks after cell implantation. Tumor formation did not occur in R-7.

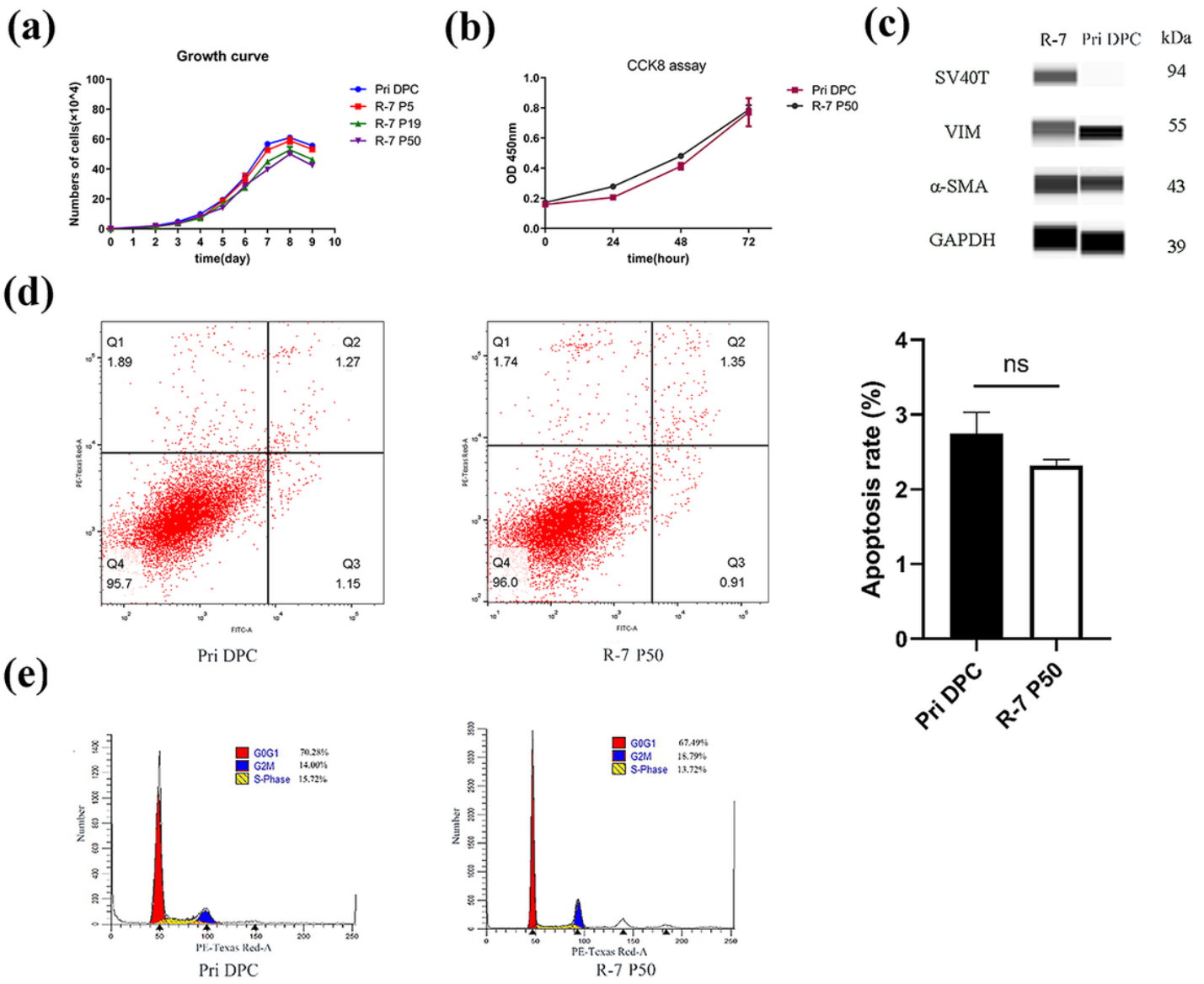

Figure 5

Cell growth activity of R-7 (passage 50) cells. (a) Growth curves of DPCs consisting of four passages, primary DPCs (passage 2) and R-7 cells (passage 5, passage 19, passage 50). (b) Primary DPCs (passage 2) and R-7(passage 50) cell proliferation estimated by CCK8 assays after 24, 48 and 72 h. (c) Western blotting of primary DPCs (passage 2) and R-7 (passage 50). (d) Cell apoptosis of primary DPCs (passage 2) and R-7 (passage 50). ns: not significant. (e) Cell cycle assays of primary DPCs and R-7. ns: not significant.

Supplementary Files

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