Gene therapy of hypoparathyroidism with TheraCyte-encapsulated stem cells

Fong-Fu Chou,MD., Shun-Chen Huang*, MD., Shun-Yu Chi,MD., Kun-Chou Hsieh, MD., Pei-Hua Hung, BA., Feng-Sheng Wang, PhD.

Department of Surgery, Department of Pathology*, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Taiwan

123, Ta-Pei Road, Niao-Sung Hsiang, Kaohsiung Hsien 833, Taiwan

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Correspondence and reprint requests: Shun-Chen Huang*MD.

Department of Pathology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Taiwan

123, Ta-Pei Road, Niao-Sung Hsiang, Kaohsiung Hsien 833, Taiwan

Tel No. 886-7-7317123 ext. 8093

Fax No. 886-7-7354309

E-mail address: choulu@ms4.hinet.net

Introduction:

Although permanent hypoparathyroidism after thyroid or parathyroid surgery rarely happens in general practice, it is a difficult clinical problem. Long-term parathyroid hormone (PTH) therapy is rarely applied nowadays because PTH has very short half life (2-4 min.) and must be injected daily [1 - 3]. Gene therapy for hypoparathyroidism through gene transfer to skeletal cells or stem cell has been previously reported [4 - 6]. Although long-term gene therapy of hypoparathyroidism with stem cells recombined with parathyroid hormone gene and retroviral vectors has been reported previously [6], we conducted this study to optimize the gene therapy of hypoparathyroidism with a stem cell-line without viral vectors.

Animals:

Male Spraque-Dawley rats (about 300 gm) were used throughout this experiment. The animals were purchased from the National Science Council animal center and housed in Chang Gung Memorial Hospital at Kaohsiung, Taiwan. This study was performed in accordance with the guidelines provided by the experimental animal laboratory and approved by the animal care and use committee. The rats could access to food and water *ad libitum* and were housed in a controlled environment at a temperature of 24±1℃, a humidity of 55±5%, and a circadian light rhythm of 12 hr. Three groups were designed as followed: parathyroidectomy treated with the

TheraCyte encapsulated $4x10^7$ cells, which were transfected with PTH (1-34) cDNA as the study group (group A, n=9); parathyroidectomy treated with the subcutaneous injection of $4x10^7$ cells, which were transfected with PTH (1-34) cDNA as the subcutaneous injection group (group B, n=9); and parathyroidectomy treated with nothing as the control group (group C, n=10).

Results

All rats in group A survived well with normal appetites and activities. Two of 9 rats in group B and also 3 of 10 rats in group C died as a result of exhaustion within one month after parathyroidectomy. Serum levels of calcium in group A were significantly higher than in both groups B and C (all $\leqq 0.003$) (Table 1). Serum levels of phosphorus in group A were significantly lower than in both groups B and C (all $p \leqq 0.002$) (Table 2). Serum levels of PTH (1-34) in group A were significantly higher than in both groups B ($p = 0.004$) and C ($p = 0.003$) (Table 3). In group A, both serum calcium and PTH (1-34) levels were significantly higher at 2, 3, and 4 months post-treatment than at baseline (0-month) (all $p \leqq 0.022$) (Table 1 and Table 3). In group A, serum phosphorus levels were lower at 1, 2, 3, and 4 months post-treatment than at baseline (all $p < 0.001$) (Table 2). Hematoxylin and eosin staining showed live cells in the TheraCyte (Fig. 1A), and immunohistochemical staining demonstrated PTH (1-34)-positive cells in the TheraCyte-encapsulated $4x10^7$ cells (Fig. 1B). No

PTH (1-34)-positive cells could be found in the subcutaneous tissue of group B (Fig. 2A, 2B). PTH (1-34) mRNA (179 bp) was detected in C3H/10T1/2 cells 48 hr after PTH (1-34) cDNA transfection by Lipofectamine and also in cells 48 hr after G148 treatment. PTH (1-34) mRNA could not be detected either in the C3H/10T1/2 cells without transfection of PTH (1-34) cDNA, nor cells after G418 treatment (Fig. 3 A,B,C,D). Before loading to the TheraCyte, many PTH (1-34)-positive C3H/10T1/2 cells survived after PTH (1-34) cDNA transfection and G418 treatment and only a few cells survived after G148 treatment without transfection (Fig. 4A, 4B).

Discussion:

Mesenchymal stem cells have been used as a carrier for conditionally replicating adenoviruses to target metastatic breast cancer in vivo [7] or as a vehicle for oncolytic adenoviruses [8,9]. Mesenchymal stem cells-mediated gene delivery of bone morphogenetic protein-2 also has been reported to induce bone formation and to heal bone fracture [10, 11]. We used the C3H/10T1/2 cell-line, which was established in 1972 from 14-and 17-day old C3H mouse embryos [12], as the gene therapy of hypoparathyroidism. These cells displayed fibroblastic morphology in cell culture and were functionally similar to mesenchymal stem cells. Inhibiting methylation with 5-azacytidine in C3H/10T1/2 cells produces cells that exhibit stable morphological and biochemical features of muscle, adipose, bone, or cartilage cells. After

transfection of PTH (1-34) cDNA into C3H/10T1/2 cells using Lipofectamine 2000, PTH (1-34) mRNA could be found in cells and they could survive after G148 treatment. TherayCyte devices consist of an inner membrane of polytetrafluoroethylene (PTFE) that is 30 μm thick and has a 0.4-μm pore size which prevents the entry of cells into the device but allows the entry of antibodies and compliment factors. The outer membrane is a laminated 15-μm-thick PTFE layer which has a 5-μm pore size that allows improved biocompatibility and induces vascularization. We used a 20-μl device to load $4x10^7$ live cells in a volume of 1 ml.

After implantation of the TheraCyte-encapsulated $4x10^7$ C3H/10T1/2 cells, which were recombined with PTH (1-34) cDNA, we found that the live cells could secrete PTH (1-34) as documented in our study of live human parathyroid cells [13]. Serum calcium and PTH (1-34) levels increased significantly 2 months later and could be maintained for up to 4 months. Serum calcium and PTH (1-34) levels in the study group were significantly higher than in the subcutaneous injection group and also higher than in the control group. Serum levels of phosphorus decrease significantly 1 month later and could be maintained for up to 4 months. Serum phosphorus levels were significantly lower in the study group than in the subcutaneous injection group and also lower than in the control group. Four months after subcutaneous injection of C3H/10T1/2 cells, which were recombined with PTH (1-34) cDNA, neither PTH

(1-34)-positive cells nor tumor cells could be found at the injection site. It was thought that C3H/10T1/2 cells transfected with PTH (1-34)-cDNA were all rejected and destroyed by the host rat after the subcutaneous injection. We therefore, conclude that stem cells can be used for gene therapy of hypoparathyroidism. After implantation of the TheraCyte-encapsulated stem cells recombined with PTH (1-34) cDNA, cells seem to function very well. The TheraCyte can prevent cells from rejection, dissemination or tumor formations.

Materials and Methods:

Plasmid DNA:

The plasmid carrying full-length human PTH (1-34) that was under the control of a human cytomegalovirus promoter (pcDNA3) was cloned. We chose pcDNA3 as a vector (Mission Biotech, Taipei, Taiwan). The PTH (1-34) gene (179 bps) was inserted into pcDNA3 (5.4 Kb) via the Hind III, Bam HI cloning sites, and competent *Escherichia coli.* cells were used to amplify the cDNA. The cDNA concentratons was measured by ultraviolet absorption at 260 nm.

Stem cells:

C3H/10T1/2 stem cells were plated at a low density, and cultured in DMEM containing 10% Fetal Bovine Serum with Antibiotic-Antimycotic (GIBCO, Grand Island, N.Y. U.S.A.) for 48-72 hr at 37℃ in an incubator containing 5% CO_2 at 75

cm^2 flasks.

Gene transfection:

C3H/10T1/2 cells were seeded at 6-well plates at 80-90 confluence in the appropriate amount of growth median without antibiotics and transfected with 2 ug/well plasmid DNA using Lipofectamine 2000 (Invitrogen, Bio Vision, Inc. Mountain View, CA), at the ratio of 1:3 (vol./vol.). After transfection for 24 hr, the cells were transferred, and cultured in DMEM containing 10% Fetal Bovine Serum with Antibiotic-Antimycotic. Forty-eight hr after transfection the transfected cells were grown in DMEM containing G418 (GIBCO, Grand Island, NY) at 30 mg/ml until all of the non-transfected cells were dead (1 week). The gene expression of stem cells was confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Live cells containing PTH (1-34) cDNA at the density of $4x10^7$ were loaded into the TheraCyte, using the centrifugation loading method according to the users' manual (TheraCyte Inc. Irvine, CA). In group A, after parathyroidectomy, the TheraCyte-encapsulated 4×10^7 live stem cells containing PTH (1-34) cDNA, were implanted to the subcutaneous tissue of rats' back. In group B, after parathyroidectomy, live stem cells containing PTH (1-34) cDNA at the density of $4x10^7$ were injected directly to the subcutaneous tissue of rats' xiphoid processes. In group C, nothing was given after parathyroidectomy.

Detection of PTH (1-34) mRNA with RT-PCR in stem cells:

Total RNA from the transfected stem cells was extracted with TRI-Zol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturers' instructions. cDNA was prepared with Superscript II Reverse Transcriptase (Invitrogen Life Technologies) and stored at -20℃. A 179 bp segment of the PTH (1-34) gene was amplified by PCR as described below.

Sequences of the primer pair of amplification of the transferred gene were 5'-CCATGTACGTAGCCATCCAG-3' and 5'-GCACGATTTCCCTCTCAGCTGT-3' (custom synthesized by Life Technologies Burlington, Ontario, Canada). Reactions were carried out under the following conditions: 94℃ for 5 min. followed by 30 cycles at 94℃ for 30 sec., 51.6℃ for 30 sec., 72℃ for 30 sec., 72℃ for 7 sec. and 4℃ to terminate the reaction. The PCR products were separated by electrophoresis on 2% agarose gels, and stained with ethidium bromide to visualize the bands. β-actin was used as an internal control.

Serum levels of calcium, phosphorus and PTH (1-34) determinations:

Blood samples were collected to determine calcium, phosphoruse and PTH (1-34) levels at baseline, 1 month, 2 months, 3 months and 4 months after treatment. Serum levels of PTH (1-34) were measured by enzyme-linked immunosorbent assay (ELISA) with a human PTH (1-34) kit (Peninsula Laboratories, San Carlos, CA). The minimal

detectable concentration was 0.04-0.06 ng/ml, and the detectable range was 0-25 ng/ml. Serum levels of calcium and phosphorus were measured by a cresophthalein complexion and phosphomolybdate UV method.

Parathyroidectomy:

Under sodium pentothal anesthesia, a longitudinal skin incision was made at the anterior part of neck. The right and left parathyroid glands were exposed and removed under microscopic examination (x16). After this procedure the rats were returned to the animal center, to recover until the experiment.

Immunohistochemical staining with PTH (1-34) antibody:

Four months after implantation, the TheraCyte-encapsulated $4x10^7$ cells was removed from the rats' back and sent for the histological examination. Four months after subcutaneous injection of $4x10^7$ cells (group B), the rats' skin and subcutaneous at xiphoid process was excised for the histological examination. After formalin-fixation, the TheraCyte and tissues were deparaffinized in xylene and dehydrated in a graded ethanol series. An endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol. After incubation of the tissue with rabbit anti-human parathyroid hormone (1-34) serum (dilated 1:6000, Phoenix Pharmaceuticals, Belmont, CA), horseradish peroxidase (Zymed Laboratories/Invitrogen, San Francisco, CA) was added, and after a wash step,

diaminobenzidine was then added. The peroxidase then catalyzed the substrate and converted the chromogen diaminobenzidine to a brown-colored deposit.

Statistical analysis was performed with the Statistical Product and Service Solution (SPSS) version 11.5 software package (SPSS, Chicago, IL). All data are expressed as mean ± SD. The paired t-test and a repeated measures of analysis of variance were used for inter-group analysis. To determine whether a group was significantly different from the others, simultaneous multiple comparison were conducted using Bonferroni techniques. A p value less than 0.05 was considered significant.

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Table 1. Serum calcium levels (mg/dl) after parathyroidectomy: at baseline and 1-month, 2-month, 3-month and 4-month after therapy in various groups.

Groups \ Month	Baseline	1-month	2-month	3-month	4-month
Group A (TheraCyte-encapsulated $4x10^7$ cells) (N=9)	4.4±0.43	5.6±0.74 ⁺	7.0±0.64#	7.9±1.10#	8.5±1.50#
Group B☆ (Subcutaneous injection of $4x10^7$ cells) (N=7)	5.0±0.82	5.1±0.97	5.2±0.51	5.7±1.07	5.1±0.44
Group C△ (Control) (N=7)	4.2±1.24	3.7±1.00	4.0±1.25	3.8±1.13	3.8±1.03

All data=mean±SD

△☆Using repeated measures ANOVA and ⁺ #paired t-test

☆P = 0.003 compared with group A

△P < 0.001 compared with group A

⁺P = 0.0012 compared with baseline

#P < 0.001 compared with baseline

Table 2. Serum phosphorus levels (mg/dl) after parathyroidectomy: at baseline and 1-month, 2-month, 3-month and 4-month after therapy in various groups.

Month / Groups	Baseline	1-month	2-month	3-month	4-month
Group A (TheraCyte-encapsulated $4x10^7$ cells) (N=9)	12.7±1.91	11.1±1.81 ⁺	10.2±0.89 ⁺	8.8±1.64 ⁺	8.4±1.94 ⁺
Group B☆ (Subcutaneous injection of $4x10^7$ cells) (N=7)	12.5±1.70	11.9±1.57	12.3±1.33	12.6±1.61	13.3±2.60
Group C△ (Control) (N=7)	11.4±1.38	13.2±1.01	12.4±1.78	13.4±1.72	12.7±1.79

All data=mean±SD

☆△Using repeated measures ANOVA and ⁺paired t-test

☆P=0.002 compared with group A

△P=0.001 compared with group A

⁺P<0.001 compared with baseline

Table 3. Serum PTH (1-34) (ng/ml) levels after parathyroidectomy: at the baseline and 1-month, 2-month, 3-month and 4-month after therapy in various groups.

Groups \ Month	Baseline	1-month	2-month	3-month	4-month
Group A (TheraCyte-encapsulated $4x10^7$ cells) (N=9)	0.13±0.03	0.39±0.48 [+]	2.1±2.07[#]	3.1±2.644[##]	2.9±2.556[###]
Group B[☆] (Subcutaneous injection of $4x10^7$ cells) (N=7)	0.13±0.037	0.15±0.049	0.28±0.277	0.21±0.140	0.20±0.152
Group C[△] (Control) (N=7)	0.12±0.029	0.11±0.026	0.10±0.04	0.13±0.045	0.11±0.019

All data=mean±SD

[☆△]Using repeated measures ANOVA and [+] [#] [##] [###]paired t-test

[☆]P=0.004 compared with group A

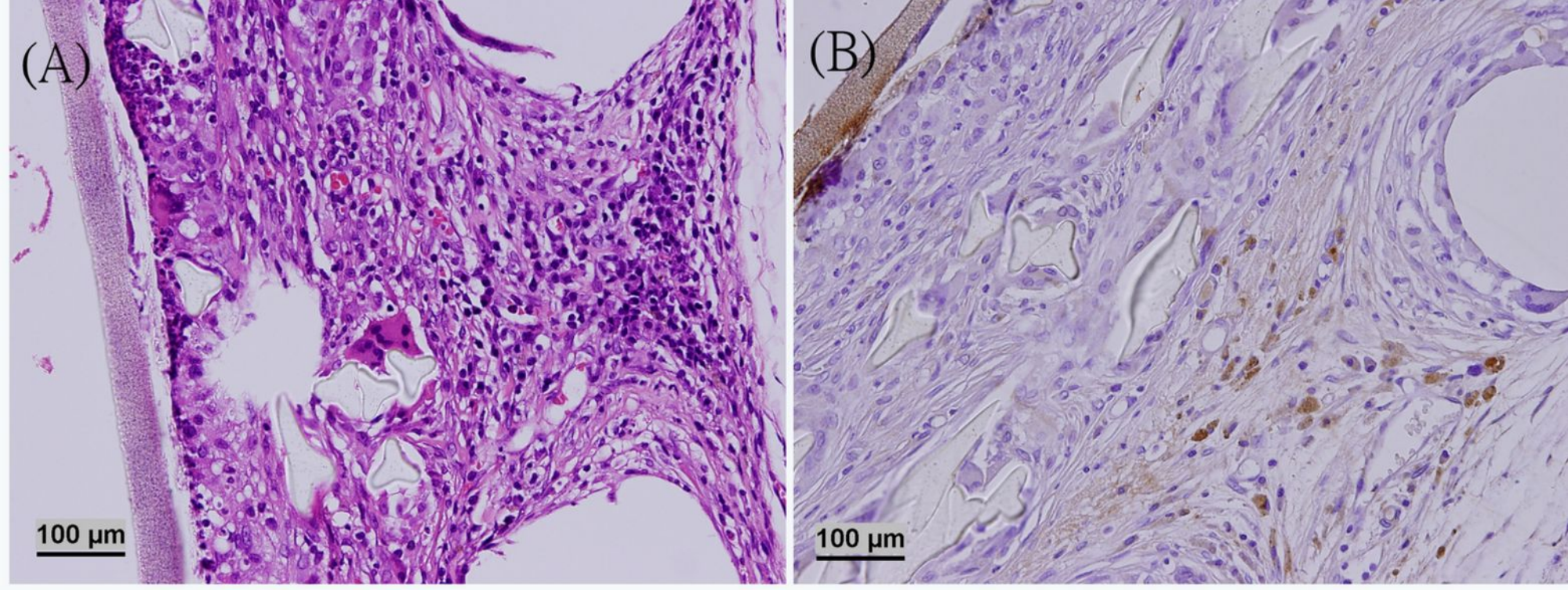
[△]P=0.003 compared with group A

[+] P=0.119 compared with baseline

[#]P=0.022 compared with baseline

[##]P=0.010 compared with baseline

[###]P=0.011 compared with baseline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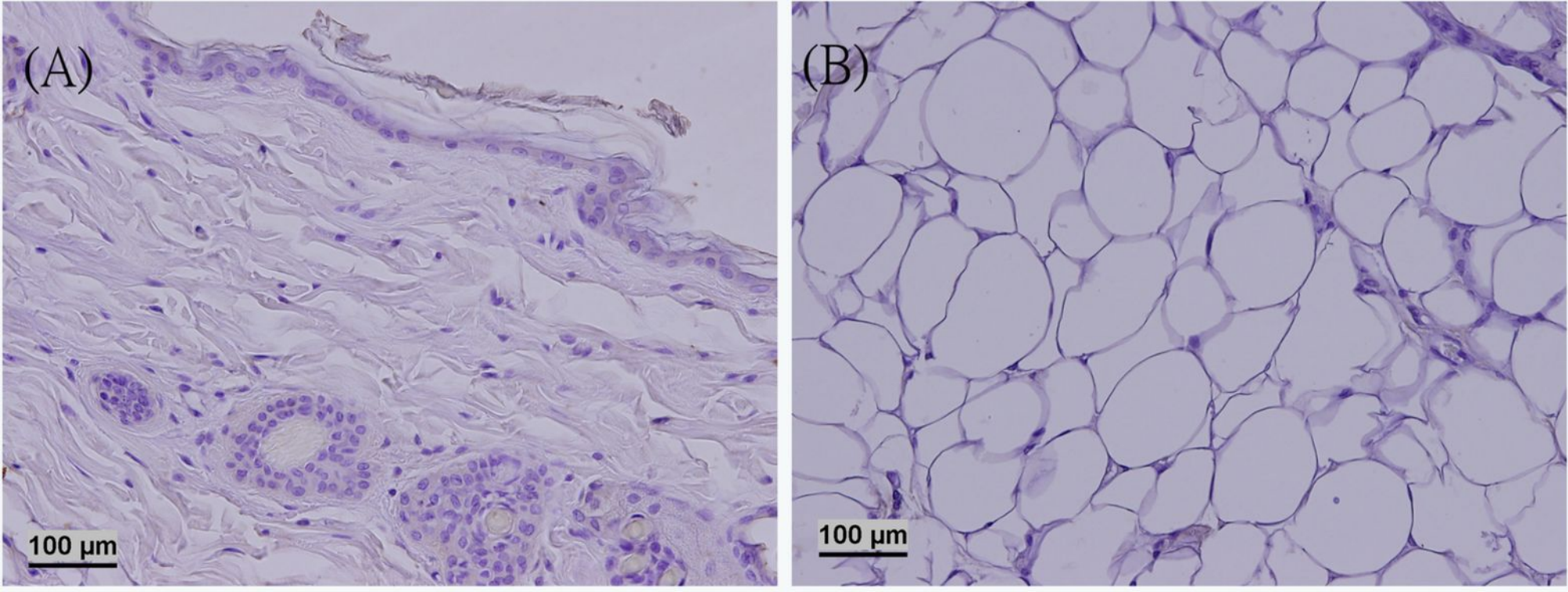
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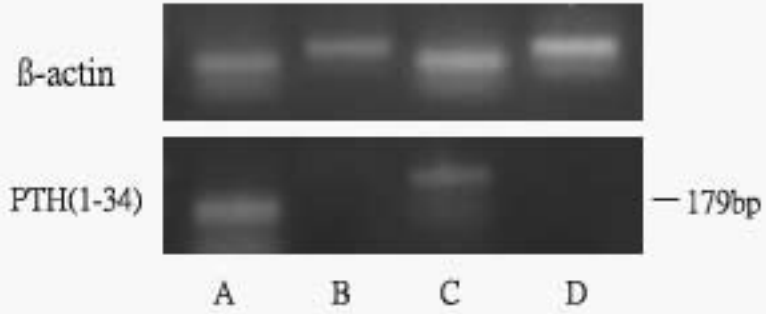
ß-actin
PTH(1-34)
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A
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C
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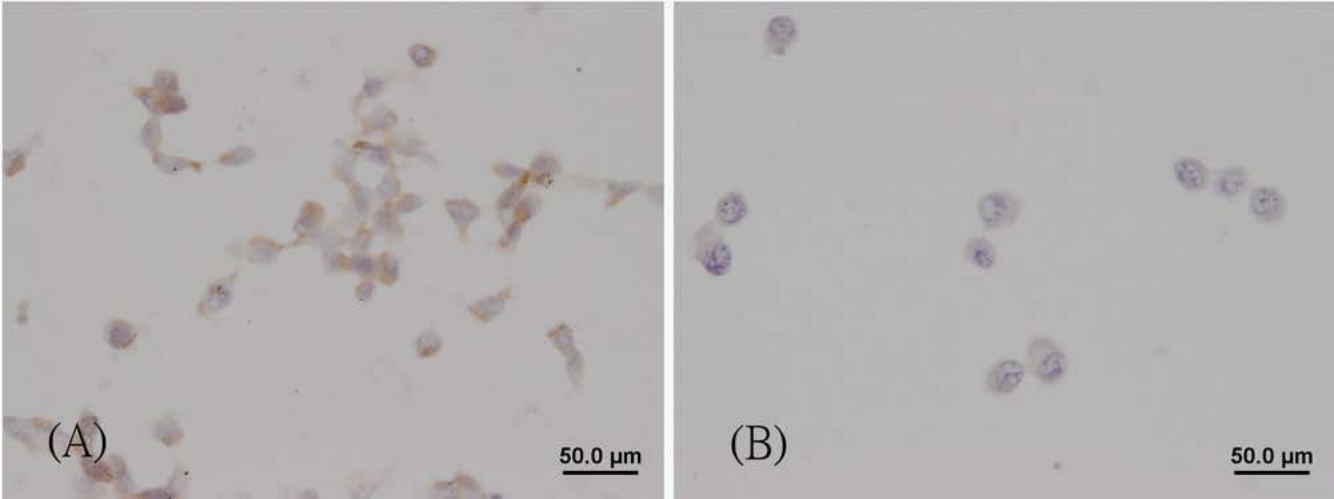
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