MicroRNA let-7b-regulated epidermal stem cell proliferation in hypertrophied anal papillae

HONG LU1,2*, XIANG HE1*, QINGMING WANG3, DE ZHENG1, YE HAN1, WEI YANG1 and TE LIU2

1Department of Anorectal Dermatology, Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 201203; 2Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200031, P.R. China

Received July 19, 2014; Accepted April 10, 2015

DOI: 10.3892/mmr.2015.4017

Abstract. The present study investigated the role of epidermal stem cell-expressed microRNA let-7b in the pathogenesis of hypertrophied anal papillae. Hypertrophied anal papillae were examined for the presence of epidermal stem cells. Epidermal stem cells were identified using flow cytometry and immuno-fluorescent staining for the cell surface markers, integrin α6 and integrin β1 subunits. Expression levels of microRNA let-7b in α6+/β1+ and α6-/β1-cells were compared using reverse transcription-quantitative polymerase chain reaction and northern blotting. Lentivirus-mediated expression of microRNA let-7b in epidermal stem cells was utilized in order to study the effects of this microRNA on the cell cycle proteins, cyclin D1 (CCND1) and cyclin-dependent kinase 4 (CDK4). MicroRNA let-7b-overexpressing cells were examined using flow cytometry, in order to determine the effects of the microRNA on cell cycle progression. α6+/β1+epidermal stem cells were identified in hypertrophic anal papillae. Following isolation and enrichment of the α6+/β1+ population, these cells were found to have a rapid rate of proliferation in vitro. The expression of cell cycle-related proteins was elevated in this population, compared with that in α6-/β1-cells. The expression of microRNA let-7b in α6+/β1+epidermal stem cells was significantly lower than that in α6-/β1-cells. Two microRNA let-7b target genes, CCND1 and CDK4, were found to be upregulated in α6+/β1+cells. When the exogenous precursor, microRNA let-7, was overexpressed in α6+/β1+ epidermal stem cells, the cell proliferation rate was significantly lower than that in cells expressing microRNA let-7 containing a mutated seed sequence. The addition of exogenous microRNA let-7 resulted in an increased expression level of mature microRNA let-7b, while the expression of CCND1 and CDK4 was reduced. Epidermal stem cells transfected with microRNA let-7b were arrested in the G2/M phase and the percentage of cells in S-phase was significantly reduced. In conclusion, let-7b expression results in upregulation of the cell cycle-related proteins, CCND1 and CDK4, resulting in the excessive proliferation that leads to the formation of hypertrophic anal papillae.

Introduction

Anal papillomas are common, benign anal cysts. Previous studies have indicated that hypertrophied anal papillae arise as a result of proliferative inflammatory disease (1-3). Hypertrophied anal papillae are, in essence, skin tags that project up from the dentate line, or from the junction between the skin and the epithelial lining of the anus. They are often found as part of the classic triad of a chronic fissure; namely the fissure itself, with hypertrophied papilla above and a skin tag below (1-3). However, the mechanisms underlying the development of human hypertrophied anal papillae are poorly understood.

Epidermal stem cells (EpSCs) are skin tissue-specific adult stem cells with a strong proliferative capacity, which undergo asymmetric division (4-7). Following induction, EpSCs are able to differentiate into a variety of epidermal lineages in order to promote self-renewal and regeneration of the epidermis, and to promote wound healing (5,6,8,9). Proliferation and differentiation of EpSCs may be regulated by a number of factors, including the stem cell microenvironment, or cellular factors and cell surface receptors (5,6). Previous studies have found that the integrin family of proteins has a significant effect on EpSC proliferation and differentiation. Integrins have been utilized for the isolation and enrichment of EpSCs from tissues in vitro (5,6,8-10). Integrins are cell surface receptors composed of α and β subunits. To date, 18 different α subunits and 8 β subunits have been identified that may be combined to
form a total of 24 different integrin receptors in mammals (9). The N-terminal region of the α subunit forms a domain that binds divalent cations and contains a highly conserved sequence, ‘KXGFFKR’, which is proximal to the cytoplasmic membrane and is involved in regulation of integrin activity (9). β1 integrins form the largest subgroup of integrins. The 12 members of this group bind a variety of ligands. α1β1, α2β1, α10β1 and α11β1 primarily interact with collagen, an interaction that is conducive to cell proliferation. α1β1, α2β1, α3β1, α6β1 and α7β1 interact primarily with laminins, which are involved in adhesion to the basement membrane. α4β1, α5β1, α8β1 and αβ1 bind fibronectin, and α9β1 binds tenascins (9). In the intact epidermis, integrin expression occurs in the basal layer and outer root sheath of hair follicles. β1 expression is predominantly confined to regions of the hair follicle bulge and epidermal progenitors, while α6 integrin expression occurs in the outer root sheath of hair follicles and the outer-most basal layer of the interfollicular epithelium, which is composed of hemidesmosomes. Integrins α6 and β1 are used as molecular biomarkers of EpSCs (9,10).

MicroRNAs (miRNAs) are a recently discovered class of naturally occurring, single-stranded, 21-23 nucleotide, non-coding RNAs (11,12), which exist in a wide range of eukaryotic organisms (11-16). Each mammalian miRNA may prevent the translation of a number of downstream target mRNAs, which ultimately results in the inhibition of target gene expression (17-20). let-7 is a well-studied miRNA, known to be involved in cell cycle regulation and development, which is underexpressed in various cancers (21). Restoration of let-7 expression has been found to inhibit cancer growth by targeting various oncopgenes and inhibiting key regulators of numerous mitogenic pathways (21-24). Yu et al (24) found that let-7 suppressed self-renewal and tumorigenicity of breast cancer cells by reducing H-RAS and high-mobility group AT-hook 2 (HMGA2) expression. Furthermore, Schultz et al (22) reported that let-7b, a member of the let-7 miRNA family, interfered with the proliferation and growth of primary malignant melanoma cells by targeting and suppressing important cell cycle molecules, such as cyclin D1 (CCND1). In addition, Dangi-Garimella et al (23) revealed that elevated let-7 expression inhibited HMGA2 expression and suppressed metastasis in breast cancer cells.

The present study aimed to establish the role of miRNA let-7b in regulation of integrin α6/β1+EpSC proliferation and define its role in the formation of human hypertrophied anal papillae.

Materials and methods

Patients and ethics. Hypertrophic anal papilla tissue samples were obtained during surgery from five patients who had been diagnosed with mixed hemorrhoids or anal fistula. Two patients (1 male and 1 female) had archosyrinx, and three patients (2 males and 1 female) had mixed hemorrhoids. The median age of this population was 39 years old. All human materials were obtained from the Department of Anorectal Dermatology (Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China). All of the patients in the present study provided written informed consent. The study was approved by the ethics committee of Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Isolation of integrin α6 and integrin β1 phenotype cells by a magnetic activated cell sorting system. Integrin α6 and Integrin β1 subpopulation cells were isolated from primary cells from hypertrophic anal papilla tissues, using 4 µl primary monoclonal antibody (rabbit anti-human Integrin α6-FITC, rabbit anti-human Integrin β1-PE, eBioscience, Inc., San Diego, CA, USA), stored at 4°C in phosphate-buffered saline (PBS) for 30 min in a volume of 1 ml, as previously described (4,25). Following this reaction, the cells were washed twice in PBS, and goat anti-rabbit secondary monoclonal antibodies conjugated to magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) were added, incubated at 10°C in PBS for 15 min and then washed twice in PBS. Single cells were plated at 1,000 cells/ml in Dulbecco's modified Eagle's medium (DMEM:F12; HyClone Laboratories, Inc., Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin and 0.5% bovine serum albumin (BSA; all Sigma-Aldrich, St. Louis, MO, USA). All cells had been cultured under the same conditions until passage three, prior to subsequent experiments.

Recombinant lentivirus second generation vector construction. All steps of recombinant lentivirus packaging were conducted as previously described (17,20). An RNAi pLL3.7 (LentiLox 3.7) retroviral system was used to create lentiviral virus vectors (GenePharma Co., Ltd., Shanghai, China). For vector pLL3.7-miR-let-7 (pre-miRNA let-7), an oligonucleotide pair for pre-miRNA of microRNA let-7, and linker sequences with HpaI and XhoI sites were chemically synthesized. The following oligonucleotides were used: Bottom strand, 5'-CGGGGGCGAGTGGAGGATAGGTTGTGGTTTCGAGGGCATGTA TGTTGGCCCTCCGGAGAATACTATACAACCTACTGGCTTCCCTGCCTGCGAGC-3'. Sequences corresponding to microRNA let-7 seed sequences are represented in capitalized and bold characters, and restriction enzyme sites are represented in lower case and bold characters. In order to produce the expression plasmid, the pairs of oligonucleotides were annealed as follows: Denaturation at 95°C for 5 min; annealing at 58°C for 45 min, then the oligonucleotides were inserted into the multiple cloning sites between the HpaI and XhoI sites in the pLL3.7 vector. The negative control plasmid, pLL3.7-miR-Mut, was constructed in a similar manner, with the exception that 22 nucleotides in sequences corresponding to microRNA let-7 seed sequences were mutated (TGAGGGATAGGTTGTGGTTGTGTTT changed to TGTCCCTAGAGCATGAGCATG). The pLL3.7-miR-let-7 and pLL3.7-miR-Mut vectors were then recombined in the package cell lines, 293T, in order to create lentiviruses. Recombinant viruses were propagated in 293T cells, and purified, and titered using standard methods, as described previously (17,20). The corresponding viruses were termed Ldv-miR-let-7 or Ldv-miR-Mut. Cotransfection of EpSCs was conducted with 4x10^5 PFU/ml Ldv-miR-let-7 or Ldv-miR-Mut lentivirus, respectively, according to the manufacturer's instructions. Cells were seeded in a 6-well plate in
DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone Laboratories, Inc.), at 37°C in a humidified atmosphere of air with 5% CO₂, until they reached 80% confluence.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from was isolated using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were treated with Dnase I (Sigma-Aldrich) and reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis kit (Toyobo, Co., Ltd., Osaka, Japan). RT-qPCR was conducted using a RealPlex4 real-time PCR detection system, obtained from Eppendorf Co., Ltd. (Hamburg, Germany), with SyBR Green RealTime PCR Master Mix (Toyobo, Co., Ltd.) used as the detection dye. RT-qPCR amplification was performed over 40 cycles with denaturation at 95°C for 15 sec and annealing at 58°C for 45 sec. Target cDNA was quantified using the relative quantification method (20,25). A comparative threshold cycle (Ct) was used to determine gene expression relative to that of a control (calibrator), and steady-state mRNA levels were reported as an n-fold difference relative to the calibrator. For each sample, the maker genes Ct values were normalized using the following formula: \( \Delta Ct = Ct(\text{gene}) - Ct(18S\text{rRNA}) \). In order to determine relative expression levels, the following formula was used: \( \Delta \Delta Ct = \Delta Ct(\text{Ldv - miR-let-7 group}) - \Delta Ct(\text{Ldv-miR-Mut group}) \). The values used to plot relative expressions of markers, were calculated using the expression 2^{−\Delta \Delta Ct}. The mRNA levels were calibrated based on levels of 18S rRNA. The cDNA of each stem cell markers was amplified using the primers shown in Table I.

Western blotting analysis. Protein extracts were resolved using 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Bedford, MA, USA). The PVDF membranes were blocked with WB blocking solution (Beyotime Biotechnology Co., Ltd, Shanghai, China) for the detection of 22-nucleotide fragments, according to the manufacturer’s instructions. After washing, membranes were exposed to a Kodak XAR-5 film (Sigma-Aldrich) at 1,000 x paraformaldehyde (Sigma-Aldrich) for 30 min. Following blocking, the cells were incubated overnight at 4°C with primary antibodies as follows: Rabbit anti-human p53 polyclonal antibody (cat. no. 12571), rabbit anti-human CCND1 polyclonal antibody (cat. no. 3300), rabbit anti-human CDK4 polyclonal antibody (cat. no. 12790), rabbit anti-human E-cadherin polyclonal antibody (cat. no. 3195), rabbit anti-human CLDN1 polyclonal antibody (cat. no. 4933), rabbit anti-human GAPDH polyclonal antibody (cat. no. 5174), all at 1:1,000 (Cell Signaling Technology, Inc.) and then incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:200; Abcam, Cambridge, UK) and 5 mg/ml DAPI (Sigma-Aldrich) at room temperature for 30 min. The cells were then thoroughly washed with TBST and viewed through a fluorescence microscope (DM13000; Leica, Allendale, NJ, USA).

Northern blotting. Northern blotting was conducted as previously described (17,20). For all cell treatment groups, 20 µg of total RNA was analyzed on a 7.5 M urea, 12% PAA denaturing gel and transferred to a Hybond N+nylon membrane (Amersham, Freiburg, Germany). Membranes were cross-linked using ultraviolet light for 30 sec at 1:1,000 (Cell Signaling Technology, Inc.) and then incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:200; Abcam, Cambridge, UK) and 5 mg/ml DAPI (Sigma-Aldrich) at room temperature for 30 min. The cells were then thoroughly washed with TBST and viewed through a fluorescence microscope (DM13000; Leica, Allendale, NJ, USA).

Flow cytometric (FCM) analysis of the cell cycle by PI staining. Each group of cells were seeded at 3x10^5 per well in 6-well plates and cultured until they reached 85% confluence. Each group of cells was washed three times with PBS, then collected by centrifugation (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 1,000 x g for 5 min. The cell pellets were

| Table I. Primers used for RT-qPCR. |
| Gene | RT-qPCR primers (5'→3') |
| p53 | F: GCTTTCACGACGGTGAC R: GCTCGACGCTAGGATCTGAC |
| CCND1 | F: TCCCTCTCAAAATGCCAGAG R: GCGGATTGGAATGAATCTT |
| CDK4 | F: TCGAGTCCACATAGCAACA R: GTCCGCTTCAGAGTTTCCAC |
| 18S rRNA | F: CAGCCACCCGAGATTGAGCA R: TAGTACGCGGCGGTTGTG |
| Let-7b | F: TGAGTATAGTGTTGTGTTGTT R: GCTGTCACAGATACGCCTACCTA |
| RT-qPCR, reverse transcription-quantitative polymerase chain reaction; F: forward; R: reverse; CCND1, cyclin D1; CDK4, cyclin-dependent kinase 4. |
the resuspended in 1 ml of PBS, fixed in 70% ice-cold ethanol, and maintained in a freezer for >48 h. Prior to FCM analysis, the fixed cells were centrifuged, washed twice with PBS, and resuspended in PI staining solution (Sigma-Aldrich) containing 50 µl/ml PI and 250 µg/ml RNase A (Sigma-Aldrich). The cell suspension, which was maintained in darkness, was incubated for 30 min at 4°C and analyzed using the FACS (FACSAria, BD Biosciences, San Jose, CA, USA). A total of 20,000 events were acquired for analysis using CellQuest software version 2.1.0.

Statistical analysis. Each experiment was performed as least three times, and data are presented as the mean ± standard error where applicable. Differences were evaluated using Student’s t-test and P<0.05 was considered to indicate a statistically significant difference. GraphPad Prism version 5.0 was also used for statistical analyses.

Results

α6+β1+EpSCs are present at low levels in hypertrophic anal papillae. The anal papillae examined in this study arose due to chronic inflammation caused by fibrous connective tissue hyperplasia. The anal papillae were cylindrical or walnut-shaped, with a large upper part and smaller lower part. The smooth surface was milky white, and no bleeding was observed (Fig. 1A and B). Pathological examination demonstrated significant endothelial hyperplasia in the anal papillae, accompanied by infiltration of inflammatory cells and vascular proliferation, although no cell heterogeneity was observed (Fig. 1C). A magnetic-activated cell sorting system was used to isolate and enrich the α6+β1+ subpopulation from the hypertrophied anal papillae. Following isolation, cells were quantified using FCM. α6+β1+EpSCs represented 0.27±0.02% of the total population in five primary samples, whereas α6-/β1-cells represented 59.51±8.31% of the total population (Fig. 1D-F). These results demonstrated that α6+β1+ epidermal stem cells (EpSCs), although occurring at a low frequency, may be successfully enriched using magnetic-activated cell sorting.

α6+β1+EpSCs express epidermal stem cell markers. FCM and immunofluorescent (IF) staining were used to compare the relative expression levels of epidermal stem cell markers in α6+β1+EpSCs and α6-/β1-cells. The IF assay confirmed that α6+β1+EpSCs expressed higher levels of the epidermal stem cell markers, integrin α6 and β1 (Fig. 2A). FCM also demonstrated that the expression of integrin α6, β1 and β-catenin was significantly higher in α6+β1+EpSCs than that in α6-/β1-cells (Fig. 2B; Table II). These results demonstrate that the α6+β1+ subpopulation possesses epidermal stem cell characteristics.

Table II. Assessment of EpSC marker expression using FCM.

| Biomarkers    | α6-/β1-cells (n=5) | α6+β1+EpSCs (n=5) |
|---------------|--------------------|-------------------|
| Integrin α6   | 1.155±0.015%       | 60.645±4.955%     |
| Integrin β1   | 1.025±0.035%       | 63.450±7.789%     |
| β-catenin     | 1.080±0.079%       | 56.905±2.965%     |

EpSC, epidermal stem cell; FCM, flow cytometry.

α6+β1+EpSCs proliferate rapidly and express low levels of miRNA let-7. The proliferation rates of α6+β1+EpSCs and α6-/β1-cells were examined for up to 72 h following passaging. Measurements were repeated in quintuplicate. No significant differences were observed in the total number of cells between the two groups at 0-12 h (Fig. 3A). However, between 24-72 h, α6+β1+EpSCs were found to divide significantly more rapidly than α6-/β1-cells (Table III). The miRNA RT-qPCR assay
Figure 2. Analysis of EpSC expression of integrin α6+β1+. (A) Immunofluorescent staining indicated that integrin α6+β1+EpSCs expressed high levels of integrin α6 and β1. (B) FCM analysis of EpSC expression of integrin α6+β1+. Expression of EpSC markers was higher in integrin α6+β1+EpSCs than that in integrin α6−β1-cells. EpSC, epidermal stem cell; FCM, flow cytometry; FITC, fluorescein isothiocyanate.

Figure 3. α6+β1+EpSCs proliferated more rapidly and expressed lower levels of microRNA let-7b than α6−β1-cells. (A) The proliferation rates of α6+β1+EpSCs and α6−β1-cells were examined 12-72 h after passaging. Between 24-72 h, α6+β1+EpSCs divided significantly more rapidly than α6−β1-cells. (B) An miRNA RT-qPCR assay demonstrated that expression of miRNA let-7b was markedly lower in α6+β1+EpSCs at 72 h compared with that in α6−β1-cells. (C) Northern blot analysis revealed strong pre-miRNA let-7b and mature microRNA let-7b hybridization signals in α6−β1-cells at 72 h compared with those α6+β1+EpSCs. (D) RT-qPCR demonstrated that the mRNA expression of p53 was markedly lower in α6+β1+EpSCs at 72 h compared with that in α6−β1-cells. By contrast, mRNA expression of the cell cycle-related factors, CCND1 and CDK4, was significantly higher in α6+β1+EpSCs at 72 h, compared with that in α6−β1-cells. (E) Western blotting confirmed that p53 protein expression was significantly reduced in α6+β1+EpSCs. The expression of CCND1 and CDK4 proteins was significantly elevated in α6+β1+EpSCs. GAPDH served as a loading control (*P<0.01, vs. α6−β1-cells and *P<0.05, vs. α6−β1-cells; n=3). EpSCs, Epidermal stem cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCND1, cyclin D1; CDK4, cyclin-dependent kinase 4.
showed that the expression of miRNA let-7b was markedly lower in α6+/β1+EpSCs at 72 h than that in α6-/β1-cells (Fig. 3B). Northern blot analysis demonstrated strong let-7b pre-miRNA and mature miRNA hybridization signals in α6-/β1-cells at 72 h, compared with levels in α6+/β1+EpSCs (Fig. 3C). RT-qPCR and western blotting were used to determine difference in the expression of cell cycle-related proteins in the different groups of cells. RT-qPCR showed that mRNA levels of p53, a protein involved in apoptosis, were markedly lower in α6+/β1+EpSCs at 72 h compared with levels in α6-/β1-cells (Fig. 3D and E). By contrast, mRNA levels of the cell cycle-related proteins, CCND1 and CDK4, were markedly lower in α6+/β1+EpSCs transfected with miR-Mut than that in α6+/β1+EpSCs transfected with miR-Mut. These results were confirmed by western blotting (Fig. 3E). These results indicate that miRNA let-7b expression is reduced in α6+/β1+EpSCs, stimulating the expression of cell cycle-related proteins and the proliferation of these cells.

Overexpression of exogenous miRNA let-7b decreases the proliferation rate of α6+/β1+EpSCs by interfering with...
CCND1 expression. In order to determine whether the addition of exogenous miRNA let-7b influences α6/β1+EpSC proliferation, recombinant lentiviruses expressing miR-let-7b or miR-Mut were transfected into α6/β1+EpSCs. Following expression of miRNA let-7b, no significant differences in the number of cells were detected between the two groups from 0-24 h (Fig. 4A). However, between 48-72 h, α6/β1+EpSCs transfected with miR-let-7b divided significantly less rapidly than α6/β1+EpSCs transfected with miR-Mut (Table III). The effects of the transfected miRNA on mRNA and protein expression were detected by RT-qPCR and northern blotting, and western blotting, respectively. The RT-qPCR results demonstrated that mRNA expression of p53 was markedly higher in α6/β1+EpSCs transfected with miR-let-7b than that in α6/β1+EpSCs transfected with miR-Mut (Fig. 4B). By contrast, mRNA expression of the cell cycle-related proteins, CCND1 and CDK4, was lower in α6/β1+EpSCs transfected with miR-let-7b than that in α6/β1+EpSCs transfected with miR-Mut (Fig. 4B). The western blotting results confirmed that p53 protein expression was significantly increased in α6/β1+EpSCs transfected with miR-let-7b compared with that in α6/β1+EpSCs transfected with miR-Mut (Fig. 4C). The expression of the CCND1 and CDK4 proteins was significantly decreased in α6/β1+EpSCs transfected with miR-Mut compared with that in α6/β1+EpSCs transfected with miR-Mut (Fig. 4C). Northern blotting demonstrated a strong let-7b hybridization signal in α6/β1+EpSCs transfected with miR-let-7 compared with the signal in α6/β1+EpSCs transfected with miR-Mut (Fig. 4D). Furthermore, FCM demonstrated significant cell cycle arrest in α6/β1+EpSCs transfected with miR-let-7b. Compared with α6/β1+EpSCs transfected with miR-Mut, α6/β1+EpSCs transfected with miR-let-7 were arrested in the G2/M phase, and the percentage of S-phase cells in this group was significantly decreased (Fig. 4E). These results indicate that proliferation of the α6/β1+EpSC subpopulation decreased when the expression of cell cycle-related proteins was suppressed by the addition of exogenous let-7b miRNA.

Discussion

Clinically, anal papillae may result in increases in local secretions, blood in the feces and anal itching (1-3). However, the pathogenesis of hypertrophic anal papillae remains unclear. The present study examined the mechanism underlying the development of hypertrophic anal papillae, with respect to the presence of EpSCs and miRNA-mediated epigenetics. In clinical practice, the growth of anal papilla in certain patients is rapid, and regrowth of new anal papilla tissue occurs in <1 year following surgical removal (1-3). It was hypothesized that the presence of cellular growth factors or stem cells, promoted cell proliferation and frequent regeneration. Due to the anatomical sites of anal papilla, it was hypothesized that EpSCs may be involved. There are various types of stem cells in the EpSC family, including follicular stem cells, hair follicle stem cells and sebaceous isthmus precursor stem cells (5,6). EpSCs exhibit three basic stem cell characteristics, which are proliferation, self-renewal, as well as the ability to differentiate into keratinocytes (5,6). In mammals, EpSCs produce a large number of mature skin cells in order to replace the epidermal and hair loss that occurs on a daily basis, and they exhibit a high metabolism. The skin is one of the most important regenerative organs in the body, and requires the rapid proliferation and differentiation potential of EpSCs. The present study found that α6/β1+EpSCs cells were present in patients with hypertrophic anal papillae. Following isolation and enrichment of these α6/β1+EpSCs, it was found that they exhibited a rapid rate of proliferation in vitro, and expressed elevated levels of cell cycle-related proteins. These results suggest that EpSCs are present in human hypertrophied anal papillae and that an increased rate of proliferation of these cells may be one of the causes of anal papilla hyperplasia.

There are a variety of mechanisms involved in the regulation of cell proliferation. Epigenetic regulation of transcription and of the expression of cell cycle-related genes is an area of current research. The miRNA, let-7b, is involved in the regulation of cell proliferation. The present study explored whether miRNA let-7b is involved in hypertrophic anal papilla hyperplasia. let-7b is a well-studied miRNA that is known to be involved in regulation of the cell cycle and development, and is underexpressed in various types of cancer (21). Restoration of normal let-7b expression has been shown to inhibit cancer growth by targeting various oncogenes and inhibiting the key regulators of a number of mitogenic pathways (21-24). The present study found that α6/β1+EpSC expression of endogenous microRNA let-7b was significantly lower than that in α6/β1-cells. Furthermore, target genes of miRNA let-7b, including the cell cycle regulatory factors, CCND1 and CDK4, were upregulated in α6/β1+EpSCs compared with α6/β1-cells. When the exogenous precursor microRNA let-7 was overexpressed in α6/β1+EpSCs, it was demonstrated that the proliferation of α6/β1+EpSCs was significantly less rapid than EpSCs transfected with miR-Mut. The expression of mature microRNA let-7b was significantly higher, while that of cyclin CCND1 and CDK4 was reduced. Compared with α6/β1+EpSCs transfected with miR-Mut, α6/β1+EpSCs transfected with miR-let-7b were arrested in the G2/M phase, and the percentage of S-phase cells in this group was significantly reduced. These data indicate that proliferation of the α6/β1+EpSC subpopulation was decreased when the level of endogenous cell cycle-related proteins was suppressed by overexpression of exogenous miRNA let-7b.

In conclusion, the present data suggest that two important mechanisms contribute to the development and recurrence of hypertrophic anal papillae. The first is hyperproliferation of EpSCs. The second is the expression of miRNA let-7b, which modulates expression of the cell cycle-related proteins, CCND1 and CDK4, and contributes to excessive cellular proliferation.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (grant nos. 81202811 and 81403401), the Project funded by China Postdoctoral Science Foundation (grant no. 2014M550250), Shanghai Municipal Health Bureau Fund (grant no. 20124320), Shanghai Municipal Health Bureau Fund (grant no. 20124Y013) and Shanghai ‘Xing Lin Xin Xing’ Fund (grant no. ZYSNXD011-RC-XLX X-20130025).
References

1. Gupta PJ: Hypertrophied anal papillae and fibrous anal polyps, should they be removed during anal fissure surgery? World J Gastroenterol 10: 2412-2414, 2004.

2. Gupta PJ and Kalaskar S: Removal of hypertrophied anal papillae and fibrous anal polyps increases patient satisfaction after anal fissure surgery. Tech Coloproctol 7: 155-158, 2003.

3. Gupta PJ: A study of the symptomatology of hypertrophied anal papillae and fibrous anal polyps. Bratisl Lek Listy 106: 30-33, 2005.

4. Reisi S, Esmaeili F and Shirazi A: Isolation, culture and identification of epidermal stem cells from newborn mouse skin. In vitro Cell Dev Biol Anim 46: 54-59, 2010.

5. Janes SM, Lowell S and Hutter C: Epidermal stem cells. J Pathol 197: 479-491, 2002.

6. Barthel R and Aberdam D: Epidermal stem cells. J Eur Acad Dermatol Venereol 19: 405-413, 2005.

7. Chen S, Takahara M, Kido M, et al: Increased expression of an epidermal stem cell marker, cytokeratin 19, in cutaneous squamous cell carcinoma. Br J Dermatol 159: 952-955, 2008.

8. Luis NM, Morey L, Mejetta S, et al: Regulation of human epidermal stem cell proliferation and senescence requires polycomb-dependent and independent functions of Cbx4. Cell Stem Cell 9: 233-246, 2011.

9. Watt FM: Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J 21: 3919-3926, 2002.

10. Nanba D, Toki F, Matushita N, Matushita S, Higashiyama S and Barrandon Y: Actin filament dynamics impacts keratinocyte stem cell maintenance. EMBO Mol Med 5: 640-653, 2013.

11. Sumazin P, Yang X, Chiu HS, et al: An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. Cell 147: 370-381, 2011.

12. Poultton JS, Huang YC, Smith L, et al: The microRNA pathway regulates the temporal pattern of Notch signaling in Drosophila follicle cells. Development 138: 1737-1745, 2011.

13. Lei P, Li Y, Chen X, Yang S and Zhang J: Microarray based analysis of microRNA expression in rat cerebral cortex after traumatic brain injury. Brain Res 1284: 191-201, 2009.

14. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.

15. Yoo AS, Sun AX, Li L, et al: MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476: 228-231, 2011.

16. Dai Y, Qiu Z, Diao Z, et al: MicroRNA-155 inhibits proliferation and migration of human extracellular trophoblast derived HTR-8/SVneo cells via down-regulating cyclin D1. Placenta 33: 824-829, 2012.

17. Liu T, Shen D, Xing S, et al: Attenuation of exogenous angiotensin II stress-induced damage and apoptosis in human vascular endothelial cells via microRNA-155 expression. Int J Mol Med 31: 188-196, 2012.

18. He L and Hannon GJ: MicroRNAs: Small RNAs with a big role in gene regulation. Nat Rev Genet 5: 522-531, 2004.

19. El Ouassaari A, Baroukh N, Martens GA, Lebrun P, Pipeleers D and van Obberghen E: miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. Diabetes 57: 2708-2717, 2008.

20. Liu T, Cheng W, Huang Y, Huang Q, Jiang L and Guo L: Human amniotic epithelial cell feeder layers maintain human iPS cell pluripotency via inhibited endogenous microRNA-145 and increased Sox2 expression. Exp Cell Res 318: 424-434, 2012.

21. Barh D, Malhotra R, Ravi B and Sindurapu P: MicroRNA let-7: An emerging next-generation cancer therapeutic. Curr Oncol 17: 70-80, 2010.

22. Schultz J, Lorenz P, Gross G, Ibrahim S and Kunz M: MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res 18: 549-557, 2008.

23. Dangi-Garimella S, Yun J, Eves EM, et al: Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7. EMBO J 28: 347-358, 2009.

24. Yu F, Yao H, Zhu P, et al: Let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131: 1109-1123, 2007.

25. Liu T, Huang Y, Guo L, Cheng W and Zou G: CD44+/CD105+ human amniotic fluid mesenchymal stem cells survive and proliferate in the ovary long-term in a mouse model of chemotherapy-induced premature ovarian failure. Int J Med Sci 9: 592-602, 2012.