Effects of recombinant human parathyroid hormone (1-34) on cell proliferation, chemokine expression and the Hedgehog pathway in keratinocytes

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Abstract. Psoriasis is an autoimmune disease involving the excessive proliferation of keratinocytes mediated by T-cells. Parathyroid hormone (PTH) has been identified as an essential factor in the treatment of psoriasis. In the present study, the mechanism underlying the effect of recombinant human parathyroid hormone (rhPTH) (1-34) in keratinocytes was investigated. The effects of rhPTH (1-34) on cell proliferation, cell cycle, and the secretion and expression of C-X-C motif chemokine 11 (CXCL11) and components of the Hedgehog signaling pathway were examined in HaCaT cells by MTT assay, flow cytometric analysis, ELISA and gene chip analysis. The data showed that rhPTH (1-34) significantly inhibited keratinocyte proliferation at concentrations >8x10^{-7} mol/l. rhPTH (1-34) induced G1 phase arrest of the cell cycle in the keratinocytes. The secretion of CXCL11 in tumor necrosis factor (TNF)-α-induced keratinocytes was downregulated by rhPTH (1-34) in a dose-dependent manner, compared with that in keratinocytes treated with TNF-α alone. It was also found that rhPTH (1-34) inhibited the expression of CXCL11 in the HaCaT cells. rhPTH (1-34) also affected the Hedgehog signaling pathway specifically by regulating the expression of associated genes. In conclusion, these data suggested that rhPTH (1-34) inhibited cell proliferation, and the secretion and expression of CXCL11 in HaCaTs. rhPTH (1-34) also altered the expression of associated genes in the Hedgehog pathway. Therefore, rhPTH (1-34) can be considered as a novel therapeutic agent for the treatment of psoriasis.

Introduction

Psoriasis is a chronic skin disease, which is characterized by the hyperproliferation and abnormal differentiation of keratinocytes, and is caused by an abnormal immune response induced by T cells. Its common symptoms include a reddish, scaly rash, with itching and scaling (1). Relapse is common in of psoriasis and the disease usually has a long course, which has serious effects on the physical and mental health of patients (2). As a novel drug for psoriasis, vitamin D analogues have been provided for treating this challenging disease. However, ~15% of patients develop dermatitis, and the majority of patients developed a resistance to vitamin D analogues following ~0.5-1 year of treatment (3,4). Therefore, a safe and effective topical therapy for psoriasis is required.

Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) have been identified as being responsible for humeral hypocalcemia of malignancy (5,6). PTHrP is also expressed by a range of normal and neoplastic tissues, including the skin and hair follicles (7,8). Although the physiological role of PTHrP in the skin remains to be fully elucidated, increasing evidence has suggested that this peptide is important in the normal physiology of skin. The regulation of PTHrP occurs shortly following the point at which keratinocyte cell growth stops. The level of PTHrP is negatively associated with the number of suprabasal cells, and the alteration of PTHrP is involved in the regulation of basal cell proliferation. Therefore, the proliferation and differentiation of keratinocytes are controlled by PTHrP and Ca^{2+}. The failure of suprabasal keratinocytes to express PTHrP can have a domino effect on the mechanisms controlling the production and differentiation of keratinocytes, and this process has been shown to occur in psoriatic lesions where PTHrP is not expressed.

Recombinant human parathyroid hormone (rhPTH) (1-34) has been found to be a potent inhibitor of epidermal

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proliferation and induce terminal differentiation *in vitro* and *in vivo*. PTH has potentially wide therapeutic applications in the treatment of hyperproliferative skin disorders. The first insight into the possible role of PTHrP in the skin was reported by Holick *et al* (9,10), in which rhPTH (1-34) was used to treat psoriasis. The psoriatic lesions treated with rhPTH (1-34) showed marked improvement in scaling, erythema and induration. None of the patients experienced hypercalcemia or hypercalciuria, and none developed any side effects to the treatment.

As a substitute of vitamin D analogues, rhPTH (1-34) may be useful in psoriasis, however, the physiological role of this peptide in human keratinocytes remains to be fully elucidated. Therefore, the present study aimed to investigate the function of rhPTH (1-34) in the HaCaT human keratinocyte cell line. The proliferation of cells, cell cycle and cell apoptosis were examined, and the expression of C-X-C motif chemokine 11 (CXCL11)/I-TAC and the Hedgehog signaling pathway were determined in the HaCaT cells.

Materials and methods

Cell culture. The human keratinocyte HaCaT cell line was obtained from the National Institutes of Health (Bethesda, MD, USA). The HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum (both from GE Healthcare, Chicago, IL, USA), 100 U/ml penicilin and 100 μg/ml streptomycin. All cells were maintained at 37°C in 5% CO_2.

Cell proliferation. The HaCaT cells were maintained in DMEM culture medium supplemented with different concentrations of rhPTH (1-34) (0.1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 5×10^{-8}, 2×10^{-7}, 8×10^{-7}, 3.125×10^{-6}, 1.25×10^{-5} and 5×10^{-5} mol/l), and seeded in 96-well plates (1×10^4/well). After cultured at 37°C for 48 h, an MTT assay was used to analyze the cell proliferation, and the optical density (OD) value at 490 nm was examined.

Cell cycle and cell apoptosis. The HaCaT cells were harvested 48 h following culture with DMEM culture medium and were added to 2.5×10^{-5} mol/l rhPTH (1-34), following which the cells were washed twice with PBS. The cells were then stained with propidium iodide. Cell cycle and cell apoptosis were analyzed using flow cytometry. The cells were analyzed using CellQuest software version 3.2 (BD Biosciences; Franklin Lakes, NJ, USA) and the DNA content was analyzed using Modifit 3.0 software (Verity Software House, Topsham, ME, USA).

CXCL11 expression assay. The HaCaT cells were seeded in 96-well plates (5×10^4/well) and divided into five groups. The concentrations of CXCL11 were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol of the human CXCL11 kit. The absorbance at 450 nm was recorded using the enzyme-labeling measuring instrument. All chemokines were calculated with the standard curve indirectly. The cells in each group were treated as follows and cultured at 37°C for 24 h: i) Control group, treated with culture medium; ii) TNF-α group, treated with 10 ng/ml TNF-α; iii) PTH1 group, treated with 10 ng/ml TNF-α and 3.125×10^{-6} mol/l rhPTH (1-34); iv) PTH2
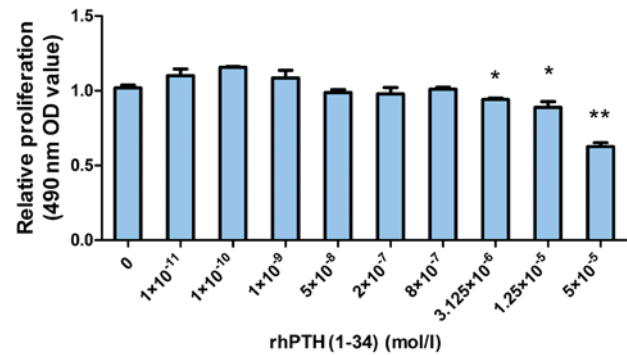

Figure 1. Cell proliferation of HaCaT cells 48 h following treatment with different doses of rhPTH (1-34). All data are presented as the mean ± standard deviation (n=3). rhPTH (1-34), recombinant human parathyroid hormone (1-34); OD, optical density.

group, treated with 10 ng/ml TNF-α and 1.25×10^{-5} mol/l rhPTH (1-34); and v) PTH3 group, treated with 10 ng/ml TNF-α and 5×10^{-5} mol/l rhPTH (1-34).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The HaCaT cells were maintained in DMEM supplemented with 2.5×10^{-5} mol/l rhPTH (1-34), and the cells were collected at 24, 36 and 48 h. Total RNA was extracted from the HaCaT cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reverse transcription was performed using a cDNA synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RT-qPCR procedure was performed using a SYBR-Green PCR kit (Fermentas; Thermo Fisher Scientific, Inc.) in an ABI-7300 Real-Time PCR system (ABI; Thermo Fisher Scientific, Inc.). Each reaction consisted of 2 μl cDNA, 12.5 pmol of each primer and 25 μl SYBR-Green Mix in a total volume of 50 μl. The thermocycling steps were as follows: 50°C for 2 min, 95°C for 5 min and 40 cycles of 95°C for 15 sec and 60°C for 45 sec. All procedures were performed in accordance with the manufacturer's protocol. β-actin served as an internal control. The primer sequences were listed as follows: CXCL11/I-TAC forward, 5'-GCTATAGCCTTGGCT GTGATATTGTG-3' and reverse, 5'-CTGCCACTTTCACTG CTTTTACC-3'; β-actin forward, 5'-ACACTGTGCCCATCTA CGAGGGG-3' and reverse, 5'-ATGATGGAGTTGAAGGT A GTTTCGTGGAT-3'.

Gene chip assay. For gene chip analysis, the cells were harvested following treatment with 2.5×10^{-5} mol/l rhPTH (1-34) for 48 h, and total RNA extraction and cDNA synthesis were performed as described above. Subsequently, 20 μg RNA was subjected to DNase I digestion in a 100-μl system. Reverse transcription was performed with 1.5 μg RNA in a 20-μl system. RT-qPCR was performed in a total volume of 1, 100 μl containing 20 μl cDNA and 550 μl 2X Super array PCR master mix. All reactions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min to obtain fluorescent signals. All data were analyzed using the $2^{-\Delta\Delta Cq}$ method (11). The computational formulas were as follows: i) ΔCq (group 1) = average Cq - average housekeeping (HK) gene Cq for group 1 array; ii) ΔCq (group 2) = average
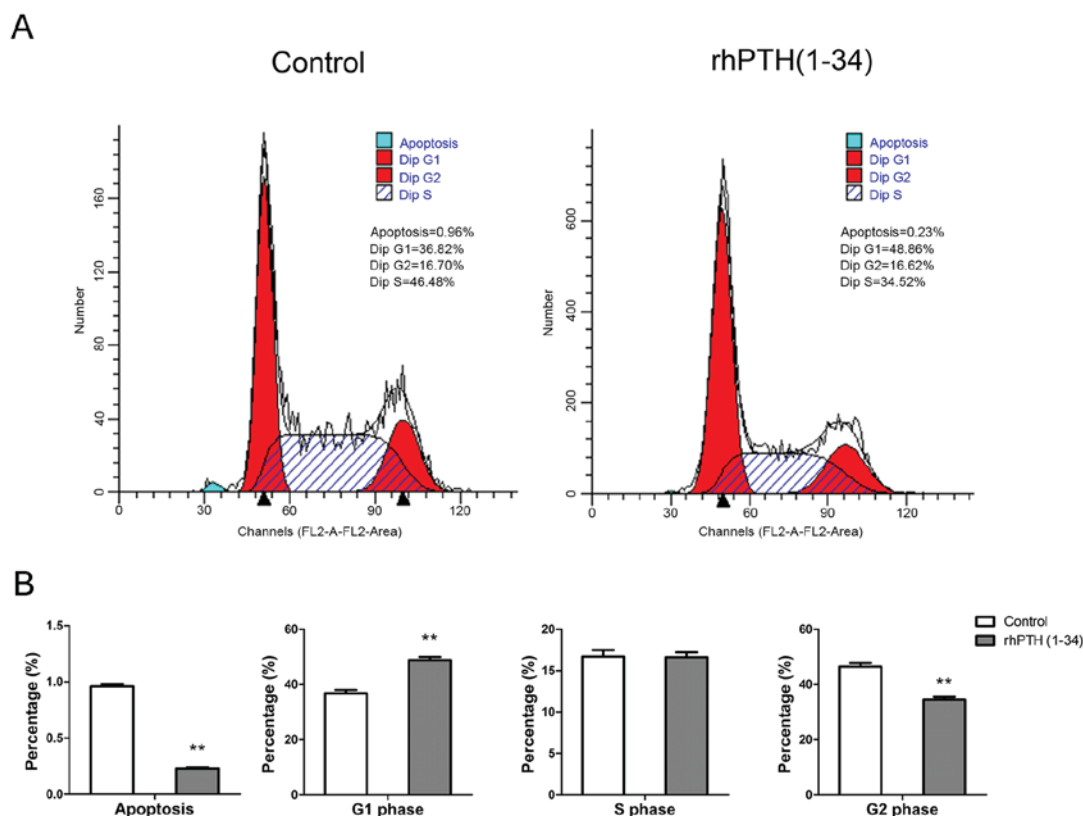
Figure 2. Cell cycle analysis. (A) Cell cycle analysis and (B) quantification was performed using flow cytometry following staining with propidium iodide staining in HaCaT cells. rhPTH (1-34) treatment induced G1 phase arrest of the cell cycle All data are presented as the mean ± standard deviation (n=3). rhPTH (1-34), recombinant human parathyroid hormone (1-34).

Cq - average HK gene Cq for group 2 array; iii) $\Delta\Delta Cq = \Delta Cq$ (group 2) - ΔCq (group 1). Group 1 represents the control group and group 2 represents the PTH-treated group.

Statistical analysis. At least three independent experiments were performed for every assay. All data are presented as the mean ± standard deviation. Statistical significance was determined using Student's t-test with GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of rhPTH (1-34) on the proliferation of HaCaT cells. In the present study, to profile the effects of rhPTH (1-34) on the growth of the HaCaT cells, the keratinocytes were treated with different concentrations of rhPTH (1-34) and cell proliferation was examined 48 h following treatment using MTT assays. As shown in Fig. 1, no significant differences were found in OD values compared with the control group when the concentration of rhPTH (1-34) was between 1×10^{-11} and 8×10^{-7} mol/l. The 490 nm OD values were significantly reduced when the concentration of rhPTH (1-34) was between 3.125×10^{-6} and 5×10^{-5} mol/l. The inhibitory effects were positively associated with the concentration of rhPTH (1-34). These data indicated that the proliferation of HaCaT cells was inhibited when the rhPTH (1-34) concentration was $>3.125\times10^{-6}$ mol/l and this inhibition was positively associated with the concentration of rhPTH (1-34).

Effects of rhPTH (1-34) on the cell cycle and apoptosis of HaCaT cells. To investigate the mechanism underlying the effect of PTH on cell proliferation, the present study investigated the effects of rhPTH (1-34) on cell cycle and cell apoptosis. As shown in Fig. 2A and B, in the rhPTH (1-34)-treated group, the proportions of cells in the G1, S and G2 phases were 48.86, 16.62 and 34.52%, respectively. In the control group, the proportions of cells in the G1, S and G2 phases were 36.82, 16.70 and 46.48%, respectively. In addition, the numbers of apoptotic cells were significantly decreased in the treatment group, compared with those in the control group. Therefore, the data obtained suggested that rhPTH (1-34) treatment markedly induced G1 phase arrest and G2 phase reduction in the cell cycle of the HaCaT cells, and also induced apoptosis of the keratinocytes.

Effects of rhPTH (1-34) on the secretion of CXCL11 in HaCaT cells induced by TNF-α. Previous studies have shown that the concentrations of TNF-α are high in skin lesions. TNF-α can induce human keratinocytes to express CXCL11, which has been identified as a cytokine associated with T cell recruitment and the maintenance of T cell infiltration (12). Therefore, the present study investigated the effects of rhPTH (1-34) on the secretion and expression of CXCL11 in TNF-α-induced cells. Following treatment of the HaCaT cells with TNF-α (10 ng/ml) and different concentrations of rhPTH (1-34) (3.125×10^{-6}, 1.25×10^{-5} and 5×10^{-5} mol/l), the results of the ELISA showed that TNF-α significantly induced the secretion of CXCL11 in the HaCaT cells. rhPTH (1-34) inhibited the

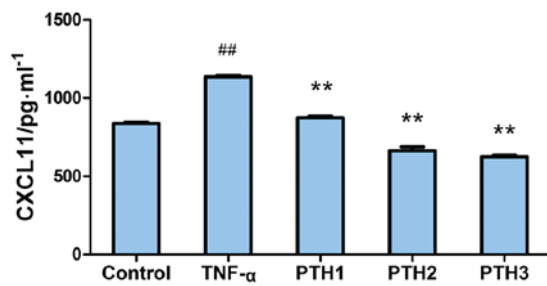Figure 3. Effects of rhPTH (1-34) treatment on the secretion of CXCL11 in TNF-α-induced HaCaT cells. rhPTH (1-34) inhibited the secretion of CXCL11 in a dose-dependent manner. All data represented as the mean ± standard deviation (n=3). PTH, parathyroid hormone; rhPTH (1-34), recombinant human parathyroid hormone (1-34); CXCL11, C-X-C motif chemokine 11; TNF-α, tumor necrosis factor-α.
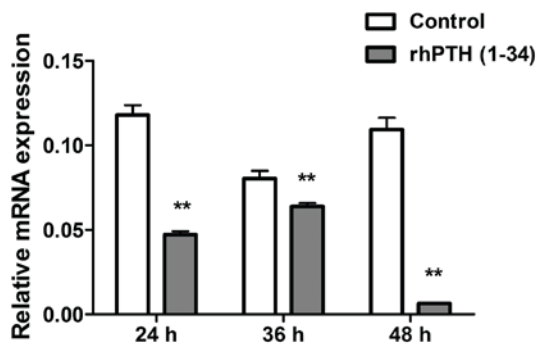

Figure 4. mRNA expression of CXCL11 in cells treated with $5x10^{-5}$ mol/l rhPTH (1-34). Cells were treated with rhPTH (1-34) for 24, 36 and 48 h. mRNA expression of CXCL11 in the treatment groups were decreased significantly. All PTH-treated groups were compared with the control group. All data are presented as the mean ± standard deviation (n=3). rhPTH (1-34), recombinant human parathyroid hormone (1-34); CXCL11, C-X-C motif chemokine 11.

secretion of CXCL11 induced by TNF-α in a dose-dependent manner (Fig. 3).

Effects of rhPTH (1-34) on the expression of CXCL11 in HaCaT cells. As the results of the ELISA analysis indicated that rhPTH (1-34) inhibited the secretion of CXCL11 induced by TNF-α, as described above, the present study analyzed the effects of rhPTH (1-34) on the expression of CXCL11 in HaCaT cells. The HaCaT cells were treated with $5x10^{-5}$ mol/l rhPTH (1-34) for 24, 36 and 48 h. As shown in Fig. 4, the results of the RT-qPCR analysis showed that the mRNA expression of CXCL11 in the rhPTH (1-34)-treated group was significantly decreased, compared with that in the control group.

Effects of rhPTH (1-34) on the Hedgehog signaling pathway. The Hedgehog signaling pathway has been suggested to be important in the development of psoriasis. Therefore, the present study investigated the effect of rhPTH (1-34) on the expression of 83 genes of the Hedgehog signaling pathway using the human Hedgehog signaling RT² Profiler™ PCR array. As shown in Fig. 5, compared with the control group, the rhPTH (1-34)-treated group exhibited lower expression of 15 genes and higher expression of 68 genes. The expression levels of PTCHD1 and C6orf138 were downregulated more

than two-fold. The expression of 23 genes in the Hedgehog pathway were upregulated more than two-fold. These genes were BMP4, BMP6, BMP7, BTRC, C18orf8, CSNK1A1, CTNNB1, DHH, ERBB4, GAS1, KCTD11, LRP2, MTSS1, PRKACA, PTCHD2, SFRP1, SIAH1, SMO, WNT10B, WNT2B, WNT5B, WNT6 and WNT9A.

Discussion

Psoriasis, induced by T cells, is characterized by the abnormal proliferation of keratinocytes. Following consideration of complications and resistance, investigations into treatment for psoriasis tend to use PTH or PTH derivatives rather than glucocorticoids and vitamin D derivatives. A previous study suggested that PTH inhibits the proliferation of keratinocytes and promote the restoration of epidermal structure (13). However, there are limited studies on its use in dermatology. In the present study, rhPTH (1-34) was used to treat HaCaT cells to examine its effects on cell proliferation, cell cycle, the expression of CXCL11 and the Hedgehog pathway.

The results of the present study indicated that rhPTH (1-34) significantly inhibited the growth of HaCaT cells, and the inhibitory effect of rhPTH (1-34) on cell proliferation was positively correlated with the concentration of rhPTH (1-34) at concentrations $>3.125x10^{-6}$ mol/l. The cell cycle assay revealed that rhPTH (1-34) markedly induced G1 phase arrest of the cell cycle and apoptosis in HaCaT cells. In the last century, studies have shown that PTH (1-34) acts on human skin fibroblasts (14). PTHrp (1-34) was previously found more likely to activate cAMP and plasminogen in osteosarcoma cells, which confirmed the existence of PTHrp in squamous cell carcinoma and in the keratinocytes of normal skin (15). It was shown that, in normal skin and several skin disorders, only the granular layer expresses PTH (34-68). The upper granular layer was shown to express PTHrp again following treatment with betamethasone or vitamin D analogue for 1-2 weeks, which indicated the potential inhibitory role of PTHrp in keratinocyte proliferation (16). This indicated a novel method of curing psoriasis with PTH. PTH (1-84) and PTH (1-34) can stimulate the activity of membrane-associated protein kinase C in keratinocytes, but cannot activate adenylate cyclase, suggesting that the response of keratinocytes to PTH in normal skin and in carcinoma is associated with peptide production and secretion (6). As an antagonist of the PTH receptor, PTH (7-34) eliminates the inhibitory effect of PTH (1-34) on cell growth. PTH and PTHrP are important in normal physiological activities, but also have potential beneficial effects in skin cell proliferation and aging, and may also be effective in hair growth (10).

In the present study, rhPTH (1-34) was used to treat HaCaT cells. The results confirmed that rhPTH (1-34) inhibited the proliferation of the keratinocytes in a dose-dependent manner, in accordance with the previous studies.

The present study also examined the effects of rhPTH (1-34) on the secretion and expression of CXCL11 in keratinocytes. Dysimmunity, induced by the abnormal secretion and expression of chemokines among lymphocytes, keratinocytes and antigen-presenting cells (17), is key in the occurrence of psoriasis (18). CXCL11 is a type I chemokine and exhibits specific chemotaxis to Th1 cells, which express
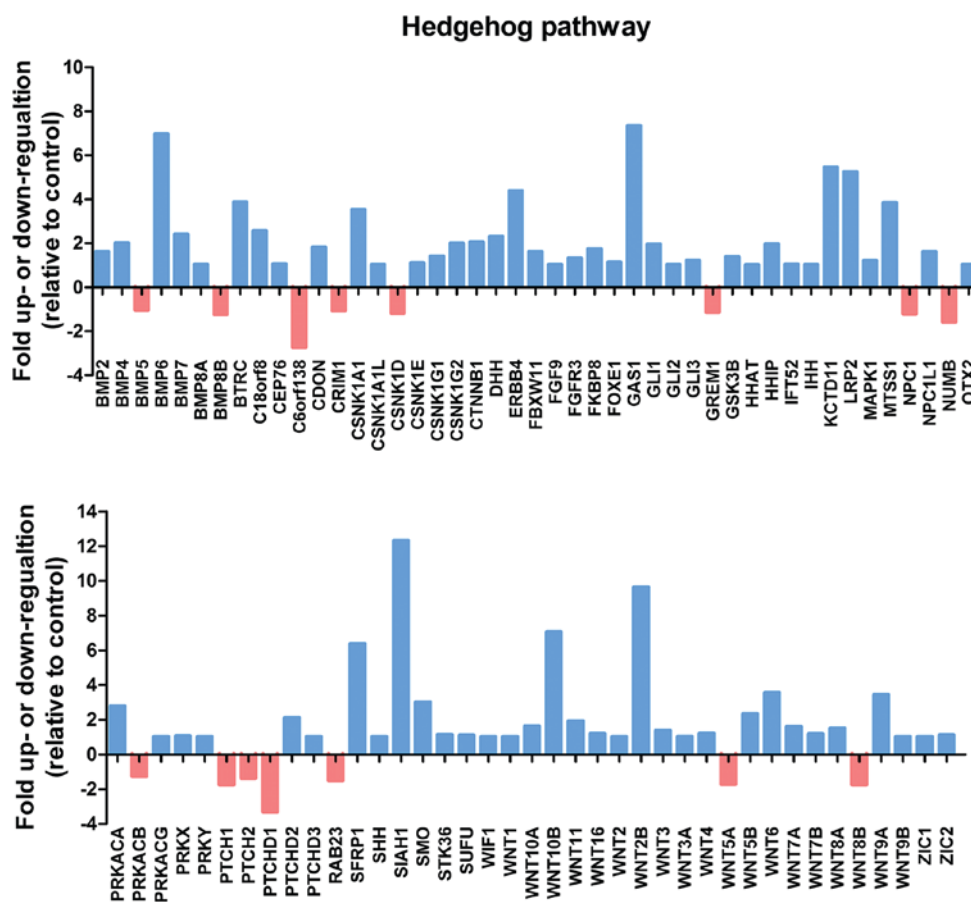
Figure 5. Effects of recombinant human parathyroid hormone (1-34) treatment on the expression of proteins associated with the Hedgehog signaling pathway.

CXCR3. Studies have shown that the expression level of CXCL11 in keratinocytes is high in skin disease, in which Th1 occupies a dominant position. It is suggested that CXCL11 and CXCR3 are important in the occurrence of this type of chronic inflammatory skin disease (19,20). The expression of TNF-α, a Th1 cytokine, is high in skin lesions of Th1 type skin diseases (21). In the present study, rhPTH (1-34) was found to inhibit the secretion of CXCL11 in a dose-dependent manner in HaCaT cells induced with 10 ng/ml TNF-α. In addition, rhPTH (1-34) significantly suppressed the mRNA expression of CXCL11 in keratinocytes and promoted the secretion of CXCL11/I-TAC. However, PTH concentrations of 3.125×10^{-6}, 1.25×10^{-5} and 5×10^{-5} mol/l inhibited the secretion of CXCL11 in the TNF-α-treated keratinocytes. These results showed that the expression of CXCL11 in HaCaT cells was upregulated by TNF-α. However, following treatment with rhPTH (1-34), the expression of CXCL11 was inhibited in a dose-dependent manner. These results indicated that rhPTH (1-34) assisted in curing psoriasis through inhibiting the expression of CXCL11 in keratinocytes to suppress inflammatory responses.

Finally, the present study examined the effects of rhPTH (1-34) on the Hedgehog signaling pathway using a genechip assay (22). The results of the genechip assay showed 15 genes with lower expression and 68 genes with higher expression in the rhPTH (1-34)-treated group, compared with that in the control group. It was shown that two genes, PTCHD1 and C6orf138, were downregulated more than two-fold. These are Hedgehog receptors and the downregulation in

their expression indicated that these two genes may be target genes in the treatment of psoriasis. A total of 23 genes were upregualted more than two-fold, including BMP4, BMP6, BMP7, BTRC, C18orf8, CSNK1A1, CTNNB1, DHH, ERBB4, GAS1, KCTD11, LRP2, MTSS1, PRKACA, PTCHD2, SFRP1, SIAH1, SMO, WNT10B, WNT2B, WNT5B, WNT6, WNT9A. PTCH is an important gene in the Hedgehog signaling pathway. It is involved in and regulates Hedgehog signal transduction by expressing transmembrane proteins of recipient cells. It can be combined with all members of the Hedgehog family and this has an effect on the Hedgehog signaling pathway. PTCH1 has been found in nevoid basal cell carcinoma syndrome, and may be a tumor suppressor gene (23,24). Whether PTCH gene mutation is involved in psoriasis requires further investigation (25). The increased activity of the Hedgehog signaling pathway can induce keratinocyte proliferation and abnormal differentiation (26). In the present study, it was shown that rhPTH (1-34) may inhibit keratinocyte proliferation by regulating the Hedgehog signaling pathway.

In conclusion, the results of the present study confirmed that rhPTH (1-34) inhibited cell proliferation through inducing G1 phase arrest and apoptosis of HaCaT cells. Treatment with rhPTH (1-34) inhibited the secretion and expression of CXCL11 in the HaCaT cells, and altered the expression of proteins in the Hedgehog signaling pathway. These results suggested that rhPTH (1-34) may be a useful target in the treatment of psoriasis and provide a foundation for the use of PTH in treating psoriasis.

Acknowledgements

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