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Treatment of Alopecia Areata in Mice by Stimulating the Hair Follicles Using Parathyroid Hormone Agonists Linked to a Collagen Binding Domain

Ranjitha Katikaneni[1*], Tulasi Ponnapakkam[2], Rohan Gulati[3], Andrew A. Seymour[4] and Robert Gensure[5]

[1]*Department of Pediatric Endocrinology, Children's Hospital at Montefiore and Albert Einstein College of Medicine, Bronx, NY, USA*

[2]*Department of Chemistry, Research Scientist, Xavier University of Louisiana, New Orleans, LA, USA*

[3]*Department of Pediatric Endocrinology, Student, Children's Hospital at Montefiore and Albert Einstein College of Medicine, Bronx, NY, USA*

[4]*Department of Dermatopathology, Clinical Fellow, Tufts Medical Center, Boston, MA, USA*

[5]*Department of Pediatric Endocrinology, Attending Physician, Children's Hospital at Montefiore and Albert Einstein College of Medicine, Bronx, NY, USA*

[*]**Corresponding Author:** Katikaneni R, Research Associate, Pediatric Endocrinology, Children's Hospital at Montefiore, 3415 Bainbridge Ave, Bronx, NY 10467, USA; Tel: 718-920-7004; Email: rkatikan@montefiore.org; rkatikaneni@gmail.com

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Abstract

Alopecia Areata is a patchy hair loss from autoimmune-mediated destruction of hair follicles, for which there is no adequate therapy. PTH-CBD is a fusion protein of parathyroid hormone and a bacterial collagen-binding domain, providing targeted delivery of a hair cycle stimulator to skin and promoting hair growth.

Objectives: We tested the effects of PTH-CBD on hair growth in an established animal model for alopecia areata, the C3H/HeJ engrafted mouse.

Methods: C3H/HeJ engrafted mice (Jackson Laboratories, Bar Harbor, ME) were treated subcutaneously for 8 weeks with either vehicle or different doses of PTH-CBD (320 mcg/kg x1, 1000 mcg/kg x1 or 1000 mcg/kg/wk).

Results: Vehicle animals showed progressive hair loss, as expected. In PTH-CBD treated animals, grey scale quantification showed a greater proportion of PTH-CBD treated animals without significant hair loss at the end of the 2 month period (18/22 PTH-CBD vs. 4/11 vehicle), with no observed differences between the different PTH-CBD treatment regimens. Histological examination revealed no change in CD8+ cells, but there was a marked increases in the number of anagen VI hair follicles in PTH-CBD treated animals. There were also increased levels of beta-catenin, a known initiator of the hair cycle, observed around the bulge region of hair follicles.

Conclusions: C3H/HeJ engrafted animals treated with PTH-CBD showed rapid and persistent improvements in hair growth in the majority of tested animals. There were marked increases in anagen hair follicles despite an ongoing immune reaction. Increased beta catenin levels suggest that PTH-CBD stimulates hair growth by activating the Wnt pathway.

Keywords: Alopecia Areata; Anagen; Hair follicles; PTH-CBD; C3H/HeJ engrafted mouse; Beta-catenin; Grey scale analysis

Introduction

Alopecia areata is an autoimmune-mediated destruction of anagen phase hair follicles which leads to patchy hair loss [1]. It is a common form of hair loss and affects 2.1% of population [2]. Alopecia areata can progress to alopecia totalis (loss of all hair on the head) or alopecia universalis (loss of all hair on body) [3,4]. It can cause significant psychological distress, affecting children and teenagers resulting in negative effects on the quality of life, psychosocial parameters, and emotional symptoms [5].

Alopecia areata is caused by T cell-mediated autoimmune destruction of hair follicles [6], with a predominantly CD8+ lymphocyte infiltrate resulting in hair loss [7,8]. A new study using human clinical samples and a mouse model demonstrates that NKG2D (+) T effector memory cells, part of CD8+ lymphocyte family mediate alopecia areata; through Janus kinase (JAK) signaling. Based on this mechanism, alopecia areata can be treated with JAK inhibitors [9, 10]. Indeed, early clinical trials with JAK inhibitors have shown a high rate of response (75%) in promoting hair growth in patients with severe alopecia areata [11]. While we now know many of the details of the immunologic pathways in the development of the autoimmune reaction for alopecia areata, the antigenic triggers for this immune reaction have not yet been identified, nor has the time course for the development of this reaction been defined.

Currently there is no adequate therapy for alopecia areata available for use in the clinic. Various drugs (steroids, topical immunotherapy, topical sensitizers, topical minoxidil, anthralin, immunosuppressant's alkylating agents) [12-14], topical immunotherapy, topical minoxidil, and anthralin) have been utilized for the treatment of alopecia areata, but these generally have limited efficacy [15]. Intralesional corticosteroids and topical immunotherapy are considered as the first

line treatment options for alopecia areata [4,16]. Corticosteroids are believed to suppress the T cell mediated immune attack on the hair follicle [17]. The response rate for intralesional corticosteroids is 60% [18]. Despite their common use, there are no randomized controlled trials supporting their use [19], and patients often relapse after treatment [20]. Diphenylcyclopropenone (DPCP) is an immunomodulating agent that works by initiating a T-cell response. It is believed to function by downregulating CD8+ activity [21]. DPCP has not only been used to treat skin cancers such as metastatic melanoma, but is also used to treat alopecia areata [22]. In a large retrospective study done by Ohlmeier et al, topical treatment with DPCP showed that 37.8% of patients had a complete response (90% regrowth of hair) and 14.8 % had a partial response (50-90% regrowth) [23]. The extent of hair loss before therapy is the main predictor for the therapeutic success of DPCP. However, DPCP therapy is associated with a high degree of relapse [24]. Because of its clinical significance, a DPCP gel formulation which has an Investigational New Drug (IND) is currently in phase 2a clinical trials for the treatment of alopecia areata [25].

Parathyroid hormone-related peptide (PTHrP) is a paracrine factor with different effects throughout the body; in skin, PTHrP functions as a hair cycle regulator [26,27]. PTHrP functions through a common receptor with that of parathyroid hormone (PTH), the PTH/PTHrP receptor, thus parathyroid hormone (PTH) agonists and antagonists can also modulate the hair cycle [28]. In skin, PTH/PTHrP receptors are located primarily in keratinocytes [29,30]. PTHrP overexpression in skin increases production of beta-catenin and LEF-1 [31]. Beta-catenin is a known activator of the hair cycle, which promotes transition of hair follicles to the anagen phase [32]. PTHrP agonists and antagonists have been shown to have beneficial effects on hair growth in animal models of chemotherapy-induced alopecia [29].

To improve delivery and retention of PTH in the skin, we synthesized a fusion protein of the active portion of parathyroid hormone, PTH (1-33), linked to a bacterial collagen binding domain (CBD) derived from ColH collagenase (*Clostridium histolyticum*). The collagen binding activity of this compound, PTH-CBD, alters its distribution pattern, resulting in long-term retention of the peptide in skin collagen [33]. We have previously shown that PTH-CBD is effective at preventing or reversing chemotherapy-induced hair loss, and causes marked increase in the number of anagen hair follicles [33,34]. We therefore hypothesized that PTH-CBD therapy could replace damaged anagen hair follicles and promote hair growth in alopecia areata. The mechanism by which PTH-CBD promote anagen response is not through immune modulation but rather by hair cycle stimulation. We therefore tested the efficacy of PTH-CBD in C3H/HeJ engrafted mouse, the mouse model for alopecia areata.

Material and Methods

33 female C3H/HeJ engrafted mice 20 week old were purchased from Jackson Laboratory, Bar Harbor, ME. They were then acclimatized for two weeks in the animal room and maintained under standard conditions, with a 12/12 hour light/dark period at a temperature of 68-70 degrees Celsius. The mice were given access to tap water and a diet consisting of 18 percent protein purchased from Harlan Company located in Barton, IL and Madison, WI. The Institutional Animal Care and Use Committee approval for these studies was obtained from Montefiore Medical Center, Bronx, NY.

Chemicals

PTH-CBD peptide was synthesized by recombinant techniques in *E-coli* [35]. Prior to injection, PTH-CBD was dissolved in a collagen binding buffer (pH 7.5, 50 mM Tris HCl, 5 mM CaCl2).

Study Protocol

The mice were observed daily and photographed weekly for 2 months, during which the majority developed partial hair loss, as expected with this model. Mice were ordered by degree of hair loss, then divided into cohorts corresponding to the number of groups. One mouse from each cohort was then randomly assigned to each group. This method of group assignment was performed to compensate for heterogeneity of hair loss normally observed in the C3H/HeJ engrafted mouse model. Mice were divided into 4 groups as follows:

Vehicle x1 (N=11)

PTH-CBD (320 mcg/kg) x1 (N=5)

PTH-CBD (1000 mcg/kg) x1 (N=11)

PTH-CBD (1000 mcg/kg) weekly (7 doses total) (N=6)

Based on previous distribution studies and persistence of effects of the test compound in bone, 1 dose can be considered full treatment for an 8 week period. The Vehicle and PTH-CBD (1000 mcg/kg) x1 groups are larger to maximize power to detect differences between the control and the most efficacious dose (1000 mcg/kg) of PTH-CBD from our previous dose-response studies in chemotherapy alopecia [36]. Animals were injected subcutaneously near the engraftment site with either vehicle control or PTH-CBD at the indicated dose and dosing schedule.

Photo- documentation

Animals were observed daily, and photo-documentation was obtained on a weekly basis. Images were captured with the KODAK Gel Logic 100 Imaging System (Eastman Kodak Company, Rochester, NY, USA), on a SpectrolineR Bi-O-VisionTM uv/white light transilluminator (Spectronics Corporation, Westbury, NY, USA). Photographs were taken with exposure 0.2 seconds, F-stop 2 mm, magnification 15 mm to keep the hair texture in the linear range for analysis. Images were analyzed quantitatively by grey scale analysis, as previously described [34]. Briefly, light absorption within specified regions was quantified using the provided software, with greater absorption corresponding to increased hair growth. In this study, an elliptical region of interest (ROI) was selected covering the entire back of the mouse. This densitometry value was normalized to the average of that obtained from a background ROI placed at the top of the mouse. On the ventral side we have two ROIs, one placed in the thigh region (accessible for self-grooming) and the other covering the chest area (not accessible for self-grooming). This densitometry value was again normalized to the average of that obtained from a background ROI placed at the top of the mouse.

Histology

Mice were sacrificed at the end of the study, day 60 after initiation of treatment. Skin samples from dorsal non-engrafted (nape of the neck), and ventral non-engrafted regions were flash frozen in Optimal Cutting Temperature Compound (Tissue-Tek, Torrance, CA). Samples were cut on long sections and processed for routine histology using Hematoxylin and Eosin (H&E) staining. Immunohistochemistry was

performed for evaluation of beta-catenin [37] by using anti-Catenin-β antibody produced in rabbit (Sigma-Aldrich Corp., St. Louis, MO) and to assess the immune response of CD4 and CD8 positive cells from dorsal non-engrafted skin samples; to assess the effects of therapy on the immune reaction to individual follicles [38].

Quantitative Assessment of Hair follicle counts

The number of anagen VI hair follicles per high power field (HPF) was determined by two independent observers in a blinded fashion [39]. The HPF with maximum follicular density was counted on cross sections, which included the hair follicles per high power field in each skin layer and were compared between groups.

Statistical Analysis

Relative absorption from grey scale analysis was analyzed by 2-way ANOVA, followed by 1-way ANOVA at each time point and post hoc Tukey's test. Statistical analyses were performed using GraphPad Prism 5.0. (GraphPad Software, Inc., La Jolla, CA, USA).

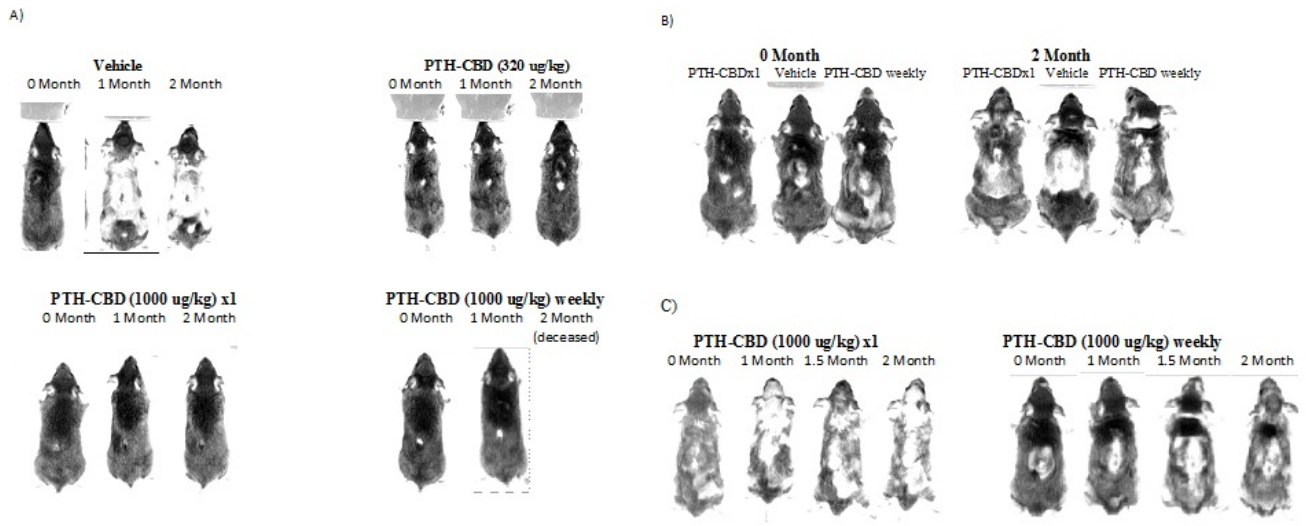

Figure 1: Gross view of hair growth on the dorsal surface of individual mice. A) Response to treatment in animals with the least hair loss at initial dosing: shown are outcomes of vehicle or PTH-CBD treated animals (at the indicated doses) which had the least amount hair loss at the start of the study. The vehicle treated animal showed expected hair loss, PTH-CBD treated animals had little or no hair loss. B) Response to treatment in animals with the most hair loss at the start of the study: Shown are outcomes of animals receiving either vehicle or PTH-CBD (1000 ug/kg, x1 or weekly as indicated) housed in the same cage. Note that the vehicle treated animal showed significantly more hair loss. C) Hair loss followed by regrowth in PTH-CBD treated animals: Shown are photographs of the same animal at different time points, with evidence of hair loss events followed by regrowth, from the groups receiving PTH-CBD (1000 ug/kg x1 or weekly as indicated).

Results

Photo-documentation

On gross examination at the engraftment site, which was also the site of drug administration, the only visible change was perhaps decreased size of the region of hair loss in some animals (Figure 1A). However, in the non-engrafted sites, there were visible improvements in hair growth (vs. vehicle control animals) in animals which had received PTH-CBD, as observed in animals with the least (Figure 1A) and most (Figure 1B) hair loss at the start of the dosing period. While control animals continued to lose hair during the experimental period, as expected, a greater proportion of PTH-CBD treated animals maintained or regrew hair on the dorsal surface, evident by gross inspection, regardless of dose or dosing frequency. Many of the treated animals showed cycles of hair loss followed by regrowth, which could be observed in either the single dose or multiple dose groups (Figure 1C). The proportion of animals receiving PTH-CBD therapy which showed continued hair loss based on visual assessment (9/22, 41%) was considerably lower than those receiving vehicle control (8/11, 72%, $p<0.01$ Chi Square test). Ventral changes were more difficult to assess by gross inspection. In all groups, there was no observable change in hair growth in regions where the hair coat is normally thin, that is, ears and tail.

We performed grey scale analysis on serial photographs of the dorsal surface of the mice, with higher light absorption corresponding to increased hair growth [40]. At the end of the study, hair regrowth or maintenance (within 20% of baseline, which is within the expected variance of this technique) was observed in 18/22 (81%) total PTH-CBD treated mice vs. 4/11 (36%) vehicle control mice ($p<0.01$, Chi Square test) (Figure 2). This confirmed the findings by visual inspection that a greater proportion of animals showed decreased overall hair loss on the dorsal surface after PTH-CBD therapy, again irrespective of the dose or dosing frequency (40% for 320 mcg/kg, 30% for single dose of 1000 mcg/kg, 50% for weekly 1000 mcg/kg of PTH-CBD). On the ventral side, grey-scale analysis showed the same findings of decreased hair loss with PTH-CBD treatment, correlating with the dorsal-side analysis for each animal, even though this was not obvious on visual inspection (Figure 2).

On histological examination, H&E staining of long sections showed that PTH-CBD treatment resulted in an apparent increase in the number of anagen VI hair follicles (Figure 3), similar to that described in models of chemotherapy alopecia [33,34]. This increase in anagen

VI hair follicles was independent of the dose and dosing frequency. There was an increased cellularity around the hair follicles, consistent with an ongoing immune response in most animals, in all groups. Quantitative assessment of hair follicle number was performed by determining the number of anagen VI hair follicles per HPF on cross sections at the region of maximum follicle density.

The number of anagen VI hair follicles was significantly increased in animals receiving PTH-CBD (42.8+/-8.1 PTH-CBD vs. 2.6+/-0.9 vehicle control, $p<0.05$) (Figure 4). Animals receiving a single dose of PTH-CBD showed dose dependent effects; however, repeat dosing showed a more modest increase in hair follicle number (Figure 4).

Immunohistochemistry showed the expected increase in CD4+ and CD8+ cells surrounding and infiltrating the hair follicles in control animals exhibiting hair loss; control animals with no hair loss did not show lymphocytic infiltration (Figure 5). In PTH-CBD treated animals, most animals showed lymphocytic infiltrate, regardless of the presence or absence of hair loss. There did appear to be more non-infiltrated hair follicles in PTH-CBD treated animals, although this may simply reflect the increase in the total number of visible hair follicles. Importantly, there were no obvious differences in the extent of lymphocytic infiltration between treated animals or controls (illustrated in Figure 5, comparing control vs. PTH-CBD 1000 mcg/kg x1).

Immuno-histochemical staining for beta-catenin revealed increased staining in the nucleus of cells surrounding bulge of the hair follicles in animals treated with PTH-CBD in spite of the underlying immune reaction, while controls animals with immune reaction showed minimal staining for beta-catenin (Figure 5).

Discussion

PTH-CBD is a fusion protein of the active portion of parathyroid hormone (PTH(1-33)) and a bacterial collagen binding domain (CBD). This compound was designed to promote distribution and retention of the active component to collagen-containing tissues, such as skin and bone. PTH and PTHrP act through the same PTH/PTHrP receptor, and agonists to this receptor are known to have positive effects on WNT signaling and cause upregulation of the intracellular regulatory protein beta-catenin in bone [41] and in skin [31]. WNT signaling and increased levels of beta-catenin can cause transition of hair follicles to the anagen, or growth, phase of the hair cycle [32]. Thus, mechanistically it is plausible that PTHrP agonists would activate the hair cycle and have the greater effects on promoting hair growth [26]. We have shown previously that PTH-CBD promotes more rapid regrowth of anagen VI hair follicles in both depilated and non-depilated mouse models of chemotherapy-induced alopecia [33,34].

Given this observed increase in anagen phase hair follicles, we hypothesized that PTH-CBD therapy would replace damaged anagen hair follicles and promote hair growth in alopecia areata. In this study, we tested the effect of PTH-CBD on hair growth in C3H/HeJ engrafted mouse; a mouse model for alopecia areata.

On gross examination, we observed that most control animals continued to lose hair during the experimental period, as expected. PTH-CBD treatment reduced the proportion of animals with ongoing hair loss by approximately 50% vs. vehicle control. Grey scale analysis on dorsal surface confirmed these findings, and on the ventral side revealed a similar pattern of change, even where these differences are not as evident on gross inspection. On histological examination, there was a marked increase in the number of anagen hair follicles in all treated groups when compared to vehicle control animals. Immunohistochemistry showed CD4+ and CD8+ cells, with a predominance of CD8+ cells surrounding and infiltrating hair follicles, as is typically seen in this animal model [42]; the only notable group differences that there appeared to be are more non-infiltrated hair follicles in PTH-CBD treated animals. Thus, there is no evidence that PTH-CBD treatment either potentiated or inhibited immune reaction to hair follicles. PTH-CBD treatment resulted in increased beta catenin staining in the bulge of the hair follicles, suggesting this is a possible mechanism for stimulation of folliculogenesis.

There was a significant proportion (81%) of C3H/HeJ engrafted mice that responded to PTH-CBD therapy with improvements in hair growth. The response appeared to be 'all-or-nothing', with clear segregation into animals with increased hair growth and animals with ongoing growth and loss events. While we were pleased to see a measurable overall positive response, we would have been more pleased to see all of the animals responding with consistent increases in hair growth.

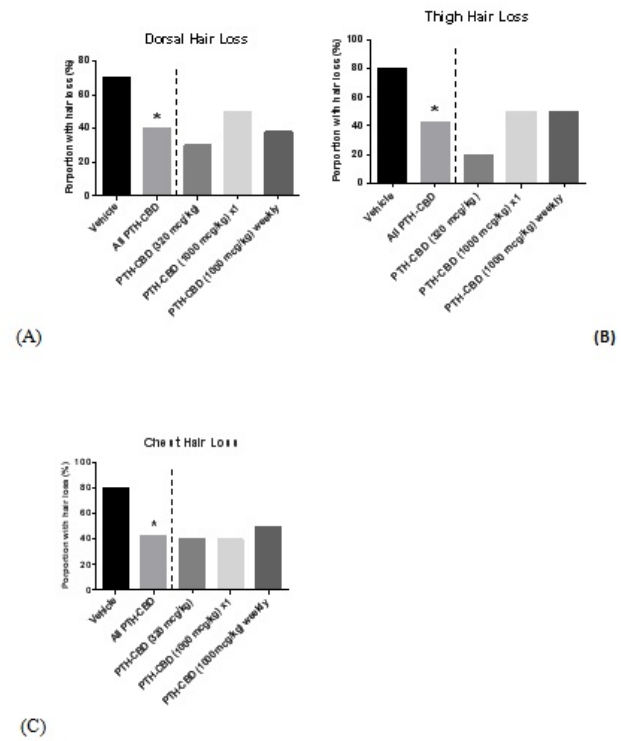

Figure 2: Grey Scale Analysis: Proportion of animals with hair loss on (A) dorsal surface, (B) ventral surface – thigh, (C) ventral surface – chest. Assessment of hair loss was based on observation (dorsal surface) or grey scale analysis (ventral surface), counting animals with evidence of continued hair loss at the end the treatment period compared to baseline assessments. Animals which had evidence of hair regrowth that was followed by additional hair loss events were counted as hair loss overall. * = $p<0.01$ vs. vehicle control by Chi square test.

Notably, this variability was not limited to treated animals; control animals also showed heterogeneous hair loss responses. Importantly,

all treated animals showed a marked increase in the number of anagen VI hair follicles regardless of the gross observations, suggesting that other factors (i.e. the virulence of the immune response) determine the net effect on hair growth. It is possible that co-therapy with PTH-CBD and an immunosuppressive agent could result in a positive response in a greater proportion of animals.

There are other medications which act by stimulating hair follicles, but their use in alopecia areata has been limited. Minoxidil (Rogaine) was developed as an antihypertensive medication but has an observed effect of causing hair growth, and is now used as a topical therapy for androgenic alopecia. However, it does not show efficacy in chemotherapy-induced alopecia [43], and minoxidil has only limited efficacy as a therapy for alopecia areata, with many subjects showing only slowing of hair loss rather than actual regrowth of hair [44].

Cyclosporine is an immunosuppressive agent that also has direct stimulatory effects on the hair cycle, promoting transition of hair follicles to anagen phase [45], although the mechanism is less likely to be the result of direct effects on WNT signaling [46]. While clinical use of cyclosporine for this purpose is limited by systemic toxicity, there are reports of effectiveness in treating alopecia areata [47].

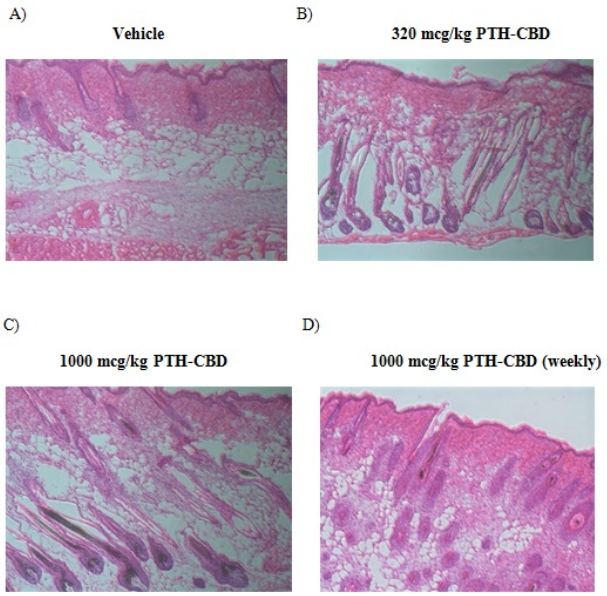

Figure 3: Histological evaluation (Hematoxylin and Eosin staining) of dorsal non-engrafted skin samples from each treatment group and the vehicle control group.

PTH-CBD is a compound which stimulates the hair cycle, and its effect in the C3H/HeJ engrafted mice was to increase the number of cycling hair follicles and in a proportion of the animals to prevent or reverse hair loss. It appears that PTH-CBD reduced hair loss not by modulation of the immune response but rather by direct stimulation of the hair cycle through increased production of beta-catenin, which suggests that PTH-CBD could act synergistically with an immunosuppressive agent. With these encouraging results, efforts are underway to develop PTH-CBD or similar analogs for further testing towards performing assessments in clinical trials.

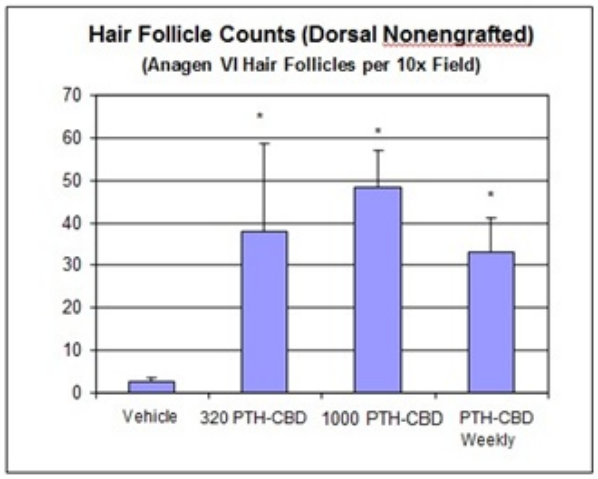

Figure 4: Counts of total anagen VI hair follicles per 10x field from histological sections, cross-sectional view (see Fig. 4). * = $p<0.05$ vs. vehicle control by Dunnett's test.

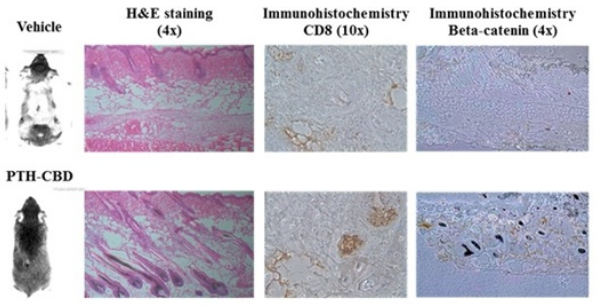

Figure 5: Immunohistochemistry was performed to assess the immune response of CD4 and CD8 positive cells from dorsal non-engrafted skin samples of vehicle control and PTH-CBD (1000 ug/kg x1) treated mice and as well as for evaluation of beta-catenin.

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Conflict of Interest

PTH-CBD is patented and exclusively licensed to Biologics MD, LLC. Robert Gensure is Chief Medical Officer of Biologics MD. Robert Gensure, Tulasi Ponnapakkam has a stock ownership in Biologics MD.

Ranjitha Katikaneni, Rohan Gulati and Andrew Seymour have none to declare.

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