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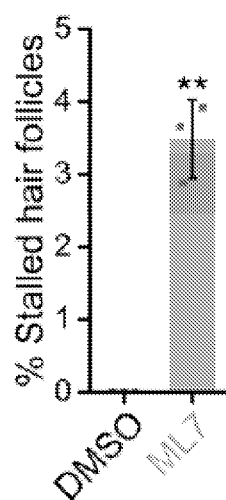


FIG. 3I

(57) Abstract: Methods and compositions useful in the prevention and treatment of hair loss. In some embodiments, compositions are applied to hair follicles to inhibit the transition of growth phases. In some embodiments, the composition is a myosin light chain kinase (MLCK) inhibitor alone, or in conjunction with other components. In some embodiments, methods are disclosed for identifying and/or selecting compositions for use in methods of preventing or treating hair loss.

TITLE**METHOD FOR PREVENTING HAIR LOSS****CROSS REFERENCE TO RELATED APPLICATIONS**

5 This application claims the benefit and priority under 35 U.S.C. § 119(e) of the filing date of United States Provisional Patent Application Serial Number 62/871,030, filed July 5, 2019.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

10 This invention was made with government support under grant numbers AR070639 and AR071047 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

15 The present specification relates to a hair loss treatment. More in particular, the present specification relates to methods, kits, and compositions for treating hair loss by regulating the hair cycle (*e.g.*, by inhibiting the progression of the catagen phase of the hair cycle).

BACKGROUND OF INVENTION

20 A defining biological characteristic of mammals is hair, *i.e.*, the threadlike outgrowths of the outer layer of the skin that provide an animal with its coat or pelage. Humans are among the most hairless of all mammals but society continues to attach a stigma to hair loss when it comes to the human scalp. As a result, men and women who suffer from hair loss often experience self-consciousness relating to the condition.

25 Hair growth is cyclical, whereby hairs are continually shed and renewed by the operation of a hair growth cycle consisting of, growth (anagen), regression (catagen), and rest (telogen), followed by a shedding of the hair shaft and a renewed growth phase. In humans, the average life of different varieties of hair varies from about four months for downy hairs to three to five years for long scalp hairs. During each anagen phase, follicles produce an entire hair shaft from tip to root. In catagen and telogen, follicles reset and prepare their stem cells so that they can
30 receive the signal to start the next growth phase and make a new hair shaft, while the previous hair shaft is shed. The hair cycle is a complex biological process that involves intricate regulation of stem cell quiescence and activation, as well as cell proliferation, cell-fate choice,

differentiation and apoptosis in a regenerative adult epithelial tissue. Each human follicle follows this cycle independently of others, so the total amount of hair remains constant so long as the follicles remain functional and/or active. Humans are born with a full complement of hair follicles, which can change in size and growth characteristics as in early baldness or can ultimately degenerate and disappear as in the late stages of baldness (*e.g.*, male-pattern baldness or alopecia).

For androgenetic alopecia, a large number of compounds have been developed as candidate treatments. Examples include 2,4-diamino-6-piperidinopyrimidine-3-oxide (also known as “minoxidil”) and finasteride. A medicament containing minoxidil as an active ingredient is commercially available under the trademark ROGAINE® (Pharmacia & Upjohn Company). A medicament containing finasteride as an active ingredient is commercially available under the trademark PROPECIA® (Merck & Co., Inc.). PROPECIA® is a pill for oral administration. Both treatments require continuous application of the compositions to the skin for a long period of time and the success rates are limited. Moreover, the use of finasteride has been associated with reduced libido, teratogenic effects and other side effects in certain individuals.

In addition, attempts have been made to extract compositions from natural plants, including medicinal herbs, to be used for the treatment of alopecia. Various extracts of crude drugs, generally known as hair growth compositions, have been used as hair growth stimulants or promoters. Even though some of these hair growth compositions may show some effects in some individuals, the treatments can be associated with adverse effects, such as skin irritation and unpleasant odors.

Surgical approaches are also available for treating hair loss, such as hair transplantation. This method typically comprises transplanting the natural hair in the scalp from regions where there is active hair growth to regions which are bald. Hair transplantation often times is costly, time consuming, painful and only shows limited success in certain individuals.

Given the significant limitations, lack of effectiveness, and disadvantages of existing treatment options for baldness, there is a need for the development of more effective methods, kits, and compositions to treat hair loss.

SUMMARY

Society continues to attach a stigma to hair loss when it comes to the human scalp, as a result, men and women who suffer from hair loss often experience self-consciousness relating to

the condition. These individuals may desire to retain as much of their hair as possible, prevent, and/or treat hair loss in an effort to avoid these consequences. Accordingly, provided herein are methods and compositions useful in preventing and/or treating hair-loss.

In a first aspect, the disclosure relates to a method of treating hair loss by inhibiting the contraction of dermal sheath cells of a hair follicle. In some embodiments, the contraction of dermal sheath cells can be inhibited or blocked by the administering of a therapeutically effective amount of an agent which inhibits the myosin light chain kinase (MLCK) pathway, *e.g.*, by inhibiting the myosin light chain kinase with a myosin light chain kinase inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In a second aspect, for hair follicles which are in anagen phase, the disclosure relates to a method of treating hair loss by inhibiting the activation of catagen phase. In some embodiments, the inhibiting of activation of catagen phase can be achieved by the administering of a therapeutically effective amount of an agent which inhibits the myosin light chain kinase (MLCK) pathway, *e.g.*, by inhibiting the myosin light chain kinase with a myosin light chain kinase inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In a third aspect, for hair follicles which are in catagen phase, the disclosure relates to a method of treating hair loss by inhibiting the further progression of catagen phase (*i.e.*, arresting catagen phase). In some embodiments, the inhibiting of catagen progression can be achieved by the administering of a therapeutically effective amount of an agent which inhibits the myosin light chain kinase (MLCK) pathway, *e.g.*, by inhibiting the myosin light chain kinase with a myosin light chain kinase inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

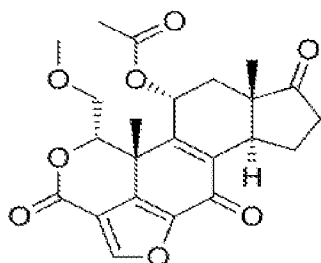
In a fourth aspect, the disclosure relates to a method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle, thereby treating hair loss. In some embodiments, the pharmacological agent is a compound, a peptide, or an antibody which inhibits myosin light chain kinase. In some embodiments, the myosin light chain kinase (MLCK) pathway can be inhibited with a myosin light chain kinase (MLCK) inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In some embodiments, the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 1 μ M or less, or 900 μ M or less, or 800 μ M, or 700 μ M, or 600 μ M or less, or 500 μ M

or less, or 400 μM or less, or 300 μM or less, or 200 μM or less, or 100 μM or less, or 50 μM or less, or 40 μM or less, or 30 μM or less, or 20 μM or less, or 10 μM or less, or 5 μM or less, or 1 μM or less. In other embodiments, the compound, peptide, or antibody has a binding inhibitory IC_{50} value of 500 nM or less, or 400 nM or less, or 300 nM or less, or 200 nM or less, or 100 nM or less, or 50 nM or less, or 40 nM or less, or 30 nM or less, or 20 nM or less, or 10 nM or less, or 5 nM or less, or 1 nM or less. In still other embodiments, the compound, peptide, or antibody has a binding inhibitory IC_{50} value of 500 pM or less, or 400 pM or less, or 300 pM or less, or 200 pM or less, or 100 pM or less, or 50 pM or less, or 40 pM or less, or 30 pM or less, or 20 pM or less, or 10 pM or less, or 5 pM or less, or 1 pM or less.

In some embodiments, the pharmacological agent is inhibitor of myosin light chain kinase (*i.e.*, an MLCK inhibitor), such as, but not limited to, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof. Many examples of such compounds are known in the art and are readily available from commercial sources, *e.g.*, from MILLIPORE SIGMA. In addition, examples of MLCK inhibitors may be found in Xiong *et al.*, “Myosin light chain kinase: a potential target for treatment of inflammatory disease,” *Front Pharmacol*, 2017, 8:292, which is incorporated herein by reference (*e.g.*, see Table 2).

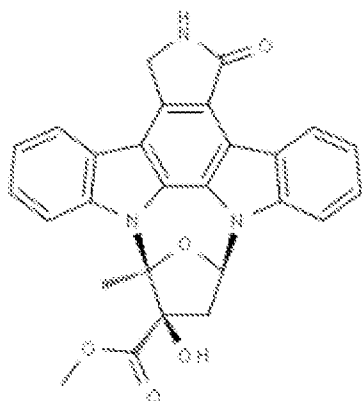
In some embodiments, the pharmacological agent is wortmannin, having the following structure,



, or a functional derivative thereof.

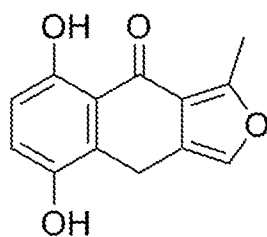
In some embodiments, the pharmacological agent is KT5926 ((8R*,9S*,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-14-n-propoxy-2,3,9,10-tetrahydro-8,11-epoxy, 1H,8H, 11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one). See *e.g.*, Nakanishi *et al.*, “KT5926, a potent and selective inhibitor of myosin light chain kinase,” *Mol Pharmacol*, 1990, 37(4): 482-8, which is incorporated herein by reference.

In some embodiments, the pharmacological agent is K-252a ((8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8, 11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one) having the chemical structure of:



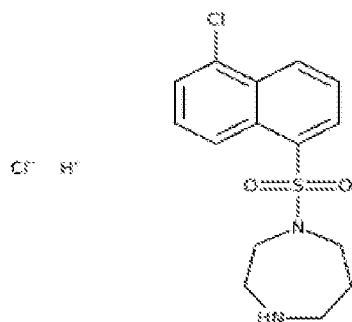
, or a functional derivative thereof. See *e.g.*, Nakanishi *et al.*, “K-252a, a novel microbial product, inhibits smooth muscle myosin light chain kinase,” *J. Biol. Chem.*, 1988, 263(13): 6215-9, the contents of which are incorporated by reference.

In some embodiments, the pharmacological agent is MS-444 having the chemical structure of:



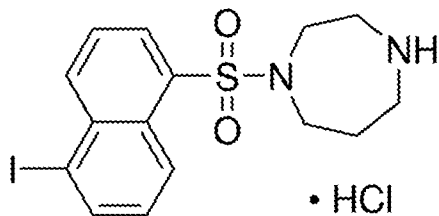
, or a functional derivative thereof. See *e.g.*, Nakanishi, *et al.* “MS-444, a new inhibitor of myosin light chain kinase from *Micromonosporasp.* KY7123,” *The Journal Of Antibiotics*, 1995, 48(9):948-951, which is incorporated herein by reference.

In some embodiments, the pharmacological agent is ML-9 ([1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine]) having the chemical structure of:



, or a functional derivative thereof. See *e.g.*, Ito *et al.*, “ML-9, a myosin light chain kinase inhibitor, reduces intracellular Ca²⁺ concentration in guinea pig trachealis,” *Euro J Pharmacol*, 2004, 486: 325-33; also see Takahashi *et al.*, “Myosin light chain kinase-independent inhibition by ML-9 of murine TRPC6 channels expressed in HEK293 cells,” *Br J Pharmacol.* 2007 Sep; 152(1):122-31, both of which are incorporated herein by reference.

In some embodiments, the pharmacological agent is ML-7 (hexahydro-1-[(5-iodo-1-naphthalenyl)sulfonyl]-1H-1,4-diazepine hydrochloride) having the chemical structure:



, or a functional derivative thereof. See *e.g.*, Xiong *et al.*, “Myosin light chain kinase: a potential target for treatment of inflammatory disease,” *Front Pharmacol*, 2017, 8:292; also see Saitoh *et al.*, “Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase,” *J Biol Chem*, 1987, 262(16) : 7796-801, both of which are incorporated herein by reference.

In some embodiments, the method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle, thereby treating hair loss, further comprises administering an agent for facilitating hair growth.

In some embodiments, the agent for facilitating hair growth is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole, each of which are well known agents in the art for treating hair loss (*e.g.*, see Hair Loss: What to do if it happens to you, Jordi B., 2010, iUniverse Books, Chapter 8: Pharmaceuticals: What’s out there and how they work, which is incorporated herein by reference). In some embodiments, the agent for facilitating hair growth is finasteride. In other embodiments, the agent for facilitating hair growth is cyproterone acetate. In some embodiments, the agent for facilitating hair growth is alfatradiol. In yet other embodiments, the agent for facilitating hair growth is minoxidil. In still other embodiments, the agent for facilitating hair growth is bimatoprost. In some embodiments, the agent for facilitating hair growth is bicalcutamide. In other embodiments, the agent for facilitating hair growth is lantoanoprost. In still other embodiments, the agent for facilitating hair growth is spironolactone. In yet other embodiments, the agent for facilitating hair growth is flutamide. In still other embodiments, the agent for facilitating hair growth is dutasteride. In some embodiments, the agent for facilitating hair growth is ketoconazole.

In some embodiments, the pharmacological agent is administered topically.

In other embodiments, the methods may further comprise administering a penetration enhancer prior to or concomitantly with the administration of the pharmacological agent.

In some embodiments, the inhibition of the MLCK pathway results in cessation of dermal sheath cell contractions, thereby halting the hair cycle in the catagen phase, which prevents further progression of the hair cycle to telogen phase.

In another aspect, the disclosure relates to a method of identifying an agent capable of treating hair loss, comprising, administering a test agent to a cell culture of dermal sheath cells, detecting the production of one or more biomarkers indicative of the inhibition of dermal sheath contraction, validating a test agent in a hair follicle model to confirm that the test agent inhibits dermal sheath contraction, thereby identifying an agent capable of treating hair loss.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGs. 1A-1J show that the dermal sheath is required for hair follicle regression. **FIG. 1A** is a schematic of catagen regression during the hair cycle. The hair shaft, inner root sheath (IRS) and matrix progenitors are eliminated by terminal differentiation and extrusion from the skin. The majority of outer root sheath (ORS) progenitors (the second layer of the follicle as counted from the outermost layer to the center of the follicle) are eliminated by apoptosis. It is unknown how the dermal papilla (DP) niche relocates to the surviving stem cell reservoir (shown as a small portion positioned at the bottom of each figure indicated by an arrow). **FIG. 1B** shows the immunofluorescence for the proteoglycan, ACAN, secreted by ITGA8+ dermal sheath (DS) cells (ITGA8 is shown as the outermost layer from the outside moving inward (moving right to left in the small dotted box of the left panel); ACAN is shown as the second outermost layer from the outside moving inward (moving right to left in the small dotted box of the left panel); DAPI is shown as the internal layer). The image on the right is a magnified view of the dotted box region of the image on the left. **FIG. 1C** is a schematic of cytotoxic ablation of DS during catagen. **FIG. 1D** shows the immunofluorescence for α SMA in control (R26^{LSL-DTA}) and DS-ablated (Acan^{CreER}; R26^{LSL-DTA}) back skin. Remnants of fragmented α SMA staining in DS-ablated follicle at the beginning of catagen are shown at P15. **FIGs. 1E-1F** show a whole mount immunofluorescence for ORS marker K14 in P20 back skins (viewed from dermis side, anterior = left). Control follicles are in telogen (FIG. 1E). Note elongated stalled follicles after DS ablation (FIG. 1F). **FIG. 1G** shows an inset (10 μ m) of the

full scan. The hair shaft and DP remain at bulb tip. **FIG. 1H** shows the quantification of percent stalled follicles with length greater than 150 μm at P20 ($n = 698$ in control and 895 in DS-ablated follicles in 5 mice). $**P = 0.003$, unpaired two-tailed t-test. **FIG. 1I** shows the quantification of follicle lengths ($n = 11$, P13 control; $n = 14$ P13 ablated; $n = 80$, P20 control; $n = 27$, P20 stalled follicles; 11 mice). $****P < 10^{-4}$, unpaired two-tailed t-test. **FIG. 1J** shows that stalled follicles have no DS (αSMA^-), but retain intact DP (LEF1+). K14+ ORS progenitor and K6+ companion (Cp) layers are present and lack apoptosis (activated CASP3) or proliferation (Ki67) markers. Differentiating cuticle (Ch), cortex (Co), medulla (Me) of hair shaft and the inner root sheath (IRS) are undetectable. Scale bars represent 50 μm in FIGs. 1B, 1E, and 1F. Scale bars represent 10 μm in FIGs. 1D and 1J.

FIGs. 2A-2J show the dermal sheath expresses the molecular machinery of smooth muscle. **FIGs. 2A-2B** show flow cell sorting of DS and DP from Sox2^{GFP}; *Lef1-RFP* P5 back skin and immunofluorescence for PDGFRA. Dermal fibroblasts (DF) were sorted for comparison. **FIG. 2C** shows qRT-PCR verification of sorted populations by known marker genes performed on biological duplicates. **FIG. 2D** is a Venn diagram of gene signatures. **FIG. 2E** shows the gene ontology (biological process) analysis of DS signature. **FIG. 2F** shows a gene set enrichment analysis (GSEA) for genes involved in smooth muscle contraction and regulation are highly enriched in DS. **FIG. 2G** is a schematic of Ca²⁺-dependent smooth muscle contraction pathway. The heatmap is of smooth muscle contraction gene expression. Ca²⁺ contraction pathway and pan-smooth muscle genes (asterisks) are highly enriched in DS. **FIG. 2H** shows 3D immunofluorescence for αSMA fibers arranged in a concentric ring-like network wrapping around the follicle. **FIG. 2I** shows the immunofluorescence of smooth muscle contraction components in DS. **FIG. 2J** shows the immunofluorescence of smooth muscle proteins in DS of human scalp follicles. Data bars are mean $\pm$ s.d. Scale bars represent 50 μm .

FIGs. 3A-3J show that DS-mediated hair follicle contraction is required for regression *in vivo*. **FIG. 3A** shows high K⁺ membrane depolarization activates Ca²⁺ influx in isolated DS cells (Fluo8 indicator). $n = 6$. Data bars are mean $\pm$ 95% C.I. $**P = 0.002$, paired two-tailed t-test. **FIG. 3B** shows that depolarization triggers DS contraction and surface area reduction in cells grown on matrigel. $n = 35$. Data bar is mean $\pm$ 95% C.I. $***P < 0.0001$, paired two-tailed t-test. **FIG. 3C** is a schematic of live imaging microdissected follicles pre-incubated with or without MLCK inhibitor ML7 and after high K⁺ depolarization. **FIGs. 3D-3E** show still images from brightfield movie at start (left-most panels) and end (middle panels; composite of start and stop is shown in the right-most panels) of high K⁺ incubation. Overlays highlight

reduction of follicle width, blocked by ML7. **FIG. 3F** shows the quantification of follicle widths during live imaging. $n = 7$ follicles for ML7 and no inhibitor pre-incubation. Data points are mean $\pm$ s.d. $**P < 0.01$, unpaired two-tailed t-test. **FIGs. 3G-3J** show the topical inhibition of MLCK by ML7 blocks hair follicle regression *in vivo*. **FIG. 3H** is a schematic of ML7 or vehicle application during catagen. P20 back skins show normal regression of follicles into telogen in control, but stalled follicles in contraction-inhibited ML7-treated regions. **FIG. 3I** shows the quantification of % stalled follicles. $n = 279$ follicles from control and $n = 288$ follicles from treated regions in 3 mice. Data bars are mean $\pm$ s.d. $**P = 0.008$, unpaired two-tailed t-test. **FIG. 3J** shows the immunofluorescence for LEF1, proliferation marker Ki67 and α SMA demonstrated full regression of follicles into telogen in DMSO treated control skin region. K14 marks bulge and germ stem cells, and LEF1 marks the relocated, adjacent DP. Absence of Ki67 in the stem cells confirms telogen quiescent state. α SMA+ DS is absent in the telogen follicle. Stalled follicles have intact DS (α SMA) and DP (LEF1) that are no longer engulfed. Epithelial cells of stalled follicles are not proliferative (Ki67-). The scale bars represent 50 μ m in FIG. 3H, and 10 μ m in FIG. 3J.

FIGs. 4A-4P show that DS cell movement pushes the hair shaft and indirectly pulls the DP. **FIG. 4A** shows the triple-fluorescent reporter follicles for intravital 3D time-lapse imaging of catagen regression in live mice (5 hours). Acan^{tdT} and Tbx18^{H2BGFP} marks DS cytoplasm and nuclei, respectively. *K14-H2BCer* highlights all epithelial nuclei (Acan^{tdT} can be seen generally as the outer DS (the first layer moving outward to inner), with Tbx18^{H2BGFP} shown as the small round markings therein); *K14-H2BCer* can be seen in the ORS/Epithelial Strand as the second layer counting from the outermost layer moving inward). The DP was recognized by surrounding epithelial and DS cells and low level Tbx18^{H2BGFP} expression. **FIG. 4B** shows single optical slices of a follicle during regression at 0 hours, 2.5 hours, and 5 hours of imaging. **FIG. 4C** shows the upward movement of hair shaft and DP during regression. **FIG. 4D** shows the individual cell tracking of ORS (second layer moving outward inward from left to right or right to left), shaft (middle portion (fourth layer counting from the outermost to innermost) shown in the upper half of the image), and DS (first layer (outer layer) counting from the outermost to innermost) and tracking of upper and lower bounds of DP during 5-hour imaging. **FIG. 4E** shows the quantifications of live cell tracking relative to ORS movement (7.5 minute intervals). Shaft and DP move upward (distally) relative to ORS and DS. Solid lines are average; shaded areas are s.d. $n = 13$ HS, $n = 8$ DP, $n = 26$ ORS, and $n = 17$ DS measurements (7 follicles, 3 imaging sessions). **FIG. 4F** shows the final relative cell movement. $****P < 10^{-8}$,

unpaired two-tailed t-test. **FIG. 4G** is a schematic showing DP niche relocation during regression and shows two historically hypothesized mechanisms. **FIGs. 4H-4I** show the DS cross-sectional diameter at DP bottom. $n = 4$ follicles. $P = 0.994$ (0 hours v. 5 hours.) **FIGs. 4J-4K** show the length of epithelial strand during mid-regression. $n = 10$ follicles. $P = 0.572$ for 0 hours vs 5 hours. **FIGs. 4L-4N** show the DS cross-sectional diameter at club hair-epithelial strand bottleneck ($n = 3$ follicles). $*P = 0.021$, $**P = 0.009$ and $P = 0.010$, unpaired two-tailed t-test. **FIG. 4O** is a schematic of the new proposed mechanism of DP niche relocation during regression by potential DS contraction on follicle bottleneck and pulling of DP via epithelial strand. **FIG. 4P** is a model for DP niche relocation during regression by DS contraction forcing upward movement of the hair shaft, which pulls the DP upward via the epithelial strand. Data bars are mean $\pm$ s.d. Scale bars represent 10 μ m.

FIGs. 5A-5B show the hair follicle cycle of dynamic stem cell niche reorganization. **FIG. 5A** is a schematic of the hair follicle bulb and its epithelial and mesenchymal lineages during anagen growth. In the mesenchymal compartment, the dermal sheath (DS) forms the outermost casing of the follicle and the central dermal papilla (DP) at the base is engulfed by epithelial matrix (Mx) progenitors. The outer root sheath (ORS), the outermost layer of epithelium contains slow cycling multipotent progenitors. Mx progenitors abutting the DP consist of rapidly dividing transit amplifying cells (TAC) that produce progeny that differentiate into cells of the outgoing shaft and its channel. Distinct markers highlight epithelial lineages: K14 for ORS; K6 for companion layer (Cp) and shaft medulla (Me); AE15 for Henle's layer (He), Huxley's layer (Hu), and cuticle (Ci) of the inner root sheath (IRS) and shaft medulla (Me); AE13 for shaft cuticle (Ch) and cortex (Co). **FIG. 5B** is a schematic of the hair cycle with focus on catagen regression. During catagen onset, matrix progenitors cease proliferation and deplete through terminal differentiation. Throughout mid- and late-catagen, ORS progenitors undergo large-scale pruning by apoptosis. During regression, the shaft and DP niche are translocated upward through an unknown mechanism. Throughout the entirety of catagen, the DS encapsulates the follicle. In late catagen, the DS extends below the DP forming an empty sleeve. At the conclusion of catagen, the DP niche resides adjacent to the stem cell reservoir, an essential position vital for the relay of short-range activating niche signals to the stem cells for induction of hair regeneration and the start of a new cycle.

FIG. 6 shows a drastic homeostatic follicle remodeling and niche relocation to the stem cell reservoir during catagen regression. Histological section of Tbx^{18Cre} ; $R26^{mT/mG}$ back skin follicles. Mesenchymal populations including DP (arrows pointing up and to the left) and

DS (arrows pointing up and to the right) are marked by membrane GFP. During catagen regression, the DP niche relocates a distance of ~90% of the follicle length (between DP and sebaceous gland, SG). At the beginning of regression, matrix progenitors degenerate, and the DP niche (now rounded appearance) is only partially engulfed. During mid-regression, the DP is connected to the upper follicle via the regressing epithelial strand. The thin DS layer becomes notably thicker around the epithelial strand and DP. During final regression, the hair shaft reaches its final position and the DP is separated from the stem cell reservoir only by the epithelial strand. The DS forms a dense trailing empty sleeve below the DP. After the conclusion of regression (telogen = rest), the DP niche resides adjacent to the stem cell reservoir. Cell nuclei are highlighted by DAPI. Scale bar represents 50 μ m.

FIGs. 7A-7C show highly selective and inductive DS targeting with Acan^{CreER}. FIG. 7A is a schematic showing the fluorescence reporter of dermal sheath genetic targeting with Acan^{CreER}; R26^{LSL-tdTomato} (Acan^{tdT}). **FIG. 7B** shows the fluorescence microscopy for tdT expression in back skin after induction with tamoxifen from P10-12 and analysis at P15. **FIG. 7C** shows the quantification of follicles with DS labelling. Data bars are means of each bin; n = 65 follicles from 3 mice. Line is normal distribution fit of data. Scale bars represent 50 μ m.

FIGs. 8A-8C show the immunofluorescence of lineage, proliferation, and apoptosis markers. Control immunofluorescence of wild-type anagen (P8), catagen (P18), and telogen (P20) hair follicles. **FIG. 8A** shows outer root sheath progenitors during anagen are K14+. The DS is marked by α SMA. Matrix progenitors express LEF1 and are Ki67+ indicative of proliferative status. LEF1 is also moderately expressed in DP. Differentiated epithelial lineage markers are detectable in inner root sheath (IRS), AE15; shaft cortex (Co) and cuticle (Ch), AE13; shaft medulla (Me), AE15 and K6; and companion layer (Cp), K6. Cell death by apoptosis is undetectable (active caspase 3, CASP3*). **FIG. 8B** shows progenitor pruning by apoptosis is detectable by CASP3*. **FIG. 8C** shows proliferation, differentiation, and apoptosis markers are absent in telogen follicles. Only K6 marks the inner bulge cells of telogen follicles. α SMA+ DS is absent during telogen. K14 is retained in bulge and germ stem cells. Scale bars represent 50 μ m.

FIG. 9 shows DP, DS, and dermal fibroblast labeling for cell isolation. Sox2^{GFP}; *Lef1RFP* mice and PDGFRA immunofluorescence for cell isolations. GFP marks DP (arrows, some representative arrows are labeled DP) and DS (indicators with circular heads (circles represent the tip of the indicator), some representative indicators are labeled DS). DF (indicators with diamond heads (diamonds represent the tip of the indicator), some representative indicators

are labeled DF) and DP have robust RFP fluorescence, which is absent in DS cells. DP and DS can be further distinguished from other GFP+ skin populations by PDGFRA staining. Scale bars represent 50 μ m.

FIGs. 10A-10E show RNA sequencing analysis and smooth muscle gene enrichment in DS. **FIG. 10A** shows the hierarchical clustering of DS, DP, DF, ORS, matrix (Mx), and melanocytes (Mc) using all differentially expressed genes identified by ANOVA (FDR < 0.05). Biological duplicates of sorted populations cluster together. DS, DP, and DF and ORS and Mx cluster together indicative of lineage relatedness. **FIG. 10B** shows the principal component analysis using all differentially expressed genes. DS, DP, and DF cluster together apart from ORS, Mx, and Mc, and DS and DP cluster closer than DF. Mc cluster separate from other populations. **FIG. 10C** shows a signature gene analysis reveals 483 DS signature genes. **FIG. 10D** shows expression levels of smooth muscle genes from RNA sequencing represented as fragments per kilobase per million mapped reads (FPKM). **FIG. 10E** shows the qRT-PCR verification of smooth muscle gene expression in DS. Data are mean $\pm$ s.d.

FIGs. 11A-11C show the post-processing of triple-color 2-photon imaging. **FIG. 11A** shows intravital imaging setup of live mice. **FIG. 11B** shows a table of fluorescence signal strength of fluorophores in each channel using a single 900 nm excitation wavelength. **FIG. 11C** shows the steps of fluorophore signal isolation. (1) Isolate second harmonic generation (SHG) signal in raw blue channel data by subtracting green channel from blue channel. (2) Isolate H2BCer-only signal by subtracting isolated SHG from raw blue channel. (3) Isolate H2BGFP and tdT signals by subtracting H2BCer-only signal from raw green and red channels, respectively. (4) Merge isolated tdT, H2BGFP and H2BCer signals and pseudocolor H2BCer to light blue for visibility. Scale bar represents 50 μ m.

FIGs. 12A-12C show the generation of *K14-H2BCer* fluorescence reporter mice. **FIG. 12A** shows the schematic of transgenesis strategy. The coding sequence for H2B-mCerulean3 fusion protein was inserted into plasmid vector containing K14 promoter, b-globin intron, and polyA (pA). **FIG. 12B** shows that cerulean fluorescence is restricted to epithelial cells in skin epidermis and hair follicles. **FIG. 12C** shows the distinct fluorescence of Cerulean (nuclei of epithelial cells) and GFP (nuclei of DS and other mesenchymal cells) in *Tbx18*^{H2BGFP}; *K14-H2BCer* anagen back skin. Far-red NucRed 647 highlighted all nuclei. Scale bars represent 50 μ m.

FIGs. 13A-13C show the dynamic movements of hair follicle populations during regression. **FIG. 13A** shows intravital 3D time-lapse imaging of catagen regression in live

mice. Triple-fluorescent Acan^{tdT}; Tbx18^{H2BGFP}; *K14-H2BCer* reporter follicles were live-imaged for 5 hours. Time courses of two optical slices capturing shaft (z-slice 8) and DP (z-slice 10) movements (slices are separated by 6 μ m). Steady upward movements of shaft and DP are outlined by the sloped dashed lines, in each set of 5 panels (leftmost 5 panels and rightmost 5 panels) respectively. **FIG. 13B** shows cell tracking for quantifying cell movements during regression. DS and ORS show minimal movement in the long axis. Bases of arrows are at starting position and tips of arrows are at ending position of 5-hour cell tracking. **FIG. 13C** shows the tracking of DS cell centripetal constriction movement at club-epithelial strand bottleneck and of hair shaft upward movement. Arrows are starting and ending positions of 5-hour tracking. Scale bars represent 10 μ m.

DETAILED DESCRIPTION

The present disclosure relates, in part, to the surprising discovery that the dermal sheath cells of human hair follicles bear the specific function of driving the upward relocation of the dermal papilla niche cells at the bottom of the hair follicle to a position that is adjacent to stem cells in the upper region of the hair follicle during catagen phase. The inventors have discovered and demonstrated it is believed for the first time that this upward movement of the niche cells during catagen phase is propelled by the smooth muscle cell-like properties of the dermal sheath cells. As proposed herein, the hair cycle may be held or “frozen” in catagen phase by inhibiting or otherwise blocking the smooth muscle cell-like contractions of the dermal sheath cells. In some embodiments, this is achieved by inhibiting the myosin light chain kinase (MLCK) pathway, which disrupts the smooth muscle cell function of the dermal sheath cells, thereby freezing the hair growth cycle in catagen phase. This state results in the minimization of further hair loss, which occurs normally at the end of the subsequent telogen phase. Thus, in various aspects, the present disclosure provides methods, compounds, and compositions for inhibiting the smooth muscle cell function of the dermal sheath cells as a means to halt the cell cycle in catagen phase.

Thus, in one aspect, the disclosure relates to a method of treating hair loss by inhibiting the smooth muscle cell function of the dermal sheath cells of the hair follicles. Such inhibition may be achieved in certain embodiments through the administration of one or more agents that inhibit smooth muscle cell function, *e.g.*, an inhibitor of the myosin light chain kinase (MLCK) pathway.

A. Definitions

A, an, the

As used herein and in the claims, the singular forms “a,” “an,” and “the” include the singular and the plural reference unless the context clearly indicates otherwise. Thus, for
5 example, a reference to “an agent” includes a single agent and a plurality of such agents.

Human hair growth cycle

As used herein, reference to the “human hair growth cycle” refers to the naturally occurring life cycle of a strand of hair grown from a hair follicle. Human hair begins growing *in utero*. In the later stages of a pregnancy around week 22, a fetus will have developed all or
10 nearly all of the hair follicles it will have throughout its lifetime. At this point, the average human will have around 5 million hair follicles on the body with approximately one hundred thousand of those follicles located on the scalp. Humans do not normally develop new hair follicles after birth, thus, at birth this number is larger than at any other point during the course of a human subject’s life. Hair loss can be defined under any medically accepted criteria, but
15 generally means any decrease in the number of live follicles, the health of the follicles, the rate at which the follicles progress through the hair growth cycle, the thinning of the hair shafts produced by the follicles, or any combination thereof, which may lead to less or thinning hair on the subject in a target area, or the appearance thereof.

Human hair has two distinct structures: (1) the follicle itself, which resides in the skin,
20 and (2); and the shaft, which is what is visible above the scalp. The hair follicle bulb is a cylinder-like epithelial segment of the epidermis that extends down into the dermis. The follicle contains several concentric layers which all have separate functions to form the differentiated hair shaft and surrounding inner root sheath (IRS). At the base of the follicle is the dermal papilla (DP), which contains capillaries, or tiny blood vessels that nourish the cells. The DP’s
25 main function is to send signals as a specialized “niche” to the surrounding hair follicle progenitors to divide every 23 to 72 hours. Dividing cells then move upwards and differentiate and die to form the outgrowing hair shaft and IRS. The outermost cell layer is the outer root sheath (ORS), that directly connects the progenitors at the base with the epidermis. In the upper portion of the ORS reside the hair follicle stem cells in a reservoir called the bulge.

30 A mesenchymal layer surrounds the epithelial part of the hair follicle, called the dermal sheath (DS). The DS follows the hair follicle and ends below the opening of a sebaceous gland (*i.e.*, oil gland), and sometimes an apocrine gland (*i.e.*, scent gland) A muscle called an erector

pili muscle attaches below the gland to a fibrous layer around the DS. When this muscle contracts, it causes the hair to stand up which also causes the sebaceous gland to secrete oil. The sebaceous gland is vital because it produces sebum, which conditions the hair and skin. After puberty our body produces more sebum but as we age we begin to make less sebum. Women have far less sebum production than men do as they age.

The hair itself, referred to as the shaft, is made of a hard protein called keratin and is comprised of three layers. Keratin is actually dead, so the visible hair strands are not living structures. The three layers of the shaft from inner to outer are: (1) medulla; (2) cortex; and (3) cuticle. The cortex makes up the majority of the hair shaft. The cuticle is a tightly formed structure made of shingle-like overlapping scales. It is both the cortex and the medulla that holds the hair's pigment, giving it its color. Hair on the scalp grows about 0.3 to 0.4 millimeters per day or about 6 inches per year. Unlike other mammals, human hair growth and shedding is random and not seasonal or cyclical. At any given time, a random number of hairs will be in one of three stages of growth and shedding: anagen, catagen, and telogen.

Anagen is the active phase of the hair growth cycle, when the cells in the root of the hair are dividing rapidly. A new hair is formed and pushes the old, mature club hair from the previous hair growth cycle (a hair that has stopped growing or is no longer in the anagen phase) up the follicle and eventually out. Scalp hair stays in this active anagen phase of growth for two to six years. Some people have difficulty growing their hair beyond a certain length because they have a short active phase of growth. On the other hand, people with very long hair have long active phases of growth. There is a correlation between the length of the active phase of hair growth and the length of a hair, for example, the hair on the arms, legs, eyelashes, and eyebrows have very short active growth phases of about 30 to 45 days, which is why they are so much shorter than scalp hair. The next phase, catagen, is a transitional stage and about 3% of all hairs are in this phase at any given time. Catagen typically lasts for about two to three weeks, during which, growth stops and the ORS shrinks and attaches to the root of the hair. This process forms what is known as a club hair. During this phase, the DP niche physically relocates from the lower region of the hair follicle up the shaft to a position that is adjacent to the bulge of stem cells near the upper part of the hair follicle. Telogen is the final phase in the process, it is known as the resting phase and usually accounts for 6% to 8% of all hairs. This phase lasts for about 100 days for hairs on the scalp and longer for hairs on the eyebrow, eyelash, arm, and leg. During this phase, the hair follicle is completely at rest and the club hair

is completely formed. Pulling out a hair in this phase will reveal a solid, hard, dry, white material at the root. About 25 to 100 telogen hairs are shed normally each day.

Different mechanisms in the niche affect the stages and transition from one stage of hair growth to the next, however, contraction of the follicle, by muscle, is required to move the club hair out of the follicle and allow the next hair shaft to begin growing and migrating out the follicle. When physiological changes occur, and one phase of the hair growth cycle is exited and another is entered, the hair shaft is pushed up the follicle. The mechanism of action required to propel the shaft upward was previously poorly characterized, however, herein it is shown that the action is at least in part due to contractions of the surrounding tissue of the follicle. Many of the biomarkers found in smooth muscle have been identified and illustrate a possible mechanism of control over the phases of the hair growth cycle. In particular, it is shown herein, that by inhibiting the myosin light-chain kinase pathway, the hair growth cycle can be modulated to prevent and/or delay the follicle from entering catagen from anagen or if in catagen already, prevent it from leaving catagen and entering telogen.

Myosin light chain kinase (MLCK) pathway

Reference to the “myosin light chain kinase (MLCK) pathway” or equivalently, the “calcium/calmodulin/myosin light chain kinase pathway” refers to the signaling pathway that activates smooth muscle cell contraction in dermal sheath cells via calcium influx-activated calmodulin and calcium binding to MLCK that phosphorylates myosin light chains for myosin/actin-mediated contraction. The blocking or inhibiting of same impedes follicle contraction, regression and niche relocation by inhibiting the smooth muscle cell-like contractions of dermal sheath cells.

Subject

As used herein, a “subject” refers to a human exhibiting hair loss, *e.g.*, male pattern baldness (alopecia), or who is genetically predisposed to hair loss, *e.g.*, male pattern baldness.

Therapeutically effective amount

As used herein, “therapeutically effective amount” refers to the amount of a pharmacological agent which is sufficient to block or otherwise slow hair loss in a subject. In various embodiments, the therapeutically effective amount may include the amount of a pharmacological agent which is sufficient to inhibit the myosin light chain kinase (MLCK) pathway in the dermal sheath cells, thereby treating hair loss. In other embodiments, the therapeutically effective amount may include the amount of a pharmacological agent which is

sufficient to arrest the natural hair cycle in the catagen phase. In still other embodiments, the therapeutically effective amount may include that amount of an agent which is sufficient to inhibit the smooth muscle cell-like contractile activity of dermal sheath cells of a hair follicle. Each of these therapeutically effective amounts may be considered as equivalents one to the other.

Treatment

As used herein, reference to “treatment” refers to the arrest or otherwise slowing of hair loss. In some embodiments, such treatment can be achieved by arresting the natural hair cycle in the catagen phase with one or more therapeutically effective agents. In other embodiments, such treatment can be achieved by inhibiting the smooth muscle cell-like contractile activity of the dermal sheath cells of a hair follicle. In still other embodiments, such treatment can be achieved by the inhibition of the myosin light chain kinase (MLCK) pathway in the dermal sheath cells. Each of these means can be considered equivalent or at least overlapping in scope because they each are achieved by the inhibition of smooth muscle cell function of the dermal sheath cells, *e.g.*, by administering a therapeutically effective amount of an inhibitor of the myosin light chain kinase (MLCK) pathway.

Pharmacological agent

As used herein, reference to a “pharmacological agent” can include a small molecule compound, a therapeutic peptide, a therapeutic polypeptide (*e.g.*, an antibody), which exhibits a therapeutic effect, *e.g.*, the inhibition of the myosin light chain kinase pathway, the arrest of the natural hair cycle in the catagen phase, or the inhibition of the smooth muscle cell-like contractile activity of dermal sheath cells in the hair follicles. In some embodiments, the compounds, peptides, and/or polypeptides can include functional derivatives of known compounds. The term “functional derivative” refers to a structurally or chemically modified variant of a known agent which has the same or substantially the same activity as the known agent.

B. Pharmacological Agents (*e.g.*, MLCK inhibitors)

The disclosure provides pharmacological agents (*e.g.*, MLCK inhibitors) which can include small molecule compounds, therapeutic peptides, or therapeutic polypeptides (*e.g.*, an antibody) (or combinations thereof) which exhibit a therapeutic effect, *e.g.*, the inhibition of the myosin light chain kinase pathway, the arrest of the natural hair cycle in the catagen phase, or the inhibition of the smooth muscle cell-like contractile activity of dermal sheath cells in the hair

follicles. Further, the disclosure provides methods of treating hair loss by administering a therapeutically effective amount of such pharmacological agents. Such therapeutic effects can include the inhibition of the myosin light chain kinase (MLCK) pathway, the arrest of the natural hair cycle in the catagen phase, or the inhibition of the smooth muscle cell-like contractile activity of dermal sheath cells in the hair follicles.

The MLCK pathway involves the interaction of multiple proteins to ultimately cause an increase in phosphorylation of the myosin light chain, and the contraction of smooth muscle cells. Without being bound by theory, the present inventors have discovered and/or shown the existence of the MLCK pathway in the dermal sheath cells of the hair follicles and their association with a smooth muscle cell-like contractility activity which is responsible for the movement of the dermal papilla (DP) cells from the lower portion of the hair follicle upwards to a position adjacent the stem cell bulb in the upper portion of the hair follicle during the catagen phase. The present methods may involve inhibiting the normal function of any suitable protein target involved in the MLCK pathway which results in inhibition of the MLCK pathway and the smooth muscle cell contractions of the dermal sheath cells. Such proteins include, but are not limited to, calmodulin, MLCK, pp60 SRC, and myosin light chain phosphatase (MYLP).

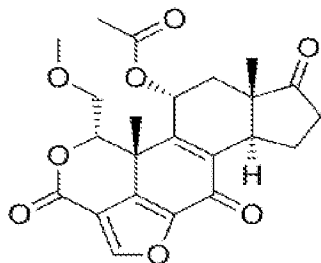
In certain embodiments, the herein methods may involve administering a pharmaceutical agent that inhibits the myosin light chain kinase (MLCK) pathway and consequently, the inhibition of the smooth muscle cell-like contractions of dermal sheath cells of the hair follicles.

In certain aspects, the pharmaceutical agents may specifically inhibit a myosin light-chain kinase (MLCK). MLCK refers to a set of serine/threonine-specific protein kinases which play an important role the mechanism of contractions in smooth muscle. MLCK is calcium cation dependent, an influx of calcium cations (Ca^{2+}) into the muscle binds to calmodulin. After binding to calmodulin, pp60 SRC (a protein kinase) phosphorylates MYLK, activating it and resulting in an increase in phosphorylation of the myosin light chain. Phosphorylation of MLCK enables the myosin crossbridge of phosphorylated myosin light chain with myosin heavy chain to bind to the actin filament and allow contractions to begin. Myosin light chain phosphorylation is balanced with dephosphorylation by myosin light chain phosphatase (MYLP). MLCK pathway-mediated smooth muscle contraction of DS cells is in at least part causative of the progression of the hair shaft migration out of the follicle and allow the follicle to progress from one phase to the next. Inhibiting MLCK, inhibits the contractions of the smooth muscle of the hair follicle. Since follicle niche physiology can at least in part affect the rate at which the follicle progresses through the hair growth cycle, this inhibition will decrease the

likelihood the follicle will leave catagen and enter telogen. As a result, it is likely to retain the mature club hair. In some embodiments, a pharmacological agent is applied to inhibit MLCK in the dermal sheath to prevent or treat (*e.g.*, inhibit) hair loss. In some embodiments the pharmacological agent is a compound, a peptide, or an antibody which inhibits myosin light chain kinase.

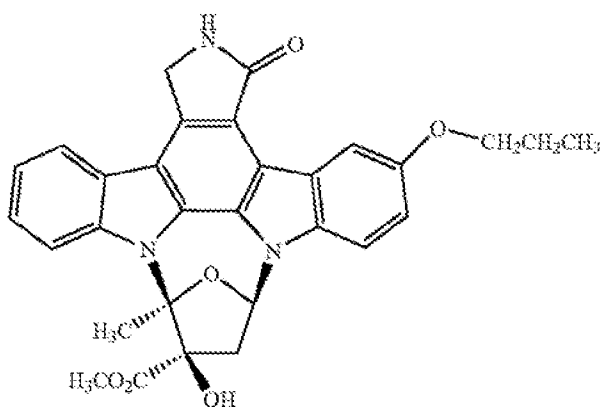
Various pharmacological agents are known in the art which inhibit MLCK activity. In some embodiments, the pharmacological agent is inhibitor of myosin light chain kinase (*i.e.*, an MLCK inhibitor), such as, but not limited to, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof, including all functional equivalents, analogs, and pharmaceutically effective derivatives of these inhibitors, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of a compound of this disclosure, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect. These compounds are known in the art and are readily available from commercial sources, *e.g.*, from MILLIPORE SIGMA. Additional MLCK inhibitors can include, for example, staurosporine (*e.g.*, US Patent No. 6,110,912), calphostin C, H-7, H-8, H-89, HA-1 00, HA-1 077, K252b, piceatannol, peptide 18, Sm-I peptide, peptide 342-352. In addition, examples of MLCK inhibitors may be found in Xiong *et al.*, “Myosin light chain kinase: a potential target for treatment of inflammatory disease,” *Front Pharmacol*, 2017, 8:292 (*e.g.*, see Table 2 of Xiong *et al.*); Luck, S., and Choh, V. (2011), “Effects of a myosin light chain kinase inhibitor on the optics and accommodation of the avian crystalline lens,” *Mol Vis*. 17, 2759–2764; Chang, et al. (2016), “Cardiac myosin light chain is phosphorylated by Ca²⁺/calmodulin-dependent and -independent kinase activities,” *Proc. Natl. Acad. Sci. U.S.A.* 113, E3824–E3833; and Saitoh, *et al.* (1987), “Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase,” *J. Biol. Chem.* 262, 7796–7801, each of which is incorporated herein by reference in their entireties. In addition, further examples of MLCK inhibitors that may be used in the accordance with the instant disclosure include any of those compounds or polypeptides disclosed in US 2008/0081078 (“Prevention of Cell Proliferation by Inhibiting Myosin Light Chain Kinase”).

In some embodiments, the pharmacological agent is wortmannin, having the following structure,



, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

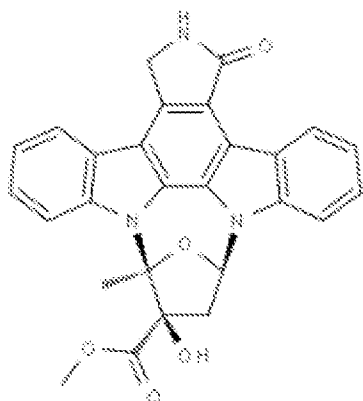
- 5 In some embodiments, the pharmacological agent is KT5926 ((8R*,9S*,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-14-n-propoxy-2,3,9,10-tetrahydro-8,11-epoxy, 1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one), and having the following structure:



, including all functional equivalents,

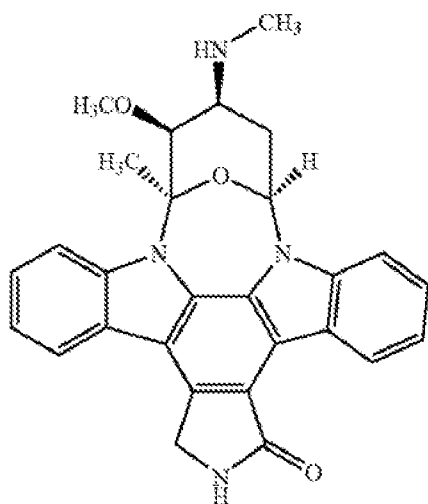
- 10 analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect. See *e.g.*, Nakanishi *et al.*, “KT5926, a potent and selective inhibitor of myosin light chain kinase,” *Mol Pharmacol*, 1990, 37(4): 482-8, which is
15 incorporated herein by reference.

In some embodiments, the pharmacological agent is K-252a ((8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one) having the chemical structure of:



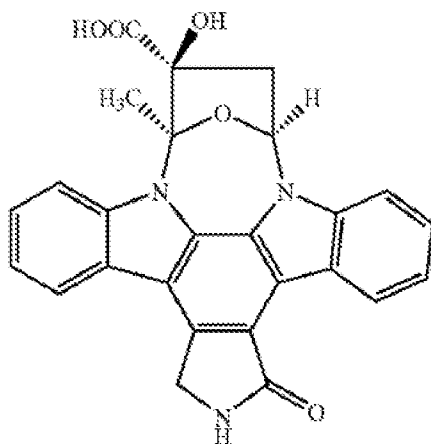
, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect. See *e.g.*, Nakanishi *et al.*, “K-252a, a novel microbial product, inhibits smooth muscle myosin light chain kinase,” *J. Biol. Chem.*, 1988, 263(13): 6215-9, the contents of which are incorporated by reference.

In other embodiments, the pharmacological agent is staurosporine, having the chemical structure:



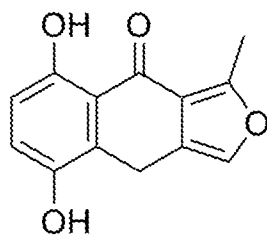
, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

In various embodiments, the pharmacological agent is K-252b, having the chemical structure:



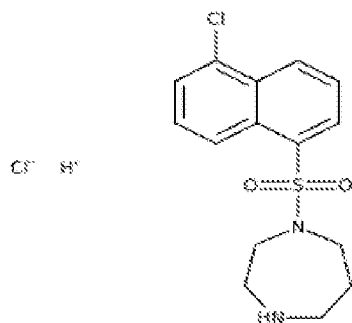
, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

In some embodiments, the pharmacological agent is MS-444 having the chemical structure of:



, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect. See *e.g.*, Nakanishi, *et al.* "MS-444, a new inhibitor of myosin light chain kinase from *Micromonosporasp.* KY7123," *The Journal Of Antibiotics*, 1995, 48(9):948-951, which is incorporated herein by reference.

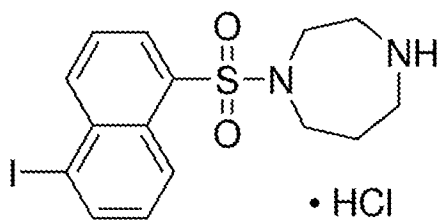
In some embodiments, the pharmacological agent is ML-9 ([1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine]) having the chemical structure of:



, including all functional equivalents, analogs, and

pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect. See *e.g.*, Ito *et al.*, “ML-9, a myosin light chain kinase inhibitor, reduces intracellular Ca²⁺ concentration in guinea pig trachealis,” *Euro J Pharmacol*, 2004, 486: 325-33; also see Takahashi *et al.*, “Myosin light chain kinase-independent inhibition by ML-9 of murine TRPC6 channels expressed in HEK293 cells,” *Br J Pharmacol*. 2007 Sep; 152(1):122-31, both of which are incorporated herein by reference.

In some embodiments, the pharmacological agent is ML-7 (hexahydro-1-[(5-iodo-1-naphthalenyl)sulfonyl]-1H-1,4-diazepine hydrochloride) having the chemical structure:

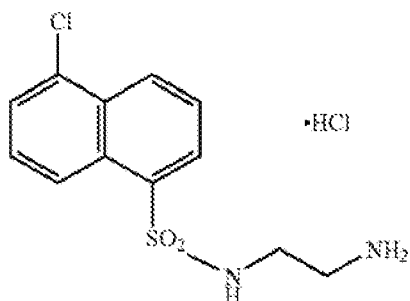


, including all functional equivalents, analogs, and

pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect. See *e.g.*, Xiong *et al.*, “Myosin light chain kinase: a potential target for treatment of inflammatory disease,” *Front Pharmacol*, 2017, 8:292; also see Saitoh *et al.*, “Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase,” *J Biol Chem*, 1987, 262(16) : 7796-801, both of which are incorporated herein by reference.

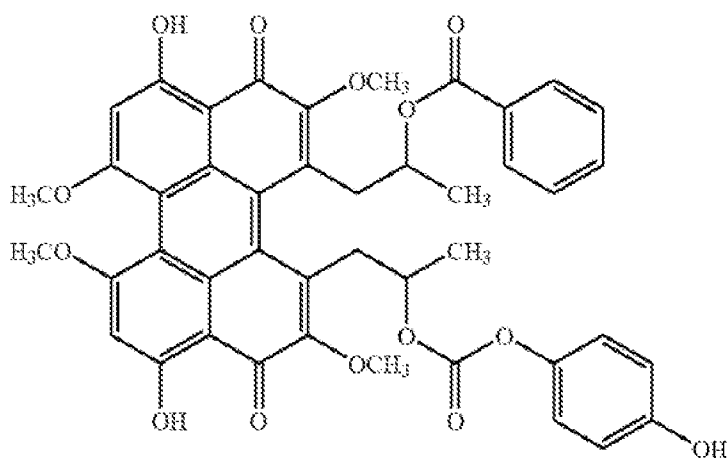
Other MLCK inhibitors may include:

A-3, having the chemical name, N-(2-aminoethyl)-5-chloronaphthalene-1-hydrochloride sulphonamide, (HCL), and having the following structure:



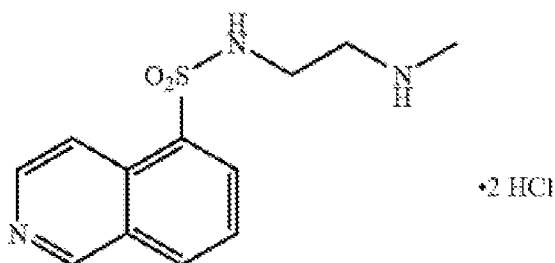
, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

Calphostin C, having the following structure:



, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

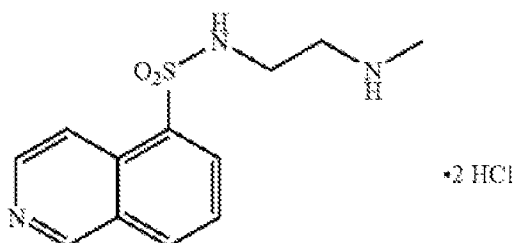
H-7, having the chemical name, N-[2-methylamino)ethyl]-5-dihydrochloride isoquinolinesulfonamide·2HCl, and having the following structure:



, including all functional equivalents,

analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

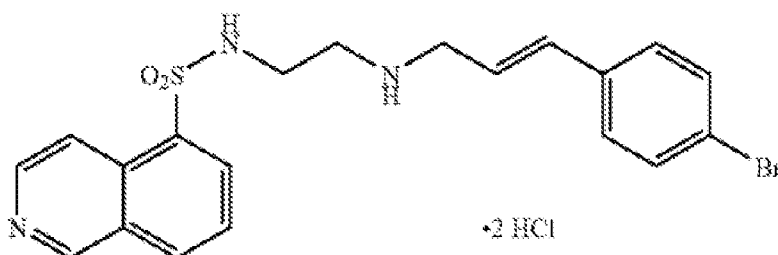
H-8, having the chemical name, N-[2-methylamino)ethyl]-5-dihydrochloride isoquinolinesulfonamide·2HCl, and having the following structure:



, including all functional equivalents, analogs,

and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

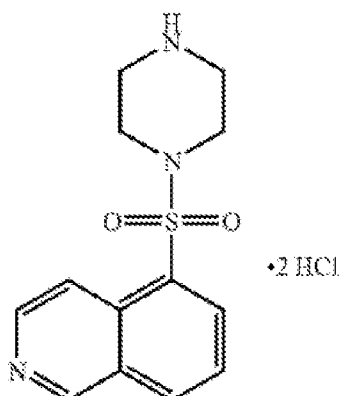
H-89, having the chemical name, N-[2-p-bromoinnamylamino)ethyl]-5]dihydrochloride isoquinolinesulfonamide·2HCl, and having the following structure:



, including all functional

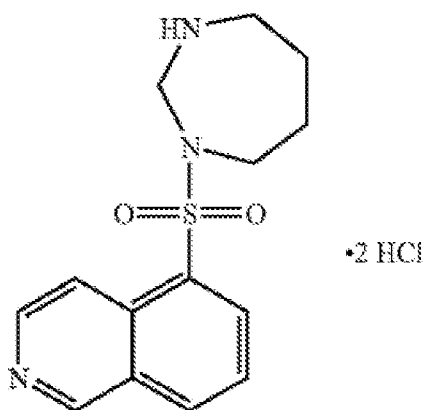
equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

HA-100, having the chemical name, 1-(5-isoquinolinylsulfonyl)piperazine·2HCl, and having the following structure:



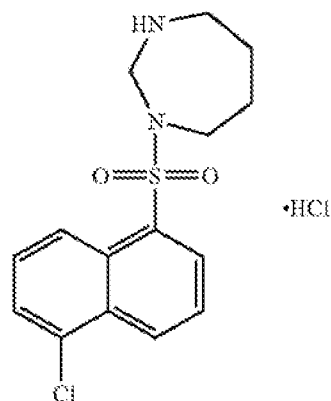
, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

HA-1077, having the chemical name, 1-(5-dihydrochloride isoquinolinylsulfonyl)homopiperazine·2HCl, and having the following structure:



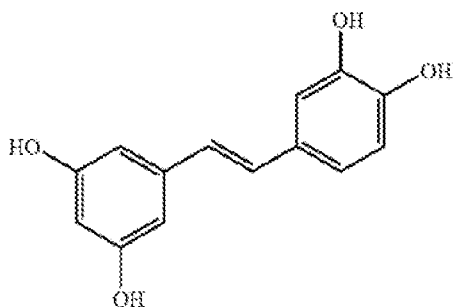
, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

ML-9, having the chemical name, 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine·HCl, and having the following structure:



, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

Piceatannol, having the chemical name *trans*-3-3'-4,5'-tetrahydroxystilbene, and having the following structure:



, including all functional equivalents, analogs, and pharmaceutically effective derivatives of this inhibitor, which include any pharmaceutically acceptable salt, ester, or salt of such ester, of the compound, or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) an MLCK inhibitory effect.

In addition, MLCK inhibitors can include any known peptide MLCK inhibitor, including the following two commercially available polypeptides:

MLCK Inhibitor Peptide 18 having an amino acid sequence of H-RKKYKYRRK-NH₂ (SEQ ID NO: 1) (see Zolotarevsky, Y., et al. 2002. *Gastroenterology* 123, 163, and Lukas, T.J., et al. 1999. *J. Med. Chem.* 42, 910, each of which is incorporated herein by reference); and

Inhibitor Polypeptides SM-1 (MLCK Inhibitor H-AKKLSKDRMKKYMARRKWQKTG-NH₂ Peptide 480-501) (SEQ ID NO: 2).

In yet another embodiment, MLCK inhibitors may include an anti-MLCK antibody. The term "antibody" refers to a monoclonal or a polyclonal antibody, immunologically effective

fragments thereof (*e.g.*, Fab, Fab', or F(ab')₂), or a single chain version of the antibodies, usually designated as Fv regions. Methods of producing polyclonal and monoclonal antibodies, including binding fragments and single chain versions are well known in the art.

C. Pharmaceutical Compositions

5 In some embodiments, it may be advantageous to have the pharmacological agent administered in a composition. For example, the subject might have need of multiple treatments of which the route of administration, dosing, or frequency of administration is similar to that of the pharmacological agent. In some embodiments, there may be treatments the subject needs or desires which affect the same tissues or area of the subject's body. In some embodiments, 10 treatments, agents, or other components may be administered to bolster, modulate, or otherwise affect the activity of the pharmacological agent. For example, agents may be added to accelerate or repress the activity of the pharmacological agent, thus making it more or less quickly acting. In other circumstances, it may be desired to add agents which make the pharmacological agent more or less potent, thus affecting dosing. Further, in some embodiments, agents may be added 15 to address, treat, or prevent side effects associated with the pharmacological agent. Accordingly, in some embodiments, the composition may have a variety of pharmaceutically acceptable carriers, excipients, or supplementary pharmacological agents as part of the composition or as part of the method of administration along with the pharmacological agent. For example, a pharmacological agent preferably suspended in a physiologically compatible carrier (*i.e.*, in a composition). 20

The compositions of the disclosure may comprise a pharmacological agent alone, or in combination with one or more other active or inactive components (*e.g.*, a second active pharmacological agent for MLCK inhibition or other agent for a separate function). In some 25 embodiments, a composition comprises 1, 2, 3, 4, 5, 6, or more different pharmacological agents, each having at least one effect on the subject related the prevention or treatment of hair loss. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the pharmacological agent directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, 30 dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present disclosure.

Optionally, the compositions of the disclosure may contain, in addition to the pharmacological agent(s) and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens. Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied. For example, and without limiting, depending on the route of administration, formulations may be as low between about 1 or 2% or more, for example, or may be greater than 70% or more (*e.g.*, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) of the weight or volume of the total formulation. Naturally, the amount of active compound in each composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For administration of an aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The pharmacological agents may be formulated such that they are suitable for topical or injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under

ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

5 The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable
10 to include isotonic agents, for example, sugars or sodium chloride. Absorption properties (*e.g.*, increased, decreased, accelerated, prolonged) of the pharmacological agent can be modulated by the use in the compositions known in the art, for example agents for the delaying absorption, for example, aluminum monostearate and gelatin.

The pharmacological agents disclosed herein may also be formulated in a neutral or salt
15 form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such
20 organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to
25 molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a subject. Delivery vehicles such as liposomes, nanocapsules, micro-particles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present disclosure into suitable subject cells or tissue.

The therapeutic agents described herein can be administered alone, or in combination
30 with one or more other suitable hair loss drugs, at any effective dose, using any effective or suitable dosing regimen, and in any suitable dosing form, and administered by any suitable dosing route.

Hair loss agents are known in the art and in some cases marketed as hair growth enhancers. They operate by a variety of mechanisms of action which are poorly defined and/or not fully understood. For example, some operate to inhibit various hormones believed to shrink hair follicles, thereby maintaining the maximum hair shaft, while others purport to enhance hair growth by facilitating the opening of potassium channels in the cell membranes in and around the hair follicles, thereby theoretically increasing the amount of oxygen, blood, and nutrients reaching the hair follicle, thus promoting hair growth. In some embodiments, such hair loss agents can include one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole, each of which are well known agents in the art for treating hair loss (*e.g.*, see Hair Loss: What to do if it happens to you, Jordi B., 2010, iUniverse Books, Chapter 8:

Pharmaceuticals: What's out there and how they work, which is incorporated herein by reference). In some embodiments, the agent for facilitating hair growth is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, each of which are well known agents in the art for treating hair loss. In some embodiments, the agent for facilitating hair growth is finasteride. In other embodiments, the agent for facilitating hair growth is cyproterone acetate. In some embodiments, the agent for facilitating hair growth is alfatradiol. In yet other embodiments, the agent for facilitating hair growth is minoxidil. In still other embodiments, the agent for facilitating hair growth is bimatoprost. In some embodiments, the agent for facilitating hair growth is bicalcutamide. In other embodiments, the agent for facilitating hair growth is lantoanoprost. In still other embodiments, the agent for facilitating hair growth is spironolactone. In yet other embodiments, the agent for facilitating hair growth is flutamide. In still other embodiments, the agent for facilitating hair growth is dutasteride. In some embodiments, the agent for facilitating hair growth is ketoconazole.

In some embodiments, the pharmacological agents described herein, and/or the hair loss agents may be administered with a penetration enhancer. Penetration enhancers are drugs, agents, compounds, or other substances, used to facilitate transport of another drug, compound, or substance (*e.g.*, pharmacological agents) across a barrier (*e.g.*, the skin, dermis, and/or epidermis). They are well known, characterized, and described in the art. The penetration enhancer can be formulated as a component of the pharmacological agent, as a component of a composition comprising the pharmacological agent and the penetration enhancer, as a component of a kit comprising the pharmacological agent and the penetration

enhancer, or administered as part of a method comprising the use of the pharmacological agent and the penetration enhancer. In such instances where the penetration enhancer is not a component of the pharmacological agent or part of a composition comprising the pharmacological agent and the penetration enhancer, the penetration enhancer may be administered prior to, contemporaneously (*i.e.*, simultaneously or nearly simultaneously) with, or subsequently to, administration of the pharmacological agent. In some embodiments, the penetration enhancer is formulated as a component of the pharmacological agent, as a component of a composition comprising the pharmacological agent and the penetration enhancer, as a component of a kit comprising the pharmacological agent and the penetration enhancer, or administered as part of a method comprising the use of the pharmacological agent and the penetration enhancer. In some embodiments, the penetration enhancer is administered prior to the pharmacological agent. In some embodiments, the penetration enhancer is administered contemporaneously (*i.e.*, simultaneously or nearly simultaneously) to the pharmacological agent. In some embodiments, the penetration enhancer is administered subsequent to the pharmacological agent.

Penetration enhancers (*e.g.*, sorption promoters or accelerants) are known in the art and will be readily apparent to one skilled in the art. Exemplary penetration enhancers are, sulfoxides (such as dimethyl sulfoxide, DMSO), Azones (*e.g.*, laurocapram), pyrrolidones (*e.g.*, 2-pyrrolidone, 2P), alcohols and alkanols (*e.g.*, ethanol, or decanol), glycols (*e.g.*, propylene glycol, PG, a common excipient in topically applied dosage forms), surfactants (also common in dosage forms), terpenes, dimethyl isosorbide, isopropyl myristate, and gabapentin. In some embodiments, the penetration enhancer is selected from the following: sulfoxides (such as dimethyl sulfoxide, DMSO), Azones (*e.g.*, laurocapram), pyrrolidones (*e.g.*, 2-pyrrolidone, 2P), alcohols and alkanols (*e.g.*, ethanol, or decanol), glycols (*e.g.*, propylene glycol, PG, a common excipient in topically applied dosage forms), surfactants (also common in dosage forms), terpenes, dimethyl isosorbide, isopropyl myristate, gabapentin, or a combination thereof. In some embodiments, the penetration enhancer is dimethyl sulfoxide. In some embodiments, the penetration enhancer is DMSO). In some embodiments, the penetration enhancer is laurocapram. In some embodiments, the penetration enhancer is 2-pyrrolidone. In some embodiments, the penetration enhancer is 2P. In some embodiments, the penetration enhancer is an alcohol. In some embodiments, the penetration enhancer is ethanol. In some embodiments, the penetration enhancer is an alkanol. In some embodiments, the penetration enhancer is decanol. In some embodiments, the penetration enhancer is propylene glycol. In

some embodiments, the penetration enhancer is a surfactant. In some embodiments, the penetration enhancer is a terpene. In some embodiments, the penetration enhancer is dimethyl isosorbide. In some embodiments, the penetration enhancer is isopropyl myristate. In some embodiments, the penetration enhancer is gabapentin.

5 **D. Methods of Use**

Without being bound by theory, the present disclosure relates to the surprising discovery that dermal sheath cells of hair follicles bear the specific function of driving the upward relocation of the dermal progenitor niche cells at the bottom of the hair follicle to a position that is adjacent to stem cells in the upper region of the hair follicle during the progression of catagen
10 phase. The inventors have discovered and demonstrated, it is believed for the first time, that this upward movement of the niche cells during catagen phase is propelled by the smooth muscle cell-like properties of the dermal sheath cells, *i.e.*, the contraction properties of the dermal sheath cells. Without being bound by theory, the hair cycle may be (a) arrested in anagen phase by inhibiting the activation of catagen phase or (b) arrested in catagen phase by inhibiting or
15 otherwise blocking the progression of catagen phase into telogen phase. Without being further bound by theory, each of these aspects of the hair cell cycle depend on the smooth muscle cell contractions of the dermal sheath cells; thus, in various embodiments, each of these effects may be induced by inhibiting the smooth muscle cell-like contractions of the dermal sheath cells. In some embodiments, this is achieved by inhibiting the myosin light chain kinase (MLCK)
20 pathway, which disrupts the smooth muscle cell function of the dermal sheath cells, thereby inhibiting the activation of the catagen phase from the anagen phase or inhibiting the progression of the catagen phase. This results in the minimization of further hair loss, which would otherwise normally occur during the telogen phase (which naturally follows catagen phase). Thus, in various aspects, the present disclosure provides methods, compounds, and
25 compositions for inhibiting the smooth muscle cell function of the dermal sheath cells as a means to inhibit the activation of catagen phase from anagen phase, or inhibit the progression of catagen phase.

Thus, in a first aspect, the disclosure relates to a method of treating hair loss by inhibiting the contraction of dermal sheath cells of a hair follicle. In some embodiments, the
30 contraction of dermal sheath cells can be inhibited or blocked by the administering of a therapeutically effective amount of an agent which inhibits the myosin light chain kinase (MLCK) pathway, *e.g.*, by inhibiting the myosin light chain kinase with a myosin light chain

kinase inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In a second aspect, for hair follicles in anagen phase, the disclosure relates to a method of treating hair loss by inhibiting the activation of catagen phase. In some embodiments, the inhibiting of activation of catagen phase can be achieved by the administering of a therapeutically effective amount of an agent which inhibits the myosin light chain kinase (MLCK) pathway, *e.g.*, by inhibiting the myosin light chain kinase with a myosin light chain kinase inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In a third aspect, for hair follicles in catagen phase, the disclosure relates to a method of treating hair loss by inhibiting the further progression of catagen phase (*i.e.*, arresting catagen phase). In some embodiments, the inhibiting of catagen progression can be achieved by the administering of a therapeutically effective amount of an agent which inhibits the myosin light chain kinase (MLCK) pathway, *e.g.*, by inhibiting the myosin light chain kinase with a myosin light chain kinase inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In a fourth aspect, the disclosure relates to a method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle, thereby treating hair loss. In some embodiments, the pharmacological agent is a compound, a peptide, or an antibody which inhibits myosin light chain kinase. In some embodiments, the myosin light chain kinase (MLCK) pathway can be inhibited with a myosin light chain kinase (MLCK) inhibitor (*e.g.*, wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or combinations thereof).

In various embodiments, the objective of preventing further hair loss (*e.g.*, by inhibiting the progression of catagen phase, or inhibiting the activation into catagen phase from anagen phase) involves the general concept of inhibiting the smooth muscle cell-like contraction of the dermal sheath cells. Any suitable approach for inhibiting smooth muscle cell-like contractions of the dermal sheath cells is envisioned and may involve, for example, an agent which inhibits a target protein or molecular pathway which is involved in the biologic process of smooth muscle cell-like contractions. As a non-limiting example, the methods envision administering an agent which inhibits the myosin light chain kinase (MLCK) pathway, which refers to the signaling pathway that activates smooth muscle cell contractions in dermal sheath cells. The blocking or

inhibiting of same impedes follicle contraction, regression and niche relocation, as demonstrated in the Examples. The disclosure provides pharmacological agents which can include small molecule compounds, therapeutic peptides, or therapeutic polypeptides (*e.g.*, an antibody) (or combinations thereof) which exhibit a therapeutic effect, *e.g.*, the inhibition of the myosin light chain kinase pathway, the inhibition of catagen activation, the arrest of the natural hair cycle in the catagen phase, or the inhibition of the smooth muscle cell-like contractile activity of dermal sheath cells in the hair follicles.

Such inhibition may be achieved in certain embodiments through the administration of one or more agents that inhibit smooth muscle cell function, *e.g.*, an inhibitor of the myosin light chain kinase (MLCK) pathway.

In yet another aspect, the disclosure relates to a method of treating hair loss by the administration of one or more agents that inhibit smooth muscle cell function in dermal sheath cells, *e.g.*, an inhibitor of the myosin light chain kinase (MLCK) pathway in dermal sheath cells. In some embodiments, the administration of one or more agents that inhibit smooth muscle cell function in dermal sheath cells, *e.g.*, an inhibitor of the myosin light chain kinase (MLCK) pathway in dermal sheath cells inhibits the catagen phase of the hair growth cycle from activating (*i.e.*, initiating).

In still another aspect, the disclosure relates to a method of treating hair loss by the administration of an inhibitor of the myosin light chain kinase (MLCK) pathway in dermal sheath cells. In some embodiments, the administration of an inhibitor of the myosin light chain kinase (MLCK) pathway in dermal sheath cells inhibits the catagen phase of the hair growth cycle from activating (*i.e.*, initiating).

The above methods of use are not intended to be limiting. The present disclosure relates to the observation that dermal sheath cells of hair follicles bear the specific function of driving the upward relocation of the dermal progenitor niche cells at the bottom of the hair follicle to a position that is adjacent to stem cells in the upper region of the hair follicle during the progression of catagen phase and that this biological process is driven by the smooth muscle cell-like contractions of the dermal sheath cells. Thus, the methods contemplated herein may include any reasonable approach that results in the inhibition of the contraction properties of the dermal sheath cells, *e.g.*, the inhibition of the MLCK pathway.

E. Methods of Administration

The pharmacological agents described herein, or compositions thereof, may be administered to a variety of subjects, generally, any subject in need of, or suspected to be in need of, prevention or a treatment for hair loss (*e.g.*, host animal, such as a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, or a non-human primate (*e.g.*, Macaque)). In some embodiments, the subject is a human subject.

Delivery of the pharmacological agents, or a compositions thereof, may be through any acceptable route known in the art. For example the pharmacological agent may be administered topically or through injection, for example microinjections to the scalp. Routes of administration may be combined, if desired. When administered topically, the administration may be by hand, by spray, by gel, by ointment, by lotion, as a shampoo or conditioner, and generally by any vehicle which can be applied to the target tissue surface (*e.g.*, scalp, hair growing area) to be treated. Applicators (*e.g.*, brush, dropper, squeeze bottle, spray bottle) may be used to facilitate that administration of the pharmacological agent to the target tissue and area. Combinations of methods may be used, for example a topical gel in combination with a spray or shampoo. In some embodiments, the pharmacological agent is administered topically. In some embodiments, the pharmacological agent is administered topically by a gel. In some embodiments, the pharmacological agent is administered topically by a shampoo. In some embodiments, the pharmacological agent is administered topically by a lotion. In some embodiments, the pharmacological agent is administered topically by a spray. In some embodiments, the pharmacological agent is administered topically by a conditioner. In some embodiments, the pharmacological agent is administered topically by an aqueous solution.

The pharmacological agents, or compositions thereof, may be administered in a variety of doses and or according to a variety of administration schedules. One of ordinary skill in the art will readily be capable and able to determine such a dosing and administration plan. In some cases, the pharmacological agent is administered once. In some embodiments, the pharmacological agent is administered more than once or in multiple doses. In some embodiments, the doses are once per calendar day, in other embodiments, the dose is once per calendar week, calendar month, calendar year, any partial portion of any period, any iteration or combination thereof, or other schedule as one of ordinary skill in the art will be able to ascertain (*e.g.*, bi-weekly, ever 2, 3, 4, 5, 6...calendar days, weeks, months, years).

The pharmacological agents can be administered in a sufficient amount to achieve the desired effect (*i.e.*, inhibition of MLCK and/or hair loss) in the subject. A “therapeutic effect,”

“effective amount,” or “therapeutically effective amount” of pharmacological agent is an amount sufficient to target and effect the desired tissue to the desired degree without undue adverse effects. The effective amount will depend primarily on factors such as the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among animal and tissue.

5 The effectiveness may be assessed through a measure of its inhibitory concentration (IC), which is a measure of the level of reduction of activity of the target by a given amount of the inhibitor. Often the IC is measured by the amount of inhibitor required to reduce the target by half, this amount is referred to as the half maximal inhibitory concentration (IC₅₀). In some
10 embodiments, an effective amount of a pharmacological agent is an amount sufficient to inhibit MLCK and/or inhibit or treat hair loss. In some embodiments, the IC₅₀ is in a range of 500 nano-moles (nM) to 1 nM. In some embodiments, the IC₅₀ is in the range of 200 nM to 50 nM. In some embodiments, the IC₅₀ is 100 nM or less. In some embodiments, the IC₅₀ of the
15 MLCK inhibitor, compound, peptide, or antibody is 500 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 450 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 400 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 350 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 300 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 250 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor,
20 compound, peptide, or antibody is 200 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 190 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 180 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 170 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 160 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 150 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 140 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 130 nM or less. In some embodiments, the IC₅₀ of the
25 MLCK inhibitor, compound, peptide, or antibody is 120 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 110 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 100 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 90 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or

antibody is 85 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 80 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 75 nM or less. In some embodiments, the IC₅₀ of the MLCK inhibitor, compound, peptide, or antibody is 70 nM or less.

5 In some embodiments, the effective amount of the pharmacological agent is adjusted according to the level of inhibition of as measured by cessation of dermal sheath cell contractions, thereby halting the hair cycle in the catagen phase. This may be done by periodic evaluations of the hair visually, or by any means in the art. Evaluation may also be made by evaluation of the treated tissue for physiological changes to the tissue. For example, biopsies
10 may be used to evaluate the follicles for cessation of dermal sheath cell contractions.

In another aspect, the disclosure relates to a method of identifying an agent capable of treating hair loss, comprising, administering a test agent to a cell culture of dermal sheath cells, detecting the production of one or more biomarkers indicative of the inhibition of dermal sheath contraction, validating a test agent in a hair follicle model to confirm that the test agent inhibits
15 dermal sheath contraction, thereby identifying an agent capable of treating hair loss.

Biomarkers are measurable substances present in a target subject or tissue which the presence, or absence of, is indicative or represents a physiological state of the target subject or tissue (*e.g.*, health, disease, infection, exposure to environmental factors, growth state).

Biomarkers which indicate a cellular slowing, regression, or halting of transition in the hair
20 follicle or dermal sheath, from one phase of growth to another, especially catagen to telogen, are target biomarkers. For example, biomarkers associated with MLCK, smooth muscle contractions, cell growth and proliferation, and phosphorylation may be helpful biomarkers. Further, biomarkers associated with the activation or inactivation of certain genes related to the transition of phases in the hair follicle, such as the presence of certain proteins, mRNA
25 transcripts, miRNA, or other nucleic acids may also be used as biomarkers for the methods described herein.

F. Kits

The pharmacological agents described herein may, in some embodiments, be assembled into pharmaceutical, therapeutic, diagnostic, or research kits to facilitate their use in therapeutic,
30 diagnostic, or research applications. A kit may include one or more containers housing the components of the disclosure and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended application and the

proper use of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

5 The kit may be designed to facilitate use of the methods described herein by subjects, medical professionals, practitioners, and/or researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a
10 suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, “instructions” can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be
15 associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, etc.), Internet, and/or web-based communications, etc. The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflect approval by the agency of manufacture, use or sale for animal administration.

20 The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The pharmacological agents may be in the form of a liquid, gel or solid (*e.g.*, powder). The agents may be prepared sterilely,
25 packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container. The kit may have one or more or all of the components required to administer the agents to an animal, such as a syringe, topical application devices, or intravenous
30 (iv) needle tubing and bag, particularly in the case of the kits for producing specific somatic animal models.

 The kit may have a variety of forms, such as a blister pouch, a shrink wrapped pouch, a vacuum sealable pouch, a sealable thermoformed tray, or a similar pouch or tray form, with the

accessories loosely packed within the pouch, one or more tubes, containers, a box, or a bag. The kit may be sterilized after the accessories are added, thereby allowing the individual accessories in the container to be otherwise unwrapped. The kits can be sterilized using any appropriate sterilization techniques, such as radiation sterilization, heat sterilization, or other sterilization methods known in the art. The kit may also include other components, depending on the specific application, for example, containers, cell media, salts, buffers, reagents, syringes, needles, a fabric, such as gauze, for applying or removing a disinfecting agent, disposable gloves, a support for the agents prior to administration etc.

Exemplary embodiments of the invention are described in more detail by the following examples. These embodiments are exemplary of the invention, which one skilled in the art will recognize is not limited to the exemplary embodiments.

EXAMPLES

In order that the invention described herein may be more fully understood, the following examples are set forth. The examples described in this application are offered to illustrate the methods, compositions, and systems provided herein and are not to be construed in any way as limiting their scope.

Example 1

Introduction

Tissue homeostasis requires the balance of growth by cell production and regression through cellular loss. In the hair cycle during follicle regression, the niche traverses the skin through an unknown mechanism to reach the stem cells and prime regeneration. Here, by cell specific ablation and *intravital* imaging in live mice, the follicle-lining dermal sheath is identified as the key driver of tissue regression and niche relocation via smooth muscle contractile machinery that generates centripetal constriction force. The calcium/calmodulin/myosin light chain kinase pathway is revealed as the mechanism of sheath contraction, that when blocked inhibits follicle contraction and impedes regression and niche relocation. Thus, the study identifies the dermal sheath as a smooth muscle that drives regression for reuniting niche and stem cells to regenerate tissue structure during homeostasis.

Multipotent progenitors produce new cells to replace those that have been lost by natural turnover and shedding in rapidly renewing tissues such as the bone marrow and hair follicle ⁽¹⁾.

However, progenitors have limited renewal capacity and need replenishing from the stem cell reservoir, coordinated by specialized niche cells ⁽²⁻⁴⁾. During hair growth, the specialized dermal papilla (DP) cluster secretes niche signals to orchestrate matrix progenitor proliferation, migration and differentiation at the base of follicle bulbs ^(5,6) (FIG. 5A). To reboot the progenitor pool throughout life, repeated cycles ⁽⁷⁾ of follicle regression (catagen), rest (telogen) and regrowth (anagen) derive new cells from the bulge/germ stem cell reservoir located in the upper follicle ⁽⁸⁻¹²⁾ (FIG. 5B). During regression, matrix progenitors shut down proliferation and finish terminal differentiation, and outer root sheath (ORS) progenitors along the entire follicle axis undergo apoptotic pruning ⁽¹³⁾ driven by extrinsic signals ^(14,15). Only the most direct stem cell progeny in the sub-bulge region of the ORS survive and retain multipotency, giving rise to a new bulge and germ stem cells ^(16,17). These cells then become reactivated by signals from the intact, surviving DP niche that needs to fully relocate from the follicle base to the stem cells to regenerate the follicle ⁽¹⁸⁻²⁰⁾ (FIG. 5B).

Drastic tissue remodeling poses a considerable challenge for stem cell regulation — precise and reliable niche relocation to the stem cell reservoir for preserving close communication lines. Indeed, erroneous detachment of the DP niche leads to stem cell dysfunction and complete hair loss ^(21,22). How the DP, while trailing the regressing epithelial strand, travels over 80% of the hair follicle length (FIG. 6) upward towards the stem cell reservoir is still unknown (FIG. 1A and FIG. 5B). Two hypotheses for DP movement have been proposed for many years ⁽²³⁾: (1) an “apoptotic force” generated by dying cells in the shrinking follicle and (2) a “contraction force” by follicle-encapsulating dermal sheath (DS) cells based on alpha smooth muscle actin (α SMA) expression identified nearly 3 decades ago ⁽²⁴⁾.

Results and Discussion

To gain insight into this longstanding question, whether and how the DS may be important for niche relocation during follicle regression was addressed. DS-specific genetic tools were first established using the promoter of the cartilage proteoglycan Aggrecan (Acan) as a driver. ACAN protein is detected in skin specifically only in the follicle basement membrane that separates the DS from epithelium ⁽²⁵⁾ (FIG. 1B). Using Acan^{CreER} mice crossed with the R26^{LSL-tdTomato} reporter (Acan^{tdT}) enabled efficient inducible targeting during catagen that within skin was restricted to the DS (FIGs. 7A-7C). To test its functional role, the DS at early catagen using R26^{LSL-DTA} (Acan^{DTA}) (FIG. 1C) was selectively ablated. After tamoxifen induction, the DS continuity was disrupted with only fragmented α SMA-stained cell remnants at the beginning

of catagen (FIG. 1D). By the subsequent telogen phase, while follicles in control skin were fully regressed (FIG. 1E), follicles with efficient DS ablation remained aberrantly long (FIG. 1F) with hair shafts extending down to the follicle base and DP remaining deep in the dermis (FIGs. 1G-1H). Stalled follicle lengths ranged from partially regressed to fully stranded deep within the dermis (FIG. 1I). This indicates that the DS is required for progression of catagen regression and niche relocation to the stem cells located in the upper follicle.

Next, the stalled follicle phenotype was analyzed more closely with lineage specific markers (FIG. 5A) to determine the degree of catagen dysfunction in hair follicles lacking DS cells. Immunofluorescence confirmed absence of the DS (α SMA loss) and widespread presence of ORS (K14) around non-regressed shafts (FIG. 1J). LEF1-staining affirmed the DP-identity of rounded cell clusters at the bulb end that were no longer engulfed by Ki67+ proliferative matrix progenitors (FIG. 1J and FIG. 8A). Likewise, hair shaft (AE13, AE15) and inner root sheath (AE15) precursors were also absent (FIG. 1J), suggesting that anagen-type differentiation (FIG. 8A) had ended and catagen within the bulb had been initiated. Staining for active Caspase 3 (FIG. 8B) was also undetectable in DS-ablated follicles suggesting that catagen did not proceed beyond the early steps (FIG. 1J). The hair shafts themselves were surrounded by a single differentiated K6+ layer (FIG. 1J), reminiscent of the companion layer in growing hair follicles (FIG. 8A) and of inner bulge cells at telogen (FIG. 8C), and by multiple layers of K14+ ORS progenitors (FIG. 1J). Together, the phenotypic analyses indicated that long, non-regressed follicles were not caused by continued matrix progenitor proliferation or erroneous differentiation but resulted from a failure of hair shaft and DP movement towards the skin surface and concomitant absence of ORS apoptosis. Thus, the DS may exert a physical force fundamental for the upward travel of shaft and niche.

To directly explore if the DS expresses the contractile molecular machinery of smooth muscles that could execute follicle regression and niche relocation, DS cells were isolated for transcriptome analysis, in comparison to DP and dermal fibroblasts (DF). DS, DP and DF cells were flow-sorted from Sox2^{GFP}; *Lef1-RFP* reporter back skins^(6, 26), also stained for mesenchymal receptor PDGFRA (FIGs. 2A-2B, and FIG. 9). DS cells expressed Sox2^{GFP} and PDGFRA and could be cleanly distinguished from GFP+RFP+PDRGFA+ DP and RFP+ DF⁽²⁷⁾ (FIGs. 2A-2B). Verification of known marker expression for DS (*Acta2*), DP (*Fgf10*), and DF (*CD34*) demonstrated their identity and purity (FIG. 2C). Comparing the RNA sequencing-generated transcriptomes of DS, DP and DF with ORS, matrix, and melanocyte transcriptomes⁽²⁷⁾ by hierarchical clustering and principal component analysis established their close lineage

relationship (FIGs. 10A-10B). ANOVA and signature gene analysis then identified unique gene expression signatures reflecting their specialized functions and revealed a DS molecular signature of 483 enriched genes (FIG. 2D and FIG. 10C; Table 1).

Table 1: Dermal Sheath Molecular Signature Genes

Dermal Sheath					
1190005I06Rik	Casq2	Epha3	Klf4	Penk	Socs2
2200002D01Rik	Cbln1	Ephb2	Klhl29	Pex5l	Sprr4
2310022B05Rik	Cbr3	Erg	Klhl30	Pfn2	Srpx
2700060E02Rik	Ccdc109b	Erp27	Krt20	Phlda3	Srpx2
3110062M04Rik	Ccdc74a	F2r	Larp6	Pil6	Sspn
4833403I15Rik	Ccdc80	Fam101b	Ldlrad4	Pitpnm3	Steap3
4833412C05Rik	Cck	Fam129a	Leprel4	Pla2g16	Stmn2
4931406P16Rik	Ccpg1	Fam189a2	Lgals3	Pla2r1	Stx11
6330403A02Rik	Cd47	Fam20c	Lims2	Plcd1	Sumf1
9130206I24Rik	Cd55	Fam211a	Lmcd1	Plcl1	Susd2
9230115E21Rik	Cdc42ep2	Fam46b	Lrrc15	Pld2	Sybu
9930012K11Rik	Cdc42ep3	Fam65b	Lrrc32	Plekha6	Syne3
A430105I19Rik	Cdc42ep5	Fam78b	Lrrk2	Plekhf1	Synpo2
A730085A09Rik	Cdh13	Fbn2	Lst1	Plekhg2	Synpo2l
Aatk	Cdkn1c	Fgf13	Ltbp4	Plekho2	Syt13
Ablim1	Cdkn2b	Fgf14	Mafb	Pls3	Syt17
Abtb2	Cdo1	Fgf18	Map3k14	Pltp	Tagln
Acan	Cgn1l	Fgl2	Map6	Plxdc2	Tango2
Acta2	Chst15	Fhl2	Mapk11	Pmepa1	Tapbp
Actg2	Chst3	Fhl5	Marveld1	Postn	Tbx15
Adam15	Cilp	Fkbp1b	Matn4	Ppap2b	Tbx18
Adam19	Ckb	Fmod	Mcam	Ppp1r3c	Tcea3
Adamts12	Clec11a	Foxd2	Medag	Prelp	Tcf7l1
Adamts18	Clec3b	Foxp4	Mettl24	Prickle1	Tcf7l2
Adamts4	Cmklr1	Fxyd5	Mfap5	Prkaa2	Tek
Adamts5	Cnn2	Galnt10	Mfge8	Prkab2	Tenm3
Adamts6	Coch	Galnt12	Mgst3	Prkcdp	Tgfbr2
Adamts7	Col11a1	Galnt9	Mkx	Prkg1	Thbs1
Adamtsl1	Col12a1	Gap43	Mme	Prr5l	Thbs4
Adamtsl5	Col27a1	Gas2	Mmp11	Prx	Timp1
Adc	Col3a1	Gas7	Mpz	Ptges3l	Tmbim1
Adcy1	Col4a1	Gfpt2	Mras	Ptprb	Tmem119
Add3	Col4a2	Ghr	Mrvi1	Ptrf	Tmem200a
Adk	Col6a6	Glpr2	Mthfr	Pygm	Tmem204
Adm	Col8a1	Glis2	Mustn1	Rab37	Tmem37
Adrb2	Col8a2	Gm10046	Mvp	Rab3il1	Tmsb10
Afap112	Colec12	Gm14005	Mxra8	Radil	Tmsb4x
Agtr1a	Cpn2	Gm527	Myadm	Rasa4	Tmtc1
Ahnak	Cpxm2	Gngt2	Myh11	Rasgrp2	Tnfrsf11b
Ak5	Crip1	Gpha2	Myl12a	Rasl11b	Tnfrsf14
Akap12	Crip2	Gpr133	Myl4	Rassf2	Tnfsf9
Akr1c14	Crispld2	Gpr176	Myl6	Rbms1	Tnmd
Alox5ap	Crtap	Gprc5b	Myl9	Reep1	Tnnc1

Amhr2	Cryaa	Gpx1	Mylk	Reep2	Tnnt1
Amotl2	Cryab	Gria3	Myo1c	Rftn1	Tnnt2
Ang.Rnase4	Crybb3	Grip2	Ncs1	Rgag4	Tpbpg
Angptl4	Cst3	Grk5	Ndr4	Rgcc	Tpm1
Anks1	Cst6	Gxylt2	Nes	Rgs6	Tpm2
Anxa2	Ctgf	Hapln3	Nexn	Rhoj	Trim16
Anxa4	Ctsk	Hcfc1r1	Nhs12	Rin3	Trim47
Anxa7	Ctnn1	Hck	Nid1	Rnasel	Tsc22d3
Anxa8	Cxcl12	Hdac7	Nid2	Rnf150	Tshz2
Aoc3	Cxcr7	Hhat	Nr1d1	Rnf19b	Tshz3
Apbb1	Cyb5r3	Hmgn3	Nradd	Rora	Tspan11
Aqp1	Cyp1b1	Hpgd	Nrxn2	Runx2	Tspan32
Arap1	Cyp2d22	Hrct1	Nt5e	S100a10	Tspo
Arf2	Dapk1	Hs6st1	Ntn4	S100a11	Tuba1a
Arhgap20	Ddah2	Hspb1	Ntrk2	S100a4	Tubb6
Arhgap23	Dennd4a	Hspb2	Ntrk3	S100a6	Tulp1
Arhgap28	Dhrs3	Iah1	Nuak1	S1pr3	Txnip
Arhgdib	Dock9	Ifi30	Numb1	Satb2	Tyropb
Arhgef16	Dpep1	Ifitm1	Nupr1	Sbspon	Vamp5
Arhgef26	Dpysl5	Ifitm2	Olfml2a	Sema3c	Vav2
Arhgef6	Drp2	Ifitm3	Osbpl5	Sema3d	Vcl
Arntl	Dse	Igfbp2	Palm	Sept9	Vim
Arvcf	Dstn	Igfbp7	Palm3	Serpinb1a	Vwa5a
Atp10a	E130310I04Rik	Il33	Pamr1	Serpinb6a	Wasf1
Atp2b4	Ecm1	Il4ra	Papolb	Serpind1	Wfdc1
Axl	Ecm2	Isg20	Papss2	Serpine1	Wisp1
B3galnt1	Ecsr	Itga11	Parp3	Serping1	Wnt9a
Bcl11b	Ednra	Itgb3	Parva	Sertad4	Wscd2
Bgn	Eef2k	Itgb5	Pawr	Sfxn3	Zbtb8a
Bicc1	Egflam	Itgb11	Pcdh9	Sgcd	Zdhhc14
Bok	Ehbp1	Jdp2	Pced1b	Sgms2	Zdhhc2
Btla	Ehd2	Jph2	Pcsk5	Shisa5	Zfp423
C1qb	Eif4ebp1	Kank2	Pdgfc	Slc27a3	Zfp524
C1qtnf5,Mfrp	Emp1	Kank4	Pdgfr1	Slc29a1	Zfp57
C1qtnf7	Emp3	Kcns1	Pdlim1	Slc6a6	Zim1
C230081A13Rik	Endod1	Kctd11	Pdlim2	Slco2a1	
Capg	Epas1	Klf10	Peg3	Slit2	
Carhsp1	Epb4.112	Klf2	Peg3as	Slit3	
Dermal Papilla					
1500015O10Rik	Chn1	Frat2	Ldb2	Pnpla3	Sod3
1700047M11Rik	Chodl	Frem1	Lepr	Ppp1r26	Sostdc1
1700085B03Rik	Chst8	Frzb	Lingo2	Ppyr1	Sox2
1810010H24Rik	Clcn2	Fst	Loxl3	Prdm1	Sparcl1
1810011O10Rik	Cldn9	Fut10	Lrrc73	Prdm5	Spf2
1810041L15Rik	Clec14a	G0s2	Lrrn1	Prkar1b	Spock1
2010001M06Rik	Clmn	Gcnt4	Lrrn2	Prkcq	Spock2
2810011L19Rik	Clstn2	Gdf10	Lrrn3	Prlr	Spock3
4833422C13Rik	Cntn1	Gdpd2	Lrrtm3	Prr5	Spon1
4930426D05Rik	Col15a1	Gem	Ltbp1	Prtr1	Spry1

4931406H21Rik	Col23a1	Gfra1	Lypd1	Prss12	Spry4
5033411D12Rik	Col26a1	Ggt7	Mad2l2	Prtg	Sqrdl
5430421F17Rik	Col9a2	Gldn	Maff	Psd2	Ss18
6030419C18Rik	Corin	Glis3	Maob	Ptgfr	St6galnac5
6330403K07Rik	Cpne2	Gm10664	Mc2r	Ptgs1	Stat5a
a	Crabp1	Gna14	Mc3r	Ptk7	Stat5b
Aard	Crabp2	Gnai1	Mc5r	Ptprz1	Steap1
Abca8a	Crispld1	Gng2	Mdk	Pygl	Steap2
Abca8b	Csrnp3	Gpr165	Meis1	Rab13	Stx1b
Abcg4	Ctbp2	Gprasp2	Meis2	Rab3b	Syt12
Ache	Cx3cl1	Gpx3	Mgst1	Rgs9	Tagln3
Acot11	Cxcl1	Grb14	Mir1897,Sox2ot	Rhod	Tceal3
Adam22	Cxxc5	Grid1	Mmp17	Rnf125	Tceal5
Adrbk2	Cygb	Grin3a	Mov10	Rorb	Tcl1
Aff2	Cyr61	Gstt1	Mterfd2	Rspo1	Tfap2c
Alpl	D430019H16 Rik	H2-DMA	Myrf	Rspo2	Thal
Alx3	Daam2	H2-DMb1	Naga	Rspo3	Thbd
Alx4	Dcc	H2-Ke6	Nav3	Rspo4	Thrb
Amot	Ddx26b	H6pd	Ncam2	Runx1t1	Thsd7a
Ampd2	Dfna5	Hcn1	Ncoa1	S100b	Tlcl1
Amph	Dgkh	Heph	Ncoa3	Samd14	Tlcl2
Aplp1	Dio2	Hey1	Ncoa7	Sapcd1	Tle2
Apoe	Dmpk	Hey2	Ndnf	Scara5	Tle4
Ar	Dnajc12	Hfe	Ndp	Scarb2	Tmem100
Arhgef28	Dnali1	Hhip	Nfam1	Scn4b	Tmem132c
Arl5c	Dram1	Hif3a	Ngfr	Scube1	Tmem132e
Armex6	Draxin	Hmgcll1	Nlgn3	Scube3	Tmem176a
Aspg	Dusp10	Hoga1	Notum	Sdk2	Tmem176b
Atoh8	Dync2li1	Hoxa10	Nox4	Sec1	Tmem2
B230217C12Rik	Ebf1	Hoxc10	Nr3c2	Serpine2	Tmem35
Bag2	Ebf4	Hoxc8	Nrg2	Sfmbt2	Tnfrsf1b
Bai1	Eda	Hoxc9	Nsg1	Sfrp1	Tnni3
Bai3	Edn3	Hs3st1	Ntng1	Sh2b2	Tril
Bambi	Efcab4b	Hs3st3a1	Olfm2	Sh3bp1	Tro
BC028528	Efna2	Hs3st3b1	Oxnad1	Sh3bp5	Trpm3
BC051142	Eid2b	Hunk	Pafah2	Shc2	Trps1
BC089491	Emilin1	Id1	Pappa	Shd	Tspan4
Bcl6	En1	Id3	Pappa2	Shisa4	Ttc39c
Bdh2	Enpp2	Ier3	Paqr8	Shisa9	Ttyh1
Bex2	Epha7	Ikzf4	Pax1	Shox2	Tubg2
Bmp4	Ephx2	Il16	Pbx1	Slc16a2	Unc5c
Bmp6	Eva1a	Inhba	Pcbp3	Slc1a1	Usp25
Brdt	Eya2	Insl3,Jak3	Pde3a	Slc22a4	Vegfa
C1s	F5	Islr	Pde4d	Slc24a6	Vip
Cachd1	Fads1	Itga9	Pde9a	Slc2a10	Vipr2
Cacna1c	Fam126a	Itprp	Pdlim4	Slc35f1	Vps37d
Cacna1d	Fam131a	Jam2	Per3	Slc43a2	Wasf3
Cacng7	Fam196b	Jazf1	Pfkfb3	Slc5a7	Wif1
Camp	Fam229b	Kat6b	Pfkm	Slc7a7	Wnt5b
Cand2	Fam5c	Kcnc4	Pgd	Slc9b2	Zc3h12b

Capn6	Fam69b	Kcnn3	Pgf	Slitrk5	Zfp37
Cblb	Fap	Kcnq3	Phactr2	Smad6	Zfp467
Ccnd3	Fbxo2	Khdrbs2	Piezo2	Snai2	Zfp503
Cd1d1	Fgf10	Kif1a	Pik3r1	Snap91	Zfp608
Cd302	Fgf7	Kifc2	Pitpnc1	Sned1	Zfp809
Cd83	Fgfr1	Klhl14	Pkdec	Snrk	Zic1
Cdh18	Fgfr11	Kng2	Plekhg6	Sobp	Zic4
Cebpa	Fli1	Ksr1	Plscr4	Socs3	Zmiz1
Cebpd	Flywch2	Lamc3			
Dermal Fibroblasts					
3110079O15Rik	Col4a5	Fndc5	Ltbp2	Pcsk6	Ssc5d
4930444P10Rik	Col7a1	Galnt16	Lum	Pde7b	Sulf2
Abca9	Cox6c	Galnt5	Meox2	Pknx2	Svil
Adam12	Cp	Gas1	Mfap4	Por	Syt4
Adams8	Cpe	Gatm	Mir669g	Ppp2r2c	Tac1
Adhl	Cpz	Glt8d2	Mmp2	Psph	Tgfb1
Agtr2	Csta	Gm16897	Mmp27	Pycr1	Thbs2
Ahr	Ctsh	Grem1	Mrgprf	Rarres2	Tlr5
Ahrr	Cyp27a1	Gria4	Mybpc1	Rbp4	Tmem150c
Asphd2	Dcn	Gsg11	Nbl1	Rgag1	Tmem26
Atp1a2	Des	Hs3st6	Nnat	Rgs10	Tmtc2
C1qtnf3	Dkk2	Hspg2	Nnmt	Rnf112	Tnfaip6
Casp12	Dpp4	Htra1	Nov	Rtp4	Tnfrsf26
Cav1	Dpt	Ifit2	Nrep	Sash1	Tnxb
Ccbe1	Eln	Itga4	Nrn1	Scg3	Ucn2
Ccdc42	Entpd1	Itih5	Olfml2b	Sct	Vash1
Ccl11	Epb4.113	Kcnj8	ORF63	Sdk1	Vax2os
Cd34	F13a1	Lhfpl2	Osr2	Sema4f	Wisp2
Cdh4	Fam198b	Limch1	P4ha2	Serpina3h	Zfp385b
Cdon	Fgfr4	Loxl1	Pam	Serpina3n	Zfp536
Clec2d	Fn1	Lpin3	Pcdh10	Sfrp4	
Col25a1	Fn3k	Lrrc17			
Dermal Sheath & Dermal Papilla					
1500009L16Rik	Crybg3	Gp1bb,Se pt5	Lrp1	Pf4	Sphk1
2610203C20Rik	Csdc2	Hip1	Magee1	Pros1	Spsb1
4632428N05Rik	Ctsf	Hoxc6	Meis3	Prr16	Stxbp1
5730409E04Rik	Ddit4l	Igf1	Mest	Prrx1	Sulf1
Adams9	Dock6	Igfbp3	Mir143hg	Prrx2	Synpo
Ak1	Efnb3	Igfbp4	Naalad2	Ptger3	Tcea2
Arhgap44	Ehbp111	Il6st	Ncam1	Sept4	Tenc1
Asap3	Eif2c4	Irak3	Ndr1	Slc43a3	Tmem200b
B4galnt1	Fmn13	Irf1	Nog	Slc4a3	Tmem98
Bmp3	Fos	Itga8	Ntng2	Slc9a3r2	Tns3
C1ra	Fosb	Itpr1	Olfml3	Slitrk6	Tppp3
Cdh11	Gadd45b	Kif13b	Pcolce	Smad3	Vcan
Chst14	Gm11627	Klhd8b	Pde4a	Snai1	Zfx4
Col5a3	Gnao1	Lama2	Pdgfra	Snrpn,Sn urf	Zfp36

Cox4i2	Gng8	Lbp	Pdlim3	Sp7	
Cpa6	Golm1	Lgals3bp			
Dermal Sheath & Dermal Fibroblasts					
6430411K18Rik,Mi r127	Col1a2	Fam114a1	Lox	Ntn1	Ramp1
9430020K01Rik	Col5a1	Fam46a	Lsp1	Ostc	Rian
Adam33	Col5a2	Fbln5	Ly6a	Pde6h	Rtn4r
Adams14	Col6a1	Fbn1	Ly6c1	Pdgfd	Scn1b
Adams2	Col6a2	Fibin	Maf	Phldb1	Sec24d
Afap1	Creb3l1	Figf	Meg3,Mir1906- 2,Mir770	Phldb2	Serpinh1
Anpep	Creld1	Fkbp11	Metnl	Plaur	Sgsm2
Anxa5	Csrp2	Fkbp9	Mex3b	Pmp22	Stx2
Aspn	Cyp4b1	Fndc1	Miat	Podn	Tgfbr3
C1qtnf2	Dab2	Fxyd6	Mir22,Mir22hg	Ppap2a	Thy1
Cadm3	Darc	Has1	Mir369,Mir410,Mir412, Mirg	Ppic	Tnfrsf23
Ccl7	Dtx4	Htra3	Mmp23	Ppp1r14a	Twist2
Cgref1	Efemp1	Impdh1	Myh10	Prss57	Vgll3
Ckap4	Eml1	Kazald1	Ndufa4l2	Ptx3	Wbscr17
Colla1	Fam101a	Lgals1	Ngf	Pxdc1	
Dermal Papilla & Dermal Fibroblasts					
Twist1	Antxr2	Ccr11	Fgd5	Rai2	Stxbp6
Apcdd1	Emid1	Speg	Tubb4a	Rab40b	Lsamp
Sfrp2	Thsd4	Rab30	Pitx2	Glis1	Pgm5
Hist1h2bc	Fam105a	Gpc6	Iglon5	Mmp19	
Dermal Sheath, Dermal Papilla, & Dermal Fibroblasts					
0610037L13Rik	Cenpv	Fat4	Lepre1	Pdgfrb	Selm
2410006H16Rik	Cercam	Fbxo17	Leprel2	Pepd	Sepw1
3110001D03Rik	Clip3	Fkbp10	Loxl4	Pfdn5	Serf1
Acads,Gm13826	Clmp	Fkbp14	Lynx1	Pgrmc1	Sgce
Acyp2	Cnrip1	Fkbp7	Lysmd2	Pias3	Sh3gl1
Adams10	Coa3	Flnc	Maged1	Pid1	Shank1
Adecy5	Col16a1	Flot1	Maged2	Pih1d1	Slc39a7
Aes	Col6a3	Fndc3b	Map1a	Pkd2	Smim11
Ahdc1	Commd5	Fndc4	Map1b	Plagl1	Snx22
AI462493	Cped1	Fstl1	Map1lc3a	Pofut2	St6galnac4,St6gal nac6
AI837181	Cpq	Fxyd1	Masp1	Pold4	Svep1
Aldh7a1	Cry2	Gemin7	Matn2	Ppapdc1b	Syne1
Antxr1	Csfl	Gm1821	Megf6	Ppib	Tmed1
Anxa6	Cuedc1	Gm266	Mfap2	Prmt2	Tmed3
Ap5s1	Cxx1b	Gng10	Mgat1	Prrt2	Tmem107
Arf5	Cys1	Gpr124	Mir1188	Psd	Tmem256
Armex4	Ddr2	Gpx7	Mpnd	Ptdss2	Tmod2
Arrb1	Dhrs7	Gpx8	Mrc2	Pth1r	Tor1a
Atf5	Dip2a	Grik5	Myof	Rabac1	Tpst1
Athl1	Dlg4	Gsdmd	Ndn	Rarres1	Tram2

Atp9a	Dnm1	Gtdc2	Ndst3	Reck	Trp53i13
Atraid	Dpm3	Gypc	Nenf	Rftn2	Tspan17
B4galt2	Dusp1	H2-K1	Nfatc4	Rhoc	Txndc5
Bet1l	Ech1	Ifi271l	Npdc1	Rnf215	Vash2
Beta-s	Efemp2	Igdcc4	Npr2	Robo2	Vcam1
Blvrb	Eif1b	Ikbip	Oaf	Ror2	Wipfl
C1qtnf1	Emc10	Itga5	Olfml1	Rpl13	Yif1a
Cacna1g	Eng	Itm2a	Osr1	Rpl37a	Ypel3
Ccdc23	Eno2	Izumo4	Ostf1	Rps29	Zcchc24
Ccdc28b	Fam189b	Klhl22	Pagr1a	Rtn2	Zfp354c
Cd248	Fam198a	Krtap22-2	Pard3b	S100a13	Zfp651
Cdk14	Fam212a	Krtcap2	Pddc1	Sar1a	Zfp11

Gene ontology analysis of the DS signature expectedly yielded extracellular matrix organization categories (FIG. 2E), as the DS is closely associated with the basement membrane that separates the mesenchyme from follicle epithelium. Importantly, the DS signature was also enriched for genes involved in “muscle filament sliding” and “smooth muscle contraction” (FIG. 2E). Gene set enrichment analysis for “regulation and process of smooth muscle contraction” (Table 2) showed significant enrichment in the DS (FIG. 2F), suggesting smooth muscle identity and function.

Table 2: Regulation and Process of Smooth Muscle Contraction Genes

ABAT	CACNA1D	GNA12	OXT	PRKG2
ACTA2	CACNA1F	GNA13	OXTR	PRKX
ACTB	CACNA1S	GNAQ	P2RX1	PROK2
ACTG1	CALCA	GNAS	PAK1	PTAFR
ACTG2	CALCRL	GPBR	PAK2	PTEN
ACTN1	CALD1	GUCY1A2	PAK3	PTGER3
ACTN2	CALM1	GUCY1A3	PAK4	PTGER4
ACTN3	CALM2	GUCY1B3	PAK6	PTGIR
ACTN4	CALM3	ILK	PAK7	PTGS1
ACTR2	CALM4	IQGAP1	PALLD	PTGS2
ACTR3	CALM5	ITGA1	PARD3	PTK7
ACTR3B	CALML3	ITGA2	PARD3B	RAC1
ADA	CALML5	ITGB5	PARD6A	RACGAP1
ADCY1	CALML6	ITPR1	PARD6B	RAF1
ADCY2	CAMK1	ITPR2	PARD6G	RAMP1
ADCY3	CAMK2D	ITPR3	PAWR	RAMP2
ADCY4	CAV1	JMJD7-PLA2G4B	PDK1	RAMP3
ADCY5	CDC42	KCNB2	PDLIM1	RAP1A
ADCY6	CDC42BPA	KCNMA1	PLA2G10	RGS2
ADCY7	CDC42BPB	KCNMB1	PLA2G12A	RHOA
ADCY8	CDC42BPG	KCNMB2	PLA2G12B	RhoC
ADCY9	CHRM1	KCNMB3	PLA2G1B	RND3
ADORA1	CHRM2	KCNMB4	PLA2G2A	ROCK1
ADORA2A	CHRM3	KIF23	PLA2G2C	ROCK2

ADORA2B	CHRNA3	KNG1	PLA2G2D	S100A4
ADRA1A	CHRNA4	LCP1	PLA2G2E	S100P
ADRA1B	CIT	LIMK	PLA2G2F	SFN
ADRA1D	CLF1	LIMK2	PLA2G3	SHROOM1
ADRA2A	CNN1	LLGL1	PLA2G4A	SHROOM2
ADRA2B	CNN2	LLGL2	PLA2G4B	SHROOM3
ADRA2C	CNN3	LMOD1	PLA2G4E	SHROOM4
ADRB2	CSNK2	LOC642076	PLA2G5	SMTN
AGTR1	CTTN	MAP2K1	PLA2G6	SOD1
ALKBH4	CYP4A11	MAP2K2	PLCB1	SORBS1
ANLN	CYP4A22	MAPK1	PLCB2	SORBS3
ARAF	DAAM1	MAPK3	PLCB3	SPHK1
ARF1	DAPK3	MARK1	PLCB4	SRC
ARF6	DAPK3	MPRIIP	PLCE1	SRF
ARHGAP18	DDR1	MRV11	PLEKHG6	STK11
ARHGAP35	DIAP1	MYBPH	PLK1	SVIL
ARHGAP5	DIAP2	MYH10	PLS1	TACR1
ARHGEF1	DIAP3	MYH11	PLS3	TACR2
ARHGEF11	DLC1	MYH14	PPP1CA	TACR3
ARHGEF12	DMPK	MYH9	PPP1CB	TBXA2R
ARHGEF17	DNM2	MYL12A	PPP1CC	TLN1
ARHGEF18	DOCK4	MYL12B	PPP1R12A	TNNI3
ARHGEF2	DOCK5	MYL6	PPP1R12B	TPM1
ARHGEF28	DSTN	MYL6B	PPP1R14A	TPM2
ARHGEF7	ECT2	MYL8P	PRKAA1	TPM3
ARPC1A	EDN1	MYL9	PRKAA2	TPM4
ARPC1B	EDN2	MYLK	PRKACA	TRPM7
ARPC2	EDNRA	MYLK2	PRKACB	VASP
ARPC3	EMD	MYLK3	PRKACG	VCL
ARPC4	F2R	MYO9B	PRKCA	WAS
ARPC5	FDAMM2	MYOCD	PRKCB	WASF2
ARPC5L	FLNA	NMU	PRKCD	WASL
ASPM	FLT1	NMUR1	PRKCE	WDR1
ATE1	FMN1	NMUR2	PRKCG	YWHAB
ATP1A2	FMN2	NPNT	PRKCH	YWHAE
AURKB	FSCN1	NPR1	PRKCI	YWHAG
AVPR1A	GHSR	NPR2	PRKCQ	YWHAH
AVPR1B	GIT1	NPY2R	PRKCZ	YWHAQ
BRAF	GIT2	NUAK1	PRKG1	YWHAZ
CACNA1C	GNA11	NUAK2		

Several core components of the Ca²⁺-dependent smooth muscle contraction pathway were highly enriched in the DS including *Calml1* (Calmodulin, CaM), *Mylk* (Myosin light chain kinase, MLCK), *Myh11* (Myosin heavy chain 11), *Myh9* (Myosin light chain 9) and *Acta2* (α SMA) (FIG. 2G). While smooth muscle is present in diverse organ systems (*e.g.* blood vessels, intestine) with varying transcriptomes to meet unique functional needs, several genes such as *Cald1* (Caldesmon 1), *Smtn* (Smoothelin) and *Tagln* (Transgelin, SM22) are considered

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pan-smooth muscle markers ⁽²⁸⁾ that were also part of the DS signature (FIG. 2G and FIGs. 10D-10E). Importantly, among those *Cald1*, *Myh11* and *Smtn* are not expressed in contractile myofibroblasts ⁽²⁹⁾, indicating that the DS expresses bona fide genes of smooth muscles. During smooth muscle contraction, mechanical forces are generated through actomyosin cross-bridges and ATP-powered myosin ratcheting action ⁽³⁰⁾ (FIG. 2G). 3-dimensional immunofluorescence revealed that the DS forms a network of α SMA stress fibers that wrap the follicle in concentric rings indicating that the potential actomyosin forces would be directed towards the center of the longitudinal axis in centripetal fashion (FIG. 2H). Actomyosin cross-bridge formation is promoted when CaM-activated MLCK phosphorylates regulatory myosin light chains (*e.g.* MYL9, FIGs. 2G and 2I) that associate with myosin heavy chain molecular motors (*e.g.* MYH10, FIGs. 2G and 2I). Immunofluorescence for phosphorylated pMYL9 confirms the active state of myosin in the DS further supporting DS functional contractile activity (FIG. 2I). Finally, immunofluorescence of human scalp hair follicles showed conservation of smooth muscle components in the DS between mice and humans (FIG. 2J). Overall, these results demonstrate that the DS harbors the contractile apparatus and its regulatory elements, long hypothesized by the presence of α SMA ⁽²⁴⁾.

Whether DS cells can functionally contract in a smooth muscle-like fashion through Ca²⁺-dependent MLCK activation was then explored. Isolated, short-term cultured DS cells were membrane depolarized by an extracellular K⁺ spike to activate voltage-gated Ca²⁺ channels in the presence of Fluo8 fluorescent Ca²⁺ indicator. The switch from high Na⁺ to high K⁺ media led to effective Ca²⁺ influx within 1.5 minutes (FIG. 3A). Tracing the cell surface area of Acan^{tdT}-marked DS cells cultured on a soft substrate further demonstrated functional contraction in high K⁺ conditions during 15 minutes (FIG. 3B). Next, whether the DS can functionally contract and compress intact hair follicles isolated by microdissection (FIG. 3C) was tested. Live imaging of freshly isolated follicles showed significant reduction of follicle widths after 3 minutes in high K⁺, consistent with contraction of concentric α SMA/myosin rings (FIGs. 3D and 3F), which was effectively blocked by preincubation with the MLCK-specific inhibitor ML7 (FIGs. 3E-3F). These data demonstrate that the DS can functionally contract by activation of voltage-gated Ca²⁺ channels in the CaM to MLCK to MYL/MYH/ α SMA pathway.

To determine whether smooth muscle-like contraction is a main function of the DS to propagate hair follicle regression *in vivo*, the MLCK pathway was blocked by twice-daily topical application of ML7 throughout catagen on the back skin of mice (FIG. 3G). On the contralateral vehicle control side, follicles regressed normally into telogen with the DP niche relocated next to

the SC reservoir (FIG. 3H). ML7-mediated smooth muscle contraction blocking in the treated skin region, however, resulted in striking failure of regression with long stalled follicles stranded deep in the dermis (FIG. 3H -3I), reminiscent of the DS-ablated phenotype (FIG. 1E). Also here, the DP remains at the tip of stalled follicles (FIG. 3J). However, in contrast to ablated follicles, the DS as expected surrounds follicles including DP. Taken together, these data demonstrate that DS contraction is functionally required for follicle regression and DP niche relocation to the stem cells.

To directly investigate where the DS contracts and uncovered the force mechanism by which the DS drives niche relocation during regression, *intravital* 2-photon microscopy of unperturbed skin in live mice ^(15, 18) was utilized (FIG. 11A). Dual-color fluorescent labeling of DS cells was first established to visualize both the flat DS cell sheet while also highlighting their nuclei (FIGs. 4A-4B). Acan^{tdT} labeled the DS cytoplasm in red, and Tbx18^{H2BGFP} strongly marked DS nuclei in green and weakly those of the DP. To contextualize any DS cell movement, neighboring follicle progenitors also needed to be labeled independently. To this end, a K14-H2BCer transgenic line was generated that labeled the nuclei of all skin epithelial cells in bright blue (FIGs. 12A-12B). The triple-color fluorescent reporter combination (FIG. 12C) made possible the simultaneous live imaging of DS, DP niche, and follicle epithelium at a single excitation wavelength (FIGs. 11B-11C) and enabled fine-mapping over time their absolute movement and relative to each other.

Cell tracking during a 5-hour span of catagen regression demonstrated upward movement of the hair shaft and DP, as expected (FIGs. 4B-4C and FIG. 13A). By contrast, the DS and ORS showed minimal movement in the long axis (FIGs. 4D-4F and FIG. 13B). Thus, it was concluded that the upward movement of the hair shaft occurs at the interface between the ORS progenitors and shaft, as previously observed ⁽¹⁵⁾. Further, the lack of movement relative to each other also demonstrated that the DS and ORS maintain strong connections through the basement membrane that separates these cellular layers. Together, live imaging in the labeling setup and timeframe demonstrated that the movements of key components during catagen regression were captured.

Then, the mechanism by which the niche becomes relocated during regression to reach its final position next to the stem cell reservoir was sought, and two long-standing hypotheses were tested. The first hypothesis states that an apoptotic force from the epithelial strand that pulls the DP ⁽²³⁾. The second hypothesis states that DS contracts to push the DP and/or hair shaft upwards ⁽²⁴⁾ (FIG. 4G). To investigate the first hypothesis, the length of the epithelial strand

during the 5-hour imaging window was measured. If the shrinking due to dying cells would generate force for pulling the DP, shortening of the strand correlating to DP movement (FIG. 4G) would be expected. However, while the epithelial strand length varies between follicles, their individual lengths remained constant throughout despite steady upward DP movement (FIGs. 2H-2I), ruling out the apoptotic force hypothesis. In support of this finding, it was also observed that shaft and DP upward movement occurred at similar rates (FIG. 4F).

To test the hypothesis that a DS-generated contractile force pushes the DP from underneath, potential DS movement below the DP was investigated. If this hypothesis is true, DS centripetal contraction underneath the DP corresponding to contraction-driven upward movement would be expected. The cross-sectional diameter of the DS at the DP bottom over time was measured and it was found, however, that the diameter of DS remained unchanged during regression (FIGs. 4J-4K) indicating that direct DS contraction on the DP does not account for its relocation. Then, a variation of the contraction hypothesis was explored in which DS contraction pushes the shaft upward, which in turn pulls both the epithelial strand and the DP upward. The observation of an unchanged epithelial strand length (FIGs. 4H-4I) is consistent with this new hypothesis in which the strand acts as a stable tether between the shaft and DP (FIG. 4G). Indeed, centripetal contraction movement of DS cells right at the “bottleneck” that is formed by the border between the shaft-containing club hair and the narrower epithelial strand indicates that DS contraction pushes the shaft upward (FIG. 4L and FIG. 13C). Measuring the cross-sectional diameter of the ring formed by follicle-wrapping DS cells at this bottleneck, a significant decrease in diameter over time coinciding with shaft upward movement was found (FIGs. 4M-4N). These data reveal that the DS moves centripetally at the bottleneck towards the center suggesting that it contracts to generate the constriction force necessary for pushing the hair shaft upward – akin to the squeezing motion of a tooth paste tube – which then pulls the DP by its connection to the hair shaft via the epithelial strand (FIG. 4O).

Here, the long-standing question of how the niche is relocated nearly the entire length of the follicle to reach its essential stem cell-adjacent position is answered. Through *intravital* imaging, contraction assays in isolated cells and intact follicles *ex vivo*, and *in vivo* inhibition of catagen skin, it has been demonstrated that DS is a smooth muscle that contracts to power the key catagen regression movements. The decades-old hypotheses that proposed apoptotic or contraction forces to propel catagen progression based on observations possible at the time were tested, and were consolidated into a new model, in which the niche relocates to the stem cells through a series of force relays (FIG. 4P). Having adapted the original “contraction hypothesis,”

the DS contracts throughout catagen at the club-strand bottleneck where, because of its angle, the centripetal constricting force gets redirected to an upward pushing force on the shaft. This force is then relayed through the tether-like epithelial strand pulling the DP. At the end stage of regression when the hair shaft has reached its final position and the DS trails below the DP as a hollow sleeve, it is possible that an epithelial strand apoptotic force then pulls in the niche next to the stem cell reservoir before launching regeneration in the next cycle.

Methods

Mice

Sox2^{GFP} (34) and *Lef1-RFP* (26) mice to label key skin populations was described previously (6, 27). *R26*^{mT/mG} (35), *Acan*^{CreER} (36), *R26*^{LSL-tdTomato} (Ai14) (37), and *R26*^{LSL-DTA} (38) were obtained from the Jackson Laboratory. Generation of *Tbx18*^{Cre} and *Tbx18*^{H2BGFP} mice was previously described (39). Generation of *K14-H2BCer* mice is described below. All animal studies were conducted in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) at ISMMS, and all mice used in this study were housed in facilities operated by the Center for Comparative Medicine and Surgery (CCMS) at ISMMS.

Acan^{CreER};*R26*^{LSL-tdTomato} and *Acan*^{CreER};*R26*^{LSL-DTA} mice were injected with tamoxifen (20mg/ml dissolved in corn oil, injections of 0.2g/kg body weight i.p.) daily from P10-P12 for DS-labeling and -ablation experiments and P9, P10 for *intravital* imaging experiments. Animals were PCR genotyped to select for control and experimental groups without blinding. All labeling, ablation, and *intravital* imaging experiments were repeated with at least three different mice; all specific n values for each experiment were listed in figure legends. All experiments were performed on both sexes with verification of all major findings present in both, but only results from females were reported to avoid any variation of measurements owing to sex.

Human scalp samples

Samples of human scalp were obtained from the Skin and Laser Center of the Mount Sinai Health System. Samples were collected during Mohs procedures, and regions of healthy tissue were separated from tumor-containing regions before processing. Additionally, selected tissue was verified for the absence of tumors by histology before use in further experimentation. Personal patient information was blinded to researchers except for age and sex. The study was conducted in accordance with guidelines outlined by the institutional review board (IRB) at ISMMS.

Generation of K14-H2BCer mice

An H2BmCerulean3 (H2BCer) insert was obtained from the mCerulean3-N1 plasmid (M. Davidson via Addgene, #54730) by BamHI/XbaI (NEB) restriction enzyme digestion and subsequently ligated into a pG3Z-K14-H2B vector (E. Fuchs) with T4 Ligase (NEB). The final K14-H2BCer cassette was obtained from the resultant plasmid by digestion with KpnI and SphI (NEB) and injected into blastocysts at the ISMMS Mouse Genetics and Gene Targeting CoRE. Transgenic mice were then screened by assessing Cer fluorescence in tail tips and PCR genotyping using primers targeting the transgene-unique K14 promoter/enhancer. A single founder was then selected from all mice positive for the transgene that had the highest and most uniform expression of H2BCer in the epidermis of F1 generation mice to establish the final transgenic line.

Immunofluorescence and microscopy

For immunofluorescence on tissue sections, back skins were harvested then embedded and fresh frozen in OCT (Tissue Tek). Sections were cut at a thickness of 8 μ m with a Leica cryostat. After drying, sections were post-fixed to slides with 4 % paraformaldehyde for 15 minutes at room temperature. After washing in PBS, sections were then permeabilized in 0.3-1.0 % Triton X-100/PBS for 15 minutes at room temperature before blocking in 0.5 % normal donkey serum (Jackson ImmunoResearch) or MOM blocking reagent for mouse primary antibodies (Vector Labs) for 1 hour at room temperature. Primary antibody labelling against ITGA8 (R&D Systems, goat 1:100), ACAN (Millipore, rabbit 1:100), α SMA (Neomarkers, mouse 1:100), PDGFRA (eBiosciences, rat 1:100), ECAD (Invitrogen, rat 1:1000), LEF1 (Cell Signaling, rabbit 1:100), K14 (Gifts from J. Segre, rabbit or chicken 1:1000), activated CASP3 (R&D Systems, rabbit 1:300), Ki67 (Leica, rabbit 1:200), AE13 (Abcam, mouse 1:100), AE15 (Santa Cruz, mouse 1:50), K6 (Gift from E. Fuchs, rabbit 1:1000), MYH10 (Cell Signaling, rabbit 1:25), MLCK (Abcam, rabbit 1:100), MYL9 (Proteintech, rabbit 1:100), phosphorylated (Ser19) MYL9 (Novus, rabbit 1:100), or SM22 (Proteintech, rabbit, 1:200) was performed at room temperature for 1 hour or 4° C overnight. Secondary labeling was done with donkey anti-goat, rabbit, rat, or mouse antibodies conjugated with AlexaFluor 488, 555, or 647 (Jackson ImmunoResearch, Invitrogen) for 30 minutes at room temperature. Nuclei were labelled with Hoechst 33342 (Thermo Fisher), and stained sections were mounted in a glycerol-based p-phenylenediamine (Sigma) antifade reagent.

For whole mount immunofluorescence of single hair follicles from mouse back skin or human scalp, tissues were harvested and fixed in 4 % PFA for 2 hours at room temperature with gentle shaking. Individual hair follicles were then separated from the skin through microdissection under a Leica S6E stereomicroscope and collected into a 96-well plate with PBS. Tissue was permeabilized in 0.5 % Triton X-100/PBS for 15 minutes, washed, then stained in primary antibody against α SMA (Abcam, rabbit 1:300), K14 (Gift from J. Segre, rabbit 1:1000), SM22 (Proteintech, rabbit 1:200), MYH11 (Proteintech, rabbit 1:100), or MYL9 (Proteintech, rabbit 1:100) at 4°C overnight. Secondary labeling was done with donkey anti-rabbit or chicken antibodies conjugated with AlexaFluor 488, 555, or 647. Nuclei were then labelled with Hoechst 33342, and follicles were then mounted in antifade reagent.

The whole mount immunofluorescence of pieces of intact back skin followed a modification of a previously published procedure ⁽⁴⁰⁾. Tissues were harvested, and fixed in 4 % PFA for 2 hours at room temperature or overnight at 4°C before peeling away the panniculus carnosus muscle and cutting into 1 cm x 2 cm strips. Subsequent steps were then performed in a 6-well plate. Skins were then treated with 0.3 % Triton X-100/PBS for 5-8 hours before primary antibody staining for K14 (rabbit 1:1000) in a 0.3 % Triton X-100, 5 % donkey serum, 20 % DMSO/PBS solution at room temperature for 3-5 days with gentle shaking. After washing in 0.3 % Triton X-100/PBS solution for 5-8 hours at room temperature with media changes every 30 minutes, tissues were then stained with a donkey anti-goat AlexaFluor 555 antibody in the Triton X-100/donkey serum/DMSO/PBS solution used similarly with primary antibody labeling for 2-3 days at room temperature with gentle shaking. Finally, tissues were washed again in 0.3 % Triton X-100/PBS solution for 5-8 hours at room temperature with media changes every 30 minutes and counter stained with Hoechst 33342. Optical clearing was then performed by dehydrating tissue in 50:50 methanol/water for 5 minutes then a series of three 100% methanol treatments for 30 minutes each at room temperature. Final clearing was performed in a BABB (benzyl alcohol/benzyl benzoate, 1:2 ratio) solution until visibly clear then mounted in residual BABB held in a chambered glass slide.

Immunofluorescence stained sections were imaged with Leica DM5500 widefield microscope, and whole mount stained tissues were imaged with a Leica SP5 DMI confocal microscope equipped with Leica LASAF software. Images were post-processed and adjusted for brightness and contrast using ImageJ/FIJI (NIH). Hair follicle length was measured from whole mount fluorescence stained, cleared back skin in FIJI and defined as length from the proximal tip of K14+ epithelium to the base of the sebaceous gland.

Isolation of DS, DP, and DF

During morphogenetic anagen, DP and DS from 1st and 2nd wave hair follicles could be distinguished based on distinct expression patterns of Sox2^{GFP}; *Lef1-RFP* reporter expression ⁽²⁷⁾.

In order to further purify the DS and DP, both were selected as PDGFRA-Brilliant Violet 421+.

5 Back skins were harvested from P5 mice and digested in dispase (Invitrogen) overnight at 4° C. Epidermis was then removed from dermis by peeling, and the dermis was then digested in a 0.2 % collagenase (Sigma-Aldrich) and DNase (20 U/μl, Roche) solution at 37° C for one hour with gentle shaking. Cell pellets were obtained by centrifugation at 350 x g for 5 minutes and further digested in 0.25 % trypsin EDTA solution for 5 minutes at 37° C. Cells were then stained
10 against PDGFRA (eBiosciences, biotinylated rat, 1:50,) followed by streptavidin-Brilliant Violet 421 (Biolegend, 1:200) secondary staining. DAPI was added for live/dead cell identification and cells were sorted using a BD Influx cell sorter at the ISMMS Flow Core facility using the following criteria: DP = GFP+, RFP+, PDGFRA+; DS = GFP+, RFP-, PDGFRA+; DF = GFP-, RFP^{mid}. FACS profiles were analyzed using FlowJo software.

qRT-PCR

Whole cell RNA was purified from sorted cells using the Absolutely RNA Nanoprep Kit (Agilent). cDNA synthesis was done with Superscript III (Invitrogen) using oligo(dT) primers. qRT-PCR was performed using SYBR Green Master Mix I (Roche) on a LightCycler 480 thermocycler (Roche). Relative mRNA levels were calculated using the 2^{-ΔΔCt} method,
20 normalized to Gapdh. All measurements were performed with biological duplicates each with technical duplicates.

RNA sequencing and analysis

Total RNA was purified from sorted cells using the Absolutely RNA Nanoprep Kit (Agilent). RNA concentrations were measured with a NanoDrop spectrophotometer (Thermo),
25 and quality was assessed using an Agilent Bio-analyzer. Samples with an RNA integrity number score of 8 or higher were further processed. 6 ng of starting material was reverse transcribed and amplified with the RNA Ovation RNaseq System V2 (NuGEN). cDNA was then sheared by sonication using a Covaris LE220. From 100 ng of sheared amplified cDNA, sequencing libraries were generating with 6 unique barcoded adaptors, one for each sample (2 biological
30 replicates x 3 populations) using the Ovation Ultralow DR Library System (NuGEN). Library concentration and quality were quantified by Qubit (Invitrogen) and Agilent Bioanalyzer.

Samples were then sequenced on the Illumina HiSeq 2000 platform using a 50-nt single-read setting at the Genome Technology Center at NYU.

To refine the DS gene signature, previously published RNA sequencing data from 3 additional follicle populations consisting of hair follicle outer root sheath, matrix, and melanocytes obtained at a similar age ⁽²⁷⁾ were included in analyses. Raw RNA sequencing reads were mapped to the mm10 mouse genome with TopHat v2.0.3 ⁽⁴¹⁾ coupled with Bowtie2 ⁽⁴²⁾ aligner with default parameters. Transcriptomes were assembled and fragments per kilobase per million reads (FPKM) for each gene were computed with Cufflinks v2.1.1 ⁽⁴³⁾. Differentially expressed genes (DEGs) were identified using Cuffdiff (with default parameters except for the library normalization method was upper quartile normalization, where FPKMs were scaled via the ratio of the 75th quartile fragment counts to the average 75th quartile value across all libraries) and ANOVA with Benjamini-Hochberg correction for multiple hypothesis testing with significance cut off FDR < 0.05. Principal component analysis and hierarchical clustering were performed on all DEGs using Origin 2019 (OriginLab) and Morpheus (Broad) respectively. Population signature genes were defined by DEGs with an FPKM ≥ 5 , and fold enrichment ≥ 2 compared to all other populations. Gene set enrichment analysis was performed on GenePattern (Broad) using a user-defined gene set for smooth muscle contraction and regulation components (Table 2).

Intravital live-imaging and analysis

Acan^{tdT}; Tbx18^{H2BGFP}; K14-H2BCer mice were injected with tamoxifen (0.15 g/kg i.p.) at P9 and P10 to label DS cytoplasm with tdT. Mice were then imaged at P15 or P16 to capture mid-regression using an Olympus FV1000 MPE 2-photon microscope equipped with a tunable Coherent Chameleon Ultra Ti:Sapphire laser. During imaging, mice were first anaesthetized with ketamine/xylazine mixture (100 mg/kg, 12.5 mg/kg i.p.) and subsequently maintained on inhaled isoflurane anesthetic (0.75 % isoflurane vapor, O2 flow rate = 0.75 L/minute, EZ Anesthesia) delivered through nose-cone. Specifications of the imaging stage set up was as previously described ^(2, 15, 18, and 44). Optical slices were captured using a single 900 nm excitation wavelength at 10 % laser power and resultant fluorescence imaged with a 25X XLPlanLN water immersion objective (NA 1.05, Olympus). Emission filters for each channel were as follows: blue = BA420-456, green = BA495-540H, red = BA575-630. Each optical slice field had a 250 x 400 μ m area captured at a resolution of 640 x 1024 px with a 12.5 μ s/px scan speed. Z-stacks

of 30-45 μm in depth were taken with 3 μm step intervals. Time intervals between acquisitions for time-lapse imaging were 7.5 minutes.

Raw images of time-lapse series were processed in FIJI to separate fluorescence signal from each fluorescent protein type, the process for which is demonstrated in FIGs. 12A-12C.

Fluorescence signal captured by each channel were as follows: blue = mCerulean3 and second harmonic generation (SHG), green = GFP and Cer, and red = tdT and mCerulean3. The excitation/emission spectra and fluorescence strength of Cer permitted its fluorescence excited at 900 nm to be strongly visible in the blue and green channels and moderately visible in the red channel. To isolate only the Cer signal in the blue channel, first the pixel values of the green channel were subtracted from the blue channel to obtain SHG signal, which was then subtracted from the blue channel so that only Cer signal remained. The resulting Cer pixel values were then subtracted from the green and red channels to isolate GFP and tdT signal respectively. The Cer stack was then pseudocolored to light blue for better visibility and was color merged with isolated GFP and tdT stacks to obtain the final triple color images.

Cells were manually tracked in FIJI by determining the x,y coordinates of the centers of nuclei. Tracking did not include the z dimension since cells rarely moved between z-sections during imaging sessions. The strong brightness of the H2BCer fluorescence enabled tracking of nuclei of differentiated hair shaft cells which were identified by anatomical location and extreme ovoid nuclear shape. Movement of DPs were measured by determining the boundaries of round DP clusters made by the surrounding epithelial or DS cells and weak H2BGFP fluorescence. Movements of cells and structures were standardized to the relative movement compared to the average movement of tracked ORS cells of each respective follicle. Any cells that underwent apoptosis during imaging were not included in movement quantifications.

In vitro intracellular Ca^{2+} and contraction assays

Acan^{tdT} mice were injected with tamoxifen (0.15 g/kg i.p.) at P6 and P7 to fluorescently label DS. Single cell suspensions were obtained from P8 back skins using a dispase/collagenase digestion procedure as described for flow isolations and plated to 24-well plates. For contraction assays, cells were plated to pre-coated Matrigel (Corning 1:50) plates. Cells were grown in DMEM (Gibco) containing 10% bovine calf serum (HyClone) and were incubated at 37°C with 5 % CO₂ for 24-48 hours to allow for attachment. Cells were then incubated in Krebs buffer + glucose for 1 hour prior to assays. Both assays were conducted on an inverted DM6000 microscope with a live cell chamber set to 37° C with 5 % CO₂. DS cells were identified and

selected by tdT fluorescence. Ca²⁺ assays were performed using the Fluo-8 No wash Calcium Assay Kit (AAT Bioquest). Fluorescence was quantified in FIJI by averaging the signal of three 25 x 25 μ m areas per cell in the cell periphery. Cell surface areas during contraction assays were measured in FIJI by quantifying areas of threshold fluorescence signal exclusive of cell-occupied areas.

Ex vivo hair follicle contraction assay

Hair follicles were microdissected from P8 back skin under a Leica S6E stereomicroscope in PBS and collected in 24-well plates. Media was then changed to Krebs buffer + glucose with or without 200 μ M ML7 HCl (Tocris) and incubated for 1 hour at 37° C. Single follicles were then transferred to a 6 cm petri dishes immersed in Krebs buffer + glucose and immobilized by embedding the hair shaft tips in a 5 x 5 mm piece of parafilm. Follicles were then imaged under brightfield illumination on a Leica inverted DM6000 microscope in an enclosed live cell chamber at 37° C with 5 % CO₂. Images were taken with a 10X air lens with time intervals of 10 seconds. During imaging, media was exchanged for Krebs buffer + glucose with KCl supplanting NaCl and follicles were refocused when necessary, which took a duration of no more than 10 seconds. Measurements of follicle width at each time point were made in FIJI at three separate locations of the follicle isthmus located above the bulb and averaging. Presented data points and statistics were performed on the means of biological replicates each consisting of technical triplicates of width measurements.

In vivo contraction inhibition

Patches of lateral back skin measuring 1 x 1 cm of P13 mice (2 patches per mouse) were clipped and then shaved with a razor blade until no external hair shafts within patches remained. Vehicle-only control DMSO or ML7 HCl (Tocris) dissolved in an equal volume of DMSO (1 mM in 20 μ l) was pipetted onto hairless patches and locally rubbed into the skin using a glass rod until visibly dry. Topical applications were performed twice daily from P13 to P19, once in the morning and once in the evening separated by 12 hours. Pieces of back skins including treated areas were then harvested at P20 for downstream analysis.

Statistics and reproducibility

Data are represented as percentages, whisker plots, bar plots, or line plots with error bars or boundaries as mean $\pm$ s. d. Two-tailed, unpaired Student's t-test was used to analyze data sets with two groups. ANOVA with Benjamini-Hochberg correction for multiple hypothesis testing was performed to generate DEGs from RNA sequencing. Generation of data plots and statistical

analyses were performed using Microsoft Excel, MATLAB (MathWorks), or Origin 2019 (OriginLab). No statistical method was used to predetermine sample sizes. P-values < 0.05 were designated as significant and symbolized in figure plots as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ with precise values supplied in figure legends.

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Conclusion

As intimate crosstalk between stem cells and their niches is vital for proper stem cell maintenance and cell fate decisions, it is unsurprising that niches are found in anatomically distinct locations for hematopoietic stem cells in the bone marrow and intestinal stem cells at the crypt base. Providing close proximities for paracrine signaling and insulation from outside influences, most stem cell niche systems under homeostasis remain structurally stable, but after injury to the niche, re-establishment is vital for the restoration of long-term function^(31, 32). While hematopoietic stem cells can mobilize, and home to their niche, such as during transplantation⁽³³⁾, the more restricted freedom of movement of epithelial stem cells may limit their homing potential. Here, a smooth muscle function was discovered to relocate a niche to its stem cell reservoir to add to the vast array of diverse roles of smooth muscles throughout the body. This example highlights the evolutionary advantage of repurposing pre-existing functionality rather than inventing new systems in wake of new adaptive challenges. Further study may identify more instances of the function of smooth muscle contraction in regulating stem cell niche signaling range in homeostasis, which could be lost or exploited in disease.

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Other Embodiments

Embodiment 1. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle, thereby treating hair loss.

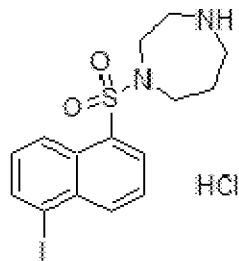
25 Embodiment 2. The method of embodiment 1, wherein the pharmacological agent is a compound, a peptide, or an antibody which inhibits myosin light chain kinase.

Embodiment 3. The method of embodiment 2, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

Embodiment 4. The method of embodiment 1, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

30

Embodiment 5. The method of embodiment 1, wherein the pharmacological agent is:



, or a functional derivative thereof.

Embodiment 6. The method of embodiment 1, further comprising administering an agent for facilitating hair growth.

Embodiment 7. The method of embodiment 6, wherein the agent for facilitating hair growth is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

Embodiment 8. The method of embodiment 1, wherein the pharmacological agent is administered topically.

Embodiment 9. The method of embodiment 1, further comprising administering a penetration enhancer prior to or concomitantly with the administration of the pharmacological agent.

Embodiment 10. The method of embodiment 1, wherein inhibiting the myosin light chain kinase (MLCK) results in cessation of dermal sheath cell contractions, thereby halting the hair cycle in the catagen phase.

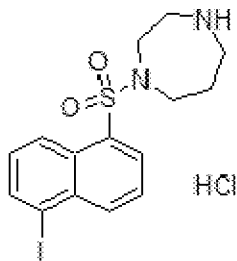
Embodiment 11. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the smooth muscle cell-like contractions of the dermal sheath cells of the hair follicles, thereby treating hair loss.

Embodiment 12. The method of embodiment 11, wherein the pharmacological agent is a compound, a peptide, or an antibody which inhibits the smooth muscle cell function of the dermal sheath cells.

Embodiment 13. The method of embodiment 12, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

Embodiment 14. The method of embodiment 11, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

Embodiment 15. The method of embodiment 11, wherein the pharmacological agent is:



Embodiment 16. The method of embodiment 11, further comprising administering an agent for facilitating hair growth.

Embodiment 17. The method of embodiment 16, wherein the agent for facilitating hair growth is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantanoanoprost, dutasteride, or ketoconazole.

Embodiment 18. The method of embodiment 11, wherein the pharmacological agent is administered topically.

Embodiment 19. The method of embodiment 11, further comprising administering a penetration enhancer prior to or concomitantly with the administration of the pharmacological agent.

Embodiment 20. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that arrests the hair cycle in catagen phase.

Embodiment 21. The method of embodiment 20, wherein the pharmacological agent is a compound, a peptide, or an antibody which arrests the hair cycle in catagen phase.

Embodiment 22. The method of embodiment 20, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

Embodiment 23. The method of embodiment 20, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

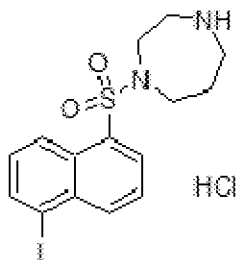
Embodiment 24. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the activation of catagen phase of the hair cycle or which inhibits the progression of the catagen phase of the hair cycle, thereby treating hair loss.

Embodiment 25. The method of embodiment 24, wherein the pharmacological agent is a compound, a peptide, or an antibody which inhibits the smooth muscle cell function of the dermal sheath cells.

Embodiment 26. The method of embodiment 25, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

Embodiment 27. The method of embodiment 24, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

5 Embodiment 28. The method of embodiment 24, wherein the pharmacological agent is:



Embodiment 29. The method of embodiment 24, further comprising administering an agent for facilitating hair growth.

10 Embodiment 30. The method of embodiment 29, wherein the agent for facilitating hair growth is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

Embodiment 31. The method of embodiment 24, wherein the pharmacological agent is administered topically.

15 Embodiment 32. The method of embodiment 24, further comprising administering a penetration enhancer prior to or concomitantly with the administration of the pharmacological agent.

Embodiment 33. The method of embodiment 24, wherein the pharmacological agent is a compound, peptide, or antibody that has a binding inhibitory IC₅₀ value of 100 nM or less.

20 Embodiment 34. The method of embodiment 24, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

Embodiment 35. A pharmaceutical composition comprising a pharmaceutical agent in a therapeutically effective amount such that the myosin light chain kinase pathway becomes inhibited in a dermal sheath cell of a hair follicle.

25 Embodiment 36. A pharmaceutical composition comprising a pharmaceutical agent in a therapeutically effective amount such that the hair growth cycle becomes arrested in the catagen phase.

Embodiment 37. A pharmaceutical composition comprising a pharmaceutical agent in a therapeutically effective amount such that the smooth muscle cell-like contractility of dermal sheath cells is inhibited.

Embodiment 38. The pharmaceutical composition of any of embodiments 35-37, further comprising a penetration enhancer.

Embodiment 39. The pharmaceutical composition of any of embodiments 35-37, wherein the pharmaceutical agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

Embodiment 40. The pharmaceutical composition of any of embodiments 35-37, further comprising an agent for facilitating hair growth.

Embodiment 41. The pharmaceutical composition of embodiment 40, wherein the agent is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

Embodiment 42. The pharmaceutical composition of any of embodiments 35-41 for topical application.

Embodiment 43. A method of identifying an agent capable of treating hair loss, comprising: (a) administering a test agent to a cell culture of dermal sheath cells; (b) detecting the production of one or more biomarkers indicative of the inhibition of dermal sheath contraction; (c) validating a test agent in a hair follicle model to confirm that the test agent inhibits dermal sheath contraction; thereby identifying an agent capable of treating hair loss.

Embodiment 44. The method of embodiment 43, wherein an antibody to myosin light chain 9 (pMyl9) is used to detect the biomarkers in step (b).

In addition to the embodiments expressly described herein, it is to be understood that all of the features disclosed in this disclosure may be combined in any combination (*e.g.*, permutation, combination). Each element disclosed in the disclosure may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, and can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS AND SCOPE

In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than
5 one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes
10 embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

Furthermore, the disclosure encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is
15 dependent on the same base claim. Where elements are presented as lists, *e.g.*, in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the disclosure, or embodiments of the disclosure, is/are referred to as comprising particular elements and/or features, certain
20 embodiments of the disclosure or embodiments of the disclosure consist, or consist essentially of, such elements and/or features. For purposes of simplicity, those embodiments have not been specifically set forth *in haec verba* herein. It is also noted that the terms “comprising” and “containing” are intended to be open and permits the inclusion of additional elements or steps. Where ranges are given, endpoints are included. Furthermore, unless otherwise indicated or
25 otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or sub-range within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

This application refers to various issued patents, published patent applications, journal articles, and other publications, all of which are incorporated herein by reference. If there is a
30 conflict between any of the incorporated references and the present disclosure, the specification shall control. In addition, any particular embodiment of the present disclosure that falls within the prior art may be explicitly excluded from any one or more of the claims. Because such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded

even if the exclusion is not set forth explicitly herein. Any particular embodiment of the disclosure can be excluded from any claim, for any reason, whether or not related to the existence of prior art.

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments described herein. The scope of the present embodiments described herein is not intended to be limited to the above Description, but rather is as set forth in the appended claims. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present disclosure, as defined in the following claims.

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CLAIMS

What is Claimed is:

1. A method of treating hair loss comprising administering a therapeutically effective
5 amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle, thereby treating hair loss.

2. The method of claim 1, wherein the pharmacological agent is a compound, a peptide, or
10 an antibody which inhibits myosin light chain kinase.

3. The method of claim 2, wherein the compound, peptide, or antibody has a binding
inhibitory IC50 value of 100 nM or less.

4. The method of claim 1, wherein the pharmacological agent is wortmannin, KT5926, K-
15 252a, MS-444, ML-9, or ML-7, or a combination thereof.

5. The method of claim 1, wherein the pharmacological agent is ML-7, or a functional
derivative thereof.

6. The method of claim 1, further comprising administering an agent for facilitating hair
20 growth.

7. The method of claim 6, wherein the agent for facilitating hair growth is one or more of
finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide,
25 spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

8. The method of claim 1, wherein the pharmacological agent is administered topically.

9. The method of claim 1, further comprising administering a penetration enhancer prior to
or concomitantly with the administration of the pharmacological agent.

10. The method of claim 1, wherein inhibiting the myosin light chain kinase (MLCK) results in cessation of dermal sheath cell contractions, thereby halting the hair cycle in the catagen phase.

11. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the smooth muscle cell-like contractions of the dermal sheath cells of the hair follicles, thereby treating hair loss.

12. The method of claim 11, wherein the pharmacological agent is a compound, a peptide, or an antibody which inhibits the smooth muscle cell function of the dermal sheath cells.

13. The method of claim 12, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

14. The method of claim 11, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

15. The method of claim 11, wherein the pharmacological agent is ML-7, or a functional derivative thereof.

16. The method of claim 11, further comprising administering an agent for facilitating hair growth.

17. The method of claim 16, wherein the agent for facilitating hair growth is one or more of finasteride, cypoterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

18. The method of claim 11, wherein the pharmacological agent is administered topically.

19. The method of claim 11, further comprising administering a penetration enhancer prior to or concomitantly with the administration of the pharmacological agent.

20. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that arrests the hair cycle in catagen phase.

21. The method of claim 20, wherein the pharmacological agent is a compound, a peptide, or an antibody which arrests the hair cycle in catagen phase.

22. The method of claim 20, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

23. The method of claim 20, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

24. A method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the activation of catagen phase of the hair cycle or which inhibits the progression of the catagen phase of the hair cycle, thereby treating hair loss.

25. The method of claim 24, wherein the pharmacological agent is a compound, a peptide, or an antibody which inhibits the smooth muscle cell function of the dermal sheath cells.

26. The method of claim 25, wherein the compound, peptide, or antibody has a binding inhibitory IC₅₀ value of 100 nM or less.

27. The method of claim 24, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

28. The method of claim 24, wherein the pharmacological agent is ML-7, or a functional derivative thereof.

29. The method of claim 24, further comprising administering an agent for facilitating hair growth.

30. The method of claim 29, wherein the agent for facilitating hair growth is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

5 31. The method of claim 24, wherein the pharmacological agent is administered topically.

32. The method of claim 24, further comprising administering a penetration enhancer prior to or concomitantly with the administration of the pharmacological agent.

10 33. The method of claim 24, wherein the pharmacological agent is a compound, peptide, or antibody that has a binding inhibitory IC₅₀ value of 100 nM or less.

34. The method of claim 24, wherein the pharmacological agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

15

35. A pharmaceutical composition comprising a pharmaceutical agent in a therapeutically effective amount such that the myosin light chain kinase pathway becomes inhibited in a dermal sheath cell of a hair follicle.

20 36. A pharmaceutical composition comprising a pharmaceutical agent in a therapeutically effective amount such that the hair growth cycle becomes arrested in the catagen phase.

37. A pharmaceutical composition comprising a pharmaceutical agent in a therapeutically effective amount such that the smooth muscle cell-like contractility of dermal sheath cells is
25 inhibited.

38. The pharmaceutical composition of any of claims 35-37, further comprising a penetration enhancer.

30 39. The pharmaceutical composition of any of claims 35-37, wherein the pharmaceutical agent is wortmannin, KT5926, K-252a, MS-444, ML-9, or ML-7, or a combination thereof.

40. The pharmaceutical composition of any of claims 35-37, further comprising an agent for facilitating hair growth.

41. The pharmaceutical composition of claim 40, wherein the agent is one or more of finasteride, cyproterone acetate, alfatradiol, minoxidil, bimatoprost, bicalcutamide, spironolactone, flutamide, lantoanoprost, dutasteride, or ketoconazole.

42. The pharmaceutical composition of any of claims 35-41 for topical application.

43. A method of identifying an agent capable of treating hair loss, comprising:

- (a) administering a test agent to a cell culture of dermal sheath cells;
 - (b) detecting the production of one or more biomarkers indicative of the inhibition of dermal sheath contraction;
 - (c) validating a test agent in a hair follicle model to confirm that the test agent inhibits dermal sheath contraction;
- thereby identifying an agent capable of treating hair loss.

44. The method of claim 43, wherein an antibody to myosin light chain 9 (pMyl9) is used to detect the biomarkers in step (b).

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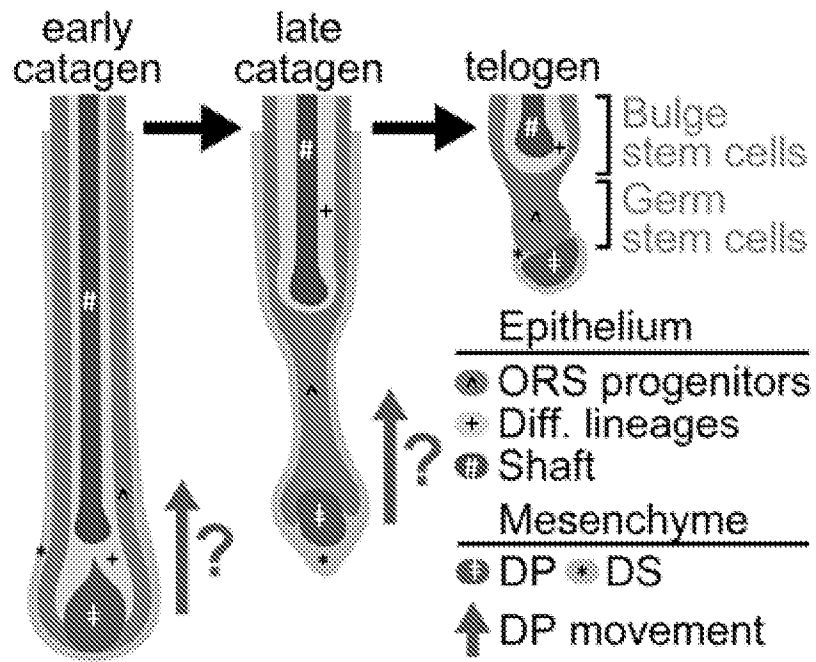


FIG. 1A

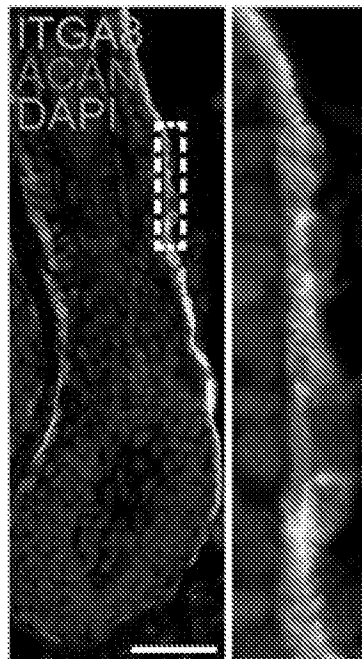


FIG. 1B

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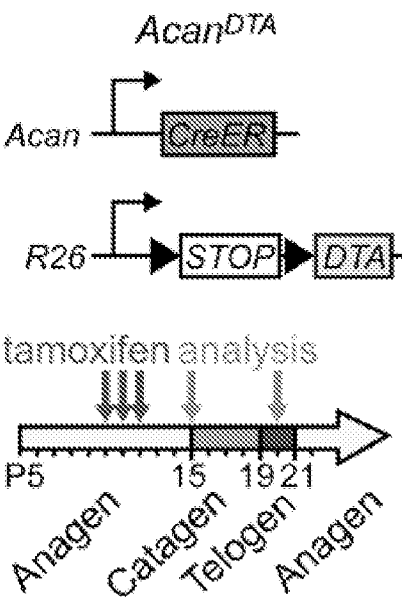


FIG. 1C

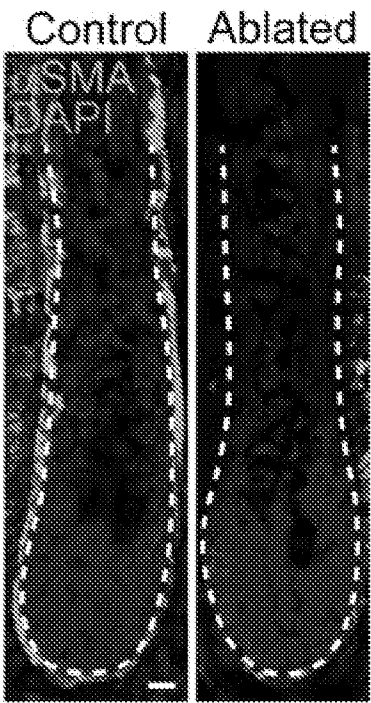


FIG. 1D

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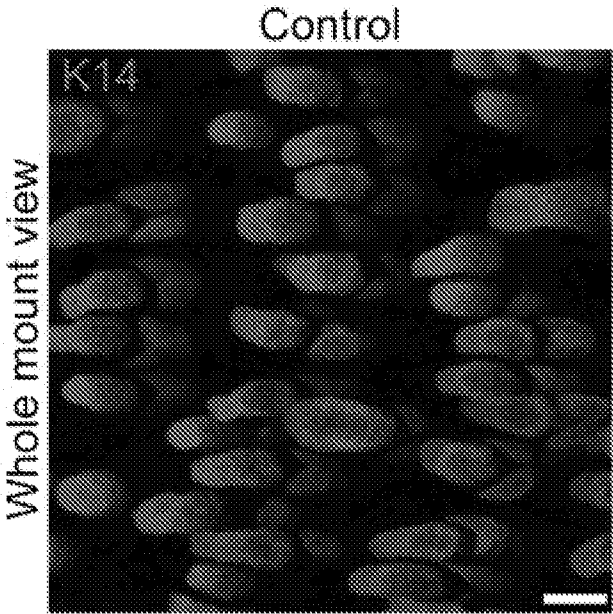


FIG. 1E

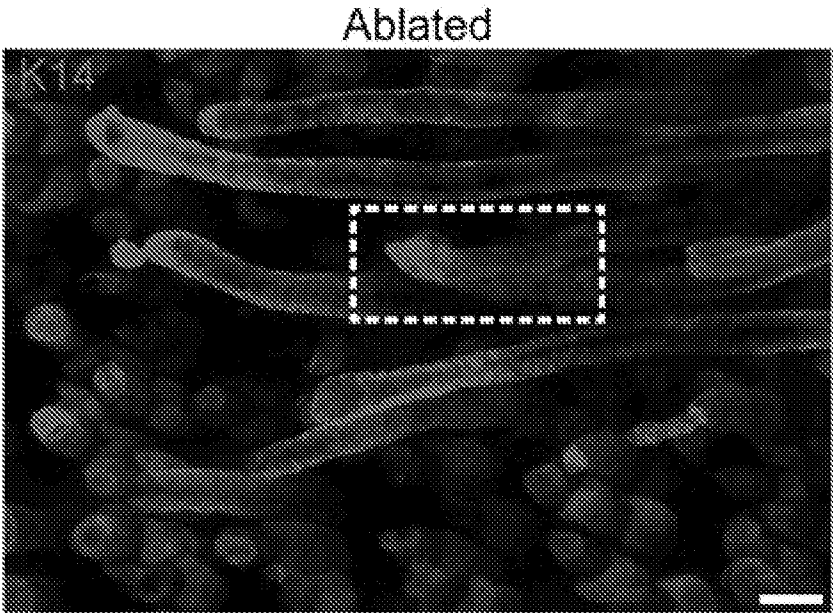


FIG. 1F

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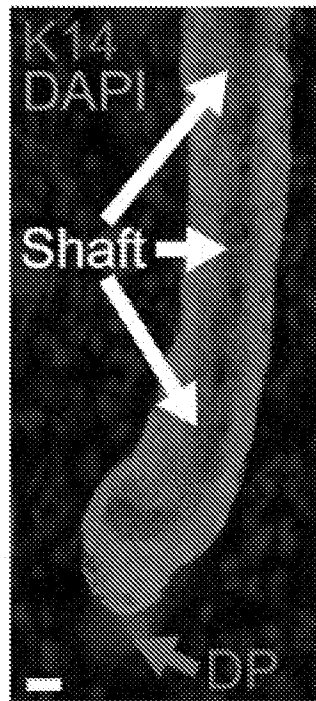


FIG. 1G

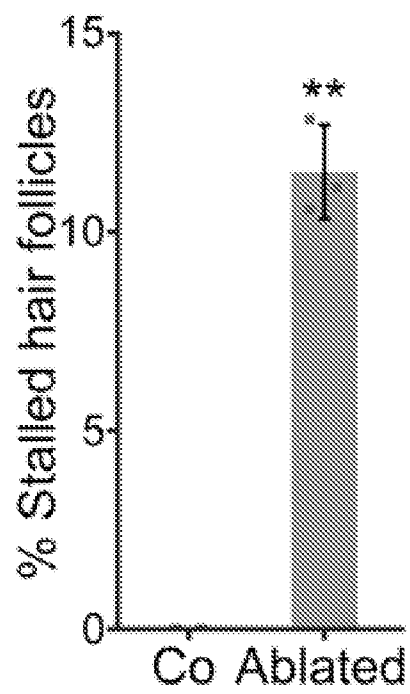


FIG. 1H

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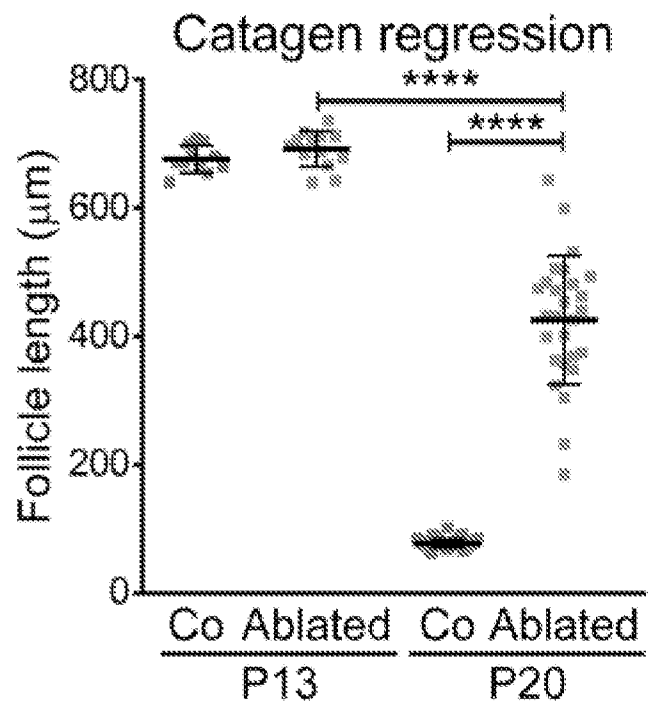


FIG. 1I

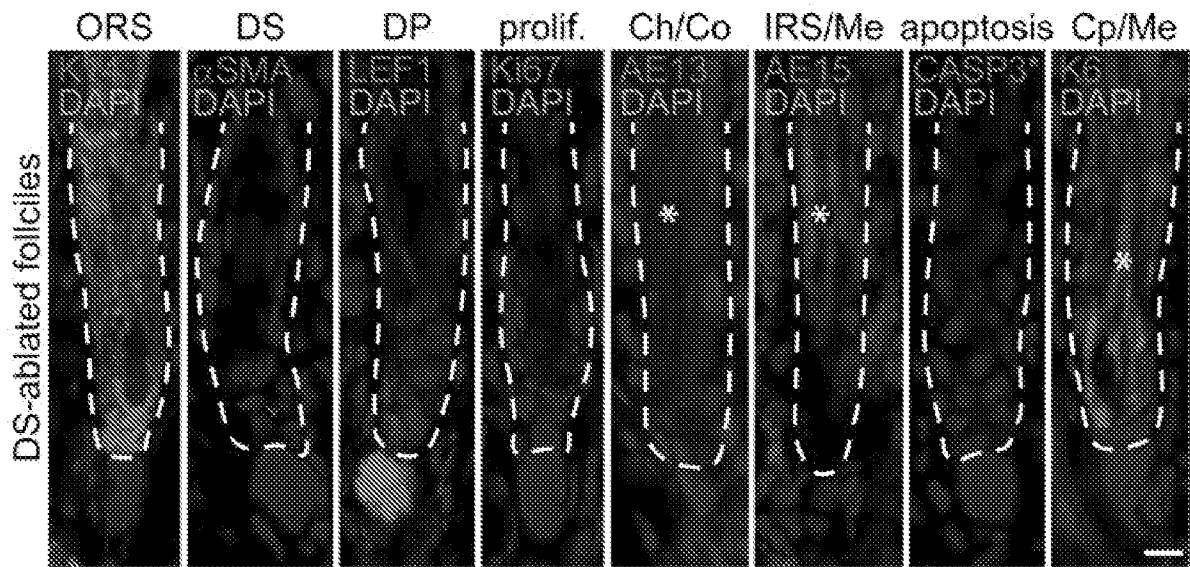


FIG. 1J

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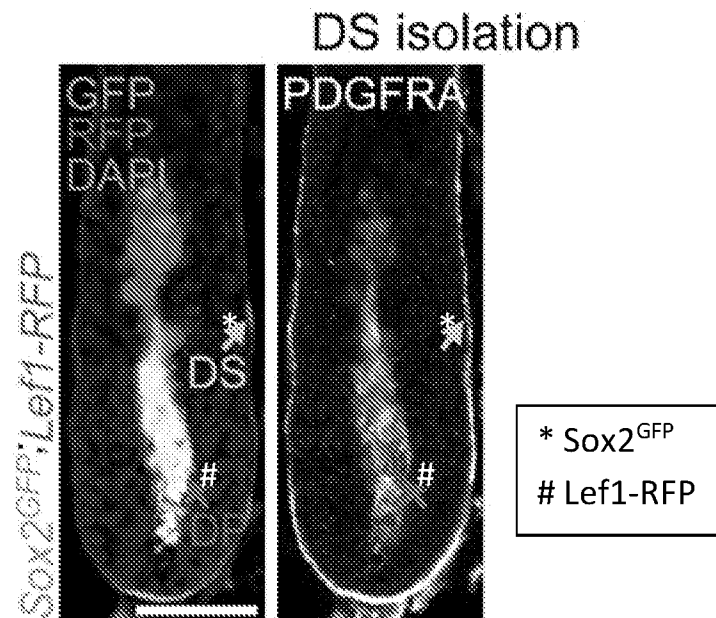


FIG. 2A

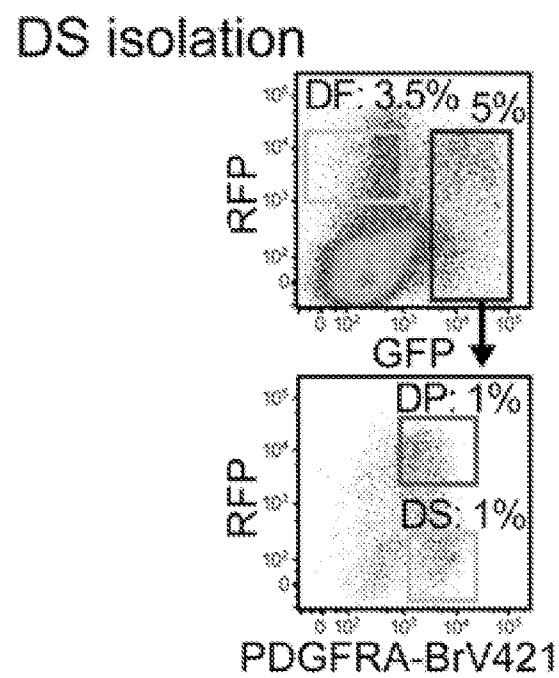


FIG. 2B

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qRT-PCR verification of DS, DP, DF

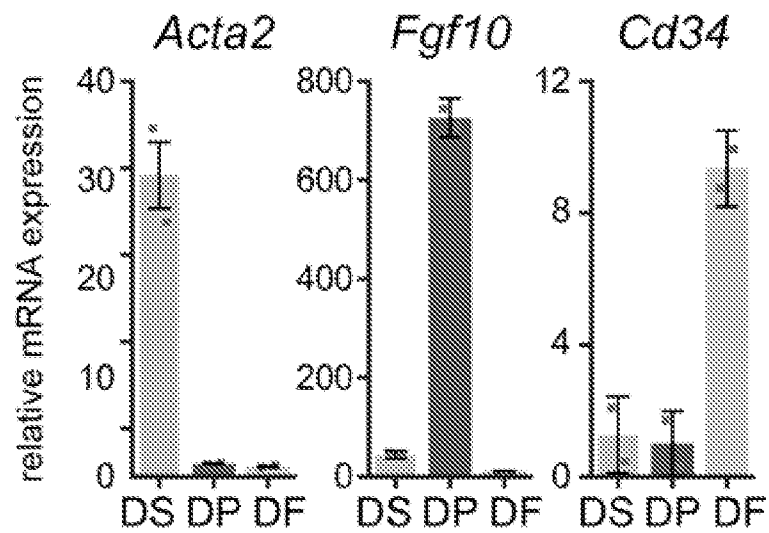


FIG. 2C

Signature genes

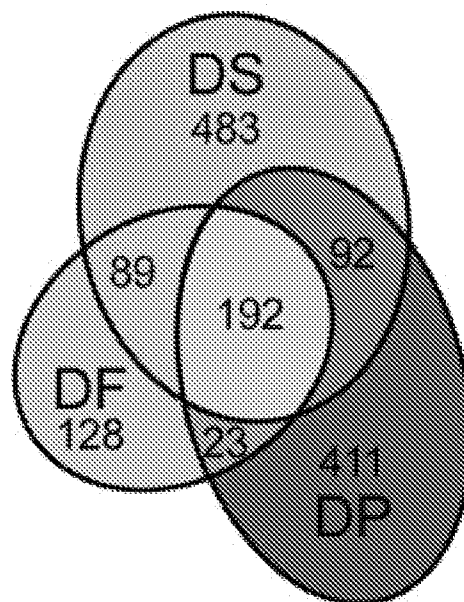


FIG. 2D

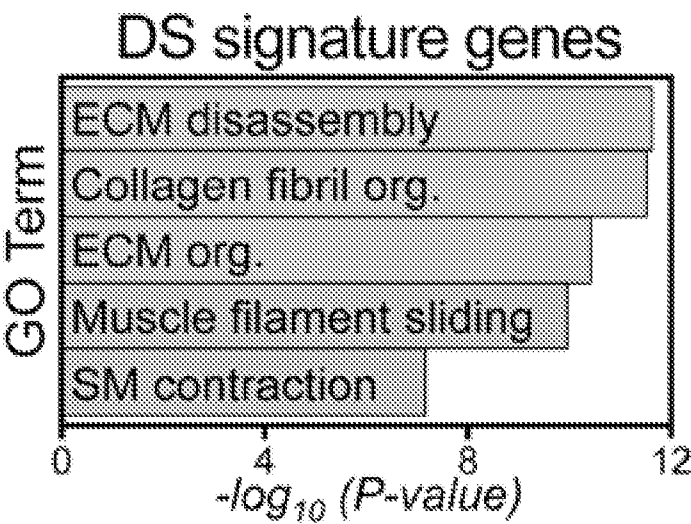


FIG. 2E

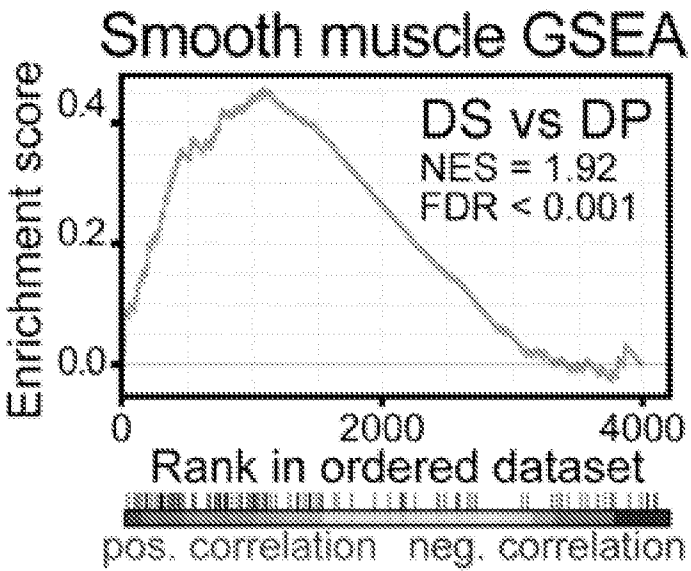


FIG. 2F

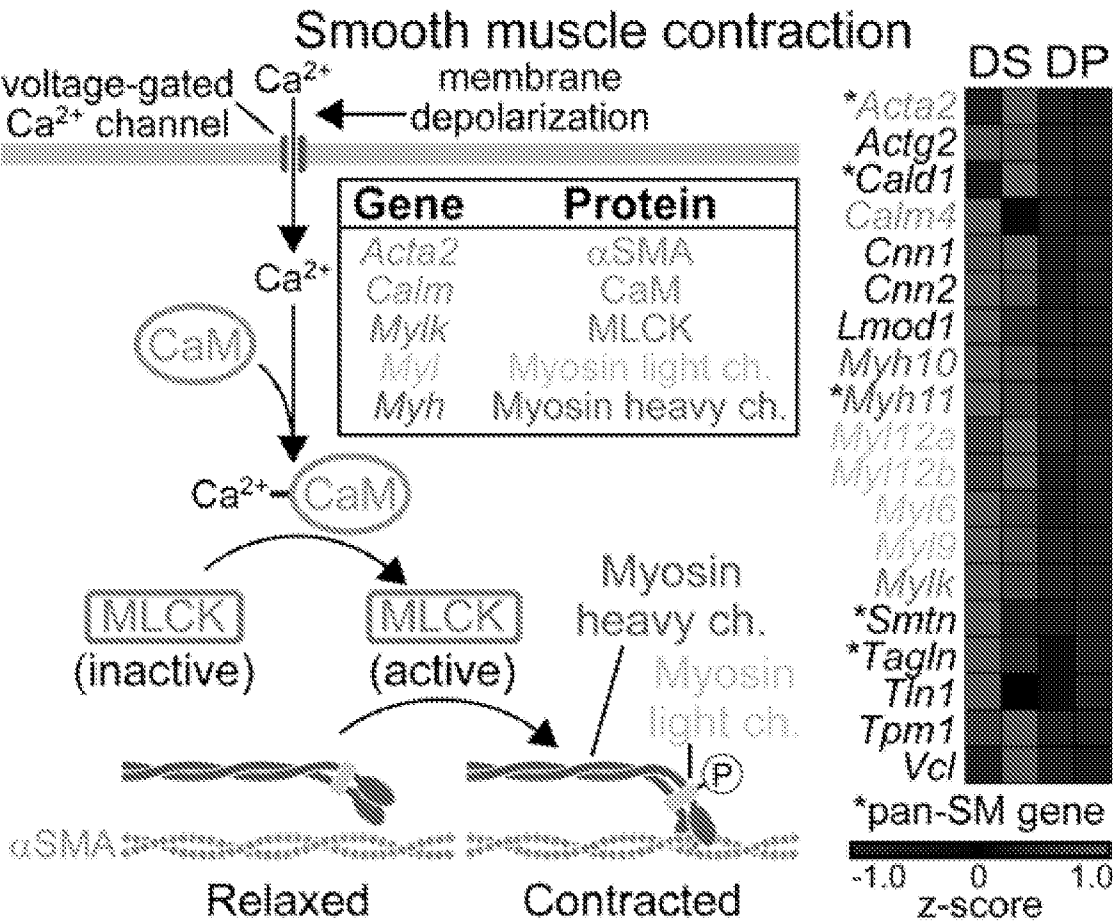


FIG. 2G

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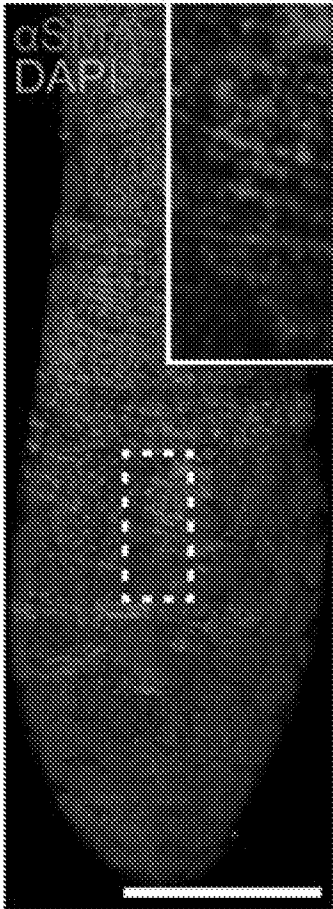


FIG. 2H

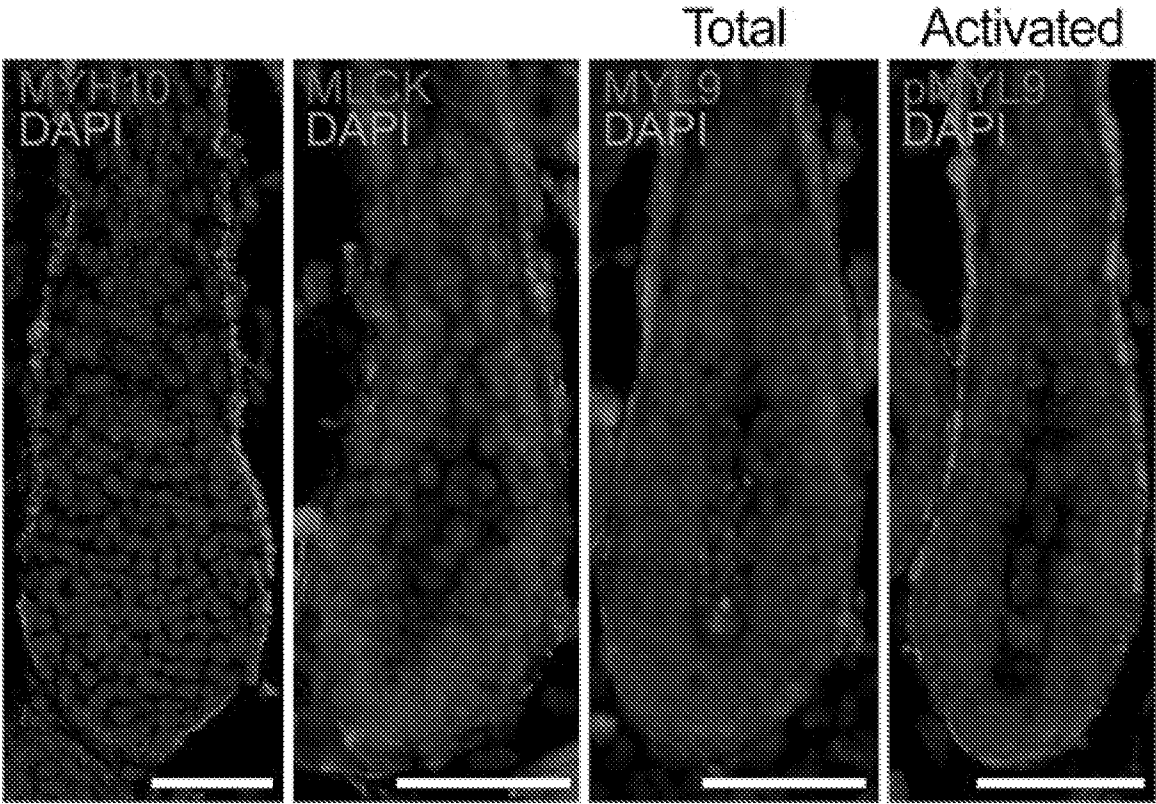


FIG. 2I

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Human scalp hair follicle

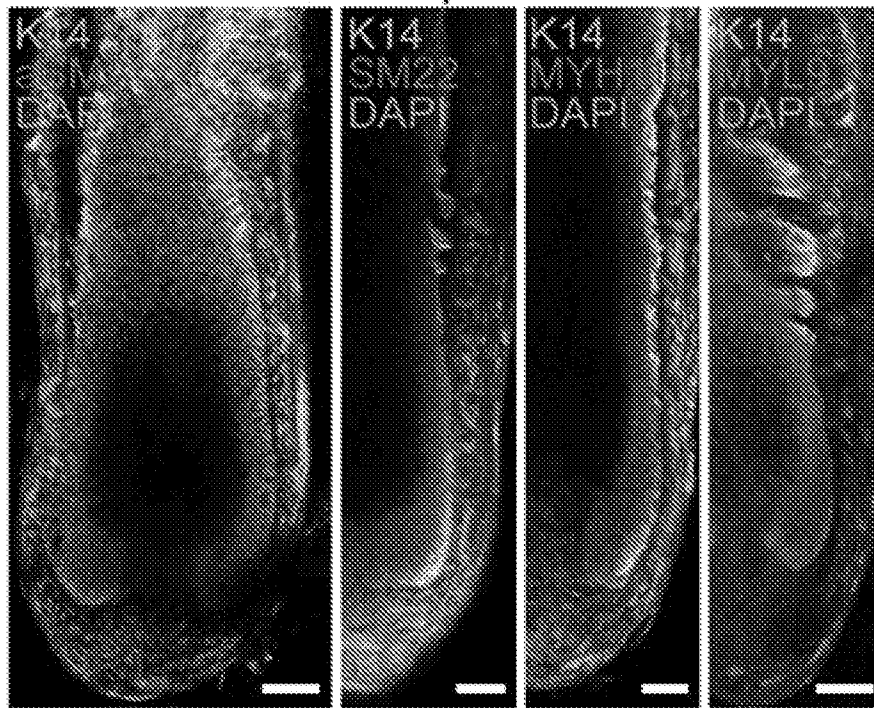


FIG. 2J

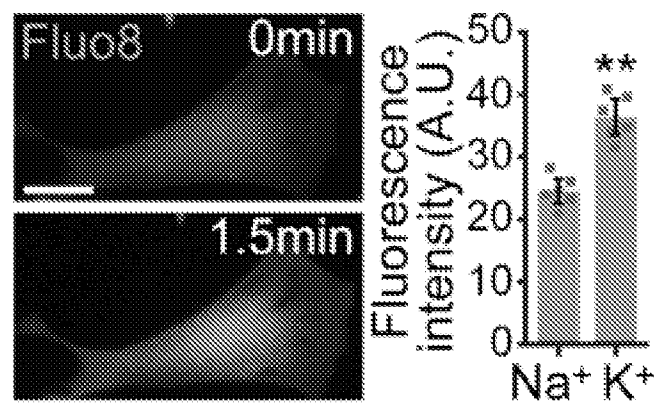
Depolarization-triggered
 Ca^{2+} influx

FIG. 3A

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Depolarization-triggered contraction

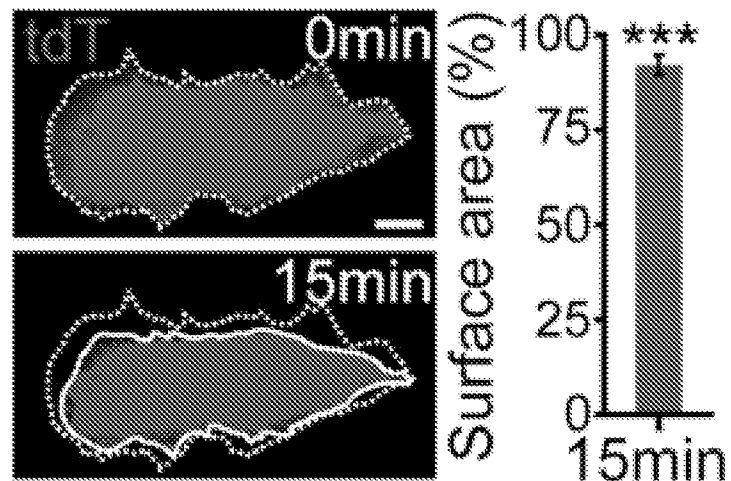


FIG. 3B

Ex vivo live imaging

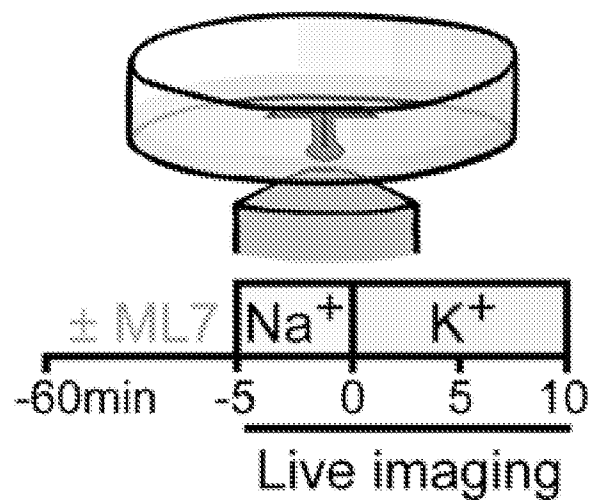


FIG. 3C

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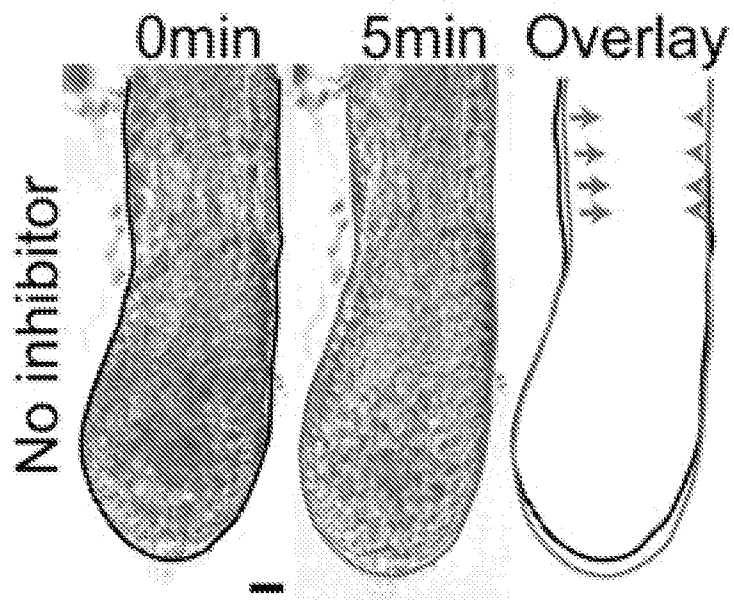


FIG. 3D

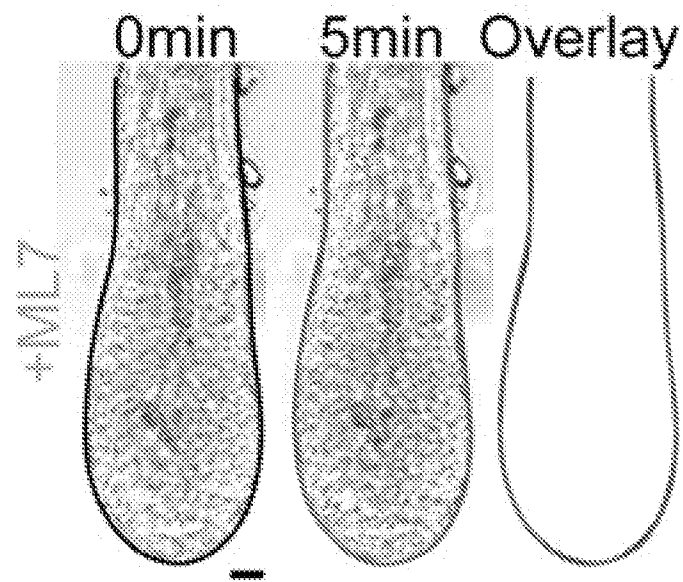


FIG. 3E

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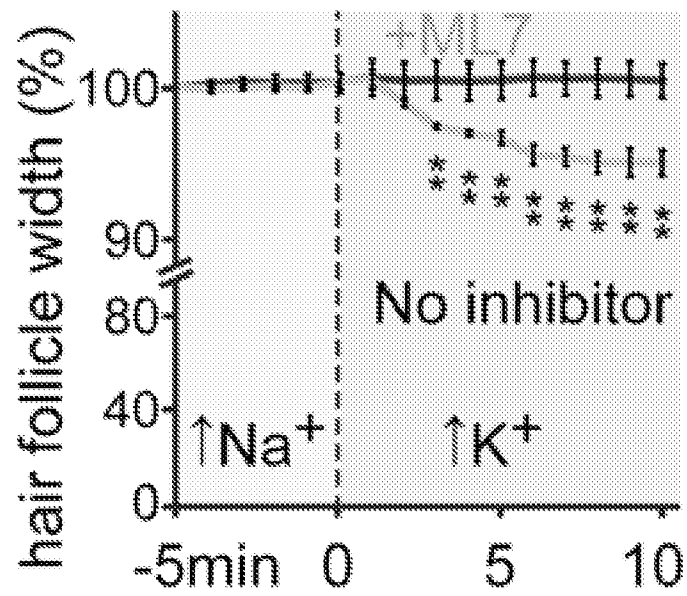


FIG. 3F

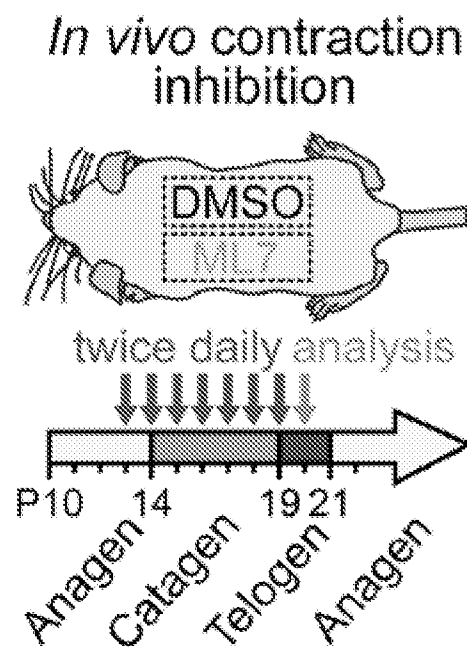


FIG. 3G

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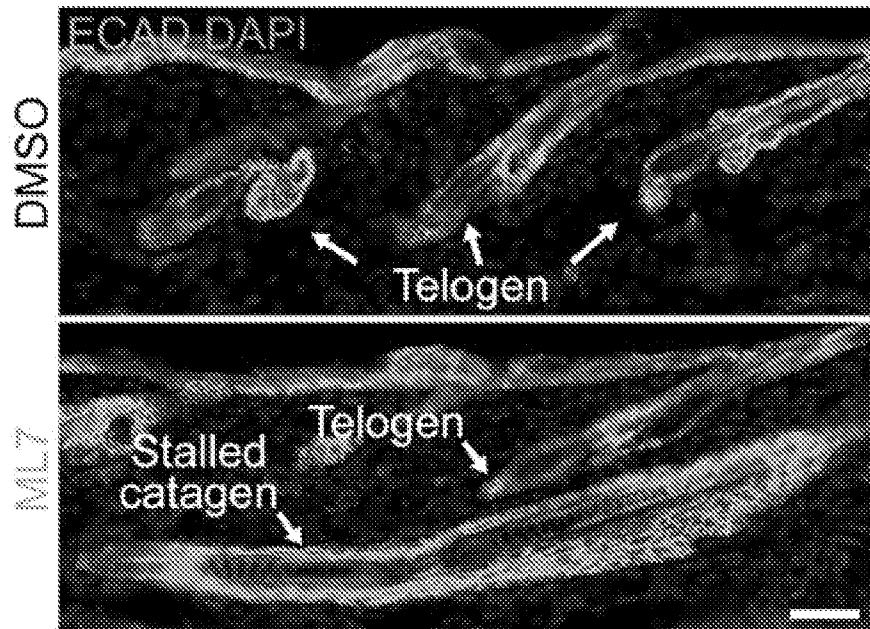


FIG. 3H

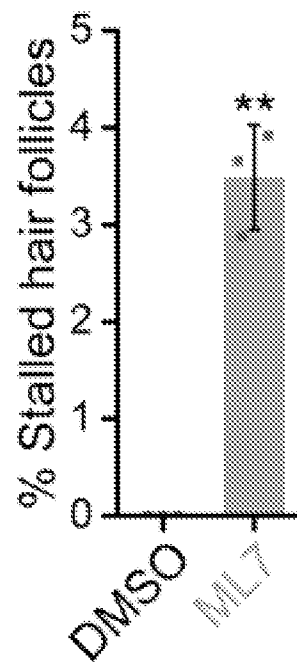


FIG. 3I

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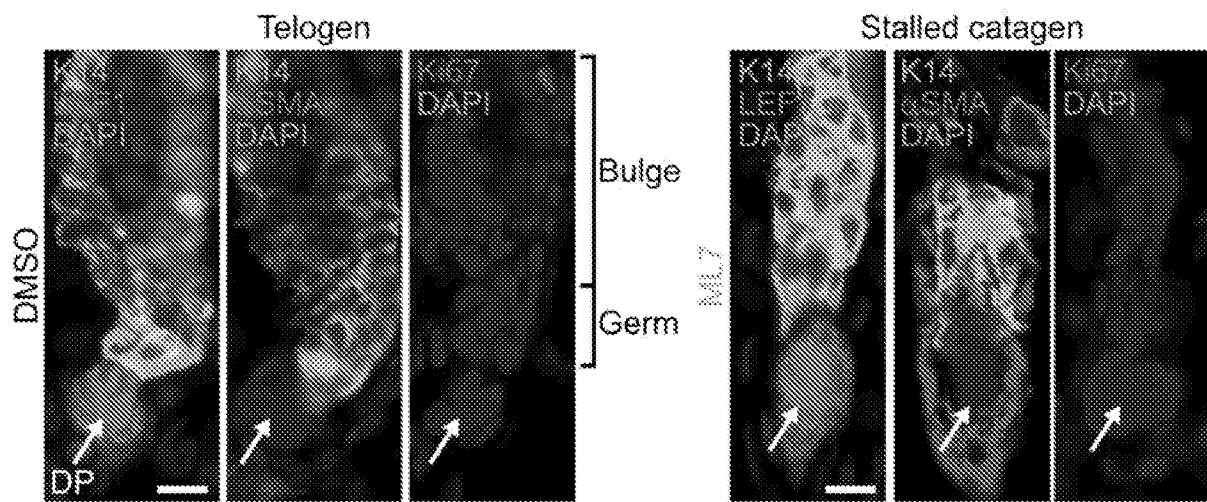


FIG. 3J

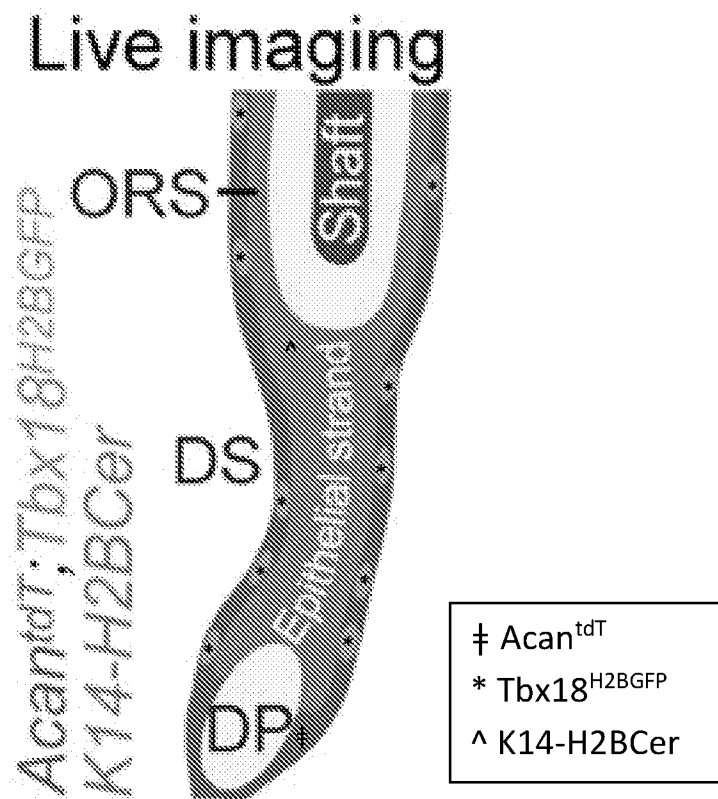


FIG. 4A

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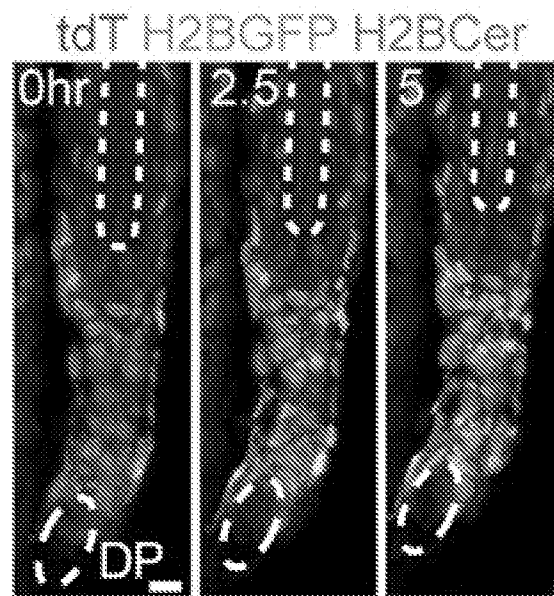


FIG. 4B

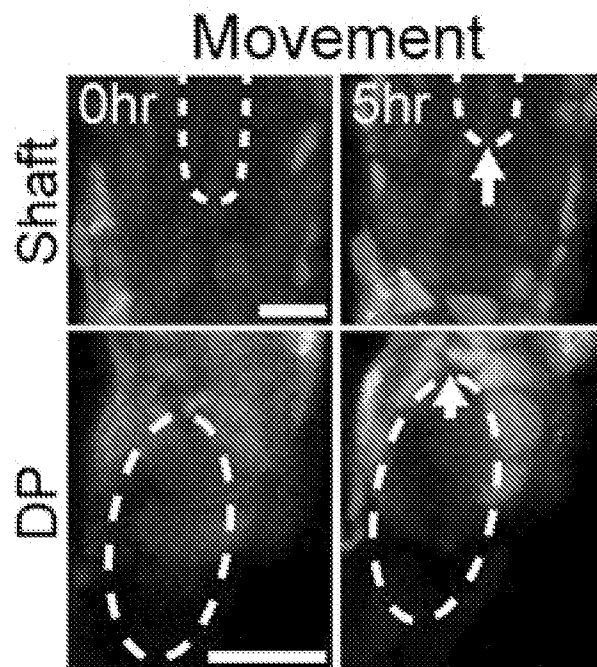


FIG. 4C

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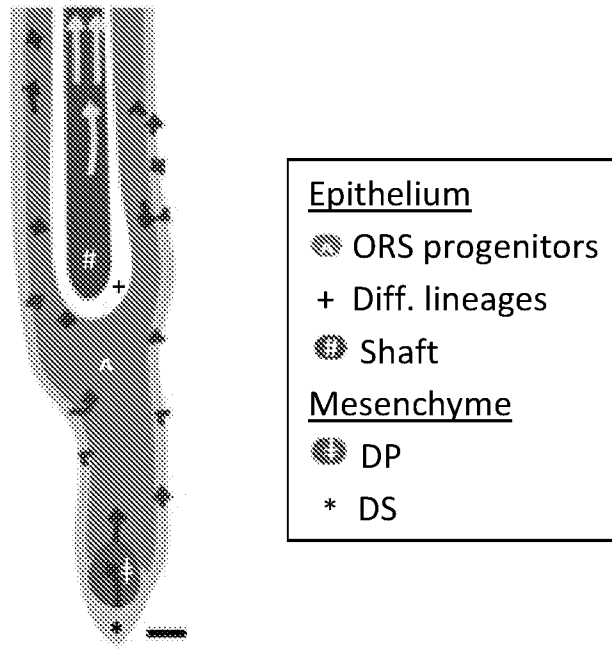


FIG. 4D

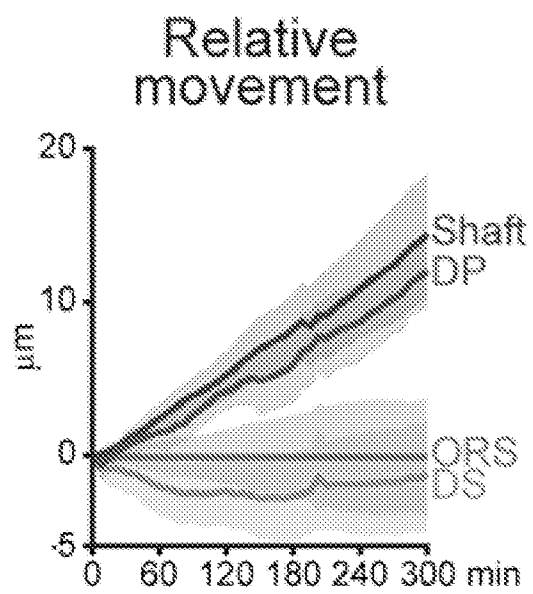


FIG. 4E

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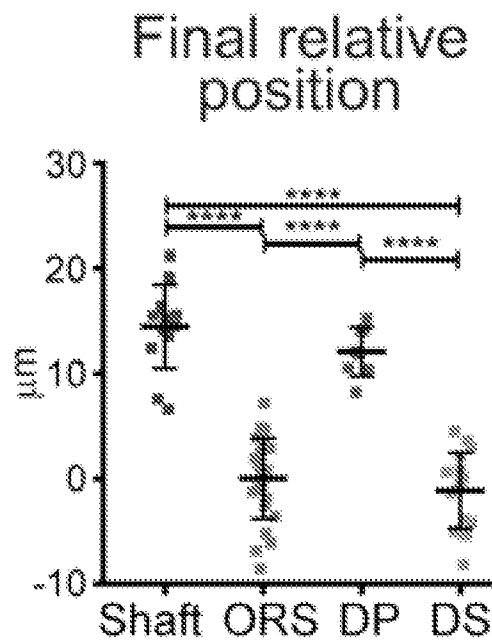


FIG. 4F

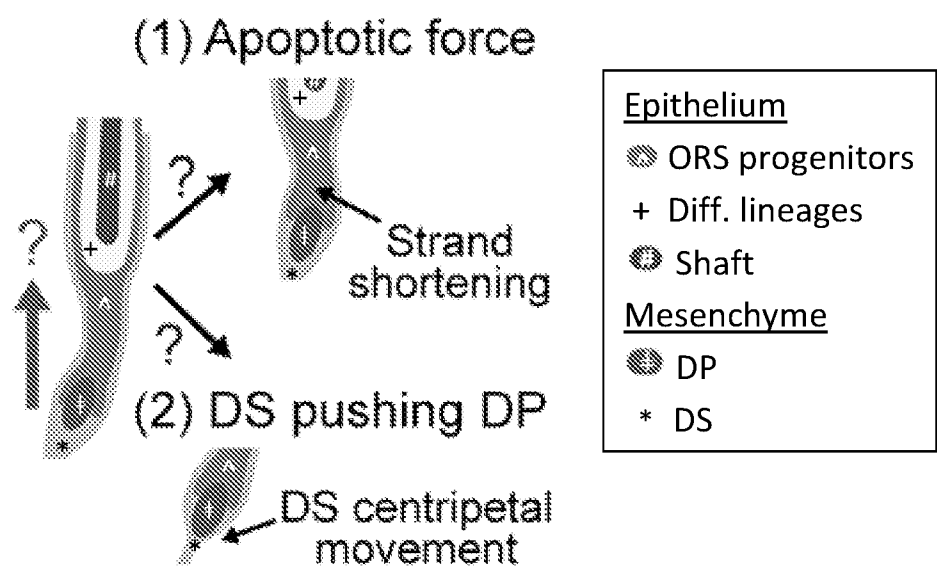


FIG. 4G

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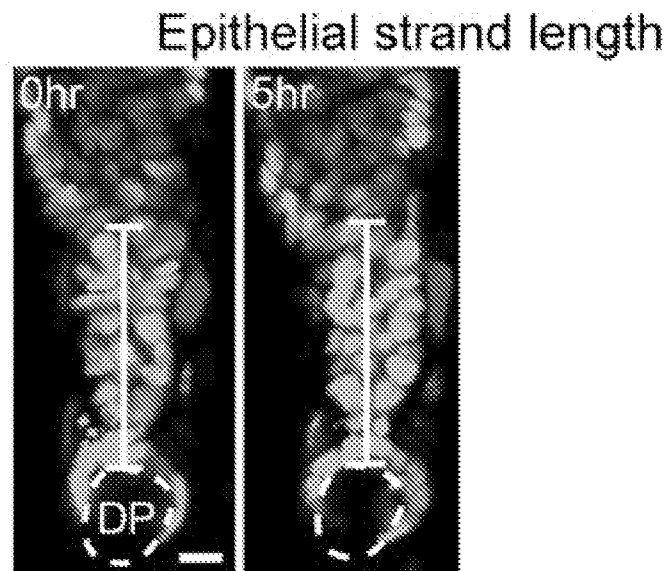


FIG. 4H

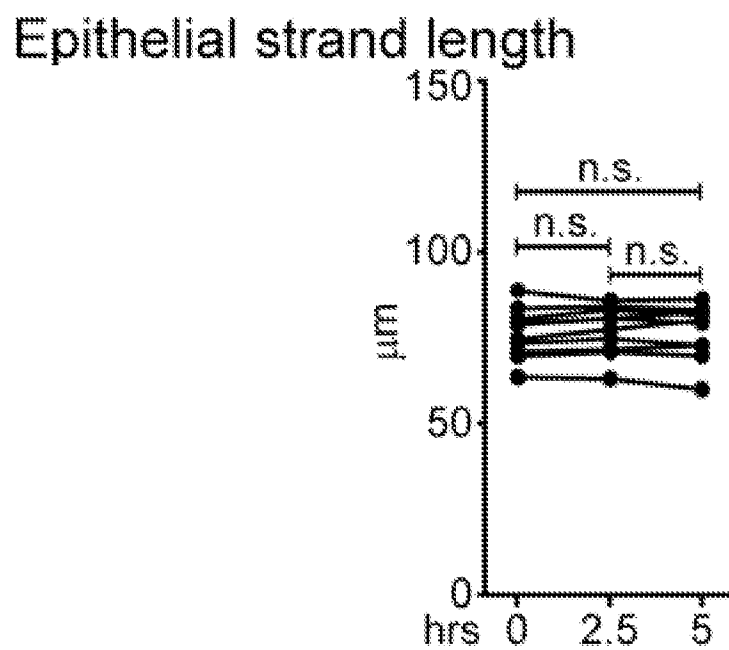


FIG. 4I

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DP bottom

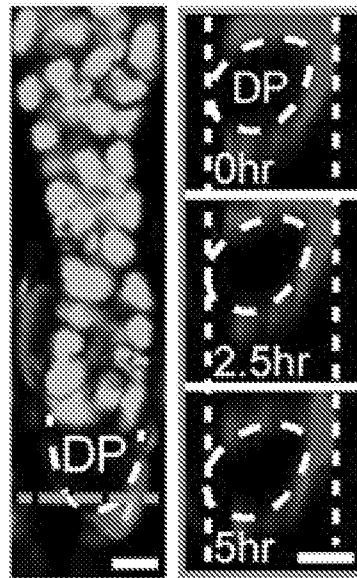


FIG. 4J

DS diameter

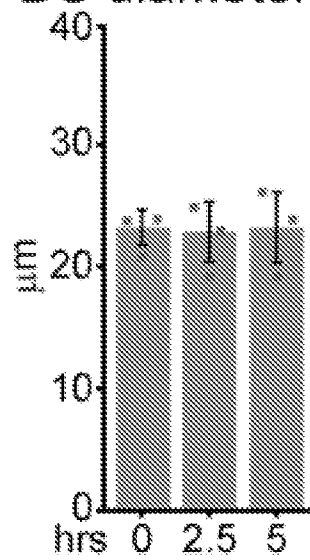


FIG. 4K

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Club-epithelial strand bottleneck

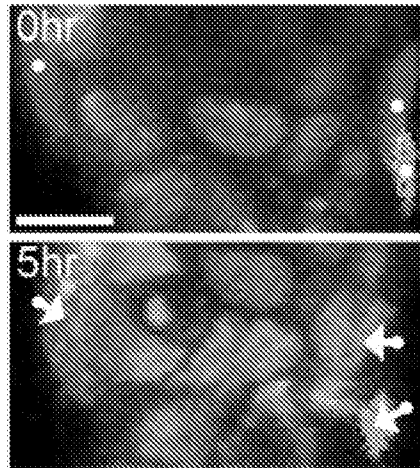


FIG. 4L

Bottleneck

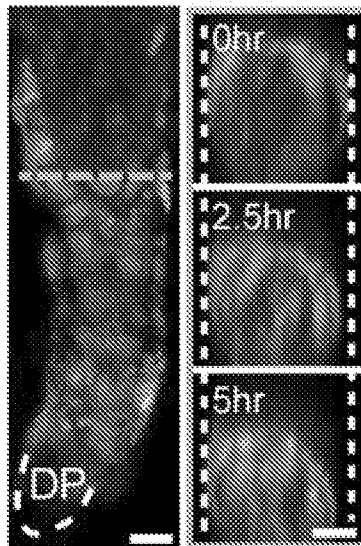


FIG. 4M

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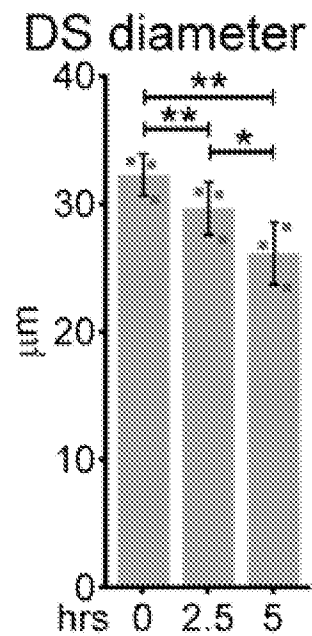


FIG. 4N

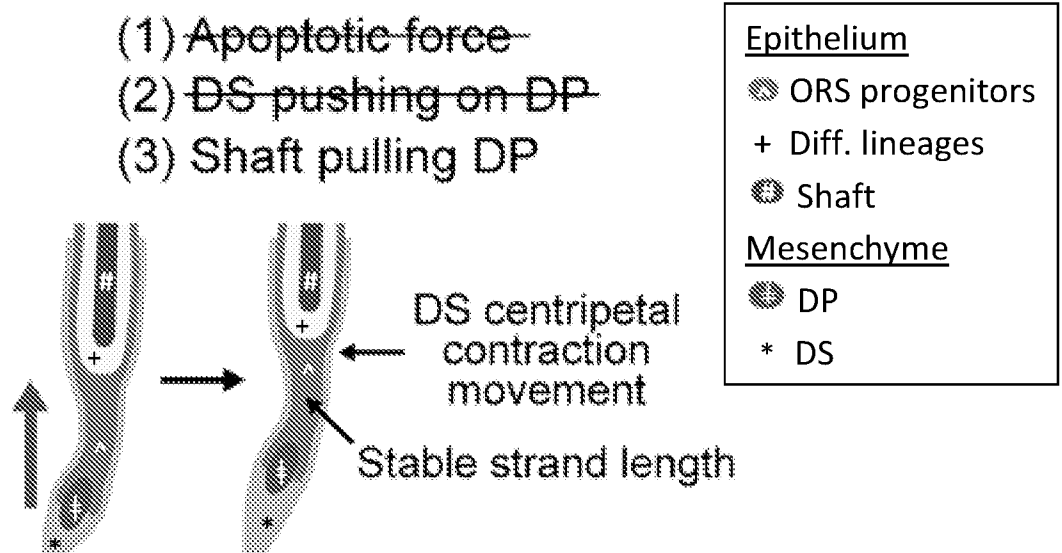


FIG. 4O

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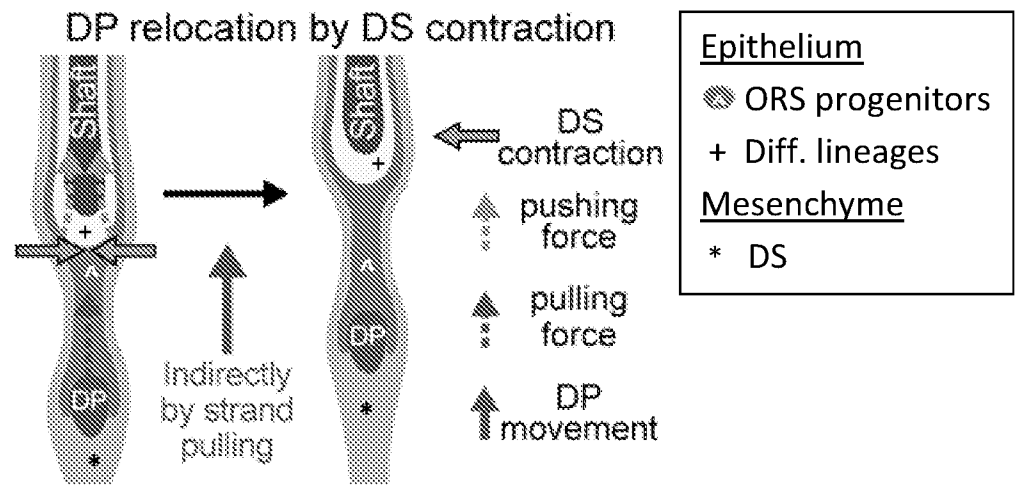


FIG. 4P

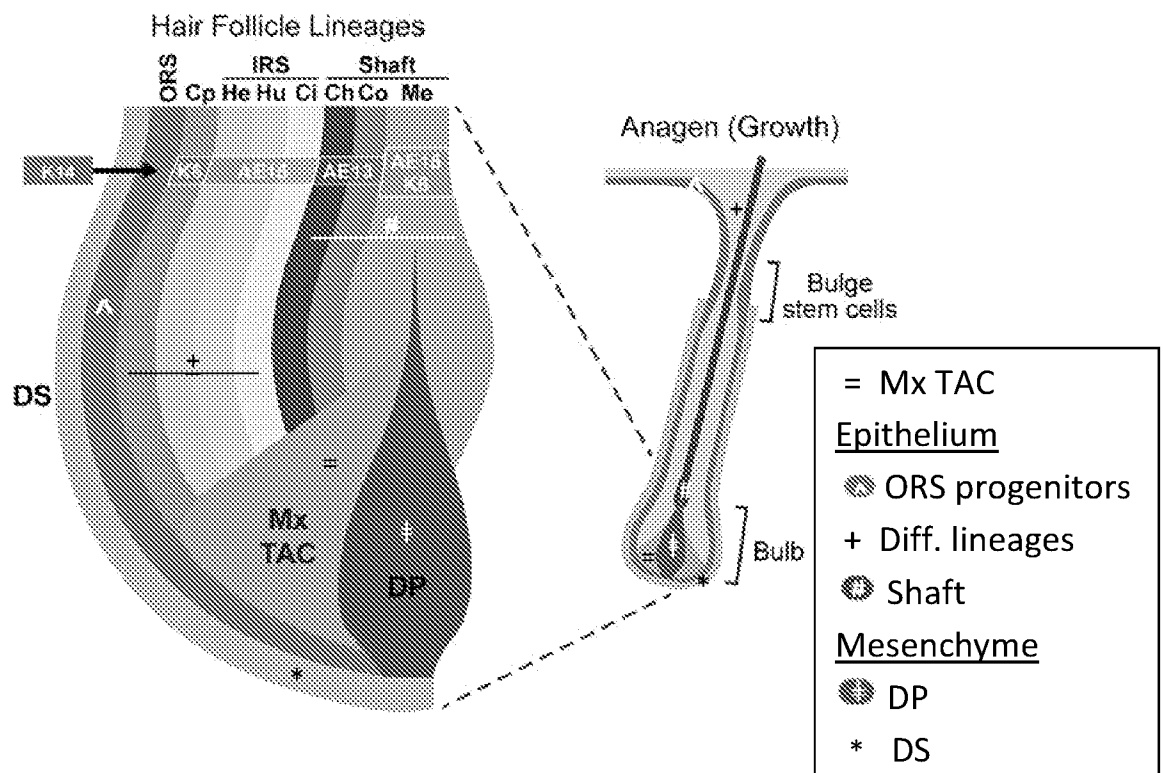


FIG. 5A

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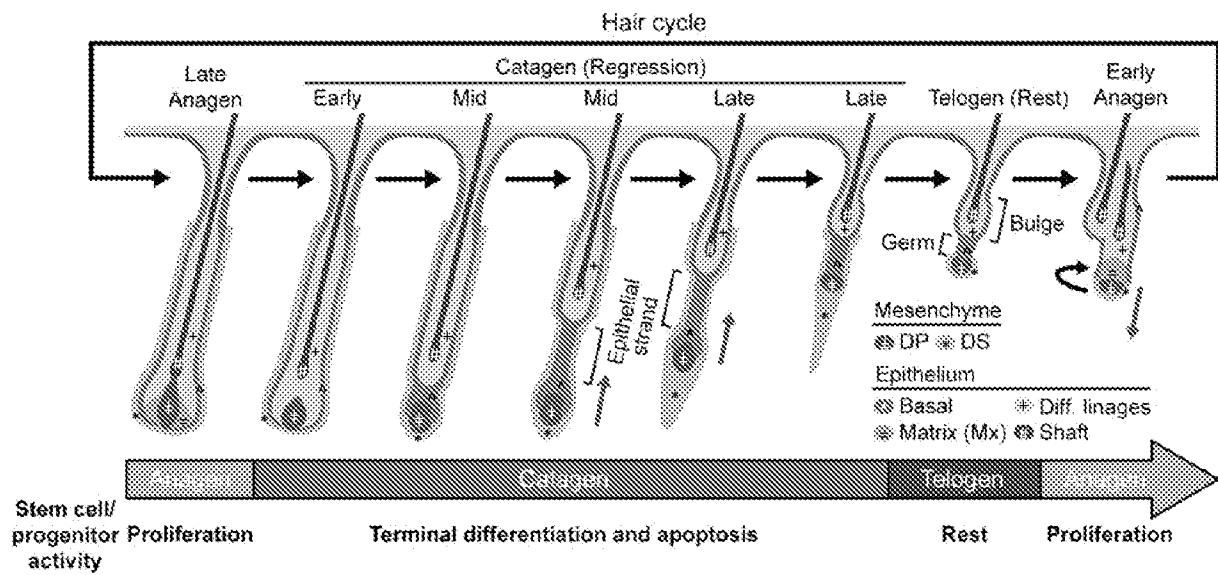


FIG. 5B

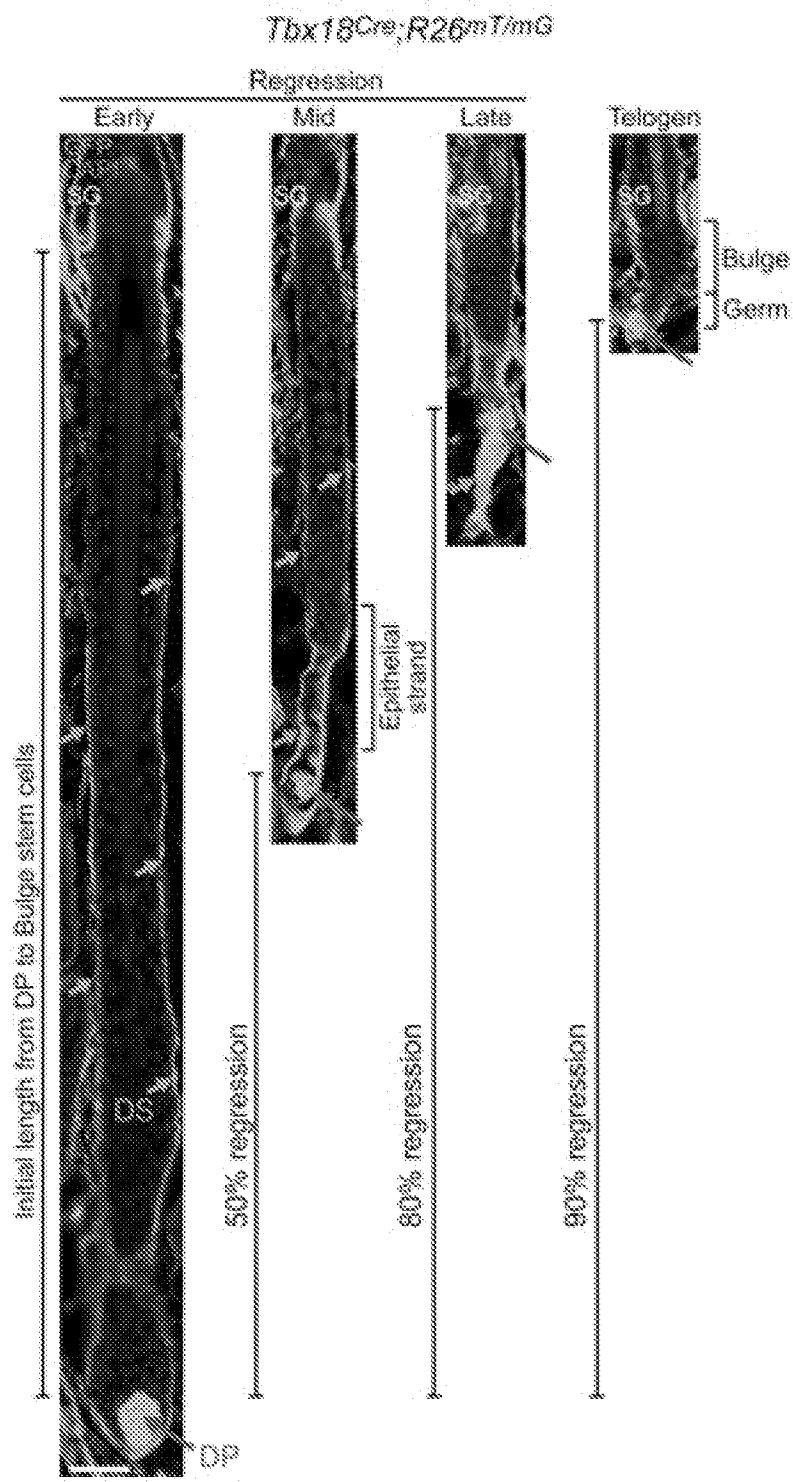


FIG. 6

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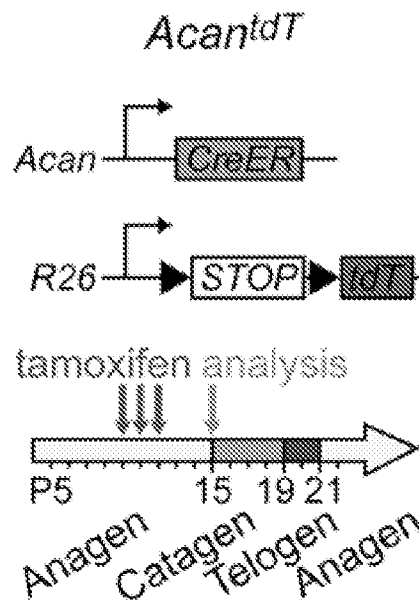


FIG. 7A

Targeting specificity

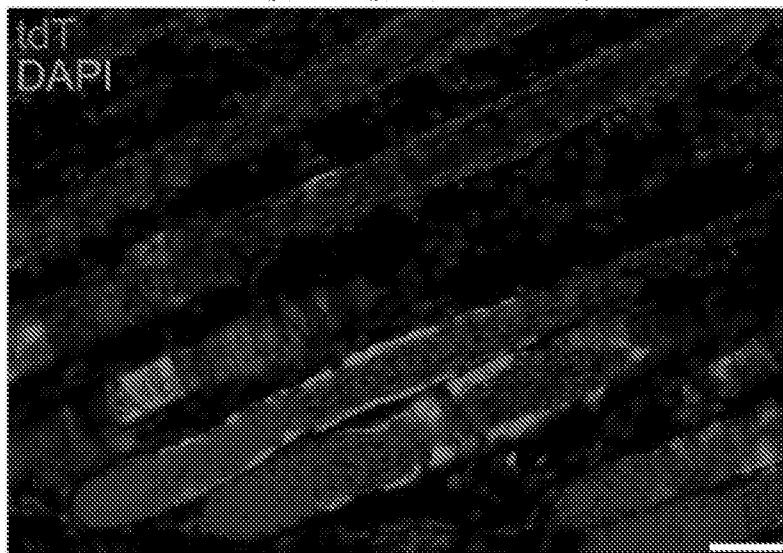


FIG. 7B

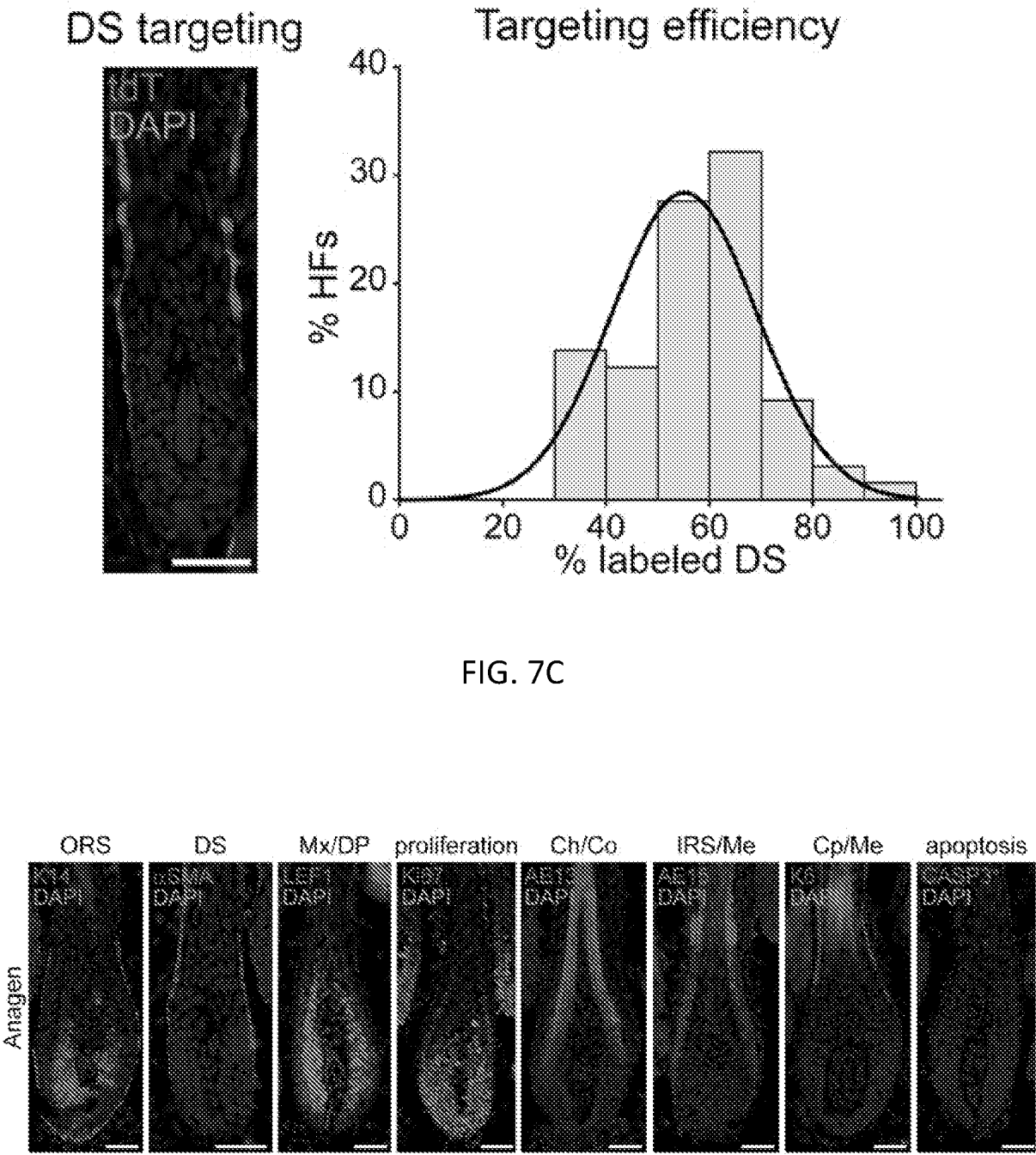


FIG. 8A

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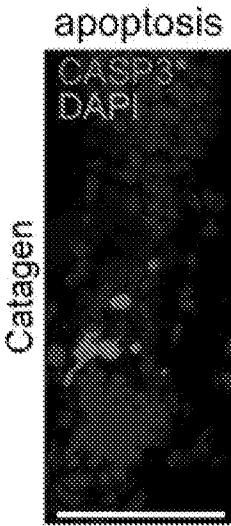


FIG. 8B

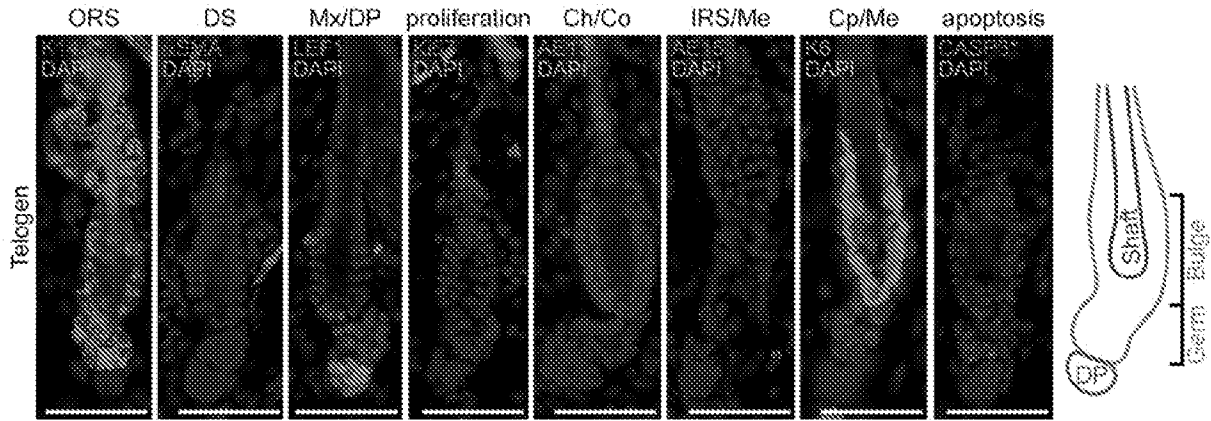


FIG. 8C

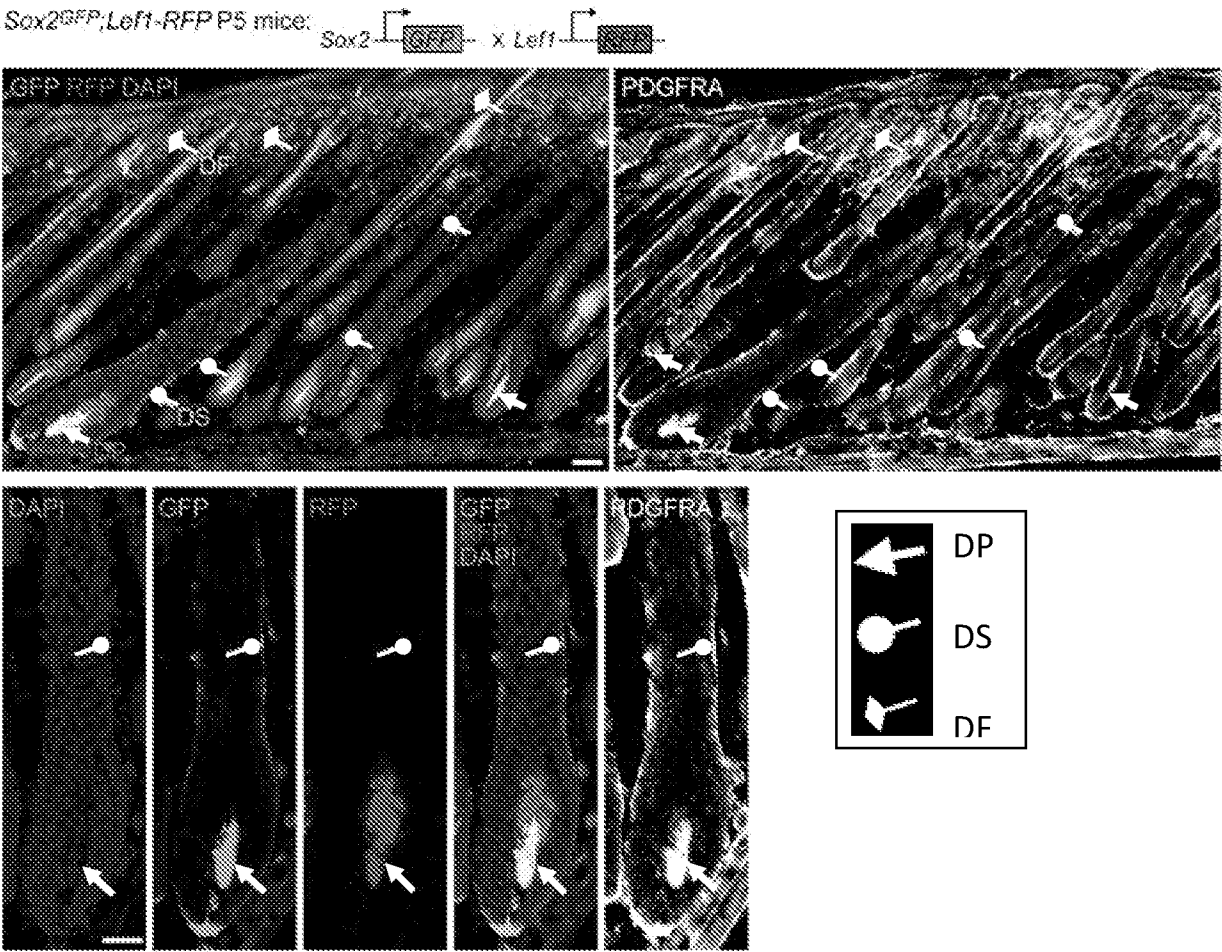


FIG. 9

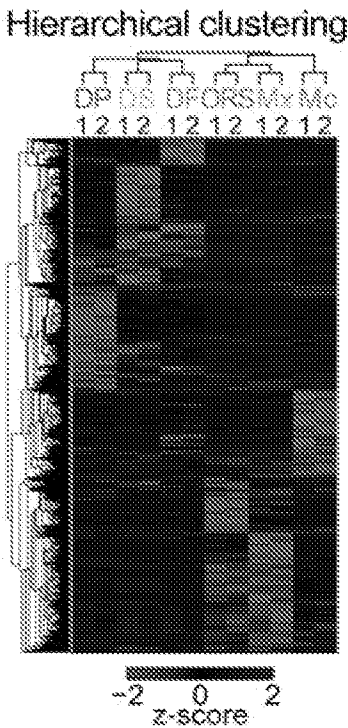


FIG. 10A

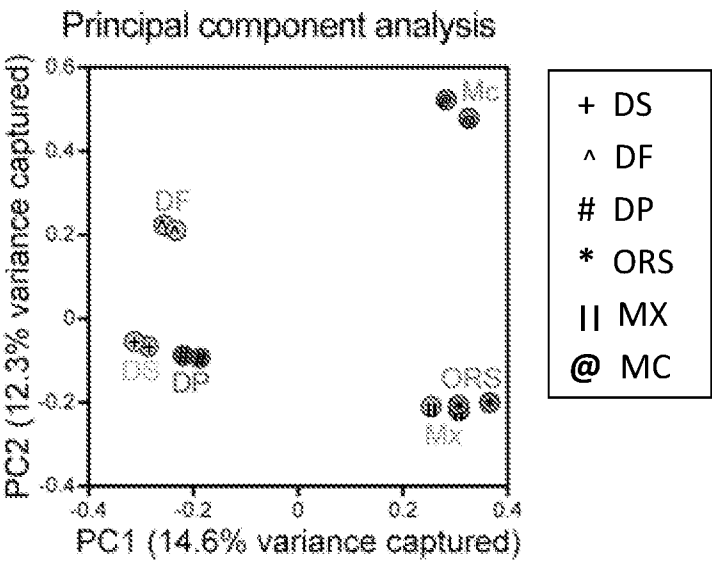


FIG. 10B

Mesenchyme population gene signature overlaps

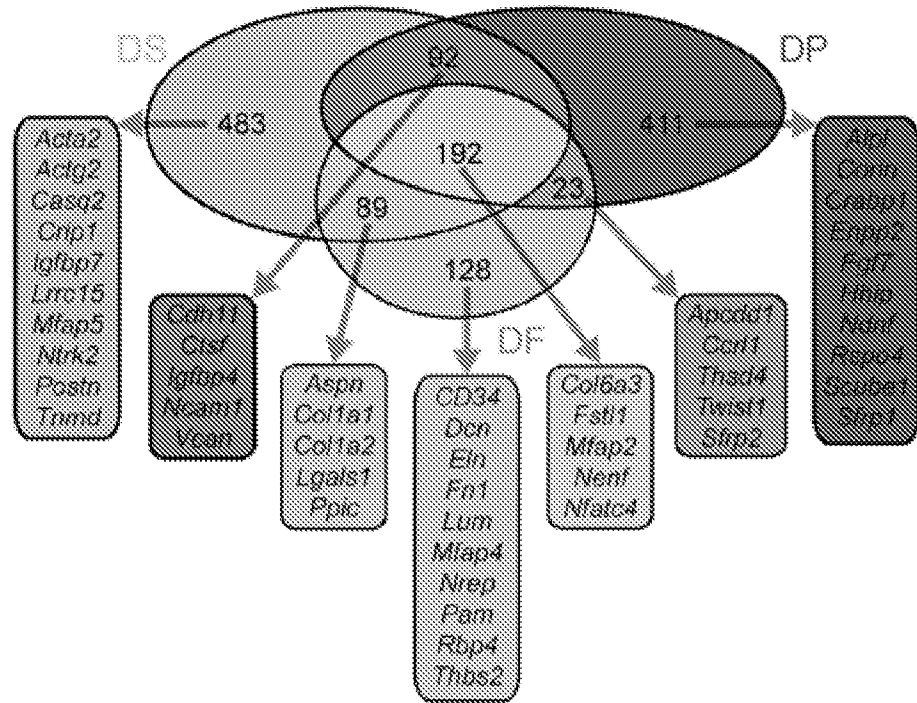


FIG. 10C

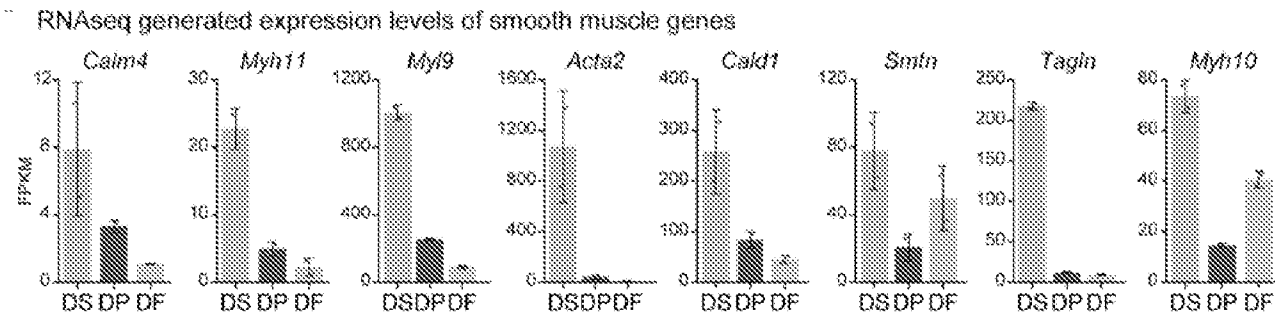


FIG. 10D

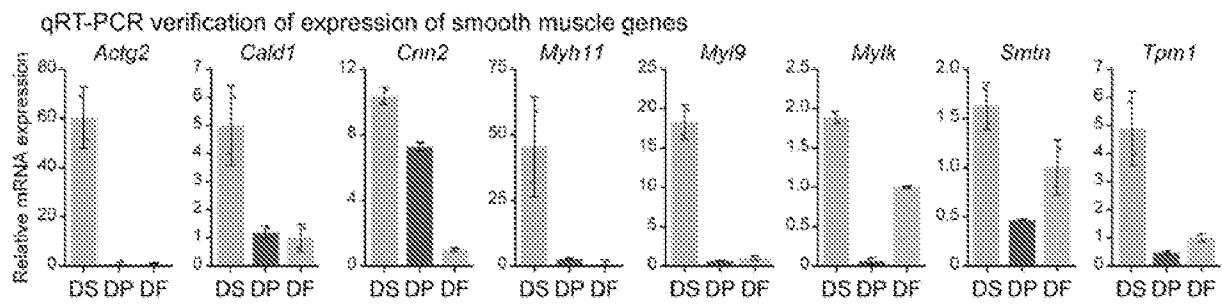


FIG. 10E

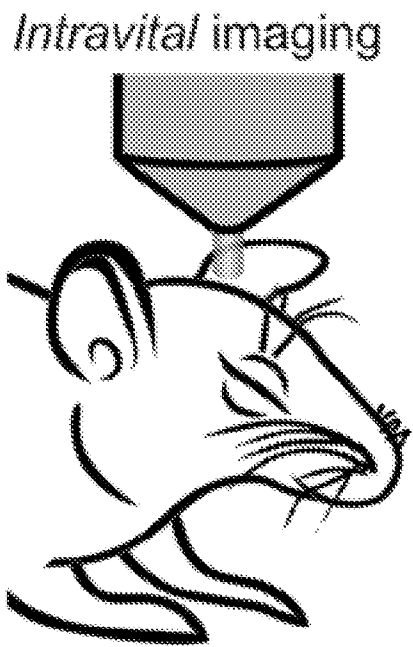


FIG. 11A

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Post-processing of triple-color fluorescence *intravital* 2-photon
imaging with single wavelength excitation:
Acan^{tdT};Tbx18^{H2BGFP};K14-H2BCer

		Channel			
		Blue	Green	Red	
Fluorophore	K14-H2BCer	Strong	Strong	Weak	Signal strength
	Tbx18 ^{H2BGFP}	None	Strong	None	
	Acan ^{tdT}	None	None	Strong	

FIG. 11B

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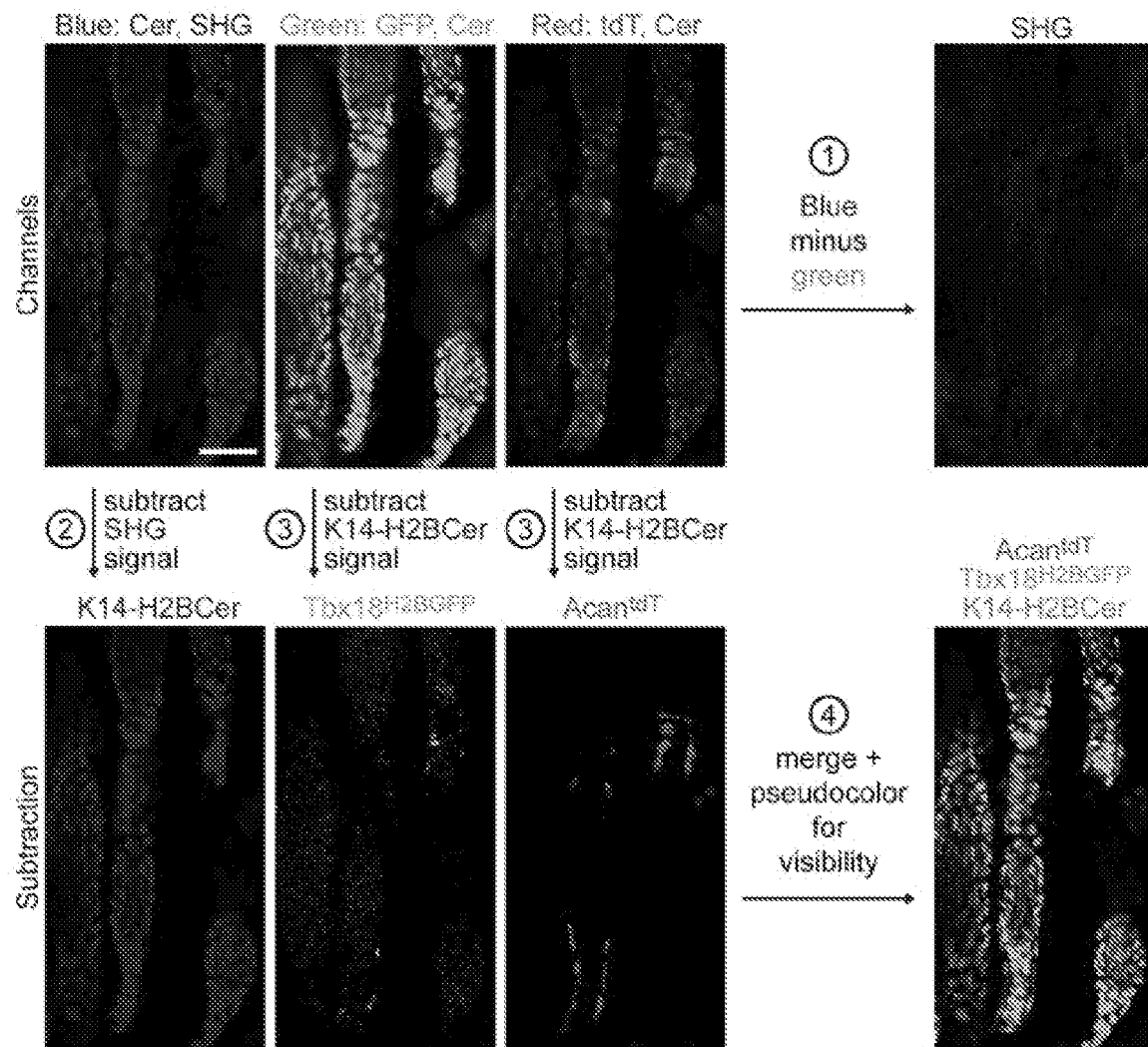


FIG. 11C

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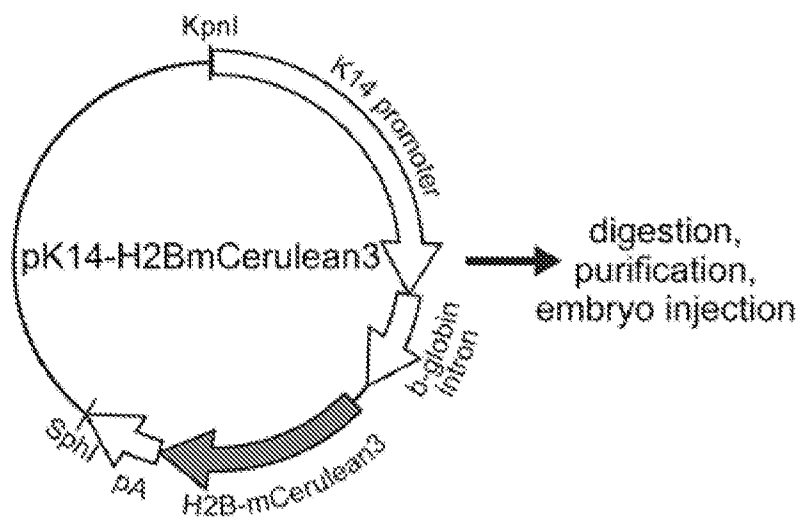


FIG. 12A

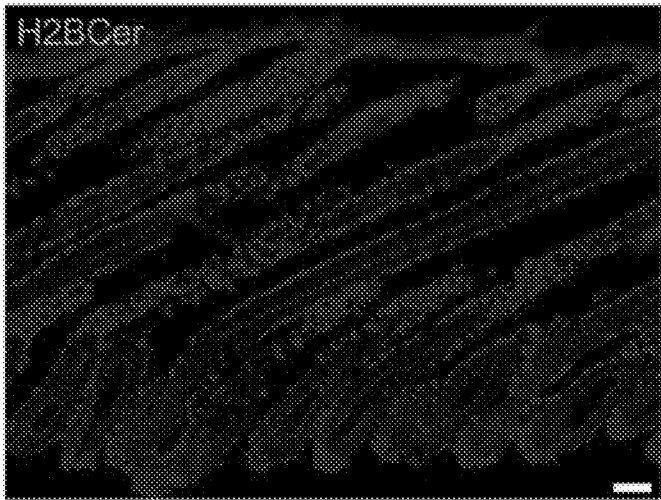


FIG. 12B

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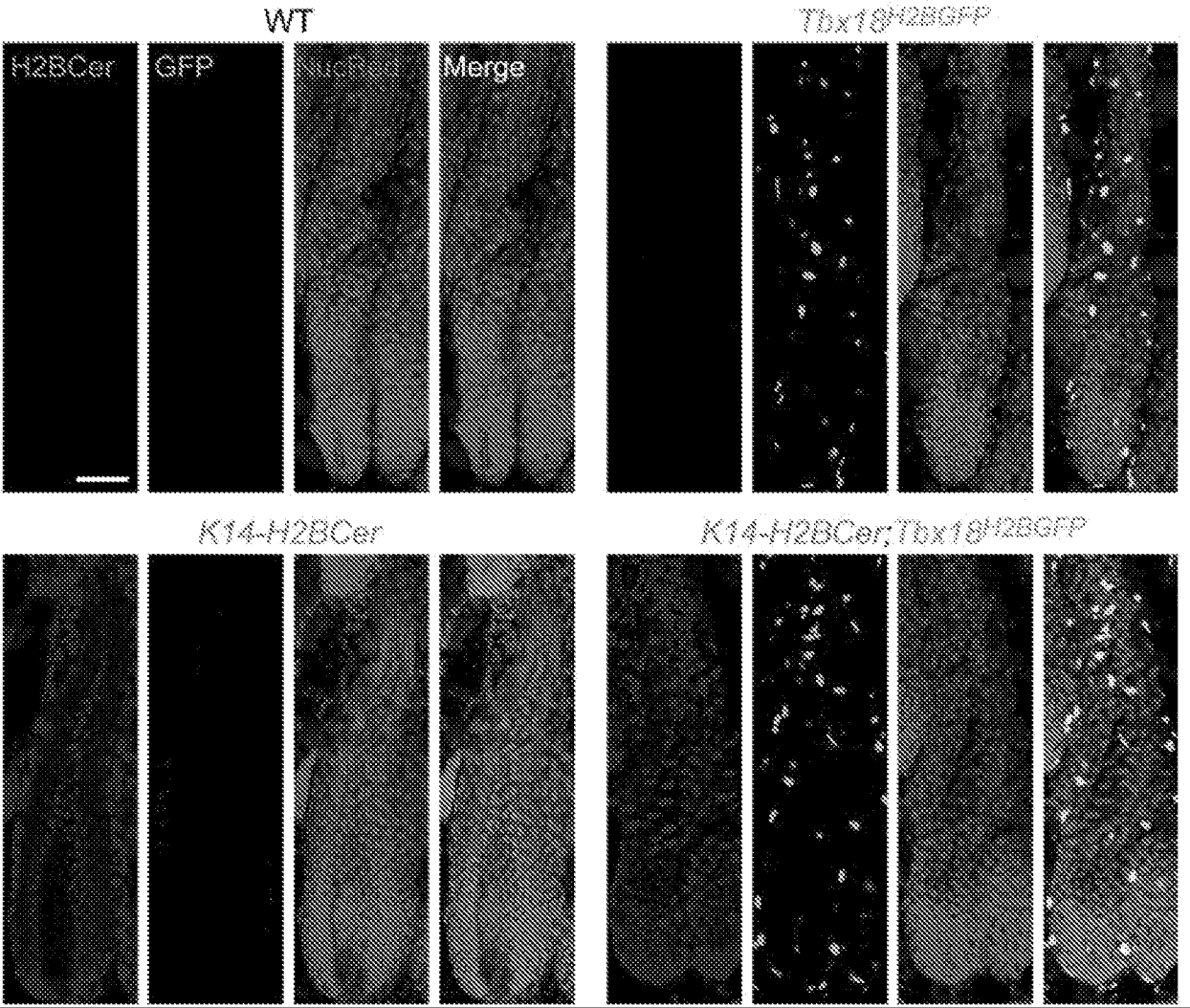


FIG. 12C

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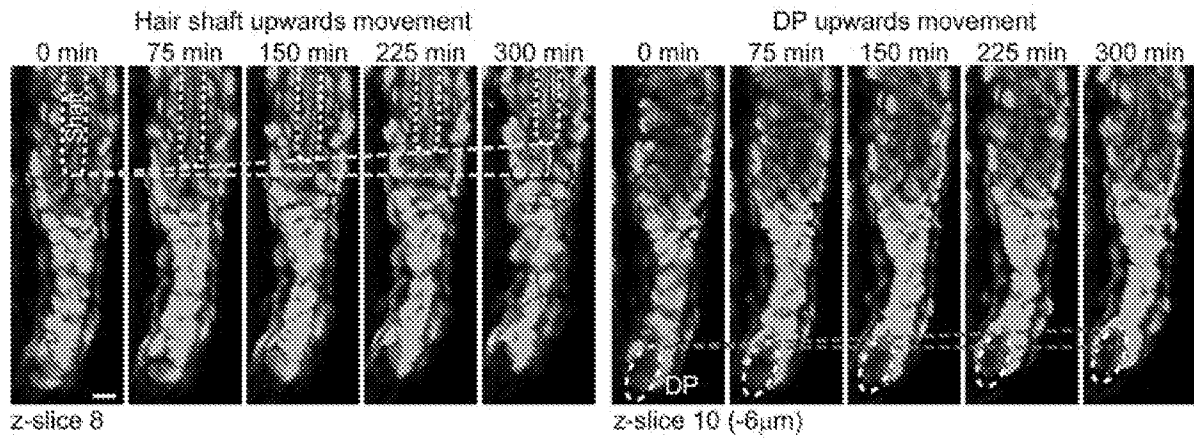


FIG. 13A

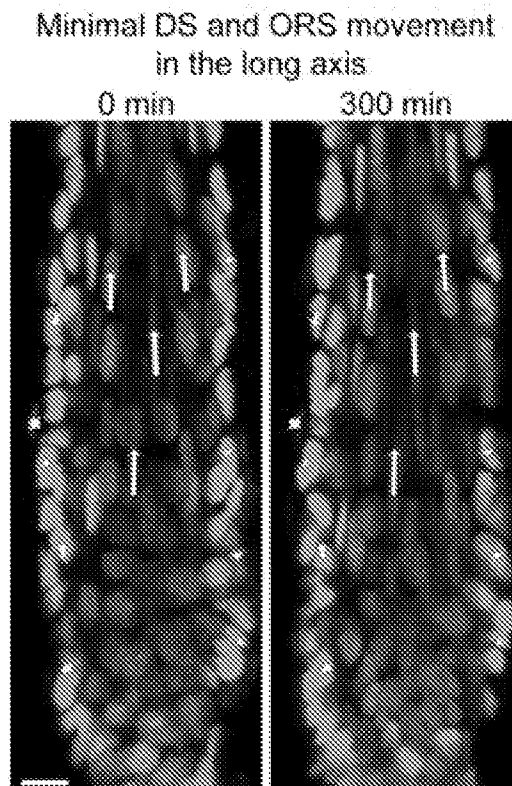


FIG. 13B

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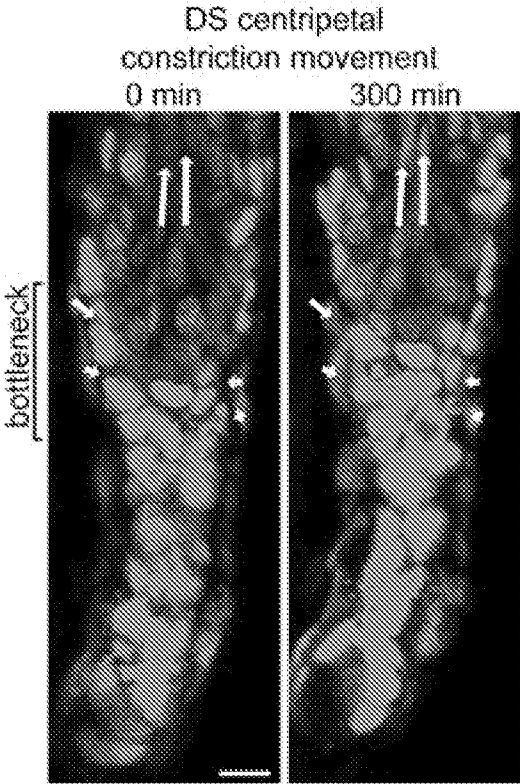


FIG. 13C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/40715

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 8/49; A61Q 7/00 (2020.01)

CPC - A61K 31/352; A61K 36/72; A61K 8/498

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- A	US 2012/0329780 A1 (Thormann et al) 27 December 2012 (27.12.2012), entire document esp abstract, claim 1, claim 18, para [0091]	1-9, 35, and (38-40, 41/40)/35 ----- 10
A	US 2008/0081078 A1 (Lanerolle) 3 April 2008 (03.04.2008), entire document esp abstract, para [0057]	1-10
A	WO 2011/039175 A1 (Melnik) 7 April 2011 (07.04.2011), entire document	1-10
A	US 2010/0098732 A1 (Tippens) 22 April 2010 (22.04.2010), entire document	1-10
A	WO 2010/068692 A1 (Purdue Research Foundation) 17 June 2010 (17.06.2010), entire document	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

4 September 2020

Date of mailing of the international search report

24 NOV 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/40715

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 42
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-10, 35, and (38-40, 41/40)/35, directed to a method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle.

Group II: Claims 11-19, 37, (38-40, 41/40, and 42)/37, and 43-44, directed to a method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the smooth muscle cell-like contractions of the dermal sheath cells of the hair follicles.

*****See supplemental box below*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10, 35, and (38-40, 41/40)/35

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 20/40715

Box III Lack of Unity

Group III: Claims 20-34, 36, and (38-40, 41/40, and 42)/36 directed to a method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that arrests or inhibits the hair cycle in catagen phase.

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I requires the technical feature of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle, not required by Groups II-III.

Group II requires the technical feature of a pharmacological agent that inhibits the smooth muscle cell-like contractions of the dermal sheath cells of the hair follicles, not required by Groups I or III.

Group III requires the technical feature of a method of a pharmacological agent that arrests or inhibits the hair cycle in catagen phase, not required by Groups I or II.

Common technical features:

Groups I-III share the technical feature of a method of treating hair loss comprising administering a therapeutically effective amount of an inhibitor

These shared technical features, however, do not provide a contribution over the prior art, as being anticipated by US 2012/0329780 A1 to Thormann et al (hereinafter Thormann).

Thormann discloses a method of treating hair loss (para [0091] .. Preferably the compounds of the present invention may be used for the treatment and/or prevention of the following conditions.. alopecia greata, male-pattern baldness,.. see instant specification pg 16 In 23-26 ..As used herein, a subject refers to a human exhibiting hair loss, e.g., male pattern 25 baldness (alopecia)..). comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle (abstract .. The present invention relates to novel compounds of formula (I).. that are capable of inhibiting one or more kinases, especially SYK (Spleen Tyrosine Kinase), LRRK2 (Leucine-rich repeat kinase 2) and/or MYLK (Myosin light chain kinase).. claim 18 .. a method of treatment of a disease mediated by kinase-activity comprising the administration, to a patient in need thereof, of a therapeutically effective amount of a compound according to claim 1.. see instant claim 2, inhibitors of MLCK are suitable agents), thereby treating hair loss (para [0091] .. Preferably the compounds of the present invention may be used for the treatment and/or prevention of the following conditions.. alopecia greata, male-pattern baldness,.. see instant specification pg 16 In 23-26 ..As used herein, a subject refers to a human exhibiting hair loss, e.g., male pattern 25 baldness (alopecia)..).

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I and II share the technical feature of a method of treating hair loss comprising administering a therapeutically effective amount of a pharmacological agent that inhibits in a dermal sheath cell of a hair follicle, thereby treating hair loss

These shared technical features, however, do not provide a contribution over the prior art, as being obvious over Thormann.

Thormann discloses a method of treating hair loss (para [0091] .. Preferably the compounds of the present invention may be used for the treatment and/or prevention of the following conditions.. alopecia greata, male-pattern baldness,.. see instant specification pg 16 In 23-26 ..As used herein, a subject refers to a human exhibiting hair loss, e.g., male pattern 25 baldness (alopecia)..). comprising administering a therapeutically effective amount of a pharmacological agent that inhibits the myosin light chain kinase (MLCK) pathway in a dermal sheath cell of a hair follicle (abstract .. The present invention relates to novel compounds of formula (I).. that are capable of inhibiting one or more kinases, especially SYK (Spleen Tyrosine Kinase), LRRK2 (Leucine-rich repeat kinase 2) and/or MYLK (Myosin light chain kinase).. claim 18 .. a method of treatment of a disease mediated by kinase-activity comprising the administration, to a patient in need thereof, of a therapeutically effective amount of a compound according to claim 1.. see instant claim 2, inhibitors of MLCK are suitable agents), thereby treating hair loss (para [0091] .. Preferably the compounds of the present invention may be used for the treatment and/or prevention of the following conditions.. alopecia greata, male-pattern baldness,.. see instant specification pg 16 In 23-26 ..As used herein, a subject refers to a human exhibiting hair loss, e.g., male pattern 25 baldness (alopecia)..). Thormann does not specifically disclose an embodiment wherein an MLCK inhibitor is used to treat hair loss. It would have been obvious to one skilled in the art to test this specific embodiment using routine experimentation.

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups. Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.