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(54) Title: METHODS USING WNT AND SHH AGONISTS TO STIMULATE HAIR FOLLICLE GROWTH

(57) Abstract: It was discovered that proliferative *Dkk1*+ progenitors transiently amplify to become quiescent dermal condensate cells by the spatiotemporal patterning of Wnt/ β -catenin and sonic hedgehog ("SHH") signaling gradients. Together, they deterministically coordinate a rapid transition from proliferation to quiescence, cell fate specification, and morphogenesis. This is useful for screening to discover compounds that can be administered to hair follicles to stimulate hair growth (new hair follicles) and restore hair follicle size. These agonists of Wnt and SHH are formulated for application to skin to grow hair.



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METHODS USING WNT AND SHH AGONISTS TO STIMULATE HAIR FOLLICLE GROWTH**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of and priority to U.S.S.N. 63/513,764 filed July 14, 2023, which is hereby incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant No. R01AR076420 awarded by NIH/NIAMS. The government has certain rights in the invention.

FIELD OF THE INVENTION

The disclosed invention is generally in the field of treatments for hair growth through proliferation and restoration of hair follicles *in situ*.

BACKGROUND OF THE INVENTION

Several conditions affect the health of hair follicles. The most common conditions include alopecia areata, baldness in women and people assigned female and male at birth, folliculitis, hidradenitis suppurativa, stress, telogen effluvium.

Most therapies to date have consisted of transplantation to create more hair follicles, treatment for infection or inflammation, and hormonal supplementation.

These efforts have met with limited to no success. It has not been understood why hair follicles do not proliferate, nor why they shrink in size over time, much less how one can stop or reverse this process.

It is therefore an object of the present invention to provide methods and systems to screen for compounds that will promote health and regeneration of hair follicles.

It is a further object of the present invention to provide compounds that are useful to promote the health and regeneration of hair follicles.

It is still further object of the present invention to provide formulations that are useful for treatment of hair follicles.

BRIEF SUMMARY OF THE INVENTION

Innovative technology was used to examine how key stem cell molecular signals can recruit cells to undergo collective growth during normal tissue regeneration such as hair growth. Understanding how stem cells are regulated to promote tissue regeneration is key to developing targeted therapies to treat human diseases that lead to either tissue damage or uncontrolled growth in cancer.

By combining scRNA-sequencing with genetic perturbation, it was discovered that proliferative *Dkk1*+ progenitors transiently amplify to become quiescent dermal condensate cells by the spatiotemporal patterning of Wnt/ β -catenin and sonic hedgehog ("SHH") signaling gradients. Together, they deterministically coordinate a rapid transition from proliferation to quiescence, cell fate specification, and morphogenesis.

This discovery and the model based on it are useful for screening to discover compounds that can be administered to hair follicles to stimulate hair growth (new hair follicles) and restore hair follicle size. These agonists of Wnt and SHH are formulated for application to skin to grow hair. The timing of application, as well as the formulation, are optimized for the site of application. In most cases, the compounds are applied topically (gel, spray, shampoo, foam, solution, transdermal patch) and incorporates at least one means for sustained or pulsed controlled delivery of the agent. In a preferred embodiment, the topical formulation includes an inert solvent, a surfactant, a viscosity modifying agent, a transdermal penetration enhancer, and soluble Wnt agonist (such as CHIR-99021) and/or SHH agonist (such as SAG dihydrochloride) either alone (CHIR 5 or 10 micromolar (μ M), SAG 50, 100 or 200 nM) or together in the following combinations of these exemplary concentrations: CHIR 5 μ M + either SAG 50, 100 or 200 nM and CHIR 10 μ M + either SAG 50, 100, or 200 nM.

This is administered in a method for enhancing hair follicle growth. The Wnt agonist is applied first, then the SHH agonist is administered as the levels of the Wnt agonist decrease. This is achieved by either applying the agonists separately or, more preferably, formulated so that the Wnt agonist is released immediately and the SHH agonist is released thereafter, either as a delayed release formulation, or as a slow release which is timed for release of the SHH agonist as the levels of the Wnt agonist is decreasing. These concentrations of CHIR (5 μ M + SAG 50 nM. combined low dose initially) can be administered topically or intradermally daily for 2-5 days to an animal such as a mouse for screening or to human skin for treatment, followed by incrementally higher dose of SAG: CHIR 10 μ M +SAG (100-200 nM) based on gene responses from *in vitro* culture experiments daily for 2-5 days (without washout period).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A-1C are graphs showing induction of high levels of SHH signaling across the entire gradient of Wnt signaling prior to morphogenesis, and not just those in the peri-DC, cause every cell within that gradient to augment Wnt signaling incrementally while increasing their proliferation and expression of DC genes, resulting in more transitioning

intermediates proliferating until they reach a level of Wnt signaling sufficient to cause arrest. Fig. 1A and 1B are graphs of the effect of SHH level in combination with Wnt level showing the change from no dermal condensate ("DC") to proliferation intermediates (transition level) to arrest. Fig. 1C shows this process graphically.

5 Fig. 2A-2E are schematics of epidermis with quiescent CDs in the dermis (2A); epithelial placode with DC (2B); proliferation of DC (2C); further invagination of the epidermis as it forms a hair follicle at the site of DC (2D), and formation of the hair follicle in the epidermis (2E).

Fig. 3A-3M show that dermal SHH activation is required for the rapid transition to quiescence and mature DC differentiation. Lef1 levels in Bmp4+ DCs at E14.5 and E15.5 (FIG. 3A). FISH showing decreased Lef1 expression in Bmp4+ SHH cKO pseudo-DCs (FIG. 3B). Proliferation rate of Dkk1+ peri-DC and upper dermal cells at E14.5 (FIG. 3C). Pseudo-order of control and SHH cKO dermal cells at E14.5 (FIG. 3D). FISH showing proliferative Bmp4+ pseudo-DCs in SHH cKO at E14.5 and E15.5 (FIG. 3E). Number of Bmp4+ cells in SHH cKO and control over time (FIG. 3F). %EdU+ of Bmp4+ population over time in control and SHH cKO (FIG. 3G). Diffusion maps of E14.5 and E15.5 control and SHH cKO dermal cells showing that SHH cKO pseudo-DCs progress only on the Wnt component by E15.5 (FIG. 3H-3K). Cartoons showing aberrant proliferation of SHH cKO Dkk1+ peri-DC cells and slow transition to quiescence (FIG. 3L, 3M). Data as mean± SEM; 10 15 20 *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, one-way ANOVA. Scale bars, 50 µm.

Fig. 4A-4H show that high dermal SHH activation in early Wnt-active cells deterministically reproduces events of DC genesis over more intermediates. E14.5 control and SmoM2YFP whole mount showing proliferative mutant Sox2+ clusters (FIG. 4A). Number of Sox2+ cells per dermal cluster in control and SmoM2YFP at E14.5 and E15.5 (FIG. 4B). Diffusion maps of E14.5 control and SmoM2YFP cells (FIG. 4C, 4D). Pseudo-order with Regions 1 and 2 demarcated (FIG. 4E, 4F). FISH of E14.5 control and mutant showing virtually all Sox2+ cells co-express Ptch1 and Lef1 (FIG. 4G, 4H). 25

Fig. 5A-5N show that high dermal SHH activation in early Wnt-active cells deterministically reproduces events of DC genesis over more intermediates. E14.5 control and SmoM2YFP whole mount showing proliferative mutant Sox2+ clusters (FIG. 5A). Number of Sox2+ cells per dermal cluster in control and SmoM2YFP at E14.5 and E15.5 (FIG. 5B). Diffusion maps of E14.5 control and SmoM2YFP cells (FIG. 5C). Pseudo-order with regions 1 and 2 demarcated (FIG. 5D). FISH of E14.5 control and mutant showing 30

virtually all Sox2⁺ cells co-express Ptch1 and Lef1 (FIG. 5E). Pseudo-order of indicated genes in mutant and control (FIG. 5F). E14.5 FISH showing EdU, Sox2, and Dkk1 (FIG. 5G). FISH at E15.5 after EdU pulse in control and SmoM2YFP skin (FIG. 5H). FISH 24 hours after EdU chase (FIG. 5I). %EdU⁺ cells by population in E14.5 mutant and control (FIG. 5J). %EdU⁺ of Sox2⁺ cells at E15.5 after EdU pulse or 24-hour chase (FIG. 5K). E14.5 SmoM2; β catf1/EX3 FISH showing Sox2⁺ clusters in upper and lower dermis that co-express Ptch1 and Lef1 (FIG. 5L). Sox2⁺ clusters with %EdU⁺ of indicated populations by condition at E14.5 (FIG. 5M). Depiction of transition rate and number of intermediates affected by modulating SHH and Wnt signaling (FIG. 5N). FIG. 5O is a graph of % EdU⁺ for UD, Dkk1+Sox2⁻ compared to Sox2⁺. FIG. 5P is a diagram of the histology in cross-section of wild type, SmoM2 and SmoM2 β^{Ex3} showing proliferating Dkk1⁺, penultimate dividing Dkk1⁺ and quiescent Dkk1⁺.

Fig. 6A-6B are graphs of %DC (6A), the ratio of periDC/Perfollicular (6B) and the progression of DC differentiation from proliferative periDC (Dkk1⁺) to quiescent corner (Dkk1⁺) to DC (Dkk1⁻) (Fig. 6C).

FIG. 7A and 7B are graphs showing dermal cells pre-treated with Wnt agonist, CHIR, significantly enhanced SHH and DC gene responses compared to without Wnt agonist pretreatment (compare blue no pretreatment to red with Wnt pretreatment).

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

A hair follicle is a tube-like structure (pore) that surrounds the root and strand of a hair. Hair follicles exist in the top two layers of the skin. People are born with over 5 million hair follicles in their body and over one million hair follicles on their head. As one ages, hair grows out of your hair follicles. The hair follicle is one of a few structures in the body that can stop functioning and begin functioning again (degenerate and regenerate).

Hair grows in cycles within the hair follicle:

Anagen: The first phase of hair growth takes between two to seven years. Growth begins at the root (dermal papilla) in your hair follicle, which gives the hair blood supply and the nutrients it needs to grow. The hair grows about 1 centimeter per month.

Catagen: The second phase of hair growth occurs when the hair transitions from a growing phase to a resting phase, which takes about two weeks. During this phase, the hair detaches from your blood supply.

Telogen: The final phase of hair growth is the inactive phase, where your hair sheds or falls out of your hair follicle. This phase takes up to four months.

Hair follicles originate in the first and second layers of your skin (epidermis and dermis). Follicles holding your terminal hair, or the hair that grows on your scalp, eyelashes and eyebrows, extend into the first and second layer of your skin and sometimes into the third layer (subcutaneous tissue).

The hair follicle dermal condensate is the precursor to the permanent mesenchymal unit of the hair follicle, the dermal papilla, which regulates hair cycling throughout life and bears hair inductive potential. Dermal condensate morphogenesis depends on epithelial

Fibroblast Growth Factor 20.

Sonic Hedgehog Protein, also referred to as SHH, HHG1, HLP3, HPE3, MCOPCB5, SMMCI, TPT, TPTPS, sonic hedgehog, Sonic hedgehog, SHHNC, and sonic hedgehog signaling molecule. This signaling molecule is key in regulating embryonic morphogenesis in all animals. SHH controls organogenesis and the organization of the central nervous system, limbs, digits and many other parts of the body. Sonic hedgehog is a morphogen that patterns the developing embryo using a concentration gradient. model has a non-uniform distribution of SHH molecules which governs different cell fates according to concentration.

Sonic hedgehog still plays a role in differentiation, proliferation, and maintenance of adult tissues. Abnormal activation of SHH signaling in adult tissues has been implicated in various types of cancers including breast, skin, brain, liver, gallbladder and many more.

The Wnt signaling pathways are a group of signal transduction pathways which begin with proteins that pass signals into a cell through cell surface receptors. The name Wnt is a portmanteau created from the names Wingless and Int-1. Wnt signaling pathways use either nearby cell-cell communication (paracrine) or same-cell communication (autocrine). They are highly evolutionarily conserved in animals, which means they are similar across animal species from fruit flies to humans.

Three Wnt signaling pathways have been characterized: the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway. All three pathways are activated by the binding of a Wnt-protein ligand to a Frizzled family receptor, which passes the biological signal to the Dishevelled protein inside the cell. The canonical Wnt pathway leads to regulation of gene transcription, and is thought to be negatively regulated in part by the SPATS1 gene. The non-canonical planar cell polarity

pathway regulates the cytoskeleton that is responsible for the shape of the cell. The non-canonical Wnt/calcium pathway regulates calcium inside the cell.

Wnt signaling was first identified for its role in carcinogenesis, then for its function in embryonic development. The embryonic processes it controls include body axis patterning, cell fate specification, cell proliferation and cell migration. These processes are necessary for proper formation of important tissues including bone, heart and muscle. Its role in embryonic development was discovered when genetic mutations in Wnt pathway proteins produced abnormal fruit fly embryos. Later research found that the genes responsible for these abnormalities also influenced breast cancer development in mice. Wnt signaling also controls tissue regeneration in adult bone marrow, skin and intestine.

This pathway's clinical importance was demonstrated by mutations that lead to various diseases, including breast and prostate cancer, glioblastoma, type II diabetes and others. In recent years, researchers reported first successful use of Wnt pathway inhibitors in mouse models of disease. Wnt comprises a diverse family of secreted lipid-modified signaling glycoproteins that are 350–400 amino acids in length. The lipid modification of all Wnts is palmitoleoylation of a single totally conserved serine residue. Palmitoleoylation is necessary because it is required for Wnt to bind to its carrier protein Wntless (WLS) so it can be transported to the plasma membrane for secretion and it allows the Wnt protein to bind its receptor Frizzled. Wnt proteins also undergo glycosylation, which attaches a carbohydrate in order to ensure proper secretion. In Wnt signaling, these proteins act as ligands to activate the different Wnt pathways via paracrine and autocrine routes.

These proteins are highly conserved across species. Wnt signaling begins when a Wnt protein binds to the N-terminal extra-cellular cysteine-rich domain of a Frizzled (Fz) family receptor. These receptors span the plasma membrane seven times and constitute a distinct family of G-protein coupled receptors (GPCRs). However, to facilitate Wnt signaling, co-receptors may be required alongside the interaction between the Wnt protein and Fz receptor. Examples include lipoprotein receptor-related protein (LRP)-5/6, receptor tyrosine kinase (RTK), and ROR2. Upon activation of the receptor, a signal is sent to the phosphoprotein Dishevelled (Dsh), which is located in the cytoplasm. This signal is transmitted via a direct interaction between Fz and Dsh. Dsh proteins are present in all organisms and they all share the following highly conserved protein domains: an amino-terminal DIX domain, a central PDZ domain, and a carboxy-terminal DEP domain. These different domains are important because after Dsh, the Wnt signal can branch off into

multiple pathways and each pathway interacts with a different combination of the three domains.

Canonical and non-canonical pathways

The three best characterized Wnt signaling pathways are the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway. As their names suggest, these pathways belong to one of two categories: canonical or non-canonical. The difference between the categories is that a canonical pathway involves the protein beta-catenin (β -catenin) while a non-canonical pathway operates independently of it.

The canonical Wnt pathway (or Wnt/ β -catenin pathway) is the Wnt pathway that causes an accumulation of β -catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional coactivator of transcription factors that belong to the TCF/LEF family. Without Wnt, β -catenin would not accumulate in the cytoplasm since a destruction complex would normally degrade it. This destruction complex includes the following proteins: Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1 α (CK1 α). It degrades β -catenin by targeting it for ubiquitination, which subsequently sends it to the proteasome to be digested. However, as soon as Wnt binds Fz and LRP5/6, the destruction complex function becomes disrupted. This is due to Wnt causing the translocation of the negative Wnt regulator, Axin, and the destruction complex to the plasma membrane. Phosphorylation by other proteins in the destruction complex subsequently binds Axin to the cytoplasmic tail of LRP5/6. Axin becomes de-phosphorylated and its stability and levels decrease. Dsh then becomes activated via phosphorylation and its DIX and PDZ domains inhibit the GSK3 activity of the destruction complex. This allows β -catenin to accumulate and localize to the nucleus and subsequently induce a cellular response via gene transduction alongside the TCF/LEF (T-cell factor/lymphoid enhancing factor) transcription factors. β -catenin recruits other transcriptional coactivators, such as BCL9, Pygopus and Parafibromin/Hyrax. The complexity of the transcriptional complex assembled by β -catenin is beginning to emerge thanks to new high-throughput proteomics studies. However, tissue-specific players might assist β -catenin to define its target genes. The extensivity of the β -catenin interacting proteins complicates the understanding: β -catenin may be directly phosphorylated at Ser552 by Akt, which causes its disassociation from cell-cell contacts and accumulation in cytosol, thereafter 14-3-3 ζ interacts with β -catenin (pSer552) and enhances its nuclear translocation. BCL9 and

Pygopus have been reported, in fact, to possess several β -catenin-independent functions (therefore, likely, Wnt signaling-independent).

The non-canonical planar cell polarity (PCP) pathway does not involve β -catenin. It does not use LRP-5/6 as its co-receptor and is thought to use NRH1, Ryk, PTK7 or ROR2.

- 5 The PCP pathway is activated via the binding of Wnt to Fz and its co-receptor. The receptor then recruits Dsh, which uses its PDZ and DIX domains to form a complex with Dishevelled-associated activator of morphogenesis 1 (DAAM1). Daam1 then activates the small G-protein Rho through a guanine exchange factor. Rho activates Rho-associated kinase (ROCK), which is one of the major regulators of the cytoskeleton. Dsh also forms a complex with rac1 and
- 10 mediates profilin binding to actin. Rac1 activates JNK and can also lead to actin polymerization. Profilin binding to actin can result in restructuring of the cytoskeleton and gastrulation.

Non-canonical Wnt/calcium pathway

- The non-canonical Wnt/calcium pathway also does not involve β -catenin. Its role is to
- 15 help regulate calcium release from the endoplasmic reticulum (ER) in order to control intracellular calcium levels. Like other Wnt pathways, upon ligand binding, the activated Fz receptor directly interacts with Dsh and activates specific Dsh-protein domains. The domains involved in Wnt/calcium signaling are the PDZ and DEP domains. However, unlike other Wnt pathways, the Fz receptor directly interfaces with a trimeric G-protein. This co-
- 20 stimulation of Dsh and the G-protein can lead to the activation of either PLC or cGMP-specific PDE. If PLC is activated, the plasma membrane component PIP2 is cleaved into DAG and IP3. When IP3 binds its receptor on the ER, calcium is released. Increased concentrations of calcium and DAG can activate Cdc42 through PKC. Cdc42 is an important regulator of ventral patterning. Increased calcium also activates calcineurin and CaMKII.
- 25 CaMKII induces activation of the transcription factor NFAT, which regulates cell adhesion, migration and tissue separation. Calcineurin activates TAK1 and NLK kinase, which can interfere with TCF/ β -Catenin signaling in the canonical Wnt pathway. However, if PDE is activated, calcium release from the ER is inhibited. PDE mediates this through the inhibition of PKG, which subsequently causes the inhibition of calcium release.

Integrated Wnt Pathway

The binary distinction of canonical and non-canonical Wnt signaling pathways has come under scrutiny and an integrated, convergent Wnt pathway has been proposed. Some evidence for this was found for one Wnt ligand (Wnt5A). Evidence for a convergent Wnt

signaling pathway that shows integrated activation of Wnt/Ca²⁺ and Wnt/ β -catenin signaling, for multiple Wnt ligands, was described in mammalian cell lines.

In order to ensure proper functioning, Wnt signaling is constantly regulated at several points along its signaling pathways. For example, Wnt proteins are palmitoylated. The protein porcupine mediates this process, which means that it helps regulate when the Wnt ligand is secreted by determining when it is fully formed. Secretion is further controlled with proteins such as GPR177 (wntless) and evenness interrupted and complexes such as the retromer complex.

Upon secretion, the ligand can be prevented from reaching its receptor through the binding of proteins such as the stabilizers Dally and glypican 3 (GPC3), which inhibit diffusion. In cancer cells, both the heparan sulfate chains and the core protein of GPC3 are involved in regulating Wnt binding and activation for cell proliferation. Wnt recognizes a heparan sulfate structure on GPC3, which contains IdoA2S and GlcNS6S, and the 3-O-sulfation in GlcNS6S3S enhances the binding of Wnt to the heparan sulfate glypican. A cysteine-rich domain at the N-lobe of GPC3 has been identified to form a Wnt-binding hydrophobic groove including phenylalanine-41 that interacts with Wnt. Blocking the Wnt binding domain using a nanobody called HN3 can inhibit Wnt activation.

At the Fz receptor, the binding of proteins other than Wnt can antagonize signaling. Specific antagonists include Dickkopf (Dkk), Wnt inhibitory factor 1 (WIF-1), secreted Frizzled-related proteins (SFRP), Cerberus, Frzb, Wise, SOST, and Naked cuticle. These constitute inhibitors of Wnt signaling. However, other molecules also act as activators. Norrin and R-Spondin2 activate Wnt signaling in the absence of Wnt ligand.

Interactions between Wnt signaling pathways also regulate Wnt signaling. As noted above, the Wnt/calcium pathway can inhibit TCF/ β -catenin, preventing canonical Wnt pathway signaling. Prostaglandin E2 is an essential activator of the canonical Wnt signaling pathway. Interaction of PGE2 with its receptors E2/E4 stabilizes β -catenin through cAMP/PKA mediated phosphorylation. The synthesis of PGE2 is necessary for Wnt signaling mediated processes such as tissue regeneration and control of stem cell population in zebrafish and mouse. Intriguingly, the unstructured regions of several oversized Intrinsically disordered proteins play crucial roles in regulating Wnt signaling.

Wnt signaling plays a critical role in embryonic development. It operates in both vertebrates and invertebrates, including humans, frogs, zebrafish, *C. elegans*, *Drosophila* and others. It was first found in the segment polarity of *Drosophila*, where it helps to establish

anterior and posterior polarities. It is implicated in other developmental processes. As its function in *Drosophila* suggests, it plays a key role in body axis formation, particularly the formation of the anteroposterior and dorsoventral axes. It is involved in the induction of cell differentiation to prompt formation of important organs such as lungs and ovaries. Wnt further ensures the development of these tissues through proper regulation of cell proliferation and migration. Wnt signaling functions can be divided into axis patterning, cell fate specification, cell proliferation and cell migration.

Cell fate specification

Cell fate specification or cell differentiation is a process where undifferentiated cells can become a more specialized cell type. Wnt signaling induces differentiation of pluripotent stem cells into mesoderm and endoderm progenitor cells. These progenitor cells further differentiate into cell types such as endothelial, cardiac and vascular smooth muscle lineages. Wnt signaling induces blood formation from stem cells. Specifically, Wnt3 leads to mesoderm committed cells with hematopoietic potential. Wnt1 antagonizes neural differentiation and is a major factor in self-renewal of neural stem cells. This allows for regeneration of nervous system cells, which is further evidence of a role in promoting neural stem cell proliferation. Wnt signaling is involved in germ cell determination, gut tissue specification, hair follicle development, lung tissue development, trunk neural crest cell differentiation, nephron development, ovary development and sex determination.

Cell proliferation

In order to have the mass differentiation of cells needed to form the specified cell tissues of different organisms, proliferation and growth of embryonic stem cells must take place. This process is mediated through canonical Wnt signaling, which increases nuclear and cytoplasmic β -catenin. Increased β -catenin can initiate transcriptional activation of proteins such as cyclin D1 and c-myc, which control the G1 to S phase transition in the cell cycle. Entry into the S phase causes DNA replication and ultimately mitosis, which are responsible for cell proliferation. This proliferation increase is directly paired with cell differentiation because as the stem cells proliferate, they also differentiate. This allows for overall growth and development of specific tissue systems during embryonic development. This is apparent in systems such as the circulatory system where Wnt3a leads to proliferation and expansion of hematopoietic stem cells needed for red blood cell formation.

Cell migration

Cell migration during embryonic development allows for the establishment of body axes, tissue formation, limb induction and several other processes. Wnt signaling helps mediate this process, particularly during convergent extension. Signaling from both the Wnt PCP pathway and canonical Wnt pathway is required for proper convergent extension during gastrulation. Convergent extension is further regulated by the Wnt/calcium pathway, which blocks convergent extension when activated. Wnt signaling also induces cell migration in later stages of development through the control of the migration behavior of neuroblasts, neural crest cells, myocytes, and tracheal cells.

Wnt signaling is involved in another key migration process known as the epithelial-mesenchymal transition (EMT). This process allows epithelial cells to transform into mesenchymal cells so that they are no longer held in place at the laminin. It involves cadherin down-regulation so that cells can detach from laminin and migrate. Wnt signaling is an inducer of EMT, particularly in mammary development.

II. HAIR FOLLICLE ANATOMY AND PHYSIOLOGY

Anatomy

The hair shaft consists of an inner core known as the medulla. This is surrounded by the cortex, which makes up the bulk of the hair. Moving outwards, there is a single layer of cells making up the shaft cuticle. The shaft cuticle is then encased in three layers that form the inner (internal) root sheath. The inner sheath is important in shaping the hair shaft as it grows upwards from the matrix. The inner sheath keratinizes from the outside-in, and will eventually disintegrate mid-follicle, around the level of the isthmus.

The outer (external) root sheath encases the entirety of the hair shaft. This layer undergoes trichilemmal keratinization around the level of the isthmus. The hair follicle begins at the surface of the epidermis. For follicles that produce terminal hairs, the hair follicle extends into the deep dermis, and sometimes even subcutis. Meanwhile, follicles producing vellus hairs extend only to the upper reticular dermis. There are three important segments of hair follicles found on the head: the infundibulum, the isthmus, and the lower follicle/inferior segment (which includes the bulb).

The infundibulum segment is the upper portion of the follicle. It begins at the surface of the epidermis and extends to the opening of the sebaceous duct. The isthmus is the area between the sebaceous duct opening and the bulge.

The bulge is an area of the follicle marked by the insertion of the arrector pili muscle. Also, the bulge contains several epidermal stem cells that are part of the outer root sheath and stain with CK19, CK15, and CD200.

The inferior segment of the hair follicle extends from the bulge to the base of the
5 follicle.

The base of the follicle includes the bulb, which contains the follicular matrix surrounding the sides and top of the dermal papilla. The dermal papilla contains capillaries. The papilla interacts with the matrix, which has the highest mitotic rate of any organ. The matrix is the part of the hair follicle where matrix keratinocytes proliferate to form the hair
10 shaft of growing hair. Melanocytes are mixed amongst the matrix cells to provide the hair shaft with color.

Sebaceous glands are holocrine glands closely associated with hair follicles, especially in certain areas of the skin such as the face. These glands open onto the hair follicles, except in areas such as the lips, where they empty directly onto the mucosa surface
15 because lips do not contain hair follicles. When stimulated by hormones such as androgens, sebaceous glands secrete a lipid-rich sebum that protects the hair and provides the skin with a hydrophobic barrier that can serve as protection.

Arrector pili muscles insert at the level of the bulge and also attach to the papillary layer of the dermis. In cold climates, sympathetic stimulation causes these muscles to
20 contract. This raises the level of the skin slightly and causes the hair to stand erect, which is commonly referred to as “goose-bumps.”

Structure and Function

The hair follicle cycles through three different growth phases: anagen, catagen, and telogen.

The anagen phase is the proliferation phase, and it occurs when the hair follicle is
25 growing a new hair shaft. The length of this phase can vary. For hair on the scalp, the growth phase can last 2 to 6 years, whereas, for eyebrows and eyelashes, hair may only need a few months to grow. This is the only phase during which the inferior segment of the hair follicle is present.

To signal the beginning of the anagen phase, the dermal papilla signals to the
30 multipotent epithelial stem cells in the bulge. Once these stem cells are stimulated, the inferior segment of the hair follicle can now grow downwards, forming a bulb around the

dermal papilla. Now, the dermal papilla can signal matrix cells in the bulb to proliferate, differentiate, and grow upwards, forming a new hair.

The catagen phase is also known as the transition or regression phase. It is the shortest of all three phases, and may only last a few weeks. During this phase, cell division in the matrix ceases, and the inferior segment of the hair follicle begins to regress. Eventually, the inferior segment of the follicle no longer exists, and the dermal papilla has moved upwards to contact the bulge once again. During this process, a club hair is formed with a white, hard node on the end.

Finally, the telogen phase ensues, which is referred to as the resting phase. Club hairs, which are essentially dead, are held. On the scalp, these club hairs are typically held for about 100 days. Eventually, these hairs are released and shed so that the anagen phase can begin again with a new hair.

At birth, all hairs begin in the anagen average; the human scalp contains over 100,000 hairs. At any one point in time, most hairs (85% to 95%) are commonly found in the anagen phase. These hairs can grow approximately 1 cm per month. Meanwhile, hair loss is continuous, with people losing about 100 hairs per day on average. With shampooing, the number of hairs lost can double.

Embryology

Around nine weeks gestation, the fetal epidermis gives rise to small buds of specialized cells that will form the hair follicle and its associated appendages. Mesenchymal cells that have accumulated within the dermis help drive this process by secreting substances such as epimorphin that signal the epithelial cells to proliferate and migrate in a downwards (cranio-caudal) direction towards the dermis. Once this process is complete, the fetus is left with a hair follicle containing a matrix derived from ectoderm and an underlying dermal papilla-derived from mesoderm. Additionally, an arrector pili muscle and a sebaceous gland form around each follicle. *In utero*, maternal androgens will stimulate the activation of fetal sebaceous glands, so that the glands can secrete a lipid-rich sebum that combines with desquamated stratum corneum cells to form the vernix caseosa.

Once the hair follicle has developed in the fetus, lanugo hairs grow in utero. These hairs are thin and short. They eventually shed by about 36 to 40 weeks gestation and are replaced by vellus hairs that cover most areas of the body. Meanwhile, thicker, coarser terminal hairs can be found on the head in certain areas such as the scalp, eyebrows, and

eyelashes. Beard hairs will arise once puberty is reached, with androgen hormones transforming the vellus hairs in this area into terminal hairs.

After birth, no new hair follicles will be made. However, new hair can grow from the existing follicles. Follicle size can still be altered after birth.

5 III. DISCOVERY OF IMPORTANCE OF WNT AND SHH SIGNALING

The signals that coordinate the events that lead to DC genesis have been identified. Data indicate that sufficient levels of SHH signaling determine when cells begin the transition to DC status, and the length of transition is delimited by a threshold level of Wnt signaling and quiescence. This transition phase is characterized by proliferation, augmented
10 Wnt signaling, and acquisition of DC markers followed by cell cycle exit. The spatiotemporal distance between threshold levels of SHH and Wnt signaling determines the number of transitioning intermediates and a cell's probability to undergo DC commitment.

Dermal SHH and Wnt signaling coordinate events of DC genesis within a short window of time and space. DCs form by the local migration of quiescent cells to form a cluster. The
15 work shows that a local pool of quiescent cells is generated by a selectively proliferative population. These proliferative progenitors express *Dkk1* and are located in a defined transition zone in the peri-DC region.

Studies have demonstrated the role of SHH in differentiated DC cells, establishing its function in DC maintenance. However, the essential function for dermal SHH signaling in
20 DC differentiation and DC expansion is difficult to examine, as the progenitors and cellular processes that lead to DC differentiation have been largely unknown. By leveraging scRNA-seq methods, a previously unrecognized role for SHH in cooperating with and augmenting Wnt signaling to drive the pre-DC-to-DC transition was identified. SHH functions in Wnt-active DC progenitors to: 1) promote their proliferation, 2) induce expression of mature DC
25 genes, 3) stimulate autonomous morphogenetic events and cell fate patterning, and 4) cause their timely arrest by augmenting Wnt signaling. In support of studies showing that high levels of Wnt signaling promote *Cdkn1a* expression and cell cycle exit, Wnt signaling levels correlated with quiescence across all mutants examined, including SHH cKO mutants. Notably, genetic co-activation of SHH and Wnt signaling induces these events independent
30 of FGF20, indicating that FGF20 likely functions upstream to modulate these two principal signals.

Fig. 1A-1C are graphs showing induction of high levels of SHH signaling across the entire gradient of Wnt signaling prior to morphogenesis, and not just those in the peri-DC,

cause every cell within that gradient to augment Wnt signaling incrementally while increasing their proliferation and expression of DC genes, resulting in more transitioning intermediates proliferating until they reach a level of Wnt signaling sufficient to cause arrest. Fig. 1A and 1B are graphs of the effect of SHH level in combination with Wnt level showing the change from no dermal condensate (“DC”) to proliferation intermediates (transition level) to arrest. Fig. 1C shows this process graphically.

The tight coupling of threshold levels of SHH and Wnt signaling in peri-DC cells should result in few transitioning intermediates prior to arrest, partly explaining why this transition is difficult to capture. Inducing high levels of SHH signaling across the entire gradient of Wnt signaling prior to morphogenesis, and not just those in the peri-DC, would cause every cell within that gradient to augment Wnt signaling incrementally while increasing their proliferation and expression of DC genes, resulting in more transitioning intermediates proliferating until they reach a level of Wnt signaling sufficient to cause arrest. Effectively, the *SmoM2YFP* trajectory represents a “transition eigenvector” with more intermediates to resolve the process in “slow-motion”.

In *SmoM2YFP;βcat^{fl/EX3}* mutants, both SHH and Wnt signaling levels are simultaneously induced at or above threshold levels, and mutant cells undergo that transition faster and more similar to the wildtype condition. Thus, two mitogenic signals can cooperate to regulate the length of transition between proliferation and arrest if they covary in a feed forward fashion, and one reins in this proliferation to cause arrest at a threshold level. This work supports a mechanism by which differentiation is coordinated with proliferation and cell cycle exit, a phenomenon utilized by other somatic stem cells, including muscle and neuronal progenitors. The data indicates the length of transition may depend upon the spatiotemporal distance between signal thresholds. This distance may define the number of divisions or duration that transit-amplifying cells divide before undergoing terminal differentiation.

Based on the *SmoM2YFP* trajectory, SHH and Wnt signaling cooperate to deterministically drive DC differentiation, and the spatial pattern follows this order. Differentiation coupled to morphogenesis may have evolved to ensure that DC cells condense upon commitment to provide rapid and precise local feedback to the epidermis to regulate placode size or the spatial segregation of placode cell fates. Spatially segregated placode populations follow branching trajectories. Time and space are necessarily coupled to establish a committed pool of quiescent DC cells. Placode Wnt and SHH signaling antagonize each

other to segregate placode cell fates, while the reciprocal convergence of these two signals in dermal cells results in accelerated signaling and DC differentiation. The factors that direct cell context-specific molecular interactions in the epidermis and dermis are unclear but indicate that signal segregation regulates cell fate trajectories.

5 Dermal SHH signaling promotes adult HF neogenesis following wounding. At the same time, forced activation of dermal β -catenin in adult skin inhibits HF neogenesis. The heterogeneous cell populations involved in wound healing likely require dermal Wnt activity to be tightly regulated. Signals have been identified which are sufficient to reproduce a major aspect of DC genesis as well as the tunable parameters that regulate the rate of DC genesis and DC size, which can help guide biomimetic methods to recreate inductive mesenchymal condensates.

These signals, using agonists and/or antagonists of Wnt and SSH, can be applied to skin to induce HF neogenesis and/or to restore HF size.

15 DCs form by the local migration of quiescent cells to form a cluster. A local pool of quiescent cells is generated by a selectively proliferative population. These proliferative progenitors express *Dkk1* and are located in a defined transition zone in the peri-DC region. Previous seminal studies demonstrated the role of SHH in differentiated DC cells, establishing its function in DC maintenance.

20 Fig. 2A-2E are schematics of epidermis with quiescent CDs in the dermis (2A); epithelial placode with DC (2B); proliferation of DC (2C); further invagination of the epidermis as it forms a hair follicle at the site of DC (2D), and formation of the hair follicle in the epidermis (2E).

Dermal SHH activation is required for the rapid transition to quiescence and mature DC differentiation. Fig. 3A-3M show that dermal SHH activation is required for the rapid transition to quiescence and mature DC differentiation. *Lef1* levels in *Bmp4*⁺ DCs at E14.5 and E15.5 (FIG. 3A). FISH showing decreased *Lef1* expression in *Bmp4*⁺ SHH cKO pseudo-DCs (FIG. 3B). Proliferation rate of *Dkk1*⁺ peri-DC and upper dermal cells at E14.5 (FIG. 3C). Pseudo-order of control and SHH cKO dermal cells at E14.5 (FIG. 3D). FISH showing proliferative *Bmp4*⁺ pseudo-DCs in SHH cKO at E14.5 and E15.5 (FIG. 3E). Number of *Bmp4*⁺ cells in SHH cKO and control over time (FIG. 3F). %EdU⁺ of *Bmp4*⁺ population over time in control and SHH cKO (FIG. 3G). Diffusion maps of E14.5 and E15.5 control and SHH cKO dermal cells showing that SHH cKO pseudo-DCs progress only on the Wnt

component by E15.5 (FIG. 3H-3K). Cartoons showing aberrant proliferation of SHH cKO Dkk1+ peri-DC cells and slow transition to quiescence (FIG. 3L, 3M).

Dermal SHH signaling is required to induce Region 2 changes, including accelerated dermal Wnt signaling. It was found that SHH cKO embryos showed expanded Wnt-active (Lef1+) placode cells at E14.5, indicating that placode Wnt ligands were preserved, but underlying dermal cells were unclustered and expressed much lower Lef1 levels than control DC cells (FIGs 3A–B). By E15.5, a small cluster of dermal cells was seen in SHH cKO embryos with Lef1 levels now reaching levels similar to E14.5 control DC cells, reflecting a slower rate of increase in Lef1 expression. Using Bmp4 as a DC marker previously shown to be retained in SHH cKO DCs, significantly fewer Bmp4+ cells were found in SHH cKO DCs than control DCs (FIGs 3D–F). Also, while control Bmp4+ DCs were quiescent (G0/G1 fraction=1) and surrounded by proliferating Dkk1+ cells, a significant proportion of SHH cKO Bmp4+ cells were aberrantly proliferating and co-expressed Dkk1 (FIGs 3D–E, 3G). At the same time, SHH cKO Dkk1+/Bmp4-peri-DC cells showed a lower rate of proliferation than control Dkk1+ peri-DC cells, suggesting a defect in the proliferation of Dkk1+ peri-DC cells as well as their transition into quiescence (FIG 3C).

The SHH cKO Bmp4+ population did not increase in size over time (FIG. 3F). Eventually SHH cKO Bmp4+ cells formed small quiescent clusters, showing that SHH cKO DC-like (pseudo-DC) cells undergo delayed entry into quiescence (FIGs 3D–G). These results were corroborated using the *in vivo* Fucci2 cell cycle reporter in which G1/G0 cells express mCherry (Abe et al., Development 140(1):237-246 (2013)). Only by E15.5, small quiescent mCherry+/Ki-67-dermal clusters were observed underneath SHH cKO placodes.

To confirm that the defects in DC differentiation were not indirectly due to a loss of epidermal SHH signaling, the transducer of SHH signaling, Smo, was ablated in epidermal cells (K14Cre;Smo^{fl/fl}) (Long et al., Development 128(24):5099-5108 (2001)). At E14.5, mutant placode cells lacked SHH activity but were associated with Sox2+ DCs that showed an unexpected modest increase in the number of Sox2+ cells per DC. While K14Cre;Smo^{fl/fl} placodes were stalled in growth due to the lack of epidermal SHH activation, their corresponding DCs expanded at a greater rate than control DCs. Mutant DCs remained in close approximation to the epidermis with a persistently high Lef1+/Ptch1+ co-positive peri-DC population, while controls showed a diminishing Lef1+/Ptch1+ co-positive peri-DC population that corresponded to the downward displacement of the DC from the epidermis. Taken together with previous work (Gritli-Linde et al., Dev. 301(2):309-326

(2007); Woo et al. *Genes & Dev.* 26, 1235-1246 (2012)) for dermal SHH signaling in DC genesis this supports the model that SHH/Wnt co-activation promotes DC differentiation.

Diffusion maps of E15.5 SHH cKO and control dermal populations showed that mutant cells progressed on a Wnt component but not a DC component. Specifically, E15.5 SHH cKO cells formed a divergent pseudo-DC branch that differed from controls by their lack of genes associated with the DC component, while their expression pattern was aligned with an attenuated Wnt component that terminates with quiescence. Consistent with this, SHH cKO pseudo-DCs express many genes found in Wnt-active dermal cells prior to DC formation (Gupta et al., *Dev. Cell* 48(1):1731 (2019)). Collectively, these results reveal a previously unrecognized mechanism by which dermal SHH signaling governs DC genesis by promoting the proliferation of Wnt-active peri-DC cells and their rapid entry into quiescence and mature DC differentiation.

SHH functions in Wnt-active DC progenitors to: 1) promote their proliferation, 2) induce expression of mature DC genes, 3) stimulate autonomous morphogenetic events and cell fate patterning, and 4) cause their timely arrest by augmenting Wnt signaling.

However, the essential function for dermal SHH signaling in DC differentiation and DC expansion was difficult to examine, as the progenitors and cellular processes that lead to DC differentiation were largely unknown. By leveraging scRNA-seq methods, a previously unrecognized role for SHH in cooperating with and augmenting Wnt signaling to drive the pre-DC-to-DC transition was discovered.

In support of studies showing that high levels of Wnt signaling promote *Cdkn1a* expression and cell cycle exit, Wnt signaling levels correlated with quiescence across all mutants examined, including SHH cKO mutants. Notably, genetic co-activation of SHH and Wnt signaling induces these events independent of FGF20, indicating that FGF20 likely functions upstream to modulate these two principal signals.

Fig. 4A-4H show that high dermal SHH activation in early Wnt-active cells deterministically reproduces events of DC genesis over more intermediates. E14.5 control and SmoM2YFP whole mount showing proliferative mutant Sox2⁺ clusters (FIG. 4A). Number of Sox2⁺ cells per dermal cluster in control and SmoM2YFP at E14.5 and E15.5 (FIG. 4B). Diffusion maps of E14.5 control and SmoM2YFP cells (FIG. 4C, 4D). Pseudo-order with Regions 1 and 2 demarcated (FIG. 4E, 4F). FISH of E14.5 control and mutant showing virtually all Sox2⁺ cells co-express *Ptch1* and *Lef1* (FIG. 4G, 4H).

Pseudo-order of indicated genes in mutant and control (FIG. 4F, 4G). E14.5 FISH showing EdU, Sox2, and Dkk1 (FIG. 4G). FISH at E15.5 after EdU pulse in control and SmoM2YFP skin (FIG. 4H). FISH 24 hours after EdU chase (FIG. 4I). %EdU+ cells by population in E14.5 mutant and control (FIG. 4J). %EdU+ of Sox2+ cells at E15.5 after EdU pulse or 24-hour chase (FIG. 4K). E14.5 SmoM2; β catfl/EX3 FISH showing Sox2+ clusters in upper and lower dermis that co-express Ptch1 and Lef1 (FIG. 4L). Sox2+ clusters with %EdU+ of indicated populations by condition at E14.5 (FIG. 4M). Depiction of transition rate and number of intermediates affected by modulating SHH and Wnt signaling (FIG. 4N).

It is believed that the tight coupling of threshold levels of SHH and Wnt signaling in peri-DC cells will result in few transitioning intermediates prior to arrest, partly explaining why this transition is difficult to capture. Inducing high levels of SHH signaling across the entire gradient of Wnt signaling prior to morphogenesis, and not just those in the peri-DC, will cause every cell within that gradient to augment Wnt signaling incrementally while increasing their proliferation and expression of DC genes, resulting in more transitioning intermediates proliferating until they reach a level of Wnt signaling sufficient to cause arrest. Effectively, the *SmoM2YFP* trajectory represents a “transition eigenvector” with more intermediates to resolve the process in “slow-motion”. In *SmoM2YFP*; β cat^{fl/EX3} mutants, both SHH and Wnt signaling levels are simultaneously induced at or above threshold levels, and mutant cells undergo that transition faster and more similar to the wildtype condition. Thus, two mitogenic signals can cooperate to regulate the length of transition between proliferation and arrest if they covary in a feed forward fashion, and one reins in this proliferation to cause arrest at a threshold level.

This supports a mechanism by which differentiation is coordinated with proliferation and cell cycle exit, a phenomenon utilized by other somatic stem cells including muscle and neuronal progenitors. The data indicates that the length of transition may depend upon the spatiotemporal distance between signal thresholds. This distance may define the number of divisions or duration that transit-amplifying cells divide before undergoing terminal differentiation.

Based on the *SmoM2YFP* trajectory, SHH and Wnt signaling cooperate to deterministically drive DC differentiation, and the spatial pattern follows this order. Coupling of differentiation to morphogenesis may have evolved to ensure that DC cells condense upon commitment to provide rapid and precise local feedback to the epidermis to regulate placode size or the spatial segregation of placode cell fates. Spatially segregated

placode populations follow branching trajectories. Time and space are necessarily coupled to establish a committed pool of quiescent DC cells. Placode Wnt and SHH signaling antagonize each other to segregate placode cell fates, while the reciprocal convergence of these two signals in dermal cells results in accelerated signaling and DC differentiation. The factors that direct cell context-specific molecular interactions in the epidermis and dermis are unclear but suggest that signal segregation may regulate cell fate trajectories.

The data support a mechanism to explain how dermal SHH signaling promotes adult HF neogenesis following wounding. At the same time, forced activation of dermal β -catenin in adult skin inhibits HF neogenesis. The heterogeneous cell populations involved in wound healing likely require dermal Wnt activity to be tightly regulated. Studies demonstrate that the signals sufficient to reproduce a major aspect of DC genesis as well as the tunable parameters that regulate the rate of DC genesis and DC size can help guide biomimetic methods to recreate inductive mesenchymal condensates.

Studies demonstrate that the DC differentiation trajectory was defined by Wnt and DC components. The data show that the combination of these two eigenvectors represents a significant aspect of DC genesis

IV. CLINICAL SIGNIFICANCE

Hair growth rate and hair density decrease with age. Hair loss, known as alopecia, is a common occurrence. It can be due to drugs, diet, hormone imbalances, altered mitotic activity, growth cycle abnormalities, among others. A thorough history, physical exam, hair pull test, daily hair counts, part width, clip tests to examine the hair shaft, hair growth windows, and hair pluck, and trichograms can all be used to diagnose hair disease. Scalp biopsies, hormone studies, and a potassium hydroxide examination for fungi may also need to be performed in certain cases. It is important to diagnose hair disease correctly, as the treatment for hair loss is dependent on the diagnosis.

Male pattern baldness, known as androgenetic alopecia, is a common condition in which hair loss occurs as men age. With increased dihydrotestosterone binding to the follicle of hair receptors, each successive anagen cycle shortens in length. In these individuals, follicles shrink and gradually produce shorter, thinner, more vellus-like hairs. Possible treatments may include slowing the conversion of testosterone to dihydrotestosterone in men with this condition.

After extensive experimentation, methods have been developed to screen for compounds for treatment of embryonic skin stem cells to grown hair follicles and to restore the size and functionality of hair follicles whose size and virulity have decreased.

Tissue homeostasis and regeneration are mediated by the coordinated growth of multiple cell types to generate tissue that sustains integrity and function of the organism. This ability to durably regenerate tissue relies on adult stem cells that reside in a specialized environment called the niche, which influences their self-renewal, growth and differentiation. Failure to maintain or mobilize stem cells results in tissue loss and dysfunction, while uncontrolled activation of these cells can fuel disorganized growth and cancer. Elucidating how key molecular signals govern stem cell behavior holds tremendous implications for designing targeted therapies to treat human diseases. A major challenge to examining how mammalian stem cells are regulated is the inability of conventional static analysis to follow the fate and behavior of cell populations *in vivo* over time.

V. COMPOUNDS ACTIVE IN WWNT AND SSH SIGNALING PATHWAYS

Studies were undertaken to determine how heterotypic signals between the skin epidermis and dermis regulate robust and organized growth hair follicle growth during embryonic development and adult regeneration, as well as how dysregulation of these signals can result in skin cancer. In particular, (1) how cellular behaviors such as cell divisions and movement are regulated by key morphogenetic signals during regeneration, (2) how these signals are spatially disseminated to a field of cells in to ensure robust but patterned and compartmentalized growth, and (3) how these mechanisms can contribute to disorganized and uncontrolled growth during tumorigenesis. The hair follicle is an ideal model to address these questions as it is exceptionally accessible and undergoes well-characterized growth and cyclical regeneration in a manner dependent on resident stem cells. This model is coupled with live imaging and single-cell genomic techniques to address these outstanding questions.

It has been determined how Wnt/ β -catenin signaling, a key molecular pathway required for hair follicle regeneration, is propagated throughout a population of undifferentiated cells to promote synchronous and coordinated growth. By live imaging, it was found that only a subset of cells is required to fuel the non-cell autonomous activation of this signal and growth behaviors throughout surrounding epithelial cells and is associated with upregulation of diffusible Wnt ligands. A second important signaling pathway uses the sonic hedgehog (SHH) pathway.

One of the goals is to understand how cooperative epithelial growth coordinates normal hair follicle regeneration. The second is to examine how this mechanism of collective behavior regulates basal cell carcinomas (BCCs), the most common human skin cancer, which utilizes hair morphogenetic signals for growth. Third, is how the mesenchyme regulates both organized regeneration and development and how this can be applied to modify tumor growth. Accomplishing these aims will provide novel insight into the principle mechanisms that ensure proper tissue regeneration and how they can also be exploited deleteriously to promote collective growth in cancer.

The dermal papilla (DP) instructs the cyclical regeneration of the adult hair follicle (HF). The HF grows in anagen, regresses in catagen and rests in telogen. The DP expands in cell number as the HF regenerates, which is critical for HF growth and regulating hair shaft size. Currently the DP is considered to be a quiescent population. The prevailing theory is that the DP expands from proliferative cells that reside outside the DP. EdU pulse chase experiments were performed to track when and where DP progenitors divide and contribute to the expanding DP. It was found that two different populations contribute to DP expansion. In early anagen, dermal cup cells that reside outside of DP proliferate and migrate into the DP. In late anagen, a subset of DP cells is reactivated to proliferate to expand the DP. The proliferative dermal cup cells trace to the lower DP and proliferative DP cells trace to the upper DP. Surprisingly, the proliferative dermal cup cells exit from DP after a hair cycle is finished while the proliferative DP cells are retained, suggesting differential behavior and potential of these two populations contributing to the DP.

VI. Model for DP Expansion and Testing of Compounds and Dosages

The following two-stage model was proposed for DP expansion. In the first stage, the DP itself is quiescent and dermal cup cells surrounding the DP proliferate to expand the DP. In the second stage, the DP starts proliferating to expand itself. This indicates that DP cells are not terminally differentiated but rather have self-renewing capacity. The results reveal a previously unknown potential for DP self-renewal. This provides a way to understand signals that regulates DP regeneration, which can help to expand DP cells *in vitro* for clinical applications.

To determine the dose response of dermal fibroblasts to Wnt and/or SHH stimulation, two molecular signals required and sufficient for DC formation in mouse embryonic skin, varying doses of soluble Wnt agonist (CHIR-99021) and/or SHH agonist (SAG dihydrochloride) were added to cultured primary embryonic dermal fibroblasts. Specifically,

dorsolateral skin was microdissected from mouse embryos in which all dermal cells genetically express nuclear GFP (PDGFRaH2BGFP). Skin is collected in 4%FCS/PBS wash buffer and dissociated into a single-cell suspension using 0.25% trypsin (Gibco, Life Technologies) for 20 minutes at 37° C. Cells are then washed in 4%FCS/PBS before filtering through a 70 mm strainer and FACS. All live GFP⁺ dermal cells are FACS sorted and collected in wash buffer. Cells are collected by centrifugation at 300g for 10 min. Cells are resuspended in media (10% FCS/DMEM) at 0.5×10^6 per well supplemented with varying doses of vehicle alone (DMSO), CHIR (5 and 10 mM), or SAG (50 or 200 nM) or both CHIR and SAG. Cells are incubated at 37°C/5% CO₂ for 24, 48 or 96 hours. At each time point, adherent cells are collected by trypsinization (0.05% Trypsin) and RNA is isolated using a Qiagen RNeasy column. RNA is quantified and reverse-transcribed into cDNA (Superscript III, Invitrogen) before analyzing relative mRNA levels by qPCR with Fast SYBR Green Master Mix and primers specific for selected marker genes of Wnt activation (e.g. Lef1, Tcf7, Axin2), SHH activation (e.g. Gli1, Ptch1) or DC differentiation (e.g. FoxD1, Sox18) to assess gene expression responses to the above culture conditions.

To assess the functional ability of the above cultured dermal fibroblasts to induce hair follicle formation, a standard hair follicle patch assay is used. Here, 2×10^6 cultured dermal cells (PDGFRaH2BGFP⁺) from the above conditions are mixed in PBS with 1×10^6 neonatal primary keratinocytes isolated from P0-P1 neonatal mice. Immunodeficient host mice (Nude) mice are injected with this mixture of dermal and epidermal cells subcutaneously (3 replicate grafts per culture condition) into the back skins of host mice. Mice are sacrificed after 4-5 weeks and grafts are harvested from skin. Under a stereomicroscope, regenerated hair follicles can be visualized volumetrically and quantified for number of hair follicles/graft, number of DC cells per hair follicle and hair fiber length and size.

There is currently no cell platform that responds to Wnt and SHH agonists to induce hair inductive DC genes. This is resolved with the use of embryonic dermal cells allows for robust and tunable wnt and SHH gene expression responses as well as DC gene responses (when both wnt and SHH are combined).

Using this regimen, Axin2CreER;SmoM2YFP embryos were examined at E14.5 and a lack of placodes noted as evidenced by the lack of epidermal Edar or Ptch1 transcripts. Sox2⁺ dermal clusters were found in the upper dermis that were bigger than control DCs at both E14.5 and E15.5 and that expressed eYFP and Ptch1. These clusters usually lacked a direct

connection to the epidermis but were confined to the upper dermis and occasionally abutted one another.

Sox9 was also expressed by control and mutant dermal clusters, while the mutant epidermis largely lacked Sox9⁺ cells normally found in SHH-active placodes. Thus, high dermal SHH activation is sufficient to induce Sox2⁺ dermal clusters in the Wnt-active upper dermis independent of placodes.

As high SHH activation is sufficient to induce DC differentiation in early Wnt-active cells, it was hypothesized that molecular changes of Region 2 are caused by sufficient levels of SHH signaling. scRNA-seq data from E14.5 control and SmoM2YFP embryos confirmed that neither placode markers nor eYFP was detected in the epidermal population of mutants. Due to the global transcriptional differences between wildtype (eYFP⁻) and mutant (eYFP⁺) dermal populations, dermal diffusion maps of mutant cells were analyzed separately. The SmoM2YFP trajectory was represented by one eigenvector, while other eigenvectors were either not significantly correlated with DC changes or contained minimal variance. Similar to Region 2 of controls, the mutant pseudo-order showed that Lef1 levels increased monotonically, and Sox2⁺ cells were concentrated near the terminus. Further, Lef1 and Ptch1 levels co-varied at a nearly constant rate along the SmoM2YFP pseudo-order.

By the SmoM2YFP trajectory, SHH/Wnt co-activation functions as a deterministic component that correlates with expression of DC and Wnt target genes. Notably, SmoM2YFP cells transition across more Dkk1⁺ proliferative intermediates that gradually acquire DC genes before quiescence. To verify these inferences, it was found that virtually all Sox2⁺ cells in mutant embryos coexpressed Ptch1 and Lef1, supporting the notion that Wnt and SHH co-activation is required for DC differentiation. As dermal Wnt signaling requires epidermal Wnt ligands, all mutant Sox2 clusters were located in the upper dermis. At E14.5, it was also found that many mutant Sox2⁺ cells were proliferating and coexpressed Dkk1⁺, but by E15.5, most Sox2⁺ cells were quiescent, lacked Dkk1 expression, and were surrounded by a ring of proliferating Dkk1⁺ cells similar to control DCs. Further, E15.5 mutant cells pulsed with EdU at E14.5 showed that many quiescent Sox2⁺ cells originated from E14.5 proliferating cells in contrast to control DCs that expanded from a quiescent pool.

These results show that Wnt and SHH co-activation reproduces DC changes without placodes represented by a longer or “slower” continuum of proliferative Dkk1⁺ intermediate states that progressively gain DC markers prior to quiescence.

Recognizing that FGF20 is a placode signal previously shown to be essential for DC differentiation and morphogenesis, the role of FGF20 in this process was assessed.

SmoM2YFP expression in Axin2CreER; SmoM2YFP; FGF20lacZ/lacZ embryos that lack FGF20 (Huh et al., 2012) was measure and it was found that mutant Sox2 clusters formed similar to SmoM2YFP embryos. FGF20lacZ/lacZ embryos largely lacked Sox2⁺ DCs at E14.5, indicating that SHH may act downstream of FGF20. Consistent with this, SHH cKO placodes showed intact Fgf20 expression. Further, there was a marked reduction in the number of Ptch1⁺ and Lef1⁺ cells in the dermis underlying FGF20lacZ/lacZ placodes, and Sox2⁺ cells that were seen co-expressed Ptch1 and Lef1. These data indicate that FGF20 modulates DC differentiation upstream of the two principal drivers of this process.

The spatial patterning of SHH and Wnt signaling gradients regulates the number of transitioning intermediates. The data show that inducing uniformly high SHH activation across a gradient of Wnt signaling results in a gradient response of DC differentiation. Thus, DC genes that are normally restricted to the distal region of the wildtype DC trajectory are now distributed across the entire gradient of Wnt signaling and decoupled from terminal quiescent cells. Based on these observations, inducing high Wnt signaling in SmoM2YFP cells could bypass earlier intermediate states. Both β -catenin and Smo were activated in Axin2CreER;SmoM2YFP; β catfl/EX3 (SmoM2YFP; β catfl/EX3) embryos (Harada et al., 1999). In contrast to SmoM2YFP embryos, Sox2⁺ clusters were now seen in the upper and lower dermis and surrounded by proliferating Dkk1⁺ cells in SmoM2YFP; β catfl/EX3 embryos. Additionally, most Sox2⁺ clusters in SmoM2YFP; β catfl/EX3 embryos were quiescent similar to control DCs, indicating a faster transition to quiescence concurrent with molecular differentiation and morphogenesis.

Fig. 5A-5N show that high dermal SHH activation in early Wnt-active cells deterministically reproduces events of DC genesis over more intermediates. E14.5 control and SmoM2YFP whole mount showing proliferative mutant Sox2⁺ clusters (FIG. 5A). Number of Sox2⁺ cells per dermal cluster in control and SmoM2YFP at E14.5 and E15.5 (FIG. 5B). Diffusion maps of E14.5 control and SmoM2YFP cells (FIG. 5C). Pseudo-order with regions 1 and 2 demarcated (FIG. 5D). FISH of E14.5 control and mutant showing virtually all Sox2⁺ cells co-express Ptch1 and Lef1 (FIG. 5E). Pseudo-order of indicated genes in mutant and control (FIG. 5F). E14.5 FISH showing EdU, Sox2, and Dkk1 (FIG. 5G). FISH at E15.5 after EdU pulse in control and SmoM2YFP skin (FIG. 5H). FISH 24 hours after EdU chase (FIG. 5I). %EdU⁺ cells by population in E14.5 mutant and control

(FIG. 5J). %EdU+ of Sox2+ cells at E15.5 after EdU pulse or 24-hour chase (FIG. 5K).

E14.5 SmoM2; β catfl/EX3 FISH showing Sox2+ clusters in upper and lower dermis that co-express Ptch1 and Lef1 (FIG. 5L). Sox2+ clusters with %EdU+ of indicated populations by

condition at E14.5 (FIG. 5M). Depiction of transition rate and number of intermediates

- 5 affected by modulating SHH and Wnt signaling (FIG. 5N). FIG. 5O is a graph of % EdU+ for UD, Dkk1+Sox2- compared to Sox2+. FIG. 5P is a diagram of the histology in cross-section of wild type, SmoM2 and SmoM2 β^{Ex3} showing proliferating Dkk1+, penultimate dividing Dkk1+ and quiescent Dkk1+.

- 10 Fig. 6A-6B are graphs of %DC (6A), the ratio of periDC/Perfollicular (6B) and the progression of DC differentiation from proliferative periDC (Dkk1+) to quiescent corner (Dkk1-) to DC (Dkk1-) (Fig. 6C).

- FIG. 7A and 7B are graphs showing dermal cells pre-treated with Wnt agonist, CHIR, significantly enhanced SHH and DC gene responses compared to without Wnt agonist pre-treatment (compare blue no pretreatment to red with Wnt pretreatment). FIG. 7A and 7B
15 show that pre-treating dermal cells with Wnt agonist, CHIR (2 micromolar for 24 hours) followed by SAG (10 nanomolar for 48 hours) significantly enhanced SHH and DC gene responses compared to without Wnt agonist pre-treatment (compare no pretreatment with vehicle (DMSO alone) to Wnt pretreatment). This indicates that Wnt activation (low dose) primes cells to respond more robustly to SHH activation.

20 VII. Formulations for Administration of Wnt and SHH modulators

- The ability to chemically modulate both signals using varying doses and timing of the two agonists using these cells shows that the timing and levels of SAG and CHIR regulate distinct aspects of DC differentiation. For example, low SAG and low CHIR induce proliferation which promotes higher number of DC progenitors capable of expressing DC
25 genes, but high SAG and high CHIR induce robust DC gene expression and stops the proliferation. Thus, there is a two step (or gradient) of these two signals required to achieve ideal size and functionality of DCs. These DC parameters control hair follicle size and regeneration (larger DCs induce larger hair follicles).

- Without a platform that can measure the effect of different Wnt/SHH agonist
30 doses/duration, there is no way to determine how to formulate these two chemicals to induce effective hair inductive DCs for human application. Such a platform is described herein. In one embodiment, a Wnt agonist is used to lower SAG to expand DC progenitors, followed by a SHH agonist. This can be achieved using sequential administration of Wnt agonist and

SHH agonist, or using a formulation in which one is present in a slow or delayed release formulation. Timing and dose is critical.

Formulations for topical administration can include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like can be necessary or desirable.

Formulations are prepared using a pharmaceutically acceptable “carrier” composed of materials that are considered safe and effective and may be administered to an individual without causing undesirable biological side effects or unwanted interactions. The “carrier” is all components present in the pharmaceutical formulation other than the active ingredient or ingredients. The term “carrier” includes but is not limited to diluents, binders, lubricants, desintegrators, fillers, and coating compositions.

“Carrier” also includes all components of the coating composition which may include plasticizers, pigments, colorants, stabilizing agents, and glidants. The delayed release dosage formulations may be prepared as described in references such as “Pharmaceutical dosage form tablets”, eds. Liberman et. al. (New York, Marcel Dekker, Inc., 1989), “Remington – The science and practice of pharmacy”, 20th ed., Lippincott Williams & Wilkins, Baltimore, MD, 2000, and “Pharmaceutical dosage forms and drug delivery systems”, 6th Edition, Ansel et.al., (Media, PA: Williams and Wilkins, 1995) which provides information on carriers, materials, equipment and process for preparing tablets and capsules and delayed release dosage forms of tablets, capsules, and granules.

Examples of suitable coating materials include, but are not limited to, cellulose polymers such as cellulose acetate phthalate, hydroxypropyl cellulose, hydroxypropyl methylcellulose, hydroxypropyl methylcellulose phthalate and hydroxypropyl methylcellulose acetate succinate; polyvinyl acetate phthalate, acrylic acid polymers and copolymers, and methacrylic resins that are commercially available under the trade name EUDRAGIT® (Roth Pharma, Westerstadt, Germany), zein, shellac, and polysaccharides.

Additionally, the coating material may contain conventional carriers such as plasticizers, pigments, colorants, glidants, stabilization agents, pore formers and surfactants, diluents, binders, lubricants, disintegrants, colorants, stabilizers, and surfactants. Stabilizers are used to inhibit or retard drug decomposition reactions which include, by way of example, oxidative reactions.

Surfactants may be anionic, cationic, amphoteric or nonionic surface active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate,

sulfonate and sulfate ions. Examples of anionic surfactants include sodium, potassium, ammonium of long chain alkyl sulfonates and alkyl aryl sulfonates such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium bis-(2-ethylthioxyl)-sulfosuccinate; and alkyl sulfates such as sodium lauryl sulfate. Cationic surfactants include, but are not limited to, quaternary ammonium compounds such as benzalkonium chloride, benzethonium chloride, cetrimonium bromide, stearyl dimethylbenzyl ammonium chloride, polyoxyethylene and coconut amine. Examples of nonionic surfactants include ethylene glycol monostearate, propylene glycol myristate, glyceryl monostearate, glyceryl stearate, polyglyceryl-4-oleate, sorbitan acylate, sucrose acylate, PEG-150 laurate, PEG-400 monolaurate, polyoxyethylene monolaurate, polysorbates, polyoxyethylene octylphenylether, PEG-1000 cetyl ether, polyoxyethylene tridecyl ether, polypropylene glycol butyl ether, Poloxamer[®] 401, stearyl monoisopropanolamide, and polyoxyethylene hydrogenated tallow amide. Examples of amphoteric surfactants include sodium N-dodecyl-.beta.-alanine, sodium N-lauryl-.beta.-iminodipropionate, myristoamphoacetate, lauryl betaine and lauryl sulfobetaine.

Extended release formulations are generally prepared as diffusion or osmotic systems, for example, as described in "Remington – The science and practice of pharmacy" (20th ed., Lippincott Williams & Wilkins, Baltimore, MD, 2000). A diffusion system typically consists of two types of devices, reservoir and matrix, and is well known and described in the art. The three major types of materials used in the preparation of matrix devices are insoluble plastics, hydrophilic polymers, and fatty compounds. Plastic matrices include, but not limited to, methyl acrylate-methyl methacrylate, polyvinyl chloride, and polyethylene. Hydrophilic polymers include, but are not limited to, methylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and carbopol 934, polyethylene oxides. Fatty compounds include, but are not limited to, various waxes such as carnauba wax and glyceryl tristearate.

Alternatively, extended release formulations can be prepared using osmotic systems or by applying a semi-permeable coating to the dosage form.

Materials to produce different release mechanisms can be combined in a final dosage form comprising single or multiple units. An immediate release portion can be added to the extended release system by means of either applying an immediate release layer on top of the extended release core using coating or compression process or in a multiple unit system such as a capsule containing extended and immediate release beads. Extended release forms

containing wax materials are generally prepared using methods known in the art such as a direct blend method, a congealing method, and an aqueous dispersion method. In a congealing method, the drug is mixed with a wax material and either spray- congealed or congealed and screened and processed. By "pulsatile" is meant that a plurality of doses are released at spaced apart intervals of time.

In one embodiment, pulsatile release profile is achieved with dosage forms that are closed and preferably sealed capsules housing at least two drug-containing "dosage units" wherein each dosage unit within the capsule provides a different drug release profile. Control of the delayed release dosage unit(s) is accomplished by a controlled release polymer coating on the dosage unit, or by incorporation of the active agent in a controlled release polymer matrix. Alternatively, each dosage unit in the capsule may comprise a plurality of drug-containing beads, granules or particles. As is known in the art, drug-containing "beads" refer to beads made with drug and one or more excipients or polymers. Drug-containing beads can be produced by applying drug to an inert support, e.g., inert sugar beads coated with drug or by creating a "core" comprising both drug and one or more excipients. As is also known, drug-containing "granules" and "particles" comprise drug particles that may or may not include one or more additional excipients or polymers. In contrast to drug-containing beads, granules and particles do not contain an inert support. Granules generally comprise drug particles and require further processing. Generally, particles are smaller than granules, and are not further processed. Although beads, granules and particles may be formulated to provide immediate release, beads and granules are generally employed to provide delayed release.

In a further alternative embodiment, a dosage form is provided that comprises an inner drug-containing core and at least one drug-containing layer surrounding the inner core. An outer layer of this dosage form contains an initial, immediate release dose of the drug. Alternatively, for dosage forms mimicking three times daily dosing, the dosage form has an outer layer and an inner layer free of drug.

As will be appreciated by those skilled in the art and as described in the literature, a number of methods are available for preparing drug-containing tablets, beads, granules or particles that provide a variety of drug release profiles. Such methods include, but are not limited to, the following: coating a drug or drug-containing composition with an appropriate coating material, typically although not necessarily incorporating a polymeric material;

increasing drug particle size; placing the drug within a matrix; and forming complexes of the drug with suitable complexing agents.

Particles can be prepared entirely from a therapeutic or diagnostic agent, or from a combination of the agent and a surfactant. The particles preferably are biodegradable and biocompatible, and optionally are capable of biodegrading at a controlled rate for delivery of a therapeutic or diagnostic agent. The particles can be made of a variety of materials. Both inorganic and organic materials can be used.

Polymeric particles may be formed from any biocompatible, and preferably biodegradable polymer, copolymer, or blend. The polymers may be tailored to optimize different characteristics of the particle including: i) interactions between the agent to be delivered and the polymer to provide stabilization of the agent and retention of activity upon delivery; ii) rate of polymer degradation and, thereby, rate of drug release profiles; iii) surface characteristics and targeting capabilities via chemical modification; and iv) particle porosity.

As used herein, the term "surfactant" refers to any agent which preferentially absorbs to an interface between two immiscible phases, such as the interface between water and an organic polymer solution, a water/air interface or organic solvent/air interface. Surfactants generally possess a hydrophilic moiety and a lipophilic moiety, such that, upon absorbing to microparticles, they tend to present moieties to the external environment that do not attract similarly-coated particles, thus reducing particle agglomeration. Surfactants may also promote absorption of a therapeutic or diagnostic agent and increase bioavailability of the agent. Surfactants known in the art can be used including any naturally occurring surfactant. Other exemplary surfactants include diphosphatidyl glycerol (DPPG); hexadecanol; fatty alcohols such as polyethylene glycol (PEG); polyoxyethylene-9-lauryl ether; a surface active fatty acid, such as palmitic acid or oleic acid; sorbitan trioleate (Span 85); glycocholate; surfactin; a poloxomer; a sorbitan fatty acid ester such as sorbitan trioleate; tyloxapol and a phospholipid.

Rapidly bioerodible polymers such as poly[lactide-co-glycolide], polyanhydrides, and polyorthoesters, whose carboxylic groups are exposed on the external surface as their smooth surface erodes, are excellent candidates for drug delivery systems. In addition, polymers containing labile bonds, such as polyanhydrides and polyesters, are well known for their hydrolytic reactivity. Their hydrolytic degradation rates can generally be altered by simple changes in the polymer backbone. Representative natural polymers include proteins, such as zein, modified zein, casein, gelatin, gluten, serum albumin, or collagen, and polysaccharides,

such as cellulose, dextrans, polyhyaluronic acid, polymers of acrylic and methacrylic esters and alginic acid.

Shampoos typically contain water or another clarifying ingredient, Fatty alcohols such as cetyl alcohol, lauryl alcohol, Cetearyl alcohol, and stearyl alcohol as moisturizers, and
5 thickeners such as stearic acid, gelatin, xanthan gum, carnauba wax, and stearyl alcohol.

Gel formulations typically include a gelling agent such as a carbomer, hydroxyethyl cellulose, and hydroxypropyl cellulose and solubilizers.

These formulations can be used to administer an effective amount of the agents *in vitro* or *in vivo* to induce an individual's cells to form new hair and/or hair follicles, or to active hair
10 formation and/or growth.

We claim:

1. A method to stimulate growth of new hair follicles and/or restore hair follicle size comprising topically applying to the site in need thereof agonists of Wnt and SHH formulated for application to skin to grow hair or hair follicles or restore hair follicle size.
2. The method of claim 1 wherein the agonists are applied topically, in a formulation selected from the group consisting of a solution, gel, spray, spray, foam, shampoo, or transdermal patch.
3. The method of any of claims 1-3 wherein the formulation comprises a surfactant, a viscosity modifying agent, and/or a transdermal penetration enhancer.
4. The method of any of claims 1 - 3 wherein the formulation incorporates at least one means for sustained or pulsed controlled delivery of the Wnt and/or SHH agonists, effective to create higher levels of Wnt agonist initially, then higher levels of SHH agonist as the Wnt agonist levels decrease.
5. The method of any of claims 1-4 comprising administering the SHH agonist after the Wnt agonist or in a formulation releasing the SHH agonist after the Wnt agonist or in higher amounts after the Wnt agonist is released and levels begin to decrease.
6. The method of any of claims 1-5 wherein the SHH agonist is an agent such as SAG dihydrochloride.
7. The method of any of claims 1-6 wherein the Wnt agonist is an agent such as CHIR-99021.
8. The method of any of claims 1-7 wherein the Wnt agonist is administered before the SHH agonist or in the same formulation where the SHH agonist is in a controlled or delayed release form so that the SHH agonist level increases as the Wnt agonist level decreases.
9. The method of any of claims 1-8 wherein the dosage of the Wnt agonist is equivalent to 5 to 10 micromolar (μM) CHIR.
10. The method of any of claims 1-9 wherein the dosage of the SHH agonist is equivalent to 50 to 200 nM SAG dichloride.
11. The method of any of claims 1-10 wherein the dosage of the Wnt agonist and SHH agonist is equivalent to 5 μM CHIR + either 50, 100 or 200 nM SAG or 10 μM CHIR + SAG 50, 100, or 200 nM. SAG.
12. The method of any of claims 1-11 wherein the Wnt agonist and SHH agonist are administered topically or intradermally for two or more days.

13. The method of any of claims 1-12 wherein the method is for screening of Wnt agonists and SHH agonists agents to restore hair, grow hair follicles, or increase hair follicle size, or the dosage and timing thereof, or formulations thereof for restoration of hair follicles or size thereof.
14. The method of claim 13 wherein a Wnt agonist and SHH agonist is administered topically or intradermally daily for 2-5 days to an animal such as a mouse, followed by incrementally higher doses based on gene responses from *in vitro* culture experiments daily for 2-5 days to assess efficacy and/or effective dosage for restoration or regrowth of hair follicles.
15. A formulation for use in the methods of any of claims 1-14.

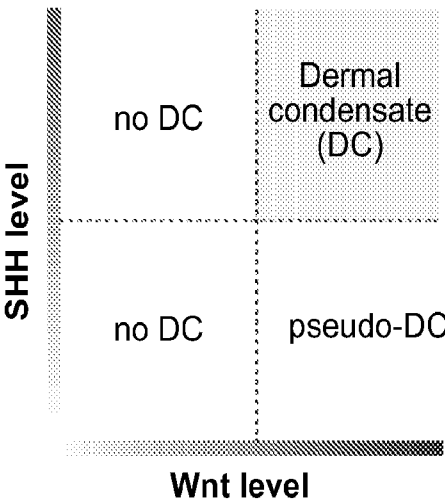


FIG. 1A

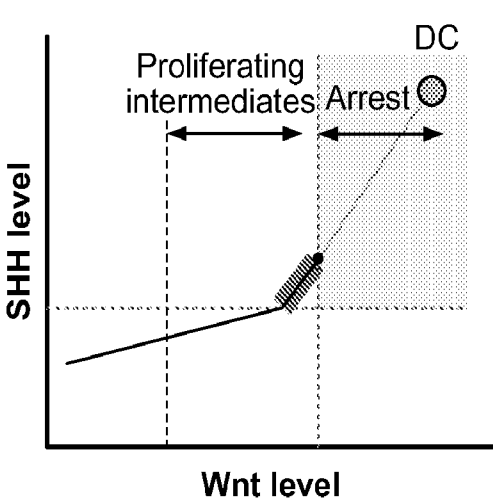


FIG. 1B

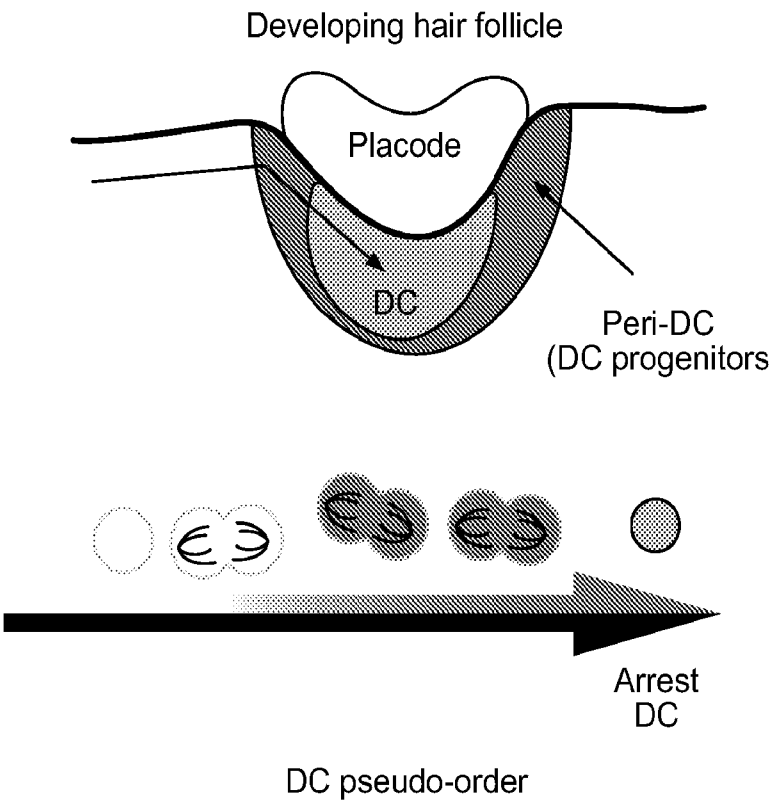
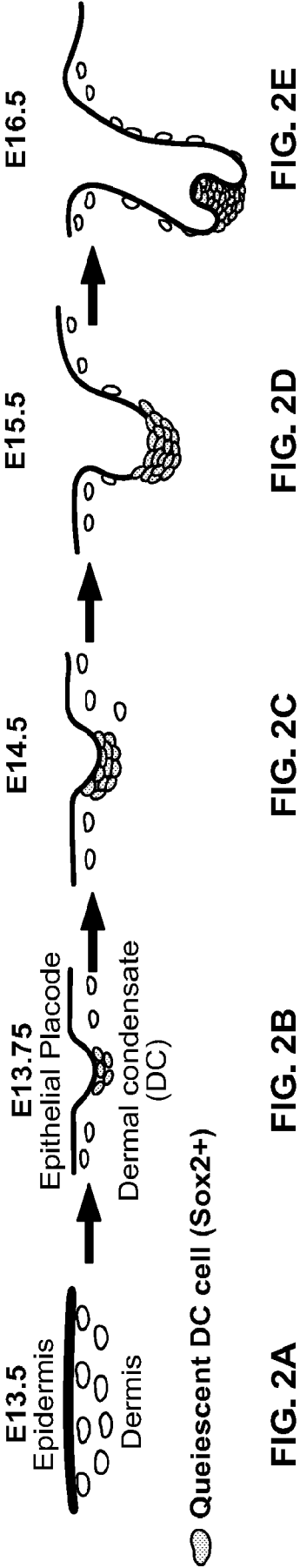
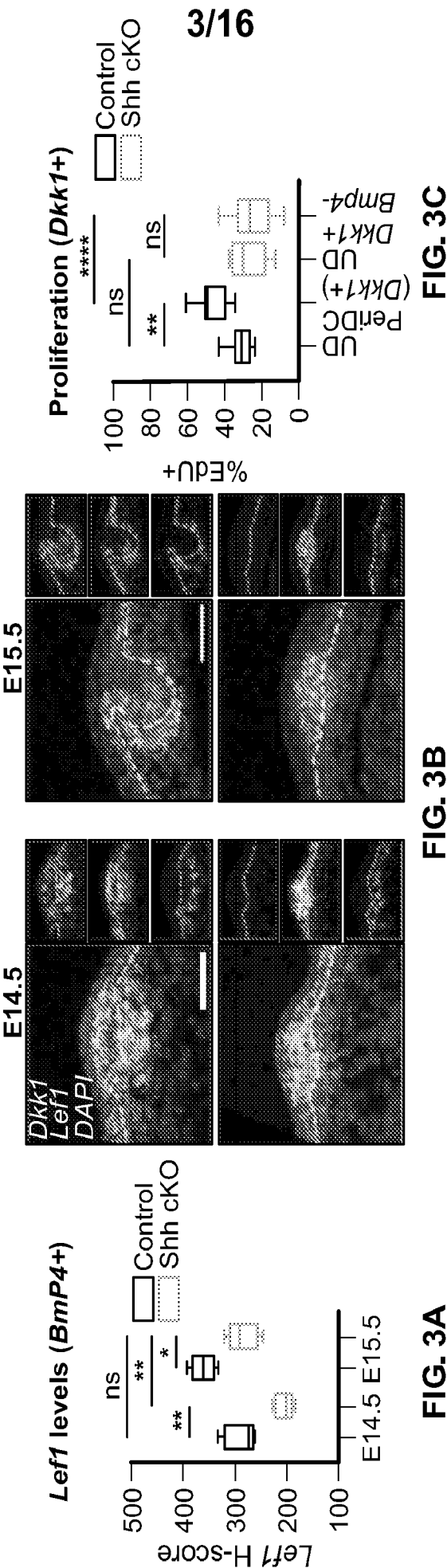


FIG. 1C





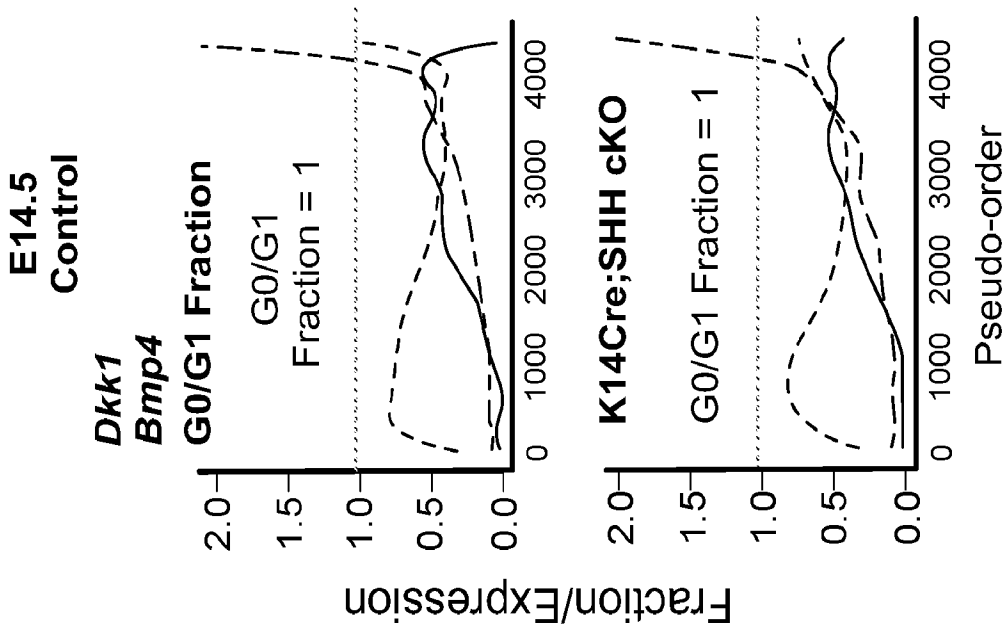


FIG. 3D

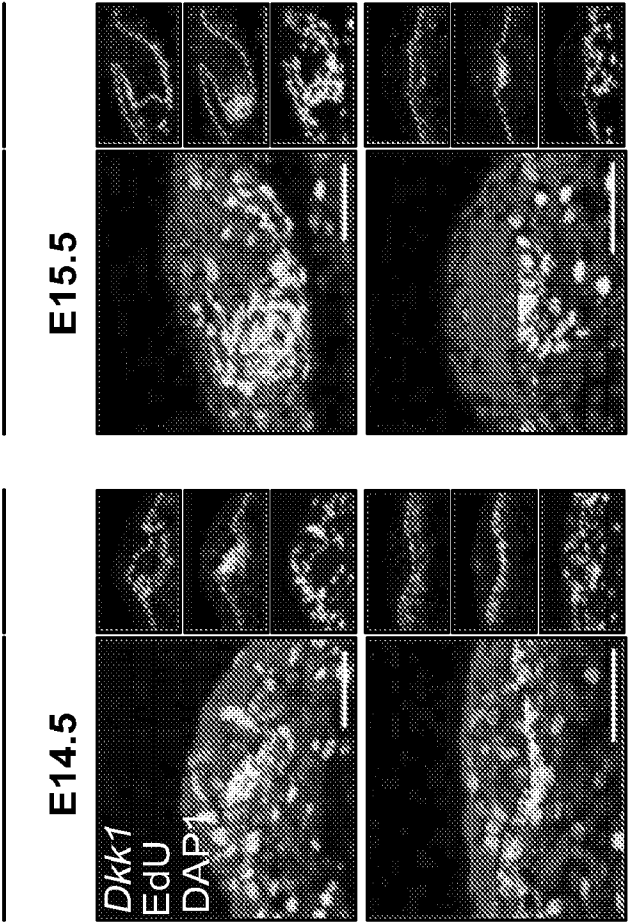


FIG. 3E

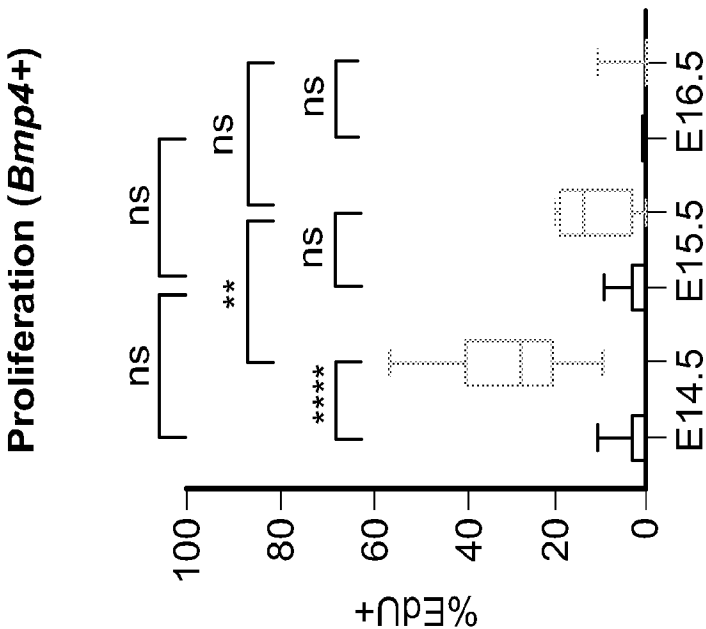


FIG. 3G

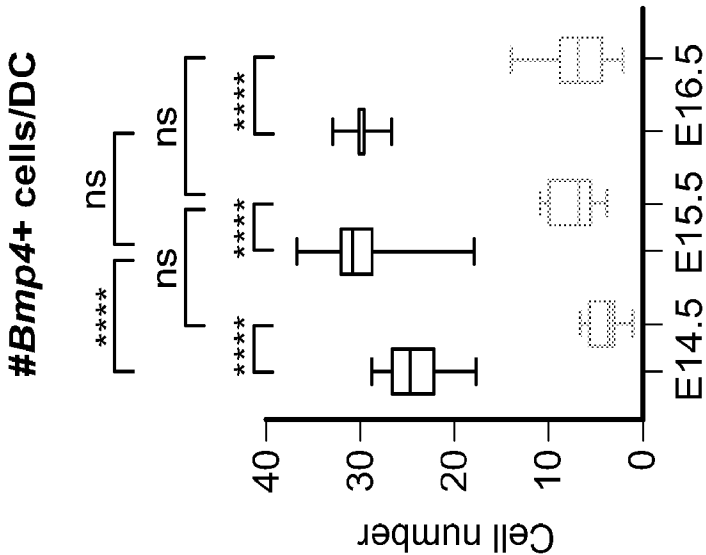


FIG. 3F

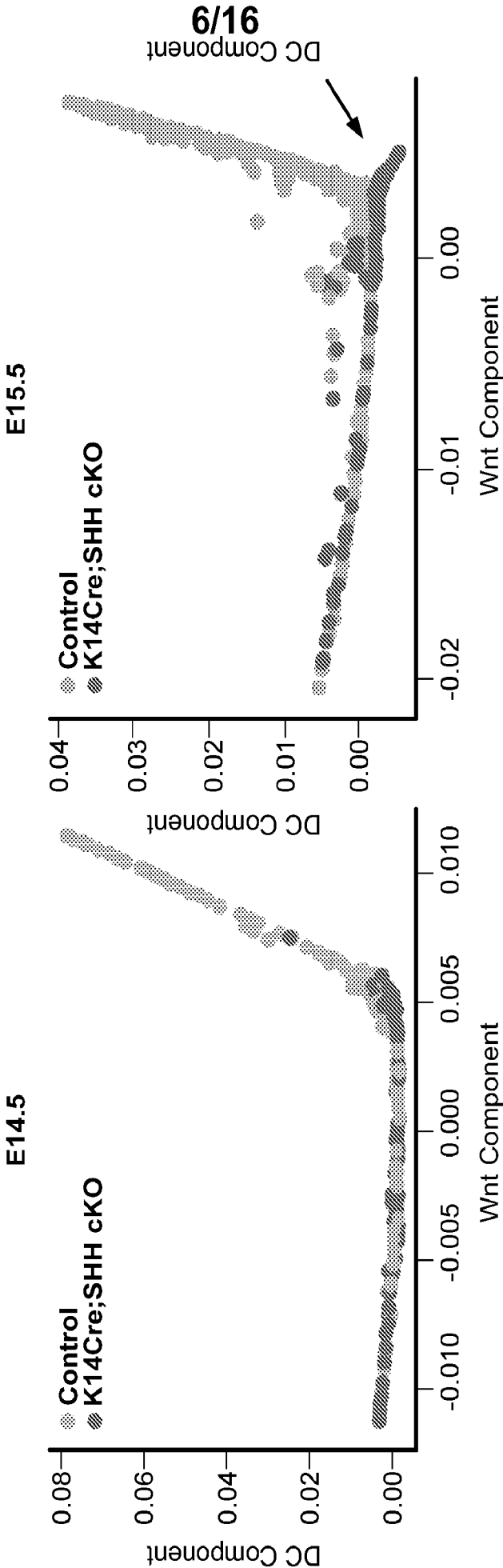


FIG. 3I

FIG. 3H

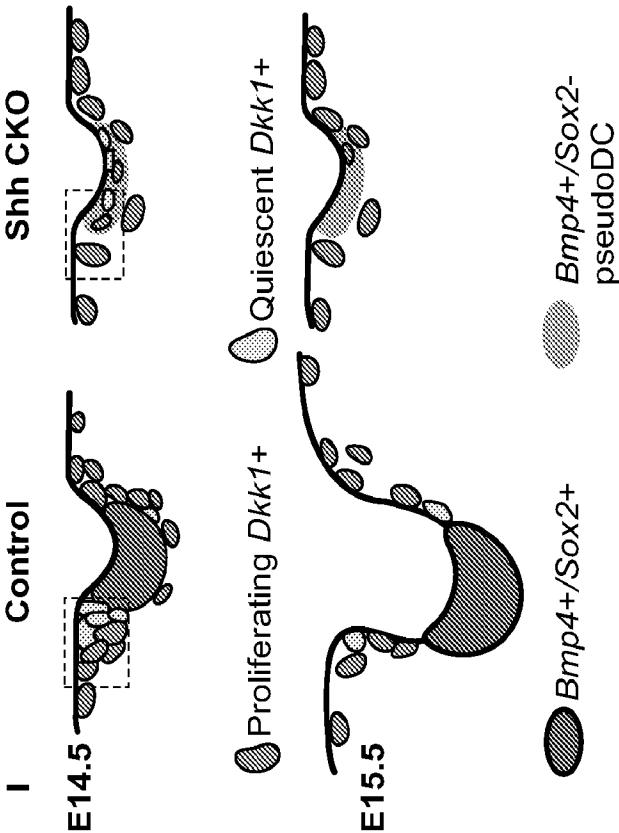


FIG. 3M

FIG. 3L

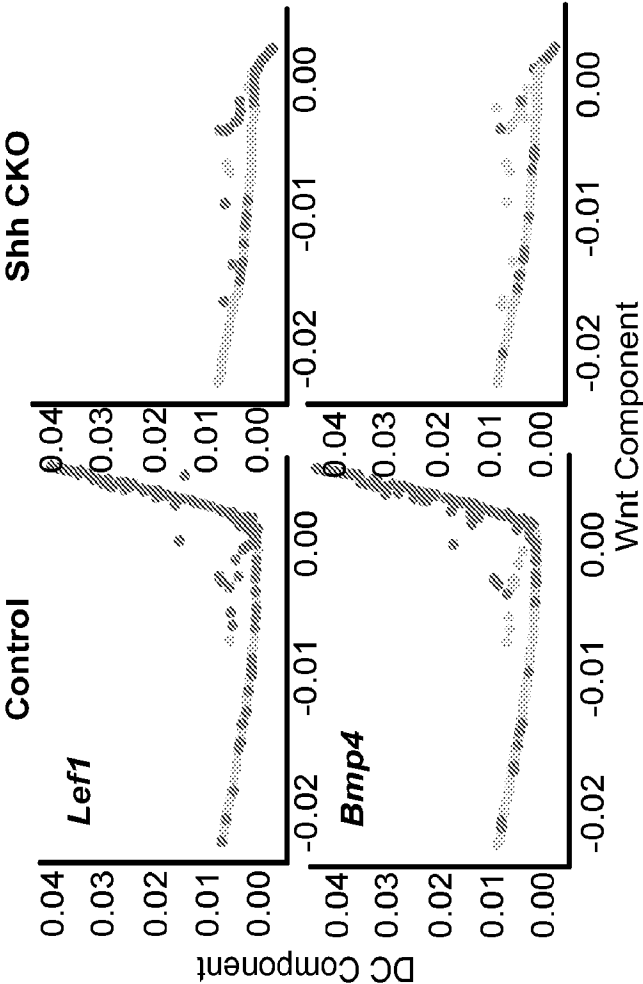
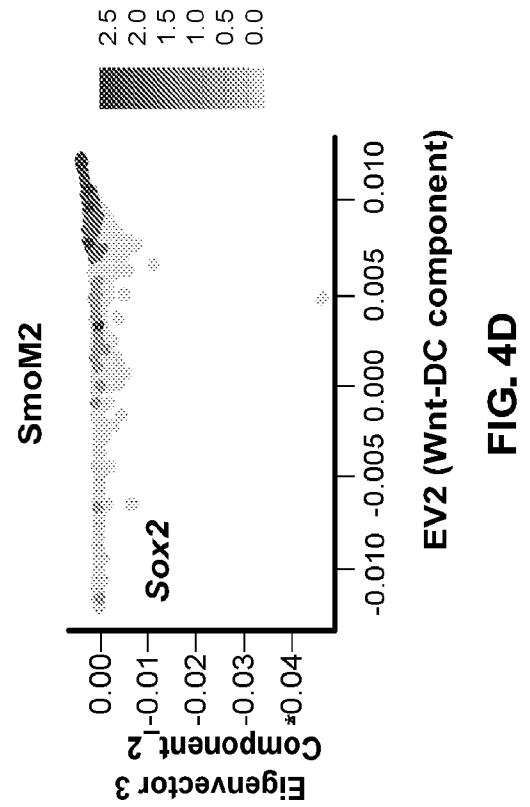
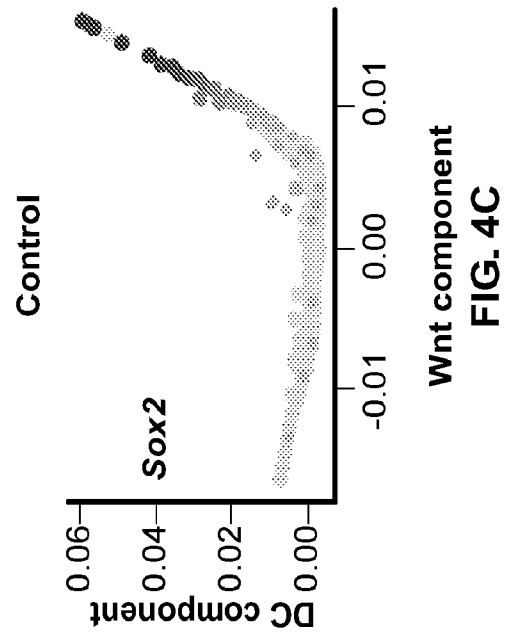
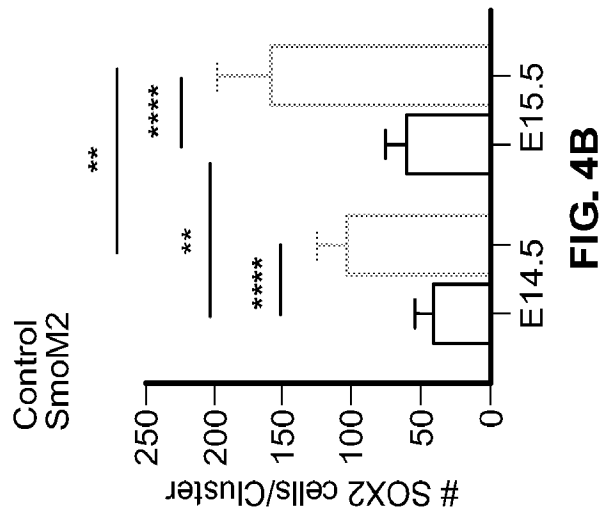
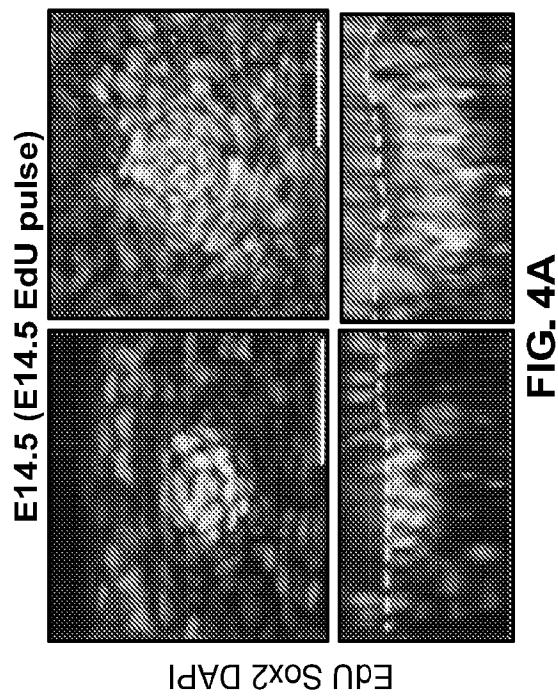
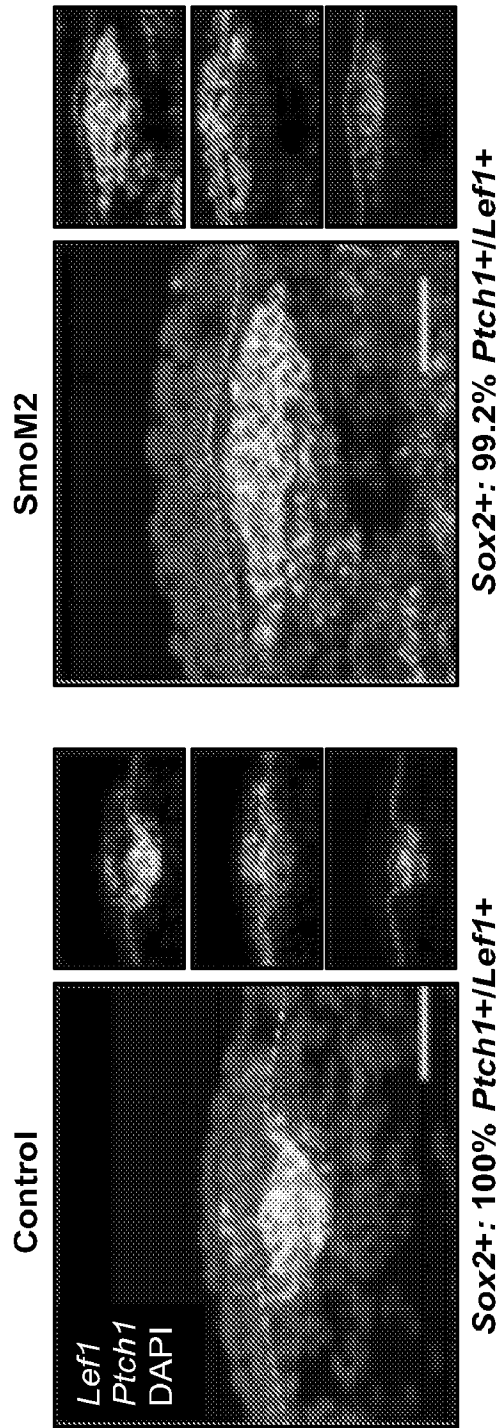
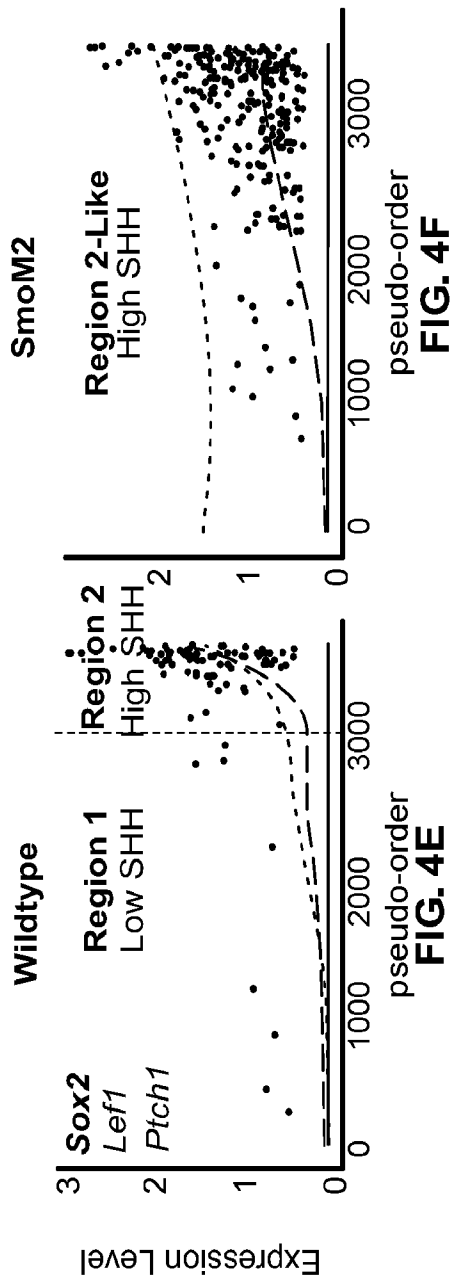
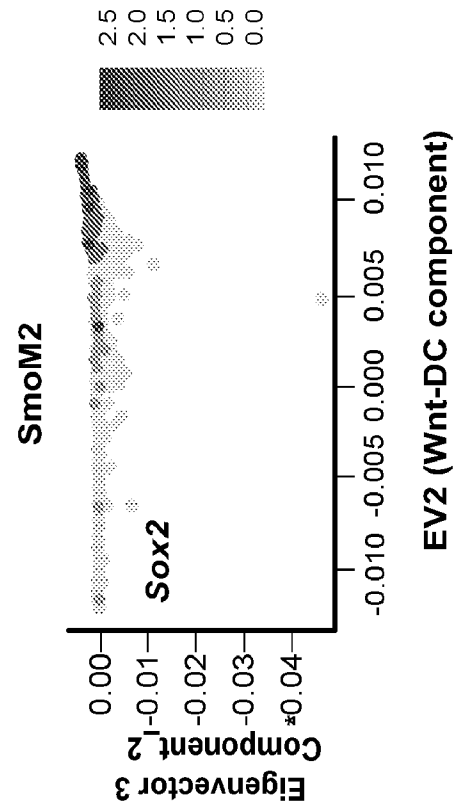
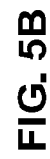
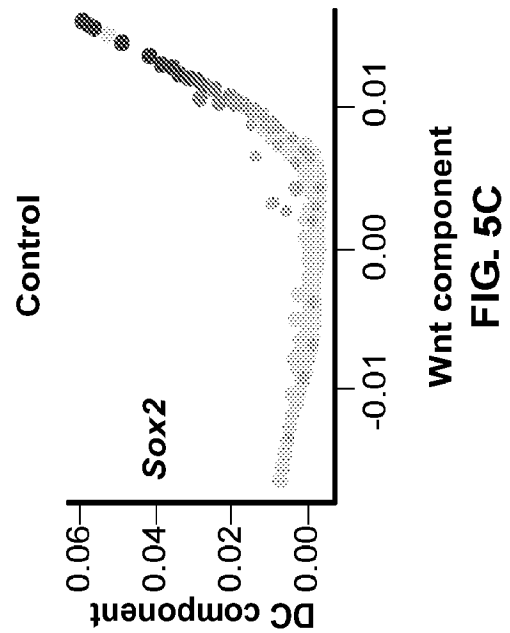
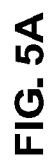
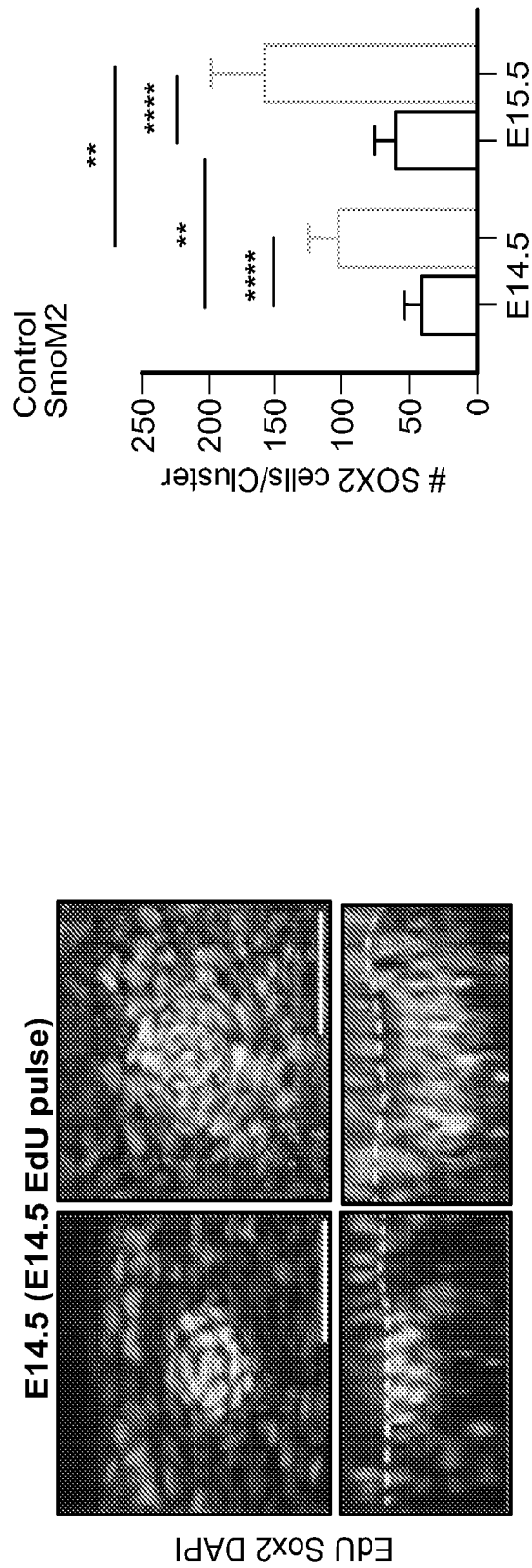


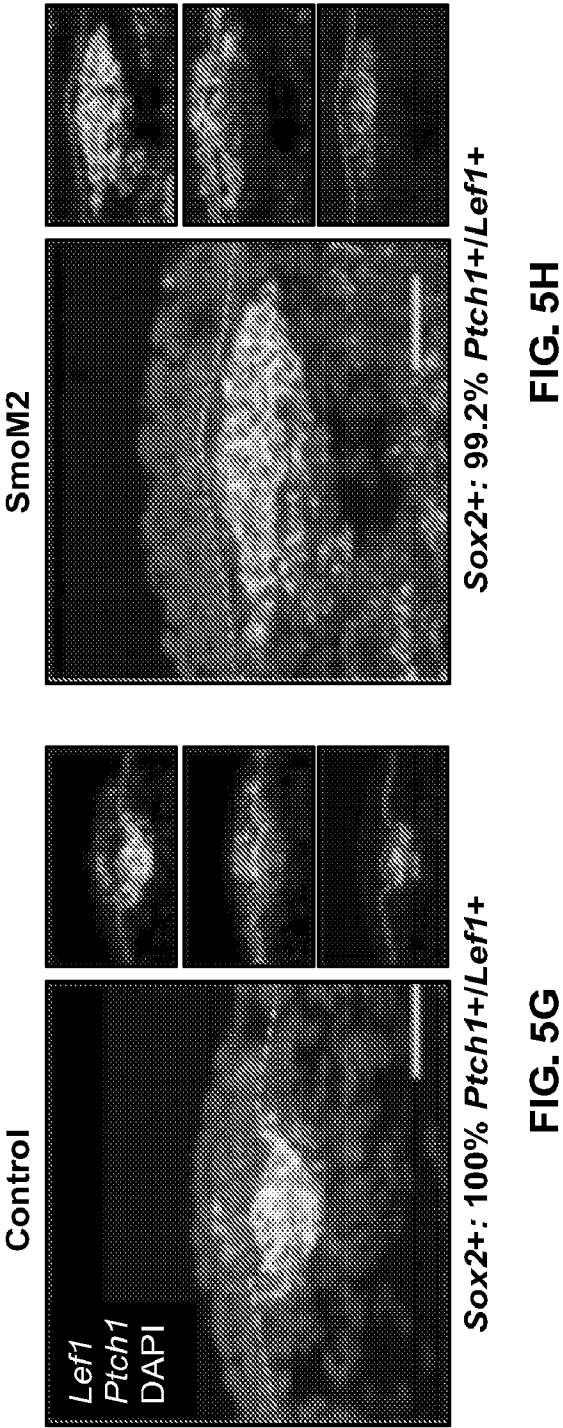
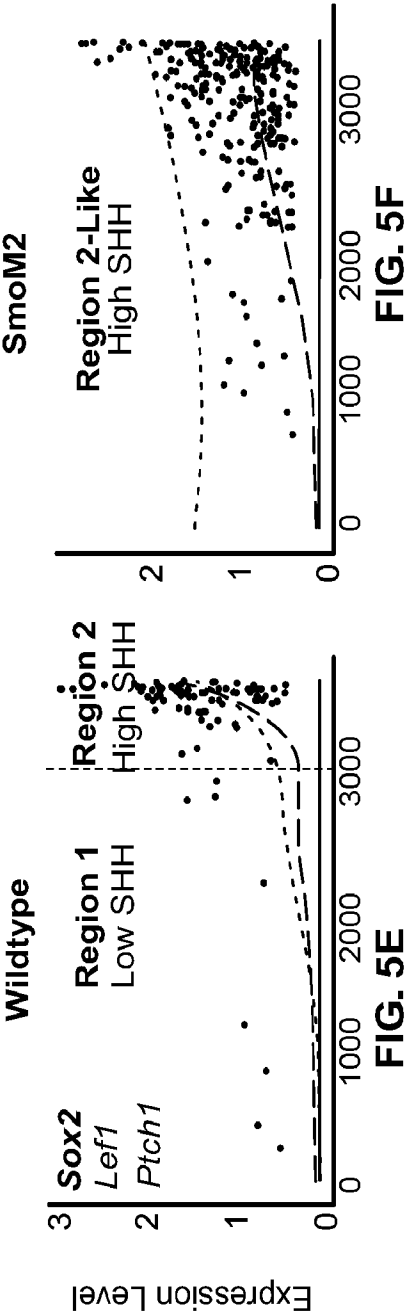
FIG. 3K

FIG. 3J









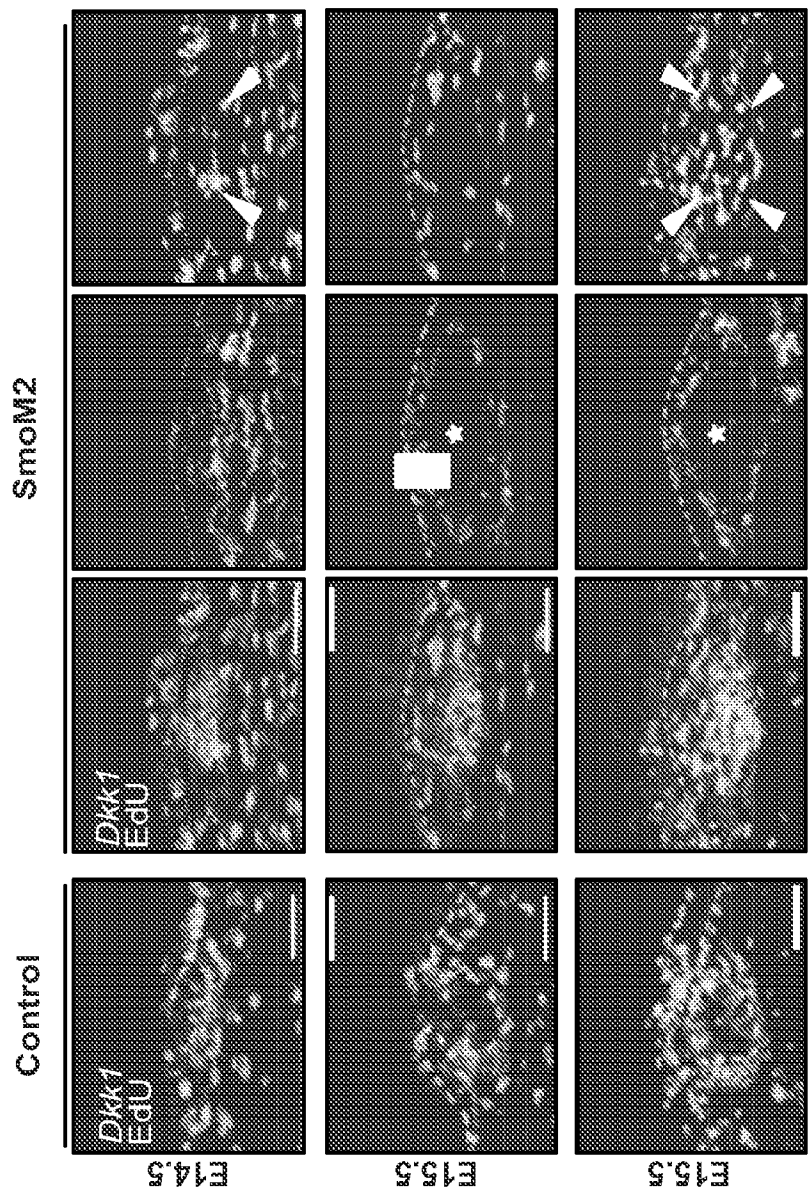


FIG. 5J

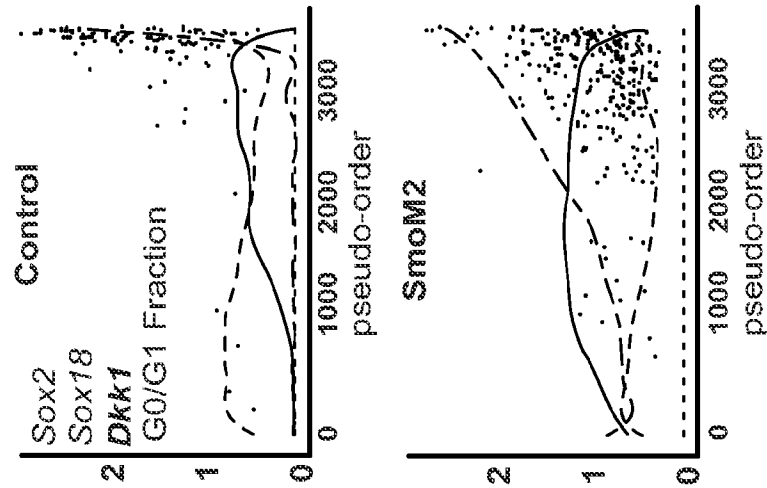


FIG. 5I

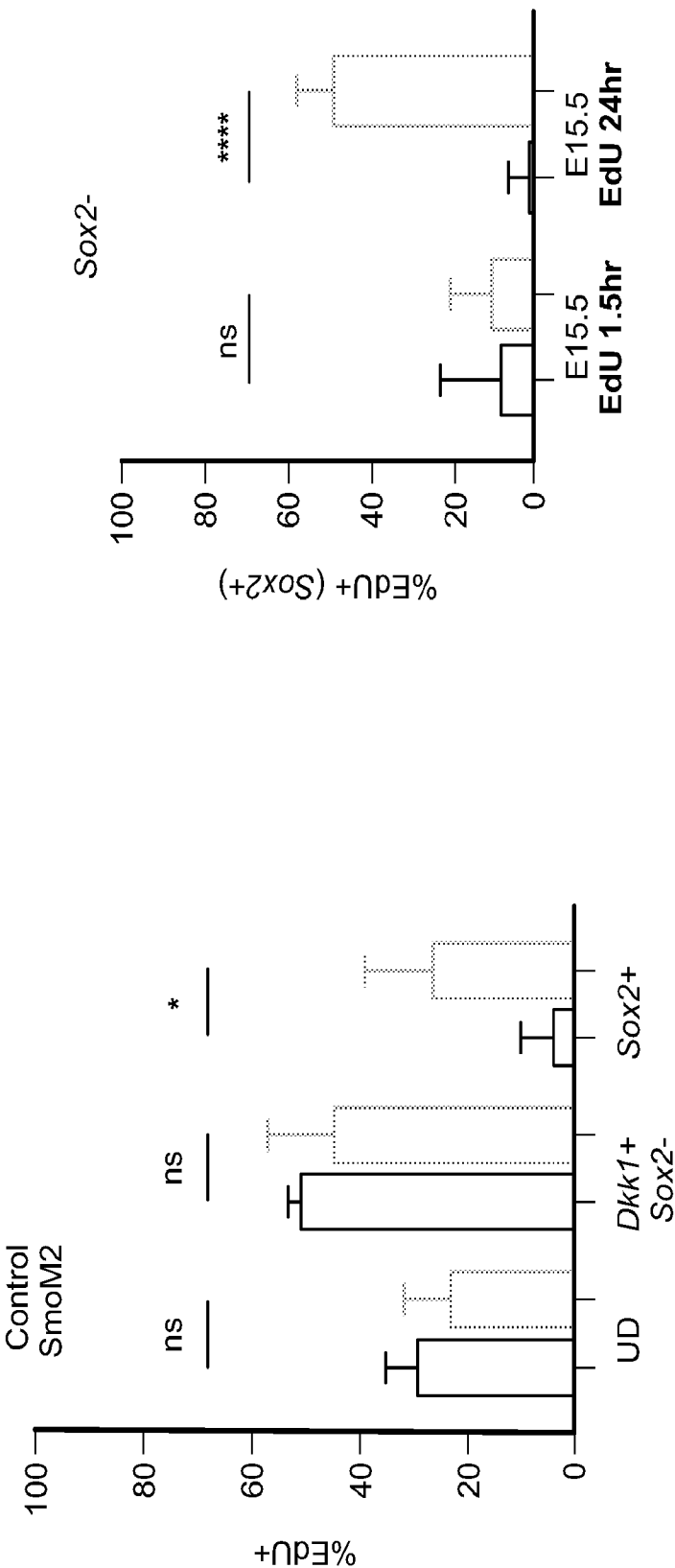
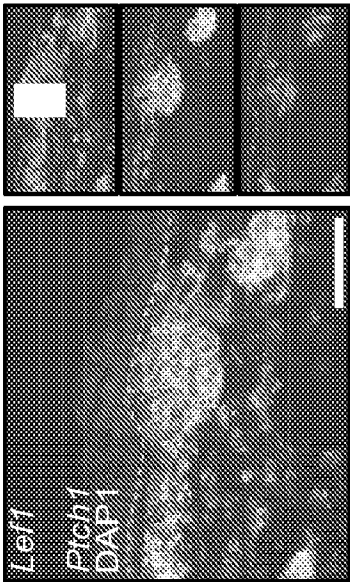


FIG. 5L

FIG. 5K

E14.5 SmoM2;βcatEX3



Sox2+; 97.9% Ptch1+; Left1+

FIG. 5M

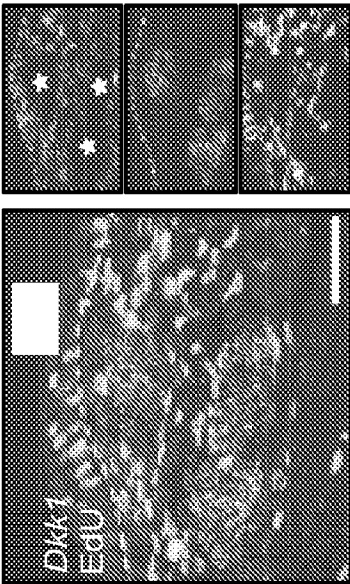


FIG. 5N



WT	
SmoM2	
SmoM2 βcatEX3	

- Proliferating *Dkk1*+
- Penultimate dividing *Dkk1*+
- Quiescent *Dkk1*+

FIG. 5P

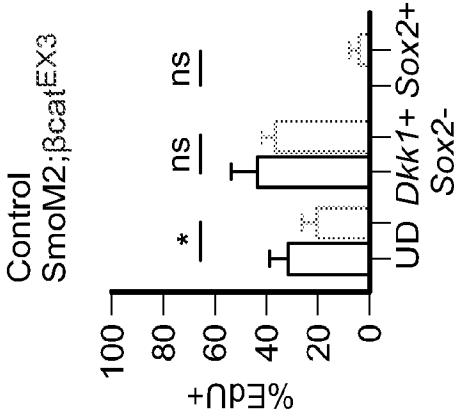
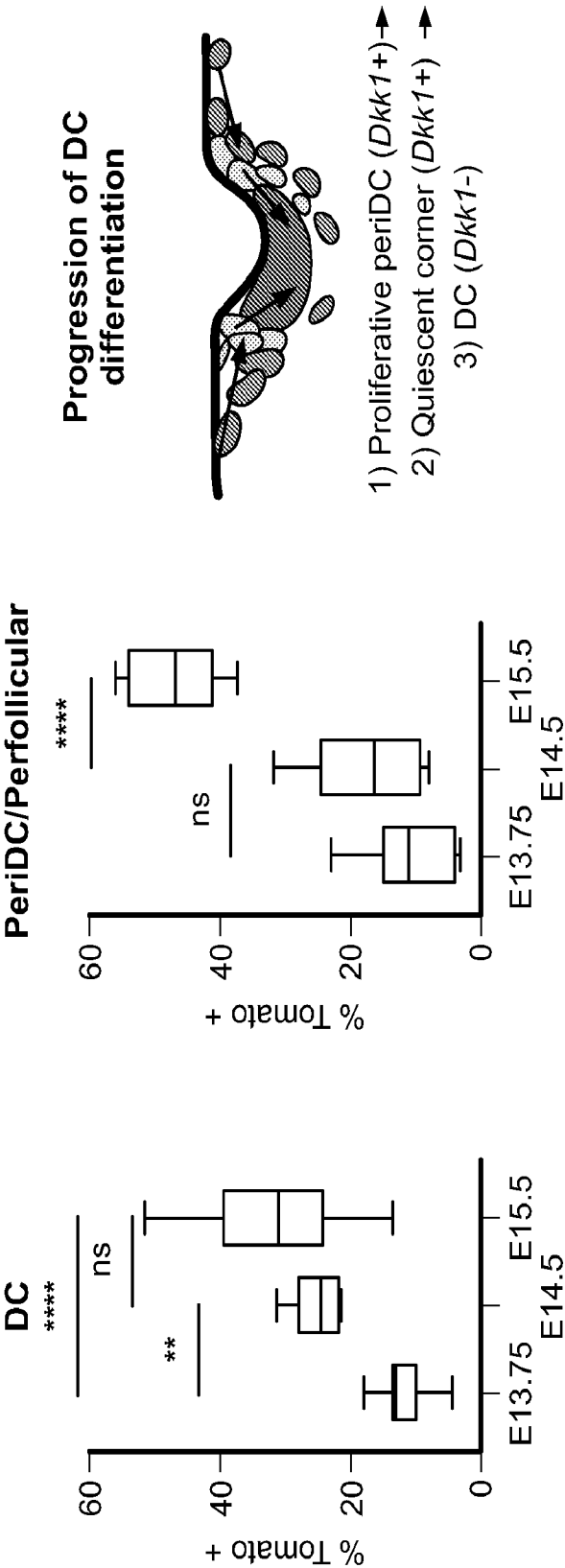


FIG. 5O



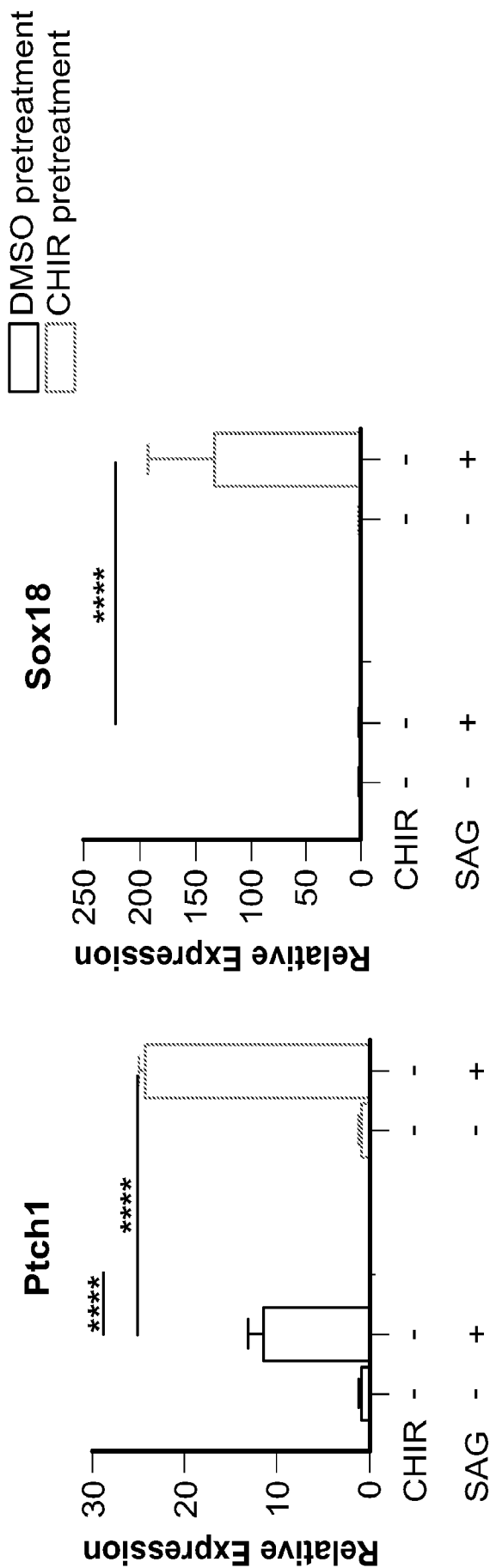


FIG. 7A

FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/037637

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A61K31/4436 A61P17/14	A61K8/00 A61K31/506 A61Q7/00
	G01N33/58	
ADD.		
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)		
A61P A61K A61Q G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
EPO-Internal, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2020/113913 A1 (LOOSE CHRISTOPHER [US]	1-3,6,7,
Y	ET AL) 16 April 2020 (2020-04-16)	9-15
	claims 61-77,212-214	1-15
	paragraph [0559] - paragraph [0560]	
	paragraph [0562]	
	paragraph [0582]	
	paragraph [0624]	
	paragraph [0626]	
	paragraph [0629]	

X	US 2020/121681 A1 (LOOSE CHRISTOPHER [US]	1-3,6,7,
Y	ET AL) 23 April 2020 (2020-04-23)	9-15
	claims	1-15
	1-7,10-11,14,17,20-21;008-9,12-13,15-16,18	
	-19	
	paragraph [0141] - paragraph [0142]	
	paragraph [0567]	
	paragraph [0632] - paragraph [0634]	

	-/-	
☒ 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		
"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		Date of mailing of the international search report
31 October 2024		13/11/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Steinheimer, K

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2024/037637

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International application No
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