UNIVERSITY OF CALIFORNIA, SAN DIEGO

Fgfr2 and Its Contribution to Hair Morphology Variation

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Biology

by

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The Thesis of Josue Rafael Gutierrez is approved and it is accepted in quality and form for publication on microfilm and electronically:

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2014

Dedication

This is dedicated to my family.

Rafael, Lila, Nikki, Gianna for supporting me and always encouraging me to pursue my passions.

Table of Contents

List of Figures

Acknowledgements

I would like to thank Dr. Benjamin Yu for accepting me into his lab and giving me an opportunity to find a new interest of mine. I have learned a lot these past four years, and have gained a tremendous amount of experience in the realm of science.

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ABSTRACT OF THE THESIS

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Genetic diversity has long been implicated as the main contributor to phenotypic variation and why some humans are susceptible to diseases and others are not. Through new advancements in sequencing technology, many of the genetic variants have already been elucidated to be associated with phenotypic variation, ignoring how these changes lead to alterations in the molecular and cellular processes that lead to these complex phenotypic variations. With high morphological variability among mammals, even within a single species, the hair follicle is a very interesting model to study how these cellular and molecular changes can lead to phenotypic variation. Generating FGFR2 null mice, we investigated how this signaling pathway influences the morphology of the hair follicle. Analyzing the effect of the absence of FGFR2 in the hair follicle, we found that it plays a major role in orchestrating the development of the hair shaft, specifically, the medullary lineage of the shaft.

I.

Introduction

Human and other Mammalian Phenotypic Variation

Humans and other mammals are remarkably diverse, displaying an extreme level of phenotypic variation. This diversity is a direct consequence of the genetic variation, and environmental factors, that provide the basis for evolution. Common epidermal features such as feathers on birds, scales on reptiles, and hair on mammals show a recurring specialization and function that surpasses the normal skin barrier function. Looking at a single epidermal derivative, the hair follicle is one of the most conspicuous, yet variable features among mammals. Even within a single species, hair morphology varies dramatically. For example, a single species of mice has 4 distinct hair types: guard, awl, auchene, and zig zag; all differing in length, diameter, medullation, and number of bends. Similarly, human hair variations are seen among different ethnicities, and even within a single individual as hair shape and structure differs depending on the region of the body. Because of the extreme morphological variation and relatively simple yet complex structure, the hair follicle as a mini-organ is an attractive model to understand how changes in the molecular basis of development can influence multiple parameters of variation within a single structure. Recent studies have elucidated some of the mechanisms that are behind these morphological differences. Ethnic-specific alleles of THCC (Martin et al, 2009), EDAR, and FGFR2, alleles that all are associated with phenotypic variations of the hair, have been identified.

Hair Follicle Biology

Hair follicle morphogenesis is unique compared to the development of other mammalian organs. Being the only mammalian organ that undergoes a continuous cycle throughout its lifespan, the hair follicle borrows many of same events and signaling elements that are characteristic embryonic development such as proliferation, cell-cell interactions, differentiation, and apoptosis to reconstruct the hair follicle. The hair-cycle begins with a proliferative phase termed anagen, followed by an induced regression of the hair follicle, catagen, and lastly, a quiescent phase known as telogen, which awaits induction into the new hair-cycle. During anagen the hair follicle undergoes a morphological process that eventually leads to the minute organ that we visualize when we think about a hair follicle. Orchestration of the development of the hair follicle is under the precise control of signaling cascades and transcription factors such as Notch, Bmp, FGF, Shh, Wnt, EDAR, lef1, Msx1, and Msx2. Together, these influential interactions lead to the instructive development of the most proximal region of the hair follicle known as the bulb. This region holds the rapid proliferating keratinocyte cells known as the matrix, which surround and receive instructions via signaling from the Dermal Papilla (DP).

Differentiation of the matrix cells give rise to 4 concentric circles, each holding a distinct cell-lineage: medulla, cortex, cuticle, and inner-root sheath (IRS). These concentric circles form the complete multi-tissue structure of the hair follicle with the exception of the outer-root sheath (ORS). The ORS is the outermost lineage of the follicle that is not derived from the matrix, but is directly connected with the basal layer of the epidermis. The IRS, adjacently inward from the ORS, is a structure that plays an important role for proper shape of the hair follicle and guides the hair shaft through the skin surface (Kaufman CK et al, 2003). The hair shaft, comprising the innermost layers of the hair follicle, is the only structure of the hair follicle that protrudes from the skin. The innermost lineage of the hair shaft is composed of the medulla cells and can be distinctly characterized by occupancy of air spaces between individual medulla cells. The next layer out holds the cortex cells. This layer is the dominant location of keratin gene expression, producing a highly keratinized structure, which functions as the major source of rigidity in the hair (Langbein et al, 2005). The outermost layer, known as the cuticle is the layer that forms the hair surface which we can visually see protruding from the skin.

FGF Signaling

Despite the complexity of development and its intricate processes to generate entire organs, only a handful of signaling pathways are responsible for orchestrating this highly regulated process. One of them is the Fibroblast Growth Factors (FGF) signaling pathway, a ubiquitous pathway with diverse roles in development and tissue homeostasis. FGF signaling comprises a family of 18 mammalian cytokines known as Fibroblast Growth Factors (FGFs).These ligands act with high affinity and specificity on a specific set of Receptor Tyrosine Kinases known as Fibroblast Growth Factor Receptor (FGFRs). The FGFRs are encoded by 4 distinct genes, generating FGFR1-4. Three of these FGFRs can be alternatively spliced to produce various isoforms, allowing additional specificity and flexibility for the recognition of different ligands producing distinct outputs in a tissue specific manner. Activation of the receptor via ligand binding induces FGFR dimerization, bringing the intracellular receptor kinase domains in close orientation, allowing for auto-transphosphorylation and further activation of the kinases (Goetz and Mohammadi et al. 2013). Activation of the receptor is known to transduce activation of the mitogen activated protein-kinase (MAPK) pathway, as well as the activation of the phosphoinositide 3-kinase (PI3K) and signal transducers and activators of transcription (STATs). These processes must be strictly orchestrated in order to prevent developmental defects or disease.

FGFR2 Role in Hair Morphology

FGFR2 signaling has been shown to be a major factor in hair morphogenesis. However, it has yet to be discovered how FGFR2 signaling is interpreted, leading to its involvement in morphogenesis of the hair follicle, and how alteration of its instructions leads to hair structural variation. To investigate the role of FGFR2 in the mouse hair follicle we created a FGFR2 null mouse. The excision of the gene is under the control of Msx2-Cre; Msx2 is expressed in the hair bulb during early anagen (Ma et al, 2003). Normally, FGFR2 is temporally expressed throughout the early to late anagen phase of the cycle. The expression is also restricted to the matrix cells, from the base of the hair follicle to the top of the bulb, and is subsequently lost during differentiation as these cells move up the hair follicle (Rosenquist and Martin et al, 1996).

Hair variation

Genetic changes leading to altered regulation of these of these FGF signaling pathways, or any of the many other signaling pathways involved in the development and morphogenesis of the hair follicle, can have severe morphological consequences. A well-known transgenic mouse model known as the Angora mouse, generates mice null for Fibroblast Growth Factor 5 (FGF5). This genetic change causes the duration of the anagen phase of the hair-cycle to be extended, producing mice with abnormally long hair (Herbert et al, 1994). Another existing transgenic mouse model produces mice that overexpress Wnt3, causing a short hair phenotype (Millar et al., 1999). Additionally, genetic variants in the THCC gene are associated with hair curliness in Europeans (Medland et al, 2009), whereas variants in the EDAR and FGFR2 genes seem to be associated with thicker and straighter hair in Asians (Fujimoto et al, 2009) (Tan et al, 2013). With vast morphological variation yet common morphology within this epidermal appendage, the hair follicle represents an excellent model to discern how changes in the molecular basis can have influential consequences that give rise to variation within a single structure.

II.

Results

Mice lacking FGFR2 have abnormal hair coat

Expression of the Fibroblast Growth Factor Receptor 2 (FGFR2) in the hair follicle is localized to matrix cells of the bulb (Rosenquist and Martin, 1996). Agonist FGF7 and FGF10, specific for FGFR2 (garashi et al., 1998; Ornitz et al., 1996), are expressed and released from the Dermal Papilla (DP), a signaling unit used to instruct development of the hair follicle. To elucidate the role of FGFR2 in the morphogenesis of the hair follicle, we generated FGFR2 null mice under the control of Msx2-cre. Msx2-cre is active postnatal in the hair follicle, specifically in the matrix cells of the hair follicle. FGFR2 null mice produced mice with an abnormal hair coat, having a shaggy appearing hair coat. This was most likely due to mutant mice having longer and thinner hair. Analyzing the hair shaft, we observed severe aberrations to the structure and organization of the inner portions of the hair shaft. Altered morphology of the regular air spacing between medulla cells is the most noticeable structural variance, as well as the irregular disbursement of melanin throughout the hair shaft (H&E).

To define the specific cell types affected by the knock out, we performed immunofluorescence using lineage specific markers such as AE15, which marks the Inner-root sheath and medulla cells, as well as AE13, which stains for cortex cells. The outcome of this experiment showed that medulla cells are completely absent from the hair shaft, while all other cell types in the hair follicle seem to be present, suggesting that FGFR2 KO exclusively affects the medulla cells of the inner hair shaft.

Underlying mechanism producing the absent medulla compartment

Hair follicles undergo cyclical waves of regular growth induction, where matrix cells are signaled to proliferate and eventually contribute to the hair follicle structure. Hair follicles also undergo a regression phase, driven by an organized apoptosis event. To understand the reason for the absence of the development of the medulla compartment, we first asked if the absence was due to a newly acquired apoptosis event. Staining both mutant and control hair follicles with pH2AX and cleaved caspase-3, cell-death markers, we failed to observe any staining for these markers, suggesting that premature apoptosis of progenitor cells or of newly differentiated medullary cells is not the cause for the absence(Immuno-pH2AX, Casp-3) of medulla cells. Another possibility we hypothesized for the absence of medullary cells was an attenuated proliferation of progenitor cells. Using various proliferative markers to stain both control and mutant, we noticed that the marker Cyclin-D in the mutant was rarely seen in cells juxtaposed to the DP. Additionally, Cyclin-D positive staining was not seen distally above the DP, whereas positive staining was frequently observed in the control. Assessing another proliferative marker, phospho-histone H3 (pH3), the control seemed to have a similar staining frequency of cells stained for this marker when compared to the mutant (Immuno-pH3). A third proliferative marker was used to assess proliferation, and again, a noticeable difference was observed. Positive staining of Proliferative Cell Nuclear Antigen (PCNA) in the lower half of the hair bulb was seen in both control and mutant. However, when analyzing the top half of the bulb and into the early segment of the hair shaft,

mutant hair follicles had negative stained cells in these areas whereas control were always stained positive (Immuno-PCNA).

These results together suggest a proliferative defect in the mutant, and support the hypothesis of an attenuated proliferation rate of the progenitors which give rise to the medullary compartment, consequently leading to the absence of the medulla cells.

Sox9 as a Pre-medulla lineage marker

In previous studies, it has been shown that a Sox9-FGF signaling modulation is used in the development and morphogenesis of many organs, in both mammals and zebra fish. With this in mind, we costained for Sox9 and AE15 in wild type and mutant hair follicles at P7. We found that wild type hair follicles stained positive in the ORS, which is already known to be expressed in that lineage. However, to our surprise, we found cells, usually in pairs, that were stained positive for Sox9. These cells seemed to represent medulla progenitors, as they were consistently found to be in the upper-middle region, distal to the DP. These Sox9 positive cells were usually found stacked in groups of three to four cells and adjacently coupled. The most distal positive cells seemed to lead into the medulla region, where frequent positive costaining for both Sox9 and medulla marker (AE15) was observed.

Fate mapping mitotically active cells and proliferative assessment of the matrix

Production of all the structures in the hair follicle, except for the outer-root sheath, originates from the lower portion of the hair follicle known as the matrix. The matrix is a highly proliferative population of progenitor's cells. As these cells exit the cell-cycle, they migrate upward and begin the process of terminal differentiation, producing many concentric circles. Each of these adopts a distinct differentiation program, producing all the various structures found in the hair follicle.

Assessing the morphological differences in the hair shaft, the pre-cortex (Immuno-Lef1) and cortex (Immuno-AE15) in the mutant seemed to have a noticeable difference. Starting from the upper region of the bulb, and well into the hair shaft, cortex positive cells seemed to exhibit a collapsed cortex structure, filling the void where medulla cells normally reside. To assess whether the observation of cortex cells in the medullary inner shaft region is due to a fate transition of medulla progenitors into a newly acquired cortex identity, we evaluated the rate of cell-cycle entry and exit into terminally differentiated lineages by EdU labeling. Co-staining for EdU and differentiation markers of the IRS and hair shaft allowed us to quantify the number of actively dividing cells in the hair bulb, which is good way to assess proliferative differences between the mutant and control. Furthermore, we were also able to quantify dividing cells that advance into the various differentiated lineages of the hair follicle.

Both control and mutant mice were injected with EdU at P4 and pulsed at two different time points: 1 hour and 36 hours. Analyzing the 1 hour mice allowed us to gain a baseline for the number of EdU positive cells in the bulb. Furthermore, the total number of cells in the bulb were also quantified so any differences in the amount of EdU positive cell in the bulb or hair shaft can either be explained by a difference in the amount of starting material, or a change in the rate of proliferation.

Both control and mutant hair bulbs at the 1 hour pulse had about the same number of cells per bulb (Graph and Immuno-EdU), and the same percentage of EdU positive cells per bulb (Graph and Immuno-EdU). To directly assess the hypothesis of the cell-fate transition of medulla progenitors into cortex cells we quantified the number of cortex cells that were stained positive for EdU. Assuming this hypothesis is correct we would expected to see an increase of cortex cells that are EdU positive, since a greater number of progenitors are contributing to that specific lineage. Measuring for this, we did not find any noticeable difference in the number of cortex cells between mutant and control (Graph and Immuno-EdU), suggesting that there is no cell-fate transition.

Measuring the number of terminally differentiated EdU positive cells, we found that mutant mice had a significantly lower number of differentiated cells. (Graph and Immuno-EdU). Since these EdU positive differentiated cells originated from the matrix of the hair follicle, one would expect a higher total

number of EdU positive cells that remain in the bulb. Assessing this hypothesis, we confirmed our assumption by quantifying a higher number of EdU positive cells that were retained in the bulb of mutant mice, suggesting a defect in its terminal division into the hair shaft.

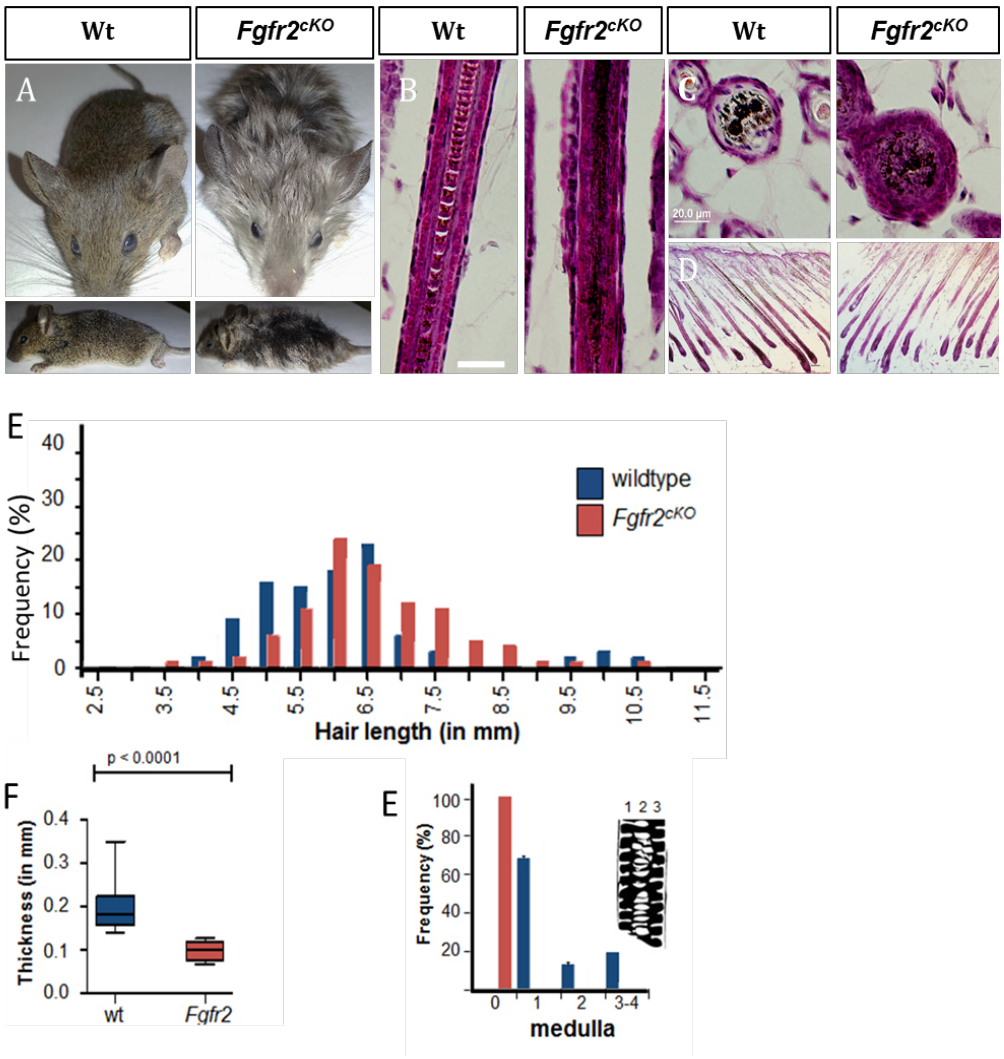

Figure 1. (A) Images of wild type and Fgfr2cKO mice. (B)(C) H&E on transverse and (D) horizontal sections of both wild type and Fgfr2cKO hair follicles. Quantification of hair length (E), diameter (F), and medullation (E) of the hair follicle.

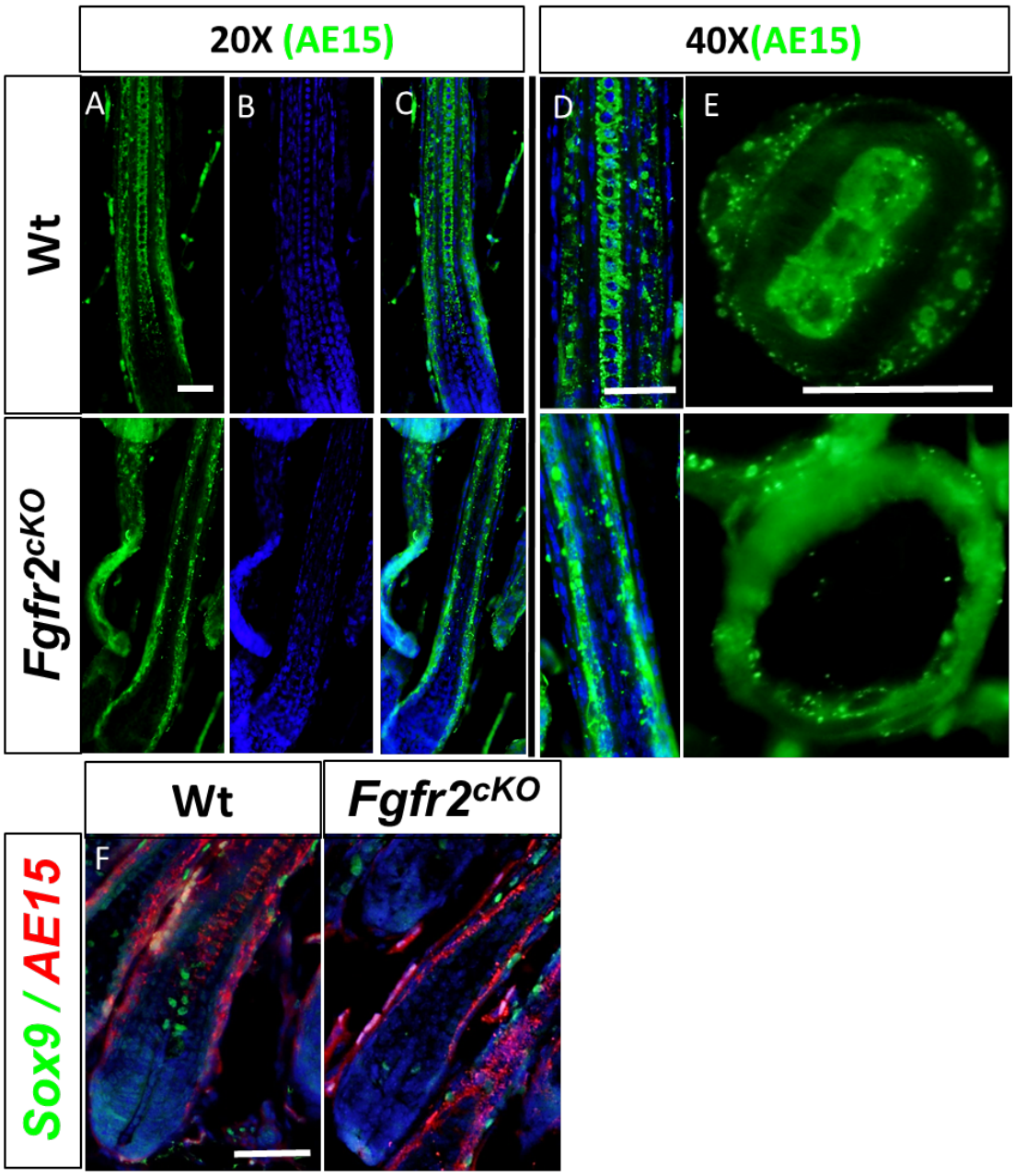

Figure 2. Assessment of the IRS and medulla lineages in the hair follicle with the use of AE15 (A), DAPI (B), and AE15+DAPI (C). Transverse sections (E) were also stained using AE15. Hair follicles were stained with a putative medulla progenitor marker (Sox9) (F).

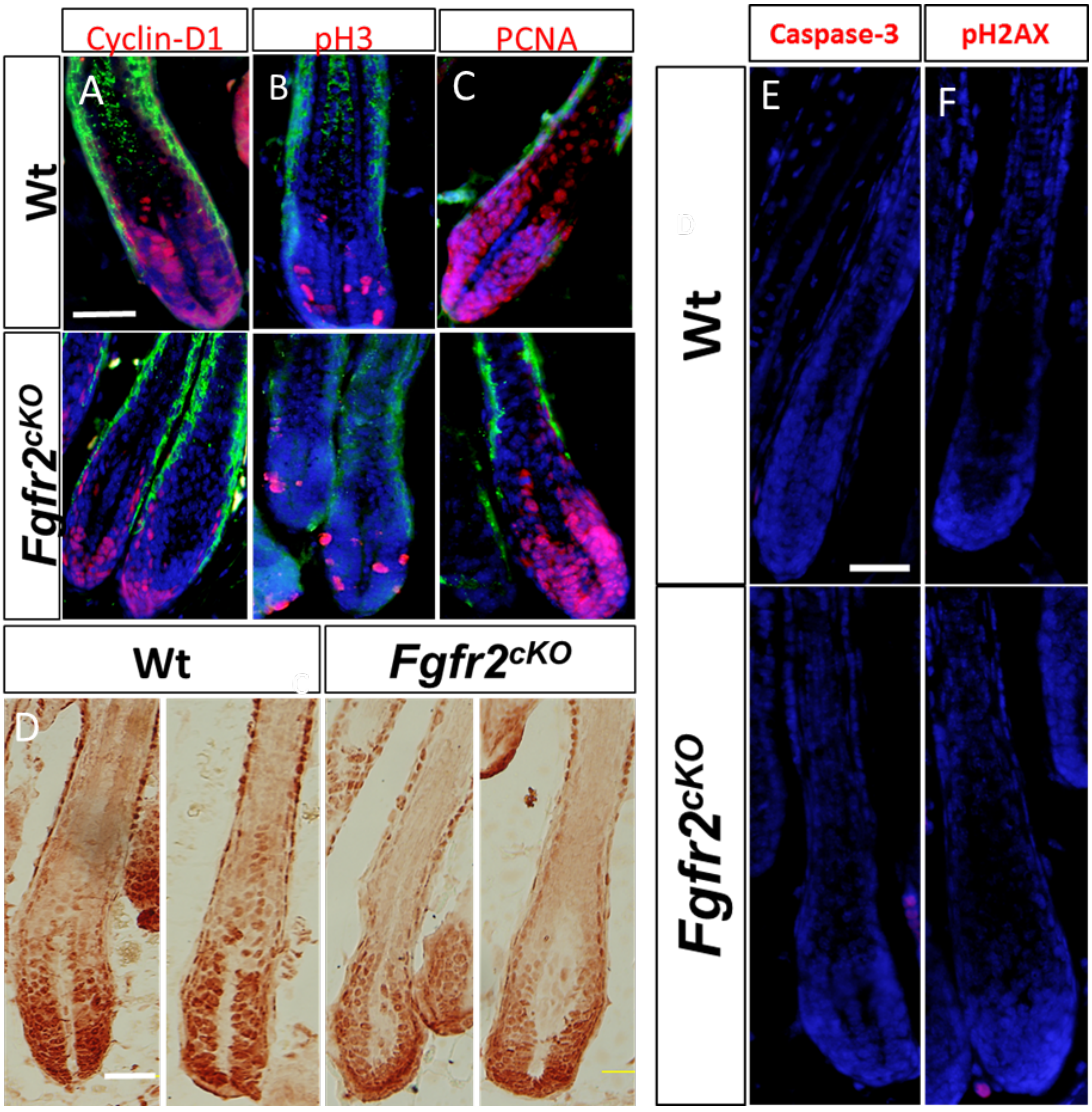

Figure 3. To asses a proliferative defect hair follicles were stained with variety of proliferative markers: Cyc-D1(A), pH3(B), and PCNA (C,D). Two cell-death markers (Casp-3 (E), and pH2AX (F)) where used to rule out any aberrant apoptosis events.

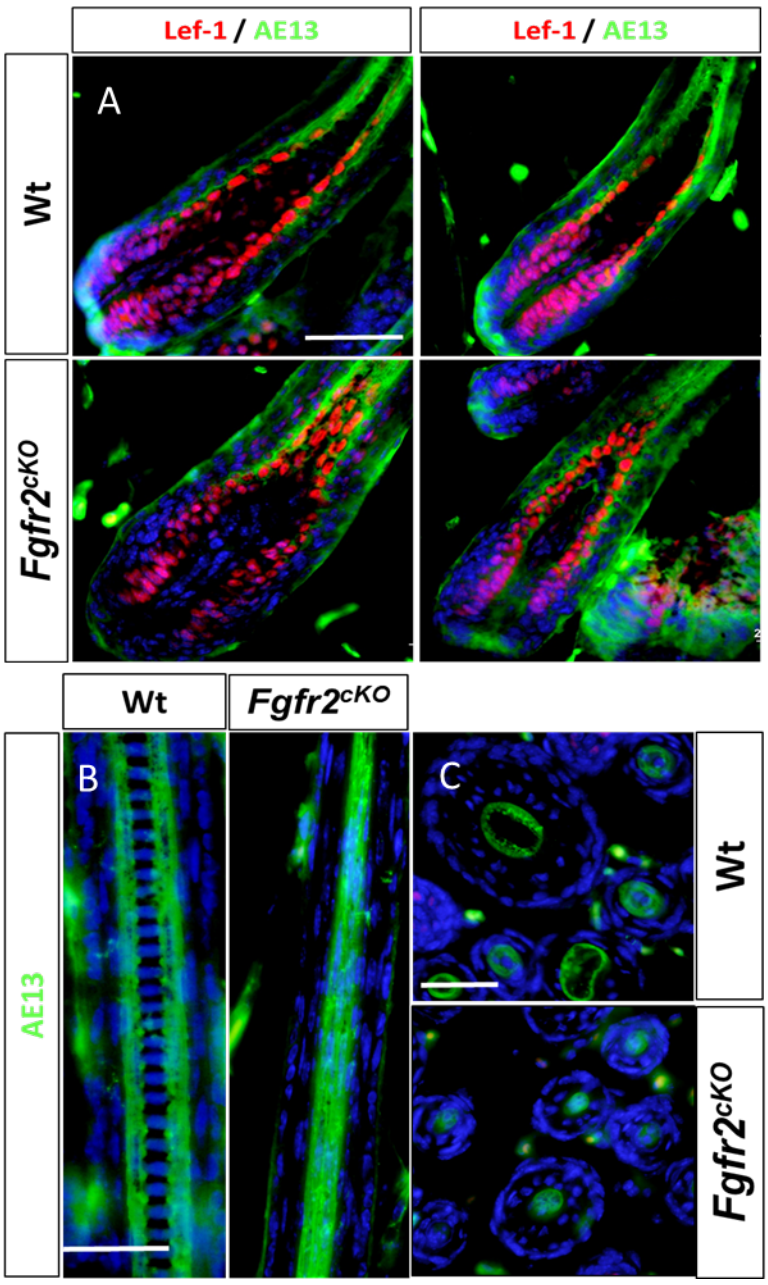

Figure 3 continued. pre-cortex and cortex lineages were analyzed using Lef-1 (A) and AE13(A, B) markers, respectively. Transverse sections of the hair follicle were also stained with AE13 (C).

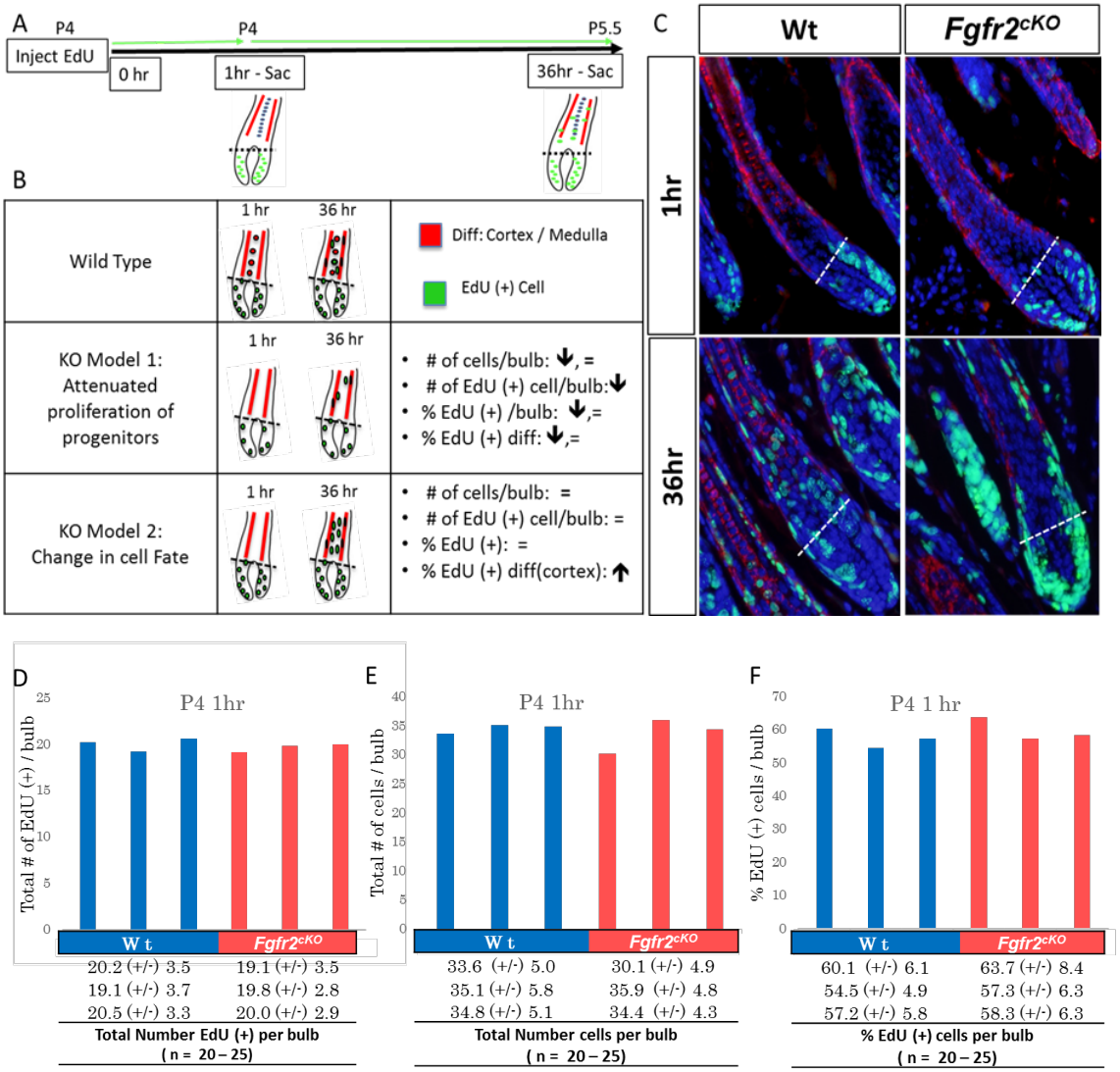

Figure 4. (A) Diagram showing the time course of EdU injection and time points of when mice were collected. (B) Table with expected results of fate map and proliferation results. (C) Transverse section of hair follicles showing EdU positive cells (in green) and costained with AE15 (in red). (D) Total number of EdU (+) cells, (E) total number of cells, (F) % of EdU (+) cells that are represented in the bulb 1hr after EdU injection.

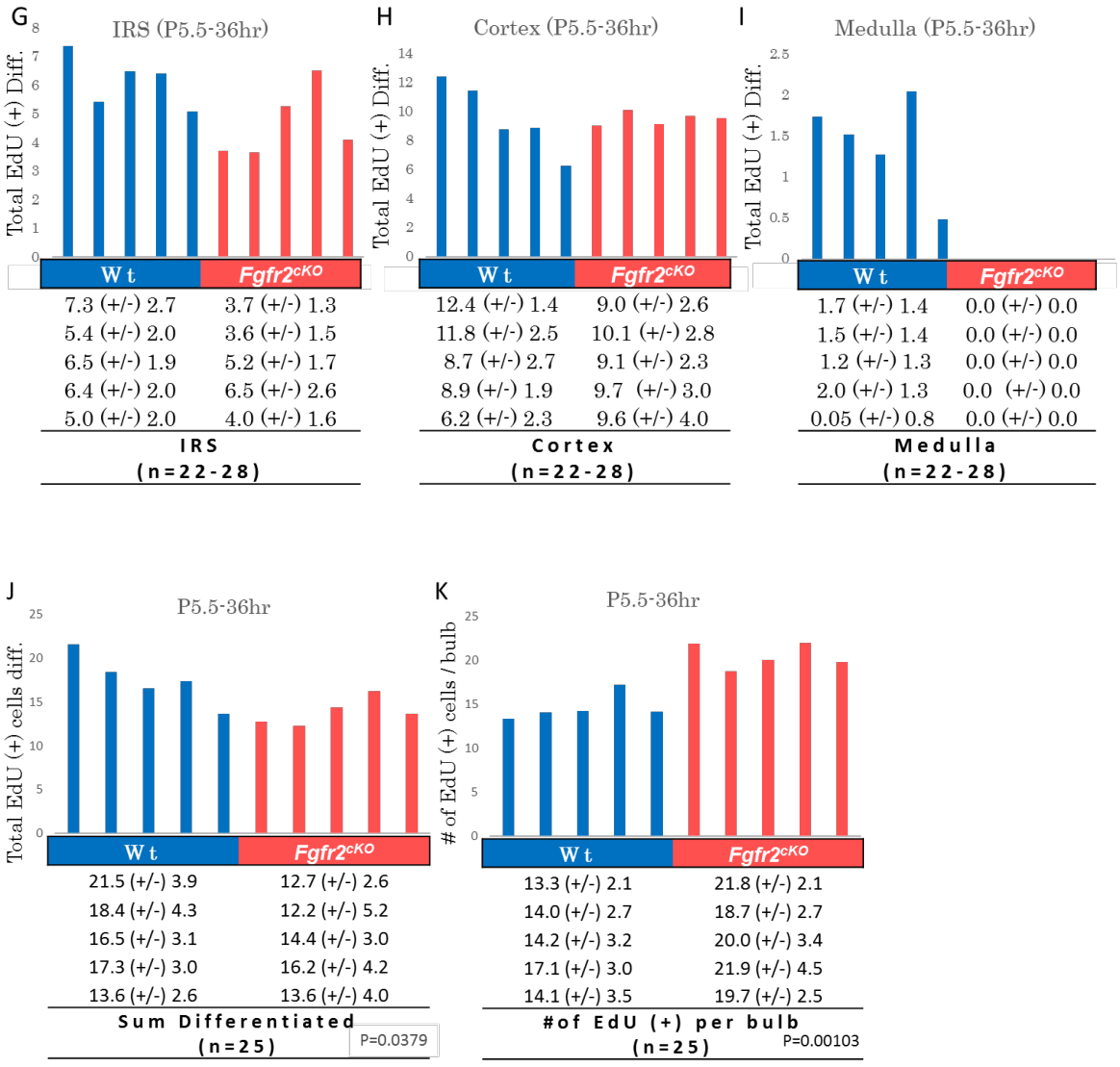

Figure 4 continued. Fate of cells were analyzed by injecting mice at P4 at and collected 36 hours post injection. (G) Total number of EdU (+) cells that were identified to be IRS cells. . (H) Total number of EdU (+) cells that were identified to be Cortex cells. (I) Total number of EdU (+) cells that were identified to be Medulla cells. (J) Total EdU (+) cells that were identified to be IRS, Cortex, or Medulla cells. (K) Total number of EdU (+) cells that were counted in the bulb of the hair follicle.

III.

Discussion

Role of FGFR2 in the morphogenesis of the Hair Follicle

This model exhibits how a change in a single signal modulator can have a substantial effect on the morphological development of the hair follicle. Our study reveals that FGFR2 plays an essential role in the formation of the hair shaft, specifically regulating the medullary lineage while leaving the cuticle and cortex unaffected. Even though we did not specifically address the cuticle lineage in our analysis, it was indirectly analyzed as we examined the cortex lineage as it has been shown that both cuticle and cortex cells share a common clonal origin and molecular marker (AE13).

Hair length is normally regulated by the duration of the anagen phase of the hair cycle. This has been shown in previous reports which indicate that loss of FGF5 leads to a longer anagen hair cycle, producing mice with longer hair (Herbert et al, 1994). In our mouse model we have quantified our FGFR2 null mice to have significantly longer hair, reflecting that FGFR2 has an influence on hair length that is independent from the cell cycle, as both transgenic and wild type hair follicles undergo the same time lapse of anagen.

The hair shaft diameter also seemed to be affected, having a smaller circumference. This is most likely due to the absence of the medulla cell, as the cortex cells collapse and fill the void where the medulla cells usually occupy. Initially, we thought that there may have been a cell fate change of pre-medulla progenitors to cortex cells, observing cells marked with a cortex marker in the inner region of the hair shaft. We expected an increase in cortex cells in the

mutant, but this hypothesis was proven wrong after our EdU fate analysis proved to have no increase in cortex cells in the hair shaft.

Proliferative Defect and Retention of the Medullary Progenitors

Assessing the hair bulb for proliferation by using proliferative markers and performing EdU labeling, we found that there was no proliferative defect on the supply of matrix cells in the mutant compared to the wild type. What was not indistinguishable is that the wild type hair follicle possessed cells adjacent to the DP, representing medullary progenitor cells. These cells extended more distally into the hair shaft, whereas mutant hair follicles lacked a positive proliferative marker in the region above the DP. These observations are in line with previous findings where a dominant negative FGFR2IIIb was used and a similar proliferation assay was performed using a ki67 antibody, which showed medulla progenitors expanding more distally in the wild type (Schlake et al, 2005). Together, both of these findings support the results from our EdU analysis, as we measured both EdU positive cells in the bulb and ones that were terminally differentiated in the hair follicle. Our findings revealed a lower number of differentiated cells in the hair follicle, and an increase of EdU positive cells in the bulb. Observing a reduction of medulla progenitors terminally dividing above

the DP and a retention of EdU positive cells in the matrix suggests a defect in the terminal division of medulla progenitors into the hair shaft. FGF signaling has been identified as a main mitogenic signal, involved in promoting proliferation and differentiation to guide developmental processes in a variety of organs (De Moerlooze et al, 2000), and could explain the total lack of division or a delayed cell-cycle event, which contribute to these observations.

Future Directions

Interestingly, mice null for ligands (FGF7, FGF10) specific for FGFR2 have modest to no effect on hair morphology. Most likely due to some overlapping roles that both ligands have. Ablation of FGF7 in mice lead to a matte? Coat appearance (Guo et al, 1996), whereas mice null for FGF10 have no obvious hair defect but do possess epidermal aberrations (Suzuki et al, 2000). Using RNA seq or qPCR approach to understand the full function that FGFR2 has or possibly orchestration with other important regulators needs to be further examined. It is known that the matrix expresses a variety of signaling components during growth and differentiation. Wnt molecules are one of the more prominent factors, as various lineages express a distinct Wnt molecule (Reddy, 2001). Additionally, numerous Bmp ligands are utilized as they function to inhibit proliferation, instructing the dividing progenitors to terminally differentiate (Rendl et al, 2005).

Sox9 is already known to have an important role in the specification of a single lineage in the hair follicle, the ORS. Ablation of this gene in mice produces mice with hair follicles very different from wild type. The most prominent change found in the ORS lineage in Sox9 null mice, is an ORS that is several layers thick, instead of the usual single cell layered. Ectopic staining is also observed using many of the epidermal and hair follicle molecular markers, suggesting that it plays an important function in maintaining the identity of the ORS (Vidal et al, 2005). On the other hand, Sox9 has not yet been shown to have any function in specifying the medullary lineage in the hair follicle. However, developmental biology has shown that both Sox9 and FGFR2 have integrated functions in specifying cell fate. This has been shown in mice in which pancreatic identity is regulated by mesenchymal signaling of FGF10, which induces FGFR2 to perpetuate Sox9 expression in pancreatic progenitors. This highly organized cascade then forms a feed-forward loop, ultimately controlling the identity of the pancreatic progenitor critical for developing the pancreas. This paradigm of Sox9-FGF signaling to coordinate morphogenesis of organs has also been seen to play a role in developing the prostate (Thomsen et al, 2008) and testes (Bagheri-Fam et al, 2008), and branching morphogenesis of the ocular gland (Chen et al, 2014). Investigating this multi-level regulatory mechanism is key to understanding the specification of the medullary lineage, and can be done by assessing the impact of FGFR2 on the levels of Sox9 in the bulb of the hair follicle.

IV.

Materials and Methods

Mice and Animal Care

Mice were generated and genotyped as previously described in (Yu et al., 2003) for FGFR2 Flox/Flox alleles. All protocols were executed following the institutional guidelines established by the University of California, San Diego, Institutional Animal care and Use Committees.

Analysis of Hair, Hair length, and Hair shaft structures

Statistical analysis was performed with Prizm5 (GraphPad Software). Hair length was determined using Image (Wayne Rasband, NIH) and the distributions of hair lengths were compared using the Kendall-Sherman test.

Histology, Immunofluorescence, Immuno-staining with HRP

All Histology and immunostaining was performed by taking mice skin at P7 and fixing it with 4% paraformaldehyde. Skins samples were then dehydrated in 30% sucrose, removed and placed in a block and frozen in OCT. The tissue sample were then cut at 8 microns. H&E was done following the protocol in (Mukhopadhyay et al., 2011). Immunostaining was performed using citrate buffer(10mM) for antigen retrieval at 95 C' for 20 min. samples were then blocked in NGST washed once and Incubated overnight with a primary antibody dilution: AE15 (1:200 Santa Cruz Biotechnologies), AE13 (1:200 Santa Cruz Biotechnologies), Lef1 (1:200 Cell Signaling), PCNA (1:100

ThermoScientific) pH2AX (1:200 Cell Signaling), Casp-3 (1:400 Cell Signaling), Cyclin D1 (1:200 NeoMarkers), pH3 (1:200 Santa Cruz Biotechnologies). All fluorescent Secondary antibodies were either 488 or 568 (1:500 Life technologies). DAPI staining was performed followed by an immediate application of Dako fluorescent mounting medium. HRP Secondary antibodies (1:500 Santa Cruz Biotechnologies), and developed using a peroxidase chromogen Kit (AEC) from Biomed Corp. Images were captured using a DP71 (Olympus) Camera, mounted on a BX51 Stereomicroscope (Olympus).

EdU injection, detection, and Analysis

Both Control and mutant mice were injected Intraperitoneally at P4 with EdU(5-ethynyl-2'-deoxyuridine) made by Invitrogen. 125 ug of EdU was injected per gram of body weight. Mice were then collected 1 hour and 36 hours later. Skin was taken and fixed in 4% paraformaldehyde. The same immunostaining protocol was followed for the sample prep to section the tissue samples as well as the costaining of the tissue. Primary antibody AE15 (1:200 Santa Cruz Biotechnologies), and secondary antibody 568 (1:500 Life Technologies) were costained with EdU, respectively. Following the fluorescent immunostaining, the samples were developed following the protocol (Salic et al, 2008). A development reaction cock-tail was made using: TBS (100mM) CuSO4 (1mM), Fluorescent Alexa Fluor Azide-488 (Invitrogen), and Ascorbic acid (100mM).

Reaction cocktail was dispersed on the tissue and allowed to incubate for 30 min. Subsequent washes were performed and the same protocol for completing the immunostaining was followed.

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