March 28, 2020, Vienna **Therese Eilertsen**

Marshall Plan Report

Investigating Molecular Control of Epidermal Development

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IMC University of Applied Sciences Krems

Abstract

During mouse embryogenesis, the epidermis stratifies from a single layer of progenitor cells to yield a functional multilayered permeability barrier at birth. To accommodate this rapid growth, basal cells execute divisions that results in either self-renewal or differentiation. The mechanism regulating this fate decision changes from being driven by density to driven by asymmetrical division (ACD) machinery at embryonic day 15.5 (E15.5) (Box et al., 2019). Intriguingly, this switch in stratification mechanism is accompanied by other changes in cell behavior in the epidermis. In cell competition – the sensing and elimination of less fit "looser cells" by neighboring "winner" cells – looser cells shift from being eliminated by engulfment by neighboring cells to elimination through an upward flux of differentiating progeny at E15.5 (Ellis et al., 2019). The coincidence of these two developmental transitions raises the possibility that E15.5 represents a critical developmental time point at which the tissue undergoes a wholesale switch in cell behavior. This have led to the hypothesis that distinct microenvironmental cues might account for this transition in development. Here, we lay a novel strategy to uncover the regulators of this transition. First, we employ an immunostaining approach to characterize potential candidates. Secondly, we purse collagen IV as a regulator and tests its functional consequence on the stratification switch by combining lentiviral mediated in vivo knock down and imaging techniques. We find no compelling evidence to suggest that collagen IV is a regulator of the switch. However, we demonstrate a strategy that can be harnessed to identify key regulators of a developmental transition which may be involved in multiple mechanisms that build vertebrate tissue.

Keywords: #epidermis, #development, #cell competition, #asymmetric division, #differentiation, #stem cells, #basal membrane, #ECM, #collagen IV

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I am sincerely honored to have been selected as a recipient of the Marshall Plan Award. Thank you for your thoughtful and generous gift that has allowed me to engage with my passion for science and knowledge. The scholarship granted me the possibility to pursue an inspiring learning-experience at The Rockefeller University, which have fueled my interest to engage with new endeavors within the life-sciences. I conducted my research in the Fuchs lab, which is a laboratory of mammalian cell biology and development, where I worked side-by-side with brilliant scientists. It was amazing to work in an environment where an enthusiasm for science was present every day. I am grateful to Elaine Fuchs for welcoming me into the lab and for making this experience a possibility. During my 6-months in the Fuchs lab I worked under the supervision of Stephanie Ellis who deserves a wholeheartedly appreciation for the huge efforts she invested in me. Many hours of teaching, discussions and advice have equipped me with the means to conduct this research. I cannot adequately thank her for this guidance which I will take with me in my future professional path. I consider myself really lucky to have got an opportunity to work with Stephanie. Thank you for all of your kindness, encouragement and support. Also, I want to thank the whole team at the IMC University of Applied Sciences for management and counselling. Particularly I want to thank Dr. Barbara Entler for navigation of the whole process, and Dr. Christoph Wiesner for feedback on my research paper.

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1. List of abbreviations

E15.5 – Embryonic day 15
K5 – Keratin 5
ACD – Asymmetrical division
PCP – Planar cell polarity
aPKC - Atypical protein kinase C
ECM – Extracellular matrix

2. Introduction

2.1 Epidermal morphogenesis creates a protective barrier

With its highly abundant and attainable biological environment, the skin serves as an attractive site for a range of therapeutics designed to advance healthcare. However, in order to come up with a potential target to improve skin health, it is important to thoroughly understand the skin in depth. The skin serves as the first line of defense towards the many physiological and pathological threats an individual encounter in daily life. As the largest organ in the body, it functions as a major reservoir of stem cells. Despite the many utile functions of the skin such as body temperature regulation and immune system engagement, the main function of the skin is to protect the internal from the external (Alonso and Fuchs, 2003). Human skin faces more frequent, direct and damaging physiological encounters with the external environment than any other tissue in the body (Cork et al., 2009; Proksch et al., 2008). Of the three primary layers; epidermis, dermis and hypodermis that make up the skin, it is the outermost of these three layers - the epidermis – that mainly accounts for the protective barrier function. A key mechanism undermining this impermeable barrier function of the epidermis is the possession of precisely tuned mechanisms that enables the skin to replace dying cells and heal wounds (Gonzales and Fuchs, 2017).

In mammals, the epidermis is comprised of stratified layers of specialized epithelial cells that fulfils the function of this barrier. These stratified layers comprise four morphologically distinct layers: the inner basal layer, the spinous layer, the granular

layer and the outer stratum corneum. The cells that comprise these layers are defined as keratinocytes and make up around 90 percent of the epidermal cells (Eckert and Rorke, 1989). The name – keratinocytes – is given because of the specific expression of different keratins. Keratins are intermediate filaments with a large role in manifesting the protective function of the epidermis. The keratins provide an extensive cytoskeletal architecture and consist of type I and type II keratins. During the course of terminal differentiation, changes in keratin synthesis results in changes in keratin composition. In the adult mammalian epidermis, keratinocytes that have exited the basal layer enter a series of biochemical and morphological changes that culminate in the production of dead squames. These dead squames are cellular ghosts that have lost all organelles, as well as the nucleus, and are composed of a cornified enveloped packed with insoluble bundles of keratin filaments. The high keratin content is largely responsible for the impermeable barrier effect of the skin. In a constant rejuvenation process, the squames are sloughed from the surface of the skin, to be replenished by differentiating cells moving upward from the proliferating basal layer (Blanpain and Fuchs, 2009; Gonzales and Fuchs, 2017). Through the remainder of life, the basal cells continue to proliferate and differentiate to maintain a functioning barrier. This proliferative capability is contained in a unique niche between the keratinocytes and the basement membrane.

Basal cells acquire the capability to differentiate during embryogenesis - as the epidermis begins to stratify. Stratification of the epidermis first begins with a monolayer of surface ectoderm. In mice, the epidermis is first specified at embryonic day 9.5 (E9.5) (Chan et al., 1994). This single layer of basal progenitor cells is distinguished by specific expression of keratinocyte 5 (K5) (type II) and K14 (type I). To drive the transition to a stratified epidermis, a stratification program is initiated to provide the embryo with a functioning barrier upon birth. Once two cell layers are established, the basal cells assemble into a polarized sheet and differentiation begins. As the basal cells differentiate upwards from the basal layer to produce stratified layers, the cells switch from expressing K5 and K14 in the basal layer, to expressing K1 (type II) and K10 (type I) in the suprabasal layer (Fuchs and Green, 1980) (Fuchs et al., 1981).

During embryogenesis, the basal cells are faced with a choice to either self-renew to contribute to the progenitor pool or differentiate upwards to produce stratified layers. Failure to properly regulate the balance between self-renewal and differentiation during skin development leads to severe barrier defects. In recent decades, the field has progressed and uncovered many intriguing mechanisms required to establish a proper

barrier function upon birth. These critical processes, underlying epidermal development, have been well-studied in the mouse model and provided valuable insights in the molecular mechanisms involved in controlling stratification of the mammalian epidermis.

2.3 Spindle orientation are coupled to establishing proper tissue architecture

A fundamental mechanism through which the mammalian epidermis arise is oriented cell division. During cell division, genetic material is correctly segregated into the daughter cell by the formation of a dedicated bipolar structure. In a profound remodeling of the microtubule network, the mitotic spindle segregate the genetic material towards opposite poles in the dividing cell (Tanaka, 2010) (Glotzer, 2009). The orientation of the spindle plays a critical role in determining the cells axis of division and is inextricably linked with cell polarity. Since the spindle angle orientation determines division axis, the axis of the spindle orientation guides the fate of the daughter cell (di Pietro et al., 2016). In the mouse epidermis, the innermost basal layer divide in both planar and perpendicular orientations (Lechler and Fuchs, 2005). The capability of the basal cells to execute divisions in both orientations is imperative for proper establishment of tissue architecture and maintenance of tissue. Planar divisions within the basal layer results in a symmetric fate of the daughter cell and contribute to increase the progenitor pool. Whereas perpendicularly dividing basal cells results in an asymmetrical fate of the daughter cell, which drive the generation of a committed suprabasal layer (Ray and Lechler, 2011). In both cases, too many of each lead to a defective adult barrier. Asymmetric cell divisions are critical regulators of stem cell biology and are facilitated by spindle orientation (Fuchs and Raghavan, 2002). The mechanisms underlying spindle orientation and asymmetric divisions have been well investigated in species such as drosophila and C. elegans, and more recently in mammals. Generally, in asymmetric cell divisions, the mitotic spindle is oriented perpendicularly to the basement membrane. To perpendicularly orient the spindle, an axis of polarity is established which localize cell fate determinants apically in the dividing basal cell (Betschinger and Knoblich, 2004). Likewise, a complementary mechanism has been demonstrated in development of the mouse epidermis.

A lot of effort has been placed into understanding the molecular mechanism controlling fate decisions in the epidermis after the differentiation program first begins at E15.5. At this timepoint, the basal cells execute a switch from primarily planar divisions to the underlying basement membrane, to perpendicular divisions. A key feature of these perpendicular divisions is the apical localization of asymmetrical division components that remain apical even after cleavage furrow formation, resulting in the generation of one basal cell and one suprabasal cell (Lechler and Fuchs, 2005; Williams et al., 2011). These intrinsic cues couple the mitotic spindle to cell polarity to orient the mitotic spindle. In mitotic basal cells, LGN co-localizes with proteins NuMA and $G\alpha_{i3}$ which selectively partition towards the apical daughter cell. Recent findings suggest that LGN is cooperatively polarized by Par3-mlnsc and $G\alpha_{i3}$ to promote perpendicular divisions (Williams et al., 2014). This interaction is thought to reorient the mitotic spindle through the cortical capture of microtubules. In the polarized cortical crescent, NuMA colocalizes with dynactin at spindle poles, which indicate that NuMA and dynein-dynactin dependent pulling forces at the apical cortex might function in asymmetric division (Lechler and Fuchs, 2005). As a downstream effect of this asymmetric division machinery, Notch signaling, which is an important effector of differentiation act genetically in a common pathway promoting the basal to suprabasal switch in differentiation (Williams et al., 2011).

2.4 Recent evidence demonstrate a two-step mechanism in stratification

Intriguingly, the machinery of apical-basal polarity cues that direct spindle orientation is not required during the early phase of stratification. In fashion with the rapidly expanding embryo the basal cell population increases three-fold between E13.5 and E15.5, undergoing mainly planar divisions. However, during this time window the monolayer of progenitor cells becomes two. Until recently, the molecular mechanics controlling this change in tissue architecture prior to E15.5 was unknown. Since polarization of asymmetrical division components guides the apical-basal spindle orientation to execute perpendicular divisions, a group from Princeton (Box et al., 2019) pursued the hypothesis that that planar cell polarity (PCP) components may guide the mitotic spindle in a planar direction. They indeed documented that the progenitor basal

cells defective for PCP gene, Vangl2, less frequently divided in a planar orientation. The mutant basal cells defective for Vanlg2 was more elongated towards its apical-basal axis than to its anterior-posterior axis. Strikingly, this alteration in basal cell geometry was not a direct consequence of defective Vangl2. Changes in cell geometry have previously been documented as a secondary consequence of an open neural tube – characteristic of several PCP mutants (Curtin et al., 2003; Wang et al., 2006). Neural tube closure involves a process where the adjoining surface ectoderm is pulled over the dorsal midline to cover the spinal cord. PCP mutants such as Vangl2 knockouts disrupts this process, which results in geometrical alterations of the cell shape. However, the correlation in basal cell elongation and perpendicular divisions maneuvered the group into another direction to unveil the mechanisms undermining early stratification.

The axis of division is increasingly skewing towards perpendicular divisions as stratification from the monolayer of progenitor cells continue. Interestingly, perpendicularly dividing basal cells occurred without the engagement of asymmetrical division (ACD) machinery components prior to E15.5. Allegedly, the increased perpendicular divisions observed in the Vangl2 mutants was a direct effect of elongation of the apical-basal axis, suggesting that the geometrical shape of the cell is tightly linked with axis of division and positional fate. According to Hertwig´s rule, a cell is likely to divide along its longest interphase axis. This phenomenon has been well documented in cells within a two-dimensional plane. Basal cells however, which reside in a three-dimensional plane, may have their longest interphase axis lying perpendicularly to the basal membrane, promoting perpendicular spindle alignment and perpendicular division (Wyatt et al., 2015). Further experiments conducted by the group demonstrated that that basal cell density was relative to the spatial location of the embryo. In areas of higher density, basal cells were more likely to perpendicularly divide, and vice versa in areas of lesser density where basal cells were more likely to undergo a planar division. Additionally, in the areas of higher density the basal cells were more elongated towards the apical-basal axis of the cell, and in areas where basal cells were less densely located, the cells were more elongated towards an anterior-posterior axis. This suggest that the geometrical shape of the cell is influenced by local density, which in turn means that density is a driver of stratification prior to E15.5. The accumulated results from the Princeton lab manifest the two-step mechanism of stratification, and compellingly demonstrates a developmental transition from density driven to ACD-machinery driven stratification.

Based on these observations, embryonic basal epidermal cells pose a flexibility during the early phase of stratification to adjust spindle orientation in response to changes in the local tissue environment to accommodate the demands of embryonic growth.

2.5 A shift in cell competition elimination mechanism occur at E15.5

This switch in stratification mechanism from density driven before E15.5 to ACD-machinery driven after E15.5, is interestingly accompanied by other changes in cell behavior in the epidermis. For example, in cell competition - the sensing and elimination of less fit 'loser' cells by neighboring 'winner' cells – we observe a change in elimination mechanism at E15.5. Cell competition is a selective mechanism that acts during development to govern and optimize vertebrate tissue. When the epidermis stratifies from the monolayer, tissue dynamically adjusts cell competition strategies to preserve fitness as its architectural complexity increases during morphogenesis. In the single-layered epithelium of the early embryonic epidermis, loser cells are eliminated by the engulfment of neighboring winner progenitors. Later, as the tissue begins to stratify, elimination mechanism changes and the basal layer instead expels losers through an upward flux of differentiating progeny. Riveting, this change in elimination mechanism of loser cells occurs namely at E15.5 (Ellis et al., 2019). This observation has led to the speculation that distinct microenvironmental cues might control not only the switch in stratification mechanism, but also account for the change in loser cell elimination in cell competition.

2.6 Microenvironmental components are involved in spindle orientation

The molecular underpinnings of the developmental transition at E15.5 is currently unknown. Although the involvement of specific microenvironmental components in regulation of development have not been well worked out, it is known that the attachment between the basal cells and the underlying basement membrane is imperative to establish polarity in the epidermal progenitor cells (Lechler and Fuchs,

2005). Also, additional polarity is imparted through the apical-lateral network of desmosomes and adherence junctions that interconnects the epithelial cells within the tissue (Green et al., 2010). These connections, between the cells and the basement membrane as well as to their neighbors, controls the orientation of the mitotic spindle by making intracellular connections that regulate polarity (Yeaman et al., 1999). All of the microenvironmental components – tight junctions, adherence junctions, adhesion molecules as well as basement membrane components – may impact a variety of molecular elements involved in stratification. Yet, the expression of these have not been well documented in the developing epidermis. All of these microenvironmental components may potentially work as regulators of the developmental transition observed at E15.5. For example, molecular components that contributes in establishing polarity may be a candidate in regulating the switch in stratification. For example, it is known that β1 integrin is an important player required to properly orient spindle and execute asymmetric divisions from the basal layer. β1 integrin does not interfere with the assembly of the Par3-LGN-mLnsc-dynactin complex but have a function in apically orienting atypical protein kinase C (aPKC) and hence dictate the direction of division. Also, α-catenin, which links actin filaments to cadherins, has a function of establishing polarity and controlling spindle orientation. Ablation of α-catenin cause a cell polarity bias which results in the loss of control of spindle orientation (Lechler and Fuchs, 2005).

2.7 The basement membrane and its influence on spatial microenvironment

Differently from other systems where asymmetric divisions have been studied, the epidermal basal cells reside on an underlying basement membrane. The basement membrane is an ubiquitous, highly specialized sheet-like extracellular matrix (ECM) composed of a distinct set of glycoproteins and proteoglycans. Whilst the dense meshwork of intracellular keratin filaments interconnected between neighboring cells in the epidermis and epithelia-specific desmosome compartments accounts for much of the mechanical strength within the tissue, the elasticity and tensile strength in the dermis is determined by the ECM. Beyond acting as a scaffold providing mechanical support, the ECM constitutes a strong proportion the crucial signals provided during embryonic development (Breitkreutz et al., 2009). Interestingly, many key functions of the

epidermis happens to be basally localized such as ECM protein secretion, growth factor signaling and integrin mediated adhesion (Fuchs and Raghavan, 2002; Mariotti et al., 2001; Schlaepfer and Hunter, 1996). The basement membrane is formed at the basal side of epithelial cells, largely by the cells that rest on it. Four main components determines the structure of the basement membrane - type IV collagen, nidogen, laminin and perlecan - and allows it to take on a rich variety of different forms with varying mechanical and biological properties (Timpl and Brown, 1996). However, the knowledge about the molecular influence of each of these basal membrane components on epidermal stratification in mammals is still unfolding.

Being responsible for overall structure, laminin and collagen IV molecules form independent networks, both of which primarily signals through integrin receptors. Integrin adhesion are crucial to mediate the attachment of the basal cells to the extracellular matrix. Several α and β integrin subunits have been identified in the mammalian epidermis, which can combine to form 24 different heterodimers with varying affinity towards specific ECM components. Whereas a proportion of the integrins are constitutively expressed, some are expressed only under certain circumstances such as during wound healing and in some pathological conditions (Hynes, 2002). Laminin mainly attach to the internal keratin cytoskeleton through integrin heterodimer α6β4, which in stratified epithelia form specialized adhesion structures known as hemidesmosomes. Differently from conventional adherence junctions, hemidesmosomes connect with the internal keratin filament, rather than the actin filament network (Dowling et al., 1996). In mural epidermal tissues devoid of hemidesmosomes, the stratified tissues display a fragile attachment to the basal lamina. The dramatically reduced adhesive properties of the skin in the absence of hemidesmosomes at the basal surface of keratinocytes display an extensive detachment of epidermis, accompanied death shortly after birth (van der Neut et al., 1996).

Collagen IV is the most abundant protein in the basement membrane where it exclusively resides. The multiple binding sites within the NC1 and triple-helical domain of collagen IV suggests the involvement of several adhesion receptors (Aumailley and Timpl, 1986) (Perris et al., 1993). Adhesion between collagen IV and the basement membrane is mediated by α1β1 integrin, as well as α2β1 and α11β1 integrins with varying affinity (Kern et al., 1993; Tiger et al., 2001; Tulla et al., 2001). Whilst its well documented that collagen is a crucial scaffold providing mechanical support in the ECM, its other influences on the epidermis have not yet been well addressed. Early

studies from the adult guinea pig epidermis indicated that epidermal cells preferentially attach and differentiate on collagen IV basement membrane substrates (Murray et al., 1979). In culture experiments, collagen IV aggregates seemed to stimulate proliferation of basal keratinocytes and improve stratification in epidermal layer in human skin equivalents (Matsuura-Hachiya et al., 2018). However, the role of collagen IV in proliferation and stratification in early development of the mural epidermis remains unaccounted for.

2.8 Specific aims of research project

Processes that begin in the embryo have consequences for organismal fitness in post-natal life and during aging. In order to identify pharmaceutical targets and improve human skin health, a major aim is to dissect the underpinnings of epidermal development relative to the fitness of the protective adult barrier. To fully understand how to manipulate skin to favor human conditions we need to thoroughly understand the skin in depth. The mouse skin epidermis offers a well investigated system to address many of the still unknown early embryonic events. Morphogenesis involves different spindle regulating genes, but little is known about the genes governing the E15.5 behavioral switch in the epidermis we have uncovered.

The coincidence of the two developmental transitions, in stratification and cell competition elimination mechanism, raises the possibility that E15.5 represents a critical developmental time point at which the tissue undergoes a wholesale switch in cell behaviour. We speculate that microenvironmental factors acquired in the developing epidermis may be important in guiding both of these changes in cell behaviour. Therefore, we hypothesised that specific changes in microenvironmental signals trigger the developmental transition we observe at E15.5 We exploit prior knowledge that several components in the early epidermal microenvironment imparts polarity through an apical-basal and apical-lateral network of adherence junctions, desmosomes, adhesion components as well as ECM components. However, the expression of many of these components are poorly documented in the developing embryo. Specifically, we tackle the following aims:

Aim 1: To generate a list of candidate regulators of the stratification switch.

Aim 2: To systematically investigate the effect of each candidate on the stratification switch and functionally address the role of each gene of interest.

Uncovering the mechanisms that govern this developmental switch will assist in establishing a better understanding of stratification at a time in development where the epithelial tissue is shaped.

4. Results

4.1 Identification of candidate regulators

We hypothesized that specific changes in microenvironmental signals trigger the developmental transition observed at E15.5. To test out hypothesis, we first generated a list of candidate regulators with a potential influence on the microenvironment at E15.5. RNA-seq provided by the Fuchs lab armed us with the expression data of several microenvironmental components which we used as guide to propose candidate regulators.

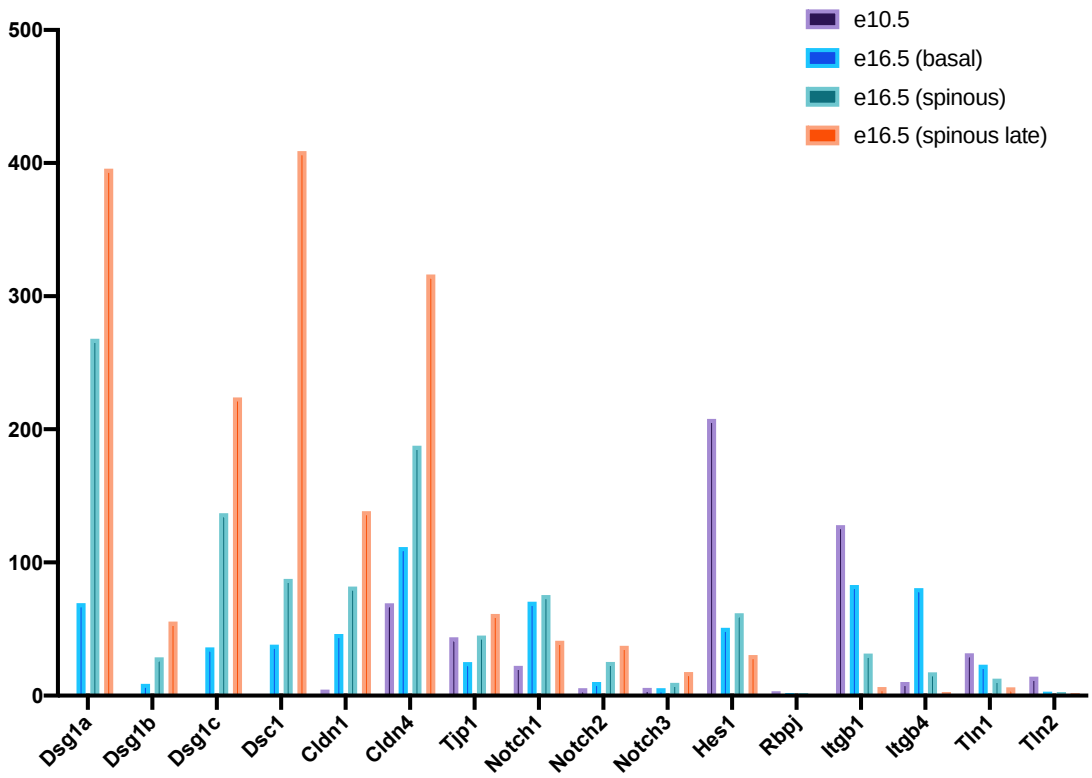

Figure 1. Lab RNA-seq data indicate potential candidate regulators of the stratification switch at E15.5. RNA-seq data provided from the Fuchs lab was used to identify expression level of potential regulators of the stratification which at E15.5.

Since the documentation of many of our candidate protein complexes is poorly documented during early stages of epidermal development, we proceeded to characterize some of them. Therefore, we sacrificed wild type embryos (CD1 background) at three consecutive days in early stratification; E11.5, E13.5 and E15.5 (Fig. 2b). By employing an immunofluorescence imaging approach, we assessed protein expression in the basal layer at these specific timepoints and mapped the location of each candidate.

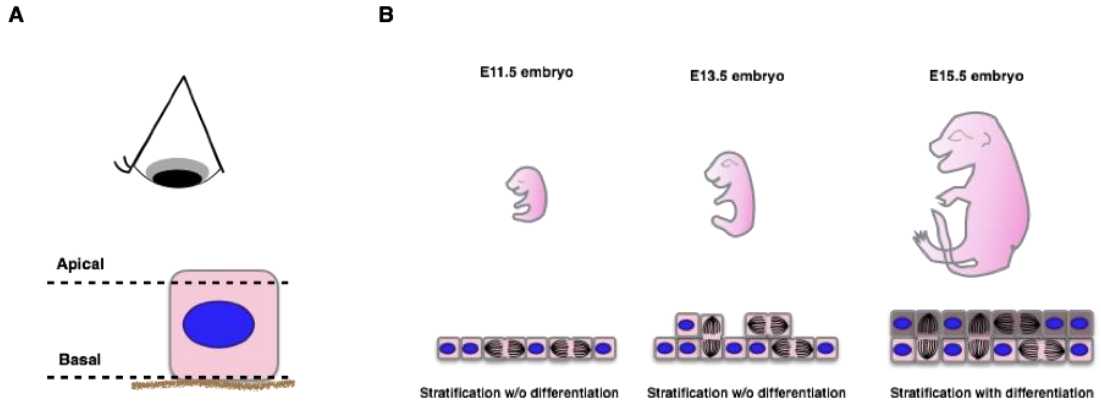

Figure 2. Early maturation of the mouse epidermis. **(A)** Schematic of skin depicting focal plane. **(B)** Schematic of mouse embryos at three stages in development depicting the maturation of epidermis.

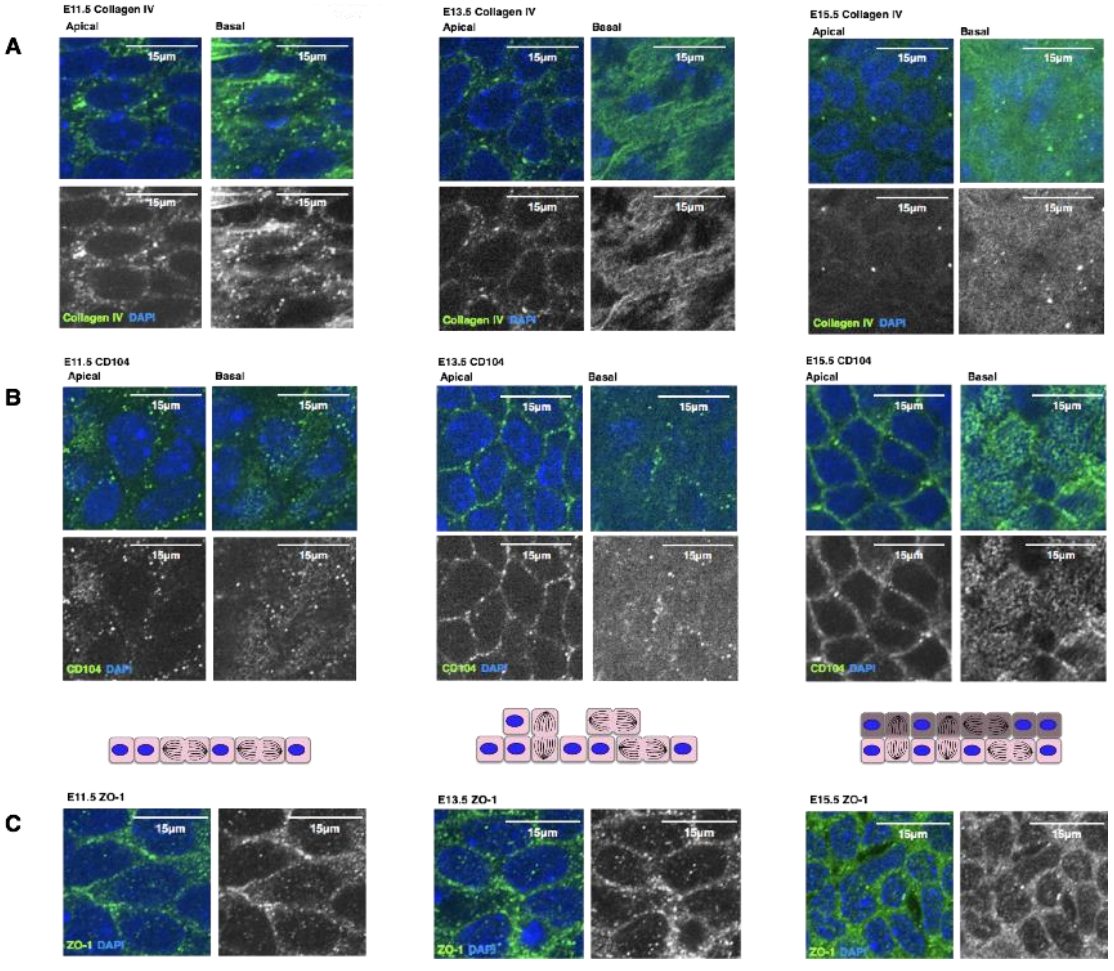

Figure 3. Characterization of candidate regulators. (A) Assembly collagen IV at E11.5, E15.5 and E15.5. **(B)** Assembly of CD104 (Integrin beta – 4) **(C)** Assembly of ZO-1 (tight junction component).

As judged by staining with 4´,6-diamidino-2-phenylindole (DAPI) to mark the nucleus and with collagen IV, immunofluorescence signal of ECM-component collagen IV dramatically increased from E11.5 till E15.5. Collagen IV was visible in the apical layer at E11.5 and E13.5, however was nearly absent in the apical layer at E15.5. The basal assembly of collagen IV was effectively increasing through each timepoint (Fig. 2a). By contrast, for adhesion component integrin beta – 4 (CD104) signal increased almost equally in the apical and basal layer from E11.5 till E15.5. Although, signal was almost absent in the basal layer at E11.5 it dramatically shifted between E13.5 and E15.5 (Fig. 2b). Tight-junction component ZO-1 was expressed though all three timepoints, with a stably stronger signal for each timepoint (Fig. 3c).

4.2 Functional consequences of col4a1 on the stratification switch

The dramatical increase in assembly of collagen IV in the basal membrane at E15.5 yielded a shift from a fibrillar-like structure to a sheet like structure. Recruitment and subsequent aggregation of collagen IV in the basal membrane may impact critical mechanisms involved in maturation of the epidermis (Murray et al., 1979) (Matsuura-Hachiya et al., 2018). Therefore, we next asked how this ECM-component works on the stratification switch. For the respective experiments, we used mice engineered to express keratin 14 (K14)- coupled to green fluorescent protein (GFP) to uniformly label the epidermis to examine embryos (Tumbar et al., 2004).

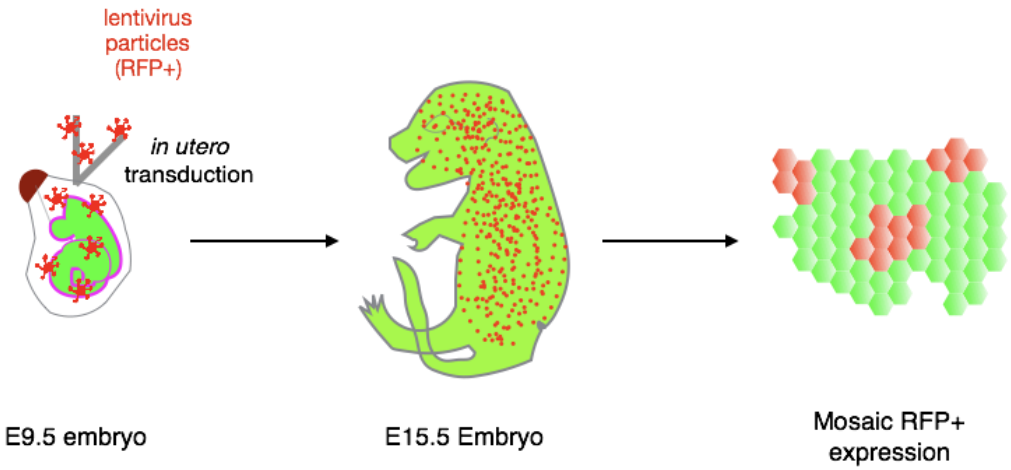

Figure 4. Lentiviral knock down construct. K14-H2B-GFP+ embryos were injected with RFP-tagged lentiviruses to knock down collagen IV production in the epidermis

The col4a1 gene provides instruction for making the alpha1 (IV) chain of collagen IV. Ablation of this gene will lead to defect collagen IV production. Using in utero ultrasound-guided delivery, we injected the amniotic sacs of E9.5 K14-H2B-GFP/+ embryos with lentivirus harboring RFP-tagged col4a1 shRNA to knock down the gene. To ensure the effect of specific shRNA transfection, the experiment was designed such that half of each litter was injected with non-targeting RFP-tagged scramble shRNA to serve as a negative control. To increase confidence of our results we additionally employed wild-type controls for some of the experiments. The lentivirus-packaged genes integrated into the single monolayer of progenitor cells which thereafter stably propagated to the epidermal basal progeny, generating a mosaic embryonic skin to investigate the functional consequences of col4a1 (Fig. 3) (Beronja et al., 2010).

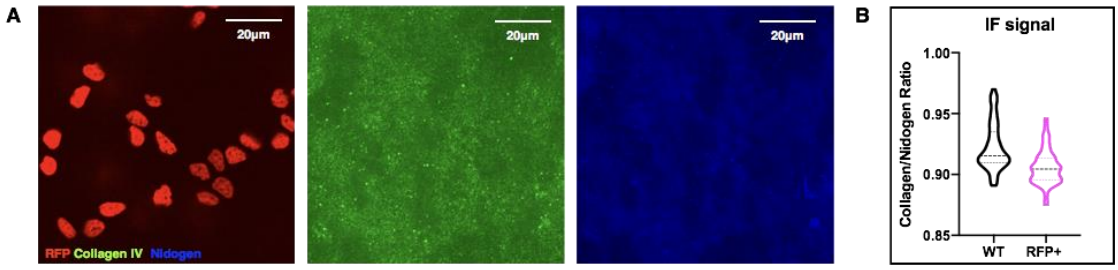

Figure 5. Immunofluorescence signal of collagen IV is decreased in the basal membrane in knock down (RFP+) basal cells. **(A)** Immunofluorescence intensity was measured in labeled collagen IV and nidogen surrounding RFP+ basal cells and WT basal cells. **(B)** Quantification of immunofluorescence signal demonstrated as a collagen IV/nidogen ratio ($p<0.0001$)

The knockdown efficiency was verified in cultured keratinocytes in vitro by qPCR. The construct proved to have a 50% knockdown efficiency (in review Fiore et al, 2020). However, to further address the physiological effect of the knock down efficiency of the lentiviral construct on the microenvironment, we quantified fluorescence intensity. We labelled ECM-components nidogen and collagen IV to detect changes in collagen IV recruitment to the basal membrane (Fig. 5a). We recorded and quantified fluorescence intensity of nidogen and collagen IV on multiple points across the basement membrane under RFP+ and wild type cells. To decrease bias in fluorescence intensity between different images, we used intensity from the labelled nidogen as a reference to generate a ratio. Notably, the fluorescent intensity of collagen IV is lower in RFP+ knock down areas when compared to signals from wild type (Fig. 5b).

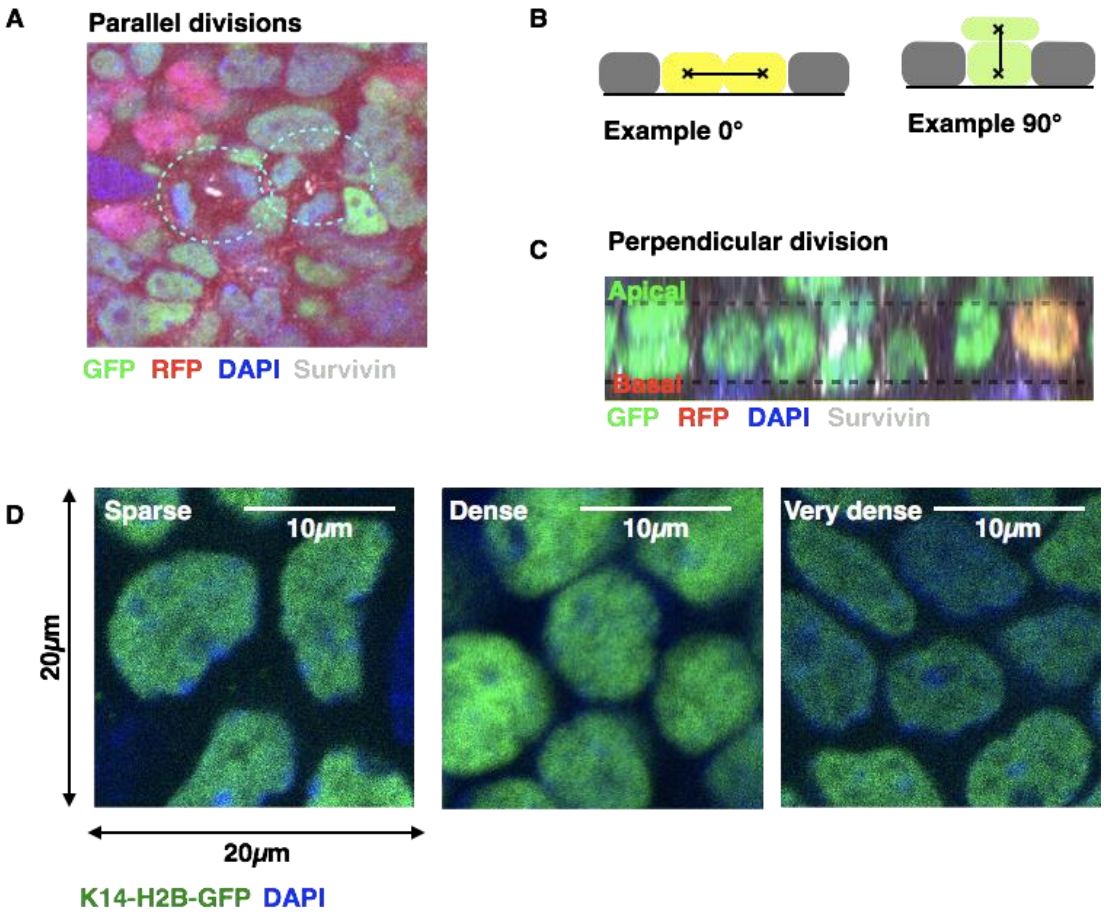

Figure 6. Methodological set-up. **(A)** Example of parallel divisions in whole-mount K14-H2B-GFP E15.5 tissue. **(B)** Schematic of measurement technique (Fiji). **(C)** Example of perpendicular divisions shown from Z-stack view. **(D)** Examples of variations in basal cell density shown in whole-mount K14-H2B-GFP E15.5 tissue.

In the epidermis, stratification shifts from being driven by density to driven by a machinery of ACD components at E15.5. Until this transition at E15.5, division angle positively correlates with density (Box et al., 2019). In order to assess if collagen IV is a regulator of this switch, we utilized this characteristic to devise a strategy. To identify a correlation between division angle and density, we quantified division angles in the genetically ablated RFP+ mitotic cells and measured the local densities surrounding the dividing cell. The model was designed such that a delayed or premature transition in stratification would model accept the candidate as a key regulator of the developmental switch e.g. a premature loss of correlation between density and division angle at E14.5 or a delayed positive correlation between density and division angle at E15.5. Since spindle orientation of mitotic cells in anaphase/telophase are considered predictive for

the ultimate division plane, we used reliable anaphase/telophase marker survivin to record the division axis (Fig. 4a, Fig 4c). We quantified division axis from whole-mount images by recording the xyz-position of the center of dividing cell and its offspring in ImageJ (Fig. 4b). The division angle between the two points was inferred using trigonometry. We then measured the local density in a constrained square (20 µm * 20 µm) surrounding the genetically ablated RFP+ mitotic cell (Fig. 4a).

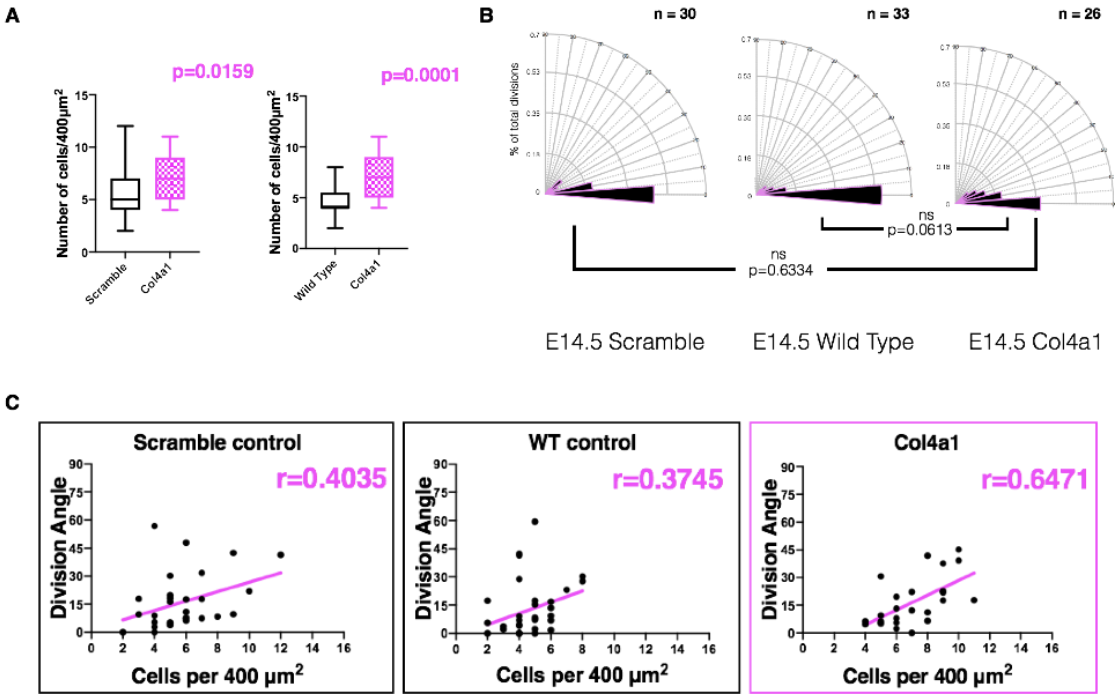

Figure 7. Density positively correlates with division angle at E14.5 (A) Quantification of local density col4a1 knock-down areas compared to scramble and wild type controls (non-parametric, with Mann Whitney two-tailed t-test) **(B)** Quantification of division axis for dividing col4a1 knock down basal cells with scramble and wild type controls (non-parametric, with Mann Whitney two-tailed t-test) (**C)** Correlation analysis of density relative to division angle of basal cells dividing within the basal plane (Spearman r-value).

Finally, we tacked the functional consequence of col4a1 ablation on the stratification switch. We first characterized density and division angles in E14.5 basal cells. Compared to scramble and wild type controls, local density in Col4a1 knock down areas significantly increased (Fig. 6a). However, the increased density of the basal layer in the col4a1 knock down areas did not lead to a statistically significant increase in perpendicular divisions. Although not statistically significant, col4a1 knock down basal cells did however lead to an increased skewing towards perpendicular division angles compared to the scramble and wild type controls (Fig. 6b). To address the correlation between density and division axis we plotted the density relative to division axis (Fig. 6c). Regression analysis revealed a positive correlation for our controls and our knock down. This effect is not unexpected and is indeed consistent with others in the field, supporting previous evidence that stratification is density driven in early stratification. Noticeably, the correlation coefficient is greater in the knockdown compared to the controls, implying a stronger correlation between density and division angle in basal cells where collagen IV production is ablated.

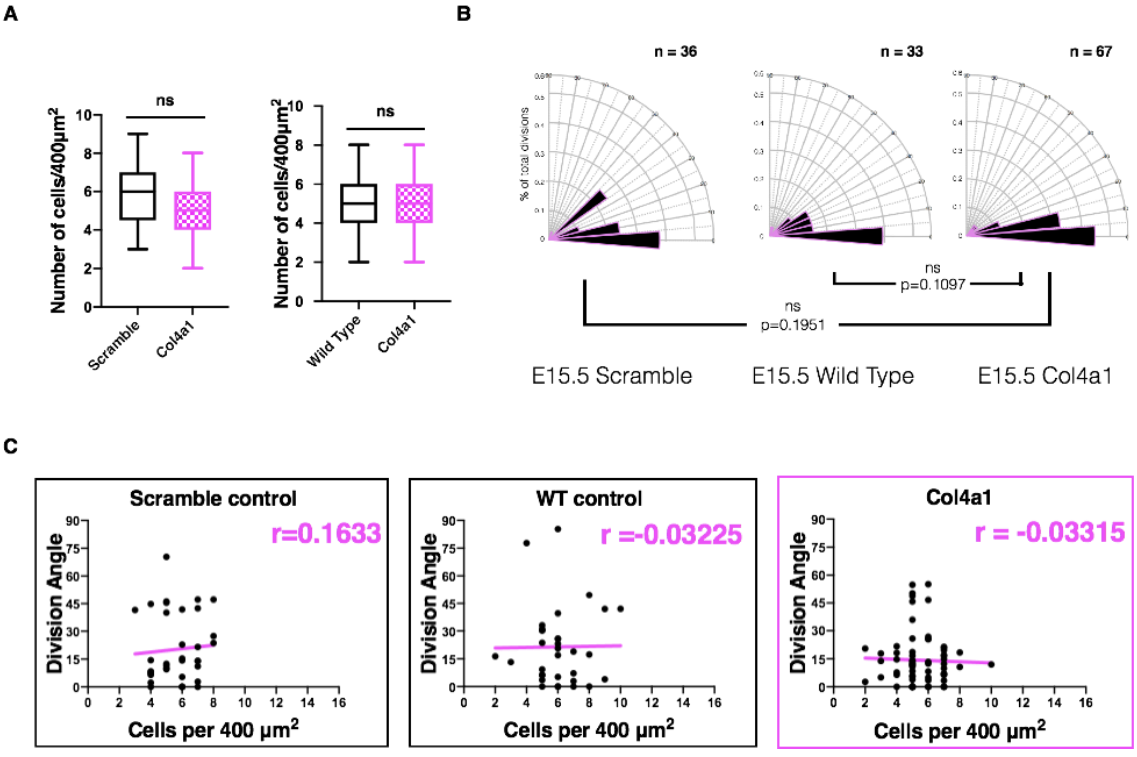

Figure 8. **Positive correlation between density and division angle is lost at E15.5** **(A)** Quantification of local density col4a1 knock-down areas compared to scramble and

wild type controls (non-parametric, with Mann Whitney two-tailed t-test) **(B)** Quantification of division axis for dividing col4a1 knock down basal cells with scramble and wild type control (non-parametric, with Mann Whitney two-tailed t-test) **(C)** Correlation analysis of density relative to division angle of basal cells dividing within the basal plane (Spearman r-value).

We next addressed the consequences of col4a1 ablation at E15.5, when stratification shift from being driven by density to being driven by ACD-machinery. In contrast to E14.5, at E15.5 col4a1 knock down did not lead to a significant decrease or increase in density when compared to controls. Nor did this the knock down induce any significant changes in division axis when compared to controls. It seemed like the epidermis was not dependent on col4a1 expression to initiate the transition to ACD-machinery driven stratification. The correlation between density and division angle was lost at E15.5 for our controls as well the col4a knock down, supporting what we know about division angle and stratification.

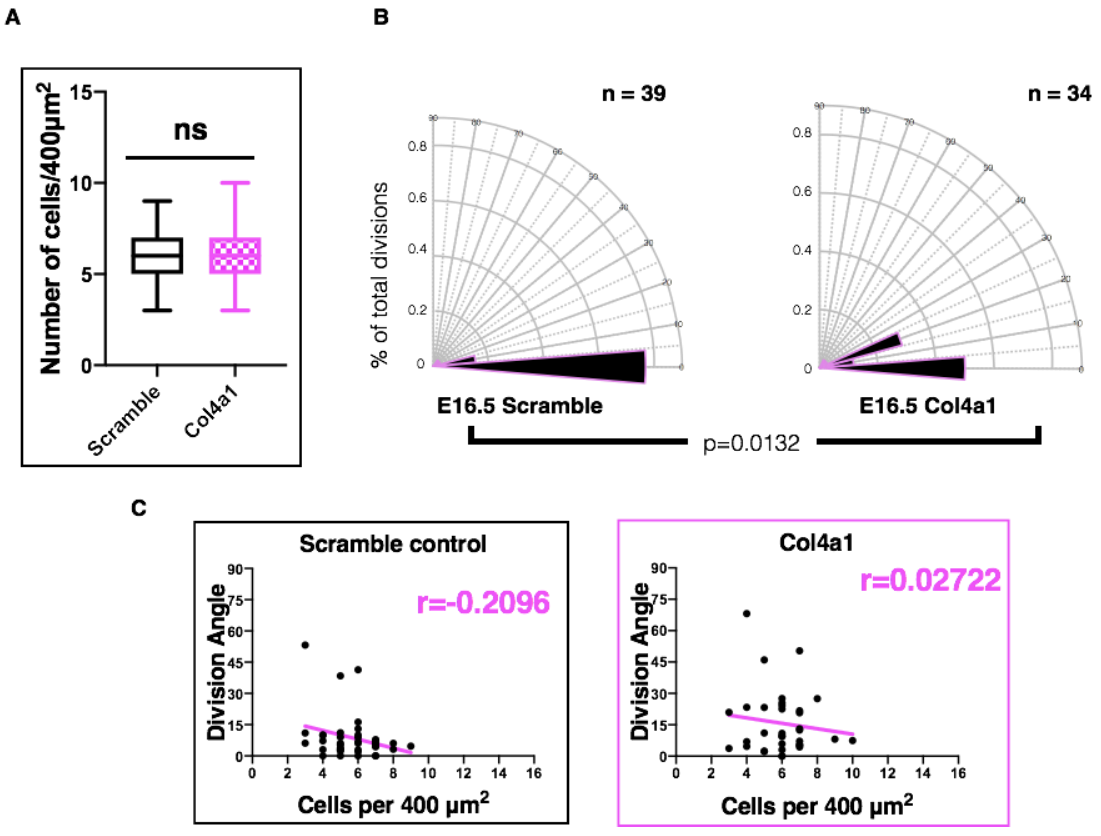

Figure 9. Quantification of density, division orientation and correlation analysis at E16.5. (A) Quantification of local density col4a1 knock-down areas compared to scramble control (non-parametric, with Mann Whitney two-tailed t-test) **(B)** Quantification of division axis for dividing col4a1 knock down basal cells with scramble and wild type control (non-parametric, with Mann Whitney two-tailed t-test) (**C)** Correlation analysis of density relative to division angle of basal cells dividing within the basal plane (Spearman r-value).

Under normal circumstances, stratification at E16.5 stratification is driven by ACD-machinery. Although the lack of alteration of stratification mechanism at E15.5 already indicated that we were not likely to have hit at a later time point, we proceeded to investigate potential alterations in stratification mechanism at E16.6. Density remained largely the same in the knock down and the control at E16.6 (Fig. 9a). Also, the correlation analysis revealed a weak negative correlation for both the scramble control and the knock down (Fig. 9c). Unexpectedly, the vast majority of the scramble control cells executed divisions in a planar orientation (Fig. 9b). Though, it is unlikely that the exaggerated increase in planar divisions actually occurs at E16.5. Also, the division axis for the knock down statistically differed from the wild type control and seemed to strongly skew towards perpendicular divisions – a peculiar observation considering the planar divisions in the control. However, whilst the increased skewing towards perpendicular divisions might would have been expected at this timepoint, it also resembles the effects observed in the epidermis upon integrin beta – 4 ablation where basal cells detach from the basal membrane and delaminate up in the dermis (van der Neut et al., 1996). Bearing in mind that both the scramble control and the knockdown exhibited peculiar and unexpected features at E16.5 it was rational to suggest that the tissue might have exerted some additional challenges at this timepoint. At E16.5 the tissue was characterized by prominent hair follicles which increased bias in measuring angles and restricted us from using many parts of the tissue.

5. Discussion

In summary, we establish a unique multidisciplinary approach to address regulators of the developmental switch in stratification mechanism. The expression of various microenvironmental components in the early developing epidermis have previously been poorly documented. Using an immunofluorescence approach, the characterization of components was a subject to availability of antibodies in the lab. Our findings have led to the characterization of some of these components, which have previously not been done. Here, we identify presence of tight junction ZO-1, integrin beta – 4 and the collagen IV at a timepoint early as E11.5. Additionally, we characterize their progression through timepoints E11.5, E13.5 and E15.5. We document remarkable changes in behavior, for particularly integrin beta - 4 and collagen IV. Whilst integrin beta – 4 markers steadily and evenly increase in the apical and the basal layer as the embryo mature, collagen IV is first roughly equally distributed in both the apical and the basal layer. Intriguingly, at E15.5 we observe a dramatical increase and recruitment of collagen IV to the basement membrane. Concomitantly, the apical layer is close to cleared for collagen IV markers at E15.5. As mentioned, collagen IV is expressed by the basal cells that rest on the basement membrane. Our observation might suggest that collagen IV secretion of the basal cells mainly occur prior to E15.5.

With this observation in mind, there was reason to suspect that collagen IV may also be important for regulation of stratification mechanisms. Therefore, we pursued collagen IV as a candidate, and endeavored to test collagen IV on the stratification switch by lentiviral mediated knocked down of the col4a1 gene, which provides instructions for making type IV collagen. In this study, we do not find compelling evidence to suggest that collagen IV is a key regulator of the switch. However, the combination of the two following factors might have influenced the outcome of this experiment: collagen IV is secreted by the basal cells, and the skin is mosaic. In our case, the lentivirus infected just some patches of cells in the epidermis. Often, RFP tagged knock down cells was neighboring a wild type cell which might have compensated for the loss of collagen IV. This compensation from neighboring wild type collagen IV producing cells might have satisfied the amount of collagen IV in the microenvironment. In this case, the effect of the knock down cells were simply enough to exert a physiological difference in the microenvironment, in the attempt to reduce local collagen IV production. To avoid compensation from neighboring wild type cells,

density and division angle measurements was repeated, and this time we recorded only mitotic cells that were neighboring other knock down cells (results not shown). Despite the effort, the phenotype remained unchanged. This may suggest that the knock down (verified to be of 50% efficiency using qPCR) itself was not sufficient to impact the microenvironment. Additionally, we observed that the RFP marked knock down cells was less rare at E16.5 than at the earlier timepoints. This effect may be a result of a cell competition mechanism (Ellis et al., 2019), where these cells with ablated col4a1 were regarded as looser cells and thus were eliminated by more fit neighboring winner cells. Although these notions might explain the absent phenotype, we do not have enough proof to either regard or disregard collagen IV as a regulator of the stratification switch at E15.5. The next steps for this study would have involved the engagement of a second, stronger hairpin to knock down collagen IV. In addition to unanswered questions regarding the role of collagen IV, it remains to investigate the functional consequence of various adherence junctions, tight junction and other ECM-components related to the stratification switch. In the case of a "hit" with any of these components, it would be interesting to investigate their consequence on basal cell geometry in the epidermis. In regard to this, we could address the microenvironmental components that carries the capability to alter geomatical cell shape. Measurements of surface area and height/width ratio would be tools to assist in this process. Thus, we could also bolster evidence for the density driven stratification mechanism in the maturing epidermis (Box et al., 2019).

The peculiar observations at E16.5 in regard to division angle and correlation to density, may illuminate a need to change imaging approach. The morphology of the tissue dramatically changes from E14.5 to E16.5, and we speculate that these differences in thickness and morphology may be the cause of bias in quantification at E16.5. Although huge efforts were being placed to carefully handle tissues with the consistent method, we found the method to be sensitive to handling. To overcome this problem, we would suggest changing imaging method from whole-mount to live-imaging. This way, divisions and densities can be observed in intact embryos without the challenges of post-dissection processes, and thus reduce bias.

This project has indeed set the baseline for a new exiting project with the establishment of this novel method to investigate the function of several microenvironmental components in the epidermis. In addition, this paper demonstrates a comprehensive collection of very recent and important advances that have been made in the field of epidermal morphogenesis.

Future directions for this study will confirm that the switch plays a role in guiding other cell behaviours we have observed, particularly in for example cell competition. Cell competition has been proposed to act as a selection mechanism to maximum fitness during growth and homeostasis and may have a bearing on the resilience of tissues later in life. The completion of this study may represent an important advance towards understanding this early developmental transition, which may be of consequences for the fitness of the tissue not just in the embryo, but also during aging. If this developmental transition indeed is a whole-sale switch in cell behavior, as we suggest, addressing the regulators which govern this transition may also guide us into understanding how development work in other tissues in the body, and pave the way for new approaches within organ development.

6. Methods

6.1 Mouse lines

Mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited Comparative Bioscience Center at the Rockefeller University. All procedures were performed using Institutional Animal Care and Use Committee (IACUC)-approved protocols and in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals. For this study, mice from mouse strain background CD1 was used to harvest embryos. Embryos was harvested at E11.5, E12.5 and E13.5 to address the presence and spatial location of potential candidate regulators; Collagen 4, ZO-1 and Integrin Beta 4. To uniformly label the epidermis, mice engineered to express GFP in the epidermis was employed (Tumbar et al., 2004). For most experiments homozygous K14-H2B-GFP$^{+/+}$ or heterozygous K14-H2B-GFP$^{-/+}$ males were bred to CD1 females to generate K14-H2B-GFP/+ embryos. K14-H2B-GFP/+ embryos were injected with lentivirus at 9.5 days post coitum (d.p.c.)

6.2 Lentiviral constructs

In order to knock down the col4a1 gene and study its consequences on the stratification switch, we employed an ultrasound-guided lentiviral in utero delivery. Col4a1 library hairpin was isolated and cloned into a lentiviral construct with a fluorescent reporter, pLKO H2B-mRFP, by restriction cloning. As an important negative control, non-targeting "scramble" shRNA was cloned into pLKO H2B-mRFP1 reporter plasmid. Samples was sequenced and RFP-tagged plasmid DNA of col4a1 and scramble was isolated using Qiagen's Endotoxin Free Maxiprep kit. Plasmids were transfected into Hek293TA cells to produce lentiviral participles that was collected and injected into embryos. Injections was performed using ultrasound-guided lentiviral microinjection into the amniotic cavity of mouse embryos at 9 d.p.c. Epidermal-specific infection are achieved as fluorescently marked shRNA were internalized by E9.5–E10.5 in the surface epithelium comprising a single layer of ectoderm. Thereafter, shRNA uptake was confined to the transient periderm layer, which developed over the embryo surface. The method yields a mosaic expression of RFP+ cells which allows the identification of transduced cells with altered col4a1 expression and the non-targeting scramble control. Embryos transduced with lentivirus was sacrificed at timepoints E14.5, E15.5 and E16.5 (Beronja et al., 2010).

6.3 shRNA sequences

Scramble shRNA (SHC002) - CAACAAGATGAAGAGCACCAA

Col4a1 shRNA (TRCN0000306536) - ATCGGACCCACTGGTGATAAA

6.4 Immunofluorescence and Antibodies

For whole-mount imaging, back skin was dissected from the E14.5, E15.5 and E16.5 embryos and cut into four quadrants before immunostaining. For younger embryos (E11.5, E12.5 and E13.5) sacrificed to address recruitment of ZO-1, Collagen IV and CD104 (beta – integrin). Where it was difficult to remove the skin, whole embryos were kept intact throughout the staining and imaging procedure. For all immunofluorescence

experiments, embryos were fixed for 1 h in 4% paraformaldehyde. Following fixation, samples were permeabilized in 0.3% PBS-Triton for 3–4 h at room temperature, and blocked in blocking buffer (5% donkey serum, 2.5% fish gelatin, 1% BSA, 0.3% Triton in PBS) for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C, samples were washed for 3–4 h in PBS-Triton at room temperature, and secondary antibodies were incubated overnight together with DAPI (to label nuclei). Whole embryos and back skins (post E11.5) were mounted in Prolong DiamondAntifade Mountant with DAPI (Invitrogen) for imaging.

Antibodies used were as follows: rat anti-RFP (Chromotek, 5F8; 1:1,000), rabbit anti-RFP (MBL, PM005; 1:1,000), chicken anti-GFP (Abcam, ab13970; 1:1,000), rabbit anti-survivin (CellSignalling, 71G4B7, 1:200), rat anti-CD104 (beta4-integrin;BD Biosciences, 1:200), anti-ZO-1 (Zymed, 2533938; 1:200), rabbit anti-colIV (abcam; ab6586, 1:500) and rat anti-nidogen (SantaCruz biotechnology, sc-33706; 1:200). All secondary antibodies used were raised in a donkey host and were conjugated to Alexafluor488, Rhodamine, or AlexaFluor647 (Jackson ImmunoResearch Laboratory; 1:500). Rhodamine phalloidin (Life Technologies) was used to label F-actin (1:500). 4′,6-diamidino-2-phenylindole (DAPI) was used to label nuclei (1:5,000).

6.5 Microscopy

Whole-mount imaging of back skin was performed at time points E14.5, E15.5 and E16.5. For the intact E11.5, E12.5 and E13.5 embryos, each embryo were placed into a 35-mm Lumox-bottom dish (Sarstedt) and mounted with Prolong Diamond Antifade Mountant with DAPI (Invitrogen) shortly before imaging. Imaging was performed on a custom-modified inverted spinning disk confocal system (Andor) (358 nm 488 and 561 nm 647 laser beams). All whole-mount immunofluorescence images were acquired using a Zensoftware-driven Zeiss LSM 780 inverted laser scanning confocal microscope a 63× oil immersion objective (NA = 1.4). All images were assembled and processed using ImageJ.

6.6 Image processing and analysis

Candidate assembly analysis. Analysis to identify candidates (ZO-1, Collagen IV and CD104) and address their recruitment was performed using Image J (Fiji). Representative images are shown, and intensity was adjusted to improve visual representation of candidates.

Spindle angle quantification. Axis of division was determined in anaphase/telophase cells at timepoint E14.5, E15.5 and E16.5, since spindle orientation is predictive of the ultimate plane of division at this stage of the cell cycle. To identify the dividing basal cells in anaphase/telophase, we utilized reliable marker survivin. To quantify spindle angle from wholemount images at timepoints the xyz-position of the start and end of each surviving-marked spindle was recorded using ImageJ (Fiji). The division axis could be inferred using trigonometry. Local density for division angle/density correlation analysis was measured by constraining a 20 μm square surrounding the mitotic cell, which was performed using Prism8 (Graphpad). For every RFP+ mitotic cell, the local percentage of RFP+ cells in a constrained square of 40 μm and 20 μm was also recorded for future analysis to investigate collagen IV compensation from neighboring cells.

Immunofluorescence intensity measurement. To quantify the differences in recruitment of collagen IV markers to the basement membrane in knock down constructs we used Image J to measure the intensity (a well-established method of measuring fluorescence intensity) at specific points across the basement membrane in the whole-mounted tissue. We recorded intensity at multiple points across the tissue, and compared the basement membrane underlying RFP+ areas to wild type. For a reliable quantification and decrease signaling bias between the various images, we used ECM-component nidogen to generate a collagen IV/nidogen ratio.

6.8 Statistics and Study Design

Generally, all experiments were conducted using embryos from minimum two different litters per experiment to ensure a liable phenotype. The experiments were designed to have wild-type littermate controls in each litter. Data sets was generated using Prism 7/8 (Graphpad). All data sets were a subject to normal distribution. In case of failed normality distribution, the data sets were subjected to non-parametric tests for further analysis. Indication of specific statistical test used is described in figure legends. No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

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