

Evolution of the human skin barrier as innovative clothing

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Zusammenfassung

Evolution der menschlichen Hautbarriere als innovative Kleidung

Die Haut ist das äußerste Organ, das den menschlichen Körper ›kleidet‹. An der Oberfläche der Haut befindet sich die Epidermis, ein Gewebe, das aus Keratinozyten besteht, die eine Schutzbarriere gegen die Umwelt bilden. Um diese schützende Epidermis zu bilden, durchlaufen die Keratinozyten eine terminale Differenzierung, die durch die Expression von Strukturproteinen gekennzeichnet ist, die von Genen im Epidermalen Differenzierungskomplex (EDC) Locus kodiert werden. Dieser Satz von Genen ist bekanntlich der sich am schnellsten entwickelnde Locus im menschlichen Genom. Dies geht aus vergleichenden Studien mit dem Schimpansengenom hervor und verdeutlicht die Bedeutung von Evolution und genetischer Variation für die Barrierefunktion der menschlichen Haut. Hier diskutieren wir unsere bahnbrechenden Erkenntnisse über die Identifizierung spezifischer EDC-Gene, die sowohl in der Phylogenie der Säugetiere als auch beim modernen Menschen einer positiven Selektion unterworfen waren. Außerdem heben wir unsere Entdeckung der jüngsten Evolution der menschlichen Hautbarriere außerhalb Afrikas hervor. Ein spezifischer Haplotyp, der mit einer Zunahme des epidermalen Proteins Involukrin und der nachgelagerten Funktion des Vitamin-D-Rezeptors in der Epidermis assoziiert ist, unterlag in nordeuropäischen Populationen im Vergleich zu afrikanischen Populationen einer nahezu selektiven Auslese. Unsere Ergebnisse zeigen eine genomisch kodierte Strategie, um die modernen Menschen, die Nordeuropa bevölkerten, mit mehr Involukrin auf ihrer Haut zu ›bekleiden‹. Wir kontextualisieren unsere Ergebnisse mit anderen neueren adaptiven Merkmalen der menschlichen Haut, einschließlich Pigmentierung und Haarlosigkeit, und betrachten die Entstehung von exogener Kleidung in diesen evolutionären Zeitskalen für die Entwicklung des modernen Menschen.

Schlagwörter Haut, Barriere, Evolution, Involukrin, Vitamin-D-Rezeptor

The human body is protected or ›clothed‹ by our skin. This protection or barrier function is imparted by epidermal cells or keratinocytes that comprise our epidermis at the surface of the skin (Fuchs 2008) (Fig. 1). The epidermis is an exquisite cellular architecture of distinct layers of kerati-

Summary

The skin is the outermost organ that ›clothes‹ the human body. At the surface of the skin is the epidermis, a tissue comprised of keratinocytes that provide a protective barrier against the environment. To form this protective epidermis, keratinocytes undergo terminal differentiation marked by the expression of structural proteins encoded by genes in the Epidermal Differentiation Complex (EDC) locus. This set of genes is known to be the most rapidly evolving locus in the human genome based on comparative studies of the chimpanzee genome, thus identifying the significance of evolution and genetic variation for the human skin barrier function. Here, we discuss our seminal findings identifying specific EDC genes that have undergone positive selection both in mammalian phylogeny and in modern humans. We further highlight our discovery of the recent evolution of the human skin barrier out-of-Africa. A specific haplotype associated with an increase in the epidermal protein, involucrin, and downstream Vitamin D receptor function in the epidermis underwent a near selective sweep in Northern European populations compared to that of African populations. Our results reveal a genome-encoded strategy to ›clothe‹ the modern humans that populated Northern Europe with more involucrin on their skin. We further contextualize our findings with other recent human skin adaptive traits, including pigmentation and hairlessness, and consider the emergence of exogenous clothing with these evolutionary timescales for modern human evolution.

Keywords Skin, barrier, evolution, involucrin, vitamin D receptor

nocytes that stratify across the entire body. The innermost layer of the epidermis is comprised of basal keratinocytes that proliferate and self-renew. During asymmetrical division, a basal keratinocyte generates two daughter cells, one that self-renews and the other that moves upward towards

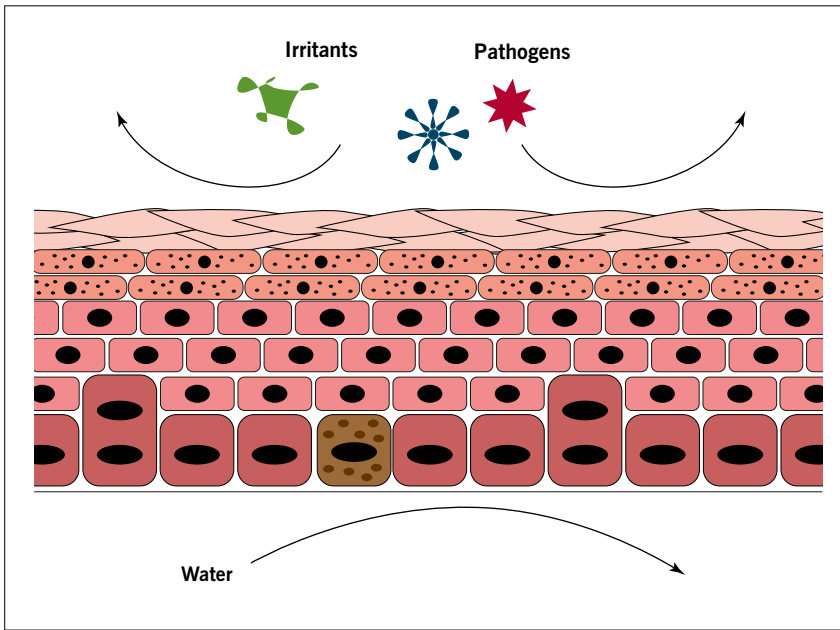


Fig. 1 Schematic of the epidermis comprised of keratinocytes that form stratified layers as a physical barrier against harsh environmental factors (e. g. irritants and pathogens) and prevent water loss.

Abb. 1 Schematische Darstellung der Epidermis, die aus Keratinozyten besteht, die stratifizierte Schichten als physische Barriere gegen raue Umweltfaktoren (z. B. Reizstoffe und Krankheitserreger) bilden und den Wasserverlust verhindern.

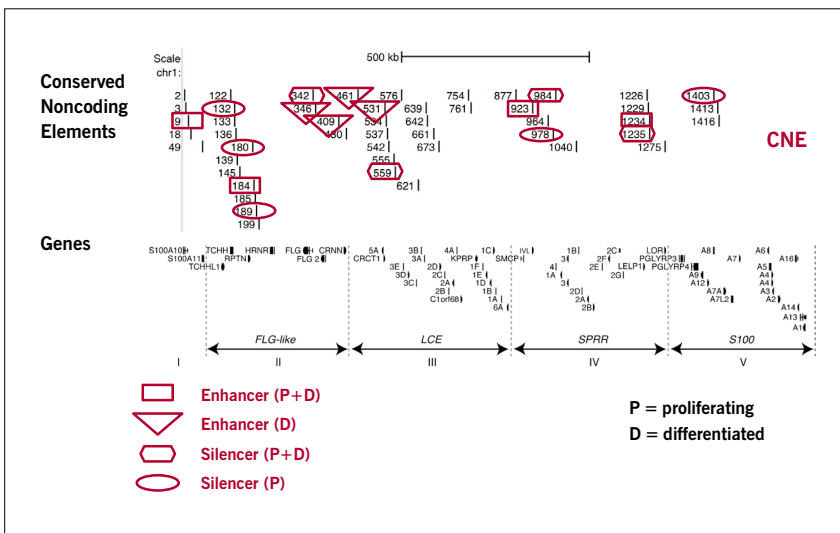


Fig. 2 Genome track of genes Epidermal Differentiation Complex (EDC) locus (bottom track) located on human chromosome 1q21 and conserved noncoding elements (CNEs) (top track). CNEs are numbered to indicate the number of kilobase away from the most 5'EDC gene, S100A10. Red geometric shapes correspond to CNEs that exhibit either enhancer or silencer activity in proliferating (P) and/or differentiated (D) keratinocytes.

Abb. 2 Genomspur des Epidermalen Differenzierungskomplex (EDC) Locus (untere Spur) der Gene auf dem menschlichen Chromosom 1q21 und konservierte nicht-kodierende Elemente (CNEs) (obere Spur). Die CNEs sind nummeriert und geben die Anzahl der Kilobasen an, die vom 5' EDC-Gen, S100A10, entfernt sind. Die roten geometrischen Formen entsprechen den CNEs, die entweder Enhancer- oder Silencer-Aktivität in proliferierenden (P) und/oder differenzierten (D) Keratinozyten aufweisen.

the surface of the skin and begins terminal differentiation (Lechler/Fuchs 2005). This reiterative process forms the differentiated layers of the epidermis to essentially ›clothe‹ the entire body and selectively prevents the penetration of irritants and pathogens and loss of water from the inside out.

As keratinocytes differentiate, they coordinately express genes encoded from the Epidermal Differentiation Complex (EDC) locus (Volz et al. 1993; Mischke et al. 1996; Zhao/Elder 1997). The EDC contains four gene families based on their protein sequence homology: FLG-like (filaggrin-like), LCE (Late Cornified Envelope), SPRR (Small Proline Rich Region), and S100 proteins (Fig. 2). Involucrin is one of the major EDC proteins whose expression is a hallmark marker for terminal differentiation in keratinocytes (Rice/1979). Involucrin is known to be a major scaffolding protein for other SPRR and LCE proteins that are cross-linked together to form the cornified envelope that surrounds and renders the keratinocyte the ›building block‹ of the skin barrier (Simon/Green 1985; Etoh et al. 1986). These individual blocks are, in turn, sealed together by lipids that are

extruded from these keratinocytes to further reinforce protection (Landmann 1986; Wertz 1991; Marekov/Steinert 1998). The collection of EDC proteins in the epidermis contributes to physical barrier function as well as the maintenance of this tissue in coordination with proper immune response.

Motivated by the coordinate gene expression during terminal differentiation, we hypothesized the presence of regulatory elements in the EDC or non-coding DNA sequences to activate gene expression. In comparison to at least 60 human EDC coding genes, we discovered 48 sequences in the EDC that are highly conserved across seven mammals (including humans) yet do not code for protein, hence referred to as conserved non-coding sequences or elements (CNEs) (de Guzman Strong et al. 2010). Conservation of these sequences suggests an element of biological function to maintain these sequences over the course of evolution. We later determined that approximately half of the CNEs exhibit regulatory activity to activate (or enhance) or repress (or silence) reporter gene expression in proliferating

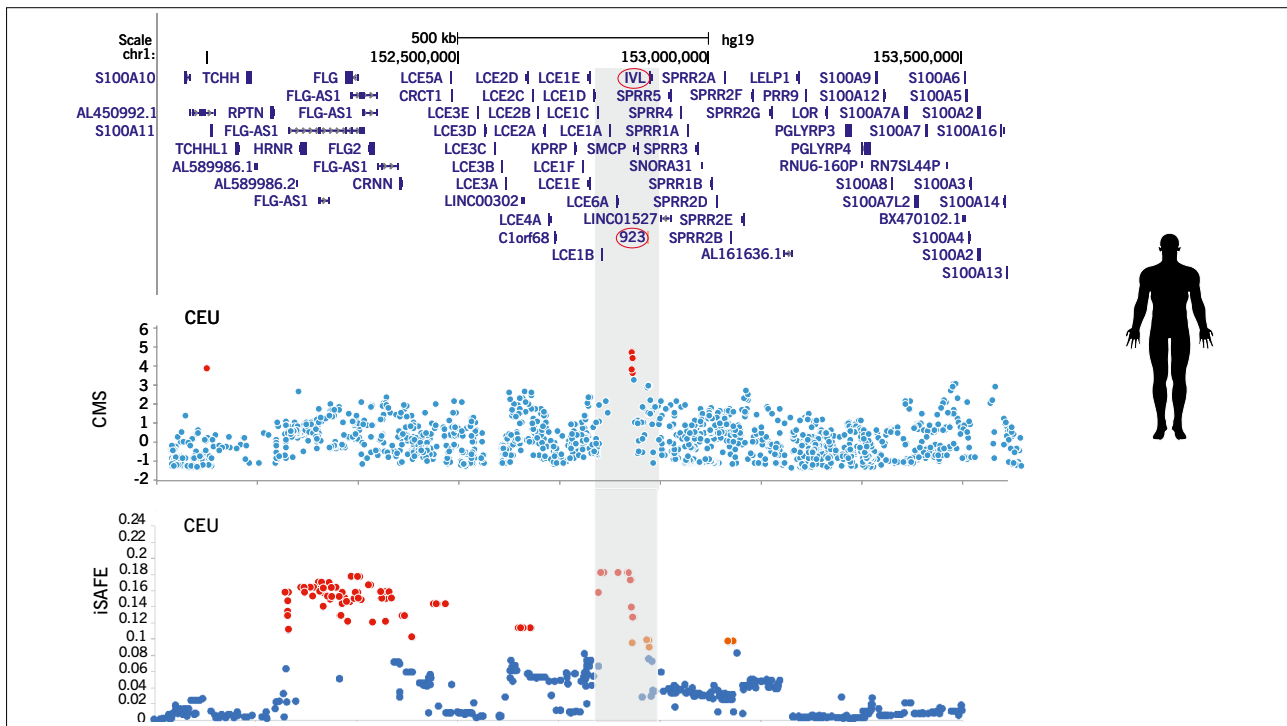


Fig. 3 Positive Selection for involucrin gene (*IVL*) in human CEU (N. European) 1KGP population as determined by the shared region of significantly high CMS and iSAFE scores (grey region).

Abb. 3 Positive Selektion für das Involucrin Gen (*IVL*) in der menschlichen CEU (N. European) 1KGP Population, bestimmt durch die gemeinsame Region mit signifikant hohen CMS- und iSAFE-Scores (graue Region).

and differentiated keratinocytes (Fig. 2). Our finding thus identified enhancers and silencers, respectively, in the EDC for proper coordination of gene expression in keratinocytes. The elucidation of these key elements was crucial for us to further decode genetic variation and functional significance in the EDC in our later studies.

The complete sequencing of the chimpanzee (*Pan troglodytes*) genome was pivotal in finding significance for genetic changes within the EDC in humans. By aligning and comparing the chimpanzee genome to the human reference genome, it was determined that the EDC was the most rapidly evolving locus in the human genome (The Chimpanzee Sequencing and Analysis Consortium 2005). In other words, the human EDC exhibited the highest number of nonsynonymous amino acid substitutions in its protein-coding genes owing to significant changes in single nucleotides compared to that of the chimpanzee. The discovery revealed positive selective pressure on these genetic changes for the skin in human evolution that is poorly understood. This finding motivated us to further examine the significance of the evolution of the EDC over timescales of mammalian phylogeny and across modern human populations that are geographically distinct.

We first sought to determine the macroevolution of the EDC in mammalian phylogeny. Is there evidence of positive selection in the EDC in mammals? Using statistical methods to determine the significance of nonsynonymous amino acid substitutions across 14 mammals via likelihood ratio tests, we identified positive selection for *FLG*, *SPRR4*, *LELP1*, and *S100A2* genes across mammalian phylogeny

(Goodwin/de Guzman Strong 2016). We also determined ongoing positive selection for *SPRR4* in primates, thus supporting more recent evolution in our closest ancestors that was ongoing.

These findings led us to ask questions about more recent microevolution of the EDC in modern humans. In other words, is our human skin barrier evolving? To address this question, we determined and examined signatures of positive selection for each EDC single nucleotide polymorphism (SNP, also known as a common single DNA base pair change) that was publicly available for each of the 1000 Genomes (1KGP) populations – CEU (Northern European), YRI (Yoruba, Africa), and CHB/JPT (pooled Han Chinese and Japanese) (Mathyer et al. 2021). We identified a genomic region in the CEU population that was shared by a set of SNPs that exhibited both significantly high CMS and iSAFE positive selection scores (Fig. 3). A closer examination of these SNPs revealed that they comprise a specific haplotype or a group of DNA variants that are inherited together for the involucrin gene. Using GTEx data, we determined the significance of this positively selected haplotype for more involucrin gene expression in non-sun-exposed human skin. By examining the allele frequency and distribution of this haplotype in other modern human populations in Africa, Southeast Asia, and Europe, we determined a near selective sweep for the selection of this haplotype in Northern Europe that correlated with an increase in northern latitude position. We sought to further demonstrate the significance of the 923 CNE as a putative enhancer for involucrin gene expression. The 923 CNE was deleted using CRISPR/

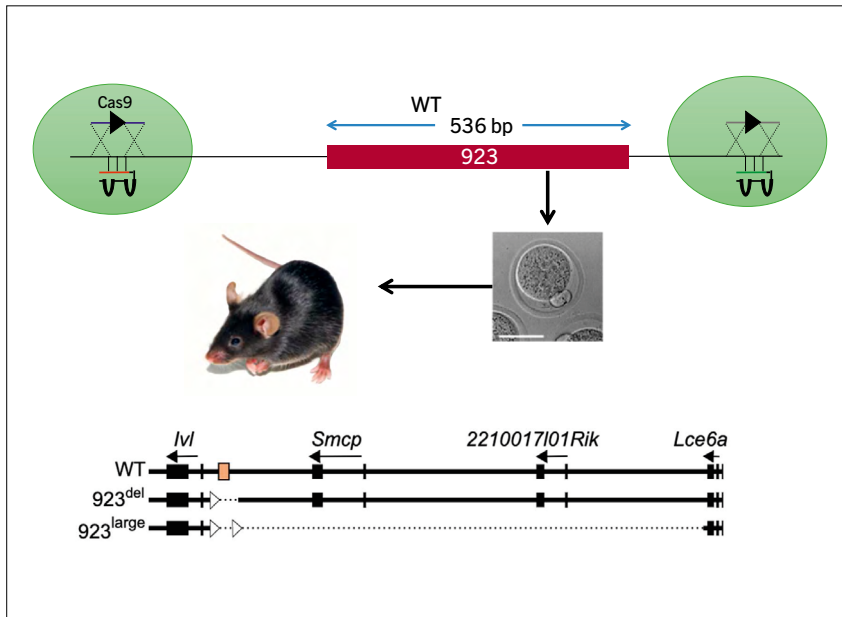


Fig. 4 Strategy to delete 923 CNE in mouse genome by CRISPR/Cas9 genome editing of mouse embryonic stem cells and injection in pseudopregnant mice resulting in two independent mouse strains for the deletion of 923 CNE, 923^{del} and 923^{large}. Arrowhead lines indicate the homologous sequence (marked by dotted X) and loxP site (arrowhead) for CRISPR-mediated recombination by Cas9 nuclease and guided by CRISPR gRNA as shown with the red and green hairpin structures below the targeted locus.

Abb. 4 Strategie zur Löschung von 923 CNE im Mausgenom durch CRISPR/Cas9 Genome Editierung von embryonalen Stammzellen der Maus und Injektion in pseudoträchtige Mäuse, was zu zwei unabhängigen Mausstämmen für die Löschung von 923 CNE führte, 923^{del} und 923^{large}. Die Pfeile zeigen die homologe Sequenz (markiert durch ein gepunktetes X) und die LoxP-Stelle (Pfeilspitze) für die CRISPR-vermittelte Rekombination durch die Cas9-Nuklease und gelenkt durch die CRISPR gRNA, die durch die roten und grünen Haarnadelstrukturen unterhalb des Zielortes dargestellt wird.

Cas9 genome editing in the mouse genome (Fig. 4). Deletion of the 923 CNE resulted in decreased involucrin gene expression in the skin of both 923^{del/del} and 923^{large/large} mice, thus identifying 923 CNE as an enhancer for the involucrin gene (Fig. 5). Thus far, we have identified a positive selection and near selective sweep for an enhancer haplotype for increased involucrin gene expression in the skin in the CEU population revealing human skin barrier evolution out of Africa.

We next examined the significance of having more involucrin on the skin by performing functional studies in the mouse. It was previously determined that involucrin-deficient mice exhibited normal skin barrier development, and it was revealed that involucrin is not required for developing the skin barrier (Djian et al. 2000). This led us to test the alternative hypothesis for involucrin to maintain skin homeostasis in the adult mouse. To test this hypothesis, we examined the skin homeostatic response in involucrin-deficient and wild-type control mice by treating their ear skin with MC903, a vitamin D agonist that induces skin inflammation (Schmidt et al. 2023). After 16 days of daily MC903 treatment, we found that involucrin-deficient mice exhibited a decreased response to MC903-induced inflammation compared to control wild-type mice. To better understand

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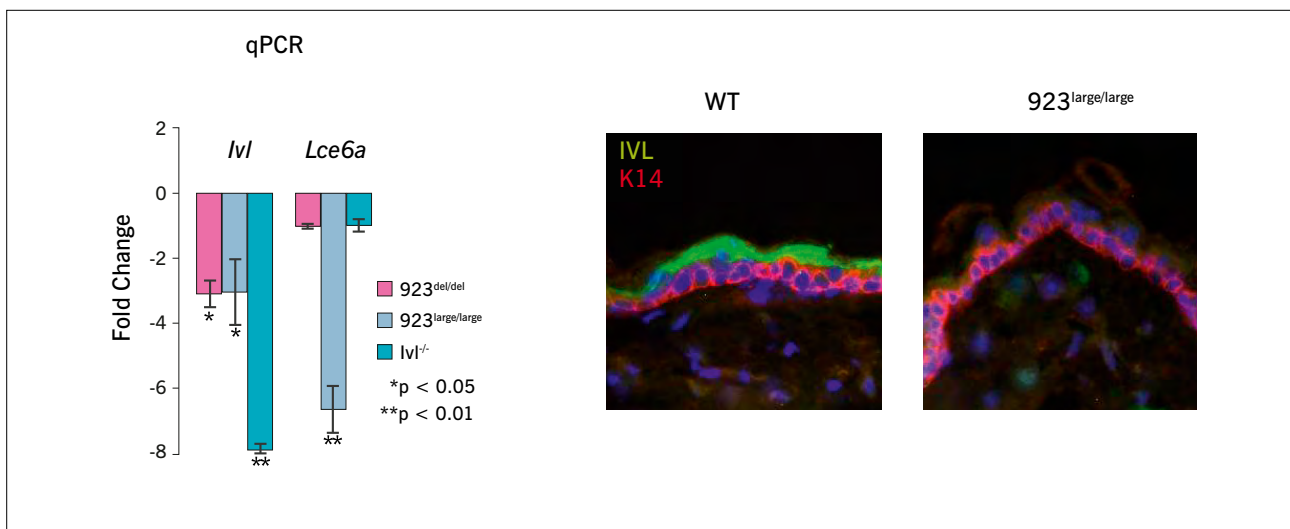
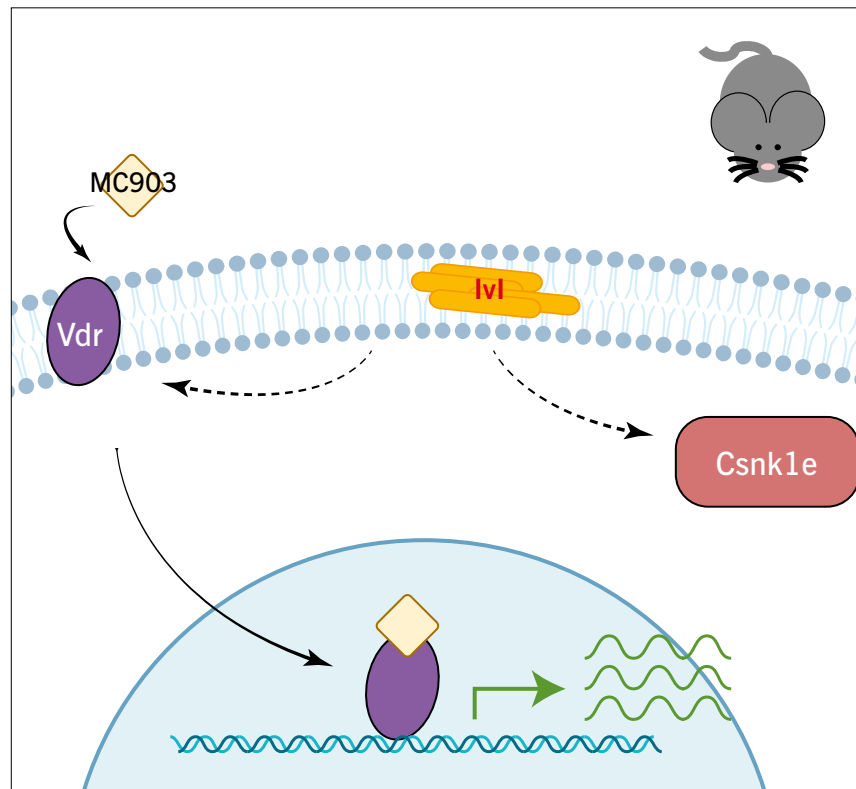


Fig. 5 Loss of involucrin gene (*Ivl*) gene expression in newborns skin of both 923^{del/del} and 923^{large/large} mice compared to control wild-type mice as determined by quantitative PCR (qPCR) and further validated by immunofluorescent microscopy for loss of involucrin protein expression (green) in 923^{large/large} mouse skin with keratin 14 (K14, red) expression. *Lce6a* gene expression was also significantly decreased in newborn skin of 923^{large/large} mice as this gene was also deleted. p values < 0.05 shown to demonstrate significance.

Abb. 5 Verlust der Involukrin (*Ivl*) [A2]-Genexpression in der Haut von sowohl Neugeborenen 923^{del/del} als auch 923^{large/large} Mäusen im Vergleich zu Wildtyp-Kontrollmäusen, bestimmt durch quantitative PCR (qPCR) und weiter validiert durch Immunfluoreszenzmikroskopie für den Verlust der Involukrin-Proteinexpression (grün) in der Haut von 923^{large/large} Mäusen mit Keratin 14 (K14, rot) Expression. Die Expression des Gens *Lce6a* war in der neugeborenen Haut von 923^{large/large}-Mäusen ebenfalls signifikant verringert, da dieses Gen ebenfalls gelöscht wurde. p-Werte < 0,05 zeigen die Signifikanz.

Fig. 6 Schematic of our MC903 mouse study that identifies a role for involucrin (*Ivl*) to positively regulate Vitamin D receptor (*Vdr*) function to localize to the nucleus and activate gene expression (shown in green wave lines), a biological activity that was lost in Involucrin-deficient mice.

Abb. 6 Schematische Darstellung unserer MC903-Mausstudie, die zeigt, dass Involucrin (*Ivl*) die Funktion des Vitamin D-Rezeptors (*Vdr*) positiv reguliert, um sich im Zellkern zu lokalisieren und die Genexpression zu aktivieren (dargestellt in den grünen Wellenlinien), eine biologische Aktivität, die bei Involucrin-defizienten Mäusen verloren gegangen ist.



why involucrin-deficient mice failed to respond to MC903, we performed single cell RNA sequencing (scRNA-seq) on MC903-treated ears to determine the gene expression profile at the single cell level and their differences between involucrin-deficient and control wild-type mice. Our bioinformatic analyses of scRNA-seq identified a significant reduction in Vitamin D receptor expression in many of the keratinocyte clusters. Our finding reveals a role for involucrin in regulating vitamin D receptor expression in keratinocytes (Fig. 6).

We next sought to determine the relevance of this putative relationship between involucrin and vitamin D receptor in light and dark-pigmented human foreskin samples with the goal of understanding the significance of having more involucrin for human skin barrier evolution. Here, we found a direct yet inverse relationship between involucrin and vitamin D receptor expression with respect to human skin pigmentation that was used as a proxy for CEU haplotype (light pigmentation). Involucrin and vitamin D receptor gene expression decreased as pigmentation increased in human foreskin samples (Fig. 7). Decreased vitamin D receptor was also observed in the normal skin and keloid scars of Black individuals compared to White individuals (Hahn/Supp 2017), which further demonstrates an inverse correlation between involucrin and vitamin D receptor with pigmentation. Indeed, future studies are needed to best determine a more direct relationship for vitamin D receptor expression with respect to involucrin CEU haplotypes.

In summary, our genomic studies in modern human populations and functional studies in mice identify evolution of the human skin in Northern Europe to have more involucrin and its significance in impacting vitamin D receptor function. Yet these results also raise an impor-

tant question: When did this occur? We performed a preliminary analysis that suggests this derived haplotype for more involucrin in European skin likely emerged in modern humans. For example, SNP rs4845327-G is found in the positively selected haplotype in Europe and is not reported in the genomes of the archaic Denisovan and four Neanderthal individuals that have been sequenced to date (Briggs et al. 2007; Green et al. 2010). However, this SNP allele is also found in modern human populations in Africa and, as described by our study, is found in a haplotype that reached a near selective sweep in European populations. These findings suggest the emergence of this allele to have occurred in modern humans after the divergence of Denisovan and Neanderthal and likely prior to the human migration out of Africa that occurred approximately 50,000 years ago. This approximation also leads us to surmise that this allele for more involucrin was present in Neolithic humans who likely had darker skin and was positively selected in a haplotype that arose over time as humans migrated into Europe. To date and to our knowledge, no other SNPs have been reported to be associated with increased involucrin and are not in this positively selected CEU haplotype. This is of great significance as it suggests that this human haplotype was likely a single event in modern human history that was positively selected in recent history. The alternative explanation is the emergence of multiple and independent genetic events for increased involucrin that is less likely. The causative SNP(s) for this increased expression for involucrin in this haplotype has yet to be determined. Indeed, it is interesting to speculate that the emergence of having more involucrin in modern humans in Africa may have coincided with and perhaps compensated for the succeeding loss of body hair.

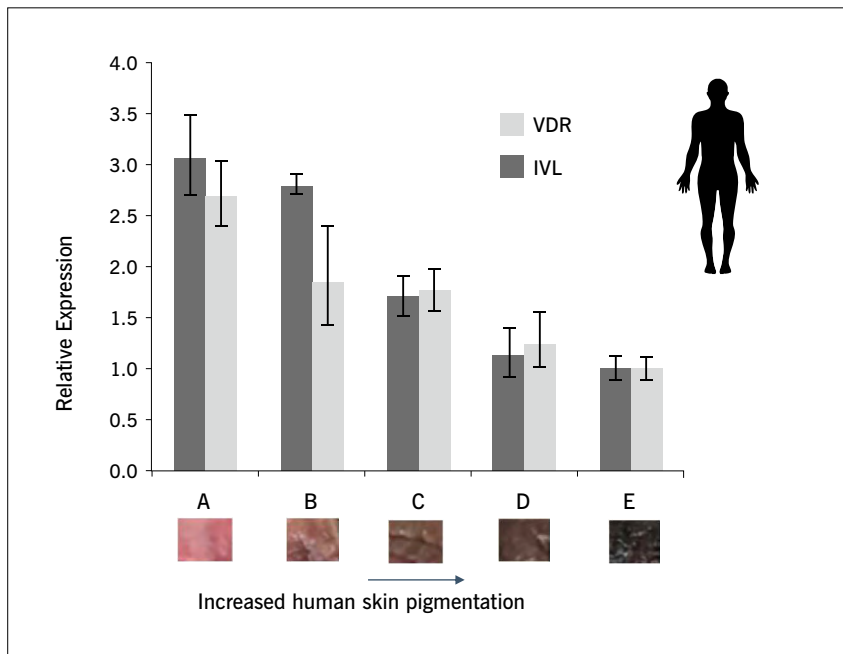


Fig. 7 A direct inverse relationship between involucrin and vitamin D receptor and human skin pigmentation (proxy for CEU vs. YRI ancestral skin). IVL and VDR gene expression decreases as pigmentation increases in human foreskin samples.

Abb. 7 Eine direkte umgekehrte Beziehung zwischen Involukrin und dem Vitamin-D-Rezeptor und der menschlichen Hautpigmentierung (stellvertretend für CEU vs. YRI-Haut menschlicher Vorfahren). Die IVL- und VDR-Genexpression nimmt mit zunehmender Pigmentierung in menschlichen Vorhautproben ab.

Furthermore, ours and several other evolutionary studies are beginning to understand that the human skin has adapted to different regions around the world. The observation of increased skin pigmentation in populations that live near the equator attests to the selection of darker pigmentation as a human adaptable trait in regions with high solar irradiation exposure (Jablonski/Chaplin 2010). Our findings for the genetics for more involucrin highlight the innovation of the human genome and its selection as an adaptive feature to the environment to appropriately ›clothe‹ the human body. As higher involucrin is correlated with higher vitamin D receptor expression, this conserved relationship in the mouse and highly associated with lighter vs dark pigmented skin further reveals a potential titration of the skin by involucrin to be environmentally responsive. Future studies are ongoing to determine the biological significance of having more involucrin ›clothing‹ with respect to environments of Northern European latitude and the mechanism by which involucrin regulates the vitamin D receptor.

Additionally, the loss of terminal body hair is another example of human skin adaptation. Two current models are proposed as negative selection pressures to retain body-wide hair in contrast to our ancestors (Wheeler 1984; Pagel/Bodmer 2003). It has been proposed that the loss of body hair prevented the ability of lice to properly inhabit the human host and thus mitigate its potential as a vector for infection (Pagel/Bodmer 2003). As modern humans became bipedal and began to hunt, another model was suggested for the loss of body-wide hair: it facilitated an increased demand for more adaptive thermal regulation (Wheeler 1984). How does the emergence of exogenous clothing factor into our evolutionary timescale for human skin adaptation? Rogers et al. posited that hairlessness likely emerged prior

to the use of clothing (Rogers et al. 2004), given that there is no evidence to date for hide scraping prior to 300 000 years ago, nor even for clothing before 20 000 years ago. This contrasts with an earlier dating of human thermoregulatory hairlessness that may have coincided with humans as hunters at least 400 000–2.5 million years ago. Thus, the likelihood of clothing predating hairlessness is low and therefore unlikely to be a selective pressure for human skin evolution. Yet as humans physically clothed themselves, their covered skin sites likely adapted (not evolved) to the frictional forces or thermoprotection of exogenous clothing. These biological responses are akin to a wound-healing response in that they are reversible and not heritable, and thus not evolutionarily constrained. Nevertheless, these current models and their dating indicate that clothing was not likely a selective pressure for hairlessness but rather an innovative strategy that probably occurred after humans lost their terminal body hair. We further reason that the near selective sweep of more involucrin ›clothing‹ likely represents an even more recent event in modern human evolution. We anticipate more accurate dating and assessments of future archaeological findings with newer technologies to better reconstruct our understanding of modern human evolution with respect to skin adaptation and clothing.

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