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MicroRNA-148a controls epidermal and hair follicle stem/progenitor cells by modulating the activities of Rock1 and Elf5

M. E. Pickup (0000-0002-6006-2540), A. Hu (0000-0001-8821-6591) H. J. Patel (0000-0002-8461-9980) and M. I. Ahmed* (0000-0002-9051-7681).

Nottingham Trent University, School of Science and Technology, Nottingham, UK

Correspondence:
*Mohammed I. Ahmed, PhD, Nottingham Trent University, School of Science and Technology, Nottingham, UK, E-mail: mohammed.ahmed@ntu.ac.uk

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Abstract

Skin and hair development is regulated by complex programs of gene activation, silencing and microRNA-dependent modulation of gene expression to maintain normal skin and hair follicle (HF) development, homeostasis, and cycling. Here, we show that miR-148a, through its gene targets, plays an important role in regulating skin homeostasis and HF cycling. RNA and protein analysis of miR-148a and its gene targets were analysed using a combination of in vitro and in vivo experiments. We show that expression of miR-148a markedly increases during telogen (bulge and hair germ stem cell compartments). Administration of anti-sense miR-148a inhibitor into mouse skin during the telogen phases of the postnatal hair cycle results in accelerated anagen development and altered stem cell activity in skin. We also demonstrate that miR-148a can regulate colony forming abilities of HF-bulge stem cells, as well as control keratinocyte proliferation/differentiation processes. RNA and protein analysis revealed that miR-148a may control these processes by regulating expression of Rock1 and Elf5, in vitro and in vivo. This data provides an important foundation for further analyses of miR-148a as a crucial regulator of these genes target in the skin and HFs and its importance in maintaining stem/progenitor cell functions during normal tissue homeostasis and regeneration.
**Introduction**

Skin development is a complex and dynamic process that results in formation of a fully formed stratified epidermis with self-renewing capabilities, including several skin appendages, such as, hair follicles (HFs) (Blanpain and Fuchs, 2009; Hsu et al., 2014). HF development and cycling processes are regulated by a well-balanced relationship between cell proliferation, differentiation, and apoptosis. These processes are controlled at several levels including epigenetic, signalling/transcription factor-mediated and microRNA mechanisms. MicroRNAs (miRNAs, miRs) are small non-coding-RNAs involved in the post-transcriptional regulation of gene expression. MiRNAs provide an additional level of regulation of important cellular processes such as growth, differentiation and remodelling of skin (Ahmed et al., 2014; Ahmed et al., 2011; Aunin et al., 2017; Mardaryev et al., 2010).

MiRNAs are ~22 nucleotide long and highly conserved, and their regulation is based on their interactions with target messenger RNAs by base-pairing between 5’ end sequences of miRNAs and mRNAs sequences located in the 3’ untranslated region (3’ UTR). This results in either mRNA destabilization, inhibition of translation initiation, or both, impacting important cellular pathways, such as cellular proliferation and differentiation. Thus, miRNAs principally contribute to the regulation of gene expression by fine tuning and buffering the activity of signalling pathways (Ambros, 2001; Lee et al., 1993; O’Brien et al., 2018; Pong and Gullerova, 2018). MiRNAs and their targets characterize remarkable regulatory networks, which have a crucial role in the regulation of gene expression programs in stem cells (SCs) and their progenies (Ambros, 2001; Inui et al., 2010; Zhang et al., 2011).
Principal studies have identified ~70 miRNAs and >200 miRNAs, which are dynamically expressed in mouse embryonic skin and during skin and HF cyclic regeneration, respectively (Andl et al., 2006; Mardaryev et al., 2010; Yi et al., 2006). In addition, epidermal-specific deletion of the miRNA processors Dicer, Dorsha or Dgcr8 results in the severe abnormalities in skin and HF development and growth (Andl et al., 2006; Teta et al., 2012; Yi et al., 2006; Yi et al., 2009). These findings highlight the crucial role miRNAs have in the control of gene expression programs during skin and hair cycle-associated tissue remodelling.

Furthermore, individual miRNAs have been identified to be involved in controlling the expression of key regulators of cutaneous-SCs and their lineage-committed progenies that control skin and HF development, homeostasis and regeneration: miR-203 controls the proliferative potential of epithelial precursor cells (Lena et al., 2008; Yi et al., 2008), miR-125b serves as a rheostat that controls SC proliferation and differentiation (Zhang et al., 2011) and miR-205 is indispensable for SC survival (Wang et al., 2013a). While, miR-214 is a key regulator of SC functions during normal tissue homeostasis and regeneration (Ahmed et al., 2014) and miR-31 controls hair cycle-associated tissue remodelling (Mardaryev et al., 2010). Recently, miR-218 was shown to regulate postnatal skin and HF development via Wnt signalling pathway (Zhao et al., 2019). These findings highlight the importance of miRNAs in the control of gene expression programs during skin development and hair cycle-associated tissue remodelling.

Although, substantial progress has been made in discovering the important regulators in skin and HF development and homeostasis, understanding the molecular mechanisms involved in the establishment of signalling/transcription networks in keratinocytes still requires additional efforts. In this study, we aimed to explore the role of miR-148a in regulating stem/progenitor cells during skin and HF development and
regeneration. MiRNA-148a has been identified as an onco-suppressor in various cancers (Takahashi et al., 2012; Wang et al., 2013b), including in skin (Luo et al., 2015; Tian et al., 2015). However, its role in the control of skin and HF development and homeostasis remains unknown.

Here, we identify miR-148a as a contributor to stem/progenitor cell activities in maintaining skin and HF development and homeostasis, at least in part, by regulating Rho-associated coiled-coil kinase (Rock)-1 and E74-like factor 5 (Elf5). We demonstrate that miR-148a shows distinct expression patterns in the epidermis and HF stem/progenitor cell compartments during hair cycle-associated tissue remodelling. Modulation of miR-148a activities can affect SC colony formation, as well as, keratinocyte proliferation and differentiation processes, in vitro. While in vivo, loss of miR-148a can lead to anagen progression and irregular expression pattern of SCs and differentiation during postnatal development.

**Results**

MiRNA-148a is expressed in distinct stem/progenitor cell population in the skin and hair follicles.

To understand the role of miR-148a in the control of skin and HF development/homeostasis and regeneration, miR-148a expression pattern was determined in C57Bl/6 wild-type mouse dorsal skin during postnatal and depilation-induced hair cycles. During spontaneous postnatal skin and HF development, we observed low miR-148a expression levels in the skin at postnatal day (P) 12, with its expression increasing significantly during HF transition to the first regressing phase (catagen, P16-P17) and elevated further during subsequent resting phase (telogen; P20-P23;Figure 1A). Similar fluctuations in miR-148a expression levels were observed in adult skin during depilation-induced hair cycle: miR-148a expression progressively
decreased during HF-transition from being its highest at telogen stage (day 0) to significantly dropping in expression during anagen and catagen stages (day 3-19, Figure 1B). As CD34 is uniquely expressed in and used to isolate mouse HF-bulge stem cells (SCs) (Blanpain et al., 2004; Morris et al., 2003), therefore we employed FACS-sorting and identified that the expression of miR-148a was significantly higher in HF-bulge SCs populations (CD34+ cells versus CD34 negative cell populations) (Figure 1C).

We followed this analysis with, in situ hybridization in postnatal and depilation induced hair cycle (Figure 1D). Consistent with our RT-qPCR data, during depilation-induced hair cycle, we observed high expression of miR-148a in telogen skin, localised predominately in the epidermis (suprabasal layer) and in the telogen HFs (bulge and secondary hair germ regions). While in subsequent stages, reduced expression was seen in early anagen (day 5), with lack of expression seen in full anagen HFs (day 12) and catagen HFs (day 18) (Figure 1D). We subsequently performed fluorescent in situ hybridization with simultaneous immunofluorescent staining, and we observed that miR-148a co-localises with SC-makers cytokeratin 15 (Krt15) and CD34 in telogen HFs (bulge and secondary hair germ regions). In skin, miR-148a is predominantly expressed in the suprabasal layer of the epidermis compared to Krt15, which is expressed in the basal layer (Figure 1E).

Inhibition of miR-148a activity in the skin accelerates telogen-anagen development and alters stem cell activity

To explore the role of miR-148a in the control of hair cycle-associated tissue remodelling, a synthetic inhibitor which specifically binds and blocks miR-148a activity was administered into the dorsal skin of wild type mice at different time-points of spontaneous hair cycle.
Due to miR-148a expression being the highest during postnatal days P20-23 (Figure 1A), we administered anti-miR-148a daily into dorsal skin during the postnatal days P20, P21 and P22, and skin was harvested at postnatal day P23 (Figure 2A-G). The efficiency of inhibiting miR-148a activity was assessed by TaqMan RT-qPCR assay and showed significant decrease of miR-148a expression in the skin treated with miR-148a inhibitors versus the controls (Figure S4).

Inhibition of miR-148a activity during P20-P22 resulted in acceleration of telogen-anagen progression compared to the controls (Figure 2A-G). In mice treated with anti-miR-148a, significantly more HFs were found in anagen III stage, characterized by enlarged dermal papilla partially enclosed by keratinocytes, whereas many HFs in the control skin reached only anagen II phase of the hair cycle (Figure 2A-G). Acceleration of telogen-anagen development in skin treated with anti-miR-148a versus controls was also associated with increase in the skin thickness (Figure 2E), a well-established parameter of the hair cycle progression (Hansen et al., 1984; Mardaryev et al., 2010).

To determine if miR-148a has an impact on skin and HF stem/progenitor cell populations, we performed immunohistochemical staining. We observed an increased expression of Krt15+ SCs in the epidermis and in HFs of miR-148a inhibited skins compared to controls (Figure 2F). These changes in expression were confirmed by quantitative immunofluorescence analysis, which revealed increased expression of Krt15 in anti-miR-148a treated versus control skins (Figure 2G). This data suggests an early or abnormal activation/expansion of SCs has occurred after inhibition. We next isolated mouse HF-bulge SCs from wild type C57Bl/6 mice skin and compared colony formation capabilities, in vitro. We observed a significant increase in the total number of colonies and the formation of larger holoclonal colonies (>50μm) after miR-148a
loss compared to controls (Figure 2H-I). Successful knockdown of miR-148a in HF-bulge SCs compared to controls was confirmed by TaqMan RT-qPCR (data not shown).

MiRNA-148a overexpression induces complex changes in gene expression programs in keratinocytes

To explore the molecular mechanisms of miR-148a in primary mouse epidermal keratinocytes (PMEKs), RNA sequencing analysis was performed after overexpression of miR-148a (pro-miR-148a) versus controls (Figure 3A). Functional ontology programs (https://usegalaxy.org/) were used to categorize differentially expressed genes into the set of 12 distinct functional categories. These were then ranked into groups with the most significantly changed genes, which included ‘differentiation’ and ‘cell cycle’ category groups (Figure 3B). To identify putative miR-148a gene targets, we performed bioinformatics analysis using well-established miRNA target prediction tools (Ahmed et al., 2019; Mardaryev et al., 2010). By overlapping predicted miR-148a targets from three different databases, we identified 408 potential genes whose expression may be regulated by miR-148a. To further refine this list, we overlapped these 408 genes with our RNA-seq data, genes that were significantly downregulated (859 genes) in pro-miR-148a versus controls (Figure 3C). We selected genes for RT-qPCR validation with predicted binding sites, as well as genes known to have important regulatory role in epithelial development (total 43 gene targets). RT-qPCR analysis revealed that only Rock1 and Elf5 were significantly up and/or downregulated of the genes analysed after gain-or-loss of miR-148a, respectively (Figure 3D-E, Figure S1).

Our analysis then focused on the effects of miR-148a on Rock1 and Elf5 protein levels: we observed protein levels increased and/or decreased after gain-or-loss of miR-148a, respectively (Figure 3F). We next confirmed the direct regulation of Rock1 and Elf5
by miR-148a using a luciferase reporter assay. Co-transfection of HaCaT cells with pro-miR-148a mimic (overexpression) and either Rock1 or Elf5 3'UTR reporter construct, caused a significant reduction in luciferase activity, respectively compared with their corresponding controls. Whereas these effects were not detected when miR-148a binding sites were mutated (Figure 3G).

Additionally, we performed fluorescent in situ hybridization for miR-148a with simultaneous immunofluorescence detection of Rock1 and Elf5 in wild type telogen skin. In the epidermis, miR-148a, Rock1 and Elf5 were expressed mutually exclusively, where miR-148a is primarily expressed in the suprabasal layer, while, Rock1 and Elf5 expression are predominately observed in the basal layer. We also observed Rock1 and Elf5 were co-expressed with miR-148a within SC-compartments of HFs (bulge and secondary hair germ) (Figure 3H-I, Figure S3).

Modulation miR-148a activities alters expression of key regulators during keratinocyte proliferation and differentiation

Our bioinformatic analysis revealed differentially expressed genes that belong to the ‘cell cycle’ and ‘differentiation’ categories (Figure 3B), which were further investigated.

Using RT-qPCR, we confirmed that the transcript levels of Cdk16, Cdk1, Ccnb2, Ccne1, Ccnd1, Ccnd2 and Cksβ1 (Cyclin dependent kinase 16, Cyclin dependent kinase 1, Cyclin B2, Cyclin E1, Cyclin D1, Cyclin D2 and CDC28 protein kinase 1β) were significantly increased and/or decreased after modulation of miR-148a activity (Figure 4A-B). To assess whether miR-148a shows any effects on cell cycle, PMEKs were stained by propidium iodide and flow cytometric analysis revealed overexpression
of miR-148a led to S-phase accumulation and prevented cells progressing into G2/M phase compared with controls (Figure 4C). Interestingly, this accumulation of cells in S-phase suggests that miR-148a allows cells to commit to S-phase from G0/G1 but prevents progression through the cell cycle in keratinocytes. Potentially acting as a checkpoint of mitosis from the S-to-G2/M phase.

We next examined the effects of miR-148a during calcium-induced keratinocyte differentiation, which revealed a significant increase and/or decrease in expression at transcript level of keratinocyte differentiation-associated genes (cytokeratin 1(Krt1), Krt10, Involucrin (Ivl)) after modulating miR-148a activities versus controls (Figure 4D-E). Additionally, Rock1 and Elf5 were also significantly increased and/or decreased in expression after modulation of miR-148a activities (Figure 4D-E). Similar impact on protein levels was observed after overexpression and/or inhibition of miR-148a for Krt1, Rock1 and Elf5 during keratinocyte differentiation (Figure 4F-G). We also attempted to determine if miR-148a could prevent nuclear localisation and activation of Rock1 and Elf5 upon PMEKs calcium induced-keratinocyte differentiation. Using immunocytochemistry and western blot analysis, we observed that Rock1 and Elf5 were significantly hindered in their ability to localize to the nucleus after overexpression of miR-148a (pro-miR-148a) during differentiation (Figure S2A-H).

MiRNA-148a Regulates Expression of Rock1 and Elf5 in Skin and Hair follicles

Expression of target genes as well as impact on proliferation and differentiation were further examined in skin treated with anti-miR-148a, in vivo (Figure 5). In the epidermis: inhibition of miR-148a resulted in increased and broader expression Krt1 compared with controls (Figure 5A-B). This data is consistent with our in vitro data demonstrating miR-148a effects on keratinocyte differentiation (Figure 4D-G).
suggesting that miR-148a is potentially required for maintaining skin integrity. However, we did not notice observable differences in Ki67 expression in miR-148a inhibited (epidermis or HFs) compared to controls (data not shown). This data suggests that loss of miR-148a in the epidermis, can lead to the transition of keratinocytes from proliferation to early differentiation, \textit{in vivo}.

We observed increased expression of Rock1 and Elf5 in the basal layer and appearance in suprabasal layers of treated skin with anti-miR-148a (Figure 5D, F) compared to control epidermis (Figure 5C, E). In the HFs, Rock1 is increased in the discrete cell populations (outer root sheath (ORS), bulge, and hair bulb regions) in developing HFs (Figure 5D) compared with control HFs (Figure 5C). Elf5 expression is observed throughout the HFs including the developing hair bulb, ORS and bulge region (Figure 5F) compared to controls HFs, where expression was lower and restricted to bulge region of HF and not present in advancing hair bulb (Figure 5E). These changes in expression were confirmed by quantitative immunofluorescence analysis, which revealed increased expression of Krt1, Rock1 and Elf5 in anti-miR-148a treated \textit{versus} control skins (Figure 5G-I).

**Discussion**

We have identified a previously undescribed role for miR-148a in the control of skin and HF maintenance and demonstrated that: (1) miR-148a shows spatiotemporal expression patterns predominately in the telogen stage of skin and HFs; (2) modulation of miR-148a in SCs and keratinocytes, affects cell proliferation, differentiation and colony forming abilities, \textit{in vitro}; (3) inhibition of miR-148a during postnatal hair cycle results in HFs progressing further into anagen (without effecting HF morphology), abnormal expression of differentiation and SCs markers and gene targets, \textit{in vivo}; and
(4) miR-148a regulates the balance of cellular processes in skin and HFs by regulating the activities of two gene transcripts in skin, Rock1 and Elf5, which are potentially required for normal development of skin and HFs. Our data suggests miR-148a through its gene targets has an important regulatory role in these cellular programs controlling skin and hair cycle-associated changes of gene expressions. Our findings add miR-148a to only a few miRNAs reported in skin and HFs in regulating gene targets associated with stem/progenitor cells, proliferation and differentiation during skin and HF development, homeostasis, and regeneration.

By analysing the gene expression changes in primary keratinocytes and demonstrating that miR-148a is involved in regulating Rock1 and Elf5 in vitro and in vivo (Figure 3D-I; Figure 5C-F), suggests that miR-148a acts, in part, by regulating suprabasal expression of basal genes, thereby potentially acting as a switch between proliferation and differentiation in keratinocytes.

MiRNA-148a was also co-expressed with Rock1 and Elf5 in stem/progenitor cell regions of telogen HFs (bulge and hair germ, Figure 3H-I), suggesting that miR-148a also has an important role in regulating SC-fate and activity during HF cycling. Of note, miRNAs and their targets tend to be mutually exclusive in neighbouring cells/tissues (Wang et al., 2019) as seen for miR-148a in the epidermis (basal versus suprabasal). However, miRNA and target mRNA co-expression have also been observed in a cell and tissue-dependent manner (Liu and Kohane, 2009), suggesting a dual functionality of miR-148a is dependent on its spatiotemporal location in skin. Our data suggests that miR-148a through Rock1 and Elf5 contributes to normal skin and HF development and homeostasis by finely regulating stem/progenitor cells activities leading to normal cell growth and homeostasis. However, currently it is still unclear what functional roles
Rock1 and Elf5 have within stem/progenitor cell populations in skin and HFs, which require further investigations.

Subsequently, we observed that modulating miR-148a effects cellular proliferation and differentiation processes, in vitro (Figure 4A-C). These findings are consistent with miR-148a known function as an anti-proliferative regulator and is downregulated in cancer tissues, including in skin (Li et al., 2019; Wang et al., 2013b). Our data revealed cell accumulation at S-phase-G2/M transition after overexpression of miR-148a (Figure 4C). We speculate that this may be caused by downregulation of Cdk1 and Cks1β as cell cycle arrest between S-phase-G2/M transition/mitosis has been attributed to impaired transcription of these genes (Martinsson-Ahlzén et al., 2008). Additionally, Cdk1 is a key regulator of S-phase and mitosis (Murray, 2004). While Cks1β promotes cell growth, invasion, and metastasis (Shi et al., 2020). Our data suggests that miR-148a is potentially regulating cell proliferation, in part, by controlling Cdk1 and Cks1β levels and contributes to a previously unreported mechanism of keratinocyte anti-proliferative mitosis-checkpoint to maintain healthy keratinocytes.

During cellular differentiation, miR-148a has been shown to regulate skeletal muscle cell differentiation (Yin et al., 2020), and monocytes differentiation into macrophages (Huang et al., 2017). Of interest, inhibition of Rock1 has been shown to result in the failure of human keratinocytes to terminal differentiate (Chapman et al., 2014; McMullan et al., 2003), which is consistent with our data in PMEKs (Figure 4D-G). While, Elf5 is essential in the control of mammary cell proliferation and differentiation processes (Zhou et al., 2005) and is expressed in differentiating keratinocytes, IRS and ORS layers of the HF (Choi et al., 2008; Singh et al., 2019). Our data suggests that in the epidermis miR-148a may function, in part, by restricting proliferative potential of...
stem/progenitors as they transitioned from basal to suprabasal layers through regulation of Rock1 and Elf5 expression. This mechanism may explain how proliferating keratinocytes commit to differentiation and how the epidermis is protected from cell growth abnormalities and disease.

We next examined longer-term effects in vivo by inhibiting miR-148a in postnatal skin and hair cycle associated tissue remodelling. We observed that the inhibitory effects of miR-148a: leads to telogen-anagen progression, modulation of stem/progenitor activities and differentiation processes (Figures 2 and 5) but not proliferation, in vivo. This data suggests that miR-148a is an important regulator of HF progression (telogen to anagen transition) via tight regulation of HF stem/progenitor cell populations. Our findings suggest that negative regulation of Rock1 by miR-148a may maintain the self-renewal and stemness characteristics of HF-SCs, which has been observed in primary skin tissue cultures after inhibition of Rock1 (An et al., 2018). In addition, studies have suggested that Rock1 signalling inhibition elevates stem/progenitor cell functions, in vitro (Centonze et al., 2022). Furthermore, Rock1 has been shown to promote embryonic SC-colony formation and maintenance of neural progenitor cells (Chang et al., 2010). While, Elf5 is expressed in differentiated epidermal and oral mouse keratinocytes as well as in the IRS and ORS of HFs (Choi et al., 2008; Oakes et al., 2008; Parikh et al., 2008; Singh et al., 2019; Tummala and Sinha, 2006). However, Elf5 functional role in these cell populations has not been determined. It is, therefore, important to understand the expression pattern of Elf5 in order to explore its potential functions in skin and HFs. We believe Elf5 (via miR-148a regulation) may have a similar functional role, as determined in other epithelial tissues (Zhou, Chehab et al. 2005, Chakrabarti, Wei et al. 2012, Grassmeyer, Mukherjee et al. 2017), in regulating
stem/progenitor cells and cell fate processes in skin and HFs, which require further investigations.

In the epidermis, miR-148a can regulate the transition of keratinocytes from proliferation to early differentiation, at least in part, through modulation of the activity and expression of Rock1 and Elf5, which are required to maintain healthy skin and HFs. Rock1 has been shown to be involved in proliferation and differentiation processes of keratinocytes and in other epithelial cells (Ma et al., 2017). In addition, reduced Rock1 function results in cellular senescence, both in vitro and in vivo (Centonze et al., 2022; Kümpér et al., 2016). This loss of Rock1 mediated cellular senescence involves downregulation of cell cycle protein Cdk1 (Diril et al., 2012). Of note, after modulating miR-148a expression we observed that Cdk1 along with Rock1 were up-and/or downregulated in PMEKs (Figure 4A-B). Our data suggests that miR-148a may regulate keratinocyte proliferation and differentiation in skin by regulating cell cycle progression either by targeting Cdk1 directly (Wang et al., 2020) or through downregulation of Rock1 leading to reduced Cdk1 (Kümper et al., 2016) or both, in keratinocytes. Interestingly, elevated Rock1 expression has also been implicated in tumour progression, deregulated differentiation, expansion of SC-populations and increased motility in keratinocytes (Benitah et al., 2004; Lefort et al., 2007; Whatcott et al., 2017; Wilkinson et al., 2005). This suggests that miR-148a regulation of Rock1 may also prevent cancer formation in skin, which is consistent with its role as a tumour suppressor. This data highlights a potential previously unknown role of miR-148a as a protector against age-related pathological conditions, such as, cellular senescence, as well as a previously undescribed mechanism of anti-tumourigenic function via Rock1, leading to the control of normal cell proliferation and differentiation processes in skin.
In summary, our data reveal that miR-148a is a key determinant that controls the activities of Rock1 and Elf5 in skin. As both Rock1 and Elf5 play crucial roles in the control of cellular proliferation and differentiation and stem/progenitor cell activity in many organs, we propose that an essential function of miR-148a in the epidermis is to restrict proliferative potential of basal cell progenitors as they transitioned from basal to suprabasal layers. While in HFs, miR-148a role also serves to regulate SC-fate and activity, in part, via regulating Rock1 and Elf5 during HF development/homeostasis and cycling (Figure 5J). These data provide an important foundation for further analyses of the role of miR-148a as a regulator of pathway activities in many areas of research, including SC and cancer biology, regenerative medicine, and ageing. Our findings also provide compelling evidence that miR-148a represents a potential powerful candidate therapeutic target, which requires further exploration.

**Material and Methods**

**Animals**

Animal studies were performed in accordance with protocols approved by the UK Home Office Project License. C57Bl/6 wild type female mice were purchased from Charles River Laboratories. Skin samples were collected at defined stages during hair cycle-associated tissue remodelling (Müller-Röver et al., 2001) and snap frozen in liquid nitrogen for histological, immunofluorescent, and RT-qPCR analysis. Skin samples were taken during postnatal (P) hair cycle days 12-23 and hair cycle was induced as described previously (Mardaryev et al., 2010) and harvested at days 0-19 post depilation for *in situ* hybridization and RT-qPCR analysis.
Cell Culture and Transfection

Cell culture and transfections of primary mouse epidermal keratinocytes (PMEKs) were prepared from C57Bl/6 wild type new-born mice at P2-3 as described previously (Ahmed et al., 2014) and grown at 33°C, 8% CO2 until 60-70% confluent. PMEKs were transfected with 200nM of synthetic miR-148a mimic (pro-miR-148a), miR-148a inhibitor (anti-miR-148) or miRNA-negative controls (ThermoFisher, UK) using Lipofectamine 3000 (ThermoFisher, UK) as done previously (Ahmed et al., 2019).

RNA Sequencing Analysis

PMEKs were cultured and transfected with pro-miR-148a and miRNA-control and RNA extracted as described in supplemental methods. Samples were sent to Novogene, UK for RNA sequencing using Illumina Novoseq 6000 platform. The sequencing data were uploaded to the Galaxy web platform, and we used the public server at https://usegalaxy.org/ to analyse the data as described previously (Afgan et al., 2016). The sequenced data was aligned to *mus musculus* genome (*mm9*). Genes that were insignificantly changed (p>0.05) were excluded from our analysis. Gene ontology analysis and heat maps were generated using Galaxy web platform.

Pharmacological Treatment of Skin

Pharmacological treatment of mice with anti-miR-148a was performed as described previously (Mardaryev et al., 2010). In brief, synthetic miR-148a inhibitor (anti-miR-148a) or miRIDIAN-negative control (ThermoFisher, UK) was administered subcutaneously to dorsal skin of C57BL/6 wild type mice in concentration 20μM using atelocollagen (Koken, Japan) for their local and sustained delivery. Anti-miR-148a treatment was performed on postnatal skin days P20, P21 and P22, and skin samples were collected on P23. In each experiment, ≥3 or 4 mice/time point were used for
analyses in both experimental and control groups. Collected samples were processed for histological, immunofluorescent, and RT-qPCR analysis.

**Fluorescent Activated Cell Sorting (FACS)**

Skins from C57Bl/6 wild type mice were harvested between 7-9 weeks of age. In brief, dissected and minced tissue was filtered using 40µm filter. The cells were centrifuged down at 300xg for 10mins and resuspended in 1ml of EMEM calcium-free medium (Lonza, UK). Cells were stained with Ly-6A/E (Sca-1) (ThermoFisher, UK), CD34 (RAM34) (ThermoFisher, UK) and CD49f (BD Pharmingen, USA) in 2% bovine serum albumin/PBS staining buffer rotating for 1 hour at 4°C. Cells were then stained with secondary antibody APC-streptavidin (Biolegend, USA) for 1 hour at 4°C. CD34+/CD49f^High/Sca-1^- HF-bulge-SCs, CD34+/CD49f^High/Sca-1^+ basal-SCs and CD34-/CD49f^Low/Sca-1^- suprabasal keratinocytes were sorted using a MoFlo-XDP cell sorter (Beckman Coulter, UK) and data was analysed using Summit software (Beckman Coulter, UK). Sorted SCs were then used for subsequent colony forming assays and RT-qPCR analysis.

**Colony forming assay**

Swiss-3T3 cells were grown in DMEM media (ThermoFisher, UK) in 24-well plates at 37°C, 5% CO2. At 70% confluence, 3T3 cells were treated with 8µg/ml mitomycin C (ThermoFisher, UK) for 2 hours. One-thousand HF-bulge SCs were seeded per 24-well plate and incubated for 48 hours with DMEM/F-12 media (3:1) supplemented with 10% chelated fetal bovine serum, 10ng/ml epidermal growth factor, 0.5µg/ml hydrocortisone, 10^{-10}M cholera toxin, 5µg/ml insulin, 1.8^{-4}M Adenine (Merck, UK), 100U/ml penicillin, 100µg/ml streptomycin and 0.3mM calcium. 48 hours post-seeding, SCs were transduced with MiRZIP-148a or shRNA control lentiviruses at a
MOI of 20 (4.0 x 10⁴ viral titre/per ml) supplemented with 8µg/ml polybrene (ThermoFisher, UK) for 4 hours at 32°C, 5% CO2. Media was replaced every 48-hours and cultured for 10 days. GFP and Phase imaging was performed using IncuCyte S3 live cell analysis instrument (Sartorius, Germany).

**Data and code availability**

The data that support the findings of this study are openly available in GEO Datasets at https://www.ncbi.nlm.nih.gov/gds, reference number: GSE197862.

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**Author contributions**

Conceptualization: M.I.A; Writing: M.I.A; Data curation: M.E.P, A.H, H.J.P and M.I.A. Formal Analysis: M.E.P and M.I.A; Funding Acquisition: M.I.A. This work was supported by funding from Nottingham Trent University, UoA03 QR and Capital Funds and Office for Students funds.

**Conflict of Interests**

Authors declare no conflict of interest.
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and mammary gland development during pregnancy and lactation. The EMBO Journal 24, 635-644.

**Figure Legends**

Figure 1. **Spatiotemporal expression analysis of miRNA-148a during skin and hair follicle development and regeneration.** (a-c) TaqMan RT-qPCR analysis of miR-148a expression. (a) MiR-148a levels in skin during the postnatal (P) hair cycle days: anagen stage (P12), catagen (P16-P17), and telogen (P20-P23). MiR-148a expression was elevated and reached its highest point during telogen stages (P20-23). n = three mice were used per stage. Data are presented as mean ± SEM (error bars). (b) MiR-148a in depilation-induced hair cycle: telogen (day 0), anagen (days 3 and 12), and catagen (days 17 and 19). MiR-148a expression was elevated at telogen stage (day 0) and significantly dropped in expression during all subsequent hair cycle stages. n = three mice were used per stage. Data are presented as mean ± SEM values. Data are presented as mean ± SEM. (c) FACS cell sorting: C57Bl/6 wild type mice aged 7-9-week-old were used to isolate hair follicle (HF) bulge stem cells. MiR-148a expression is elevated in CD34+ HF-bulge stem cells compared to CD34− negative cells. n = >three mice were used per experiment. Data are presented as mean ± SEM values from three independent experiments. (d) MiR-148a *in situ* hybridization. Depilation-induced hair cycle: miR-148a expression was detected in the stem cell compartments of telogen HFs (bulge and secondary hair germ). Furthermore, miR-148a expression in the epidermis was restricted to the differentiated, outermost suprabasal layer (arrowheads). Expression of miR-148a is dramatically reduced in early anagen skin (day 5, asterisks) and hair bulb (arrows) of HFs and expression is absent from subsequent hair cycle stages (anagen and catagen). (e-f) MiRNA-148a fluorescent *in situ* hybridization with immunofluorescent staining of miR-148a (red) and stem cell markers cytokeratin 15 (Krt15, green) and CD34 (pseudo-coloured cyanine) and DAPI (blue) in telogen stage
of hair cycle. We observed co-localisation of miR-148a expression with both stem cell markers in the bulge (miR-148a+/Krt15+/CD34+) and secondary hair germ stem cell (miR-148a+/Krt15+/CD34+) compartments (arrowheads) of telogen HFs. While, in the epidermis, Krt15 (basal layer) and miR-148a (suprabasal layer, asterisk) were predominantly mutually exclusive expressed. (g-h) The scrambled negative controls using fluorescent (Panel g) and chromogenic (Panel h) detections. Images are representative microphotographs of each stage and staining. The broken lines demarcate the epidermal-dermal border. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student’s $t$-test. Scale bars: 50µm.

Figure 2. Inhibition of miRNA-148a accelerates telogen-anagen transition and alters stem cell activity. (a-f) Anti-miR-148a (inhibitor) or miRIDIAN-negative control were administered daily subcutaneously at postnatal (P) skin days P20-P22. Skin was harvested at P23. Each experiment, ≥ three or four mice/time point were used for analyses in both anti-miR-148a and control groups. (a-c) Representative microphotographs of skin examples of control (Panel A(i)) and anti-miR-148a-treated skin (Panel A(ii-iii)) at P23; sections were stained for the detection of endogenous alkaline phosphatase activity to visualize the morphology of dermal papilla as an important indicator of the defined stages in hair follicle cycle. In control skins, epidermis remained thin (Panel a, asterisk) compared to anti-miR-148a treated skins (Panel b-c, asterisks). HFs of anti-miR-148a treated skins observable had progressed to anagen III stage, characterized by the presence of enlarged dermal papilla partially enclosed by keratinocytes (Panels b-c, arrows), whereas the majority of HFs in the control skin reached only anagen II phase in control treated skins (Panel a, arrows). (d) Percentage of HFs in defined stages of postnatal development was evaluated in cryostat sections of the skin of control or anti-miR-148a-treated mice by quantitative
histomorphometry using established morphological criteria (Müller-Röver et al., 2001); there was a significant increase in the percentage of HFs in anagen III stage in anti-miR-148a-treated skins, compared with the controls, which we predominately in anagen II. Data are presented as mean ± SEM values from 100 hair follicles counted per treatment. (e) Skin thickness after anti-miR-148a treatment is significantly increased, compared to the controls. Data are presented as mean ± SEM values. (f) Cytokeratin 15 (Krt15) expression analysis by immunofluorescence. In anti-miR-148a treated skin, increased expression of Krt15 is observed in the bulge region and developing hair bulb (Panel f, arrows) compared to controls (Panel f, arrows). In the epidermis, Krt15 expression is also increased and continuous throughout the basal layer of anti-miR-148a treated skin (Panel f, asterisks) compared to control epidermis where expression is lower and irregular (Panel f, asterisk). Images are representative microphotographs of staining observed. (g) Quantitative immunofluorescence analysis: immunodetection of Krt15+ cells in the epidermis and HFs (red fluorescence) of control versus anti-miR-148a treated skin samples normalised to DAPI+ cells. n = three mice/treatment. Data are presented as mean ± SEM (error bars). (h) Colony forming assay: inhibition of miR-148a expression using lentiviruses increases the capacity of HF-bulge stem cells to form colonies. Successful transduction of lentiviral particles was confirmed by the presence of green fluorescent protein (GFP) in cultured HF-bulge stem cells. (i) Quantification of the size and number stem cell colonies formed after anti-miR-148a inhibition versus controls. An increase in total number of colonies and larger colonies (>50µm in diameter) were observed in miR-148a inhibited stem cells compared to controls. Data are presented as mean ± SEM values from five independent experiments. *, P <0.05; **, P <0.01; ***, P<0.001 by Student’s t-test. Scale bars: 50µm.
Figure 3. **Global gene expression profiling of keratinocytes after overexpression of miRNA-148a.** (a) Heat map representing the gene changes associated with overexpression of miR-148a (pro-miR-148a) in primary mouse epidermal keratinocytes (PMEKs). (b) A bar chart depicts the ontology of the up- and down-regulated genes and the actual number of genes with more than two-fold expression change in control versus pro-miR-148a treated PMEKs. (c) Venn diagram depicting the predicted miR-148a gene targets from three established databases miRanda, TargetScan and miRDB. These targets were overlapped with our RNA sequencing data (859 genes that were significantly, *p*<0.05, downregulated following miR-148a overexpression) revealed 43 gene targets. (d-e) Validation of RNA sequencing data; RT-qPCR analysis focusing on two specific gene targets Rock1 and Elf5 in PMEKs; modulation of miR-148a activities, leads to an increase and/or a decrease in expression of *Rock1* and *Elf5*, respectively. Data are presented as mean ± SEM values from three independent experiments. Additional target validations of our RNA sequencing and predicted gene targets are shown in Supplemental Figure S1 (supplemental material). (f) Western blot analysis: modulation of miR-148a activities leads to an increase and/or a decrease in protein levels of Rock1 and Elf5 in PMEKs. Data shown are from a single representative experiment from three independent experiments. (g) A significant reduction in luciferase activity was observed when HaCaTs cells were co-transfection with pro-miR-148a (overexpression) and mouse *Rock1* and *Elf5* 3′-UTR construct containing miR-148a binding site. This reduction was not observed following miR-148a binding site mutation and co-transfection with pro-miR-148a. Green letters denote miR-148a ‘seed’ region with 3′-UTR wild-type (black bold letters) and mutated (red letters) binding sites of each gene target. Each sample was normalised to pFirefly luciferase activity. Data is presented as mean ± SEM values from five independent experiments. (h-i). Representative microphotographs of miR-148a fluorescent *in situ*
hybridization with immunofluorescent staining of: miR-148a (red), Rock1 (green), Elf5 (green) and DAPI (blue) in telogen stage (day 0) of depilation-induced hair cycle. We observed co-localisation of miR-148a with both Rock1 and Elf5 primarily in the bulge and secondary hair germ (SHG) stem cell compartments of telogen hair follicles. In the epidermis, miR-148a was predominately expressed in the suprabasal layer (Panels H and I, asterisks) whereas Rock1 and Elf5 expression is primarily observed in the basal layer with some overlapping expression between miR-148a and Rock1 in the basal layer (Panel H, arrow). Negative controls are included in Supplemental Figure S3 (supplemental material). The broken lines demarcate the epidermal-dermal border. The dotted box denotes area, which has been magnified (right side panels). *, P <0.05, **, P <0.01, ***, P <0.001; Student’s t-test. Scale bars: Panels h and i: 50µm; Panels h and i (right side panels): 25µm.

Figure 4. **MiRNA-148a regulates keratinocyte proliferation and differentiation.** (a-b) RT-qPCR analysis of cell cycle gene markers in primary mouse epidermal keratinocytes (PMEKs) showed an increase and/or decrease in expression of genes analysed following modulation of miR-148a activities, respectively. Data are presented as mean ± SEM values from three independent experiments. (c) Flow cytometric analysis by propidium iodide (PI) in PMEKs showed accumulation in S-phase and subsequently, a reduction of cells entering G2/M phase of cell cycle following miR-148a overexpression (pro-miR-148a). Graphs shown are from a single representative experiment. Percentages are presented as mean values from three independent experiments. (d-e) RT-qPCR analysis during calcium-induced (1.8mM) keratinocyte differentiation in PMEKs, showed an increase and/or decrease in expression of keratinocyte differentiation-associated markers cytokeratin 1 (Krt1), Krt10 and Involucrin (Ivl) and gene targets Rock1 and Elf5 after modulating miR-148a activity,
respectively. Data are presented as mean ± SEM values from three independent experiments. (f-g) Western blot analysis of Krt1, Rock1 and Elf5 during calcium-induced keratinocyte differentiation in PMEKs: overexpression of miR-148a reduces expression of Krt1, Rock1 and Elf5, while anti-miR-148a (inhibition) leads to an increase in protein levels. Data shown are from a single representative experiment out of three experimental repeats. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$; Student’s $t$-test.

Figure 5. Inhibition of miRNA-148a leads to elevated expression of Rock1 and Elf5 in skin and hair follicles. Anti-miR-148a (inhibition) or miRIDIAN-negative controls were administered daily subcutaneously at postnatal (P) skin days P20-P22. Skin was harvested at P23. (a-c) Representative microphotographs of control and anti-miR-148a-treated mice sections: immunohistochemistry staining for cytokeratin 1 (Krt1), Rock1 (green), Elf5 (green) and DAPI (blue) of skin sections. Krt1 immunohistochemistry in the epidermis: increased expression of Krt1 in the basal layer after anti-miR-148a treatment (Panel B, arrowheads), compared to the control skins (Panel A, asterisk). Immunohistochemistry staining of Rock1 and Elf5: increased expression was observed for Rock1 in the epidermis (Panel d, arrowheads) compared to controls (Panel c, asterisk). In the hair follicles (HFs), Rock1 is elevated in the distinct cell populations (ORS, bulge, and hair bulb regions) (Panel d, arrows) compared to control HFs (Panel c, arrows). Elf5 expression was elevated in both the basal and suprabasal layer of the epidermis (Panel f, arrowheads) compared with control skins (Panel e, asterisk). In the HFs, Elf5 expression is elevated in the hair bulb, and ORS, and bulge regions (Panel f, arrows) compared to control HFs where expression was restricted to the bulge region (Panel e, arrows). The broken lines demarcate the epidermal-dermal border. (g-i) Quantitative immunofluorescence analysis: immunodetection of Krt1 (Panel g), Rock1
(Panel h) and Elf5 (Panel I) in the epidermis and HFs (green fluorescence) of control versus anti-miR-148a treated skin samples normalised to DAPI+ cells. n = three mice/treatment. Data are presented as mean ± SEM (error bars). (j) A working model of the involvement of miR-148a in the regulation of skin and HF stem cells and their lineage-committed progenies that controls skin and HF development and homeostasis via its gene targets Rock1 and Elf5. These effects may also be linked to the activation/repression of specific signalling pathways in keratinocytes that result from the regulation of Rock1 and Elf5 by miR-148a, which are yet to be determined (created with BioRender.com). Scale bars: 50µm.
Figure 1

**a**

Relative expression of miR-148a during the postnatal hair cycle (days) and induced hair cycle (days).

**b**

Relative expression of Shh during the induced hair cycle (days).

**c**

Relative expression of miR-148a in CD34+ cells.

**d**

Images of different stages of hair development: Telogen, Early Anagen, Anagen, and Catagen. SHG images showing bulge areas.

**e**

Immunofluorescence images showing DAPI, Krt15, and miR-148a expression in Telogen stage.

**f**

Immunofluorescence images showing DAPI and CD34 with miR-148a expression in Early Anagen stage.

**g**

Negative controls for immunofluorescence images.

**h**

Scrambled probe control for DAPI staining in an unspecified stage.
Figure 2
Figure 3

Samples:

<table>
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Gene Count

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<td>Elf5</td>
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Relative expression (fold change)

- **Control**
- **Pro-miR-148a**

- Rock1
- Elf5

- **Control**
- **Anti-miR-148a**

- Rock1
- Gapdh
- Elf5
- Gapdh

Luciferase activity (Percentage fold change)

- **Control**
- **Pro-miR-148a**

- Wild type Rock 3' UTR
- Mutant Rock 3' UTR
- Wild type Elf 3' UTR
- Mutant Elf 3' UTR

Downregulated (p*<0.05)
Figure 4
Figure 5
Supplemental Figure S1
Supplemental Figure S2
Supplemental Figure S4
Supplemental Information

**Materials and Methods**

**Cell Culture and Transfection**

Cell culture and transfections of primary mouse epidermal keratinocytes (PMEKs) were prepared from C57Bl/6 wild type new-born mice at P2-3 as described previously (Ahmed et al., 2014). In brief, PMEKs were grown in EMEM calcium-free medium (Lonza, UK) supplemented with 0.05mM calcium (ThermoFisher, UK), 4% chelated heat-inactivated fetal bovine serum (ThermoFisher, UK), 0.4µg/ml hydrocortisone (Merck, UK), 5µg/ml insulin (Merck, UK), 10ng/ml epidermal growth factor (Merck, UK), 10^{-10}M cholera toxin (Merck, UK), 2x10^{-9}M T3 (Merck, UK), 100U/ml penicillin (ThermoFisher, UK), 100µg/ml streptomycin (ThermoFisher, UK), and 2mM L-glutamine (ThermoFisher, UK), at 33°C, 8% CO2 until 60-70% confluent. PMEKs were transfected with 200nM of synthetic miR-148a mimic (pro-miR-148a), miR-148a inhibitor (anti-miR-148) or miRNA negative controls (ThermoFisher, UK) using Lipofectamine 3000 (ThermoFisher, UK) as done previously(Ahmed et al., 2019). Cells were harvested 48-hours post-transfection and used for further analyses. For calcium-induced keratinocyte differentiation analysis, PMEKs were differentiated following transfection by media supplementation with 1.8mM calcium. PMEKs were cultured for an additional 48 hours post-transfection before collection for further analysis.

**Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)**

Total RNA was isolated using the Zymo Direct-zol RNA kit (Cambridge Biosciences, UK). For gene expression analysis, 100ng of total RNA was converted into cDNA using the qPCRBIO cDNA Synthesis Kit system (PCR Biosystems, UK). Gene expression analysis was performed on QuantStudio5 Real Time PCR System (ThermoFisher, UK). Gene expression was analysed using qPCRBIO SyGreen mix (PCR Biosystems, UK) at the following
conditions: 95°C for 2mins, followed by 40 cycles of denaturation (95°C for 5s), annealing and extension (30s at temperature experimentally determined for each primer pair). RT-qPCR primers (Supplemental Table S1) were designed using primer3 (https://primer3.ut.ee/) and further validated using UCSC genome browser (https://genome.ucsc.edu/) and NCBI primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Amplification differences between samples and controls were calculated based on the Ct ($\Delta\Delta$Ct) method and normalized to mouse Actin gene (Actb). Data from triplicates were pooled, standard error of the mean (± SEM) was calculated, and statistical analysis was performed using unpaired student’s t-test.

For detection of microRNA-148a (miR-148a), TaqMan quantitative reverse transcriptase PCR was performed using TaqMan Real Time PCR Assay (ThermoFisher, UK) as described previously (Ahmed et al., 2014; Ahmed et al., 2019). In brief, miR-148a was amplified under the following cycling conditions: 95°C for 10mins, followed by 40 cycles of 95°C for 15s and 60°C for 60s. Differences between samples and controls were calculated based on the Ct ($\Delta\Delta$Ct) method and normalized to the U6 snRNA values. Data from triplicates were pooled, ± SEM was calculated, and statistical analysis was performed using unpaired Student’s t-test.

Western blot

Whole cell protein lysates were extracted from cultured cells using RIPA lysis buffer (50mm Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150mm NaCl, and 1mm EDTA; pH 7.4) and cOmplete ULTRA Protease Inhibitor Cocktail (Merck, UK). Nuclear protein was extracted from cultured cells using NE-PER Nuclear and Cytoplasmic extraction kit (ThermoFisher, UK). Western blot was performed as described previously (Ahmed et al., 2019). In brief, 10μg (micrograms) of protein were processed for western blot analysis, followed by membrane incubation with primary antibody (Supplemental Table S2) overnight at 4°C. Horseradish peroxidase tagged IgG antibodies were used as secondary antibodies (1:3000, ThermoFisher,
UK). Antibody binding was visualized with an enhanced chemiluminescence system (SuperSignal West Pico Kit, ThermoFisher, UK) on the FL1000 iBright GelDoc Imager (ThermoFisher, UK).

Flow Cytometry

PMEKs were cultures and transfected with pro-miR-148a or miRNA control as described above. 48 hours post-transfection, cells were trypsinized, washed and fixed in 70% ethanol/PBS at -20°C for 30 mins. Fixed cells were incubated with RNase A (100μg/ml) for 30mins at 37°C. Cells were subsequently incubated with 20μg/ml Propidium iodide (ThermoFisher, UK) for 30mins at 4°C. The percentage of cells at distinct phases of the cell cycle was analysed by flow cytometry with a Beckman Coulter Gallios (Beckman Coulter, UK). For each sample, 10,000 events were collected and analysed on Beckman Coulter Kaluza Analysis Software (Beckman Coulter, UK).

In Situ Hybridization and Fluorescent In Situ Hybridization

In Situ hybridization was performed as described previously (Pickup and Ahmed, 2020). In brief, skin cryosections (10µm) were fixed in 4% paraformaldehyde (ThermoFisher, UK) for 10 mins at room temperature. Slides were hybridized with 320nM double-DIG-labelled miR-148a probe (Qiagen, USA) diluted in hybridization buffer (50% formamide DI (Merck, UK), 5x saline-sodium citrate (SCC; ThermoFisher, UK), 50μg/ml yeast RNA (ThermoFisher, UK), 1% SDS (ThermoFisher, UK), 50μg/ml heparin (ThermoFisher, UK)) for 16-18 hours at 60°C overnight. Slides were subsequently washed and blocked for 60 mins in blocking solution (2% blocking reagent, Merck, UK), 5% heat inactivated sheep serum (Merck, UK), 1% Tween-20 and levamisole in 1x TBST). Slides were incubated for 2 hours at room temperature with sheep alkaline-phosphatase-conjugated anti-DIG antibody (1:2500; Merck, UK).
Fluorescent *In Situ* Hybridization: was performed with the following modifications. Slides were blocked for 30 mins in blocking solution (0.1M Tris-HCl (pH 7.5), 0.15M NaCl and 0.5% blocking reagent (Merck, UK). Immunodetection of miR-148a was performed with sheep Rhodamine-conjugated-anti-DIG antibody (1:100; Merck, UK) alongside antibodies for Rock1, Elf5, CD34 or Krt15 (Supplemental Table S2). Slides were incubated overnight at 4°C. Slides were then washed and incubated with corresponding Alexa-Fluor-488 antibodies or Alexa-Fluor-647 (1:200; ThermoFisher, UK) for 1 hour. Slides were either developed by NBT/BCP solution (ISH, Merck, UK) or mounted with mounting medium with DAPI (FISH, Vectashield, 2B Scientific, UK). Images were taken using THUNDER imager 3D cell culture (Leica, Germany).

**MiRNA-148a Gene Target Predictions**

Putative miR-148a target genes were aligned from three different prediction algorithms as done previously (Ahmed et al., 2014): TargetScan ([http://www.targetscan.org/](http://www.targetscan.org/)) predicts biological targets of microRNAs by searching for the presence of conserved sites that match the ‘seed’ region of each microRNA, miRanda ([http://microrna.sanger.ac.uk](http://microrna.sanger.ac.uk)) uses an algorithm to predict microRNA-mRNA pairs, and miRDB ([http://mirdb.org/](http://mirdb.org/)) uses miRNA-target interactions from high-throughput sequencing experiments.

**Luciferase Reporter Assay**

HaCaT cells were grown in DMEM media (ThermoFisher, UK) as described previously (Ahmed et al., 2014; Ahmed et al., 2011; Aunin et al., 2017; Mardaryev et al., 2010) in at 37°C, 5% CO2, until 70% confluent. 3’UTR fragments of *Rock1* and *Elf5* incorporating miR-148a binding site were amplified from C57Bl/6 wild type mouse genomic DNA using forward and reverse primers containing XhoI and NotI restriction sequences. *Elf5* Sequences: 5’-
CTCGAGACACACGATCGATCTCTCTCTCT-3’ and 5’-
GCGGCCAGTTAGGTTCCAGGCACTC-3’, and Rock1 sequences: 5’-
CTCGAGTTGGGAAGTTGGGAGAAGG-3’ and 5’
GCGGCCGCGGGTAATGCAACTTCCACTGA-3’ were used. PCR products were
incorporated into psiCHECK2 plasmid (Promega, UK) using Zero blunt cloning kit
(ThermoFisher, UK). Mutated binding sequences were generated using Q5 Site-directed
mutagenesis kit (New England Biolabs, UK) as per manufactures instruction. All plasmids
were verified by Sanger sequencing (Source Biosciences, UK).
HaCaTs were co-transfected with 100ng generated plasmid and 200nM pro-miR-148a or
miRNA control using lipofectamine 3000 into 96-well-plates. 24 hours post-transfection
relative luciferase activities were measured using Dual-Glo luciferase assay system (Promega,
UK) as per manufacturers instruction. Luminescence was measured using CLARIOStar plate
reader (BMG Labtech, Germany). Data from five independent experiments was pooled, ± SEM
was calculated, and statistical analysis was performed using unpaired Student’s t-test.

Alkaline Phosphatase Staining
Detection of endogenous alkaline phosphatase was performed as described previously (Ahmed
et al., 2014; Mardaryev et al., 2010). In brief, acetone-fixed cryosections (10µm) were
incubated in developing solution (100mM NaCl, pH8.3, 100mM Tris, pH 9.5, 20mM HCl,
0.05% Naphtol ASBI phosphate, 0.5% DMF, 25mM sodium-nitrite, and 5% New fuchsin) for
15 mins, washed and immersed into Haematoxylin nuclear counterstain solution (Vector
Laboratories, USA) for 45s at room temperature. Images were captured using Leica
THUNDER imager 3D cell culture (Leica, Germany).
Immunohistochemistry

For immunohistochemical analysis, 4% paraformaldehyde-fixed cryostat sections (10μm) were blocked with 0.2% Triton-X-100/PBS, 5% fetal calf serum, 2% bovine serum albumin and 10% normal goat and/or donkey serum (Merck, UK) and incubated with primary antibodies against Rock1, Elf5, Krt1 or Krt15 (Supplemental Table S2) overnight at 4°C. The following day, slides were incubated with the corresponding Alexa-Fluor-488 or Alexa-Fluor-A555 (Supplemental Table S2) secondary antibodies for 1 hour at room temperature. Incubation steps were interspersed with wash steps with 0.2% Triton-X-100/PBS. Sections were mounted with mounting medium with DAPI. Images were taken using Leica THUNDER imager 3D cell culture (Leica, Germany).

Immunocytochemistry

For immunocytochemical analysis, PMEKs were transfected with pro-miR-148a and miRNA control as described earlier. This was followed by treatment with high calcium (1.8mM) for a further 48 hours post-transfection. Cells were fixed with 4% paraformaldehyde and blocked with 0.2% Triton-X-100/PBS, 5% fetal calf serum, 2% bovine serum albumin and 10% normal goat and/or donkey serum. Cells were incubated with primary antibodies against Elf5 and Rock1 (Supplemental Table S2) overnight at 4°C. Slides were then incubated with the corresponding Alexa-Fluor-488 secondary antibody for 1 hour at room temperature. Incubation steps were interspersed with wash steps with 0.2% Triton-X-100/PBS. Cells were mounted with mounting medium with DAPI. Images were taken using Leica THUNDER imager 3D cell culture (Leica, Germany).
Immunofluorescence Quantification Analysis

Immunofluorescence intensity analysis was determined using ImageJ software (https://imagej.nih.gov/ij/), as described previously (Ahmed et al., 2014). In brief, red or green fluorescent signals were collected from experimental tissues in RGB format using the same exposure conditions. To measure the fluorescence intensity at each pixel, the RGB images were converted to 16-bit grayscale format. Regions of interest (cells/epidermis and/or HFs) of distinct size within the controls, anti-miR-148a and/or pro-miR-148a were selected, and the mean values of intensity were calculated for each selected areas followed by the normalization relative to the number of DAPI+ cell fluorescence intensity.

Production of Lentiviruses

HEK293T cells were cultured in DMEM (ThermoFisher, UK) supplemented with heat-inactivated 10% fetal bovine serum, 1% L-glutamine and 5% non-essential amino acids (ThermoFisher, UK) at 37°C, 5% CO2. For production of MiRZIP-148a (inhibition) or shRNA control lentiviruses: at 40% confluence HEK293T cells were co-transfected with MiRZIP-148a plasmid (Cambridge Biosciences, UK) or pGFP-C-shLenti shRNA vector control (Insight Biotechnology, UK) with packaging plasmid using Lenti-vpak packaging kit (Insight Biotechnology, UK) following manufacturer’s instructions. Cell culture medium containing viruses was collected 24 hours and 48 hours post-transfection, followed by precipitation of the viral particles using 80µg/ml polybrene (ThermoFisher, UK) and 80µg/ml chondroitin sulphate C (Merck, UK) for 18-hours at 4°C before centrifugation at 10,000xg. Titration of lentiviral particles was performed using qPCR lentivirus titration kit (NBS Biological, UK) as per manufacturer’s instruction.
References


Supplemental Figure Legends

Supplemental Figure S1. RNA sequencing data validation. (a-b) RT-qPCR analysis of predicated target genes that were highly conserved between human and mouse genomes from our RNA sequencing data and which had predictive binding sites from miRNA target databases (from Figure 3). No significant effects were observed following the modulation of miR-148a activity in primary mouse epidermal keratinocytes. Data are presented as mean ± SEM values from three independent experiments.

Supplemental Figure S2. MiRNA-148a inhibits nuclear localisation of Rock1 and Elf5 in keratinocytes. (a-d) Immunocytochemistry staining of Rock1 and Elf5 during calcium-induced (1.8mM) keratinocyte differentiation after overexpression of miR-148a in primary
mouse epidermal keratinocytes (PMEKs). A reduction of Rock1 and Elf5 expression in the nucleus as well as cytosol was observed after pro-miR-148a (overexpression) treatment versus control cells. (e-f) Quantitative immunofluorescence intensity analysis after pro-miR-148a versus control treatments in PMEKs. A significant inhibitory effect of miR-148a on Rock1 (green) and Elf5 (green) expression is observed in the nucleus and cytosol compared with control cells normalised to DAPI+ cells. Data are presented as mean ± SEM values from 100 cells counted per treatment from three independent experiments. (g-h) Nuclear localisation of Rock1 and Elf5 was analysed by western blot during calcium-induced keratinocyte differentiation in PMEKs: overexpression of miR-148a reduced Rock1 (g) and Elf5 (h) nuclear protein levels compared to controls. Data shown are from a single representative experiment out of three experimental repeats. Fraction purity of western blots was confirmed by using antibodies against nuclear Histone H3 and Lamin B1 marker proteins. ***, P<0.001 by Student’s t-test. Scale bars: 50µm.

Supplemental Figure S3. Controls of MiRNA-148a fluorescent in situ hybridization with immunofluorescent staining. (a-c) The negative controls using scrambled probe (red) and Alexa-fluor-488 (A488, green) and counter stained with DAPI (blue) detections for Figure 3h-i. No specific staining was observed for negative controls. Images are representative microphotographs of staining. The broken lines demarcate the epidermal-dermal border. Scale bars: 50µm.

Supplemental Figure S4. Confirmation of MiRNA-148a Inhibition, In Vivo. (a) Inhibition of miR-148a was confirmed by TaqMan RT-qPCR analysis of pharmacologically inhibited miR-148a skin samples, which revealed a significant reduction in miR-148a
expression in anti-miR-148a treated skins compared to control skins. \( n = \) three mice/treatment.

Data are presented as mean ± SEM (error bars). ***, \( P<0.001 \) by Student’s t-test.