Inducible deletion of epidermal Dicer and Drosha reveals multiple functions for miRNAs in postnatal skin

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SUMMARY
MicroRNAs (miRNAs) regulate the expression of many mammalian genes and play key roles in embryonic hair follicle development; however, little is known of their functions in postnatal hair growth. We compared the effects of deleting the essential miRNA biogenesis enzymes Drosha and Dicer in mouse skin epithelial cells at successive postnatal time points. Deletion of either Dicer or Drosha during an established growth phase (anagen) caused failure of hair follicles to enter a normal catagen regression phase, eventual follicular degradation and stem cell loss. Deletion of Drosha or Dicer in resting phase follicles did not affect follicular structure or epithelial stem cell maintenance, and stimulation of anagen by hair plucking caused follicular proliferation and formation of a primitive transient amplifying matrix population. However, mutant matrix cells exhibited apoptosis and DNA damage and hair follicles rapidly degraded. Hair follicle defects at early time points post-deletion occurred in the absence of inflammation, but a dermal inflammatory response and hyperproliferation of interfollicular epidermis accompanied subsequent hair follicle degradation. These data reveal multiple functions for Drosha and Dicer in suppressing DNA damage in rapidly proliferating follicular matrix cells, facilitating catagen and maintaining follicular structures and their associated stem cells. Although Drosha and Dicer each possess independent non-miRNA-related functions, the similarity in phenotypes of the inducible epidermal Drosha and Dicer mutants indicates that these defects result primarily from failure of miRNA processing. Consistent with this, Dicer deletion resulted in the upregulation of multiple direct targets of the highly expressed epithelial miRNA miR-205.

KEY WORDS: Dicer (Dicer1), Drosha, Skin, miRNA, Hair follicle, Epidermis, Mouse

INTRODUCTION
The epidermis and hair follicles provide an accessible system for studying the molecular mechanisms controlling proliferation, differentiation and maintenance of adult progenitor cells. The interfollicular epidermis (IFE) is composed of a continuously proliferating basal layer, which self-renews and gives rise successively to a non-proliferating suprabasal layer, a granular layer, and a cornified terminally differentiated layer that is cross-linked to form the epidermal barrier (Blanpain and Fuchs, 2009). Hair follicles (HFs) are composed of epithelial cells and a dermal papilla (DP). Throughout postnatal life, HFs undergo cycles of growth (anagen), regression (catagen) and rest (telogen) that are linked to form the epidermal barrier (Blanpain and Fuchs, 2009). Hair follicles (HFs) are composed of epithelial cells and a dermal papilla (DP). Throughout postnatal life, HFs undergo cycles of growth (anagen), regression (catagen) and rest (telogen) that depend on a specialized population of label-retaining epithelial stem cells residing in their permanent bulge region (Cotsarelis et al., 1990). At the end of each resting phase, bulge stem cells are stimulated to divide transiently, possibly by signals from dermal cells (Oliver and Jahoda, 1988; Plikus et al., 2008). Bulge cell progeny proliferate rapidly, establishing a matrix population that differentiates to form a keratinized hair shaft (HS) and an inner root sheath (IRS) that molds the HS as it emerges from the skin (Millar, 2002; Fuchs, 2007). An outer root sheath (ORS) that is contiguous with basal epidermis surrounds each follicle. MicroRNAs (miRNAs) are non-coding RNAs that regulate ~30% of mammalian genes (Lewis et al., 2005). Primary miRNA precursors are transcribed in the nucleus and are processed into 19–22 nt mature miRNAs by the nuclear RNase III enzyme Drosha and its co-factor Dgcr8, and the cytoplasmic RNase III enzyme Dicer. miRNAs bind imperfectly complementary sequences that are usually located in 3′ untranslated regions of target mRNAs, resulting in mRNA degradation and/or translational inhibition (Hendrickson et al., 2009; Djuranovic et al., 2011). The repressive effects of miRNAs on individual target mRNAs are typically relatively mild (Baek et al., 2008; Selbach et al., 2008), and deletion of individual or even multiple related miRNAs often results in subtle phenotypes that sometimes require stress conditions such as tissue injury for their expression.

Although Drosha and Dicer process most miRNAs, the biogenesis of certain miRNA subclasses is either Drosha- or Dicer-independent (Ruby et al., 2007; Cheloufi et al., 2010; Chong et al., 2010; Cifuentes et al., 2010), and both endonucleases also possess miRNA-independent functions (Wu et al., 2000; Tan et al., 2008; Watanabe et al., 2008). Analysis of the effects of Drosha or Dicer deletion alone might therefore fail to reveal the full scope of miRNA functions or could produce phenotypes unrelated to miRNA processing.
Approximately seventy miRNAs are expressed in embryonic skin (Andl et al., 2006; Yi et al., 2006). Constitutive epidermis-specific deletion of either Dicer or Dgcr8 during embryonic development causes defects in HF proliferation and HS formation, and evagination, rather than downgrowth, of a subset of HFs (Andl et al., 2006; Yi et al., 2006; Yi et al., 2009). The similarities in these effects suggest that these phenotypes are primarily related to miRNA biogenesis rather than to miRNA-independent functions of Dicer and the Dgcr8-Drosha complex (Yi et al., 2009).

In vitro studies suggest functions for miRNAs in epidermal keratinocyte differentiation (Hildebrand et al., 2011; Xu et al., 2011). miR-205 is highly expressed in epithelia and promotes keratinocyte migration in vitro (Ryan et al., 2006; Yu et al., 2010). Known targets of miR-205 include regulators of proliferation, apoptosis and the epithelial-mesenchymal transition (Gregory et al., 2008; Paterson et al., 2008; Dar et al., 2011; Majid et al., 2011), suggesting possible additional roles for this miRNA in epidermal development and homeostasis. In vivo, overexpression of miR-125b in adult skin results in HF, sebaceous gland and epidermal differentiation defects (Zhang, L. et al., 2011). miR-203 is specifically expressed in suprabasal epidermal cells and its overexpression restricts proliferative potential and suppresses expression of the stem cell-associated transcript p63 (Trp63 – Mouse Genome Informatics) (Yi et al., 2008), and miRNA regulation of HF cycling is suggested by the observation of accelerated anagen in mice treated with miR-31 antagonomir (Mardaryev et al., 2011).

Given the profound effects of global miRNA depletion on embryonic HF development, and the similar mechanisms that control HF morphogenesis in embryos and cyclical regeneration in the adult (Millar, 2002), we hypothesized that miRNAs play additional key roles in the postnatal HF growth cycle. To test this, we determined the effects of inducible epidermal deletion of Dicer (Dicer1 – Mouse Genome Informatics) or Drosha at successive stages of postnatal life. We found that Dicer or Drosha loss does not affect the maintenance of resting HFs, but produces dramatic defects in cell survival in the rapidly proliferating matrix population during early anagen, blocks transition to a normal regression phase, and prevents long-term maintenance of follicular structures and their associated stem cells. An inflammatory response is observed concomitant with follicular degradation and epidermal hyperplasia, but is preceded by initial HF defects including matrix cell apoptosis and DNA damage. These data demonstrate specific requirements for Drosha and Dicer in maintaining the ability of adult HFs to undergo normal cycles of growth and regression. The similar phenotypes observed in induced Drosha and Dicer epidermal mutants suggest that these functions are largely associated with miRNA processing. Consistent with this, Dicer deletion resulted in upregulation of multiple direct targets of the highly expressed epithelial miRNA miR-205, which is depleted in Drosha- and Dicer-deficient skin.

MATERIALS AND METHODS

Mouse strains, Cre induction and wound healing assays

Floxed mice carrying Krt5-rtTA tetO-Cre (Zhang et al., 2008) were placed on doxycycline chow (formulated at 6 g/kg chow; Bio-Serv, Laurel, MD, USA) to induce recombination. Genotyping primers are listed in supplementary material Table S1. For specific deletion of Dicer in bulge cells, DicerflGI22-23 Rosa26R mice carrying the Krt5-CrePR1 transgene (Ito et al., 2005) were topically treated with 0.1 g Mifepristone (Sigma-Aldrich, St Louis, MO, USA) in ethanol. For wound healing assays, 48 mice were anesthetized with ketamine/xylazine followed by full-thickness excision of a 1 cm² area of dorsal skin. All experimental procedures involving mice were performed according to the guidelines of the IACUC committee of the University of Pennsylvania.

RESULTS

miRNAs are depleted in inducible epidermal Dicer and Drosha mutants

To elucidate the role of Drosha and Dicer in postnatal HFs and epidermis we paired a conditional Dicer allele and two independent conditional Drosha alleles with a bi-transgenic Krt5-rtTA tetO-Cre system that permits inducible recombination in basal epidermis and HF epithelial cells, including stem cells, on dosage with oral doxycycline (Zhang et al., 2008). The first Drosha allele (DroshaflGI), contains a floxed gene inactivation cassette (GIC) inserted between exons 3 and 4, upstream of the essential RNase III domains. Cre-mediated recombination promotes irreversible inversion of the GIC (Xin et al., 2005) resulting in premature truncation of Drosha mRNA (supplementary material Fig. S1A,B). In the second conditional Drosha allele (DroshaflEx9), loxp sites flank exon 9 and recombination results in a null allele (Chong et al., 2008) (supplementary material Fig. S1C,D). Inducible inactivation of the two Drosha alleles produced similar effects, with a slightly less severe phenotype observed for the recombined DroshaflGI allele, possibly owing to splicing of a subset of transcripts around the GIC. The results described below were obtained using the conventional DroshaflEx9 allele, except where noted otherwise. In the conditional Dicer allele (DicerflEx22-23), two loxp sites flank exons 22 and 23, which encode the majority of the RNase III domain, and Cre recombinase-mediated recombination generates a null mutation (Murchison et al., 2005) (supplementary material Fig. S1E).

Analysis of epidermal genomic DNA from induced Krt5-rtTA tetO-Cre DroshaflEx9/flEx9 (DroshaflGI) mutant mice confirmed inversion of the GIC (supplementary material Fig. S1B), and RT-PCR analysis of epidermal mRNA and immunohistochemistry with anti-Drosha antibody showed reduced expression of Drosha mRNA and protein in mutants compared with controls (supplementary material Fig. S2A-C). In line with negative regulation of Dgcr8 by Drosha (Shenoy and Blelloch, 2009), Dgcr8 mRNA levels were increased in DroshaflGI mutant skin (supplementary material Fig. S2A).

PCR using primers that amplify the recombinated DicerflEx22-23 locus (supplementary material Fig. S1E) confirmed excision in epidermal and HF cells of induced Krt5-rtTA tetO-Cre DicerflEx22-23flEx22-23 (DicerflEx22-23) mutant mice (supplementary material Fig. S1F,G). Induced DicerflEx22-23 mutants carrying the
**Drosha or Dicer deletion during an established anagen phase reveals essential functions in HF regression and maintenance**

To determine whether Drosha or Dicer is required to maintain anagen or for entry into catagen, Drosha<sup>GI</sup> or Dicer<sup>Ex22-23</sup> deletion was initiated during ‘embryonic’ anagen at embryonic day (E) 18 or postnatal day (P) 1, and analyzed at successive time points when control littermate HFs were in mid-anagen, catagen or telogen (Fig. 2U). In both mutants, external hair became wavy between P12 and P14 (Fig. 1J,N), followed by hair loss (Fig. 1K,L,O,P), a temporary phenotype of dry scaly skin which resolved in an anterior-posterior direction (Fig. 1L,Q, arrows), and permanent failure of hair regrowth (Fig. 1M,Q). These phenotypes appeared more rapidly in Dicer than in Drosha mutants, possibly owing to more efficient excision of the Dicer<sup>Ex22-23</sup> allele and/or the more downstream activity of Dicer relative to Drosha in miRNA biogenesis. Given the faster appearance of overt phenotypes in Dicer mutants, further analyses were conducted at slightly earlier stages post-induction in Dicer than in Drosha mutants. Drosha<sup>GI</sup>, Drosha<sup>Ex9</sup> and Dicer<sup>Ex22-23</sup> mutants were generally smaller than littermate controls, which was potentially related to Krt5 promoter-driven deletion in the forestomach and esophagus (Yagi et al., 2007).

Skin histology revealed abnormal HS structures in Drosha<sup>Ex9</sup> and Dicer<sup>Ex22-23</sup> mutants compared with littermate controls (Fig. 2A,F,K,P). At P14, when control HFs were in anagen, Dicer<sup>Ex22-23</sup> mutant follicles displayed elevated numbers of TUNEL-positive cells compared with controls (supplementary material Fig. S3A,B), suggesting that defects in HS formation might result from matrix cell apoptosis.

At P20, despite the presence of apoptotic cells, Drosha<sup>Ex9</sup> and Dicer<sup>Ex22-23</sup> mutant follicles failed to regress and remained in an abnormal growth phase (Fig. 2G,Q). By contrast, catagen occurred normally in doxycycline-treated control littermates of genotype Krt5-rtTA tetO-Cre Dicer<sup>+/+</sup> and Krt5-rtTA tetO-Cre Dicer<sup>Ex22-23</sup>/+ (Fig. 2B,L; supplementary material Fig. S3C,D), and these controls also showed normal histology at every other stage examined. Expression of Fgf5, a major regulator of the
anagen-catagen transition (Hebert et al., 1994), was reduced in P17 Drosha<sup>flEx9</sup> and Dicer<sup>Rex22-23</sup> mutants induced from E18 and P1, respectively, consistent with failure of catagen (supplementary material Fig. S4A-D). Dicer deletion induced during the first postnatal anagen phase, starting from P20, produced similar phenotypes to those observed when Dicer or Drosha was deleted in embryonic anagen (supplementary material Fig. S5A-H).

In both Drosha<sup>flEx9</sup> and Dicer<sup>Rex22-23</sup> mutants, failed catagen was followed by follicular degradation (Fig. 2C,D,H,I,M,N,R,S) and apparent extrusion of abnormally keratinized cellular material from degrading HFs (Fig. 2H,R), consistent with the gross phenotype of epidermal scaling (Fig. 1L,Q). By P32, only HF remnants persist (Fig. 2E,J,O,T). Krt15-positive stem cells were present in mutant follicles at P20-21 (supplementary material Fig. S4E-H) but were lost by P50-59 (supplementary material Fig. S4I-L). TUNEL staining and expression of the DNA damage response marker pH2A.X in the bulge regions of degrading mutant follicles at P32 (supplementary material Fig. S4M-T) suggested that apoptosis and DNA damage might contribute to stem cell disappearance.

At P17, when Drosha<sup>Rex9</sup> and Dicer<sup>Rex22-23</sup> mutant HFs displayed significant abnormalities, but prior to follicle degradation, mutant and control IFE displayed similar histology, proliferation rates and expression of p63 (Fig. 2A,F,K,P, Fig. 3A-H) and lacked evidence of dermal inflammation (Fig. 3I-L). Importantly, the onset of HF abnormalities in the absence of inflammation indicated that these were a primary consequence of Drosha or Dicer depletion.

By P20, concomitant with follicular degradation, the IFE became markedly thickened (Fig. 2G-J,Q-T, yellow arrows) and was hyperproliferative at P32 (Fig. 3Q-T), with expanded p63 expression (Fig. 3M-P) and increased numbers of p63-positive cells in Krt10-expressing suprabasal layers (Fig. 3M-P, arrows), consistent with previous results (Yi et al., 2008). By P32, mutant dermis displayed increased numbers of CD11b (Itgam)-positive inflammatory cells, particularly surrounding degrading HFs (Fig. 3U-X).

Given this increase in basal levels of inflammation, we asked whether the inflammatory response to acute wounding was altered in mutant skin by assaying for the presence of CD11b-positive cells 8 days after excision of a 1 cm<sup>2</sup> area of dorsal skin in induced Drosha<sup>Rex9</sup> and Dicer<sup>Rex22-23</sup> mutants and control littermates. All samples displayed increased inflammation compared with unwounded skin. However, the numbers of CD11b-positive cells at wound margins were not statistically different between Drosha or Dicer mutants and their respective littermate controls (supplementary material Fig. S6).
**Drosha and Dicer are not required for maintenance of stem cells in telogen HFs**

To determine whether *Drosha* or *Dicer* is required to maintain resting HFs, *Drosha*^GI^ and *Dicer*^flEx22-23^ mutant mice were induced during the second postnatal telogen and assayed 8-20 days later. Epidermal miRNA levels were depleted following induction in telogen (supplementary material Fig. S7A,B). However, HF structures appeared grossly normal by histological analysis (Fig. 4A-D), Krt15- and CD34-expressing bulge stem cells were maintained (Fig. 4E-H; supplementary material Fig. S8A-D), and TUNEL and pH2A.X staining revealed no increases in apoptosis or DNA damage relative to controls (supplementary material Fig. S8E-L). However, at later time points, after HFs had spontaneously re-entered anagen, degradation of mutant follicular structures and loss of stem cells were observed (Fig. 4I,J). Thus, *Drosha* and *Dicer* are not required for maintenance of stem cells or follicular structures in resting HFs, suggesting that overt defects might be associated with entry into anagen.

**Drosha and Dicer mutant HFs initiate anagen following hair plucking but fail to sustain normal growth**

To test directly whether *Drosha* or *Dicer* mutant follicles become defective upon anagen entry, we induced *Drosha*^flEx9^ and *Dicer*^flEx22-23^ deletion in telogen and initiated a synchronous hair growth cycle by plucking dorsal hair (Fig. 5M). External hair regrowth was observed in littermate controls by 14 days post-plucking (DPP), but was absent in plucked mutant skin. Histological analysis and Ki67 staining at 2 DPP revealed that both control and mutant HFs entered anagen and proliferated in response to plucking, but were defective in maintaining anagen growth.
(supplementary material Fig. S9A-D). However, by 5 DPP, mutant keratinocytes failed to completely surround the DP (supplementary material Fig. S9E-H). The sizes and extent of downgrowth of mutant follicles were reduced by 12 DPP in Drosha\textsuperscript{flEx9} and by 8 DPP in Dicer\textsuperscript{flEx22-23} mutants as compared with controls (Fig. 5A,B,D,E,G,J; see also supplementary material Fig. S11A,B). In Dicer\textsuperscript{flEx22-23} mutant follicles at 8 DPP, the number of DAPI-positive nuclei in the matrix, defined as the region beneath the line of Auber (Peters et al., 2003), was 61±6% of that in control follicles (600-1700 DAPI-positive cells counted per mouse; n=4 controls and n=4 mutants; P=0.0006). The developing HS appeared irregular in both mutants (Fig. 5A,D,G,J). Signs of follicular degradation were observed in Drosha\textsuperscript{mutants by 12 DPP and in Dicer mutants by 8 DPP, and HFs subsequently started to degrade in both mutants.

Fig. 5. Anagen defects in Drosha\textsuperscript{flEx9} and Dicer\textsuperscript{flEx22-23} mutant HFs. (A-L) Drosha\textsuperscript{mutants} and Dicer\textsuperscript{mutants and littermate controls were placed on doxycycline at P38, 10 days prior to hair plucking. Skin was biopsied from the lower dorsal region at successive days post-plucking (DPP) as indicated and analyzed by H&E staining. Defects in HF downgrowth were observed in Drosha mutants by 12 DPP and in Dicer mutants by 8 DPP, and HFs subsequently started to degrade in both mutants. (M) Schematic depiction of the plucking-induced HF growth cycle indicating time points at which doxycycline administration was initiated and skin was biopsied.

Drosha and Dicer mutant HF retain stem cells in early anagen

To determine whether loss of bulge stem cells could account for the decreased size of the early anagen matrix in Drosha\textsuperscript{mutants and Dicer\textsuperscript{mutants}, we assayed for expression of the stem cell markers Krt15 and CD34 at 12 or 8 days after plucking, respectively, when mutant follicles showed severe histological defects (Fig. 5B,E,G,J). Interestingly, similar levels of Krt15 and CD34 staining were observed in mutant follicles and littermate controls (Fig. 6A-D; supplementary material Fig. S10A-D). Bulge cells are slow cycling and retain labeled deoxyribonucleotides (Cotsarelis et al., 1990). To determine whether label-retaining cells are affected by Dicer deletion, Dicer\textsuperscript{mutants and littermate control mice were injected with chloro-deoxyuridine (CldU) for the first 3 days after birth, and were doxycycline treated from P38, followed by hair plucking at P48. Mice were injected with iododeoxyuridine (IdU) to label proliferating cells, 2 hours before skin biopsy at 8 DPP. The numbers of CldU-positive label-retaining cells, IdU-positive proliferating cells, and double-positive proliferating label-retaining cells, as assayed by immunofluorescence, were not significantly different in Dicer\textsuperscript{mutants and control HFs (Fig. 6E-G). Similarly, significant differences were not observed in the numbers or proliferation of Sox9-positive ORS cells, which are necessary for maintenance of the matrix (Nowak et al., 2008), in Dicer\textsuperscript{mutants and control HFs at 3 DPP (Fig. 6H-J). Taken together, these data indicate that loss or failure of proliferation of bulge stem cells and Sox9-positive ORS cells is unlikely to account for matrix defects in early anagen.

Specific deletion of Dicer in bulge stem cells does not prevent their contribution to the matrix population

To assay more directly the requirements for miRNAs in early anagen stem cells, we generated Krt15-CrePR1 Dicer\textsuperscript{flEx22-23/Rosa26R} mice in which deletion of Dicer is specifically induced in stem cells by application of topical RU486, and X-Gal staining can be used to track the fates of Cre-active cells and their progeny. Experimental mice and control littermate Krt15-CrePR1 Dicer\textsuperscript{mutants and Rosa26R mice were treated daily with topical RU486 for 4 days...
starting at P46. Hair was plucked at P50 and dorsal skin was biopsied 7 days later. In both mutant and control HFs, mosaic X-Gal staining was observed in the ORS, matrix, precortex and IRS (Fig. 6K,L). These results are consistent with our observation that stem cells are not depleted immediately following Krt5-rtTA tetO-Cre-mediated inactivation of Drosha or Dicer, and suggest that Dicer-deleted stem cells can contribute to the matrix and differentiate into HS and IRS.

**Drosha and Dicer mutant HF matrix cells differentiate appropriately**

It was possible that mosaic Cre reporter activity did not correlate perfectly with Dicer deletion in Krt15-CrePR1 DicerflEx9/+/R26R mice. To determine whether Cre-active cells and their progeny could also contribute to HF lineages in the more completely deleted Krt5-rtTA tetO-Cre system, we analyzed X-Gal expression in induced Krt5-rtTA tetO-Cre DicerflEx22-23/R26R dorsal skin at 8 DPP. X-Gal staining was present in the ORS, IRS and precortex of mutant follicles at 8 DPP (n=2 controls and n=2 mutants; more than 6 HFs analyzed per sample), consistent with this, immunofluorescence of DroshaflEx9 or DicerflEx22-23 mutant and control HF section at 3 DPP (n=2 controls and n=2 mutants; more than 6 HFs analyzed per sample). These results suggest that Dicer-deleted stem cells can contribute to the matrix and differentiate into HS and IRS.
smaller than controls (supplementary material Fig. S11A,B), contained fewer matrix and differentiating cells, and these did not assemble into normal differentiating structures (Fig. 7A-H; supplementary material Fig. S11A-D).

**Dicer and Drosha mutant HF matrix cells display normal rates of proliferation**

As defective differentiation did not appear to account for Drosha\textsuperscript{flox}\textsuperscript{Ex9} and Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant HF phenotypes, we asked whether Drosha or Dicer deletion affected proliferation rates. Drosha\textsuperscript{flox}\textsuperscript{Ex9} and Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant hair bulbs contained fewer Ki67-positive cells than littermate controls at 12 DPP and 8 DPP, respectively; however, mutant hair bulbs also contained fewer cells than controls (Fig. 7I-L). Proliferation rates, estimated by calculating the percentage of Ki67-positive DAPI-stained cells in at least eight hair bulbs from control and mutant mice, were not significantly altered by Drosha or Dicer deletion (Drosha\textsuperscript{flox}\textsuperscript{Ex9} control, 42±4% Ki67-positive cells; Drosha\textsuperscript{flox}\textsuperscript{Ex9} mutant, 37±6%; Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} control, 50±12%; Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant, 47±5%; not statistically significant), indicating that Drosha and Dicer are not required for normal rates of matrix proliferation (Fig. 7U, V).

**Deletion of Drosha or Dicer causes matrix cell apoptosis and a DNA damage response**

We next asked whether the diminished size of Drosha\textsuperscript{flox}\textsuperscript{Ex9} and Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant hair bulbs following hair plucking was due to increased cell death. TUNEL-positive cells were rarely observed in the bulge or matrix of control HFs at 12 DPP or 8 DPP (Fig. 7M,O). By contrast, at 12 DPP and 8 DPP, respectively, Drosha\textsuperscript{flox}\textsuperscript{Ex9} and Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant follicles displayed increased apoptosis, particularly in the matrix (Fig. 7M-P), consistent with elevated Dicer mutant matrix cell death in embryonic anagen (supplementary material Fig. S3A,B). At 12 DPP, 2.8±0.7% of Drosha\textsuperscript{flox}\textsuperscript{Ex9} mutant matrix cells were TUNEL positive compared with 0.2±0.1% of control matrix cells (P=0.0003). Likewise, at 8 DPP, 1.1±0.3% of cells were TUNEL positive in Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant follicles compared with 0.02±0.007% in control follicles (P=0.005) (Fig. 7U, V). Interestingly, Drosha\textsuperscript{flox}\textsuperscript{Ex9} and Dicer\textsuperscript{flox}\textsuperscript{Ex22-23} mutant matrix compartments also displayed elevated expression of the DNA damage response marker pH2A.X at 12 DPP and 8 DPP, respectively (Fig. 7Q-T). Quantification of pH2A.X-positive cells as a percentage of the bulb population revealed that these increases were statistically significant (Drosha\textsuperscript{flox}\textsuperscript{Ex9} control 0.0±0.0%,}
These data indicate that miRNA roles in postnatal skin levels were normalized to 1.0. Relative expression levels are shown (arbitrary units).

**Notch signaling is downregulated and Tslp levels are elevated in Drosha and Dicer mutant skin**

Epidermal loss of Notch signaling produces phenotypes similar to those observed following miRNA depletion, including: severe abnormalities at later stages of HF morphogenesis; the ability of Drosha/Dicer-depleted and Notch-depleted bulge stem cells to contribute to the matrix, IRS and ORS; failure of HF maintenance; interfollicular epidermal hyperproliferation; and inflammation (Pan et al., 2004; Vauclair et al., 2005; Lee et al., 2007; Demehri et al., 2008; Demehri and Kopan, 2009). Consistent with these, levels of full-length (FL) Notch1 and Notch1 intracellular domain (NICD) were reduced by P17 in DicerflEx22-23 and DroshaflEx9 mutants compared with control skin following induction from E18 (Fig. 8A,B).

Inflammation in epidermal Notch-deficient mutants is thought to occur as a result of defective barrier formation and is associated with elevated levels of the keratinocyte-derived cytokine Tslp (Demehri et al., 2008). We observed a more than 10-fold elevation of Tslp mRNA levels in DicerflEx22-23 mutant compared with control epidermis from mice induced from E18 and biopsied at P15 (Fig. 8C), suggesting that barrier function might be impaired as a result of intrinsic defects in the IFE (Yi et al., 2008) and/or loss of HF integrity. Consistent with these data, treatment of epidermal Dicer-depleted mice with MC903, a vitamin D3 analog, triggers increased levels of Tslp production relative to those observed in MC903-treated control mice (Hener et al., 2011). In this latter study, however, epidermal Dicer-depleted mice had a normal phenotype in the absence of MC903 treatment, suggesting that Dicer deletion was incomplete. Although Tslp levels are directly and positively controlled by miR-375 in intestinal cells (Biton et al., 2011), decreased miR-375 levels in Dicer-deficient epidermis and HF s (supplementary material Fig. S2M,N) suggest that epidermal Tslp is regulated by other, less direct, mechanisms.

**Direct targets of miR-205 are upregulated in Dicer mutant skin**

To identify direct effects of miRNA depletion on epidermal target miRNAs we focused on miR-205, which is one of the most abundant epithelial miRNAs (Andl et al., 2006; Yi et al., 2006) and is dramatically depleted in epidermal Drosha and Dicer mutants (Fig. 1E-I; supplementary material Fig. S7). Of particular interest was the direct miR-205 target E2f1 (Dar et al., 2011), a key regulator of the G1/S transition and p53 (Trp53)-mediated apoptosis (DeGregori, 2002). Krt5 promoter-driven overexpression of E2f1 in transgenic mouse skin epithelial cells results in apoptosis of early anagen HF matrix cells and IFE hyperproliferation (Pierce et al., 1998), replicating phenotypes observed in epidermal Dicer and Drosha mutants. qPCR analysis of dorsal skin samples from P15 DicerflEx22-23 mutants and control littersmates induced from E18 revealed a statistically significant increase in the levels of E2f1 mRNA in mutants relative to controls (n=2 mutants and n=2 controls; P=0.038) (Fig. 8D). These data suggest that miR-205-mediated E2f1 mRNA degradation might be important in preventing matrix cell apoptosis in early anagen and IFE hyperproliferation.

The transcriptional repressor Sip1 is another direct target of miR-205 (Paterson et al., 2008) that is elevated in DicerflEx22-23 mutant compared with control skin at P15 in a statistically significant manner (n=2 mutants and n=2 controls; P=0.022) (Fig. 8D). Among other roles, Sip1 regulates the epithelial-mesenchymal transition (Gregory et al., 2008). Dicer deletion in embryonic epidermis results in an unusual phenotype of HF evagination (Andl et al., 2006; Yi et al., 2006), and we observed apparent extrusion of keratinized HF cells in induced postnatal epidermal Drosha and Dicer mutants (Fig. 2H,R). These phenotypes might be related to abnormal control of intercellular adhesion and cell movements by dysregulated Sip1.

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**Fig. 8. Decreased levels of full-length Notch1 and NICD and increased expression of Tslp and the miR-205 direct targets E2f1, Sip1 and Src in miRNA-depleted skin.** (A,B) Immunoblotting for full-length (FL) Notch1, Notch1 intracellular domain (NICD), Gapdh or β-actin using DicerflEx22-23 (A) and DroshaflEx9 (B) mutant and littermate control skin extracts at the time points indicated, following doxycycline treatment from E18. (C,D) qPCR analyses of Tslp (C) or E2f1, Sip1 and Src (D) mRNAs extracted from P15 DicerflEx22-23 mutant and control skin following doxycycline treatment from E18 (n=2 control and n=2 mutant samples for each experiment; qPCR replicated three times for each sample). Control expression levels were normalized to 1.0. Relative expression levels are shown (arbitrary units). P-values were calculated using a two-tailed Student’s t-test. Asterisks indicate statistically significant differences.
A third direct miR-205 target, the non-receptor intracellular tyrosine kinase Src (Majid et al., 2011), was also upregulated in Dicer mutant skin, although this did not reach statistical significance \((n=2\) mutants and \(n=2\) controls; \(P=0.073\)) (Fig. 8D). Elevated levels of epidermal Src cause hyperproliferation, HF defects and chronic inflammation (Matsumoto et al., 2003; Yagi et al., 2007) and could contribute to these abnormalities in miRNA-depleted skin.

Taken together, these results suggest that upregulation of multiple miRNA target genes contributes to the complex phenotypes of epidermal Drosha and Dicer mutants.

**DISCUSSION**

Here we use inducible epidermal-specific deletion of two independent components of the miRNA biogenesis pathway, Drosha and Dicer, to investigate the global functions of miRNAs at successive stages of the postnatal HF growth cycle and in adult epidermis. HF and epidermal phenotypes resulting from postnatal epidermal deletion of Drosha and Dicer were virtually identical at each stage examined, suggesting that miRNA-independent functions of these enzymes play only minor roles in postnatal HFs and epidermis. A similar conclusion was reached following comparison of the effects of Dgcr8 and Dicer deletion in embryonic skin (Yi et al., 2009). Thus, it seems likely that the expression of co-factors that modulate the actions of Drosha-Dgcr8 and Dicer in the epidermis does not change substantially after birth. The largely miRNA-related functions of the Drosha-Dgcr8 complex and Dicer in skin epithelial cells at multiple developmental and postnatal stages contrast with the prominent miRNA-independent roles of these enzymes in certain other cell types, such as early-stage thymocytes (Chong et al., 2010), but are similar to what has been observed in regulatory T-cells (Chong et al., 2008).

Dicer deletion in epidermal cells during embryonic development leads to a failure in establishment of the HF bulge stem cell compartment (Andl et al., 2006). Interestingly, however, deletion of either Drosha or Dicer in established, resting, telogen stage HFs did not cause marked histological abnormalities or loss of HF stem cells prior to entry into a natural or depilation-induced anagen stage. Thus, miRNAs are required for establishment of the HF stem cell compartment, but not for its maintenance in resting follicles.

In contrast to the lack of gross abnormalities during the resting phase, mutant HFs were unable to engage in normal growth. HF stem and matrix cells in either mutant proliferated following plucking, and stem cells were not initially depleted in mutant follicles. However, matrix cells exhibited marked increases in cell death that led to failed HF downgrowth and rapid follicular degradation. Interestingly, matrix cell death in Drosha and Dicer mutants was accompanied by a DNA damage response, indicated by a significantly increased percentage of cells expressing the DNA damage marker pH2A.X compared with controls. At early stages following deletion, pH2A.X expression was specific to matrix cells; however, once HFs started to degrade, apoptosis and a DNA damage response were also observed in the bulge.

Initiation of Drosha or Dicer deletion in established anagen HFs also resulted in matrix cell apoptosis and defective HS assembly. Strikingly, HFs were maintained in an abnormal proliferative state instead of undergoing programmed regression, revealing essential functions for miRNAs in the anagen-catagen transition. Expression of Fgf5, a key regulator of catagen (Hebert et al., 1994), was reduced in mutant follicles at this stage. As most direct miRNA targets are predicted to be upregulated following Drosha or Dicer deletion, it is likely that an inhibitor of Fgf5 expression, rather than Fgf5 itself, is a direct target of miRNA action during catagen.

Failed catagen in Drosha and Dicer mutants was followed by follicular degradation, an inflammatory response and epidermal hyperplasia. Defects in mutant HFs, including HS abnormalities and failure of regression, were observed soon after induction of Drosha or Dicer gene inactivation and prior to the appearance of an inflammatory infiltrate, indicating that these were caused directly by miRNA depletion and were not a consequence of inflammation.

By contrast, epidermal hyperproliferation in Drosha and Dicer mutants was comitant with inflammation. This suggests that degrading HFs trigger a dermal inflammatory response and might cause breaks in the epidermal barrier, further promoting inflammation and IFE hyperproliferation. In addition, IFE-intrinsic mechanisms associated with miRNA depletion could contribute to IFE hyperplasia and inflammation. These mechanisms include loss of direct repression of p63 by miR-203 (Yi et al., 2008; Lena et al., 2008), depletion of the cell-cycle-inhibitory miRNAs miR-34a and miR-34c (Antonini et al., 2010), decreased Notch signaling, and possibly increased levels of Src tyrosine kinase. Whether depletion of miRNAs contributes directly or indirectly to decreased levels of Notch1 and Notch1 NICD in Drosha and Dicer mutants, and enhances susceptibility to tumorigenesis as is seen in Notch pathway mutants (Demehri et al., 2009b), will be important areas for future investigation.

The delay in the appearance of IFE phenotypes relative to HF defects might be due to the relatively slow turnover of IFE cells compared with the HF matrix. As there is extensive cross-talk between skin epithelial cells, dermal fibroblasts and skin adipose tissue (Pliks et al., 2008; Festa et al., 2011), additional secondary effects of epithelial miRNA depletion on dermal and adipose cells, including activation of stromal fibroblasts (Demehri et al., 2009a), might contribute to epidermal phenotypes in Drosha and Dicer mutants.

The most immediate and striking phenotypes in Drosha or Dicer mutants were observed in the early anagen HF matrix, one of the most rapidly proliferating adult cell populations, which displayed increased apoptosis and DNA damage. The extremely rapid proliferation of matrix cells might render them particularly susceptible to double-stranded breaks, resulting in increased requirements for genomic repair. We observed increased expression of the direct miR-205 target E2f1, a key regulator of the G1/S transition and p53-mediated apoptosis (Dar et al., 2011), in Dicer mutant skin. Interestingly, forced expression of E2f1 in transgenic mouse skin epithelial cells causes a similar phenotype of HF matrix cell apoptosis and IFE hyperproliferation (Pierce et al., 1998), suggesting that miR-205-mediated E2f1 mRNA degradation might be important in preventing matrix cell apoptosis in early anagen and IFE hyperplasia. E2f1 is post-translationally modified in response to DNA damage, localizes to DNA strand breaks, recruits repair factors and promotes DNA repair (Degregori, 2011), and thus might also contribute to the abnormal DNA damage response observed in miRNA-depleted matrix cells.

Another possible mechanism contributing to our observation of elevated phosphorylated histone H2A.X in mutant matrix cells is decreased production of miR-24, which is known to directly target the H2ax (H2afx – Mouse Genome Informatics) transcript (Lal et al., 2009). In addition, accumulating data suggest key roles for miRNAs in the DNA damage response (Hu et al., 2010; Leung and Sharp, 2010; Hu and Gatti, 2011). Biogenesis of a subset of miRNAs is involved in genomic repair. We observed increased expression of several direct miRNA targets, including Fgf5, which is a key regulator of catagen (Hebert et al., 1994), and decreased Notch signaling and apoptosis in mouse skin epithelial cells causes a similar phenotype of HF matrix cell apoptosis and IFE hyperproliferation (Pierce et al., 1998), suggesting that miR-205-mediated E2f1 mRNA degradation might be important in preventing matrix cell apoptosis in early anagen and IFE hyperplasia. E2f1 is post-translationally modified in response to DNA damage, localizes to DNA strand breaks, recruits repair factors and promotes DNA repair (Degregori, 2011), and thus might also contribute to the abnormal DNA damage response observed in miRNA-depleted matrix cells.
regulatory protein (Ksrp; Khsrp – Mouse Genome Informatics), which associates with both Drosha and Dicer, facilitating recruitment and processing of specific miRNA precursors (Liu and Liu, 2011). Other, Ksrp-independent miRNAs, such as miR-34a/c, are transcriptionally upregulated by p53 in response to DNA damage (He et al., 2007). The functions of DNA damage-induced miRNAs have been suggested to include facilitating DNA repair, cell cycle arrest and apoptosis; alternatively, they could act in a negative-feedback loop to dampen the DNA damage response once repair is completed (Liu and Liu, 2011). Our data showing increased apoptosis and pH2A.X levels in Drosha- and Dicer-depleted matrix cells are consistent with the latter role. The identification and functional analysis of the individual miRNAs and their targets that are important for suppressing the DNA damage response in matrix cells are likely to provide mechanistic insights into this process that are relevant well beyond the HF.

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