Rapid and widespread suppression of self-renewal by microRNA-203 during epidermal differentiation

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SUMMARY
MicroRNAs (miRNAs) play important roles in differentiation of stem cells. However, the precise dynamics of miRNA induction during stem cell differentiation have not been visualized and molecular mechanisms through which miRNAs execute their function remain unclear. Using high-resolution in situ hybridization together with cell lineage and proliferation markers in mouse skin, we show that miR-203 is transcriptionally activated in the differentiating daughter cells upon the asymmetric cell division of interfollicular progenitor cells. Once induced, miR-203 rapidly promotes the cell cycle exit within 6 hours and abolishes self-renewal of the progenitor cells. With an inducible mouse model, we identify numerous miR-203 in vivo targets that are highly enriched in regulation of cell cycle and cell division, as well as in response to DNA damage. Importantly, co-suppression of individual targets, including p63, Skp2 and Msi2 by miR-203 is required for its function of promoting the cell cycle exit and inhibiting the long-term proliferation. Together, our findings reveal the rapid and widespread impact of miR-203 on the self-renewal program and provide mechanistic insights into the potent role of miR-203 during the epidermal differentiation. These results should also contribute to understanding the role of miR-203 in the development of skin cancer.

KEY WORDS: Epidermal differentiation, miRNA, Self-renewal, Mouse

INTRODUCTION
Differentiation of somatic stem cells is a complex process that is orchestrated by multiple mechanisms leading to changes in gene expression. In turn, these changes at the molecular level drive stem cells to abandon their state of self-renewal and produce terminally differentiated cells with specific physiological functions (Blanpain and Fuchs, 2009; He et al., 2009). Although extensive effort has been devoted to identify master regulators that directly drive differentiation programs, little is known about the kinetics of such transition, e.g. when is a differentiation-related gene induced and how does its expression compromise self-renewal and initiate differentiation? Among many regulators, miRNAs are implicated important roles in stem cell differentiation (Ivey and Srivastava, 2010; Yi and Fuchs, 2011). miRNAs are a family of small, noncoding RNAs that are widely expressed in most animal species ranging from sponge to human (Grimson et al., 2008). These tiny riboregulators function by recruiting the RNA-induced silencing complex (RISC) to their target mRNAs usually through base-pairing between 5’ end sequences of miRNAs and mRNA sequences located in the 3’ untranslated region (3’UTR) (Bartel, 2009). In turn, miRNAs repress their target expression by inhibiting translation initiation and destabilizing mRNAs (Bazzini et al., 2012). Despite the evidence for functional significance of miRNAs in stem cell differentiation (Melton et al., 2010; Murchison et al., 2005; Yi et al., 2008), the dynamics of the molecular events that are controlled by miRNAs remain poorly defined. In particular, the precise timing of miRNA induction during differentiation is not known. Although many distinct miRNAs have been detected in stem cell lineages, these results are usually obtained by quantifying miRNAs in isolated cell populations that lack spatiotemporal resolution to distinguish the exact expression pattern of miRNAs at the single-cell level. Furthermore, the underlying mechanism for the role of miRNAs in differentiation, namely how many targets are regulated by an miRNA and how the regulation of many targets by an miRNA contributes to its function, has yet to be elucidated.

In the epidermis of mouse skin, stem/progenitor cells reside in the basal layer in touch with the basement membrane (Blanpain and Fuchs, 2009). These progenitor cells divide either horizontally to give rise to two basal cells (symmetric cell division, SCD) or perpendicularly to one basal cell and one suprabasal cell (asymmetric cell division, ACD) (Lechler and Fuchs, 2005). The basal cell retains the stem cell/progenitor property, whereas the suprabasal cell embarks upon epidermal differentiation (Lechler and Fuchs, 2005). Thus, the epidermis is an excellent model for examining kinetics and mechanisms that underlie differentiation processes.

We and others have identified miR-203 as a skin-specific miRNA that plays an important role in the epidermal differentiation both in vivo and in vitro (Lena et al., 2008; Yi et al., 2008). To distinguish whether the induction of miR-203 is an early event that drives the epidermal differentiation, we have developed a high-resolution in situ hybridization technique and precisely define spatiotemporal expression patterns of miR-203 together with protein markers for skin lineages and proliferation during the epidermal differentiation. We have also established an inducible mouse model that enables us to control the expression level and timing of miR-203. We show that miR-203 has an immediate impact on the cell cycle exit and also abolishes long-term cell proliferation. We further identify a large number of in vivo mRNA targets of miR-203 that are highly enriched in the regulation of self-renewal. By enhancing the expression of the targets of miR-203 (including Skp2, a cell cycle...
miR-203 inhibits self-renewal

regulator; Msi2, a RNA-binding protein; and p63, a transcription factor required for skin stem cells) in the presence of miR-203 both individually and combinatorially, we demonstrate that co-repression of these targets is required to mediate the widespread inhibition of self-renewal by this miRNA. Together, our studies provide mechanistic insights into the activation and function of miR-203 during the epidermal differentiation.

MATERIALS AND METHODS

Animals

miR-203-inducible mouse was generated by standard pronuclear injection of pTRE2-miR-203 expression plasmid in a FVB background. This strain was subsequently bred to K14-rtTA to create the inducible mouse model. Two independent pTRE2-miR-203/K14-rtTA founder lines were generated and validated for the experiments. Mice were bred and housed according to the guidelines of IACUC at a pathogen-free facility at the University of Colorado (Boulder, CO, USA).

In situ hybridization, Immunofluorescence and antibodies

In situ hybridization of miRNAs was performed as previously described (Yi et al., 2008) with modifications to signal development. Briefly, double DIG-labeled miR-203 probe (Exiqon, Denmark) was used for hybridization at 46°C for 2 hours and the signal was developed with the TSA amplification systems with FITC-conjugated reagent (PerkinElmer, USA). For co-staining with other protein markers, the developed images were treated with DNease I (25 units/ml; Sigma, USA) for 1 hour at 37°C, then incubated with primary antibodies against BrdU (1:500; Abcam, USA), Krt5 (1:500; Covance, USA), PH3 (1:1000; Cell Signaling, USA) or β4 integrin (1:200; BD Biosciences, USA). Subsequent antibody co-staining was performed as described previously (Yi et al., 2008).

5' RACE and luciferase assay

5' RACE for miR-203 primary transcripts was carried out with the SMART miRNA Amplification kit (Clontech, USA) following the manufacturer’s instruction. Four independent clones were sequenced for the identification of the 5' end of miR-203. The promoter region was amplified with mouse genomic DNA and cloned into a pGL3-basic vector (Promega, USA). The luciferase assay was carried out by transfecting 20 ng of the luciferase reporters as indicated together with 380 ng of an empty pGL3-control vector (Promega, USA). The luciferase activity was measured 48 hours post-transfection.

For the 3' UTR luciferase assay, 3' UTR fragments of individual targets were obtained by PCR amplification (supplementary material Table S2) from total skin cDNA and cloned into the 3' end of a pGL3-control vector (Promega, USA). The luciferase assay for wild-type and mutant 3' UTRs was carried out as described previously (Yi et al., 2008).

Microarray and target analyses

Total RNA (500 ng) isolated from the basal epidermis of two pairs of miR-203 induced and control mice were used for microarray analysis with Mouse Genome 430 2.0 array (Affymetrix, USA) following the manufacturer's instruction. Microarray data were analyzed with the Bioconductor software (Gentleman et al., 2004). Probesets that were called 'present' and had known annotations were collected for downstream analysis.

The 5'UTR, CDS and 3'UTR of all genes annotated in the Knowgene table of the mouse genome database (mm9) were downloaded from the UCSC Genome Browser (Kent et al., 2002). The sequences obtained were used for miR-203 target analysis by searching for perfect matches for 7–mer motifs that are derived from mature miR-203 sequences.

Microarray data have been deposited in Gene Expression Omnibus with accession number GSE45121.

Cell culture and cell cycle analysis

Primary keratinocytes were cultured as previously described (Yi et al., 2008). Inducible keratinocytes were isolated from pTRE2-miR-203/K14-rtTA transgenic mice. miR-203 expression was induced by adding doxycycline to the media at a concentration of 3 μg/ml. For induction for longer than 24 hours, fresh doxycycline was added daily. The cell cycle profile was determined by following the instruction of the BD Pharmingen BrdU kit.

Characterization of the functions of miR-203 targets

The rescue experiments were conducted using retroviral MIGR constructs containing cDNA of miR-203 targets [Msi2, p63 (Trep6- Mouse Genome Informatics), Skp2 and Vav3], as well as an empty MIGR vector control. The knockdown of p63, Msi2 and Skp2 was carried out with two shRNA constructs (Sigma-Aldrich, USA) for each gene with cultured keratinocytes. Puromycin-resistant cells (3000 cells) were plated for the colony formation assay.

RNA-Seq and quantification

Total RNA (~0.5-2 μg) isolated from the basal and suprabasal cells was used for RNA-Seq. The RNA-Seq experiment was performed as previously described (Wang et al., 2013). The relative expression was calculated by normalizing to Hpirt.

Statistical analysis

P values were calculated using an unpaired t-test using the online calculator from GraphPad software. Error bars indicate s.d.

RESULTS

miR-203 is inversely correlated with epidermal proliferation

miR-203 is thought to have an important role during epidermal differentiation (Yi et al., 2008). However, the precise dynamics of the upregulation of miR-203 remain unclear. We set out to determine the correlation between the expression and epidermal differentiation of miR-203 with high-resolution in situ hybridization. During mouse embryonic skin development, a highly proliferative stem/progenitor cell population resides at the basal layer and gives rise to differentiating daughter cells, collectively called suprabasal cells (Blanpain and Fuchs, 2009). We first examined the expression of miR-203 in the developing epidermis by correlating its in situ signal with the proliferative cells, as marked by phosphorylated (Ser-10) Histone H3 (PH3) at embryonic day 15 (E15), a period when the basal progenitor cells begin to stratify and produce the differentiated suprabasal cells (Blanpain and Fuchs, 2009; Lechler and Fuchs, 2005). The signals of miR-203 were largely restricted to the cytoplasm of the differentiated suprabasal cells (Fig. 1). The punctate localization of miR-203 signals throughout the cytoplasm was reminiscent of the established patterns for the RISC, as determined by immunofluorescence staining of argonaute proteins (Liu et al., 2005). Furthermore, the specificity of in situ signals was confirmed by the absence of signals in the Dicer knockout skin (supplementary material Fig. S1), where all miRNAs, including miR-203, are absent (Yi et al., 2008). These observations confirm that our in situ hybridization specifically detects miR-203 with subcellular resolution. At E15, although most proliferative cells were already restricted in the basal layer, limited cellular proliferation did occur at the suprabasal layer immediately adjacent to the basal layer (Fig. 1A-C). By doing so, it allows rapid expansion of developing epidermis at this stage (Lechler and Fuchs, 2005). When examined closely, these proliferative cells at the suprabasal layer expressed a lower level of miR-203 than did the cells located further away from the basal layer (Fig. 1A-C). Moreover, the expression of miR-203 appeared to vary among individual suprabasal cells at this stage, suggesting an ongoing transition from proliferation to terminal differentiation (Fig. 1A-C). When examined at later stages, e.g. E17 and postnatal day 6 (P6), the expression of miR-203 was uniformly high in all suprabasal layers when all proliferative cells are restricted in the basal layer (Fig. 1D-E). Together, these results demonstrate that the temporal
induction of miR-203 is inversely correlated with cell proliferation in the epidermis.

Unlike the interfollicular progenitor cells, which constantly progress through the cell cycle, hair follicle stem cells are quiescent (Blanpain et al., 2004; Nowak et al., 2008; Tumbar et al., 2004). To distinguish whether miR-203 is associated simply with the lack of proliferation or specifically with the differentiation, we characterized its expression in hair follicle stem cells. As shown in Fig. 1F, miR-203 was absent from the hair follicle stem cells that are attached to the basement membrane marked by β4 integrin but was highly expressed in the suprabasal differentiated cells located in the adjacent companion layer (Hsu et al., 2011). Together, these results demonstrate that miR-203 is intimately correlated with the epidermal differentiation in a spatiotemporally specific manner.

**miR-203 is transcriptionally activated in differentiating cells upon asymmetric cell division**

When the basal cells divide, they divide either symmetrically or asymmetrically to balance maintenance and differentiation of the epidermal lineages (Poulson and Lechler, 2010). To define precisely the spatial induction pattern of miR-203, we next examined the expression of miR-203 more closely in postnatal skin. Because epidermal cell division becomes increasingly rare postnatally, a relatively long BrdU pulse can label both daughter cells derived from a single cell division. The two daughter cells should be easily recognized as dual-labeled cells adjacent to each other, whereas the neighboring cells are mostly unlabeled because of the overall scarcity of dividing cells. Indeed, when we pulse P6 animals with BrdU for 12 hours, we observed many dual-labeled cells in the epidermis either horizontally (Fig. 2A-C) or perpendicularly localized (Fig. 2D-F). When we co-stained miR-203 with BrdU as well as keratin 5 (K5), we found that miR-203 is not expressed in the daughter cells of the SCD but is robustly induced in the suprabasal cell of the ACD (Fig. 2A-F). These results provide novel insights to the precise correlation between the induction of miR-203 and the onset of epidermal differentiation. In particular, the simultaneous detection of miR-203 and dividing cells provides a high-resolution picture for the rapid induction of miR-203 following the ACD in the epidermis.

Because the expression of miRNAs can be controlled by transcriptional regulation (Krol et al., 2010), we characterized the transcription start site (TSS) and the promoter region of miR-203. The miR-203 gene is located at an intergenic region on chromosome 12. With 5′ RACE, we determined that the TSS of miR-203 is located ~100 bp upstream of the miR-203 hairpin, which is immediately downstream of three highly conserved DNA sequences in vertebrates (Fig. 2G). The conserved DNA fragments are located within a CpG island characteristic of mammalian promoters. To test whether the upstream sequences from the TSS are indeed the promoter of miR-203, we cloned a fragment of ~3 kb DNA sequences upstream of miR-203 into a promoterless luciferase reporter (pGL-basic) and tested the luciferase activity in undifferentiated primary keratinocytes (LowCa), where miR-203 is expressed at low levels, and in differentiated primary keratinocytes (HiCa), where miR-203 is expressed at high levels (Yi et al., 2008). As expected, the promoter activity was significantly upregulated in the differentiated primary keratinocytes (HiCa), while demonstrating a basal level of expression in the undifferentiated primary keratinocytes (LowCa) (Fig. 2H). Importantly, the approximately ninefold upregulation of the promoter activity is consistent with the expression pattern of mature miR-203 reported in these cells (approximately eight- to 10-fold activation).

The promoter assay suggests that the ~3 kb DNA sequences upstream of miR-203 contain binding sites for transcription factors to activate miR-203 during the epidermal differentiation. We then performed bioinformatic analysis to identify transcription factors that have conserved binding sites in this region by using ECR Browser (Ovcharenko et al., 2004). Intriguingly, we have identified numerous conserved binding sites for transcription factors that are directly involved in the epidermal differentiation (supplementary material Fig. S2). Among them, C/EBP (Lopez et al., 2009), Hes1 (Moriyama et al., 2008), SRF (Luxenburg et al., 2011), AP2 (Wang et al., 2008), IRF (Ingraham et al., 2006; Richardson et al., 2006) and Blimp1 (Magnúsdóttir et al., 2007) have been reported to have crucial roles in promoting epidermal differentiation, whereas E2F (Dicker et al., 2000) and LeF1/Tcf3/Tcf4 (Nguyen et al., 2009) are important for maintaining the progenitor fate of the basal epidermal cells. Taken together, these observations suggest that miR-203 is repressed in the basal progenitor cells and activated in the suprabasal cells through the control of many transcription factors.

**miR-203 has immediate and long-term impact on cell proliferation**

Having determined the precise dynamics of miR-203 induction in the skin, we sought to determine the functional significance of the rapid induction of miR-203. To achieve this, we established an inducible
miR-203 inhibits self-renewal

Fig. 2. miR-203 is transcriptionally activated in differentiating cells following asymmetric cell division. (A-C) miR-203 remains repressed in both basal cells (marked by BrdU) following symmetric cell division in the basal layer (marked by K5). (D-F) miR-203 is rapidly induced in the differentiating daughter cell upon asymmetric progenitor cell division in the epidermis. Two daughter cells of the asymmetric cell division are marked by BrdU in D and F. The daughter cell remaining in the basal layer is marked by K5 in E and F. Scale bars: 10 μm. (G) The TSS of miR-203, located ~100 bp upstream of the miR-203 hairpin, is determined by 5′ RACE. Conserved DNA patterns and a CpG island surrounding the conserved DNA sequences are identified in the upstream region, indicating the existence of a putative promoter. (H) The promoter activity of the DNA fragment spanning ~3 kb region upstream of the TSS is tested in undifferentiated (LowCa) and differentiated (HiCa) keratinocytes. A basal-level promoter activity is observed in the undifferentiated condition (compared with the pRL-basic and the positive control pGL-control). A significant activation (approximately ninefold) is detected in the differentiated condition. n=3, *P≤0.01. Data are mean±s.e.m.

system to control miR-203 in the skin (Fig. 3A). Previously, skin-specific inducible transgenic mouse models driven by Keratin14-rtTA (K14-rtTA) have been established to study transcription factors (Nguyen et al., 2006). We adapted this approach by using the K14-rtTA line crossed with pTRE2-miR-203 lines (Fig. 3A).

To characterize the immediate impact of miR-203 on the epidermal differentiation, we isolated and established keratinocytes from the K14-rtTA/pTRE2-miR-203 double positive (DP) skin. We further validated an eightfold induction of miR-203 24-48 hours after the addition of doxycycline to the DP cells (Fig. 3B). The induced level of miR-203 is consistent with the levels of endogenously expressed miR-203 during Ca²⁺-induced keratinocyte differentiation (Lena et al., 2008; Yi et al., 2008).

Next, we examined the impact of miR-203 on the epidermal proliferation. When we analyzed cell cycle progression with the BrdU analysis, we observed a rapid cell cycle withdrawal (Fig. 3C). Within 6 hours of the doxycycline addition, the percentage of cells in S phase was reduced from 48% to 37%. By 24 hours, S-phase proliferation. When we analyzed cell cycle progression with the BrdU analysis, we observed a rapid cell cycle withdrawal (Fig. 3C).

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Upon differentiation, the epidermal stem/progenitor cells lose proliferative potential. To further define the role of miR-203 during the process, we asked whether a short exposure to miR-203 was sufficient to drive the cell cycle exit and dampen the proliferative potential. We induced miR-203 transiently and terminated the induction by washing away doxycycline after 24, 48 or 72 hours of induction. Although the inhibitory effect on the colony formation by a 24-hour exposure to miR-203 could be reversed, longer exposure resulted in permanent loss of proliferation (Fig. 3E). In particular, after a 72-hour induction, colony formation was completely abolished, similar to cells under continuous miR-203 induction (Fig. 3E). These data provide compelling evidence for a potent role of miR-203 in restricting the proliferative potential during the epidermal differentiation. Because an extended induction of miR-203 can lead to the permanent loss of the proliferative capacity, it suggests that miR-203 causes widespread and irreversible changes to the gene expression in the epidermis.

**miR-203 has a widespread impact on the self-renewal program**

To provide molecular insights on the role of miR-203 in the epidermal differentiation, we identified *in vivo* miR-203 targets by microarray profiling with the inducible mice. Recent studies directly link miRNAs to the destabilization of mRNAs, and microarray analysis has been successfully employed to identify miRNA targets (Giraldez et al., 2006; Guo et al., 2010; Lim et al., 2005). Because miR-203 is rapidly induced when the basal cells divide to give rise to the suprabasal cells (Figs 1, 2) and plays a potent role in the differentiation (Fig. 3), we reasoned that we could identify the targets of miR-203 by examining downregulated genes shortly after the induction of miR-203. We first validated the Dox-mediated induction of miR-203 *in vivo*. As shown in Fig. 4A, 6 hours after the Dox injection, miR-203 was induced in the basal cells only in the K14-rtTA/TRE-miR-203 double-positive (DP) skin but not in the TRE-miR-203 single-positive (SP) skin. Importantly, the induced miR-203 was comparable with the endogenous expression in the suprabasal cells, as determined by *in situ* hybridization (Fig. 4A). To establish the optimal timing for detecting the changes to the targets of miR-203 and minimizing secondary effects, we monitored the mRNA level of...
miR-203 is a well-characterized miR-203 target (Lena et al., 2008; Yi et al., 2008), at 12, 24 and 48 hours after Dox treatment. Overall, we determined that the 24-hour time-point was ideal, which is the shortest time-point that allows us to reliably detect the downregulation. Under this condition, miR-203 was induced ~2.5 fold and p63 mRNA was downregulated ~30% in the epidermis (Fig. 4B,C).

We used two pairs of biological duplicates to perform the microarray analysis from the epidermal samples harvested from DP and SP littermates at P4, 24 hours after the Dox injection. The expression of H2BGFP is used for sorting epidermal cells specifically. Collectively, we have identified 424 genes that are consistently downregulated more than 19% in both experiments by using p63 as the cut-off. We first determined whether the genes that contain the matches to miR-203 seed sequences in their 3'UTRs were enriched in the downregulated group. We also carried out three analyses as control. First, we used all genes as the control for the downregulated genes. Second, we used the 5'UTRs and the coding regions as the control for the 3'UTRs. Finally, we used all possible 7-mer motifs from mature miR-203 sequences, e.g. 1-7, 2-8, 3-9, etc., as the control for the seed region (2-8). As expected, we observed very strong (approximately threefold) enrichment of perfect matches to the seed region (2-8) in the 3'UTRs but not in the 5'UTR or coding regions of the downregulated genes (Fig. 4D). We also observed strong enrichment (2-fold) of matches to the 7-mer motifs of number 1-7 and number 3-9 nucleotides of miR-203, whereas no other 7-mer motifs of miR-203 are significantly enriched (Fig. 4E; supplementary material Fig. S4). These observations are consistent with the notion that miRNAs preferentially destabilize their target mRNAs through the recognition of the seed sequences (1-7, 2-8 and 3-9) at the 3'UTRs (Bartel, 2009). Therefore, our analysis has successfully enriched miR-203 targets in the downregulated genes.

We next examined the correlation between the match to seed sequences [e.g. 7-mers, including 1-7, 2-8 and 3-9, and an 8-mer (2-9)] and the level of mRNA downregulation as a measurement for the strength of seed matches. Consistent with previous bioinformatic analyses (Grimson et al., 2007; Lewis et al., 2005), 8-mer (2-9) matches showed the strongest effect on the downregulation of target mRNAs, whereas 7-mer matches (1-7, 2-8 and 3-9) also showed strong effects (Fig. 4F). Overall, the 2-8 and 3-9 matches showed stronger effects on the mRNA downregulation than did the 1-7 matches. We also examined the correlation between the number of seed matches and the degree of mRNA downregulation. As expected, mRNAs containing more than two seed matches showed the greatest reduction followed by mRNAs with two and one seed match (Fig. 4G). All together, these genome-wide analyses further support the robustness of our target identification.

Among these miR-203 target candidates, we next focused on the transcripts that contain perfect matches to 1-7, 2-8 and 3-9 seed sequences of miR-203 in their 3'UTRs as the candidates for the
miR-203 inhibits self-renewal

Fig. 4. Systematic identification of miR-203 targets by microarray and bioinformatic analyses with the inducible mouse model. (A) Six hours after Dox injection, miR-203 is readily induced in the basal progenitor cells only in the DP but not in the SP control skin. All epidermal cells are marked by H2BGFP; the basement membrane is marked by β4 integrin; miR-203 is marked by red cytoplasmic signals. Scale bars: 10 μm. (B) miR-203 is induced at the physiological level in the basal progenitor cells. The expression of miR-203 is not affected by the induction of miR-203 by Dox treatment. (C) Twenty-four hours after Dox treatment, p63 is downregulated ~30% at the mRNA level, as measured by qPCR. (D) High enrichment of miR-203 seed sequences is detected only in the 3’UTR of the downregulated genes upon miR-203 induction. Neither 5’UTR nor the CDS is enriched for the miR-203 seed sequences. (E) Perfect matches to 5’ sequences of miR-203, including numbers 1-7, 2-8 and 3-9 nucleotides but not other regions of miR-203, e.g. numbers 16-22, are highly enriched in the 3’UTRs of the downregulated genes. (F) Effectiveness of seed matches on the downregulation of target mRNAs. Changes in the mRNA level after miR-203 induction are plotted with the cumulative distributions. Repression from UTRs containing an 8-mer (2-9) site is most significant, whereas 7-mer sites (2-8, 3-9, 1-7) also show strong repression, compared with all expressed UTRs. (G) Effectiveness of the number of seed matches on the downregulation of target mRNAs. Changes in the mRNA level after miR-203 induction are plotted with the cumulative distributions. Repression from UTRs containing more than two seed matches is strongest, whereas repression from UTRs containing 2 or 1 seed matches is also significant, compared with UTRs without any seed match.

targets of miR-203. Among these genes, we have identified numerous miR-203 targets that are experimentally validated in the skin, including p63 (Lena et al., 2008; Yi et al., 2008), Bmi1 (Wellner et al., 2009) and Cav1 (Ørom et al., 2012). Overall, we identified 261 out of 424 downregulated genes (61.6%) that contain at least a single match to the seed sequences of miR-203 in their 3’UTRs (supplementary material Table S1). To provide molecular insights into the functions of the targets of miR-203, we performed a pathway analysis to classify these genes (Huang et al., 2009). Strikingly, the processes of cell cycling, cell division and response to DNA damage are the three events that produce the most increases in gene function (Table 1). In our functional studies, miR-203 had strong inhibitory effects on both cell cycle progression and long-term proliferation (Fig. 3). Therefore, these results strongly argue that miR-203 directly targets many factors for the control of cell proliferation.

miR-203 directly regulates a large number of targets

Having demonstrated that miR-203 may regulate many genes that are crucial for cell cycle and cell division by our bioinformatic analysis, we set out to determine the accuracy of our target identification experimentally. We selected a group of 15 genes that are downregulated by miR-203 at the mRNA level (from 19% to 65% downregulation). We cloned the 3’UTR from each of these 17 genes and performed the heterologous luciferase assay by testing their response to the expression of miR-203. As a positive control, we engineered a miR-203 reporter construct with 2x perfectly matched sites at the 3’UTR of the luciferase gene. Perfect matched sites usually lead to miRNA cleavage and, in turn, to significant downregulation of the luciferase activity (Zeng et al., 2003). Indeed, we observed a 95% repression in the luciferase activity, indicating the efficacy of our assay (Fig. 5A). Among the 15 3’UTRs that we tested, 12 of them (80%) showed statistically significant downregulation of the luciferase activity. Notably, the lack of repression by three of the 3’UTRs did not correlate with the level of mRNA downregulation detected by microarray analysis. This observation suggests that: (1) target sites may locate in other regions of the mRNA, e.g. the coding region, which may be responsible for the regulation; and (2) the extent of mRNA downregulation may not be used as a sole determinant to validate individual targets.

To further validate the direct regulation of these candidates by miR-203, we tested whether the predicted miR-203 target sites are responsible for the observed repression. Among the 12 3’UTRs that confer downregulation on the luciferase activity, we targeted eight genes for point mutations at the predicated miR-203 binding sites (supplementary material Table S1). Seven out of eight tested 3’UTRs showed robust derepression of the luciferase activity when the miR-203 sites were mutated (Fig. 5B). Together, these results validate that the downregulated genes bearing miR-203 target sites in the 3’UTR are highly enriched for bona fide miR-203 targets. They also confirm that our combinatorial approach with the inducible mouse model and bioinformatic analysis is highly effective for identifying the targets of miR-203. Furthermore, these results support the observations that miR-203 targets many genes in the epidermis.

Co-regulation of multiple targets is required for the function of miR-203

We and others have previously identified p63 as a target of miR-203 (Lena et al., 2008; Yi et al., 2008). However, it is not clear whether the function of miR-203 is simply mediated through the downregulation of p63 or whether the regulation of other targets also contributes to the function of miR-203. In this study, we greatly expanded the pool of miR-203 targets using a genome-wide approach. It raises the issue of whether miR-203 functions by targeting a single crucial gene, e.g. p63, or multiple targets...
concurrently. We reasoned that if a single target is crucial for the function of miR-203 function, then derepression of the target should be sufficient to suppress the proliferation defects caused by the expression of miR-203. In addition to p63, we selected Skp2 as a representative gene for cell cycle regulation. We also chose Msi2, a RNA-binding protein that has recently been implicated in the self-renewal of hematopoietic stem cells (Hope et al., 2010; Kharas et al., 2010), and Vav3, an oncogene with possible roles in cytokkeleton organization and cell signaling (Hornstein et al., 2004; Tybulewicz, 2005), for the functional test.

We first examined whether a single miR-203 target could recover the cell cycle and proliferation defects. We infected the miR-203 inducible keratinocytes with MSCV constructs for p63, Skp2, Msi2, Vav3 or empty vector control. Twenty-four hours after infection, we induced miR-203 expression by adding Dox (Fig. 6A). Forty-eight hours after miR-203 induction, cell cycle was determined by BrdU incorporation miR-203 expression by adding Dox (Fig. 6A). Forty-eight hours after miR-203 induction, cell cycle was determined by BrdU incorporation.

As shown in Fig. 6B, Msi2, p63 and Vav3 were unable to rescue the impaired cell cycle progression, whereas Skp2 increased the S-phase population in the presence of miR-203. We next examined the ability of individual targets to compromise the inhibition of long-term cell growth caused by miR-203 with colony formation assay (Fig. 6C). Compared with the empty vector-infected cells, where no colony formation was observed, cells infected with Msi2, p63 and Skp2 formed small colonies, whereas Vav3 showed a weaker effect (Fig. 6C). These results indicate that the targets of miR-203 may have distinct functions in the regulation of proliferation and co-suppression of multiple targets is required for the function of miR-203.

To further test this hypothesis, we performed combinatorial experiments in which we infected the cells with a cocktail of Msi2+p63, Msi2+Skp2, p63+Skp2 or Msi2+p63+Skp2. Interestingly, the slight recovery of the cell cycle progression was only observed when Skp2 was included and no synergistic effect was observed when Skp2 is combined with Msi2 and/or p63 (Fig. 6D). Intriguingly, when Msi2, Skp2 and p63 were simultaneously delivered to the cells in the presence of miR-203 induction, we observed the best phenotypes in long-term cell proliferation, e.g. larger colonies were formed (Fig. 6E). Taken together, these results provide further evidence that co-regulation of multiple targets is crucial for the function of miR-203 in potently driving the cell cycle exit and restricting the proliferative potential.

**Targets of miR-203 are required for the self-renewal in vitro**

To provide more insights about p63, Skp2 and Msi2, we characterized their expression patterns in the basal and suprabasal cells. Using E14 epidermis from K14-H2BGFP animals where all epidermal cells are marked by the expression of H2BGFP, we purified the basal and suprabasal cells as the H2BGFP<sup>α</sup>6<sup>hi</sup> and H2BGFP<sup>α</sup>6<sup>hi</sup> populations, respectively. Using RNA-Seq analysis, we quantified their expression levels in the basal and suprabasal cells (Fig. 7A).

Both p63 and Skp2 were highly enriched in the basal cells with over twofold basal/suprabasal ratio, whereas Msi2 was mildly enriched in the basal cells with 1.23-fold basal/suprabasal ratio (Fig. 7A). As control, Krt14 was over 20-fold enriched in the basal cells, whereas Krt10 was ~50-fold enriched in the suprabasal cells (Fig. 7A). These results suggest that these targets are highly enriched in the basal progenitor cells and inversely correlated with the expression of miR-203 in the epidermis. Furthermore, when we induced miR-203 expression in vivo with a single Dox injection, we observed 19%, 32%, 43% and 38% reduction of miRNAs for p63, Msi2, Skp2 and Vav3, respectively (supplementary material Fig. S5). These results further validate that miR-203 suppresses these targets in vivo.

Because of the basal enrichment of p63, Skp2 and Msi2 and the fact that their repression is required for the inhibition of self-renewal in vitro.
by miR-203, we investigated their function in the proliferation of keratinocytes. We used two independent shRNAs to knockdown each gene and test with colony formation assay. Consistent with previous studies (Senoo et al., 2007), knockdown of p63 abolished colony formation (Fig. 7B). Interestingly, knockdown of Skp2, which is crucial for the cell cycle progression, also abolished colony formation (Fig. 7B), whereas knockdown of Msi2 showed relatively modest yet significant reduction in the long-term proliferation (Fig. 7B). When all three genes were knocked down concurrently, the cells failed to give rise to any colonies, as expected (data not shown). These results demonstrate the crucial role of each target in the epidermal proliferation and further highlight the potent impact of miR-203 on the self-renewal by simultaneously repressing these targets.

**DISCUSSION**

miRNAs are thought to have important roles in stem cell differentiation (Ivey and Srivastava, 2010; Yi and Fuchs, 2011). In somatic stem cells, the roles of miRNAs in promoting differentiation have been reported in numerous tissues, including skin (Botchkareva, 2012; Lena et al., 2008; Ning and Andl, 2012; Yi et al., 2008), brain (Cheng et al., 2009; Zhao et al., 2009) and skeletal muscle (Chen et al., 2010). However, two important questions about the role of miRNAs in the differentiation of stem cells remain unanswered. First, it is not clear when miRNAs are activated during differentiation. A pertinent question is what is the role of miRNA-mediated regulation during the transition of stem cells towards their differentiating daughter cells? Our current study has provided an important insight by revealing the rapid activation of miR-203 during the epidermal differentiation. To visualize the expression patterns of miR-203 at single-cell resolution, we have developed a high resolution in situ hybridization technique. The ability to co-stain miR-203 together with skin lineage markers e.g. K5 and β4 integrin, and proliferation markers, e.g. BrdU and PH3, allows us to demonstrate the immediate activation of miR-203 in the differentiating daughter cell following the ACD of the epidermal progenitor cells. We also characterize the promoter of miR-203 and
provide evidence for the transcriptional activation of miR-203 during the differentiation. These observations argue that the activation of miR-203 is one of the early events after the ACD. To address the functional significance of the rapid induction of miR-203, we have generated a miR-203 inducible model that allows us to define the immediate impact of the activation of miR-203. We show that within 6 hours of the transcriptional activation of miR-203, the inducible cells begin to exit the cell cycle. This result demonstrates that miR-203 acts rapidly to promote cell cycle exit, a hallmark of epidermal differentiation. Finally, the inducible system also allows us to examine the impact of transient miR-203 expression on self-renewal. Interestingly, cultured keratinocytes are unable to re-gain their proliferative potential after a 72-hour exposure to miR-203. This suggests that miR-203 profoundly switches the balance towards the terminal differentiation. All together, these observations highlight the potent function of miR-203 in promoting the transition of stem cells towards the differentiation.

The second question we have addressed in this study is how many mRNAs are regulated by miR-203 and how does the regulation of individual targets contribute to the function of miR-203? With the inducible mouse model, we are able to examine the changes of the transcriptome 24 hours after the Dox injection that activates miR-203. This approach minimizes secondary effects by miR-203 on the transcriptome and detects the direct targets of miR-203. Indeed, among 424 genes whose expression is suppressed more than 18% (the level of suppression that is observed for the established miR-203 targets), 261 genes (61.6%) are likely to be bona fide targets containing the matches to the miR-203 seed sequences. Furthermore, by bioinformatic analyses, we demonstrate that these genes are significantly enriched for the regulators in the cell cycle, cell division and response to DNA damage, supporting the potent role of miR-203 in the regulation of self-renewal. To further dissect the underlying mechanism, we examine the impact on the cell cycle and long-term proliferation by a group of miR-203 targets either individually or combinatorially. Interestingly, each target only partially recovers the proliferation in the colony formation assay, indicating the functional requirement of coordinated suppression of these targets by miR-203. Indeed, the best proliferation is observed when Skp2, p63 and Msi2 are delivered to the miR-203-inducible cells simultaneously. Taken together, these results argue that the rapid and widespread regulation of the self-renewal by miR-203 is mediated by a large number of targets involved in several key downstream pathways.

Finally, our study has yielded a large collection of miR-203 in vivo targets. We note that we have identified most, if not all, previously published miR-203 targets, including p63 (Lena et al., 2008; Yi et al., 2008), Bmi1 (Wellner et al., 2009) and Cav1 (Ørom et al., 2012). In addition, we identify many novel targets that are involved in the regulation of the cell cycle and self-renewal of stem cells. Among them, Skp2 is implicated important roles in tumor development (Chen et al., 2012; Lin et al., 2010); Msi2, a RNA-binding protein, is shown to regulate the self-renewal of hematopoietic stem cells and leukemia cells (Hope et al., 2010; Ito et al., 2010; Kharas et al., 2010). Given the widespread inhibition of these genes by miR-203, our results thus establish a molecular basis to dissect the potential tumor suppressor role of miR-203 in the future.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References

miR-203 inhibits self-renewal


