A Comprehensive Analysis of MicroRNA Expression During Human Keratinocyte Differentiation *In Vitro* and *In Vivo*

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Here, we report a comprehensive investigation of changes in microRNA (miRNA) expression profiles on human keratinocyte (HK) differentiation *in vitro* and *in vivo*. We have monitored expression patterns of 377 miRNAs during calcium-induced differentiation of primary HKs, and have compared these patterns with miRNA expression profiles of epidermal stem cells, transient amplifying cells, and terminally differentiated HKs from human skin. Apart from the previously described miR-203, we found an additional nine miRNAs (miR-23b, miR-95, miR-210, miR-224, miR-26a, miR-200a, miR-27b, miR-328, and miR-376a) that are associated with HK differentiation *in vitro* and *in vivo*. *In situ* hybridization experiments confirmed miR-23b as a marker of HK differentiation *in vivo*. Additionally, gene ontology analysis and functional validation of predicted miRNA targets using 3′-untranslated region–luciferase assays suggest that multiple miRNAs that are upregulated on HK differentiation cooperate to regulate gene expression during skin development. Our results thus provide the basis for further analysis of miRNA functions during epidermal differentiation.

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INTRODUCTION

The human epidermis continuously renews itself by a process of proliferation and differentiation. Basal epidermal stem cells generate transient amplifying cells, which move outward from the basal membrane, migrate through the epidermis, and undergo terminal differentiation (Fuchs, 2008; Blanpain and Fuchs, 2009). MicroRNAs (miRNAs) are thought to have an important role during this process, as epithelium-specific depletion of miRNA biogenesis by genetic disruption of *dicer* or *dgcr8* genes lead to barrier defects, abnormal hair follicle development, and hyperproliferation of basal interfollicular keratinocytes (KCs) (Andl *et al.*, 2006; Yi *et al.*, 2006, 2009). To date, global miRNA expression analysis during skin differentiation has been restricted to murine models, in which several studies suggest the existence of at least 70 highly expressed miRNAs (Andl *et al.*, 2006; Yi *et al.*, 2006, 2008, 2009). The miR-203 was identified as a skin-specific miRNA expressed in differentiated murine and human epidermal KCs (Sonkoly *et al.*, 2007; Yi *et al.*, 2008). By regulating p63 protein levels, miR-203 controls the switch from basal progenitor skin cells to suprabasal differentiated cell types (Lena *et al.*, 2008; Yi *et al.*, 2008).

Except for miR-203, the knowledge about miRNAs expressed during human skin differentiation is poor. Here, we report a comprehensive approach to analyze the expression of human skin miRNAs and their regulation during KC differentiation *in vitro* and *in vivo*. We have studied the expression patterns of 377 miRNAs during calcium-induced differentiation of primary human KCs (HKs) *in vitro* and compared these patterns with miRNA expression profiles of epidermal stem cells and transient amplifying and terminally differentiated KCs isolated from human skin to analyze changes in miRNA expression during KC differentiation *in vivo*. We identified 10 miRNAs associated with HK differentiation *in vitro* and *in vivo*, confirming the function of miR-203 and identifying miR-23b as a differentiation marker for HKs *in vivo*. Gene enrichment analysis of predicted miRNA targets and functional analysis using 3′-untranslated region (UTR)–luciferase assays suggest that multiple miRNAs cooperate to regulate gene expression in HKs.

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Abbreviations: Ct, cycle threshold; EDNRA, endothelin A receptor; HK, human keratinocyte; EYA4, eyes absent homolog 4; GDAP1, ganglioside-induced differentiation-associated protein 1; KC, keratinocyte; miRNA, microRNA; NA, nonadherent; NHEK, neonatal normal human epidermal keratinocytes; RA, rapid adherent; RT-PCR, real-time PCR; UTR, untranslated region

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RESULTS

In vitro differentiation of KCs and isolation of differentiation stage-specific human skin KC populations

Calcium-induced KC differentiation is a widely used model system to study skin differentiation processes *in vitro*. Although being robust and relatively easy to carry out, similar to all *in vitro* models, this system is also bound to produce artifacts. We therefore followed a dual approach to stringently screen for miRNAs associated with human skin differentiation: first, to analyze KC differentiation *in vitro,* we monitored miRNA expression in calcium-treated neonatal normal human epidermal KCs (NHEK). Second, to investigate changes *in vivo,* we performed differentiation stage-specific isolation of KCs from human skin and subjected the resulting populations to miRNA profiling. By comparing the results from both model systems, we should be able to identify those miRNAs that are most likely to be of functional importance for the differentiation of HKs.

For our *in vitro* studies, we induced differentiation of NHEK cells by increasing calcium concentration in the medium to a concentration of 1 mM. A total of six independent experiments were performed, and samples were collected after 3 and 7 days of calcium treatment. In each experiment, successful induction of the differentiation process was verified using quantitative real-time PCR (RT-PCR) analysis of the differentiation markers keratin 14 and keratin 10 (Supplementary Figure S1A online). In addition to the expected keratin expression patterns, the cultures showed a marked reduction in proliferation activity, culminating in a near complete growth arrest at day 7 post-treatment (Supplementary Figure S2 online). Thus, all markers indicated the induction of differentiation in the calcium-treated cultures.

To study miRNA expression patterns during KC differentiation in human skin, we isolated cell fractions according to the *in vivo* differentiation gradient in this tissue. We employed collagen IV adherence assays to achieve separation of the cell populations. The method makes use of differences in the ability of the cells to adhere to collagen-coated solid surfaces: the fraction that is able to adhere within minutes (accordingly termed rapidly adhering (RA) cells) is highly enriched in epidermal stem cells. In contrast, cells that are devoted to differentiation but are still proliferation competent (transient amplifying cells) can be isolated on culturing of the nonadherent (NA) fraction. The cultivation procedure eliminates proliferation-incompetent, terminally differentiated KCs that otherwise account for $\sim$98% of the original cell suspension; the total cell fraction is therefore used as a proxy for differentiated KCs (Jones and Watt, 1993).

A total of five human skin biopsies from different donors were subjected to the above procedure, and the efficiency of cell separation was monitored by quantitative RT-PCR expression analysis of marker genes indicative of differentiation (keratin 5/14 and keratin 1/10) or epidermal stemness (integrins and epidermal stem cell markers). Compared with the RA (that is, the stem cell) fraction, the transient amplifying NA cells and the differentiated total fraction expressed all investigated marker genes in the expected manner (see Supplementary Figure S1B–C online for details). As all investigated RA cell fractions exhibited a higher clonogenic potential compared with corresponding NA cells in colony formation efficiency assays (Supplementary Figure S3 online), we conclude that our separation procedure was successful, allowing us to isolate representative epidermal stem, transient amplifying, and terminally differentiated cell populations for our further analysis.

miRNA profiling of undifferentiated and differentiated HKs

The samples described above were used to study the expression of 377 miRNAs during human epidermal differentiation using TaqMan low-density arrays (Applied Biosystems, Foster City, CA). A total of 33 samples were analyzed (samples collected at days 0, 3, and 7 from six individual *in vitro* differentiation assays, as well as RA, NA, and total fractions from the five skin biopsies). In both differentiation models, hierarchical clustering revealed highly distinct miRNA expression profiles (Supplementary Figure S4 online), indicating that miRNAs can serve as differentiation markers of HKs *in vitro* and *in vivo*.

Identification of miRNAs linked to calcium-induced differentiation of NHEKs *in vitro*

We used significance analysis of microarrays to identify miRNAs that are induced or repressed during KC differentiation. To qualify as a putative differentiation-associated miRNA in our *in vitro* assays, a miRNA had to (i) show at least twofold change of expression when compared with untreated NHEK cells and (ii) had to be expressed at significant levels in at least one of the end points of the analysis, as indicated by a cycle threshold (*C*t) value below 30 in at least five out of six independent experiments. According to these criteria, we identified 13 upregulated and 1 downregulated miRNA (miR-376a) after 3 days of calcium treatment (Table 1). Among the five miRNAs that showed a more than threefold upregulation during this early time point of calcium-induced differentiation, miR-203 revealed the strongest increase. The number of differentially expressed miRNAs further increased to 55 upregulated and 8 downregulated miRNAs after 7 days of cultivation under high calcium conditions (Table 1). All miRNAs that had been identified as differentially expressed at 3 days of calcium treatment also registered after 7 days. Furthermore, for all of these miRNAs, a consistently higher degree of induction or repression, respectively, was observed at the later time point.

Identification of miRNAs linked to KC differentiation in human skin

We analyzed RA, NA, and total fractions from human skin in an analogous manner, as described above (at least twofold expression change, *C*t values <30 in at least four of the five total samples), to detect miRNAs that are significantly regulated during skin differentiation. First, we compared the miRNA expression profiles of the stem cell-containing RA cell fractions with those of the NA cell fractions (that is, the transient amplifying cells). We identified seven miRNAs as being significantly upregulated in transient amplifying cells, whereas no miRNAs were repressed (Table 2). When comparing miRNA expression of the total cell fractions,

Table 1. miRNAs found to be up- or downregulated during calcium-induced differentiation of NHEKs 3 and 7 days after calcium treatment

miRNA	Average fold change[1]	*q*-Value (%)[2]	miRNA	Average fold change[1]	*q*-Value (%)[2]
miRNAs regulated 3 days after calcium treatment					
miR-203	13.38	<0.01	miR-375	2.52	<0.01
miR-95	5.96	<0.01	miR-34a	2.27	<0.01
miR-210	3.94	<0.01	miR-98	2.26	2.42
miR-34c-5p	3.13	<0.01	miR-200a	2.08	<0.01
miR-132	3	<0.01	miR-26a	2.02	<0.01
miR-328	2.76	<0.01	miR-27b	2	0.62
miR-224	2.65	<0.01	miR-376a	0.35	<0.01
miRNAs regulated 7 days after calcium treatment					
miR-203	68.75	<0.01	miR-500	3.03	<0.01
miR-95	20.35	<0.01	miR-328	2.98	<0.01
miR-212	19.09	1.51	miR-491-5p	2.8	<0.01
miR-145	13.72	<0.01	let-7b	2.68	<0.01
miR-224	12.62	<0.01	miR-26b	2.66	<0.01
miR-23b	12.45	0.72	miR-362-5p	2.6	<0.01
miR-132	12.2	<0.01	miR-193b	2.57	<0.01
miR-210	10.49	<0.01	miR-342-3p	2.5	<0.01
miR-194	8.05	<0.01	let-7g	2.49	<0.01
miR-146b-5p	7.66	<0.01	miR-365	2.4	<0.01
miR-505	6.8	0.38	miR-183	2.31	<0.01
miR-375	6.76	<0.01	let-7a	2.31	<0.01
miR-660	5.82	<0.01	miR-429	2.27	<0.01
miR-192	5.45	<0.01	miR-27b	2.21	<0.01
miR-141	4.82	<0.01	let-7e	2.2	<0.01
miR-21	4.6	<0.01	let-7d	2.18	<0.01
miR-34a	4.54	<0.01	miR-195	2.18	<0.01
miR-886-3p	4.45	<0.01	miR-98	2.16	<0.01
miR-34c-5p	4.33	<0.01	miR-455-5p	2.15	<0.01
miR-181c	4.07	<0.01	miR-31	2.13	<0.01
miR-532-5p	4.07	<0.01	let-7f	2.1	<0.01
miR-22	3.69	<0.01	miR-152	2.07	<0.01
miR-200a	3.61	<0.01	miR-425	2.06	<0.01
miR-452	3.61	<0.01	miR-221	0.42	0.42
miR-181a	3.6	<0.01	miR-345	0.41	<0.01
miR-200b	3.58	<0.01	miR-127-3p	0.39	<0.01
let-7c	3.58	<0.01	miR-671-3p	0.38	<0.01
miR-26a	3.45	<0.01	miR-18a	0.33	<0.01
miR-532-3p	3.41	<0.01	miR-339-5p	0.23	0.85
miR-301a	3.38	<0.01	miR-503	0.14	2.71
miR-193a-5p	3.16	<0.01	miR-376a	0.11	<0.01
miR-182	3.05	<0.01			

Abbreviations: miRNA, microRNA; NHEK, neonatal normal human epidermal keratinocyte; SAM, significance analysis of microarrays.
[1]Average fold change values are given relative to undifferentiated NHEK cultures.
[2]q-Value according to significance of differential expression (SAM) analysis (see Materials and Methods for details).
MicroRNAs below the separating lines were downregulated.

Table 2. miRNAs up- or downregulated in transient amplifying (nonadherent) and terminally differentiated keratinocytes (total cell fractions) of human skin

miRNA	Average fold change[1]	*q*-Value (%)[2]	miRNA	Average fold change[1]	*q*-Value (%)[2]
miRNAs regulated in nonadherent cell fractions					
miR-218	4.58	9.24	miR-203	2.7	7.56
miR-138	4.26	9.24	miR-27a	2.06	7.56
miR-424	3.67	7.56	miR-31	2.05	7.56
miR-135b	2.86	7.56			
miRNAs regulated in total cell fractions					
miR-223	95.83	2.93	miR-30c	3.16	3.24
miR-508-3p	38.12	2.37	miR-342-3p	3.15	<0.01
miR-9	32.93	5.29	miR-193b	2.95	3.11
miR-146a	32.13	2.68	miR-16	2.82	3.03
miR-95	31.34	<0.01	miR-152	2.76	4.02
miR-203	26.43	2.68	miR-335	2.75	2.68
miR-181c	25.12	2.93	let-7d	2.68	4.76
miR-210	21.66	<0.01	miR-429	2.67	3.11
miR-150	20.16	<0.01	miR-30b	2.66	2.89
miR-23b	18.57	3.81	miR-200b	2.63	2.68
miR-517a	13.23	5.05	miR-224	2.56	4.76
miR-146b-5p	12.63	2.68	miR-324-3p	2.34	2.73
let-7c	11.36	2.68	let-7f	2.33	3.24
miR-340	8.46	2.68	miR-574-3p	2.32	2.6
miR-27b	7.9	2.68	miR-455-3p	2.3	5.29
miR-181a	7.51	2.8	miR-222	2.2	2.89
miR-328	7.41	2.68	miR-345	2.15	2.5
miR-141	7.17	3.24	miR-500	2.13	5.92
miR-26a	7.04	2.5	miR-140-5p	2.11	5.29
miR-101	6.98	2.93	miR-27a	2.07	7.14
miR-149	6.95	<0.01	miR-183	0.5	<0.01
miR-195	6.8	2.9	miR-99b	0.49	3.24
miR-26b	6.41	2.89	miR-125a-5p	0.41	<0.01
miR-361-5p	6.23	5.09	miR-330-3p	0.36	2.37
let-7a	5.17	2.8	miR-130b	0.31	<0.01
miR-491-5p	4.71	<0.01	miR-218	0.25	<0.01
miR-125b	4.25	2.37	miR-886-3p	0.21	<0.01
miR-15a	4.01	2.37	miR-135b	0.13	3.03
miR-200a	3.94	2.68	miR-18a	0.11	<0.01
miR-455-5p	3.89	5.83	miR-485-3p	0.11	<0.01
miR-148a	3.84	4.76	miR-376c	0.06	2.46
let-7b	3.46	2.37	miR-127-3p	0.06	2.68
miR-452	3.46	2.12	miR-31	0.04	<0.01
let-7g	3.43	3.03	miR-376a	0.04	3.24
miR-29c	3.29	3.11	miR-886-5p	0.03	<0.01
miR-660	3.24	2.93	miR-424	0.01	3.38
let-7e	3.23	4.76			

Abbreviations: miRNA, microRNA; SAM, significance analysis of microarrays.
[1]Average fold change values are given relative to rapidly adhering epidermal stem cell fractions.
[2]*q*-Value according to significance of differential expression (SAM) analysis (see Materials and Methods for details).
MicroRNAs below the separating line were downregulated.

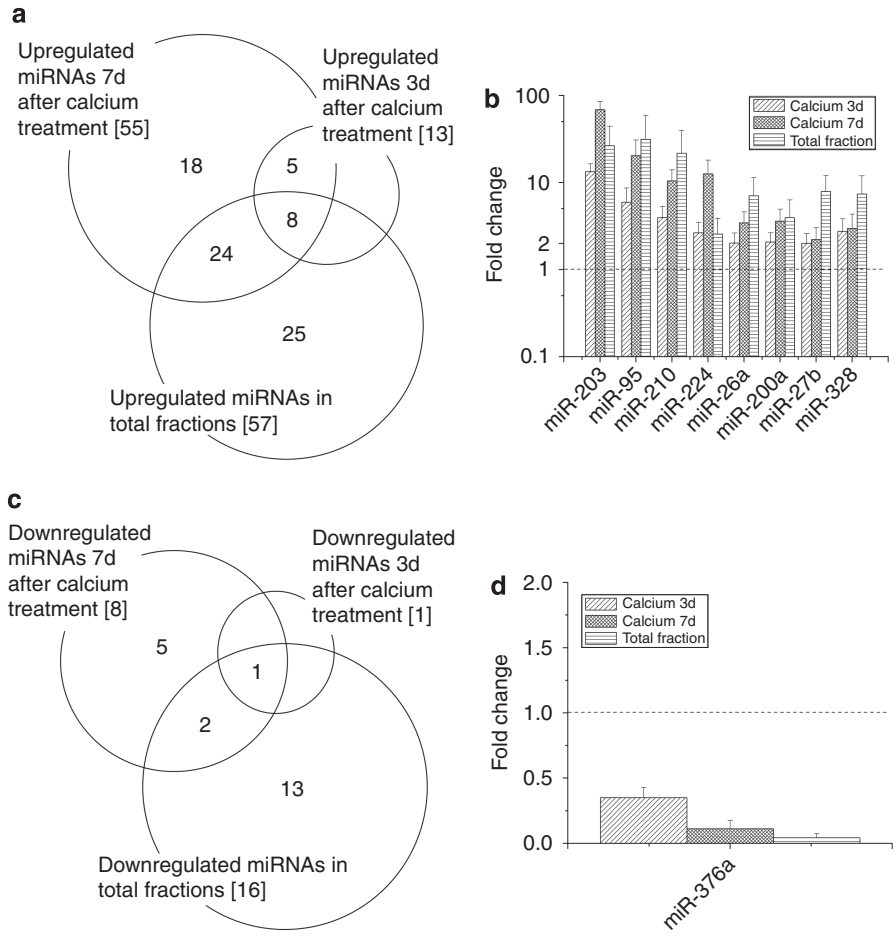

Figure 1. MicroRNAs (miRNAs) commonly up- or downregulated during keratinocyte (KC) differentiation *in vitro* or *in vivo*. (**a, c**) Venn diagrams of up- (**a**) and downregulated (**c**) miRNAs in neonatal normal human epidermal KCs (NHEKs) at 3 days (3d) and 7 days (7d) after calcium-induced differentiation, and in terminally differentiated skin KCs (total fractions). The total number of miRNAs subject to significant expression changes (see Tables 1 and 2 for details) in each entity is shown in square brackets. (**b, d**) Expression of commonly up- (**b**) or downregulated (**d**) miRNAs in calcium-treated relative to undifferentiated NHEK cells (set to 1), or in terminally differentiated (total fraction) KCs relative to epidermal stem cell fractions from skin. Expression was measured using TaqMan low-density arrays and data were normalized to U6 small nuclear RNA levels. Error bars represent standard deviations between six independent calcium-induced differentiation experiments or between skin biopsies from five different donors.

mainly composed of terminally differentiated KCs, with the RA/stem cell fractions, a total of 57 upregulated and 16 repressed miRNAs were detected (Table 2). Thus, a total of 73 miRNAs undergo significant changes in their expression levels on terminal differentiation of KCs in human skin.

Identification of functionally important miRNA candidates during differentiation of KCs *in vitro* and in human skin

Our analysis of terminally differentiated skin KCs, that is, total cell fractions from human skin and NHEK cells after 7 days of calcium treatment, revealed large numbers of up- or downregulated miRNAs. Out of a total of 55 positively regulated miRNAs in NHEK cells and 57 in human skin KCs, 32 miRNAs (or roughly 60%) were upregulated in both entities (Figure 1a), suggesting a direct link between their enhanced expression and the differentiation process. Among the negatively regulated miRNAs, three were shared between the 8 and 16 downregulated miRNAs seen in fully differentiated NHEK and total skin fractions, respectively (Figure 1c). This overlapping set of regulated miRNAs is likely to include miRNAs that are functionally involved in KC differentiation, as well as miRNAs that change expression merely as a secondary result thereof. We reasoned that one criterion that sets apart the former from the latter is the time frame of expression changes, that is, functionally important miRNAs are likely to change their expression early during the differentiation process. Accordingly, we determined the set of miRNAs that are upregulated in differentiated skin KCs and NHEK cells after 7 days, as well as 3 days of differentiation induction. Besides miR-203, another seven miRNAs satisfied this criterion, suggesting that these miRNAs may cooperate during skin differentiation (Figure 1a and b). To further substantiate this hypothesis, we performed a bioinformatic screen for those mRNA transcripts that may be targeted by the full complement of above miRNAs. For this purpose, we used the latest implementation (v5.1) of the TargetScan algorithm (Grimson *et al.*, 2007; Friedman *et al.*, 2009) to predict targets of individual miRNAs, and for each of

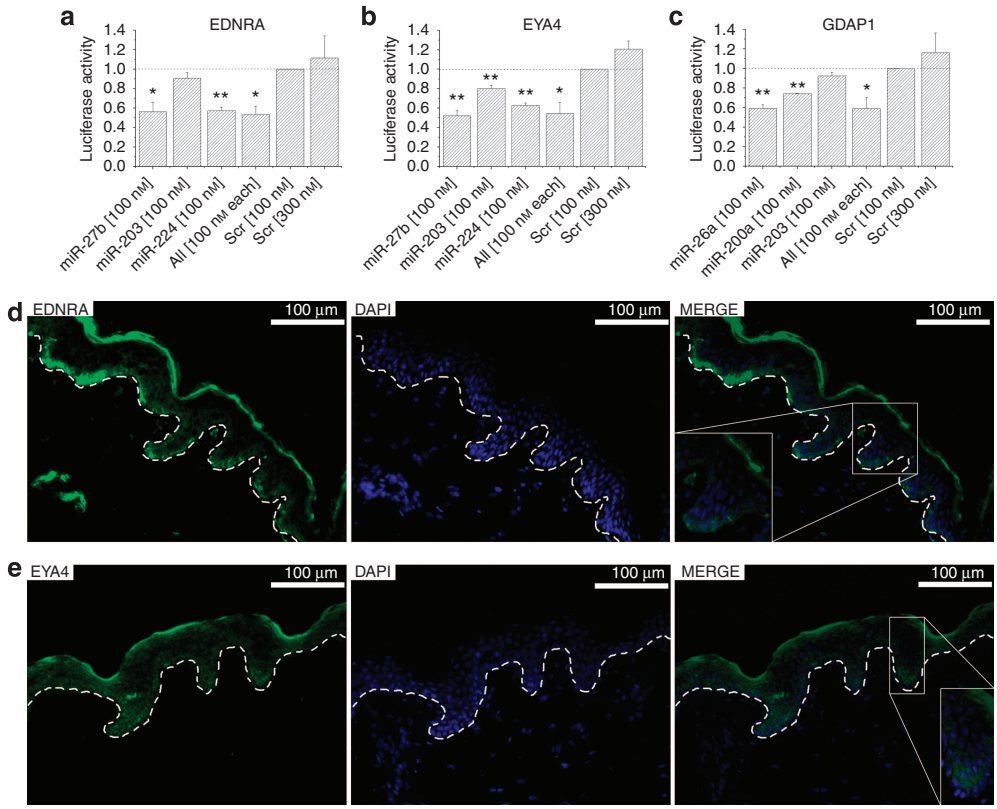

Figure 2. Functional validation of target genes regulated by multiple microRNAs (miRNAs). (**a–c**) 3′-Untranslated region (UTR)–luciferase assays revealing repression of the endothelin A receptor (EDNRA) (**a**), eyes absent homolog 4 (EYA4) (**b**), and ganglioside-induced differentiation-associated protein 1 (GDAP1) 3′-UTRs (**c**) by miRs-27b, -224, -26a, -200a, or -203. The miRNAs were transfected at a concentration of 100 nM each either individually (leftmost bars in each panel) or in combination (bars labeled ''all''). A scrambled miRNA mimic (Scr) served as a negative control. Repression is indicated as the percentage of luciferase activity seen with the negative control (100 nM). Error bars represent standard deviations between three independent experiments. $*P<0.05$ and $**P<0.01$ (*t*-test). (**d, e**) Immunofluorescence staining of human cryofixed skin sections. EDNRA (**d**) localizes to basal cells, and EYA4 (**e**) is predominantly detected in keratinocytes located in niches of papillae. Bar = 100 µm.

the obtained putative targets, calculated a cumulative score to predict the outcome of regulation by all miRNAs (see Supplementary Materials and Methods online for details). The complete list of predicted targets is given in Supplementary Dataset 1 online and includes many factors with possible roles in differentiation and KC biology among the top-scoring candidates (for example, Abelson-related gene (ABL2) (Klosner *et al.*, 2008), stem cell factor/kit ligand (SCF/KITLG) (Katayama *et al.*, 1995; Hussein, 2007), or cyclin-dependent kinase 6 (Ezhevsky *et al.*, 1997)). Strikingly, when we subjected all predicted targets with a score of at least −1 (corresponding to an expected negative regulation of at least twofold) to a gene annotation enrichment analysis (see Supplementary Materials and Methods online for details), the top-scoring annotation cluster was development- and cell differentiation related, with a highly significant enrichment score corresponding to a *P*-value of 1.8×10^{-6} (see Supplementary Dataset 2 online). Notably, this cluster was not observed when predictions made for the individual miRNAs (including miR-203) were analyzed, thus suggesting that multiple miRNAs contribute to gene regulation during KC differentiation.

For functional validation of this hypothesis, we used 3′-UTR–luciferase assays to analyze the miRNA-induced repression of the endothelin A receptor (EDNRA), the protein phosphatase eyes absent homolog 4 (EYA4), and the ganglioside-induced differentiation-associated protein 1 (GDAP1). The EDNRA and EYA4 transcripts harbor two predicted target sites for miR-27b and one site each for miRs-203 and -224. GDAP1 contains two sites for miR-203 and single sites for miRs-26a and -200a (Supplementary Figure S5 online). As shown in Figure 2a, both miR-27b and miR-224 mediated significant repression of the EDNRA reporter, whereas miR-203 had only a mild effect. The EYA4 3′-UTR was targeted by all three tested miRNAs (Figure 2b), and GDAP1 was repressed by both miRs-26a and -200a, but not by miR-203 (Figure 2c). These data show that our bioinformatic analysis was able to identify functional targets of multiple miRNAs that may cooperate during skin differentiation. Interestingly, at least under the experimental conditions used here, we did not observe additive effects when all three miRNAs were cotransfected (rather, the resulting repression was equivalent to that of the most potent miRNA). Although we have not titrated the amounts of transfected miRNAs, it seems possible

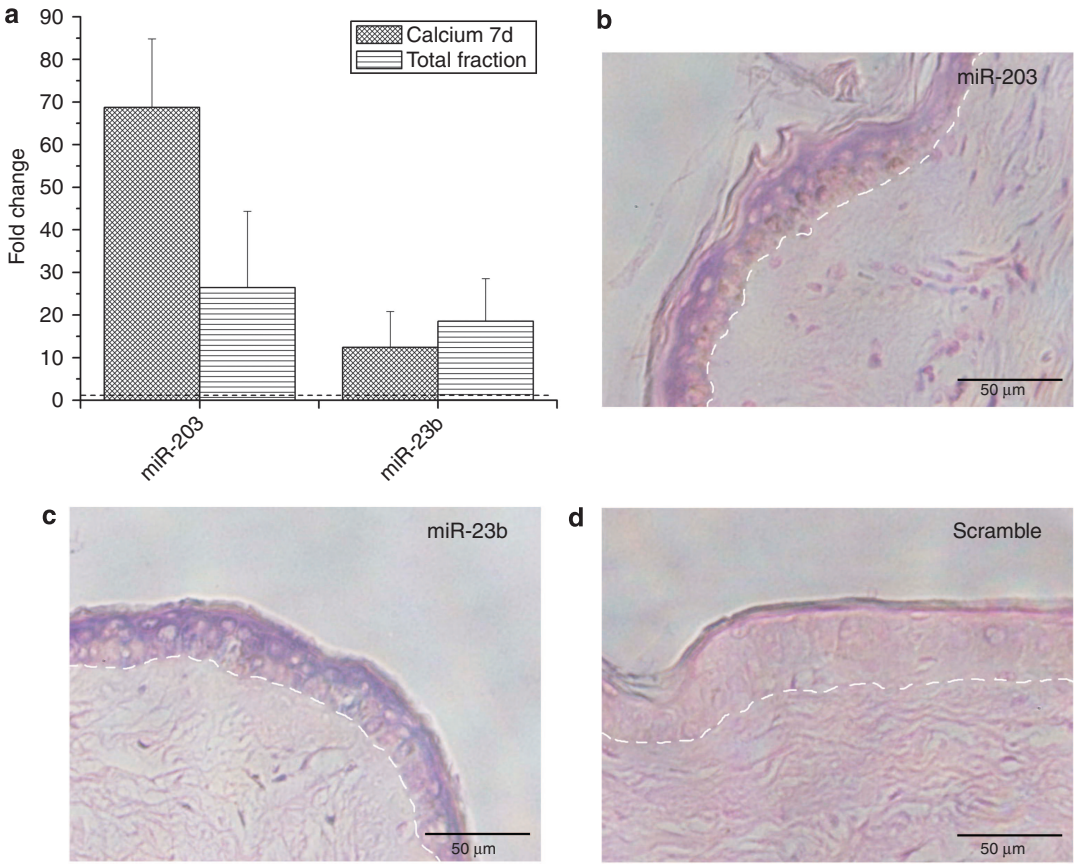

Figure 3. MicroRNA-203 (miR-203) and miR-23b are differentiation markers of human skin keratinocytes (KCs) *in vivo*. (**a**) Expression of miR-203 and -23b in calcium-treated neonatal normal human epidermal KCs (NHEKs) relative to undifferentiated NHEK cells, and in terminally differentiated (total fraction) KCs relative to epidermal stem cell fractions from human skin. Error bars represent standard deviations between six independent calcium-induced differentiation experiments or skin biopsies from five different donors, respectively. (**b, c**) Digoxigenin-labeled LNA-modified nucleotide probes were used to detect miR-203 (**b**) and miR-23b (**c**) transcripts in human skin by *in situ* hybridization. A scrambled probe (**d**) was used as a control. Skin sections were treated with anti-digoxigenin–alkaline phosphatase conjugates to detect miRNA expression (blue) and counterstained with nuclear fast red. Representative pictures are shown. Bar = 50 μm. 7d, 7 days.

that additive effects may be more pronounced at lower miRNA levels.

EDNRA and EYA4 are predominantly expressed in basal KCs in human skin

As the EDNRA and EYA4 3′-UTRs can be targeted by differentiation-associated miRNAs, we sought to investigate the expression levels of these proteins in human skin. As shown in Figures 2d and e, immunofluorescence staining of skin sections revealed that expression of both proteins indeed was mainly restricted to basal KCs. EDNRA was expressed in all basal cells, whereas EYA4 was especially abundant in those KCs located in niches of papillae. Hence, the observed expression patterns of EDNRA and EYA4 are in line with our hypothesis that these genes represent targets of multiple miRNAs that are upregulated during skin differentiation.

In situ hybridization of miR-203 and miR-23b identifies miR-23b as a differentiation marker of HKs

Along with miR-203, miR-23b was the most stringently upregulated miRNA in differentiated KCs after 7 days of calcium treatment and in total cell fractions from human skin (Figure 3a). Furthermore, both miRNAs were amplified at low *C*t values in the total cell fractions of human skin (Supplementary Dataset 3 online; note that miR-95b is more strongly upregulated than mir-23b and is also ~10-fold less abundant). We therefore performed *in situ* hybridization with LNA-modified nucleotide probes to monitor expression of miR-203 and miR-23b in human skin sections from three different donors. In agreement with earlier *in situ* hybridization experiments (Sonkoly *et al.*, 2007; Yi *et al.*, 2008), we detected miR-203 transcripts predominantly in suprabasal cell layers of human skin (Figure 3b). Our investigation furthermore revealed near-exclusive expression of miR-23b transcripts in suprabasal cells (Figure 3c), thus identifying miR-23b as a differentiation marker for human skin KCs *in vivo*.

DISCUSSION

Although miR-203 has been reported to have a crucial role during KC differentiation (Sonkoly *et al.*, 2007; Lena *et al.*, 2008; Yi *et al.*, 2008; Yi and Fuchs, 2010), the knowledge

about global miRNA expression changes during human skin differentiation remains limited. We therefore used a systematic and carefully controlled approach to identify miRNAs involved in the process of HK differentiation *in vitro* and the previously unreported miRNA profiles of epidermal stem cells, transient amplifying cells, and terminally differentiated KCs isolated from human skin.

We were able to identify a total of 32 miRNAs that were upregulated and 3 miRNAs that were downregulated in KCs fully differentiated *in vitro* (that is, after 7 days of calcium treatment), as well as in terminally differentiated KCs isolated from skin. Besides miR-203, miR-23b was found as a second miRNA that was highly expressed in differentiated KCs. This observation was confirmed by performing *in situ* hybridization of both miRNAs on human skin sections, thus identifying miR-23b as a differentiation marker of human skin. Of note, another study has recently investigated miRNA expression changes in KCs (Sonkoly *et al.*, 2009) and identified eight miRNAs that were differentially regulated 48 hours after calcium treatment *in vitro*. In agreement with our data, miR-203 was the most stringently upregulated miRNA under high calcium conditions. However, we do find significantly more miRNAs that change expression during HK differentiation. This is likely to be due to technical and conceptual differences between the two studies: first, we used the latest generation of TaqMan low-density arrays (v2.0), allowing us to perform a preamplification step during miRNA–cDNA generation that increases specificity and sensitivity of the assay (Chen *et al.*, 2009). Second, whereas Sonkoly *et al.* investigated changes *in vitro* at a single time point after induction of differentiation, the miRNAs we report here represent a cross-section of miRNAs regulated during several time points *in vitro* as well as *in vivo*.

It is also noteworthy that the contingent of differentially expressed miRNAs in NA/transient amplifying skin cell fractions showed little overlap with any of the other samples investigated here (see Tables 1 and 2). Only miR-203 was shared between transient amplifying fractions and calcium-treated NHEK cells or terminally differentiated skin KCs. As the NA fraction represent a poorly defined entity, it is possible that our results are indicative of some inherent level of heterogeneity within this population, despite the consistent marker gene expression, as shown in Supplementary Figure S1B–D online. However, at least in part, the diversity may also be a consequence from the experimental procedure required to isolate these cells, as they need to be sub-cultured for a total of 14 days (see Materials and Methods for details) to eliminate terminally differentiated KCs. As the total and RA fractions are harvested after a maximum of 5 days, some of the heterogeneity may arise during the extended culturing period. It is thus unclear whether the profiles shown at the top of Table 2 are representative of the original cell population. However, we have included the results here for completeness.

In contrast, a total of eight miRNAs (including miR-203) were found not only to be induced in terminally differentiated KCs *in vitro* and *in vivo* but also already upregulated after 3 days of calcium treatment, suggesting that they were functionally involved in the differentiation process. This hypothesis is furthermore supported by a functional annotation analysis of the genes predicted to be commonly targeted by these miRNAs. The targets are highly enriched in genes involved in processes relevant for skin differentiation, with 'multicellular organismal development' and 'anatomical structure development' (*P*-value = 0.0000018) being the top-scoring functional terms (Supplementary Dataset 2 online). Importantly, this signature was not observed when the predicted target sets from the eight miRNAs were analyzed individually.

We were able to confirm that three predicted target transcripts (EDNRA, EYA4, and GDAP1) were subject to miRNA-mediated repression in 3′-UTR–luciferase assays. All transcripts were furthermore repressed by at least two different miRNAs, a finding that is in line with several recent studies that have reported regulation of single genes by multiple miRNAs (Jiang *et al.*, 2009; Wu *et al.*, 2010). The observation that EDNRA and EYA4 expression is largely restricted to basal KCs in human skin furthermore supports our *in vitro* findings. Of note, although EDNRA and GDAP1 harbor putative target sites for miR-203, only miRs-27b, -224, -26a, or -200a had a significant effect, underlining the notion that additional miRNAs besides miR-203 may be involved in the regulation of gene expression during skin differentiation. The regulation and expression patterns of EDNRA are of particular interest, given the established role of endothelin-1 is that of a potent positive regulator of KC proliferation (Tsuboi *et al.*, 1994; Bagnato *et al.*, 1995). Thus, although the miR-203-dependent ablation of p63 expression undoubtedly is of critical importance for the cell cycle exit of differentiated KCs (Yi *et al.*, 2008), downregulation of EDNRA via miRs-27b and -224 may be an additional factor that contributes to the negative regulation of KC proliferation.

Our analysis has created a starting point for the detailed investigation of the combined effects that the upregulation of skin differentiation-associated miRNAs may have. A number of questions remain to be answered. For example, miR-203 clearly is the most dramatically upregulated miRNA and also accumulates to significantly higher levels than the remaining miRNAs. Although it is difficult to judge absolute miRNA copy numbers on the basis of the comparison of *C*t values for the individual miRNAs with those for U6 small nuclear RNA, we estimate that a terminally differentiated cell contains ~400,000 copies of miR-203, whereas the numbers for the remaining miRNAs are, on average, two orders of magnitude lower (see Supplementary Table 1 online). To date, there is no consent on the minimum miRNA copy numbers that are required to elicit a biological effect, as this also depends on other factors such as target transcript abundance and site efficacy. In differentiated KCs, however, we observed detectable signals for miR-26, miR-27, and miR-203 family members on northern blots, indicating that they should be sufficiently abundant to be of functional importance (Supplementary Figure S6 online). Ultimately, however, this issue will have to be investigated in experimental systems employing miRNA inhibitors or reporters expressed at authentic levels in KCs undergoing active differentiation.

Similarly, despite of the results of our functional annotation analysis, it is to be expected that the predicted target genes listed in Supplementary Dataset 1 online will contain a significant number of false positives. We are currently performing unbiased high-throughput screens to experimentally identify transcripts that are downregulated and/or associated with miRNAs during KC differentiation. In combination with the data presented here, these studies will help to identify functionally important targets of miR-203, as well as other miRNAs that may cooperate to regulate gene expression during human skin development.

MATERIALS AND METHODS

Samples were collected according to the Declaration of Helsinki Principles and institutional approval and written informed patient consents were obtained.

Cell culture and *in vitro* differentiation

NHEKs (Lonza, Basel, Switzerland) were used in passage two and cultured in EpiLife serum-free KC growth medium (Cascade Biologics, Portland, OR) containing EpiLife defined growth supplement (Cascade Biologics) at a final calcium concentration of 0.06 mM and GIBCO antibiotic–antimycotic (Invitrogen, San Diego, CA) at 37°C and 7% CO_2. Cells were trypsinized and sub-cultured when approaching 80% confluency. For *in vitro* differentiation, cells were seeded in six-well plates at a density of 10^5 cells per well and cultured under standard conditions to ~70% confluency. Differentiation was induced by increasing the calcium concentration of the growth medium to 1 mM (the cultures also become confluent over the ensuing days, which is another factor that contributes to differentiation). HEK293T cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (PAA Laboratories, Pasching, Austria).

Isolation of epidermal cell fractions from human skin biopsies

Human skin biopsies from five different healthy female donors were used to isolate epidermal stem, transient amplifying, and terminally differentiated cells by collagen IV adherence assays, as described previously (Jones and Watt, 1993). A description of the procedure is given in the Supplementary Information online.

RNA extraction and reverse transcription

Total RNA was prepared using the *mir*Vana miRNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to generate cDNA from total RNA in a RT-PCR reaction using 1 μg of total RNA.

Custom TaqMan RT-PCR low-density array

A TaqMan Custom Array (Applied Biosystems) containing 48 gene-specific reactions in a 384-well format was used to analyze the expression of epidermal stem cell and differentiation markers (for assay accession numbers see Supplementary Table S1 online). PCR reactions were carried out as recommended by the manufacturer, using 200 ng RT product for each loading port. Quantitative RT-PCR was performed using Applied Biosystems 7900HT Fast Real Time PCR system. *Ct* values were calculated by the RQ Manager software v.1.2 (Applied Biosystems, Foster City, CA). Target gene expression was normalized based on the expression values of 18S RNA, and fold change values were calculated using the comparative *Ct* method (Livak and Schmittgen, 2001).

miRNA profiling and data analysis

miRNA-specific cDNA were generated with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in combination with the Megaplex RT Primers Human Pool A (Applied Biosystems) according to the manufacturer's instructions. Data analysis was carried out using TIGR Multiexperiment viewer (v4.4, Dana-Farber Cancer Institute, Boston, MA). A detailed overview of the experimental procedure and data analysis is given in the Supplementary Information online.

3′-UTR–luciferase assays

HEK293T cells were transfected with 75 ng of 3′-UTR reporter plasmids (SwitchGear Genomics, Menlo Park, CA), 25 ng pMIR-REPORT beta-gal control vector (Ambion, Austin, TX), and 100 nM of each of the miRIDIAN miRNA mimics (Dharmacon, Lafayette, LA) or an equivalent amount of the miRIDIAN miRNA mimic negative control (scramble, Dharmacon). Luciferase activity was determined using the Luciferase assay system and β-galactosidase enzyme assay system (Promega, Madison, WI) as per the manufacturer's instructions and normalized to β-galactosidase activity to control for differences in transfection efficiency.

In situ hybridization and immunofluorescence staining of human skin

In situ hybridization experiments to detect miR-203 and miR-23b were performed in cooperation with Phylogeny, Columbus, OH. Skin tissues from three female donors were procured by Phylogeny. The 5′-digoxigenin-labeled miRCURY LNA detection probes for has-miR-203, has-miR-23b, and scrambled sequences (Exiqon, Vedbaek, Denmark) were prepared according to the manufacturer's recommended conditions. The *in situ* hybridization procedure was performed as previously reported (Nuovo, 2008). Sections were photographed using an Olympus IX71 microscope (Olympus, Hamburg, Germany). Immunofluorescence staining of human cryofixed skin sections was done using standard protocols and polyclonal rabbit antibodies against EDNRA (Abcam, Cambridge, UK) and EYA4 (Bethyl Laboratories, Montgomery, AL).

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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