miR-203 modulates epithelial differentiation of human embryonic stem cells towards epidermal stratification

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The molecular mechanisms controlling the differentiation of human basal keratinocyte stem cells towards the epidermis are well characterized, whereas the earliest process leading to the specification of embryonic stem cells into keratinocytes is still not well understood. MicroRNAs are regulators of many cellular events, but evidence for microRNA acting on the differentiation of human embryonic stem cells into a specific lineage has been elusive. By using our recent protocol for obtaining functional keratinocytes from hESC, we attempted to analyze the role of microRNAs in the early stages of epidermal differentiation. Thus, we identified a set of 5 microRNAs, namely miR-200a, miR-200b, miR-203, miR-205 and miR-429, that are specifically overexpressed during the early stages of the differentiation process. Interestingly, our functional analyses revealed an instrumental role of miR-203, which had been previously shown to play a key role during the formation of the pluristratified epidermis by basal keratinocyte stem cells, in the early keratinocyte commitment. These results highlight the determinant and unique role of miR-203 during the entire process of epidermal development by extending its spectrum of action from the early commitment of embryonic stem cells to ultimate differentiation of the organ.

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Introduction

Formation of the epidermis during ontogeny involves two major developmental steps: first the differentiation of keratinocyte stem cells from primitive ectodermal precursors and second the establishment of the pluristratified epithelium by a combined process of proliferation of those keratinocyte stem cells and migration of their progressively maturing post-mitotic progeny.

The molecular mechanisms underlying the second step of this process are readily available in adults, including humans, because post-mitotic keratinocytes are continuously produced, and all strata of epidermis above the basal layer are completely replaced about every 4 weeks (Blanpain and Fuchs, 2009). A major role in those phenomena has been attributed to the transcription factor p63, a member of the p53 oncogene family (Truong et al., 2006). Keratinocyte differentiation and epidermal stratification may be regulated by p63 via the transactivation of the Notch ligand JAGGED-1 (Okuyama et al., 2008) and the control of IKKα (IκB kinase-α) expression (Candi et al., 2006). Recently, miRNAs have also been implicated in the regulation of mouse skin development (Yi et al., 2006). Thus, miR-203 has been specifically identified in keratinocytes (Sonkoly et al., 2007) and its role in skin morphogenesis has been emphasized by its repressive action on p63 expression (Lena et al., 2008; Yi et al., 2008).

Molecular mechanisms of the earlier developmental stages leading to keratinocyte stem cells specification have remained more elusive (Aberdam et al., 2007). In the mouse, a role for p63 has been identified because the knock out of this gene results in the total absence of epidermis and epithelial appendages (Mills et al., 1999). In mouse embryonic stem cells BMP-4 treatment has also been shown, to induce both an ectodermal fate and a specific isoform of p63, DeltaNp63, which is required for the transition from epithelial progenitor cells to keratinocytes (Lena et al., 2008). However, in humans nothing is known except that ectodermal dysplasia syndromes can be correlated to mutations of the p63 gene (Trp63) (Rinne et al., 2007).

Human embryonic stem cells (hESC) are being increasingly used to model in vitro human embryogenesis since they are pluripotent, amenable to differentiation along any cell lineage, and their unlimited self renewal allows procurement of any required quantity of cells. Recent studies have, for example, provided important clues on the role of various families of transcription factors in neural induction (Chambers et al., 2009) or in the progression toward the hematopoietic lineage (Navarro et al., 2009). By taking advantage of our recent development of a protocol to derive fully functional keratinocyte stem...
cells from human embryonic stem cells (Guenou et al., 2009) – i.e. capable of forming a pluristratified epidermis – we then explored the molecular mechanisms involved in the earlier stages of their differentiation more precisely by focusing more particularly on the role of p63 and miRNAs. In this study, we demonstrate that the p63-regulator miR-203 is a key actor not only in the final differentiation of the pluristratified epidermis, but also in the ectodermal to keratinocyte transition during human skin embryogenesis. This extends the spectrum of action of these miRNAs at all the stages of epidermal specification.

Materials and methods

hESC culture

The human embryonic stem cell lines H9 (WA09, WiCell Research Institute) and SA01 (Cellartis, Sweden) were used in this study. Cells were maintained on a feeder layer of mitomycin C-inactivated murine STO (Sim’s Thioguanine Ouabaine Resistant) fibroblasts in Knock-Out (KO)-DMEM supplemented with 20% KO Serum Replacement (KSR, Invitrogen, Cergy, France), 1 mM L-glutamine, 0.1% penicillin-streptomycin, 1% non-essential amino acids (NEAA) and 10 ng/ml FGF2 (all from Invitrogen). The culture medium was changed by half daily, and supplemented with 10 ng/ml FGF2. For cellular expansion, cells were manually cut and placed into new dishes every 5 to 6 days at a 1:4 ratio.

hESC differentiation into keratinocytes

To engage hESC toward epithelial lineage, clumps were seeded onto mitomycin C-treated 3T3 fibroblasts in FAD medium as previously described by Guenou et al. (2009). The induction of ectodermal differentiation was induced by the treatment with 0.5 nM of human recombinant BMP-4 (R&D Systems Europe, UK) and 0.3 mM ascorbic acid (Sigma-Aldrich, St Louis, USA). Cells were grown in the same medium until clones of epithelial cells were isolated. The latter were then seeded into the same feeder layer in FAD medium devoid of BMP4 and ascorbic acid. As a control, primary human keratinocytes (HK) were grown on mitomycin C treated 3T3 fibroblasts in FAD medium.

hESC differentiation into MSCs

Mesenchymal differentiation was induced by plating 2 × 10^4 hESC/cm^2 on 0.1% gelatin-coated dishes in the presence of KO-DMEM medium supplemented with 20% Fetal Bovine Serum (FBS), 1 mM L-glutamine, 1% non-essential amino-acid, 1% penicillin-streptomycin and 0.1 mM β-mercaptoethanol (all from Invitrogen) as adapted from the literature (Mateizel et al., 2008).

hESC differentiation into NPCs

Neural progenitor cells derived from hESC (NPC-hESC) were obtained using a protocol adapted from the literature (Perrier et al., 2004). hESC cells were dissociated from STO by manual dissociation and plated in a density of approximately 10^5 cells/cm^2 on a confluent layer of mitotically inactivated murine stromal feeder (MS5). Cells were cultured in KSR medium (Knock-out Serum Replacement, 15% KSR; 1% Glutamax; 1% NEAA and 0.1% BM, (all from Invitrogen) for 14 to 16 DIV (days in vitro), replaced by Neurobasal medium, N2 (DMEM-F12 + Glutamax, 1% N2 supplement and 1% P/S) until DIV21. Cells were harvested at DIV21 using TrypLE Express (Invitrogen) and approximately 5.10^6 cells were suspended in PBS 2% Fetal Calf Serum containing 1% of 7-amino-actinomycin D (7AAD) from Sigma and then incubated with IgG1x Direct conjugated Phyco-erythrin (PE) monoclonal anti human Neural Cell Adhesion Molecules (HNCA M) antibody diluted 1/10 (BD Biosciences Pharmingen™, France). This antibody recognizes an extracellular immunoglobulin-like domain common to three molecular weight forms – Mr 120, 140 and 180 kDa – of the NCAM protein. Cell sorting was performed using a MoFlow Cell Sorter Cytometer from Cytomation, and alive positive and negative fractions were collected in 1 ml of N2 medium with 1% P/S.

Human keratinocytes culture

Human keratinocytes (HK) were isolated from human foreskin cultured on confluent feeder layers of lethally irradiated mouse J2-3T3 fibroblasts in keratinocyte FAD medium. Cells were passed each week using trypsinization and seeded at a density of 5000 cells/cm^2 into 100 mm petri dishes.

Epidermis reconstruction assay

Organotypic epidermis was generated as detailed elsewhere (Guenou et al., 2009; Poumay et al., 2004). Keratinocytes cultures were grown on polycarbonate culture inserts (Thermo scientific, Illkirch, France). These cells were maintained for 3, 6, 9, 12 or 15 days in Epilife medium (Invitrogen) supplemented with 1.5 mM CaCl2 and 50 μg/ml ascorbic acid. The cells were exposed to the air–liquid interface by removing the culture medium after 2 days for a period of 10 days.

Cell transfection

Cells were transfected by Lipofectamine RNAimax according to the manufacturer’s protocols (Invitrogen) using AntimiR or PremiR (Applied Biosystems, Courtaboeuf, France) at 100 nM each. The transfection medium was removed after 6 h and replaced with appropriate culture medium.

miRNAs extraction and qPCR

The total RNA was isolated from samples using RNAeasy extraction kits (Qiagen, Courtaboeuf, France) according to the manufacturer’s protocol. The purity and concentration of RNA were checked using Nanodrop technology (Agilent). Reverse transcription was performed using the Superscript III reverse transcription kit (Invitrogen). Real-time RT-PCR was performed using a LC480 real time system (Roche, Basel Switzerland) and SYBR Green PCR Master Mix (Roche) following the manufacturer’s instructions. Quantification of gene expression was based on the Ct (Cycle threshold) value calculated using LC1.5 software. Melting curve analysis and electrophoresis were performed to control PCR products specificities and exclude non-specific amplification. PCR Primers were designed using Primer3 software and are listed in Supplementary Table 3. Samples were normalized against 18S.

MicroRNAs extraction and TaqMan assay

MicroRNAs were extracted using a miRVana extraction kit (Applied Biosystems) according to the manufacturer’s protocol after phenol-chloroform and column purification. For cell sorting expression experiments, microRNAs were extracted using the RecoverAll extraction kit (Applied Biosystems).

Individual TaqMan microRNA assays were performed on ABI 7900 (Applied Biosystems) with a No AmpErase® UNG Master Mix (Applied Biosystems) according to the manufacturer’s protocol. Results were normalized against RNU 48 a small nuclear RNAs (snRNAs).
miR-371, miR-372 and miR-373 expression gradually decreased non-cellular (MSC-hESC) and neural progenitor cells- (NPC-hESC) derived hESC-derived-epithelial cells were compared to mesenchymal stem cells. MiR-203 expression during pluristratification, the distinction into biological clusters: a self-organizing map, miRNAs distributions clearly showed two highly distinguishable clusters: a first cluster associated to the pluripotent stage, miR-302, miR-367, and miR-429; and a second cluster associated to the epithelial lineage commitment specific markers, DeltaNp63 and keratin 14 (KRT14) (Fig. 1D). In order to demonstrate that miR-203 was induced in the earliest progenitor cells and not by only a few keratinocytes derived at this stage of differentiation, we confirmed that no K14 positive cells were present in culture by means of immunostaining and flow cytometry analysis. In contrast, more than 70% of the cells expressed K18 (Sup. Figs. 2A–C). K18 positive cells were purified by cell sorting and quantitative PCR analysis; this revealed an overexpression of miR-203 in those cells compared to undifferentiated hESC (Sup. Fig. 2D). Finally, to characterize the knockdown miR-203 effect on hESC differentiation towards the epithelial lineage, quantitative PCR analysis was performed to quantify key genes of other lineages (Fig. 4B). Results showed that the increase in keratinocyte markers (DeltaNp63, KRT5 and KRT14) in Anti-miR-203-treated-cells was associated with a decrease in self-renewal and pluripotency markers (OCT4 and NANOG), of a neural crest-specific marker (SOX10), and a neural-specific marker (PAX6) (Fig. 4C). Immunostaining for keratin 14 (Fig. 4D) confirmed its increase at the protein level after Anti-miR-203 treatment.

miR-203 expression during pluristratified epidermis formation

The expression of key markers (mRNAs and miRNAs) of the differentiation process was analyzed by quantitative PCR in order to compare the molecular events that occurred in the in vitro keratinocytes differentiation with in vivo human fetal skin development from 8 weeks (unstratified epidermis) to 33 weeks of development (fully functional epidermis). The stratification of keratinocytes was stimulated according to a published protocol (Poumay et al., 2004) with 2 days of culture in immersion phase followed by 13 days in emersion phase. Keratinocytes derived from hESC strictly followed the chronobiology of epidermis formation by first overexpressing markers of basal keratinocytes (such as DeltaNp63 KRT5 and KRT14), then markers of differentiated keratinocytes (such as keratin 10 (KRT10) and Involucrin (IVL)) (Fig. 5A) during the emersion phase. MiR-203 expression levels demonstrated a significant increase in pluristratified epidermis (Figs. 5B and C). These data suggested that miR-203 was a key regulator of human epidermis stratification; its functional role was evaluated using specific AntimiR and PremiR. Quantitative PCR analysis revealed that keratinocytes overexpressing miR-203 exhibited a significantly increased expression of KRT1 and deposited miRNAs expression profiles were compared in undifferentiated hESC, keratinocytes derived from hESC (K-hESC) and human adult keratinocytes (HK). In K-hESC, miRNA profiles appeared very similar to that observed in adult human keratinocytes (HK) (Fig. 3A), with a coefficient of linearity of 0.92, taking hESC as baseline (0.46) (Fig. 3B). Comparison of expression profiles showed miR-200a, miR200b, miR-203, miR-205 and miR-429 among the top 10 most upregulated miRNAs in both K-hESC and HK (Supplemental Table 1). Conversely, miR-302, miR-367, miR-371, miR-372 and miR-373 were undetectable in HK and K-hESC (Supplemental Table 2).

To investigate the role of these five miRNAs associated with epithelial commitment, overexpression or downregulation, experiments were carried out using specific AntimiRs and PremiRs on hESC treated by BMP4 during 8 days. After 3 days of treatment, only changes in the expression of miR-203 were accompanied by alteration in the cell phenotype. MiR-203 knockdown gave rise to an increased expression of DeltaNp63 and KRT14 (Fig. 4A), whereas no variations were detectable in the other four miRNAs. The effect of the anti-miR-203 treatment was also confirmed at the protein level by immunostaining for keratin 14 (K14) (Fig. 4D). In order to demonstrate that miR-203 was induced in the earliest progenitor cells and not by only a few keratinocytes derived at this stage of differentiation, we confirmed that no K14 positive cells were present in culture by means of immunostaining and flow cytometry analysis. In contrast, more than 70% of the cells expressed K18 (Sup. Figs. 2A–C). K18 positive cells were purified by cell sorting and quantitative PCR analysis; this revealed an overexpression of miR-203 in those cells compared to undifferentiated hESC (Sup. Fig. 2D). Finally, to characterize the knockdown miR-203 effect on hESC differentiation towards the epithelial lineage, quantitative PCR analysis was performed to quantify key genes of other lineages (Fig. 4B). Results showed that the increase in keratinocyte markers (DeltaNp63, KRT5 and KRT14) in Anti-miR-203-treated-cells was associated with a decrease in self-renewal and pluripotency markers (OCT4 and NANOG), of a neural crest-specific marker (SOX10), and a neural-specific marker (PAX6) (Fig. 4C). Immunostaining for keratin 14 (Fig. 4D) confirmed its increase at the protein level after Anti-miR-203 treatment.

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miR-203 strongly promoted the differentiation of basal keratinocyte stem cells into more mature phenotypes (Fig. 5D). This effect was correlated with the results of the reconstructed epidermis assay, which showed a reduction of the stratification for the epidermis generated from keratinocytes overexpressing miR-203 (Fig. 5E). In contrast, treatment of keratinocytes with the AntimiR-203 had no apparent effect on either the maturation of keratinocytes or the stratification of the epidermis (Figs. 5D and E).
miRNAs profiling of hESC engagement into epithelial lineage. (A) TaqMan array of 367 miRNAs during hESC differentiation into epithelial commitment at 0 (hESC), 3, 8 and 25 days of differentiation and into fully differentiated cell types such as keratinocytes (K-hESC), neural progenitor cells (NPC-hESC), and mesenchymal stem cells (MSC-hESC). Human keratinocytes (HK) are used as positive control of keratinocyte lineage. Data are expressed in Log2 Delta CT (Log2(DCT)) and were normalized to RNU48, and Array Assist 4.1 software (Stratagen) was used to achieve the clustering distribution following a “self organizing organization” and using a Euclidian distance metric. (B) Quantitative PCR analysis of hESC-specific miRNAs miR-302, miR-367, miR-371, miR-372 and miR-373. Results are expressed in relative expression to hESC. Data are normalized to RNU48 and are presented as a mean ± SD of 3 independent experiments. (C) Quantitative PCR analysis of miRNAs specifically increased in epithelial commitment: miR-200a, miR-200b, miR-203, miR-205 and miR-429. Results are expressed in comparison to the relative expression of hESC. Data are normalized to RNU48 and are presented as a mean ± SD of 3 independent experiments.
Discussion

The main result of this study is the demonstration that, in humans, one miRNA – miR-203 – plays a determinant role in the commitment of hESC to the keratinocyte lineage. Together with previous data showing that this miRNA is implicated in the formation of the pluristratified epidermis by basal keratinocyte stem cells, this result highlights the role of miR-203 as a major determinant for the epidermal development. This agrees with recently published results suggesting the key role of a small subset of tissue-specific miRNAs during development.

miR-203 controls the early commitment of ES cells to the keratinocyte lineage in the human

In order to decipher the molecular pathways involved in the earliest stages of keratinocyte commitment from human embryonic stem cells, we took the advantage of our recently published protocol, which leads to the production of a homogeneous population of basal keratinocyte stem cells. This protocol, based on the treatment of hESC with BMP4 and ascorbic acid, induces cells to mimic the succession of differentiation stages observed in the embryo over a period of 40 days. Thus, by recapitulating the entire chronobiology of ontogenesis, we were able to reconstitute a pluristratified ES-derived epidermis comparable to that produced by adult basal keratinocytes (Guenou et al., 2009). By analyzing the expression of miRNAs systematically, our results point to miR-203 as a candidate actor in this keratinocyte differentiation.

In a recent study in which mouse embryonic stem cells were guided toward epithelial commitment by means of the forced expression of p63, Lena et al. (2008) suggested that miR-203 may play such a key role because its expression increased parallel to the differentiation. By decreasing the expression of the miRNAs by exogenous means, such a key role of miR-203 is additionally observed here during the early stages of epidermal differentiation, because it does exert a potent regulatory function on the expression of key markers of keratinocyte differentiation, DeltaNp63 and keratin 14. MiR-203 and one of its direct targets, DeltaNp63, increase in parallel with the expression of markers of keratinocyte differentiation.
Fig. 4. Role of miR-203 in epithelial cells differentiation. (A) Quantitative PCR analysis of DeltaNp63 and KRT14 expression of 8 days differentiated hESC. Cells were transfected with PremiRs and AntimiRs specific of miR-203, miR-205, miR-200a, miR-200b and miR-429 and analyzed 3 days after transfection. Results are expressed in comparison to the relative expression of hESC. Data are normalized to 18S and are presented as a mean ± SD of 3 independent experiments. (B) Schematic representation of hESC fate choice during epithelial engagement induced by BMP4 stimulation. (C) Quantitative PCR analysis of the effect of miR-203 knockdown using AntimiR-203 on pluripotency markers OCT4 and NANOG, neural progenitor marker PAX6, neural crest progeny markers SOX10 and keratinocyte lineage markers DeltaNp63, KRT14 and KRT5 expression in hESC committed along epithelial lineage. Results are expressed in comparison to the relative expression of hESC. Data are normalized to 18S and are presented as a mean ± SD of 3 independent experiments. (D) Immunofluorescence of keratin 14 (K14) in 8-days-differentiated hESC derived from H9, transfected with the AntimiR-203, and fixed 3 days after treatment. Nuclear staining was done with DAPI (in blue). Scale bar is 100 μm.

to the course of hESC differentiation to keratinocyte. Based on this observation, one may hypothesize that miR-203 plays a dual role. On the one hand, at low DeltaNp63 levels, it may regulate epithelial commitment through other targets transitory more expressed in early epithelial cells. On the other hand, at high levels of DeltaNp63, miR-203 may act to repress “stemness”-related genes in keratinocytes. Powerful algorithms permit identification of several hundreds of potential targets for microRNAs. Through computational analysis, more than 400 targets were found to be putatively regulated by miR-203 (440 for PicTar http://pictar.mdc-berlin.de/ and 558 for TargetScan http://www.targetscan.org). Among them, only the direct target DeltaNp63 and the suppressors of cytokine signaling 3 (SOCS3) have been described as being implicated in the epidermis development and stratification directly to date (Lena et al., 2008; Wei et al., 2010). Some of the other putative targets such as the proto-oncogenes ABL1 (Bueno et al., 2008; Craig et al., 2011) and BCL (Bo et al., 2011) as well as a cohort of pro-metastatic genes including ZEB2 (Saini et al., 2010), Survivin, Runx2, and the polycomb repressor Bmi (Saini et al., 2010; Vitecchie et al., 2011) have been described in cancer cell lines. Accordingly, such an in vitro protocol of differentiation could provide a unique opportunity to study the functions of these genes in a developmental context or identify new targets for this microRNA. Quite interestingly, a similar effect on keratinocyte differentiation was not observed for 4 other miRNAs, namely miR-200a, miR-200b and miR-429 and miR-205 in humans, as it was in the mouse (Yi et al., 2006). For those miRNAs, exogenously induced expression failed to alter DeltaNp63 and keratin14. The functions of the other 4 miRNAs during this differentiation process are a matter of speculation at this stage. However, it may be interesting to mention that they have been associated with several different epithelial phenotypes (Sand et al., 2009) since this may reveal a less specific action on keratinocyte differentiation than for miR-203. Instead, a role of all three miRNAs during this differentiation process is a matter of speculation at this stage. However, it may be interesting to mention that they have been associated with several different epithelial phenotypes (Sand et al., 2009) since this may reveal a less specific action on keratinocyte differentiation than for miR-203. Instead, a role of all three miRNAs during this differentiation process is a matter of speculation at this stage.
signaling pathway by means of SHIP2 inhibition (Yu et al., 2008). The miR-200 family also regulates epithelial differentiation during Epithelial Mesenchymal Transition (Burk et al., 2008; Korpal et al., 2008). Altogether, these data suggest a role of miR-205 and the miR-200 family in the maintenance of an epithelial phenotype.

**miR-203 is a key actor at all stages of keratinocyte differentiation in humans**

With regard to the second step of the epidermal differentiation, i.e. the formation of a pluristratified epithelium, the results of this study with miR-205 are agree with those obtained in the mouse by Yi et al. (2006). During epidermal development in the mouse, miR-203 is specifically expressed in the suprabasal layer and continuously up-regulated. Its functional role has been established in vivo because transgenic mice overexpressing miR-203 under a K14 promoter exhibited a significantly thinner pluristratified epidermis than the wild-type. It was further demonstrated that this skin-specific miRNA promotes epidermal differentiation in vitro by restricting the proliferative potential of keratinocytes and inducing cell cycle exit via the control of p63 expression (Yi et al., 2006). Human primary keratinocytes overexpressing miR-203 were also characterised by a lower proliferation potential, as revealed by a significant decrease in their clonogenic capacities (Lena et al., 2008). We have confirmed these results using our pluristratified epidermis reconstruction assay based on hESC progenies. Indeed, overexpression of miR-203 resulted in increased expression of differentiation markers (Keratins 1 and 10) and a reduced stratification, which indicates that this miRNA negatively controls the stemness of basal keratinocytes. Molecular mechanisms involved in this control in humans have not been specifically sought. However, in the mouse Lena and colleagues have demonstrated that miR-203 represses stemness in keratinocytes via a direct negative regulation of DeltaNp63 (Lena et al., 2008).

**miR-203 belongs to a subset of miRNAs that are tissue-specific developmental regulators**

The present observation that miR-203 is a key regulator of the entire process between the early steps of epithelial cell fate commitment and the terminal keratinocyte differentiation in the epidermis accompanies a similar description of key roles in specific tissue development for a small subset of miRNAs. Regulatory
functions of miRNAs during development have been acknowledged since the earliest studies in invertebrates. Lin-4 and Let-7 were thus shown to control timing of larval development in *Caenorhabditis elegans* (Lee and Ambros, 2001) as mutations in those miRNAs led to the reiteration of larval cell fates and delayed final differentiation of several cell types. In the same organism, miR-16 and miR-84 affect vulva development through the regulation of vav and ras (Jannot and Lee, R.C., Ambros, V., 2001. An extensive class of small RNAs in *Caenorhabditis elegans*?/em? controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. Cell 113 (1), 25–36.


