miR-193b/365a cluster controls progression of epidermal squamous cell carcinoma

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Incidence of cutaneous squamous cell carcinomas (cSCCs) constantly increases in the Caucasian population. Developing preferentially on precancerous lesions such as actinic keratoses due to chronic sunlight exposure, cSCCs result from the malignant transformation of keratinocytes. Although a resection of the primary tumor is usually curative, a subset of aggressive cSCCs shows a high risk of recurrence and metastases. The characterization of the molecular dysfunctions involved in cSCC development should help to identify new relevant targets against these aggressive cSCCs. In that context, we have used small RNA sequencing to identify 100 microRNAs (miRNAs) whose expression was altered during chemically induced mouse skin tumorigenesis. The decreased expression of the miR-193b/365a cluster during tumor progression suggests a tumor suppressor role. Ectopic expression of these miRNAs in tumor cells indeed inhibited their proliferation, clonogenic potential and migration, which were stimulated in normal keratinocytes when these miRNAs were blocked with antisense oligonucleotides. A combination of in silico predictions and transcriptome analyses identified several target genes of interest. We validated KRAS and MAX as direct targets of miR-193b and miR-365a. Repression of these targets using siRNAs mimicked the effects of miR-193b and miR-365a, suggesting that these genes might mediate, at least in part, the tumor-suppressive action of these miRNAs.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer affecting the Caucasian population, and it displays a constantly increasing incidence estimated to 700,000 new cases diagnosed each year in the USA (http://www.aad.org/skin-conditions/dermatology-a-to-z/squamous-cell-carcinoma). cSCC is a malignant neoplasm of epidermal keratinocytes that occur preferentially on precancerous lesions such as UV-induced actinic keratoses, burns, mucous papillomavirus (human papillomavirus) lesions, chronic wounds or ulcers.

Currently, most of the cSCCs are considered as low-risk tumors and are efficiently treated by curative resection of primary tumors. There is however a subset of more aggressive cSCCs that have a substantial risk of recurrence and a propensity to develop lymph node or even distant metastases (1,2). Some features of ‘high-risk’ cSCCs include size, growth rate, tumor thickness or depth, perineural invasion, lymphatic or vascular vessel invasion, immunosuppression (lymphoproliferative or auto-immune disorders, solid-organ transplants), human papillomavirus infection, histologic subtypes (desmoplastic, adenosquamous, etc.), anatomic locations and some chronic blistering genodermatoses such as severe forms of recessive dystrophic epimolysis bullosa (1,2). These situations represent a significant number of cases that are responsible for up to 25% of all skin cancer deaths. It is thus essential to identify key molecular or genetic defects responsible for the malignant transformation of the epidermis and to develop new treatments.

One option for the development of future antitumoral therapies in cSCCs that has been recently suggested corresponds to target microRNAs (miRNAs) (3). miRNAs are small (~22 nt) non-coding RNAs that regulate many important processes in cancer biology (4). Altered expression of many tumor-suppressive or oncogenic miRNAs has been reported in various cancers, but few studies have reported the expression profiling of miRNAs in the context of cSCCs (5–8).

We report here a characterization of the miRNA signature at different stages of cSCC development in a mouse model of two-stage chemically induced skin carcinogenesis by small RNA sequencing (9). The expression of 112 miRNAs was significantly altered in papillomas and/or tumors versus normal skin. We further characterized the miR-193b/365a locus that is repressed in human and mouse cSCCs. In line with a tumor suppressor function, we demonstrated using gain-of-function and loss-of-function approaches, that miR-193b and miR-365a indeed regulate the proliferative, clonogenic and migratory properties of mouse and human SCC cell lines as well as normal human keratinocytes (NHKs). A molecular characterization of their functional targets suggests that these effects can be mediated, at least in part, through the direct targeting of KRAS and MAX.

Materials and methods

Animals

Animal experiments were carried out in accordance with the Declaration of Helsinki and were approved by the local ethical committee. Six-week-old female FVB/N mice (n = 45) (Janvier, Le Genest-Saint-Isle, France) were subjected to 7,12-dimethylbenz[a]anthracene (DMBA) two-hit multistage skin carcinogenesis protocol as described previously (9). Mice were topically treated with 200 nmol of DMBA in 0.2 ml acetone, then twice weekly for 6 weeks with 5 nmol phorbol 12-myristate 13-acetate (PMA) stock solution delivered on the right flank. Half of the mice received a single treatment of PMA in the right flank. Control mice (n = 20) were only topically treated with 0.2 ml acetone vehicle. Mice were monitored weekly for papillomas and tumors counting. Healthy skins (acetone treated), hyperplastic skins, papillomas and tumors were harvested throughout the protocol, and biopsies were frozen for protein and RNA extractions, and were formalin fixed/paraffin embedded for histological analysis.

Clinical samples

Four millimeter punch biopsies were taken, after informed consent, from skin of healthy donors (n = 21), actinic keratosis (n = 13) and cSCC patients (n = 13) at the Dermatology and Venereology Unit, Karolinska University Hospital, Stockholm, Sweden. The clinical diagnosis was made by a dermatologist and confirmed by histopathological evaluation. The study was approved by the Regional Ethics Committees and conducted according to the Declaration of Helsinki Principles. RNA was extracted from frozen biopsies as described previously (8).

Abbreviations: BrdU, 5-bromo-2'-uridine; cSCC, cutaneous squamous cell carcinoma; DMBA, 7,12-dimethylbenz[a]anthracene; EGF, epidermal growth factor; FCS, fetal calf serum; HNSCC, head and neck squamous cell carcinomas; IPA, Ingenuity Pathway Analysis; miRNA, microRNA; NHK, normal human keratinocyte; PMA, phorbol 12-myristate 13-acetate.
Cell culture and transfection
NHKs isolated from healthy neonatal foreskins were cultured on a feeder layer of lethally irradiated 3T3-J2 fibroblasts as described previously (10). The human CAL27 and CAL60 oral SCC cell lines were kindly provided by Dr. J.L. Fischel (Centre Antoine Lacassagne, Nice, France) (11). The human HaCaT immortalized keratinocyte cell line was a gift from Dr. P. Boukamp (Heidelberg, Germany). The A431 human cSCC and the COS-7 monkey kidney cell lines were from ATCC (Rockville, MD). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS; Perbio Science AB, Helsingborg, Sweden). Pre-miRNAs (pre-miR-193b-3p, pre-miR-365a-3p and negative control pre-miR-NC1), anti-miRNAs oligonucleotides (LNA-NC, LNA-193b-3p and LNA-365a-3p) and ON-Targetplus siRNAs [for KRAS, MAX and irrelevant control (Ctl)] were purchased from Ambion (Austin, TX), Exiqon (Vedbaek, Denmark) and Dharmacon (Thermo Fisher Scientific, Courtaboeuf, France), respectively. Cells were plated and transfected 24h later at 30–50% confluency, with pre-miRNAs (2–5 nM), anti-miRNAs (10 nM) or siRNAs (25 nM) using Lipofectamine™ RNAiMAX reagent (Life Technologies, Carlsbad, CA). NHKs were seeded and transfected in defined KGM2 medium (Lonza, Basel, Switzerland). Feeder cells and fresh medium were added 24h after transfection.

Establishment of murine cSCC cell lines
Murine mSCC-20 and mSCC-38 cell lines were established from two distinct DMBA/PMA-induced cSCCs. Briefly, tumors were dissected to remove necrotic areas and the covering skin, then cut into 1–2 mm³ pieces and incubated with collagenase A (1.5 mg/ml) and dispase II (5 mg/ml) for 1h at 37°C under orbital shaking. Cell slurries were passed through a 70 µm mesh and pelleted at 1800 r.p.m. for 5min. Cells were plated on culture flasks coated with type I collagen (1.5 mg/ml) and dispase II (5 mg/ml) for 1h at 37°C under orbital shaking. Cell slurries were passed through a 70 µm mesh and pelleted at 1800 r.p.m. for 5min. Cells were plated on culture flasks coated with type I collagen (40 µg/ml) in the presence of lethally irradiated feeder cells in William’s medium E without calcium but containing antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone). Cells were progressively cultured under classical conditions (in Dulbecco’s modified Eagle’s medium containing 10% FCS without antibiotics, collagen coating and feeder cells) and used between passages 30 and 50 in our experiments.

RNA extraction and small RNA-Seq profiling of miRNAs
Total RNA containing the small RNA fraction were isolated from healthy skins, hyperplastic skins, papillomas and cSCCs biopsies with TRIzol™ reagent (Invitrogen, Life Technologies) according to the manufacturer’s instructions. Small RNA libraries were generated from total RNA (300 ng) with the SOLiD Small RNA Expression Kit (SREK; Applied Biosystems Foster City, CA) and sequenced on the Applied Biosystems SOLiD System following the manufacturer’s instructions. Reads (5–20 × 10⁶ per sample) were matched to both known miRNA precursors and to the mouse genome (NCBI37/mm9) using the RNA2MAP software (Applied Biosystems). The significance of the difference between the experimental and control conditions was estimated by an empirical Bayes method using the DEseq package from Bioconductor. The experimental data have been deposited in the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under series GSE52299. Unsupervised hierarchical clustering was done with the MultiExperiment...
Quantitative reverse transcription–PCR analysis of mature miRNAs and pri-miRNAs expression

Expression of mature mmu-miR-193b-3p, hsa-miR-193b-3p and mmu-miR-365-3p or mmu-pri-miR-365-1 and mmu-pri-miR-365-2 was evaluated using TaqMan MicroRNA and Pri-microRNA Assays (Applied Biosystems) and the Lightcycler 480 detection system (Roche Applied Science, Indianapolis, IN). Expression levels were normalized to mmu-SnoR55 or hsa-RNU44 and calculated using the comparative CT method ($2^{-\Delta\Delta C T}$).

miRNA in situ hybridization

Hsa-miR-193b-3p and hsa-miR-365a-3p in situ hybridizations were done as described previously (10). Briefly, formalin-fixed optimal cutting temperature compound-embedded frozen human skin sections (7 µm) were acetylated in phosphate-buffered saline containing 0.1 M triethanolamine and 0.5% acetic anhydride for 30 min at 25°C, washed in phosphate-buffered saline and incubated overnight at 58°C in hybridization buffer (5x saline-sodium citrate buffer, 50% deionized formamide, 20% Dextran Sulfate, 0.5 mg/ml Yeast tRNA) containing 25 nM of double Digoxigenin-labeled hsa-miR-193b-3p, hsa-miR-365a-3p or scramble-miR miRCURY LNA detection probes (Exon). After washing, samples were incubated with alkaline phosphatase-conjugated anti-Digoxigenin (Roche) and developed for 24–48 h in nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Roche) containing 2 mM levamisole (Vector). Images were done on a Leica DMD108 microscope.

Migration, proliferation and clonogenic assays

For proliferation assays, 5-bromo-2’-deoxyuridine (BrdU) was added to the cell culture medium at the biotindicated time and BrdU incorporated into the DNA was revealed using a detection kit (Roche, Penzberg, Germany). Boyden’s chamber assays were performed as described previously (10). Briefly, cells were detached 48 h after transfection, seeded in the upper chambers of Transwell inserts and attracted in the lower chambers by epithelial growth factor (EGF, 2 ng/ml). After 24 h, cells on the lower side of the filters were stained with 4’,6-diamidino-2-phenylindole and scored in eight independent fields. For clonogenic assays, transfected NHKs were seeded (50 cells/cm$^2$) and scored in eight independent fields. For clonogenic assays, transfected NHKs were seeded (50 cells/cm$^2$) and scored in eight independent fields.

Cell cycle analysis

Exponentially growing HaCaT cells were transfected, then collected at different time, fixed with 70% ethanol and stained with propidium iodide solution (Sigma) containing RNase A. Data were acquired on a LSRRForessta flow cytometer and analyzed using FACSDiva software (Becton-Dickinson, Franklin Lakes, NJ).

Antibodies

Anti-MAX rabbit polyclonal antibody, anti-KRAS and anti-E-cadherin mouse monoclonal Abs and anti-Hsp60 goat pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit pAb to Keratin 14 and Vimentin were from Covance (Princeton, NJ) and Epitomics (Burlingame, CA), respectively. The secondary peroxidase-conjugated pAb were from Dakopats (Hamburg, Germany).

Microarrays hybridization and miR-193b-3p/miR-365a-3p target genes analyses

Transcriptome analyses of mSCC-20 cells transfected with 5 nM mmu-pre-miR-193b-3p, pre-miR-365a-3p or pre-miR-NC1 for 30 h were performed using mouse GE 4x44K v2 microarrays from Agilent Technology (Santa Clara, CA). Typical biologic replicates were used for each comparison. Experimential data and associated microarray designs are deposited in the NCBI GEO under series GSE52381. Normalization was performed using the limma package available from Bioconductor (http://www.bioconductor.org). Interslide normalization was performed using the quantile methods. Means of ratios from all comparisons were calculated and B-test analysis was performed. Differentially expressed genes were selected based on an absolute log₂-fold change of >0.6 and a P value of <0.05 and 0.01 for mmu-193b-3p and miR-365-3p, respectively. mir-193b-3p and miR-365a-3p targets were predicted using in-house tool MironTop (http://www.microarray.fr:8080/mirontop/index) as described previously (12).

Plasmid constructs, site-directed mutagenesis and luciferase assays

CAL27 cDNA fragments corresponding to human KRAS and MAX 3’UTR miRNAs were cloned into the Xhol and NotI restrictions sites downstream the Renilla luciferase gene of psiCHECK™-2 vector (Promega, Madison, WI) as described previously (10). Mutagenesis of the putative miR-193b-3p and miR-365a-3p binding sites was performed using the QuickChange Multi Site-Directed Mutagenesis kit from Stratagene (Agilent, La Jolla, CA) according to the manufacturer’s protocol. Luciferase assays were performed in COS-7 cells as described previously (10). The primers used for 3’-UTR cloning and mutagenesis are described in Supplementary data, available at Carcinogenesis Online.

Statistical analysis

Statistical evaluations were performed by Student’s t test for paired data, and data were considered significant at a P value inferior to 0.05.

Results

Profiling of miRNA signatures at various stages of cSCC development

We used a mouse model of chemically induced skin carcinogenesis to characterize the miRNA signature specific to each stage of tumor development. Tumor initiation was induced by topical application of the chemical mutagen DMBA on the skin of FVB/N mice. Then, clonal expansion of the mutated cells was performed during the promotion step using repeated application of PMA, leading to epidermis hyperplasia. This resulted in the development of pretumoral outgrowths called papillomas. We performed a second DMBA-hit in order to increase the malignant conversion efficiency of papillomas (Figure 1A) (9). The first papillomas arose around the eighth week and were monitored until the maximum response was achieved, which coincides with progression to invasive cSCC after ~20 weeks (Figure 1B).

The analysis of miRNAs expression profiles by small RNA sequencing was performed on biopsies from each stage of tumor development: (i) acetone-treated healthy skins, (ii) DMBA/PPMA-treated hyperplastic skins, (iii) papillomas and (iv) cSCCs (Figure 1C). Data showed a significant alteration of 112 miRNAs during epidermis tumorigenesis. Twenty-eight of them were upregulated and 84 were repressed by at least 2-fold relative to healthy skins (Supplementary Table 1, available at Carcinogenesis Online). A recent study from Xu et al. identified, using TaqMan Low Density Arrays, 58 miRNAs that were significantly modulated in human cSCCs (8). Thirty-five of these miRNAs were common between the human and mouse models, suggesting the pathophysiologic relevance of our observations for the human disease.

Most of the miRNAs were already deregulated at the papilloma stage, although some of them such as miR-203-3p or miR-211-5p were only altered in tumors (Figure 1D). Some of the identified miRNAs have been reported to be deregulated in other cancers. This is the case for miR-21-5p, miR-31-5p or miR-135b-5p, which were upregulated, and for miR-143/miR-145 cluster as well as let-7a/b/c/d/g and miR-99a/miR-100 families members, which were downregulated. Interestingly, the first were previously demonstrated to exert onco- genic functions, whereas the latter were demonstrated to exert tumor suppressor functions (5,6). Outside the behavior of these reference miRNAs, the function of the rest of the cSCC-modulated miRNAs was poorly described and nothing was established regarding the role of tumor suppressor miRNAs in the control of epidermis homeostasis.

The miR-193b/365-1 cluster is repressed during cSCC development

We initially focused our attention on miR-193b-3p whose expression dropped 3- to 10-fold, in papillomas and in cSCCs (Supplementary Table 1, available at Carcinogenesis Online). miR-193b being clustered with miR-365-1, we also observed that miR-365-3p was downregulated by 3-fold in papillomas and cSCCs (13,14). These parallel decreased expressions of miR-193b-3p and miR-365-3p in papillomas and tumors were further confirmed on a larger number of samples using quantitative real-time PCR assays (Figure 2A).

It turns out that there are indeed two distinct miR-365 genes in mouse: miR-365-1 located on chromosome 16 and miR-365-2 located on chromosome 11. In order to determine the origin of miR-365-3p downregulation, we assessed the expression status of the two mouse genes, in healthy skin, papilloma and cSCC samples using a Taqman assay designed to discriminate between pri-miR-365-1 and pri-miR-365-2. We showed that only pri-miR-365-1, clustered with miR-193b, was indeed repressed during epidermal tumorigenesis (Supplementary Figure 1, available at Carcinogenesis Online).


Expression of mature mmu-miR-193b-3p, hsa-miR-193b-3p and mmu-miR-365-3p or mmu-pri-miR-365-1 and mmu-pri-miR-365-2 was evaluated using TaqMan MicroRNA and Pri-microRNA Assays (Applied Biosystems) and the Lightcycler 480 detection system (Roche Applied Science, Indianapolis, IN). Expression levels were normalized to mmu-SnoR55 or hsa-RNU44 and calculated using the comparative CT method ($2^{-\Delta\Delta C T}$).
We next measured the expression of miR-193b-3p and miR-365-3p in human specimen of healthy skins, actinic keratoses and cSCCs (Figure 2B). We also observed a significant decrease in expression of these miRNAs in human cSCC compared with healthy skin.

The analysis of miR-193b-3p and miR-365-3p expression in human skin by in situ hybridization revealed that the two miRNAs are exclusively located in the suprabasal layers of the epidermis (Figure 2C). Consistent with this localization, we observed an increased expression of these miRNAs during the terminal differentiation of human keratinocytes in vitro (data not shown).

Taken together, these data suggest that the miR-193b/365a cluster could act as a tumor suppressor in the epidermis.

Functional role of miR-193b/365a in normal and malignant keratinocytes

Because the function of miR-193b and miR-365a in the epidermis was completely unknown, we investigated their role using gain-of-function and loss-of-function approaches.

We showed that the ectopic expression of miR-193b-3p or miR-365-3p strongly inhibited the proliferation of NHK and of immortalized (HaCaT) or tumoral keratinocyte cell lines (CAL27, CAL60 and A431), measured by BrdU incorporation (Figure 3A). These antiproliferative effects were confirmed by cell numeration experiments (Supplementary Figure 2A, available at Carcinogenesis Online). We then investigated the impact of miR-193b-3p and miR-365-3p on serum-induced cell cycle commitment. Pre-miR-NC, pre-miR-193b-3p and pre-miR-365-3p-transfected HaCaT cells were synchronized in G0/G1 phase by serum starvation and released from quiescence by FCS addition. HaCaT cells transfected with miR-193b-3p and miR-365-3p did not reach the S and/or G2/M phases upon FCS addition (35 and 39% versus 62% in S + G2/M phases after 24h) (Figure 3B), indicating that these miRNAs block cells in the G1 phase. In line with this observation, we showed that the ectopic expression of miR-193b-3p or miR-365-3p markedly reduced the colony forming efficiency of NHK (Figure 3D).

We then assessed the effect of the miR-193b/365a cluster on cell migration. NHK, HaCaT and SCC cells transfected with
Fig. 3. Effects of miR-193b-3p and miR-365a-3p on normal and tumoral keratinocytes functions. HaCaT, CAL27, CAL60, A431 cell lines and NHK were transfected with pre-miR-193b-3p, pre-miR-365a-3p or pre-miR-NC (2 nM) (A, B, D and F) or with anti-miR-193b-3p (LNA-193b), anti-miR-365a-3p (LNA-365a) or anti-miR-control (LNA-NC) (10 nM) (C, E and G). (A and C) Transfected cells were grown for 48 h, pulsed overnight with BrdU, then harvested, labeled with anti-BrdU and nuclei counterstained with 4′,6-diamidino-2-phenylindole. Data are expressed as percentage of BrdU-positive cells determined in 10 independent microscope fields and represent three independent experiments performed in duplicate. (B) Cell cycle analysis of transfected HaCaT cells synchronized in G₀/G₁ by FCS starvation and launched into the cell cycle for 24 h by FCS addition as described in Materials and methods. Data are typical of one experiment chosen among four. (D and E) Colony forming efficiency (CFE) analysis of transfected NHK seeded at 50 cells/mm² 24 h after transfection and allowed to form colonies during 10 days. Colonies were stained with rhodamine-B and counted. Data are means ± SD from three independent experiments performed in triplicate. (F and G) Comparative analysis of migration of transfected cells using Boyden’s chambers 48 h after transfection. Nuclei of migrating cells were stained with 4′,6-diamidino-2-phenylindole and counted. Data are means ± SD from three independent experiments performed in triplicate. Statistically significant differences are indicated (*P < 0.05; **P < 0.001).
Identification of miR-193b/365a target genes

To go further into the mechanism of action of miR-193b and miR-365a, we undertook the identification of their putative target genes by transcriptome analysis of murine SCC-20 cells overexpressing these miRNAs (10). We found that miR-193b-3p and miR-365-3p significantly altered the expression of 504 (271 up- and 233 downregulated) and 821 genes (299 up- and 522 downregulated), respectively. The functional annotation using the Ingenuity Pathway Analysis software (IPA; http://www.ingenuity.com) indicated that the lists of miR-193b-3p- and miR-365-3p-modulated genes were associated with Gene Ontology terms such as cell death and survival (20 and 22%), cell growth (22.5 and 21.5%), cell cycle (12 and 10%) and cell migration (15 and 13%).

We monitored the extent of overrepresentation of specific miR-193b-3p or miR-365-3p target genes among the set of downregulated transcripts (12). Using TargetScan target prediction algorithm analysis, we observed significant enrichments of miR-193b-3p or miR-365-3p putative targets (Figure 4A). Based on these predictions, 96 and 186 putative target genes were proposed for miR-193b-3p and miR-365-3p, respectively (Supplementary Table 2, available at Carcinogenesis Online). Only five common predicted targets could be found for these two miRNAs, as visualized by the Venn diagram on Figure 4B.

Given the potential tumor suppressor properties of the miR-193b/365a cluster, we focused on the predicted target genes associated with terms such as cell proliferation and/or tumorigenesis: the KRAS oncogene and the MYC partner MAX. Signaling pathways involving RAS and MYC are frequently exacerbated in cSCC due to activating mutations or gene amplification (15,16). Interestingly, IPA gene networks annotations revealed that miR-193b-3p and miR-365-3p ectopic expression in mSCC-20 cells altered the expression of numerous genes regulated by MYC, including KRAS itself (Figure 4C).

According to TargetScan, the 3’UTR of KRAS contains binding sites for miR-193b-3p and miR-365-3p that are well conserved between human and mouse (Figure 5A). While TargetScan identified only one conserved binding site for miR-193b-3p on MAX, a miR-365-3p seed-complementary region was conserved in the 3’UTRs of mouse and human MAX mRNAs (Figure 5B).

Direct targeting of these genes by miR-193b-3p and miR-365-3p was experimentally investigated by cloning the 3’UTR region of each of the human transcripts containing the putative binding sites into the psiCHECK™-2 vector, downstream to a luciferase reporter gene. miR-193b-3p markedly decreased the luciferase activity of the KRAS and MAX constructs in a range of 60% (Figure 5B). These inhibitions were abolished after modification of the binding sites. As shown in Figure 5B, miR-365-3p inhibited the luciferase activity of the human KRAS construct, harboring three putative miR-365-3p binding sites, by 80%. This inhibition was abrogated by simultaneous mutation of these binding sites. In contrast, miR-365-3p reduced the luciferase activity of the MAX construct by 30% but this effect was not reverted by mutation of the putative binding site.

Western blot analysis showed that the expression levels of KRAS and MAX were strongly reduced by miR-193b-3p and miR-365-3p in NHK, HaCaT and CAL27 cells (Figure 5C). Similar results were obtained in mouse mSCC-20 and mSCC-38 cell lines (Figure 5E). Conversely, blocking endogenous miR-193b-3p and miR-365-3p with LNAs significantly increased the expression of KRAS and MAX in keratinocytes (Figure 5D).

Taken together, these data indicate that KRAS represents a bona fide target gene of both miR-193b-3p and miR-365-3p, whereas MAX is directly targeted by miR-193b-3p and indirectly by miR-365-3p.

We also analyzed the level of expression of Kras and Max in healthy skin and cSCC biopsies of DMBA/PMA-treated mice. An inverse relationship was observed between the expression of miR-193b-3p/miR-365-3p (Figure 5A) and the level of Kras transcript (Figure 5F). Max protein levels were markedly increased in tumors relative to healthy skins (Figure 5G) but, in contrast, no change in mRNA expression was observed in cSCCs compared with healthy skins (Figure 5F). The same situation exists in mouse epidermis and mSCC-20 and mSCC-38 cell lines (Supplementary Figure 5, available at Carcinogenesis Online).

Role of KRAS and MAX on normal and tumoral keratinocyte functions

NHK, HaCaT and CAL27 cells were transfected with siRNAs specific to KRAS and MAX (Figure 6D), and the proliferation rate was significantly reduced by BrdU incorporation. Inactivation of KRAS and MAX significantly reduced cell proliferation by ~20–50% and 30–40%, respectively (Figure 6A). In these conditions, MAX silencing blocked HaCaT keratinocytes in the G0 phase (40 versus 58% in S + G2/M phases after 24 h of FCS stimulation), whereas KRAS silencing appeared less efficient to block cell cycle progression (45 versus 58% in S + G2/M phases) (Figure 6B). The clonogenic potential of NHK was also markedly affected by the inactivation of MAX to a lesser extent by that of KRAS (Figure 6C). Finally, we showed that the inactivation of KRAS strongly decreased migration of the three cell lines, while siRNAs against MAX significantly affected NHK and HaCaT cells but not CAL27 SCC cells (Figure 6D).

Inactivation of KRAS and MAX mimics most of the effects of miR-193b and miR-365a, suggesting a direct involvement in their biological effects.

Discussion

The functional impact of miRNAs in cSCC is still largely underappreciated. Recent studies using microarray hybridizations or Taqman low density arrays identified, respectively, 31 and 58 miRNAs that were significantly altered in human cSCC biopsies relative to adjacent healthy skin but the functional roles of these miRNA candidates were poorly explored (7,8). In the present study, we combined a mouse skin model of multistage chemical carcinogenesis that recapitulates the sequential and stepwise development of cSCC, with a small RNA-Seq approach, to identify the miRNA signature corresponding to the different stages of tumor development. Deep-sequencing has several advantages compared with other miRNA profiling technologies: it
C. Gastaldi et al. has very low background signal, a large dynamic range of expression levels over which transcripts can be detected, high levels of reproducibility and is overall not restricted to a panel of miRNAs.

We found that 112 miRNAs are altered in pretumoral papillomas and cSCCs. In accordance with the recent characterization of the human cSCC miRNome, we observed that most of the altered miRNAs are downregulated during tumorigenesis (84 versus 28 upregulated miRNAs) (8). We noted the significant upregulation of miR-21, miR-31, miR-135b and miR-210 and the repression of miR-99a/miR-100 family members, miR-101, miR-143/miR-145 cluster and miR-204. This profile is consistent with the respective oncogenic or tumor suppressor functions of these different miRNAs (5, 6, 17–21). Interestingly, miR-203, which exerts an antitumoral action in basal cell carcinomas, was also repressed in mouse cSCC (22).

Among the downregulated miRNAs, we found the miR-1/133a and miR-206/133b clusters that exert tumor-suppressive

![Fig. 4. Determination of miR-193b-3p and miR-365a-3p target genes. mSCC-20 cells were transfected with pre-miR-193b-3p, pre-miR-365-3p or pre-miR-NC (n = 2). RNA samples were harvested 30 h post-transfection and mRNA profiles were determined with pan genomic arrays. (A) Overrepresentation of miR-193b-3p and miR-365a-3p seed complementary sequences in the 3′-UTR of downregulated transcripts. Representation of TargetScan predicted targets in the set of downregulated genes was compared with the set of all expressed genes using the bioinformatic tool MiRonTop (12). For each miRNA seed, a fold enrichment value and an associated P value were calculated. Representation is based on log2 ratio >0.6 and adjusted P value <0.05 or <0.01 for miR-193b and miR-365, respectively. (B) Venn diagram comparing the number of miR-193b-3p and miR-365a-3p targets among the set of downregulated genes according to TargetScan target prediction tool. (C) The most significant IPA network assembled around MYC in mSCC-20 cells overexpressing miR-193b-3p or miR-365a-3p. IPA results showing the network of MYC and their close interactions with MAX and KRAS genes. Genes repressed in the signature are in green.](https://academic.oup.com/carcin/article-abstract/35/5/1110/272493)
properties in many cancers and notably in head and neck squamous cell carcinomas (HNSCCs) (23). However, these miRNA clusters are known to be strongly expressed in striated muscles (24). Because mouse full-skin biopsies contain more subcutaneous striated muscle (panniculus carnosus) than papillomas and tumors, the observed decrease in miR-1/133a and miR-206/133b could be explained by the lower proportion of muscle tissue in these samples. This hypothesis is supported by the lack of modulation of these miRNAs in human cSCCs (8).

We and others found that miR-211 expression was strongly decreased in cSCC in mouse as well as in human, arguing for a potential tumor suppressor role in the development of these carcinomas (8). This observation is supported by data showing that miR-211 represses melanoma invasion (25). However, depending on tissue context,

![Fig. 5](https://academic.oup.com/carcin/article-abstract/35/5/1110/272493)
C.Gastaldi et al. miR-211 could also be associated with tumor progression as it is the case in HNSCC (26).

Other studies have reported a decrease in miR-124/miR-214, in line with a targeting of ERK1/2 that could contribute to abnormal keratinocyte proliferation (27). However, these miRNAs were not modulated in our mouse model of skin carcinogenesis.

We thus focused our interest on the miR-193b/365a cluster that is highly expressed in epidermis and markedly repressed during tumorigenesis, already at the papilloma stage, suggesting that it could intervene in the control of the early steps of cancer development. In line with this hypothesis, we demonstrated that miR-193b-3p and miR-365a-3p are endowed of antiproliferative and antimigratory properties.

Fig. 6. Effect of miR-193b-3p/miR-365a-3p cluster targets knockdown on normal and tumoral keratinocytes functions. (A–E) NHK, HaCaT and CAL27 cells were transfected with KRAS, MAX or control (Ctl) ON-Target Plus siRNAs (25 nM). (A) Transfected cells were grown for 48 h and pulsed overnight with BrdU, and percentage of BrdU-positive cells was determined as described in Figure 3A in 10 independent microscope fields. Data represent three independent experiments performed in duplicate. (B) Cell cycle analysis of transfected HaCaT cells synchronized in G0/G1 by FCS starvation and launched into the cell cycle for 24 h by FCS addition. Data are typical of one experiment chosen among four. (C) Colony forming efficiency analyses of transfected NHK were done as described in Figure 3D. Data are means ± SD from three independent experiments performed in triplicate. (D) Comparative analysis of migration of transfected cells using Boyden’s chambers as depicted in Figure 3F. Data are means ± SD from three independent experiments performed in triplicate. (E) Cell lysates were analyzed by western blot hybridization to check the efficiency of RNA interference. Statistically significant differences are indicated (**P < 0.001).
consistent with potential tumor suppressor functions in the epidermis. Interestingly, although mir-193b and mir-365 have been shown to be decreased in melanoma and hepatocellular carcinoma or in non-small-cell lung and colon carcinomas, respectively, their role in skin carcinoma has never been investigated (28–31). mir-193a and mir-193b inhibit cell proliferation, migration, invasion and survival in melanoma, prostate, breast and liver cancer cell lines, likely by targeting CCND1, ETS1, PLAU, STMN1, KRAS and/or MCL1 (28, 29, 32–35). Similarly, mir-365 represses cell proliferation and induces apoptosis by silencing CCND1, BCL2, NXX2-1, HMGAA2 and TTF-1 in lung and colon cancer cell lines (30,31,36).

The antiproliferative properties of mir-193b and mir-365 are consistent with their involvement in various cell differentiation programs. Indeed, the mir-193b/365a cluster regulates brown adipose tissue differentiation notably through repression of myogenesis (14). Moreover, induction of mir-365 by mechanical strains favors chondrocyte differentiation, whereas mir-193b upregulation is involved in aging-associated senescence of chondrocytes (37,38). mir-365 also controls the maturation of colon epithelial cells through the direct targeting of Myb2, a transcription factor crucial for the maintenance of stemness (39). However, it remains to clarify which of mir-365a or mir-365b is involved in these processes. Interestingly, an increase in mir-193b and/or mir-365 expression has been reported in NHK upon calcium-induced differentiation (40,41). This observation is in agreement with our results showing that these miRNAs are located in the skin suprabasal layers and suggests their role in the terminal differentiation program of keratinocytes. Further experiments would be needed to clarify this hypothesis.

Although mir-193b appears mostly as a tumor suppressor, it was reported to be oncogenic in HNSCC. This effect was mediated through the targeting of neurofibromin 1 (NF1), i.e. a factor which is not significantly altered in cSCC cells that overexpress mir-193b-3p (42). mir-193b-3p could thus exert paradoxical effects on tumor development depending on cellular context, as observed for other miRNAs (25,26,43). Incidentally, mir-365b might also favor tumor development through an uncharacterized mechanism (44).

We verified that the ectopic expression of mir-193b-3p or mir-365a-3p in murine cSCC cells repressed the expression of some validated targets such as Ets1 and Stmn1 (for mir-193b-3p) or Cnd1 and Hmg2 (for mir-365a-3p). We also searched for additional target genes that could play a role in epidermis tumorigenesis. Despite the low rate of mutant RAS genes, an increase in levels of RAS with active GTP is observed in most spontaneous epithelial cancers including cSCCs (45). Such an activation results either from (i) a genomic amplification like KRAS in HNSCC, (ii) an overactivation of RAS upstream regulators such as EGF receptor or (iii) a loss of specific miRNAs targeting RAS (46–49). In this context, it has recently been reported in isogenic models of cellular transformation that KRAS is a direct target of mir-193a (33). We show in the present study that KRAS is also targeted by mir-193b-3p, which shares the same seed with mir-193a-3p. Moreover, we validated KRAS as a bona fide target of mir-365-3p. These observations show that two miRNA paralogs generated from the same pri-miRNA can act together to silence the same gene. We found that other Kras-targeting miRNAs, such as let-7 family members, mir-143 and mir-181a, are also repressed during epidermis carcinogenesis in mouse (49–51). Additionally, cSCCs are characterized by high expression of miR-21, a well-established inhibitor of negative regulators of the RAS/MEK/ERK pathway in KRAS-dependent lung cancer (52). From that perspective, the decrease in cSCC of several Kras-targeting miRNAs, including mir-193b-3p and mir-365a-3p, is able to favor tumor development through exacerbation of Ras-dependent signaling. Given that pharmacological targeting of RAF/MEK/ERK and PI3K/akt/mTOR RAS downstream pathways has been shown to suppress the growth of cSCC, one can hypothesize that modulation of the mir-193b/365a cluster could affect these pathways (53).

MYC, which is also frequently overexpressed in cSCC, represented an alternative pathway of interest in the context of our study (16). It contributes to tumorigenesis notably through repression of multiple tumor suppressor miRNAs including miR-30 and let-7 families members, miR-99a, miR-125b, miR-26a, miR-146a, miR-150 and miR-195 (54). Interestingly, most of these miRNAs were downregulated in our experimental model, suggesting that MYC axis is activated in DMBa/PlaMA tumor and play a role in tumor development. Although it is described as ubiquitous and constitutively expressed, we observed a significant upregulation of Max, the Myc partner, in cSCC compared with healthy skin. This upregulation is consistent with MAX being directly targeted by mir-193b-3p and altered by mir-365-3p. The consequences of MAX upregulation deserve to be further studied, but it could contribute to the activation of MYC pathway. A miRNA-dependent regulation of MAX has already been described in the HL-60 promyelocytic leukemia cell line. In that context, the induction of miR-22 represses MAX expression, thus favoring cell differentiation through inhibition of cell proliferation (55).

Interestingly, MAX has recently been shown to repress mir-193a expression (33). We have identified potential CACGTG MYC/MAX DNA binding sites in the promoter regions of mouse and human mir-193b/365a clusters. Thus, it is tempting to speculate that regulatory feedback loops involving MAX and mir-193b/365a could participate in tumor development. Further experiments would be needed to check these hypotheses.

Using a siRNAs strategy, we show that MAX and KRAS play a central role in the antitumoral effects of mir-193b and mir-365a. Interestingly, MAX knockdown markedly altered the clonogenic potential of NHK, which is consistent with its implication in maintenance of self-renewal and undifferentiated state of murine embryonic stem cells (56). The impact of KRAS invalidation was stronger on cell migration than on KHN proliferation and clonogenicity may be because of redundancy between RAS family members.

In conclusion, our findings identified numerous miRNAs that are dysregulated during epidermis carcinogenesis in mouse, some of which have not yet been reported to be altered in human cSCCs. Focusing on the mir-193b/365a cluster, we confirmed that these miRNAs are endowed of tumor suppressor functions and we showed that they can act in concert to repress skin carcinogenesis through repression of multiple targets including KRAS and MAX. This post-transcriptional regulation of KRAS and MAX provides an additional level of control of the oncogenic potential of RAS and MYC pathways. Because of their small size, miRNA replacement therapies appear particularly suitable for the treatment of cancers (3). In light of the sensitivity of cSCC cells to restored miR-193b-3p or miR-365-3p expression, delivery of these miRNAs could provide promising future adjuvant antitumoral strategies.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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