Telomere crisis in kidney epithelial cells promotes the acquisition of a microRNA signature retrieved in aggressive renal cell carcinomas

Luis Jaime Castro-Vega, Karina Jourvarleva, Win-Yan Liu, Carolina Martinez, Pierre Gestraud, Philippe Hupe, Nicolas Servant, Benoît Albaud, David Gentien, Sophie Gad, Stéphane Richard, Silvia Bacchetti and Arturo Londoño-Vallejo

1UMR3244, Telomeres and Cancer Laboratory, Institut Curie, 26 rue d’Ulm, Paris 75248, France, *UPMC University, Paris 06, P F-75005, France, 2INSERM U932, Immunity and Cancer, Institut Curie, 26 rue d’Ulm, Paris 75248, France, 3INSERM U900, Institut Curie, 26 rue d’Ulm, Paris 75248, France, 4 Mines ParisTech, Centre for Computational Biology, 35 rue Saint Honoré, Fontainebleau, Paris 77305, France, 5 CNRS-UFR414, Institut Curie, 26 rue d’Ulm, Paris 75248, France, 6Department of Translational Research, Institut Curie, 26 rue d’Ulm, Paris 75248, France, 7INSERM U753, Génomique Oncologique EPHÉ, Institut de cancérologie Gustave Roussy, 114 rue Edouard Vaillant, Villejuif 94800, France, and 8Department of Experimental Oncology, Istituto Regina Elena, Rome 00158, Italy

Telomere shortening is a major source of chromosome instability (CIN) at early stages during carcinogenesis. However, the mechanisms through which telomere-driven CIN (T-CIN) contributes to the acquisition of tumor phenotypes remain uncharacterized. We discovered that human epithelial kidney cells undergoing T-CIN display massive microRNA (miR) expression changes that are not related to local losses or gains. This widespread miR deregulation encompasses a miR-200-dependent epithelial-to-mesenchymal transition (EMT) that confers to immortalized pre-tumoral cells phenotypic traits of metastatic potential. Remarkably, a miR signature of these cells, comprising a downregulation of miRs with conserved expression in kidney, was retrieved in poorly differentiated aggressive renal cell carcinomas. Our results reveal an unanticipated connection between telomere crisis and the activation of the EMT program that occurs at pre-invasive stages of epithelial cancers, through mechanisms that involve miR deregulation. Thus, this study provides a new rational into how telomere instability contributes to the acquisition of the malignant phenotype.

Introduction

Telomere shortening is frequently detected at early stages of human epithelial cancers (1,2) and likely contributes to chromosome instability (CIN), a hallmark of cancer cells (3,4). Recent studies on the evolution of genome instability in cancer have identified distinctive patterns of CIN that could be explained by a mechanism of breakage-fusion-bridge (BFB) cycles following telomere dysfunction (5). However, the contribution of such rearrangements to tumor progression remains largely uncharacterized. On the other hand, mouse models with short telomeres have demonstrated that telomere-driven CIN (T-CIN) plays a central role in the promotion and progression of epithelial cancers through amplifications and losses of cancer genes (6–10). Moreover, it has been recently shown that a transient period of T-CIN followed by the reactivation of telomerase contributes to the acquisition of the metastatic phenotype, presumably by the selection of clones carrying mutations in specific loci (11). However, the genetic elements impinged upon by T-CIN in human tumors, particularly from epithelial origin, remain largely undetermined.

MicroRNAs (miRs) are small non-coding RNAs that play a critical role in gene expression regulation through post-transcriptional silencing (12). As miRs participate in key cellular processes that are disrupted in cancer cells, it is not surprising that the altered expression of these molecules has been found in several types of human cancers (13). Interestingly, it has been shown that miR genes are located in cancer-associated genomic regions that are preferentially affected by genome instability events (14). In this study, we have used human epithelial kidney cells (HEK cells), a well-established in vitro model of progressive telomere instability, to study the impact of T-CIN in the expression of miRs and the biological consequences that might be relevant for the acquisition of tumor phenotypes.

Materials and methods

Cell culture

HEK cells were maintained under standard culture conditions in a humidified 5% CO2 atmosphere at 37°C in modified Eagle’s culture media without deoxyribonucleases (Invitrogen) and supplemented with 10% fetal bovine serum, essential amino acids and sodium pyruvate. Cell growth was monitored by cell counting and population doublings (PDs) were calculated by using the formula: number of PDs = (log [final count] – log [initial count])/0.301.

Cell transfection

For plasmid transfections, DNA was purified by standard methods using the Maxiprep kit (Qiagen) and 20 µg of plasmids were used to transfect the cells. The following plasmids were used: pSV3 Neo (simian virus 40 SV40), pLXSP-hTERT Puro (human telomerase reverse transcriptase [hTERT]), pBabe Puro (rat sarcoma viral oncogene homolog [RAS]) and pRetro-Super (shATM). Medium was changed 6 h after transfections and antibiotic selection was initiated in the following 24–48 h. For HEK cells, 0.5 µg/ml of puromycin and up to 450 µg/ml of G418 were used. Selection is completed in 1 week after which non-transfected controls have died off. Transfections of miRs were carried out using synthetic miR Precursor Molecules (Ambion Pre-miR) and Lipofectamine 2000 (Invitrogen) as transfection reagent. Cells were transfected with a mixture of pre-miR-200a, pre-miR-200b, pre-miR-141 and pre-miR-200c at a final concentration of 100nM. An equivalent amount of Precursor Negative Control (Ambion) was used as control. Cells were transfected twice with an interval of 48 h and then analyzed.

Western blot

Cells were harvested at 75% confluence and lysed in RIPA buffer with protease (Roche) and phosphatase (Thermo scientific) inhibitors. Protein quantifications were performed using the Pierce BCA Protein Assay kit (Thermo scientific), and 20–40 µg protein was analyzed either in 3–8% Tris-acetate or in 4–12% Bis-Tris gels. Broad range protein marker (Fermentas) was used as ladder. Transfer was performed for 2h at 25V or for 10min when an iBlot gel transfer system (Invitrogen) was used. Blocking was performed with 5% milk or 5% bovine serum albumin for 1–2h at room temperature for phosphorylated proteins and primary antibodies were incubated at 4°C overnight. Detection was performed using the ECL plus kit (Amersham) and photos were taken at different exposition times. The following antibodies with their respective specifications were used: mouse monoclonal 419 (LT-SV40; provided by Dr Silvia Bacchetti) dilution 1:1000; mouse monoclonal (ataxia telangiectasia mutated [ATM]) (Abcam; Ref. ab2618) dilution 1:500; mouse monoclonal pATM (Rockland; Ref. 200-301-400 dilution 1:500; rabbit polyclonal CHK23 (Abcam; Ref. ab8108) dilution 1:500; rabbit polyclonal pCHK2 (Cell Signalling; Ref. 2661) dilution 1:500; rabbit polyclonal H2AX (Abcam; Ref. ab1175) dilution 1:500; mouse monoclonal pH2AX (Millipore; Ref. 05-636) dilution 1:500; rabbit polyclonal (telomeric repeat binding factor 2 [TRF2]) (Novus Biologicals; Ref. NB110-57130) dilution 1:500; mouse monoclonal Pan-cytokeratin (Sigma; Ref. C2562) dilution 1:500 00; mouse monoclonal E-cadherin (BD Biosciences; Ref. 610182) dilution 1:5000; rabbit polyclonal transforming growth factor-β1/2/3 (Santa Cruz Biotechnology; Ref. sc-7892).

Abbreviations: BFB, breakage-fusion-bridge; CIN, chromosome instability; DDR, DNA damage responses; ER-SV40, early region of SV40; EMT, epithelial-to-mesenchymal transition; HEK cells, human epithelial kidney cells; hTERT, human telomerase reverse transcriptase; miR, microRNA; RT-qPCR, real-time–quantitative PCR; SSC, standard saline citrate; SV40, simian virus 40; T-CIN, telomere-driven CIN.
DNA extractions were performed by using the method of phenol–chloroform previous digestion with proteinase K. For teloblot, 5–10 μg DNA was digested with 50U HindIII and RsaI enzymes overnight. Then, samples and DNA marker (Low Range PFK marker, Biolabs) were loaded onto an 1% agarose gel in 0.5X Tris–Borate–EDTA buffer. The clamped homogeneous electric field (CHEF) electrophoresis conditions are as follows: initial pulse: 0.1s; final pulse: 2.5s; time: 8h and voltage: 200V or 65V/cm². Gels were stained with ethidium bromide for 10min and destained with Nanopure water for 20min. DNA was broken with a ultraviolet crosslinker set to 1800x100J/K (about 1 min). Gels are denatured and transferred in alkaline conditions (0.6 M NaCl, 0.4 M NaOH) for up to 48h. Fixation and neutralization steps were performed in 0.4 N HCl, 3× SSC, 10% Tris and 250μg/ml of RNAse A at 42°C for 30min. After washing steps, membranes were exposed for 1–2 days and scanned for analysis.

**Immunofluorescence**

The day before, 20 000 cells per well were seeded on microscope slides (Thermo scientific). For F-actin staining, cells were washed with phosphate-buffered saline 1× and fixed in 3% formaldehyde and 2% sucrose for 15min. Next, cells were washed twice and permeabilized for 10min. Stock solutions of phallolidin–tetramethylrhodamine isothiocyanate (TRITC) (Sigma) conjugates have been made in dimethyl sulfoxide at 0.5μg/ml and used at a concentration of 1:400 in phosphate-buffered saline for 40min at room temperature. Cells were washed several times to remove the conjugate and stained with 0.5μg/ml 4,6-diamidino-2-phenylindole.

**Migration/invasion assays**

Wound healing assays were performed in cells at 75% confluence by scratch- ing with a p200 pipette tip. Images were captured at 0, 6 and 12h. At least 10 images at each time point in two independent experiments were used for analysis and the percentage of invaded area at each time was estimated for quantifications. For matrigel invasion assays, chambers were rinsed with media without serum 4h before the assay. Then, a coat of matrigel (diluted one-third in media without serum) is applied in the upper side of the chamber and incubated for 1h. A total of 200 000 non-starved cells were seeded for analysis; 10% fetal bovine serum was used as chemoattractant on the bottom side of the chamber. Cells were allowed to traverse the matrigel and filter for a period of 72h after which they were fixed with methanol for 15min and stained with 0.2% crystal violet in water for 1h. Cells from the upper side of the chamber were removed with cotton swabs. A total of 10 images from two independent experiments were used for quantifications. For matrigel invasion assays, chambers were rinsed with media without serum 4h before the assay. Then, a coat of matrigel (diluted one-third in media without serum) is applied in the upper side of the chamber and incubated for 1h. A total of 200 000 non-starved cells were seeded for analysis; 10% fetal bovine serum was used as chemoattractant on the bottom side of the chamber. Cells were allowed to traverse the matrigel and filter for a period of 72h after which they were fixed with methanol for 15min and stained with 0.2% crystal violet in water for 1h. Cells from the upper side of the chamber were removed with cotton swabs. A total of 10 images from two independent experiments were used for quantifications.

**Array-comparative genomic hybridization**

To characterize genome instability in HEK cells, human CGH 4×72K Whole- Genomic Tiling Arrays ( NimbleGen) were used. This platform carries syn- thetic probes representing loci spaced on average of every 40 Kb. H1A-Early cells were used as normal controls of hybridizations. DNAs for analyses were digested at 100–200ng/ml and quality was checked by Nanodrop and Qubit (Invitrogen). Labeling reactions (Cy3 normal/Cy5 tumoral) were performed by random priming method using the BioPrime kit (Invitrogen) and 500ng DNA. After precipitation, DNA was resuspended in hybridization buffer and hybridizations were performed at 42°C overnight in hybridization chambers. After washing steps, slides were scanned using Genepix 400B (Axin scanner). Data were uploaded to the VAMP (Visualization and Analysis of array-CGH, Transcriptome and other molecular profiles) platform at the Curie Institute (12) and CGH data were normalized by using Micro-Array Normalization of array-CGH data (MANOR) (13) and segmented by using Gain and Loss Analysis of DNA (GLAD) (14).

**RNA sequencing**

Total RNA was purified from immortalized and non-immortalized CIN– and CIN+ HEK cells using miRNA easy (Qiagen). Quality of preparations was checked using Agilent Bioanalyzer 2100 and only RNAs with a RNA integrity number (RIN) > 8.5 were used for subsequent analyses. Small (20–22 nt) RNA libraries were prepared in duplicate with size-purified RNA isolated from different batches of cells. Approximately, 100 μg of total RNA was submitted to the InqGel platform (Gif-sur-Yvette, France) for Illumina sequencing. This procedure was performed by fractionating total RNA using polyacrylamide gel electrophoresis to isolate 18–30nt small RNAs. 3’ and 5’ adapters were ligated to the small RNAs and constructs were amplified following real-time–PCR (RT–PCR) following the conditions specified in the 36 cycles Sequencing Kit v5 (FC1045001, Illumina) protocol. The small RNA library was sequenced using a Solexa/Illumina GA-Ix Genome analyzer. Small RNA sequences were analyzed using the ncPRO-seq pipeline ( http://ncproseq.sourceforge.net) (15). Briefly, reads were mapped to the human genome (hg19) using the Bowtie aligner (16) allowing up to 20 genomic match positions. The genomic positions of mature miRs and miR* were obtained from the mirBase database (17). Aligned reads were then considered to corre- spond to a mature miR (or miR*) only if the aligned position did not differ from the annotated position of the mature miR (or miR*) by more than 2bp. In order to be able to compare miR expression levels and profiles between libraries, miR read counts were normalized using a two-step procedure. The normalized miRs read counts were then assessed for differential analysis using the R/Bioconductor package DESeq (18) and miRs with an adjusted P value (adj P < 0.05) were reported as significant.

**Expression profiling of mature miRs**

Screening for miR expression was performed using the Affymetrix miRNA Galaxy arrays following the manufacturer’s protocol. Raw data were uploaded onto the VAMP platform, normalized using standard procedures and analyzed. Differentially expressed miRs between two independent RT–qPCR reactions, and qPCR reactions were carried out using miRNA locked nucleic acid (LNA) primers in a Roche LightCycler 480. The expression of hsa-miR-31 was used as endogenous control for normalization and relative quantities were calculated with the 2−ΔΔCT method (19).

**Expression profiling of pre-miRs**

A total of 178 precursors were analyzed using the MiRmaid miRNA Precursors RT-qPCR primer set (Eurogentec). Universal RT reactions were purified and then amplified using specific pre-miR primers. The qPCR reaction was performed in ABI Prism 7500 thermal cycler (Applied Biosystems). Amplifications with Ct values > 38 and unspecific amplifications (more than two peaks in the melting curve) were considered not reliable for analysis. Analysis of two independent controls (5S, TBP, HBMS) was used for normalization (20). A second method of normalization was performed using the average of the whole set of miRs present in the same PCR plate (21). Analysis of differentially expressed miR was performed using a two-tail t-test. The false discovery rate was controlled using the Benjamini–Hochberg procedure, and a P value <0.05 was considered significant. All the analyses were performed using R software with Bioconductor packages and custom functions defined at the Institut Curie Bioinformatics group (http://bioinfo. curie.fr/projects/ema).

**Expression profiling of primary miRs**

RNA samples were treated with DNase using TURBO-DNA-free (Ambion). The protocol includes a universal RT reaction step using the high-capacity RNA-to- cDNA kit (Applied Biosystems) followed by detection of primary miR by specific Tagman Pri-miRNA assays (Applied Biosystems). In addition, a Taqman assay for RNU24 was used for normalization. The qPCR reaction was performed in ABI Prism 7500 thermal cycler (Applied Biosystems). Relative quantitations were calculated with the 2−ΔΔCT method (19).

**Transcriptome analysis**

Gene expression profiling was performed using the U133 Plus 2.0 Array platform that contains 47 000 transcripts. Complementary DNA synthesis, fragmentation and hybridization were carried out following an optimized protocol at the Institut Curie Translational Platform. After scanning, data were normalized using GC robust multi-array average (GCRMA) and non-detected probesets were removed (using a threshold of 3.5 in log scale on the average value for each probeset), leaving 25 022 probesets for the analysis. Clustering analyses were performed using Pearson’s correlation as distance and Ward’s linkage. Differentially expressed genes between pre-crisis and post-crisis samples were assessed with r-test and false discovery rate was controlled by Benjamini–Hochberg procedure.

**Clinical samples**

A series of 33 human renal clear cell carcinomas were obtained from the French Kidney Cancer Consortium co-ordinated by S.Richard. This study was approved by the ethical committee of Le Kremlin-Bicêtre University Hospital, France. All patients had provided informed consent before surgery for use of their tumors for further investigation, and samples were frozen immediately in liquid nitrogen. RNAs were extracted using Trizol reagent (Invitrogen).
Telomere crisis induces miR deregulation and EMT

Results

HEK cells model of T-CIN

In this model, primary HEK cells are transfected with the early region of SV40 (ER-SV40), which drives the expression of both large T and small t antigens, to allow them to divide 80–90 times before entering crisis, a period characterized by massive cell death due to rampant CIN (22) (Figure 1A). We have shown previously that T-CIN takes place around population doubling 50 (PD50) and is driven by the shortest telomeres in these cells (23). Using a particular clone (HA1), isolated after introduction of ER-SV40, it is possible to reproducibly recover telomerase positive post-crisis cells with critically short telomeres, whereas other clones (HA2–HA5) never give rise to survivors (22) (Figure 1A–C). On the other hand, the introduction of hTERT in clones HA1 and HA5 before PD50 allows the immortalization of cells without CIN (HA1-early and HA5-early), whereas introduction of hTERT after this point in clone HA5 leads to immortalization of karyotypically abnormal CIN+ cells (HA5-late) as determined by array-comparative genomic hybridization (Figure 1A and Supplementary Figure S1, available at Carcinogenesis Online). This system provides a perfect experimental setup to study the impact of T-CIN on gene expression and the derived biological consequences by comparison of CIN+ versus CIN− cells.

HEK cells with T-CIN exhibit massive miR deregulation

To explore the extent to which T-CIN impacts the gene expression landscape, we chose to monitor miRs, as it has been suggested that genomic regions carrying these genes may be preferentially affected by genome instability events (24). We performed a comparison of the miR expression profiles between CIN+ and CIN− cells derived from the same HEK-ER-SV40 clone using a next-generation sequencing approach (Supplementary Figure S2A, available at Carcinogenesis Online). We found that of about 1008 miRs that are detected in HA5-derived cells, 538 (53.3%) were significantly deregulated (adj P < 0.05) in HA5-late versus HA5-early cells (Figure 2A and Supplementary Table S1, available at Carcinogenesis Online). A comparison between the 1038 miRs expressed in HA1-derived cells revealed a similar result, with 395 miRs (38.1%) significantly deregulated (adj P < 0.05) in post-crisis PC1 versus HA1-early cells (Supplementary Figure S2B and Table S2, available at Carcinogenesis Online). In all, 138 miRs presented variations in the same direction (Supplementary Figure S2C, available at Carcinogenesis Online), suggesting that a significant proportion of miRs were similarly affected, independently of the genome instability history of both clones. This assumption was confirmed using array hybridization in additional HEK cells, in which around 16% of miRs were deregulated in CIN+ versus CIN− cells (P < 0.05) (Supplementary Figure S3A and Table S3, available at Carcinogenesis Online). Although array hybridization is less sensitive than massive sequencing, there is a significant correlation between the expression changes detected by both methods (Supplementary Figure S3B, available at Carcinogenesis Online). Furthermore, a fraction of the differentially expressed miRs was confirmed by RT–qPCR (Supplementary Figure S3C, available at Carcinogenesis Online).

Intriguingly, these validated miRs have been found deregulated in human renal cell carcinomas (25,26).

To determine whether the miR deregulation exhibited by cells with T-CIN is restricted to mature strands, we examined the expression of pre-miRs using the same set of RNA samples. A total of 172 pre-miRs were measured by RT–qPCR, from which 102 were expressed across the HEK cells. An unsupervised clustering analysis readily distinguished CIN+ from CIN− cells and, similarly to the sequencing
data for mature strands, this deregulation was significant for half of the pre-miRs analyzed (adj $P < 0.05$) (Supplementary Figure S4A and Table S4, available at Carcinogenesis Online). There is also a significant correlation between the expression changes detected for pre-miRs and mature strands (Supplementary Figure S4B, available at Carcinogenesis Online). To further test whether this miR deregulation is also reflected at the level of transcription, we measured the expression of some primary strands (pri-miRs) in HA5-late versus HA5-early cells (Supplementary Figure S4C, available at Carcinogenesis Online). Most, albeit not all, tested miRs showed a co-deregulation at the levels of both primary and mature strands, suggesting that transcription contributes to miR deregulation in the context of T-CIN. Collectively, these data indicate a massive deregulation of miR expression associated with T-CIN. Notably, changes in miR expression are completely prevented if telomerase is introduced before, but not after, the initiation of T-CIN (Supplementary Figures S3A and S4A, available at Carcinogenesis Online), demonstrating that telomere instability is responsible for the observed miR deregulation. As this miR deregulation could be related to genomic aberrations induced by T-CIN, we closely examined the array-comparative genomic hybridization profiles of immortalized CIN+ HEK cells. Intriguingly, only 12 miR-altered loci in at least three out of six CIN+ cells overlap a significant change in the expression of pre-miRs (Supplementary Figure S4D and Table S5, available at Carcinogenesis Online), suggesting that genomic gains and losses had a limited contribution to the miR deregulation in cells that underwent T-CIN.

Ongoing T-CIN triggers transcriptional miR deregulation in the absence of an overt DNA damage response

To determine the time at which T-CIN induces the miR deregulation, we monitored proliferating, non-immortalized HEK cells before and after the initiation of CIN. We found that changes in the expression of most of the mature miRs examined were similar in clones HA1 and HA5 (Supplementary Figure S5A, available at Carcinogenesis Online), with increased deregulation with increasing passages, and a maximum fold change in crisis. When pre-miRs were monitored, a shift in the global expression profile was detected in cells undergoing active T-CIN, a few passages after the initiation of BFBs (Figure 2B and C). This massive deregulation was similar in different clones as well as in polyclonal populations and did not induce changes in cell growth kinetics (Figure 2C). Interestingly, the detected changes affected in a similar way miRs belonging to the same family or to the same genomic cluster suggesting a concerted transcriptional response. In fact, we also detected changes in the expression of pri-miRs, which also tend to accumulate soon after the initiation of CIN (Supplementary Figure S5B, available at Carcinogenesis Online), demonstrating that T-CIN directly impacts the expression of miRs at the transcriptional level.

It has been shown that DNA damage responses (DDR) impact miR expression (25). To test whether the miR transcriptional deregulation induced by T-CIN is linked to a DDR due to telomere shortening (26,27), we evaluated the presence of marks for DDR activation by western blot. HEK cells examined after the initiation of T-CIN did not exhibit the classic marks for DDR activation by the time we detected the first changes in pri-miR or pre-miR expression levels (PD55-60) (Supplementary Figure S6A, available at Carcinogenesis Online), suggesting that the miR deregulation induced by T-CIN did not require a strong DDR. Supporting this interpretation is the fact that a partial depletion of ATM did not prevent the pre-miR changes exhibited by cells undergoing T-CIN (Supplementary Figure S6B–D, available at Carcinogenesis Online).
Telomere crisis induces miR deregulation and EMT

To determine whether the miR deregulation induced by T-CIN can be also found in situations of telomere dysfunction that are independent of telomere shortening, we evaluated miR expression changes upon depletion of TRF2, an essential component of the telomeric shelterin complex that prevents the recognition of chromosome extremities by the DNA damage machinery (28). As complete loss of TRF2 induces growth arrest (29), we aimed at achieving a partial depletion to allow cells to cycle and to be chronically exposed to telomere dysfunction. Three rounds of transfections with a specific small interfering RNA against TRF2 led to 70% depletion of the protein (Supplementary Figure S7A, available at Carcinogenesis Online). This depletion was associated with a 3-fold increase in the percentage of cells with more than two telomere dysfunction-induced foci, as compared with cells transfected with the small interfering RNA control, indicating the presence of DNA damage due to uncapped telomeres (Supplementary Figure S7B and C, available at Carcinogenesis Online).

Although TRF2 depletion leads to changes in expression of some mature miRs that were also observed in cells undergoing T-CIN (Supplementary Figure S7D, available at Carcinogenesis Online), the impact was quite different at the level of pri-miRs, which showed lower expression upon TRF2 depletion (Supplementary Figure S7E, available at Carcinogenesis Online), whereas cells reaching crisis pri-miRs tend to be upregulated. This experiment, while supporting the notion that telomere dysfunction influences miR transcription, suggests that different mechanisms of telomere uncapping may have a different biological outcome.

**T-CIN leads a miR-200-dependent transdifferentiation**

In order to examine the biological consequences of the miR deregulation induced by T-CIN on cell phenotypes, we focused on the miR-200 because members of this family were downregulated in CIN+ cells in most of the comparisons we performed. We confirmed by RT–qPCR that the cluster located on 1p36.33 (miR-200a and miR-200b), which is robustly expressed in primary and CIN− cells, was significantly downregulated in CIN+ cells. Similarly, the miR-200 cluster located on 12p13.31 (miR-141 and miR-200c), which is poorly expressed in primary and CIN− cells, was further downregulated (Figure 3A). The miR-200 family has been directly implicated in the induction of epithelial-to-mesenchymal transition (EMT) (30,31), a physiological phenomenon during organismal development, which is also considered relevant for metastasis (32). This prompted us to examine the phenotypical features of CIN+ HEK cells.

Microscopic evaluation revealed that CIN+ cells have lost the rounded, cobblestone morphology typical of epithelial cells and display a more elongated, spindle-like shape typical of fibroblasts with a characteristic orientation of actin microfilaments (Figure 3B). In addition, all— but one—CIN+ cells showed modified expression of epithelial markers, E-cadherin and cytokeratins, and enhanced expression of transforming growth factor-β and ZEB1, two well-known EMT inducers (Figure 3C). We also detected a change in the proportion of cells expressing CD24 (epithelial) or CD44 (mesenchymal) surface markers (33), which becomes evident in crisis (Figure 3D), as well as enhanced migration and invasion capacities in CIN+ cells (Supplementary Figure S8A–C, available at Carcinogenesis Online), suggesting that CIN+ HEK cells underwent transdifferentiation. In keeping with these observations, transcriptome analyses identified 878 genes differentially expressed between CIN+ and CIN− cells that are involved in EMT-related signaling pathways (Supplementary Figures S9A and B and Table S6, available at Carcinogenesis Online); indeed several genes downregulated in

![Fig. 3.](https://academic.oup.com/carcin/article-abstract/34/5/1173/2463270/1177)
CIN+ cells are involved in kidney development (Supplementary Table S7, available at Carcinogenesis Online), supporting the notion that T-CIN impinges on the differentiation program of renal epithelial cells. To test whether the downregulation of the miR-200 family in CIN+ HEK cells is responsible for the observed EMT, as described for other epithelial systems (30–32), we transfected these cells with a mixture of pre-miRs representing the miR-200 family (Figure 4). This experiment shows that re-expression of these miRs in CIN+ HEK cells is sufficient to restore the epithelial phenotype (Figure 4).

The miR expression signature of post-crisis HEK cells is displayed by advanced renal cell carcinomas

Several studies have shown a deregulated expression of miRs in renal cell carcinomas when compared with normal tissues (34,35). Some of the miR changes frequently found across published studies include overexpression of members of the miR-17–92 cluster, miR-224 and miR-34a, as well as downregulation of members of the miR-200 family and the miR-143/145 cluster, the latter being associated with early renal cancer relapse (26). Remarkably, all these miRs are deregulated in HEK cells undergoing T-CIN, and this deregulation persists in immortalized CIN+ cells. Because short telomeres are associated with a high incidence of chromosome abnormalities (36) and higher risk of renal cancer (37), we examined the in vivo relevance of the miR expression pattern acquired by HEK cells upon T-CIN. To this end, we defined a signature composed of a set of 20 miRs that were significantly downregulated in post-crisis HEK cells and which belong to a core signature of miRs expressed in normal kidney tissues (38). This signature was used to interrogate a set of human renal cell carcinomas (Gad et al., in preparation). Unsupervised clustering analysis indicated that this miR signature correctly classifies 28 out of 33 renal tumors (Figure 5 and Supplementary Table S8, available at Carcinogenesis Online). Outstandingly, advanced renal carcinomas with the highest Fuhrman’s nuclear grade and tumor stage are considered poorly differentiated and exhibit, as post-crisis HEK cells, low expression levels of these miRs.

Discussion

This work provides unprecedented evidence indicating that T-CIN in human epithelial cells induces widespread changes in miR expression ultimately leading to a major perturbation in the differentiation program. Interestingly, T-CIN impacts miR expression at the transcriptional level soon after the initiation of BFB cycles in the absence of a strong DDR. Given the reproducibility of miR expression changes in clonal and polyclonal cell populations in which chromosome rearrangements accumulate stochastically, it is very likely that this transcriptional activity is independent of local rearrangements. Alternatively, there is a possibility, although this remains to be shown, that changes in miR expression are linked to pervasive epigenetic changes. Such changes could be related either to the spreading in cis of chromatin changes that reach far away from the point of double-strand breaks or to the activation in trans of chromatin remodeling complexes able to impact the transcription program of the cell.

Remarkably, the miR deregulation induced by T-CIN recapitulated the most common miR expression changes described in renal cancers that have been proposed to be associated with tumor progression. Specifically, we showed that a downregulation of the miR-200 family is directly responsible for the activation of the EMT program in CIN+ cells. Of note, although telomere crisis and EMT have been proposed
Telomere crisis induces miR deregulation and EMT

Conclusions

This study shows that in the context of telomere instability, there is a vast deregulation in miRs transcription that is not connected with corresponding DNA copy number changes. We demonstrated that HEK cells that have traversed a period of T-CIN both display low expression levels of genes and miRs that are normally expressed in kidney tissue and undergo some instability is arrested in immortal cells which express telomerase activity. Additionally, we observed that miRs with altered expression in HEK post-crisis cells were found in high-grade undifferentiated aggressors and that belong to a core signature of normal kidney tissue. The heatmap was generated using a miR expression pattern displayed by transdifferentiated post-crisis HEK cells was also retrieved in poorly differentiated, high-grade renal tumors. The EMT program has been fully documented as being required for the acquisition of metastatic potential (41). Intriguingly, however, post-crisis HEK cells are poorly tumorigenic (data not shown), as opposed to immortalized HEK cells expressing oncogenic RAS (42). Nevertheless, as circulating tumor cells can be detected at early stages during tumorigenesis (40), our data support a model whereby telomere shortening at early stages of human epithelial cancers might promote the acquisition of the malignant phenotype, perhaps with post-crisis cells reflecting an intermediate step in the road to oncogenic transformation. Therefore, we provide a new mechanistic rational to understand the long-standing association among aging, short telomeres and the progression of carcinomas toward the metastatic disease.

Supplementary material

Supplementary Tables 1–8 and Figures 1–9 can be found at http://carcin.oxfordjournals.org/

Acknowledgement

We thank the Somatic Genetics team at the Curie Institute for their help with array-comparative genomic hybridization experiments. We thank Eric Gilson, Martha Stumper and Judy Campisi for providing reagents and their helpful discussions on this study, and we thank members of the Telomere & Cancer lab for their useful comment on the manuscript. We also thank the French Kidney Cancer Consortium (especially Vincent Moliné, Virginie Verkarre, Sophie Ferlicot and Arnaud Méjean) and the Tumorothèques Necker-Enfants Malades et Antoine Béclère for providing the tumor samples. We are also grateful to the Genomics Platform of Gustave Roussy Institute for their technical assistance.

Conflict of Interest Statement: None declared.

References


Received September 18, 2012; revised December 16, 2012; accepted January 19, 2013