Epigenetic deregulation of *TCF21* inhibits metastasis suppressor *KISS1* in metastatic melanoma

Khelifa Arab¹, Laura T.Smith², Andreas Gast^{3,7},
Dieter Weichenhan¹, Joseph Po-Hsien Huang¹,
Rainer Claus¹, Thomas Hielscher⁴, Allan V.Espinosa⁵,
Matthew D.Ringel⁵, Carl D.Morrison⁶, Dirk Schadendorf⁷,
Rajiv Kumar³ and Christoph Plass^{1,2,*}

¹Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany,

²Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, 43210 OH, USA, ³Division of Molecular Genetic Epidemiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, ⁴Division of Biostatistics, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, ⁵Department of Medicine, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, The Ohio State University, Columbus, 43210 OH, USA and ⁶Division of Molecular Pathology, Roswell Park Cancer Institute, Buffalo, 14263 NY, USA

⁷Present address: Department of Dermatology, University Hospital Essen, 45147 Essen (former address: Skin Cancer Unit, German Cancer Research Center (DKFZ), University Hospital Mannheim, 68167 Mannheim) Germany

*To whom correspondence should be addressed. Tel: +49 6221 423330;
Fax: +49 6221 423359;
Email: c.plass@dkfz.de

Metastatic melanoma is a fatal disease due to the lack of successful therapies and biomarkers for early detection and its incidence has been increasing. Genetic studies have defined recurrent chromosomal aberrations, suggesting the location of either tumor suppressor genes or oncogenes. Transcription factor 21 (TCF21) belongs to the class A of the basic helix-loop-helix family with reported functions in early lung and kidney development as well as tumor suppressor function in the malignancies of the lung and head and neck. In this study, we combined quantitative DNA methylation analysis in patient biopsies and in their derived cell lines to demonstrate that TCF21 expression is downregulated in metastatic melanoma by promoter hypermethylation and TCF21 promoter DNA methylation is correlated with decreased survival in metastatic skin melanoma patients. In addition, the chromosomal location of TCF21 on 6q23–q24 coincides with the location of a postulated metastasis suppressor in melanoma. Functionally, TCF21 binds the promoter of the melanoma metastasis-suppressing gene, *KISS1*, and enhances its gene expression through interaction with E12, a TCF3 isoform and with TCF12. Loss of TCF21 expression results in loss of *KISS1* expression through loss of direct interaction of TCF21 at the *KISS1* promoter. Finally, overexpression of TCF21 inhibits motility of C8161 melanoma cells. These data suggest that epigenetic downregulation of TCF21 is functionally involved in melanoma progression and that it may serve as a biomarker for aggressive tumor behavior.

Introduction

The World Health Organization reported in 2008 an incidence of almost 2 million melanoma patients per year worldwide with an estimated mortality of 46 000 cases, classifying melanoma as the prime cause of death from skin cancers (1). Metastatic melanoma is a fatal disease with rapidly increasing incidence over the recent years. Although limited localized melanomas can be successfully treated surgically, this is not the case for more advanced melanomas resulting in poor survival rates (2).

Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; cDNA, complementary DNA; mRNA, messenger RNA; LOH, loss of heterozygosity; PCR, polymerase chain reaction; SFM, survival following the first metastasis.

Metastasis suppressor genes have been identified through low-resolution genomic scans, but to date, only few such genes have been identified (3). Loss or translocations of chromosome 6q have been described in many human malignancies including melanoma (4–6). In particular, loss of heterozygosity at 6q22 and 6q23–q24 has been associated with malignant melanomas, lung, head and neck, breast and ovarian tumors and was more frequently observed in metastasized tumors (7–9). Microcell-mediated chromosome transfer of chromosome 6q, specifically, a 45 Mbp region along 6q16.3–q23, was able to suppress metastasis of the highly invasive C8161 melanoma cell line, suggesting the presence of a metastasis suppressor gene in this region (10–13). Further support for a definitive link between 6q16.3–q23 and *KISS1* transcriptional downregulation was demonstrated by a loss of heterozygosity (LOH) scan in primary melanomas, where LOH of 6q16.3–q23 was found to be significantly correlated with loss of *KISS1* expression (11). An epigenetic scan identified silencing of a novel tumor suppressor gene, *TCF21* which is located in 6q23 and involved in repression of epithelial–mesenchymal transition markers (14). During normal embryogenesis, TCF21, a basic helix-loop-helix transcription factor, is important for mesenchymal to epithelial transitions (15). Although the reverse process, epithelial–mesenchymal transition, has proven vital for dissemination of carcinoma cells, *TCF21* silencing and its function in human malignancies have only recently been examined.

Here, we investigate the silencing of *TCF21* by DNA methylation in metastatic melanoma patients. We demonstrate that *TCF21* directly interacts with the promoter of the metastasis suppressor *KISS1* and regulates its expression. Furthermore, our results suggest that the status of epigenetic silencing of TCF21 could be a useful marker for poor prognosis.

Materials and methods

Tissue DNA, cell cultures and patient data

DNA was extracted from 79 biopsies of metastatic tumors from melanoma patients recruited at the Skin Cancer Unit at the Mannheim University Hospital, Germany, according to the eligibility criteria that included histologically confirmed melanoma of skin, The American Joint Committee on Cancer (AJCC), stage I–IV (supplementary Table S1 is available at *Carcinogenesis* Online). Median and mean age of the melanoma cases at diagnosis was 52 and 51.2, respectively (ranging from 12 to 85 years old). The mean Breslow thickness of the tumors was 3.35 mm, ranged from 0.22 to 17 mm. The ethical approval for the study was granted by the Ethics Commission of the Faculty for Clinical Medicine at the University of Heidelberg. Characterization of 40 tumor-derived cell lines has been described previously (16). Corresponding to these, DNA from 31 samples of peripheral blood mononuclear cells was available (Supplementary Table S2 is available at *Carcinogenesis* Online). Wild-type C8161 (17) was kindly provided by Dr Mary Hendrix and C8161neo6q clones (C8161neo6q.1, C8161neo6q.2 and C8161neo6q.3) were provided by Dr Bernard Weissman. Cell lines were cultured in RPMI 1640 media with 10% fetal calf serum, 5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded and cultured for 24 h, then treated with 1.5 µM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich, Steinheim, Germany). Media was refreshed and 5-aza-dC was resubstituted daily for during 7 days.

DNA copy number analysis

For copy number (CN) analysis, we performed genotyping with the 250k StyI SNP array GeneChip® human mapping 500K array set (Affymetrix, Santa Clara, CA). CN analysis was conducted as described previously (16).

Quantitative DNA methylation analysis

High resolution quantitative assessment of DNA methylation was conducted using the MassARRAY system (Sequenom, San Diego, CA). Sodium bisulfite conversion was performed on 0.5 µg genomic DNA using the EZ DNA

Methylation Kit (Zymo Research, Freiburg, Germany). Polymerase chain reaction (PCR) amplification of target regions was followed by treatment with shrimp alkaline phosphatase, reverse transcription and subsequent base-specific cleavage as described previously (18). Cleavage products were subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry-based analysis. DNA methylation levels were quantified from mass spectra using the Epytyper software v1.0 (Sequenom). All primers used in this study were purchased from Sigma–Aldrich (all primer sequences available in supplementary Table S3, available at *Carcinogenesis* Online).

Gene expression with quantitative reverse transcription–PCR

One microgram of total RNA was used to generate oligo dT-primed complementary DNA (cDNA) using the Superscript II RT kit (Invitrogen, Darmstadt, Germany). Relative messenger RNA (mRNA) expression was assessed by TaqMan assay using Roche universal probes (Roche, Mannheim, Germany). Three housekeeping genes, *HPRT1*, *GAPDH* and β -*ACTIN*, were used for the $\Delta\Delta CT$ normalization method (all primer sequences are available in supplementary Table S3, available at *Carcinogenesis* Online).

C8161 fluorescence in situ hybridization

For fluorescence *in situ* hybridization (FISH), bacterial artificial chromosome clone RP11-465P13 containing human *TCF21* genomic DNA was labeled with Spectrum Orange deoxyuridine triphosphate (Vysis, Downers Grove, IL). The chromosome 6-specific probe CEP6 was labeled with Spectrum Green (Vysis). Hybridization was performed on C8161 cells using standard conditions (19).

KISS1 luciferase constructs

Genomic segments upstream of the *KISS1* transcription start site were PCR amplified using DNA derived from peripheral blood of a healthy donor. Purified PCR products were digested with KpnI and XhoI and directionally cloned into pGL4.10[luc2] (Promega, Mannheim, Germany). To generate a clone that deleted the putative *TCF21*-binding site [chromatin immunoprecipitation (ChIP) region 6], the plasmid was digested with SacI and religated. Mutagenesis constructs were generated according to the manufacturer's instructions (Agilent-Genomics, Waldbronn, Germany). For the luciferase assay, in brief, cells were incubated in the presence of the transfection mixture for 4 h followed by normal culture medium for 48 h. Subsequently, the luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega).

Cloning of the coding sequences genes and transfection

A *TCF21* cDNA clone was purchased from imaGENES (Berlin, Germany)® and recloned into vector pCS2+ which provides an HA-tag sequence at the 3' end of the cloned gene. Cloned cDNAs of transcription factor 4 (TCF4), transcription factor 12 (TCF12) and E12 and E47, the two isoforms of transcription factor 3 (TCF3) were kind gifts from Drs Y.S.Kim (Medical Genomics Research Center, KRIBB, South-Korea), M.Koegl (DKFZ core facility), M.Nakamura (Tokyo Medical and Dental University, Tokyo, Japan) and C.Murre (University of California, San Diego, CA), respectively. The cDNAs were cloned into the mammalian expression vector p-Flag-CMV using Gateway technology (Invitrogen). The corresponding proteins were flag-tagged at the N-terminus. All cDNA constructs were transfected into a melanoma cell line Mel-66a by electroporation (Amaza, Lonza Verviers, Belgium).

Coimmunoprecipitation, immunoblotting and chromatin immunoprecipitation

Pairwise combinations of cDNA constructs were transfected into Hek293 cells, followed 48 h later. The protocols for immunoblotting and immunoprecipitation have been described previously (20). ChIP assays were performed as previously published (20). Briefly, viable cells were washed with phosphate-buffered saline and crosslinked with 1% formaldehyde at room temperature for 10 min. Chromatin was fragmented and immunoprecipitation was carried out using specific antibodies. Enriched genomic DNA fragments were then quantified by real-time PCR.

TCF21 retroviral infection of C8161 cell lines

The viral particles production and the transductions are done as described previously (14). In brief, 24 h after the transduction, the infectious medium was removed; cells were washed with phosphate-buffered saline and recultivated. The next day, C8161 cells were washed, trypsinized and split 1:5 in selection culture medium with 5 μ g/ml Puromycin (Sigma–Aldrich).

Cell migration assay

About 5×10^4 of mycTCF21 or pBABE positive C8161 cells were seeded into the upper section of a Boyden chamber containing serum-free medium. The lower section, separated from the upper one by a membrane with 8 μ m pores, contained medium with serum. Cells were grown at 37°C for 15 h, fixed in methanol and stained with 0.05% crystal violet solution. Cells on top of the

membrane were photographed before and after incubation. To analyze migration ability, absorbance at 560 nm of stained cells in both chambers was measured.

Y2H library screening

Automated yeast two hybrid screens using human *TCF21* as bait encoded by full-length cDNA were performed by the DKFZ core facility essentially as described (21). Human cDNA libraries from kidney and lymph nodes (Clontech, Saint-Germain-en-Laye, France) and a full-length human cDNA library of 10,070 different genes were used (22).

Statistics and survival analysis

Association between MassARRAY-derived DNA methylation data and clinical covariates was assessed univariately for amplicon mean methylation levels. Survival analysis included 56 cases of cutaneous melanoma stage I and II out of the total of 79 cases of the investigated patient cohort. The association between methylation and overall survival and metastasis-free survival was tested in a Cox PH regression model based on mean DNA methylation levels of amplicon a1. Overall survival was defined as time (in years) from date of diagnosis till death. Metastasis-free survival was the time from diagnosis until the first metastasis (either regional or distant). For the survival analysis, only stage I and II were selected. For Kaplan–Meier analysis, patients were distributed in two groups according to their normal distributions by mean methylation. The follow-up of patients without metastases or who did not decease has been censored at the latest visit/last contact.

Results

Aberrant DNA methylation of the *TCF21* promoter and association with survival of patients with metastatic melanomas

Human *TCF21* encodes two transcripts that share the same 5' untranslated region but have different 3' untranslated regions. The open reading frames are identical (Figure 1A). Using the Epytyper assay, we quantitatively analyzed methylation of the *TCF21* promoter CpG island and flanking sequences (Figure 1B) in metastatic melanoma tissue biopsies from 79 patients (for patient datasupplementary Table S1 is available at *Carcinogenesis* Online) and in 61 low passed melanoma cell lines established from the same metastatic tumors. In the patient tumors, (upper panel of Figure 1B) all three examined amplicons (a1–a3) showed a widespread distribution of DNA methylation among the samples ranging from levels of >0.9 to <0.1. Analysis of the patient methylation data by the mean values of amplicon a1 and a2 identified two groups, high- and low-methylated patients (supplementary Figure S1 is available at *Carcinogenesis* Online and Figure 1B). In the melanoma cell lines (lower panel Figure 1B), the cell lines exhibit an overall increase of DNA methylation compared with the tumors. Analysis of DNA methylation and patient data showed no significant differences of clinical parameters including age at diagnosis, stage at diagnosis, location of tumor, Breslow thickness between the two groups. However, survival following the first metastasis (SFM) was significantly different ($P_{\log\text{-rank}} = 0.05$) between patients with low DNA methylation at amplicon a1 and those with high methylation (Figure 1C). Median SFM was 3.4 years for patients with low levels of DNA methylation, whereas median SFM was 1.8 years and the hazard ratio 2.05 for those with high methylation (Table I). There was a trend that patients with low methylation status had higher overall survival than those with high methylation status ($P = 0.055$). Moreover, methylation levels in tumor biopsies and corresponding cell lines exhibited positive correlation (Spearman coefficient $\rho = 0.59$, $P = 0.0001$; Figure 1D), indicating that the cell lines maintain and enforce the methylation patterns.

We used single-nucleotide polymorphism (SNP) microarray data available from 40 of the cell lines to identify CN variations in the *TCF21* gene region using peripheral blood mononuclear cells-DNA from the corresponding patients as a reference (16). Seventeen cell lines (42%) showed a normal diploid *TCF21* region, two (5%) had an additional copy and 21 (53%) had only one copy (supplementary Table S2 is available at *Carcinogenesis* Online). Ten of the 17 cell lines with diploid CN showed copy neutral LOH (also referred to as uniparental disomy). We also analyzed *TCF21* expression in 16 cell lines

Fig. 1. *TCF21* promoter methylation and transcriptional gene silencing in metastasis melanoma. (A) Composition of the human *TCF21* gene on the chromosome 6q23.3. (B) Heat maps display quantitative DNA methylation data. Positions of corresponding amplicons a1–a3 are shown relative to the TSS(+1) of *TCF21* and a CpG island (CGI, green bar) located in exon 1. Each column represents a CpG unit (consisting of a single CpG or several CpG dinucleotides), each row represents a sample. Relative methylation levels (mCpG) range from 0.0 (light yellow) to 1.0 (dark blue). Gray indicates unavailable data. Upper part, data of 79 metastasis melanoma skin cancers biopsies sorted by the highest to lowest mean value of amplicon a1 and separated into high methylated ($\geq 50\%$) and low methylated ($< 50\%$). In the bottom part, data of 61 metastatic melanoma cell lines derived from the same metastasized biopsies sorted by the highest to lowest mean value of amplicon a1. (C) Kaplan–Meier curves indicating survival after diagnosis of first metastasis (SFM). Patients were grouped according to amplicon a1 methylation levels. Patients with lower methylation level ($< 50\%$) of amplicon a1 had a significantly increased SFM ($P = 0.05$, log-rank test) as compared with patients with higher methylation level ($\geq 50\%$). (D) Positive correlation (Spearman coefficient $\rho = 0.59$, $P = 0.0001$, $n = 61$) of mean methylation levels in amplicons a1–a3 of metastatic melanoma tissue (MM tissue) and corresponding cancer cell lines (MM cell). (E) Positive correlation (Spearman coefficient $\rho = 0.79$, $P = 0.0003$, $n = 16$) between *TCF21* mRNA re-expression and the degree of loss of DNA methylation on *TCF21* promoter assessed by amplicon a1 (mean value). The 16 metastatic cell lines were treated by 5-aza-dC (1.5 μM) for 1 week.

Table I. Patient survival correlated with the methylation status of *TCF21* amplicon a1

	Methylation status ^a	<i>n</i>	Median survival ^b (95% CI)	No. of deaths (%)	Hazard ratio ^c (95% CI)	<i>P</i> -value ^d
OS	Low	32	6.7 (2.0–8.4)	23 (71.9)	Reference	0.055
	High	24	6.2 (3.8–9.6)	23 (95.8)	1.92 (0.99–3.73)	
SFM	Low	29	3.4 (1.4–5.7)	20 (69.0)	Reference	0.04
	High	24	1.8 (1.1–3.0)	23 (95.8)	2.05 (1.05–4.02)	

OS, overall survival; *n*, patients number.

^aMean methylation status. Low $< 50\%$; High $\geq 50\%$.

^bIn years.

^cAdjusted for age, gender and Breslow thickness.

^d*P*-value of the Cox regression.

(supplementary Table S4 is available at *Carcinogenesis* Online) of which four displayed normal diploid CN in the *TCF21* locus, eleven showed loss of one copy and one an additional copy. Compared with adult primary skin melanocytes and primary skin fibroblasts used as normal controls and displaying low methylation and high expression, all 16 cell lines showed much lower *TCF21* expression ranging from 3-fold to almost total silencing (supplementary Table S4 is available at *Carcinogenesis* Online). Low *TCF21* expression coincides with high promoter methylation. Treatment of the cell lines with 1.5 μM 5-aza-dC for 7 days decreased the methylation levels by 7–26% (supplementary Table S4 is available at *Carcinogenesis* Online). Efficient general genomic demethylation was controlled by monitoring a decrease in LINE1 methylation of $> 10\%$ except for cell line Mel-80a (4%), which was already lowly methylated in the untreated state. The reduction of DNA methylation of the *TCF21* promoter by 5-aza-dC treatment correlated with mRNA re-expression (Figure 1E), suggesting that *TCF21*

promoter hypermethylation is responsible for *TCF21* silencing.

TCF21 transactivates *KISS1* expression in metastasis suppression

The metastatic ability of the melanoma cell line C8161 is inhibited by microcell-mediated chromosome transfer of portions of chromosome 6q where *TCF21* is located (13, 16). FISH on C8161 metaphase spreads with a *TCF21* containing bacterial artificial chromosome (BAC) as probe indicated the presence of two *TCF21* gene copies (Figure 2A). Examination of the methylation level of *TCF21* amplicon a1 revealed 100% mean amplicon methylation in C8161 compared with only 20 and 23% methylation in primary skin melanocytes and primary skin fibroblasts, respectively (Figure 2B). 5-Aza-dC treatment but not the treatment with the histone deacetylase inhibitor trichostatin A restored *TCF21* expression in C8161. Moreover, there was no synergistic effect by the two substances (Figure 2C). Transcription of *TCF21* and *KISS1*,

Fig. 2. *TCF21* metastasis suppressor activity is mediated by transactivation of *KISS1*. (A) Dual color FISH on a metaphase spread of cell line C8161 using chromosome 6-specific probe CEP 6 (green signal) and bacterial artificial chromosome RP11-465P13 which contains the genomic sequence of *TCF21* (red signal). The two colors indicate the presence of both chromosomes 6. (B) Complete methylation across part of the *TCF21* promoter in cell line C8161 as compared with much lower methylation in normal primary skin melanocytes (PSM) and normal primary skin fibroblasts (PSF). (C) Reactivation of *TCF21* mRNA expression in C8161 cells upon treatment with 5-aza-dC but not with Trichostatin A (TSA). Treatment with the indicated concentrations for 48 h, values are means $\pm$ standard deviation from three independent experiments and shown as a fold re-expression (normalized to the solvent as a control). (D) Quantitative reverse transcription-PCR shows silencing of *TCF21* and *KISS1* in C8161 but not in PSM and PSF. (E) Restoration of *KISS1* transcription upon retroviral introduction of *TCF21* cDNA into cell line C8161 (mycTCF21). 1.neo6q, 2.neo6q and 3.neo6q are C8161 derivatives harboring additional 40 cM of chromosome 6q including *TCF21*. pBABE is empty vector. (F) Binding of TCF21 5' to the *KISS1* transcription start site. Sketch shows the location of several potential TCF21-binding sites (E-box sequence CANNTG). ChIP with cell lines C8161 pBABE (negative vector control) and C8161 pBABEmycTCF21 (TCF21 positive) and antibodies raised against the myc-tag expressed by vector myctcf21 revealed binding of TCF21 in amplicon regions 1–5 (bands in rightmost lanes of composite gel picture below the sketch). No bands were visible in lanes corresponding to the negative control pBABE and amplicon region 6 harboring no E-box sequence. (G) ChIP-quantitative PCR with cell line C8161 myctcf21 and antibodies against TCF21 shows different binding strengths at putative binding sites in the *KISS1* promoter, the primer number indicating the position order as in Figure F. Values were normalized to input and are shown as fold enrichment.

a putative metastasis suppressor gene, was silenced in C8161 cells but not in primary skin melanocytes or fibroblasts (Figure 2D).

We analyzed whether retroviral introduction of *TCF21* cDNA can induce *KISS1* expression in C8161 cells as observed in C8161neo6q.1, C8161neo6q.2 and C8161neo6q.3 cell lines which harbor additional $\sim$ 40 cM of chromosome 6q including *TCF21* (10). Presence of the *TCF21* cDNA led to a 46-fold induction of *KISS1* transcription measured by reverse transcription-qPCR, compared with C8161pBABE cells containing retroviral vector only (Figure 2E). In the C8161neo clones, *TCF21* mRNA levels were linearly correlated with *KISS1* mRNA levels (supplementary Figure S2A is available at Carcinogenesis Online). Re-expression of *TCF21* in C8161 cells by 5-aza-dC treatment correlated with increased *KISS1* mRNA levels (supplementary Figure S2B is available at Carcinogenesis Online). Positive correlation between *TCF21* and *KISS1* expression was also observed in the 16 metastatic melanoma cell lines upon 5-aza-dC treatment (supplementary Table S5 is available at Carcinogenesis Online).

TCF21 binds to the sequence motif 5'-CANNTG-3'-designated E-box (23). An *in silico* search identified various E-boxes in the upstream region of *KISS1*. Performing ChIP using C8161 mycTCF21 cells expressing myc-tagged TCF21 and antibodies against myc, several of the predicted TCF21-binding regions in the *KISS1* promoter could be verified (Figure 2F). ChIP-quantitative PCR revealed differential binding of TCF21 at its binding sites in the *KISS1* promoter (Figure 2G). Using luciferase reporter constructs in Hek293 cells, we narrowed down the *KISS1* core promoter to a region ranging from

–2804 to +48 bp relative to the *KISS1* transcriptional start site (Figure 3A). We then systematically deleted putative TCF21-binding sites and identified two vicinal regions whose deletion in construct Δ CHIP6 completely abolished promoter activity (Figure 3B). Deleting the two sites individually revealed that only loss of the first E-box (CAGCTG) located at position –2144 reduced promoter activity to $\sim$ 50% as compared with non-mutated control. Other deletion constructs displayed even higher promoter activity than control suggesting the existence of additional, still unknown *KISS1* regulators. Therefore, we performed an interaction partners search using Yeast two hybrid screens (Y2H) with full-length TCF21 as bait to search for interaction partners. Only three binding partners were identified: TCF3 (E12/E47), TCF4 and TCF12, also designated E2A, E2-2 and HEB, respectively. All of the identified partners belong to class B of the basic helix-loop-helix proteins (24). The protein interactions identified by Y2H screens were confirmed by coimmunoprecipitation using Flag-tagged E12, E47, TCF4 and TCF12 and HA-tagged TCF21 coexpressed in HEK293 cells (Figure 3C). Stimulation of the *KISS1* promoter by the diverse interaction partners was analyzed in a reporter assay. TCF21 stimulated the *KISS1* promoter 1.5-fold, whereas E12 lead to a 2-fold decrease (Figure 4D). Cotransfection of TCF21 with E12 and TCF12 revealed a further increase of the promoter activity by 1.8- and 1.9-fold, respectively. A synergistic effect could not be observed with E47 or TCF4. Thus, our data suggest that TCF21 co-operates with E12 and TCF12 to sustain the promoter activity of *KISS1*.

Fig. 3. TCF21 binds to the core promoter of *KISS1* (A) Promoter reporter assay in HEK293 cells with overlapping segments located 5' to the *KISS1* transcription start site revealed sequence Δ2 (−2804 to +48) as having the highest promoter strength relative to cells with vector pGL4 only (RLU, relative luminescence units). (B) Promoter reporter assay with deletion derivatives of sequence Δ2 in which various E-box protein sequences had been eliminated. Deletion of 234 bp including the two most 3' located E-box protein sequences completely abolished promoter activity, whereas elimination of other E-box protein sequences lead to only slight decrease or even strong increase in promoter strength. (C) TCF21 physically interacts with class B basic helix-loop-helix proteins. Coimmunoprecipitation of flag-tagged class B basic helix-loop-helix candidates (E12/E47, TCF4, TCF12) with HA-tagged TCF21 48 h after transfection in Hek293. Equal amounts of proteins were loaded onto an sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-HA or anti-flag antibody. Negative controls were HA-TCF21 and Flag-E12 cotransfected cells and IgG immunoprecipitation as a negative antibody. (*) indicating that the negative control as input: HA-TCF21 and flag-E12 as protein lysates; IP: IgG. Molecular weights in kilodaltons are indicated on the right. (D) Increased *KISS1* promoter strength (construct pGL4-KISS1, −2804 to +48) by synergistic interaction between TCF21 and TCF12. MM-66a cells were transfected with pGL4-KISS1 and basic helix-loop-helix Class B cDNA alone or together with TCF21. Values are means ± standard deviations of four independent experiments (* $P < 0.05$; Student's *t*-test, compared with pGL4-KISS1).

Fig. 4. Reduced motility of TCF21 expressing C8161 cells (A) Upper panels show initially seeded cells (i) of control pBABE and of pBABEmycTCF21 cells, cells migrated through Boyden Chamber pores (ii). (B) Quantitation of migrated cells transfected with control vector pBABE or pBABEmycTCF21. Given values are means ± standard deviations from triplicates.

Suppression of cell migration by TCF21

To determine if TCF21 functionally regulated cell motility *in vitro*, we performed a cell migration assay using C8161 cells overexpressing TCF21. Compared with the control cells harboring vector only, the TCF21-expressing cells displayed a clear reduction in their migration ability (Figure 4A and B) as demonstrated in three independent experiments. This indicates that TCF21 suppresses the motility of these melanoma cells. When considered together, the expression, clinical and *in vitro* data suggest that TCF21 may function as a metastasis suppressor gene by transactivating *KISS1* mRNA in melanoma. Conversely, the epigenetic silencing of TCF21 mRNA would be predicted to cause an increase of the metastatic potential of the tumors.

Discussion

We have uncovered potential antimetastatic properties of TCF21 in a melanoma. TCF21, a gene encoding a basic helix-loop-helix transcription factor, is localized within a recognized region of LOH for melanoma on chromosome 6. Our studies demonstrate that TCF21 is epigenetically regulated and a direct activator of the known metastasis suppressor *KISS1* on chromosome 1q32.

Metastasis is the leading cause of cancer-related deaths and results from cells that have migrated to distant organs and are resistant to conventional therapies. Metastatic cells have acquired the ability to detach from their original tissue and migrate to a new location. Metastatic dissemination occurs either through the lymphatic system or the blood stream. Therefore, a prerequisite for the metastatic ability of cells is the deregulation of genes that mediate cell–cell interactions and the ability to proliferate in new host environments. Two possible scenarios have been postulated: (i) progression of cells accumulating genetic alterations until they reach the potential to disseminate and migrate or (ii) the separate evolution of metastatic cells parallel to primary tumor cells. An important step during the transition from non-invasive cells to invasive cells is the downregulation of *E-cadherin* (CAD1), either by genetic or epigenetic events (25,26). CAD1 is a transmembrane glycoprotein involved in calcium-dependent cell adhesion. Currently, only 17 metastasis suppressor genes have been identified with normal functions in cell motility, invasion, adhesion and tumor growth (27).

TCF21 is involved in maintenance of the epithelial integrity through promotion of differentiation via mesenchymal to epithelial transition (23). Mesenchymal to epithelial transition occurs normally during endodermal lung branching, kidney and mammary gland developments (28–30). In fact, migratory mesenchymal cells provide

structural support for the epithelium. KISS1 is the natural ligand of Gpr54 (KISS1 receptor) a G protein-coupled receptor-54, Gpr54 was recently reported in viable knockout mice to play a critical role in glomerular development and kidney morphogenesis (31). *Tcf21* knockout is lethal, as a result of the lack of kidney and lung branching. In kidney, absence of *Tcf21* or *Gpr54* showed similar defect phenotypes despite the knowledge that *Tcf21* in the development of the kidney is expressed earlier than *Gpr54*. Furthermore, heterozygous point mutations in the *Gpr54* have been linked to small testes in male and an absence of follicular maturation in female mice. In addition, in human, these particular *GPR54* mutations have been identified in patients with familial or sporadic isolated hypogonadotropic hypogonadism (32). An autosomal active dominant *GPR54* mutation appears to be associated with a central precocious puberty phenotype (33). Whereas the lack of *TCF21* cause congenital and sex determination defects by the absence of cell fate decisions in the progenitors (34). All together, these data are in line with the idea that *TCF21* may regulate the KISS1–GPR54 pathway.

KISS1 was first identified as a melanoma metastasis suppressor gene localized on chromosome 1 and regulated by a gene on chromosome 6 (11,35). Although the mechanism for the function of *KISS1* remains an area of active research, there is some evidence that *KISS1* is a ligand for an orphan G-protein-coupled receptor. *KISS1* is processed to a 54 amino acid protein that is secreted and binds to a G protein-coupled receptor (h0T7T175 or GPR54), which induces a block in chemotaxis, invasion and metastasis (36,37). Expression of both *KISS1* and its receptor have been correlated with reduced metastasis (38). Alternatively, *KISS1* has been shown to function as a metastasis suppressor independent of *GPR54* *in vivo* (13). Although the mechanism is uncertain, *KISS1* appears to be a bona fide metastasis suppressor gene in melanoma and our data suggest that *TCF21* may play a key role in regulating its expression.

The development of novel diagnostic and prognostic biomarkers as well as more efficient therapies is urgently needed. Microarray expression profiling-based approaches (39) or genetic markers including mutations in *BRAF* or *N-RAS* identified clinical subgroups (40,41); however, their applicability is not well established. In this study, we show that epigenetic silencing of *TCF21* is a frequent event in melanoma and that this particular epigenetic event can predict the outcome of melanoma patients in that *TCF21* promoter methylation is able to subgroup metastatic skin melanoma patients and define a high-risk group independent of the disease stage. This finding will need to be confirmed on larger studies, however, indicates the potential for a biomarker in melanoma.

Supplementary material

Supplementary Tables S1–S5 and Figures S1 and S2 can be found at <http://carcin.oxfordjournals.org/>

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