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Absence of keratins 8 and 18 expression in rodent epithelial cell lines associates with keratin gene mutation and DNA methylation: cell line selective effects on cell invasion

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Abstract

Epithelial-mesenchymal transition (EMT) in carcinoma is associated with dramatic up-regulation of vimentin and down-regulation of the simple-type keratins 8 and 18 (K8/K18), but the mechanisms of these changes are poorly understood. We demonstrate that two commonly-studied murine (CT26) and rat (IEC-6) intestinal cell lines have negligible K8/K18 but high vimentin protein expression. Proteasome inhibition led to a limited increase in K18 but not K8 stabilization, thereby indicating that K8/K18 absence is not due, in large part, to increased protein turnover. CT26 and IEC-6 cells had <10% of normal K8/K18 mRNA and exhibited decreased mRNA stability, with K8 being higher in IEC-6 versus CT26 and K18 being higher in CT26 versus IEC-6 cells. Keratin gene sequencing showed that *KRT8* in CT26 cells had a 21-nucleotide deletion while K18 in IEC-6 cells had a 9-amino acid in-frame insertion. Furthermore, the *KRT8* promoter in CT26 and the *KRT18* promoter in IEC-6 are hypermethylated. Inhibition of DNA methylation using 5-azacytidine increased K8 or K18 in some but all the tested rodent epithelial cell lines. Restoring K8 and K18 by lentiviral transduction reduced CT26 but not IEC-6 cell matrigel invasion. K8/K18 re-introduction also decreased E-cadherin expression in IEC-6 but not CT26 cells, suggesting that the effect of keratin expression on epithelial to mesenchymal transition is cell-line dependent. Therefore, some commonly utilized rodent epithelial cell lines, unexpectedly, manifest barely detectable keratin expression but have high levels of vimentin. In the CT26 and IEC-6 intestinal cell lines, keratin expression correlates with keratin gene insertion or deletion and with promoter methylation, which likely suppress keratin transcription or mRNA stability.

Keywords

Vimentin; CT26 cells; IEC-6 cells; keratin 8; keratin 18

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INTRODUCTION

Intermediate filaments (IFs) are one of the 3 major components of the cytoskeleton, with the other two prominent constituents being microfilaments and microtubules [1]. IFs regulate the distribution and function of several cellular organelles, and are important for the maintenance of cell morphology and mechanical integrity [2-5]. IFs are classified into six types that make up more than 70 proteins that manifest cell-selective expression. Examples of the six types of IFs include keratins of epithelial cells (Types I and II), vimentin of mesenchymal cells (Type III), neurofilaments of neuronal cells (Type IV), lamins of the nuclear lamina (Type V) and the beaded filament structural proteins of the lens (Type VI) [6-8]. Mutations in most IF proteins either cause or predispose to more than 70 human diseases [9-12]. Keratins (K) are the largest subgroup of IFs and, in humans, keratins include 37 functional genes (excluding the 17 hair keratin genes) that encode type-I (K9-K28) and type-II (K1-K8 and K71-K80) keratins [13-15]. All epithelial cells typically express at least one type-I and one type-II keratin, which form obligate heteropolymers. For example, simple-type epithelia (as found in intestine and liver) express K8 and K18 with levels of K7, K19 and K20 depending on the cell type [16]. In simple-type epithelia, K8 is the major type II keratin and its absence or mutation has a severe phenotype [e.g., colitis and colonic hyperplasia in K8-null mice [17] [18]] because of the dominant-negative consequences of heteropolymeric keratin type I-II interactions [19]. K18 is the only type-I keratin in adult hepatocytes, together with its partner K8, and is the major type-I keratin in many colonic tumor cell lines [16].

In addition to their well-documented disease association, the importance of IFs in a variety of cell functions is becoming increasingly understood. For example, IFs play a critical role in protecting cells from injury and are important in subcellular organelle function and in protein targeting to different subcellular compartments [20] and also play a role in cell migration [21]. The tissue-specific expression of IFs renders them very useful markers of specific cell types, for defining the tissue origin of poorly differentiated cancers [22] [16], or as prognostic markers for cancer progression [23]. For example, loss of K8 is associated with poor prognosis in colorectal cancer [24]; and keratin expression correlates inversely with vimentin expression, with vimentin positivity being associated with high histological and nuclear grades in invasive breast tumors [25]. Furthermore, keratin down-regulation via RNA interference in nodal cells of squamous cell carcinoma metastases results in decreased cell proliferation and migration [26-27]. The reciprocal changes of keratin and vimentin in epithelial tumors is part of the process of epithelial-mesenchymal transition (EMT) that occurs when epithelial cells lose their non-migratory and polarized features and transform into fibroblastic, migratory cells acquiring mesenchymal characteristics [28-30]. These functional characteristics include the loss of basolateral polarity and cell adhesion as well as a gain in migratory potential and invasiveness [28-30]. Among the molecular alterations that occur during EMT are induction of mesenchymal markers, such as vimentin, and down-regulation of the cell adhesion marker E-cadherin [30]. However, the mechanism(s) that account for the changes in keratin and vimentin expression during EMT are not known. As a first step towards understanding such mechanisms, we tested several epithelial cell lines for the expression of keratins and vimentin. We were surprised to observe that several highly

utilized simple-type epithelial cell lines, including mouse colon CT26 and rat small intestine IEC-6 cells, express high levels of vimentin but negligible and barely detectable amounts of keratin mRNA and protein. Some genetic alterations were noted in K8 or K18, and differences in DNA methylation were also observed, depending on the cell line tested. In CT26 but not the IEC-6 cells, re-introduction of K8/K18 decreased cell invasion. Our findings indicate that epigenetic alterations account, at least in part, for the down-regulation of keratins that is observed in some tumor cell lines. This mechanism may extend to what is seen in human tumors and to epithelial cell lines that lack keratins.

MATERIALS AND METHODS

Cell lines, antibodies, plasmids

Mouse CT26.WT (colon, N-nitroso-N-methylurethane-induced), BNL CL.2 and NMuLi (liver, derived by methylcholanthrene epoxide transformation, and without viral transformation, respectively), 266-6 (acinar pancreas, derived upon expression of an elastase SV40 T-antigen), and rat IEC-6 (non-transformed cells) and IA-XsSBR (radiation-induced cell line) small intestinal cells and CRL-1439 (normal rat liver cell line) cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). All cells were cultured (37 °C) in media recommended by the supplier. Mouse (FVB/N) and rat (Sprague Dawley) liver and colon were used as control tissue for keratin expression. The antibodies (Ab) used in this study included rabbit Ab 8592 (specific to K8/K18; [31]); rabbit Ab to a conserved K8 R341-containing peptide [32]; Troma-I rat anti-mouse/human K8 (Developmental Studies Hybridoma Bank); E-cadherin (Abcam); vimentin V9 Ab (Sigma); and actin (Labvision). For plasmid preparation, K8 was cloned into the pLentiloxEV vector using the restriction sites Not I/BamHI; and K18 was cloned into the same plasmid using the cloning sites Not I/XbaI. pLentiloxEV without insert was used as control. Virus packaging and transduction were carried out as described [33].

Biochemical methods

A well-established high salt extraction (HSE) method was used to obtain cytoskeletal IF-enriched preparations from tissues (100 mg/tissue) and cell lines (1×10^7 cells) [34]. Samples were analyzed by SDS-PAGE then Coomassie staining or were transferred to polyvinylidene fluoride (PVDF) membranes for immunoblotting and visualization using enhanced chemiluminescence.

PCR, Quantitative real-time PCR and determination of mRNA half-life

DNA fragments were amplified with a AccuPrime Pfx DNA polymerase (Invitrogen). DNA fragments were purified with a QIAquick PCR purification kit (Qiagen) and sequenced in both directions using 3730 XL sequencer (Applied Biosystems). All PCR and quantitative real-time PCR (qPCR) primers (Supplemental Table 1) were designed using DNASTAR's Lasergene version 7 software. Total mRNA from different cell lines or tissues was extracted using RNeasy Mini Kit (Qiagen), then reverse transcribed into cDNA using Taqman reverse transcription kit (Applied Biosystems). qPCR was performed in triplicates with Mastercycler ep realplex (Eppendorf) and iQ SYBR-Green supermix mix (Biorad). The cycling parameters (40 cycles) were 95 °C (2 min), 95 °C (15 seconds), then 55 °C (15 seconds).

Relative mRNA fold-change compared to control was calculated using the comparative Ct method [35]. mRNA half-life was estimated after treating cells with 5 µg/ml actinomycin-D (Sigma) for 0, 15, 30, 60 and 120 min. Total RNA was extracted and the relative keratin mRNA was normalized to β-actin at the zero time point of actinomycin-D treatment.

Methylation-specific PCR and bisulfite sequencing

Genomic DNA was isolated from cells using the DNeasy Blood & Tissue Kit (QIAGEN). The DNA (0.5 µg) was treated with sodium bisulfite using EZ DNA Methylation-Gold Kit (Zymo Research). Approximately 50 ng of bisulfite-converted DNA was used as template for PCR amplification of the entire CpG island in the K8 and K18 proximal gene promoters. All primers (Supplemental Table 1) were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems). The presence of CpG islands was determined using Methyl Primer Express v1.0 software. Bisulfite PCR products were amplified with a AccuPrime Pfx DNA polymerase (Invitrogen) and cloned into pGEM-T Easy vector (Promega) and sequenced in both directions using 3730 XL sequencer (Applied Biosystems).

Inhibition of DNA methylation

5-aza-cytidine (Sigma-Aldrich) was prepared at a 5 mg/ml stock concentration in 1:1 water/glacial acetic acid solution. Cells were treated for 72 h with either vehicle or 1 µM 5-aza-cytidine, and the media was replaced every 24 h. Cells were lysed in homogenization buffer (0.187 M Tris, pH 6.8, 3% SDS, and 5 mM EDTA) for the analysis of keratin protein expression. RNA from treated cells was also prepared using the RNeasy Mini Kit (Qiagen).

Cell invasion assay

A matrigel invasion assay was performed using BioCoat matrigel invasion chamber with 8 µm pore membrane and control chambers (BD Biosciences) according to manufacturer's instructions. Briefly, 1.25×10^5 cells per well in serum-free DMEM medium were seeded in the matrigel invasion chamber or a control chamber. 10% FBS in DMEM was added to the bottom well as a chemoattractant. The chamber was incubated for 22 h in a humidified incubator (37 °C, 5 % CO₂). Invading cells on the lower surface of the membrane were stained using the CAMCO staining kit (Modern Laboratory Services), and the percentage of invading cells was calculated by dividing by the number of cells that migrated in the absence of matrigel in the control chamber.

Immunofluorescence staining and confocal imaging

K8, K18 and K8/K18 transfected IEC-6 cells were fixed by methanol (10 min, -20°C). After fixation, the cells were air dried and non-specific binding was blocked by incubation in blocking buffer (PBS with 2.5% w/v BSA and 2% normal goat serum). Primary antibodies were diluted 1:500 (Ab 8592) in blocking buffer and incubated with the cells for 1 h at room temperature, followed by 3 washes in PBS and a 1 h incubation (in the dark) with secondary Alexa-conjugated antibodies (1:1000 dilution). Following secondary antibody incubation, the cells were rinsed 3 times in PBS, mounted with Prolong Gold containing DAPI (Invitrogen) and viewed using an Olympus FluoView 500 confocal microscope with a 60x

oil-immersion (1.4 NA) objective and magnified 2.5 times with FluoView software (version 5.0).

Statistical Analysis

All data obtained from the qPCR and invasion assays were analyzed using t-test from GraphPad Prism 5 or Microsoft Excel. P values less than 0.05 were considered significant.

RESULTS

Absence of keratins in non-transformed and tumor cell lines

We initially used IEC-6 rat small intestine cultured cells [36] as a presumed source for rat K8/K18 given the known abundance of keratins in rodent intestine [19]. However, we were surprised that there were no detectable keratins and this was confirmed upon testing an independent stock of cells from ATCC (not shown). The absence of keratins in IEC-6 cells was particularly surprising since this cell line is commonly used as a “normal” non-transformed small intestine cell line in many studies [37-39]. This led us to examine in detail the presence of K8/K18 and vimentin in rat IEC-6 cells, and in mouse colon CT26 cells and hepatic BNL and NMuLi cells, using a well-established method to enrich for keratin IFs from tissues or cultured cells [34]. This analysis showed that IEC-6 and CT26 cells do not express detectable K8 or K18 protein as assessed by Coomassie staining or by the more sensitive immunoblotting, while abundant levels of K8/K18 are isolated from the liver cell lines BNL and NMuLi (Fig.1A,B; overexposed blots are shown to make the point regarding lack of keratin expression in CT26 and IEC-6 cells). Notably, the normally abundant keratins [which typically make up nearly 5% of total cellular proteins in cultured cells or 0.2-0.5% of total mouse liver or intestine protein [40]] were replaced by high levels of vimentin (that is readily seen by Coomassie staining, Fig.1A,B).

The absence of K8/K18 in IEC-6 and CT26 cells suggests that these cells lack a prototype feature of simple-type epithelial cells; namely, the expression of a major epithelial cell marker. We examined whether the apparent absence of keratins in IEC-6 and CT26 cells is related to instability of one of the two partner K8 or K18 protein since all epithelial cells express at least one type-I and one type-II keratin that bind in a non-covalent pair-like manner [13]. The type I and type II keratins stabilize each other via their association to avoid proteasomal degradation [41, 42]. For this, we treated IEC-6 and CT26 cells with the proteasome inhibitor MG132 which showed limited change in K8 or K18 levels (Fig.1C, D), albeit there was marginal stabilization of K18 protein in CT26 and IEC-6 cells, while K8 protein remained either undetectable or decreased. Similar to the findings in IEC-6 and CT26 cells, the mouse pancreatic acinar tumor cell line 266-6 also lacks K8/K18 and manifested similar results after treatment with MG132 although there appeared to be basal proteasomal degradation of both keratins in these cells (Supplemental Fig.1A). Therefore, several rodent simple-type epithelial cell lines lack the expected keratin expression but have marked induction of vimentin. Based on the proteasome inhibitor findings, the lack of keratin expression in these cells does not appear to be related to selective absence of either K8 or K18 (e.g., via nonsense mutation).

K8 and K18 mRNA relative levels and stability

The limited effect of proteasome inhibition on stabilization of K8/K18 suggests that the absence of keratins in CT26 and IEC-6 cells is likely due to limited keratin transcription or limited keratin mRNA stability. To address these possibilities, we measured K8 and K18 mRNA in CT26 and IEC-6 cells, and in the BNL and NMuLi cell lines as controls. Compared to the control group, CT26 and IEC-6 have barely detectable K8 and K18 mRNA expression (Fig.2A; for K8 mRNA: IEC-6 > CT26, and for K18 mRNA: CT26 $\approx$ IEC-6), and similar findings are noted in the 266-6 cell line which also lacked K8/K18 protein (Supplemental Fig.1). The mRNA for actin and tubulin (controls) was either slightly increased or unchanged in the CT26 and IEC-6 cell lines. These data are consistent with the protein analysis findings shown in Fig.1.

We then evaluated keratin mRNA stability by measuring changes in mRNA levels over time using quantitative PCR after inhibiting mRNA transcription with actinomycin-D (Fig.2B). Notably, K8 and K18 mRNA in CT26 and IEC-6 cells have different responses to actinomycin-D. CT26 K8 mRNA has a very short half-life, about 15 min, whereas IEC-6 K8 mRNA has a half-life of nearly 60 min (Fig.2B). By comparison, K18 mRNA in IEC-6 cells has the shortest half-life as compared with the other cell lines where, except for CT26 which exhibited a more modest level of K18 mRNA decay, K8 and K18 levels do not change even after 120 min of exposure to actinomycin-D (Fig.2C).

Identification of aberrant transcripts of K8 and 18 genes in CT26 and IEC-6 cells

Genetic mutations, insertions or deletions may ultimately result in aberrant, absent or defective proteins [43]. Given the markedly low levels of K8 and K18 transcripts in CT26 and IEC-6 cells, we sequenced the entire K8 and K18 genes in the two cell lines and also reverse transcribed the K8 and K18 mRNA to obtain cDNA sequence. In IEC-6 cells, there is a 27-nucleotide insertion identified in K18 exon-2 (453-475 bp) (Fig.3A), but no deletions or insertions were found in the K8 gene. The CT26 K8 gene has a 21-nucleotide TAA-repeat intronic deletion in base pairs (bp) 4558-4578 of the K8 gene compared to the control gene (Fig.3B). Sequencing of reverse transcribed CT26 mRNA showed exonic silent point mutations in K8 (A374A in exon-7) and K18 (K93K and F134F in exon-2) (Fig.3C). The instability of CT26 K8 mRNA (Fig.2B) may be related to the intronic K8 deletion (Fig.3B) and possibly to the exon-7 point mutation, while the instability of CT26 K18 mRNA (Fig. 2B) may be related to the point mutations in K18 exon-2. Silent mutations have been shown to affect RNA stability in other systems [44] [45]. The nucleotide insertion in the IEC-6 K18 gene (Fig.3A) and a L203L silent mutation may be contributors to the instability of K18 mRNA in IEC-6 cells, but this was not formally tested.

Identification of DNA methylation in CT26 and IEC-6 keratin promoter regions

DNA methylation plays a central role in gene regulation of mammalian promoter regions [46, 47]. The markedly decreased keratin mRNA in CT26 and IEC-6 cells suggests that enhanced DNA methylation of the promoter regions of the keratin genes and consequent decreased transcription of the keratin genes may be a contributing factor to the observed decreased in K8/K18 mRNAs. We examined this possibility using methylation specific primers (MSPs) and bisulfite sequencing. Notably, the mouse K8 promoter region (0-500

bp) contains two CpG islands (see Materials and Methods) for which MSP and control non-MSP primers were designed. Compared to the control group, DNA methylation was identified by the MSPs only in the promoter region of CT26 (Fig.4A). DNA methylation was identified by MSP in the K18 promoter region of IEC-6 cells but not in normal rat liver (Fig. 4B). Three CpG islands are predicted in the mouse K18 promoter region (0-500 bp) but no DNA methylation was identified by MSP (not shown but summarized in Fig.4C). In rat K8/K18, K18 is predicted to include one CpG island in the proximal promoter region (-500 to -1000) and no CpG islands are predicted in the K8 promoter region. Therefore, only rat K18 was tested by the two MSPs and a control non-MSP (Fig.4B).

Subsequently, genomic DNA was isolated from CT26 (with rat liver as control) and IEC-6 cells (with NMuLi cells as control) and used to analyze the methylation status of a 1000-bp region within the CpG island of the K8 or K18 promoter regions by bisulfate conversion, followed by nested PCR, cloning and sequencing (results are summarized in Fig.4C). The K8 CpG island is unmethylated in NMuLi cells. Similarly, the K18 CpG island was unmethylated in rat liver. However, there was hypermethylation in CT26 K8 CpG island (13/20, 13 methylated sites of 20 predicted sites) and IEC-6 K18 CpG island (11/30).

We then treated IEC-6 and CT26 cells with the DNA methylation inhibitor 5-aza-cytidine to determine if the observed promoter and other regulatory genomic methylation is functionally significant for K8/K18 transcription. 5-azacytidine treatment increased K8 transcript levels by over 50 fold and K18 transcript levels by approximately 3 fold in CT26 cells (Fig.5A). Despite the derepression of K8 gene expression in CT26 cells after 5-aza-cytidine treatment, we did not observe a commensurate increase in K8 protein (not shown). We also observed derepression of K8 transcript expression in two other epithelial cell lines that do not express K8 and K18 (Fig.5B). Specifically, 5-azacytidine increases K8 mRNA expression by 2.5 and 5 fold in IA-XsSBR and CRL-1439 cells, respectively, indicating that K8 promoter methylation likely plays a role in the lack of K8 mRNA expression in these cell lines as well (Fig.5B). In contrast, 5-azacytidine had no effect on K18 mRNA (Fig.5C) and protein expression in IEC-6 cells (not shown), despite the observed K18 promoter methylation in this cell line. No significant changes in K18 mRNA in IA-XsSBR and CRL-1439 cells were noted after 5-azacytidine treatment (not shown).

Cell Invasion is inhibited upon restoring K8 and K18 expression in CT26 but not IEC-6 cells

The loss of regulatory proteins can be associated with the promotion of EMT [48, 49]. We tested the hypothesis that the restoration of K8 and K18 might alter EMT in CT26 and IEC-6 cells by assessing cell invasion. We designed K8, K18 and control (GFP) lentiviral constructs and tested their expression in the two cell lines. K8 and K18 become highly expressed in IEC-6 (Fig.6A) and CT26 (Fig.7A) cells upon transduction with their corresponding lentiviral vectors as determined biochemically or by immunofluorescence staining. Next, we tested the migratory potential of IEC-6 cells that were transduced with either GFP or K8+K18 lentiviruses. Although there was a trend towards reduced cell invasion, we did not observe a significant difference between GFP and K8/K18 transduced IEC-6 cells (Figure 6B,C and data not shown). In contrast to IEC-6 cells, K8/K18

transduction in CT26 cells (Fig.7A) reduced % cell invasion by approximately two-fold compared with GFP transduced cells (Figure 7B,C).

We also investigated the effect of forced keratin overexpression in IEC-6 cells on EMT using the surrogate markers E-cadherin and vimentin. We examined these two markers since E-cadherin down-regulation and vimentin upregulation are noted in EMT while E-cadherin up-regulation and vimentin down-regulation accompany mesenchymal-to-epithelial transition (MET) [48, 49]. K8/K18 re-introduction into IEC-6 cells had no effect on vimentin expression and unexpectedly caused E-cadherin levels to decrease (Fig.6D), without any significant change in mRNA levels of vimentin or E-cadherin (not shown). Similar findings for CT26 cells showed a lack of change in vimentin and E-cadherin mRNA after K8/K18 reintroduction by lentiviral transduction (not shown).

DISCUSSION AND CONCLUSIONS

Mechanism of the loss of keratin expression in simple-type epithelial cultured cell lines

Our findings (summarized in Fig.8) demonstrate the near-complete loss of K8/K18 with concomitant high levels of vimentin in CT26 cells, a chemically-induced mouse colonic tumor [50]; and in IEC-6 cell, a “normal” epithelial cell line derived from rat small intestine [36]. In addition to CT26 and IEC-6 cells, two additional epithelial tumor cell lines, 266-6 (mouse acinar pancreas) and IA-XsSBR (rat small intestine), also lack the expected K8/K18 expression but express high levels of vimentin (Supplemental Fig.2). We have not observed a similar pattern of keratin^{loss}/vimentin^{gain} in several human colonic, pancreatic and hepatoma cell lines we tested (not shown) but this may be related to the specific cell lines we examined or it may be unique to rodent cell lines. However, limited expression of K8/K18, with high vimentin expression, has also been described in the human breast epithelial cell line MDA231 [51]. In addition, and consistent with our findings, there is a loss of keratin expression in human left-sided colonic tumors [52] as well as loss of K8 in colorectal cancers that carry poor prognosis [24]. Furthermore, breast tumors in patients have an inverse expression of keratin versus vimentin, with the more aggressive tumors being keratin^{Low}/vimentin^{High} [25].

The mechanism of keratin down-regulation in cultured cells or in the human epithelial cancers has not been examined. Our findings suggest that the causes of keratin absence in the cell lines are likely to be multi-factorial and to depend on the specific cell line. Several lines of evidence support this conclusion. First, CT26 cells have low baseline levels of K18 but no detectable K8 while IEC-6 cells have low baseline levels of K8 but no detectable K18 (Fig.1C,D). Second, the K8 and K18 mutations in the keratin cDNAs that are reverse transcribed from the low levels of detectable mRNAs are unique (e.g., insertion in K18 exon-2 in IEC-6 cells and an intronic deletion in the K8 gene in CT26 cells, Fig.7). Notably, both the deletion and insertion in these two cell lines have a repeat sequence. The function of repeat sequences is unclear, but they may contribute to genomic instability. For example, two-thirds of deletions remove a repeat sequence, and over 80% of insertions create a repeat in mammalian cells [53]. Third, the mRNA instability of K8 and K18 in CT26 and IEC-6 cells is different. The relatively long half-life of keratin mRNAs in NMuLi and BNL (Fig.2) are consistent with previous studies showing that metabolic and structural proteins typically

have long mRNA half-lives [54–55]. Fourth, the nature of gene silencing via DNA methylation is distinct for the individual keratins and within the two cell lines. For example, the K8 promoter but not the K18 promoter in CT26 cells was hypermethylated whereas the opposite was the case in IEC-6 cells. 5-aza-cytidine treatment greatly increased K8 transcription in CT26 cells but had no effect on K8 transcription in IEC-6 cells. However, 5-aza-cytidine treatment had no effect on K8 expression levels in CT26 cells.

Surprisingly, 5-azacytidine treatment had no effect on K18 transcript levels in IEC-6 cells, despite the observed K18 promoter methylation in IEC-6 cells. It is possible that the 27 base pair insertion in K18 exon-2 of IEC-6 K18 or the L203L silent mutation that we observed destabilizes the K18 transcript, preventing the derepression of K18 expression by 5-azacytidine treatment. With regard to K8 transcript levels in IEC-6 cells, which are very low compared to that in NMuLi and BNL cells, no mutations or changes in K8 promoter methylation were observed that could account for low K8 transcript levels. It is possible that other mechanisms are involved such as changes in local histone acetylation or microRNA-mediated destabilization of K8 mRNA. We cannot formally exclude the latter possibility although we did test for the induction of several potential microRNAs but were not able to implicate this mechanism (not shown). Taken together, insertional mutations, deletions, promoter methylation, and proteasome activation are all likely contributors to the observed low levels of K8/K18 protein in the cell lines we analyzed. We hypothesize that one or more of these mechanisms account for the well-described low levels of keratins in human carcinomas.

Functional significance of keratin and vimentin genetic alterations

K8 and K18 are important cytoprotective and abundant IF proteins in mammalian digestive organs [16–56]. Mutations in K8 or K18 pose a risk factor for human acute and chronic liver disease progression [16–57] but K8/K18 mutation or keratin absence in mouse models are not associated with predisposition to cancer development [16–56]. The reciprocal change from keratin^{High}/vimentin^{Absent-Low} in normal simple-type epithelial cells to keratin^{Absent-Low}/vimentin^{High} in the cancer situation is part of the process of EMT [28–30]. We demonstrate that the loss of K8/K18 expression in CT26 cells enhances their invasiveness since the reintroduction of K8/K18 by lentiviral transduction reduces their matrigel invasiveness by two-fold. The reduction of in vitro invasiveness is similar to 50% reduction in invasiveness observed upon knockdown of the key regulators of EMT, Twist and Snail [58]. Similarly, downregulation or upregulation of N-cadherin, an adhesion molecule found on mesenchymal cells, inhibited or enhanced, respectively, the invasiveness of prostate cancer cells by nearly two-fold [59]. Thus, alterations in the ratio of keratin and vimentin by lentiviral transduction can reduce cell invasiveness, which represents a functional characteristic of EMT.

In contrast to CT26 cells, the ectopic expression of K8 and K18 into IEC-6 cells had no effect on their invasiveness. Moreover, K8/K18 lentiviral transduction also decreased E-cadherin expression in IEC-6 cells. Thus, it is likely that the inhibitory effects of keratins on cell invasion are cell line dependent. This is supported by other studies that indicate that K8/K18 knockdown or ectopic expression have different effects on migration and invasion

depending on the cell system studies. For example, aberrant expression of K8 and K18 in squamous cell carcinomas is associated with increased invasiveness [60-61]. Knockdown of K8/K18 in the squamous cell carcinoma cell line AW13516 decreased its tumorigenic potential and invasiveness [62]. Similarly, hepatocytes (which do not express vimentin) isolated from K8-null mice migrate slower than their wild-type counterparts [63] while K6-null keratinocytes migrate faster than wild-type cells [64]. Interplay with vimentin is likely to be a contributing factor as to how keratins affect cell invasiveness. For example, co-expression of K8/K18 in L cells (which expresses vimentin but not keratins under basal conditions) leads to an enhanced cell migration and invasion potential [65]. However, as observed in our experiments, the cell type and other changes observed in many cell lines alter how cells respond to gain or loss of keratins and vimentin.

The inverse relationship between vimentin and keratin expression, and how the expression of one appears to down-regulate the other [25-27] and alter the migration potential of the involved cell, are likely to be critical determinants in the progression towards or away from EMT. Our findings provide insights regarding K8/K18 down-regulation in simple-type epithelial rodent tumor cell lines. The dramatic decrease in K8/K18 expression in the cell lines takes place via several pathways which may reflect similar findings in human carcinomas and other epithelial cell lines that lack keratin expression. Our findings also make the point that non-transformed and transformed cell lines can be remarkably different from their normal cell origin by lacking a critical group of IF cytoskeletal proteins that define a particular epithelial origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody
ATCC	American Type Culture Collection
EMT	epithelial-to-mesenchymal transition
HSE	high salt extraction
IFs	intermediate filaments
K	keratin
MET	mesenchymal-to-epithelial transition
MSP	methylation specific primer

PVDF	polyvinylidene fluoride
qPCR	quantitative real-time PCR

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Figure 1.

Changes in keratin and vimentin protein expression in rodent epithelial cell lines. (A) High salt extraction was carried out to obtain a cytoskeletal preparation of K8/K18 and vimentin from rodent liver and colon, mouse normal liver cell lines BNL and NMuLi, mouse colon carcinoma CT26, and the rat normal intestinal cell line IEC-6. (B) A duplicate gel to that shown in Panel A was transferred to a PVDF membrane then analyzed by immunoblotting using antibodies to the indicated antigens. (C) CT26 cells were cultured for 24 h in the presence of absence of MG132 (10μM). Equal fractions of total cell lysates were analyzed by immunoblotting. (D) Immunoblot of IEC-6 protein expression after treatment with the proteasome inhibitor MG132 (10μM; 24 h).

Figure 2.

K8 and K18 mRNA levels and stability. (A) qPCR analysis of actin, tubulin, and keratin mRNA expression in NMuLi, BNL, CT26 and IEC-6 cells. Equal numbers of cells were used to isolate mRNA from each of the cell lines. Note that for each transcript, the mRNA level is normalized to 1.0 based on NMuLi levels but the absolute values of the actin, tubulin, K8 and K18 levels in each cell line are different. (B) Half-life analysis of K8 mRNA expression after actinomycin-D treatment (10 μ g/mL, 0-120 min). For each transcript, the mRNA level is normalized to 1.0 at the zero time point. An unpaired t-test was performed to determine statistical significance. A significant difference was observed for CT26, t=0 and t=120 (*, $p < 0.0001$) and for IEC-6, t=0 and t=120 (+, $p < 0.001$) (C) Half-life analysis of K18 mRNA expression after actinomycin-D treatment. The same samples were used as in (B). A

significant difference was observed for CT26, t=0 and t=120 (*, $p<0.0001$) and for IEC-6, t=0 and t=120 (+, $p<0.0001$).

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Figure 3. Comparison of DNA sequences of K8 and K18 among rat liver, CT26, NMuLi, and IEC-6 cells. (A) IEC-6 cells have a 27 base pair insertion in K18 exon-2 (453-475 bp). This insertion results in the in-frame addition of 9 amino acids (shown using single letter abbreviation: TMPAPTCRS). (B) Genomic DNA sequencing demonstrates that CT26 has a 21 base-pair intronic deletion in K8 between exons 3 and 4, while no mutations were identified in K18. (C) Summary of point mutations identified in the coding regions of the K8 and K18 genes in CT26 (compared with NMuLi) and IEC-6 cells (compared with rat liver).

Figure 4.

Methylation-specific PCR and bisulfite sequencing analysis. (A) MSP-1, MSP-2 and control MSP (directed towards the promoter region of mouse K8) were designed to test methylation of the K8 promoter region in BNL, NMuLi and CT26 cells. MSP amplified bands were only found in CT26 genomic DNA (lanes 1 and 2 on right side of panel). (B) Rat (r) MSP-1, MSP-5 and control MSP (directed towards the promoter region of rat K18) were used in rat liver tissue and IEC-6 cells. Notably, MSP-amplified bands were only found in IEC-6 genomic DNA. (C) Bisulfite sequencing of the promoter region of K8 and K18 in the indicated tissues and cell lines showed different level of methylation in NMuLi, CT26, rat liver and IEC-6 cells, with K18 being hypermethylated in IEC-6 cells (11 of 30 in IEC-6 versus 0 of 30 potential sites in rat liver). Similarly, K8 is hypermethylated in CT26 cells (13 of 20 in CT26 versus 4 of 20 potential sites in mouse liver).

Figure 5.

Effect of the inhibition of DNA methylation on keratin mRNA expression. (A) qPCR analysis of K8 and K18 expression in CT26 cells after treatment with 1 mM 5-azacytidine for 72 h. (B) qPCR analysis of K8 expression in IA-XsSBR, CRL-1439, and IEC-6 cells after 72 h of 1 mM 5-azacytidine. (C) qPCR analysis of K18 expression in IEC-6 cells after 72 h of 1 mM 5-azacytidine. Each graph shows the mean of 3 independent experiments, and the error bars represent S.E.M. An unpaired t-test was performed to test for statistical significance.

Figure 6.

Expression of K8/K18 in IEC-6 cells has no effect on cell invasion. (A) Validation of K8/K18 expression after transduction with K8/K18 lentiviruses by Coomassie staining, K8/K18 immunoblotting, and immunofluorescence (B) Brightfield images of IEC-6 cells transduced with GFP (vector) or K8/K18 lentiviruses after Matrigel invasion assay. The panel shows the cells that have invaded through Matrigel versus the total cells that migrate in the absence of Matrigel (Control). (C) The percentage of IEC-6 cells that invaded through Matrigel versus the total number of cells that migrate in the absence of Matrigel (Control) was calculated for a representative experiment. Analysis of this and of 3 additional experiments indicates that there is no statistical significance between GFP- versus K8/K18-transduced cell invasion. (D) IEC-6 cells transduced with GFP or K8/K18 lentiviruses exhibit differential expression of EMT markers. Note that K8/K18 transduction reduces E-cadherin expression but has no effect on vimentin expression.

Figure 7.

Expression of K8/K18 in CT26 cells inhibits cell invasion. (A) Validation of K8/K18 expression after transduction with K8/K18 lentiviruses by Coomassie staining and immunofluorescence (B) Brightfield images of CT26 cells transduced with GFP (vector) or K8/K18 lentiviruses after Matrigel invasion assay. The panel shows the cells that have invaded through Matrigel versus the total cells that migrate in the absence of Matrigel (Control). (C) The percentage of CT26 cells that invaded through Matrigel versus the total number of cells that migrate in the absence of Matrigel (Control) was calculated for a combination of 2 representative experiments. An unpaired t-test indicates that K8/K18-transduced CT26 cells are significantly less invasive than GFP-transduced cells.

	<u>CT26</u>	<u>IEC-6</u>	<u>Normal</u>
Vimentin	++++	++++	—
K8 protein/mRNA	+/-	+	++++
K18 protein/mRNA	+	+/-	++++
K8 genomic mutation	21 bp deletion	No	No
K18 genomic mutation	No	27 bp insertion	No
K8 promoter methylation	yes	No	No
K18 promoter methylation	No	yes	No

Figure 8.

Summary of the protein changes in keratins and vimentin and the molecular and genetic alterations of keratins in CT26 and IEC-6 cells as compared with control cells.