Opposing Effects of KLF5 on the Transcription of MYC in Epithelial Proliferation in the Context of Transforming Growth Factor β

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The proto-oncogene MYC plays a critical role in cell proliferation and tumorigenesis, and its down-regulation by transforming growth factor β (TGFβ) signaling is necessary for TGFβ to inhibit cell proliferation. KLF5, on the other hand, is a pro-proliferative basic transcription factor that reverses function to become an anti-proliferative TGFβ cofactor upon TGFβ stimulation in epithelial homeostasis. In this study we investigated whether KLF5 directly regulates MYC transcription in epithelial cells in the context of TGFβ. Knockdown of KLF5 significantly reduced MYC expression in the HaCaT epidermal epithelial cells. When TGFβ was applied, however, whereas MYC expression was significantly inhibited, knockdown of KLF5 increased MYC expression. Furthermore, re-expression of KLF5 restored the inhibitory effect of TGFβ on MYC expression in two cancer cell lines. Chromatin immunoprecipitation and oligo pulldown experiments demonstrated that whereas binding of KLF5 to both KLF5 binding element (KBE) and TGFβ inhibitory element (TIE) DNA elements was necessary for MYC transcription, binding to KBE was decreased by TGFβ, and binding to TIE was increased by TGFβ. These results suggest that KLF5 is not only essential for MYC transcription in proliferating epithelial cells but also mediates the inhibitory effect of TGFβ on MYC transcription. Furthermore, different binding sites mediate different effects of KLF5 in the context of TGFβ.

The c-myc (MYC) gene encodes a short-lived transcription factor (MYC) which heterodimerizes with Max. MYC/Max heterodimers activate or repress two distinct pools of target genes that elicit a variety of biological responses, including cell cycle progression, cellular growth, differentiation, and apoptosis/survival (1–3). Physiologically, MYC is broadly expressed during embryogenesis and in the compartments of adult tissues that possess high proliferative capacity, including skin epidermis and gut, and its role in the regulation of cell proliferation, differentiation, and apoptosis has been demonstrated (3). In epithelial homeostasis, MYC has a positive function in cell proliferation which involves its interaction with the zinc finger protein Miz-1 and Smads to repress the cyclin-dependent kinase inhibitor p15Ink4b (4, 5). More specifically, MYC appears to promote the switch from stem cell to transit amplifying cell (6–9), and ectopic expression of MYC disrupts terminal differentiation of epithelial cells (7). During TGFβ2-induced epithelial differentiation, MYC is rapidly down-regulated, and its down-regulation is necessary for TGFβ to induce cyclin-dependent kinase inhibitors p15Ink4b and p21Cip1 and to inhibit cell cycle progression from G1 to S phase (10, 11). Although the down-regulation of MYC involves a number of transcription factors including Smads and E2F4/5 (12–15), regulation of MYC in proliferating cells is not well understood.

Human Krüppel-like factor 5 (KLF5, also named IKLF or BTEB2) belongs to the Sp/KLF zinc finger transcription factor family, which is composed of about 20 mammalian members that share three C2H2-type zinc fingers at the carboxyl terminus (16, 17). KLF5 is a basic transcription factor necessary for cell proliferation in epithelial homeostasis. For example, it is highly expressed in proliferating epithelial cells including immortal but untransformed epithelial cell lines as well as proliferating primary cultures of epithelial cells, which mostly represent progenitor cells (18–20). Consistently, KLF5 is expressed at a higher level in basal rapidly proliferating cells of normal intestine, but its expression is reduced in mature and differentiated cells (21). In addition, knock-out of one KLF5 allele significantly reduced the size of villi in mouse intestine, whereas knock-out of both KLF5 alleles was embryonically lethal (21). Furthermore, overexpression of KLF5 in the epidermis of transgenic mice caused hyperplasia of basal cells (22), further indicating a pro-proliferative role of KLF5 in epithelial homeostasis.

Our recent study demonstrates that, although essential for cell proliferation, KLF5 reverses function and becomes a cofactor for TGFβ to inhibit cell proliferation upon TGFβ-mediated acetylation in epithelial cells (23). Based on the correlation between KLF5- and MYC-proliferating epithelial cells and that between the functional reversal of KLF5 and down-regulation of MYC in TGFβ-induced cell cycle arrest in epithelial cells, we hypothesize that, as a basic transcription factor, KLF5 could function in the transcription of MYC in epithelial homeostasis. In this study we tested this hypothesis using an in vitro model of epithelial homeostasis. We found that KLF5 binds to MYC pro-

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2 The abbreviations used are: TGFβ, transforming growth factor β; ChIP, chromatin immunoprecipitation; KBE, KLF5 binding element; TIE, TGFβ inhibitory element; KLF5, Krüppel-like factor 5; hTERT, human telomerase reverse transcriptase; siRNA, small interfering RNA; GFP, green fluorescent protein.
moter to induce MYC expression, and the induction is reversed when the TGFβ signaling is activated.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Other Materials**—The HaCaT epidermal epithelial cell line was established by Dr. Norbert E. Fusenig of the German Cancer Research Center (24) and was kindly provided to us by Dr. Robert A. Swerlick of Emory University. It was maintained following established procedures (24). The MDA-MB-231 breast cancer cell line, PC-3 prostate cancer cell line, and HepG2 hepatoma cell line were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and propagated following the ATCC instructions. The TGFβ used in this study was TGFβ1 from R&D Systems (Minneapolis, MN). The siRNA for KLF5 had a sequence of 5′-AAGCTCAC-CTGAGGACTCA-3′ and was described previously (25). The antibody against KLF5 was prepared as described previously (26).

**Promoter-Luciferase Reporter Assay**—Both wild type and mutant plasmids were transfected into HaCaT cells using Lipo-fectamine reagent (Invitrogen) following the manufacturer’s instructions. Twenty-four hours later different concentrations of siRNA for KLF5 and a negative control, 5′-AAGUCCCA-GUAAAGCAUGG CCTT-3′ (Dharmacon, Lafayette, CO), were transfected using siPORT Amine (Ambion, Austin, TX). For HepG2 cells and MDA-MB-231 cells, plasmids of MYC promoter reporter and pcDNA3-FLAG-KLF5 (26), pcDNA3-FLAG-KLF5-K369R (23), or pcDNA3-FLAG (Invitrogen) were transfected into cells with the Lipofectamine 2000 reagent (Invitrogen). On the following day, 100 pmol (2 ng/ml) TGFβ was added and incubated with cells for 20 h. Luciferase assay was carried out using the Promega luciferase assay kit as previously described (27). Three wells of cells were used for each data point, and each experiment was repeated at least once.

**Chromatin Immunoprecipitation (ChIP) Assay**—HepG2 cells were transfected with pcDNA3-FLAG-KLF5, pcDNA3-FLAG-KLF5-K369R, or pcDNA3-FLAG plasmid. Forty hours after transfection cells were incubated in the presence or absence of 5 ng/ml TGFβ for 1 h. ChIP was performed according to the protocol from Upstate Biotechnology (Lake Placid, NY). FLAG antibody-agarose beads (Sigma) or antibody against Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA) were used to precipitate the protein-DNA complex. Precipitated DNA was analyzed by PCR with the following primers specific to the MYC promoter: F3, 5′-TCTTGAGGAAAAATGTGTAGGC-GCCG-3′; F4, 5′-TTTCTCAGAGCTCCATACGGGCCC-CTC-3′; F5, 5′-TTTCTCAGAGCTCCATACGGGCCC-CTC-3′; F6, 5′-TTTCTCAGAGCTCCATACGGGCCC-CTC-3′; F7, 5′-TTTCTCAGAGCTCCATACGGGCCC-CTC-3′; F8, 5′-TTTCTCAGAGCTCCATACGGGCCC-CTC-3′; R3, 5′-TTTAAAGCTTGAGGACCTGCCTTCCGT-3′; R4, 5′-TTTAAAGCTTGAGGACCTGCCTTCCGT-3′; R5, 5′-TTTTAAGCTTGAGGACCTGCCTTCCGT-3′; R6, 5′-TTTTAAGCTTGAGGACCTGCCTTCCGT-3′.

The procedure for real-time PCR was the same as described previously (27), and the same primers as those for regular PCR were used. The value for each sample, as shown in Fig. 4, F–H, was normalized by the value of respective input.

**RESULTS**

KLF5 Plays a Necessary Role in the Transcription of MYC in Proliferating Epithelial Cells—The HaCaT epidermal epithelial cell line was used in this study because it is highly proliferative without TGFβ and its proliferation is inhibited upon TGFβ activation, it has been used in the study of multiple factors including TGFβ signaling and MYC in epithelial homeostasis (10, 28, 29), and both KLF5 and MYC are expressed at higher levels in these cells (13, 30). To examine whether KLF5 is required for basal transcription of MYC, siRNA targeting KLF5 (siKLF5) was used to knock down endogenous KLF5 in HaCaT cells, and expression analysis was conducted. As shown in Fig. 1A, expression of MYC mRNA was significantly reduced upon the knockdown of KLF5, as detected by real-time PCR. Further-
translated regions of KLF5 (23) also decreased MYC and hTERT levels (Fig. 1C). These results indicate that KLF5 is required for basic MYC transcription in proliferating epithelial cells.

**Inductive Function of KLF5 in MYC Transcription Is Reversed to Suppressive in TGFβ-induced Cell Cycle Arrest**—TGFβ induces cell cycle arrest in HaCaT cells through gene regulation including suppression of MYC and induction of p15. To determine whether KLF5 is also involved in MYC regulation when TGFβ is present, we analyzed MYC expression in HaCaT cells treated with TGFβ and transfected with siRNA against KLF5. Although knockdown of KLF5 reduced MYC mRNA levels in the absence of TGFβ (Fig. 2A, white bars), as detected by real-time PCR, it showed an opposite effect by increasing MYC transcription in TGFβ-treated HaCaT cells (Fig. 2A). Consistent with previous findings, TGFβ still inhibited MYC transcription when HaCaT cells were transfected with negative control siRNA, but the inhibitory effect of TGFβ was diminished by 50 nM siRNA for KLF5 and even reversed by 100 nM siRNA for KLF5 (Fig. 2A). Consistent with RNA expression, Western blot analysis showed that the protein level of MYC was down-regulated upon the knockdown of KLF5 in HaCaT cells without TGFβ (Fig. 2B). Considering that MYC is rapidly down-regulated by TGFβ (10, 11), we selected two time points of TGFβ treatment, 1 and 4 h, for detecting MYC protein expression, so that the effect of TGFβ on MYC protein expression could be more accurately revealed. MYC protein expression was obviously reduced by TGFβ without the knockdown of KLF5, but TGFβ increased MYC protein levels in cells where KLF5 was knocked down (Fig. 2B), further suggesting that knockdown of KLF5 reverses TGFβ-caused inhibition of MYC expression in HaCaT cells.

To further clarify whether KLF5 plays a necessary role in the inhibition of MYC induced by TGFβ, we modulated the expression of KLF5 in the MDA-MB-231 breast cancer cell line and the PC-3 prostate cancer cell line and treated them with TGFβ. Both MDA-MB-231 and PC-3 cell lines express KLF5 in transfected with siRNA against KLF5, siRNA for luciferase (SiLuc, negative control), or a mixture of 100 nM SiLuc and 100 nM siKLF5 (SiL + SiK), as detected by real-time PCR assay. B, protein expression of KLF5 and MYC in HaCaT cells transfected with different concentrations of siRNA for KLF5, as detected by Western blotting. Total concentration of siRNA (SiKLF5 and SiLuc) is 200 nM for each lane. C, expression of KLF5, MYC, and hTERT in HaCaT cells transfected with a 1:1 mixture of two siRNAs targeting the 5′- and 3′-untranslated regions UTR of KLF5, established in a previous study (23). The concentration for SiLuc is 100 nM, whereas those for SiKLF5 mixtures are indicated. The same method of real-time PCR as in panel A was used. Asterisks indicate statistically significant differences (p < 0.05) in gene expression between knockdown (SiKLF5) and control (SiLuc).
expression without TGFβ but rescues the inhibitory effect of TGFβ on MYC expression in tumor cells.

**KLF5 and TGFβ Co-regulate MYC Promoter in the Same Pattern as Their Effect on MYC Expression**—To further understand the role of KLF5 in MYC regulation in the context of TGFβ, we examined the effect of KLF5 and TGFβ on the activities of MYC promoters. As a basic transcription factor, KLF5 usually binds to GC-rich and CACCC element (CA-box) of promoter DNA (17). Recently, the CA-box was extended to CCCCACCC in the Nanog enhancer region that was shown to bind to KLF5 (32). We analyzed MYC promoter and found that the sequence 5’-CCCACCTCCCACCTC-
CCCACCTCCCACCTC-3’ (nucleotides −319 to −288) in the MYC promoter contains two tandem CCCCACCC motifs or tandem CA boxes. We defined these two motifs as KLF5 binding elements. In addition, the TGFβ inhibitory element, which is at nucleotides −92 to −63 relative to the P2 transcription initiation site of MYC promoter, has been established as an essential region for TGFβ to suppress MYC expression (13–15). The core sequence of the TIE (5’-GGCTTGCCG-3’, nucleotides −84 to −75) contains GC-rich elements that could also be bound by KLF5.

Fig. 3A illustrates the promoter of MYC with relative locations of potential KLF5 bindings KBE (CA boxes) and TIE, the P1 and P2 initiation sites, and PCR primers that were used in both the ChIP assay and the construction of different sizes of MYC promoter. We cloned four MYC promoter fragments into the pGL3-basic vector to construct four promoter-reporter plasmids, including pMyc9 (containing KBE and TIE), pMyc10 (containing TIE), pMyc11 (containing TIE but is 188-bp shorter than pMyc10), and pMyc3 (containing only KBE) (Fig. 3B). We first investigated the response of these promoters to TGFβ signals by transfecting pMyc3, pMyc9, pMyc10, and pMyc11 into HaCaT cells individually and performing luciferase reporter assays. Regardless of TGFβ, pMyc9 showed the highest promoter activity, whereas pMyc3 showed the weakest (Fig. 3C). TGFβ treatment at 2 ng/ml for 20 h suppressed promoter activity in pMyc9, pMyc10, and pMyc11 but not in pMyc3 in HaCaT cells (Fig. 3C).

We then determined whether KLF5 regulates MYC promoter activity in the context of TGFβ using pMyc3, pMyc9, pMyc10, and pMyc11. Reporter plasmids were co-transfected with siRNA for KLF5 (siKLF5) or siRNA for scramble sequence (SiS, negative control) into HaCaT cells (Fig. 3D), and TGFβ treatment was applied at 2 ng/ml for 20 h. In control cells without TGFβ treatment, knockdown of KLF5 significantly reduced the promoter activity of pMyc9 and pMyc11, and the reduction in pMyc3 and pMyc10 was not statistically significant. TGFβ had no effect on the activity of pMyc3 vector. Treatment with TGFβ, on the other hand, significantly inhibited the promoter activity for pMyc9, pMyc10, and pMyc11 in cells with normal levels of KLF5 but significantly induced the promoter activity for each of the of promoter constructs in cells where KLF5 was knocked down. Consistent with endogenous MYC expression results in HaCaT cells (Fig. 2A), these results further indicate that, whereas KLF5 alone transactivates MYC transcription,
Opposite Effects of KLF5 on MYC Transcription

KLF5 reverses function when TGFβ is present and is essential for TGFβ to inhibit MYC transcription.

KLF5 Directly Binds to MYC Promoter, and Bindings to Different Sites Are Affected Differently by TGFβ—As a basic transcription factor, KLF5 most likely binds to MYC promoter directly to regulate its transcription. To test this hypothesis, we conducted a ChIP assay to scan the proximal region of MYC promoter (~414 to +54 nucleotides) for KLF5 binding. HepG2 cells were transfected with pcDNA3-FLAG-KLF5 or pcDNA3-FLAG vector and treated with TGFβ at 2 ng/ml for 20 h, as detected by luciferase activity assay. 

As shown in Fig. 3A, ChIP assay detected the binding of KLF5 to the regions between F6 and R6 (~318 to −208), F6 and R3 (~318 to −88), and F5 and R4 (~108 to +54) but not those between F3 and R5 (~414 to −318), F7 and R3 (~208 to −88), and F8 and R4 (~71 to +54). In addition, binding of KLF5 to MYC promoter containing the KBE site, as shown in fragments between F6 and R6 (~318 to −208) and between F6 and R3 (~318 to −88), was decreased by TGFβ treatment, whereas the binding to MYC promoter containing the TIE site, as shown in fragment between F5 and R4 (~108 to +54), was increased by TGFβ treatment. Therefore, KLF5 binds to different regions of MYC promoter, and bindings to different regions respond differently to TGFβ.

As illustrated in Fig. 3A, the MYC promoter has two potential KLF5 binding sites, KBE and TIE. In addition, DNA fragments containing KBE and TIE had different patterns of binding to KLF5 in the context of TGFβ, as shown in the ChIP assay (Fig. 4A). To further investigate whether KLF5 directly binds to KBE and TIE on MYC promoter, we performed oligo pulldown assays for both of them. We incubated the lysates from HaCaT cells treated with or without TGFβ with biotin-labeled oligonucleotides for wild type and mutant KBE or TIE and performed immunoprecipitation and Western blotting to detect KLF5 in the DNA-protein complexes pulled down by the oligos. As shown in Fig. 4B, whereas binding of Smad4 to KBE was undetectable, binding of KLF5 to KBE was detected, and the binding was reduced by TGFβ, which is consistent with the ChIP data. The mutation in KBE decreased its binding to KLF5, whereas mutation of CGG to TAT in the TIE site abolished the binding (Fig. 4C). Consistent with a previous report (13), we also detected the binding of Smad4 to wild type TIE, and the binding was increased by TGFβ (Fig. 4C).

Unlike KLF5, Smad4 showed no binding to mutant 1 (CGG > TAT) but a weak binding to mutant 2 (GGCT > TTAA) (Fig. 4C).

To examine whether the binding of KLF5 to MYC promoter is direct or mediated by other proteins, we performed oligo pulldown assays using in vitro translated 35S-labeled full-length KLF5 protein, with a truncated form of KLF5 lacking the car-
Our previous study showed that TGFβ causes the acetylation of KLF5, and the acetylation alters the bindings of KLF5 to p15 promoter and other transcription factors to reverse the function of KLF5 in p15 regulation and cell proliferation (23, 33). The same mechanism could be also responsible for TGFβ-determined opposing effects of KLF5 on MYC transcription. To investigate whether KLF5 acetylation affects the binding of KLF5 to KBE and TIE of MYC promoter, we conducted a ChIP assay in HepG2 cells transfected with pcDNA3-FLAG-KLF5, pcDNA3-FLAG-KLF5K369R (acetylation deficient KLF5), or pcDNA3-FLAG vector. The cells were also treated with TGFβ at 5 ng/ml for 1 h. Antibodies against FLAG or Smad4 were used to immunoprecipitate the protein-DNA complexes. Real-time PCR was performed with different primer pairs from the MYC promoter, including F5 and R4 flanking TIE, F6 and R6 flanking KBE, and F7 and R3 not flanking either. As a positive control, Smad4 bound to the TIE-containing region (−108 to +54) at a higher level upon TGFβ treatment (Fig. 4G). Consistent with the results from ChIP assay combined with regular PCR, whereas KLF5 bound to both the TIE-containing region (−108 to +54) and the KBE-containing region (−318 to −208) (Fig. 4, F and H), TGFβ dramatically increased the binding of KLF5 to the TIE-containing region (Fig. 4F) but decreased the binding of KLF5 to the KBE-containing region (Fig. 4H). KLF5 did not bind to the region between TIE and KBE (−208 to −88) (data not shown).

Interestingly, although the K369R mutation of KLF5 increased the binding of KLF5 to the TIE-containing fragment without TGFβ, the same mutation dramatically decreased the binding of KLF5 to the same element when TGFβ was present (Fig. 4F). The K369R mutation also decreased the binding of Smad4 to the TIE-containing fragment when TGFβ was present (Fig. 4G). For the KBE-containing promoter fragment, although wild type KLF5 bound to
Opposite Effects of KLF5 on MYC Transcription

KLF5 Regulates MYC Promoter Activity in the Context of TGFβ through Different Binding Sites—To further evaluate the role of KBE and TIE in KLF5-mediated MYC transcription in the context of TGFβ, we mutated KBE and/or TIE in pMyc9 MYC promoter reporter plasmid, transfected resultant reporter plasmids and siRNA of KLF5 into HaCaT cells, and performed luciferase reporter assays. In the control HaCaT cells where control siRNA (SiS) was transfected and no TGFβ treatment was applied, whereas the activity of MYC promoter was at a higher level and mutation 1 of TIE (CGG/TAT) did not change the activity (Fig. 5A), the activity was significantly reduced by mutation 2 of TIE (GGCT/TTAA), the mutation in KBE (A/TTCCCTCCC) and the combination of KBE mutation with either TIE mutation (Fig. 5A). In the same cells treated with TGFβ, however, mutation 1 of TIE made the response of promoter to TGFβ insignificant, whereas reporter activity for MYC promoter with mutation 2 of TIE or KBE mutation was still inhibited by TGFβ (Fig. 5A). When both TIE and KBE were mutated, the inhibitory effect of TGFβ on promoter activity was abolished (Fig. 5A). Knockdown of KLF5 decreased the activity of wild type and mutant pMyc9 (Fig. 5B). Moreover, KLF5 knockdown decreased the activity of wild type and mutant pMyc10 (Fig. 5B). These results indicate that KLF5 is necessary for MYC transcription and that both KBE and
Opposite Effects of KLF5 on MYC Transcription

TIE are important for MYC transcription regulated by KLF5 and TGFβ.

The MDA-MB-231 breast cancer cell line was used to investigate the effect of KLF5 overexpression on MYC promoter activity in the context of TGFβ (Fig. 5C). Wild type pMyc9 MYC promoter reporter plasmid and its mutants were transfected with either pcDNA3-KLF5 or pcDNA3 vector into MDA-MB-231 cells, and luciferase reporter assays were performed. When TGFβ was absent, overexpression of KLF5 significantly increased reporter activities in wild type pMyc9 and the mutant with mutation 1 of TIE but did not cause significant changes in pMyc9 mutants with TIE mutation 2, KBE mutation, or combined KBE and TIE mutations (Fig. 5C). When TGFβ was present, only the wild type pMyc9 but not any of the pMyc9 mutants showed a TGFβ-mediated inhibition of promoter activity (Fig. 5C). These results are consistent with those from HaCaT cells (Fig. 5, A and B), further indicating that both KBE and TIE are important for MYC transcription regulated by KLF5 and TGFβ.

To determine whether and how acetylation of KLF5 affects KLF5-mediated MYC transcription in the context of TGFβ, we transfected different MYC promoter reporter plasmids, including pMyc9 that contained both TIE and KBE, pMyc11 containing only the TIE, and pMyc3 containing only the KBE into HepG2 cells along with plasmids for wild type KLF5 (pcDNA3-FLAG-KLF5), acetylation-deficient mutant of KLF5 (pcDNA3-FLAG-KLF5K369R), or vector control (pcDNA3-FLAG). Luciferase reporter assays were then performed (Fig. 5D). Without TGFβ, expression of wild type KLF5 but not its mutant significantly increased promoter reporter activity in each of the three MYC reporter plasmids (Fig. 5D). Interestingly, when TGFβ treatment was applied, inhibition of reporter activity was achieved only in cells with wild type KLF5 for promoters with TIE (i.e. pMyc9 and pMyc11) but not in cells with mutant KLF5 or in cells with only the KBE (i.e. pMyc11) (Fig. 5D). These results not only indicate that KLF5 is necessary for transcriptional activation of MYC; they further indicate that acetylation of KLF5 is indispensable for KLF5 to modulate MYC transcription in the context of TGFβ.

DISCUSSION

KLF5 Is Required for the Maintenance of MYC Transcription during KLF5-mediated Epithelial Proliferation—Recently, different groups including our own demonstrated that KLF5 is required for the proliferation of non-tumorigenic epithelial cells from different tissues (20, 23, 33, 34). MYC is also a pro-proliferative factor in different types of cells including epithelial cells, as demonstrated in previous studies (3, 4). In this study we demonstrate that knockdown or overexpression of KLF5 inhibits or induces, respectively, the transcription of MYC in epithelial cells and that KLF5 directly binds to the promoter of MYC to regulate MYC transcription (Figs. 1–5). These results indicate a necessary role of KLF5 in the maintenance of MYC transcription in proliferating epithelial cells. We noticed that in MDA-MB-231 cells KLF5 did not induce an obvious increase in MYC transcription (Fig. 2C), which could be caused by the higher level of endogenous MYC expression and/or rapid degradation of MYC mRNA in this cell line (35, 36). Several studies have shown that KLF5-mediated cell proliferation involves the regulation of different genes including the induction of cyclin D1 (20, 34) and growth factor platelet-derived growth factor α (25) as well as the inhibition of cyclin-dependent kinase inhibitors p15 and p27 (23, 33, 37). Our results in this report suggest that MYC, which itself plays a role in the regulation cell cycle-related genes (3, 4), is another functional mediator of KLF5 in epithelial proliferation.

Recently, expression of MYC and three other transcription factors (KLF4, Oct4, and Sox2) was shown to reverse differentiated cells to a pluripotent state (38–40). On the other hand, KLF5 is involved in self-renewal and pluripotency maintenance in stem cells, which may be through regulating the transcription of Nanog and Oct3/4 (32, 41, 42). Regulation of MYC by KLF5 could also play a role in the maintenance of self-renewal and pluripotency of embryonic stem cells.

Down-regulation of MYC in TGFβ-induced Cell Cycle Arrest Also Requires KLF5—As a potent inducer of cell cycle arrest, TGFβ down-regulates the expression of MYC in different types of human cells including epithelial cells (14), and down-regulation of MYC is necessary for TGFβ to inhibit cell cycle progression by inducing cyclin-dependent kinase inhibitors p15 and p21 (10, 11, 43, 44). In addition, overexpression of MYC abrogates the growth inhibition induced by TGFβ in epidermal epithelial cells (13). Our findings in this study, including those showing the necessity of KLF5 for TGFβ to suppress MYC transcription (Fig. 2), different effects of KLF5 on the transcription and promoter activity of MYC with and without TGFβ (Figs. 2–5), and different bindings of KLF5 to MYC promoter in the context of TGFβ signaling (Fig. 4) indicate that KLF5 is also necessary for the down-regulation of MYC mediated by TGFβ in epithelial cells. When the knockdown of KLF5 was more extensive, the inhibitory effect of TGFβ on MYC transcription could be even reversed to a stimulatory effect (Fig. 2A). These results are consistent with our previous study establishing KLF5 as an essential cofactor for TGFβ signaling in the regulation of p15 (23). Furthermore, these results indicate that the function of KLF5 in MYC regulation reverses upon the activation of TGFβ signaling, which is consistent with our previous study demonstrating the reversal of KLF5 function in cell proliferation and p15 regulation in TGFβ-treated epithelial cells (23).

Two KLF5 Binding Elements of MYC Promoter Respond Differently to TGFβ Signaling and Are Necessary for MYC Transcription Regulated by KLF5 and TGFβ—Based on previously characterized MYC promoter (12–15) and consensus sequences for KLF5 binding, we constructed reporter plasmids covering different sizes of MYC promoter, analyzed their promoter activities under different treatments of TGFβ and KLF5 (Fig. 3), performed ChIP analysis to locate KLF5 binding elements on MYC promoter (Fig. 4), and carried out oligo pull-down assays to characterize the binding sites of KLF5 (Fig. 4). These experiments led to the identification of two KLF5 binding sites, KBE and TIE, on the MYC promoter that function in transcriptional regulation of MYC mediated by TGFβ and KLF5 (Fig. 5). These findings indicate that KLF5 binds to specific elements of MYC promoter to regulate MYC transcription.
Binding of KLF5 to the KBE element, which also occurred to in vitro translated KLF5 (Fig. 4D), was decreased by TGFβ (Fig. 4, A, B, and H). In addition, Smad4, a typical component of the TGFβ signaling, did not bind to KBE (Fig. 4B), and binding of acetylation-deficient mutant KLF5 to KBE was not reduced by TGFβ (Fig. 4H). These results suggest that the KBE element is more important for KLF5 to maintain MYC expression without TGFβ. For the TIE, the binding of both KLF5 and Smad4 was increased by TGFβ, as demonstrated in the ChIP and oligo pulldown experiments (Fig. 4, A, C, and F), and the TIE-containing promoter showed a TGFβ-dependent decrease in activity (Fig. 5D). Therefore, the TIE element is more critical for KLF5 to mediate TGFβ-suppressed MYC transcription. However, both KBE and TIE are clearly necessary for either MYC maintenance or MYC repression by TGFβ, as demonstrated in the luciferase reporter assays where mutation of either KBE or TIE could attenuate the promoter activities of MYC without TGFβ, and it was necessary to mutate both KBE and TIE to abolish the inhibitory effect of TGFβ on MYC promoter (Fig. 5, A–C).

TGFβ induces a transcriptional complex on the TIE element to repress MYC transcription (12–15), which involves Smads including Smad4. In our oligo pulldown experiment for TIE (Fig. 4C), although both KLF5 and Smad4 were detected in the protein complex precipitated with wild type TIE oligo, mutant 1 of TIE only pulled down KLF5, and mutant 2 of TIE only pulled down Smad4, suggesting that KLF5 and Smad4 bind to TIE at different nucleotides. In addition, in vitro translated KLF5, which does not contain other DNA or transcription factors, still bound to TIE and mutant 1 of TIE in the oligo pulldown assay (Fig. 4E), and KLF5 enhanced the binding of Smad4 to TIE in the presence of TGFβ, as demonstrated by ChIP assay (Fig. 4G). Taken together with our previous finding that KLF5 and Smad4 interact with each other in epithelial cells (33), our results suggest that KLF5 and Smad4 co-exist in the same transcriptional complex on MYC promoter.

Acetylation of KLF5 at Lysine 369 Is Necessary for TGFβ and KLF5 to Regulate MYC Transcription—Previously we found that TGFβ induces the acetylation of KLF5 and that acetylation of KLF5 is essential for TGFβ and KLF5 to inhibit the transcription of p15 and cell proliferation, primarily by modulating the assembly of transcriptional complex (23, 33). Our results in this paper further demonstrate that acetylation of KLF5 is also involved in TGFβ-KLF5-regulated MYC transcription. For example, the K369R mutation of KLF5 significantly decreased TGFβ-induced binding of KLF5 to TIE (Fig. 4F) but interrupted TGFβ-mediated inhibition of KLF5 binding to KBE (Fig. 4H). Consistently, the mutation also abolished the inhibitory effect of TGFβ on MYC promoter activities (Fig. 5D).

We noticed that without TGFβ, mutant KLF5 still bound to MYC promoter (Fig. 4, F and H), and the binding to the TIE-containing region was even at a higher level for mutant KLF5 than for the wild type (Fig. 4F), suggesting that the K369R mutation does not reduce the binding of KLF5 to MYC promoter DNA. Increased binding of mutant KLF5 to the TIE-containing fragment (Fig. 4F) could be because of structural changes in KLF5 caused by the mutation. We also noticed that although the K369R mutation did not reduce the binding of KLF5 to MYC promoter, it reduced MYC promoter activities in the luciferase assay (Fig. 5D). One explanation is that lysine 369 might undergo other TGFβ-independent modifications that modulate the transactivation activity of KLF5, as suggested by a previous study (45).

Loss of KLF5 in Tumor Cells May Attenuate the Function of TGFβ in Proliferation Inhibition and MYC Down-regulation—MYC is a proto-oncogene activated in various animal and human tumors through different mechanisms (14, 46). Upon TGFβ treatment, MYC expression decreases rapidly, relieving MYC-mediated repression of p15 to inhibit cell proliferation (10). Artificial avert of MYC down-regulation blocks the ability of TGFβ to induce p15 and inhibit cell proliferation (13). Thus, down-regulation of MYC is a key event in the TGFβ program of growth inhibition.

Selective loss of MYC down-regulation in response to TGFβ was identified as a mechanism for the loss of TGFβ growth inhibitory effect in cancer cells, including MDA-MB-231 breast cancer cells in which TGFβ still induces the binding of Smad4 to the Smad binding element but has no effect on MYC expression (13). Mechanistically, TGFβ induces the formation of a Smad complex on the TIE element of MYC promoter, and the formation of this complex is selectively interrupted in breast cancer cells, leading to the loss of TGFβ-mediated MYC down-regulation (13). In the present study we found that re-expression of KLF5 rescued the inhibitory effect of TGFβ on MYC expression in both MDA-MB-231 and PC-3 cancer cells (Figs. 2, C and D, and 5C). While further confirming that KLF5 is essential for TGFβ to function in inhibiting the proliferation of epithelial cells, these results also suggest that loss of KLF5 in cancer cells, which could be mediated by frequent genomic deletion and excessive protein degradation in prostate and breast cancers (18, 19, 26, 47), is a mechanism that attenuates the inhibitory effect of TGFβ on proliferation inhibition and MYC down-regulation.

In summary, we found that KLF5 is a necessary factor for the transcription of MYC in proliferating epithelial cells and that KLF5 is also necessary for TGFβ-mediated MYC down-regulation. The opposing functions of KLF5 in MYC regulation are mediated by its bindings to different elements on MYC promoter, which are modulated differently by TGFβ signaling. These findings will help us understand the role of KLF5, MYC, and TGFβ in the development and progression of human cancer.

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Opposite Effects of KLF5 on MYC Transcription

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