A critical role of MYC for transformation of human cells by HPV16 E6E7 and oncogenic HRAS

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Human papillomaviruses (HPVs) are the primary causal agents for development of cervical cancer, and deregulated expression of two viral oncogenes E6 and E7 is considered to contribute to disease initiation. Recently, we have demonstrated that transduction of oncogenic HRAS (HRASG12V) and MYC together with HPV16 E6E7 is sufficient for tumorigenic transformation of normal human cervical keratinocytes (HCKs). Here, we show that transduction of HRASG12V on the background of E6E7 expression causes accumulation of MYC protein and tumorigenic transformation of not only normal HCKs but also other normal primary human cells, including tongue keratinocytes and bronchial epithelial cells as well as hTERT-immortalized foreskin fibroblasts. Subcutaneous transplantation of as few as 200 HCKs expressing E6E7 and HRASG12V resulted in tumor formation within 2 months. Dissecting RAS signaling pathways, constitutively active forms of AKT1 or MEK1 did not result in tumor formation with E6E7, but tumorigenic transformation was induced with addition of MYC. Increased MYC expression endowed resistance to calcium- and serum-induced terminal differentiation and activated the mammalian target of rapamycin (mTOR) pathway. An mTOR inhibitor (Rapamycin) and MYC inhibition a level not affecting proliferation in culture both markedly suppressed tumor formation by HCKs expressing E6E7 and HRASG12V. These results suggest that a single mutation of HRAS could be oncogenic in the background of deregulated expression of E6E7 and MYC plays a critical role in cooperation with the RAS signaling pathways in tumorigenesis. Thus inhibition of MYC and/or the downstream mTOR pathway could be a therapeutic strategy not only for the MYC-altered but also RAS-activated cancers.

Introduction

A subset of human papillomaviruses (HPVs), the so called high-risk types such as type 16 and 18, are associated with >90% of all cervical carcinomas as primary causal agents (1), with deregulated expression of the HPV viral oncogenes E6 and E7 as the main contributors to an etiology (2). However, epidemiological studies and experimental data indicate that the viral presence is not enough to induce cervical cancer and additional genetic and epigenetic events (to alter the cellular factors) are presumably required (3). To address this, we have established an in vitro model for cervical cancer with normal human cervical keratinocytes (HCKs) focusing on sequential transduction of defined genetic elements (4) and succeeded in the creation of highly potent cancer initiating cells by introduction of c-MYC (MYC) and oncogenic HRASG12V (HRAS) on a background of HPV16 E6 and E7 expression (4). However, since the cells having been cultivated in the differentiating medium were used for the assays, we could not exclude the possibility that genetic and/or epigenic alterations during the selection might be critical for the transformation. In the present study, by directly examining transformed phenotype of the cells without such selection, we could demonstrate that oncogenic HRAS, without overdoseexpression of exogenous MYC, is sufficient for tumorigenic transformation of normal human cells expressing E6 and E7. Nonetheless, endogenous MYC stabilized by HRAS was revealed to be a critical player in tumor-initiating potential.

Materials and methods

Cell culture and cell lines

Normal HCKs were obtained with written consent from patients who underwent abdominal surgery for a gynecological disease other than cervical cancer. HCK1, HCK4 and HCK8 cells derived from different donors were maintained in low-calcium serum-free keratinoctye growth medium (KGM) (Epiline-KG2 KURABO Industries, Ltd, Osaka, Japan) unless otherwise described. HCK1T cells were established by transduction of hTERT into HCK1 cells (4). These HCK cells were then further transduced with HPV16 E6E7 followed by the oncogene(s) of interest. Normal human bronchial epithelial cells (HBEcs) were purchased from Cell Applications (San Diego) and cultivated in KGM. Normal human foreskin fibroblasts (HFFs) purchased from BioWhittaker (Walkersville) were immortalized by transduction of hTERT. HFFs and cervical cancer cell lines, SiHa, CaSki and HeLa and C33A, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) containing 10% fetal bovine serum. The source, authentication and methods of maintenance of the cell lines are described in the Supplementary Materials and Methods, available at Carcinogenesis Online.

Vector construction and retroviral infection

Construction of the retroviral expression vectors, pCLXSN-16E6E7, pCLXSH-tERT, pCMSCVpuro-MYC, pCLMSCVpuro-myr-AKT1, pCMSCVb multidrug-resistant gene (16), which encodes the C-terminal 92 amino acids of MYC with four amino acid substitutions (E410T, E417I, R423Q and R424N) and MYCT58A were made by in vitro mutagenesis and cloned into a lentiviral vector, CSII-TRE-Tight-RfA, in which the elongation factor promoter (EF1α) served as a dominant-interfering MYC mutant (6), which encodes the C-terminal 92 amino acids of MYC with four amino acid substitutions (E410T, E417I, R423Q and R424N) and MYC T58A were made by in vitro mutagenesis and cloned into a lentiviral vector, CSII-TRE-Tight-RfA, in which the elongation factor promoter in CSII-EP-RfA (a gift from Hiroyuki Miyoshi, RIKEN, BioResource Center) was replaced with the tetracycline-responsive promoter from pTRE-Tight (Clontech). CSII-TRE-Tight-16E6E7-2A-MYCT58A-2A-HRASG12V was constructed by inserting the 16E6E7, MYCT58A and HRASG12V segments separated by the sequences encoding the autonomous ‘self-cleaving’ 2A peptide derived from foot-and-mouth disease virus (7) into CSII-TRE-Tight-RfA. CSII-TRE-Tight-MYCMir-1, -2 and -3 were constructed by inserting the short RNA sequence (3) into CSII-TRE-Tight-RfA. The target sequence for MYCMir-1, -2 and -3 were constructed by inserting the short RNA sequence (3) into CSII-TRE-Tight-RfA. The target sequence for MYCMir-1, -2 and -3 was described previously (4,5). OmmMYC, a human version of the dominant-interfering MYC mutant (6), which encodes the 92 amino acid substitutions (E410T, E417I, R423Q and R424N) and MYC T58A were made by in vitro mutagenesis and cloned into a lentiviral vector, CSII-TRE-Tight-RfA, in which the elongation factor promoter in CSII-EP-RfA (a gift from Hiroyuki Miyoshi, RIKEN, BioResource Center) was replaced with the tetracycline-responsive promoter from pTRE-Tight (Clontech). CSII-TRE-Tight-16E6E7-2A-MYCT58A-2A-HRASG12V was constructed by inserting the 16E6E7, MYCT58A and HRASG12V segments separated by the sequences encoding the autonomous ‘self-cleaving’ 2A peptide derived from foot-and-mouth disease virus (7) into CSII-TRE-Tight-RfA. CSII-TRE-Tight-MYCMir-1, -2 and -3 were constructed by inserting the short RNA sequence (3) into CSII-TRE-Tight-RfA. The target sequence for MYCMir-1, -2 and -3 was described previously (4,5).

Western analysis

Western blotting was conducted as described previously (4). Antibodies used were listed in the Supplementary Material and Methods, available at Carcinogenesis Online.

Colony formation in soft agar medium

Cells were seeded at 5 × 103 cells per 35 mm dish (BD Falcon 3046) in an appropriate medium. Colonies over 50 μm in diameter were counted after 3 weeks as described previously (4).

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; E6, embryonic stem cell; HBEc, human bronchial epithelial cell; HCK, human cervical keratinocyte; HFF, human foreskin fibroblast; HPV, human papillomavirus; KGM, keratinocyte growth medium; mTOR, mammalian target of rapamycin.

These authors contributed equally to this work.
Clonogenic assay
Aliquots of 500 cells were seeded on 35 mm dishes under sparse conditions. After cultivation for 2 weeks, the cells were stained with Giemsa’s dye, and the number of colonies was counted.

Tumorigenesis in nude mice
All surgical procedures and care administered to the animals were in accordance with institutional guidelines. A 100 μl volume of cells in a 1:1 mixture of Matrigel (BD Biosciences) was subcutaneously injected into female BALB/c nude mice (Clea Japan). The expression of human involucrin in all tumors was determined by western blots with antibodies against human involucrin that do not react with mouse epidermis to confirm that the tumors were derived from implanted HCKs (data not shown).

Quantitative reverse transcription–PCR analysis
Quantitative reverse transcription–PCR was performed as described previously (8). Amplified products were detected with a TaqMan Gene Expression Assay (Applied Biosystems). The expression level of the MYC gene was then normalized to RNA content for each sample using beta-2-microglobulin messenger RNA as a control.

Results
Oncogenic HRAS is sufficient for tumor initiation with normal human cells expressing HPV16 E6E7
Previously, we demonstrated that introduction of HPV16 E6 and E7 (E6E7), H-RASG12V (HRAS) and c-MYC (MYC) to normal HCKs transduced with hTERT (HCK1T) resulted in the creation of highly potent tumor-initiating cells capable of forming tumors in nude mice when only 10 cells were transplanted subcutaneously (4). Since the cells having been cultivated in the differentiating medium containing high calcium and serum (DMEM + 10% fetal bovine serum; DMEM hereafter) were used for the assays, it is possible that such adaptation or selection was required for the tumor-initiating potential by adding further epigenetic or even genetic alteration(s). To exclude the possibility, we directly examined transformed phenotype of the cells soon after transduction of oncogenes cultivated in KGM and revealed that HRAS addition was sufficient for tumorigenic transformation of primary HCKs and HCKTs (HCKT where T is for hTERT) expressing HPV16 E6E7 (Table I, A). In the presence of HRAS, endogenous MYC protein levels were markedly elevated (Figure 1A). When 1 million cells were subcutaneously transplanted into nude mice, HCK4T-E (E is for E6E7) expressing HRAS formed large tumors within 2 weeks, irrespective of the presence of an exogenous MYC transgene although growth was marginally faster with the latter (Figure 1B; P > 0.05). A high proportion of tumor-initiating cells in populations expressing E6E7 and HRAS was confirmed by injecting only 200 cells of different batches of HCKTs expressing the same set of genes into nude mice, resulting in tumor formation within 2 months (Table I, B). Thus, we examined whether HPV16 E6E7 and HRAS with or without exogenous MYC could confer tumor formation properties on other human cell types, including HBEC and HFF immortalized with hTERT (Figure 1D and E). Although, exogenous MYC expression resulted in the faster tumor-forming ability of HBEC (P < 0.0005), E6E7 and HRAS was sufficient for tumorigenic potential of these human cells with increased endogenous MYC levels (Figure 1C).

Then, we examined the effect of induced expression of MYCT58A, which is a form resistant to FBWX7-dependent proteasomal degradation (9), on the tumorigenic potential of HCK1T-E with HRAS cells. Although MYCT58A accumulation was observed in doxycycline-treated cells both in vitro and in vivo, it did not result in increased tumorigenic potential in this setting (Supplementary Figure I is available at Carcinogenesis Online), indicating the possibility that certain threshold levels of MYC stabilized by HRAS might be sufficient.

Table 1. Summary of xenograft transplantation of HCKs

(A) Tumor formation using E6E7 expressing HCKs with HRASG12V

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of tumors per sites of injection 1 × 10⁶ cells per site</th>
<th>Weight of tumors (mg) at the end of the experiment (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCK1T</td>
<td>4/4</td>
<td>460 ± 137 (3 weeks)</td>
</tr>
<tr>
<td>HCK1Ta</td>
<td>4/4</td>
<td>640 ± 479 (2 weeks)</td>
</tr>
<tr>
<td>HCK1Tb</td>
<td>4/4</td>
<td>500 ± 338 (2 weeks)</td>
</tr>
<tr>
<td>HCK1Tc</td>
<td>4/4</td>
<td>140 ± 50 (2 weeks)</td>
</tr>
<tr>
<td>HCK4T</td>
<td>4/4</td>
<td>523 ± 148 (2 weeks)</td>
</tr>
<tr>
<td>HCK4</td>
<td>4/4</td>
<td>225 ± 50 (3 weeks)</td>
</tr>
<tr>
<td>HCK8</td>
<td>4/4</td>
<td>555 ± 140 (3 weeks)</td>
</tr>
</tbody>
</table>

(B) Tumor formation using E6E7 expressing HCKs with HRASG12V

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of tumors per sites of injection 2 × 10³ cells per site (weeks)</th>
<th>Weight of tumors (mg) at the end of the experiment (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCK1T</td>
<td>6/6 (4)</td>
<td>6/6 (4)</td>
</tr>
<tr>
<td>HCK4T</td>
<td>4/4 (4)</td>
<td>4/4 (6)</td>
</tr>
<tr>
<td>HCK8</td>
<td>4/4 (4)</td>
<td>4/4 (6)</td>
</tr>
</tbody>
</table>

(C) Tumor formation using E6E7 expressing HCK1T cells with MYC or other downstream signals of HRASG12V

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of tumors per sites of injection 1 × 10⁶ cells per site</th>
<th>Tet-inducible-MYCT58A</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCK1T-E6E7-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vect (weeks)</td>
<td>MYC</td>
<td>MYCT58A</td>
</tr>
<tr>
<td>BCL2</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>AKT</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>MEK1DD</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>HRASG12V</td>
<td>12/12 (2)</td>
<td>10/10 (6)</td>
</tr>
</tbody>
</table>

(B) Latency was determined as the time taken before a palpable mass could be detected and indicated in parentheses (weeks). (C) Incidence of tumor formation within 20 weeks of observation period was scored otherwise observation was terminated at the time indicated in parentheses (weeks). No description indicates not determined.

*Only small tumors developed as in Figure 3B and C.

10⁶ (n = 4) or 10³ (n = 6) cells were transplanted.
To confirm that tumorigenicity is readily induced by expression of E6E7, HRAS and MYC (endogenous/exogenous) without further genetic changes and is reversible on cessation of such gene expression, E6E7, MYCT58A and HRAS were cloned into a single lentiviral vector in which expression of transgenes was regulated by doxycyclin (Tet-off) (Figure 1F, G and H). HCK1T cells transduced with this vector together with a Tet-off (tTA) vector were transplanted into nude mice. When tumors had started to grow (the volume of the tumor exceeded 100 mm³), the gene expression was terminated by adding doxycyclin in the drinking water. This resulted in halted tumor growth followed by complete regression (Figure 1H). These data support the idea that E6E7, HRAS and MYC are sufficient for tumor-forming ability of human cells without additional genetic alterations.

MYC stabilization by HRAS

We have reported that endogenous as well as exogenous MYC protein stability is increased in the presence of HRAS in exponentially growing HCK1T cells adapted to calcium and serum (grown in DMEM) (4). Because most of the data in this report were prepared with cells kept in KGM, which does not contain serum and high calcium, endogenous MYC protein stability was determined using HCK1T-E cells with a vector, AKT (myr-AKT1), MEK1DD [constitutively active form of MEK1 which activates the extracellular signal-regulated kinase (ERK) pathway (10,11)] or HRAS in KGM. Endogenous MYC protein levels were increased in the order of the genes listed above (vector, AKT, MEK1DD and HRAS) (Figure 2A). In parallel with the MYC levels, Survivin (12), phosphorylated 4EBP1 (13) and phosphorylated p70S6K levels were increased and TSC2 levels were decreased (14). Increased ERK phosphorylation was observed in MEK1DD- and HRAS-expressing cells. Although similar trends were observed in subconfluent culture (data not shown), they were more evident under post-confluent culture conditions (Figure 2A).

Furthermore, increased MYC protein stability was found in the presence of HRAS (Figure 2C, also with post-confluent cells; data not shown) without significant increase in MYC messenger RNA.
MYCT58A alone did not give rise to tumors. Though MYCT58A mutant HRAS-induced tumorigenic potential, HCK1T-E with induction of MYCT58A showed weak tumor-forming ability upon MYCT58A induction (Table I, Fig. 3B and C). Although without induction of MYCT58A they eventually started to form small tumors at the end of observation period (7–9 weeks), this might have been due to leakage of MYCT58A from the Tet-regulated expression system because HCK1T-E with AKT or MEK1DD alone did not form tumors within 20 weeks (Table I, C). Since tumorigenic potential was less than with HCK1T-E-RAS cells, it is evident that multiple RAS signaling pathways other than simply MYC stabilization are cooperatively involved in tumorigenic transformation of HCK1T-E cells.

**MYC confers resistance to calcium- and serum-induced terminal differentiation and activates the mTOR pathway in HCK cells**

Then, the biological effects of MYC on HCK were examined. Upon induction of MYCT58A in HCK1T cells with BCL2, the expression levels of carbamoyl phosphate synthase/aspertate transcarbamoylase/dihydroorotase, a bona fide MYC target gene (18) and survivin (12) were increased, whereas the levels of a differentiation marker, involucrin, and a key inducer of keratinocyte differentiation, NOTCH1 (19), were decreased (Figure 4A). Furthermore, repression of TSC2 accompanied by activation of the mammalian target of rapamycin (mTOR) pathway was observed upon MYCT58A induction. Activation of NOTCH1 and accumulation of involucrin induced by exposure to calcium and serum were largely canceled by MYCT58A expression (Figure 4A). Similar effects of MYCT58A induction were also observed in HCK1T-E cells with AKT or MEK1DD, although they were less marked, probably because E6E7 and AKT or MEK1DD influenced MYC regulation (17,20) (Figure 4B; data not shown). Induction of MYCT58A significantly supported the growth of these cells in differentiating medium containing serum and high calcium, whereas no significant effects were observed in KGM (Figure 4C and D). Thus, MYC confers resistance to calcium- and serum-induced terminal differentiation and activates the mTOR pathway in HCK cells.

**Inhibition of tumorigenic potentials of HCK1T-E–HRAS cells by inhibition of MYC or mTOR**

Finally, we examined the role of endogenous MYC with HRAS in tumorigenic potential of HCK cells and cervical cancer cell lines (CaSki, SiHa, HeLa and C33A; Supplementary Figure 3B and C is available at Carcinogenesis Online; data not shown) by introducing the MYC inhibitor, OmoMYC (6), with Tet-regulated expression system, for which potential tumor-suppressive effects were recently reported in a mouse lung cancer model featuring KRAS mutation (21). OmoMYC induction levels were determined with an anti-MYC monoclonal antibody that recognizes both endogenous MYC and OmoMYC (Figure 5A). In contrast to the observations with MYCT58A induction (Figure 4A and B), OmoMYC induction resulted
in increased involucrin and TSC2 (Figure 5A), further supporting regulation of these molecules through MYC in HCK cells. With induction of OmoMYC not exceeding endogenous MYC levels, HCK1T-E and HCK8T-E with HRAS cells did not result in significant reduction of growth (Figure 5B). Anchorage-independent growth ability of these cells was dramatically reduced with OmoMYC induction (Figure 5C) and tumorigenic potential was also profoundly reduced (Figure 5D). We obtained essentially the same result by moderate silencing of endogenous MYC in HCK1T-E with HRAS (Supplementary Figure 3A is available at Carcinogenesis Online). The induction of OmoMYC in cervical cancer cell lines also resulted in the suppression of their transforming abilities (Supplementary Figure 3B and C is available at Carcinogenesis Online). Although overexpression of MYC was not obvious in these cell lines, even in HeLa cells with a low level of MYC amplification (22), MYC might also play a critical role in these cells.

Because we found activation of the mTOR pathway in HRAS-transduced HCK cells, effects of an mTOR inhibitor, Rapamycin, on their transformation were tested. The clonogenicity of HCK1T-E with HRAS cells was reduced with Rapamycin in a dose-dependent manner (Figure 5E). Although either 10 nM Rapamycin or OmoMYC induction alone did not result in complete repression of clonogenic potential, simultaneous use of them blocked clonogenicity completely (Figure 5E), while strongly suppressing tumorigenic potential in nude mice (Figure 5F). These data indicate that the mTOR pathway is a major downstream effector activated by HRAS through MYC.

Discussion

MYC and RAS oncogenes can cooperatively induce full transformation of mouse cells but cause apoptosis and senescence, respectively, when expressed individually. Unlike the mouse cell case, transformation of MYC and RAS oncogenes into human cells does not suffice for full transformation, possibly because of more sophisticated tumor-suppressive failsafe mechanisms. However, we recently demonstrated that MYC and RAS can cooperatively transform human cells (HCKs) with the help of HPV16 E6 and E7 (4). In the development of cervical cancer, deregulated expression of E6 and E7 precedes disease progression, and E6 and E7 can immortalize HCKs and alleviate both MYC-induced apoptosis and RAS-induced senescence, mainly through inactivation of p53 and pRB. Here, we showed that oncogenic RAS on a background of E6E7 expression can induce full transformation of HCKs, and that stabilization of MYC by RAS is critical for tumorigenic transformation. Many mechanisms have been reported to be involved in MYC stabilization. A major ubiquitin ligase of MYC, FBXW7, preferentially recognizes and induces degradation of MYC with phosphorylated Thr58 and unphosphorylated Ser62, and thus the MYC$^{T58A}$ mutant is very stable (23). Phosphorylation of Ser62 by ERK1/2 and inhibition of Thr58 phosphorylation through inactivation of GSK3β by AKT/PDK1 are reported to be involved in RAS-induced MYC stabilization (17). Recently, CDK2 and downstream target(s) of PDK1 were also documented to phosphorylate Ser62 (24,25). Activities of these kinases can be regulated by multiple RAS signaling pathways as well.

If we could identify core gene sets, which promote reprogramming of normal human cells into cancer-generating cells, it would be of great advantage to understanding the complicated molecular mechanisms of carcinogenesis. In this study, we clarified that transduction of only three factors, namely oncogenic HRAS, E6 and E7, is sufficient for tumorigenic transformation of HCKs, though early studies have already suggested cooperation between E6E7 and oncogenic RAS (26–29). Thus, E6, E7 and HRAS might constitute one such core gene set. It also proved sufficient to induce full transformation of other normal human cell types, including human tongue keratinocytes, HBECs and HFFs even though the HBECs and HFFs had been transduced with hTERT. Our recent study indicates that the role of E6E7 could be largely but not completely replaced by the blockade of the pRB and p53 pathways in human tongue keratinocytes (30). We previously found that ovarian surface epithelial cells could not be fully transformed by transduction of oncogenic KRAS and MYC with blockade of the pRB and p53 pathways by CDK4/CYCLIN D1 and a dominant-negative form of p53 (5). Other than inactivation of p53 and pRB, E6 and E7 proteins have many functions and it is very conceivable that these could be involved in full transformation. Indeed, in HCKs, the PDZ-binding motif of E6 is critical for full transformation of HCKs through degradation of several PDZ-containing proteins (our unpublished results).

Increased tumorigenic potential by exogenous MYC was observed with variation (Figure 1), indicating certain threshold levels of MYC are required for tumorigenesis depending on the cell type. However,
transduction of four factors, E6, E7, RAS and MYC, proved sufficient for tumorigenic transformation of normal human cells tested here and broader cell types, including colon epithelial cells and pancreatic duct epithelial cells (data not shown), though hTERT might be additionally required for cells, such as HFFs, in which E6 cannot activate telomerase. In our previous study, HCK1T-E–HRAS–MYC cells adapted to DMEM showed much higher MYC expression than those kept in KGM (Supplementary Figure 4 is available at Carcinogenesis Online) with higher tumorigenicity, i.e. 10 DMEM-adapted cells formed huge tumors in 50 days in contrast to the same cell number kept in KGM forming tiny tumors after 100 days [(4) and data not shown]. DMEM-adapted cells might have gained the capacity to permit high levels of MYC and might give us a clue to understand further malignant conversion. Our preliminary data indicate that the DMEM-adapted cells exhibit epithelial–mesenchymal transition like changes, as determined by immunoblotting and microarray analysis (Supplementary Figure 4 and Supplementary Table 1 are available at Carcinogenesis Online).

We tried to dissect the RAS signaling pathways in order to define the critical factors for the promotion of cancer and found that activation of AKT or ERK pathway alone on the background of E6 and E7 expression was insufficient for full transformation. However, with additional induction of MYC, the cells acquired tumorigenicity in nude mice (Figure 3 and Table I, C). These results allow us to hypothesize that one critical player to promote cancer ‘stemness’ downstream of HRAS signaling is elevated function of MYC. In normal HCK1T, induced expression of MYC inhibited terminal differentiation and increased expression of Survivin, which is implicated as a cancer stem cell marker (31) (Figure 4A). Furthermore, we found that TSC2 expression was repressed with induction of MYC, as reported recently for another cell type (14), accompanied by activation of the mTOR pathway.

Fig. 5. Inhibition of MYC and/or mTOR pathway repressed tumorigenic potential of HCK cells with E6E7 and HRAS. (A) Induction of OmoMYC, an MYC inhibitor (Dox 1 μg/ml 5 days) and alteration of involucrin and TSC2 in HCK and CaSki cells were determined by western blotting. (B) Effects of OmoMYC induction on growth of HCK cells with E6E7 and HRAS were determined as for Figure 4C. (C) For assessment of anchorage independent growth of HCK cells with OmoMYC induction, aliquots (5 × 10⁴ cells) were seeded in 35 mm dishes. After 3 weeks, the numbers of colonies (≥50 μm in diameter) were counted. (D) Effects of OmoMYC on tumor-forming ability of HCK cells were determined as for Figure 3B. (E) Clonogenic potential of HCK1T cells with OmoMYC or Rapamycin was determined (5 mg/kg Rapamycin administered by intraperitoneal injection twice a week).
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Supplementary Figure 1 is available at Carcinogenesis Online and remaining cells form tumors (Figure 3). We did not observe significant differences in the tumorigenic potential with the induction of MYCT58A in HCKIT-E with HRAS cells (Supplementary Figure 1 is available at Carcinogenesis Online). It is also reported that low levels of deregulated MYC are competent to drive ectopic proliferation of somatic cells and oncogenesis, but overexpression of MYC wakes up the apoptotic and ARF/p53 intrinsic tumor surveillance pathways (33). These results clearly indicate that a certain threshold level of MYC is sufficient for tumor development, which is not affected by further overexpression, though such surplus expression of MYC might affect other pathological features such as metastasis.

MYC has been identified as one of four genes, which can reprogram fibroblasts into ES cells (34). Analysis of the ES cell-specific gene expression signature revealed that core pluripotency factors such as OCT4 and SOX2 are active in ES and induced pluripotent stem cells but not in cancer stem cells (35), but MYC regulatory networks are activated in both ES and cancer stem cells. Thus, MYC seems to play a role in normal ES cell biology and also cancer stem cells. MYC expression is deregulated in a wide range of human cancers and the rate of overexpression is generally more than the level of amplification (36). Cancers without amplification of MYC but with alterations in other oncogenes, such as RAS and growth factor receptors, which activate the function of MYC, could also be considered as MYC deregulated. Here, inhibition of endogenous MYC functions with Omo-MYC resulted in significant reduction of tumor formation and when the mTOR pathway activated by MYC was suppressed with Rapamycin, the tumorigenic potential of HCK cells was suppressed profoundly (Figure 5). To our knowledge, this is the simplest in vitro carcinogenesis model for human cancer and the first report indicating that endogenous MYC is a critical regulator of HRAS-induced tumor formation by human cells. The contribution of MYC to the cancer stemness might be broader than generally considered, and attempts to inhibit MYC functions with small molecules (37) as cancer therapy might be applicable to a wide range of malignancies.

Supplementary material

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

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Conflict of Interest Statement: None declared.

References


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