

ORIGINAL ARTICLE

Critical role of Myc activation in mouse hepatocarcinogenesis induced by the activation of AKT and RAS pathways

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MYC activation at modest levels has been frequently found in hepatocellular carcinoma. However, its significance in hepatocarcinogenesis has remained obscure. Here we examined the role of Myc activation in mouse liver tumours induced by hepatocytic expression of myristoylated AKT (AKT) and/or mutant HRAS^{V12} (HRAS) via transposon-mediated gene integration. AKT or HRAS alone required 5 months to induce liver tumours, whereas their combination generated hepatocellular carcinoma within 8 weeks. Co-introduction of AKT and HRAS induced lipid-laden preneoplastic cells that grew into nodules composed of tumour cells with or without intracytoplasmic lipid, with the latter being more proliferative and associated with spontaneous Myc expression. AKT/HRAS-induced tumorigenesis was almost completely abolished when MadMyc, a competitive Myc inhibitor, was expressed simultaneously. The Tet-On induction of MadMyc in preneoplastic cells significantly inhibited the progression of AKT/HRAS-induced tumours; its induction in transformed cells suppressed their proliferative activity with alterations in lipid metabolism and protein translation. Transposon-mediated Myc overexpression facilitated tumorigenesis by AKT or HRAS, and when it was co-introduced with AKT and HRAS, diffusely infiltrating tumours without lipid accumulation developed as early as 2 weeks. Examination of the dose-responses of Myc in the enhancement of AKT/HRAS-induced tumorigenesis revealed that a reduction to one-third retained enhancing effect but three-times greater introduction damped the process with increased apoptosis. Myc overexpression suppressed the mRNA expression of proteins involved in the synthesis of fatty acids, and when combined with HRAS introduction, it also suppressed the mRNA expression of proteins involved in their degradation. Finally, the MYC-positive human hepatocellular carcinoma was characterized by the cytoplasm devoid of lipid accumulation, prominent nucleoli and a higher proliferative activity. Our results demonstrate that in hepatocarcinogenesis induced by both activated AKT and HRAS, activation of endogenous Myc is an enhancing factor and adequate levels of Myc deregulation further facilitate the process with alterations in cellular metabolism.

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INTRODUCTION

As a prototypic oncogene, MYC actively participates in the development of various human cancers, including hepatocellular carcinoma (HCC).^{1,2} Although a significant copy number gain of 8q24 containing MYC has been reported to be positive in a relatively small fraction (4–24%) of HCC cases,³ modest multiplication (two to sixfold) has been found in up to 44% of cases.⁴ MYC gene expression is known to be increased due to hypomethylation of its promoter early in hepatocarcinogenesis.⁵ Furthermore, the MYC (or a mouse orthologue Myc) gene product, MYC (or mouse Myc), could be posttranslationally modified and its stability is increased through its cooperation with activated PI3K and RAS-mitogen activated protein kinase (MAPK) pathways.⁶

MYC has been shown to contribute to cell proliferation and tumorigenesis in various ways.⁷ It directly affects the cell cycle machinery by increasing the expression of E2F, as well as cyclin D and cyclin-dependent kinase (CDK) 4, which then activates cyclin E and CDK2 via sequestration of p27.⁸ MYC is involved in transcriptional elongation of many genes with E-boxes in their promoter.¹ MYC also affects posttranscriptional gene expression mechanisms via positive feedback activation of an eukaryotic translation initiation factor (eIF4E)⁹ and ribosome biogenesis by regulating RNA polymerases I, II and III.¹⁰ Furthermore, prior to

G1-S transition, MYC induces metabolic reprogramming, involving glycolysis and glutaminolysis, which is necessary for efficient cell proliferation.^{1,2} Using liver-specific transgenic mouse systems with the albumin enhancer/promoter, deregulated MYC expression has been demonstrated to induce liver tumours, with synergistic interactions with mutant Hras¹¹ or overexpression of E2F1 or transforming growth factor- α .¹² However, there has been a new confounding report showing that liver-specific overexpression of MYC does not promote hepatocarcinogenesis triggered by conditional expression of mutant Hras.¹³ Thus, at present, the precise roles of deregulated Myc expression in hepatocarcinogenesis have not yet been clarified.

By combining the Sleeping Beauty (SB) transposon system¹⁴ and hydrodynamic tail vein injection (HTVi),¹⁵ long-term *in vivo* expression of exogenous genes in mouse hepatocytes is possible,¹⁶ and the introduction of various oncogenes has generated liver cancers.¹⁷ Recently, the transposon-mediated gene integration of both activated (myristoylated) AKT (*myrAKT*) and mutant NRAS (*NRAS^{V12}*) has been shown to induce multiple HCC in as little as 1 month,¹⁸ contrasting with the time course of tumorigenesis induced by *myrAKT* alone, which usually takes several months.¹⁹ Interestingly, a spontaneous increase of Myc has been demonstrated during the tumorigenic process.¹⁸ Although it

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has been shown that MYC induces liver tumours with the appearance of cancer stem-like chemoresistant cells,²⁰ the interaction of MYC with other oncogenic pathways has not been examined.

In this study, we explored the effects of spontaneous expression and forced overexpression of Myc on tumorigenesis by transposon-mediated activation of phosphatidylinositol 3-kinase (PI3K) and/or RAS-MAPK pathways. Our results show that both activation of endogenous Myc and deregulated Myc expression significantly contribute to hepatocarcinogenesis.

RESULTS

Introduction of activated *AKT* or mutant *HRAS* into hepatocytes induces hepatocellular carcinoma after long periods of incubation. Hydrodynamic tail vein injection (HTVi)-mediated introduction of either *myrAKT* (*AKT* henceforward) or mutant *HRAS* (*HRAS* henceforward) alone induced multiple tumours after 20–28 weeks

(Figure 1a). Kang *et al*²¹ reported that similar introduction of mutant *NRAS* (*NRAS*^{G12V}) did not induce liver tumours due to oncogene-induced senescence with immune response. However, in our experiments using mutant *HRAS*, even at 5 weeks after HTVi, numerous transduced hepatocytes were present and invoked only focal and limited immune reaction (Supplementary Figure 1a). Furthermore, these transduced hepatocytes and subsequent tumour cells did not express senescence markers (p16 and p21) (Supplementary Figures 1b, c). Although the reasons for this discrepancy are unclear, it might reflect functional differences between these RAS isoforms.^{22,23}

The tumours induced by either oncogenes showed histological features of HCC (Figure 1b). However, *AKT*-induced tumours demonstrated more marked lipid accumulation than *HRAS*-induced tumours, which was confirmed to be triglyceride by Sudan III staining (Figure 1b). Immunohistochemical studies revealed the highly phosphorylated status of *AKT* in *AKT*-induced tumours but not in *HRAS*-induced tumours (Figure 1c). In contrast, the presence of highly phosphorylated ERK in the nucleus and

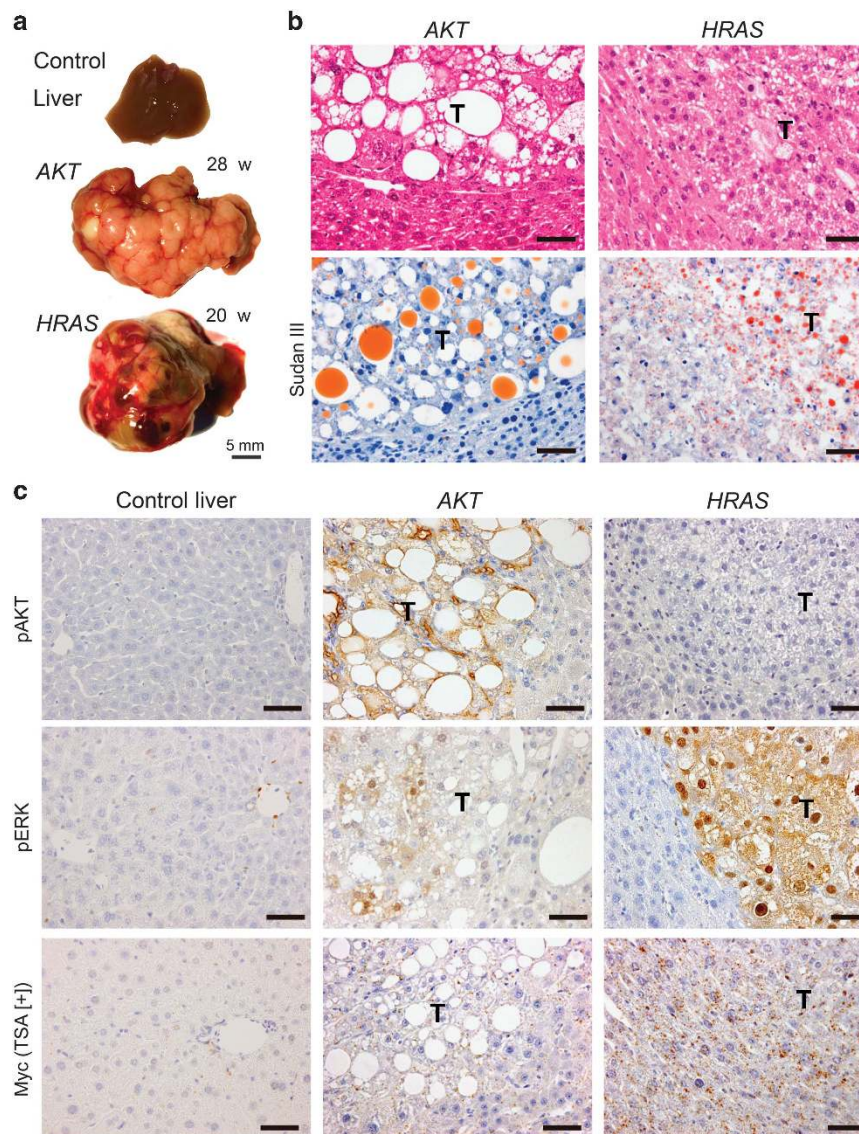


Figure 1. Introduction of *AKT* or *HRAS* into mouse hepatocytes induces liver tumours after long periods of incubation. The pathological features of the liver tumours induced by activated *myrAKT* (*AKT*) or *HRAS*^{V12} (*HRAS*) (incubation periods: *AKT*-induced tumours, 28 weeks; *HRAS*-induced tumours, 20 weeks). (a) Gross appearances of the livers. (b) Hematoxylin and eosin (H&E) and Sudan III stainings of the liver. The tumours show features of HCC associated with lipid accumulation. T, tumours. Scale bar, 50 μm. (c) Immunohistochemistry for phosphorylated *AKT* (pAKT), phosphorylated ERK1/2 (pERK1/2) and Myc (with TSA). T, tumours. Scale bar, 50 μm.

cytoplasm was demonstrated in *HRAS*-induced tumours, whereas there was weak and heterogeneous phosphorylation of ERK in *AKT*-induced tumours (Figure 1c). Myc immunohistochemistry with tyramide signal amplification (TSA) did not detect Myc activation in either tumours (Figure 1c).

Activation of both *AKT* and *HRAS* facilitates hepatocarcinogenesis with decreased lipid accumulation and Myc activation

Following the concomitant introduction of *AKT* and *HRAS*, multiple large tumours appeared as early as 8 weeks (Figure 2a). Microscopically, preneoplastic hepatocytes with lipid accumulation were readily detected after 2 weeks and they grew into HCC composed of tumour cells with or without intracytoplasmic lipid accumulation (Figure 2b). Ki-67 immunohistochemistry revealed that the tumours induced by *AKT* and *HRAS* (*AKT/HRAS*) were more proliferative than those induced by *AKT* or *HRAS* alone (Figures 2c and d). In the *AKT/HRAS*-induced tumours, tumour cells without discernible lipid accumulation showed higher proliferative activity than those with abundant lipid (Figure 2e). Although the preneoplastic cells observed after 2 weeks of injection were negative for Myc, some tumour cells observed after 8 weeks showed weak nuclear Myc expression (Figure 2f). Myc-positive tumour cells were more frequently found in cells without lipid accumulation (Figure 2g). Double immunohistochemistry confirmed that Myc-positive tumour cells were more frequently

positive for Ki-67 (Figures 2h and i). The expression of Myc was not accompanied with an increase of its mRNA (Supplementary Figure 3).

Myc inhibition by MadMyc suppressed hepatocarcinogenesis induced by concomitant introduction of *AKT* and *HRAS*

To examine the significance of the activation of endogenous Myc in *AKT/HRAS*-induced tumours, we concomitantly introduced *MadMyc*, which inhibits Myc activity in a dominant-negative manner.^{24,25} *MadMyc* introduction almost completely abolished *AKT/HRAS*-induced tumorigenesis (Figure 3a). There were only scattered swollen hepatocytes with lipid accumulation in the liver in which *MadMyc* was introduced (Figure 3b). All the *AKT/HRAS*-induced tumour cells, as well as the swollen hepatocytes in the *AKT/HRAS/MadMyc*-treated liver, were positive for HA, which was tagged to both *AKT* and *MadMyc*, indicating that they were transduced cells (Figure 3b). Quantitative analysis of HA-positive area demonstrated that *MadMyc* dramatically inhibited *AKT/HRAS*-induced hepatocarcinogenesis (Figure 3c).

Next we examined the effect of *MadMyc* on progression of *AKT/HRAS*-induced preneoplastic hepatocytes. *MadMyc* was induced by doxycycline (Dox) using the Tet-On system 2 weeks following the introduction of the plasmids, when only preneoplastic hepatocytes were present. The induction of *MadMyc* significantly suppressed the enlargement of the liver at 4 weeks (Figure 3d,

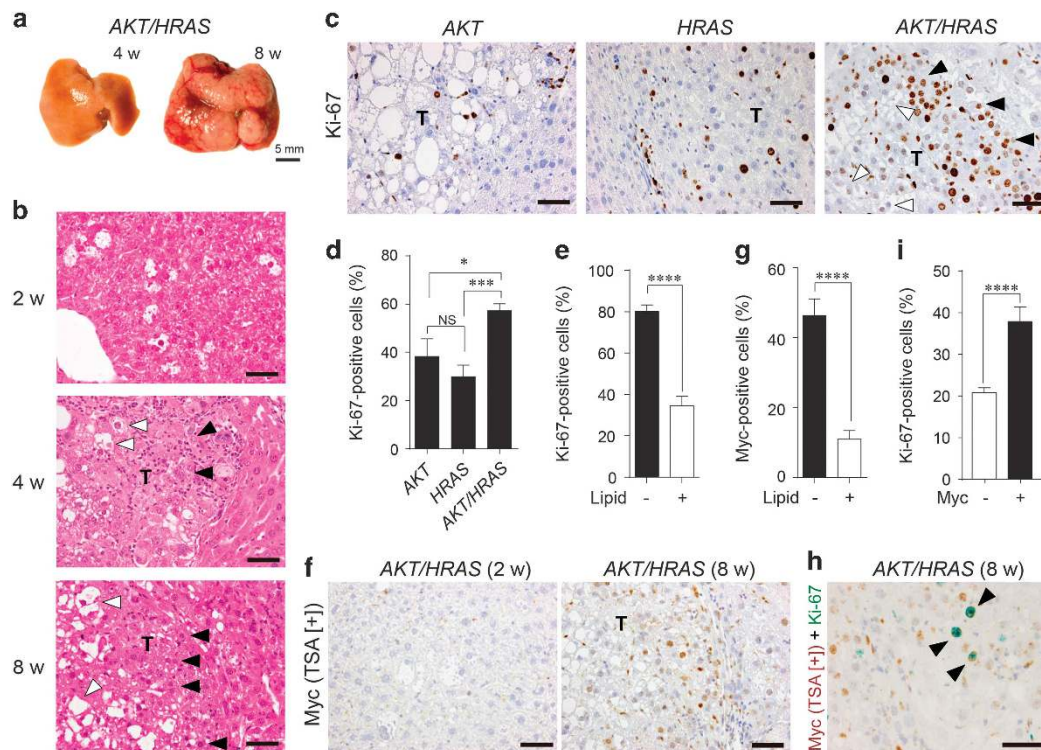
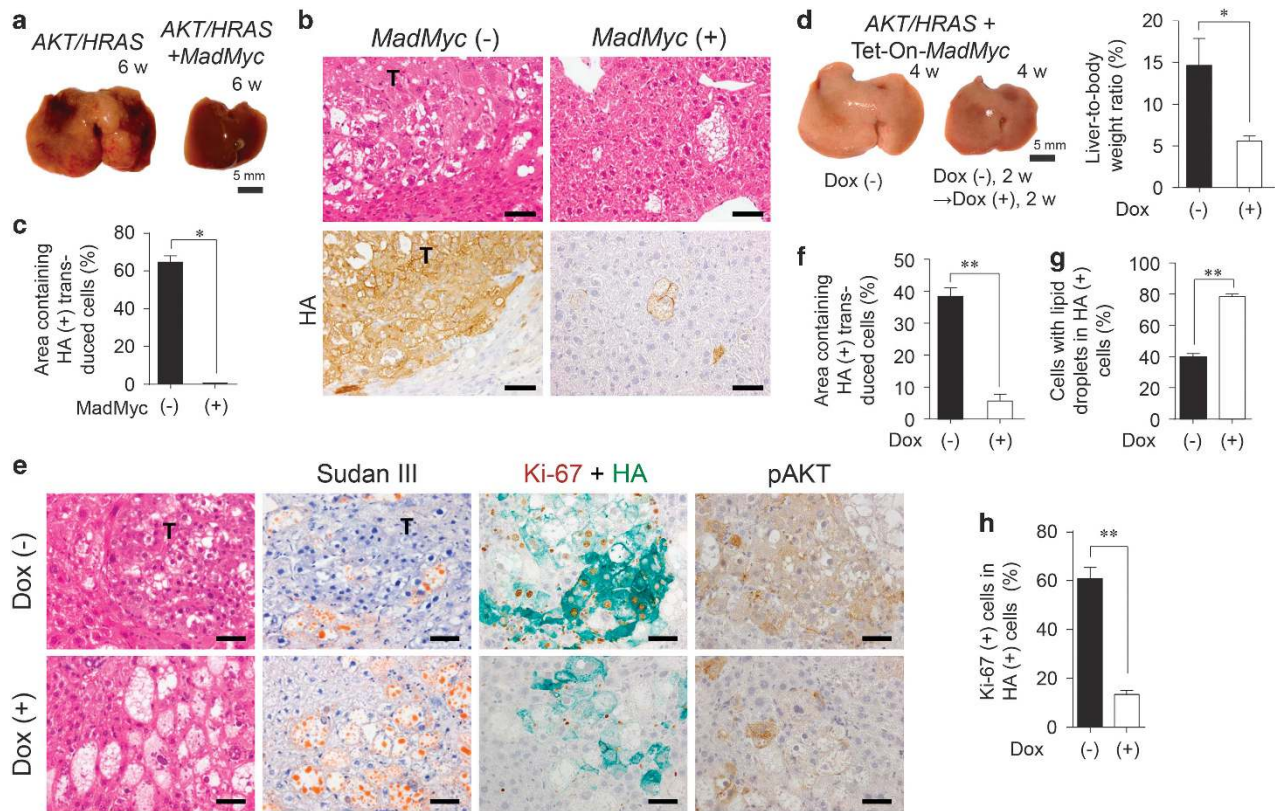


Figure 2. Co-introduction of *AKT* and *HRAS* into mouse hepatocytes rapidly induces liver tumours with spontaneous Myc expression. (a) Gross appearances of the livers. Note the white discoloration and multiple large tumours 4 and 8 weeks after introduction of both *AKT* and *HRAS*. (b) H&E staining of the liver. Premeoplastic hepatocytes containing abundant intracytoplasmic lipid found after 2 weeks develop into microscopic (4 weeks) and macroscopic (8 weeks) tumours with typical histology of HCC. The tumours consist of cells with (white arrowheads) and without (black arrowheads) lipid accumulation. (c) Immunohistochemistry for Ki-67 comparing *AKT*-, *HRAS*- and *AKT/HRAS*-induced tumours. The cells in *AKT/HRAS*-induced tumours, particularly those without lipid accumulation, show higher levels of Ki-67 labelling. (d) Quantitative analysis of Ki-67-labelled tumour cells in *AKT*-, *HRAS*- and *AKT/HRAS*-induced tumours ($n=10, 12$ and 14 , respectively). (e) Quantitative analysis of Ki-67-labelling in tumour cells with or without intracytoplasmic lipid accumulation in *AKT/HRAS*-induced tumours. (f) Myc immunohistochemistry with TSA of *AKT/HRAS*-induced tumours. Some of the tumour cells without intracytoplasmic lipid accumulation show nuclear Myc expression. (g) Quantitative analysis of Myc protein expression in tumour cells with or without intracytoplasmic lipid accumulation in *AKT/HRAS*-induced tumours. (h) Myc/Ki-67 double immunohistochemistry of *AKT/HRAS*-induced tumours. Note the many tumour cells positive for both Myc (brown) and Ki-67 (green) (black arrowheads). (i) Quantitative analysis of Ki-67-labelling in tumour cells with or without Myc expression in *AKT/HRAS*-induced tumours. T: tumours. Scale bar, 50 μm . Statistical analyses: Kruskal–Wallis test (d), Mann–Whitney *U*-test (e, g, i). NS, not significant. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.



Supplementary Figure 2a). Microscopically, in contrast to multiple coalescent tumour nodules in the Dox (-) control, there were scattered large cells with lipid accumulation in the livers of Dox-treated mice (Figure 3e). These cells were positive for both HA and phosphorylated AKT, although the staining intensity of the lipid-rich cells were relatively low (Figure 3e). Dox treatment greatly reduced the area containing HA-positive transduced cells (Figure 3f). HA-positive cells in Dox-treated animals tended to be more abundant in cytoplasmic lipid (Figure 3g) and less proliferative (Figures 3e and h) as compared with those in the Dox (-) control. No significant apoptotic cell death was induced by Dox treatment (Supplementary Figure 4). In another set of experiments, whereas most of the Dox (-) control mice were dead or severely ill due to massive liver tumours within 7 weeks, all of Dox-treated mice survived and appeared well (Supplementary Figures 5a, b). The tumours in Dox-treated mice were smaller in number and size and mainly composed of cells with marked lipid accumulation (Supplementary Figure 5c).

We also explored whether MadMyc suppressed the proliferation of neoplastic hepatocytes by the induction of MadMyc 4 weeks after the introduction of AKT and HRAS, when incipient tumour nodules emerged. Although Dox treatment for 2 weeks did not affect the liver weight (Figure 4a, Supplementary Figure 3b), it induced lipid accumulation in tumour cells (Figure 4b). This was associated with the suppression of proliferative activity (Figures 4b and c). The induction of MadMyc mRNA by Dox treatment was

confirmed by reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis of the tumour tissues (Figure 4d). A cDNA microarray analysis was performed to compare gene expression profiles of tumours obtained from mice with or without Dox treatment. Partial lists of differentially expressed genes are shown in Figure 4e (including the data of the control liver) and Supplementary Table S2. Interestingly, Dox treatment reduced the mRNA expression of *Afp*, *Slpi*, *Gpc3* and *H19*, which are expressed in the fetal/neonatal liver and activated in CCl_4 - or diethylnitrosamine-induced liver tumours.²⁶ The induction of MadMyc suppressed the mRNA expression of *S100a9* and *S100a8*, which encodes calprotectin,²⁷ a heterodimeric protumorigenic protein, as well as that of *Psat1* and *Phgdh* whose products are involved in serine biosynthesis. The detection of transcripts of the genes for immunoglobulin and immune functions in the tumours was attributable to inflammatory cell infiltrates frequently found in the tumours with induced MadMyc (Figure 4b). *Ppp1r3g*, *Slc1a2* and *Cyp1a2* were identified as the genes that were suppressed in AKT/HRAS-induced tumours, but partially recovered by MadMyc induction. Gene Set Enrichment Analysis (GSEA) revealed that the expression dataset of the tumours was significantly enriched with the genes enumerated in the gene sets listed in the Myc Target Gene Database (Figure 4f, Supplementary Tables S3a and S4). Furthermore, the expression dataset was closely associated with the 'lipid metabolism' (Figure 4g, Supplementary Tables S3b, S3c and S5) and

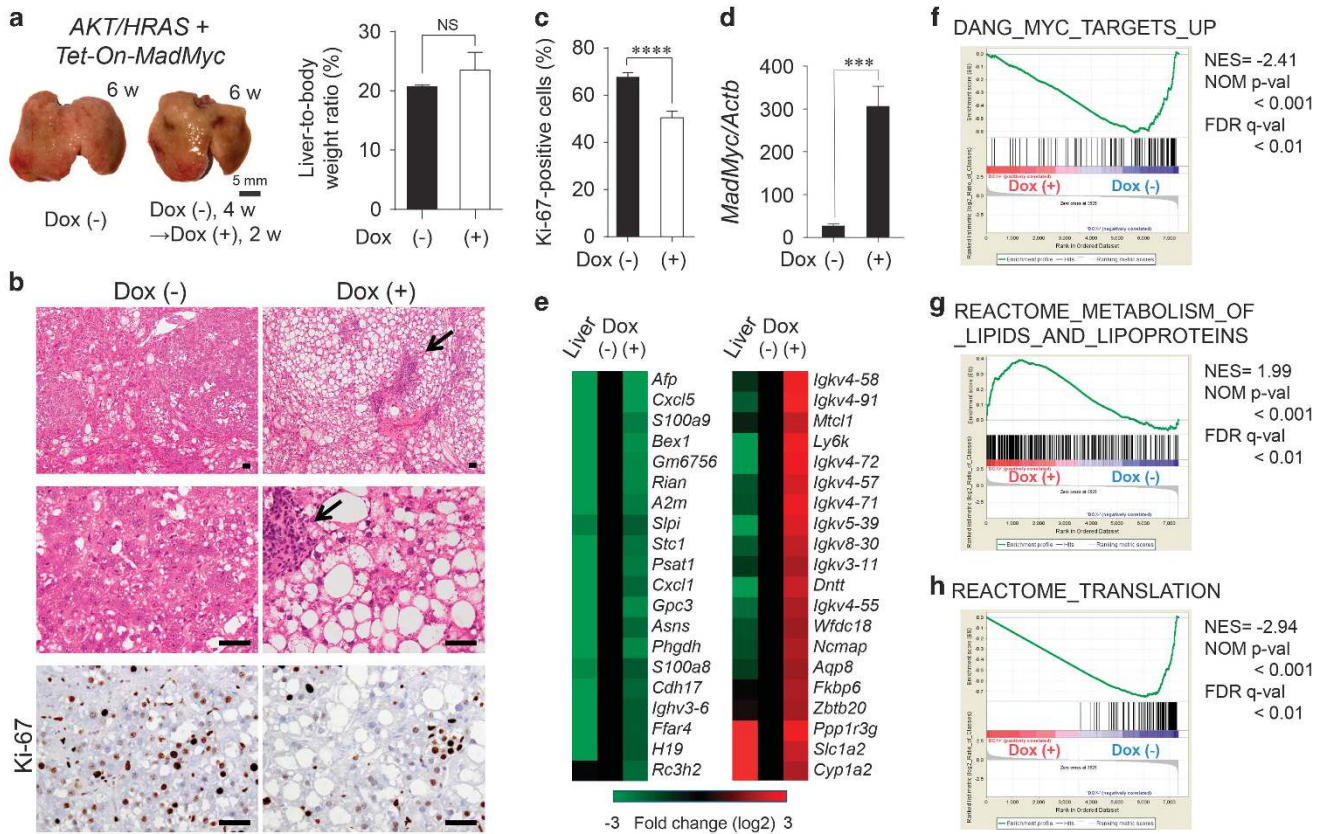


Figure 4. Competitive inhibition of Myc inhibits the proliferative activity of *AKT/HRAS*-induced tumours. **(a)** Gross appearances of the livers and liver-to-body weight ratios 6 weeks after the introduction of *AKT/HRAS/Tet-On-MadMyc*. In the Dox (+) group, at 4 weeks after plasmid injection, Dox was administered during a period of 2 weeks to induce *MadMyc* ($n = 5$ for Dox [-] group, $n = 3$ for Dox [+] group). **(b)** H&E staining and immunohistochemistry for Ki-67 of the liver. Intracytoplasmic lipid accumulation is more prominent in tumour cells in the Dox (+) group. There are scattered foci of lymphoplasmacytic infiltration in the stroma (arrows). **(c)** Quantitative analysis of Ki-67-labelling in tumour cells (10 tumour nodules were examined in each group). **(d)** RT-qPCR analysis of *MadMyc* mRNA expression in tumours ($n = 5$ for Dox [-] group, $n = 8$ for Dox [+] group). **(e)** cDNA microarray analysis of control liver and tumours in the Dox (-) and Dox (+) groups. **(e–h)** GSEA results showing significant correlations of the expression dataset of the tumours with a 'Myc target' gene set **(f)**, a 'lipid metabolism' gene set **(g)**, and a 'translation' gene set **(h)**. For the Reactome gene sets and GO gene sets, those with subset size less than 100 were excluded. From 10 837 probe sets with gene expression value greater than 25, 7348 known genes were selected by the symbol identifier and used as the expression dataset. Scale bar, 50 μ m. Statistical analyses: unpaired *t*-test **(a, c and d)**. NS, not significant. *** $P < 0.001$, **** $P < 0.0001$.

'translation' (Figure 4h, Supplementary Tables S3b, S3c and S6) gene sets.

Overexpression of Myc enhances *AKT*- and/or *HRAS*-mediated hepatocarcinogenesis with suppression of lipid accumulation in tumour cells

We then co-introduced *Myc* in combination with *AKT* (*AKT/Myc*), *HRAS* (*HRAS/Myc*), and *AKT* and *HRAS* (*AKT/HRAS/Myc*). Although injection of the *Myc*-expressing plasmid alone did not produce any tumours during the period of one year (data not shown), the co-introduction of *Myc* markedly enhanced tumorigenesis induced by *AKT* or *HRAS*, generating multiple liver tumours within 7–8 weeks (Figure 5a). The combination of these three oncogenes (*AKT/HRAS/Myc*) induced numerous small liver tumours, which replaced whole livers as early as 2 weeks (Figure 5a). Increased levels of *Myc* mRNA in these tumours were confirmed by RT-qPCR analysis (Supplementary Figure 2). All these tumours showed the histology of HCC with prominent nucleoli (Figures 5b and c). *AKT/HRAS/Myc*-induced tumours were the least differentiated and diffusely infiltrative. Although some cells in *AKT/Myc*-induced tumours showed marked lipid accumulation, *HRAS/Myc*- and *AKT/HRAS/Myc*-induced tumour cells were almost devoid of lipid (Figure 5b). The tumour cells showed highly proliferative activities, with Ki-67-labelling indices of 71.3,

80.7 and 82.2% in *AKT/Myc*-, *HRAS/Myc*- and *AKT/HRAS/Myc*-induced tumours, respectively (Figure 5d). In contrast to the heterogeneous expression levels of Myc in *AKT/Myc*-induced tumours, *HRAS/Myc*- and *AKT/HRAS/Myc*-induced tumour cells demonstrated strong and homogeneous Myc expression (Figure 5b). In *AKT/Myc*-induced tumours, cells without lipid accumulation showed higher proliferative activity than those with abundant intracytoplasmic lipid (Figure 5e). *HRAS/Myc*-induced tumours contained many apoptotic cells that were highlighted by cleaved caspase-3 immunohistochemistry (Figures 5b, c and f).

Adequate levels of Myc activation are required for the promotion of *AKT/HRAS*-induced hepatocarcinogenesis

We further explored the dose-response relationship between the levels of Myc activation and its carcinogenic effect. In the *AKT/HRAS/Myc*-induced hepatocarcinogenesis model, the amount of introduced *Myc*-expressing plasmid was decreased to one-third ($1/3 \times$) or increased three times ($3 \times$) and compared the extent of tumour formation with the original ($1 \times$). Myc immunohistochemistry without TSA demonstrated that the introduction of more ($3 \times$) *Myc* increased Myc expression in the individual transduced hepatocytes at 3 days after the introduction of the plasmids (Supplementary Figures 6a, b), although the total number of the

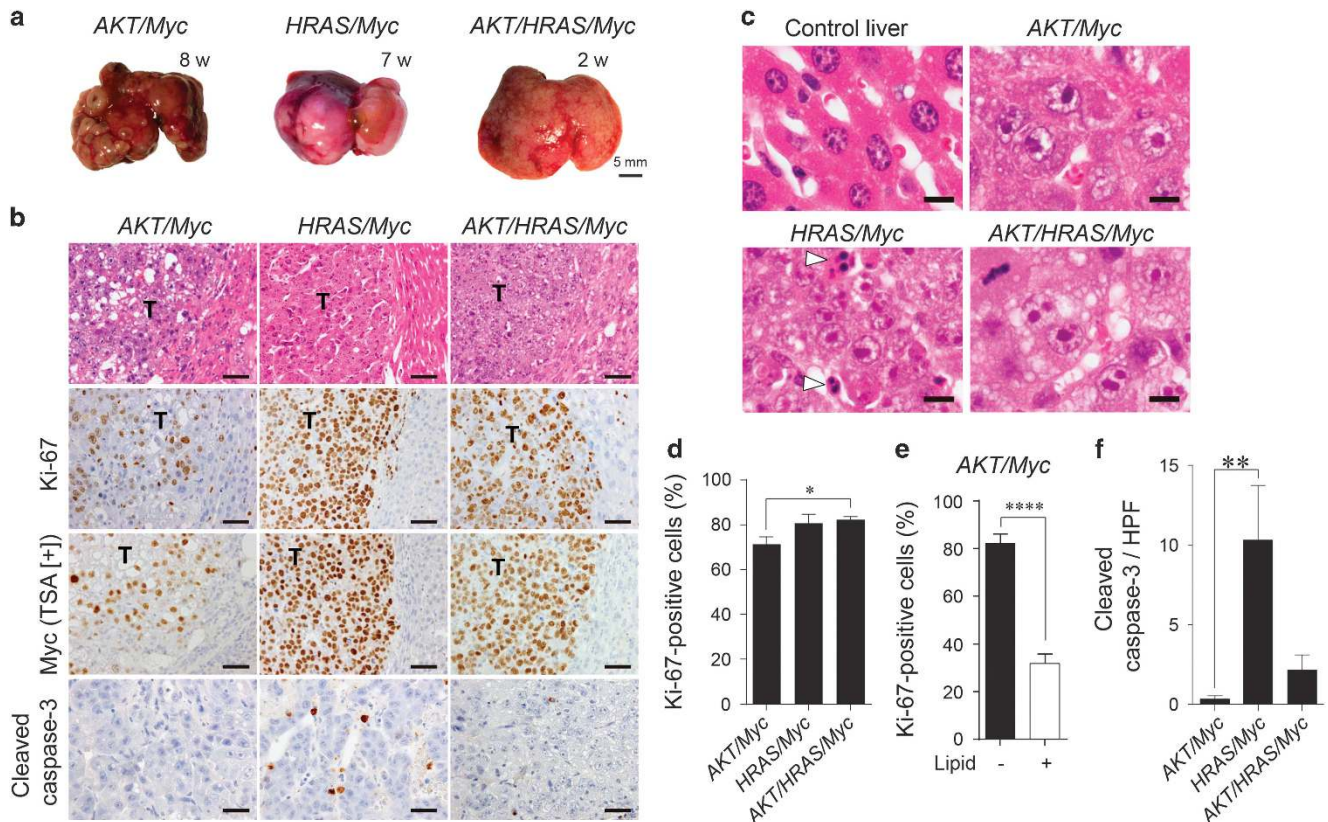


Figure 5. Overexpression of *Myc* enhances *AKT*- and/or *HRAS*-mediated hepatocarcinogenesis with suppression of lipid accumulation in tumour cells. **(a)** Gross appearances of the livers after introduction of *AKT/Myc*, *HRAS/Myc* and *AKT/HRAS/Myc*. Although multiple liver tumours were generated after 7–8 weeks when *AKT/Myc* or *HRAS/Myc* was introduced, the combination of three oncogenes (*AKT/HRAS/Myc*) induced diffuse liver tumours as early as 2 weeks. **(b)** H&E staining and immunohistochemistry for Ki-67, *Myc* (with TSA), and cleaved caspase-3 of the livers 8, 7 and 2 weeks after the introduction of *AKT/Myc*, *HRAS/Myc* and *AKT/HRAS/Myc*, respectively. T: tumours. Scale bar, 50 μ m. **(c)** H&E staining of the normal liver and tumours with *Myc* overexpression (higher magnification). White arrowheads indicate apoptotic cells in *HRAS/Myc*-induced tumours. Scale bar, 20 μ m. **(d)** Comparison of cell proliferative activities (percentages of Ki-67-positive cells) among *AKT/Myc*-, *HRAS/Myc*- and *AKT/HRAS/Myc*-induced liver tumours ($n=9$, 11 and 15, respectively). **(e)** Relationship between the proliferative activity and lipid accumulation in *AKT/Myc*-induced tumours. **(f)** Quantitative analysis of the number of cleaved caspase 3-positive tumour cells per high power field. Statistical analyses: Kruskal–Wallis test (**d**, **f**), unpaired *t*-test (**e**). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

HA-positive transduced cells was comparable to that seen in the mice in which 1 \times or 1/3 \times *Myc* was introduced (Supplementary Figures 6a, c). Interestingly, the introduction of 3 \times *Myc* inhibited its tumour-promoting effect, whereas the effect was not affected by the decreased dosage (1/3 \times) of *Myc* (Figure 6a), which was clearly exemplified by changes in relative liver weight (Figure 6a, Supplementary Figure 2c). Although HA-positive transduced cells formed coalescent tumours of poorly differentiated HCC when 1 \times or 1/3 \times *Myc* was introduced, the introduction of 3 \times *Myc* reduced the size and the number of tumours, resulting in discrete tumour nodules, which were composed of a thick trabecular structures surrounded by well-developed sinusoids, suggesting a more differentiated state (Figure 6b). Quantitative analysis of areas of HA-positive cells showed a biphasic dose response (Figure 6c). *Myc* immunohistochemistry without TSA revealed that *Myc* immunoreactivity was significantly higher in the tumours induced by the introduction of 3 \times *Myc* as compared with those induced by 1 \times or 1/3 \times *Myc* (Figure 6b, Supplementary Figure 7a). *Myc* overexpression did not affect the phosphorylation state of *AKT* (Figure 6b, Supplementary Figure 7b). Because *Myc* has also been shown to trigger apoptosis when activated, we suspected that *Myc*-induced apoptosis might be involved in dampening of the tumour-promoting effect when *Myc* was applied at high doses. Indeed, the average number of cleaved caspase-3-positive apoptotic cells 3 days following plasmid injection was twice as

high in the liver infused with *Myc* (3 \times) as in the liver infused with *Myc* (1 \times) (Figures 6d and e; Supplementary Figure 6a).

Myc inhibits fatty acid synthesis and β -oxidation with potential interaction with *HRAS*

We also explored the mRNA expression of genes relevant to fatty acid synthesis and degradation. In tumours induced by *AKT*, *HRAS* and *AKT/HRAS*, the mRNA expression of proteins involved in fatty acid synthesis (*Acly*, *Acaca*, *Fasn*, *Scd1*, *Scd2* and *Srebf1*) was increased compared with the control liver (Figure 7). However, the increased expression was suppressed by the co-introduction of *Myc* particularly when *Myc* was combined with *HRAS* (Figure 7). As for proteins related to transport of long-chain fatty acids (*Cpt1a*) and β -oxidation (*Acadm*, *Hadha*, *Hadhb*), the mRNA levels were markedly decreased in *HRAS/Myc*- and *AKT/HRAS/Myc*-induced tumours, but not in *AKT/Myc*-induced tumours (Figure 7). These results suggested a potential synergistic interaction between *Myc* and *HRAS* in the suppression of fatty acid synthesis, transport and β -oxidation. Compatible with the RT-qPCR data, proteomic analysis revealed that the expression of the critical enzymes in fatty acid synthesis, *Acly* and *Fasn*, was elevated in *AKT/HRAS*-induced tumours, but decreased in *AKT/HRAS/Myc*-induced tumours (Supplementary Figure 8). The expression of many enzymes in fatty acid degradation was decreased in both tumours (Supplementary Figure 8).

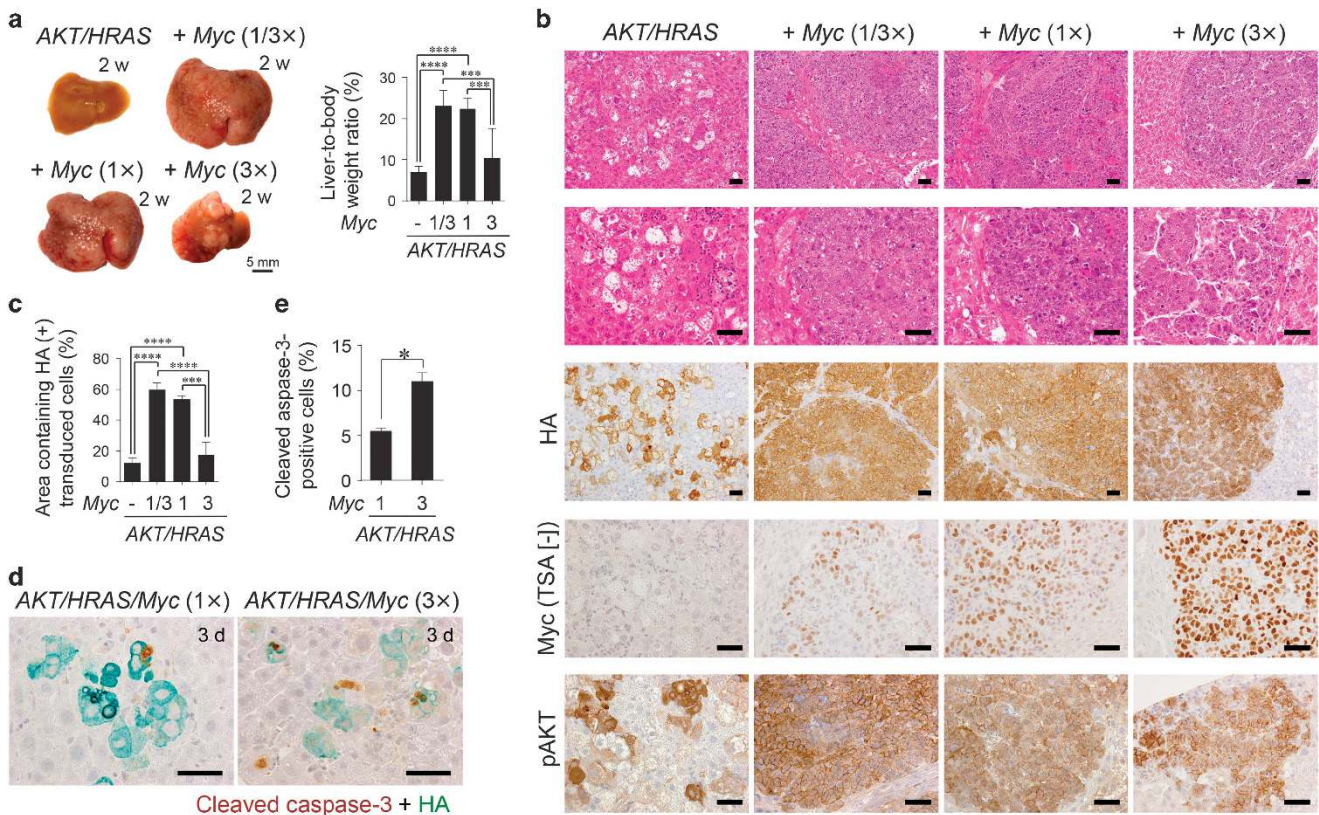


Figure 6. The tumour-promoting effect of Myc overexpression is dependent on its degree of deregulation. **(a)** Gross appearances of the livers and liver-to-body weight ratios 2 weeks after introduction of *AKT/HRAS* along with various doses of Myc (0, 1/3 \times , 1 \times and 3 \times of the original dose used in the previous experiments). **(b)** H&E staining and immunohistochemistry for HA, Myc (without TSA), and phosphorylated AKT of the livers (2 weeks after the introduction of the indicated oncogenes). **(c)** Quantitative analyses of the area containing HA-positive transduced cells (2 weeks after the introduction of the indicated oncogenes) ($n=6$ in each group). A total area of approximately 32 mm² was analysed in each animal. **(d)** Double immunohistochemistry for cleaved caspase-3 and HA of the livers 3 days after the introduction of *AKT/HRAS/Myc* (1 \times) or *AKT/HRAS/Myc* (3 \times). **(e)** Quantification of cleaved caspase-3 and HA double-positive cells in *AKT/HRAS/Myc* (1 \times)- and *AKT/HRAS/Myc* (3 \times)-induced tumours. Approximately 500 HA-positive cells were analysed in each animal. Scale bar, 50 μ m. Statistical analyses: one-way ANOVA with Tukey *post-hoc* test (**a**, **c**), Mann–Whitney *U*-test (**e**). * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

MYC-expressing human HCC is devoid of lipid accumulation and highly proliferative

Finally, we inquired whether human HCC with MYC expression might demonstrate the phenotype similar to MYC-expressing mouse tumours. Among the 33 HCC cases examined, nine cases demonstrated distinct nuclear MYC expression (Figures 8a and b), although there was intratumoural heterogeneity. Tumours in 11 cases showing diffuse intracytoplasmic lipid accumulation of various degrees were completely negative for MYC (Figures 8a and b). No MYC-positive tumour areas were accompanied with intracytoplasmic lipid accumulation (Figure 8a) and a statistical analysis suggested that there was a non-random association between Myc expression and the lack of lipid accumulation (Figure 8b; $P=0.015$, Fisher's exact test). Interestingly, similar to Myc-overexpressing mouse tumours (see Figure 5c), the nuclei of tumour cells with MYC expression contained prominent nucleoli (Figure 8a). This tendency was confirmed by quantitative analysis (Figure 8c; nucleolar size of control hepatocytes: $2.78 \pm 0.17 \mu\text{m}^2$). Furthermore, Ki-67 immunohistochemistry revealed that MYC-expressing HCC were more proliferative as compared with MYC-negative HCC with or without lipid accumulation (Figures 8a and d).

DISCUSSION

Spontaneous posttranscriptional Myc activation occurred in *AKT/HRAS*-induced tumours. This was compatible with previous

reports,¹⁸ in which secondary Myc activation was described in liver tumours induced in *AKT/NRAS*-induced tumours. The Ras/MEK/ERK pathway has been shown to phosphorylate Ser-62 of MYC protein and enhance its stability.⁶ The PI3K-mediated activation AKT has been demonstrated to inhibit glycogen synthase kinase-3, which phosphorylates MYC at Thr-58 and facilitates its proteasomal degradation.⁶ The significance of these posttranslational regulation has been confirmed in mammary gland tumorigenesis *in vivo*.²⁸ It has also been demonstrated that RAS and AKT induced both phosphorylation-dependent and -independent suppression of MAD, which inhibits MYC-mediated transcriptional activity.²⁹ In the present study, we found that increased Myc expression was closely associated with higher proliferative activity of tumour cells, suggesting an important role of Myc in the progression of mouse liver tumours. Intriguingly, malignant conversion of human hepatocarcinogenesis has been demonstrated to be associated with activation of the MYC transcription signature without concomitant overexpression of the MYC gene.³⁰

AKT/HRAS-induced tumorigenesis was almost completely abrogated by the simultaneous introduction of *MadMyc* with these oncogenes, suggesting that low or basal levels of Myc expression facilitated the initiation step of *AKT/HRAS*-induced tumorigenesis. Interestingly, *KRAS*-driven mouse lung cancers, in which Myc is not amplified, have been reported to be contained and eradicated by the induction of a dominant-negative Myc mutant.³¹ Furthermore, recent experiments using conditional Myc-knockout mice have

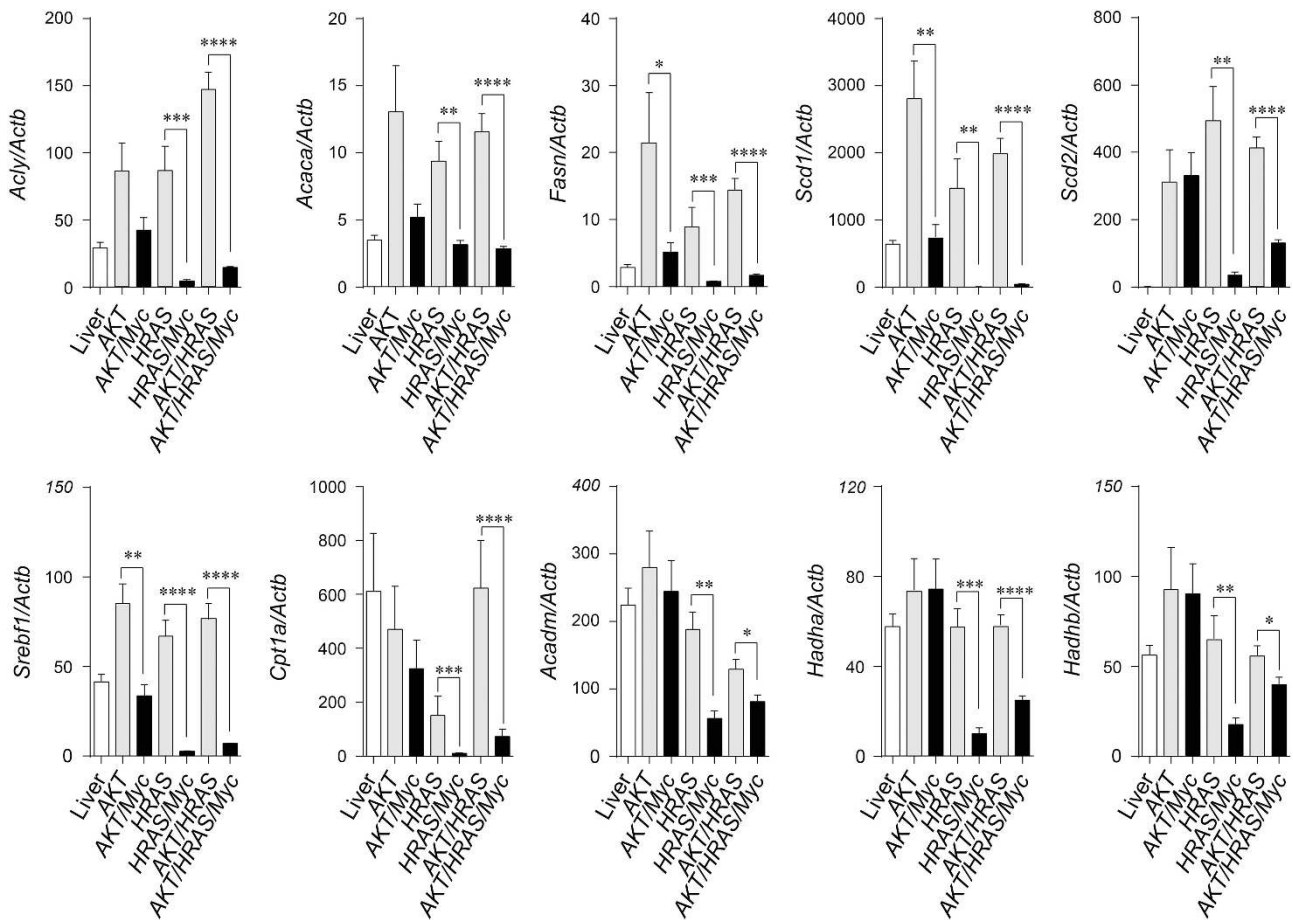


Figure 7. The combination of *Myc* and *HRAS* inhibits mRNA expression of proteins involved in fatty acid synthesis and degradation. RT-qPCR analyses of mRNA expression of proteins involved in fatty acid synthesis (*Acl1*, *Acaca*, *Fasn*, *Scd1*, *Scd2*, *Srebf1*), transport of long-chain fatty acids (*Cpt1a*) and fatty acid degradation (*Acadm*, *Hadha*, *Hadhb*) in control liver (Liver) and liver tumours induced by the introduction of various oncogenes (*AKT*, *AKT/Myc*, *HRAS*, *HRAS/Myc*, *AKT/HRAS*, *AKT/HRAS/Myc*) ($n = 7-8$ in each group). Statistical analyses: Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

shown the importance of expression of endogenous *Myc* in hepatocytic proliferation induced by a PPAR- α agonist and diethylnitrosamine-induced hepatocarcinogenesis.³² We also showed that inhibition of secondary *Myc* activation by inducible MadMyc efficiently suppressed the progression of preneoplastic hepatocytes into fully proliferative neoplastic hepatocytes. Furthermore, MadMyc-mediated *Myc* suppression in *AKT/HRAS*-induced neoplastic hepatocytes decreased their proliferative activity. It has been shown that conditional *Myc* inactivation induces differentiation and tumour dormancy in mouse HCC induced by overexpression of *Myc*.³³ These results suggest that, although therapeutic interventions through *Myc* suppression are effective in the earlier phases of hepatocarcinogenesis, they might be also applicable to the established tumours.

MadMyc-mediated inhibition of proliferation of *AKT/HRAS*-induced tumours was associated with the reduced expression of several genes (*Afp*, *Sipi*, *Gpc3* and *H19*) with oncofetal features.²⁶ Although the exact functions of these genes in hepatocarcinogenesis remain elusive, our results suggest that the regulation of the expression of fetal/neonatal liver genes by *Myc* may be important in HCC cell proliferation. *S100a8* and *S100a9*, encoding calprotectin, were also identified to be suppressed by MadMyc induction. Calprotectin has recently been shown to play a protumorigenic function in diethylnitrosamine-induced mouse hepatocarcinogenesis.²⁷ Our results also demonstrated that the mRNA expression of *Psat1* and *Phgdh* was suppressed by MadMyc induction, suggesting that serine biosynthesis, which is crucial in

glutathione synthesis,³⁴ could be a therapeutic target. On the other hand, MadMyc induction recovered the mRNA expression of *Cyp1a2* and *Ppp1r3g*. Interestingly, cytochrome P450 1A2, the product of *CYP1A2*, which metabolizes 17- β -estradiol to 2-methoxyestradiol, has been demonstrated to be decreased in human HCC.³⁵ *Ppp1r3g*, encoding a glycogen-targeting subunit of protein phosphatase 1 that dephosphorylates and activates glycogen synthase, is involved in glucose homeostasis and lipid metabolism.³⁶ Furthermore, GSEA revealed that MadMyc induction was closely correlated with enhanced lipid synthesis and reversely correlated with protein translation, highlighting the importance of therapeutic reversal of metabolic reprogramming induced by *Myc*.

When *Myc* was overexpressed simultaneously with *AKT*, *HRAS* or *AKT/HRAS*, it dramatically facilitated tumorigenesis. The synergistic interaction between transgenic *Myc* and *Hras* has been shown in mouse hepatocarcinogenesis.¹¹ Furthermore, in a transgenic mouse model overexpressing both *Myc* and transforming growth factor- α , liver tumorigenesis has been reported to be facilitated due to the generation of an oxidative stress environment that causes the accumulation of DNA damage.³⁷ However, rapid induction of liver tumours in our experiments suggests more direct interactions of *Myc* and these oncogenic pathways, such as cross signalling between epidermal growth factor receptor and *Myc*, which has been found in mutant *KRAS*-induced pancreatic tumours.³⁸ Although deregulated *Myc* is also a well-known inducer of apoptosis in cells with intact ARF/p53 function,³⁹ the

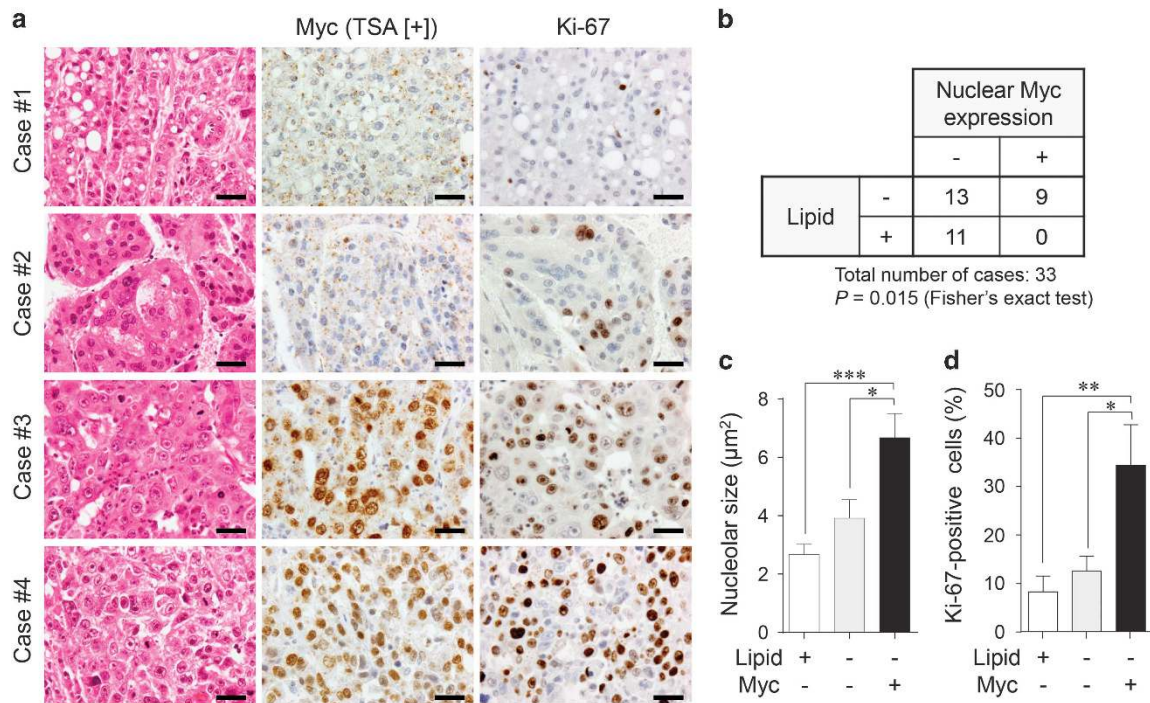


Figure 8. MYC-expressing human HCC lacks intracytoplasmic lipid accumulation and is highly proliferative. **(a)** H&E staining and immunohistochemistry for MYC and Ki-67 of human HCC. Most tumour cells in the case #1 contain intracytoplasmic lipid droplets, while tumours in the cases #2–4 lack intracytoplasmic lipid accumulation. MYC is negative in the cases #1 and 2, while positive in the cases #3 and 4. Scale bar, 50 μm . **(b)** The relationship between lipid accumulation and nuclear MYC expression in HCC. **(c, d)** Comparison of nucleolar sizes **(c)** and cell proliferative activities (percentages of Ki-67-positive cells) **(d)** among lipid-positive/MYC-negative HCC ($n = 13$), lipid-negative/MYC-negative HCC ($n = 11$), and lipid-negative/MYC-positive HCC ($n = 9$). Statistical analyses: Fisher's exact test **(b)** and Kruskal-Wallis test **(c, d)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

activation of AKT signalling has been shown to inhibit apoptosis induced by Myc,⁴⁰ sparing its growth-promoting effects.

Our results also revealed that concomitant *Myc* overexpression above specific threshold levels suppressed *AKT/HRAS*-induced hepatocarcinogenesis with an increase in early apoptotic cell death of transduced hepatocytes. Consistent with our findings, Murphy *et al*⁴¹ reported that low levels of deregulated Myc induced ectopic proliferation of somatic cells including hepatocytes and that higher levels of Myc expression triggered ARF/p53 apoptotic pathways. Similar differential dose effects of MYC were found in human HCC cell lines with various levels of induced Myc expression.⁴² Our data show for the first time that the extent of hepatocarcinogenesis is significantly influenced by the levels of deregulated Myc expression, with lower levels being more efficient.

Intracytoplasmic lipid was lost in highly proliferative cells with Myc expression in *AKT/HRAS*-induced tumours and in tumour cells with forced *Myc* expression. Although the mRNA expression of proteins involved in fatty acid synthesis was increased in *AKT*- and/or *HRAS*-induced tumours, it was strongly suppressed by forced Myc expression. The mRNA expression, as well as protein expression, of the major enzymes participating in β -oxidation was suppressed in tumours, especially in those induced by the combination of *HRAS* and *Myc*. Although Myc has been known to stimulate lipogenesis and β -oxidation,² our results clearly demonstrated a novel interaction of these oncogenes in metabolic reprogramming in hepatocytic tumours. Interestingly, in *MYCN*-amplified neuroblastoma cell lines, inhibition of N-Myc results in the accumulation of lipid droplets.⁴³ Because the proliferative activity observed in *AKT/HRAS/Myc*-induced tumours, in which both fatty acid synthesis and β -oxidation were profoundly suppressed, was the highest, this metabolic reprogramming might be beneficial for tumour cell proliferation.

MYC-positive human HCC cells lacked intracytoplasmic lipid accumulation and were more proliferative as compared with those without MYC expression. A previous report demonstrated that MYC gene amplification in human HCC was positively correlated with proliferative activity and associated with shorter disease-free survival in patients.⁴⁴ Furthermore, MYC-positive tumour cells characteristically possessed prominent nucleoli, which were similar to those found in the nuclei of Myc-overexpressing mouse tumours. MYC is known to be involved in synthesis of the precursor rRNA and its processing.⁴⁵ Interestingly, adenovirus-mediated overexpression of Myc in mouse hepatocytes *in vivo* has been reported to induce enlargement of their nucleoli, as well as nuclei, with increased mRNA levels of genes that encode proteins engaged in ribosomal biogenesis and protein translation.⁴⁶ In conclusion, our findings in this experimental study, which have been at least partly validated in human specimens, highlight the critical importance of Myc in hepatocarcinogenesis.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). The mice were killed under deep anesthesia. The protocols used for animal experimentation were approved by the Animal Research Committee, Asahikawa Medical University, and all animal experiments adhered to the criteria outlined in the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. No blinding was used for animal experiments. All animals used were included in the analyses.

Transposon-mediated introduction of oncogenes into mouse hepatocytes

To introduce genes into hepatocytes *in vivo*, the combination of the SB transposon system and HTVi was performed.⁴⁷ The plasmids were

co-injected with an SB13 transposase-expression plasmid into male C57BL/6J mice (8–12-week-old) via HTVi. An SB13 transposase-expressing vector (pT2/C-Luc/PGK-SB13, Addgene plasmid #20207) and a myrAKT-HA-expressing transposon cassette vector (pT3-EF1 α -myrAKT-HA, Addgene plasmid #31789) were obtained from Addgene (Cambridge, MA, USA). cDNA fragments of FLAG-HRAS^{V12}, EGFP, Venus-IRES-rtTA3 and MadMyc-HA were amplified from pTomo-Ras (Addgene plasmid #26292), pCMV-EGFP (Takara Bio, Kusatsu, Shiga, Japan), TtRMPVIR (Addgene plasmid #27995) and MadMyc-HA (Addgene plasmid #16557),²⁵ respectively. A full length Myc fragment was amplified from cDNA of diethylnitrosamine-induced mouse liver tumours. These cDNA fragments were cloned into the pT3-EF1 α transposon cassette plasmid using the Gateway cloning system (ThermoFisher Scientific, Waltham, MA, USA). For construction of the tetracycline-controlled transcriptional activation system (Tet-On), the EF1 α promoter of the destination vector of the Gateway cloning system was replaced with the TRE-tight promoter, which was amplified from TtRMPVIR, using the GeneArt Seamless Cloning and Assembly Kit (ThermoFisher Scientific).

For HTVi, plasmids were dissolved in 2.5 ml Ringer solution and rapidly injected via the lateral tail vein of mice within 8 s. The total amount of plasmid DNA were 16, 25, 36 and 43 μ g for mixtures of 2, 3, 4 and 5 different plasmids (including the transposase-expressing vector), respectively. The molar ratio of the transposase-expressing vector to each transposon cassette plasmid containing various genes was 1:2. To induce MadMyc expression in the Tet-On system, mice were given Dox *ad libitum* as drinking water (2 mg/ml Dox and 1% sucrose in tap water). The animals were randomly assigned to Dox (-) and Dox (+) groups.

Microscopic examination and immunohistochemistry

The livers were fixed in phosphate-buffered 4% paraformaldehyde for 24 h, dehydrated, cleared and embedded in paraffin. Immunohistochemical staining was performed using the EnVision/HRP system (DAKO, Carpinteria, CA, USA) on deparaffinized sections treated with Target Retrieval Solution (DAKO). The antibodies used were as follows: anti-GFP (A-11122, ThermoFisher Scientific), anti-p-AKT (#3787, Cell Signaling Technology, Danvers, MA, USA), anti-p-ERK1/2 (#4370, Cell Signaling Technology), anti-Myc (Abcam 32072, Cambridge, UK), anti-Ki-67 (Nichirei, Tokyo, Japan), anti-cleaved caspase-3 (#9661, Cell Signaling Technology), anti-hemagglutinin (HA) (Roche Diagnostics, Mannheim, Germany), anti-p16 (M-156) (sc-1207, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p21 (F-5) (sc-6246, Santa Cruz). The chromogen 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA, USA) and HistoGreen (LINARIS Biologische Produkte, Dossenheim, Germany) were used for signal detection. For the detection of Myc in some experiments, TSA was performed using the TSA Plus DIG Kit (PerkinElmer, Waltham, MA, USA). Morphometric analyses were performed using ImageJ software. Frozen sections were stained with Sudan III.

Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from frozen liver tissues and subjected to RT-qPCR analyses, which were performed using the $\Delta\Delta$ Ct method with the FastStart Universal SYBR Green Master Mix (Roche Diagnostics). Each reaction was performed in duplicates, and the mRNA levels were normalized against β -actin (*Actb*). The sequences of the specific primers are listed in Supplementary Table 1.

Microarray and Gene Set Enrichment Analysis

Total RNA was prepared from snap-frozen tissues using a Sepasol reagent (RNA I Super G; Nacalai Tesque, Kyoto, Japan) and samples with an RNA integrity number > 7.0 were used for microarray analyses. Samples of AKT/HRAS/Tet-On-MadMyc tumours (a mixture of three independent tumours) either from mice treated or not treated with Dox were analysed and compared by one-colour microarrays (3D-Gene Microarray, TORAY, Tokyo, Japan). After background subtraction, the raw microarray data were normalized using a standard global normalization technique. GSEA was performed using the GSEA v2.3 software using gene set collections of 'MYC Target Gene Database' (C2), 'Reactome gene sets' (C2) and 'GO gene sets' (C5) from the Molecular Signature Database provided by the Broad Institute.⁴⁸

Proteomic analysis of the enzymes involved in fatty acid metabolism

Samples for soluble proteins were extracted from the tumours induced by AKT/HRAS (a mixture of four tumours) and AKT/HRAS/Myc (a mixture of five tumours), as well as control liver tissues (a mixture of tissue fragments from four mice). The samples were digested by trypsin and subjected to triplicate liquid chromatography-tandem mass spectrometry analyses (LTQ-Orbitrap XL, ThermoFisher Scientific, San Jose, CA, USA). Proteins were identified by the Mascot software (Matrix Science, London, UK) and the spectral counting data were semiquantitatively represented as exponentially modified protein abundance index (emPAI).

Human liver samples

The retrospective analysis of surgical specimens was approved by the internal review board of Asahikawa Medical University (approved number: 14004). A total of 33 HCC samples from patients who had curative hepatectomy were collected. Myc and Ki-67 immunohistochemistry was performed as described above using serial paraffin sections.

Statistical analysis

Results from experiments were expressed as mean \pm SEM. Unpaired two-tailed *t*-test, Mann-Whitney *U*-test, Kruskal-Wallis test, one-way analysis of variance (ANOVA) with Tukey *post-hoc* test, or Mantel-Cox test (for a Kaplan-Meier plot) were performed to compare differences. Fisher's exact test was used to evaluate human HCC samples. Statistical analyses were performed using Prism 6.07 (GraphPad Software, La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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