Myc regulates VEGF production in B cells by stimulating initiation of VEGF mRNA translation.

Pau Mezquita, Sean S. Parghi, Kimberly A. Brandvold and Alanna Ruddell*.

Division of Basic Science, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

1. To whom correspondence and reprint requests should be addressed: Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N., PO Box 19024, MS C2-023, Seattle, Washington, WA 98109-1024, USA. (phone 206-667-5496, fax 206-667-6523, aruddell@fhcrc.org

Key words: Myc, VEGF, translational control, mammalian target of rapamycin, protein synthesis

Running title: Myc regulates initiation of VEGF mRNA translation

ABSTRACT

De-regulated *c-myc* gene expression is associated with many human and animal cancers. Myc overexpression promotes the growth of blood and lymphatic vessels, which is due in part to induction of growth factors including vascular endothelial growth factor (VEGF). We determined that the P493-6 human B cell line increases VEGF production 10-fold upon Myc overexpression. Myc overexpression in avian B cells similarly resulted in high level VEGF production. Real-time RT-PCR analyses showed that Myc did not alter the VEGF mRNA content of these cell lines, indicating that a post-transcriptional mechanism regulates VEGF production. VEGF mRNA translation was examined by RT-PCR analysis of monosome and polysome sucrose gradient fractions from Myc-on and Myc-off P493-6 cells. Myc increased VEGF mRNA translation initiation, as VEGF mRNA loading onto polysomes increased 14-fold in Myc-on cells, and the number of ribosomes loaded per VEGF mRNA increased 3-fold. This translational regulation is specific to VEGF mRNA, as total polysomes show the same sucrose gradient profile in Myc-on and Myc-off cells, with no change in the percent ribosomes in polysomes, or in the number of ribosomes per polysomal mRNA. Myc stimulates VEGF production by a rapamycin- and LY294002-sensitive pathway, which does not involve alteration of eIF4E activity.

INTRODUCTION

Increased expression of the c-Myc transcription factor is a common feature of many human cancers, including colon, breast, prostate, and lung cancers, as well as leukemias and lymphomas (reviewed by (Nesbit et al., 1999)). Myc overexpression in various cell types in mice or chickens rapidly induces tumors which show increased cell growth and delayed differentiation (Brandvold et al., 2001; Iritani & Eisenman, 1999; Pelengaris et al., 1999). These tumors also feature intensive growth of blood vessels at early stages, which could support their rapid growth (Brandvold et al., 2000; Pelengaris et al., 1999; Ruddell et al., 2003). Conditioned media from B cell lines overexpressing Myc strongly induces endothelial cell proliferation (Brandvold et al., 2000), supporting the idea that Myc induces the production of vascular growth factors. Myc overexpression in B cells of Eμ-*c-myc* transgenic mice also induces extensive growth of lymphatic sinuses within lymph nodes, which is associated with greatly increased lymph flow (Ruddell et al., 2003). Some or all of these functions may contribute to the ability of Myc overexpression to rapidly induce tumors. The strong induction of angiogenesis and lymphangiogenesis could also contribute to the association of Myc overexpression with metastasis and poor prognosis in human cancers (Nesbit et al., 1999).

Myc influences the transcription of a variety of target genes that could promote oncogenesis, including genes involved in cell growth and metabolism (Coller et al., 2000; Neiman et al., 2001). Myc has also been shown to increase VEGF production in several cell types (Baudino et al., 2002; Pelengaris et al., 1999; Ruddell et al., 2003). For example, Myc-overexpressing B cells show increased VEGF production during early stages of lymphomagenesis in Eμ-*c-myc* mice (Ruddell et al., 2003) VEGF promotes angiogenesis and lymphangiogenesis (Nagy et al., 2002; Sundberg et al., 2001), so that this factor could contribute to Myc induction of vessel growth. Hypoxia and other cellular stresses such as glucose deprivation strongly induce VEGF mRNA transcription, and this pathway is thought to be important to drive angiogenesis in hypoxic tumors (Satake et al., 1998; Shweiki et al., 1992). The VEGF promoter contains a consensus Myc binding site, although thus far there is little evidence that Myc directly induces VEGF mRNA transcription (Baudino et al., 2002).

Myc influences the transcription of genes involved in RNA metabolism and protein synthesis (Coller et al., 2000), so that it is also possible that Myc influences VEGF production by a post-transcriptional mechanism. Previous studies have shown that hypoxia-induced VEGF production can be augmented by stabilization of VEGF mRNA (Levy et al., 1996; Stein et al., 1995). VEGF production can

also be regulated at the translational level. Overexpression of the eIF4E translation initiation factor in Chinese hamster ovary (CHO) cells greatly increased VEGF mRNA translation (Kevil et al., 1996). In a breast carcinoma cell line, integrin-mediated signaling, and increased VEGF mRNA translation on polysomes (Chung et al., 2002). In this latter example, the mammalian target of rapamycin (mTOR) kinase induced phosphorylation of eIF4E binding protein (4E-BP1), which can release eIF4E and increase its translation initiation activity (reviewed by (Gingras et al., 2004)). The mTOR inhibitor rapamycin blocked 4E-BP1 phosphorylation and VEGF production, indicating that mTOR is an important mediator of VEGF production in this cell line.

We used a human B cell line featuring a tetracycline-repressible c-*myc* gene (Pajic et al., 2000), to determine whether and how Myc influences VEGF expression in B cells. This P493-6 cell line is particularly useful because proliferation can also be driven by an estrogen-inducible Epstein Barr virus (EBV) EBNA-2 gene (Kempkes et al., 1995), so that c-Myc expression can potentially be turned on or off without altering proliferation of these cells. P493-6 cells grown in tetracycline do not express detectible Myc, while cells grown in the absence of tetracycline express high Myc levels (Pajic et al., 2000). This cell line allowed us to determine that Myc overexpression strongly induces VEGF production in proliferating B cells. This potent effect is

entirely mediated by an increase in VEGF mRNA translation initiation, while VEGF mRNA levels remain constant.

RESULTS

The P493-6 human B cell line was developed as a model to examine the mechanism by which Myc induces VEGF production. This cell line was derived by infection of primary human B cells with an Epstein Barr virus (EBV) expressing an estrogen-regulated EBNA-2 gene (Kempkes et al., 1995). These cells were then transfected with a plasmid driving tetracycline-repressible c-*myc* gene expression (Pajic et al., 2000). Myc-on P493-6 cells grown in 1 μM β-estradiol showed high Myc expression by immunoblotting, while Myc was not detectable in Myc-off cells treated with 1 μM β-estradiol and 100 ng/ml tetracycline (Figure 1a). The endogenous *c-myc* gene is not expressed at detectible levels in estrogen- and tetracycline-treated P493-6 cells. VEGF expression in each condition was examined by ELISA assay of conditioned media collected from Myc-on and Myc-off P493-6 cells. Myc-on B cells produced 10 times as much VEGF compared to Myc-off cells, where Myc had been repressed by the use of tetracycline (Figure 1b). These results indicate that Myc induces VEGF production from P493-6 B cells.

FIG. 1

Varying doses of tetracycline were used to modulate Myc expression levels in P493-6 cells. The tetracycline repressor expression cassette is quantitatively sensitive to tetracycline dose so that Myc expression can be calibrated from zero to a maximum, by using decreasing concentrations of tetracycline from 100 down to 0 ng/ml (Figure 1c). Measurement of VEGF production in conditioned media from these cells demonstrated that VEGF production increases coordinately with the level of Myc expression (Figure 1d). This finding suggests that Myc influences VEGF production in a dose-dependent fashion.

Myc can stimulate cell division, which involves altered expression of a number of genes (Grandori et al., 2000), so that Myc-induced proliferation could potentially mediate the observed increase in VEGF production in Myc-on cells. However, cell division in P493-6 cells is also driven by estrogen-induced EBNA2 expression (Kempkes et al., 1995; Pajic et al., 2000), so that tetracycline shutoff of c-Myc expression may not influence the cell cycle in these cells. The growth of P493-6 cells under Myc-on and Myc-off conditions was compared by counting log phase cells over 3 days, which revealed that Myc-on and Myc-off cells proliferate at a similar rate, with Myc-on cells dividing somewhat faster (Figure 2a). Cell cycle analysis by flow cytometry confirmed that cell cycle phases are similar in Myc-on and Myc-off cells

(Figure 2b). These findings indicate that the increase in VEGF production in Myc-on cells is not due to cell proliferation, but instead reflects some other effect of Myc on these cells. Interestingly, Myc overexpression visibly increases the size of these cycling cells (Figure 2c). This increase in the size of Myc-on cells is also detected by flow cytometric analysis of the forward scatter-height plot (Figure 2d). These findings indicate that Myc can increase cell growth in proliferating cells.

FIG. 2

Myc overexpression does not alter VEGF mRNA levels.

c-Myc could induce VEGF production in P493-6 cells by increasing VEGF mRNA transcription, as Myc is a transcription factor which influences the expression of a variety of genes (Coller et al., 2000). This hypothesis was tested by measuring VEGF mRNA levels in Myc-on and Myc-off cells by real-time RT-PCR, using GAPDH mRNA levels as an internal control. GAPDH mRNA levels are unaffected by Myc expression in P493-6 cells, as determined by Northern blot analysis (data not shown, and Figure 7). Surprisingly, P493-6 cells showed similar VEGF mRNA levels whether or not Myc was expressed (Figure 3). Standard RT-PCR and agarose gel electrophoresis confirmed that VEGF mRNA levels are the same in Myc-on and Myc-off cells (data not shown). These findings suggest that Myc regulates

VEGF production by a post-transcriptional mechanism. Moreover, hypoxia or stress effects are not likely to account for Myc induction of VEGF, as those responses are primarily mediated by increased VEGF mRNA transcription (Satake et al., 1998; Shweiki et al., 1992), and by increased VEGF mRNA stability (Levy et al., 1996; Stein et al., 1995).

FIG. 3

The relation of Myc to VEGF production was further investigated in P493-6 cells during induction of Myc expression. Myc expression was inactivated in P493-6 cells by adding tetracycline for 48 h, and then re-induced following its removal. Conditioned media was collected at various times, and VEGF production was measured by ELISA assay. Addition of tetracycline to cells resulted in a rapid decline of Myc expression, and in the amount of VEGF collected in the media during the first 8 h (data not shown). Upon removal of tetracycline, Myc expression achieved maximum levels within 8 h (Figure 4a). In contrast, the rate of VEGF production increased more slowly, reaching maximal production 12-24 h after removal of tetracycline (Figure 4b). VEGF mRNA levels did not change during Myc shut-off or re-induction as measured by real-time RT-PCR (Figure 4C), in agreement with our finding that VEGF mRNA levels are not affected by chronic Myc overexpression (Figure 3). These findings are consistent with a relatively direct effect of Myc to regulate VEGF production at a post-

transcriptional level, which operates within a few hours after the onset of c-Myc overexpression.

FIG. 4

Myc also regulates VEGF production in avian lymphocytes

The failure to detect an increase in VEGF mRNA in Myc-on P493-6 cells was surprising, given the fact that Myc regulates gene expression at the transcriptional level. Moreover, most studies of VEGF regulation have focused on strong transcriptional induction of VEGF mRNA by stresses such as hypoxia (Shweiki et al., 1992). We examined additional cell lines to determine how Myc influences VEGF production in other situations. The DT40 avian bursal lymphoma cell line expresses high c-Myc levels (Figure 5a) under the influence of a retrovirus integration upstream of the c-Myc gene (Humphries & Baba, 1984). Conditioned media collected from DT40 cells contains high levels of VEGF detected by ELISA assay (Figure 5b), similar to that produced by P493-6 cells (Figure 1a). In contrast, the E3 cell line, a bone marrow-derived B cell precursor cell line transformed by an REV retrovirus (Lewis et al., 1981) expressed undetectable Myc levels in 20 μg of cell lysate (Figure 5a), and produced low levels of VEGF (Figure 5b). Similar findings were obtained using the 1W41 REV-transformed B cell line, which also showed background VEGF levels (data not shown). The E3 cell line was infected with a replication-competent HB1

retrovirus (Enrietto et al., 1983; Smith et al., 1985), which expresses a 108 kD gag-v-Myc fusion protein, as well as smaller proteolytic products (Figure 5a). These E3 + Myc cells produce high levels of VEGF, similar to that produced by DT40 cells (Figure 5b). This study demonstrates that Myc overexpression in avian hematopoietic cell lines also increases VEGF production.

FIG. 5

Measurement of VEGF transcript by real-time RT-PCR showed the same VEGF mRNA levels in all 3 cell lines, relative to control GAPDH mRNA (Figure 5c). These findings indicate that Myc regulates VEGF at a post-transcriptional level in these avian cells, as was observed for human P493-6 B cells. The increased production of VEGF by the Myc-overexpressing DT40 and E3 + Myc cells correlates with the ability of conditioned media from these cells to induce proliferation of human microvascular endothelial cells, while that from E3 or 1W41 cells does not induce endothelial proliferation (Brandvold et al., 2000). VEGF is a potent endothelial growth factor (Nagy et al., 2002) so that VEGF alone or in concert with other vascular growth factors could mediate this angiogenic phenotype.

Myc increases VEGF mRNA translation in P493-6 cells

Our finding that Myc overexpression does not affect VEGF mRNA levels suggests that Myc could induce VEGF protein production by increasing VEGF mRNA translation. This hypothesis was investigated by measuring the distribution of VEGF mRNA on monosomes and polysomes separated by sucrose gradient centrifugation, using P493-6 cells grown in the presence or absence of tetracycline. Cells were lysed in buffer containing 1% PET and 1% DOC detergents to release membrane-bound as well as cytoplasmic polysomes (Davies & Abe, 1995). Sucrose gradient fractions were analyzed by A_{254} profile as illustrated in Figure 6a, which shows similar profiles for Myc-on and Myc-off cells, although Myc-off cells display somewhat lower RNA content. RNA was purified from each gradient fraction, and subjected to real-time RT-PCR assay to determine VEGF mRNA levels. Actin mRNA was used as an internal control. Analysis of VEGF mRNA distribution normalized to actin mRNA, revealed that VEGF mRNA is mainly found in the monosome (≤ 80S) fractions in Myc-off cells (Figure 6b). However, in Myc-on cells VEGF mRNA is distributed throughout the polysome fractions (>80S), indicating that Myc induces VEGF mRNA translation.

FIG. 6

Comparison of the uncorrected VEGF mRNA levels in each gradient fraction with those corrected as a VEGF:actin mRNA ratio yielded a similar profile (data not shown), indicating that VEGF and actin mRNA levels could be directly analyzed. This allowed us to calculate the total VEGF mRNA units in each fraction, by multiplying the relative VEGF units in each sucrose gradient fraction by their RNA content. This analysis confirmed that a significant portion of the VEGF mRNA is translated in Myc-on cells, with distribution throughout the polysome fractions, while VEGF mRNA is almost entirely associated with the untranslated monosome fraction in Myc-off cells (Figure 6c). In contrast, actin mRNA distribution in monosomes and polysomes is similar in Myc-on and Myc-off cells (Supplementary Figure 1).

The percent VEGF mRNA in polysomes was calculated by comparison of the VEGF mRNA content of the monosomes (≤80S) and polysomes (>80S). This measurement shows that 2% of the VEGF mRNA is associated with polysomes in Myc-off cells (Table 1). In contrast, 28% of the VEGF mRNA is associated with polysomes in Myc-on cells. Analysis of the loading of ribosomes onto VEGF mRNA also identified an increase in VEGF mRNA translation efficiency, from an average of 2 ribosomes translating each polysomal mRNA in Myc-off cells, to 6 ribosomes per mRNA in Myc-on cells. By contrast, measurement of actin mRNA levels in gradient fractions revealed a

two-fold increase in loading onto polysomes in Myc-on cells, while the average number of ribosomes translating each actin mRNA, remained constant at 6 ribosomes per mRNA (Table 1). These findings indicate that, while Myc modestly increases actin mRNA translation, it has a much larger effect over VEGF mRNA translation, by increasing VEGF mRNA loading onto polysomes 14-fold, and also by increasing the number of ribosomes translating each VEGF mRNA 3-fold. These observations also indicate that VEGF mRNA translation initiation is the rate-limiting step influenced by Myc in B cells.

Table 1

Myc exerts modest effects on total protein synthesis in cycling cells

Previous studies indicated that Myc overexpression increases total protein synthesis in P493-6 cells (Iritani & Eisenman, 1999; Schuhmacher et al., 1999), although this effect could also be attributed to the ability of Myc to induce proliferation in quiescent EBNA 2-off and Myc-off cells (Pajic et al., 2000). Our analysis of polysomes in EBNA 2-expressing Myc-on and Myc-off cells allowed us to perform a detailed analysis of the effects of Myc on translation in cycling cells. As Table 2 illustrates, the cellular RNA content (consisting mainly of rRNA) increases by about 40% in Myc-on cells. Comparison of the RNA yield from monosomes and polysomes showed

that Myc-on and Myc-off cells contain the same percentage of ribosomes in polysomes. Moreover, the average polysome loading for all mRNAs remained constant at 5 ribosomes per polysome on average. This finding that Myc does not influence the percent ribosomes in polysomes, or the average number of ribosomes per polysome, suggests that Myc does not significantly influence overall translational efficiency. In fact, measurement of the rate of protein synthesis in Myc-on and Myc-off cells showed a two-fold increase in ^{35}S methionine incorporation (Table 2). This is in good agreement with previous measurements of protein synthesis in untreated Myc-off P493-6 cells that are not cycling due to Myc and EBNA-2 shutoff, versus estrogen-treated EBNA-2-expressing Myc-on P493-6 cells that proliferate continuously (Schuhmacher et al., 1999). Our similar finding of a two-fold increase in protein synthesis upon Myc activation in cycling P493-6 cells, suggests that the moderate effects of Myc on overall translation are independent of the cell cycle. This increment in protein synthesis elevates the protein content of Myc-on cells by about 25% (Table 2), which may support the increase in size of these cells (Figure 2c,d). This modest effect of Myc on total protein synthesis suggests that the translation of most cellular mRNAs is not significantly altered in Myc-on cells, in contrast to the potent effect of Myc to increase VEGF mRNA translation.

Table 2

c-Myc expression in P493-6 cells does not affect the levels or activity of translation initiation factor eIF4E

Increased expression of translation initiation factor eIF4E can result in enhanced VEGF mRNA translation (De Benedetti & Harris, 1999; Kevil et al., 1996). Myc overexpression has been associated with increased eIF4E mRNA expression (Rosenwald et al., 1993). The eIF4E gene promoter contains a consensus Myc-binding site (Jones et al., 1996), suggesting that Myc could increase VEGF mRNA translation by increasing eIF4E levels. However, Northern blot analysis revealed only a 1.4-fold increase in eIF4E mRNA levels in Myc-on cells relative to control GAPDH mRNA (Figure 7). eIF4E protein levels were also similar in Myc-on and Myc-off cells, as determined by immunoblotting (Figure 8a). We also found that Myc does not affect eIF4E mRNA abundance in avian DT40 or E3 cells (data not shown). These findings show that the increased VEGF mRNA translation observed in Myc-overexpressing P493-6 cells is not mediated by increased eIF4E expression.

Fig. 7

eIF4E activity can be modulated via phosphorylation of serine residue 209 by the Mnk1 kinase, which has been associated with cell growth (Scheper, 2002; Tuxworth et al., 2004; Waskiewicz et al.,

1999). An antibody specific for phosphorylated eIF4E was used to show that eIF4E is phosphorylated in Myc-on and Myc-off P493-6 cells (Figure 8a). The Mnk1 kinase is also constitutively phosphorylated in Myc-on and Myc-off cells (data not shown). These findings indicate that eIF4E or Mnk1 activity is not directly affected by Myc overexpression.

Fig. 8

One other example of VEGF mRNA translational control has been identified in the MDA-MB-435 human breast carcinoma cell line, which requires VEGF for survival (Chung et al., 2002). Integrin $\alpha 6\beta 4$ expression in this cell line allows survival in low serum, by increasing the translation of VEGF mRNA. This autocrine signalling pathway involves activation of the phosphatidylinositol-3 kinase/Akt (PI-3K/Akt) pathway, and the associated mTOR-dependent phosphorylation of 4E-BP1, which releases eIF4E from an inhibitory eIF4E- 4E-BP1 complex (Lawrence & Abraham, 1997). We tested whether 4E-BP1 phosphorylation status is altered by Myc expression in P493-6 cells. We were unable to detect phosphorylated 4E-BP1 in P493-6 Myc-on or Myc-off cells by immunoblotting, even though phosphorylated 4E-BP1 was readily detected in serum–induced 293 cells (Fig 8b). m^7GTP-sepharose beads were used to purify 4E-BP1 associated with cap-binding eIF4e (Arsham et al., 2003; Rajasekhar et al., 2003).

Immunoblotting with a 4E-BP1 antibody determined that equal amounts of 4E-BP1 are associated with eIF4E in Myc-on and Myc-off cells (Figure 8b). Taken together, these findings indicate that mTOR phosphorylation of 4E-BP1 does not account for increased VEGF mRNA translation initiation in B cells.

The integrin-induced VEGF mRNA translation observed in MDA-MB-435 breast carcinoma cells was inhibited by rapamycin and by LY294002 (Chung et al., 2002), two inhibitors of the PI3K/Akt pathway that act upon the mTOR and PI-3 kinases, respectively. We tested whether these pathways could mediate Myc induction of VEGF mRNA translation. P493-6 cells were treated with tetracycline to shut Myc off, and then Myc was induced after tetracycline washout, with or without rapamycin or LY294002 treatment for 30 h. ELISA analysis of VEGF secretion into the media showed that rapamycin treatment of Myc-on cells blocks induction of VEGF production, so that VEGF levels are low and similar to those of Myc-off cells treated continuously with tetracycline (Figure 8c). This was a surprising finding in light of the failure of Myc to induce mTOR-mediated 4E-BP1 phosphorylation (Figure 8B). However, rapamycin also inhibits mTOR-mediated S6 kinase phosphorylation of the S6 ribosomal protein, which could potentially affect translation of VEGF mRNA in addition to the translation of 5'TOP sequence-containing mRNAs (Jefferies et al.,

1997). Immunoblotting using an antibody to phosphorylated S6 ribosomal protein showed similar phosphorylation levels in Myc-on and Myc-off cells (Figure 8d), so that this pathway does not mediate Myc induction of VEGF production. S6 ribosomal protein phosphorylation is however strongly inhibited by rapamycin treatment (Figure 8d) indicating that this drug is effective in these cells. As a further control, immunoblotting for Myc determined that rapamycin treatment does not affect Myc protein expression (Figure 8d). These findings indicate that Myc-induced VEGF production is regulated by a rapamycin-sensitive pathway, which does not involve phosphorylation of 4EBP-1 or S6 kinase.

The PI-3K inhibitor LY294002 also prevented VEGF production in Myc-on P493-6 cells (Figure 8C). PI-3K phosphorylates and activates Akt, which then phosphorylates mTOR to increase kinase activity (Vivanco & Sawyers, 2002). However, Akt phosphorylation was not affected by Myc expression in P493-6 cells, indicating that phosphorylation of this kinase is not involved in Myc-induced VEGF production (Figure 8d). Akt phosphorylation was also not altered by LY294002 treatment of P493-6 cells (Figure 8d), suggesting that PI3-K does not regulate Akt phosphorylation in these cells. The LY294002 treatment was effective to inhibit kinase activity, as S6 ribosomal protein phosphorylation was inhibited at the dose used, while Myc

protein expression was unaffected (Figure 8D). These findings indicate that Myc acts on an LY294002-sensitive kinase other than Akt to induce VEGF mRNA translation.

DISCUSSION

Myc is thought to stimulate protein synthesis by increasing the biogenesis of ribosomes and translation factors (Schmidt, 1999). Our analysis of the effects of Myc in cycling human P493-6 B cells demonstrates that Myc overexpression produces a two-fold increase in overall protein synthesis. This effect is comparable to that observed during Myc-induced proliferation in quiescent (EBNA 2-off and Myc-off) P493-6 cells (Schuhmacher et al., 1999). This increased translation supports cell growth, as Myc-on P493-6 cells are visibly larger than Myc-off cells, while they proliferate at a similar rate. These findings demonstrate that Myc effects on protein synthesis are related to cell growth, rather than proliferation. Overexpression of Myc not only enhances translation, but also elevates the RNA content of P493-6 cells by about 40%. This increase in the ribosome pool could account for the modest increase in protein synthesis. A 2-fold increase in protein synthesis and increased cell size was also observed in Myc-expressing B cells from Eμ-*c-myc* transgenic mice (Iritani & Eisenman, 1999), suggesting that Myc performs similar roles *in vivo*.

Myc can enhance the transcription of a number of target genes that participate in protein synthesis, including eIF4E and eIF2A (Coller et al., 2000; Rosenwald et al., 1993). Elevated levels of eIF4E in particular are thought to increase the rate-limiting step of translation initiation (De Benedetti & Harris, 1999). However, we found that Myc does not significantly change eIF4E mRNA or protein levels, or its phosphorylation status in B cells. Moreover, we found that Myc does not affect overall translation initiation, the rate-limiting step in protein synthesis, as the percent ribosomes in polysomes, and the average loading of ribosomes onto polysomal mRNAs, were not altered by Myc overexpression. These findings suggest that the major effect of Myc to stimulate protein synthesis in P493-6 cells involves a general increase in ribosome content, rather than an increase in the rate of translation initiation or loading of ribosomes onto polysomes. However, our finding that Myc strongly increases the initiation of VEGF mRNA translation suggests that Myc could also influence the efficiency of translation of a subset of cellular mRNAs. Shiio *etal* (Shiio et al., 2002) used a proteomics approach to show that Myc increases the expression of some proteins in fibroblasts without affecting their mRNA abundance, supporting the idea that Myc could selectively increase the translation of certain mRNAs in addition to VEGF mRNA. The Ras oncogene has also been found to influence the translation of a subset

of mRNAs in glioblastoma cells (Rajasekhar et al., 2003), supporting the idea that translational control is an important component of oncogene function.

Myc may regulate VEGF expression in a variety of cell types, as we found that Myc increases VEGF production in human and avian B cells, while others found that Myc increases VEGF levels in keratinocytes (Pelengaris et al., 1999) and embryonic stem cells (Baudino et al., 2002). Myc-induced VEGF production from B cells appears to be entirely mediated by an increase in VEGF mRNA translation initiation. We also observed enhanced VEGF production by murine lymphocytes overexpressing Myc in Eμ-*c-myc* transgenic mice (Ruddell et al., 2003), while VEGF mRNA levels remained constant (data not shown), suggesting that Myc also influences VEGF mRNA translation in B lymphocytes *in vivo*. However, the finding that Myc is required for VEGF expression at the mRNA level in murine embryonic stem cells (Baudino et al., 2002), and the finding that Myc inhibits VEGF expression in lung cancer cells (Barr et al., 2000), suggest that Myc could regulate VEGF gene expression by more than one mechanism.

Most studies of VEGF gene regulation have focused on the strong effect of hypoxia, glucose deprivation, or other stresses to rapidly induce VEGF mRNA transcription (Satake et al., 1998; Shweiki et al.,

1992). Our observation that Myc expression does not affect VEGF mRNA levels indicates that VEGF production is not a result of Myc-induced stress or hypoxia in B cells. Moreover, our finding that Myc increases VEGF production 10-fold by a translational mechanism demonstrates that post-transcriptional mechanisms can exert strong effects on VEGF production. Previous studies identified translational regulation of VEGF production by increased expression of eIF4E, or by mTOR-mediated 4E-BP1 phosphorylation, which stimulate VEGF mRNA translation initiation (Chung et al., 2002; Kevil et al., 1996). In our model of VEGF regulation, Myc does not affect the level of eIF4E expression or phosphorylation, or the phosphorylation of 4E-BP1. These findings suggest that several mechanisms can influence the initiation of VEGF mRNA translation, depending on the cell type involved.

Transcripts whose translation is tightly controlled, such as those that encode for regulatory proteins involved in cell growth and differentiation, often exhibit complex secondary structures in their 5' untranslated regions (UTR; reviewed by (Gingras et al., 2004)). This makes the conventional ribosome scanning process difficult, so that these mRNAs are weakly translated under ordinary circumstances. To circumvent this limitation, many of these transcripts feature internal ribosome entry sites (IRES) in their 5' UTRs that can promote

translation initiation at internal start codons (Hellen & Sarnow, 2001). The VEGF mRNA belongs to this group of transcripts, as its 5' UTR contains two IRES elements (Akiri et al., 1998; Huez et al., 1998). Alternative initiation of VEGF mRNA transcription produces an mRNA with a shorter 5' UTR truncating one of the IRES elements, which could potentially influence VEGF mRNA translation initiation (Akiri et al., 1998). A mechanism involving the VEGF IRES could potentially explain the specific induction of VEGF mRNA translation by Myc, while overall protein synthesis is only modestly stimulated by Myc overexpression.

Myc induction of VEGF production is inhibited by the mTOR kinase inhibitor rapamycin, and by the PI3K inhibitor LY294002, indicating that a kinase signalling pathway is involved in Myc induction of VEGF mRNA translation. We were surprised to find that this does not involve mTOR phosphorylation of 4E-BP1, or phosphorylation of Akt, indicating that one or more unknown kinases mediate Myc-induced VEGF mRNA translation. Rapamycin exerts additional effects to inhibit translation initiation (Gingras et al., 2004), for example inhibiting eIF4G phosphorylation, although it is not known how this influences translation initiation activity (Raught et al., 2000). Further studies will be required to characterize the rapamycin- and LY294002-sensitive pathway by which Myc induces VEGF mRNA translation

initiation, and to determine whether Myc selectively increases the translation of additional mRNAs.

Myc-induced VEGF expression appears to make important contributions to the development of the vasculature during embryogenesis (Baudino et al., 2002). Myc -/- embryos die at day 10.5 after defective formation of the heart and blood vessels, which is due at least in part to reduced VEGF expression. The mechanism by which Myc induces VEGF gene expression during embryogenesis has not been identified (Baudino et al., 2002). We found that the initiation of VEGF mRNA translation in B cells is dependent on the level of Myc expression, and that VEGF secretion is strongly stimulated at high but physiological Myc levels. These findings suggest that fluctuations in Myc expression could influence VEGF expression, to modulate vessel growth during normal organ growth in embryos or in the adult.

The level of VEGF production induced by Myc overexpression in B cells is similar to that produced by tumor cell lines (Bellamy et al., 1999), consistent with a role of Myc in tumor angiogenesis. In fact, Myc strongly induces growth of blood vessels *in vivo* at early stages of Myc-induced tumor formation in mice and chickens (Brandvold et al., 2000; Pelengaris et al., 1999; Ruddell et al., 2003). Myc-induced VEGF production could also contribute to the strong angiogenic phenotype observed in Myc-associated multiple myeloma and Burkitt's

lymphoma (Vacca A, 1994; Vacca et al., 1999; Willis A.E., 1997), or in human solid tumors featuring increased Myc expression (Nesbit et al., 1999). Further analysis of Myc-regulated VEGF mRNA translation will establish the contribution of this pathway to normal development and cancer.

MATERIALS AND METHODS

Cell culture

The human P493-6 cell line was maintained in RPMI 1640 medium containing 10% tetracycline system-approved fetal calf serum (BD Biosciences, Palo Alto, CA), 2 mM L-glutamine, and 1 μM β-estradiol (Sigma, Saint Louis, MO), as previously described (Pajic et al., 2000). For repression of c-Myc, 100 ng/ml tetracycline (Sigma, Saint Louis, MO) was added to the culture. For re-induction of Myc, cultures were washed 3 times to remove tetracycline, and were incubated in media with β-estradiol. The DT40 and E3 avian B cell lines were cultured, and E3 cells were infected with the HB1 retrovirus, as previously described (Brandvold et al., 2000). Conditioned media was collected from cells washed and resuspended in fresh media at 0.7 x 10^6 cells/ml.

For cell cycle analysis, 2 x 10^6 cells were fixed in 70% ethanol, and DNA content was determined by a FACScan flow cytometer (BD Biosciences, Mountain View, CA), as previously described (Iritani & Eisenman, 1999). The percentage of cells in each phase of the cell cycle was calculated by using M-cycle software (Phoenix Flow Systems, San Diego, CA). Cell size was assessed by flow cytometry using CellQuest software (BD Biosciences).

For measurement of protein synthesis, P493-6 cells (3 x 10^6) grown in the presence or absence of tetracycline for 72 h, and equal numbers of cells were metabolically labeled for 1 h with 0.2 mCi of [^{35}S]methionine-cysteine (1175 Ci/mmol) (NEN) in 1 ml of methionine-free medium, supplemented with dialyzed 10% tetracycline-system approved fetal calf serum. Samples were precipitated with 10% trichloroacetic acid and filtered for liquid scintillation counting.

Immunoblot analysis

Cell extract samples containing equal amounts of protein (20 μg) were heat denatured in SDS sample buffer containing 100 mM DTT, resolved by SDS-PAGE, and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were probed with a polyclonal antibody raised against the N-terminal half of human c-Myc

(a gift from Dr. Robert Eisenman) or with one of the following antibodies (Cell Signaling Technology, Beverly, MA): eIF4E, phosphorylated eIF4E (Ser209), 4E-BP1, phospho-4E-BP1 (Ser65), phospho-Akt (Ser473), phospho-S6 Ribosomal Protein (Ser 235/236), or phospho-Mnk1 (Thr197/202), followed by detection with HRP-labelled secondary antibody and Supersignal West Pico ChemiLuminescence reagent (Pierce, Rockford, IL). Blots were then stripped and re-probed with actin antibody (Sigma) to confirm equal protein loading.

For detection of 4E-BP1, m^7GTP-conjugated Sepharose beads were used to purify eIF4E and 4E-BP1 from the cell lysate, as previously described (Arsham et al., 2003). Briefly, cells were lysed in an affinity purification buffer and incubated with m^7GTP-Sepharose beads (Amersham), which were then washed 3 times and resolved by SDS PAGE and immunoblotting.

ELISA assay

VEGF concentrations were measured in conditioned media from P493-6 cells using a quantitative sandwich enzyme immunoassay (DuoSet human VEGF ELISA kit; R&D Systems Inc., Minneapolis, MN), which detects the soluble isoforms of human VEGF (VEGF 165 and 121). For ELISA detection of avian VEGF, a rabbit antibody was raised

against chicken VEGF 165. The mRNA encoding the 165-amino acid chicken VEGF isoform (Flamme et al., 1995) was reverse-transcribed using primers 5'-GAGCGGAAGCCCAATGAAG and 3'-CGCTGCTCACCGTCTCGG, and then expressed in a pET expression plasmid (Novagen, Madison, WI) in BL21-DE3 *E. coli* (Stratagene, La Jolla, CA). The resulting 6xHis-tagged VEGF protein was purified from cell pellets by Nickel-NTA chromatography (Qiagen, Valencia, CA), renatured by dialysis to remove urea (Christinger et al., 1996), and concentrated using Centricon-10k filters (Millipore, Billerica, MA). The resulting protein was used to immunize rabbits (R&R Rabbitry, Stanwood, WA). Antibody was purified by NH_4SO_4 precipitation, and by passage through a column of E. coli proteins linked to Sepharose 4B (Bowers et al., 1996). The purified antibody was then used to capture VEGF from chicken cell conditioned media, with detection by the pan-species VEGF antibody provided in the Duoset ELISA kit. At least three independent conditioned media samples were examined in each experiment.

Real-time RT-PCR and Northern blot analysis

Total RNA was isolated from cells with Trizol (Gibco BRL, Rockville, MD) and reverse-transcribed by Moloney Murine Leukemia Virus reverse transcriptase, using random hexamer primers (Gibco

BRL). Real-time PCR was performed using the Taqman detection protocol in an ABI Prism 7700 thermocycler (Applied Biosystems, Foster City, CA). The results for VEGF real-time RT-PCR assays were normalized to those obtained for the corresponding beta-actin or GAPDH mRNAs, providing a relative quantitation value. Actin and GAPDH mRNAs showed relatively small variation under the conditions tested. Primers were designed using the Primer Express™ program (Applied Biosystems) and the following sequences were used: human VEGF, forward 5'-GCACCCATGGCAGAAGGA, reverse 5'-GCTGCGCTGATAGACATCCA; human beta-actin, forward 5'-ATCAAGATCATTGCTCCTCCTGAG, reverse 5'- AGCGAGGCCAGGATGGA; human GAPDH, forward 5'-GGTGAAGGTCGGAGTCAACG, reverse 5'-CACCAGGGCTGCTTTTAACTCT; chicken VEGF, forward 5'-GCAGAGCGCGGAGTTGTC, reverse 5'- GTCCACCAGGGTCTCAATTGTC; chicken beta-actin, forward 5'- CGAGAGAGAAATTGTGCGTGAC, reverse 5'- TGACCTGACCATCAGGGAGTT. Taqman probes were obtained from Synthegen (Houston, TX) and the following sequences were used: human VEGF, 5'-AACTTCACCACTTCGTGATGATTCTGCCCT; human beta-actin, 5'-CCGCCGATCCACACGGAGTACTTG; human GAPDH, 5'-CCAGGCGCCCAATACGACCAAA; chicken VEGF, 5'-CAAATTCCTGGAAGTCTACGAACGCAGC; chicken beta-actin, 5'-TGCTACGTCGCACTGGATTTCGAGC. RT-PCR reactions using the

primers yielded specific products of the expected size. A standard curve of cDNA was tested in each real time RT-PCR experiment, to confirm the dynamic range for quantitation.

For Northern blot analysis of eIF4E expression, RNA was probed with eIF4E or GAPDH cDNA probes. Data from four independent samples was analyzed by phosphoimaging, and was quantitated using ImageQuant software (Molecular Dynamics).

Polysome analysis

P493-6 cells were grown in the presence or absence of tetracycline (100 ng/ml) for 72h. Upon harvesting, cells were treated with 100 μg/ml cycloheximide (Sigma, Saint Louise, MO) for 15 min at 37°C, washed once with cold PBS containing 100 μg/ml cycloheximide, and stored at –70'C. Aliquots of 2 x 10^7 cells were lysed with 10-12 strokes using a loose Dounce homogenizer in 1.25 ml of cold lysis buffer (200 mM Tris pH 8.8, 25 mM $MgCl_2$, 150 mM KCl, 2% polyoxyethylene 10-tridecy ester (PET), 1% Na deoxycholate (DOC), 5 mM DTT, 0.5 U/μl RNAsin, 1 mg/ml heparin, 100 μg/ml cycloheximide, 5 mM EGTA pH 8.0 and 200 mM sucrose), as previously described (Davies & Abe, 1995). These conditions allow the dissociation of membrane-bound polysomes. The lysates obtained were centrifuged 5 min at 10,000 g, 4°C to remove nuclei and the cytoplasmic extracts

were layered onto 11 ml 15%-50% sucrose gradients (100 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 20 mM HEPES KOH pH 7.4) and centrifuged 1h 50 min at 36,000 g, 4°C. Twelve fractions (1 ml each) were collected from the top of the gradient using an ISCO fraction collector with continuous monitoring of absorbance at 254 nm and placed into tubes containing 100 μl of 10% SDS for storage at -80 °C. After proteinase K digestion, total RNA from each fraction was prepared by phenol-chloroform extraction and equal aliquots (2 μg) were reverse transcribed for use in real-time RT-PCR analysis.

ACKNOWLEDGEMENTS

We thank Adam Geballe, Robert Eisenman, Carla Grandori, Stephanie Child, and Paul Neiman for their valuable expertise and advice, and Dirk Eick for providing P493-6 cells. This work was supported a grant from the Elsa U. Pardee Foundation and by Public Health Service Grant CA68328 from the National Cancer Institute.

REFERENCES

Akiri, G., Nahari, D., Finkelstein, Y., Le, S.Y., Elroy-Stein, O. & Levi, B.Z. (1998). *Oncogene*, 17, 227-36.

Arsham, A.M., Howell, J.J. & Simon, M.C. (2003). *J Biol Chem*, 278, 29655-60.

Barr, L.F., Campbell, S.E., Diette, G.B., Gabrielson, E.W., Kim, S., Shim, H. & Dang, C.V. (2000). *Cancer Research*, 60, 143-149.

Baudino, T.A., McKay, C., Pendeville-Samain, H., Nilsson, J.A., Maclean, K.H., White, E.L., Davis, A.C., Ihle, J.N. & Cleveland, J.L. (2002). *Genes Dev*, 16, 2530-43.

Bellamy, W.T., Richter, L., Frutiger, Y. & Grogan, T.M. (1999). *Cancer Research*, 728-33.

Bowers, W.J., Baglia, L.A. & Ruddell, A. (1996). *J Virol*, 70, 3051-9.

Brandvold, K.A., Ewert, D.L., Kent, S.C., Neiman, P. & Ruddell, A. (2001). *Oncogene*, 20, 3226-34.

Brandvold, K.A., Neiman, P. & Ruddell, A. (2000). *Oncogene*, 19, 2780-5.

Christinger, H.W., Muller, Y.A., Berleau, L.T., Keyt, B.A., Cunningham, B.C., Ferrara, N. & de Vos, A.M. (1996). *Proteins*, 26, 353-7.

Chung, J., Bachelder, R.E., Lipscomb, E.A., Shaw, L.M. & Mercurio, A.M. (2002). *J Cell Biol*, 158, 165-74.

Coller, H.A., Grandori, C., Tamayo, P., Colbert, T., Lander, E.S., Eisenman, R.N. & Golub, T.R. (2000). *Proc Natl Acad Sci U S A*, 97, 3260-5.

Davies, E. & Abe, S. (1995). *Methods Cell Biol*, 50, 209-22.

De Benedetti, A. & Harris, A.L. (1999). *Int J Biochem Cell Biol*, 31, 59-72.

Enrietto, P.J., Payne, L.N. & Hayman, M.J. (1983). *Cell*, 35, 369-79.

Flamme, I., von Reutern, M., Drexler, H.C., Syed-Ali, S. & Risau, W. (1995). *Dev Biol*, 171, 399-414.

Gingras, A.C., Raught, B. & Sonenberg, N. (2004). *Curr Top Microbiol Immunol*, 279, 169-97.

Grandori, C., Cowley, S.M., James, L.P. & Eisenman, R.N. (2000). *Annu Rev Cell Dev Biol*, 16, 653-99.

Hellen, C.U. & Sarnow, P. (2001). *Genes Dev*, 15, 1593-612.

Huez, I., Creancier, L., Audigier, S., Gensac, M.C., Prats, A.C. & Prats, H. (1998). *Mol Cell Biol*, 18, 6178-90.

Humphries, E.H. & Baba, T.W. (1984). *Curr Top Microbiol Immunol*, 113, 47-55.

Iritani, B.M. & Eisenman, R.N. (1999). *Proc Natl Acad Sci U S A*, 96, 13180-5.

Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B. & Thomas, G. (1997). *Embo J*, 16, 3693-704.

Jones, R.M., Branda, J., Johnston, K.A., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L. & Schmidt, E.V. (1996). *Mol Cell Biol*, 16, 4754-64.

Kempkes, B., Spitkovsky, D., Jansen-Durr, P., Ellwart, J.W., Kremmer, E., Delecluse, H.J., Rottenberger, C., Bornkamm, G.W. & Hammerschmidt, W. (1995). *Embo J*, 14, 88-96.

Kevil, C.G., De Benedetti, A., Payne, D.K., Coe, L.L., Laroux, F.S. & Alexander, J.S. (1996). *Int J Cancer*, 65, 785-90.

Lawrence, J.C., Jr. & Abraham, R.T. (1997). *Trends Biochem Sci*, 22, 345-9.

Levy, A.P., Levy, N.S. & Goldberg, M.A. (1996). *J Biol Chem*, 271, 2746-53.

Lewis, R.B., McClure, J., Rup, B., Niesel, D.W., Garry, R.F., Hoelzer, J.D., Nazerian, K. & Bose, H.R., Jr. (1981). *Cell*, 25, 421-31.

Nagy, J.A., Vasile, E., Feng, D., Sundberg, C., Brown, L.F., Detmar, M.J., Lawitts, J.A., Benjamin, L., Tan, X., Manseau, E.J., Dvorak, A.M. & Dvorak, H.F. (2002). *J Exp Med*, 196, 1497-506.

Neiman, P.E., Ruddell, A., Jasoni, C., Loring, G., Thomas, S.J., Brandvold, K.A., Lee, R., Burnside, J. & Delrow, J. (2001). *Proc Natl Acad Sci U S A*, 98, 6378-83.

Nesbit, C.E., Tersak, J.M. & Prochownik, E.V. (1999). *Oncogene*, 18, 3004-16.

Pajic, A., Spitkovsky, D., Christoph, B., Kempkes, B., Schuhmacher, M., Staege, M.S., Brielmeier, M., Ellwart, J., Kohlhuber, F., Bornkamm, G.W., Polack, A. & Eick, D. (2000). *Int J Cancer*, 87, 787-93.

Pelengaris, S., Littlewood, T., Khan, M., Elia, G. & Evan, G. (1999). *Mol Cell*, 3, 565-77.

Rajasekhar, V.K., Viale, A., Socci, N.D., Wiedmann, M., Hu, X. & Holland, E.C. (2003). *Molecular Cell*, 12, 889-901.

Raught, B., Gingras, A.C., Gygi, S.P., Imataka, H., Morino, S., Gradi, A., Aebersold, R. & Sonenberg, N. (2000). *Embo J*, 19, 434-44.

Rosenwald, I.B., Rhoads, D.B., Callanan, L.D., Isselbacher, K.J. & Schmidt, E.V. (1993). *Proc Natl Acad Sci U S A*, 90, 6175-8.

Ruddell, A., Mezquita, P., Brandvold, K.A., Farr, A. & Iritani, B.M. (2003). *Am J Pathol*, 163, 2233-45.

Satake, S., Kuzuya, M., Miura, H., Asai, T., Ramos, M.A., Muraguchi, M., Ohmoto, Y. & Iguchi, A. (1998). *Biol Cell*, 90, 161-8.

Scheper, G.C., Proud, C.G. (2002). *Eur J Biochem*, 269, 5350-5359.

Schmidt, E.V. (1999). *Oncogene*, 18, 2988-96.

Schuhmacher, M., Staege, M.S., Pajic, A., Polack, A., Weidle, U.H., Bornkamm, G.W., Eick, D. & Kohlhuber, F. (1999). *Curr Biol*, 9, 1255-8.

Shiio, Y., Donohoe, S., Yi, E.C., Goodlett, D.R., Aebersold, R. & Eisenman, R.N. (2002). *Embo J*, 21, 5088-96.

Shweiki, D., Itin, A., Soffer, D. & Keshet, E. (1992). *Nature*, 359, 843-5.

Smith, D.R., Vennstrom, B., Hayman, M.J. & Enrietto, P.J. (1985). *J. Virology*, 56, 969-976.

Stein, I., Neeman, M., Shweiki, D., Itin, A. & Keshet, E. (1995). *Mol Cell Biol*, 15, 5363-8.

Sundberg, C., Nagy, J.A., Brown, L.F., Feng, D., Eckelhoefer, I.A., Manseau, E.J., Dvorak, A.M. & Dvorak, H.F. (2001). *Am J Pathol*, 158, 1145-60.

Tuxworth, W.J., Jr., Saghir, A.N., Spruill, L.S., Menick, D.R. & McDermott, P.J. (2004). *Biochem J*, 378, 73-82.

Vacca A, R.D., Roncali L, Ranieri G, Serio G, Silvestris F, Dammacco F. (1994). *Br J Haematol*, 87, 503-508.

Vacca, A., Ribatti, D., Ruco, L., Giacchetta, F., Nico, B., Quondamatteo, F., Ria, R., Iurlaro, M. & Dammacco, F. (1999). *British Journal of Cancer*, 79, 965-970.

Vivanco, I. & Sawyers, C.L. (2002). *Nature Reviews Cancer*, 2, 489-501.

Waskiewicz, A.J., Johnson, J.C., Penn, B., Mahalingam, M., Kimball, S.R. & Cooper, J.A. (1999). *Mol Cell Biol*, 19, 1871-80.. *Top. Microbiol. Immunol.*, 224, 269-276.

Table 1. Myc increases VEGF mRNA translation initiation in P493-6 cells.

	Myc-ON	**Myc-OFF**	**Myc-On/Myc-OFF**
Percent VEGF mRNA In polysomes[a]	28 +/- 3%	2 +/- 0 %	14
Number of ribosomes per VEGF mRNA[b]	6	2	3
Percent actin mRNA in polysomes[c]	47 +/- 3%	22 +/- 4 %	2
Number of ribosomes per actin mRNA[d]	6	6	1

a. Calculated by comparing the VEGF mRNA content of monosome and polysome sucrose gradient fractions, in 3 experiments.

b. Determined by measuring the median position of VEGF mRNA distribution across sucrose gradient polysome fractions, and identification of the average number of ribosomes per polysome in that median fraction, by examination of the A_{254} polysome profile. Three experiments were analyzed with similar results.

c. Calculated by comparing the actin mRNA content of monosome and polysome sucrose gradient fractions.

d. Determined by measuring the median position of actin mRNA distribution across sucrose gradient polysome fractions, and by identification of the average number of ribosomes per polysome in that median fraction.

Table 2. Myc-induced alterations in RNA/protein content and protein synthesis in P493-6 cells.

	Myc-ON	Myc-OFF	Myc-On/Myc-OFF
Total RNA content/cell[a]	10.4 +/- 0.9 pg	7.6 +/- 1.1 pg	1.4
Protein content/cell[b]	38.7 +/- 2.5 pg	31.3 +/- 3.2 pg	1.2
Protein synthesis[c]	19.1×10^6 +/- 1.8×10^6 cpm	9.8×10^6 +/- 1.9×10^6 cpm	2.0
Percent ribosomes In polysomes[d]	33%+/- 3%	35%+/- 3%	1.0
Number of ribosomes per mRNA[e]	5	5	1.0

a. Measured by A_{260} absorbence analysis of RNA purified from equal numbers of cells in 5 experiments. Standard errors are shown.

b. Measured by Bradford assay of protein content in equal numbers of cells in 7 experiments.

c. Measured by ^{35}S-methionine-cysteine incorporation in 3 experiments.

d. Calculated by comparing RNA content of sucrose gradient monosome (<=80S) and polysome (>80S) fractions.

e. Determined by measuring the median position of RNA content across sucrose gradient polysome fractions, and by identification of the average number of ribosomes per polysome in that median fraction.

LEGENDS TO FIGURES

Figure 1. Myc-dependent production of VEGF in P493-6 cells. (a) Myc and actin protein expression in P493-6 cells grown in the absence (Myc-on) or presence (Myc-off) of tetracycline for 72 h, as determined by immunoblotting. (b) ELISA analysis of media from equal numbers of Myc-on cells grown in media, or from Myc-off cells treated with tetracycline for 72 h. Standard errors are shown. (c) Immunoblot expression of Myc protein following 72 h administration of tetracycline at decreasing concentrations. (d) ELISA assay of the media from these cells demonstrates Myc dose-dependent induction of VEGF secretion.

Figure 2. Myc-on cells grow larger while they proliferate at a similar rate as Myc-off P493-6 cells. (a) Cells were seeded at 0.7 x 10^6 cells/ml and grown for three days. Cells were stained with Trypan Blue and counted each day. Standard errors from 3 experiments are shown. (b) Cell cycle analysis by flow cytometry of PI-stained cells. The percent cells in each phase of the cell cycle are given. (c) Myc-on cells are larger than Myc-off cells, shown at 400x magnification. (d) Forward scatter-height flow cytometry analysis of cell size.

Figure 3. Myc does not affect VEGF mRNA abundance in P493-6 cells. Real-time RT-PCR was performed on equal amounts of total RNA from P493-6 cells grown in Myc-on or Myc-off conditions for 3 days. Myc expression does not affect the level of VEGF transcript, in triplicate assays. Standard errors are shown for triplicate assays.

Figure 4. VEGF production increases within hours after Myc induction. (a) Myc expression achieves maximum levels within 8 h following removal of tetracycline, as determined by immunoblotting. (b) Maximal production of VEGF is re-established within 12-24h, as determined by ELISA assay. Cells were incubated with tetracycline for 48h and then were washed and grown in media plus β-estradiol for varying times (Tetracycline Removal). Control Myc-on cells (No Tetracycline) were treated similarly, except that they were not incubated with tetracycline. Conditioned media were collected at intervals as indicated. (c) Real-time RT-PCR analysis of these cells shows similar VEGF mRNA levels, which are not affected by Myc expression.

Figure 5. Myc induces VEGF production in avian hematopoieitic cells. (a) DT40 B lymphoma cells show high Myc expression, while E3 progenitor cells show low Myc expression, as determined by

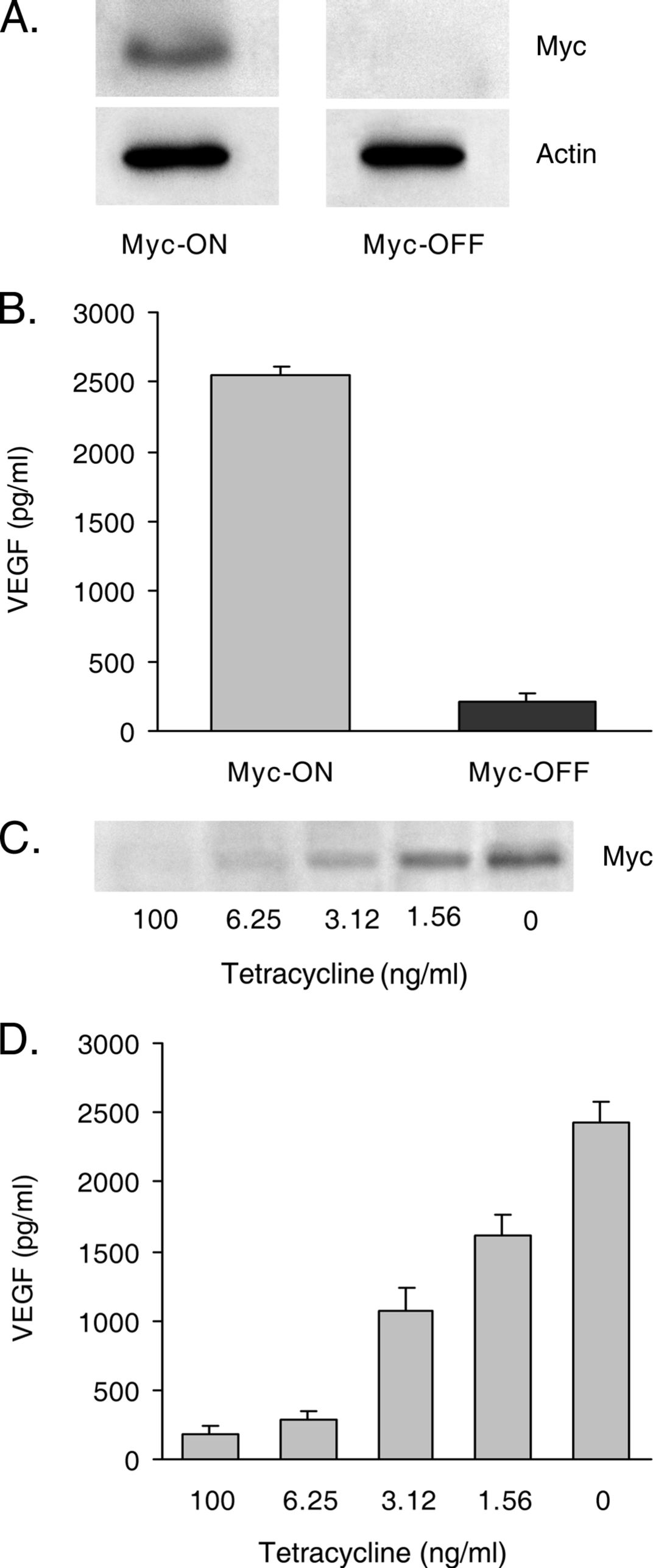
immunoblotting. E3 + Myc cells infected with HB1 retrovirus show increased expression of *v-myc-gag* fusion proteins. Twenty micrograms of protein were loaded on each gel lane. (b) Myc-overexpressing DT40 and E3 + Myc cells show high levels of VEGF production, while low Myc E3 cells produce background levels of VEGF, as determined by ELISA assay. Standard errors are shown. (c) VEGF transcript abundance is the same in high and low Myc cell lines, as determined by real-time RT-PCR. VEGF mRNA data was normalized to internal control GAPDH mRNA.

Figure 6. Myc selectively increases VEGF mRNA translation on polysomes. (a) Representative A_{254} gradient profiles of lysates from equal numbers of Myc-on and Myc-off P493-6 cells. (b) Distribution of VEGF mRNA in each fraction normalized to actin mRNA, as measured by real-time RT-PCR assay in three experiments. Standard errors are shown. (c) Distribution of VEGF mRNA content in each fraction (VEGF mRNA units x RNA content per fraction).

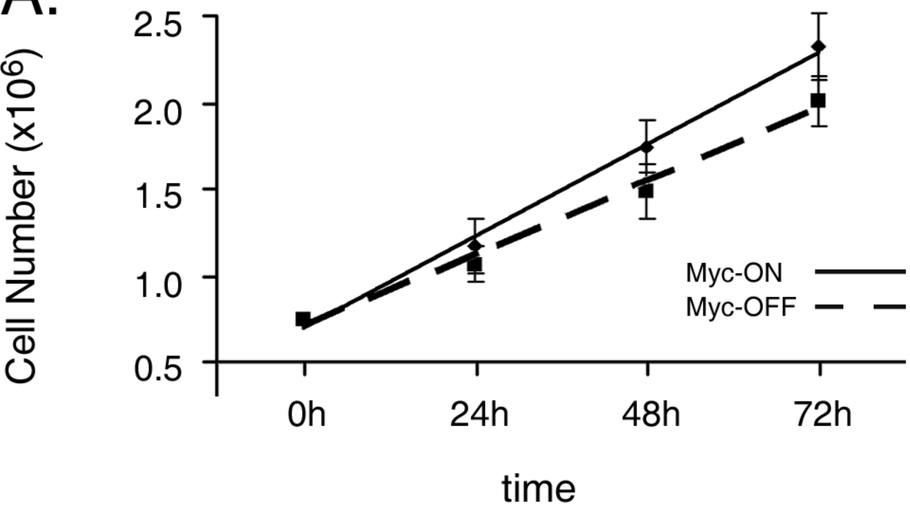
Figure 7. Myc does not affect eIF4E mRNA levels. eIF4E mRNA abundance is similar in Myc-on and Myc-off P493-6 cells, as determined by Northern blot analysis. GAPDH mRNA levels were measured as an internal control.

Figure 8. A rapamycin- and LY294002-sensitive signalling pathway mediates VEGF production from P493-6 cells. (a) eIF4E protein levels are the same in Myc-on and Myc-off P493-6 cells. eIF4E is constitutively phosphorylated in Myc-on and Myc-off cells, as determined by immunoblotting with an antibody to phosphorylated eIF4E. (b) 4E-BP1 phosphorylation is undetectable in Myc-on or Myc-off cells, while serum-induced 293 cells show 4E-BP1 phosphorylation. m^7GTP affinity purification method captures similar amounts of 4E-BP1 from equal numbers of Myc-on and Myc-off cells. (c) Rapamycin or LY294002 treatment for 30 h prevents Myc induction of VEGF production in P493-6 cells, as shown by ELISA analysis. Standard errors are shown. (d) Rapamycin or LY294002 treatment during Myc induction inhibits S6 ribosomal protein phosphorylation, while Akt phosphorylation in not affected, as shown by immunoblotting. Myc expression is also not affected by these drugs.

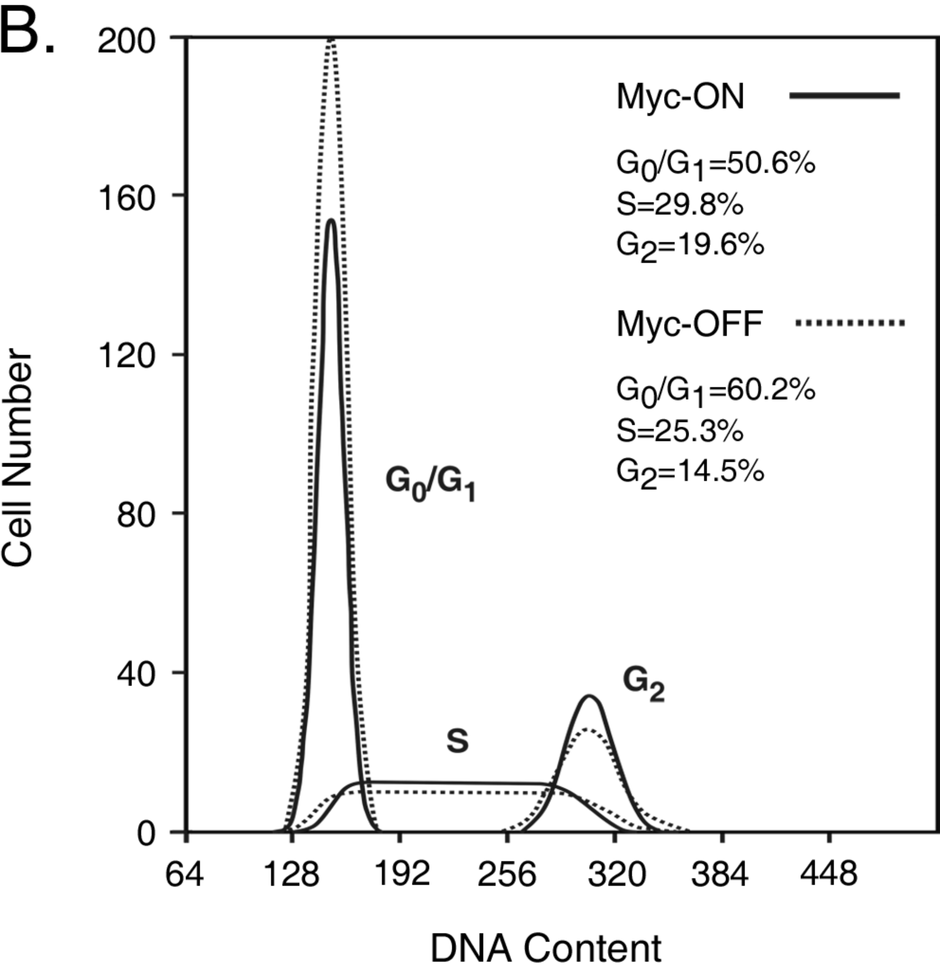
Supplementary information is available at Oncogene's website.


A.
Myc
Actin
Myc-ON
Myc-OFF
B.
VEGF (pg/ml)
3000
2500
2000
1500
1000
500
0
Myc-ON
Myc-OFF
C.
Myc
100
6.25
3.12
1.56
0
Tetracycline (ng/ml)
D.
VEGF (pg/ml)
3000
2500
2000
1500
1000
500
0
100
6.25
3.12
1.56
0
Tetracycline (ng/ml)

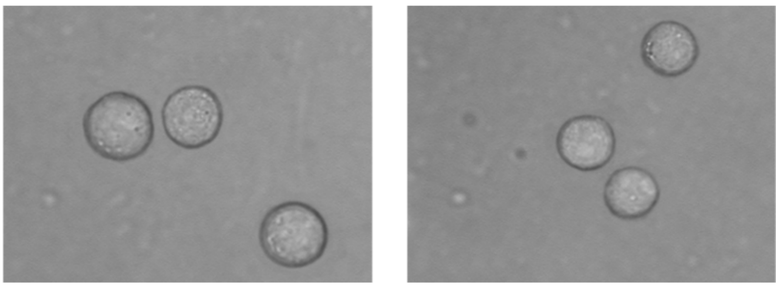
A.



B.

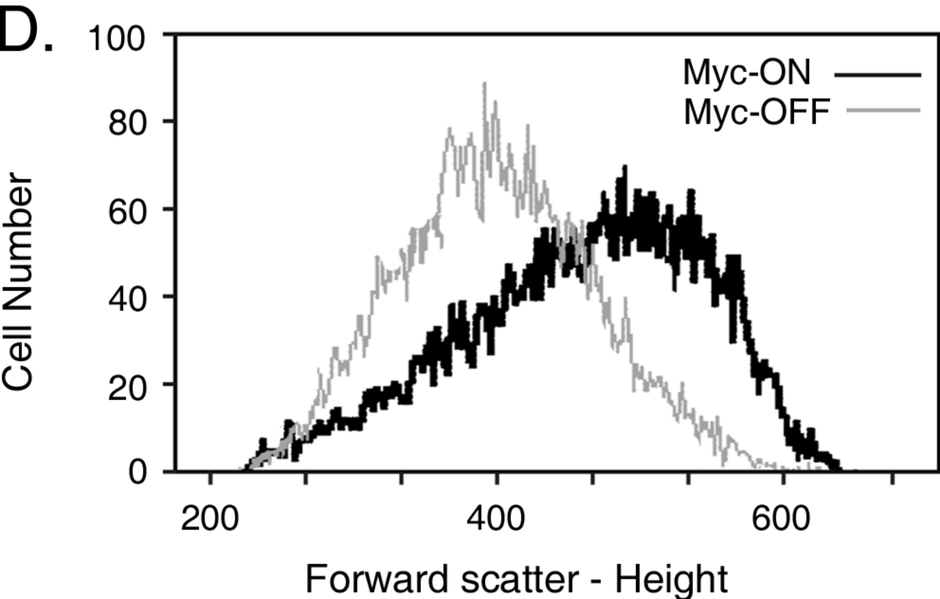


C.

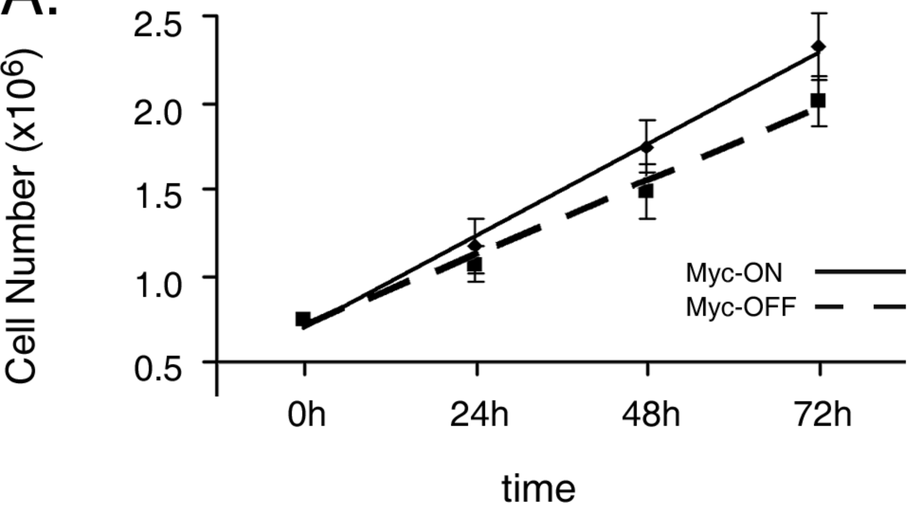


Myc-ON Myc-OFF

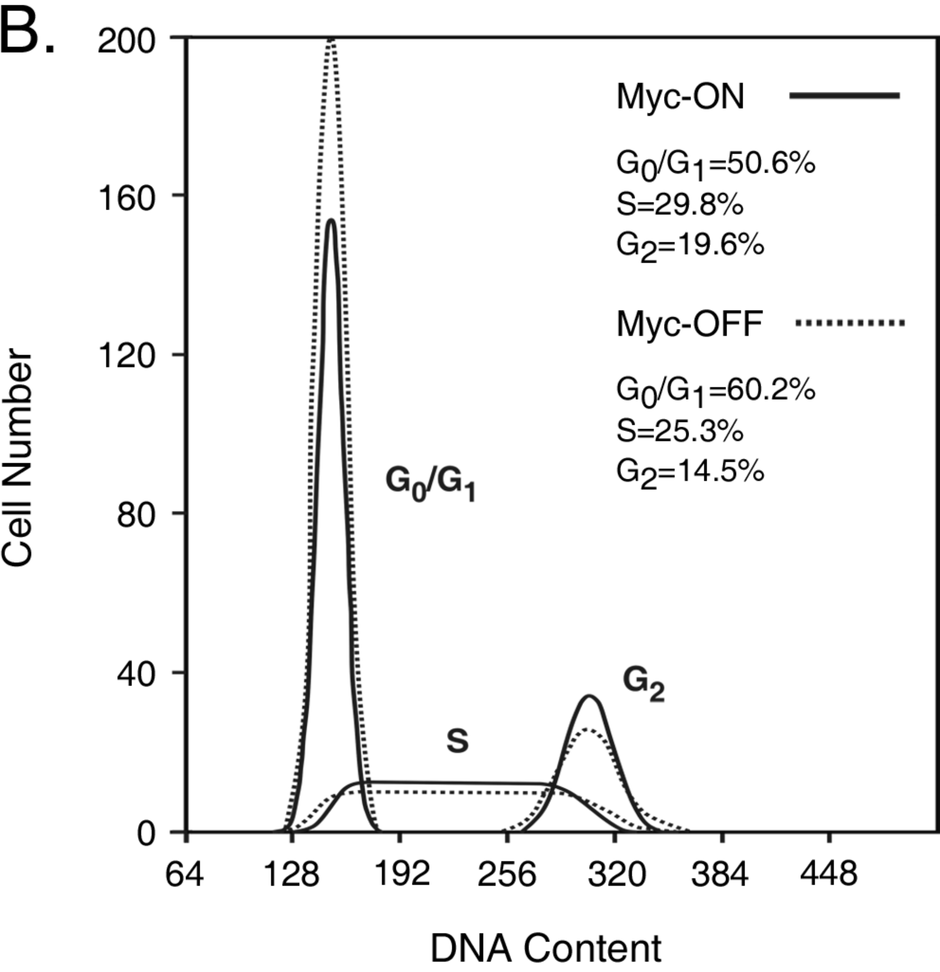
D.



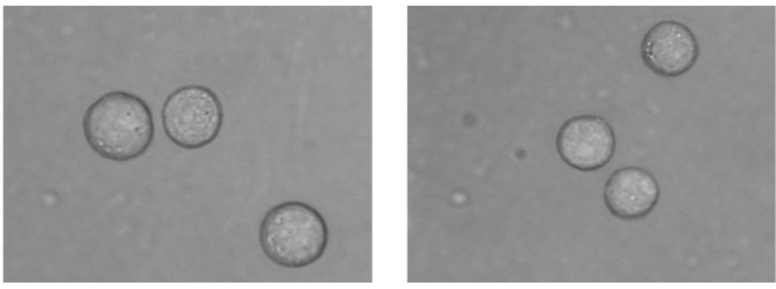
A.



B.

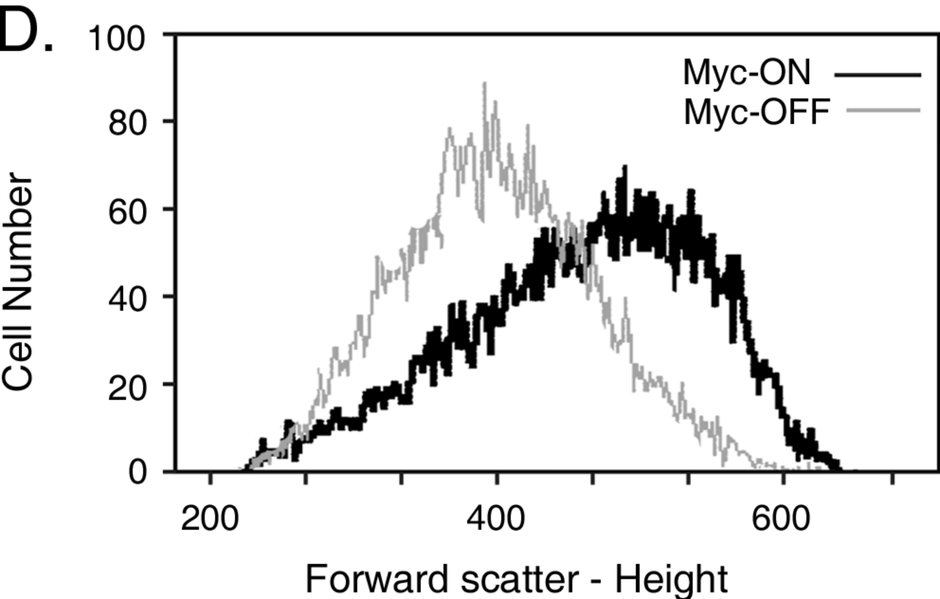


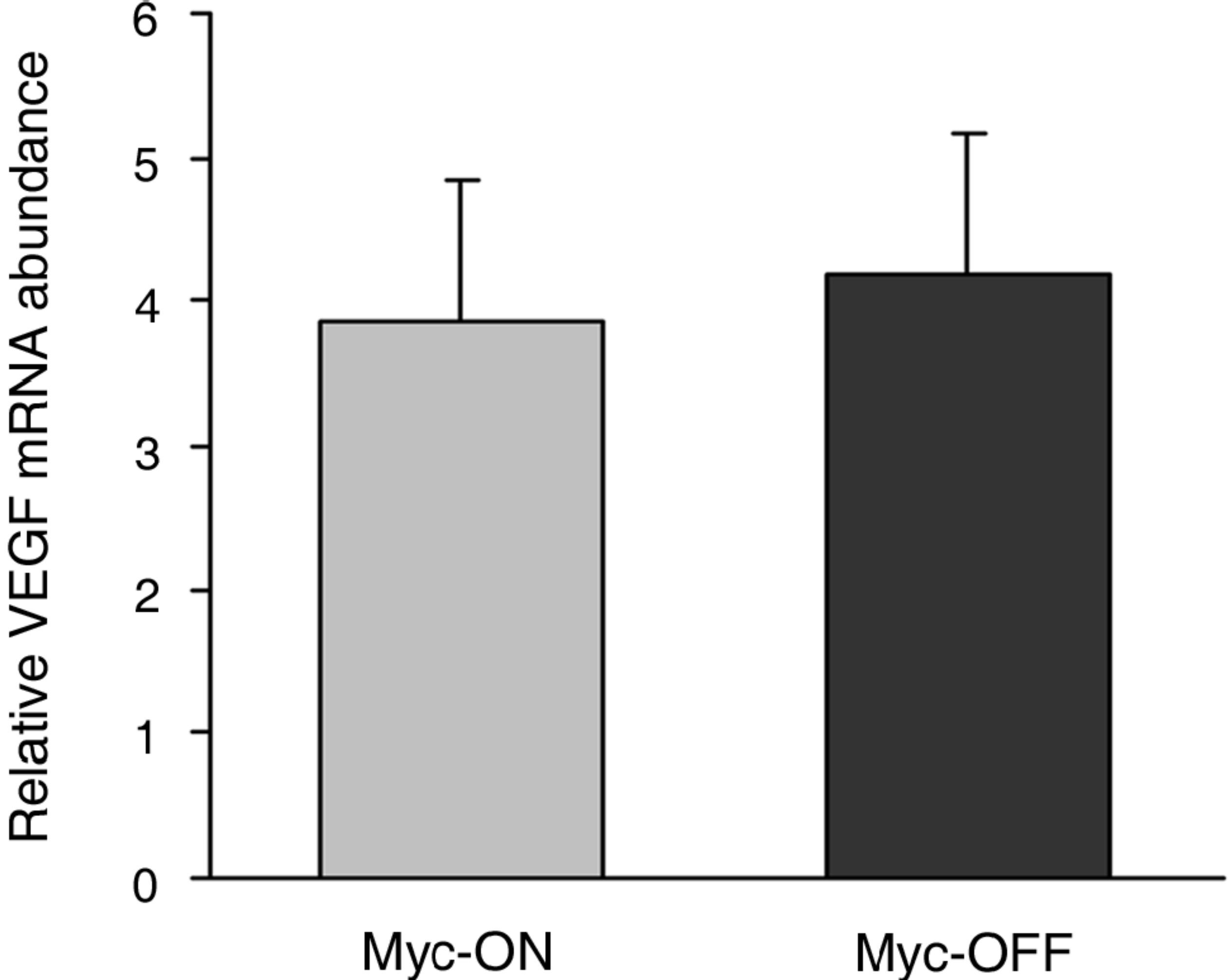
C.



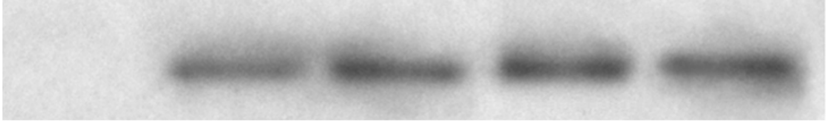
Myc-ON Myc-OFF

D.




Relative VEGF mRNA abundance
0
1
2
3
4
5
6
Myc-ON
Myc-OFF

A.

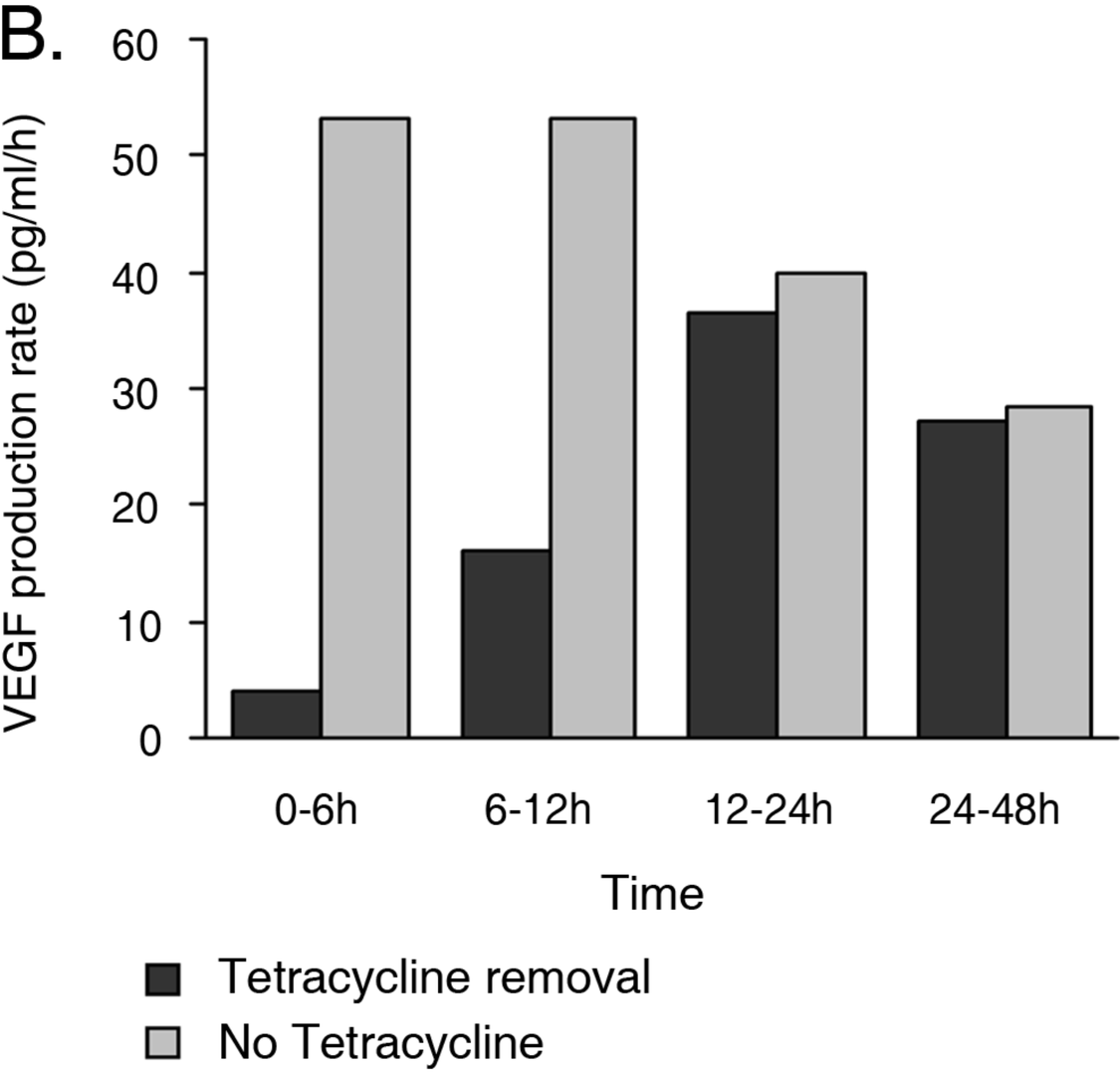


Myc

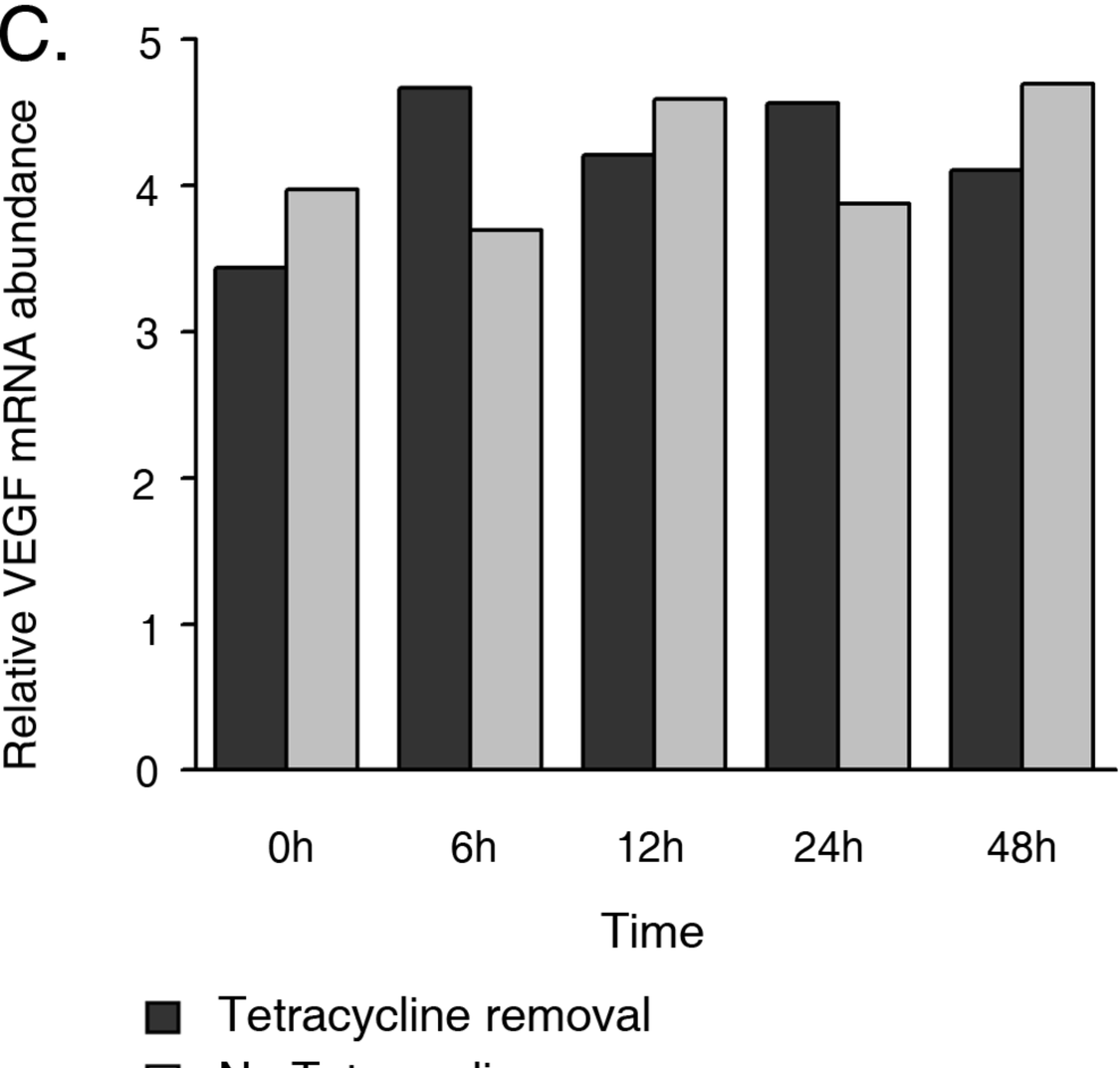
0h 4h 8h 24h 48h

Hours after tetracycline removal

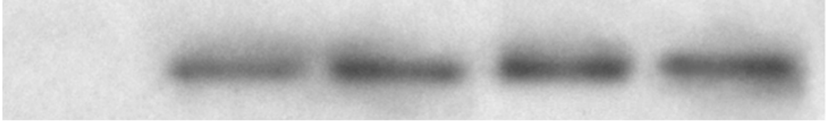
B.



C.



A.

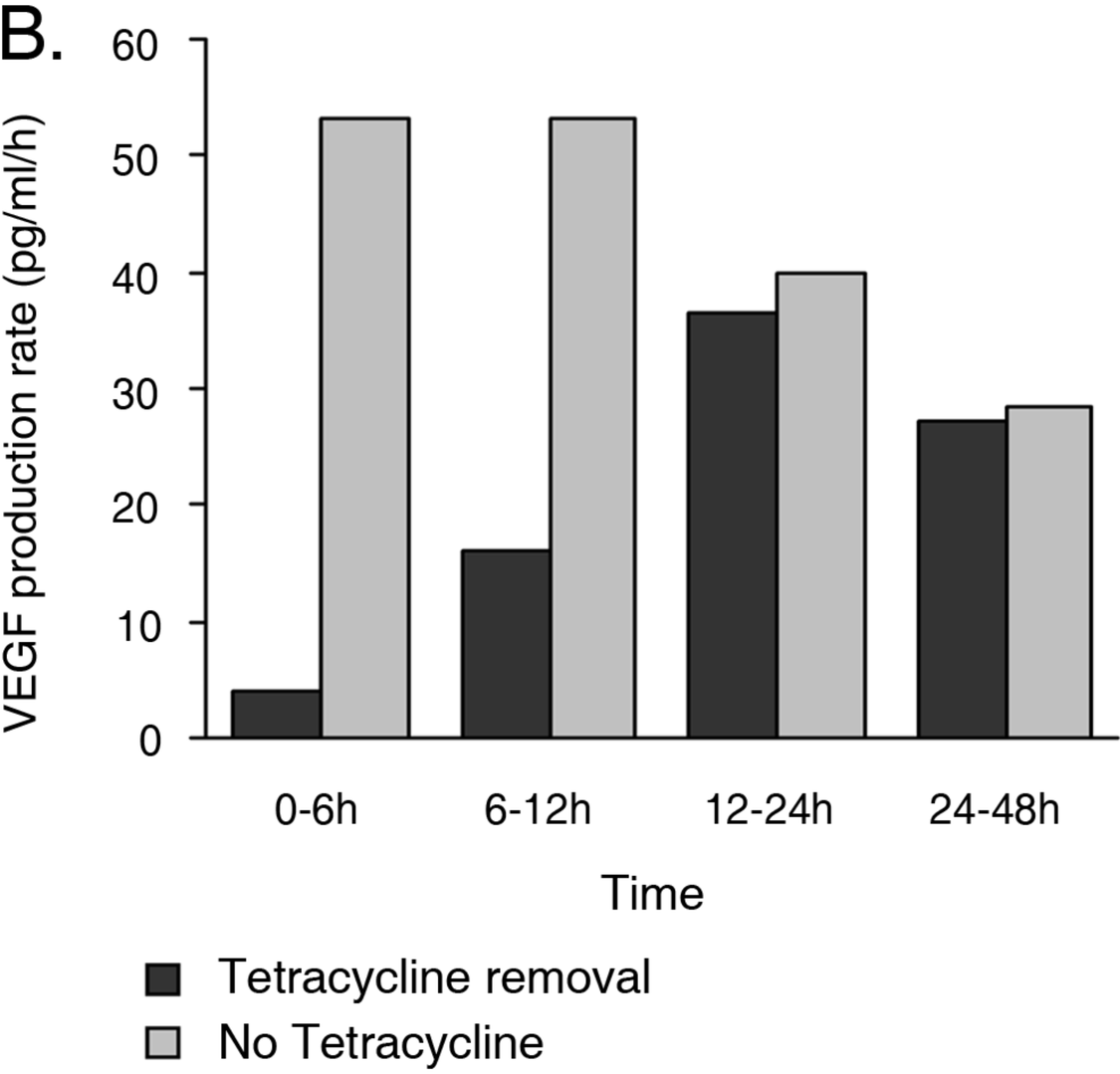


Myc

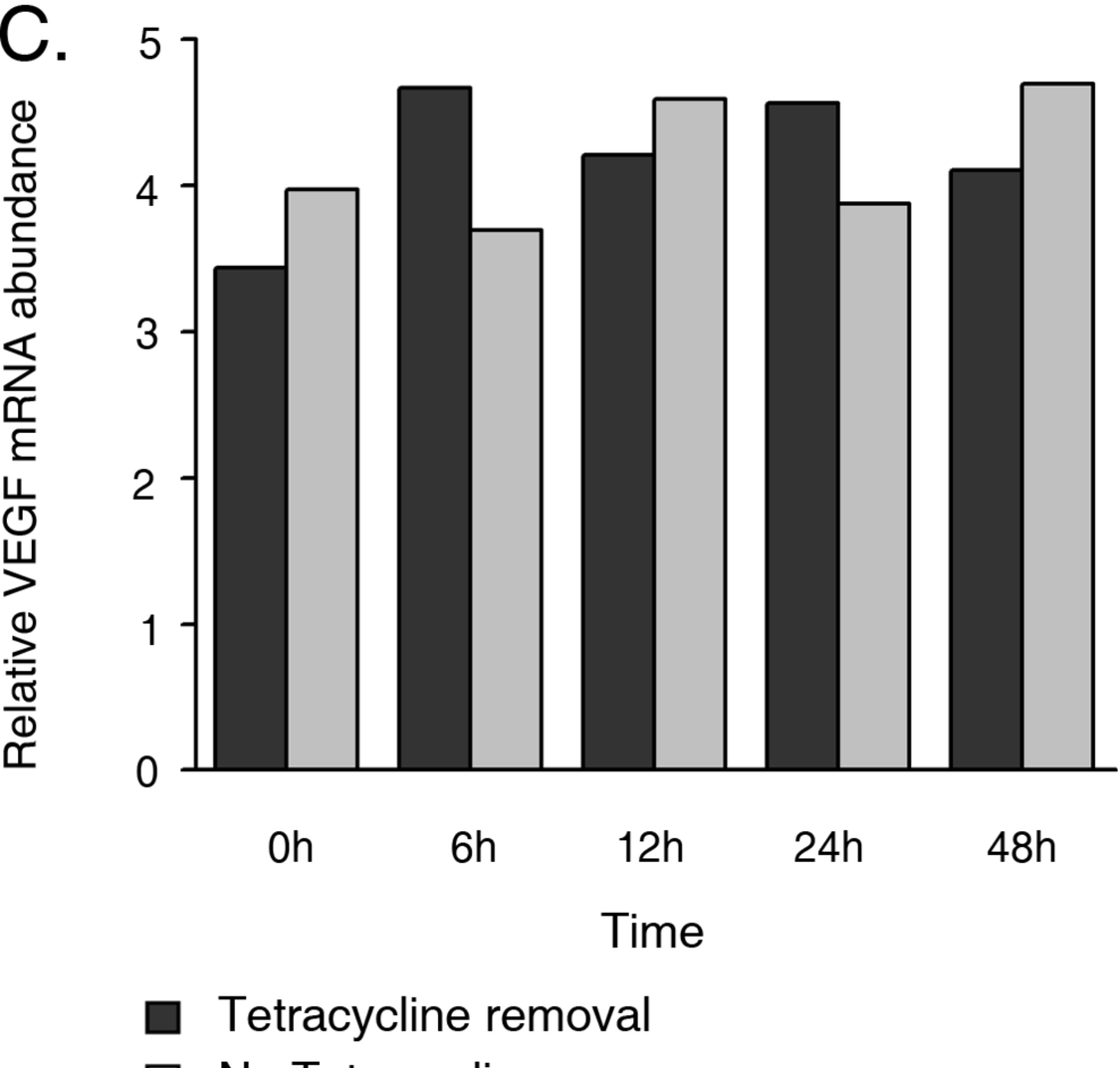
0h 4h 8h 24h 48h

Hours after tetracycline removal

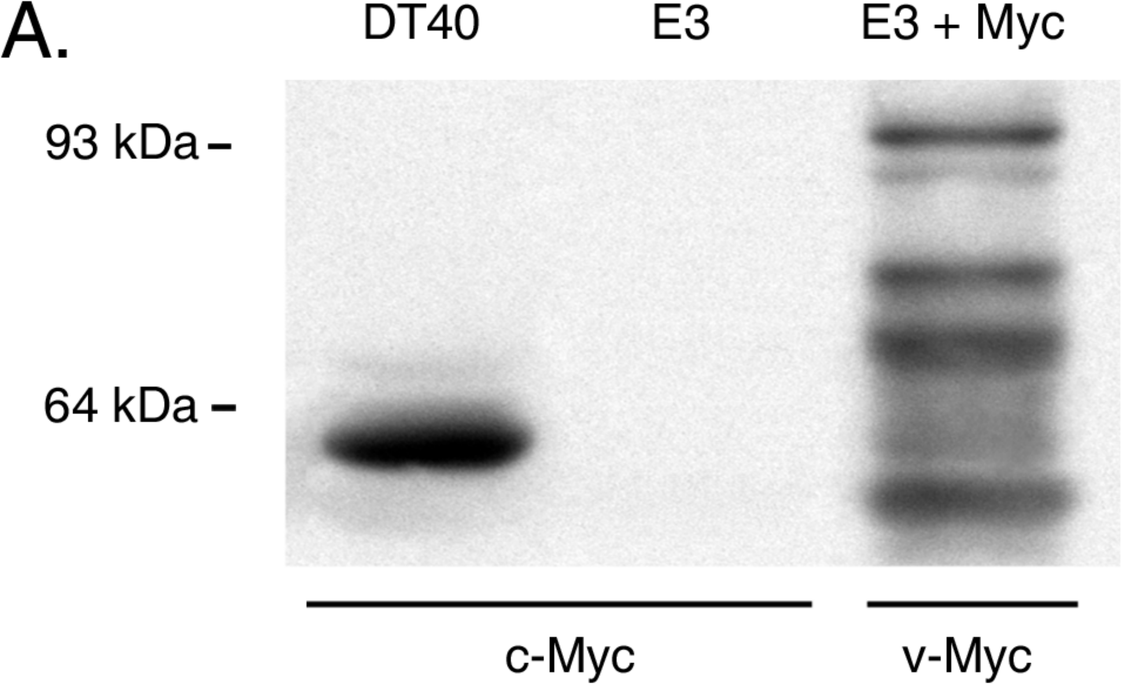
B.



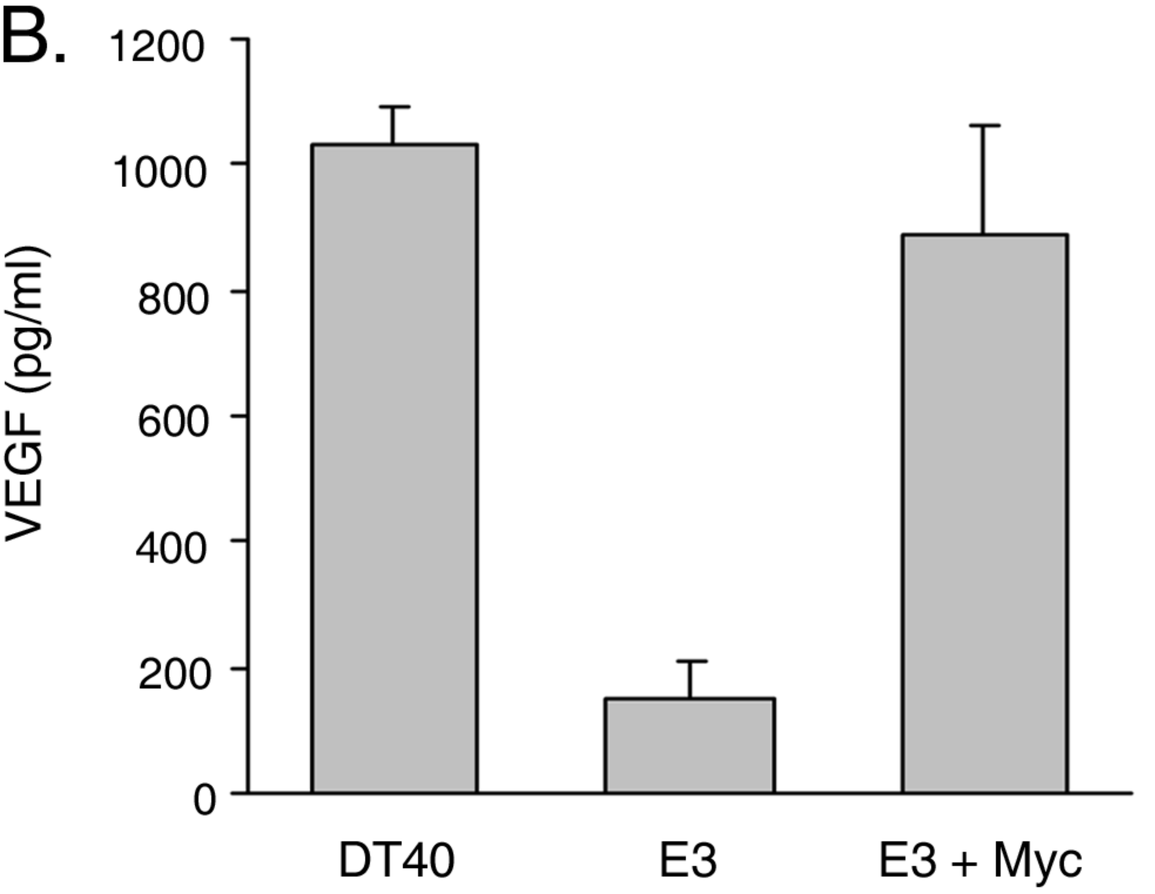
C.



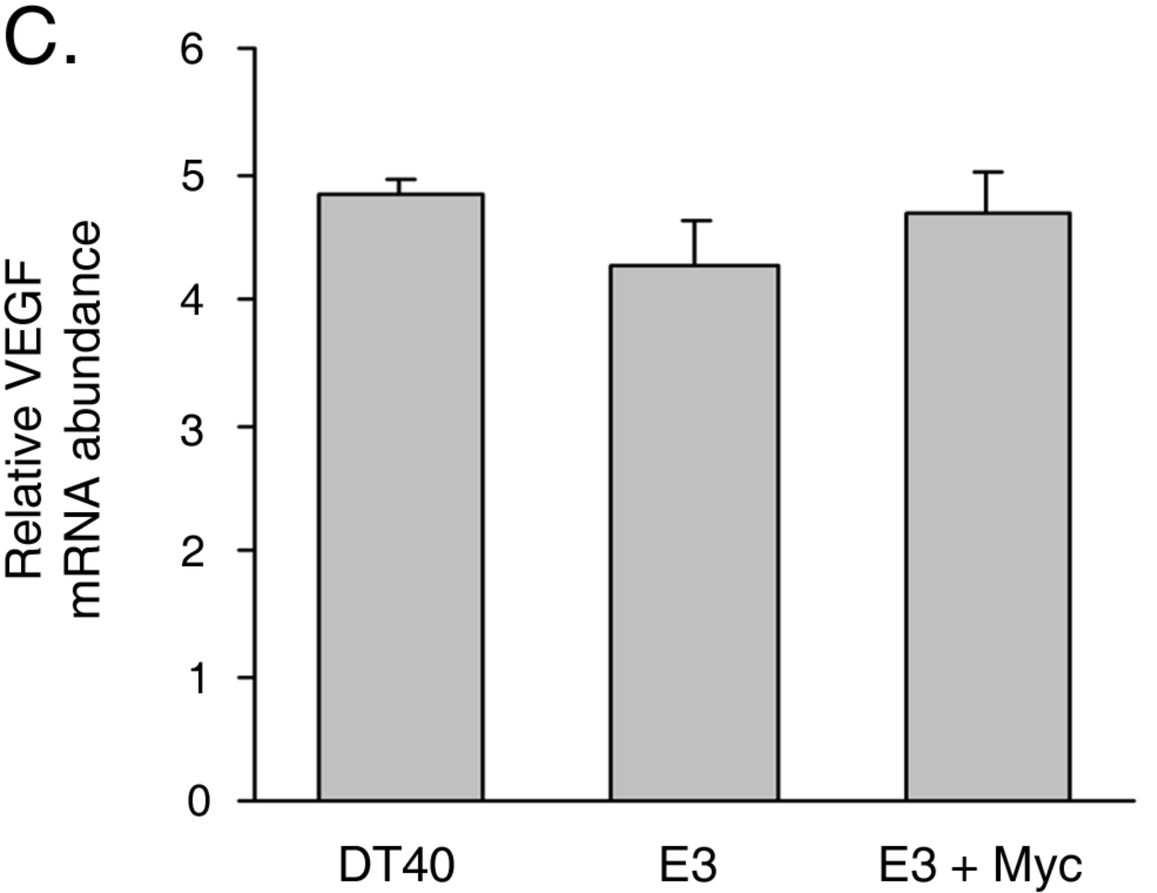
A.

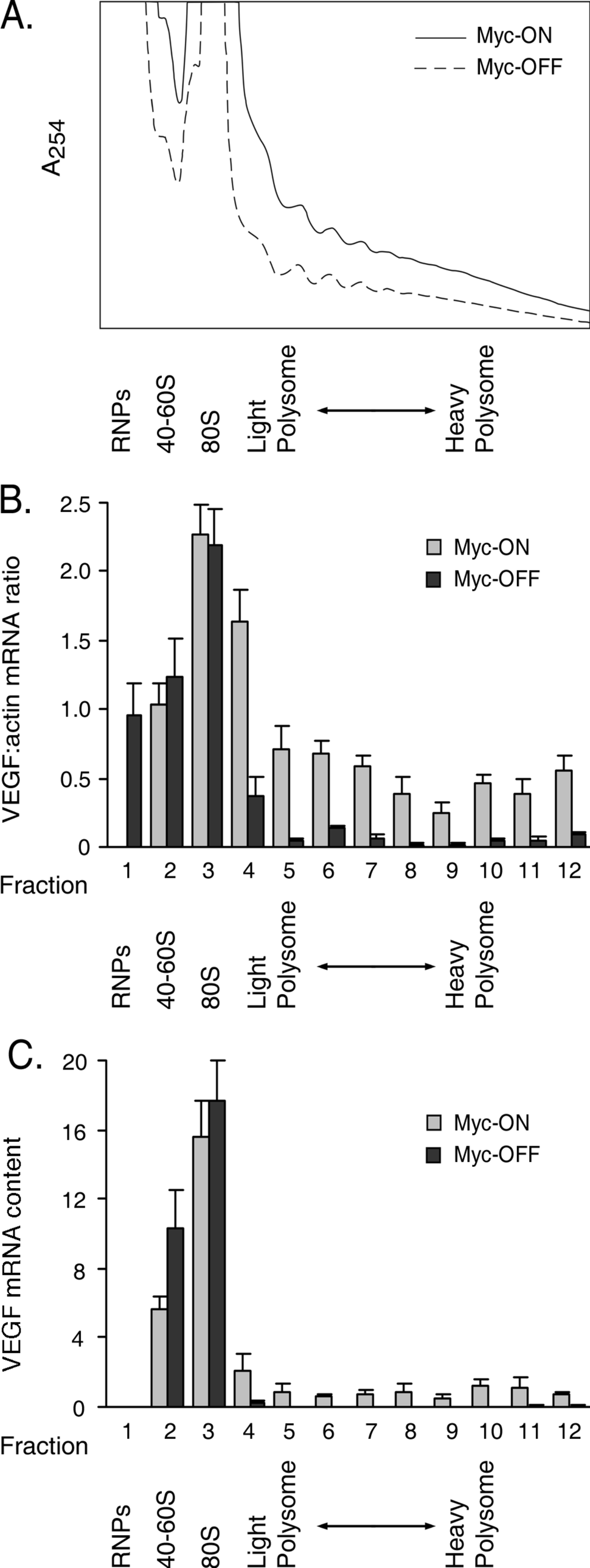

DT40
E3
E3 + Myc
93 kDa
64 kDa
c-Myc
v-Myc

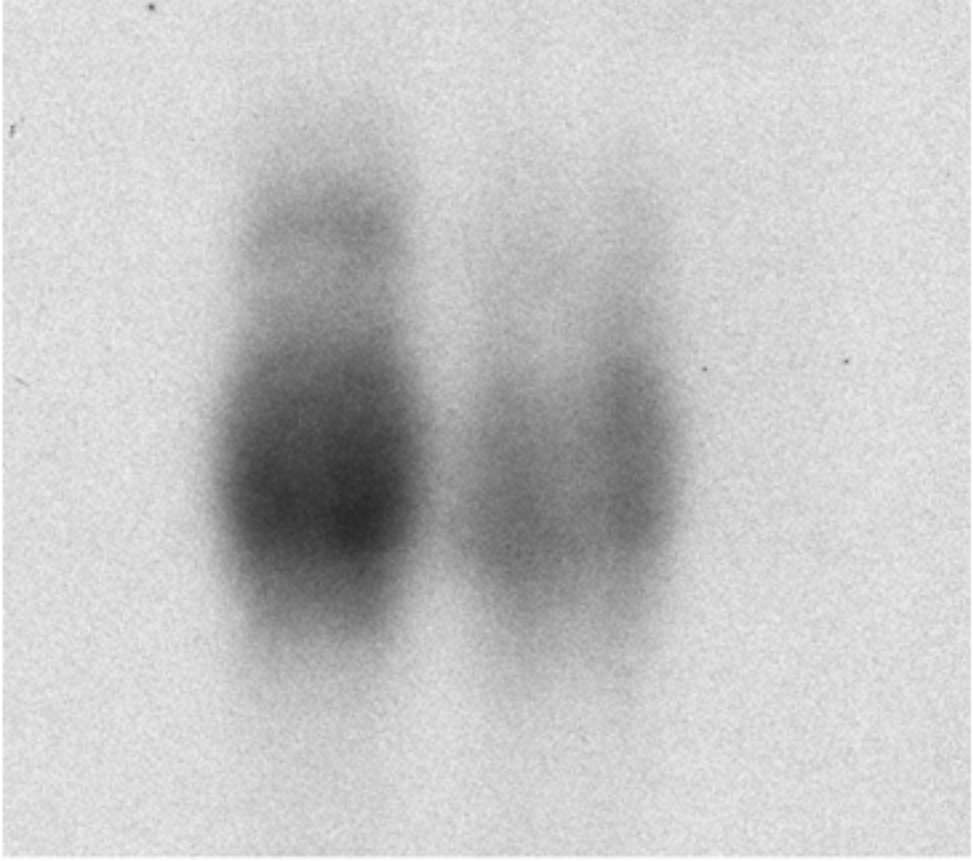
B.


VEGF (pg/ml)
1200
1000
800
600
400
200
0
DT40
E3
E3 + Myc

C.


Relative VEGF mRNA abundance
6
5
4
3
2
1
0
DT40
E3
E3 + Myc

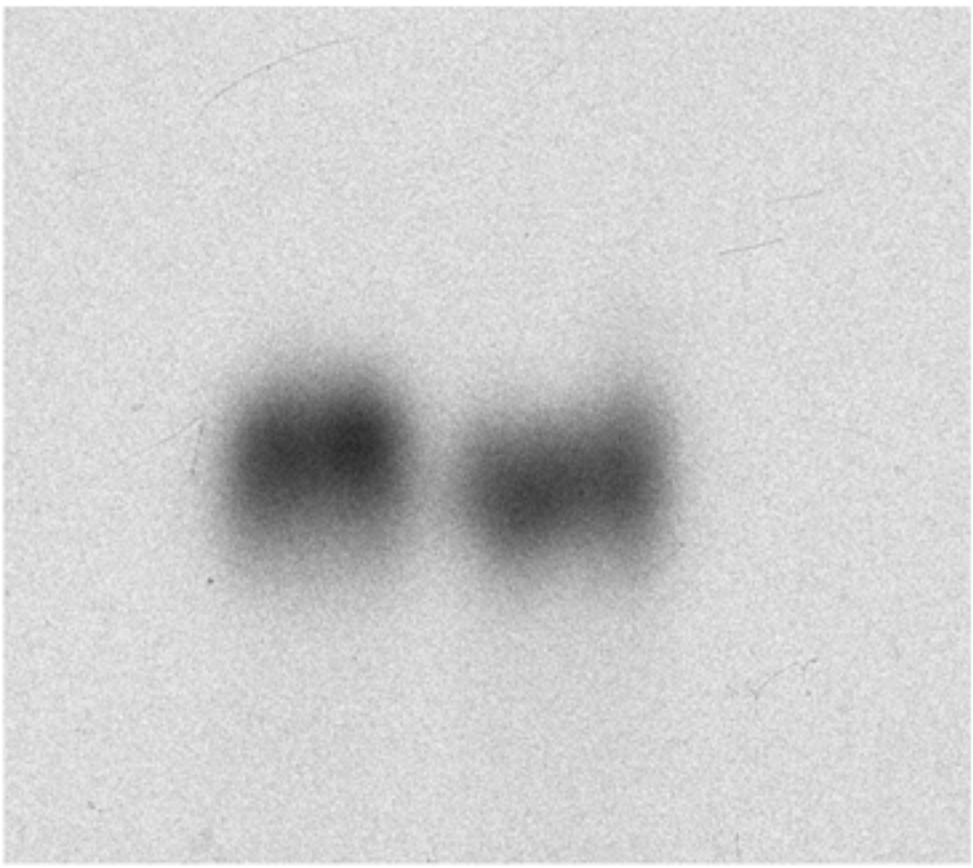

A.
Myc-ON
Myc-OFF
A254
RNPs
40-60S
80S
Light Polysome
Heavy Polysome
B.
VEGF:actin mRNA ratio
2.5
2.0
1.5
1.0
0.5
0
Myc-ON
Myc-OFF
1 2 3 4 5 6 7 8 9 10 11 12
Fraction
RNPs
40-60S
80S
Light Polysome
Heavy Polysome
C.
VEGF mRNA content
20
16
12
8
4
0
Myc-ON
Myc-OFF
1 2 3 4 5 6 7 8 9 10 11 12
Fraction
RNPs
40-60S
80S
Light Polysome
Heavy Polysome



eIF4E

Myc-ON

Myc-OFF



GAPDH

Myc-ON

Myc-OFF

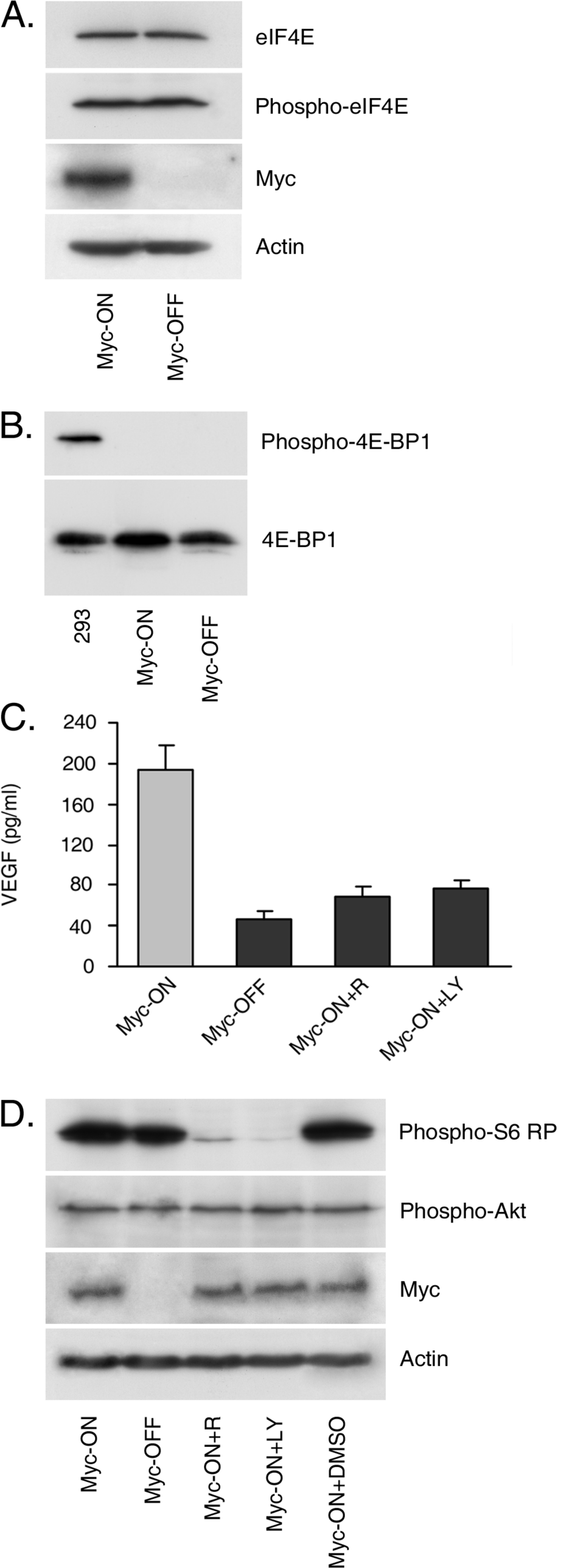

A.
eIF4E
Phospho-eIF4E
Myc
Actin
Myc-ON
Myc-OFF
B.
Phospho-4E-BP1
4E-BP1
293
Myc-ON
Myc-OFF
C.
VEGF (pg/ml)
240
200
160
120
80
40
0
Myc-ON
Myc-OFF
Myc-ON+R
Myc-ON+LY
D.
Phospho-S6 RP
Phospho-Akt
Myc
Actin
Myc-ON
Myc-OFF
Myc-ON+R
Myc-ON+LY
Myc-ON+DMSO

Table 1. Myc increases VEGF mRNA translation initiation in P493-6 cells.

	Myc-ON	**Myc-OFF**	**Myc-On/Myc-OFF**
Percent VEGF mRNA In polysomes[a]	28 +/- 3%	2 +/- 0 %	14
Number of ribosomes per VEGF mRNA[b]	6	2	3
Percent actin mRNA in polysomes[c]	47 +/- 3%	22 +/- 4 %	2
Number of ribosomes per actin mRNA[d]	6	6	1

a. Calculated by comparing the VEGF mRNA content of monosome and polysome sucrose gradient fractions, in 3 experiments.

b. Determined by measuring the median position of VEGF mRNA distribution across sucrose gradient polysome fractions, and identification of the average number of ribosomes per polysome in that median fraction, by examination of the A_{254} polysome profile. Three experiments were analyzed with similar results.

c. Calculated by comparing the actin mRNA content of monosome and polysome sucrose gradient fractions.

d. Determined by measuring the median position of actin mRNA distribution across sucrose gradient polysome fractions, and by identification of the average number of ribosomes per polysome in that median fraction.

Table 2. Myc-induced alterations in RNA/protein content and protein synthesis in P493-6 cells.

	Myc-ON	Myc-OFF	Myc-On/Myc-OFF
Total RNA content/cell[a]	10.4 +/- 0.9 pg	7.6 +/- 1.1 pg	1.4
Protein content/cell[b]	38.7 +/- 2.5 pg	31.3 +/- 3.2 pg	1.2
Protein synthesis[c]	19.1×10^6 +/- 1.8×10^6 cpm	9.8×10^6 +/- 1.9×10^6 cpm	2.0
Percent ribosomes In polysomes[d]	33%+/- 3%	35%+/- 3%	1.0
Number of ribosomes per mRNA[e]	5	5	1.0

a. Measured by A_{260} absorbence analysis of RNA purified from equal numbers of cells in 5 experiments. Standard errors are shown.

b. Measured by Bradford assay of protein content in equal numbers of cells in 7 experiments.

c. Measured by ^{35}S-methionine-cysteine incorporation in 3 experiments.

d. Calculated by comparing RNA content of sucrose gradient monosome (<=80S) and polysome (>80S) fractions.

e. Determined by measuring the median position of RNA content across sucrose gradient polysome fractions, and by identification of the average number of ribosomes per polysome in that median fraction.