Oncogene (2010) 29, 356–367

www.nature.com/onc

ORIGINAL ARTICLE

Transglutaminase 2 suppresses apoptosis by modulating caspase 3 and NF-κB activity in hypoxic tumor cells

G-Y Jang[1,5], J-H Jeon[2,5], S-Y Cho[1], D-M Shin[1], C-W Kim[1], EM Jeong[1], HC Bae[3], TW Kim[3], S-H Lee[1], Y Choi[1], D-S Lee[4], S-C Park[1] and I-G Kim[1]

[1]Department of Biochemistry and Molecular Biology/Aging and Apoptosis Research Center (AARC), Seoul National University College of Medicine, Seoul, Korea; [2]Department of Physiology, Seoul National University College of Medicine, Seoul, Korea; [3]Laboratory of Infection and Immunology, Graduate School of Medicine, Korea University, Seoul, Korea and [4]Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea

The expression of hypoxia-inducible factor-1 (HIF-1) correlates with poor clinical outcomes and confers resistance to the apoptosis of the tumor cells that are exposed to hypoxia. Presently, the mechanism underlying this phenomenon is poorly understood. In this study we provide evidence that transglutaminase 2 (TG2), an enzyme that catalyses protein crosslinking reactions, is a transcriptional target of HIF-1 to enhance the survival of hypoxic cells. We found that hypoxia induces TG2 expression through an HIF-1 dependent pathway and concurrently activates intracellular TG2. The hypoxic cells overexpressing TG2 showed resistance to apoptosis. Conversely, the hypoxic cells treated with either TG2 inhibitor or small interfering RNA (siRNA) became sensitive to apoptosis. Activation of TG2 in response to hypoxic stress inhibited caspase-3 activity by forming crosslinked multimer, resulting in insoluble aggregates. TG2 also activates nuclear factor (NF)-κB pathway after hypoxic stress, and thereby induces the expression of cellular inhibitor of apoptosis 2. The anti-apoptotic role of TG2 was further confirmed *in vivo* using xenografts in athymic mice. Our results indicate that TG2 is an anti-apoptotic mediator of HIF-1 through modulating both apoptosis and survival pathways and may confer a selective growth advantage to tumor cells. These findings suggest that the inhibition of TG2 may offer a novel strategy for anticancer therapy.
Oncogene (2010) **29,** 356–367; doi:10.1038/onc.2009.342; published online 19 October 2009

Keywords: apoptosis; caspase 3; hypoxia-inducible factor-1; NF-κB; transglutaminase 2

Introduction

Hypoxia is commonly found in poorly vascularized regions of rapidly growing solid tumors. To adapt to microenvironmental hypoxia, tumor cells undergo many phenotypic changes, such as induction of angiogenesis and upregulation of glycolysis (Brown and Wilson, 2004; Pouyssegur *et al*., 2006). These phenotypes, which are hallmarks of most tumors, promote tumor cells to be more malignant and resistant to both radiotherapy and chemotherapy due to a selective growth advantage (Brown and Wilson, 2004; Reed, 2006).

The adaptive responses to hypoxia are achieved by the transcriptional changes of a number of genes mediated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcriptional factor that consists of a closely regulated HIF-1α and a constitutively expressed HIF-1β subunits (Pouyssegur *et al*., 2006). An overexpression of HIF-1α is a common feature of many solid tumors, showing a close correlation with a poor prognosis (Zagzag *et al*., 2000; Maxwell, 2005). Moreover, HIF-1 inhibition noticeably suppresses tumor growth (Giaccia *et al*., 2003; Semenza, 2003), indicating that HIF-1 confers considerable growth advantages to tumor cells. However, HIF-1 is reported to promote apoptosis by transactivating pro-apoptotic genes, such as BNIP3 and NIX (Piret *et al*., 2002), suggesting that HIF-1 provides a driving force to promote tumor cell heterogeneity and clonal selection.

Transglutaminase 2 (TG2) is a calcium-dependent enzyme that mediates the post-translational modification of a variety of proteins by catalysing the transamidation reaction, resulting in crosslinked, polyaminated or deamidated proteins (Fesus and Piacentini, 2002). TG2 is also known to show guanosine-5'-triphosphate hydrolysing activity (Lee *et al*., 1989), protein disulfide isomerase activity (Hasegawa *et al*., 2003) and kinase activity (Mishra and Murphy, 2004). Thus, TG2 has been suggested to be involved in a diverse range of biological processes, including apoptosis, membrane signaling, cell adhesion and extracellular matrix formation (Fesus and Piacentini, 2002; Lorand and Graham, 2003). However, TG2-deficient mice showed no apparent physiological and developmental defects

Correspondence: Professor I-G Kim, Department of Biochemistry and Molecular Biology/Aging and Apoptosis Research Center (AARC), Seoul National University College of Medicine, 28 Yongon Dong, Chongno Gu, Seoul 110-799, Korea.
E-mail: igkim@plaza.snu.ac.kr
[5]These authors contributed equally to this work.
Received 3 December 2008; revised 31 August 2009; accepted 17 September 2009; published online 19 October 2009

(De Laurenzi and Melino, 2001), suggesting that TG2 is not critical in the normal physiological processes.

In contrast, TG2 expression has been implicated in pathological consequences, particularly in several cancers. The increased expression of TG2 is observed in drug-resistant and metastatic breast cancer cells (Mehta *et al*., 2004; Herman *et al*., 2006). The level of TG2 expression is higher in glioblastoma multiforme than in nonmalignant human brain tissue or in low-grade astrocytoma (Zhang *et al*., 2003a). Additional evidences indicate that the overexpression of TG2 increases tumor cell viability by preventing apoptosis (Antonyak *et al*., 2004), whereas the inhibition of TG2 can induce apoptosis (Choi *et al*., 2005; Yuan *et al*., 2005). At present, little is known about the underlying mechanism by which TG2 exerts anti-apoptotic effect in cancer cells.

Both HIF-1 and TG2 are upregulated in malignant tumor cells that are resistant to apoptosis (Zhang *et al*., 2003a; Mehta *et al*., 2004; Maxwell, 2005; Herman *et al*., 2006). The expression of TG2 is repressed by von Hippel-Lindau tumor suppressor that has a crucial role in oxygen-sensing pathways through oxygen-dependent polyubiquitination of HIF-1 (Wykoff *et al*., 2000). The evidence that TG2 is activated by reactive oxygen species implies TG2 as a stress responder (Shin *et al*., 2004). These reports lead us to analyse the possibility of whether TG2 confers a selective growth advantage to the tumor cells, which are exposed to hypoxia, through an HIF-1 pathway. In this report, we show that TG2 is a transcriptional target of HIF-1, and it suppresses apoptosis in hypoxic cells by inhibiting caspase 3 and by also activating nuclear factor (NF)-κB.

Results

Induction of transglutaminase 2 (TG2) expression by hypoxia-inducible factor-1 (HIF-1) under hypoxic conditions

To test the effect of hypoxia on the expression of TG2, we screened several cell lines for TG2 level after a treatment with $CoCl_2$. Western blot analysis showed that TG2 expression level of SH-SY5Y, U373MG and HeLa cells increased, but not of SK-N-SH, human embryonic kidney 293 (HEK293) and MCF7 cells, and that the extent of the increase varied depending on the cell type (Supplementary Figure 1). The increase in TG2 expression was confirmed in the experiments carried out under the low oxygen conditions using western blot and quantitative reverse transcriptase–PCR analyses (Figures 1a and b). Hypermethylation of DNA is generally associated with an inhibition of transcription in tumor cells (Baylin, 1997). To test this possibility, we treated SK-N-SH, HEK293 and MCF7 cells with 5-aza-2 deoxycytidine for 4 days and found that TG2 expression increased in these cell lines, indicating that DNA methylation inhibits TG2 expression under hypoxic condition (Figure 1c). We next examined the distribution of expressed TG2. Western blot analysis showed that TG2 expression increased in both the cytosol and the nuclear fractions of U373MG cells that were exposed to hypoxic stress (Figure 1d). These results were confirmed in the immunocytochemical observation, in which both the cytosol and the nucleus of hypoxic U373MG cells manifested an increase in TG2 expression (Figure 1e).

The sequence analysis revealed that TG2 has six putative hypoxia-response elements (HRE1 to HRE6; 5′-RCGTG-3′) in the promoter region (Figure 2a). To determine whether TG2 is regulated by HIF-1 at the transcriptional level in hypoxia, we generated the promoter deletion constructs and transfected them into the U373MG cells. Reporter assay showed that the deletion of HRE2, located 367-bp upstream from the translation initiation site of TG2, failed to show luciferase activity in response to both hypoxic stress and HIF-1α overexpression (Figure 2b). Only the reporter constructs including HRE2 reacted with HIF-1α in a dose-dependent manner (Figure 2c). The site-specific mutation (GCGTG to GGAAT) of HRE2 failed to respond under hypoxic stress and with overexpressed HIF-1α (Figure 2d). To confirm these results, the chromatin was precipitated with an HIF-1α antibody. PCR analysis using primer set that covered the HRE2 (−273/−429) region showed the binding of HIF-1α to TG2 promoter (Figure 2e). Moreover, TG2 expression decreased in U373MG cells treated with small interfering RNA (siRNA) for HIF-1α under hypoxic conditions (Figure 2f). A comparison of TG2 promoter sequences of human, mouse and guinea pig revealed that the position of HRE2 is well conserved among these species (Supplementary Figure 2). These results indicate that hypoxia induces TG2 expression in U373MG through HIF-1.

Suppression of apoptosis by activation of intracellular transglutaminase 2 (TG2) under hypoxia

Intracellular TG2 is known to be inactive under the normal physiological condition (Jeon *et al*., 2004; Shin *et al*., 2004). We examined whether TG2 is activated by hypoxic stress using 5′-(biotinamido)pentylamine (BP) incorporation method and found that TG2 activity increased under hypoxic conditions, which was inhibited by treatment with cystamine or monodansylcadevaline (MDC), a competitive inhibitor of TG2 (Figure 3a). Moreover, TG2 activity was also effectively suppressed by the treatment with N-acetylcysteine or BAPTA-AM under hypoxic condition (Figures 3b and c), indicating that the TG2 activation might be because of an increase in the reactive oxygen species (Dirmeier *et al*., 2004; Shin *et al*., 2004), and intracellular calcium generated under hypoxic stress (Hui *et al*., 2006).

Transglutaminase 2 has been implicated to be a participant in apoptotic process (Antonyak *et al*., 2006; Yamaguchi and Wang, 2006; Verma and Mehta, 2007). To understand the role of increased TG2 activity in hypoxia, we established HEK293 cell line overexpressing TG2 (293^{TG2}), and examined the cell death induced by hypoxia. Under the normal culture condition, 293^{TG2} cells are viable without apparent apoptotic phenotypes.

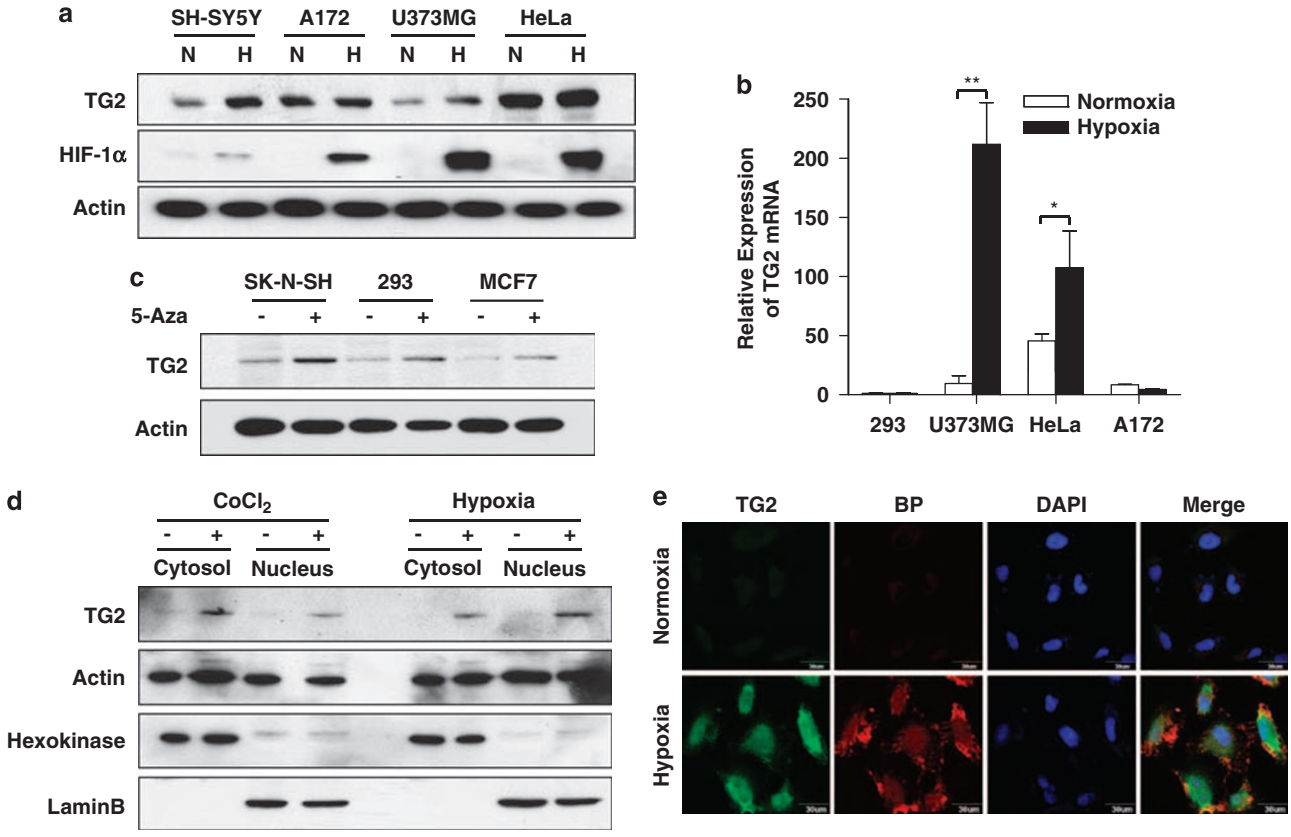

Figure 1 Induction of transglutaminase 2 (TG2) expression by hypoxic stress. (**a**) Cell lines were cultured for 24 h under normoxic or hypoxic (1% oxygen (O_2)) conditions. Cell extracts were analysed using western blotting using antibodies to TG2 and hypoxia-inducible factor-1 (HIF-1α) respectively. N, normoxia; H, hypoxia. (**b**) quantitative reverse transcriptase–PCR (qRT–PCR) analysis of TG2 expression. Cells were cultured in hypoxic condition (0.1% O_2) for 5 h. For analysis, 1 μg of total RNA was used. (**c**) Cell lines were cultured in Dulbecco's modied Eagle' s medium (DMEM) containing 5-aza-2 deoxycytidine (5-aza-dC, 5 μM) for 4 days, and changing medium every 24 h with fresh 5-aza-dC. Cell extracts were analysed using western blotting. (**d**) U373MG cells exposed to $CoCl_2$(200 μM) or hypoxia (1% O_2) for 24 h were fractionated into the cytosolic and the nuclear fractions. The extracts were probed with antibodies to TG2, actin, hexokinase and lamin B, respectively. (**e**) U373MG cells cultured on glass coverslip were incubated in the presence of 0.2 mM 5′-(biotinamido)pentylamine (BP) for 24 h under normoxic or hypoxic (1% O_2) conditions. Expression and intracellular activity of TG2 were assessed using a TG2/fluorescein isothiocyanate (FITC)-labeled immunoglobulin G (IgG) and Texas Red-conjugated streptavidin, respectively. To visualize the nucleus, 4,6-diamidino-2-phenylindole (DAPI) was used. Data represent mean ± s.d. based on three independent experiments. $*P \leqslant 0.05$; $**P \leqslant 0.001$.

In normoxia, *in situ* TG2 activity was not detectable in both 293^{vec} and 293^{TG2} cells. In contrast, in hypoxic condition, *in situ* TG2 activity increased more than 30-fold in 293^{TG2} cells after 72 h, although no significant change was observed in 293^{vec} cells (Figure 4a). It should be noted that in 293^{TG2} cells, a marked increase in TG2 protein appeared after 48 h of hypoxia despite ectopic expression (Figure 4a, inset). Possibly, the hypoxia-induced increase in TG2 is mediated at the level of protein turnover, in addition to regulation at the transcriptional level. When cells were cultured under 0.1% oxygen (O_2), most of the 293^{vec} cells died 3 days after exposure to hypoxia. In contrast, 293^{TG2} cells were resistant to hypoxia-induced cell death. Similar results were obtained with 293^{TG2} cells when the experiments were carried out under hypoxia with 1% O_2 (Figure 4b).

To confirm the anti-apoptotic effect of TG2 in hypoxia, we examined hypoxia-induced cell death of U373MG cells, of which TG2 expression was down-regulated. Under hypoxic condition, the treatment of U373MG cells with TG2-siRNA resulted in a significantly higher cell death rate than those treated with green fluorescent protein (GFP)-siRNA (Figure 4c). This finding was verified using fluorescence-activated cell sorting analysis of cells stained with propidium iodide (Supplementary Figure 3). Moreover, when U373MG cells were treated with MDC, cell death rate was increased in a dose-dependent manner (Figure 4d). Taken together, these results indicate that the reduced cell death rate under hypoxia correlates with the increase in *in situ* TG2 activity in HEK293 and U373MG cells.

Inhibition of caspase-3 activity by transglutaminase 2 (TG2) through formation of insoluble aggregates

To analyse the mechanism by which TG2 exerts anti-apoptotic activity, we examined the effect of hypoxia on caspase activity, a key executioner in apoptosis, in both 293^{vec} and 293^{TG2} cells. When both cells were exposed to hypoxia, caspase-3 activity of 293^{vec} cells increased threefold after 48 h and fivefold after 72 h. In contrast, the caspase-3 activity of 293^{TG2} cells did not vary for 48 h

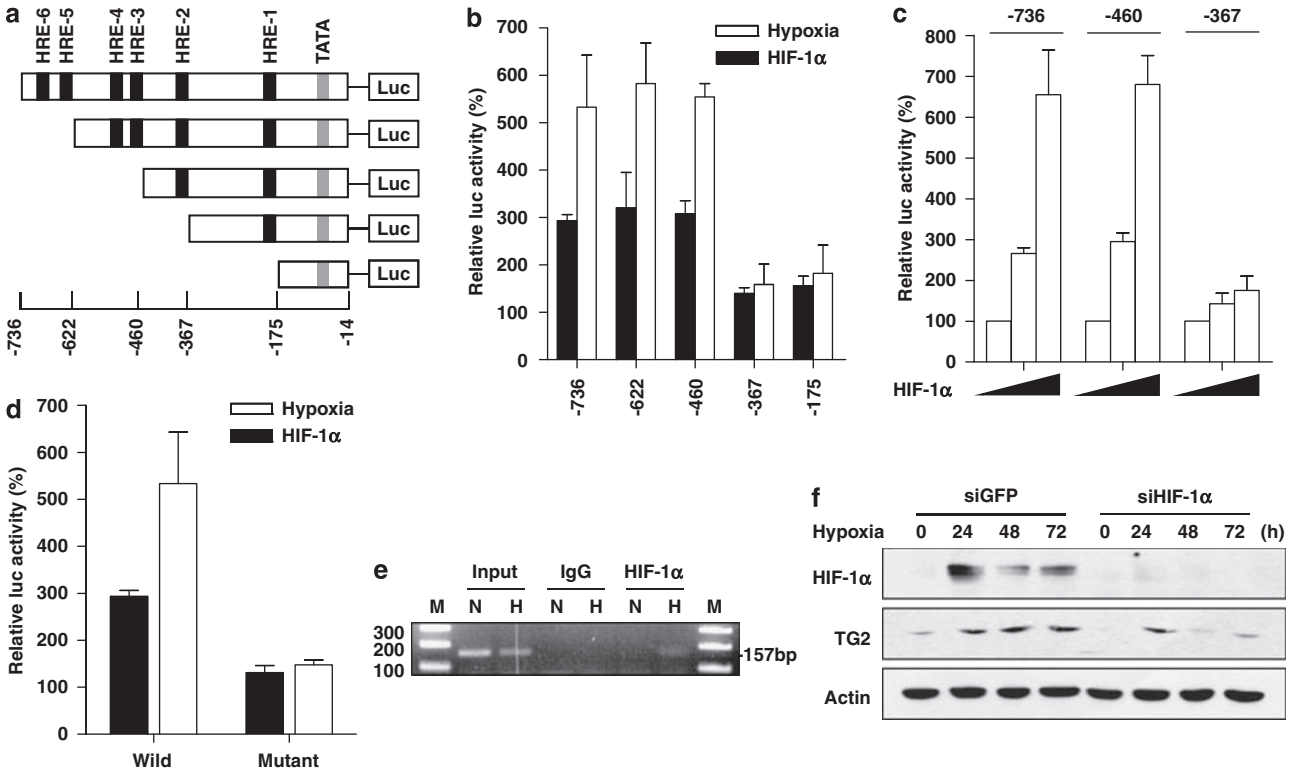

Figure 2 Hypoxia-induced transglutaminase 2 (TG2) expression mediated by hypoxia-inducible factor-1α (HIF-1α). (**a**) Schematic representation of deletion constructs for human TG2 promoter showing the location of hypoxia-response elements (HRE, 5′-RCGTG-3′). (**b**) Comparison of the ability of HIF-1α or hypoxia to transactivate TG2 promoter. U373MG cells were transfected with different deletion constructs (molar equivalence of 0.5 μg of full-length TG2 promoter) together with 0.5 μg pCMV (HIF-1α). The cells were incubated in normoxic or hypoxic (1% oxygen (O_2) conditions for 24 h and assayed for luciferase activity. (**c**) Dose dependence of HIF-1α on the reporter activity. U373MG cells were transfected with promoter/reporter constructs together with increasing amount of pCMV (HIF-1α) (0, 0.5 and 2.5 μg) (**d**) Effect of site-specific mutagenesis of HRE-2 (GCGTG to GGAAT) on the reporter activity. Luciferase activity is expressed as a relative value to that of normoxic cells. The figures denote mean ± s.d. based on three independent experiments. (**e**) Chromatin immunoprecipitation (ChIP) analysis of the TG2 promoter. Protein-DNA complexes from U373MG cells exposed to 0.1% O_2 for 24 h were subjected to immunoprecipitation with antibodies against immunoglobulin G (IgG) or HIF-1α. The DNA was amplified using PCR and resolved in 2% agarose gel. N, normoxia; H, hypoxia. (**f**) U373MG cells treated with siGFP or siHIF-1α were cultured under hypoxic conditions (1% O_2), and analysed using western blotting.

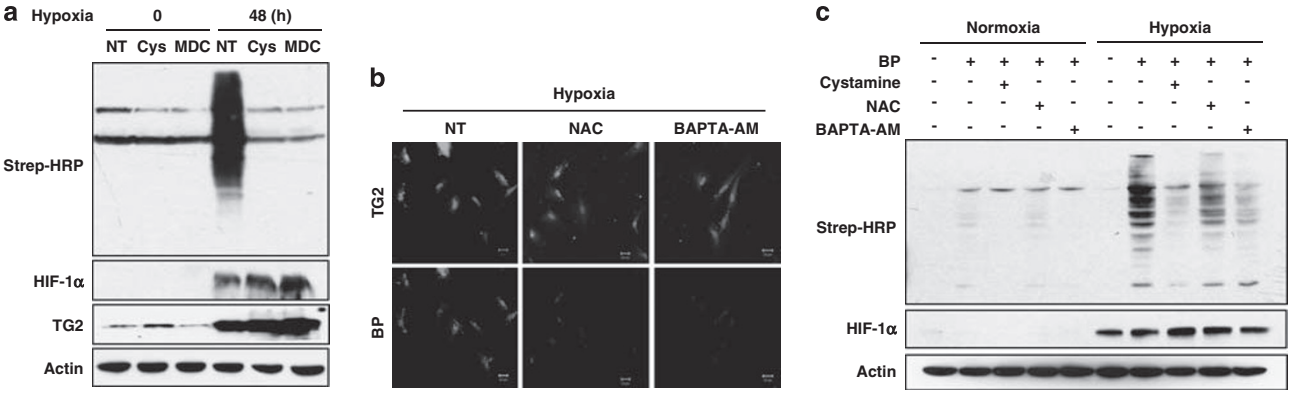

Figure 3 Activation of intracellular transglutaminase 2 (TG2) under hypoxic conditions. (**a**) Intracellular activity and expression of TG2 of the cells cultured under normoxic or hypoxic conditions. Cystamine (Cys) and monodansylcadaverine (MDC) were used to inhibit TG activity. (**b**, **c**) Effect of 1 mM N-acetylcysteine (NAC) or 10 μM BAPTA-AM on the TG2 expression and activity of U373MG cells under hypoxic conditions assessed by immunocytochemical analysis using a TG2/fluorescein isothiocyanate (FITC)-labeled immunoglobulin G (IgG) and Texas Red-conjugated streptavidin, respectively (**b**) or by western blotting using a streptavidin-horseradish peroxidase (HRP) (**c**).

and increased mostly after 72 h. These results correlated closely with those of western blot analysis, in which a cleavage of poly(ADP-ribose) polymerase appeared only in 293^{vec} cells (Figure 5a). HEK293 cells transfected with active-site mutant for TG2 (C277S) showed a similar increase in caspase-3 activity as cells with empty vector

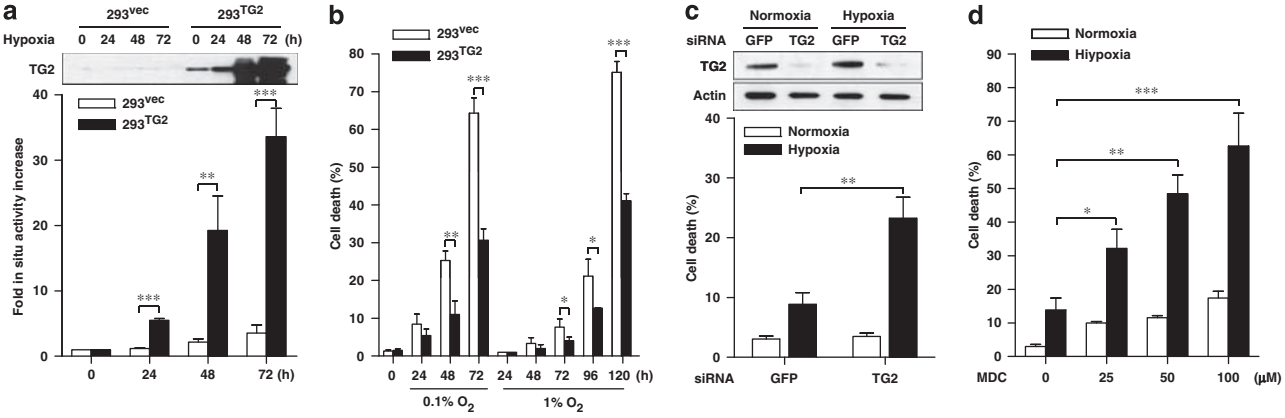

Figure 4 Suppression of hypoxia-induced apoptosis by activated transglutaminase 2 (TG2). (**a**) Vector-transfected (293^{vec}) or TG2-expressing 293 (293^{TG2}) cells were maintained under hypoxic condition (0.1% oxygen (O_2) for 0–72 h. Cells were harvested and assayed for *in situ* TG activity. The level of increase in TG2 activity is expressed as a ratio with respect to the TG2 activity of normoxic 293^{vec} cells. (**b**) Cell death rate was evaluated using Trypan blue exclusion assay method and expressed as a percentage of the stained cells. (**c**, **d**) U373MG cells were treated with TG2-specific small interfering RNA (siRNA) (**c**) or monodansylcadaverine (MDC) (**d**) under normoxic or hypoxic (0.1% O_2) conditions. Cell death rate was analysed as in (**b**). Green fluorescent protein (GFP)-specific siRNA was used as a control in the experiments. The figures represent mean ± s.d. based on three independent experiments. $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$.

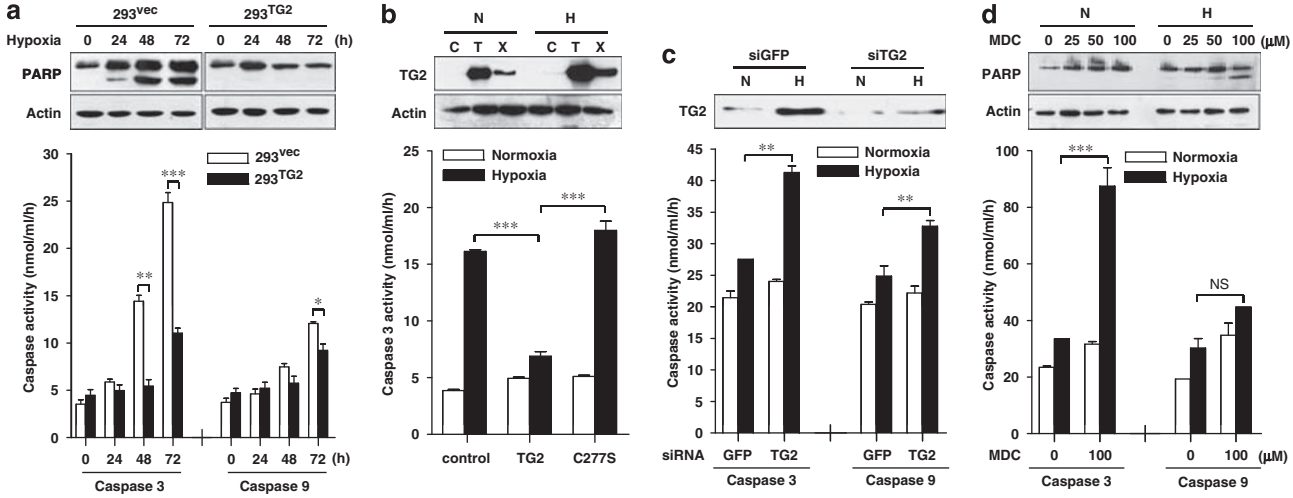

Figure 5 Inhibition of caspase 3 and 9 activities in HEK293 and U373MG cells by transglutaminase 2 (TG2) under hypoxic conditions. (**a**) 293^{vec} or 293^{TG2} cells were maintained under hypoxic condition (0.1% oxygen (O_2) for 0–72 h. Cell lysates were assayed for caspase-3 (left) and caspase-9 (right) activities, respectively. (**b**) HEK293 cells stably expressing pcDNA (C), pcDNA-TG2 (T) or active-site mutant pcDNA (X) were exposed to hypoxic condition (0.1% O_2) for 48 h and assayed for caspase-3 activity. (**c**, **d**) U373MG cells were cultured in the presence of TG2-specific small interfering RNA (siRNA) (**c**) or monodansylcadaverine (MDC) (**d**) under hypoxic (0.1% O_2) conditions. Cell lysates were assayed for caspase-3 and caspase-9 activity, respectively. Poly(ADP-ribose) polymerase (PARP) cleavage was monitored using western blot analysis (**a, d**, top). The figures represent mean ± s.d. based on at least three independent experiments. $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$; NS, not significant.

under hypoxic condition (Figure 5b), indicating that transamidation activity is required to inhibit the caspase-3 activity. Consistent with these results, the downregulation of TG2 expression in U373MG cells increased the activity of caspase 3 (Figure 5c). Treatment with MDC significantly increased the caspase-3 activities of U373MG cells under hypoxic conditions. This finding was further confirmed using western blot analysis for poly(ADP-ribose) polymerase cleavage (Figure 5d). The assay for caspase 9 also showed higher activity in 293^{vec} cells when compared with 293^{TG2} cells under the same experimental conditions (Figure 5a). Similarly, treatment of TG2-siRNA increased the caspase-9 activities of U373MG cells under hypoxic conditions (Figures 5c and d). These results indicate that TG2 suppresses hypoxia-induced cell death through inhibition of caspases.

TG2 modifies proteins by catalysing transamidation of glutamine residues, which results in the formation of protein crosslinking (Fesus and Piacentini, 2002). To test whether caspase 3 is inhibited by TG2-mediated crosslinking, we analysed its electrophoretic mobility pattern. Western blot analysis showed that the active form of caspase 3 increased with the extended period

of hypoxia in 293^{vec} cells, whereas a cleaved caspase 3 decreased in 293^{TG2} cells with concomitant increase in high MW caspase 3 compared with that of 293^{vec} cells (Figure 6a). Moreover, multimeric form of caspase 3 decreased when U373MG cells were treated with MDC (Figure 6b), indicating that the active form of caspase 3 is crosslinked by TG2 under the hypoxic conditions.

As TG2-mediated modification of proteins tends to undergo a change in solubility (Shin *et al*., 2004; Shin *et al*., 2008), we separated the homogenate of 293^{TG2} cells into detergent-soluble and detergent-insoluble fractions. Most of caspase 3 was found in the detergent-insoluble fraction as a dimer, trimer or cross-linked form of cleaved products (Figure 6c). The polymeric forms of caspase 3 were further analysed by allowing 293^{TG2} cells to culture in the media containing BP, followed by separation of BP-incorporated proteins using streptavidin-conjugated bead. Western blot analysis showed that a smear pattern of caspase 3 in the upper region of separating gel and also in the stacking gel appeared in 293^{TG2} cells by hypoxic stress, whereas no smear pattern was observed in HEK293 cells transfected with active-site mutant for TG2 (Figures 6d and e), indicating that TG2 indeed inhibits caspase 3 by forming insoluble aggregates in response to a hypoxic stress. In contrast, caspase 9 was not found in the BP-incorporated proteins under the same experimental conditions (data not shown).

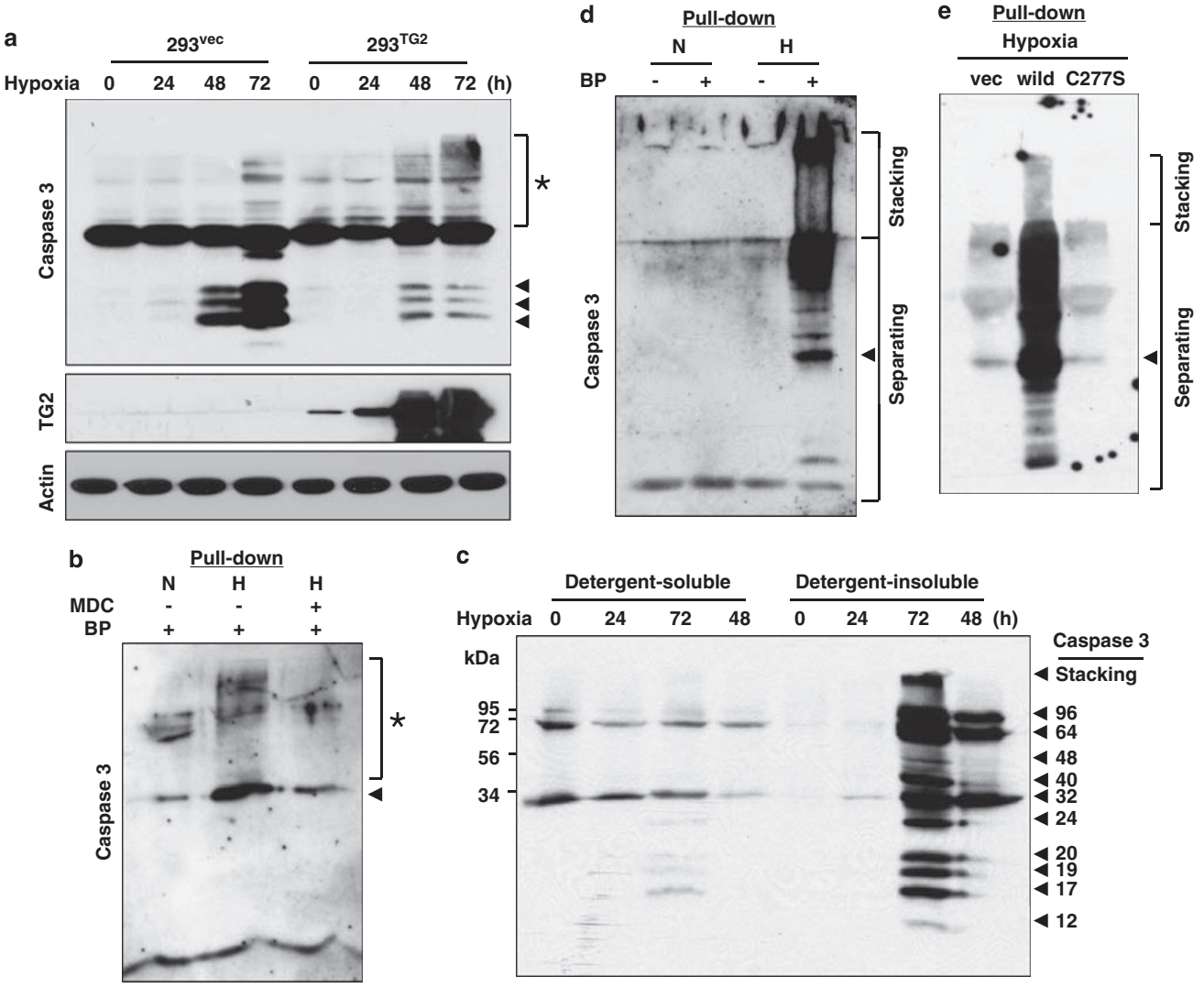

Figure 6 Formation of insoluble caspase 3 aggregates by transglutaminase 2 (TG2) under hypoxic conditions. (**a**) 293^{vec} or 293^{TG2} cells were cultured under hypoxic condition (0.1% oxygen (O_2) for 0–72 h. Cell lysates were analysed using western blotting. The arrowheads and asterisk denote cleavage and multimers of caspase 3, respectively. (**b**) U373MG cells were cultured under hypoxia (0.1% O_2 for 48 h) in the presence of 0.2 mM 5′-(biotinamido)pentylamine (BP) together with 100 μM monodansylcadaverine (MDC). The fraction purified by streptavidin-conjugated magnetic beads was separated by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and probed using anti-caspase 3 antibody. The arrowhead and asterisk denote pro-caspase 3 and multimers of caspase 3, respectively. (**c**) Detergent-soluble and detergent-insoluble fractions, extracted from 293^{TG2} cells exposed to hypoxic condition (0.1% O_2), were subjected to western blot analysis. (**d**, **e**) 293^{TG2} cells (**d**) or HEK293 cells transiently transfected with pcDNA (vec), pcDNA-TG2 (wild) and active-site mutant of TG2 (C277S) (**e**) were cultured under hypoxia (0.1% O_2 for 48 h) in the presence of 0.2 mM BP. Caspase 3 was analysed as in (**b**). The arrowhead denotes pro-caspase 3. N, normoxia; H, hypoxia.

Activation of nuclear factor (NF)-κB pathway by transglutaminase 2 (TG2) in hypoxic cells

It was previously reported that NF-κB signal pathway is activated by hypoxic stress (Antonyak *et al*., 2006), and TG2 is involved in the activation of NF-κB signaling (Park *et al*., 2006). We examined the activation of NF-κB signal pathway using reporter constructs containing NF-κB responsible elements (3κB-Luc, and cellular inhibitor of apoptosis 2 luciferase (cIAP2-Luc)). When cultured in hypoxic condition, the co-transfection of TG2 complementary DNA construct showed an increase in reporter activity in a dose-dependent manner in HEK293 cells. In contrast, the co-transfection of active-site mutant for TG2 failed to increase the reporter activity (Figure 7a), indicating that transamidation activity of TG2 is required for activation of NF-κB pathway. Moreover, the treatment of U373MG cells with MDC abrogated the increased reporter activity that had been induced by the hypoxic stress. Similarly, TG2 knockdown with siRNA also resulted in a decrease in reporter activity in U373MG cells (Figure 7b).

We next examined the effect of TG2 silencing on nuclear translocation of RelA/p65. To this end, HeLa cells were treated with siRNA for TG2 or GFP and the knockdown of TG2 was confirmed by measuring the extent of BP incorporation. Immunocytochemical analysis showed that hypoxia induced nuclear translocation of the RelA/p65, which was abrogated by a treatment with siRNA for TG2 (Figure 7c). These findings were verified using western blot analysis, in which an induction of cIAP2 expression under hypoxic conditions was observed in 293^{TG2} cells, but not in 293^{vec} cells (Figure 7d). These results show that TG2 promotes the activation of NF-κB signal cascades, and thereby induces cIAP that can inhibit caspases.

Decrease of in vivo *tumorigenecity by transglutaminase 2 (TG2) knockdown*

To analyse the *in vivo* significance of TG2 in tumor cell survival under hypoxia, we prepared TG2-knockdown HeLa cells ($HeLa^{shTG2}$) for xenoplantation in athymic mice. Mice injected with $HeLa^{shGFP}$ cells formed a detectable tumor nodule 5 days after injection, whereas mice injected with $HeLa^{shTG2}$ cells formed a tumor nodule 8 days after injection. It was notable that tumor

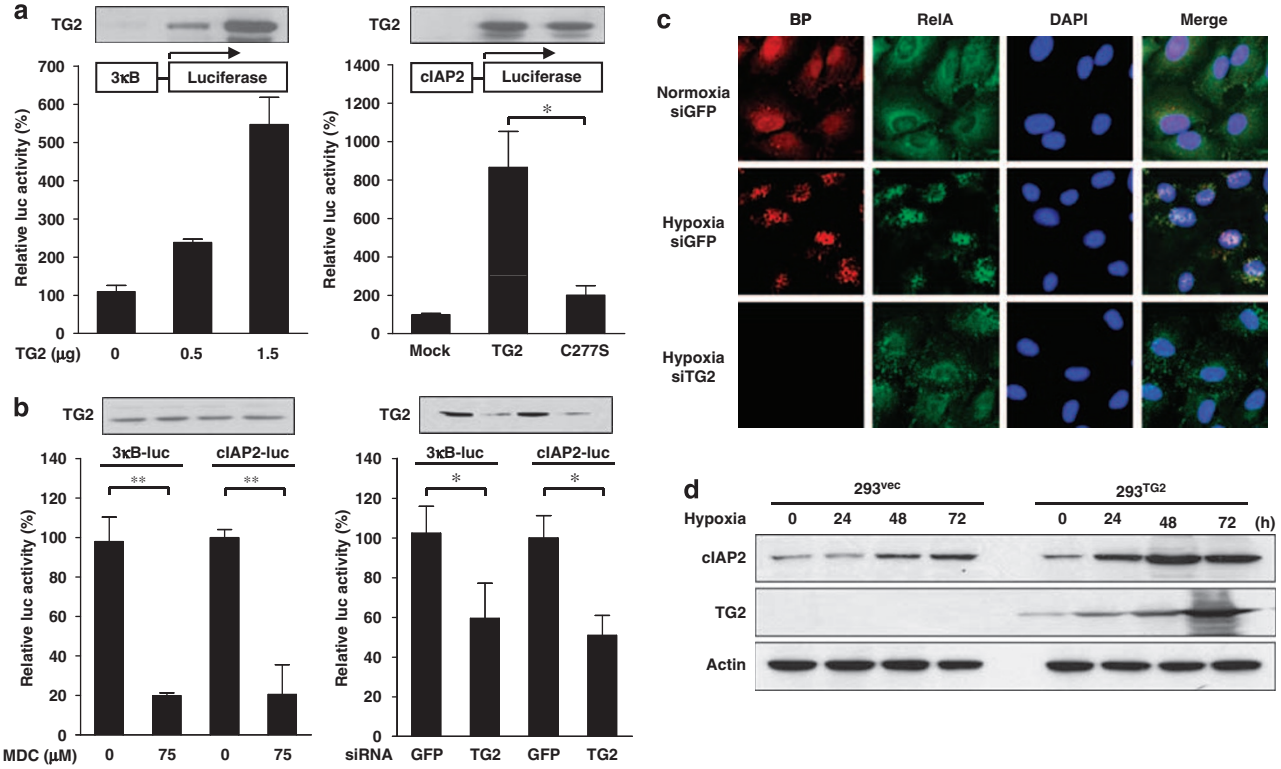

Figure 7 Activation of nuclear factor (NF)-κB signal pathway by transglutaminase 2 (TG2) in hypoxic cells. (**a**) HEK293 cells were transiently transfected with 3κB promoter/luciferase construct together with an increasing amount of pcDNA-TG2 or cellular inhibitor of apoptosis 2 (cIAP2) promoter/luciferase construct together with pcDNA, pcDNA-TG2 or active-site mutant pcDNA ($TG2^{C277S}$). Cells were exposed to hypoxic condition (0.1% oxygen (O_2) for 24 h and assayed for luciferase activity. (**b**) U373MG cells were transiently transfected with 3κB promoter/luciferase or cIAP2 promoter/luciferase construct. Cells were exposed to hypoxic condition (0.1% O_2) for 24 h in the presence of 75 μM monodansylcadaverine (MDC) or small interfering RNA (siRNA) specific for TG2 and assayed for luciferase activity. (**c**) HeLa cells were cultured under hypoxic condition (0.1% O_2) for 48 h in the presence of 0.2 mM 5′-(biotinamido)pentylamine (BP). Intracellular activity of TG2 and localization of RelA were assessed using immunocytochemical staining with Texas Red-conjugated streptavidin and RelA antibody/fluorescein isothiocyanate (FITC)-labeled immunoglobulin G (IgG), respectively. To visualize the nucleus, 4,6-diamidino-2-phenylindole (DAPI) was used. (**d**) 293^{vec} or 293^{TG2} cells were cultured under hypoxic condition (0.1% O_2) for 0–72 h. The expression of cIAP2 was analysed using western blotting. The figures represent mean ± s.d. based on three independent experiments. $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$.

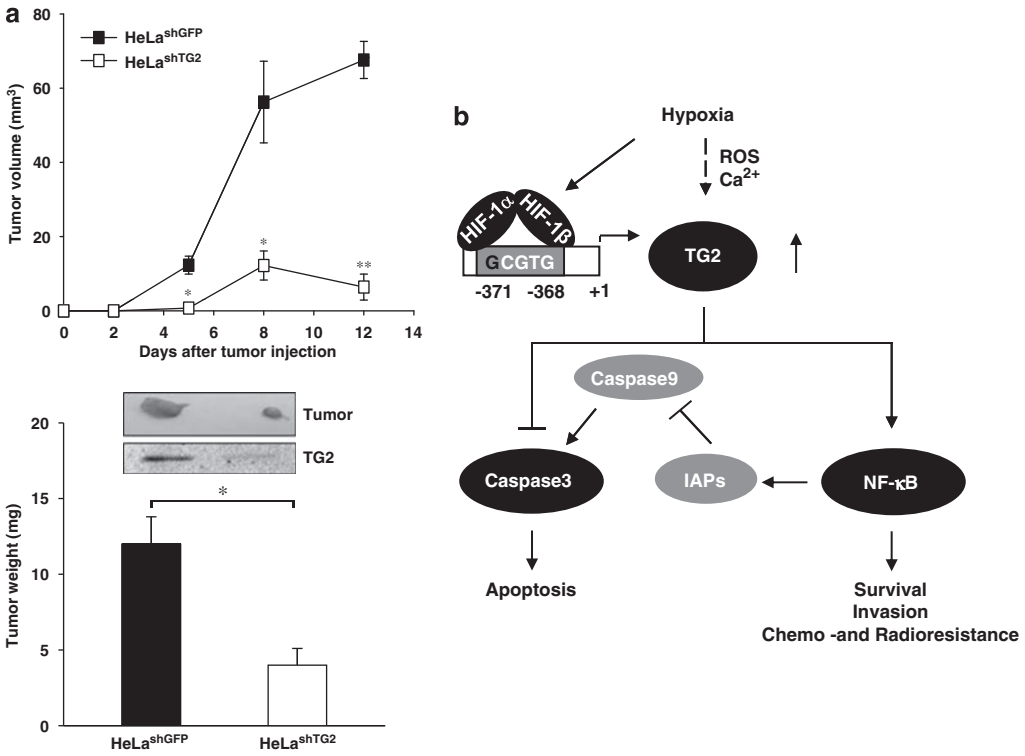

Figure 8 Decrease in *in vivo* tumorigenecity by transglutaminase 2 (TG2) knockdown. (**a**) Athymic mice ($n=3$) was injected with $HeLa^{shGFP}$ or $HeLa^{shTG2}$ cells. Tumor size was measured twice a week. Tumor weight was measured on day 12 after killing. The figures represent mean ± s.d. from two independent experiments. $*P \leqslant 0.01$; $**P \leqslant 0.001$. (**b**) A proposed mechanism depicting the role of TG2 in hypoxia-induced apoptosis.

volume of $HeLa^{shGFP}$ continuously increased, whereas tumor volume of $HeLa^{shTG2}$ started to decrease after day 8. On killing the mice at 12 days after injection, tumor weight of $HeLa^{shTG2}$ cells (4.1 ± 1.2) was significantly lower than that of $HeLa^{shGFP}$ cells (11.8 ± 4.2; $P<0.006$; Figure 8a). These data show that TG2 knockdown decreases *in vivo* tumorigenecity of cells, suggesting that TG2 has a key role in the tumor survival under hypoxic conditions (Figure 8b).

Discussion

Hypoxic stress induces a variety of cellular response through transcriptional regulation mainly mediated by HIF-1, including metabolic adaptation, angiogenesis, proliferation and apoptosis (Zagzag *et al*., 2000; Piret *et al*., 2002; Maxwell, 2005). In this study, we showed that HIF-1 upregulates TG2 expression under hypoxic conditions, and the TG2 in turn modulates caspase 3 as well as NF-κB signal pathway. Although TG2 has been regarded as a pro-apoptotic factor, recent studies provide evidence that TG2 is overexpressed in several cancers and exerts an anti-apoptotic effect (Zhang *et al*., 2003a; Antonyak *et al*., 2004; Mehta *et al*., 2004; Choi *et al*., 2005; Yuan *et al*., 2005). Our results that are presented in this study also show that TG2 acts as an anti-apoptotic mediator of HIF-1, and that TG2 may confer a growth advantage to cancer cells to survive in microenvironmental hypoxia.

Screening of tumor cell lines indicates that hypoxia-induced TG2 expression seems to be cell-type specific, even though HIF-1 was induced in all types of hypoxic cells. In HEK293 cells, HIF-1 was not able to transactivate TG2, suggesting that additional factor(s) other than HIF-1 may be involved in the TG2 expression of hypoxic tumor cells. Our data showed that the failure to induce TG2 expression is due to hypermethylation of DNA. Moreover, experiments using siRNA for HIF-1 showed that HIF-1 is necessary for TG2 expression in hypoxic cells. In addition, our results suggest that TG2 expression is also regulated at the post-transcriptional level. We observed that when TG2 is expressed ectopically in HEK293 cells, the protein level of TG2 is increased after a prolonged hypoxia. The molecular mechanism for this observation remains to be elucidated. As an increase in TG2 expression has been observed in drug-resistant, metastatic breast cancers, and malignant glioblastoma multiforme (Zhang *et al*., 2003a; Mehta *et al*., 2004; Herman *et al*., 2006), our findings may provide a clue for the further analysis regarding the role of TG2 in cancer cells.

Resistance to apoptosis is a hallmark of cancer cells under hypoxic conditions, which permits the progression

of atypical cell behaviors and desensitizes the cells to anticancer therapeutics (Brown and Wilson, 2004; Pouyssegur *et al*., 2006). HIF-1 triggers apoptosis through intrinsic pathways (Brunelle and Chandel, 2002). Our data show that TG2 suppresses the activity of a final executioner caspase, possibly neutralizing pro-apoptotic function of HIF-1. Indeed, TG2 modifies and inhibits caspase-3 activity in thapsigargin-treated cells (Yamaguchi and Wang, 2006). Moreover, a recent report showed that TG2 attenuates the expression of BNIP3, an HIF-1-dependent pro-apoptotic gene, through interacting HIF-1β that results in suppression of neuronal cell death (Filiano *et al*., 2008). In addition, TG2-mediated activation of NF-κB was suspected to be partly involved in the suppression of hypoxia-induced apoptosis through cIAPs and X-linked inhibitor of apoptosis protein, which inhibit caspase activity (Baldwin, 2001). Thus, TG2 induction by hypoxic stress may be a crucial factor in determining the fate of only cells exposed to hypoxia, which eventually contributes to tumor cell invasion and chemoresistance.

Under hypoxic conditions, NF-κB is known to be activated by less characterized noncanonical signaling pathway (Perkins, 2007). We showed that TG2 activates NF-κB signal pathway that allows induction of cIAP2 expression and postulates a mechanism for NF-κB activation under hypoxic conditions. NF-κB activation was implicated as a part of mechanisms for survival, metastasis and chemoresistance of tumor cells (Aggarwal, 2004). In fact, TG2 was regarded as a pro-inflammatory factor and the inhibition of TG2 was found to suppress inflammatory response (Sohn *et al*., 2003). Moreover, transgenic mice overexpressing TG2 showed upregulation of cyclooxygenase2 (Zhang *et al*., 2003b), which is a target of HIF-1 (Kaidi *et al*., 2006), suggesting the association between TG2 and cyclo-oxygenase2 in hypoxic cells. Our results indicate that TG2 is a molecular link between inflammation and malignant behavior of cancer cells under hypoxic conditions.

Our data showed that the changes of intracellular milieus are essential for activating TG2 under hypoxic conditions. Experiments with N-acetylcysteine or BAPTA-AM manifested a decrease in intracellular TG2 activity without affecting the protein concentration, indicating that increases in intracellular reactive oxygen species and/or calcium concentration under hypoxic conditions are critical for activating TG2 (Shin *et al*., 2004). Moreover, the pharmacological inhibition with MDC showed that the transamidation activity of TG2 has a key role in both caspase-3 inactivation and NF-κB activation, which are the mechanisms closely associated with a failure of chemotherapy or radiotherapy in the treatment of cancer patients (Aggarwal, 2004; Bubici *et al*., 2006; Reed, 2006). These findings suggest that TG2 may be a potential molecular target for the effective treatment of many advanced and metastatic cancers.

In summary, the results of this study provide evidence that TG2 suppresses hypoxia-induced apoptosis through caspase-3 inactivation and NF-κB activation. Our data suggest that the inhibition of TG2 may offer a new strategy for anticancer therapy.

Materials and methods

Cell lines and culture conditions

SH-SY5Y, SK-N-SH, A172, U373MG, HeLa, MCF7 and HEK293 cells were grown in humidified atmosphere with 5% carbon dioxide at 37 °C under the standard culture conditions. The cells were incubated under either normoxic (20% O_2) or hypoxic (0.1 or 1% O_2 balanced with molecular nitrogen) conditions for indicated period of time in a hypoxic chamber. $CoCl_2$ at 200 μM was used to induce chemical hypoxia. The HEK293 cells overexpressing TG2, active-site mutant TG2 or empty vector (pcDNA3) were established as previously described (Jeon *et al*., 2003a). The U373MG and HeLa cells downregulating TG2 were established by co-transfection with pSuper-shTG2 and pcDNA3, and selection with G418 (600 μg/ml). For demethylation study, cells (5×10^5) were cultured in the medium containing 5-aza-2 deoxycytidine (5 μM) for 4 days.

Western blot analysis

Western blot analysis and subcellular fractionation experiments were performed as previously described (Jeon *et al*., 2003b). To detect caspase 3, the samples were treated with urea-containing lysis buffer (50 mM Tris-Cl, pH 6.8, 6 M urea, 40 mM dithiothreitol and 2% sodiumdodecyl sulphate). Monoclonal antibody to TG2 was prepared as previously described (Jeon *et al*., 2003b). Monoclonal antibodies to actin and HIF-1α were purchased from Sigma (Carlsbad, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively. Polyclonal antibodies to poly(ADP-ribose) polymerase, caspase 3 and 9 were supplied by Cell Signaling (Beverly, MA, USA). Polyclonal antibodies to lamin B and cIAP2 were obtained from Santa Cruz (Santa Cruz, CA, USA) and R&D systems (Minneapolis, MN, USA), respectively.

In situ *transglutaminase (TG) assay*

Colorimetric microtiter plate assay was performed to monitor intracellular TG activity (Shin *et al*., 2004). In brief, the cells were labeled with BP (Pierce, Rockford, IL, USA) at 0.2 mM under normoxic or hypoxic condition for 0–72 h before harvesting. After washing, the cells were suspended in phosphate-buffered saline (PBS) that contained protease inhibitors, and were sonicated and centrifuged for 10 min at 20 000 *g* at 4 °C. The 96-well microtiter plates were coated with cell extracts for 16 h at 4 °C, and then overcoated with 5% bovine serum albumin in PBS for 1 h at room temperature. The BP-incorporated cellular proteins were captured by incubating with horseradish peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 45 min at 37 °C. After washing with PBS, the plate was developed with *O*-phenylenediamine dihydrochloride for 5–15 min at room temperature before stopping the reaction with 1 M sulfuric acid. The color developed was quantitated by measuring the absorbance at 490 nm with microplate spectrophotometer (Molecular Devices). Cystamine (250 μM; Sigma) and monodansylcadaverine (100 μM; Sigma) were used to inhibit TG activity (Jeon *et al*., 2004).

Immunocytochemical analysis

Cytochemical analysis was performed to determine the expression and *in situ* activity of TG2 or RelA/p65. Cells were plated onto glass coverslips, placed in a 24-well plate, and

cultured for 24 h at 37 °C. The cells were labeled with 0.2 mM BP under normoxic or hypoxic condition for 24–48 h, fixed with 4% formaldehyde in PBS for 15 min, and then permeabilized by treating with 0.1% Triton X-100 in PBS for 5 min at room temperature. After blocking with 1% bovine serum albumin in PBS at room temperature for 30 min, the cells were incubated for 16 h at 4 °C with specific antibodies to either TG2 or RelA/p65 (Santa Cruz). The BP-incorporated cellular proteins were assessed using Texas Red-conjugated streptavidin (Jackson Immuno-Research Laboratory, West Grove, PA, USA). The expression level of TG2 or RelA/p65 was probed by using fluorescein isothiocyanate-labeled anti-mouse or rabbit immunoglobulin G antibody (Molecular Probes, Eugene, OR, USA), respectively. The nucleus was visualized using 4,6-diamidino-2-phenylindole solution (Roche, Palo Alto, CA, USA). The cells were photographed with LSM510META confocal laser-scanning microscope (Zeiss, Oberkochen, Germany).

Generation of promoter constructs
TG2 promoter region (GenBank accession number U13920) flanking from −736 to −14 was amplified from genomic DNA using PCR (Roche). Five deletion mutants of TG2 promoter, flanking from −736, −622, −460, −367 and −175 to −14, were generated using PCR and cloned into pGL2-Basic (Promega, Madison, WI, USA) for the luciferase assays. Site-directed mutagenesis was performed following the manufacture's instruction (Stratagene, La Jolla, CA, USA). All constructs were verified using DNA sequencing (Applied Biosystems 3730 × l DNA Analyser, Applied Biosystems, Foster City, CA, USA).

Reporter assay
Cells were transfected with different TG2 promoter-luciferase reporter constructs, and then incubated under normoxic or hypoxic culture conditions for 24 h. The cells were harvested and assayed for luciferase activity using the kit (Promega). The cells were co-transfected with pCMV-β-Gal as the internal control. The luciferase activity was normalized using β-galactosidase activity. NF-κB activity was monitored using 3κB and cIAP2 reporter constructs.

Apoptosis assay
Apoptotic cell death was determined by Trypan blue exclusion assay or fluorescence-activated cell sorting analysis using propidium iodide after incubating the cells under normoxic or hypoxic conditions for the indicated period of time. MDC or TG2-specific siRNA was included in the cell culture system at various concentrations. Trypan blue exclusion assay was performed using the kit (Invitrogen, Carlsbad, CA, USA).

Caspase assay
The cells were extracted with lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1% Triton X-100) for 30 min on ice and centrifuged for 10 min at 12 000 *g* at 4 °C. The cell extract (50 μg per well) was added to a microtiter plate and mixed with 100 μl reaction buffer per well. The reaction buffer contained 100 mM HEPES, pH 7.4, 0.1% CHAPS, 10 mM dithiothreitol, 10% glycerol and 2% (v/v) dimethylsulfoxide. Ac-DEVD-*P*-nitroaniline and Ac-LEHD-*P*-nitroaniline (AG scientific Co., San Diego, CA, USA) at 2 mM were used for caspase 3 and 9 assay, respectively. Absorbance at 405 nm was measured on microplate spectrophotometer (Molecular Devices). *P*-nitroaniline (Sigma) was used to generate the standard curve to evaluate the concentration of the products.

Analysis of transglutaminase 2 (TG2) substrates
Cells were labeled with BP at 0.2 mM under normoxic or hypoxic condition for 72 h before harvesting. The cell pellets were resuspended in the lysing buffer (50 mM Tris-Cl, pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 0.02% sodiumdodecyl sulphate, 0.5% sodium deoxycholate and 1 mM EDTA). After dialysis against 1 × PBS overnight at 4 °C, the cell lysates were incubated with streptavidin-conjugated magnetic beads (Dynal Biotech, Oslo, Norway) on a rocking platform for 3 h at 4 °C. The beads were washed with PBS (containing 1% Tween-20). The proteins bound to the beads were eluted by boiling for 10 min in sodiumdodecyl sulphate-gel loading buffer, and subjected to western blot analysis.

Knockdown experiment
Knockdown experiments were performed as previously described (Herman *et al*., 2006). In brief, TG2-specific siRNA (5′-GGGCGAACCACCUGAACAAdTdT-3′ and 5′-UUGU UCAGGUGGUUCGCCCdTdT-3′) was chemically synthesized (Dharmacon, Lafayette, CO, USA). siRNA for GFP and HIF-1α was purchased from Santa Cruz. The siRNA was transfected into U373MG cells using Oligofectamine or Lipofectamine 2000 reagent (Invitrogen). The knockdown effect was verified using western blot analysis.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation assays were performed as described (Kuhlicke *et al*., 2007) using 2×10^7 U373MG cells exposed to normoxic or hypoxic conditions. In brief, cells were fixed with 1% formaldehyde and resuspended in buffer A (5 mM PIPES, pH 8.0, 85 mM potassium chloride and 0.5% NP-40) to isolate nuclei. After shearing the chromatin by sonication in buffer B (1% sodiumdodecyl sulphate, 10 mM EDTA, 100 mM Tris, pH 8.1 and protease inhibitor), the supernatant was incubated with either anti-HIF-1α (Novus Biologicals, Littleton, CO, USA) or control immunoglobulin G (Santa Cruz) at 4 °C for 4 h in the presence of protein G agarose beads. Immune complexes were eluted from the beads at 65 °C for overnight and treated with proteinase K at 45 °C for 1 h. DNA fragments were purified using ethanol precipitation. The DNA was amplified in 30 PCR cycles using the following primers (−273/−429 of TG2 gene promoter): 5′-CCACATCTGTGTGTCCAGGTGCAC-3′ and 5′-GGAC ACACAACTAGCCCAGG-3′. PCR products were separated on a 2% agarose gel.

Real-time quantitative PCR
Cells were cultured in normoxic or hypoxic condition (1% O_2) for 5 h. To perform the quantitative real-time reverse transcriptase–PCR using the predesigned gene-specific TaqMan (Applied Biosystems), 1 μg of purified RNA was used. Actin was used as internal control for normalization (Livak and Schmitten, 2001).

Tumor xenografts
HeLa ($HeLa^{shGFP}$) or TG2-knockdown HeLa cells ($HeLa^{shTG2}$; 2×10^6) were injected subcutaneously into athymic nude mice (Charles River, Wilmington, MA, USA). Tumor size was measured twice a week with a caliper. Mice were killed on day 12 to measure tumor weight. The tumor volume was calculated using the following equation. Tumor Volume (mm^3) = width × length^2/2 (Dachs *et al*., 1997).

Statistical analysis
Statistical significance was analysed using Student's *t*-test.

Abbreviations

HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-response element; TG, transglutaminase; BP, 5′-(biotinamido)pentylamine; PBS, phosphate-buffered saline; MDC, monodansylcadaverine.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Dr YD Kim for critical comments on this paper. We also thank Dr Shigetaka Kitajima (Tokyo Medical and Dental University, Japan) for providing cIAP2 reporter construct. This work was supported by the grants from Korea Science and Engineering Foundation (R11-2002-097-09005-0 and R01-2005-000-10364-0) and also by the Research Program for New Drug Target Discovery (M10748000296-07N4800-29610). GYJ, SYC, EMJ, SHL and YC were supported by the graduate program of BK21, Korea Ministry of Education, Science and Technology.

References

Aggarwal BB. (2004). Nuclear factor-kappaB: the enemy within. *Cancer Cell* **6**: 203–208.

Antonyak MA, Miller AM, Jansen JM, Boehm JE, Balkman CE, Wakshlag JJ *et al*. (2004). Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. *J Biol Chem* **279**: 41461–41467.

Antonyak MA, Jansen JM, Miller AM, Ly TK, Endo M, Cerione RA. (2006). Two isoforms of tissue transglutaminase mediate opposing cellular fates. *Proc Natl Acad Sci USA* **103**: 18609–18614.

Baldwin AS. (2001). Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. *J Clin Invest* **107**: 241–246.

Baylin SB. (1997). Tying it all together: epigenetics, genetic, cell cycle, and cancer. *Science* **277**: 1948–1949.

Brown JM, Wilson WR. (2004). Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer* **4**: 437–447.

Brunelle JK, Chandel NS. (2002). Oxygen deprivation induced cell death: an update. *Apoptosis* **7**: 475–482.

Bubici C, Papa S, Dean K, Franzoso G. (2006). Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* **25**: 6731–6748.

Choi K, Siegel M, Piper JL, Yuan L, Cho E, Strnad P *et al*. (2005). Chemistry and biology of dihydroisoxazole derivatives: selective inhibitors of human transglutaminase 2. *Chem Biol* **12**: 469–475.

Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ *et al*. (1997). Targeting gene expression to hypoxic tumor cells. *Nat Med* **3**: 515–520.

De Laurenzi V, Melino G. (2001). Gene disruption of tissue transglutaminase. *Mol Cell Biol* **21**: 148–155.

Dirmeier R, O'Brien K, Engle M, Dodd A, Spears E, Poyton RO. (2004). Measurement of oxidative stress in cells exposed to hypoxia and other changes in oxygen concentration. *Methods Enzymol* **381**: 589–603.

Fesus L, Piacentini M. (2002). Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends Biochem Sci* **27**: 534–539.

Filiano AJ, Bailey CDC, Tucholski J, Gundemir S, Johnson GVW. (2008). Transglutaminase 2 protects against ischemic insult, interacts with HIF1β, and attenuates HIF1 signaling. *FASEB J* **22**: 2662–2675.

Giaccia A, Siim BG, Johnson RS. (2003). HIF-1 as a target for drug development. *Nat Rev Drug Discov* **2**: 803–811.

Hasegawa G, Suwa M, Ichikawa Y, Ohtsuka T, Kumagai S, Kikuchi M *et al*. (2003). A novel function of tissue-type transglutaminase: protein disulphide isomerase. *Biochem J* **373**: 793–803.

Herman JF, Mangala LS, Mehta K. (2006). Implications of increased tissue transglutaminase (TG2) expression in drug-resistant breast cancer (MCF-7) cells. *Oncogene* **25**: 3049–3058.

Hui AS, Bauer AL, Striet JB, Schnell PO, Czyzyk-Krzeska MF. (2006). Calcium signaling stimulates translation of HIF-alpha during hypoxia. *FASEB J* **20**: 466–475.

Jeon JH, Kim CW, Shin DM, Kim K, Cho SY, Kwon JC *et al*. (2003a). Differential incorporation of biotinylated polyamines by transglutaminase 2. *FEBS Lett* **534**: 180–184.

Jeon JH, Choi KH, Cho SY, Kim CW, Shin DM, Kwon JC *et al*. (2003b). Transglutaminase 2 inhibits Rb binding of human papillomavirus E7 by incorporating polyamine. *EMBO J* **22**: 5273–5282.

Jeon JH, Lee HJ, Jang GY, Kim CW, Shin DM, Cho SY *et al*. (2004). Different inhibition characteristics of intracellular transglutaminase activity by cystamine and cysteamine. *Exp Mol Med* **36**: 576–581.

Kaidi A, Qualtrough D, Williams AC, Paraskeva C. (2006). Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* **66**: 6683–6691.

Kuhlicke J, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK. (2007). Hypoxia inducible factor (HIF)-1 coordinates induction of Toll-like receptors TLR2 and TLR6 during hypoxia. *PLos ONE* **2**: e1364.

Lee KN, Birckbichler PJ, Patterson Jr MK. (1989). GTP hydrolysis by guinea pig liver transglutaminase. *Biochem Biophys Res Commun* **162**: 1370–1375.

Livak KJ, Schmitten TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$ method. *Methods* **25**: 402–408.

Lorand L, Graham RM. (2003). Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* **4**: 140–156.

Maxwell PH. (2005). The HIF pathway in cancer. *Semin Cell Dev Biol* **16**: 523–530.

Mehta K, Fok J, Miller FR, Koul D, Sahin AA. (2004). Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. *Clin Cancer Res* **10**: 8068–8076.

Mishra S, Murphy LJ. (2004). Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. *J Biol Chem* **279**: 23863–23868.

Park SS, Kim JM, Kim DS, Kim IH, Kim SY. (2006). Transglutaminase 2 mediates polymer formation of IκBα through C-terminal glutamine cluster. *J Biol Chem* **281**: 34965–34972.

Perkins ND. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* **8**: 49–62.

Piret JP, Mottet D, Raes M, Michiels C. (2002). Is HIF-1alpha a pro- or an anti-apoptotic protein? *Biochem Pharmacol* **64**: 889–892.

Pouyssegur J, Dayan F, Mazure NM. (2006). Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* **441**: 437–443.

Reed JC. (2006). Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat Clin Pract Oncol* **3**: 388–398.

Semenza GL. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**: 721–732.

Shin DM, Jeon JH, Kim CW, Cho SY, Kwon JC, Lee HJ *et al*. (2004). Cell type-specific activation of intracellular transglutaminase 2 by oxidative stress or ultraviolet irradiation: implications of transglutaminase 2 in age-related cataractogenesis. *J Biol Chem* **279**: 15032–15039.

Shin DM, Jeon JH, Kim CW, Cho SY, Lee HJ, Jang GY *et al*. (2008). TGFβ mediates activation of transglutaminase 2 in response to oxidative stress that leads to protein aggregation. *FASEB J* **22**: 2498–2507.

Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY. (2003). Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. *J Clin Invest* **111**: 121–128.

Verma A, Mehta K. (2007). Tissue transglutaminase-mediated chemoresistance in cancer cells. *Drug Resist Updat* **10**: 144–151.

Wykoff CC, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. (2000). Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling. *Oncogene* **19**: 6297–6305.

Yamaguchi H, Wang HG. (2006). Tissue transglutaminase serves as an inhibitor of apoptosis by cross-linking caspase 3 in thapsigargin-treated cells. *Mol Cell Biol* **26**: 569–579.

Yuan L, Choi K, Khosla C, Zheng X, Higashikubo R, Chicoine MR *et al*. (2005). Tissue transglutaminase 2 inhibition promotes cell death and chemosensitivity in glioblastomas. *Mol Cancer Ther* **4**: 1293–1302.

Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL. (2000). Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* **88**: 2606–2618.

Zhang R, Tremblay TL, McDermid A, Thibault P, Stanimirovic D. (2003a). Identification of differentially expressed proteins in human glioblastoma cell lines and tumors. *Glia* **42**: 194–208.

Zhang Z, Vezza R, Plappert T, McNamara P, Lawson JA, Austin S *et al*. (2003b). COX-2-dependent cardiac failure in Gh/tTG transgenic mice. *Circ Res* **92**: 1153–1161.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)