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Overexpression of phospho mutant forms of transglutaminase 2 downregulates epidermal growth factor receptor

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ABSTRACT

Simultaneous upregulation of transglutaminase 2 (TG2) and epidermal growth factor receptor (EGFR) have been reported in a number of systems. Moreover, TG2 has been identified as a downstream target gene for EGF/EGFR. However, it is not known whether the relationship between EGFR and TG2 is only one-way or collaborative. Using embryonic fibroblasts derived from TG2 null mice ($MEF^{tg2-/-}$), co-overexpressing native TG2 and EGFR, here we report that TG2 differentially regulates EGFR protein in the presence and absence of EGF. In the absence of EGF, TG2 facilitates EGFR downregulation whereas in the presence of EGF, TG2 has opposite effect on EGFR and facilitates Akt phosphorylation. TG2 mediated ligand-independent downregulation of EGFR was not observed in $MEF^{tg2-/-}$ cells overexpressing Ser212Ala phospho mutant form of TG2 suggesting a role of TG2 phosphorylation in this process. However, similar to native TG2, Ser212Ala-TG2 mutant was also able to attenuate ligand-dependent down regulation of EGFR in $MEF^{tg2-/-}$ cells. Interestingly, overexpression of Ser216Ala-TG2 mutant led to downregulation of EGFR in $MEF^{tg2-/-}$ cells irrespective of the ligand. These results were further confirmed in breast cancer cells expressing high levels of EGFR. Collectively, data presented here suggests that the relationship between EGFR and TG2 is collaborative and phosphorylation of TG2 play a key role in it. Phospho mutant forms of TG2 reported in this study may be utilized as a part of a novel strategy to down-regulate EGFR in cancers with enhanced EGFR signaling.

1. Introduction

Transglutaminase 2 (TG2) is a protein crosslinking enzyme with diverse biological function [1]. Simultaneous upregulation and/or activation of TG2 and epidermal growth factor receptor (EGFR) have been reported in a number of cell/tissue types including drug resistance breast cancer cells, proliferating hepatocytes, basophilic leukemia cells and human glioblastoma cell lines and tumors [2–5]. In addition to TG2, another member of TG family of proteins, TG1 has also been identified as a target gene for EGFR in the differentiation of the hair follicle and a synchronized upregulation of EGFR and TG1 have been reported during hair morphogenesis [6,7]. Furthermore, TG2 has been reported as a downstream effector of prosurvival function of epidermal growth factor (EGF) which requires phosphatidylinositol 3-kinase (PI3K) activity [8]. EGF induces TG2 expression and activation in human breast cancer cells which in turn contributes to their oncogenic potential [2]. Furthermore, inhibition of TG2 expression or activity in cancer cells has been found to be associated with reversal of drug resistance [9,10]. Taken together these studies suggest that an intimate association exists between TG2 and EGFR in both normal and the disease processes. However, it is not known whether TG2 works only downstream of EGFR or also payback to EGFR in a way which further facilitates EGFR signaling thus constitute a vicious cycle in favor of invasive and drug resistance phenotype of cancer cells.

In addition to EGFR signaling, TG2 has been reported to play a role in the activation of nuclear factor-kappa B (NF-κB) and subsequently downregulation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [10,11]. PTEN is a phosphatase that dephosphorylates phosphatidylinositol tri-phosphate (PIP3) that is produced by PI3K and function as a ubiquitous inhibitor of PI3K-dependent signaling [12,13]. Recently we have found that phosphorylation of TG2 by protein kinase A (PKA) has a role in TG2 mediated activation of NF-κB and in the downregulation of

Abbreviations: Akt, protein kinase B; Ala, alanine; EGF, epidermal growth factors; EGFR, epidermal growth factor receptor; pEGFR, phospho-EGFR; MEF, mouse embryonic fibroblasts; PTEN, phosphatase and tensin homologue deleted on chromosome 10; pPTEN, phospho-PTEN; Ser, serine; PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor-kappa B; PKA, protein kinase A; mTOR, mammalian target of rapamycin; TG2, transglutaminase 2; Thr, threonine.

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PTEN which in turn contribute to the cell proliferative and invasive potential of TG2 in cancer [14]. Moreover, we have shown that the loss of TG2 phosphorylation prevents TG2 mediated activation of NF-κB and downregulation of PTEN [14]. Phosphoproteomic analyses of cancer signaling networks have consistently identified TG2 as a phosphoprotein in a number of cancer cell types indicating a role of TG2 phosphorylation in the oncogenic potential of TG2 in cancer cells [[15,16]; http://www.phosphosite.org/proteinAction.do?id=4135&showAllSites=true]. However, the identity of upstream kinase(s) responsible for TG2 phosphorylation and the functional consequences of TG2 phosphorylation in cancer cells are not known. Previously we have reported that TG2 contains PKA consensus serine phosphorylation sites at position 212 and 216 in TG2 protein (^{209}RDC**S**RRS**S**PVYVGRV^{223}) and undergoes phosphorylation in response to PKA activation [17,18]. In addition, we have shown that phosphorylation of TG2 facilitates protein–protein interaction and modulates TG2 activity [17,18]. The PKA has a role in EGFR distribution on the plasma membrane [19]. It has been shown that the predominant distribution of inactive EGFR at the plasma membranes is not simply by default but involves a PKA-dependent restrictive condition resulting in receptor avoidance of endocytosis until it is stimulated by ligand [19]. Furthermore, inhibition of basal PKA activity has been shown to induce internalization of unoccupied EGFR [19]. However, the underlying mechanism involved remains elusive. As TG2 is a common target for both PKA and EGFR, it is possible that TG2 has a role in fine-tuning of EGFR function in response to cellular demands and crosstalk with other signaling receptors. In this study, we have investigated the functional impact of loss of TG2 phosphorylation at Ser212 and Ser216 on EGFR in embryonic fibroblasts derived from TG2 null mice (MEF$^{tg2-/-}$) and in breast cancer cells.

2. Materials and methods

2.1. Reagents

MDA468 and T47D breast cancer cell lines and cell culture reagents were obtained from American Type Culture Collection (Danvers, MA) and fetal bovine serum from Invitrogen (Carlsbad, CA). Akt, EGFR and PTEN sampler kit and anti-Myc antibodies were purchased from Cell Signaling Technology (Danvers, MA) and horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) reagents were purchased from Promega (Madison, WI). Other reagents were purchased from Sigma (Oakville, ON) or as otherwise stated.

2.2. Animals

MEF$^{tg2-/-}$ cells were obtained from TG2 null mice as described elsewhere [18]. TG2 knockout mice were generously provided by Dr. Nikolaos Frangogiannis (Baylor College of Medicine, Houston, TX) with permission from Dr. Gerry Melino (University of Leicester, UK). Experiments involving mice were performed as approved by the Animal Care Committee of the University of Manitoba.

2.3. TG2 constructs

Full length EGFR construct was obtained from Addgene (Cambridge, MA), a non-profit plasmid repository. The pCMV vector containing Myc tagged human TG2 (Myc-TG2) obtained from Origene Technology (Rockville, MD) was used to generate TG2 mutants lacking Ser212 and Ser216 phosphorylation sites. TG2 mutants were made by a site-directed mutagenesis kit (Stratagene, Santa Clara, CA) using two complementary nucleotide primers (Ser212Ala: forward, 5′-GCCGTGACTGCGCCCGCCGCAGC-3′ and reverse 5′-GCTGCGGCGGGCGCAGTCACGGC-3′; Ser216Ala: forward, 5′-CTCCCGCCGCAGCGCCCCCGTCTACGTG-3′ and reverse, 5′-CACGTAGACGGGGGCGCTGCGGCGGGAG-3′ containing desired mutation and Myc-TG2 as the template. Authenticity of all the constructs was confirmed by DNA sequencing.

2.4. Cell culture and transfection

MEF and breast cancer cell culture and treatments were performed as described before [17,18]. Transfections with native and mutant-TG2 constructs were performed using FuGENE HD Transfection Reagent (Roche, Laval, QC) according to the manufacturer's instructions. Two days post-transfection, cells were serum starved for 6 h and subsequently treated with EGF (100 ng/ml) for 10 min. Cell lysates were prepared as described before and processed for further analysis by immunoblotting [18].

2.5. Western blotting

Protein concentrations of lysates were determined by the Bradford protein assay (Bio-Rad, Mississauga, ON) with bovine serum albumin as the standard. Proteins were resolved on 10% sodium dodecyl sulfate gel-electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked in 5% fat free milk and processed for incubation with primary and HRP-conjugated secondary antibodies [18]. Protein band was visualized using ECL and Kodak BioMax film.

2.6. Statistical analysis

Experimental results are shown as means ± SEM. One-way ANOVA with Dunnett's test was used for multiple comparisons. *P* values <0.05 were considered significant difference.

3. Results and discussion

3.1. TG2 differentially regulates EGFR with and without ligand

TG2 facilitates Akt mediated prosurvival effect of EGF in breast cancer cells [2]. To explore whether TG2 mediated effect occurs at the level of EGFR and involves TG2 phosphorylation, MEF$^{tg2-/-}$ cells were co-transfected with EGFR (as these cells express very low levels of EGFR) and various TG2 constructs. Post-transfection cells were challenged with or without EGF and cell lysates were processed for immunoblot analysis.

A ligand-dependent decrease in EGFR protein level was found in the vector transfected cells in comparison to without EGF treated cells (Fig. 1). Surprisingly TG2 overexpression resulted in a significant downregulation of EGFR in the absence of EGF, whereas in the presence of EGF TG2 completely blocked EGFR downregulation (Fig. 1). TG2 mediated ligand-independent downregulation of EGFR protein was not observed in MEF$^{tg2-/-}$ cells expressing Ser21Ala-TG2 (Fig. 1). However, Ser212Ala-TG2 was fable to block ligand-dependent downregulation of EGFR in comparison to vector transfected control group (Fig. 1). This would imply that most likely two different mechanisms are involved in TG2 mediated regulation of EGFR in the presence and absence of ligand. Interestingly, EGFR was found to be significantly downregulated in Ser216Ala-TG2 expressing MEF$^{tg2-/-}$ cells irrespective of the ligand suggesting a critical role of Ser216 phosphorylation in the regulation of EGFR level (Fig. 1). Taken together, these data suggest that TG2 differentially regulates EGFR protein in the presence and absence of the ligand and phosphorylation of TG2 has a role in this process.

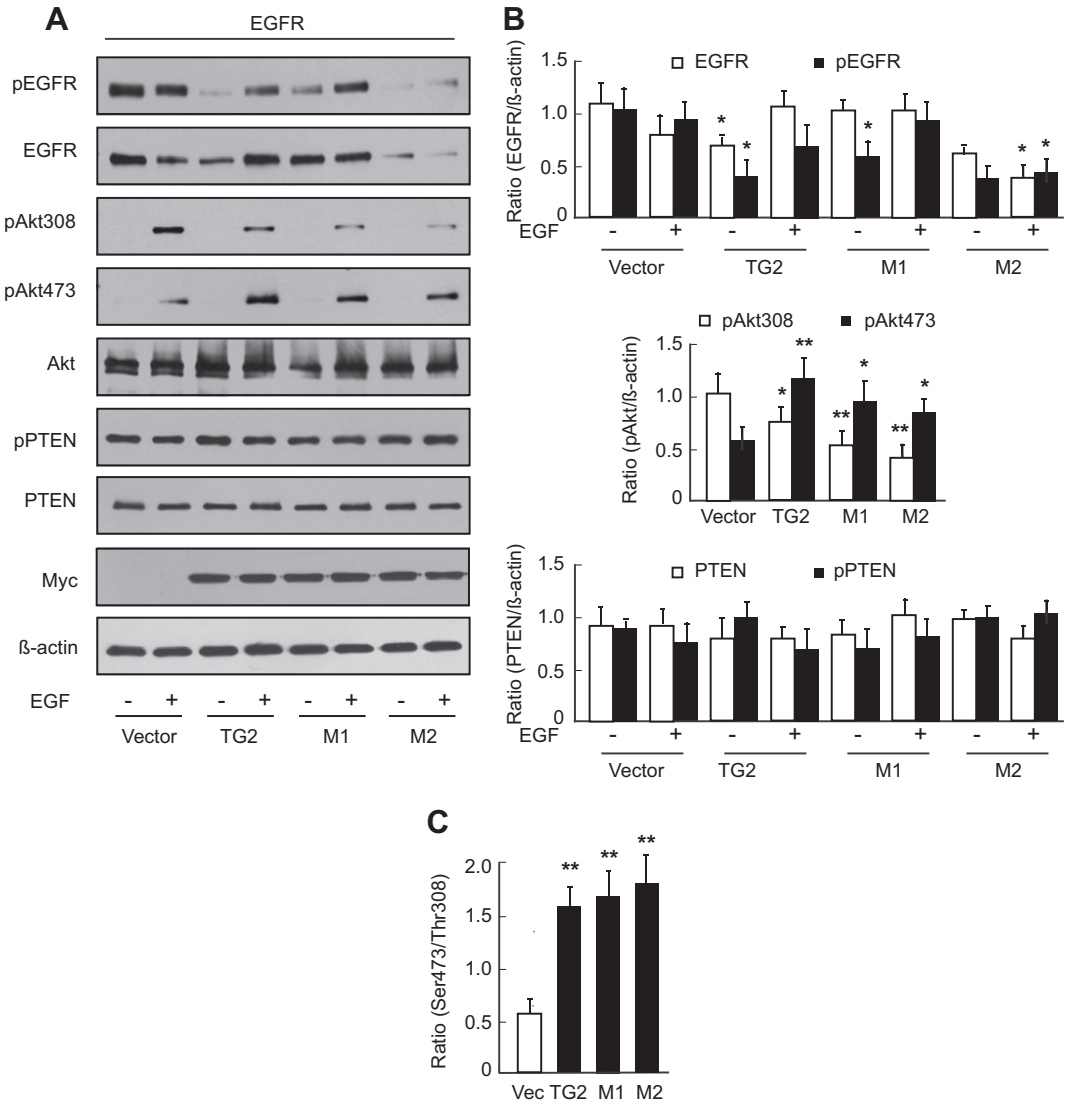

Fig. 1. TG2 differentially regulates EGFR with and without ligand. (A) MEF$^{tg2-/-}$ cells were co-transfected with EGFR and various TG2 constructs. Forty eight hours post-transfection cells were serum starved and incubated with and without EGF (100 nM) for 10 min. Cells were harvested and equal amount of proteins (25 μg) were analyzed by Western immunoblotting using protein and phospho-specific antibodies. Representative immunoblots of four different experiments are shown. β-Actin immunoblot is shown as a loading control. (B) Histograms showing relative quantification of protein/phospho-proteins as shown in A. (C) Histogram showing TG2 induced selective increase in Akt phosphorylation at Ser473 in EGFR overexpressing cells in comparison to vector control group. Data are represented as mean ± SEM, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$. M1, Ser212Ala-TG2; M2, Ser216Ala-TG2.

3.2. Overexpression of phospho mutant forms of TG2 downregulates EGFR in breast cancer cells

To establish that the effect of TG2 on EGFR is not specific to MEF cells; we determined the effect of native and TG2 mutants' overexpression on EGFR in MDA468 and T47D breast cancer cells. These cells lines were selected because in our preliminary experiments we noticed that both cell lines express high levels of EGFR and low levels of TG2 (Fig. 2). A similar pattern on EGFR protein level was found in TG2 transfected MDA468 and T47D cells as in the case of MEF$^{tg2-/-}$ cells with or without ligand, though to a lesser extent than MEF$^{tg2-/-}$ cells (Fig. 3). Interestingly, unlike MEF$^{tg2-/-}$ cells, in breast cancer cells both Ser212Ala and Ser216Ala phospho mutants of TG2 were equally able to downregulate EGFR irrespective of the ligand (Fig. 3). These differences may be attributed to the transcriptional or other regulatory differences in relation to EGFR in MEF$^{tg2-/-}$ and breast cancer cells. Collectively, these data suggest that TG2 attenuates ligand-dependent downregulation of EGFR and phosphorylation of TG2 at Ser212 and Ser216 has a role in this process.

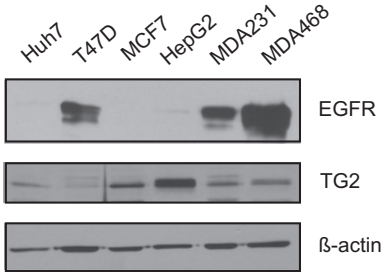

Fig. 2. Expression levels of TG2 and EGFR in cancer cells. Equal amount of proteins harvested from various cell lines were analyzed by gel-electrophoresis and immunoblotting using anti-TG2 and anti-EGFR antibodies as per manufacturer's protocols. β-Actin immunoblot is shown as a loading control.

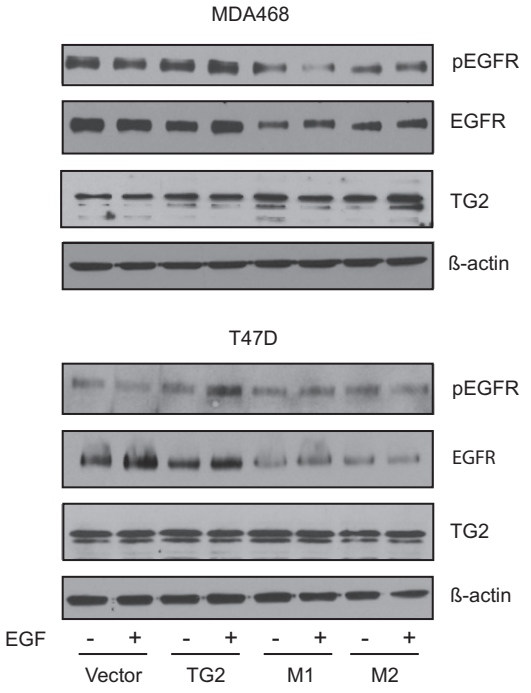

Fig. 3. Expression of phospho mutant forms of TG2 downregulates EGFR in breast cancer cells. MDA468 and T47D breast cancer cells were transfected with various TG2 constructs. Forty eight hours post-transfection cells were serum starved and incubated without and with EGF (100 nM) for 10 min. Cells were harvested and equal amount of proteins (25 μg) were analyzed by Western immunoblotting using protein and phospho-specific antibodies. Representative immunoblots of three different experiments are shown. M1, Ser212Ala-TG2; M2, Ser216Ala-TG2.

3.3. TG2 selectively increase Akt phosphorylation at Ser^{473}

To determine the functional consequence of TG2 mediated changes in EGFR on EGFR signaling we determined the phosphorylation status of Akt in MEF$^{tg2-/-}$ cell lysates with and without EGF (as TG2 has been shown to facilitate EGF induced Akt effect). As expected EGF stimulation leads to increased Akt phosphorylation at both residues (i.e., Thr^{308} and Ser^{473}) in the vector transfected control group in comparison to non-stimulated cells (Fig. 1). Akt phosphorylation at Ser^{473} was further enhanced with EGF stimulation in MEF$^{tg2-/-}$ cells overexpressing TG2, whereas phosphorylation at Thr^{308} was reduced in comparison to vector transfected control group (Fig. 1). Phosphorylation of Akt at Thr^{308} was further reduced in MEF$^{tg2-/-}$ cells overexpressing phospho mutant forms of TG2 despite the protection of EGFR downregulation in Ser212Al-TG2 expressing cells (Fig. 1). Unlike Thr^{308} phosphorylation, phosphorylation of Akt at Ser^{473} in TG2 mutants expressing cells although reduced in comparison to TG2 expressing cells but remained high in comparison to vector transfected control group. No apparent difference in PTEN protein levels were found in cells co-transfected with EGFR and TG2 with or without EGF suggesting that enhanced Akt phosphorylation occurs in a PTEN-independent manner (Fig. 1). Taken together these data suggest that TG2 facilitates EGF induced phosphorylation of Akt in a site specific manner.

As Thr^{308} and Ser^{473} in Akt are phosphorylated by two different kinases, it is likely that TG2 mediated stabilization of EGFR leads to preferential activation of signaling pathway leading to Ser^{473} phosphorylation or preventing its dephosphorylation whereas this may be opposite in the case of Thr^{308} phosphorylation. Recently, the mTOR complex 2 (mTORC2) has been identified as the kinase responsible for the Akt-Ser^{473} phosphorylation [20]. It is believed that the mTOR kinase activity towards Akt Ser^{473} phosphorylation is also dependent on PI3K activation, although the underlying molecular mechanism remains elusive [21]. To determine whether TG2's facilitatory effect on EGF induced Akt phosphorylation involves PI3K, we analyzed Akt phosphorylation by immunoblotting in the vector and TG2 transfected cells in the presence and absence of PI3K specific inhibitor LY294002. In both groups, Akt phosphorylation was completely blocked in the presence of LY294002 suggesting involvement of PI3K in this process (Fig. 4).

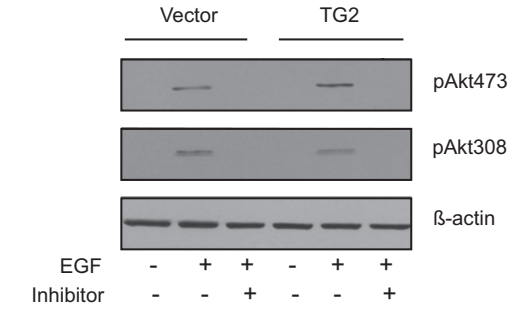

Fig. 4. TG2's facilitatory effect on EGF induced Akt phosphorylation involves PI3K. MEF$^{tg2-/-}$ cells transfected with empty vector and TG2 constructs. Forty eight hours post-transfection cells were serum starved and incubated with and without EGF (100 nM) for 10 min in the presence and absence of PI3K specific inhibitor LY294002 (100 μM). Cells were harvested and equal amount of cell lysates were analyzed by Western immunoblotting using protein and pospho specific antibodies as described in materials and methods. Representative immunoblots of three different experiments are shown. β-Actin immunoblot is shown as a loading control.

In summary, data presented here unraveled a new role of TG2 and its phosphorylation in the regulation of EGFR protein suggesting a mutual relationship between TG2 and EGFR in breast cancer cells. As EGFR and EGFR signaling is a key target in the current treatment of several neoplastic diseases our serendipitous discovery may provide a novel strategy to downregulate EGFR in cancers with enhanced EGFR signaling.

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References

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

[2] B. Li, M.A. Antonyak, J.E. Druso, L. Cheng, A.Y. Nikitin, R.A. Cerione, EGF potentiated oncogenesis requires a tissue transglutaminase-dependent signaling pathway leading to Src activation, Proc. Natl. Acad. Sci. USA 107 (2010) 1408–1413.

[3] Y. Ohtake, T. Kobayashi, A. Maruko, et al., Norepinephrine modulates zonally different hepatocyte proliferation through the regulation of transglutaminase activity, Am. J. Physiol. Gastrointest. Liver Physiol. 299 (2010) G106–G114.

[4] Z. Li, X. Xu, L. Bai, et al., Epidermal growth factor receptor-mediated tissue transglutaminase overexpression couples acquired tumor necrosis factor-related apoptosis-inducing ligand resistance and migration through c-FLIP and MMP-9 proteins in lung cancer cells, J. Biol. Chem. 286 (2011) 21264–21272.

[5] R. Zhang, T.L. Tremblay, A. McDermid, P. Thibault, D. Stanimirovic, Identification of differentially expressed proteins in human glioblastoma cell lines and tumors, Glia 42 (2003) 194–208.

[6] L.A. Hansen, N. Alexander, M.E. Hogan, J.P. Sundberg, A. Dlugosz, D.W. Threadgill, T. Magnuson, S.H. Yuspa, Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development, Am. J. Pathol. 150 (1997) 1959–1975.

[7] X. Cheng, J. Jin, L. Hu, et al., TRP channel regulates EGFR signaling in hair morphogenesis and skin barrier formation, Cell 141 (2010) 331–343.

[8] M.A. Antonyak, A.M. Miller, J.M. Jansen, et al., Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells, J. Biol. Chem. 279 (2004) 41461–41467.

[9] D.S. Kim, S.S. Park, B.H. Nam, I.H. Kim, S.Y. Kim, Reversal of drug resistance in breast cancer cells by transglutaminase 2 inhibition and nuclear factor-kappaB inactivation, Cancer Res. 66 (2006) 10936–10943.
[10] L. Yuan, M. Siegel, K. Choi, et al., Transglutaminase 2 inhibitor, KCC009, disrupts fibronectin assembly in the extracellular matrix and sensitizes orthotopic glioblastomas to chemotherapy, Oncogene 26 (2007) 2563–2573.
[11] A. Verma, S. Guha, H. Wang, et al., Tissue transglutaminase regulates focal adhesion kinase/Akt activation by modulating PTEN expression in pancreatic cancer cells, Clin. Cancer Res. 4 (2008) 1997–2004.
[12] T. Maehama, J.E. Dixon, The tumor suppressor, PTEN/ MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol3, 4, 5-trisphosphate, J. Biol. Chem. 273 (1998) 13375–13378.
[13] V. Stambolic, A. Suzuki, J.L. de la Pompa, Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN, Cell 95 (1998) 29–39.
[14] Y. Wang, S.R. Ande, S. Mishra, Phosphorylation of transglutaminase 2 (TG2) at serine-216 plays a role in TG2 mediated activation of nuclear factor-kappa B and in the downregulation of PTEN, Unpublished results.
[15] K. Rikova, A. Guo, Q. Zeng, et al., Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer, Cell 131 (2007) 1190–1203.
[16] A. Guo, J. Villén, J. Kornhauser, et al., Signaling networks assembled by oncogenic EGFR and c-Met, Proc. Natl. Acad. Sci. USA 105 (2008) 692–697.
[17] S. Mishra, L.J. Murphy, Phosphorylation of transglutaminase 2 by PKA at Ser216 creates 14–3-3 binding sites, Bichem. Biophy. Res. Commun. 347 (2006) 1166–1170.
[18] S. Mishra, G. Melino, L.J. Murphy, Transglutaminase 2 kinase activity facilitates protein kinase A induced phosphorylation of retinoblastoma protein, J. Biol. Chem. 282 (2007) 18108–18115.
[19] G. Salazar, A. González, Novel mechanism for regulation of epidermal growth factor receptor endocytosis revealed by protein kinase A inhibition, Mol. Biol. Cell. 13 (2002) 1677–1693.
[20] D. Sarbassov, D. Guertin, S. Ali, D. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, Science 307 (2005) 1098–1101.
[21] Y.-T. Wu, W. Ouyang, A.S. Lazorchak, et al., J. Biol. Chem. 286 (2011) 14190–14198.