

Chapter 2

A Role for eNOS in Oncogenic Ras-Driven Cancer

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Abstract Nitric oxide (NO) is a highly diffusible gas that is generated by the family of nitric oxide synthases and is increasingly associated with tumorigenesis. While both pro- and anti-tumorigenic properties have been ascribed to NO signaling, recent evidence suggests that eNOS or endothelial nitric oxide synthase promotes tumor formation through its effects on proliferation, cell survival, and angiogenesis. In this chapter we discuss recent evidence that eNOS promotes tumorigenic growth through the activation of the Ras family of proteins.

Keywords Nitric oxide · eNOS · Tumorigenesis · Pancreatic cancer · Ras

eNOS

Endothelial nitric oxide synthase (eNOS) is a member of the nitric oxide synthase (NOS) family of enzymes comprised of eNOS, neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS). NOS enzymes catalyze the conversion of L-arginine and oxygen to citrulline and the highly diffusible gas nitric oxide (NO) (Knowles and Moncada 1994). eNOS differs from the other two NOS family members in regard to its tissue distribution, intracellular localization, regulation by intracellular signaling pathways, and the relative levels of NO generated. Specifically, eNOS is expressed in endothelial cells, but it has been detected at lower levels in other tissues, whereas nNOS concentrates in neuronal cells and iNOS is induced in a wide variety of cell types (Alderton et al. 2001). eNOS is localized to the caveolae of plasma membranes and Golgi through myristoylation at an N-terminal glycine residue and palmitoylation at two N-terminal cysteine residues (Liu et al. 1995; Liu et al. 1997; Michel 1999). nNOS is also localized to the plasma membrane, but through protein–protein interactions vis-à-vis a unique

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N-terminal PDZ domain, whereas iNOS is a cytosolic protein (Brenman et al. 1996; Alderton et al. 2001). eNOS is activated by Ca^{2+} release, but, as discussed below, also by phosphorylation at serine 1177 (in human eNOS) by the kinase AKT (PKB). Although nNOS is also activated by Ca^{2+} , it is not activated by AKT, whereas iNOS activity is independent of Ca^{2+} signaling (Bredt and Snyder 1990; Abu-Soud et al. 1994; Gachhui et al. 1996; Salerno et al. 1997; Gachhui et al. 1998). Lastly, both eNOS and nNOS typically generate nanomolar levels of NO while iNOS can produce micromolar levels (Xie et al. 1992; Xie and Nathan 1994). Thus, eNOS is distinct from nNOS and iNOS by a number of criteria.

eNOS Activation by AKT

Recently, it was discovered that eNOS is regulated independent of elevated Ca^{2+} in endothelial cells via phosphorylation of S1177 by the kinase AKT. Specifically, AKT co-immunoprecipitates with eNOS (Dimmeler et al. 1999; Michell et al. 1999) and phosphorylates the bovine eNOS at the amino acid's corresponding to the human protein, S633 and S1177 (Dimmeler et al. 1999; Fulton et al. 1999; Michell et al. 1999), leading to eNOS activation as measured by an increase in NO production (Dimmeler et al. 1999; Fulton et al. 1999; Michell et al. 1999). However, only activating (S→D) and inactivating (S→A) mutation at S1177 (and not S633) enhance (Dimmeler et al. 1999) and abolish (Dimmeler et al. 1999; Fulton et al. 1999) catalytic activity, respectively, indicating that S1177 is the target of AKT signaling. Activation depends on AKT kinase activity (Fulton et al. 1999) and is inhibited with a dominant-negative AKT protein (Dimmeler et al. 1999) or the PI3K inhibitor wortmannin (Dimmeler et al. 1999; Michell et al. 1999). eNOS must associate with the plasma or Golgi membranes to be phosphorylated by AKT, as S1177 phosphorylation is reduced by mutating the palmytolation (Fulton et al. 1999; Gonzalez et al. 2002) or myristoylation (Gonzalez et al. 2002) sites of eNOS, and this effect is rescued if eNOS is artificially re-targeted to membranes (Fulton et al. 2004). AKT phosphorylation does not change the subcellular distribution of eNOS (Fulton et al. 2002), but instead appears to both enhance the electron flux during catalysis and reduce Ca^{2+} dependency (Dimmeler et al. 1999; Michell et al. 1999; McCabe et al. 2000). Thus, AKT phosphorylates S1177 of membrane-bound eNOS, leading to generation of NO.

PI3K–AKT: Signaling and Cancer

The PI3K–AKT signal is inappropriately activated in many cancers to promote tumorigenesis (Luo et al. 2003; Engelman et al. 2006). Activation of the canonical p110/p85 (class IA) family of phosphatidylinositol 3-kinase (PI3K) enzymes begins with engagement of growth factors with receptor tyrosine kinases. The activated receptor activates PI3K either by recruiting the regulatory p85 subunit, via direct binding to autophosphorylation sites on the receptor or an adaptor protein, or through activation of the small GTPase Ras, which when in the active

GTP-bound state binds directly to the Ras-binding domain of the p110 subunit. Once recruited to the membrane, the p85/p110 PI3K complex, hereafter referred to simply as PI3K, phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂), generating PIP₃. PIP₃, in turn, recruits other proteins such as the PDK1 kinase and the family of AKT kinases composed of AKT1, AKT2, and AKT3. Recruitment of AKT kinases and phosphorylation by PDK1 activates AKT, which in turn phosphorylates a large number of diverse proteins, including eNOS. This pathway is, in turn, negatively regulated by conversion of PIP₃ to PIP₂ by the phosphoinositide phosphatase PTEN, a tumor suppressor (Fig. 2.1) (Luo et al. 2003; Engelman et al. 2006).

The PI3K pathway can be illegitimately activated via activating mutations or amplification of *AKT1*, *AKT2*, *p85*, *PDK1*, or more often, *PI3K3CA* in a subset of cancers (Samuels and Velculescu 2004; Hennessy et al. 2005; Parsons et al. 2005; Karakas et al. 2006). More commonly, an upstream activator of AKT, Ras, is mutated to remain in the oncogenic active GTP state in one-third of human cancers (Bos 1989), and loss of expression of the negative regulator of PI3K signaling, *PTEN*, occurs at a frequency second only to *p53* (Eng 2003). Activating mutations in *Ras* are mutually exclusive with loss of *PTEN* expression in experimental tumor models (Mao et al. 2004) and human cancers (Ikeda et al. 2000; Tsao et al. 2000; Mizoguchi et al. 2004). Thus, together alterations in Ras or PTEN account for a large amount of PI3K–AKT activation in human cancer (Fig. 2.1) that promotes tumor growth (Hennessy et al. 2005).

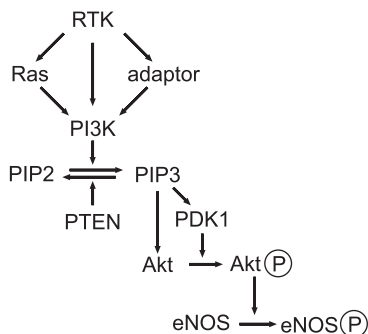


Fig. 2.1 PI3K–AKT signaling to eNOS. Simplified diagram of receptor tyrosine kinase (RTK) activation of PI3K, leading to activation of the AKT family of kinases, and in turn phosphorylation of eNOS. Oncogenic mutations to Ras, loss of PTEN expression, and amplifications or mutations to PI3K (PIK3CA) represent the most common events leading to AKT activation in cancer. P: phosphorylated amino acid

Links Between eNOS and Cancer

While the role of NO in cancer is controversial and conflicting – NO has been found to both promote and inhibit tumor growth depending on the experimental design and setting (Lechner et al. 2005; Fukumura et al. 2006) – mounting

circumstantial evidence suggests that eNOS may play some role in cancer by affecting cell proliferation, apoptosis, and angiogenesis.

In regard to the link of eNOS with cell proliferation, a phenotype of cancer cells (Vogelstein and Kinzler 1993), eNOS has been detected in tumor cells. Specifically, eNOS-positive tumor cells have been reported in colorectal (Yagihashi et al. 2000), breast (Mortensen et al. 1999a; Martin et al. 2000; Loibl et al. 2002; Tse et al. 2005), brain (Colasanti et al. 1997; Broholm et al. 2001; Broholm et al. 2003), pancreatic (Nussler et al. 1998), Kaposi sarcoma (Weninger et al. 1998), and melanoma (Tu et al. 2006) tumors, as well as in mammary carcinoma (Zeillinger et al. 1996; Mortensen et al. 1999b), pancreatic adenocarcinoma (Nussler et al. 1998), and choriocarcinoma (Kiss et al. 1998) cancer cell lines. Moreover, *eNOS*^{-/-} mice show reduced keratinocyte proliferation resulting in reduced wound margin epithelia (Stallmeyer et al. 2002). Additionally, exposure to NO donors such as GSNO, SNAP, and DEA-NO has been shown to increase DNA synthesis, cell proliferation, and migration of endothelial cells in a number of cell culture models including rabbit aortic endothelial cells (RAECs) (Oliveira et al. 2003), bovine aortic endothelial cells (BAECs) (Zaragoza et al. 2002; Kawasaki et al. 2003), and both human saphenous vein endothelial cells (HSVECs) and human aortic endothelial cells (HAECs) (Kawasaki et al. 2003). Similarly, inhibition of the eNOS pathway with the inhibitor L-NAME has been shown to inhibit proliferation in the oral squamous cancer cell line TSCCa, as measured by the MTT assay and crystal violet staining (Shang et al. 2006).

eNOS has also been found to play a pro-survival role in some settings, which has relevance to cancer as evading apoptosis is a hallmark of cancer cells (Vogelstein and Kinzler 1993). Specifically, in the prostate cancer cell line PC-3, ectopic expression of wild-type or the activated S1177D form of eNOS reduced tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by 50 and 75%, respectively (Tong and Li 2004). Additionally, in rat Nb2 lymphoma cells, which express eNOS, but not iNOS or nNOS, addition of exogenous NO through treatment with L-arginine or the NO donor DEA-NO, or generation of endogenous NO, through an FGF-induced increase in eNOS expression, provided protection against apoptosis through the upregulation of Bcl-2 (Dodd et al. 2000; Murphy et al. 2001). In CCD 1106 KERTr keratinocytes, treatment with the NOS inhibitor L-NAME or the sGC inhibitor ODQ led to an increase in apoptosis whereas treatment with the NO donor SNAP led to protection against UV-B-induced apoptosis (Weller et al. 2003). eNOS has also been shown to have a role in the modulation of apoptosis in the breast cancer cell line MCF-7, where blocking eNOS-generated NO using the scavenger PTIO leads to an increase in apoptosis, whereas low concentrations (but not high concentrations) of the NO donor sodium nitroprusside are protective (Mortensen et al. 1999). Finally, relatively low levels of NO, consistent with the amounts produced by eNOS, but not high levels of NO more commonly associated with iNOS, result in the activation of the pro-survival and pro-proliferative protein kinase C (PKC), extracellular signal-related protein kinase (ERK) and Jun in endothelial cells (Jones et al. 2004; Ridnour et al. 2005).

The most established role of eNOS in cancer is in regard to angiogenesis, the process by which tumors become vascularized (Duda et al. 2004; Ying and Hofseth

2007). *First*, eNOS is detected by immunohistochemistry (IHC) in endothelial cells of tumor stroma from a wide spectrum of cancers (Cobbs et al. 1995; Tschugguel et al. 1996; Takahashi et al. 1997; Nussler et al. 1998; Weninger et al. 1998; Doi et al. 1999; Iwata et al. 1999; Klotz et al. 1999; Mortensen et al. 1999; Yagihashi et al. 2000; Broholm et al. 2001; Kruse et al. 2002; Broholm et al. 2003; Lin et al. 2003; Tse et al. 2005; Wang et al. 2005; Tu et al. 2006), and in some (Takahashi et al. 1997; Nussler et al. 1998; Iwata et al. 1999; Klotz et al. 1999; Yagihashi et al. 2000; Broholm et al. 2001; Kruse et al. 2002; Broholm et al. 2003; Tse et al. 2005; Wang et al. 2005), but not all cases (Rajnakova et al. 1997; Doi et al. 1999; Mortensen et al. 1999; Lin et al. 2003), at elevated levels compared to normal tissue. *Second*, pro-angiogenic molecules such as vascular endothelial growth factor (VEGF) and prostaglandin E₂ (PGE₂) are known to activate eNOS through a calcium–calmodulin-dependent mechanism or through activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which results in the Akt-mediated phosphorylation of eNOS (Duda et al. 2004; Namkoong et al. 2005). *Third*, loss of eNOS function inhibits angiogenesis, as *eNOS*^{-/-} mice are defective in angiogenesis following tissue ischemia (Murohara et al. 1998) and display a ~2-fold reduction in vessel density in implanted collagen gels (Fukumura et al. 2001). A similar level of reduction of angiogenesis has also been found in tumor xenografts derived from B16 murine melanoma (Kashiwagi et al. 2005) and Lewis lung carcinoma (LLC) (Gratton et al. 2003) cell lines when implanted into *eNOS*^{-/-} mice. *Fifth*, loss of eNOS in tumor stroma inhibits tumor growth as *eNOS*^{-/-} mice exhibit a ~2-fold reduction in tumor growth of xenografts of B16 (Kashiwagi et al. 2005) or LLC (Gratton et al. 2003) cell lines. A similar reduction of LLC and human HepG2 hepatocellular carcinoma tumor cell growth was also observed in wild-type mice treated with a peptide inhibitor of eNOS, an effect that was ascribed to inhibiting stromal eNOS activity (Gratton et al. 2003).

Hypothesis: AKT Promotes Tumorigenesis by Activating eNOS in Cancer Cells

Because (1) AKT is commonly activated in human cancers to promote tumorigenesis, (2) eNOS is activated by AKT, at least in endothelial cells, (3) eNOS has been detected in cancer cells, and (4) perturbing eNOS expression in a number of settings disrupts tumor phenotypes, such as cell proliferation, apoptosis, and angiogenesis, we propose that illegitimate activation of AKT in cancer cells may act through eNOS to promote tumorigenesis (Fig. 2.1).

eNOS Activation in Pancreatic Cancer

As mentioned, Ras is mutated to remain in the active oncogenic state in one-third of human cancers, and 90% of pancreatic cancers (Bos 1989), and it is well established that oncogenic Ras activates the PI3K–AKT pathway to promote cancer

(Campbell and Der 2004). AKT, in turn, has been shown to activate eNOS through phosphorylation of S1177 in endothelial cells. We thus tested whether eNOS is phosphorylated and thereby activated at the AKT site S1177 in the cancer most characterized by oncogenic Ras mutations, human pancreatic cancer (Bos 1989). A panel of nine pancreatic cancer cell lines and two normal pancreatic tissue specimens were immunoblotted with an α -phospho(S1177)-eNOS antibody (Chen et al. 1999; Michell et al. 1999), with the finding that S1177 phosphorylation of eNOS was elevated in a subset of the tumor cell lines samples compared to the normal tissue control (Fig. 2.2a and (Lim et al. 2008)). We extended these results to actual pancreatic cancer tumor specimens, only this time finding an increase in S1177 phosphorylation in all tumor samples compared to the matched and unmatched normal controls (Fig. 2.2b and (Lim et al. 2008)). eNOS is thus phosphorylated at the AKT site S1177 in Ras-driven human pancreatic cancer specimens.

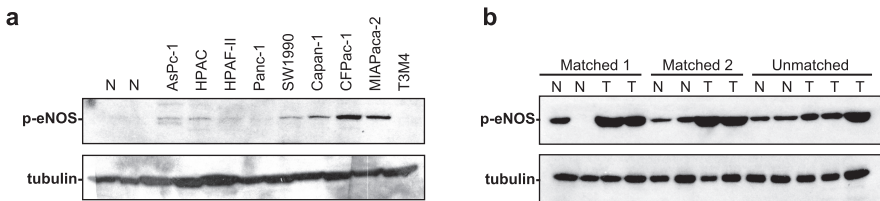
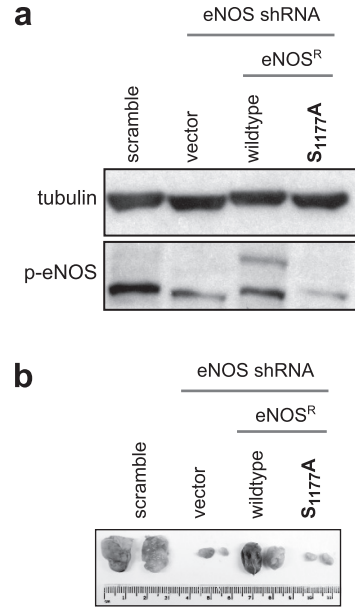


Fig. 2.2 eNOS is phosphorylated at S1177 in oncogenic KRas-driven tumors. Immunoblot of (a) indicated human pancreatic cancer cell lines or (b) pancreatic biopsies of non-malignant (N) and tumor (T) tissue with an α -phospho(S1177)-eNOS antibody. Actin and tubulin: loading controls. Adapted from (Lim et al. 2008) and used with permission from the publisher

Phosphorylated eNOS is Required for Ras-Mediated Tumor Growth

To assess the biological consequence of oncogenic Ras-mediated phosphorylation of the AKT site of eNOS during tumorigenesis, we tested whether inhibiting AKT phosphorylation of eNOS impeded tumor growth of Ras-transformed cells. For this analysis we employed the genetically defined HEK-TtH cell line engineered to express oncogenic Ras (Ras^{G12V}). Such Ras^{G12V}-TtH cells were chosen because they are human, an important consideration as Ras oncogenesis can differ between human and murine cells (Hamad et al. 2002; Rangarajan et al. 2004), the cells have a defined genetic background, thereby simplifying analysis (O'Hayer and Counter 2006), and are absolutely dependent on Ras^{G12V} for tumor growth (Hahn et al. 1999). Ras^{G12V}-TtH cells were stably infected with a retrovirus encoding no transgene (vector) or eNOS shRNA, and appropriate knockdown of eNOS expression was confirmed by immunoblot (Fig. 2.3a). To assess whether phosphorylation of eNOS was required for tumor growth, the latter cells were stably infected with a retrovirus encoding human eNOS engineered to be resistant to shRNA by introduction of silent point mutations (eNOS^R) in either the S1177A mutant or wild-type

Fig. 2.3 eNOS phosphorylation is required for tumor growth. RasG12V-TtH cells expressing the indicated transgenes or shRNAs were (a) immunoblotted with an α -phospho(S1177)-eNOS Ab to detect phosphorylated endogenous (P-eNOS) and ectopic HA-tagged (P-eNOS^R) eNOS, and (b) injected into the flanks of four mice, of which representative mice and excised tumors are shown. Adapted from Lim et al. (2008) and used with permission from the publisher



(WT) configuration, and expression validated by immunoblot (Fig. 2.3a). All four cell lines were then each injected into the flanks of four immunocompromised mice and tumor growth monitored.

The first important observation of these studies was that knockdown of eNOS clearly reduced the tumor growth of the cells compared to scramble control (Fig. 2.3b), arguing that in addition to a role for eNOS in stromal tissue, the enzyme is also required in cancerous cells for Ras-mediated tumor growth. This loss of tumor growth was rescued by expressing wild-type eNOS^R, ruling out any off-target effects of the eNOS shRNA, but not by the S1177A mutant version of eNOS^R (Fig. 2.3b). Thus, not only is eNOS required for Ras-driven tumor growth, but eNOS must be phosphorylated at the AKT site S1177 to promote tumor growth.

These results were validated in two different model systems. First, stable shRNA-mediated knockdown of eNOS reduced the tumorigenic growth potential of the two pancreatic cancer cell lines characterized by oncogenic KRas mutations, MiaPaCa2 and CFPac1 (Lim et al. 2008), when injected into immunocompromised mice. Second, the polycyclic aromatic hydrocarbon carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) followed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was topically applied on *eNOS*^{-/-} mice and, as a control, *eNOS*^{+/+} mice (Iversen 1991; Stern and Conti 1996). This approach has been extensively used to induce benign skin papillomas characterized by *HRas* oncogenic mutations that progress to adenocarcinomas (Balmain et al. 1984; Quintanilla et al. 1986; Kemp et al. 1994; Stern and Conti 1996). We found that there was a ~3-fold reduction in the number

of tumors per mouse in *eNOS*^{-/-} compared to control *eNOS*^{+/-} mice (Lim et al. 2008). Thus, in three different model systems, loss of eNOS expression reduced oncogenic Ras-driven tumor growth.

How Does eNOS Promote Tumorigenesis?

Although eNOS plays a role in oncogenic Ras-driven tumorigenesis, the mechanism of action remains to be resolved. Presumably eNOS promotes tumorigenesis by generating NO, as the S1177A mutant of eNOS, which cannot be phosphorylated by AKT but is an otherwise wild-type protein, cannot rescue the loss of tumor growth upon knockdown of endogenous eNOS, whereas wild-type eNOS can rescue this effect (Lim et al. 2008). NO produced by eNOS could have many effects, as NO can be converted to a number of other molecules depending on the chemical environment in which it is generated (Alderton et al. 2001). One possibility is that NO activates the canonical target of NO signaling, sGC. This enzyme could very well be activated in the stromal tissue, especially in regard to angiogenesis. However, knockdown of eNOS can reduce proliferation of cancer cell lines (Zaragoza et al. 2002; Kawasaki et al. 2003; Oliveira et al. 2003; Shang et al. 2006), suggesting a cell autonomous defect. Thus, perhaps eNOS targets sGC in cancer cells, or even more intriguingly, it may target other molecules aside from sGC to promote tumor phenotypes.

One attractive substrate for NO in cancer is the Ras protein itself. The Ras family of small GTPases is composed of HRas, NRas, and KRas. All three Ras family members contain a conserved cysteine at position 118. Aside from the well-established effect of NO with redox active metal centers, as exemplified in the case of sGC (Davis et al. 2001), NO can also lead to modification of the thiol group of cysteines. Derived reactive nitrogen species from NO, primarily $\bullet\text{NO}_2$ and ONOO⁻, can lead to S-nitrosylation, S-glutathionylation (in the presence of glutathione), and disulfides or sulfenic, sulfinic, or sulfenic acid derivatives of the thiol group of cysteines (Cooper et al. 2002; Hess et al. 2005). At least in the case of HRas, C118 has been shown to be S-nitrosylated and S-glutathionylated in vitro and in vivo, which leads to an increase in the active GTP-bound state. Consistent with this, mutating C118 to serine in HRas, a minor change that exchanges the sulfur atom for oxygen, abolishes both modifications and leaves the protein in an inactive GDP-bound state or reduced Ras signaling (Lander et al. 1996; Ji et al. 1999; Clavreul et al. 2006). Thus, we indirectly measured S-nitrosylation of HRas using the biotin switch assay (Jaffrey and Snyder 2001) in immortalized human epithelial cells and confirmed that HRas is nitrosylated and that it is specific for C118 (Fig. 2.4a). Additionally, we directly measured the GTP loading of HRas by capture of active Ras via the RBD of Raf1 followed by immunoblot with an HRas-specific antibody (Taylor et al. 2001) in two independent pancreatic cancer cell lines (MiaPaCa2 and CFPac1) in which eNOS expression was reduced by shRNA. We found that reducing eNOS

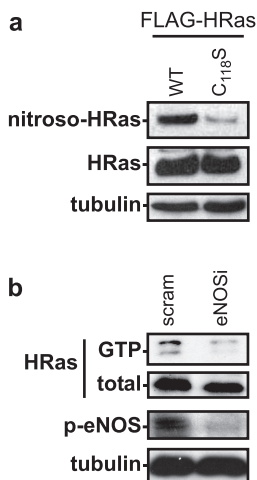


Fig. 2.4 Nitrosylation leads to an increase in GTP-bound HRas. **(a)** Immortalized human embryonic kidney cells expressing either wild type or C118S mutant flag-tagged HRas were analyzed by the biotin switch method to determine the levels of nitrosylated HRas. Tubulin: loading control. **(b)** CFPac-1 cells expressing shRNA specific for either eNOS (eNOSi) or a scrambled control sequence (scram) were assayed for levels of GTP-bound and total HRas. Tubulin: loading control. Adapted from Lim et al. (2008) and used with permission from the publisher

expression in these cell lines led to a reduction of GTP-bound HRas (Fig. 2.4b). Similar results were found when wild-type NRas was also assayed (Lim et al. 2008). Taken together, these results suggest that the modification detected on C118 of HRas is most consistent with S-nitrosylation, and further, that the eNOS-dependent modification of this cysteine activates HRas and NRas.

To assess the biological impact of eNOS-dependent activation of HRas (and NRas) on tumorigenesis, HRas was stably knocked down in oncogenic KRas mutation-positive CFPac1 pancreatic cancer cells and complemented with (i) RNAi-resistant HRas as a positive control to rescue the loss of HRas expression, (ii) no transgene as a negative control, and lastly, (iii) RNAi-resistant HRas in the C118S mutant configuration (Fig. 2.5a). The four cell lines were then each injected into the flanks of four immunocompromised mice and tumor growth monitored over time. We found that knockdown of wild-type HRas in the oncogenic KRas-driven tumor cell line CFPac1 reduced tumor growth, and this was rescued by the wild-type HRas, but not the C118S mutant (Fig. 2.5b). Similar results were observed in another cancer cell line, and when NRas was similarly knocked down and complemented with wild-type or C118S mutant versions of RNAi-resistant NRas (Lim et al. 2008). These results argue that the eNOS-dependent modification detected on C118 by the biotin switch assay, most consistent with S-nitrosylation, is required for tumor growth, which in turn suggests that HRas and NRas act downstream of eNOS in tumorigenesis.

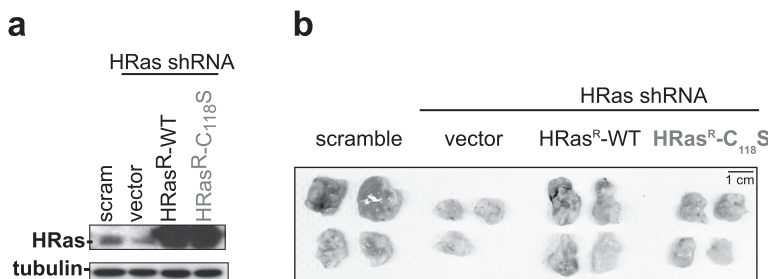


Fig. 2.5 C→S mutation on C118 of wild-type HRas inhibits tumor growth. **(a)** Immunoblot analysis of HRas expression and **(b)** excised tumors from CFPac1 cells stably infected with retrovirus encoding a scramble sequence or eNOS shRNA sequence and complemented with no transgene or RNAi-resistant HRas in a wild type or C118S mutant configuration. Adapted from Lim et al. (2008) and used with permission from the publisher

More Questions than Answers

These experiments have only just begun to shed light on what is surely to be a much more complex picture for how eNOS promotes tumorigenesis. First, the nature of the eNOS-dependent chemical modification at C118 of HRas *in vivo* remains to be resolved. Specifically, while detection of this modification by the biotin switch assay in cells (Raines et al. 2006; Lim et al. 2008), and mass spectrometric identification of S-nitrosylation of this site *in vitro* (Lander et al. 1997) suggest that C118 is modified by S-nitrosylation, this remains to be independently validated *in vivo*. Second, production of NO by eNOS could have widespread consequences, and hence it remains to be determined if Ras proteins are the only targets of NO in cancer cells. In this regard, proteins known to be modified by NO such as sGC (Denninger and Marletta 1999) and caspases (Mannick et al. 2001) are interesting candidates. Third, why a cancer cell that already has one Ras family member activated vis-à-vis an oncogenic mutation would rely on activation of the other Ras family members remains a mystery. This is especially confusing insofar as the catalytic and effector-binding domains of HRas, NRas, and KRas are nearly identical at the amino acid level. Fourth, AKT is activated by a variety of changes in cancer cells, not just by oncogenic Ras mutations, and hence it will be interesting to test whether other changes, for example, loss of *PTEN*, also rely on eNOS for tumor growth. Fifth, whether these studies can be translated into therapies remain to be determined, but it is encouraging that cavtratin, a peptide derived from caveolin-1, inhibits eNOS and displays anti-tumor activity, and general NOS inhibitors such as L-NAME and L-NMMA have been tested in clinical trials for other diseases. Lastly, the exact nature of a requirement for eNOS in tumor growth is unknown, and since eNOS can effect cell proliferation, apoptosis, and angiogenesis, there is surely more here than meets the eye.

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