Cancer Cell
Article

Control of Cyclin D1 and Breast Tumorigenesis by the EglN2 Prolyl Hydroxylase

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DOI 10.1016/j.ccr.2009.09.029

SUMMARY

2-Oxoglutarate-dependent dioxygenases, including the EglN prolyl hydroxylases that regulate HIF, can be inhibited with drug-like molecules. EglN2 is estrogen inducible in breast carcinoma cells and the lone *Drosophila* EglN interacts genetically with Cyclin D1. Although EglN2 is a nonessential gene, we found that EglN2 inactivation decreases Cyclin D1 levels and suppresses mammary gland proliferation in vivo. Regulation of Cyclin D1 is a specific attribute of EglN2 among the EglN proteins and is HIF independent. Loss of EglN2 catalytic activity inhibits estrogen-dependent breast cancer tumorigenesis and can be rescued by exogenous Cyclin D1. EglN2 depletion also impairs the fitness of lung, brain, and hematopoietic cancer lines. These findings support the exploration of EglN2 inhibitors as therapeutics for estrogen-dependent breast cancer and other malignancies.

INTRODUCTION

Most successful drugs are small organic molecules that bind to, and inhibit, specific cellular proteins. Proteins that serve as enzymes have proven to be particularly tractable as drug targets. Establishing additional classes of enzymes that can be manipulated with small organic molecules opens new avenues for drug discovery.

The 2-oxoglutarate and iron-dependent dioxygenase superfamily includes the collagen prolyl and lysyl hydroxylases, the FTO and AlkB DNA demethylases, the JmjC-containing histone demethylases, the FIH1 asparaginyl hydroxylase, and the EglN family prolyl hydroxylases (Aravind and Koonin, 2001; Klose et al., 2006; Pollard et al., 2008; Taylor, 2001). These enzymes can be inhibited with drug-like small molecules that compete with 2-oxoglutarate or interfere with iron utilization, both in vitro and in vivo (Bruegge et al., 2007; Mole et al., 2003; Ozer and Bruick, 2007; Safran et al., 2006).

There are three EglN (also called PHD or HPH) family members in humans, called EglN1, EglN2, and EglN3 (Kaelin and Ratcliffe, 2008). All three enzymes are capable of hydroxylating the α subunit of the heterodimeric transcription factor HIF (hypoxia-inducible factor). Prolyl hydroxylated HIFα is recognized by a ubiquitin ligase complex containing the pVHL tumor-suppressor protein, leading to its polyubiquitinylation and subsequent proteasomal degradation. EglN family members exhibit Km values for oxygen that exceed the oxygen concentrations found in mammalian tissues (Kaelin and Ratcliffe, 2008).

SIGNIFICANCE

Cyclin D1 plays an important role in many cancers, including breast cancer. The observations described herein predict that inhibiting EglN2 catalytic activity will diminish Cyclin D1 levels in cancer cells and impair their ability to proliferate in vivo. Notably, EglN2 is estrogen inducible and loss of either EglN2 or Cyclin D1 leads to mammary gland hypoproliferation. Therefore the relationship between EglN2 and Cyclin D1 might be especially relevant in hormone-sensitive breast cancer, in which new therapies are needed for women who become refractory to estrogen antagonists. EglN2 appears to be an attractive drug target because EglN2 is not essential in mammals and it has already been established that enzymes of this class can be inhibited with drug-like small organic molecules.

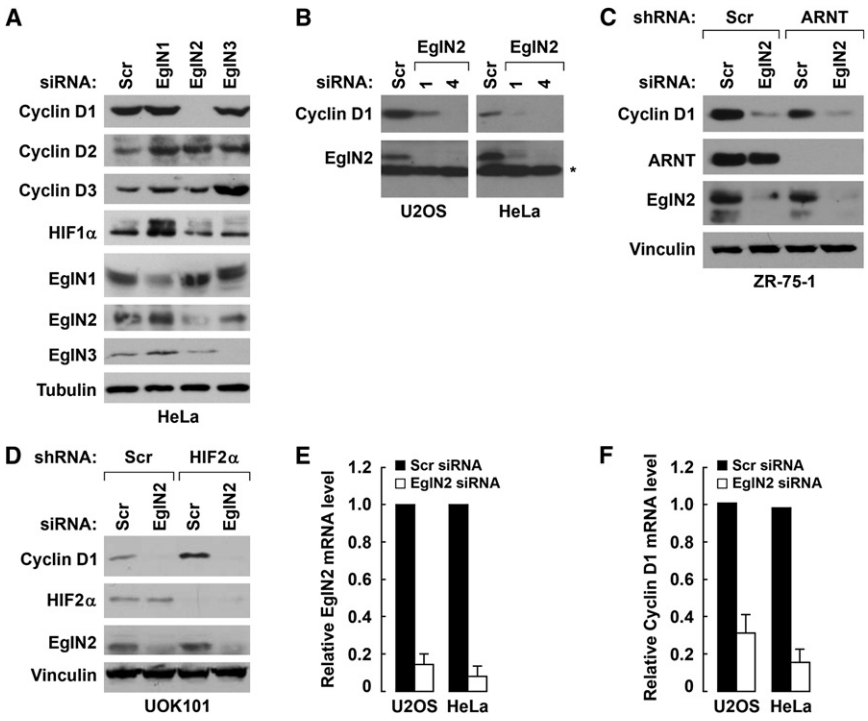

Figure 1. EglN2 Regulates Cyclin D1

(A) Immunoblot analysis of HeLa cells 48 hr after transfection with siRNAs targeting EglN1, EglN2, EglN3, or a scrambled control siRNA.

(B, E, and F) Immunoblot (B) and qRT PCR (E and F) analysis of U2OS and HeLa cells transfected with two independent siRNAs (#1 and #4) targeting EglN2 (asterisk indicates nonspecific bands). Error bars represent one SEM.

(C) Immunoblot analysis of ZR 75 1 cells infected with a lentivirus encoding an ARNT shRNA or scrambled control (Scr) followed by transfection with siRNA against EglN2 or scrambled control.

(D) Immunoblot analysis of UOK101 cells infected with a lentivirus encoding an HIF2α shRNA or scrambled control (Scr) followed by transfection with siRNA against EglN2 or scrambled control.

Accordingly, these enzymes are highly sensitive to decrements in oxygen availability, such as might occur following an interruption in blood supply. HIF regulates a program of gene expression that facilitates survival under hypoxic conditions through cell-intrinsic changes in metabolism and cell-extrinsic changes affecting oxygen delivery. For example, HIF activates the transcription of genes such as erythropoietin that enhance red blood cell production and hence blood oxygen carrying capacity. EglN antagonists stimulate red blood cell production in mammals and are currently undergoing phase II testing for different forms of anemia (Hsieh et al., 2007; Safran et al., 2006).

EglN1 (also called PHD2) is the primary prolyl hydroxylase responsible for HIF regulation (Berra et al., 2003; Minamishima et al., 2008; Takeda et al., 2008). EglN2 (also called PHD1) and EglN3 (also called PHD3) might also regulate HIF under certain conditions (Appelhoff et al., 2004). For example, EglN3 is itself a HIF target, is induced by hypoxia, and has a lower oxygen Km than EglN1 (Appelhoff et al., 2004; Minamishima et al., 2009). Cell culture and animal experiments support that EglN3 partially compensates for EglN1 when the latter is inactivated by hypoxia (Appelhoff et al., 2004; Minamishima et al., 2009). Whether EglN2 and EglN3 have HIF-independent functions is less clear, although recent studies support a HIF-independent role for EglN3 in the control of apoptosis (Rantanen et al., 2008; Schlisio et al., 2008).

Polyak and coworkers reported that EglN2 mRNA accumulates in breast cancer cells that have been stimulated to proliferate with estrogen and that EglN2 overexpression promotes estrogen-independent growth and tamoxifen resistance (Seth et al., 2002). Frei and Edgar (2004) noted that certain phenotypes observed in flies engineered to overproduce Cyclin D1 were abrogated by concurrent inactivation of Egl9, which is the lone ancestral EglN family member in *Drosophila*. Since Cyclin D1 plays an important role in many forms of cancer, including breast cancer, and is induced by estrogen in estrogen-receptor positive breast cancers (Bartkova et al., 1994; Landis et al., 2006; Roy and Thompson, 2006; Yu et al., 2001), we asked whether EglN2 activity affects Cyclin D1 activity.

RESULTS

Toward this end, we transiently transfected HeLa cervical carcinoma cells, U2OS osteosarcoma cells, and both T47D and ZR-75-1 breast carcinoma cells with previously validated siRNAs that are specific for EglN1, EglN2, or EglN3 (Appelhoff et al., 2004). Downregulation of EglN2, but not EglN1 or EglN3, decreased Cyclin D1 protein levels (Figure 1A, Figure S1A [available online], and data not shown). Similar results were observed with a second, independent, EglN2 siRNA and downregulation of Cyclin D1 by the two different EglN2 siRNAs mirrored their ability to downregulate EglN2 (Figure 1B and Figure S1B). In some experiments Cyclin D3 was also decreased (data not shown). As expected, suppression of EglN1, but not EglN2 or EglN3, induced HIF1α (Figure 1A). These results suggest that Cyclin D1 is specifically regulated by EglN2 among the EglN family members and that EglN2 regulates Cyclin D1 in a HIF-independent manner.

In further support of the latter conclusion, downregulation of Cyclin D1 after EglN2 loss was not affected by concurrent inactivation of the HIFα heterodimeric partner ARNT (HIF1β) (Figure 1C and Figure S2A). In addition, EglN2 loss decreased Cyclin D1 in UOK101 and 769-P *VHL*$^{-/-}$ renal carcinoma cells, which constitutively produce HIF2α protein due to the absence of pVHL and produce neither HIF1α mRNA nor protein (Maxwell et al., 1999) (Figure 1D, Figure S2B, and data not shown). Moreover, elimination of HIF2α in these cells with a highly effective short hairpin RNA (shRNA) did not prevent the loss of Cyclin D1 in cells depleted of EglN2 (Figure 1D and Figure S2B). Collectively, these results strongly suggest that the regulation of Cyclin