MOLECULAR ONCOLOGY XXX (2015) 1–14

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The transcription factors Slug (SNAI2) and Snail (SNAI1) regulate phospholipase D (PLD) promoter in opposite ways towards cancer cell invasion

Ramya Ganesan[b], *Elizabeth Mallets*[b], *Julian Gomez-Cambronero*[a,b,*]

[a]*Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women Hospital and Harvard Medical School, Boston, MA 02115, USA*

[b]*Wright State University Boonshoft School of Medicine, Department of Biochemistry and Molecular Biology, Dayton, OH 45435, USA*

ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form
8 December 2015
Accepted 9 December 2015
Available online ■

Keywords:
Mammalian cells
Gene expression
Cell signaling
Breast cancer
RNA role

ABSTRACT

Slug (*SNAI2*) and Snail (*SNAI1*) are master regulatory transcription factors for organogenesis and wound healing, and they are involved in the epithelial to mesenchymal transition (EMT) of cancer cells. We found that the activity of phospholipase D isoform 2 (PLD2) is highly increased in cancers with larger size and poor prognosis (MDA-MB-231 versus MCF-7 cells), so we determined if Snail or Slug were responsible for PLD2 gene transcription regulation. Unexpectedly, we found that PLD2 expression was positively regulated by Slug but negatively regulated by Snail. The differential effects are amplified in breast cancer cells over normal cells and with MDA-MB-231 more robustly than MCF-7. Slug putatively binds to the PLD2 promoter and transactivates it, which is negated when Slug and Snail compete with each other. Meanwhile, PLD2 has a negative effect on Snail expression and a positive effect on Slug, thus closing a feedback loop between the lipase and the transcription factors. Further, PA, the product of PLD2 enzymatic reaction, has profound effects on its own and it further regulates the transcription factors. Thus, we show for the first time that the overexpressed PLD2 in human breast tumors is regulated by Slug and Snail transcription factors. The newly uncovered feedback loops in highly invasive cancer cells have important implications in the process of EMT.

Abbreviations: DMEM, Dulbecco's modified eagle media; EGF, epidermal growth factor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; PLD, phospholipase D; PLD2, phospholipase D2; PVDF, polyvinylidene fluoride; qCPR, quantitative polymerase chain reaction; siRNA, silencing RNA; PA, phosphatidic acid; DOPA, di-oleoyl phosphatidic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMEC, human mammary epithelial cells.

* *Corresponding author*. Wright State University Boonshoft School Medicine, Department of Biochemistry and Molecular Biology, 3640 Colonel Glenn Highway, Dayton, OH 45435, USA. Tel.: +1 937 775 4767; fax: +1 937 775 3730.

E-mail address: julian.cambronero@wright.edu (J. Gomez-Cambronero).

http://dx.doi.org/10.1016/j.molonc.2015.12.006

Please cite this article in press as: Ganesan, R., et al., The transcription factors Slug (SNAI2) and Snail (SNAI1) regulate phospholipase D (PLD) promoter in opposite ways towards cancer cell invasion, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.12.006

1. Introduction

SNAI1 (Snail) is a zinc-finger transcription factor that belongs to a larger superfamily known as *SNAI* and participates in cell differentiation and survival (Nieto, 2002). Snail's main action mode is by inducing epithelial-to-mesenchymal transition (EMT) by suppression of E-cadherin transcription, which is responsible for cell adhesion and migratory capabilities (Bolos et al., 2003). EMT plays a major role in cancer progression and invasion (Choi et al., 2013). Snail repression via silencing RNA (siRNA) results in decreased tumor metastasis and immunosuppression. In addition, there is an increased T-cell response in the tumor due to suppression of Snail (Kudo-Saito et al., 2009). Snail deregulation confers resistant to radiation and certain therapeutic drugs specific for ovarian cancer by impeding p53 apoptosis (Kurrey et al., 2009; Mani et al., 2008).

SNAI2 (Slug) is also a zinc-finger transcription factor that has activities similar to Snail, including E-cadherin transcriptional repression and anti-apoptotic activity, and it plays a crucial role in organogenesis and neuralization. Furthermore, Slug deficiencies are present in newborns with neural tube defects. Slug expression is increased in patients with melanoma, lung, colon and ovarian cancers (Elloul et al., 2005). More aggressive forms of breast cancer, such as basal-like carcinoma, are a phenotype that expresses higher levels of Slug activity (Storci et al., 2008).

Snail and Slug are linked to tumor progression and invasiveness by their ability to alter E-cadherin and vimentin gene expression (Bolos et al., 2003; Come et al., 2004; Lee et al., 2008). Both Snail and Slug are direct repressors of E-cadherin and act by binding to the specific E-boxes of E-cadherin's proximal promoter (Seki et al., 2003). When Snail was overexpressed in epidermoid cancer cells, E-cadherin expression was lost with a concomitant change in cell morphology to a fibroblastic phenotype and vimentin gene expression was upregulated, which indicated that Snail induced an EMT (Yokoyama et al., 2003). Overexpression of Slug and Snail in MDCK cells down-regulates Claudin-1 at both protein and mRNA levels. In addition, Snail and Slug are able to effectively repress human Claudin-1-driven reporter gene constructs (Martinez-Estrada et al., 2006).

Expression of Slug and Snail are downstream mediators of epidermal growth factor receptor (EGFR)-stimulated re-epithelization (Kusewitt et al., 2009). The levels of Slug and Twist (another transcription factor) increased in cancerous tumors, whereas Snail levels are reduced (Yu et al., 2010). The human MCF-7 breast cancer cell line normally has low levels of Snail or Slug. However, when these cells are ectopically transfected with Snail or Slug, several genes involved in cell migration/invasion through the transforming growth factor β (TGF-β) signaling pathway are upregulated, while genes responsible for differentiated morphology (i.e. cell junction molecules, E-cadherin) are downregulated (Dhasarathy et al., 2011).

Phospholipase D (PLD) is a membrane protein that has specific lipid hydrolase activity that breaks phosphatidylcholine (PC) into choline and phosphatidic acid (PA) (Frohman et al., 1999). PA is used by cells for the regulation of chemotaxis and cell proliferation, as a lipid second messenger. PLD also has an important role in cell signaling through protein–protein interactions with GTPases, kinases and phosphatases, as well as through the production of PA (Hammond et al., 1997).

There are at least 4 PLD mammalian isoforms, PLD1, PLD2, PLD4 and PLD6 (Ipsaro et al., 2012; Nishimasu et al., 2012; Park et al., 1998a, 1998b; Yoshikawa et al., 2010). PLD2 has been shown to be heavily involved in tumorigenesis and cancer metastasis, especially as PLD2 expression and activity are drastically increased in many cancers (Chen et al., 2003; Cho et al., 2008; Kantonen et al., 2011; Knapek et al., 2010; Nishikimi et al., 2009; Noh et al., 2000; Riebeling et al., 2003; Saito et al., 2007; Sanematsu et al., 2013; Yamada et al., 2003; Zhao et al., 2000). Additionally, there also is a correlation between high PLD2 expression, increased tumor size and poor patient prognosis (Saito et al., 2007).

We set out in this study to investigate if the PLD2 promoter activity was regulated by two zinc-finger transcription factors, Slug or Snail. We show here that PLD2 expression was negatively regulated by the transcription factor Snail and positively regulated by Slug, which is important for low levels of breast cancer cell invasiveness. We also show that Snail suppressed PLD2, unless PA levels in cells was sufficiently abundant in which case the situation was reversed. Therefore, if PLD2 was found in high levels within the tumor cells, then this correlated to the more metastatically invasive cancer cells.

2. Materials and methods

2.1. Plasmid DNAs

The plasmids were as follows: pcDNA3.1-myc-PLD2-WT, which was designed in our lab, pEGFP-C2-Snail-WT was from Addgene.com (Plasmid #16225) (Zhou et al., 2004), pcDNA3.1+-myc Slug-WT was from Addgene.com (Plasmid #31698) (Kajita et al., 2004).

2.2. Cell culture and transfection of cells

The highly aggressive human breast cancer cell line MDA-MB-231 and the less aggressive MCF-7 cell line were obtained from ATCC (Manassas, VA, USA). MDA-MB-231 cells have the phenotype ER−/PR−/HER−, and are endocrine non-responsive. MCF-7 cells are luminal, have the phenotype ER+/PR+/−/HER− and are endocrine responsive. COS-7 cells are monkey kidney fibroblasts and HMECs are non-cancerous human mammary epithelial cells. All cell lines were obtained from ATCC (Manassas, VA, USA). COS-7, MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). HMECs were cultured in complete human mammary epithelial cell media. Cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. Cells were seeded in 6-well plates with an equal number of cells per well. Cells were then allowed to grow for 12–24 h prior to transfection. Plasmid transfection reactions included 1–2 μg of DNA plasmid and 1 μg DNA:2 μl volume of Transit-2020 transfection reagent in 300 μl of Opti-Mem Serum-Free media.

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