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14. ABSTRACT
We hypothesize that the transcriptional repressor protein SLUG down regulates some key components of the dynein/dynactin pathway of RTK internalization in human breast tumor cells resulting in increase in the surface levels of these receptors, thus adding to the mechanisms of malignant transformation of these cells. The specific aims of the research are (a) To evaluate the nuclear levels of SLUG in relation to the surface levels of RTKs in human breast cancer cells of different grade and pathology by tissue microarray analysis; (b) To evaluate the levels of mRNAs of the member proteins of the dynein and dynactin complexes in breast cancer cells with different levels of SLUG protein; and, (c) To evaluate whether knock down of individual SLUG-target proteins in the dynein/dynactin pathway increases the levels of RTKs on the cell surface in the SLUG-positive human breast cancer cells and thus increases the rate of proliferations of these cells.

15. SUBJECT TERMS
Breast cancer, EGFR, SNAI2 (SLUG), DCTN5 (dynactin 5)

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Repression of RTK Recycling Pathway by SLUG in Human Breast Tumor Cells

Gautam Chaudhuri, PhD

INTRODUCTION

Internalization and subsequent trafficking of receptor tyrosine kinases (RTKs) play an important role in the modulation of growth factor–stimulated signaling events that affect different cellular processes, from cell growth and mitosis to motility and invasion [1]. For example, repressions of the key components of the RTK recycling pathways will increase in the retention of RTKs on the cell surface leading to the increased rate of proliferation of the cells. This application postulates that the transcriptional repressor protein SLUG (also known as SNAI2) [2] inhibits the expressions of some of the key components of the receptor recycling pathways [3] in human breast cancer cells and thus adds to the increased invasiveness and malignancy of these cells. SLUG is often associated with the invasive and malignant properties of human breast tumor cells

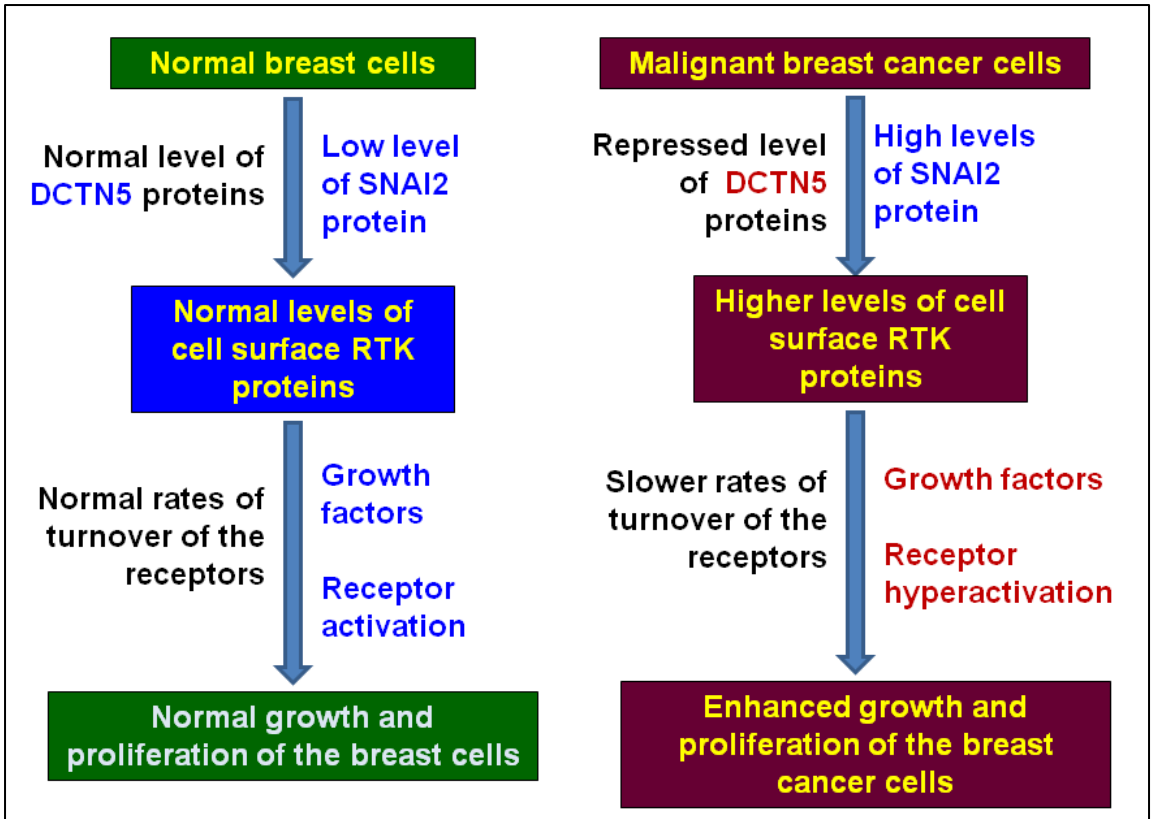

Fig. 1. Summary of our hypothesis.

but its potential to add to the retention of cell surface RTKs in these or other cells have never been explored. The focus of the proposal is to explore the role SNAI2 in the modulation of the dynactin/dynein pathway of vesicular trafficking of RTKs specifically those of EGFR family. Both dynein and dynactin are large protein complexes with multiple subunits [4]. To modulate

the functioning this complex, SNAI2 perhaps needs the down regulation of the expression of one or few of the critical components of these complex proteins.

Hypothesis/Rationale/Purpose: The hypothesis for the proposed research is that the transcriptional repressor protein SLUG down regulates some key components of the dynein/dynactin pathway of RTK internalization in human breast tumor cells resulting in increase in the surface levels of these receptors, thus adding to the mechanisms of malignant transformation of these cells. The anticipation is that SLUG will not compromise with the critical housekeeping functions of the cancer cells by repressing some major functional components of the pathway. Rather it will down regulate some minor regulatory subunits such as dynactin 5 to control the internalization of the RTKs (see Fig. 1).

Objectives: The specific aims of the proposed research are (a) To evaluate the nuclear levels of SLUG in relation to the surface levels of RTKs in human breast cancer cells of different grade and pathology by tissue microarray analysis; (b) To evaluate the levels of mRNAs of the member proteins of the dynein and dynactin complexes in breast cancer cells with different levels of SLUG protein; and, (c) To evaluate whether knock down of individual SLUG-target proteins in the dynein/dynactin pathway increases the levels of RTKs on the cell surface in the SLUG-positive human breast cancer cells and thus increases the rate of proliferations of these cells.

Significance/Relevance: Breast cancer is a collection of diseases caused by misregulations of multiple pathways in the breast cells [5]. The proteins of the SNAI family of transcriptional repressors such as SLUG are often associated with the aggressiveness of breast cancer [2]. This proposal will evaluate the potential role of SLUG over expression in the breast cancer cells in increasing the retention of RTKs on the cell surface by the modulation of the dynein/dynactin mediated endocytic pathway of receptor recycling. This study thus, has the potential in identifying new prognostic markers for aggressive breast cancer and will add to the development of rational combative measures against this fatal disease.

BODY

Task outlined in the approved Statement of Work for this period of the project

Task#1

To evaluate the nuclear levels of SLUG in relation to the surface levels of RTKs in human breast cancer cells of different grade and pathology by tissue microarray analysis (Months 1-3)

Task#2

To evaluate the levels of mRNAs of the member proteins of the dynein and dynactin complexes in breast cancer cells with different levels of SLUG protein (Months 4-7)

Task#3

To evaluate whether knock down of individual SLUG-target proteins in the dynein/dynactin pathway increases the levels of RTKs on the cell surface in the SLUG-positive human breast cancer cells and thus increases the rate of proliferations of these cells (Months 8-12)

Research accomplishments: We studied EGFR (ErbB1) as the model RTK for this study. Some of the data generated from this pilot study are highlighted below. Standard techniques, as described in our recent publications [6, 7], were employed for the experiments described

1. **EGFR (ErbB1) level is higher on the surface of the SNAI2-high breast cancer cells. Dynactin** or **Dynein activator complex** is a multi-subunit protein found in eukaryotic cells that aids in bidirectional intracellular organelle transport by binding to dynein and Kinesin II and linking them to the organelles to be transported. The dynactin complex visualized by deep etch electron microscopy appears as a short filament 37-nm in length, which resembles F-actin, plus a thinner, laterally oriented filament that terminates in two globular heads.The dynactin complex consists of three major structural domains: (1) sidearm-shoulder: DCTN1, DCTN2/ dynamitin, DCTN3/p22/p24;(2)the Arp1 rod: Arp1/centractin, actin, CapZ; and (3) the pointed end complex: Actr10/ Arp11, DCTN4/p62, DCTN5/p25, and DCTN6/p27. Cytoplasmic dynein, which has a molecular mass of about 1.5 Megadaltons (MDa), contains approximately twelve polypeptide subunits: two identical "heavy chains," 520 kDa in mass, which contain the ATPase activity and are thus responsible for generating movement along the microtubule; two 74 kDa intermediate chains which are believed to anchor the dynein to its cargo; four 53-59 kDa intermediate chains and several light chains which are less understood. Cytoplasmic dynein has the following component proteins: (1) heavy chain: DYNC1H1, DYNC2H1; (2) intermediate chain: DYNC1I1, DYNC1I2; (3) light intermediate chain: DYNC1LI1, DYNC1LI2, DYNC2LI1; and (4) light chain: DYNLL1, DYNLL2, DYNLRB1, DYNLRB2, DYNLT1, DYNLT3. While working on several human breast cancer cell lines obtained from ATCC, we noticed that the cells that have high levels of the transcriptional repressor protein SNAI2 seem to multiply in a higher rate than those that have no or low SNAI2. We postulated that the SNAI2-high cells may have higher levels of RTKs that promote their aggressive proliferations (see Fig. 1). We chose to explore the surface expressions of EGFR (ErbB1) as representative RTK for our study. We evaluated the surface levels of these proteins by flow cytometry, as described [8] as well as by immunofluorescence microscopy [9-11]. Our data suggest that indeed there is a direct correlation between the levels of the SNAI2 and the surface levels of EGFR (Figs. 2-4, Table 1). Of interest is the observation that over expression of SNAI2 in the SNAI2-negative breast cancer cells MCF7 and T47D increased the surface levels of EGFR and PAR1 while knockdown of both SNAI1 and SNAI2 (using a siRNA targeting the common SNAG domain sequence) in SNAI2-positive BT549 and MDA-MB-231 cells decreased the levels of such receptor proteins (Table 1).

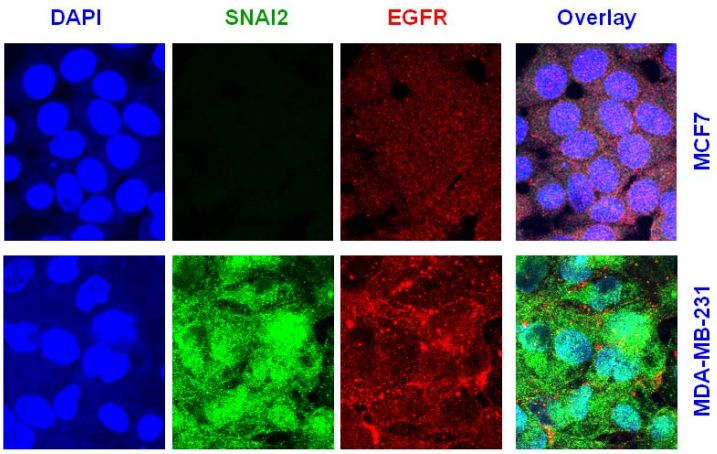

Fig. 2. Wide-field immunofluorescence microscopy showing levels of SNAI2 and EGFR (ErbB1) in MCF7 and MDA-MB-231 cells.

2. ChIP-DSL analysis suggests several targets for SNAI2 including DCTN5. In order to identify correlations between SNAI2 and high levels of growth factor receptors, we postulated that SNAI2 must be somehow preventing the turnover of the RTKs through the repression of the proteins responsible for the turnover mechanisms. Rather than checking for possible candidates, we determined the gene promoters that bind to SNAI2 in human breast cancer cells by promoter array analysis following ChIP-DSL (Aviva) techniques [12] [Mittal, M. and Chaudhuri, G, communicated]. We further validated the regulations of 30 of those genes by SNAI2 in several human breast cancer cells through qRT-PCR, Western blotting and promoter activity analyses (see Table 2). Among the genes that are regulated by SNAI2 is a protein implicated in the retrograde turnover of ligand activated growth factor receptors, dynactin 5 (DCTN5). Another interesting aspect of the finding is that DCTN5 is expressed from the reverse activity of the bidirectional promoter of the PALB2 (partner and localizer of BRCA2) gene whose product regulates the tumor suppressor protein BRCA2. Both PALB2 and DCTN5 are repressed by the SNAI2 protein (Table 2).

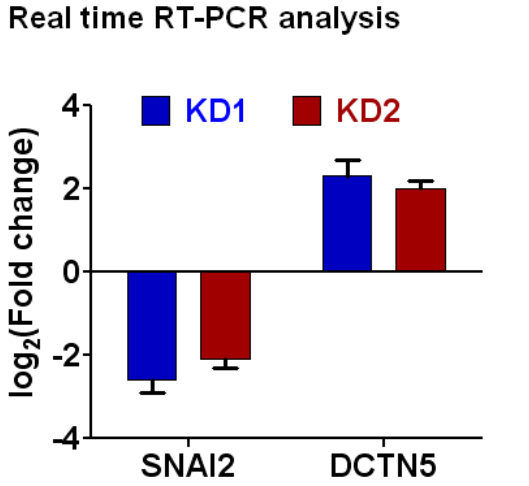

Fig. 3. Effect of knockdown of SNAI2 on the level of DCTN5 mRNA in BT549 cells. Two different stealth siRNAs (KD1 and KD2 were used along with scrambled siRNAs as controls.

3. Further validation that DCTN5 gene expression is regulated by the SNAI-proteins in human breast cancer cells. SNAI2 regulates the DCTN5 gene promoter (Fig. 5) that contains E2-box sequences (CAGGTG/CACCTG), the classical binding site of SNAI2 [13-16]. We evaluated DCTN5 levels in the SNAI2 knocked down cells; we found that DCTN5 mRNA and protein levels are increased significantly in these cells (Fig. 3 and Fig. 4). We found that the promoter of human DCTN5 gene has at least one potential SNAI2 binding E2-box. We cloned these promoters in front of *Renilla* luciferase gene in pRL-Null vector and evaluated the promoter activities with or without knock down of SNAI2 mRNA in the BT549 cells. As expected, DCTN5 promoter activity went up when SNAI2 mRNA was knocked down (Fig. 6). We also have shown by ChIP assay that SNAI2 is specifically recruited at the DCTN5 gene promoter in BT549 cells (Fig. 7). Similar observations were made with SNAI1

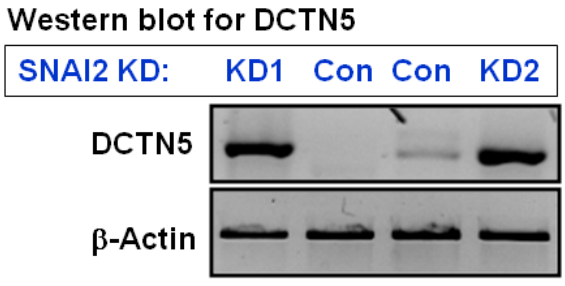

Fig. 4. Effect of knockdown of SNAI2 on the level of DCTN5 protein in BT549 cells. Two different stealth siRNAs (KD1 and KD2 were used along with scrambled siRNAs as controls.

knocked down BT549 cells. Our additional analysis of several human breast cancer cell lines indicated direct correlation among SNAI2 protein level and DCTN5 protein levels of these cells (Table 1). Protein levels were determined by Western blotting. Bands were developed using donkey anti-rabbit IR Dye 800 (LI-COR biosciences) secondary antibody, and visualized using LI-COR's Odyssey Infrared Imaging System. Quantitation and analysis of bands were performed using Odyssey's software. Of particular relevance to this proposal is our observation that expression of SNAI2 in SNAI2-negative breast cancer cells (MCF7 and T47D) or siRNA-mediated knockdown of the SNAI2 protein in SNAI2-positive cells (MDA-MB-231 and BT549) altered the levels of DCTN5 in these cells, as expected (Table 1).

4. Breast cancer tissues expressing SNAI2 protein are low in DCTN5. We evaluated the levels of SNAI2 and DCTN5 in normal and tumor tissue of breast cancer patients by tissue microarray analysis (http://www.biomax.us/tissue-arrays/Breast/). Variable expressions of these three genes was detected in all 60 matched tumor and normal tissues studied. SNAI2 was

Table I. Relative levels of SNAI2, EGFR (ErbB1), and DCTN5 in difference breast cancer cell lines.

Cell Line	SNAI2 level	EGFR level	DCTN5 level
MDA-MB-231	+++++	++++	-
BT549	++++	++++	-
Hs578T	+++	+++	-
MDA-MB-468	+	+	++++
MDA-MB-175	-	-	+++
MCF7	-	-	+++
MDA-MB-436	+++	+++	-
ZR-75-1	-	-	+++
T47D	-	-	+++
BT474	-	-	+++
SKBR3	-	-	+++
MCF7/SNAI2	++++	++++	-
T47D/SNAI2	++++	++++	-
549/SNAIKD	+	+	+++
231/SNAIKD	+	+	+++
AU565	-	-	+++
HCC1806	+++	+++	+
HCC70	++++	+++	-
UACC812	-	-	+++
HCC1937	+	+	+++
HCC1954	+	+	+++
ZR-75-30	-	-	+++

expressed at higher level in tumor *versus* normal tissue (T:N>2.0) in 68% of patients, while DCTN5 was down regulated (T:N<0.3) in 73%. Consistently with data obtained in cultured cells, we found a statistically significant inverse correlation between SNAI2 and DCTN5 levels (Spearman correlation coefficient r = -0.27, p = 0.0026).

5. Knockdown of DCTN5 in T47D cells increased the surface levels of EGFR in these cells. To validate that DCTN5 are involved in the surface expression of the EGFR receptors, we knocked down this protein in the SNAI2-negative T47D cells and evaluated the levels of EGFR

on their surface by flow cytometry. Scrambled RNAs were used as controls. Our data verified our notion that DCTN5 is indeed involved in the surface expressions of EGFR (Fig. 8).

Human DCTN5 gene promoter 866 bp (-171 to +695)

cgcgccctaaactccggccaattaaatccaccattcggcctgcgcacgcgc
agccgtagacggccgagcagtggagcgaggccgcctaaaggagagccgcgc
tgtcgatgagtcccagtgggc**cacctg**gagcggaagtggaggagcggccgg
aagtagccggaatctctg**AAAGACTGACCGACTGACTCTGACAGGATCCGG**
GGCTGAGGGAAGGAGGCGGCGGCCATGGAGTTGGGCGAGCTGCTCTACAAC
AAGTCTGAGTACATCGAGACGGTGCGCGGGTCCAGATATGTATCCTCCTCT
TTCCAACCCTGCGTCCCTTTGAGGCCTGGTCGGCGTTCCCAACCTGCCCCT
ACCCCACCAACCCCTGTCCCTTTGGCCATTAGTCCCGGATTATCTAGCGAT
GCCCCGTGTACCGTCTGGCTTTGCTGTTTACTCCGCGCTTGGCCAGTTGAG
GCCTTTTGTATTTATTCCTGATTTTCTCATAGGGGTAAAGTGCCTTCGGGA
GGATAGGACAAGTCCCATCCTGTTCATACGAATTACAGCTCGGACTTCGGG
CCCTTTTACACTGCCTTTTGTATCTGTTAACTTGCGCTAAAAACGATTCGG
TTCTTTTTTTTTGAGGAAGGGGGTTGGGGGGCGGAGACTCTGTCGCCCAGT
CCTGAGGGCCGCGGCGCGATCTCTGCTCACTGCAACCTCCGCCTCCCGGGT
TCAAGCGATTCTCCTGCCTCAGCGTCTCGAGTAGCTGGGATTACAGGCGCC
CGCCACCACGCCCGGCTAATTTTTGTATTTTTTGGTAGAGACGGGGTTTCA
CCGTGTTGGCCAGGCAGGTCCCTAACTCTTGACCT**CAGGTG**ATCGACCCG

Fig. 5. Nucleotide sequences of the 866 bp promoter of human DCTN5 gene (NM_032468). The upstream sequences are shown in lower case letters. Exon 1 is highlighted in yellow. Intron 1 (part) is shown non-highlighted in upper case letters. The SNAI2-binding E2-boxes are highlighted in red. The translational start codon (ATG) is highlighted in green.

6. Knockdown of DCTN5 in T47D cells increased the proliferation rates of these cells. Knockdown of DCTN5 gene expression in T47D cells increased the rate of their growth in the EGF-containing growth medium (Fig. 9). This data further support the validity of our hypothesis.

REPORTABLE OUTCOMES: We have not yet published or presented the research performed in this grant project. But the research performed in this project directly or indirectly contributed to the following publications and poster abstracts.

Publication:

1. Misra, S. Sharma, S., Agarwal, A., Khedkar, S. V., Tripathi, M. K., Mittal M. K., and **Chaudhuri, G**. (2010) Cell cycle-dependent regulation of the bi-directional overlapping promoter of human BRCA2/ZAR2 genes in breast cancer cells. ***Molecular Cancer* 9**, 50.

2. Mittal M. K., Singh, K., Misra, S. and **Chaudhuri, G.** (2010) SLUG-induced elevation of D1 cyclin in breast cancer through the inhibition of its ubiquitination. ***J. Biol. Chem***. (In Press)

Meeting abstracts:

The current research on this project directly or indirectly affected the studies performed in the following poster presentations from our lab during 2009-2010:

1. Mittal, M. K., Singh, K. and **Chaudhuri, G.** (2009) Mechanisms of SLUG-induced drug resistance development in breast cancer cells. **Presented as poster at the 2009 San Antonio Breast Cancer Symposium (SABCS) held in Henry B. Gonzalez Convention Center, San Antonio, Texas, USA, December 9-13, 2009.**

2. Bailey, C. K., Mittal, Mukul, Misra, Smita and **Chaudhuri, G.** (2010) Reduction of the invasive phenotype of SNAI-over expressing human breast cancer cells by peptide aptamer-mediated inhibition of SNAI protein functions. **Presented at the AACR Annual meeting in Washington DC on April 17-21, 2010.**

3. Mittal, M. K., and **Chaudhuri, G.** (2010) Repression of alpha-, beta- and gamma-catenin gene expressions by SNAI2 in human breast cancer cells. **Presented at the AACR Annual meeting in Washington DC on April 17-21, 2010.**

4. Hall, Mack III, Misra, Smita, and **Chaudhuri, G.** (2010) Molecular analysis of the physical interactions of *Trypanosoma brucei* BRCA2 with different RAD51 isoforms. **Presented at the ASBMB Annual meeting in Anaheim, CA on April 24-28, 2010.**

Table 2. Genes that are directly regulated by SNAI2 in human breast cancer cells.

Gene Name	Enriched/ control	Gene Name	Enriched/ control	Gene Name	Enriched/ control
KRT8	6.2	ITGA3	4.7	PGDH	5.0
KRT18	5.9	PUMA	4.5	PALB2	4.9
KRT19	5.8	BRCA2	4.3	DCTN5	4.9
CDH1	5.6	CLDN1	4.2	SNAI1	4.4
OCLN	5.4	CLDN3	4.1	JUP	3.7
BAX	5.4	CLDN5	4.1	UbE2D3	3.7
VDR	5.3	CLDN7	4.1	MTCH2	3.4
DSG1	5.2	CLDN11	4.0	BicD1	5.1
DSG2	5.1	CYP2R1	4.1	CAV1	5.2
PCNA	5.1	CYP27B1	3.3	BCAS4	4.8

5. Misra, Smita, and **Chaudhuri, G.** (2010) Regulation of BRCA2 gene expression through CpG methylation of its bi-directional promoter induced by endogenous siRNAs. **Presented at the ASBMB Annual meeting in Anaheim, CA on April 24-28, 2010.**

Conclusion:

The metastatic modulator protein SNAI2 (SLUG) downregulates the expression of the DCTN5 gene in human breast cancer cells through direct binding to its promoter. Relative levels of the cell surface RTK EGFR is directly correlated to the levels of SNAI2 in the breast cells. As DCTN5 a critical component of the dynactin complex which is responsible for

RTK recycling, we postulate that increase in the SNAI2 levels in the metastatic breast cancer cells cause repression of DCTN5 and thus increases the levels of RTKs on breast cell surface. This may explain the increase in the growth rate of aggressive breast cancer cells.

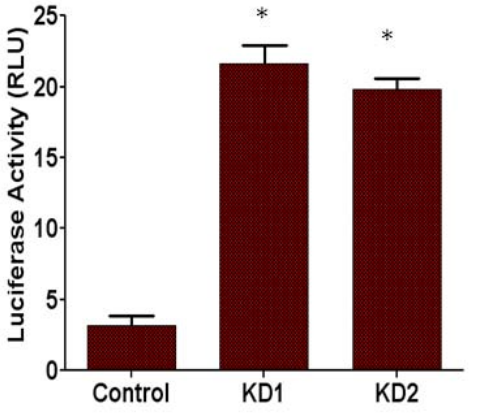

Fig. 6. Effect of SNAI2 knockdown on DCTN5 promoter activity. Results are mean ± SEM (n=6). *p<0.001.

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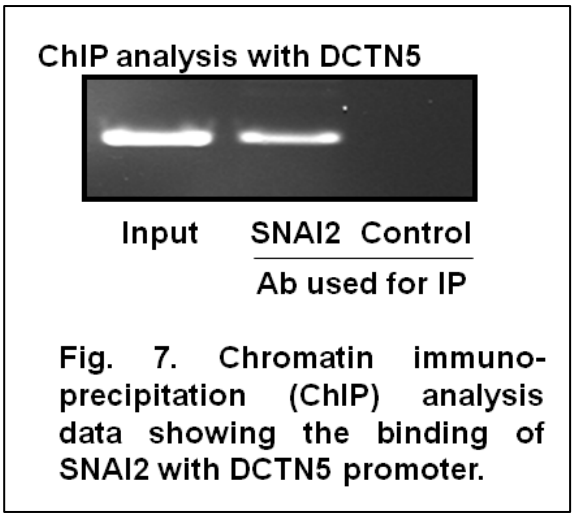

Fig. 7. Chromatin immuno-precipitation (ChIP) analysis data showing the binding of SNAI2 with DCTN5 promoter.

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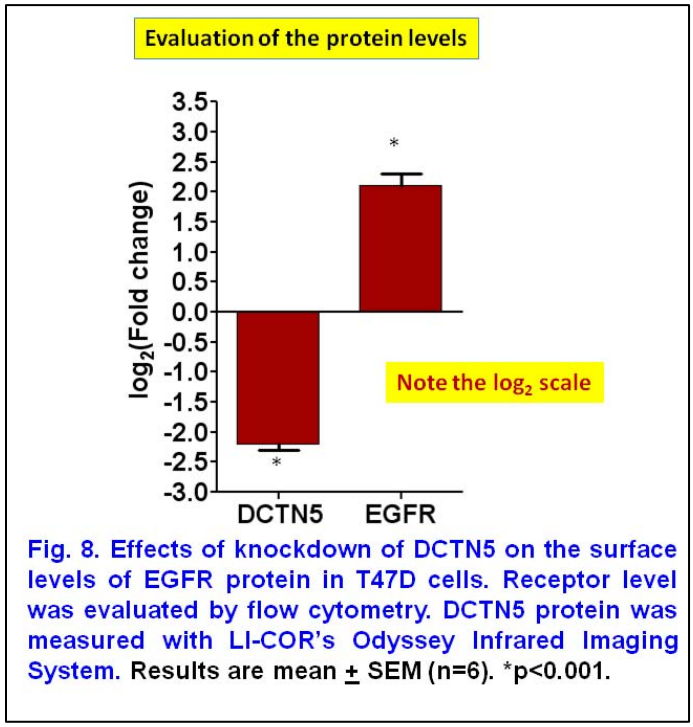

Fig. 8. Effects of knockdown of DCTN5 on the surface levels of EGFR protein in T47D cells. Receptor level was evaluated by flow cytometry. DCTN5 protein was measured with LI-COR's Odyssey Infrared Imaging System. Results are mean ± SEM (n=6). *p<0.001.

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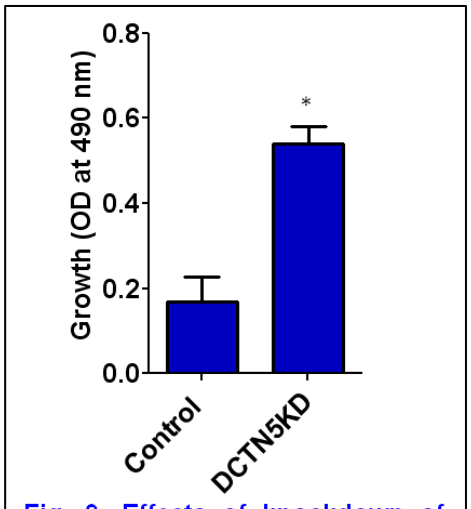

Fig. 9. Effects of knockdown of DCTN5 on the growth rate of T47D cells. Results are mean ± SEM (n=6). *p<0.001.

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APPENDICES: None.