UNIVERSITÀ DEL PIEMONTE ORIENTALE

SCUOLA DI ALTA FORMAZIONE

DOTTORATO IN SCIENZE E BIOTECNOLOGIE MEDICHE

PHD PROGRAM IN SCIENCES AND MEDICAL BIOTECHNOLOGY

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SCUOLA ALTA FORMAZIONE

ANNUAL REPORT

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1. BACKGROUND

Colorectal cancer (CRC) is one of the most common type of cancer in western countries, ranking third leading cause of cancer-related death in both sexes.

Despite improvements in prevention, early detection and life expectancy nowadays still not exist a curative therapy for most of the patients with advanced and metastatic CRC.

In the last decades, basic cancer research tried to pursue a better understanding of cancer progression; a major field of investigation are molecular pathways and micro-environmental factors leading to cancer progression/invasiveness.

Solid tumors, as CRC, are functionally heterogeneous and hierarchical, they are composed of cells that can initiate tumors (Cancer stem cells -CSCs) and cells that arise from CSCs but cannot initiate tumors; this small population of CSCs may have different sensitivities to radiation or chemotherapy. Nowadays, cancer therapy, consisting in radiation and/or chemotherapy, leads to kill the population of differentiated cells that originate from CSCs, but does not eradicate the population of CSCs that can survive and relapse the tumor months/years later. CSCs are a highly tumorigenic rare population of undifferentiated cells, with stem cell-like properties, which is thought to sustain tumor initiation and progression. Like normal adult stem cells, they perpetuate themselves by asymmetric division; feature a high resistance to apoptosis and invasive properties. CSCs are identified experimentally as a CD133-, CD44-enriched cell able to form spheroid in serum-free cultures and to form secondary tumors identical to the original tumor. The elucidation of the molecular circuits sustaining such features i.e. self-renewal, chemo-resistance and invasiveness, is crucial to develop novel therapeutical strategies targeting specifically CSCs.

Diacylglycerol kinases: Diacylglycerol kinases (DGKs) are a multigenic family grouped in five classes of enzymes; each DGK subtype has distinct regulatory motifs that suggest the existence of diverse regulatory mechanisms and/or participation in different signaling complexes. DGKs are characterized by a highly conserved catalytic domain preceded by two C1 domains and distinct regulatory domain[1].

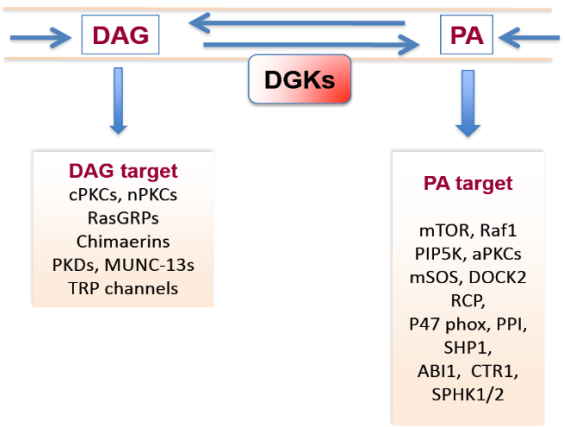

Figure 1: Signaling functions of lipids involved in the diacylglycerol kinase reaction.

DGKs holds the balance of power of two important lipid second messenger (Fig. 1): diacylglycerol (DG) and phosphatidic acid (PA). Indeed, DGKs regulate the levels of both DG and PA and DGKs KO frequently results in constitutive activation of DG-regulated PKCs and Ras-Guanyl nucleotide Releasing Proteins (Ras-GRP) [2]. Furthermore, PA binds and modulate several signaling proteins including:

- protein kinases (aPKCs and mTOR);
- Guanine nucleotide Exchange Factors (GEFs);
- GTPase Activating Proteins (GAPs) for Ras, Arfs, and other signaling enzymes[3].

Type I DGKs is a class of enzymes regulated by extracellular signaling, in fact their structure consist of an amino-terminal calcium binding EF motifs, making these isoforms calcium-responsive to slightly different extents[1]. DGKα is classified as a type I DGKs, is a cytosolic enzyme that, when is active, translocate to the plasma membrane to phosphorylate diacylglycerol. In addition to calcium generation, activation of tyrosine kinases is required for membrane stabilization of DGKα[4].

Activation and membrane localization of DGKα in epithelial cells occurs through interaction of Proline-rich sequence with SH3 domain of Src-family tyrosine kinase followed by its phosphorylation of Y335 located between the C1 and catalytic domain of the human sequence. DGKα mutants in Y335 unable to interact with Src accumulate in intracellular vesicles, suggesting that activation requires Src-dependent vesicular trafficking through the Golgi[5].

In the past years, our laboratory studied the role of DGKα through its inhibition obtained either with isoform-specific DGK inhibitors, or by expression of dominant-negative mutant or by RNA interference in endothelial and epithelial cells. These studies demonstrate that inhibition or down-regulation of DGKα impairs:

- HGF- and VEGF-induced chemotaxis, proliferation and tubular networks of endothelial cells
- cell migration, scattering, invasion, proliferation, anchorage independent growth and tubulogenesis induced by either HGF, vSrc, chemokine or estrogen in either untransformed epithelial cells and carcinomas [5,6]
- matrix invasion sustained by α5β1 integrin recycling and induced by either expression of p53 273H pro-metastatic mutant or inhibition of αvβ3 integrin binding to the ECM[7]

Finally, several screenings identified DGKα as a gene required for distinct biological features. In a siRNA screening, DGKα was required for IGF-1-induced anchorage-independent survival in MCF10A mammary epithelial cells[8]. Additionally, DGKα was also identified as a Klf5-regulated gene required for LIF-induced undifferentiated state of embryonic stem cells [9] leading us to suppose that DGKα may play an important role in self-renewal.

Thymus, spleen, peripheral T lymphocytes, testis, lung[4] and central nervous system are cells/tissues/organs particularly enriched in isoform α of diacylglycerol kinases [10]. Moreover, DGKα is also highly expressed in some cancer type such as:

- **lymphoma**, where it is constitutively activated[11];
- **leukemia**, DGKα consistently overexpressed
- **melanoma**, where it is implicated in suppression of TNFα-induced apoptosis via NFkB[12];
- **breast cancer**, DGKα has been reported to participate in tumor migration and invasion through RCP-dependent integrin trafficking[7]; furthermore there are evidences that DGKα is significantly overexpressed in invasive ductal breast carcinoma[13]
- **glioblastoma**, DGKα play a crucial role in malignant transformation due to its ability to suppress caspase-mediated apoptosis[14];
- **X-linked proliferative disease (XLP)**, studies have reported DGKA inhibition by the adaptor protein SAP. Impaired regulation of DGKα activity in SAP-deficient lymphocytes may contribute to their defective TCR-induced responses[15].

2. PRELIMINARY RESULTS/PURPOSE OF THE PROJECT

Preliminary results:

Based on the role of DGKα in mediating RTKs response and invasion, our laboratory in collaboration with Giorgio Stassi lab addressed the question whether it is required for tumorigenesis and metastasis in orthotopic xenografts in vivo.

Breast cancer stem cells have been infected with lentivirus carrying constitutive shRNA- DGKα/GFP to knock-down protein expression and then orthotopically injected in NON-SCID mice. Results shown in Fig. 2 indicate no tumor growth detected 4 weeks post-injection compared with controls, indicating that DGKα is an absolute requirement for tumor growth of mammary CSCs, moreover CSCs population could be detected in the gland suggesting that they underwent massive apoptosis.

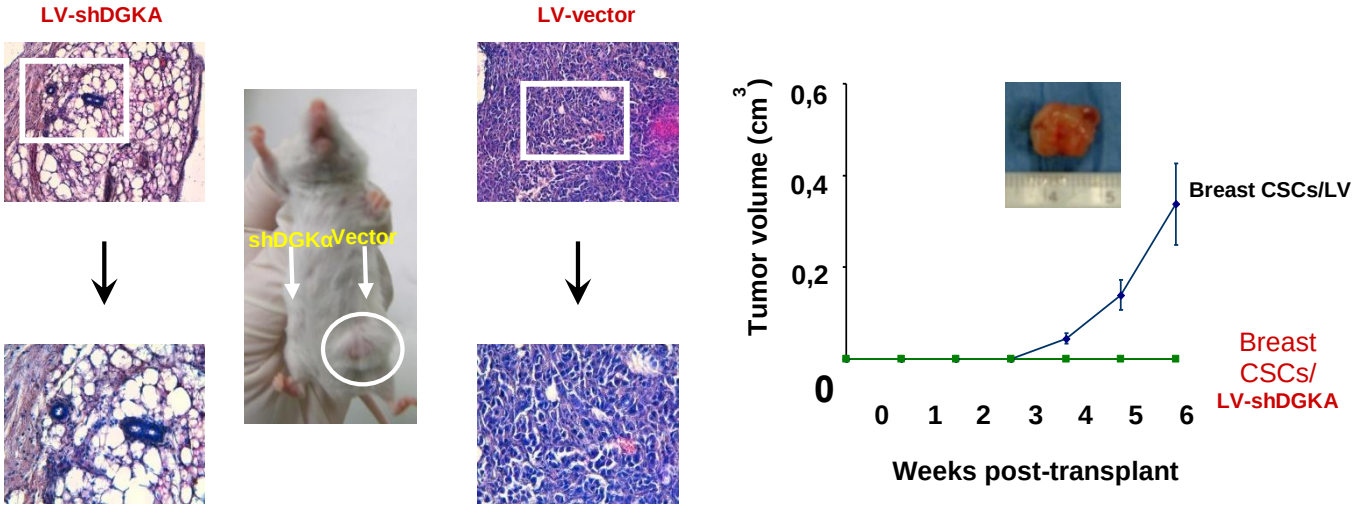

Fig. 2 Down-regulation of DGKα impairs tumor growth

To support these data in parallel we conducted an *in vitro* study of breast cancer stem cells transduced with LV-shDGKα to evaluate migration behavior (Fig. 3A) , epithelial mesenchymal transition-related markers expression compared with controls (Fig 3B) and IL4-mediated chemo-resistance (Fig 4A and 4B).

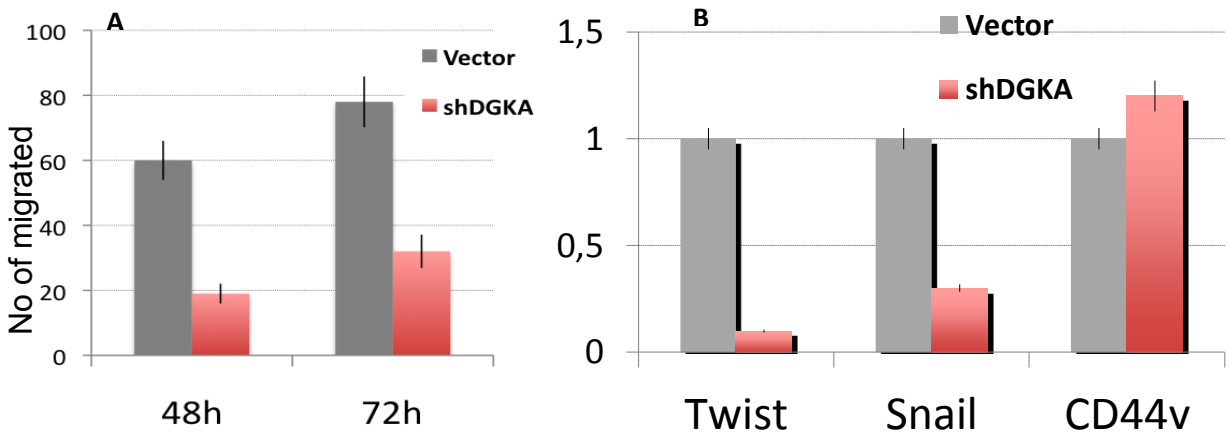

Fig 3: *in vitro* studies on mammary CSCs. A) Transwell migration assay 10.000 cell/well. B) Real time PCR mRNA expression levels of EMT-related transcription factors Twist and Snail in samples extracted 96h post transduction.

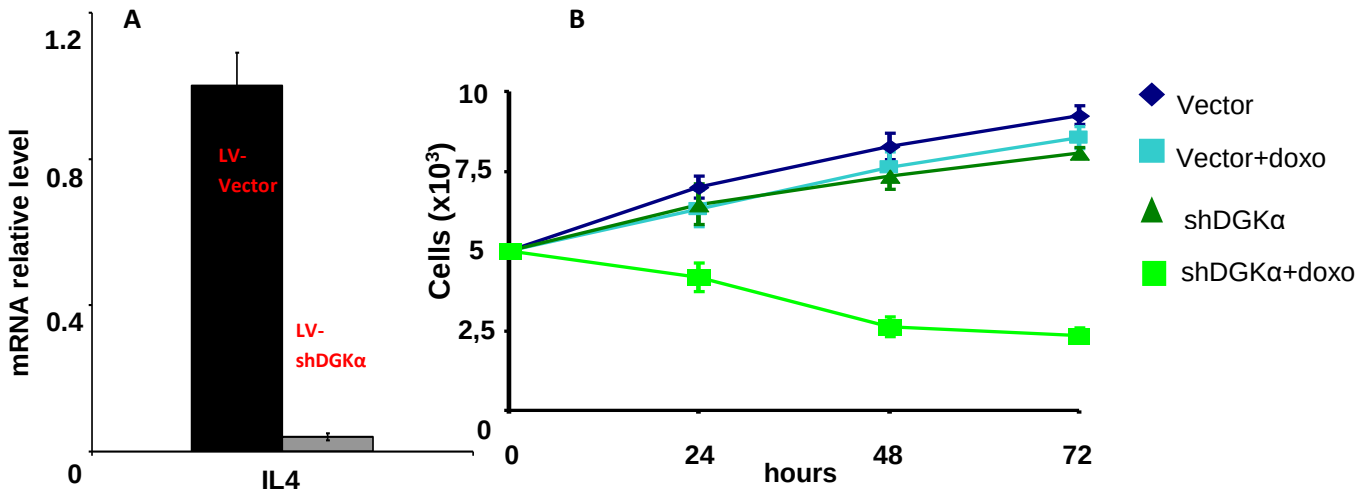

Figure 4: Down-regulation of DGKα impairs IL4 chemo-resistance. A) Real time PCR for IL4 from breast cancer stem cells transduced with LV-shDGKα. B) Chemo-resistance assay of breast cancer stem cells transduced with LV-shDGKα to doxorubicin.

Purpose of the project:

Diacylglycerol kinases (DGK) balance the levels of diacylglycerol and phosphatidic acid (PA), two lipids that lie at the core of several metabolic and signaling pathways involved in cancer progression. Our overall objective is to characterize the implication of the DGK pathway in colorectal cancer physiopathology. We are intent upon achieving this aim through the following objectives:

1. further investigate the signaling network regulated by DGKα in colorectal cancer with particular attention to the pathways regulating integrin signaling and function both in cell adhesion, migration, invasion and survival in epithelial colorectal cell line/CSCs
2. prove in vivo that DGKα indeed plays a role in colorectal cancer stem cells

3. MATERIALS AND METHODS

CELL LINES: primary colorectal cancer stem cell provided by Stassi Lab, cultered in Advanced DMEM/F12 supplemented with B27 50x, N2 100x, EGF, FGF, 0,5%, ultralow attachment flask. Colorectal cell line HCT-116 purchased from the ATCC, cultured in DMEM 10% FBS supplemented 1% AbAm. All cell lines were mainained at 37°C and 5% CO_2.

WESTERN BLOT: for western blot analysis, cells are washed twice with ice cold PBS 1x and lysed in RIPA Buffer (50mM Tris HCl pH7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EDTA, 50mM NaF + protease inhibitor 10x+ 1mM sodium orthovanadate). Lysates are collected in a tube and incubate for 30 min 4°C in a rotating wheel, then centrifuged at 12000 g x 15 min, 4°C. The supernatants are collected in a new tube and quantified with Bradford protein assay kit (Thermo scientific). Samples are denatured in Laemmli buffer 3x and resolved by SDS-PAGE. Proteins are transferred to PVDF membrane and incubated with appropriate antibodies.

ANTIBODIES: Total Erk, Akt, Src, Fak, mTOR, p38, Paxillin and their phosporilate form. Loading control GAPDH.

PLASMIDS AND TRANSFECTIONS: commercial Tet inducible vectors, pTRIPZshDGKα-710, pTRIPZsh-non targeting, pMD2, psPAX2 purchased from Dharmacon.

GENERATION OF VIRAL PARTICLES AND TRANSDUCTION: HEK293T cells are seeded $3{,}5x10^6$ in 10 cm dishes and are transfected with the appropriate lentiviral vector through DNA co-precipitation with calcium phosphate ($Ca_3(PO_4)_2$. Four hours prior transfection media is changed with OptiMEM, the cells are transfected using Calcium phosphate kit (Invitrogen, Life technologies). After overnight transfection, cell medium was replaced with IMDM 10% and incubated for 24h, after which the medium contining viral particles is collected and centrifuged 1500 g, 5 min, 4°C. The supernatant is filtered through 0.45 µm filter. Lentivirus titering is assayed with a transduction efficiency test on cell line HCT116, testing different dilutions. The preferred dilution is selected and then used to infect cell lines, after 24h post infection media is replaced with fresh media plus

500 ng/ml doxycycline, 48h post infection transduction efficiency is controlled with FACS analysis of RFP-positive cells. Establishment of stable and inducible cell lines for DGK knockdown is achieved by western blot analysis and real time PCR.

CLONOGENIC ASSAY: HCT-116 inducted shDGK/shC and HCT-116 wt are seeded in a multiwell-96 ultra-low attachment in CSC media to induce EMT and form colonosphere with stem cell like properties. Cells are detached with Trypsin, resuspended in CSC media and counted. Serial dilutions are prepared to have 100, 60 30, 15 cell/well in 100 µl/well. HCT-116 wt are treated with DMSO/DGKa inhibitor 2 µM. Cells are incubated for 6 days at 37 °C and 5% CO_2 and their growth is followed every day.

MIGRATION ASSAY: 10^5 HCT-116 cells shC/shDGKa or HCT116 Wt cells treated with DGKs inhibitor 2 µM, resuspended in DMEM Serum free, are seeded into transwell (Corning) coated human fibronectin 10 µg/ml, in the lower chamber 10% FBS is used as chemo-attractant. The cells are incubate for at least 24h at 37°C at 5% CO_2 and then the inserts are stained with Diff Quick. Total number of invaded cells are counted under optical microscope.

INVASION ASSAY: 10^5 HCT-116 induced cells shC/shDGKa or HCT116 Wt cells treated with DGKs inhibitor 2 µM, resuspended in DMEM Serum free, are seeded into transwell coated matrigel (BD Biosciences), in the lower chamber 10% FBS is used as chemo-attractant. The cells are incubate for at least 24h at 37°C at 5% CO_2 and then the inserts are stained with Diff Quick. Total number of invaded cells are counted under optical microscope.

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5. ATTENDED SEMINARS XXIX Cycle A.A. 2013-2014

SPEAKER	TITLE	DATE
Prof. SALVATORE OLIVIERO Department of Life Sciences and System Biology, Università di Torino & HuGeF	" ***Epigenetic modifications that control stem cell differentiation***"	19/02/2014
Prof. EMILIO HIRSCH Dipartimento di Biologia Molecolare e Cellulare e Genetica Molecolare, Università di Torino	" ***Role of Phosphoinositides-3-kinase C2-alpha, a Class II PI 3-kinase, in development and cancer***"	19/03/2014
Centro congressi FAST Milano	***"Intellectual property rights: innovation in the life science"***	08/05/14
Miltenyi Biotec Mini MACS Simposio Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	***"Il ruolo emergente delle vescicole extracellulari in fisiopatologia: da mediatori cellulari e biomarker"***	12/05/2014
Prof. Fabrizio Loreni	**"Ribosome alteration in cancer: effect or cause?"**	11/06/14
Dr. Gianni Cesareni	**"Metformin rewires the signaling network of breast cancer cells and changes their sensivity to growth and apoptotic stimuli"**	12/06/14
Prof. Antonia Follenzi Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	"***Terapia Genica"***	19/06/2014
Prof. GIANNI DEL SAL Department of Life Sciences, Università di Trieste LNCIB	"***Disarming mutant P53 in Cancer***"	26/06/2014
Dott. Diego Cotella Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	***"The C-value paradox, junk DNA and ENCODE"***	30/06/2014
Prof. Antonia Follenzi Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	"***Applicazioni Terapia Genica"***	15/07/2014
Prof. John F. McDonald	**"The potential of small regulatory RNAs for the treatment of ovarian cancer"**	16/07/14
Prof. Steven R. Ellis Department of Biochemistry and Molecular Biology, University of Louisville (Kentucky, USA)	***"The Borghese Sessions"***	8-22/09/14
Ciardullo Carmela	**"Clonal evolution and clinical relevance of subclonal mutations in chronic lymphocytic leukemia"**	1/10/14
Famà Rosella	**"The Krüppel-like factor 2 transcription factor is a novel tumor suppressor gene recurrently mutated in Splenic Marginal Zone Lymphoma"**	20/10/14