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Enclosures

## Diacylglycerol Kinase is Required for HGF-induced Invasiveness and Anchorage-independent Growth of MDA-MB-231 Breast Cancer Cells

NICOLETTA FILIGHEDDU<sup>1</sup>, SANTINA CUTRUPÌ<sup>2,3</sup>, PAOLO ETTORRE PORPORATO<sup>2</sup>, FRANCESCA RIBONI<sup>1</sup>, GIANLUCA BALDANZI<sup>2,4</sup>, FEDERICA CHIANALE<sup>2</sup>, ELISABETTA FORTINA<sup>1</sup>, PAOLA PIANTANIDA<sup>1</sup>, MICHELE DE BORTOLI<sup>3</sup>, GIOVANNI VACCA<sup>5</sup>, ANDREA GRAZIANI<sup>2</sup> and NICOLA SURICO<sup>1</sup>

<sup>1</sup>Laboratory of Oncological Gynecology, and <sup>5</sup>Laboratory of Physiology, Department of Clinical and Experimental Medicine, and

<sup>2</sup>Laboratory of Biochemistry, Department of Medical Sciences, University of Piemonte Orientale "Amedeo Avogadro", Novara;

<sup>3</sup>Department of Human and Animal Biology, University of Torino, Torino;

<sup>4</sup>Centro di ricerca E. Menni, Fondazione Poliambulanza-Istituto Ospedaliero, Brescia, Italy

**Abstract.** *Background:* Estrogen receptor (ER)-negative breast cancers have a worse prognosis than ER-positive cancers, being more aggressive and over-exposed to stimuli leading to their progression. Hepatocyte growth factor (HGF) has been associated with proliferation, migration and invasion of tumor cells, and several tumors, including those of breast cancer, produce HGF and overexpress its receptor. Diacylglycerol kinases (Dgks), which phosphorylate diacylglycerol to phosphatidic acid, are key regulators of cell signaling. Our research was focused on their role in HGF-induced invasion of MDA-MB-231 cells, a model of ER-negative breast cancer. *Materials and Methods:* Dgk activity was evaluated with a kinase assay, MDA-MB-231 cell invasion via culturing of cells in matrigel-coated transwells, and anchorage-independent growth was assessed using a soft agar assay. *Results:* HGF induces Dgk activation in MDA-MB-231 cells that is required for cell invasiveness. Moreover, Dgks are involved in MDA-MB-231 anchorage-independent growth. *Conclusion:* Dgks could be a target for ER-negative breast cancer therapy.

Breast tumors expressing estrogen receptors (ER) are generally responsive to therapeutic strategies using selective

*Correspondence to:* Nicoletta Filigheddu, Department of Clinical and Experimental Medicine, University of Piemonte Orientale "A. Avogadro", Via Solaroli 17, 28100 Novara, Italy. Tel: +39 0321660676, Fax: +39 0321620421, e-mail: nicoletta.filigheddu@med.unipmn.it

*Key Words:* Breast cancer, cMet, diacylglycerol kinase, HGF, MDA-MB-231 cell line, invasiveness.

ER modulators like tamoxifen and have a better prognosis than ER-negative ones (1). ER-negative breast cancer represents about 30% of invasive breast cancer and is more aggressive (2). The ER-negative tumors, unresponsive to anti-estrogens, are often overexposed to stimuli leading to their progression.

Hepatocyte growth factor (HGF) plays a well-known role in the process of tumor invasion and metastasis. HGF stimulates proliferation, dissociation, migration and invasion in a wide variety of tumor cells, and is a potent angiogenic factor (3, 4). Stromal fibroblasts are the main source of HGF, however, several tumor cells have been shown to produce HGF (5, 6). HGF elicits its biological functions through the binding to its specific receptor, c-Met. The role of HGF and c-Met in human cancer metastasis is well established: the presence of c-Met has been reported in tumors of the thyroid, ovary, pancreas, breast, prostate and gastrointestinal tract (7-10) and its level of expression has been correlated with tumor progression and poor outcome in breast cancer patients (11, 12). In addition, c-Met has also been reported to be an independent prognostic factor in breast cancer (13).

Diacylglycerol kinases (Dgks), which phosphorylate diacylglycerol to generate phosphatidic acid (PA), comprise a family of ten distinct enzymes, grouped in five classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich C1 domains (14). It has been shown that Dgk- $\alpha$ , an isoform of class I Dgk, is activated by several growth factors: HGF and VEGF in epithelial and endothelial cells (15, 16), and IL-2 activation in T-cells (17). Inhibition of Dgk- $\alpha$  activity, obtained either pharmacologically or by expression of dominant negative mutant or by RNA interference, impairs

HGF-, VEGF- and ALK-induced chemotaxis and proliferation in several cell types (15, 16, 18), as well as angiogenesis in endothelial cells (16). Similarly, in T-cells, pharmacological inhibition of Dgk- $\alpha$  severely impairs IL-2-induced G1 to S-phase transition (19).

Here we investigated whether HGF stimulation could induce activation of class I Dgks in the MDA-MB-231 breast cancer cell line and whether Dgk is involved in the invasiveness of MDA-MB-231 cells and their anchorage-independent growth.

### Materials and Methods

**Reagents.** Cell culture medium and reagents were from Gibco (Invitrogen, Carlsbad, CA, USA), recombinant HGF was from Peprotech (London, UK), class I Dgk pharmacological inhibitor R59949 was from Sigma (St. Louis, MO, USA). All reagents were from Sigma, unless otherwise indicated.

**Cell cultures.** MDA-MB-231 (ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco), penicillin (100 u/ml), streptomycin (100  $\mu$ g/ml) and an antimycotic (0.25  $\mu$ g/ml).

**Dgk assay.** Cells were starved in 0.2% FCS for 24 h, treated with 100 ng/ml HGF for 15 min and homogenated with a buffer containing 25 mM Hepes (pH 8), 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM ZnCl<sub>2</sub>, 50 mM ammonium molybdate, 10 mM NaF, 1 mM sodium orthovanadate and Protease Inhibitor Cocktail. Homogenates were collected, passed through a 23 G syringe and centrifuged at 500xg for 15 min at 4°C. Protein concentration was determined using the BCA method (Pierce, Rockford, IL, USA). Homogenates were incubated for 5 min with a saturating substrate concentration (1 mg/ml diolein, Fluka), 5 mM ATP, 3  $\mu$ Ci/ $\mu$ l ( $\gamma$ -<sup>32</sup>P)-ATP (GE Healthcare), 10 mM MgCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, 1 mM EGTA in 25 mM Hepes (pH 8) in the presence or absence of 1  $\mu$ M R59949. Lipids were extracted as described elsewhere (20), and PA was separated using TLC in chloroform:methanol:water: 25% ammonium hydroxide (60:47:11:4). TLC plates had been previously coated with potassium oxalate 1.3%, EDTA 5 mM:methanol (3:2) and dried. (<sup>32</sup>P)-PA was identified by co-migration with non-radioactive PA standards stained by incubation in an iodine chamber. Radioactive signals were detected and quantified with the GS-250 Molecular Imager and Phosphor Analyst Software (Bio-Rad, Hercules, CA, USA).

**Cell invasion assay.** MDA-MB-231 cells were plated in the upper part of a transwell, the surface of which was coated with Matrigel (BD Biosciences, Erembodegem, Belgium). The lower chamber was filled with DMEM 0.2% FCS in the presence or absence of 1  $\mu$ M R59949. After 15 min, 50 ng/ml HGF was added and cells were incubated for further 16 h. Non-migrated cells were removed from the upper part of the filters, while cells which migrated through the Matrigel in the lower part of the transwell were stained with Diff-Quik (Baxter, Deerfield, IL, USA) and counted.

**Soft agar assay.** MDA-MB-231 cells were suspended in 0.35% agar in DMEM 1% FCS and then plated (5x10<sup>4</sup> cells/well, 12-well plate) on a layer of 0.7% agar in DMEM 1% FCS in presence of 1 or 10  $\mu$ M

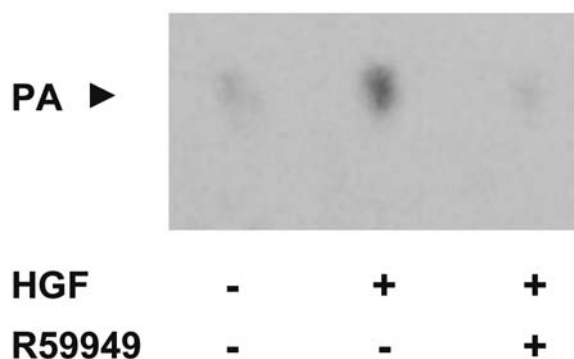


Figure 1. HGF activation of Dgk in MDA-MB-231 breast cancer cells (representative TLC plate) (<sup>32</sup>P)-phosphatidic acid (PA) was separated using TLC and identified by co-migration with non-radioactive PA standards. HGF: hepatocyte growth factor; R59949: Dgk inhibitor.

R59949. Upon 20 days of treatment, the colonies of living cells were stained with MTT and counted with the Quantity One software (Bio-Rad).

**Statistical analysis.** Where appropriate, data are presented as the mean  $\pm$  SEM and the statistical significance was assessed using Student's *t*-test.

### Results

**HGF induced the activation of class I Dgk in the MDA MB-231 breast cancer cell line.** The MDA-MB 231 human breast cancer cell line is often used as a model of ER-negative breast cancer. This cell line is considered particularly suitable for pre-clinical studies since it is highly aggressive both *in vitro* and *in vivo* (21).

As we previously demonstrated that HGF activated Dgk- $\alpha$  in epithelial cells (15), we investigated whether HGF would induce the activation of Dgk in MDA-MB-231 breast cancer cells (Figure 1). Indeed, 100 ng/ml HGF stimulated the activation of Dgk, as measured by its specific kinase activity *in vitro*. The Dgk activity up-regulated by HGF was inhibited when assayed in the presence of 1 mM R59949, a pharmacological inhibitor of class I Dgk.

**HGF-induced invasiveness of the MDA-MB-231 breast cancer cell line.** One of the peculiarities of aggressive tumors is their ability to metastasize. MDA-MB-231 cells, both spontaneously and upon growth factor stimulation, are able *in vitro* to pass through a matrigel layer, mimicking their ability to invade extracellular matrices *in vivo* (22).

In order to provide evidence that Dgk may be involved in the HGF-induced invasiveness of MDA-MB-231 cells, we investigated whether the pharmacological inhibition of Dgk impairs HGF invasive activity in these cells. Indeed, 50 ng/ml HGF in the lower chamber of the transwells promoted the

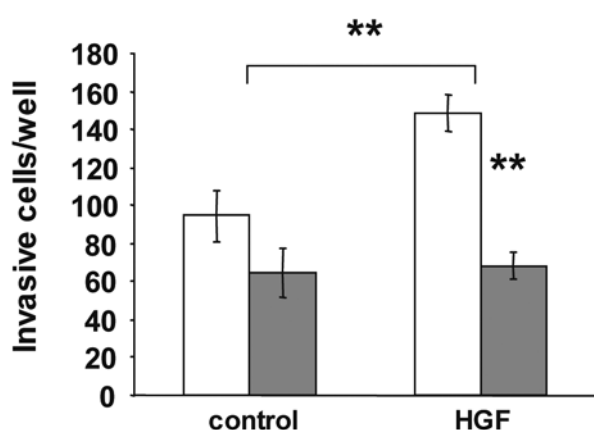


Figure 2. HGF-induced invasion of MDA-MB-231 cells, as shown using a Matrigel assay. Empty bars: without R59949, solid bars: with R59949. \*\* $p < 0.01$  vs. control.

invasion of MDA-MB-231 through a matrigel layer, while pretreatment with 1  $\mu\text{M}$  R59949 for 15 min abolished the HGF-induced invasive capability of the cells (Figure 2).

*Soft agar growth of the MDA MB-231 breast cancer cell line.* The soft agar assay evaluates the ability of cells to form colonies in the absence of adhesion in a semi-solid medium, a feature of neoplastic cells. MDA-MB-231 cells are able to form colonies in soft agar when cultured in 1% FCS. Simultaneous treatment with 1  $\mu\text{M}$  or 10  $\mu\text{M}$  R59949 reduced the number of colonies formed by ~15% and 95% respectively (Figure 3).

## Discussion

Treatment of breast cancer with selective ER modulators is an example of a successful therapy targeting estrogen receptor expression (23). However, its efficacy is limited to ER-positive breast tumors, which generally have a better prognosis (1) than ER-negative tumors. Indeed, ER-negative breast tumors are more aggressive (2), although the processes determining local invasion and the formation of metastases, responsible for their aggressiveness, are not completely understood at the molecular level. Revealing the molecular pathways involved in ER-negative hormone-independent breast cancer progression and metastasis may offer suitable targets for the development of new efficient anticancer therapies.

In recent years, diacylglycerol kinases have been intensively investigated either as negative or positive regulators of cell signaling. It has been shown, for instance, that activation of Dgk- $\alpha$  is required for growth factor-induced proliferative and chemotactic signaling (15-18), as well as for negative feedback in TCR signaling (24, 25).

Here we showed that HGF stimulation induced the activation of class I Dgks in MDA-MB-231, an ER-negative

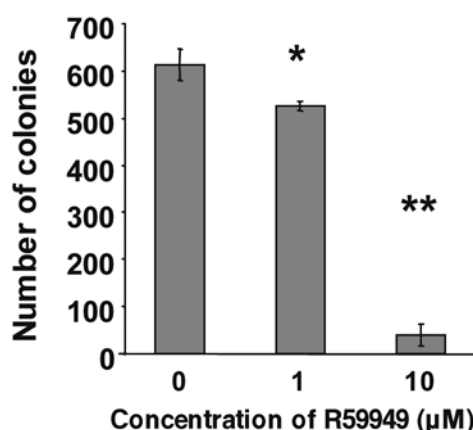


Figure 3. Soft agar growth of MDA MB 231 breast cancer cells (MTT assay). \*\* $p < 0.01$  and \* $p < 0.05$  vs. control.

human breast cancer cell line considered particularly suitable for pre-clinical studies since it is highly aggressive both *in vitro* and *in vivo*. HGF-induced Dgk activation in turn mediated the passage of MDA-MB-231 cells through a matrigel layer, a peculiarity of aggressive cancer cells which mimics the invasion of extracellular matrices and the ability of these cells to metastasize; the pharmacological inhibition of Dgk activity abolished the effects elicited by HGF. Moreover, we showed that Dgk was involved in the anchorage-independent growth of MDA-MB-231 cells, a typical feature of tumor cells, as the inhibition of Dgk activity drastically reduced the number of colonies formed in soft agar.

These results fully demonstrate the biological relevance of Dgk in signaling pathways leading to cell migration elicited by growth factors, and suggest that class I Dgks could be a suitable target for the development of efficient therapies of ER-negative breast cancer.

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## References

- Osborne CK: Steroid hormone receptors in breast cancer management. *Breast Cancer Res Treat* 51: 227-238, 1998.
- Sheikh MS, Garcia M, Pujol P, Fontana JA and Rochefort H: Why are estrogen-receptor-negative breast cancers more aggressive than the estrogen-receptor-positive breast cancers? *Invasion Metastasis* 14: 329-336, 1995.

- 3 Grant DS, Kleinman HK and Goldberg ID: Scatter factor induces blood-vessel formation *in vivo*. Proc Natl Acad Sci USA 90: 1937-1941, 1993.
- 4 Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffe A and Comoglio PM: Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 119: 629-641, 1992.
- 5 Jin L, Fuchs A, Schnitt SJ, Yao Y, Joseph A, Lamszus K, Park M, Goldberg ID and Rosen EM: Expression of scatter factor and c-met receptor in benign and malignant breast tissue. Cancer 79: 749-760, 1997.
- 6 Lamszus K, Laterra J, Westphal M and Rosen EM: Scatter factor/hepatocyte growth factor (SF/HGF) content and function in human gliomas. Int J Dev Neurosci 17: 517-530, 1999.
- 7 Di Renzo MF, Narsiman RP, Olivero M, Bretti S, Giolano S, Medico E, Gaglia P, Zara P and Comoglio PM: Expression of the MET/HGF receptor in normal and neoplastic human tissues. Oncogene 6: 1997-2003, 1991.
- 8 Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pilotti S, Belfiore A, Costantino A, Vigneri R, Pierotti MA and Comoglio PM: Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. Oncogene 7: 2549-2553, 1992.
- 9 Di Renzo MF, Olivero M and Katsaros D: Overexpression of the met/HGF receptor in ovarian cancer. Int J Cancer 58: 658-662, 1994.
- 10 Di Renzo MF, Poulson R and Olivero M: Expression of the met/hepatocyte growth factor receptor in human pancreatic cancer. Cancer Res 55: 1129-1138, 1995.
- 11 Beviglia L, Matsumoto K, Lin CS, Ziober BL and Kramer RH: Expression of the c-met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer 74: 301-309, 1997.
- 12 Camp RL, Rimm EB and Rimm DL: Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer 86: 2259-2265, 1999.
- 13 Ghossein RA, Dillon DA, D'Aquila T, Rimm EB, Fearson ER and Rimm DL: Expression of c-met is a strong independent prognostic factor in breast carcinoma. Cancer 82: 1513-1518, 1998.
- 14 Topham MK and Prescott SM: Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. J Biol Chem 274: 11447-11450, 1999.
- 15 Cutrupi S, Baldanzi G, Gramaglia D, Maffè A, Schaap D, Giraudo E, van Blitterswijk WJ, Bussolino F, Comoglio PM and Graziani A: Src-mediated activation of alpha-diacylglycerol kinase is required for hepatocyte growth factor-induced cell motility. EMBO J 19: 4614-4622, 2000.
- 16 Baldanzi G, Mitola S, Cutrupi S, Filigheddu N, van Blitterswijk WJ, Sinigaglia F, Bussolino F and Graziani A: Activation of diacylglycerol kinase alpha is required for VEGF-induced angiogenic signaling *in vitro*. Oncogene 23: 4828-4838, 2004.
- 17 Flores I, Casaseca T, Martinez-A C, Kanoh H and Merida I: Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. J Biol Chem 271: 10334-10340, 1996.
- 18 Bacchiocchi R, Baldanzi G, Carbonari D, Capomagi C, Colombo E, van Blitterswijk WJ, Graziani A and Fazioli F: Activation of alpha-diacylglycerol kinase is critical for the mitogenic properties of anaplastic lymphoma kinase. Blood 106: 2175-2182, 2005.
- 19 Flores I, Jones DR, Cipres A, Diaz-Flores E, Sanjuan MA and Merida I: Diacylglycerol kinase inhibition prevents IL-2-induced G1 to S transition through a phosphatidylinositol-3 kinase-independent mechanism. J Immunol 163: 708-714, 1999.
- 20 Graziani A, Gramaglia D, Cantley LC and Comoglio PM: The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. J Biol Chem 266: 22087-22090, 1991.
- 21 Price JE, Polyzos A, Zhang RD and Daniels MD: Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 50: 717-721, 1990.
- 22 Jeffers M, Rong S and Vande Woude GF: Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. J Mol Med 74: 505-513, 1996.
- 23 Jordan VC: Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 5: 207-213, 2004.
- 24 Sanjuan MA, Jones DR, Izquierdo M and Merida I: Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. J Cell Biol 153: 207-220, 2001.
- 25 Sanjuan MA, Pradet-Balade B, Jones DR, Martinez-A C, Stone JC, Garcia-Sanz JA and Merida I: T-cell activation *in vivo* targets diacylglycerol kinase alpha to the membrane: a novel mechanism for Ras attenuation. J Immunol 170: 2877-2883, 2003.

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