Filamin-A is involved in stabilization, signal transduction and angiogenesis regulation mediated by Somatostatin Receptor 2 (SST2) in pancreatic neuroendocrine tumors (P-NETs)

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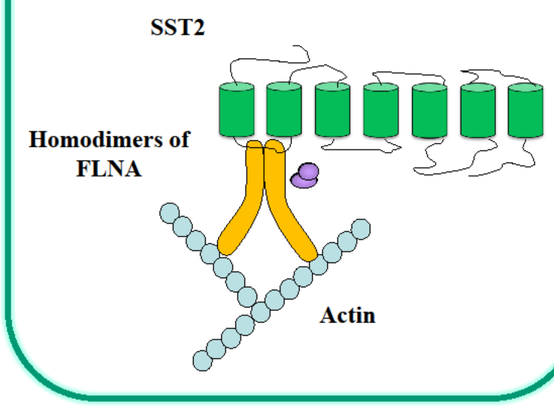
Somatostatin (SS) is an ubiquitous peptide that physiologically inhibits hormone secretion and cell proliferation in neuroendocrine cells (1). These effects are mediated by five receptor subtypes (SSTR1-5) belonging to the G protein coupled receptors superfamily (GPCR) that differ in tissue distribution, affinity to ligands and regulation (2, 3). Somatostatin receptor type 2 (SST2) is the main pharmacological target of long-acting somatostatin analogues (SSA) widely used in patients with pancreatic neuroendocrine tumors (P-NET) (1, 4), this treatment being ineffective in a subset of patients (5), but the mechanisms involved in the resistance are still unknown.

Several studies demonstrated that GPCRs expression and signalling are mediated by different cytoskeleton proteins, including filamin A (FLNA) (6, 7). FLNA is an ubiquitous actin binding protein, that acts as a molecular scaffold of several proteins, including transmembrane proteins and signalling molecules. Recently, FLNA/SST2 interaction has been found to play a critical role for SST2 stabilization and cell signalling (8, 9). Moreover , the involvement of FLNA in angiogenesis has been suggested as a target for anti-neovascular cancer therapy in vitro. In fact, a positive relationship between FLNA and vascular endothelial growth factor (VEGF) was found in patients with lung cancer (10), suggesting that FLNA is implicated in angiogenesis through links with VEGF. Interestingly, it has been demonstrated that VEGF pathway is overexpressed in neuroendocrine tumors (11), this pathway being inhibited by somatostatin analogues (12).

Aim of the present study was to investigate the role of FLNA in the regulation of SST2 stabilization, signaling and angiogenesis in pancreatic neuroendocrine tumours.

FLNA directly interacts with SST2



FLNA is not involved in SST2 expression in P-NETs

Fig. 1

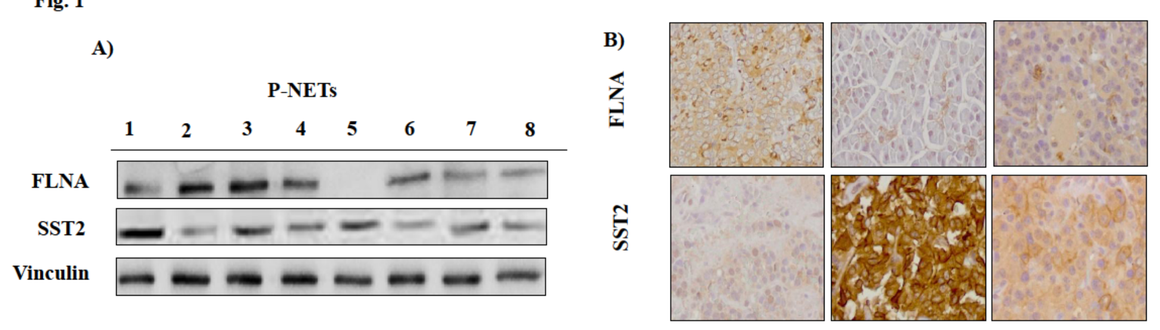


Fig.1 FLNA expression in P-NETs. A) Immunoblots of FLNA and SST2 performed on eight different neuroendocrine tumor samples. FLNA and SST2 antibodies were from Abnova (Taiwan) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The equal amount of protein was confirmed by stripping and reprobing with an anti-vinculin antibody. B) Representative pictures of immunohistochemistry for FLNA and SST2 in different GEP-NETs (20X magnification). FLNA and SST2 pictures in the same column correspond to the same tumor.

MATERIAL AND METHODS

- **Cell culture and silencing:** Short interfering RNA (SiRNA) were purchased from Invitrogen. QGP-1 cells were transfected with 200 pmol of siFLNA, or negative control siRNA (C- siRNA) for 72h, using Lipofectamine 2000 according to the instruction of the manufacturer.
- **cAMP assay:** QGP1 cells transfected with FLNA siRNA or C- siRNA were preincubated with 0.5 mM 3-isobutyl-1-methylxantine (IBMX) for 30 min, and subsequently with 1 mM forskolin with or without the SST2 selective agonist BIM23120 (10 nM) for 30 min at 37°C. Intracellular cAMP was measured by enzymatic immunoassay (Promega, Madison, WI USA).
- **Immunohistochemistry:** was performed on sections from 29 P-NETs retrieved from the archives of Pathology Unit of IRCCS Humanitas Research Hospital, Rozzano, Milan Italy. After dewaxing in Bioclear and rehydrating in ethanol, the sections were pretreated in a water bath set to 98°C in 0.01 M citrate buffer for 25 minutes. FLNA antibody (Millipore, 1:600 diluition) and SST2 (UMB-1; Abcam; 1:200 dilution) was used, and antigen-antibody detection was performed with the MACH1 universal polymer detection *kit* (Biocare Medical).
- **VEGF secretion study:** silenced or non silenced QGP1 cells were treated with or without BIM23120 10 nM in serum free RPMI-1640 medium for 72h at 37°C. Collected supernatants were used to measured VEGF concentration with ELISA kit (Invitrogen, Camarillo, CA), according to manufacturer instructions.
- **Western Blot Analysis:** All samples were separated on SDS-PAGE, and the proteins were detected by Western Blotting using antibodies against FLNA (AbNova), SST2 (Santa Cruz), GAPDH (Ambion), CD1 (Millipore), pERK/tot ERK (Cells Signalling), Vinculin (Cell signalling), VEGF (Abcam). The ratio of Immunoblotting signalling intensity was measured using NIH ImageJ software.

FLNA knockdown reduces SST2 expression after long-term agonist stimulation in QGP1 cells

Fig. 2

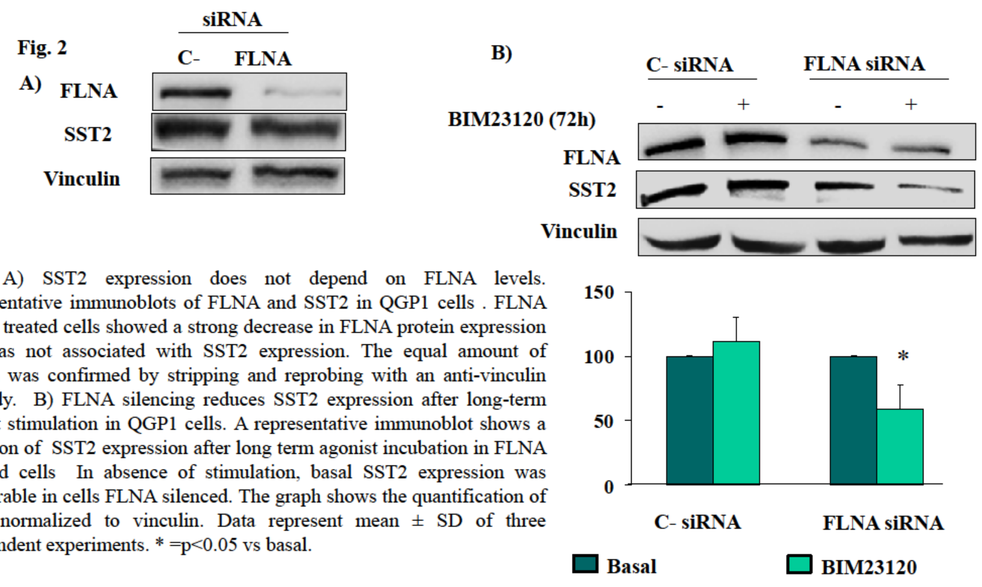
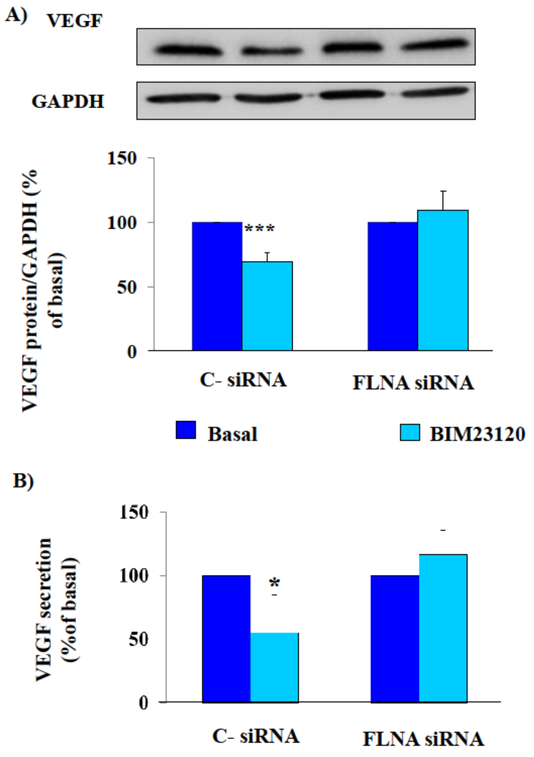


Fig.2 A) SST2 expression does not depend on FLNA levels. Representative immunoblots of FLNA and SST2 in QGP1 cells . FLNA siRNA treated cells showed a strong decrease in FLNA protein expression that was not associated with SST2 expression. The equal amount of protein was confirmed by stripping and reprobing with an anti-vinculin antibody. B) FLNA silencing reduces SST2 expression after long-term agonist stimulation in QGP1 cells. A representative immunoblot shows a reduction of SST2 expression after long term agonist incubation in FLNA silenced cells In absence of stimulation, basal SST2 expression was comparable in cells FLNA silenced. The graph shows the quantification of SST2 normalized to vinculin. Data represent mean ± SD of three independent experiments. * =p<0.05 vs basal.

FLNA is involved in SST2-mediated angiogenesis inhibition

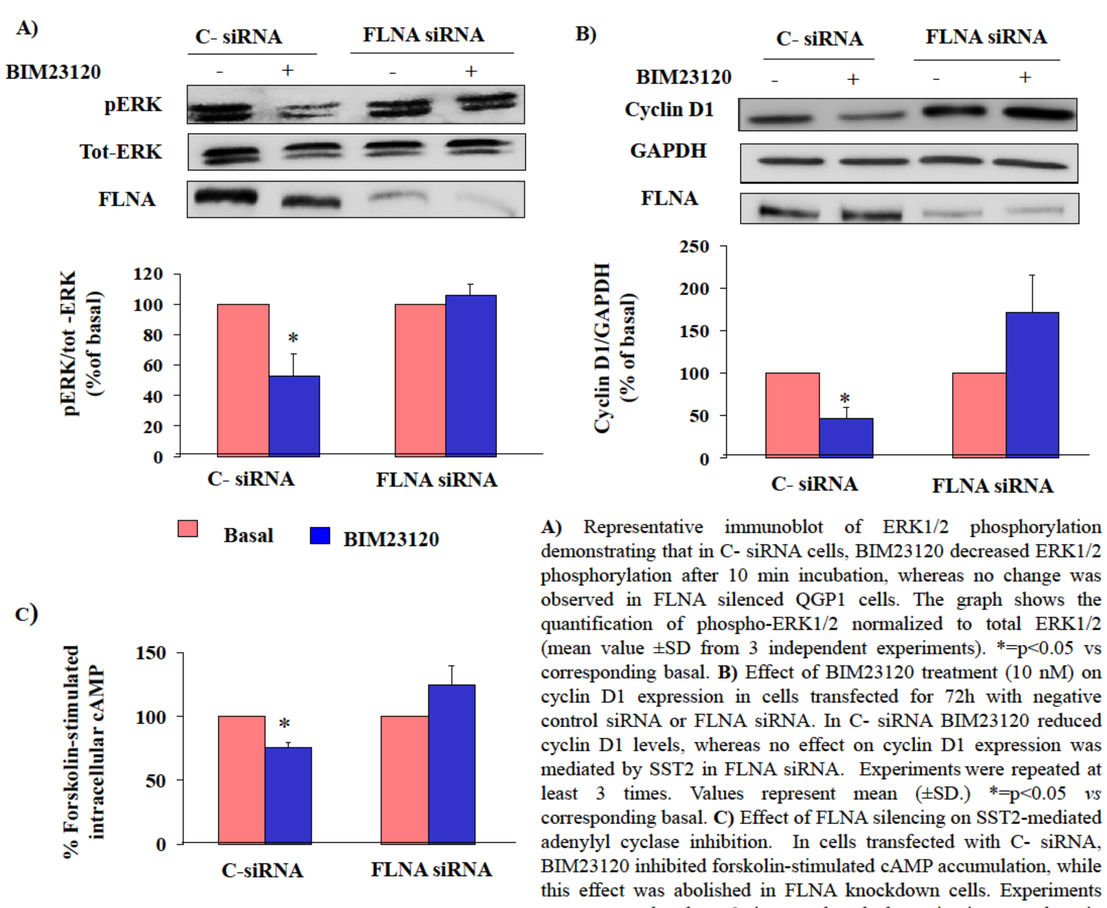
Fig.4



Effects of FLNA silencing on angiogenesis inhibition SST2 mediated in QGP1 cells A) Representative immunoblot of VEGF demonstrating that SST2 inhibitory effect on VEGF mediated by BIM23120 (10nM for 72 h) was present in C- siRNA and abolished in FLNA siRNA transfected QGP1 cells. The graph shows the quantification of VEGF normalized to GAPDH (mean value ±SD from 3 independent experiments). ***=p<0.001 vs corresponding basal. B) Effects of FLNA silencing on SST2 mediated VEGF secretion inhibition. In cells transfected with C- siRNA, BIM23120 (10nM) inhibited VEGF secretion after 72h incubation, while this effect was abrogated in FLNA silenced cells. Experiments were repeated at least 4 times and each determination was done in triplicate. Values represent mean ±SD *=p<0.05 vs corresponding basal

FLNA is required for SST2 signaling in QGP1 cells

Fig.3



A) Representative immunoblot of ERK1/2 phosphorylation demonstrating that in C- siRNA cells, BIM23120 decreased ERK1/2 phosphorylation after 10 min incubation, whereas no change was observed in FLNA silenced QGP1 cells. The graph shows the quantification of phospho-ERK1/2 normalized to total ERK1/2 (mean value ±SD from 3 independent experiments). *=p<0.05 vs corresponding basal. **B)** Effect of BIM23120 treatment (10 nM) on cyclin D1 expression in cells transfected for 72h with negative control siRNA or FLNA siRNA. In C- siRNA BIM23120 reduced cyclin D1 levels, whereas no effect on cyclin D1 expression was mediated by SST2 in FLNA siRNA. Experiments were repeated at least 3 times. Values represent mean (±SD.) *=p<0.05 *vs* corresponding basal. **C)** Effect of FLNA silencing on SST2-mediated adenylyl cyclase inhibition. In cells transfected with C- siRNA, BIM23120 inhibited forskolin-stimulated cAMP accumulation, while this effect was abolished in FLNA knockdown cells. Experiments were repeated at least 3 times and each determination was done in quadruplicate. Values represent mean ±SD. *=p<0.05 *vs* corresponding basal.

CONCLUSIONS

- **FLNA is not required for basal SST2 expression but it stabilizes the receptor expression after long-term agonist stimulation**
- **FLNA is required for SST2-mediated cell proliferation and cAMP accumulation inhibition**
- **FLNA is crucial for SST2-mediated angiogenesis inhibition**

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