Ligand-Dependent Activation of EGFR in Follicular Dendritic Cells Sarcoma is Sustained by Local Production of Cognate Ligands

William Vermi1,7, Emanuele Giurisato2, Silvia Lonardi1, Piera Balzarini1, Elisa Rossi2, Daniela Medicina1, Daniela Bosisio1, Silvano Sozzani1,8, Wilma Pellegrini1, Claudio Doglioni3, Antonio Marchetti4, Giulio Rossi5, Stefano Pileri6, and Fabio Facchetti1

Abstract

Purpose: The aim of this study was to investigate the biological and clinical significance of epidermal growth factor receptor (EGFR) signaling pathway in follicular dendritic cell sarcoma (FDC-S).

Experimental Design: Expression of EGFR and cognate ligands as well as activation of EGFR signaling components was assessed in clinical samples and in a primary FDC-S short-term culture (referred as FDC-AM09). Biological effects of the EGFR antagonists cetuximab and panitumumab and the MEK inhibitor U0126 on FDC-S cells were determined in vitro on FDC-AM09. Direct sequencing of KRAS, BRAF, and PI3KCA was conducted on tumor DNA.

Results: We found a strong EGFR expression on dysplastic and neoplastic FDCs. On FDC-AM09, we could show that engagement of surface EGFR by cognate ligands drives the survival and proliferation of FDC-S cells, by signaling to the nucleus mainly via MAPK and STAT pathways. Among EGFR ligands, heparin-binding EGF-like growth factor, TGF-α and Betacellulin (BTC) are produced in the tumor microenvironment of FDC-S at RNA level. By extending this finding at protein level we found that BTC is abundantly produced by FDC-S cells and surrounding stromal cells. Finally, direct sequencing of tumor-derived genomic DNA showed that mutations in KRAS, NRAS, BRAF, and PI3KCA, which predicts resistance to anti-EGFR MoAb in other cancer models, are not observed in FDC-S.

Conclusion: Activation of EGFR by cognate ligands produced in the tumor microenvironment sustain viability and proliferation of FDC-S indicating that the receptor blockade might be clinically relevant in this neoplasm. 

Cancer Therapy: Preclinical

Introduction

EGFR, a well-characterized member of the ErbB receptor family is normally expressed by epithelial and mesenchymal cells and plays a major role in their proliferation and differentiation by binding a large set of endogenous ligands. The intracellular signal induced through EGFR activation is transmitted by 3 main synergetic cascades including Ras/Raf which activate mitogen-activated protein kinases (MAPK), PI3K/AKT, and JAK/STAT (2). These intracellular pathways ultimately translate extracellular stimuli into a nuclear message to sustain a large set of biological activities, including cell differentiation, survival, and proliferation. As largely showed by numerous preclinical models, unchecked increase of EGFR signaling might also support the transformation of different cell types, as well as progression of carcinomas and gliomas (3–5). In these neoplasms, aberrant EGFR signaling is perpetuated in a ligand-independent fashion by genetic variants inherent to transformed cells (e.g., gene amplification or activating mutations) of the EGFR allele (6, 7). Alternatively, activation of the receptor might also occur in EGFR overexpressing transformed cells with a wild-type configuration of the EGFR allele via binding to endogenous ligands abundantly produced in the tumor microenvironment, as recently showed in soft tissue sarcomas (8).

In the hematopoietic system EGFR is functional only on subsets of monocyte-derived cells, namely osteoblasts and osteoclasts (9). In contrast, all the remaining lineages are...
**Translational Relevance**

Follicular dendritic cell sarcoma (FDC-S) is treated with surgery however a consistent fraction of them relapse. Adjuvant radiation and/or chemotherapy provides no significant benefit in term of disease-free survival. The extreme difficulty to purify human FDC and the rarity of FDC-S have represented a limitation in the understanding of the FDC-S biology. Here, we report that EGF receptor (EGFR) overexpression marks the entire spectrum of FDC-derived neoplastic lesions. By using blocking experiments on a newly developed patient-derived cellular system, we show that the engagement of surface EGFR by EGF drives the survival and proliferation of FDC-S via MAPK and STAT pathways. Among EGFR ligands, heparin-binding EGF-like growth factor, TGF-α, and Beta-cel lulin are produced in the tumor microenvironment. Lack of somatic mutations of KRAS, NRAS, BRAF, and PI3KCA in FDC-S tumor samples predicts sensitivity to anti-EGFR monoclonal antibody and suggests possible candidates for EGFR blockade in this neoplasm.

**Materials and Methods**

**Patients and tissues**

Samples included formalin-fixed paraffin-embedded (FFPE) tissue blocks. Clinical data were obtained from patients’ records. Reactive human lymph nodes and tonsils as well as normal spleen and pancreas were used as control tissues. Pathologic cases were represented by 16 FDC-S cases and 5 cases of hyaline vascular Castleman’ disease (HV-CD). The study has been conducted in compliance with the Helsinki Declaration and with policies approved by the local Ethics Board (Spedali Civili di Brescia, Italy).

**Immunostaining procedures**

Four-micrometer tissue sections from FFPE blocks were used for immunohistochemical staining. Primary antibodies are listed in the Supplementary Material. Proper isotype control or irrelevant antibodies have been included in the protocol. Double immunohistochemistry were conducted as previously reported (18). Immunohistochemistry was interpreted by WV, SL, and FF. For phosphorylated proteins, cases were scored positive when showing main reactivity in the nucleus (for pSTAT3, pAKT, and pERK) or cell membrane (for pEGFR) in >5% of tumor cells. Positive cases were scored based on the percentage of positive cells as follow: + = 5% to 20% of neoplastic cells; ++ = 20% to 50%; +++ = >50%.

**Culture procedure and cytogenetic analysis**

Part of lesion from an FDC-S case was removed under sterile conditions and transferred in RPMI 1640 medium without serum kept at 4°C until transfer to the cell culture facility. For culture, the tissue was finely minced and digested for 24 hours at 37°C in RPMI 1640 containing 0.2% of Collagenase type II. The cells suspension was washed with PBS, plated on T25 flask with complete RPMI 1640 supplemented with 10% FCS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO2. The medium was changed twice a week and after trypsinization, the cells were passed in a new flask for cells expansion and medium was changed twice a week and after trypsinization, the cells were passed in a new flask for cells expansion and cytogenetic analysis. The cytogenetic analysis was conducted on total of 20 cells in G-banded metaphases cells until transfer to the cell culture facility. For culture, the tissue was finely minced and digested for 24 hours at 37°C in RPMI 1640 containing 0.2% of Collagenase type II. The cells suspension was washed with PBS, plated on T25 flask with complete RPMI 1640 supplemented with 10% FCS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO2. The medium was changed twice a week and after trypsinization, the cells were passed in a new flask for cells expansion and morphological analysis.

**FISH**

FISH for EGFR/CEP7 was applied as previously reported (19), by using LSI EGFR Spectrum Orange/CEP7 Spectrum Green probes (Abbott Molecular). Tumor samples were examined with an epifluorescent microscope (Nikon, Eclipse 90i). In each tumor sample, signals were counted in a total of 100 nonoverlapping tumor-cell nuclei. The mean signal number of the EGFR gene as well as CEP7 was calculated and the EGFR/CEP7 ratio determined. Gene amplification was considered for EGFR/CEP7 ratio ≥ 2.

**qPCR**

RNA was extracted using TRIzol reagent (Cat. No. 15 596; Invitrogen) according to the manufacturer’s instructions.
After RNA purification, samples were treated with DNase to remove contaminating genomic DNA (DNaseI amplification grade, Cat. No. 18 068; Invitrogen). Reverse transcription was conducted using random hexamers (Cat. No. 48 190; Invitrogen) and Superscript II reverse transcriptase (Cat. No. 18 964; Invitrogen). Primers are listed in the Supplementary Material. iQTM SYBR Green Supermix (Cat. No. 170-8882; Bio-Rad) was used to run relative quantitative real-time PCR of the samples according to the manufacturer's instructions. Reactions were run in triplicate on an iCyclerTM (Bio-Rad Laboratories Inc.) and generated products analyzed with the iCyclerTM iQ Optical System Software (Version 3.0a; Bio-Rad). Gene expression was normalized based on both GAPDH mRNA and 18S rRNA contents. Data are displayed as 2-ΔΔCT values.

Flow cytometry analysis of EGFR

The cell surface expression of EGFR was determined using flow cytometry analysis using anti-EGFR mAb (BD Transduction Laboratories). Details are reported in the Supplementary Material.

Cell stimulation and immunoblotting

A total of 15 × 10^4 tumor cells were seeded in 6-well plate containing RPMI/10% FBS. After 24 hours cells were starved for 48 hours in RPMI/0.1% FBS and then stimulated with indicated concentration of EGF (Sigma) for 10 minutes at 37°C. Where indicated, FDC-S were incubated in starvation medium with and without anti-EGFR mAb cetuximab (20 μg/mL) for 4 hours at 37°C, before stimulation with 40 ng/mL of EGF. In some experiments, FDC-S were pretreated with the MEK inhibitor UO126 (1 and 10 μmol/L) at 37°C for 30 minutes. After washing cells were lysed and analyzed by immunoblotting as reported in the Supplementary Material.

FDC-S cell-proliferation assay

Cells were assayed for cell-cycle progression by monitoring the incorporation of 5-ethyl-2'-deoxyuridine (EdU) and its subsequent detection by a fluorescent azide through a Cu(1)-catalyzed [3 + 2] cycloaddition reaction (“click” chemistry; Invitrogen) as described in Supplementary Material.

Cell growth analysis

FDC-S cells (5 × 10^3/well) into a 24-well plate) were incubated with cetuximab (0.1, 1, 10, 20, 40, and 100 μg/mL) or left untreated in RPMI/0.1% FBS. Where indicated, FDC-S were pretreated with the MEK inhibitor UO126 (1 and 10 μmol/L) at 37°C for 30 minutes. After 48 hours, cells were stimulated without (-EGF) or with indicated concentration of EGF. After 5 days, FDC-S growth was analyzed by microscopy as described in the Supplementary Material.

Double staining with acridine orange (AO) and ethidium bromide (EB) for apoptosis detection

FDC-S cells (4 × 10^3/well of a 24-well plate) were seeded on gelatin-covered glasses in RPMI/10% FBS for 24 hours. Cells were incubated without (controls) or with anti-EGFR mAb cetuximab (20 μg/mL) in RPMI/0.1% FBS. At day 4, cells were stimulated with 40 ng/mL of EGF without remove the medium. At day 7, plates were centrifuged and cell apoptosis was detected by EB/AO staining procedures as reported in the Supplementary Material.

Direct sequencing

Formalin-fixed paraffin-embedded blocks were reviewed for quality control and tumor content. A representative block, containing from 30% to 80% of malignant cells was selected for each case. Genomic DNA was extracted using QIAamp DNA FFPE Tissue kit (Qiagen). Direct sequencing of EGFR (exons 18, 19, and 21), KRAS (exon 1), NRAS (exons 1 and 2), BRAF (exon 15), and PI3KCA (exons 9 and 20) was conducted on all samples as previously reported (20, 21). All samples were subjected to automated sequencing by ABI PRISM 3130 (Applied Biosystems). Controls were represented by wild-type and mutated cases obtained from colon and lung carcinoma cases as well as from melanoma.

Results

EGFR is strongly expressed by FDCs and their neoplastic counterpart

By characterizing a cohort of FDC-S on the basis of their phenotype, we and others have recently identified EGFR as a highly sensitive marker of neoplastic FDC (10, 17, 22, 23). To further confirm this finding, we applied immunohistochemistry to a more extended cohort of clinical samples including a total of 16 FDC-S as well as reactive lymph nodes and tonsils by using 2 different anti-EGFR antibodies. All FDC-S cases (16/16; 100%) showed strong and diffuse EGFR reactivity in the cytoplasm and on the cell membrane (Fig. 1A and B). The level of tumor heterogeneity of EGFR expression was very limited and most FDC-S cases expressed this marker on more than 75% of neoplastic cells. Remarkably, only a faint reactivity on occasional neoplastic cells was observed for HER2 (not shown), whereas HER4 was weakly expressed on 8 of 15 FDC-S cases (53.3%; Fig. 1C). We tested whether EGFR expression is acquired during neoplastic transformation by extending this characterization to normal and dysplastic FDC. In reactive tonsils, lymph nodes, and spleen, anti-EGFR antibodies specifically stain the 2 major stromal cells populations, namely FDC and fibroblastic reticulum cells (FRC; Fig. 1D and E), as also confirmed by staining of serial sections of reactive lymph node with the FDC marker CD21 and the FRC marker anti-smooth muscle actin (SMA; Supplementary Fig. S1); expect- edly, a strong signal was also observed on surface and crypt epithelium in the tonsils (not shown). These data indicated that EGFR can be expressed by nontransformed lymphoid organ stromal cells. Dysplastic FDCs are commonly observed in the HV-CD, the putative precursor of FDC-S (24–26). We found diffuse expression of EGFR on FDCs, including those characterized by evident dysplastic features, in all HV-CD–tested cases (5 of 5; Fig. 1F). Overall, these data
confirm that in lymphoid organs EGFR expression is restricted to the stromal cell compartment including FDC and FRC and is maintained during neoplastic transformation of FDC.

**High expression of EGFR in FDC-S is not associated to numerical alteration of chromosome 7 or amplification of the EGFR alleles**

Oncogenic addition to EGFR is observed in a large variety of human malignancies (27). In these cases, overexpression of the protein is frequently associated to genetic variants intrinsic to cancer cells. Increased EGFR expression in cancer cells at the protein level might depend on the occurrence of gene amplification. Molecular studies on FDC-S are limited to sporadic cytogenetic analysis reporting multiple chromosomal aberrations. Among the latter, abnormalities involving the chromosome 7 containing the EGFR locus have been reported in form of translocations (28) or tetrasomy (29). By using FISH, we could show that all cases showed eusomy of the chromosome 7 with normal gene copy number of EGFR as measured by EGFR/CEP7 ratio (Fig. 2A–D).

**FDC-S cells proliferation and survival is induced by the activation of EGFR-mediated signaling**

To our knowledge, stable FDC-S cell lines are not available for *in vitro* or *in vivo* studies. To characterize the biological effects associated to EGFR signaling, we generated a short-term culture from a FDC-S case (from here referred as FDC-AM09). Culture cells showed an FDC-S-like morphology (Fig. 3A and B) and expressed the FDC-S marker CD21 (Fig. 3C). By cytogenetic analysis conducted on G-banded metaphases cells, numerical and structural abnormalities were observed (Fig. 3E–H), including gain of chromosome 21, chromosome ring, chromosome marker, derivative of chromosome 1, gain of chromosome 11, and chromosome marker, but not including the chromosome 7. On flow cytometry analysis, FDC-AM09 expressed EGFR on the surface (Fig. 3D). Significantly, EGF-induced FDC-AM09 cell growth is dose
dependent and ERK phosphorylation is already detectable at low concentration of EGF (e.g., 1 ng/mL; Supplementary Fig. S2). To test whether EGFR engagement induced FDC-S survival and proliferation, cells were treated with EGF (40 ng/mL) for 48 hours and S-phase entry was measured by nucleoside analog EdU incorporation into DNA. EGF significantly promoted S-phase entry in at least 40% of FDC-S cells (Fig. 4A) associated to ERK1/2 activation and phosphorylation of AKT and STAT3 (Fig. 4B). To further confirm that this effect was dependent on EGFR, FDC-S cells were preincubated with the blocking anti-EGFR monoclonal antibody cetuximab at different concentrations for 48 hours followed by treatment with 40 ng/mL EGF for 5 days. As shown in Fig. 4C, preincubation with cetuximab inhibited FDC-S cell growth in a dose-dependent manner. Because treatment with anti-EGFR monoclonal antibody not only affects cell proliferation but also results in a strong induction of cell death in several tumor cells lines, we investigated whether cetuximab induced FDC-S cell death. Using fluorescent DNA binding dyes, we found that pretreatment with 20 μg/mL of cetuximab induced apoptosis in more than 40% of FDC-S cells (Fig. 4D–F). To determine whether cetuximab inhibits EGFR-induced signaling pathways, subconfluent cells were serum starved, treated with cetuximab or control IgG, before EGF incubation. As shown in Fig. 4B, in contrast to EGF stimulation, pretreatment with cetuximab inhibited ligand-induced ERK1/2 activation, AKT phosphorylation, and reduced STAT3 phosphorylation, indicating that Cetuximab inhibits EGFR-induced signaling cascades. Blocking of EGF-dependent signaling and biological effects was similar when using an alternative anti-EGFR monoclonal antibody Panitumumab (not shown). The role of EGFR-MAPK signaling on FDC-S growth was also investigated after inhibition of ERK activation. Preincubation with the specific MEK inhibitor UO126 resulted in a significant reduction of EGF-dependent FDC-AM09 cell growth (Supplementary Fig. S3), indicating that EGFR-induced ERK activation is required for FDC-S tumor growth.

All together, these results indicate that in vitro, FDC-S cells proliferation and survival can be induced by the activation of EGFR-mediated signaling cascades via cognate ligands.

Local production of EGFR ligands in FDC-S

Based on the results from the previous set of experiments, we hypothesized that local production of endogenous EGFR ligands could sustain viability of reactive and neoplastic FDC-S. In 4 cases of FDC-S, frozen material was available for nucleic acid extraction. By qPCR we found messenger RNA for a set of EGFR ligands including HBEGF, TGF-α, and BTC, whereas no or very low signal was observed for EGF, amphiregulin (AREG), epiregulin (EREG), and epithelial mitogen homolog (EPGN; Fig. 5A). To validate this finding at protein level and identify the cellular source of these ligands, we took advantage on an anti-BTC antibody which specifically recognizes the protein on FFPE tissues. BTC binds different combinations of ErbB receptors including EGFR (30, 31) and was identified on endocrine α cells in the pancreatic islets where it promotes proliferation and differentiation of pancreatic β cells (32, 33). Accordingly, in FFPE sections of human pancreas, anti-BTC antibody recognizes

Figure 2. FISH analysis of EGFR gene in FDC-S cases. Comparative analysis of EGFR expression (A and B) and EGFR/CEP7 FISH (C and D) conducted on 2 FDC-S case. By immunohistochemistry, FDC-S cells are strongly positive for EGFR (A and B), whereas no gene amplification or chromosome 7 numerical abnormalities are detectable (C and D). Sections are counterstained with Meyer’s hematoxylin (A and B) and DAPI (C and D). FISH is conducted with LSI EGFR Spectrum Orange and CEP7 Spectrum Green probes. Magnification/Scale bar: 400x/50 micron (A and B), 1000x (C and D).
normal and neoplastic α cells as also confirmed by double stain for BTC coupled with insulin and glucagon (Supplementary Fig. S4). In reactive lymph nodes and tonsils, BTC stain germinal center macrophages and FRC, whereas no signal is observed in lymphoid cells (Fig. 5B and C). Remarkably, in the large majority of FDC-S–tested cases, expression of BTC was observed in FDC-S cells (13 of 16; 81.25%) and in microenvironment stromal cells (16 of 16; 100%), the latter more likely representing FRC surrounding neoplastic FDC (Fig. 5D and E). Interestingly, in HV-CD cases, BTC signal was observed in CD11c+ germinal center macrophages and also on dysplastic FDC (Fig. 5F–H). All together, these data indicate that the EGFR ligands are locally produced in the tumor microenvironment of FDC-S and the major cellular sources are represented by neoplastic cells or tumor-associated stromal cells.
EGFR and downstream pathways are activated in primary FDC-S

To substantiate the activation of the EGFR pathway in vivo in FDC-S, sections were stained for antibodies directed against phosphorylated forms of EGFR, AKT [Ser473], STAT3 [Tyr705], and ERK [Thr202/Tyr204]. Detection of phosphorylated proteins in archival FFPE sections by immunohistochemistry is characterized by a significant level of heterogeneity (34–36). We validated the expression of pEGFR [Tyr1068] and pEGFR [Tyr1173] on EGF-treated and -untreated fixed cells as well as in FFPE sections obtained from cases of lung adenocarcinoma with L858R and exon 19 delE746 activating mutations of the tyrosine kinase domain of EGFR. This set of positive controls gave a recognizable cytoplasmic and membrane stain (Supplementary Fig. S5).

Summary of the data on FDC-S are reported in a Supplementary Table S6. The combination of pEGFR [Tyr1173] and pSTAT3 [Tyr705] on EGFR-fixed and -untreated fixed cells as well as in FFPE sections obtained from cases of lung adenocarcinoma with L858R and exon 19 delE746 activating mutations of the tyrosine kinase domain of EGFR. This set of positive controls gave a recognizable cytoplasmic and membrane stain (Supplementary Fig. S5). Summary of the data on FDC-S are reported in a Supplementary Table S6. The combination of pEGFR [Tyr1173] and pSTAT3 [Tyr705] expression was clearly detected in the nuclei in 9 of 16 cases (56.25%), whereas pERK [Thr202/Tyr204] was detected in the nuclei and cytoplasm of 12 of 16 cases (75%; Fig. 6C and D). By double immunohistochemistry, we could detect coexpression of pEGFR [Tyr1173] with pSTAT3 [Tyr705] and pERK [Thr202/Tyr204] (Fig. 6E and insert). pAKT [Ser473] showed a faint-to-moderate stain in 7 of 16 FDC-S (43.75%; Fig. 6F).

These findings suggest that EGFR activation might occur in vivo in FDC-S cells. Persistent activation of EGFR downstream signaling pathways in other cancers might depend on mutations of EGFR or other intracellular components. By direct sequencing of genomic DNA purified from all FDC-S cases, we tested for the occurrence of the most common genetic variants of EGFR (substitutions in exons 18 and 21 and deletion in exon 19), KRAS (exon 1), NRAS (exons 1 and 2), BRAF (exon 15), and PI3KCA (exons 9 and 20). No mutations of these genes were found in all cases tested (16 of 16; 100%). Overall these data indicate that sustained activation of EGFR-downstream pathways is independent from somatic events in major oncogenes and likely result from ligand-dependent activation of the receptor.

Discussion

Among pathways involved in cell transformation and tumor progression, the most extensively characterized are those associated with receptors tyrosine kinase, in particular...
those belonging to the ErbB family. Abnormalities in key components of the ErbB receptors, including EGFR, are particularly relevant to epithelial cells and derived carcinomas. However, EGFR signaling has also been implicated in the transformation and progression of nonepithelial cancer including soft tissue sarcomas (8, 37). Here, we provide evidences for a role of the EGFR pathway in the biology of human FDC and their neoplastic counterpart. This finding uncovers a new key molecule in the organization of secondary lymphoid organs and suggests that EGFR activation might sustain survival and proliferation of neoplastic FDC (FDC-S). In particular, EGFR is strongly expressed on FDC-S cells and signal through downstream pathways in vitro and in vivo. Receptor activation in FDC-S is dependent on the availability of cognate endogenous ligands in the tumor microenvironment, particularly BTC, that is produced by tumor cells and by the surrounding stroma.

Figure 5. Production of EGFR ligands in primary FDC-S and HV-CD. Message RNA for the EGFR-ligands BTC, HBEGF, and TGF-α is detected in 4 FDC-S cases by qPCR (A). Sections are from reactive lymph nodes (B and C), 2 FDC-S cases (D and E) and 2 HV-CD cases (F–H) stained for BTC (brown in B–H) and CD11c (blue, H and insert in B). In reactive lymph nodes, BTC is expressed by CD11c+ germinal center macrophages (B) and fibroblastic reticulum cells (C). In FDC-S, BTC is expressed by neoplastic (D) and surrounding FRC (E). In HV-CD, BTC reactivity is found in dysplastic FDC (F) and in CD11c+ germinal center macrophages (G) as confirmed by double immunohistochemistry (H). Sections are counterstained with Meyer’s hematoxylin. Magnification/ scale bar: 200 x/100 μm (B, C, F, and G) and 400 x/50 μm (D, E, and H).
The biology of normal and transformed human FDC is still poorly understood. This limitation is mainly due to the rarity of this neoplasm and to difficulties in establishing cell purification and culture systems strategies (14). This has severely hampered the development of molecularly based therapeutic strategies for FDC-S. Previous studies have suggested that the transformation process of FDC can be paralleled by the overexpression of EGFR (11). In this study, we have found that EGFR expression trace the development of all major subsets of human lymphoid organ stromal cells including FDC and FRC and is maintained during neoplastic transformation of FDC. Based on FISH analysis of FDC-S cases, we could exclude that overexpression of EGFR is associated to an increased copy number of the gene or numerical abnormalities of chromosome 7. As alternative, EGFR protein overexpression might derive from increased transcription of the wild-type allele or impairment of receptor homeostasis, the latter likely due to somatic abnormalities in genes that regulate receptor endocytosis and degradation.

By deriving the short-term culture FDC-AM09 from an FDC-S case, we could show that the engagement of EGFR provides a sufficient amount of intracellular signal that support survival and proliferation of FDC-S and activates downstream pathways. Although this finding requires validation on a larger set of cells lines, it was further confirmed in vivo in a set of clinical samples by immunohistochemistry, showing frequent coexpression of the phosphorylated forms of EGFR, ERK, and STAT3 by FDC-S. This activation is not dependent on the occurrence of somatic mutations in major molecular drivers downstream to the EGFR pathways such as KRAS, BRAF, and PI3KCA as showed by direct sequencing of tumor-derived genomic DNA. The latter results point toward a ligand-dependent activation of EGFR in FDC-S. Accordingly, message RNA for HBEGF, TGF-α, and BTC was found in FDC-S samples. The latter finding was limited to 4 FDC-S cases with available frozen material. However, we could extend this observation at the protein level showing that BTC expression, by tumor cells and by surrounding stromal cell, is found in the majority of FDC-S.

Figure 6. Expression of phosphorylated EGFR, AKT, STAT3, and ERK in primary FDC-S. Sections are from a FDC-S case and stained for pEGFR[Tyr1068] (brown in A and F), pEGFR[Tyr1173] (brown in B), pSTAT3[Tyr705] (brown in C; blue in F), pERK[Thr202/Tyr204] (brown in D), and pAKT[Ser473] (brown in E). Membrane expression of the 2 pEGFR clones is diffuse in FDC-S cells (A and B); whereas pSTAT3, pERK, and pAKT can be observed in the cytoplasm and in the nucleus (C, D, and F). Coexpression of pEGFR with pSTAT3p (E) and pERK (insert in E) is observed in a FDC-S case. Sections are counterstained with Meyer’s hematoxylin. Magnification/scale bar: 400×/50 μm (A–F).
BTC is synthesized in different tissues and by a large number of mesenchymal and epithelial cells in culture (38, 39). Its biological activity in various cellular systems is mediated by binding to many different combinations of ErbB receptors (31, 40). Significantly, we could document that BTC is also produced in reactive lymph nodes and HV-CD by germinal center macrophages. The latter population might thus support viability and proliferation of normal EGFR-expressing FDC during the life cycle of a B-cell follicles, including those de novo generated in inflamed peripheral tissues. In addition, germinal center macrophages-derived BTC might also provide a sufficient amount of signal to initially transformed FDC before their outgrowth outside the germinal center. Although not investigated in this study, how FDC acquire the capability of producing high level of BTC upon transformation is of relevance and might represent also to other cancer models.

In different form of carcinomas, a meaningful clinical benefit has been showed with the addition of therapies targeting EGFR to standard treatments (27). Four anti-EGFR compounds have been approved for the clinical use in the last decade. Two small molecule inhibitors, gefitinib and erlotinib, have shown an unprecedented clinical benefit in a subset of lung adenocarcinoma with activating somatic mutation of EGFR (41, 42). In addition, 2 monoclonal antibodies to extracellular portion of EGFR (EGFR-MoAbs), cetuximab and Panitumumab, have proven to be effective in combination with chemotherapy or as single agents for treatment of colorectal carcinomas (43–45); whereas no EGFR mutation are detected in FDC-S, addition of Cetuximab to FDC-AM09 resulted in a significant blockade of the proliferation and induction of cell death. Although preclinical models in soft tissue sarcomas have indicated a potential clinical benefit for EGFR-MoAbs, a recently conducted phase II trial has concluded that cetuximab is not an active compound in EGFR-expressing advanced sarcoma, indicating that additional biomarkers of response have to be unequivocally identified (46). It is now well established that KRAS mutated cases have a scarce probability to achieve an objective response to EGFR-MoAbs, as well as to obtain a benefit in terms survival. Additional negative predictors of response to EGFR-MoAbs such as somatic mutations of NRAS, BRAF, and PI3KCA, has recently proposed (47–49). By direct sequencing of genomic DNA obtained from FDC-S, this study shows that FDC-S lack mutations in KRAS, NRAS, BRAF, and PI3KCA indicating integrity of the intracellular pathways downstream to EGFR. Increased production of endogenous EGFR ligands in the tumor microenvironment positively correlate with disease control in colorectal cancer patients treated with EGFR-MoAbs (50). By immunohistochemistry on routine biopsies, we could show that EGFR and downstream pathways are activated in FDC-S and EGFR ligands, particularly BTC, are locally produced. It is conceivable that a combination of these biomarkers might represent a useful strategy to identify FDC-S patients eventually responding to EGFR-MoAbs.

The majority of FDC-S is treated with surgery. However, a consistent fraction of them relapse with limited curative options. Findings from this study suggest that EGFR signaling induced by locally produced cognate ligands sustain viability and proliferation of FDC-S cells by activating intracellular pathways. To this end, gene-targeting experiments of the recently proposed FDC-precursors might significantly contribute to the generation of valuable preclinical models mimicking FDC transformation (12). As proved for other EGFR-dependent neoplasms, functional inhibition of this receptor might provide an additional clinical benefit to FDC-S patients.

Disclosure of Potential Conflicts of Interest
S.A. Pileri is a consultant/advisory board member in Takeda, CelGene, and TopoTarget. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: W. Vermi, E. Giurisato, F. Facchetti
Development of methodology: E. Giurisato, S. Lonardi, P. Balzarini, D. Medicina, D. Bosisio
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Balzarini, E. Rossi, D. Medicina, W. Pellegrini, C. Doglioni, G. Rossi, S.A. Pileri
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Vermi, E. Giurisato, S. Lonardi, P. Balzarini, E. Rossi, D. Medicina, D. Bosisio, S. Sozzani, W. Pellegrini, A. Marchetti, F. Facchetti
Writing, review, and/or revision of the manuscript: W. Vermi, E. Giurisato, S. Lonardi, E. Rossi, C. Doglioni, G. Rossi, S.A. Pileri, F. Facchetti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Lonardi, E. Rossi, W. Pellegrini, G. Rossi
Study supervision: W. Vermi, F. Facchetti, S. Sozzani

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References
EGFR in FDC Sarcoma


Ligand-Dependent Activation of EGFR in Follicular Dendritic Cells
Sarcoma is Sustained by Local Production of Cognate Ligands

William Vermi, Emanuele Giurisato, Silvia Lonardi, et al.


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