

# Activated EGFR induce trans-phosphorylation of EphA2 in cancer cells



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## Background

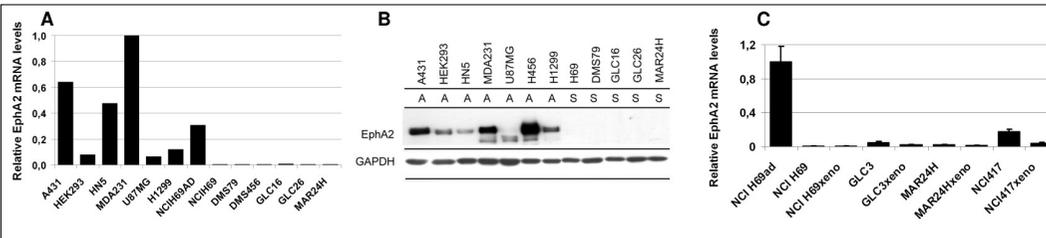
- The epidermal growth factor receptor (EGFR) regulates cell growth, survival, and migration.
- The receptor tyrosine kinases EGFR and EphA2 are frequently overexpressed in advanced cancers and contributes to tumor angiogenesis and metastasis.
- EphA2 is an EGFR transcriptional target in cancer cells (1;2) that contributes to EGF-induced cancer cell migration (2).

## Aim

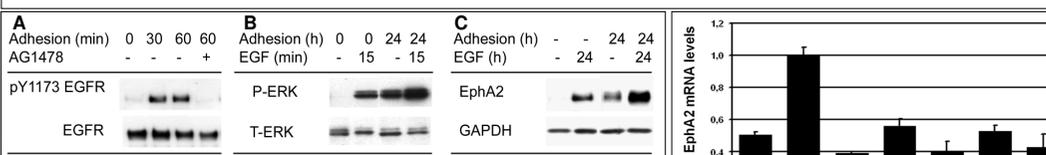
To investigate the regulation of EphA2 and its interaction with EGFR in cancer cells

## Materials and Methods

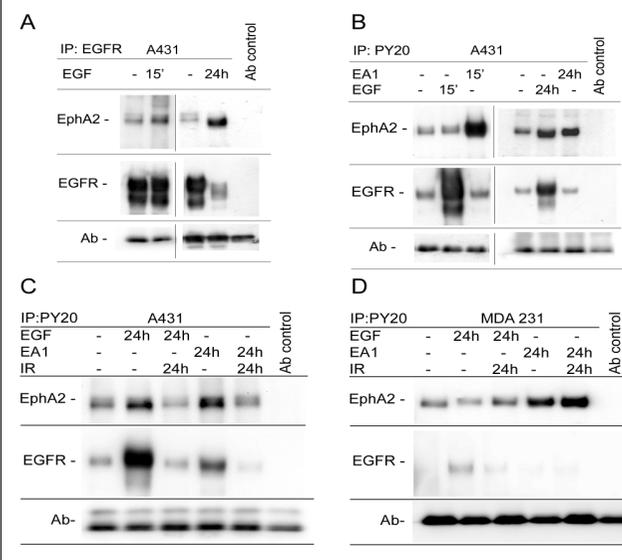
- Cell lines:** Small cell lung cancer (SCLC) cell lines: DMS79, DMS456, GLC16, GLC26, NCI 417, NCI H69 and MAR24H; non-SCLC cell lines: H456, H1299; other cell lines: A431 (skin carcinoma), HN5 (head and neck carcinoma), MDA231 (breast carcinoma), U87MG (astrocytic astrocytoma gr. III) and HEK293 (embryonic kidney). Cells were maintained in DMEM (Invitrogen) or RPMI (Invitrogen) supplemented with 10% FCS and pen/strep.
- Adhesion assay:** Adherent cells were grown in suspension by plating on agarose coated plates or Hydrocell plates (Nunc). Adherent cells were acquired from suspension cell lines by multiple passaging and continuously selections. Adhesion experiments were done with serum starved HN5 cells keep in suspension on Hydro cell plates for 4 hours and subsequently plated on adhesion plates with media containing molecular stimulators or inhibitors.
- Immunoprecipitation:** Serum starved A431 and MDA231 were left unstimulated or stimulated with EGF, EA1 (Ephrin-A1/Fc) and/or gefitinib (Iressa, AstraZeneca). Cell lysates were precleared and immunoprecipitated with 5 µg Ab-1 mouse anti-EGFR (Calbiochem) or 5 µg mouse PY-20 anti-phosphotyrosine (Zymed Lab.). Immunocomplexes were precipitated with protein G agarose beads and analyzed by immunoblot.
- Immunoblot:** 5 µg protein lysate was resolved by SDS-PAGE, electroblotted onto nitrocellulose membranes, and incubated with antibodies to EphA2, EGFR, P-ERK, T-ERK, P-FAK, T-FAK or GAPDH.
- Quantitative Real Time PCR:** The reaction was carried out using Trizol extracted RNA and SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen). The EphA2 products were normalized to RPL13A and GAPDH levels.



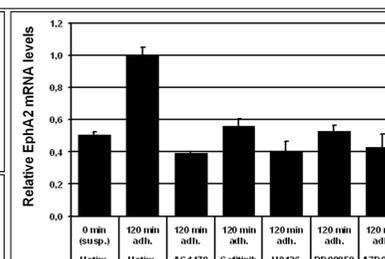
**Figure 1: EphA2 expression in cancer cells correlates with cell adhesion.** A and C) Relative EphA2 mRNA levels detected by qRT-PCR in a panel of human cancer cell lines growing either as adherent, in suspension or as xenografts. B) Detection of EphA2 protein levels by Western blot analysis in the human cancer cell lines from A). A: adherent, S: suspension



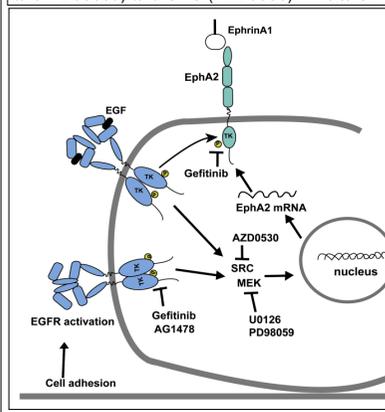
**Figure 2: EGF-induced ERK activation and EphA2 expression is enhanced by cell adhesion.** A) Adhesion of HN5 cells induce phosphorylation of EGFR (Y1173). B) and C) EGF-induced ERK-phosphorylation and EphA2-expression in HN5 cells is enhanced by cell adhesion.



**Figure 4: Activated EGFR co-immunoprecipitates with EphA2 and EphA2 phosphorylation is dependent on activated EGFR.** A) EphA2 co-immunoprecipitates with EGFR after 24h EGF-stimulation. B) EA1- and EGF-stimulation for 15 min. induce phosphorylation of the respective receptors. EGF-stimulation for 24h induce trans-phosphorylation of EphA2. C) EA1- and EGF-induced EphA2 phosphorylation is inhibited by Gefitinib (Iressa, IR) in A431 cells. D) In MDA231 cells, the EGFR TKI has no effect on EphA2 phosphorylation.



**Figure 3: EphA2 expression is dependent on EGFR, MEK and SRC activity.** EphA2 mRNA levels in HN5 cells analyzed by qRT-PCR. Adhesion induced EphA2 expression is inhibited by EGFR (AG1478 and Gefitinib), MEK (U0126 and PD98059) and SRC (AZD0530) inhibitors.



**Figure 5: Illustration showing adhesion-induced activation of EGFR leading to induction of EphA2 transcription through SRC and/or MEK signalling pathways.** EphA2 and EGFR co-localize and interact on the cell surface. Activation of EGFR induce EphA2 trans-phosphorylation and EphA2 phosphorylation is dependent on EGFR tyrosine kinase activity.

## Results

- EphA2 mRNA and protein levels are upregulated in adherent cell lines compared with suspension cell lines (Fig. 1A and 1B).
- Xenotransplants of suspension cell lines maintain a low EphA2 expression level (Fig. 1C).
- EGF-induced ERK activation and EphA2 expression is enhanced by cell adhesion (Fig. 2).
- Adhesion induced EphA2 expression is dependent on activation of EGFR, MEK and SRC (Fig. 3).
- EGF-stimulation for 24 hours induce EphA2 and EGFR co-immunoprecipitation (Fig. 4A) and trans-phosphorylation of EphA2 (Fig. 4B).
- EA1- and EGF-induced phosphorylation of EphA2 is dependent on EGFR tyrosine kinase activity (Fig. 4C).
- Effects of EGF and Gefitinib on EphA2 phosphorylation is dependent on a high EGFR expression level (Fig 4C/4D).

## Conclusions

- EphA2 expression is dependent on cell adhesion and activation of EGFR, MEK and SRC
- Activated EGFR associates with EphA2 and phosphorylation of EphA2 is dependent on EGFR activation

## References:

- 1 Pedersen MW *et al.* J Cell Biochem. 2005;96:412-427
- 2 Larsen AB *et al.* Mol Cancer Res. 2007;5(3):283-293,