EphA2 Binds to Activated EGFR and Modulates EGF-induced Cancer Cell Migration

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Background

- **The epidermal growth factor receptor (EGFR) is involved in regulation of cell growth, proliferation, survival, and migration**
- **Overexpression of EGFR and/or expression of a constitutively active variant of EGFR (EGFRvIII) is frequently found in human cancers**
- **Our laboratory has identified the receptor tyrosine kinase EphA2 in a search for EGFR and EGFRvIII regulated genes[1]**
- **EphA2 and its ligands have been associated with repulsion and attraction of neurons and endothelial cells during development**
- **EphA2 is frequently overexpressed in advanced cancers, and increasing evidence suggest that EphA2 contributes to tumour angiogenesis and metastasis**

Aim

To investigate the role of EphA2 in EGFR expressing cancer cells

Materials and Methods

- **Cell lines:** The murine fibroblast NR6, NR6wtEGFR (EGFR), NR6M (EGFRvIII), human head and neck carcinoma cell line HN5, and human skin carcinoma cell line A431
- **Immunofluorescence:** Cells plated on chamber slides were fixated, permeabilized, non-specific binding was blocked, and stained with anti-EphA2 antibody, anti-EGFR antibody and To-Pro-3. Images were acquired with confocal microscope (20x or 40x objectives)
- **Immunoprecipitation:** Cell lysates were precleared and 1000 µg protein were immunoprecipitated with 5 µg Ab-1 mouse anti-EGFR (Calbiochem) or 5 µg mouse PY-20 anti-phosphotyrosine (Zymed Lab.). The immunocomplexes were precipitated with protein G agarose beads. Antibody control was prepared by incubating pre-cleared RIPA lysisbuffer with mouse anti-EGFR antibody
- **Immunoblot:** 5 µg whole-cell protein lysate was resolved by SDS-PAGE, electroblotted onto nitrocellulose membranes, and incubated with primary antibodies to EphA2, EGFR or EGFRvIII
- **Wound-healing assay:** Confluent cells were was wounded, and the remaining cells treated with EGF and/or Ephrin-A1/Fc in low serum media. Migration of cells was observed at 0, 24 and 48h. Random selected images was acquired with a phase contrast microscope (Nikon) with 10x objective
- **Spheroid motility assay:** Spheroids of cells generated using the hanging drop method[2] and transferred to fibronectin coated six-well plates and cells allowed to adhere and migrate for 48 hours in the presence of EGF and/or Ephrin-A1/Fc

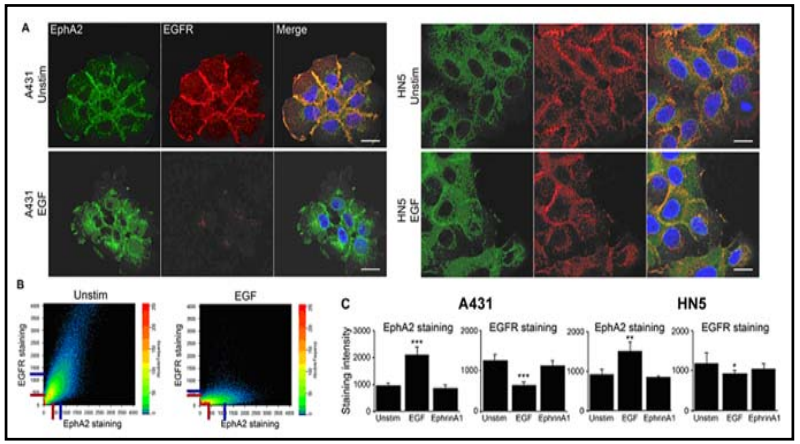

Figure 1: A) Confocal images of A431 and HN5 cells stained for EphA2 (green), EGFR (red) and nuclei (blue) after incubation with or without EGF for 48h. Bars: 20 µm. **B)** Scatter diagrams of pixel intensities of EphA2 and EGFR staining. Red lines: background pixels. Blue lines: The mean staining intensity above the background threshold. **C)** Histograms of the mean of mean staining intensities of cells stimulated with EGF or Ephrin-A1/Fc. Bars indicate 95% confidence interval and asterisks indicate significant difference compared to unstimulated cells (*: P<0.05, **: P<0.001, ***: P<0.00001).

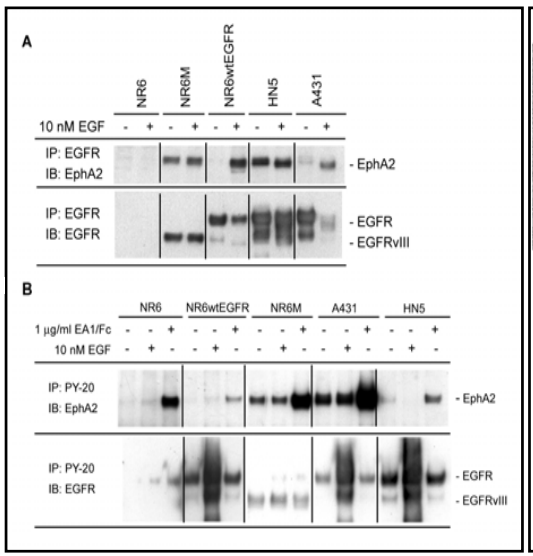

Figure 2: A) Immunoprecipitation of EGFR from NR6, NR6wtEGFR, NR6M, A431, and HN5 cells untreated or stimulated with EGF for 24h and subjected to Western blot analysis. **B)** Immunoprecipitation using anti phosphotyrosine antibody (PY-20) on NR6, NR6wtEGFR, NR6M, A431, and HN5 cells stimulated with EA1/Fc (Ephrin-A1/Fc, EphA2 ligand) or EGF for 15m and subjected to Western blot analysis.

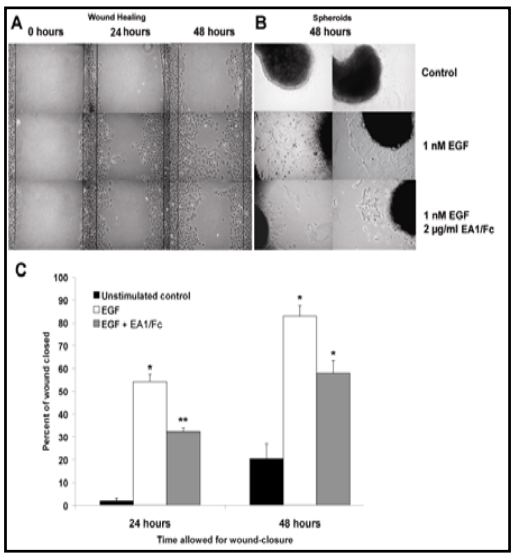

Figure 3: A) Wound healing assay with HN5 cells stimulated with EGF and/or EA1/Fc. **B)** Spheroid motility assay using HN5 cells stimulated with EGF alone or in combination with EA1/Fc. **C)** Quantification of cell motility expressed as percent of wound closure as compared to the zero time point. Bars indicate standard error of mean and asterisks indicate significant difference (*: P<0.05, **:P<0.001).

Results

- **The confocal images show that EGFR and EphA2 colocalize at the plasma membrane of A431 and HN5 cancer cells (Figure 1A)**
- **EGF stimulation increase EphA2 staining intensities due to increased expression of EphA2 and reduces EGFR staining intensities caused by EGFR internalization and downregulation (Figure 1C)**
- **Ligand activated EGFR co-immunoprecipitates with EphA2 indication an interaction between the two receptors (Figure 2A)**
- **EGF stimulation induces EGFR phosphorylation and Ephrin-A1/Fc (EphA2 ligand) induces EphA2 phosphorylation, but either ligand fails to induce tyrosine phosphorylation of the other receptor (Figure 2B) showing that the outcome of EGFR and EphA2 association is distinct from transphosphorylation**
- **EGFR is involved in motility and we inquired if activation of EphA2 could influence EGF-induced motility in a wound-healing assay (Figure 3A) and a three-dimensional spheroid motility assay (Figure 3B). Ligand activation of EphA2 significantly inhibited EGF-induced motility (Figure 3C) and altered the organization of the cells**

Conclusions

- **EGFR colocalizes with EphA2 at the plasma membrane of A431 and HN5 cells**
- **Activated EGFR and EGFRvIII associates with EphA2**
- **Ligand stimulation of EphA2 modulates EGF-induced cancer cell motility indicating a significant role for EphA2 in EGFR mediated tumorigenesis**

References:

1 **Pedersen M.W. *et al*. J Cell Biochem. 2005; 96: 412-427**

2 **Del Duca D. *et al.* J Neurooncol. 2004; 67: 295-303**