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## Short Communication

# Tight Junction Protein Junctional Adhesion Molecule-A Regulates the Expression of Receptor Tyrosine Kinase EPHA2 In Triple-Negative Breast Cancer Cells

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### ABSTRACT

Breast tumors lacking expression of the human epidermal growth factor receptor-2 (HER2), progesterone receptor (PR) and estrogen receptor (ER $\alpha$ ) are defined as triple negative breast cancers (TNBC). A lack of targeted therapies has impaired TNBC patient prognosis. It has previously been shown that high expression of Junctional Adhesion Molecule-A (JAM-A) correlates with aggressive breast cancer patient phenotypes, and that JAM-A regulates the expression of HER2 in breast cancer cells. Accordingly, we hypothesized that JAM-A might regulate the expression of other receptor tyrosine kinases. We show for the first time that JAM-A may regulate the expression of the EPHA2 receptor in TNBC cells and propose that this pathway merits deeper investigation for its therapeutic value in TNBC settings.

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## Introduction

Tumors lacking the expression of HER2, PR and ER $\alpha$  are defined as triple negative breast cancers (TNBC). Due to a lack of targeted therapies the standard of care for treating TNBC patients during early and advanced stage of the disease is chemotherapy [1]. However, TNBC patients frequently develop resistance to chemotherapeutic drugs relatively quickly, leading to an early demise. The lack of targeted therapies and poor prognosis of patients with TNBC have fostered a major effort to discover actionable molecular targets to treat patients with these tumours [1]. In the present study we extend prior work proposing that the tight junction protein Junctional Adhesion Molecule-A (JAM-A) is a potential therapeutic target in TNBC settings. High JAM-A high expression has previously been correlated with aggressive disease and poor outcome in breast cancer patients [2, 3]. Furthermore, we have shown that JAM-A regulates the expression of the receptor tyrosine kinase HER2 in breast cancer cells [4].

We therefore hypothesized JAM-A might regulate the expression of other oncogenic receptor tyrosine kinases. In this manuscript we describe the EPHA2 receptor as a target of JAM-A in TNBC cells. EPHA2 belongs to the Ephrin family of receptor tyrosine kinases and is enriched in TNBC cells; in which EPHA2 targeting impairs cell cycle progression and inhibits cell growth [5, 6]. The epidermal growth factor receptor (EGFR) which is highly expressed in TNBC cells has been shown to interact with and activate EPHA2, and activated EPHA2 has been described to amplify pro-tumorigenic MAPK, AKT and Rho family GTPase signalling [5, 7, 8]. In our study, both the JAM-A-downregulating natural compound Tetrocarcin-A and siRNA against JAM-A downregulated EPHA2 protein expression [9, 10]. JAM-A and EPHA2 were further found to co-localize and co-precipitate. Since JAM-A regulates the expression of EPHA2 in TNBC cells, and EPHA2 regulates cell survival pathways, we propose that JAM-A merits further investigation as a potential target in TNBC settings.

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## Methods

### I Cells

The human breast cancer cell line HCC38 was cultured in RPMI medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin.

### II Transfections

HCC38 cells were transfected for 72h with 25nM of control siRNA (siGENOME non-targeting siRNA #1, Dharmacon) or JAM-A siRNA (SASL\_Hs01\_00048785, Sigma-Aldrich) to silence JAM-A gene expression. Transfections were carried out using Lipofectamine-2000 (ThermoFisher Scientific) as per manufacturer's instructions.

### III Drug treatment

HCC38 triple negative breast cancer cells were plated in 6-well plates and treated 24h later with 2.5µM Tetrocarcin-A or vehicle (0.01 % v/v DMSO) for 24h.

### IV Electrophoresis and Western blot analysis

Whole cell lysates were prepared from breast cancer cells using lysis buffer composed of 0.1M KCl, 2.5mM NaCl, 3.5mM MgCl<sub>2</sub>, 10mM HEPES pH7.4, 1% Triton-X100, protease and phosphatase inhibitor cocktails (Sigma). Cells were lysed via trituration, whereupon extracts were centrifuged at 1,500xg for 5min at 4°C and supernatants stored at -20°C. Protein content was quantified via bicinchoninic (BCA) assay (Thermo Scientific), whereupon 15µg protein/lane was subjected to reducing SDS-PAGE, transferred to nitrocellulose membranes at 100V for 75min and immunoblotted with the antibodies to human JAM-A (BD Bioscience), EPHA2 (R&D Systems) and β-actin (Abcam).

### V Immunofluorescence staining

HCC38 cells were fixed in 100% ice cold ethanol (Sigma), blocked for 30 min with 3% BSA and incubated with primary antibodies to human JAM-A (1:100, Santa Cruz), EPHA2 (1:100, R&D Systems) and ZO-1 (1:100, Life Technologies). These were subsequently detected with secondary anti-mouse 488/anti-goat 568 or anti-mouse 488/anti-rabbit 568 or anti-goat 488/anti-rabbit 568 and mounted in medium containing DAPI (Vectashield, Vector Laboratories). Laser scanning confocal microscopy was performed using the Zeiss LSM 710 confocal system. Images were acquired using a 40X oil-immersion objective, and image averaging was applied to reduce noise.

### VI Co-immunoprecipitation

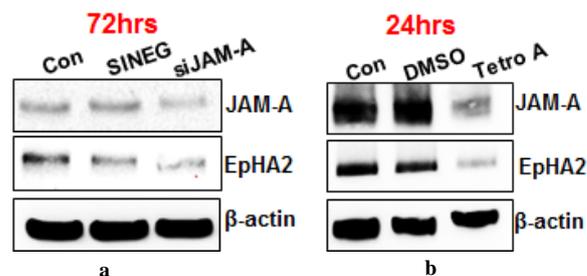
HCC8 cell lysates were prepared from T75 flasks using lysis buffer composed of 0.1M KCl, 2.5mM NaCl, 3.5mM MgCl<sub>2</sub>, 10mM HEPES pH 7.4, 1% Triton-X100, protease and phosphatase inhibitor cocktails (Sigma). Cells were lysed via trituration, whereupon extracts were centrifuged at 1,500xg for 5min at 4°C and supernatants stored at -20°C. Protein content was quantified via bicinchoninic (BCA) assay (Thermo Scientific). 1 mg of total protein was used for immunoprecipitation.

Protein G beads (Sigma), anti-human JAM-A (Santa Cruz) and IgG (Sigma) were used for immunoprecipitation of the HCC38 cell lysate. Precipitates were then subjected to reducing SDS-PAGE, transferred to nitrocellulose membranes at 100V for 75min and immunoblotted with the antibodies to human JAM-A (BD Bioscience), EPHA2 (R&D Systems) and probed with secondary goat anti-mouse HRP (Sigma) or donkey anti-goat HRP (R&D Systems) antibodies.

## Results

### Targeting of JAM-A caused downregulation of EPHA2

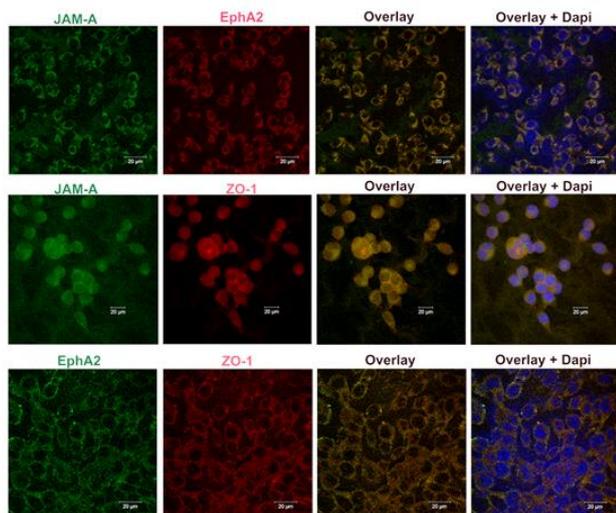
Several studies have shown that expressional or functional reductions in the adhesion protein JAM-A inhibit proliferation of several types of cancer cells [3, 11]. In the TNBC setting, we previously noted that JAM-A silencing or exposure to the JAM-downregulating compound Tetrocarcin-A reduced the proliferation of HCC38 TNBC cells [10]. Since JAM-A expression has been shown to regulate the expression of HER2 in breast cancer cells, in this manuscript we sought to uncover if JAM-A regulates the expression of other receptor tyrosine kinases relevant to TNBC settings [4].



**Figure 1: Targeting of JAM-A downregulates EPHA2**

HCC38 cells were seeded in 6 well plates and either gene-silenced for 72h with 25nM siRNA to JAM-A versus negative control siRNA (a) or treated for 24h with the JAM-downregulating compound Tetrocarcin-A (The NCI/DTP Open Chemical Repository; 2.5µM) versus vehicle (0.01 % v/v DMSO) (b). Cell extracts were prepared and Western blotted for human JAM-A, EPHA2 and β-actin.

Interrogation of a receptor tyrosine kinase RT-PCR array (data not shown) revealed parallel changes in EPHA2 expression upon manipulation of JAM-A levels. Protein level reductions in EPHA2 receptor expression secondary to JAM-A silencing were confirmed in HCC38 TNBC cells (Fig. 1a). Similarly, the JAM-A-degrading compound Tetrocarcin-A caused downregulation of EPHA2 in HCC38 TNBC cells (Fig. 1b). Double-labeling immunofluorescence experiments revealed that JAM-A and EPHA2 colocalize at the plasma membrane (Fig. 2). Since both JAM-A and EPHA2 contain a PDZ binding motif, we reasoned that their colocalization at the cell membrane may depend upon interactions with the PDZ domain-containing scaffolding protein zona occludens-1 (ZO-1) [12, 13]. Accordingly, JAM-A, EPHA2 and ZO-1 were found to colocalize at the plasma membrane (Fig. 2) and preliminary data (not shown) indicated that EPHA2 and JAM-A co-precipitated with each other. As JAM-A and ZO-1 are interacting partners, it is possible that JAM-A may interact with EPHA2 in a ternary complex involving ZO-1 and regulate the signalling/expression of other oncogenic receptor tyrosine kinases [14].



**Figure 2: JAM-A and EPHA2 colocalize at the cell membrane**  
HCC38 cells were plated on 13 mm coverslips and subjected to double immunofluorescence staining with antibodies to JAM-A/EPHA2, JAM-A/ZO-1 or EPHA2/ZO-1. Images were acquired on an LSM710 confocal microscope.

## Discussion and Conclusions

Breast cancer incidence is increasing throughout the world every year, although the mortality rates continually drop due in part to the availability of targeted therapies for HER2 or ER $\alpha$  positive breast cancer patients [15]. However, TNBCs, which comprise 10-15% of all breast cancer cases, retain high mortality rates due to the lack of actionable targets on the surface of their tumor cells. In the current study we propose the tight junction protein JAM-A merits consideration for potential therapeutic value in TNBC settings. Under normal circumstances JAM-A plays important physiological functions in numerous processes including intercellular tight junction assembly, cellular polarity, leukocyte transmigration, platelet activation, angiogenesis and cell morphology [16]. However, dysregulation of JAM-A expression or function has been increasingly implicated in various pathologies including cancer progression [17].

Importantly, high JAM-A expression has been correlated with aggressive disease and poor outcome in breast cancer patients [2, 3]. Since JAM-A regulates the expression of the receptor tyrosine kinase HER2 in breast cancer cells, we hypothesized that JAM-A might regulate other receptor tyrosine kinases relevant to TNBC settings [4]. A receptor tyrosine kinase PCR array identified the oncogenic receptor tyrosine kinase EPHA2 as a target of JAM-A. EPHA2 belongs to the Ephrin family, is enriched in TNBC cells, and its targeting has been shown to impair cell cycle progression and inhibit TNBC cellular growth [5, 6]. Having validated this at protein level and noted that both JAM-A and EPHA2 contain a PDZ-binding motif, we reasoned that JAM-A and EPHA2 might interact through PDZ domain-containing proteins at the tight junction [12, 13]. Accordingly, JAM-A and EPHA2 were noted to colocalize with the PDZ protein ZO-1; which was further validated by immunoprecipitation (preliminary data not shown). Since JAM-A regulates the expression of EPHA2 in TNBC cells and EPHA2 regulates cell survival pathways, we submit that JAM-A merits further

investigation as a potential target in TNBC settings [5].

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## Conflicts of Interest

The authors declare no conflict of interest

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