Epidermal Growth Factor Receptor activation regulates stemness in HNSCC - A possible route for the targeting of cancer stem cells

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INTRODUCTION

► **HNSCC**: Head and Neck Squamous Cell Carcinoma (HNSCC) comprises more than 90% of all head and neck cancers. Despite the many recent advancements in cancer therapy, HNSCC survival has had little improvement with a 5-year survival rate of less than 50%. HNSCC is currently the 6th most common form of cancer in the United States and is rapidly becoming one of the most frequent cancers in developing nations

► **Cancer stem cell (CSC) hypothesis**: there is a growing body of evidence that supports the idea that malignant tumors are initiated and maintained by a population of tumor cells that share similar biologic properties to normal adult stem cells. This model is based on the observation that tumors, like adult tissues, arise from cells that exhibit the ability to self-renew as well as give rise to differentiated tissue cells.

► **EGFR**: Overexpression and inappropriate activation of epidermal growth factor receptor (EGFR) occur in a variety of solid tumors, including the majority of HNSCC. EGFR signaling has been shown to have a direct role in cancer progression, including tumor proliferation, angiogenesis, metastasis, and tumor invasiveness, making EGFR an attractive target for cancer therapies. [2,3,4,5,6]

► In this study, we demonstrate that in HNSCC cell lines, activation or overexpression of EGFR results in induction of BMI-1, a proto-oncogene critical for self-renewal. Conversely, inhibition of EGFR using small molecule inhibitors resulted in downregulation of BMI-1. In addition, we demonstrate that EGFR activation or overexpression results in increased ability to form spheres in suspension, both in terms of size and number. Taken together, these results suggest that in HNSCC, EGFR overexpression or activation results in acquisition of stem cell properties.

MATERIALS AND METHODS

Quantitative Polymerase Chain Reaction: Quantitative polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the primers for BMI-1 and beta-actin (Eurofins MWG Operon). Each reaction was performed with 5 ul of cDNA made using 1x buffer, 5mM MgCl2, 500uM dNTP, random nonamers and 1.25U/ul reverse transcriptase. RNA was extracted from cell lines using RNeasy Mini Kit (QIAGEN). Beta-actin was used as the internal control. Reactions were performed in triplicate and analyzed using AB 7300 Real Time PCR System (Applied Biosystems) calibrated for use with SYBR green. Fold difference was calculated using the CT method normalized to beta-actin and expressed as a fold difference compared to untreated cells.

Flow Cytometry: Dual staining of the cell culture with CD44 conjugated to APC and CXCR4 conjugated to PE was performed after cells were trypsinized and counted. Cells were tested to confirm a single cell population which was furtheranalyzed for CD44+ expression. Results are plotted as a scatter plot representing the single cell population which was analized. Quadrant Q1 represents CD44- CXCR4- cells while Q2 represents CD44+ CXCR4- cells. Q3 represents CD44+ CXCR4+ cells and Q4 represents CD44- CXCR4+ cells. For this experiment, Quadrants 2 and 3 are combined to represent the population of interest.

Sphere Formation: Cells were plated in a 12-well low adhesion plate (Corning) at a density of 1000 cells/well in serum free DMEM as described by *Dontu et al* (2003). [7] Samples were subsequently treated with 0, 10, 50, or 100 ng/ml of epidermal growth factor (R&D Biosystems). Spheres were counted and photographed after 10 days of continuous ligand exposure. Spheres were monitored microscopically daily to ensure that they were derived from single cells and that they did not become confluent during the experiment.

ACTIVATION OF EGFR INDUCES INCREASED STEMNESS IN UMSCC-22B

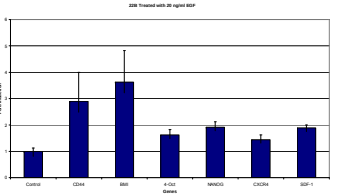


Figure 1. Cells were treated with either 0, 20, or 40 ng/ml EGF ligand for 24 hours in serum free DMEM. Cells were harvested for RNA and qPCR was used to measure mRNA levels. The results indicate that the addition of EGF ligand stimulates increased expression of stem cell genes *in vitro*.

INHIBITION OF EGFR ACTIVITY DECREASES STEMNESS IN UMSCC-22B

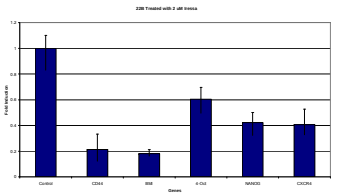


Figure 2. Cells were exposed to increasing doses of Gefitinib and incubated for 24 hours in serum free DMEM. Cells were harvested for RNA and qPCR was used to measure mRNA levels. The results indicate that the inhibition of the EGFR kinase downregulates the expression of cancer stem cell genes *in vitro*.

ISOLATION OF PUTATIVE CANCER STEM CELL CULTURE

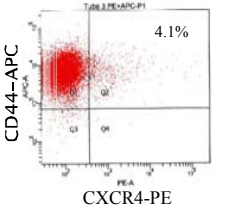


Figure 3. Primary tissue was disassociated using collagenase and plated onto Laminin treated plates supplemented with 20 ng/ml EGF and 20 ng/ml bFGF. Plates were placed under hypoxia and cells which had formed colonies were selected and allowed to expand. After several weeks of growth, cells were harvested and analyzed for CD44 expression by Flow Cytometry.

ACTIVATION OF EGFR RESULTS IN INCREASED SELF-RENEWAL CAPACITY

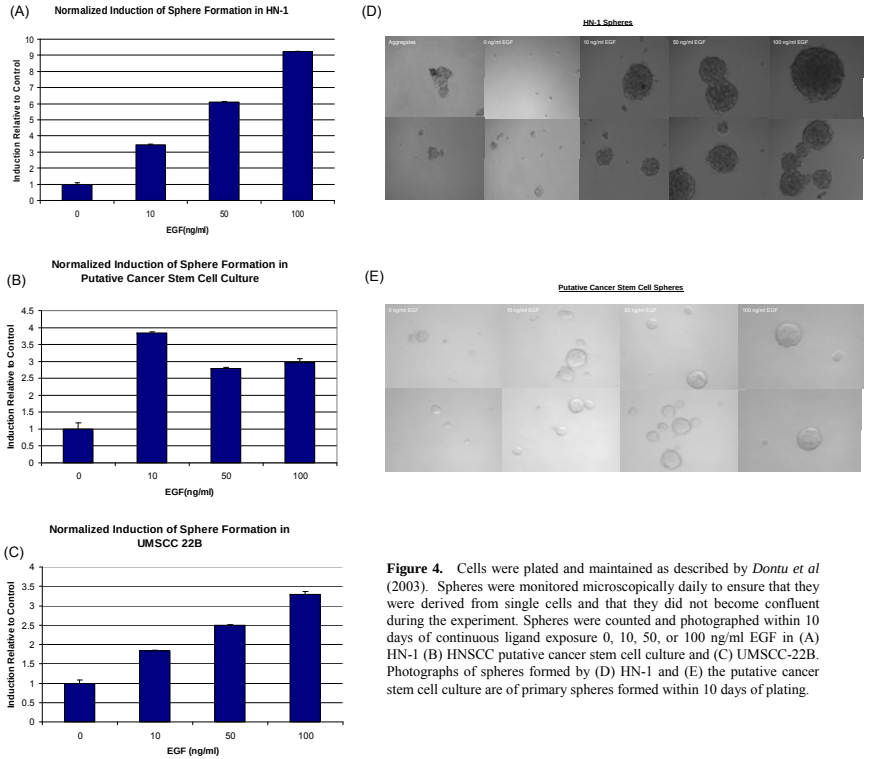
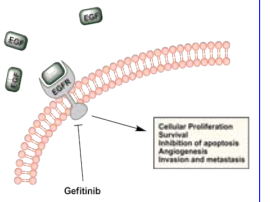


Figure 4. Cells were plated and maintained as described by *Dontu et al* (2003). Spheres were monitored microscopically daily to ensure that they were derived from single cells and that they did not become confluent during the experiment. Spheres were counted and photographed within 10 days of continuous ligand exposure 0, 10, 50, or 100 ng/ml EGF in (A) HN-1 (B) HNSCC putative cancer stem cell culture and (C) UMSCC-22B. Photographs of spheres formed by (D) HN-1 and (E) the putative cancer stem cell culture are of primary spheres formed within 10 days of plating.

CONCLUSION

► In HNSCC cell lines, Gefitinib (Iressa), an EGFR small molecule inhibitor, downregulated stem cell genes CD-44, BMI-1, Oct-4, Nanog, and CXCR-4.

► Activation of EGFR using EGF ligand induces expression of stem cell genes CD-44, BMI-1, Oct-4, Nanog, and CXCR-4.



► EGFR activation results in increased ability to form spheres in suspension, both in terms of size and number, in HNSCC cell lines and a putative cancer stem cell culture.

► **Taken together, these results suggest that in HNSCC, EGFR overexpression or activation results in acquisition of stem cell properties.**

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