Dsg2 increases exosome release and enhances EGFR/c-Src content: A mechanism for intercellular mitogenic effect

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ABSTRACT

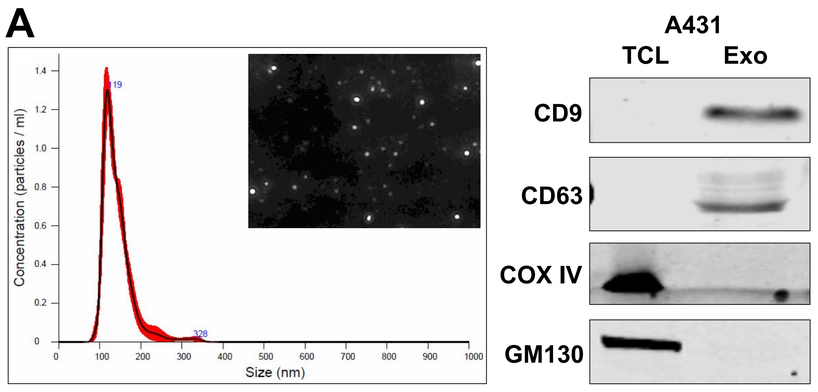
Exosomes are nanoscale membrane-derived vesicles that are secreted by cancer cells and play a critical role in modulating the tumor microenvironment and disease pathogenesis. Dsg2, a desmosomal cadherin often overexpressed in skin malignancies including squamous cell carcinoma (SCC), can activate EGFR/c-Src signaling and promote oncogenesis. We sought to address the potential role of Dsg2 in exosome biogenesis and intercellular signaling in SCC. Here, purified exosomes from SCC cells and head/neck SCC patient sera were enriched with a processed 65 kDa membrane-associated C-terminal fragment of Dsg2. Cells overexpressing Dsg2 had increased exosome release and protein content and produced particles enriched with EGFR/c-Src, enhancing proliferation in recipient fibroblasts, compared to parental cell exosomes. This study suggests a mechanism by which SCC cells can promote intercellular signaling and modulate the tumor microenvironment through enhanced Dsg2 levels.

INTRODUCTION

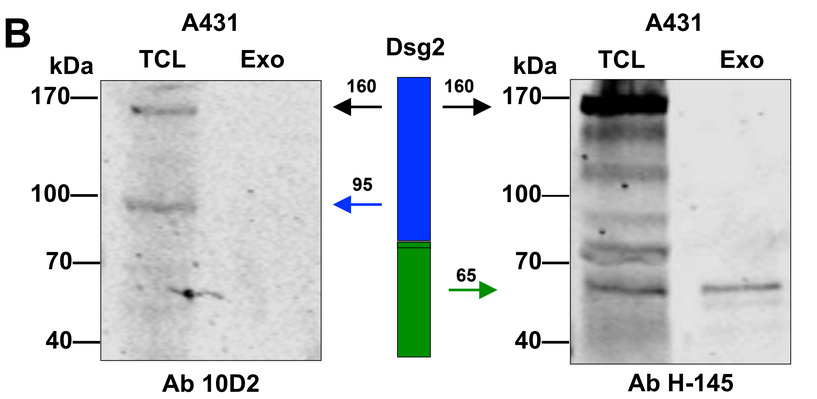
Desmoglein 2 (Dsg2) is a transmembrane desmosomal cadherin expressed in the basal epidermis, hair follicles, intestinal epithelia, and cardiac tissue[1-3]. Expression in adult interfollicular epidermis is low, but overexpression is often observed in multiple skin malignancies, including squamous cell carcinoma (SCC), and malignantly-derived cell lines[4,5]. Dsg2 can enhance activation of EGFR and c-Src to enhance both proliferation and migration in keratinocytes[6]. Transgenic mice overexpressing Dsg2 in the suprabasal epidermis have markedly increased proliferation in the *basal* cells[7]. Furthermore, Dsg2 can enhance chemically-induced skin carcinogenesis and activate the MAPK pathway in the dermis underlying the tumors, suggesting that Dsg2 is modulating intercellular signaling pathways[8]. A powerful mechanism for paracrine signaling are cell-derived exosomes—30-150nm-sized vesicles that transport protein, RNA, and DNA intercellularly[9]. Exosomes have been shown to promote tumorigenic modulations in the local and distant microenvironments, including stimulating angiogenesis and priming distant sites for metastases[10,11]. The role of exosomes in tumorigenesis is largely unknown for SCCs of the head and neck (HNSCC). HNSCC is the 6th most common cancer diagnosed worldwide, with an estimated 50,000 new cases and 10,000 deaths per year in the U.S.[12,13]. Like any solid tumor, the tumor microenvironment is critical for HNSCC progression, and cancerous epithelia cells seem to promote oncogenesis in adjacent epithelial and dermal cells[14]. The connection between Dsg2 and exosomes and the interaction of the two to contribute to HNSCC disease progression is unknown. Given that Dsg2 can enhance the functional activation of mitogenic signaling pathways in a cell, this work sought to delineate the role of Dsg2 as a modulator of intercellular mitogenic signaling.

RESULTS

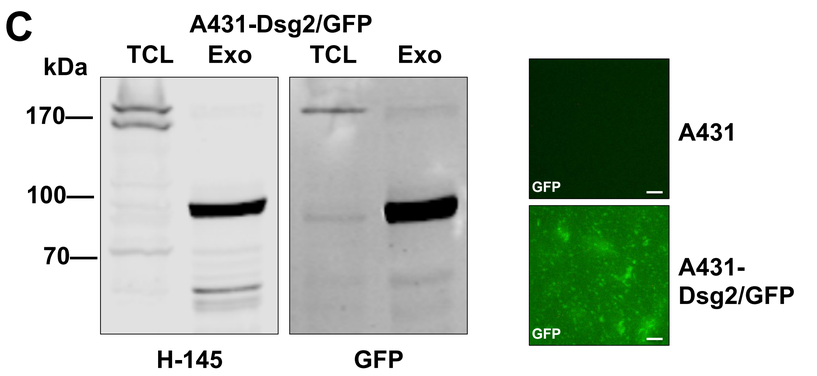
Exosomes Isolated from SCC-derived Cells are Enriched with Dsg2



(A) Exosomes were isolated from conditioned media of A431 SCC-derived cell line via sequential ultracentrifugation (similar results observed with ExoQuick polymer precipitation). Inset shows example image from NTA. *Left*, NanoSight nanoparticle tracking analysis (NTA) showing enrichment of 119 nm particles in exosome preparation (Exo). *Right*, total cell lysate (TCL) and Exo were lysed and immunoblotted for exosome-enriched markers CD9 & CD63 and exosome-negative markers COX IV and GM130. n=3.



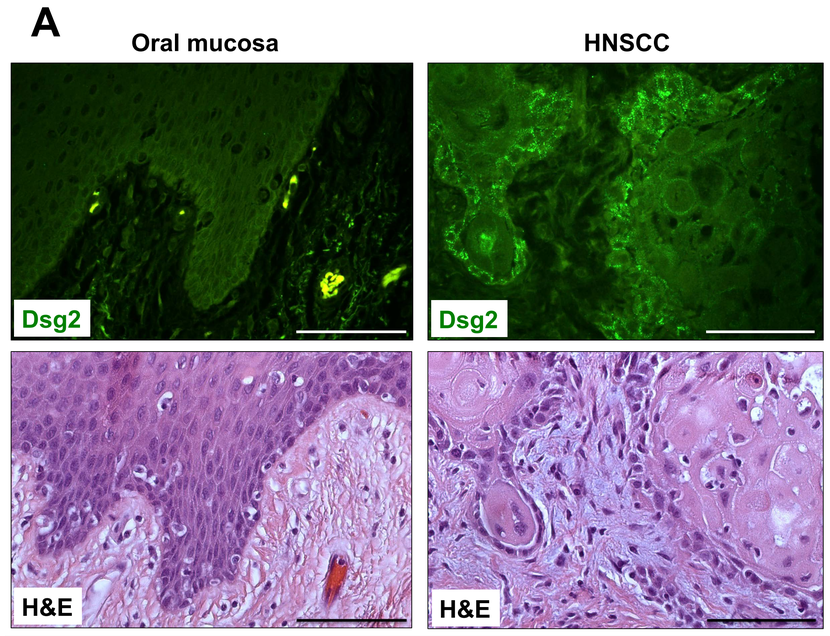
(B) A431 TCL and Exo were immunoblotted for Dsg2 with antibodies 10D2 and H-145 which detect extracellular (blue) and intracellular (green) epitopes, respectively. n=3.



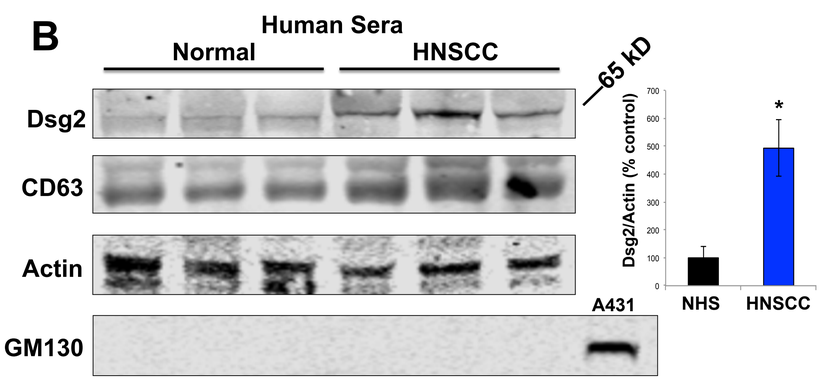
(C) Exosomes were harvested from conditioned media of A431-Dsg2/GFP cells. *Left*, immunoblot of A431-Dsg2/GFP TCL and Exo for H-145 and GFP. C-terminal GFP tag adds 27kD to Dsg2 CTF (heavy band, 100 kD). *Right*, exosomes from A431 and A431-Dsg2/GFP were coverslipped and visualized fluorescently for GFP tag. n=3. Scale bar: 10 µm

- **Exosomes isolated from A431 cells are enriched with the C-terminal fragment (CTF) of Dsg2**

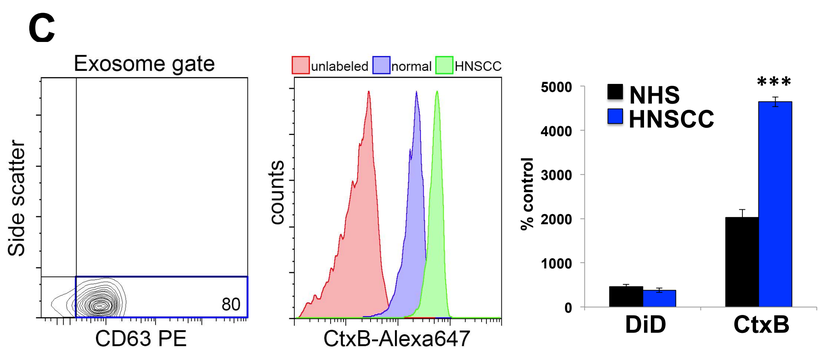
Dsg2 is Overexpressed in HNSCCs and Detected in Serum Exosomes



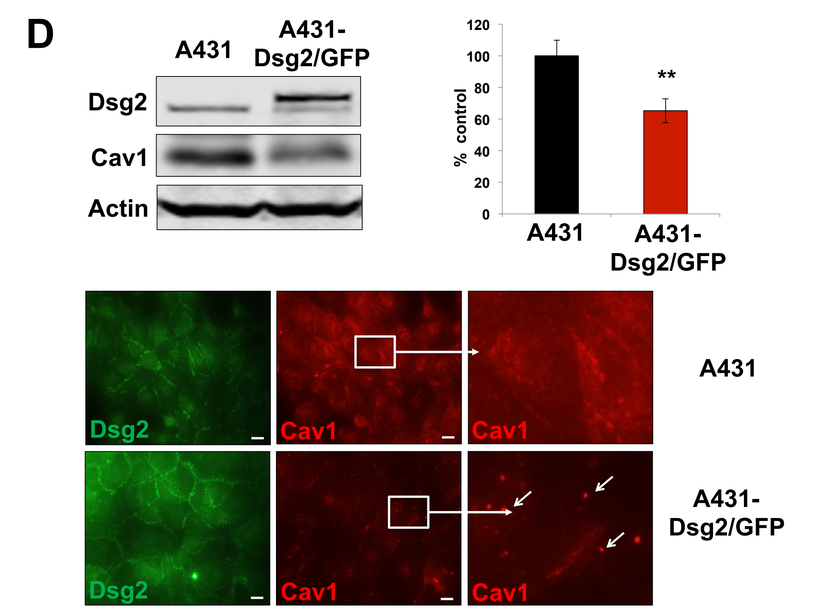
(A) Representative normal oral mucosa (left) and T1 HNSCC (right) immunostained for Dsg2; minimal basal staining in normal tissue and overexpression in invading tumor edge. Corresponding H&E staining below. Scale bar: 50 µm



(B) Exosomes were isolated from normal and HNSCC patient sera with ExoQuick. Exosomes were lysed and immunoblotted for Dsg2 (H-145), CD63, Actin, and GM130. A431 TCL immunoblotted for GM130 to show absence from exosome lanes. Only Dsg2 CTF (65 kD) band was enriched in serum-derived exosomes. Dsg2 CTF signal was quantified, normalized to Actin, and expressed as percent of normal human serum control (graph on right). *P≤0.05; n=3.



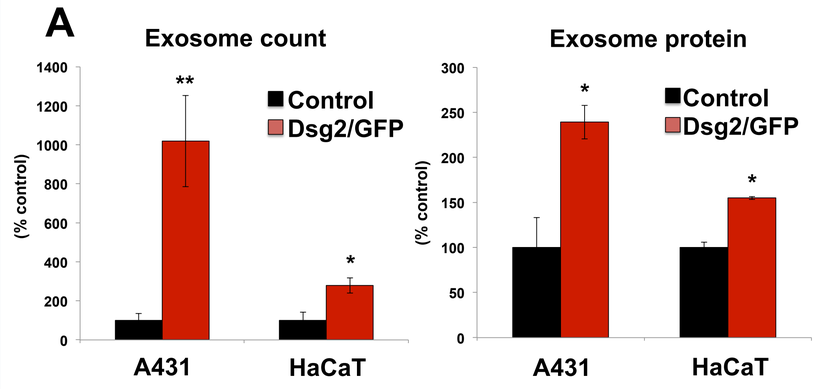
(C) Sera from healthy and HNSCC patients were stained with CD63 antibody and either fluorescently labeled lipid raft dye cholera toxin subunit B (CtxB; Alexa 647 conjugated) or lipophilic tracer DiD and analyzed by flow cytometry. Serum exosomes were gated by CD63-positivity. Fluorescence intensity was quantified for DiD- and CtxB-labeled particles and normalized to unlabeled serum control (graph on right). *** P≤0.001; n=3.



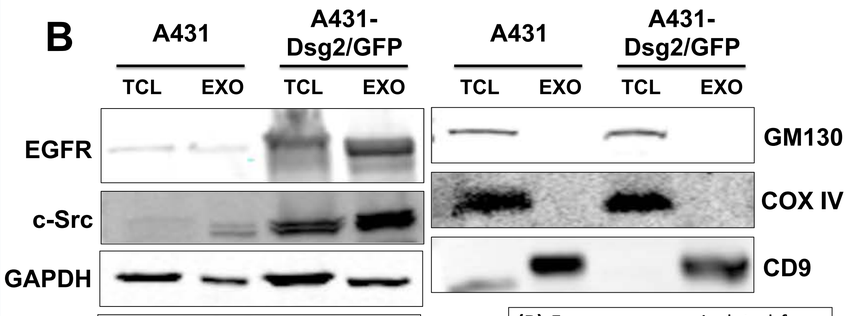
(D) *Top*, A431 and A431-Dsg2/GFP cells were lysed and immunoblotted for Dsg2, Caveolin 1 (Cav1) and Actin. Caveolin 1 signal was quantified, normalized to Actin, and plotted as % of A431 signal (n=3). *Bottom*, A431 and A431-Dsg2/GFP cells seed on slides and immunostained for Dsg2 and Cav1. Boxes demarcate enlarged section. Arrows show intracellular Cav1 puncta. ** P≤0.01; n=3. Scale bar: 20 µm

- **Dsg2 overexpression in HNSCCs most prominent in low-grade tumors and at the invasive leading edges**
- **Dsg2 CTF enriched in HNSCC serum exosomes**
- **More lipid-raft containing species circulating in blood; less Cav1 in cells overexpressing Dsg2 – secreted as exosomes?**

Dsg2 Modulates Exosome Biogenesis and Stimulates Fibroblast Proliferation



(A) Exosomes were isolated from the conditioned media of A431 (control), A431-Dsg2/GFP, HaCaT (control), and HaCaT-Dsg2/GFP cells via ultracentrifugation. *Left*, exosome count was measured by NTA and normalized to the number of secreting cells by counting the cells on the plate after harvesting conditioned media. *Right*, exosome protein measured by the bicinchoninic acid assay (BCA). Data are expressed as % control of parental cells (A431 and HaCaT). *P≤0.05, ** P≤0.01; n=3.



(B) Exosomes were isolated from the conditioned media of A431 and A431-Dsg2/GFP cells via ultracentrifugation. TCL and Exo samples were lysed and immunoblotted for EGFR, c-Src, GM130, COX IV, CD9, GAPDH, and GFP. n=3.

(C) *Left*, exosomes isolated from the conditioned media of A431 cells were labeled with the lipophilic dye PKH26. Primary human fibroblasts were seeded onto glass slides and treated with PKH26-labled exosomes (red) for 4 h prior to fixation and immunostaining for β tubulin (green). Fibroblasts visualized by confocal microscopy, Z-stack shows exosome internalization; at least 30 cells were confirmed to have PKH26 foci. PKH26 dye alone did not produce intracellular foci in recipient cells (data not shown). White arrows show internalized exosomes. Scale bar: 20 µm. *Right*, primary human fibroblasts were treated with exosomes isolated from conditioned media of A431 and A431-Dsg2/GFP cells for 24 h Cell proliferation was assayed via WST-1 assay. *P≤0.05, **P≤0.01; n=3.

- **Dsg2 overexpression increases exosome release and protein content**
- **Dsg2 overexpression enhances amount of EGFR/c-Src in A431-derived exosomes, increasing recipient fibroblast proliferation**

SUMMARY & CONCLUSIONS

Overexpression of Dsg2 is commonly observed in cutaneous skin cancers and can drive activation of EGFR-mediated signaling. Proteolytically-processed Dsg2 is exported in cell-derived and circulating exosomes. Dsg2 decreases Cav1 level in cells, potentially by promoting the endocytosis of caveolae and enhancing release of lipid-raft enriched exosomes. Dsg2 overexpression drives release of exosomes with more EGFR/c-Src that can be taken up by recipient fibroblasts, enhancing proliferation. This work demonstrates that Dsg2 can regulate exosome biogenesis and protein loading to elicit a mitogenic effect in recipient mesenchymal cells.

References: (1) Holthöfer et al. Int Rev Cytol 2007; 264: 65-163. (2) Mahoney et al. Exp Dermatol 2006; 15: 101-109. (3) Wu et al. J Invest Dermatol 2003; 120: 1052-1057. (4) Brennan et al. Cell Adh Migr 2009;3:148-154. (5) Denning et al. Exp Cell Res 1998; 239: 50-59. (6) Overmiller et al. Oncotarget 2016; doi18632/oncotarget.7675. (7) Brennan et al. J Cell Science 2007; 120: 758-771. (8) Brennan-Crispi et al. Oncotarget 2015; doi18632/oncotarget.3309. (9) Colombo et al. Annu Rev Cell Dev Biol 2014; 23: 255-298. (10) Peinado et al. Nat Med 2012; 18: 883-891. (11) Hood et al. Cancer Res 2011; 71: 3792-3801. (12) Jamal et al. CA Cancer J Clin 2011; 61: 69-90. (13) Siegel et al. CA Cancer J Clin 2014; 64: 9-29. (14) Leemans et al. Nat Rev Cancer 2011; 11: 9-22.

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