N-Glycosylation Induces the CTHRC1 Protein and Drives Oral Cancer Cell Migration*

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Background: Increased protein N-glycosylation and aberrant Wnt signaling are features of oral cancer.

Results: Overexpression of pro-migratory protein CTHRC1 is due to hyperglycosylation and transcriptional activation by canonical Wnt.

Conclusion: N-Glycosylation collaborates with canonical Wnt to induce CTHRC1 and drive OSCC cell migration.

Significance: Elucidating how N-glycosylation impacts tumor-promoting proteins is critical to understand cancer development and progression.

Oral squamous cell carcinoma (OSCC) is one of the most pernicious malignancies, but the mechanisms underlying its development and progression are poorly understood. One of the key pathways implicated in OSCC is the canonical Wnt/β-catenin signaling pathway. Previously, we reported that canonical Wnt signaling functions in a positive feedback loop with the DPAGT1 gene, a principal regulator of the metabolic pathway of protein N-glycosylation, to hyperglycosylate E-cadherin and reduce intercellular adhesion. Here, we show that in OSCC, DPAGT1 and canonical Wnt signaling converge to up-regulate CTHRC1 (collagen triple helix repeat containing 1), an N-glycoprotein implicated in tumor invasion and metastasis. We found that in human OSCC specimens, amplification of the levels of CTHRC1 was associated with its hyperglycosylation. Partial inhibition of DPAGT1 expression in OSCC CAL27 cells reduced CTHRC1 abundance by increasing protein turnover, indicating that N-glycosylation stabilizes CTHRC1. Additionally, canonical Wnt signaling promoted β-catenin/T-cell factor transcripational activity at the CTHRC1 promoter to further elevate CTHRC1 levels. We demonstrate that DPAGT1 promotes cell migration and drives the localization of CTHRC1 to cells at the leading edge of a wound front coincident with drastic changes in cell morphology. We propose that in OSCC, dysregulation of canonical Wnt signaling and DPAGT1-dependent N-glycosylation induces CTHRC1, thereby driving OSCC cell migration and tumor spread.

Head and neck squamous cell carcinoma ranks as the sixth most common cancer in the world and is associated with high morbidity and poor survival rates (1–3). Oral squamous cell carcinoma (OSCC),2 which involves epithelial neoplasms of the oral cavity and oropharynx, accounts for the majority of head and neck squamous cell carcinomas (1). Although whole-exome sequencing studies have identified components of signaling pathways whose aberrant expression and/or activity has been linked to head and neck squamous cell carcinoma pathogenesis (4, 5), there is a critical need for a better understanding of the molecular events that drive early stages of its development and progression.

The canonical Wnt signaling pathway has acknowledged roles in cell proliferation and cell fate, and its dysregulation is pivotal in the early pathogenesis of many cancers, including head and neck squamous cell carcinoma (4–10, 13). Aberrant activation of canonical Wnt signaling leads to accumulation of β-catenin, which translocates to the nucleus and, through binding to the T-cell factor (TCF)/lymphoid enhancer-binding-factor family of transcription factors, regulates the expression of a wide range of genes, including several oncogenic factors, such as cyclin D1 and c-myc.

Canonical Wnt signaling functions within a network of signaling pathways and is intimately linked to cell adhesion receptors and cellular metabolism (11–14). Our previous studies have identified asparagine-linked protein glycosylation (N-glycosylation) as a key metabolic pathway that is integrated with canonical Wnt signaling (15). Evolutionarily conserved and essential for viability, N-glycosylation controls a wide spectrum of activities, including protein folding, sorting, targeting, and secretion, as well as cell adhesion and signaling events (16). We have shown that the first N-glycosylation gene, DPAGT1, which functions at a rate-limiting step in the N-glycosylation pathway, interacts with canonical Wnt signaling via a positive feedback loop (15, 17). DPAGT1 encodes the first enzyme in the dolichol pathway, dolichol-P-dependent N-acetylgalactosamine-

2 The abbreviations used are: OSCC, oral squamous cell carcinoma; TCF, T-cell factor; GPT, dolichol-P-dependent N-acetylgalactosamine-1-phosphate transferase; AE, adjacent epithelia; TTL, total tissue lysate; PNGase F, peptide N-glycosidase F; PCP, planar cell polarity; RTK, receptor tyrosine kinase.

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1-phosphate transferase (GPT), which initiates the synthesis of the lipid-linked oligosaccharide precursor for protein N-glycosylation in the endoplasmic reticulum. Many components of canonical Wnt signaling are N-glycoproteins, and their N-glycosylation status controls Wnt signaling by regulating secretion and membrane localization of a canonical Wnt ligand, Wnt3a, and a co-receptor, LRP6 (17). In turn, canonical Wnt activates DPAGT1 expression via β-catenin at a transcriptional level. Furthermore, N-glycosylation inhibits E-cadherin adhesion and interferes with E-cadherin antagonism of canonical Wnt signaling (17, 18). Aberrant activation of canonical Wnt and N-glycosylation pathways has been associated with tumorigenesis (9, 19–21).

Recently, we reported that OSCC specimens display an aberrantly amplified DPAGT1/canonical Wnt feedback loop (6), where overexpression of DPAGT1 is due to increased occupancy of β-catenin at the DPAGT1 promoter. This leads to hyperglycosylation of E-cadherin and reduced intercellular adhesion, resulting in a continuous activation of N-glycosylation and canonical Wnt signaling.

Inappropriate activation of N-glycosylation and canonical Wnt signaling early in OSCC pathogenesis is likely to trigger downstream events leading to cancer progression. For OSCC tumors to spread, cells must migrate off the primary tumor epithelium. Thus, another consequence of amplified cancer cell migration and invasion (24–26).

Here, we report that human OSCC specimens display dramatic up-regulation of CTHRC1, which coincides with its hyperglycosylation. We show that N-glycosylation stabilizes the CTHRC1 protein by decreasing its turnover rate. At the same time, canonical Wnt signaling further induces CTHRC1 expression at a transcriptional level via increased recruitment of β-catenin to the CTHRC1 promoter. In scratch wound assays, N-glycosylation drives the localization of CTHRC1 to cells at the migrating wound front and promotes cell migration, in part, through a non-canonical Wnt pathway. Collectively, our studies show that in OSCC, CTHRC1 is up-regulated by the amplified DPAGT1/canonical Wnt feedback loop. We propose that this aberrant up-regulation of CTHRC1 drives OSCC tumor cell migration and the subsequent invasion and metastasis.

EXPERIMENTAL PROCEDURES

Reagents—Anti-CTHRC1 monoclonal antibody was used for immunoblotting and immunoprecipitation analyses. A polyclonal antibody to the last 11 amino acids of the C terminus of GPT was prepared commercially (Covance). For immunofluorescence studies, anti-CTHRC1 antibody was purchased from Abcam, as was anti-Wnt5a polyclonal antibody. Monoclonal antibodies to β-catenin, Rac1, and the IgG isotype control were obtained from BD Biosciences. Polyclonal antibodies to TCF3/4 and Fzd6 were from Exalphi Biosciences and R&D Systems, respectively. Anti-RhoA antibody was from Santa Cruz Biotechnology, and antibody to active GFP-bound RhoA was from NewEast Biosciences. Anti-Dvl2 polyclonal antibody was from Cell Signaling. Antibodies to GAPDH and FLAG were from Sigma and anti-pan-actin antibody was from NeoMarkers.

Cell Culture and Transfections— Cultures of OSCC CAL27 cells were grown to 70% confluence in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin as described (6). Total cell lysates were prepared by extraction with Triton X-100/β-octyl glucoside buffer. When indicated, CAL27 cells were treated with 50% Wnt3a-containing conditioned medium for 24 h as described (18). CAL27 cells expressing recombinant DPAGT1 were obtained by transfection of passage 2 CAL27 cells with full-length DPAGT1 (RefSeq NM_001382) or transcript variant (Refseq NM_203316) cDNA clones (OriGene) at 80–90% confluence using Lipofectamine 2000. Controls included untransfected cells and cells transfected with a control pcMV6-Entry vector. After 14 h, the media were changed, and cells were divided into several plates and grown in the presence of G418. The media were changed every 2–3 days and supplemented with G418. After 2 weeks, cells were processed for RNA isolation and preparation of total cell lysates. For immunofluorescence analyses, stable transfectants were plated in chamber slides at a density of $5 \times 10^5$ cells/cm² and processed as described (32).

RNA Interference and Quantitative Real-time PCR—SMARTpool siRNAs targeting CTHRC1 and DPAGT1 (referred to as S siRNA) were obtained from Dharmacon. The non-silencing negative control siRNA (referred to as NS siRNA) was from Qiagen. CAL27 cells were transfected at 6% confluence with either NS or S siRNA (150 nM) using Lipofectamine 2000. Controls included untransfected cells and cells transfected with a control pcMV6-Entry vector. After 14 h, the media were changed, and cells were divided into several plates and grown in the presence of G418. Media were changed every 2–3 days and supplemented with G418. After 2 weeks, cells were processed for RNA isolation and preparation of total cell lysates. For immunofluorescence analyses, stable transfectants were plated in chamber slides at a density of $5 \times 10^5$ cells/cm² and processed as described (32).

Cell Migration and Scratch Wound Assay—For cell migration assays, serum-free medium containing 1 x 10⁵ cells was placed into the upper compartment of Transwell inserts (Corning), and the lower compartment was filled with medium containing 10% FBS. Cells in Transwells were then incubated for 20 h in 5% CO₂ at 37 °C. Cell migration was quantified by counting crystal violet-positive cells (Fisher). For wound healing studies, CAL27 cells transfected with either NS or S siRNAs, as well as CAL27 cells transfected with DPAGT1 cDNA, were grown to confluence in P60 plates and wounded with a sterile 200- or 1000-μl pipette tip, washed three times with growth medium, and returned to the incubator. At the indicated times, wound edges were photographed using a phase-contrast Nikon Eclipse TE300 microscope and 10x objective. For immunofluorescence analyses of wounded cells, confluent cultures of CAL27...
cells transfected with non-silencing RNAs or siRNAs to DPAGT1 were grown in chamber slides. At 18 h post-wounding, cells were fixed and processed for immunofluorescence localization of CTHRC1 and for F-actin organization by counterstaining with rhodamine-phalloidin. Cells were then examined on a Zeiss LSM 510 META confocal microscope.

**Tissue Specimens**—All studies with surgical OSCC specimens were approved by the Institutional Review Board at the Boston University Medical Campus. Fresh tissues were obtained from patients with moderately differentiated to poorly differentiated OSCC of the tongue, maxillary gingiva, and floor of mouth. Regions of OSCC and adjacent epithelia (AE), defined by an on-site pathology analysis, were snap-frozen at −80 °C. Tissues were divided for H&E analyses, biochemistry, and immunofluorescence staining. OCT-embedded fresh tumor tissues were used for preparation of frozen sections (5 μm). One frozen section was set aside for H&E staining, whereas the remaining sections were processed for immunofluorescence analyses as described below. For biochemical analyses, total tissue lysates (TTLs) from AE and OSCC were prepared by extraction with Triton X-100/β-octyl-glucoside buffer as described previously (24). Protein concentrations were determined using the BCA assay (Pierce).

**Immunoblotting and Immunoprecipitation**—Cell and tissue lysates were fractionated on 7.5 or 10% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membranes, blocked with 10% nonfat dry milk, and incubated with primary antibodies to selected proteins. Protein-specific detection was carried out with horseradish peroxidase-labeled secondary antibodies and an ECL Plus kit (Amersham Biosciences). For quantification of TTLs, bands were cut out with a scalpel and incubated in 1 ml of protein G beads (Sigma). The resulting supernatants were incubated with anti-CTHRC1 antibody (2 μg) and protein G beads (50 μl) of protein G beads for 2 h at 4 °C. The beads were washed with lysis buffer, and samples were analyzed by immunoblotting. Due to the similar migration of CTHRC1 and the 25-kDa IgG chain, CTHRC1 immunoprecipitates were fractionated on 10% nonreducing gels. Immunoblots were quantified using ImageJ software.

**Peptide N-Glycosidase F Digestion**—Tissue lysates were digested with 100 units of peptide N-glycosidase F (PNGase F; New England Biolabs) for 1 h at 37 °C and analyzed by immunoblotting. Control samples were incubated without the enzyme.

**Protein Stability**—CAL27 cells were stimulated with conditioned medium overexpressing Wnt3a for 24 h and then transfected with either NS siRNA or siRNA to DPAGT1 and grown for 48 h. Next, cycloheximide (20 μg/ml) was added to NS and S siRNA-transfected cells, and cells were harvested at 0, 4, 8, and 12 h. CTHRC1 protein levels were examined by immunoblotting and quantified with ImageJ. The half-life of CTHRC1 in NS and S siRNA-transfected cells was determined by calculating the time when 50% of CTHRC1 remained using a line of best fit in Excel.

**Immunofluorescence**—For indirect immunofluorescence analyses, CAL27 cells were grown to 80% confluence, fixed in 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum, and incubated with primary antibodies. Cells were then incubated with FITC-tagged secondary antibodies (Invitrogen), counterstained for F-actin with rhodamine-phalloidin, mounted in VECTASHIELD, and analyzed with a Zeiss LSM 510 META confocal microscope. For indirect immunofluorescence analyses of human AE and OSCC specimens, tissue sections were blocked with 10% goat serum and incubated with antibodies against selected proteins, followed by secondary antibodies conjugated with FITC. Negative controls lacked primary antibodies. The slides were mounted in VECTASHIELD, and optical sections were analyzed with either a Nikon Eclipse TE300 microscope or a Zeiss LSM 510 META confocal microscope.

**Chromatin Immunoprecipitation**—ChIP assays were carried out with chromatin cross-linked with 1% formaldehyde from CAL27 cells. For tissue ChIP analyses, AE and OSCC were incubated in DMEM containing 1% formaldehyde, homogenized, and sonicated at 4 °C. The sonicated extract was immunoprecipitated, and genomic DNA was isolated from the complex after reverse cross-linking. The primers and the TaqMan probe surrounding the Wnt-responsive element of human CTHRC1 were designed using the Primer Express program (Applied Biosystems). They were as follows: forward primer, 5′-TGC AGT GAT CCC TTT ACC ATT ATA TAA-3; reverse primer, 5′-TGC AAT CCT AGT CTC TGA TAA AAC AGA-3; and TaqMan probe, 6Fam-TCTCTTTGTCTTTC-CTT-GMB.

**Statistical Analyses**—Data are presented as the mean ± S.E. Statistical significance was determined using two-tailed Student’s t tests. A p value <0.05 was considered significant.

**RESULTS**

**Augmented DPAGT1 Levels in Human OSCC Specimens Are Associated with Up-regulation of CTHRC1 Expression**—Previously, we reported that aberrantly amplified DPAGT1 leads to hyperglycosylation of E-cadherin and consequently reduced intercellular adhesion (6, 20). Recent studies have identified an N-glycoprotein, CTHRC1, as a key regulator of cell motility in cancer (25, 26). Thus, we hypothesized that increased DPAGT1-mediated N-glycosylation may promote additional molecular events that influence OSCC progression, such as cell migration. We therefore examined the expression and localization of the protein encoded by the DPAGT1 gene (GPT), a canonical Wnt effector (β-catenin), and a pro-migratory N-glycoprotein (CTHRC1) in nine moderately to poorly differentiated human OSCC tongue tumor specimens and corresponding cytologically normal AE. A representative H&E image of AE and OSCC used in this study is shown in Fig. 1A. As reported previously (6), immunoblots of TTLs showed that β-catenin was up-regulated by ~2-fold in OSCC compared with AE (Fig. 1B). Immunofluorescence analyses indicated that in AE, β-catenin was highly expressed in the proliferating basal layer of the epithelium, with diminished levels in the cytodifferentiating spinous layer. A similar localization pattern was observed for GPT (Fig. 1C). In contrast, in OSCC, β-catenin and GPT exhibited robust expression throughout tumor regions (Fig. 1C). Furthermore, immunoblot analysis confirmed that similar to β-catenin, GPT was more abundant in OSCC compared with AE (Fig. 1E). Although GPT overexpression in these OSCC tumors averaged 4-fold, even a modest increase in DPAGT1
expression has been shown to result in a robust activation of canonical Wnt pathway activity and hyperglycosylation of proteins (17). Immunofluorescence analyses revealed low levels of CTHRC1 in AE, but prominent expression in OSCC (Fig. 1D), with the majority of CTHRC1 localizing to cells at the invasive tumor front (data not shown). Immunoblots confirmed that AE had very low levels of CTHRC1 compared with OSCC, which exhibited increases in CTHRC1 abundance of >50-fold (n = 9) (Fig. 1F).

Next, we examined the N-glycosylation status of CTHRC1 in OSCC. Immunoblot analyses revealed that in TTLs from AE, CTHRC1 migrated mostly as a 30-kDa species, whereas in TTLs from OSCC, a higher molecular mass isoform was observed in addition to the 30-kDa species (Fig. 1G, arrows). Treatment of TTLs with PNGase F, an N-glycanase that cleaves most N-glycans at asparagine residues, converted both the high molecular mass and 30-kDa isoforms to deglycosylated species of 27 kDa (Fig. 2C), indicating that both are modified with N-glycans. Furthermore, the ratios between CTHRC1 in AE and OSCC before and after PNGase F treatment were similar, confirming that changes in mobilities were due to differences in N-glycosylation. We conclude that a hyperglycosylated CTHRC1 glycoform exists in OSCC. Because CTHRC1 has only one N-glycan consensus sequence, and activation of
Increased N-Glycosylation Drives Overexpression of CTHRC1 in OSCC—Because CTHRC1 is linked to the pathogenesis of several human cancers (25, 26), we next examined the mechanisms responsible for its up-regulation in OSCC. CTHRC1 is an N-glycoprotein with one N-glycan consensus addition sequence in the C-terminal region at Asn-188, and N-glycosylation was shown to promote its tethering to the membrane (23). Thus, it was possible that N-glycosylation contributed to up-regulation of CTHRC1 in OSCC.

To determine whether N-glycosylation impacts CTHRC1 abundance, we examined the effects of knocking down DPAGT1 levels on CTHRC1 in human OSCC CAL27 cells. We note that because DPAGT1 is essential for viability (27), we used conditions to achieve only partial knockdown of its expression (28). As shown in Fig. 2A, partial inhibition of DPAGT1 with S siRNA reduced its mRNA levels by 40% compared with NS control siRNA. This corresponded to a 40% reduction in GPT abundance (Fig. 2B). Because DPAGT1 functions at a rate-limiting step in the N-glycosylation pathway, even modest changes in its expression have pronounced effects on the N-glycosylation and function of proteins. Indeed, immunoblot analyses demonstrated that inhibition of GPT in S siRNA-transfected cells led to reduced levels of CTHRC1 compared with NS siRNA-transfected cells (Fig. 2C). Immunofluorescence staining of CTHRC1 in NS siRNA-transfected CAL27 cells showed that it localized primarily to the cell membrane, and this staining was greatly diminished in S siRNA-transfected cells (Fig. 2D).

Our observations that down-regulation of DPAGT1 reduced CTHRC1 levels in CAL27 cells suggested that N-glycosylation plays a role in CTHRC1 stability. To examine this possibility, we examined the turnover rate of CTHRC1 in CAL27 cells following siRNA-mediated DPAGT1 knockdown and subsequent inhibition of protein synthesis with cycloheximide. We note that following DPAGT1 knockdown, CAL27 cells express very modest levels of CTHRC1. Therefore, to efficiently detect CTHRC1 at different time points, CAL27 cells were first treated with Wnt3a-conditioned medium for 24 h to enhance CTHRC1 abundance (Fig. 3). The results show that in NS siRNA-transfected cells, the half-life of CTHRC1 was ~10 h (Fig. 2E). In contrast, in S siRNA-transfected cells, the half-life of CTHRC1 was reduced by 50% to ~5 h (Fig. 2E). Therefore, down-regulation of DPAGT1 increased the turnover of CTHRC1 in CAL27 cells, indicating that N-glycosylation regulates CTHRC1 stability.

Canonical Wnt/β-Catenin Signaling Induces CTHRC1 Transcription in OSCC—We previously reported that overexpression of DPAGT1 in OSCC promotes canonical Wnt/β-catenin signaling (6). Thus, we examined whether increased levels of CTHRC1 in OSCC may also be a consequence of its activation at a transcriptional level by Wnt-induced β-catenin-TCF complexes. The human CTHRC1 promoter has two TCF-binding sites spanning sequence −1946 to −1952 and sequence −1963 to −1969 (Fig. 3A). To determine whether these sites are Wnt-responsive, we examined the consequences of treating CAL27 cells with the canonical Wnt ligand, Wnt3a. The results show

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**FIGURE 2. N-Glycosylation increases CTHRC1 abundance by protein stabilization.** A, quantitative PCR of DPAGT1 transcript levels in NS (non-silenced) and S (DPAGT1-silenced) siRNA-transfected CAL27 cells. **, p < 0.01. B, left, immunoblot (IB) of GPT expression in NS and S siRNA-transfected CAL27 cells. Right, -fold change in GPT levels after normalization to GAPDH, ***, p < 0.001. C, left, immunoblot of CTHRC1 in NS and S siRNA-transfected CAL27 cells. Right, -fold change in CTHRC1 levels after normalization to GAPDH, ***, p < 0.001. D, immunofluorescence localization of CTHRC1 (green) and F-actin (red) in NS and S siRNA-transfected cells. E, comparison of CTHRC1 protein decay rates in NS and S (DPAGT1) siRNA-transfected cells. CAL27 cells were stimulated with conditioned medium overexpressing Wnt3a for 24 h, and total cell lysates were collected from cells grown in the presence of 20 μg/ml cycloheximide at 0, 4, 8, and 12 h.
that there was a modest but significant 1.5-fold increase in the CTHRC1 transcript level (Fig. 3B). Accordingly, we detected a 2-fold up-regulation of CTHRC1 protein abundance by immunoblotting (Fig. 3C). Up-regulation of CTHRC1 by Wnt3a was further confirmed by immunofluorescence staining, which showed a pronounced increase in CTHRC1 membrane localization (Fig. 3D). This was associated with increased immunofluorescence staining for F-actin (Fig. 3D), as reported previously (29, 30).

To confirm that β-catenin was present at the CTHRC1 promoter in CAL27 cells in vivo, we carried out ChIP assays with antibodies to TCF and β-catenin. The results show that compared with the IgG control, there was a >2-fold increase in β-catenin bound to the CTHRC1 promoter (Fig. 3E). We also examined the recruitment of β-catenin to the CTHRC1 promoter by ChIP in fresh human AE and OSCC specimens. We found a 4-fold increase in β-catenin at the TCF sites at the CTHRC1 promoter in OSCC tumor specimens compared with AE (Fig. 3F). Therefore, in addition to increased N-glycosylation, overactive canonical Wnt signaling in OSCC contributes to increased expression of CTHRC1 at a transcriptional level.

**DPAGT1 Promotes Cell Migration and Localization of CTHRC1 to the Wound Front**—Given that the knockdown of DPAGT1 strongly impacted CTHRC1 levels, we wanted to examine whether increasing DPAGT1 expression would have a similar effect. Therefore, we transfected CAL27 cells with Myc-

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**FIGURE 3. Wnt/β-catenin signaling increases CTHRC1 transcription.** A, TCF-binding sites in the human CTHRC1 promoter. B, quantitative PCR of CTHRC1 transcript levels in CAL27 cells grown either in conditioned medium (CM) or in conditioned medium containing Wnt3a. **, p < 0.01. C, left, immunoblot (IB) of CTHRC1 and actin expression in CAL27 cells stimulated with either conditioned medium alone or from cells overexpressing Wnt3a. Right, -fold change in CTHRC1 levels after normalization to GAPDH. ***, p < 0.001. D, immunofluorescence localization of CTHRC1 (green) and F-actin (red) in conditioned medium- or Wnt3a-stimulated cells. E, ChIP analyses of β-catenin and TCF at the CTHRC1 promoter in CAL27 cells compared with the IgG control. ***, p < 0.001. F, ChIP analyses of β-catenin and TCF at the CTHRC1 promoter after normalization to the IgG control in AE and OSCC. *, p < 0.05; **, p < 0.01.
tagged \(\text{DPAGT1}\) cDNA. Following selection of stable \(\text{DPAGT1}\) transfectants, CTHRC1 expression was compared in cells over-expressing \(\text{DPAGT1}\) with empty vector-transfected cells by immunoblotting. Because CAL27 cells are already along a tumorigenic pathway and have increased \(\text{DPAGT1}/\text{canonical Wnt signaling}\), transfection of \(\text{DPAGT1}\) cDNA resulted in only a 25% increased \(\text{DPAGT1}\) transcript abundance (Fig. 4A). These increased \(\text{DPAGT1}\) transcript levels corresponded to a robust expression of the \(\text{DPAGT1}\) protein product, GPT, as determined by immunoblotting using anti-Myc antibody (Fig. 4B). We note that the immunoblot analyses detected two GPT isoforms, previously reported to be the products of the \(\text{DPAGT1}\) gene (31). This augmented \(\text{DPAGT1}\) expression caused a >8-fold increase in CTHRC1 abundance (Fig. 4C). Accordingly, knockdown of \(\text{DPAGT1}\) expression resulted in diminished CTHRC1 abundance (Fig. 4D). CTHRC1 from the

FIGURE 4. Overexpression of \(\text{DPAGT1}\) is associated with increased cell migration and augmented levels and localization of CTHRC1 to the wound front. A, quantitative PCR of \(\text{DPAGT1}\) transcript levels in control empty vector (E) and \(\text{DPAGT1}\) cDNA-transfected CAL27 cells. **, \(p < 0.01\). B, immunoblot (IB) of recombinant GPT isoforms (Myc tag) from empty vector and \(\text{DPAGT1}\) transfectants. C, left, immunoblot of CTHRC1 from empty vector and \(\text{DPAGT1}\) transfectants. Right, -fold change in CTHRC1 levels after normalization to GAPDH. **, \(p < 0.01\). D, left, immunoblot of CTHRC1 and \(\text{DPAGT1}\) from CAL27 cells treated with either NS or S (\(\text{DPAGT1}\)) siRNA. Right, -fold change in \(\text{DPAGT1}\) levels after normalization to GAPDH. ***, \(p < 0.001\). E, immunoblot of control (C) or PNGase F (F)-treated CTHRC1 from empty vector and \(\text{DPAGT1}\) transfectants. F, scratch wound assay of empty vector- and \(\text{DPAGT1}\) transfected CAL27 cells at 0, 6, and 12 h (×4 magnification). G, scratch wound assay of \(\text{DPAGT1}\) transfectants treated with either NS or S (\(\text{DPAGT1}\)) siRNA at 0, 25, and 43 h (×4 magnification). H, immunofluorescence localization of CTHRC1 (green) and F-actin (red) at the edge of a wound in empty vector- and \(\text{DPAGT1}\)-transfected cells at 18 h.
**DISCUSSION**

Canonical Wnt/β-catenin signaling plays key roles in cancer initiation and progression (7, 33). Likewise, increased protein N-glycosylation is linked to neoplasia (34, 35). Previously, we showed that human OSCC specimens display aberrant amplification of the DPAGT1/canonical Wnt signaling positive feedback loop that leads to hyperglycosylation of E-cadherin and diminished intercellular adhesion (6). In this study, we have provided evidence that increased activity of this loop leads to overexpression of CTHRC1, an N-glycoprotein shown to drive tumor progression and metastasis (26). We have demonstrated that in OSCC, DPAGT1 and canonical Wnt synergize to upregulate CTHRC1 through hyperglycosylation/protein stabilization and transcriptional activation, respectively. Because changes in CTHRC1 in OSCC CAL27 cells are associated with corresponding changes in cell migration, our studies suggest that increased levels of CTHRC1 in human OSCC tumors drive cell migration and promote oral cancer spread. Several lines of evidence support this notion. First, overexpression of DPAGT1 promotes cell migration. Second, increased expression of CTHRC1 via up-regulation of DPAGT1 results in a more prominent localization of CTHRC1 to cells at the migrating wound edge. Moreover, DPAGT1 expression results in distinct changes in the morphology of cells at the migrating wound front. Third, inhibition of CTHRC1 in OSCC CAL27 cells reduces migration and wound closure.

In mouse cochlear sensory hair cells, CTHRC1 stabilizes the Wnt-Fzd complex to activate non-canonical Wnt/PCP signaling (30). Consistent with these observations, we found that in
CAL27 cells, CTHRC1 forms a complex with Fzd6/Fzd3 and Wnt5a. Furthermore, increased expression of CTHRC1 in DPAGT1-expressing CAL27 transfectants correlates with augmented levels of active RhoA, a downstream effector in the Wnt/PCP pathway (23). Also, overexpression of DPAGT1 promotes cell migration in a RhoA-dependent manner. These data suggest that in CAL27 cells, CTHRC1 activates a non-canonical Wnt/PCP pathway to drive cytoskeletal reorganization and migration. Activation of a non-canonical Wnt/PCP pathway in OSCC is further supported by increased levels of this pathway’s effectors in fresh tumor specimens, including Wnt5a, RhoA, Rac1, and active JNK, all known to be up-regulated in human cancers (36). Moreover, Wnt/PCP signaling has been shown to drive breast cancer cell migration (37). We note that CTHRC1 can also activate other oncogenic signaling pathways, including Src/focal adhesion kinase and MEK/ERK (26). Further studies are needed to determine the details of signaling events activated by CTHRC1 in OSCC.

N-Glycosylation has been shown to be required for effective secretion of CTHRC1 and for its anchorage at the cell surface (23). Here, we show for the first time that N-glycosylation stabilizes the CTHRC1 protein. Comparison of CTHRC1 abundance in DPAGT1 siRNA-treated CAL27 cells in the presence of cycloheximide revealed that the half-life of CTHRC1 was diminished in cells with attenuated DPAGT1 expression. Moreover, forced expression of DPAGT1 in CAL27 cells led to increases in the levels of CTHRC1 and its N-glycosylation status. Because overexpression of DPAGT1 in OSCC cells is associated with increased abundance and hyperglycosylation of CTHRC1, our results indicate that N-glycosylation promotes the accumulation of CTHRC1 in OSCC by enhancing protein stability. Furthermore, increased N-glycosylation was also asso-
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FIGURE 6. CTHRC1 is up-regulated by DPAGT1 and canonical Wnt signaling in OSCC. The schematic shows the interactions between DPAGT1 and canonical Wnt signaling and their downstream effects on CTHRC1 expression and non-canonical Wnt pathway-dependent tumor spread.

N-Glycosylation and canonical Wnt signaling represent highly conserved core pathways whose dysregulation is likely to drive early events in OSCC pathogenesis. Both pathways play pivotal roles in embryogenesis and are overactive in many malignancies. The initial aberrant activation of the DPAGT1/canonical Wnt feedback loop may involve a mutation in any of the components of the canonical Wnt signaling pathway. Likewise, up-regulation of DPAGT1 either by activating mutations or by increased availability of the GPT substrate UDP-GlcNAC from glycosylation and the hexosamine pathway will promote activation of canonical Wnt signaling (21). Once either DPAGT1 or canonical Wnt signaling is increased, it sets off a sequence of events that involve hyperglycosylation and transcriptional activation of downstream targets with key roles in tumorigenesis, such as CTHRC1. We note that CTHRC1 has been shown to be up-regulated by promoter demethylation and in response to such as CTHRC1. We note that CTHRC1 has been shown to be up-regulated by promoter demethylation and in response to TGFβ signaling in gastric cancer (38). Thus, we cannot exclude that additional mechanisms drive CTHRC1 expression in OSCC, although TGFβ receptors have also been shown to be regulated by N-glycosylation (39).

In addition to E-cadherin and CTHRC1, other important targets of the dysregulated DPAGT1/canonical Wnt signaling loop are receptor tyrosine kinases (RTKs), such as EGF receptor, ErbB2, ErbB3 and insulin-like growth factor-1 receptor, which play pivotal roles in cell proliferation, survival, and migration. These RTKs are N-glycoproteins that require N-glycosylation for activity, and some RTKs are also transcriptional targets of canonical Wnt (40). Because aberrant activation of RTKs promotes tumor cell proliferation, survival, and migration, RTKs have served as therapeutic targets for cancer treatment. Indeed, tunicamycin, a specific inhibitor of the DPAGT1 protein product, GPT, disrupts RTK signaling in tumor cells (41). However, because tumor cells develop resistance to chemotherapy by the activation of parallel signaling pathways, RTKs have not proven effective for sustained anticancer therapy. Here, our studies have identified CTHRC1 as a key downstream target of the DPAGT1/canonical Wnt feedback loop involved in OSCC cell migration and provide novel insights into the mechanisms driving its overexpression. The network formed between DPAGT1/canonical Wnt signaling and CTHRC1 highlights important upstream signaling events in OSCC pathogenesis. We propose that targeting this network may represent an effective strategy for therapeutic intervention for OSCC. Moreover, because CTHRC1 influences tumor invasion and metastasis of many aggressive neoplasms, our findings may reveal relevant mechanisms for the pathogenesis of other cancers.

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