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Nuclear localization of β-catenin and expression of target genes are associated with increased Wnt secretion in oral dysplasia

Montserrat Reyes[a,b,c], Daniel Peña-Oyarzun[a,b], Andrea Maturana[c], Vicente A. Torres[a,b,*]

[a] Institute for Research in Dental Sciences, Faculty of Dentistry, Universidad de Chile, Santiago, Chile
[b] Advanced Center for Chronic Diseases (ACCDiS), Universidad de Chile, Santiago, Chile
[c] Department of Pathology and Oral Medicine, Faculty of Dentistry, Universidad de Chile, Santiago, Chile

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ABSTRACT

Objectives: To evaluate the localization of β-catenin in oral dysplastic cells, the expression of target genes up-regulated in oral dysplasia, and the role of Wnt ligands in these events.
Materials and methods: Subcellular localization of total and non-phosphorylated (transcriptionally active) β-catenin was evaluated by immunofluorescence and biochemical fractionation in dysplastic oral keratinocytes (DOK), non-dysplastic oral keratinocytes (OKF6), oral squamous carcinoma cells (CAL27) and primary oral keratinocytes. Tcf/Lef-dependent transcription was measured by luciferase reporter assays. Expression of target genes, survivin and cyclin D1, was evaluated by RT-qPCR and Western blotting. Wnt secretion was inhibited with the inhibitor of porcupine, C59. Wnt3a and β-catenin were evaluated in biopsies by tissue immunofluorescence.
Results: Immunofluorescence and fractionation experiments showed augmented nuclear β-catenin (total and transcriptionally active) in DOK, when compared with OKF6 and CAL27 cells. Intriguingly, conditioned medium from DOK promoted nuclear accumulation of β-catenin and Tcf/Lef-dependent transcription in OKF6 and primary oral keratinocytes, suggesting the participation of secreted factors. Treatment of DOK with C59 decreased Wnt3a secretion, nuclear β-catenin and the expression of survivin and cyclin D1 at both mRNA and protein levels. Accordingly, DOK secreted higher Wnt3a levels than OKF6, and inhibition of Wnt3a secretion prevented DOK-induced Tcf/Lef-dependent transcription in OKF6. These observations were confirmed in clinical samples, since tissue immunofluorescence analysis showed simultaneous expression of Wnt3a and nuclear β-catenin in oral dysplasia, but not in healthy mucosa biopsies.
Conclusion: These data indicate that secretion of Wnt ligands is critical for β-catenin nuclear localization and expression of target genes in oral dysplasia.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, and its early diagnosis is essential to improve patient survival and to reduce mortality rates [1]. This malignancy is commonly preceded by lesions with histopathological diagnosis of epithelial dysplasia, which are associated with higher rates of progression towards invasive cancer [1,2]. However, reported rates of progression remain disparate, with variations ranging between 2–36%, which is due to the context of each study and the follow-up of these lesions [2,3]. Moreover, the histopathological diagnosis of oral dysplasia is complex and subjective [4], since no specific markers dedicated to identify potentially malignant lesions in the oral mucosa are available [5]. The latter is added to the limited knowledge on molecular alterations and the evolution of these lesions from early to advanced stages [5]. In this respect, recent studies identified a subset of signaling molecules that are deregulated in oral dysplasia and OSCC, namely components of the Wnt/β-catenin pathway [6,7].

The Wnt/β-catenin signaling pathway is involved in several biological functions, including cell differentiation, migration and proliferation [8]. β-catenin, the central player in this pathway [9], is constitutively degraded via phosphorylation on specific residues, including Ser33/Ser37/Thr41, and subsequent proteasome-dependent proteolysis, in a process mediated by the multiprotein destruction complex formed by CK1α, GSK-3β, Axin, and the tumor suppressor protein APC [9,10]. Binding of Wnt with Frizzled and LRP5/6 receptors triggers the

* Corresponding author at: Institute for Research in Dental Sciences, Faculty of Dentistry, Universidad de Chile, Calle Sergio Livingstone 943, Independencia, Santiago, Chile.
E-mail address: vatorres@med.uchile.cl (V.A. Torres).

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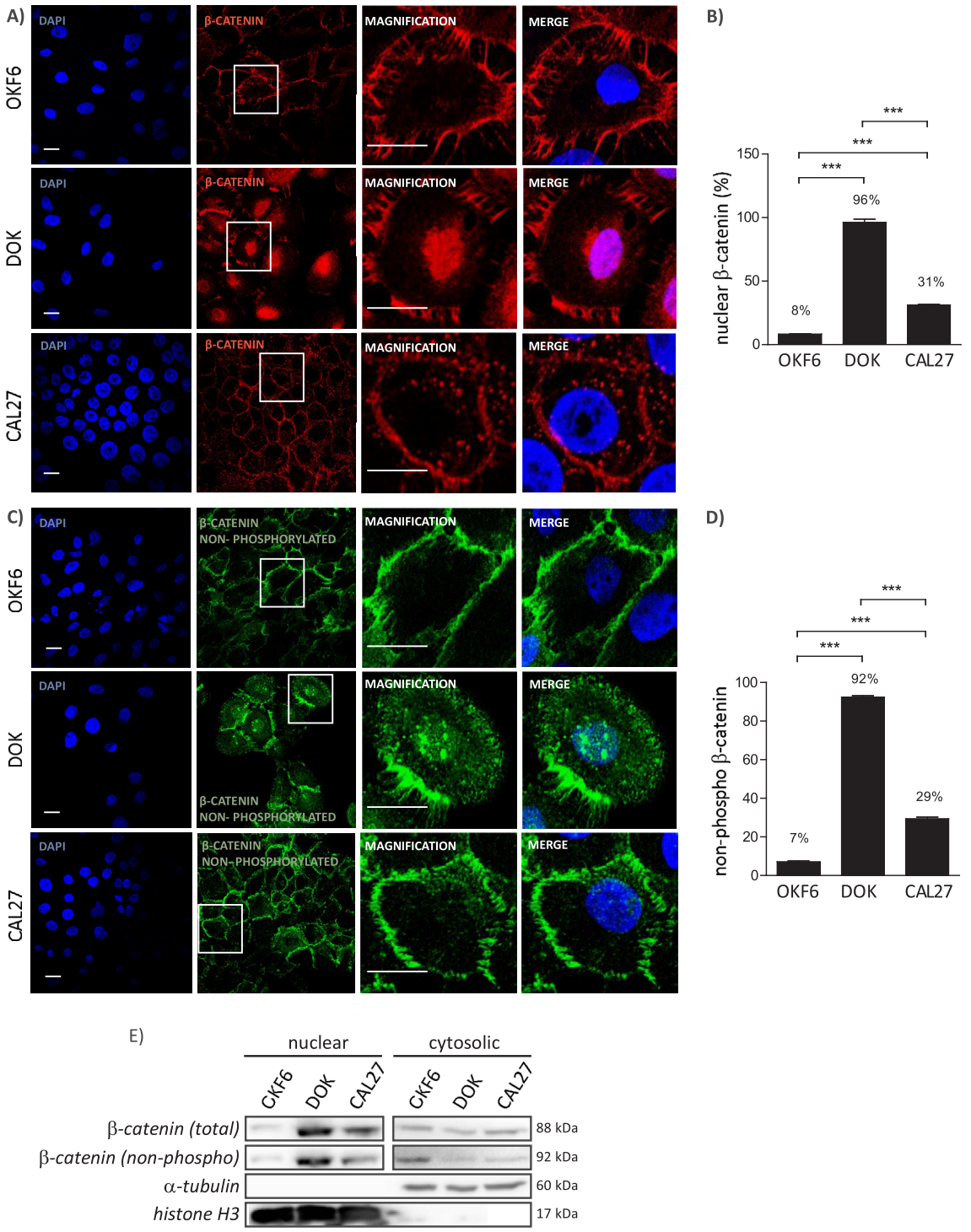

Fig. 1. Subcellular localization of β-catenin in oral keratinocytes, dysplastic oral keratinocytes and oral squamous carcinoma cells. (A and C) Immunofluorescence of total (A) and non-phosphorylated β-catenin (C) in oral keratinocytes OKF6/Tert2 (OKF6), dysplastic oral keratinocytes (DOK) and oral squamous carcinoma cells (CAL27) cells. Representative confocal microscope images are shown. (B, D) Percentage of cells with nuclear localization of total (B) and non-phosphorylated β-catenin (D), obtained as in A and B. Quantifications were obtained as described in the materials and methods, using the *Image J* software, and data represent the average from three independent experiments (mean ± s.e.m.; one-way ANOVA; $^{***}p \leq 0.001$). (E) Subcellular fractions of OKF6, DOK and CAL27 cells were obtained as described in the materials and methods and levels of total and non-phosphorylated β-catenin were evaluated by Western blot. Histone H3 and α-tubulin were used as nuclear and cytosolic markers, respectively. Images are representative from two independent experiments.

inhibition of the destruction complex, leading to β-catenin cytoplasmic stabilization and subsequent translocation to the nucleus, where it binds to Tcf/Lef transcription factors, allowing the expression of target genes, including survivin and cyclin D1 [11]. Although aberrant activation of this pathway is a recurrent mechanism in several malignancies, including melanoma, colon, gastric and cervical cancer [10], its relevance in potentially malignant oral lesions remains poorly known.

Early studies reported nuclear accumulation of β-catenin in oral dysplastic biopsies [12,13], and more recent studies by our group

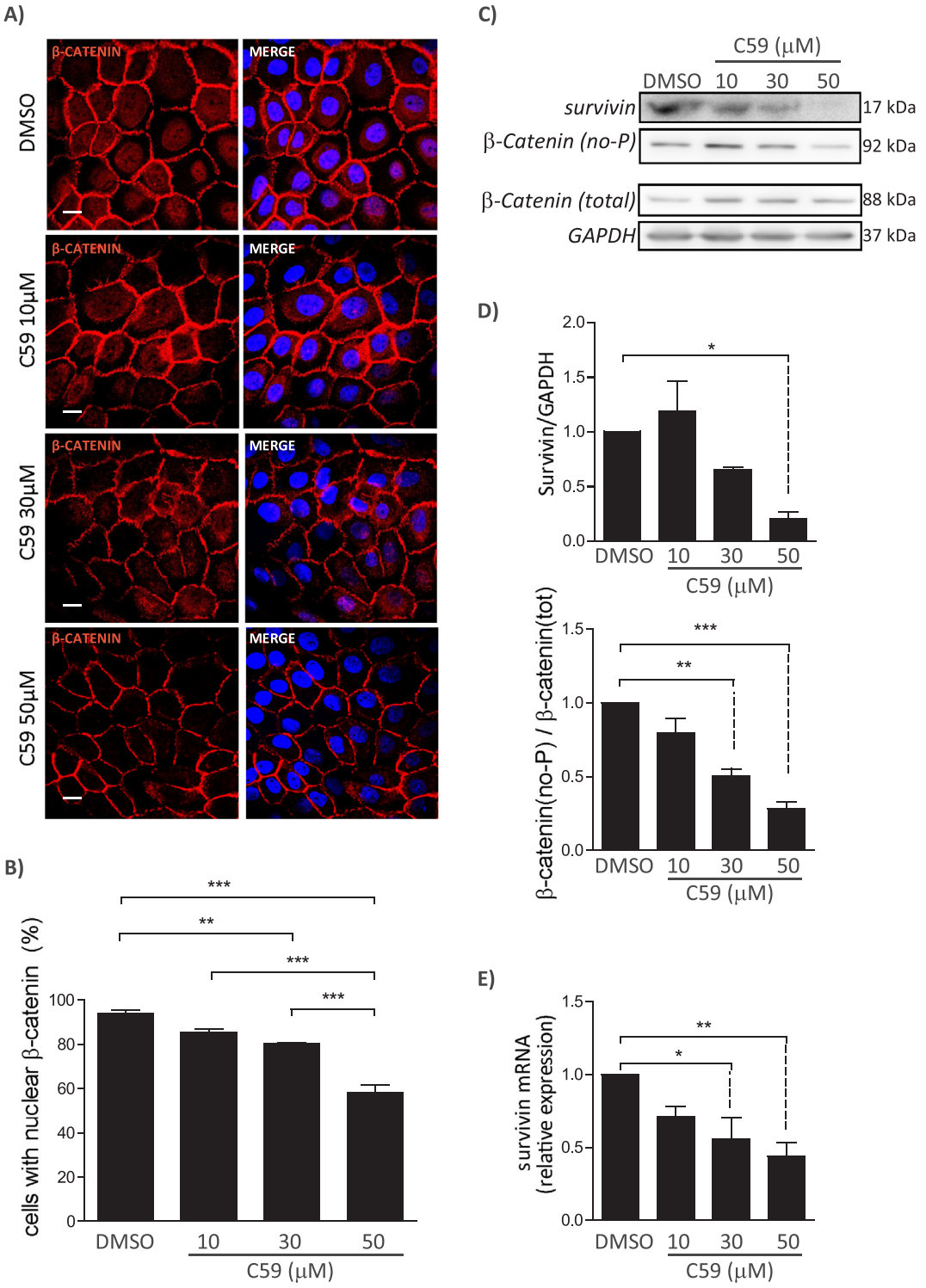

Fig. 2. Effects of Wnt secretion on β-catenin localization and the expression of target genes in dysplastic oral keratinocytes. Dysplastic oral keratinocytes (DOK) were incubated for 72 h with the inhibitor of Wnt secretion, C59 (DMSO as vehicle control), and used for subsequent analysis. (A) Subcellular localization of total β-catenin in DOK cells exposed to 10, 30 and 50 μM of C59. Representative confocal microscope images are shown. DAPI was used for nuclear staining. (B) Percentage of cells with nuclear localization of β-catenin, obtained from (A). Quantifications were obtained as described in the materials and methods, using the *Image J* software, and data are shown as the average from three independent experiments (mean ± s.e.m.; one-way ANOVA; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$). (C) Western blot analysis of DOK cells treated with increasing concentrations of C59. Survivin, total and non-phosphorylated β-catenin, and GAPDH were blotted with specific antibodies (see materials and methods for details). Representative images are shown from 3 independent experiments. (D) Relative levels of survivin and non-phosphorylated β-catenin were quantified by scanning densitometry and normalized with respect to GAPDH and total β-catenin, respectively. Graphs represent the average of 3 independent experiments (mean ± s.e.m.; one-way ANOVA; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$). (E) Relative survivin mRNA levels were quantified by RT-qPCR. Data represent the average from three independent experiments (mean ± s.e.m.; one-way ANOVA; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$).

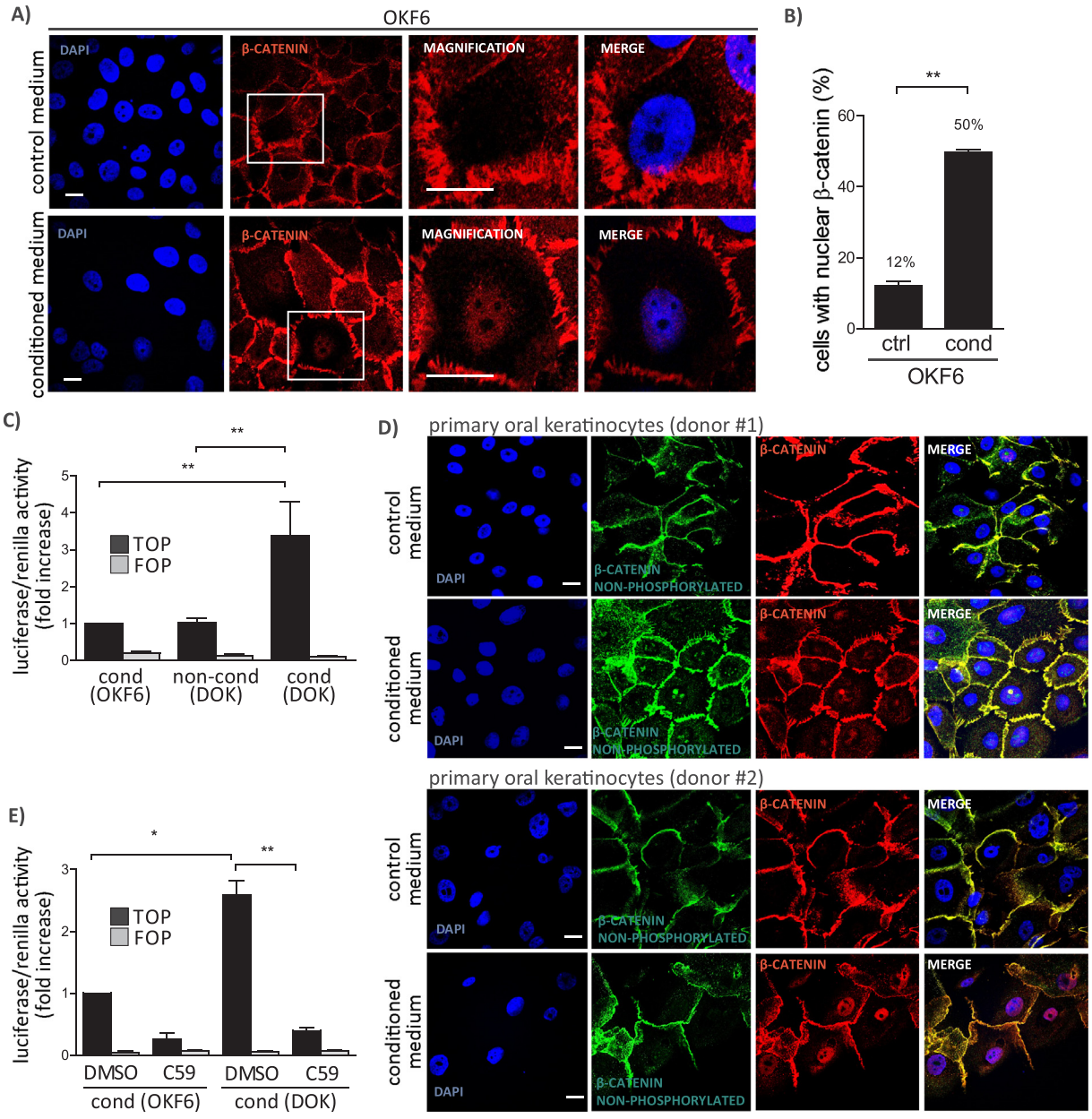

Fig. 3. Secreted factors in DOK conditioned medium promote nuclear localization of β-catenin in OKF6 and primary oral keratinocytes. Conditioned medium was obtained from 3-day cultures of dysplastic oral keratinocytes (DOK) and used for subsequent analysis. (A) Oral keratinocytes OKF6/Tert2 were incubated with DOK-derived conditioned medium (non-conditioned DOK medium used as control) for 24 h and analyzed by immunofluorescence of β-catenin. Representative images are shown. (B) Percentage of cells with nuclear localization of β-catenin, obtained from (A). Quantifications were obtained as described in the materials and methods, using the *Image J* software, and data are shown as the average from three independent experiments (mean ± s.e.m.; *t*-test; ** = $p \leq 0.01$). (C) OKF6 cells were co-transfected with the plasmids pTOP-FLASH (functional Tcf/Lef binding sites, black bars) or pFOP-FLASH (mutated, non-functional Tcf/Lef binding sites, grey bars), along with the constitutively active vector encoding renilla luciferase, and incubated in control or conditioned medium, as indicated. After 24 h, cells were homogenized and prepared for Tcf/Lef reporter assays (see materials and methods for details). Data were obtained as the ratio luciferase/renilla activity, presented as relative values with respect to control (non-conditioned medium) cells, and averaged from 3 independent experiments (mean ± s.e.m.; one-way ANOVA; ** = $p \leq 0.01$). (D) Primary cultures of oral keratinocytes were obtained as indicated in the materials and methods and characterized for their enrichment with respect to fibroblasts (Supplementary Figure 2). Subsequently, primary oral keratinocytes were exposed to DOK's conditioned medium (non-conditioned DOK medium used as control) for 24 h and analyzed by immunofluorescence of β-catenin. Representative images are shown. (E) OKF6 cells were incubated for 24 h with conditioned medium obtained from non-treated or C59-treated DOK cells, and Tcf/Lef reporter activity was measured as indicated in (C). Data are shown as the average of 3 independent experiments (one-way ANOVA; **$p \leq 0.01$; *$p \leq 0.05$).

showed that nuclear β-catenin is detected in 100% of biopsies with histopathological diagnosis of moderate and severe oral dysplasia, when compared with healthy mucosa [14]. Accordingly, immunohistochemical analyses in oral dysplastic biopsies showed increased expression of β-catenin target genes commonly upregulated in cancer, including survivin [15], cyclin D1 [16] and COX-2 [17], raising their suitability as potential markers in early oral lesions [15,16]. Nevertheless, although these studies suggest a role for β-catenin and downstream target genes in the progression of oral dysplasia, they remain correlative, and their implication in oral carcinogenesis cannot be assumed, since no causal studies are available, and mechanisms accounting for nuclear β-catenin accumulation in oral dysplastic cells are missing.

Unlike OSCC, where Wnt signaling has been widely studied

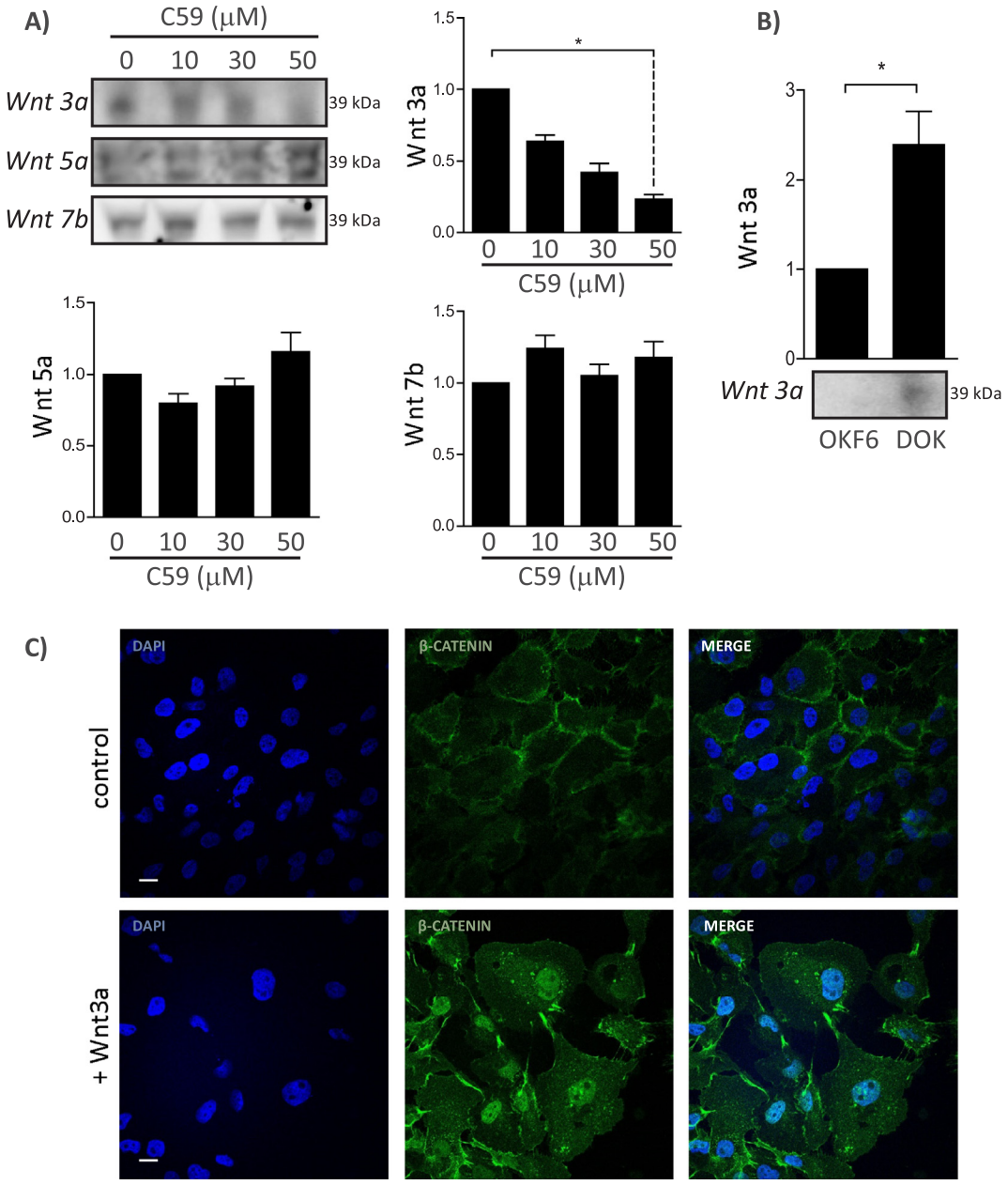

Fig. 4. Wnt secretion in oral dysplastic keratinocytes. Dysplastic oral keratinocytes (DOK) were either treated or not with increasing concentrations of C59 and then, conditioned media were obtained for subsequent assays. (A) Wnt3a, Wnt5a and Wnt7b levels were analyzed by Western blotting with specific antibodies. Representative images are shown. Levels of Wnt3a, Wnt5a and Wnt7b were measured by scanning densitometry and shown as the average of 3 independent experiments (mean ± s.e.m.; one-way ANOVA;* = $p \leq 0.05$). (B) Wnt3a levels were measured in equal amounts of conditioned medium obtained from oral keratinocytes OKF6/Tert2 (OKF6) and DOK cells. Representative Western blot images are shown and the graph represents the average of 3 independent experiments (mean ± s.e.m.; one-way ANOVA; * = $p \leq 0.05$). (C) OKF6 cells were treated with recombinant Wnt3a obtained from HEK293-overproducing cells (described in J Cell Sci 119, 2006, 1812–1823) and nuclear β-catenin assessed by immunofluorescence. Representative confocal microscopy images are shown.

[7,13,18–20], evidence addressing the role of Wnt ligands in oral dysplasia is limited. Specifically, although Wnt3a is increased in oral dysplasia biopsies when compared with healthy mucosa [13], no studies showing the effects of this Wnt in oral dysplastic cells are available. On the other hand, although Wnt7b is known to activate Wnt/β-catenin in OSCC cells [18] and Wnt5a promotes cell migration and invasion in OSCC [19,20], the expression and role of these Wnt ligands in oral dysplasia remains unknown. Hence, as for β-catenin localization and function, the role of Wnt ligands in oral dysplasia remains poorly understood, mostly due to the lack of appropriate in vitro models. In this study, we evaluated the expression, subcellular localization and transcriptional activity of β-catenin in oral dysplastic cells, and assessed the role of Wnt ligands in these events, by using a panel of cell lines that included dysplastic oral keratinocytes (DOK), non-dysplastic oral keratinocytes OKF6/Tert2 (OKF6) and oral squamous carcinoma cells (CAL27), as well as primary cultures of oral keratinocytes. The establishment of these in vitro models will permit to get a comprehensive characterization of the molecular players involved in the progression of oral dysplasia.

Table 1
Clinicopathological and demographical features of biopsies diagnosed with mild, moderate or severe dysplasia.

Total cases	Mild dysplasia 20 (100%)	Moderate dysplasia 20 (100%)	Severe dysplasia 5 (100%)
Gender			
Female	11 (55%)	12 (60%)	3 (60%)
Male	9 (45%)	8 (40%)	2 (40%)
Average age (year)	60	54	63
Clinical diagnosis			
Leukoplakia	17 (85%)	18 (90%)	4 (80%)
Erythroplasia	3 (15%)	2 (10%)	1 (20%)
Smoking			
Yes	10 (50%)	14 (70%)	4 (80%)
No	2 (10%)	3 (15%)	0 (0%)
No Records	8 (40%)	3 (15%)	1 (20%)
Alcohol			
Yes	6 (30%)	3 (15%)	2 (40%)
No	4 (20%)	4 (20%)	1 (20%)
No Records	10 (50%)	13 (65%)	2 (40%)
Location			
Tongue	4 (20%)	9 (45%)	3 (60%)
Palate	4 (20%)	1 (5%)	0 (0%)
Floor of mouth	1 (5%)	6 (30%)	1 (20%)
Gingiva	9 (45%)	3 (15%)	1 (20%)
Cheek	2 (10%)	1 (5%)	0 (0%)
No records	0 (0%)	0 (0%)	0 (0%)
Time of evolution			
< 1 year	10 (50%)	4 (20%)	5 (100%)
1–5 years	3 (15%)	6 (30%)	0 (0%)
> 5 years	2 (10%)	3 (15%)	0 (0%)
No Records	5 (25%)	7 (35%)	0 (0%)

Materials and methods

Reagents

Monoclonal anti-β-catenin (M3539) and anti-non-phosphorylated β-catenin Ser33/37/Thr41 (88145) were from DAKO (DAKO, USA) and Cell Signaling Technology, respectively. Monoclonal antibodies against Wnt3 (sc-74537), Wnt5a (sc-365370), cyclin D1 (sc-246), survivin (sc-17779) and GAPDH (sc-365062) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Wnt7b (AF3460) was from R&D systems. Monoclonal antibodies against Wnt3a (ab219412), Vimentin (377M-16) and Cytoqueratin AE1/AE3 (313M-16) were from Abcam (Cambridge, UK) and Cell Marque (St Louis, MO), respectively. Alexa-Fluor-488 and Alexa-Fluor-568-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). Goat anti-rabbit and anti-mouse antibodies coupled to horseradish peroxidase were from Bio-Rad Laboratories (Hercules, CA). Tissue culture medium, antibiotics and fetal bovine serum (FBS) were from Corning Mediatech.

Cell culture

Dysplastic oral keratinocytes (DOK) were obtained from Sigma–Aldrich, (European Collection of Authenticated Cell Cultures, *ECACC* General Collection #94122104). DOK were isolated from a 57-year old man diagnosed with squamous-cell carcinoma in the dorsal tongue. Remnant dysplasia was graded as mild to moderate, removed, and used to initiate primary cultures that originated DOK (ECACC #94122104). DOK were maintained in DMEM-high glucose, supplemented with penicillin (10,000 U/ml) and streptomycin (10 μg/ml), 20% FBS, and 5 μg/ml Hydrocortisone.

The oral keratinocyte cell line, OKF6/TERT2 was kindly donated by Dr. Denisse Bravo (Universidad de Chile). These cells were obtained from the mouth floor, immortalized by expressing the catalytic subunit of telomerase (hTERT) and shown to retain differentiation characteristics in culture [21]. OKF6/TERT2 were cultured in K-SFM medium containing penicillin (10,000 U/ml) and streptomycin (10 μg/ml) and supplemented with bovine pituitary extract and recombinant human epidermal growth factor (both contained as the K-SFM kit), plus 0.3 mM $CaCl_2$.

The oral squamous cell carcinoma (OSCC) line CAL27 (ATCC CRL-2095) were established from a lesion in the middle of the tongue, and show epithelial, polygonal morphology in culture. These cells were maintained in DMEM-high glucose containing penicillin (10,000 U/ml) and streptomycin (10 μg/ml) and supplemented with 10% FBS.

All cells were incubated at 37 °C and 5% CO_2. For Wnt secretion inhibition experiments, the C59 inhibitor of porcupine (inhibitor II C59, Calbiochem) was used as previously described [22]. For immunocytochemistry analysis, cells were grown on glass coverslips and following each treatment, samples were fixed and processed for indirect immunofluorescence, as previously described [23]. Samples were visualized in a Nikon C2 Plus confocal microscope.

Primary culture

Tissues were obtained from healthy human oral mucosa of non-smoking donor subjects. Informed consents were signed before tissue collection and this study was approved by the Medical Ethics Committee, Faculty of Dentistry, Universidad de Chile. Tissues were treated with trypsin (0.25%), subsequently disintegrated and cultured in keratinocyte-serum free media (K-SFM; GIBCO, Carlsbad, CA, USA) containing penicillin (10,000 U/ml) and streptomycin (10 μg/ml) and supplemented with bovine pituitary extract and recombinant human epidermal growth factor (K-SFM kit), plus 0.3 mM $CaCl_2$. Fibroblasts were routinely removed from cultures by trypsinization (0.05% trypsin/0.02% EDTA) and keratinocyte enrichment was evaluated by immunofluorescence, using E-cadherin, cytokeratin, β-catenin and vimentin, as markers.

Tissue immunofluorescence

Case selection. The study was approved by the Ethical Committee from the Faculty of Dentistry. Forty-eight formalin-fixed paraffin-embedded samples were obtained from the Laboratory of Pathological Anatomy, Faculty of Dentistry (Universidad de Chile), and Clinical Records were reviewed to collect the patient demographic data and clinical features of the lesions. Histological classification was assessed by an oral pathologist in sections previously stained with hematoxylin-eosin. Dysplasia was classified as mild (20 cases), moderate (20 cases) and severe (5 cases), and subsequent analyses were performed in representative areas of each degree of dysplasia. Three healthy oral mucosa samples were included as controls.

Biopsy processing. Biopsy sections (3 μm) in paraffin blocks were deparaffinized in xylol and rehydrated in decreasing alcohol, for antigenic recovery. Samples were permeabilized with 0.3% triton X-100/PBS for 15 min, washed twice and then incubated with 5% bovine serum albumin for 30 min. Samples were then incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies for 1 h and subsequently mounted with DAKO. Samples were visualized by confocal microscopy (Nikon C2 Plus).

Subcellular fractionation

Cells were washed twice with ice-cold PBS and homogenized in fractionation buffer supplemented with protease inhibitors and phosphatases. The cell homogenate was shaken at 30–50 rpm, for 30 min at 4 °C. Subsequently, samples were centrifuged at 720*g*, 4 °C for 5 min. The pellet was separated from the supernatant, to obtain nuclear and cytoplasmic proteins. Pellet was then washed with fractionation buffer and centrifuged at 720*g*, 4° C for 10 min, removing the supernatant, and resuspending the pellet in lysis buffer. Samples were resolved by

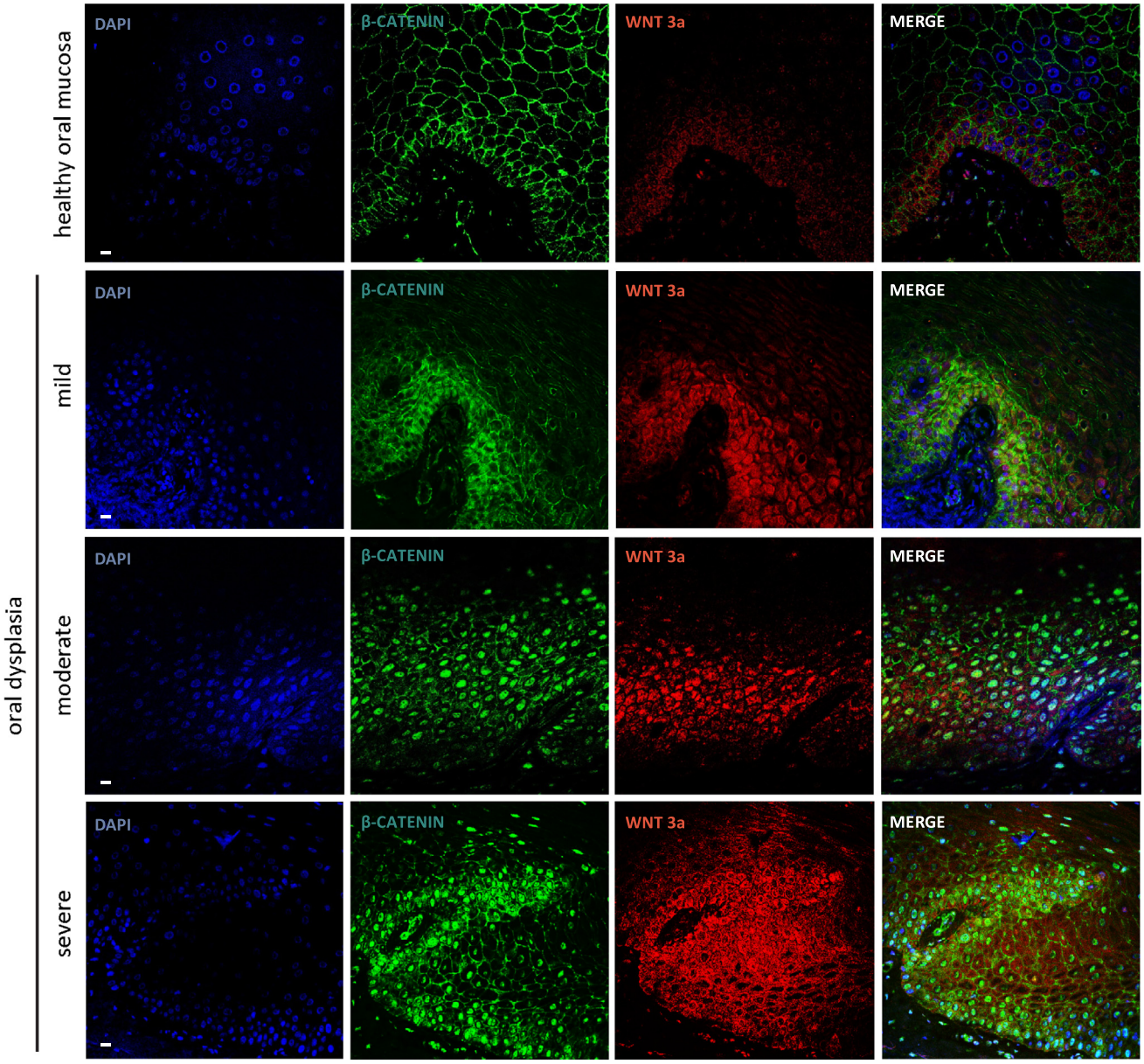

Fig. 5. Tissue immunofluorescence of β-catenin and Wnt3a in healthy oral mucosa, mild, moderate and severe oral dysplasia. Formalin fixed paraffin-embedded samples were obtained from the Laboratory of Pathological Anatomy (Faculty of Dentistry, Universidad de Chile) and histologically classified by an oral pathologist as mild, moderate and severe dysplasia (see materials and methods for details). Biopsy sections (3 μm) were deparaffinized in xylol, rehydrated and then permeabilized with 0.3% triton X-100/PBS for 15 min, for subsequent indirect immunofluorescence analysis. Wnt3a and β-catenin were detected with primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies and mounted for confocal microscopy imaging (Nikon C2 Plus, image capture at 60X magnification). Representative images are shown for healthy oral mucosa, mild, moderate and severe oral dysplasia. Of note, given the heterogeneity of severe and moderate dysplasia cases, all image acquisitions were made in high-grade areas. Membranous, cytoplasmic and nuclear β-catenin, as well as Wnt3a levels were scored for each sample and data are summarized in Table 2.

Table 2
Localization and expression of β-catenin and Wnt3a in healthy oral mucosa, and mild, moderate and severe dysplasia.

Groups	Total cases	Membranous β-catenin (intensity)		Cytoplasmic β-catenin		Nuclear β-catenin		Intensity of Wnt3a	
		Low	High	Positive	Negative	Positive	Negative	Low	High
Healthy oral mucosa	3	0 (0%)	3 (100%)	0 (0%)	3 (100%)	0 (0%)	3 (100%)	3 (100%)	0 (0%)
Mild Dysplasia	20	4 (20%)	16 (80%)	20 (100%)	0 (0%)	16 (80%)	4 (20%)	5 (25%)	15 (75%)
Moderate Dysplasia	20	11 (55%)	9 (45%)	20 (100%)	0 (0%)	20 (100%)	0 (0%)	2 (10%)	18 (90%)
Severe Dysplasia	5	3 (60%)	2 (40%)	5 (100%)	0 (0%)	5 (100%)	0 (0%)	1 (20%)	4 (80%)

Western blotting, as previously described [23].

β-catenin-Tcf/Lef reporter assay

Tcf/Lef transcriptional activity was measured with the luciferase reporter activity system, which is based on transfection with the plasmids pTOP-FLASH (encoding for the luciferase gene preceded by 3 Tcf/Lef binding sites) and pFOP-FLASH (containing point mutations on these sites) [24]. The constitutively active vector encoding for Renilla luciferase (Promega, Madison, WI, USA) was used as co-transfection control. Cells were transfected with 1.5 μg of each plasmid for 24 h and homogenized in a buffer containing 0.1 M KH_2PO_4 (pH 7.9), 1 mM DTT, 0.5% Triton X-100. Then, 50 μl of KTME buffer (100 mM Tris HCl pH 7.8, 10 mM $MgSO_4$, 2 mM EDTA) containing the substrates luciferin 0.073 mM and ATP 5.5 μM, were added. The luciferase activity was quantified in a luminometer.

RNA isolation and RT-qPCR

RNA was extracted with TRIzol Reagent™ (Invitrogen) and cleared with 1U DNase RQ1 (Promega). RNA (1 μg) was used for reverse transcription with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative amplification of cDNA templates was measured with the chemical Fast SYBR® Green Master Mix (Applied Biosystems), using a StepOne Real-Time PCR system (Applied Biosystems). Genes evaluated included: *ciclin-D1* (primers 5′-CCACCT GTCCCACTCCTACGAT-3′; 5′-GCAGGGCCGTTGGGTAGAAA-3′), *survivin* (primers 5′-GCTTCGCTGGAAACCTCTGGA-3′; 5′-TCTGGGCAGAT GGCTGTTGG-3′), *GAPDH* (primers 5′-ACCCACTCCTCCACCTTTGA-3′; 5′-CTGTTGCTGTAGCCAAATTCGT-3′).

Statistical analysis

An exploratory data analysis was performed using descriptive statistics. T-test and ANOVA were used. Values averaged from at least three independent experiments were compared. A value of significance of 5% or less ($p < 0.05$) was accepted as statistically significant. All statistical tests were performed using Stata 11.0 software.

Results

Increased nuclear β-catenin in oral dysplastic keratinocytes

Previous immunohistochemical analyses showed increased nuclear β-catenin in oral dysplasia, when compared with healthy mucosa and oral cancer samples [12–14]. Hence, we sought to corroborate these clinical observations and evaluate them in an in vitro model, using a panel of cell lines that included immortalized oral keratinocytes OKF6/Tert2 (OKF6), dysplastic oral keratinocytes (DOK) and oral squamous cell carcinoma (CAL27). In accordance with those previous observations, we detected elevated nuclear β-catenin levels in DOK cells, when compared with OKF6 and CAL27 cells, as judged by confocal microcopy analysis (Fig. 1A–D). Specifically, both total (Fig. 1A and B) and non-phosphorylated (Ser33/Ser37/Thr41, transcriptionally active) β-catenin (Fig. 1C and D) were substantially accumulated in the nucleus of DOK cells (96% and 92% of total and active β-catenin, respectively), when compared with OKF6 (8% and 7% of total and active β-catenin, respectively) and CAL27 cells (31% and 29% of total and active β-catenin, respectively) (Fig. 1B and D). These observations were further confirmed by subcellular fractionation, as both total and non-phosphorylated β-catenin were substantially enriched in nuclear fractions of DOK cells, and to a lesser extent in CAL27 cells but not in OKF6 cells (Fig. 1E). Collectively, these data indicate that β-catenin is accumulated in the nucleus of dysplastic oral keratinocytes in vitro.

Nuclear localization of β-catenin and expression of target genes depend on Wnt secretion in dysplastic oral cells

Unlike cancer, it remains unclear whether nuclear localization of β-catenin and transcription of target genes depend on autocrine secretion of Wnt ligands in oral dysplasia. Thus, we evaluated the role of Wnt secretion on nuclear localization of β-catenin in DOK cells, by using the inhibitor of Porcupine, C59, which precludes Wnt secretion [22]. Since the effects of this inhibitor in oral dysplastic cells have not been assessed, we first performed a dose-response assay by determining β-catenin localization. Increasing concentrations of C59 caused a progressive decrease of nuclear β-catenin in DOK cells (Fig. 2A), with significant effects starting at 30 μM, and reaching the highest effects at 50 μM (Fig. 2B). These observations were supported by biochemical data, since C59 decreased non-phosphorylated, transcriptionally active β-catenin levels in DOK cells (Figure 2C and D). Accordingly, C59 caused a dose-dependent inhibition in the expression of β-catenin target genes, including survivin (Fig. 2C and E) and cyclin D1 (Supplementary Figure 1), at both mRNA and protein levels. Taken together, these results indicate that increased nuclear β-catenin and expression of target genes in oral dysplastic cells require Wnt secretion.

To further evaluate the requirement of Wnt secretion on nuclear localization of β-catenin and transcription of target genes, we assessed the effect of DOK-derived conditioned medium on OKF6 cells. Intriguingly, DOK-derived conditioned medium induced nuclear accumulation of β-catenin (Fig. 3A and B) and stimulated Tcf/Lef-dependent transcription in OKF6 cells (Fig. 3C). To exclude the possibility that these effects were restricted to cell line-based models, we established primary cultures of oral keratinocytes. Upon previous enrichment and characterization of primary oral keratinocyte cultures (Supplementary Figure 2), these cells were treated with control or DOK-derived conditioned medium for 24 h, and subcellular localization of β-catenin was determined. In agreement with cell line data (Fig. 3A), treatment of primary oral keratinocytes with DOK-derived conditioned medium, but not control medium, promoted nuclear localization of β-catenin in primary oral keratinocytes (Fig. 3D).

Our observations indicate that -yet unknown- factors are secreted by dysplastic oral cells and that these factors promote nuclear accumulation of β-catenin and transcription of target genes in non-dysplastic oral keratinocytes. Intriguingly, we observed that DOK-derived conditioned medium stimulated Tcf/Lef-dependent transcription in OKF6 cells, and that these events required Wnt secretion, since C59 prevented DOK-induced Tcf/Lef-dependent transcription in OKF6 cells (Fig. 3E). Therefore, we screened for different Wnt ligands with reported expression in oral dysplasia and OSCC samples [13,18,19] and measured the effects of C59 on their secretion in dysplastic oral keratinocytes. Remarkably, Wnt3a, Wnt5a, and Wnt7b were readily detected in DOK-derived conditioned medium. However, C59 prevented secretion of Wnt3a, but not Wnt5a or Wnt7b (Fig. 4A). Indeed, DOK cells secreted higher levels of Wnt3a, when compared with OKF6 cells (Fig. 4B). These data suggest that DOK-derived Wnt3a accounts for β-catenin nuclear localization and transcription of target genes. In support of this, Wnt3a alone was able to recapitulate the effects of DOK-derived conditioned medium, since the sole treatment with recombinant human Wnt3a promoted nuclear accumulation of β-catenin in OKF6 cells (Fig. 4C).

Nuclear β-catenin and augmented Wnt3a expression in oral dysplasia

So far, our in vitro data suggest a causal relationship between Wnt3a and nuclear localization of β-catenin in oral dysplastic keratinocytes. To validate these observations in a clinical setup, we performed simultaneous evaluation of Wnt3a and nuclear β-catenin by tissue immunofluorescence in oral dysplasia and healthy mucosa samples. Biopsies were obtained from patients diagnosed with mild, moderate or severe dysplasia, and from healthy donors. All diagnoses were

performed by an oral pathologist and 85% of dysplasia cases were diagnosed as leukoplasia (for clinicopathological features, see Table 1). Of note, the most frequent location of mild dysplasia was in the gingiva, while most moderate and severe dysplasia cases were found in the tongue (Table 1). As expected, all healthy oral mucosa samples showed membranous, cell-to-cell contact restricted β-catenin, along with low expression of Wnt3a. Conversely, all oral dysplasia cases showed positive cytoplasmic immunostaining of β-catenin (Fig. 5). Intriguingly, nuclear β-catenin detection differed according to the grade of dysplasia, since severe and moderate dysplasia showed higher accumulation of nuclear β-catenin, when compared with mild dysplasia and healthy mucosa (percentage of samples with nuclear labeling of β-catenin: severe, 100%; moderate, 100%; mild, 75%; healthy mucosa, 0%; Table 2 and Fig. 5). Collectively, these observations indicate that both Wnt3a and nuclear accumulation of β-catenin are simultaneous events that evolve increasingly during oral dysplasia.

Discussion

Although aberrant activation of the Wnt/β-catenin pathway is commonly associated with the development and progression of several malignancies [10], the role that it plays in oral carcinogenesis is not clear yet. This work evaluated the role of Wnt secretion on β-catenin localization and the expression of target genes commonly deregulated in oral dysplasia. The latter is relevant, since limited information is available regarding the contribution of the canonical Wnt pathway in the progression of oral dysplasia, which is due to the lack of in vitro cell models. In agreement with previous immunohistochemistry studies from our group [14] and others [12,13,25] we showed that dysplastic oral keratinocytes (DOK) depict nuclear accumulation of β-catenin, when compared with non-dysplastic oral keratinocytes (OKF6) and OSCC cells (CAL27) in vitro. In addition, these in vitro models permitted assessing the mechanisms involved in nuclear localization of β-catenin in oral dysplasia. Specifically, by using immunocytochemical and biochemical approaches, we demonstrated for the first-time that nuclear accumulation of both total and non-phosphorylated (or transcriptionally active [26]) β-catenin involves secretion of Wnt ligands in oral dysplastic keratinocytes.

Activation of the Wnt pathway leads to inhibition of the β-catenin destruction complex, allowing the stabilization and nuclear translocation of β-catenin [9]. Not surprisingly, aberrant activation of this signaling pathway in cancer has been associated with mutations in components of the destruction complex, such as APC and Axin [27,28], or β-catenin itself [29]. Intriguingly, no mutations in this pathway have been reported during oral carcinogenesis [30,31], which suggests that mutation events are unlikely associated with nuclear accumulation of β-catenin oral dysplasia. Alternatively, increased activation of Wnt/β-catenin has been associated with increased secretion of Wnt ligands in different cancers, and shown to promote the acquisition of malignant traits, including augmented cell proliferation, migration and invasion [32,33]. Specifically, increased Wnt3a levels were observed at the invasive front of oral carcinoma biopsies [34,35]. Conversely, limiting studies have evaluated the expression and role of Wnt3a in oral dysplasia. Specifically, immunohistochemical analyses showed membrane-associated expression of Wnt3a in oral dysplasia, which correlated with nuclear β-catenin [13]. On the other hand, increased expression of Wnt5a was correlated with advanced stages of oral dysplasia, with the highest expression in OSCC, but not in healthy oral mucosa [19]. Although little is known about the expression of Wnt7b in OSCC samples [18], no studies address the expression of this Wnt in oral dysplasia. Thus, we investigated the role of these Wnt ligands in nuclear localization of β-catenin in oral dysplasia, using the inhibitor of Wnt secretion, C59 [22]. Our findings revealing that C59 decreased secretion of Wnt3a, but not Wnt5a or Wnt7b, along with data showing that C59 reduced nuclear localization of β-catenin, transcriptionally active β-catenin, and the expression of β-catenin target genes, survivin and cyclin D1, support our model that secretion of Wnt3a is required for nuclear localization of β-catenin and downstream signaling in oral dysplasia. These observations were further supported by Tcf/Lef reporter assays in non-dysplastic oral keratinocytes, since C59 prevented the effects of DOK-derived conditioned medium on OKF6 cells. The observation that DOK-derived conditioned medium recapitulates nuclear accumulation of β-catenin and Tcf/Lef-dependent transcription in OKF6 cells and primary keratinocytes is remarkable, as this supports the idea that progressive (perhaps paracrine) secretion of Wnt3a accounts for early-to-late stage progression in oral carcinogenesis [13]. In support of this, we observed augmented secretion of Wnt3a, but not other Wnts in conditioned medium obtained from DOK, but not OKF6 cells.

Although our in vitro observations showing that DOK cells depict higher accumulation of nuclear β-catenin, when compared with CAL27 cells, are consistent with previous reports in clinical samples [13,14] these findings are somewhat counterintuitive. It might be possible that transient activation of this pathway is required at early stages of oral carcinogenesis, namely moderate/severe dysplasia, where proliferation is actively demanding [1,36–38], while it downregulates in the transition to OSCC. Future studies, based on preclinical models, will be required to address this intriguing possibility.

The dependency of survivin expression on Wnt3a secretion is intriguing, because survivin is overexpressed in most malignancies, including OSCC [15,39,40] and it has been proposed as an early predictor of potentially malignant lesions of the oral cavity, since it is upregulated in 94% of oral dysplasia samples that progressed to carcinoma [15,41].

In summary, this study assessed the relationship between Wnt3a secretion and nuclear localization of β-catenin in oral dysplasia, in vitro, thus congregating earlier findings obtained in separate studies, showing increased Wnt3a secretion on the one hand [42,43], and nuclear accumulation of β-catenin on the other hand [12,13]. In fact, simultaneous expression of Wnt3a and β-catenin was confirmed in tissue biopsies of oral dysplasia, when compared with healthy oral mucosa. Specifically, Wnt3a and nuclear β-catenin correlated with dysplasia grading, since severe/moderate dysplasias showed higher levels of these molecules in comparison with mild dysplasia. Indeed, given the heterogeneity of oral dysplasia, we observed that both Wnt3a and nuclear β-catenin decreased in low-grade areas of severe/moderate dysplasia, supporting the view that dysplasia grading is associated with the expression of these markers (data not shown). In summary, this study shows that the presence of nuclear β-catenin and the expression of survivin in oral dysplasia, which are due to the activation of Wnt/β-catenin via Wnt3a, are feasible candidates to be explored as possible therapeutic targets and markers to the progression of early oral lesions.

Declaration of Competing Interest

No potential conflicts of interest are disclosed.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.05.010.

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