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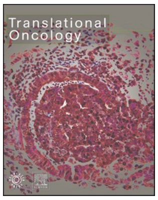

IDKK1 inhibits canonical Wnt signaling in human papillomavirus-positive penile cancer cells

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ABSTRACT

Penile squamous cell cancer (PSCC) is the most frequent penile malignant disease. Infections with human papillomaviruses (HPV) are a major etiologic driver of PSCC. However, the molecular details of the underlying carcinogenesis are understudied because of rare clinical specimens and missing cell lines. Here, we investigated if the expression of high-risk HPV16 oncogenes causes an augmentation of the Wnt pathway using unique HPV-positive penile cancer (PeCa) cell lines in monolayer and organotypic 3D raft cultures as well as tissue micro arrays containing clinical tissue specimens. The HPV oncoproteins enhanced the expression of Leucine-rich repeat-containing G-protein coupled receptor 6 (*LGR6*) and the HPV-positive PeCa cells expressed a signature of Wnt target and stemness-associated genes. However, the notable lack of nuclear β-catenin *in vitro* and *in situ* raised the question if the enhanced expression of Wnt pathway factors is tantamount to an active Wnt signaling. Subsequent TOP-flash reporter assays revealed Wnt signaling as absent and not inducible by respective Wnt ligands in PeCa cell lines. The HPV-positive PeCa cells and especially HPV-positive PeCa specimens of the tumor core expressed the Wnt antagonist and negative feedback-regulator Dickkopf1 (DKK1). Subsequent neutralization experiments using PeCa cell line-conditioned media demonstrated that DKK1 is capable to impair ligand-induced Wnt signaling. While gene expression analyses suggested an augmented and active canonical Wnt pathway, the respective signaling was inhibited due to the endogenous expression of the antagonist DKK1.

Abbreviations: CTNNB1, Catenin Beta 1; DAPI 4′, 6-Diamidin-2-phenylindol; DKK1, dickkopf; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed paraffin embedded; HFF, human foreskin fibroblasts; HPV, human papillomavirus; IF, immunofluorescence; IHC, immunohistochemistry; IRS, immune-reactive scores; LGR, Leucine-rich repeat-containing G-protein coupled receptor; NFK, normal foreskin keratinocytes; MDSC, myeloid derived suppressor cells; PeCa, penile cancer; PFA, paraformaldehyd; PMN, polymorphonuclear cells; RT-PCR, real-time polymerase chain reaction; RPL13A, ribosomal protein L13A; RSPO, R-spondin; SOX2, sex determining region Y (SRY)- box 2; TMA, tissue micro array.

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Subsequent TMA stainings indicated Dkk1 as linked with HPV-positivity and metastatic disease progression in PeCa suggesting potential as a prognostic marker.

Introduction

Penile cancer (PeCa) displays a rare malignant disease associated with low survival rates, limited therapeutic options and increasing incidence [1–3]. While in the USA or Germany, about 0.5–1.0% of all cancers among men are PeCa, it constitutes up to 10% of male malignancies in areas of Africa, South America, and Asia [3–5]. The majority of patients present with PSCC [1,3]. Besides typical risk factors, such as phimosis, chronic inflammation, poor hygiene, and smoking, one major risk factor with rising relevance is an infection with high-risk HPV [1,2,4]. HPV, a highly frequently sexually transmitted infection, drives the incidence rates of cancers at transformation susceptible sites, such as cervix uteri, tonsils, and penis [4,6–10]. The prevalence of HPV in PeCa can vary depending on the study design, the underlying HPV methods as well as histological subgroups. It was ranged globally in two large-scale reports between 33 [2] and 50% [4] with a high prevalence in subtypes of basaloid- and warty-like histology [2,4,5,8,11]. Both large-scale studies reported notable geopraphic differences with high prevalences in Africa, Latin America, followed by Europe and a lower prevalence in Asia [2,4]. The predominant subtype of HPV is the high-risk HPV16, detected in up to 79% of PeCa and 69.3% of these with active oncogene expression [2]. PeCa patients present with histological different cancers resulting from HPV-dependent or -independent pathways of carcinogenesis while both, viral oncoprotein- and or chronic inflammation and genetic mutation-driven malignant transformations may affect similar molecular pathways with potential therapeutic implications [12]. Thus, establishing prognostic markers will have a high clinical impact for subsequent patient stratification [5].

The HPV oncogenes encode for proteins with high potential to cause the malignant transformation of the infected cell [12–15]. Two HPV oncoproteins, E6 and E7, degrade and affect cell cycle regulators and tumor suppressors, such as p53 and pRB, unlock central regulatory pathways from their control mechanisms causing sustained proliferative signaling. Besides, both oncoproteins support autocrine supply with mitogenic stimuli by augmenting EGFR pathway activation [16,17]. There is growing experimental evidence that HPV oncoproteins interfere with the canonical Wnt signaling pathway [18–20]. For instance, E6 was shown to bind to the E6AP ubiquitin-protein ligase (E6AP) and disheveled segment polarity protein 2 (DVL2) and both, E6 and E7, repress the expression of seven in absentia homolog (Siah-1). In total, this has the potential to cause an active canonical Wnt pathway that further can promote cancer progression, dissemination and metastatic outbreaks [18–20].

The Wnt signaling pathway displays a key regulator of organ development and tissue renewal and was repeatedly shown to be critically involved in carcinogenesis [21,22]. In brief, the Wnt ligands bind to Frizzled (FZD) receptors and subsequent signaling causes the accumulation of stabilized and nuclear translocation of β-catenin that binds to TCF/LEF motifs enhancing target gene expression [22]. Co-factors, enhancers, and regulators fine-tuning the pathway activity accompany these basic factors. Of those, members of the LGR protein family, LGR4, LGR5 and LGR6, mark adult stem cells in charge for tissue self-renewal. While LGR5 seems to mark stem cells of internal organs [23–26], LGR6 marks stem cells in the skin [27]. LGR proteins are receptors for roof plate-specific spondin (RSPO) ligands that can amplify Wnt signaling [23]. Activation of the Wnt pathway leads to the expression of Dickkopf 1, DKK1, a target gene and negative feedback regulator of the Wnt pathway [28,29]. DKK1 desensitizes cells from Wnt ligands and is capable to suppress Wnt pathway signaling by interfering with the LRP5/6 co-receptors. Thus, it remains questionable if an increased expression of Wnt pathway components and target genes is tantamount to an actually active Wnt signaling. Moreover, we may hypothesize, that a continuously activated canonical Wnt pathway in HPV-positive cancers may cause the negative feedback regulator DKK1 to keep the cancer cells in a stem cell-like state while abrogating Wnt signaling.

Current experimental data on Wnt pathway activation in HPV-positive cancers were generated using cervical cancer (CxCa) or head and neck squamous cell cancer (HNSCC) cell lines or mouse models expressing the HPV oncoproteins under control of the cytokeratin 14 promoter [18–20,30] or designed on the assumption that Wnt pathway activation is mediated by HPV oncoproteins [3]. On the other side, investigations on the molecular mechanisms of the penile carcinogenesis have long been impaired due to the rareness of clinical specimens and appropriate cell culture models. Here we report the characterization of HPV-positive PeCa cell lines [11,24,31] and tissue microarrays with HPV-positive and negative specimens [5,11] for the expression of Wnt pathway associated genes to investigate the presence of an active canonical Wnt signaling in PeCa.

Materials and methods

Ethical statement, cohort and study design, material identifiers

The local Ethics Committee of the Saarland (Ärztekammer des Saarlandes, Saarbrücken, Germany) in accordance with the Declaration of Helsinki approved experiments with human material used in this study and written informed consent by study participators. The TMA cohort consists of patients derived from Russia and Germany between 1992 and 2015. Data on clinical outcome and HPV status were published previously [5,11]. Briefly, DNA was isolated from FFPE tissue sections by QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following manufacturers protocol and the HPV PCR was conducted using the GP5+/6+ primers as described previously [5]. HPV status was further determined by p16$^{\mathrm{INK4a}}$ immunohistochemistry, a surrogate marker for HPV oncoproteins as previously recommended [32], using a published protocol [5]. HPV status was considered as positive in case of both PCR and IHC were positive. Sections of all cases were reviewed by two experienced uropathologists and histological subtypes as well as tumor grade were defined according to the 2016 WHO classification and the 8th editions of TNM classification of malignant tumors. PeCa tissue was punched to generate four different TMAs reflecting the tumor center (TMA TC, $n = 70$), the invasion front (TMA IF, $n = 68$), lymph node metastases (TMA LM, $n = 21$) and adjacent normal foreskin ($n = 49$) with duplicates (TMAs TC, IF, NO) and triplicates (TMA LM) of the individual specimens, respectively.

Cell lines and culture conditions

Three HPV-positive PeCa cell lines were generated previously from a primary carcinoma and lymph node metastases, including the particularly rare case of one primarius- (named P2) and one metastasis-derived (named L2) cell line originating from the same patient and one further metastasis-derived cell line of an additional patient (named L3). The cell lines were authenticated by Multiplexion in 2018 using the originating biopsies obtained from patients that underwent penectomy and metachronous radical inguinal lymph node dissection for metastatic squamous cell carcinoma of the penis at the University Hospital Schleswig-Holstein, thus representing a validated cell culture system [28]. The HPV status of the cell lines was investigated recently [11]. Cells were cultivated in PeCa medium (1:1 mixture of keratinocyte growth medium 2 (KGM2) containing all supplements (C-20,011, bovine pituitary extract 0.004 ml/ml, EGF 0.125 ng/ml, insulin 5 μg/ml, hydrocortisone

0.33 μg/ml, epinephrine 0.39 μg/ml, transferrin 10 μg/ml, calcium chloride 0.06 M, PromoCell, Heidelberg, Germany) and RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FCS), 1% sodium pyruvate and 1% penicillin and streptomycin (Merck, Schnelldorf, Germany). The human cervical carcinoma cell line C33a was kindly provided by Prof. Knebel-Doeberitz (Institute of Pathology, University Hospital Heidelberg, Germany) in 2011, authenticated by Multiplexion in 2018 and maintained in DMEM containing 10% FCS, 1% sodium pyruvate and 1% penicillin and streptomycin (D10+/+). Normal foreskin keratinocytes (NFK) and human foreskin fibroblasts (HFF) were isolated from foreskin tissue (Saarland University Medical Center), tested negative for HPV using PCR [11] and expanded in KGM2 (C-20, 011, PromoCell) and D10+/+, respectively. NFK were cultured in PeCa medium for experiments. We conducted mycoplasma-specific PCRs on a regularly basis of once per month. Cell lines were used below passage 20, NFK up to passage 4 and HFF up to passage 7. Organotypic three dimensional (3D) cultures were generated using HFF (5×10^5 cells, passage 3–5) embedded in 1 ml of rat collagen (as described previously, 11) in 24 well plate in D10+/+. The day after, medium was exchanged to PeCa medium for 1 h before 7×10^5 PeCa cells were seeded on top of the collagen-fibroblast matrix. Next day, the cultures were transferred onto a metal grid in six well plates to allow multilayered growth at the air-liquid interface. 14 d later, supernatants were harvested, organotypic 3D cultures fixed in 4% paraformaldehyde (Merck) and embedded in paraffin. Retroviral gene transfer was conducted as described previously [33,34]. HPV status of PeCa cell lines was published earlier [11]. Cell lines for producing Wnt3a (L-Wnt3A (ATCC®CRL-2647™)- and RSPO1 (Cultrex® HA-R, Spondin 1-Fc 293T Cells)-conditioned media were obtained in 2018 by ATCC (Manassas, USA) and R&D (Minneapolis, USA), respectively. Conditioned media were generated as previously described [35]. Suppl. Table 1 includes material identifiers.

Immunohistochemistry and immunofluorescence

FFPE tissue (TMA) and slides of organotypic 3D cultures were stained by immunohistochemistry (IHC). Antigen retrieval was performed by heating the sections in 1 mM citrate buffer pH 6.0 at 95°C for 10 min and endogenous peroxidase activity was blocked with 3% H_2O_2/TBS for 10 min (for DAB staining). FFPE sections were incubated with the indicated antibodies (Suppl. Table 1) overnight followed by HRP-conjugated secondary anti-mouse or AP-conjugated anti-rabbit antibody incubation and developed with DAB or AP-substrates (Suppl. Table 1). After counter-staining with hematoxylin, the slides were covered with Vectamount and documented. Staining was documented using a Leica DMI6000 with LAS X software.

Immunofluorescence (IF) staining of monolayer cultured cells were conducted in black μ-clear flat bottom 96-well plates (IF). Cells (5×10^4 cells/well) were seeded in triplicates and 24 h later washed twice with 1x PBS, fixed in methanol and permeabilized using 0.2% Triton in 1x PBS for 5 min. After blocking with 3% BSA in 1x PBS + 0.002% Triton for 30 min, cells were incubated with primary antibodies (Suppl. Table 1) diluted 1:200 in 0.5% BSA in 1x PBS overnight at 4 °C. Cells were then washed and incubated with secondary antibody anti-mouse/rabbit Alexa Fluor 546 antibodies 1:200 in 0.5% BSA in 1x PBS 60 min in the dark. Nuclear counter-staining was conducted with DAPI in methanol for 2 min. After a final wash step, cells were covered in 50 μl 1x PBS and documented using a Leica DMI6000 with LAS X software.

Gene expression analysis, RT-PCR and enzyme-linked immunosorbent assay (ELISA)

Gene expression NFK, P2, L2, and L3 cells was measured with SurePrint G3 Human Gene Expression 8 × 60Kv2 Microarray in duplicates (Cat. no. G4851B, Agilent Technologies, Santa Clara, CA, USA) as previously described [36]. Bioinformatics analysis were performed using Agilent Feature Extraction image analysis software generated the raw data, which were quantile-normalized and log2-transformed using R v3.5.1. Only transcripts expressed in at least 50% of each group (cancer (L2, L3, P2) vs. control samples (NFK)) were considered for further analysis. For each HPV-positive cell line separately, fold-changes were calculated in comparison to the mean expression in the NFK samples. Quantitative RT-PCR was conducted as previously described (33, primers and probes, Suppl. Table 2). Expression levels were normalized to ribosomal protein L13A (*RPL13A*). Conditioned media of 2D-cultured cells were generated by seeding cells (1×10^6) in 6 cm dishes (Sarstedt) and collecting supernatants 24 h later. Conditioned media of 14 d grown organotypic 3D cultures were generated as described above. DKK1 was quantified using Human DKK-1 ELISA Kit RayBiotech, Norcross, USA.

TOP-flash assay

The TOP-flash Assay was conducted as previously described [35] with minor modifications. Briefly, 1.5×10^5 (PeCa) or 3×10^5 (C33a) cells were seeded one day prior to transfection into a 12-well plate. The transfection with the M50 or M51 and pEYFP-C1 plasmid was performed using Lipofectamine LTX with Plus reagent. After 24 h the cells were stimulated with Wnt3a-conditioned medium and additionally RSPO1-conditioned medium. 30 mM LiCl served as a positive control. The cells were harvested, transfection efficacy evaluated by flow cytometry (32 +/- 5%), and luciferase as well as Bradford assay conducted as previously described [33]. The luciferase activity was normalized to the protein concentration detected by Bradford assay.

Data processing and statistical analyses

Data were generated from at least three independent experiments with triplicates or duplicates as indicated. Graphical and statistical analyses were performed using Graph Pad Prism 9.0 (Graph Pad Software, San Diego, USA). Group data are reported as mean ± SEM. Data of multiple experiments were illustrated as box and whiskers blots showing individual results with minimum and maximum. Significance was determined by two-way or one-way ANOVA repeated measures test with Tukey's correction and Fisher`s exact test as indicated. Significance was accepted when p-values were ≤ 0.05.

Results

The Wnt transcriptomic signature in HPV-positive PeCa cells

We conducted a mRNA microarray of two donors of HPV-negative non-malignant NFK and three different HPV-positive PeCa cell lines, P2, L2 and L3, whose HPV status has been described previously [11], to analyze the expression of Wnt pathway-associated genes during the HPV-driven penile carcinogenesis. After normalization on NFK expression levels, we identified a signature of differently expressed genes (Fig. 1A). Of the WNT paralogues, *WNT3A, WNT7B, WNT9A* and *WNT10B,* inductors of the canonical Wnt/β-catenin signaling, displayed an enhanced expression in PeCa cells than in NFK. Other ligands such as *WNT4, WNT5A*, and *WNT5B,* inductors of the non-canonical β-catenin-independent Wnt signaling [21], were lower expressed in PeCa than in NFK cells. We detected a notable enhanced expression of *RSPO4*, a ligand for LGR proteins that may potentiate Wnt signaling [23], in PeCa cells while *RSPO3* displayed a reduced expression in PeCa cells compared to NFK. Of the Wnt receptor proteins, the FZD paralogues *FZD2, FZD4, FZD9* together with *LRP5, LRP6* displayed an elevated expression in PeCa cell lines. On the other side, *FZD7* showed a reduced expression in PeCa vs. non-malignant cells. The RSPO ligand receptors exhibited an altered expression as well. *LGR6* displayed an elevated expression in PeCa than in NFK cells while *LGR4* was lower expressed in the two lymph metastases-derived cell lines L2 and L3. Wnt pathway and signaling associated components displayed an up- and downregulated

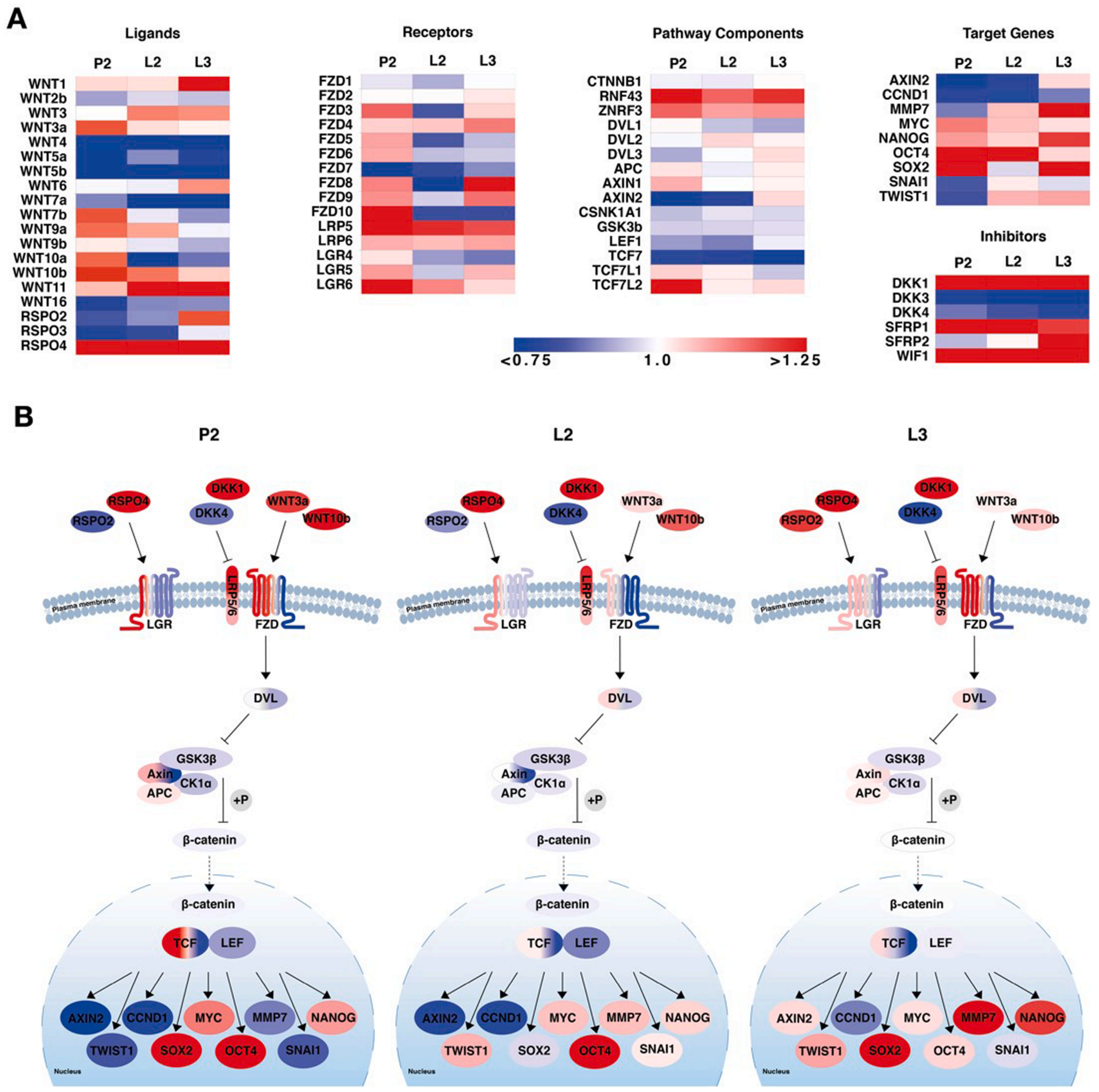

Fig. 1. Transcriptomic profiling of Wnt pathway associated genes in HPV-positive PeCa cells: (A) Gene expression was analyzed using a SurePrint G3 Human Gene Expression 8 × 60Kv2 Microarray and GenescriptSX software. Illustrated by color code are the fold change of expression to the NFK for each PeCa cell line. (B) Schematic illustration of the canonical Wnt pathway with genes identified as differently expressed in each PeCa cell line compared to NFK using the same color code as in A.

expression pattern with *RNF43, ZNRF3, CTNNB1, AXIN1* and *TCF7L2* elevated expressed in PeCa cells than in NFK. Others such as *TCF7, GSK3B* and *CCND1* displayed a reduced expression in the HPV-positive PeCa cell lines than in NFK cells. A reduced expression of GSK3B, a central participant in the β-catenin-destruction complex, may suggest an enhanced canonical Wnt signaling by an increased level of stabilized β-catenin protein [22]. Next, we analyzed potential target genes of the canonical Wnt pathway [21,22] and identified a notably enhanced expression of *MYC, NANOG, OCT4* and *SOX2*. The antagonists of Wnt signaling were differently expressed as well, with an elevated expression of *DKK1, SFRP1–1* and *WIF1* and a reduced expression of other *DKK* family members such as *DKK3* and *DKK4* in HPV-positive PeCa cells compared to NFK. In summary, transcriptomic profiling revealed extensive changes and a signature of elevated expressed Wnt pathway-related genes in HPV-positive PeCa cells compared to NFK (Fig. 1B). Since especially the two PeCa cells, P2 and L3, with high oncoprotein levels [11] displayed a Wnt signature these results suggest an HPV oncoprotein related increment in canonical Wnt signaling.

Elevated expression of Wnt pathway enhancers and target genes in HPV-positive PeCa cells

The data retrieved from the mRNA microarray indicated an elevated expression of Wnt pathway enhancing factors of the LGR-RSPO axis. We subsequently investigated if these results could be confirmed using qRT-PCR (Fig. 2A). Indeed, *LGR6* and *RSPO4* mRNA were increasingly detectable in HPV-positive PeCa cell lines P2 and L3 while *LGR4* was higher expressed in NFK than in all PeCa cell lines. Both PeCa cell lines P2 and L3 displayed an elevated expression of *LGR4, LGR5*, and *LGR6* than the PeCa cell line L2 with low oncogene expression [11]. From all RSPO ligands, *RSPO3* expression was reduced in P2 and L2 compared to NFK and L3. The expression of *RSPO2* was similar low in P2, L2 and NFK and *RSPO1* expression was not significantly different between these three cells. *RSPO4* was elevated expressed in the PeCa cell lines P2 and L3. Notably, the cell line L3 expressed the highest mRNA levels of all four LGR ligands, *RSPO1–4*. These results suggest that the HPV oncoproteins E6 and E7, that are elevated expressed in P2 and L3, may induce *RSPO4*, and that the additional oncoproteins expressed in the cell line L3, such as E2 and E5 [11], may induce all four ligands, *RSPO1–4*.

Organotypic three-dimensional (3D) raft cultures have been recently described to provide a closer picture of the physiological situation regarding the HPV oncoprotein driven malignant transformation including effects of a multilayered epithelial growth [37]. 3D raft cultures composite of HFF embedded in extracellular matrix with keratinocytes or tumor cells seeded on top at the air-liquid interface that allows them to grow to a multilayered epithelium. This cell culture model creates an environment that mirrors intercellular regulatory networks of non-malignant and cancerous tissue, thus enabling studies of the role of Wnt enhancers LGR4–6 in a more physiological context of epithelial cell differentiation and dysplastic development. IHC staining for LGR4–6 on FFPE slides of these 3D cultures (Fig. 2B) revealed that the LGR4 staining in P2 and L3 was more intense than in L2 and NFK. LGR5 staining in PeCa cell lines was more diffuse throughout all cell layers while in NFK, basal cells were stained more prominently suggesting an expansion of a LGR5$^+$ cell population. The strongest staining was detected for LGR6 and the LGR6-positive cell population expanded to the uppermost cell layer in organotypic 3D raft cultures of all three HPV-positive cancer cells while in NFK rather basal layers were stained only. These results indicated that HPV oncoproteins might enhance the expression of LGR proteins, particularly of LGR6. Subsequent qRT-PCR analyses on NFK transduced by retroviral vectors encoding the HPV oncoproteins E6 and E7 revealed a significantly reduced expression of *LGR4*, a partially but not significantly increased expression of *LGR5* and that the oncoproteins significantly drive the expression of *LGR6* (Fig. 2C). Notably, *LGR4* displayed the highest mRNA levels in NFK and PeCa cells while those for *LGR5* were the lowest. The mRNA levels of *LGR6* were in the median range but displayed the most significant alteration upon oncogene expression with a fold change of 4.07 +/- 2.16 ($p = 0.0004$) compared to *LGR4* (fold change 0.82 +/- 0.09, $p = 0.011$) and *LGR5* (fold change = 2.79 +/- 1.66, $p = 0.1384$). In summary, we detected an elevated expression of Wnt pathway enhancers in HPV-positive PeCa cell lines that in turn may lead to an elevated Wnt target gene expression in particular in PeCa cells with high levels of active viral oncoproteins.

The signature of enhanced Wnt target gene expression in HPV-positive PeCa

Subsequent analyses of Wnt target genes by qRT-PCR, IF and IHC revealed an elevated expression of *OCT4* [11], *TWIST, MYC, NANOG* and *SOX2* (Fig. 3A–C) in the PeCa cell lines, with especially SOX2 and OCT4 predominantly in the cell lines P2 and L3, those with high viral oncoprotein levels [11]. Notably, the SOX2$^+$ cell population expanded similarly to the LGR6$^+$ population in organotypic 3D raft cultures further indicating an expansion of cells with a stemness-like status and active Wnt signaling, while in NFK, the basal cells were stained (Fig. 3C). The expression of *CCND1*, encoding for CYCLIN D1, was significantly reduced compared to HPV-negative NFK confirming the results retrieved by the gene expression analyses above (Fig. 1). Together with previous data, our results suggest that HPV16 oncoproteins support the expression of Wnt target genes and stemness markers [11,38,39] further pointing to an active canonical Wnt signaling in the HPV-positive PeCa cells.

HPV-positive PeCa cells express elevated levels of CTNNB1/β-catenin

Our data suggest an active canonical Wnt signaling in HPV-positive PeCa cells. The central and critical step in this signaling cascade is the stabilization and nuclear translocation of β-catenin [22]. Initially, we tested if the PeCa cells with high oncoprotein levels express elevated levels of β-catenin as well. Indeed, both cell lines, P2 and L3, expressed significantly higher levels of *CTNNB1* mRNA than HPV-negative non-malignant control cells, especially P2 with a 2-fold increase ($p < 0.0001$) compared to NFK (11, Fig. 4A) and the PeCa cell line L2 with low oncoprotein activity [11]. Next, we conducted indirect IF (Fig. 4B) staining on adherent in monolayer growing PeCa cells to investigate the protein expression and cellular localization. B-catenin was detected in all cell lines with a stronger detection of the protein in P2 and L3 compared to L2. However, β-catenin displayed a rather exclusively cytosolic than nuclear staining that would not indicating an active canonical Wnt signaling. Next, IHC on FFPE slides of organotypic 3D raft cultures of PeCa cell lines were stained (Fig. 4C) to evaluate the β-catenin expression in a physiological cell culture model. While NFK displayed a strong but membranous β-catenin staining, P2 and L3 showed a more intense and diffuse staining. Notably, we detected only exceptional β-catenin$^+$ nuclei (black arrows) with the hematoxylin counterstaining giving the impression that almost all nuclei were negative for β-catenin. Thus, β-catenin is elevated expressed in HPV-positive PeCa cell lines with high expression levels of both oncoproteins HPV16 E6 and E7 [11]. Since both oncoproteins did not induce *CTNNB1* mRNA expression in NFK transduced by viral vectors (Suppl. Fig. 1) our results point to a stabilization of the protein by the reduced expression of factors involved in the destruction complex, as shown above for *GSK3B*, together with the previously described downregulation of the Siah-1 E3 ligase by HPV oncoproteins [20]. Nevertheless, our *in vitro* data showed that the central indicant for an active canonical Wnt signaling, the nuclear translocation of β-catenin, is missing. We then examined the expression of β-catenin in clinical PeCa specimens using four TMAs reflecting the tumor center (TMA TC, $n = 75$), the invasion front (TMA IF, $n = 64$), lymph node metastases (TMA LM, $n = 23$) and adjacent normal foreskin ($n = 78$). Slides we stained by IHC for β-catenin and immune-reactive scores (IRS) were determined according to Remmele & Stegner. HPV-positive was defined as reactive in both PCR and p16INK4a IHC [5, 11]. Briefly, nuclear translocated β-catenin was not detected in HPV$^+$ and HPV$^-$ PeCa specimens (Fig. 4D, E). There was a noteworthy but not significant higher mean IRS of HPV$^-$ as for HPV$^+$ PeCa lymph node metastases (TMA LM), while IRS were similar for HPV$^+$ and HPV$^-$specimens of the TMA TC and IF (Fig. 4F). In summary, our data using clinical PeCa specimens confirmed the *in vitro* results regarding the missing nuclear β-catenin raising the question if there is an active canonical Wnt signaling in PeCa cells.

No inducible Wnt signaling in HPV-positive PeCa cell lines

Next, we conducted reporter gene assays to analyze Wnt signaling in HPV-positive PeCa cell lines. The TOP-flash assay is the standard and widely used luciferase reporter assay to measure active Wnt signaling [40]. It is based on TCF/LEF multimerized motifs that, upon active Wnt signaling related nuclear translocation and binding of β-catenin, initiate the transcription of the encoded reporter gene [40]. As a positive control, we used conditioned media containing WNT3a either alone or in

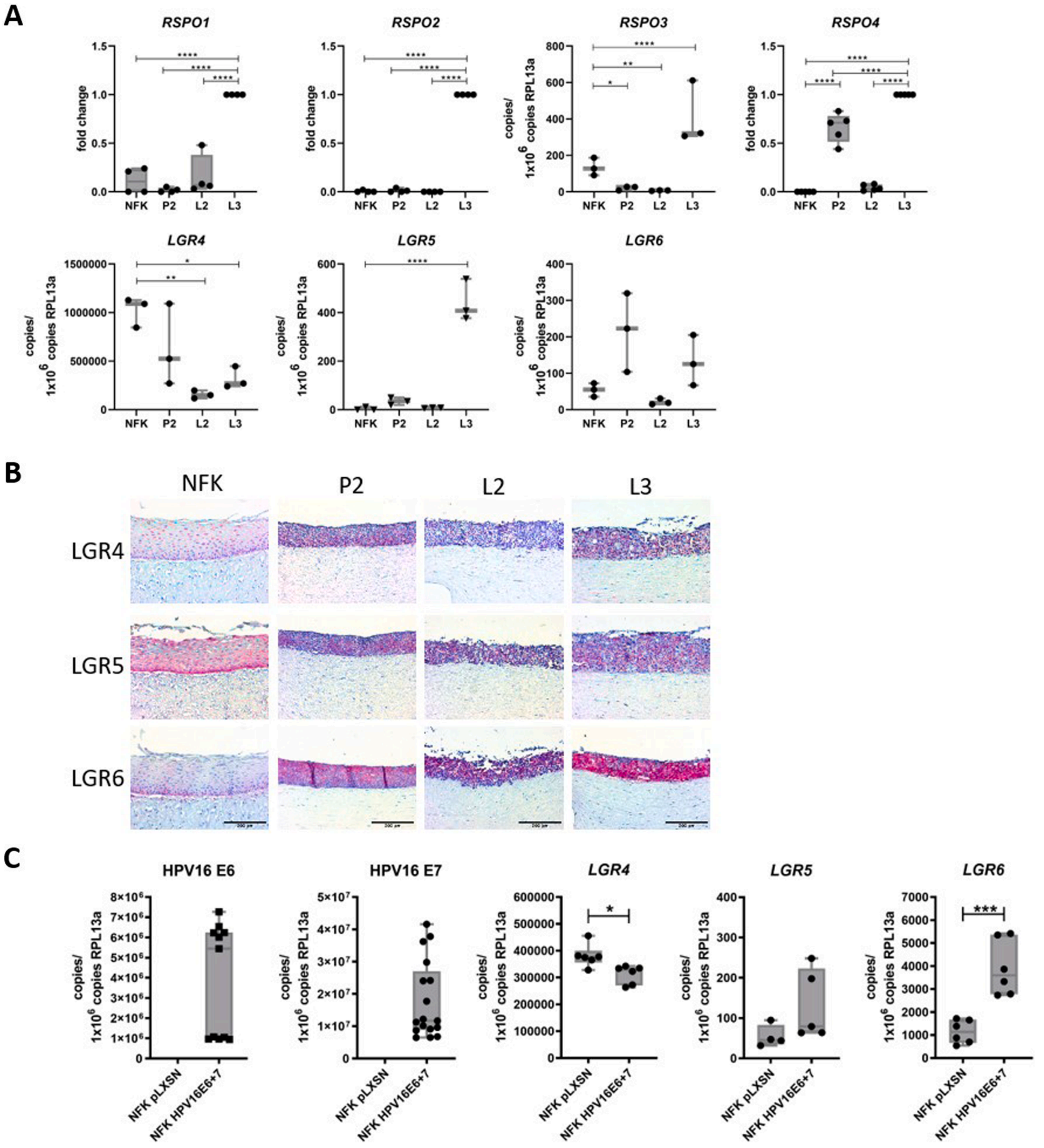

Fig. 2. The expression of the LGR-RSPO axis in HPV-positive PeCa cells: (A) Gene expression of Wnt signaling enhancers (*LGR, RSPO*) was analyzed by qRT-PCR. (B) Expression of LGR4–6 was analyzed using IHC and FFPE of organotypic 3D cultures of NFK and PeCa cell lines. (C) NFK were transduced with retroviral vectors encoding HPV16 E6 and E7 or empty plasmid (pLXSN) and assayed by qRT-PCR for the expression of E6, E7 and *LGR4–6* of individual NFK donors. Pictures were recorded with 20x magnification, 0.5 cm = 100 μM (10x). Images are representative pictures of three independent experiments of three independent cultures run in duplicates (B). Significant differences were calculated by one-way Anova and Tukey's multiple test and illustrated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$: ****$p < 0.0001$). NFK = normal foreskin keratinocytes.

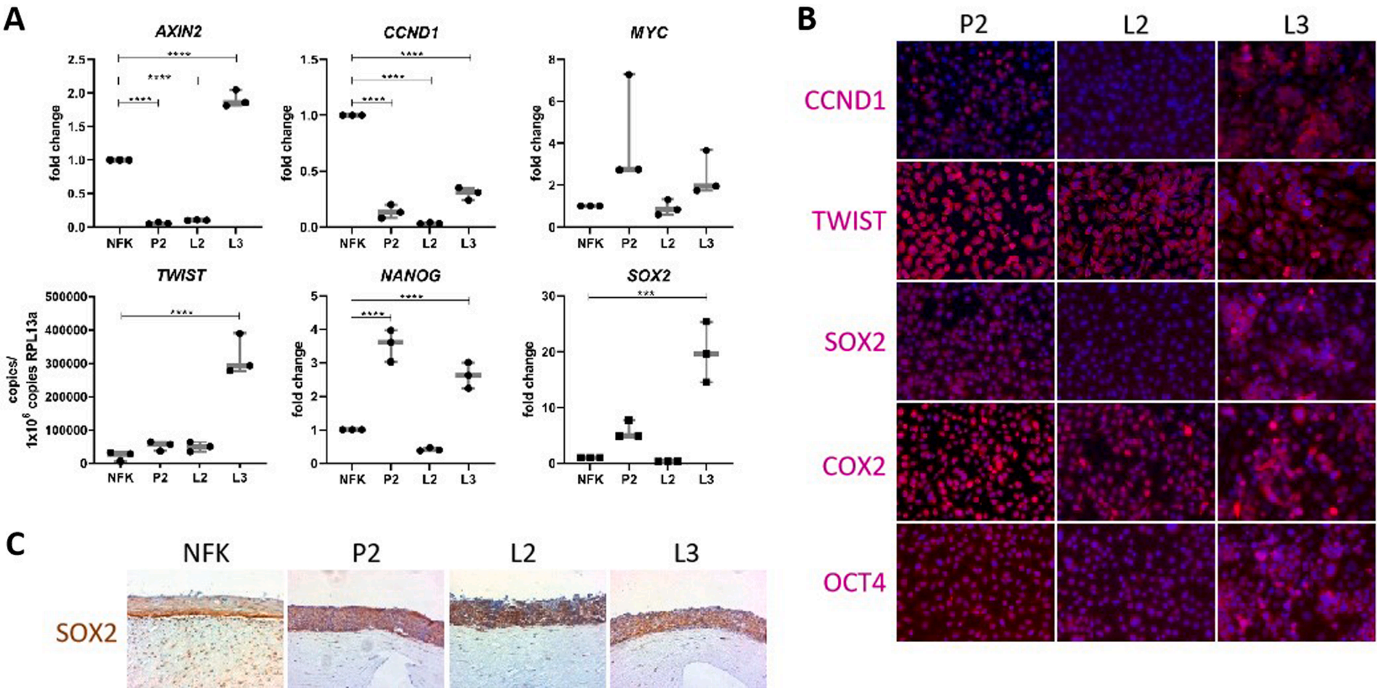

Fig. 3. The Wnt target gene expression in HPV-positive PeCa cells: Gene expression of Wnt target genes was analyzed by qRT-PCR (A), indirect immunofluorescence of monolayer cultured PeCa cell lines (B) and IHC on FFPE slides of organotypic 3D cultures of NFK and PeCa cell lines (C). Data in A were generated of three independent experiments of three independent cDNA/cell line and are illustrated as fold change (ddCt method) or as copies/1 × 10^6 copies *RPL13a*. Images are representative pictures of three independent cultures run in triplicates (B) and duplicates (C). Significant differences were calculated by one-way Anova and Tukey's multiple test and illustrated by asterisks (**p* < 0.05; ***p* < 0.01; ****p* < 0.001: *****p*<0.0001). NFK = normal foreskin keratinocytes.

combination with RSPO1 and LiCl [40]. Respective reporter plasmids were transfected into HPV-positive PeCa and C33a cells. This HPV-negative cervical cancer cell lines served as a control [41] because HPV-negative PeCa cell lines are not available. While treatment with LiCl and conditioned media significantly induced luciferase activity in C33a (Fig. 5A), PeCa cells did solely respond to LiCl without any reporter activity using the conditioned media (Fig. 5B–D). Subsequently, we stained adherent growing PeCa cell lines using indirect IF (Fig. 5E) and FFPE slides of organotypic 3D cultures using IHC (Fig. 5F) for β-catenin after stimulation with LiCl or conditioned media. In all cases, treatment with conditioned media did not result in an increase of nuclear β-catenin although P2 and L3 expressed β-catenin protein at high levels as shown above. In conclusion, it was not possible to gain a ligand-induced activation of the Wnt signaling indicating that this is actively inhibited.

DKK1 inhibits canonical Wnt signaling in HPV-positive PeCa cells

The lacking reporter gene activation in response to the stimulation with WNT3a and RSPO1-conditioned media together with the missing nuclear β-catenin raised the question about the expression of Wnt antagonists. DKK proteins inhibit the Wnt signaling cascade depending on the family member and the cellular context [28,29,41,42]. While DKK1 and DKK4 inhibit Wnt signaling, DKK3 was described as not involved in modulating Wnt signaling and the effect of DKK2 can be inhibitory and activating depending on the cellular context [29,41,42]. Interestingly, *DKK2* was not detectable on mRNA level neither in HPV-positive PeCa cells nor in HPV-negative NFK (Fig. 6A). HPV-positive PeCa cell lines expressed significantly lower amounts of *DKK3* and *DKK4* mRNA compared to NFK (Fig. 6A). In sharp contrast, *DKK1* mRNA was highly expressed in HPV-positive PeCa cell lines, with an elevated expression detected in P2 and L3 (Fig. 6A). *DKK1* has been reported as a direct target of SOX2 and of the miRNA-203 [43,44]. We previously reported that the HPV-positive PeCa cells expressed significantly reduced amounts of this miRNA compared to NFK that together with the elevated expression of the DKK1-inductor SOX2 as shown provide an explanation for the elevated expression of DKK1 protein [11].

Subsequent indirect IF on adherent growing PeCa cells confirmed this (Fig. 6B). A DKK1-specific ELISA demonstrated significantly elevated levels of DKK1 released by all PeCa cell lines compared to NFK, with slightly increased levels for P2 and L3 compared to L2 (Fig. 6C). IHC staining for DKK1 on FFPE slides of organotypic 3D cultures further underlined an elevated protein level in HPV-positive PeCa cell lines, with a more prominent staining for P2 and L3 (Fig. 6D). Since the conditioned media of PeCa cell lines contained significant amounts of DKK1, we tested their capacity to inhibit ligand-induced Wnt signaling in C33a cells (Fig. 6E–G). Remarkably, all media conditioned by HPV-positive PeCa cells reduced Wnt3a-mediated Wnt signaling (Fig. 6F) as well as the RSPO1-dependent amplification of Wnt signaling in C33a cells (Fig. 6G). Subsequent TOP-flash assays using a neutralizing antibody confirmed DKK1 as responsible factor (Fig. 6H–J). In summary, HPV-positive PeCa cells, particularly those with elevated HPV oncoprotein activity [11], expressed significant amounts of the Wnt antagonist DKK1 capable to inhibit the ligand-dependent activation of the Wnt signaling even with elevated expressed enhancers.

Elevated expression of DKK1 in HPV-positive PeCa is linked to higher TNM classification

We further investigated the expression of DKK1 in clinical PeCa specimens to test its relevance as biomarker using four TMAs reflecting the tumor center (TMA TC, $n = 70$), the invasion front (TMA IF, $n = 68$), lymph node metastases (TMA LM, $n = 21$) and adjacent normal foreskin ($n = 49$). Slides we stained by IHC for β-catenin and immune-reactive scores (IRS) were determined according to Remmele & Stegner. HPV-positive was defined as reactive in both PCR and p16INK4a IHC [5, 11]. DKK1 was expressed on normal tissue and in PeCa specimens of each TMA with individual specimens indicating an expansion of the

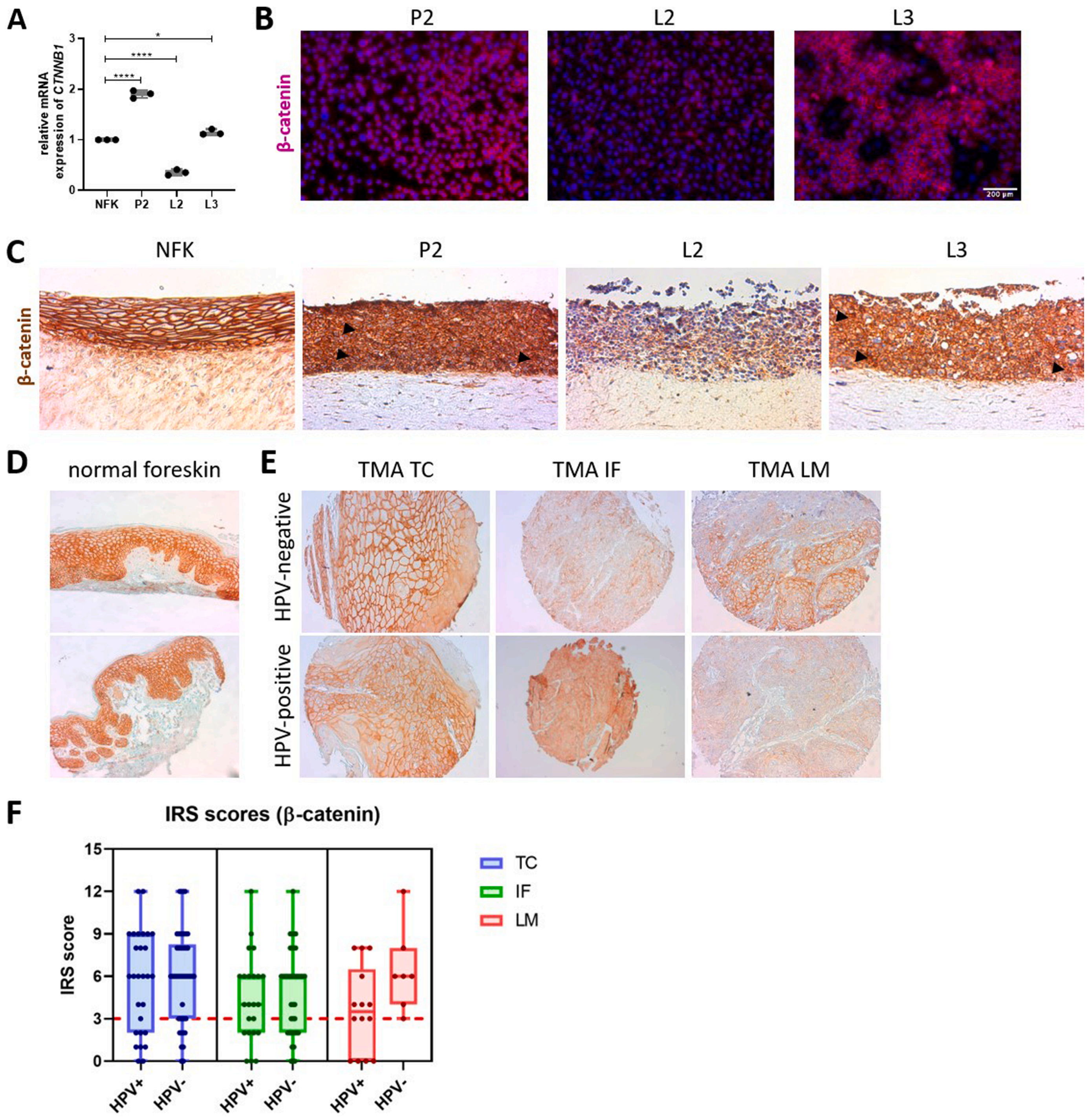

Fig. 4. β-catenin expression in HPV-positive PeCa cells and cancer specimens: (A) mRNA expression of the *CTNNB1* gene detected by qRT-PCR and normalized to NFK. (B) Protein expression of *CTNNB1* was analyzed on monolayer cultured PeCa cell lines using indirect immunofluorescence. The cell nuclei were counterstained using DAPI. The staining was documented in 20x magnification. (C) IHC for β-catenin of FFPE-slides derived from organotypic 3D cultures. Black arrows indicate nuclear β-catenin. Data in A were generated of three independent experiments of three independent cDNA/cell line. Pictures are representative of three independent experiments run in triplicates (B), or of three independent cultures run in duplicates (D). Significant differences were calculated by one-way Anova and Tukey's multiple test and illustrated by asterisks (*$p < 0.05$; ****$p<0.0001$). NFK = normal foreskin keratinocytes. FFPE slides covering TMA for normal foreskin tissue (D) and PeCa specimens (E) retrieved from tumor center (TC), invasion front (IF) and lymph node metastasis (LM) were stained for β-catenin using IHC. Representative pictures were illustrated out of 78 (A) and 75 (TC), 64 (IF) and 23 (LM) specimens. F) IRS scores of individual HPV-positive (HPV+) and HPV-negative (HPV-) PeCa specimens by TMA.

DKK1$^+$ cell population (Fig. 7A). Notably, the IRS were significantly higher in HPV-positive than negative specimens (TMA TC, $p = 0.0066$) with a similar trend for the TMA LM as well (Fig. 7B). Bunches of the invasion front had a significantly lower IRS than those of the tumor center and lymph node metastases, in particular for HPV$^+$ specimens (TMA TC/LM vs. IF HPV$^+$: $p < 0.0001$, TMA LM vs. IF HPV$^-$: $p = 0.0422$). Total counts of DKK1$^+$ specimens were enriched on TMA TC in the HPV$^+$ than HPV$^-$ subgroup ($p = 0.0003$, Fig. 7C) and in the HPV$^-$ than HPV$^+$ group regarding the TMA IF ($p = 0.0209$, Fig. 7D). With one exception, all lymph node metastases were positive for DKK1 (TMA LM,

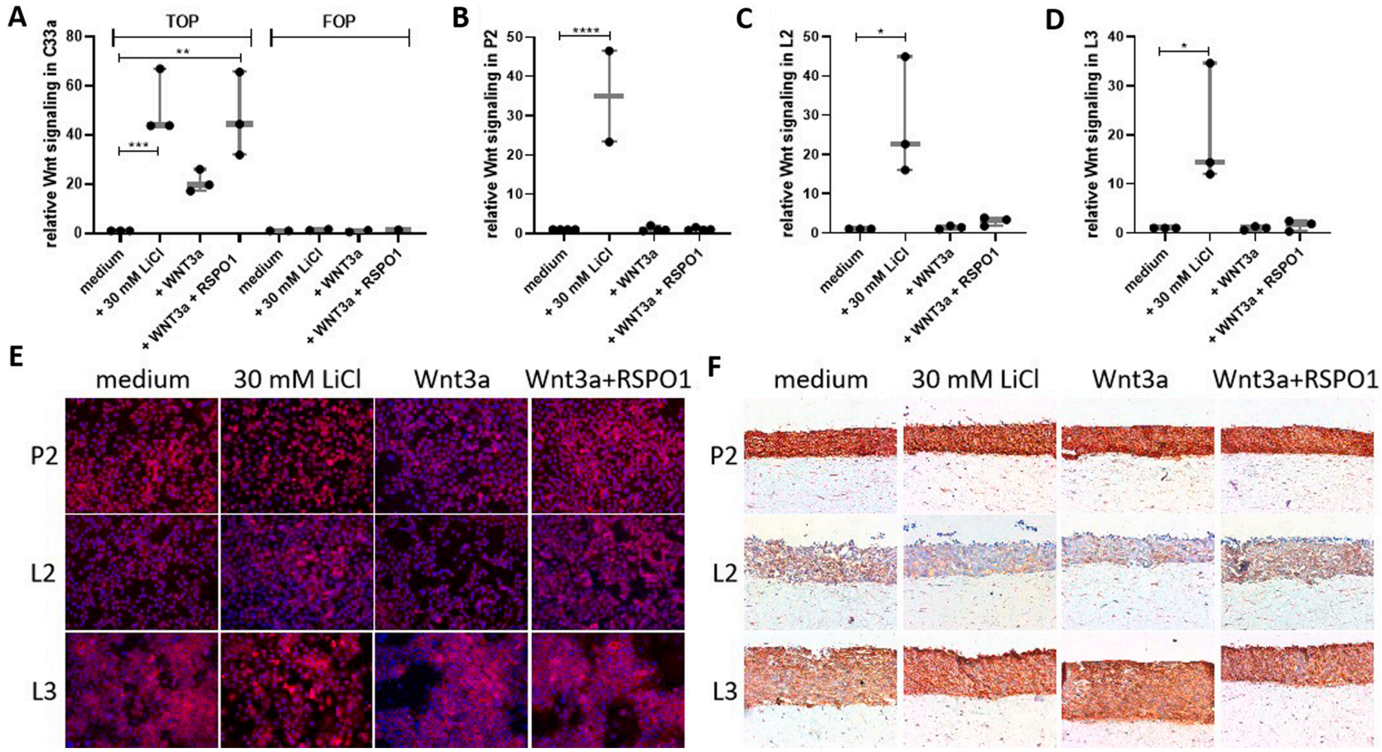

Fig. 5. Wnt signaling in PeCa cell lines: Wnt signaling was analyzed using TOP-flash Assays in C33a (A), P2 (B), L2 (C), L3 (D) cells. Cells were transfected with M50 TOP plasmid or M51 FOP plasmid. Cells were treated with 30 mM LiCl, 50% (v/v) WNT3a-conditioned media or WNT3a- and (10% (v/v)) RSPO1-conditioned media 24 h after. Next day, Wnt signaling activity was measured by luciferase assay and results normalized to the total protein amount measured by Bradford assay. localization of β-catenin after Wnt signaling stimulation was analyzed in monolayer cultured PeCa cell lines using indirect immunofluorescence (E) and IHC on FFPE slides of organotypic 3D cultures (F). The 3D cultures were stimulated 24 h prior to 4% PFA fixation. Results are displayed of three independent experiments run in triplicates. Significant differences were calculated by one-way Anova and Tukey's multiple test and illustrated by asterisks (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

HPV$^+$: 13/13, HPV$^-$: 7/8, Fig. 7E). Notably, DKK1$^+$ PeCa specimens of the tumor core had with a higher frequency a higher IRS, were less differentiated ($p = 0.0003$, Fig. 7F) and more likely associated with invasive (pT1b-pT4, HPV$^+$ vs. HPV$^-$: $p = 0.0044$) and metastatic (pN1–3, HPV$^+$ vs. HPV$^-$: $p = 0.0451$) growth in the HPV$^+$ than in the HPV$^-$subgroup (Fig. 7G). Our data suggest that DKK1 expression of the tumor core together with an active HPV transformation mark patients at higher risk for more dedifferentiated and aggressively growing PeCa.

Discussion

There is mounting evidence that the role of DKK1 in cancer progression is multimodal, organ- and context specific [45–49]. DKK1 expression has been linked to metastatic colonization and poor prognosis depending on the affected organ [48–50]. While low serum levels were linked to lung metastases, high levels increased the risk for bone metastasis [49]. Other data suggested a pro-tumorigenic role for DKK1 as it promoted the proliferation, migration, invasion, and growth of tumor cells with stem cell-like properties [51]. Moreover, DKK1 mediated autocrine Wnt inhibition led to latency-competent cancer cells expressing a SOX2-dependent stem cell-like state resistant to Wnt pathway activation [43]. The combined effect of an HPV oncoprotein dependent elevated expression of SOX2 as inductor [43,52] and the reduced expression of the miRNA-203 as repressor [11,44] can explain the elevated expression of DKK1 while Wnt signaling is abrogated. This can fuel disease progression by causing recurrent metastatic outbreaks by keeping cancer stem cells in an ambivalent hybrid state between proliferation and quiescence [43]. Our results point to similar mechanisms during the HPV-driven penile carcinogenesis with a role of DKK1 in driving metastatic dissemination. Notably, while DKK1 expression was prominent in HPV$^+$ cell lines and specimens, DKK1 was expressed in HPV-negative PeCa specimens as well, and almost all lymph node metastases were DKK1-positive indicating similar or compensatory mechanisms in HPV$^+$ and HPV$^-$ PeCa and a general role of DKK1 in lymph node metastasis development. PeCa cell lines negative for HPV would certainly help to elucidate the different mechanisms of Wnt pathway and DKK1 deregulation during the HPV-dependent and -independent penile carcinogenesis side by side. The lack of HPV-negative PeCa cells together with the limited number of HPV-positive PeCa cells display the main limitations of this study.

Recently, successful attempts of generating HPV-negative PeCa cell lines were published [53]. Subsequent gene expression analyses were performed but an elevated expression of Wnt pathway associated factors and target genes was not reported as here for HPV-positive PeCa cell lines. Moreover, gene expression analyses of PeCa specimens without a stratification on the HPV status may lead to biased results if the majority of specimens included are HPV-negative [54]. Based on these data we may hypothesize that an enrichment of Wnt pathway and target genes happens more likely in HPV-positive PeCa as it seems to be predominantly driven by the viral oncoproteins. Besides, our data from this and a previous report [11] demonstrated that gene expression signatures in PeCa cell lines are highly dependent on an extended HPV status. This includes the expression of additional viral oncoproteins, expression levels of the viral oncoproteins E6 and E7, integrated vs. episomal viral genome, integration site (affected open reading frame vs. enhanced transcription), type of integration (concatemeric vs. truncated viral genome) and methylation of the viral gene promoter. Thus, HPV-positive PeCa cells that derived from different primary tissues can display the same gene expression patterns but not necessarily. The detailed mechanism of how DKK1 is regulated and how the observed stem cell-like state in the individual HPV-positive PeCa cells is maintained is yet to be delineated, but DKK1 could display a potential target

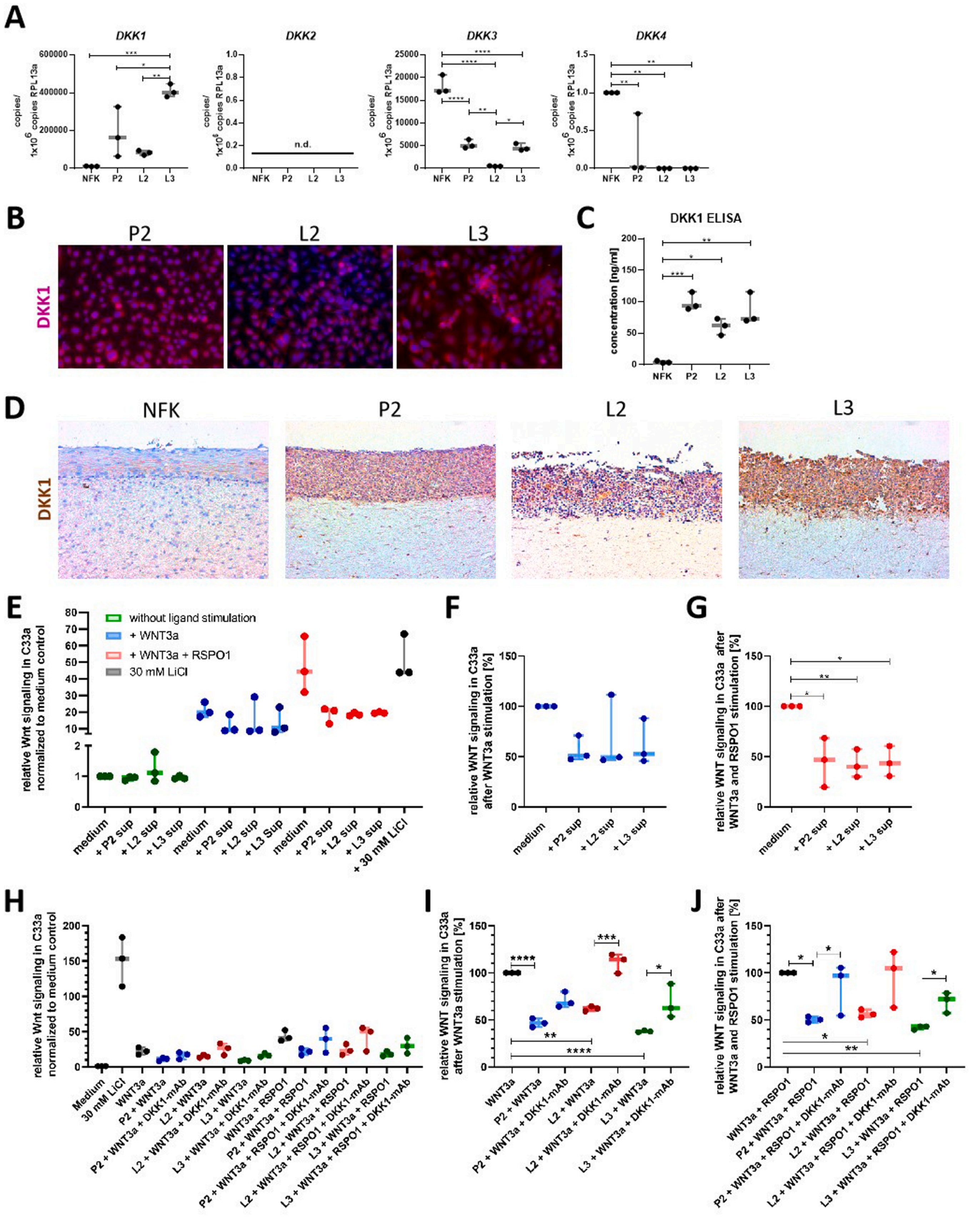

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Fig. 6. Expression of functional DKK1 in PeCa cell lines: The expression of DKK genes was analyzed using qRT-PCR (A). Protein expression of DKK1 was analyzed by indirect immunofluorescence (B) on monolayer-cultured PeCa cell lines, ELISA (C) of supernatants and IHC (D) on FFPE-slides of organotypic 3D cultures. Nuclei were counterstained with hematoxylin (D) and DAPI (B). The staining was documented in 20x magnification. (E) Impact of PeCa cell line-conditioned media on Wnt signaling in C33a cells was analyzed. C33a cells were transfected with M50 TOP plasmid and pEYFP-C1 plasmid using $CaCl_2$. Next day, cells were treated with conditioned media and stimuli. Luciferase activity was measured 24 h later. Impact of PeCa-conditioned media on Wnt signaling during Wnt3a stimulation (F) and WNT3a+RSPO1 stimulation (G). H) TOP-flash Assays using a DKK1 targeting antibody (DKK1-mAb, 0.75 μg/ml) to neutralize DKK1 in PeCa-cell line conditioned media. Impact of DKK1-mAb on Wnt signaling during Wnt3a (I) and WNT3a + RSPO1 stimulation (J). Data in A were generated of three independent experiments of three independent cDNA/cell line. Results are shown as mean +/- SEM of copies/1 × 10^6 copies *RPL13a* (A), concentration (ng/ml) in (C) and relative luciferase activity (normalized to medium control) in (E-J) of three independent experiments, representative pictures of three independent experiments run in triplicates (B) and of three independent cultures run in duplicates (D). Color-coding indicates treatment in E-G and cell lines in H-J. Significant differences were calculated by one-way Anova and Tukey's multiple test and illustrated with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$: $****p < 0.0001$). NFK = normal foreskin keratinocytes, n.d. = not detected, sup = supernatant.

for subsequent immunotherapeutic interventions in metastasizing (HPV-positive) PeCa.

Previous studies have reported an elevated expression of stemness associated factors driven by HPV-oncoproteins [11,52,55,56]. Predominantly E7 augments the expression of transcription factors, such as SOX2, OCT4 and NANOG, that are critically involved in the maintenance of pluripotent stem cells [11,52,55]. Our data provide further evidence for this observation since especially PeCa cells with high levels of E7 [11] expressed a stemness-associated signature. Besides, PeCa cells displayed an enhanced expression of LGR6 and this seems to result from an oncoprotein-dependent induction. LGR6 marks stem cells in the skin and hair follicle able to generate all cell lineages and an expansion of an LGR6-positive population during field cancerization has been observed [46,56]. We may hypothesize that HPV oncoproteins promote the expansion of a tissue-residing stem cell-like reserve cell population that is susceptible to an HPV oncoprotein driven transformation. While our data provide evidence for this hypothesis regarding the HPV-driven penile carcinogenesis, HPV oncoproteins seemed to enhance the expression of stemness markers as well. Further more sophisticated cell culture models are required to provide insights to which extend the stemness signature relates to HPV-infection and transformation susceptibility of a tissue-residing stem cell type or oncoprotein-related enhanced expression.

Cancers cells expressing high levels of DKK1 self-impose a broad downregulation of ULBP ligands for NK cells that causes the evasion of NK-cell-mediated clearance [43]. Moreover, DKK1 was shown to contribute to the lack of effective T cells in the tumor microenvironment generating "cold" tumors with low levels of infiltrating T cells [57]. This may effect T cell targeting therapeutic approaches in the treatment of PeCa, especially in advanced settings with involved lymph nodes, as DKK^+ specimens could display a reduced therapeutic responsiveness. Noteworthy, DKK1 antagonizing Wnt signaling has recently been described to increase the burden of neutrophil myeloid-derived suppressor cells (PMN-MDSC) [43]. We recently published that both PeCa cell lines with high HPV oncoprotein expression, P2 and L3, were susceptible to EGFR-directed IgA antibody-dependent neutrophil-mediated cytotoxicity due to an elevated endogenous expression of CXCL8 [11]. Either the CXCL8-related signaling may compensate the recently described DKK1-mediated immune evasion or neutrophil-targeting antibody-based immunotherapeutic approaches are not affected in contrast NK-cell targeting concepts from a DKK1-loaded tumor microenvironment [11,43]. Neutrophil-engaging IgA-based approaches could have the potential to reprogram PMN-MDSC to tumor cell killing neutrophils that in turn may recruit T cells by releasing lipid mediators [58,59]. Thus the IgA-neutrophil axis could display a promising immunotherapeutic concept for PeCa [11,58,59].

Gao and colleagues supported the hypothesis that elevated serological DKK1 levels are an independent unfavorable prognostic marker in head and neck cancer, a different HPV-associated entity, and suggested its implementation into prospective clinical trials [47]. Moreover, a retrospective study including 906 patients with cancers of the pancreas, stomach, liver, bile duct, breast and cervix, one further HPV-associated cancer entity, showed elevated levels of DKK1 in the sera of these patients [60]. At a certain point of cancer progression, elevated systemic levels of DKK1 become detectable and are linked to metastasising cancer [30,43,60]. Our data suggest similar mechanisms underlying the HPV-driven penile carcinogenesis and that serum levels of DKK1 could display a promising marker to monitor disease progression in PeCa. Future prospective long-term multicenter studies are required to collect sera of this rare group of cancer patients to align the serum levels to the *in situ* expression of DKK1 along with matching clinic-pathological data. In particular, the subsequent calculations on the correlation with overall or disease-free survival could help to decipher the role of DKK1 as a contribution to penile carcinogenesis and its potential as a prognostic marker.

Conclusions

Our data demonstrate that HPV-positive PeCa cells, especially those with active oncogene expression, displayed a stemness gene signature that is associated with disease progression and chemoresistance [3]. Furthermore, while expression analyses suggested an enhanced activation of the Wnt pathway, this was not tantamount to an active Wnt signaling. Our data suggest a DKK1-driven autocrine Wnt inhibition in cancer cells that maintain a stem cell-like state and link the $DKK1^+HPV^+$ status with more aggressively growing cancers. Finally, DKK1 could display a promising prognostic marker of disease progression in HPV-driven entities.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

CRediT authorship contribution statement

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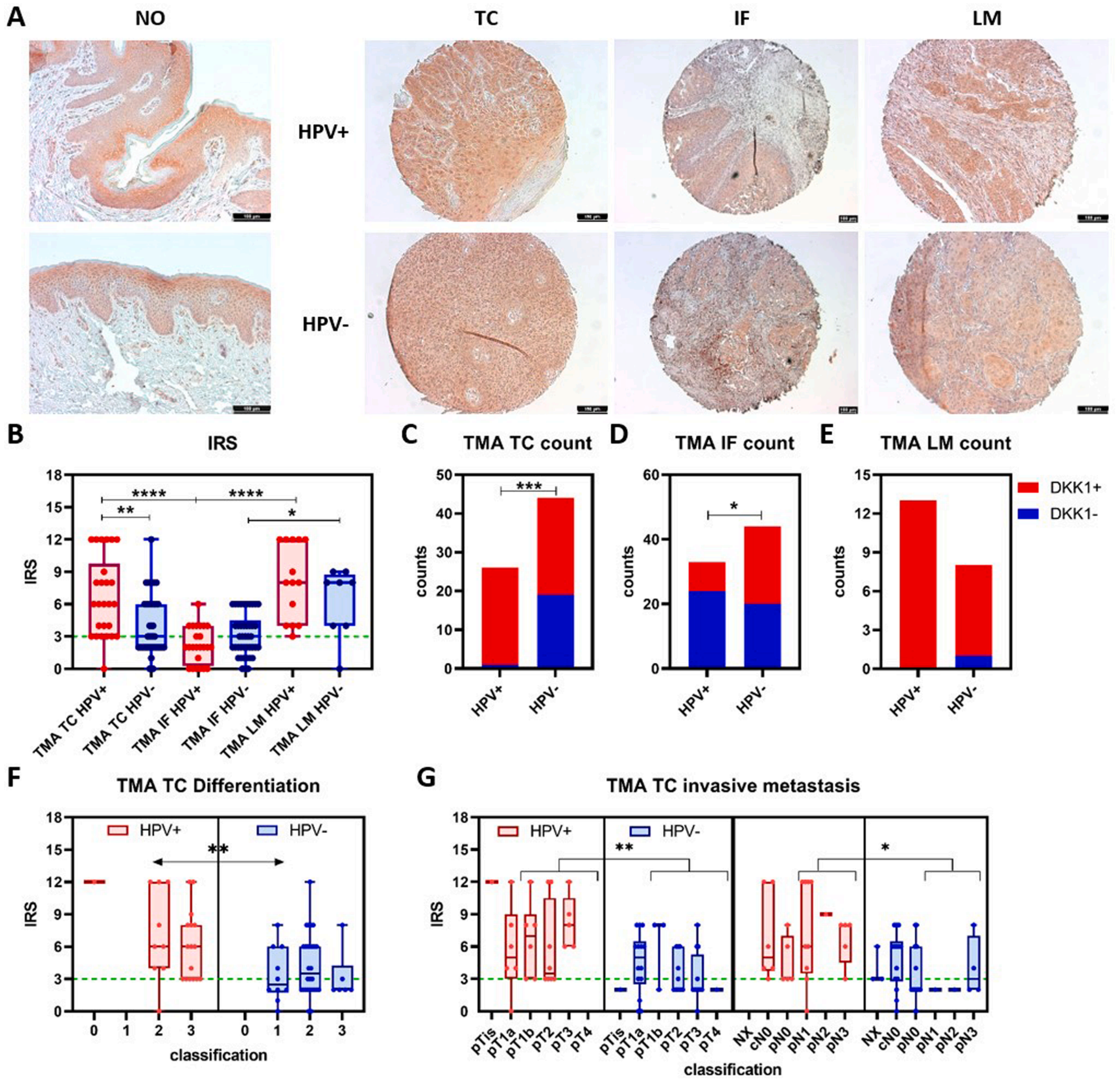

Fig. 7. Elevated DKK1 expression in HPV-positive PeCa: (A) FFPE slides covering TMA for adjacent normal foreskin (NO) and PeCa specimens retrieved from tumor center (TC), invasion front (IF) and lymph node metastasis (LM) were stained for DKK1 using IHC. Representative pictures were illustrated out of 49 (NO), 70 (TC), 68 (IF) and 21 (LM) specimens. (B) IRS of individual HPV-positive (HPV+) and HPV-negative (HPV-) PeCa specimens by TMA. (C) Amount of DKK1-positive and -negative PeCa specimens depending on HPV status for TMA TC (C), IF (D) and LM (E). Individual IRS of HPV-positive and -negative PeCa specimens classified by the 8th edition of the TNM regarding differentiation (F) and invasive and metastatic growth (G). Significant differences were calculated with an ordinary one-way Anova with Tukey correction for multiple comparison (A), two-sided Fisher exact test (C–E) and nested one-way Anova with Tukey correction for multiple comparison (F, G) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$: ****$p < 0.0001$).

Resources, Data curation. **Heiko Wunderlich:** Resources, Data curation. **Carsten Maik Naumann:** Investigation, Writing – review & editing. **Holger Kalthoff:** Investigation, Writing – review & editing. **Kerstin Junker:** Validation, Supervision, Investigation, Writing – review & editing. **Sigrun Smola:** Investigation, Writing – review & editing. **Stefan Lohse:** Conceptualization, Validation, Supervision, Investigation, Writing – review & editing, Funding acquisition, Writing – original draft, Project administration.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101267.

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