Aberrant expression of a β-catenin gain-of-function mutant induces hyperplastic transformation in the mouse cornea

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Accepted 5 February 2010
Journal of Cell Science 123, 1285-1294

doi:10.1242/jcs.063321

Summary

β-catenin signaling has been shown to play a fundamental role in embryonic development and tumorigenesis. In this study, we investigated the role of β-catenin (*Ctnnb1*) in corneal homeostasis and tumorigenesis. Conditional expression of a murine *Ctnnb1* gain-of-function mutation alone caused corneal neoplasia and neovascularization, resembling human ocular surface squamous neoplasia (OSSN). These corneas displayed an upregulation of cell proliferative markers (PCNA and p63), while presenting downregulation of both the Pax-6 transcription factor and the corneal differentiation marker cytokeratin 12. In addition, the expression of limbal-type keratin 15 ectopically extended to cornea, but the pattern of conjunctival keratin 4 and epidermal keratin 10 were unchanged. Moreover, epithelial E-cadherin and laminins decreased concomitantly with elevated levels of MMP-7. We also noticed a dramatic upregulation of pro-angiogenic factors (Vegf-A, Vegfr1) and angiopoietins in these corneas. Interestingly, all human OSSN specimens examined revealed nuclear β-catenin immunoreactivity. Taken together, these results argue that β-catenin activation is a crucial step during OSSN pathogenesis. Thus, inhibition of β-catenin might be beneficial for treating this disease.

Key words: β-catenin, Corneal epithelium, Tumorigenesis, OSSN

Introduction

In mammalian cells, β-catenin is a highly conserved ubiquitous protein that shows at least two functions. It is a component of a adherent complex of cadherin that mediates cell-cell adhesion (Kemler, 1993; Orsulic et al., 1999). It can also act in conjunction with the transcription factor TCF/LEF to regulate specific gene expression upon the activation of Wnts-dependent signaling cascades (He et al., 1998; Tetsu and McCormick, 1999; Lin et al., 2000; Nelson and Nusse, 2004), resulting in dramatic alterations of many cellular events, such as proliferation, differentiation, migration. In the absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK-3β), becomes labile and is degraded by the ubiquitin-proteasome system. By contrast, when Wnt ligand binds to its receptors Frizzled and Lrp5 or Lrp6, the assembly of proteins responsible for this phosphorylation state is abolished. Consequently, the unphosphorylated β-catenins accumulate in the cytoplasm and, subsequently, translocate into the nucleus to perform a variety of functions. In addition, β-catenin nuclear translocation can also be induced by inactivation of GSK-3β through wide variety of Wnt-independent pathways, such as fibroblast growth factors (FGFs) (Eblaghie et al., 2004; Wahl et al., 2007), epidermal growth factor (EGF) (Musgrove et al., 2004), insulin-like growth factor (IGF) (Richard-Parpaillon et al., 2002; Zhu et al., 2008), nuclear factor-κB (NF-κB) (Katoh and Katoh, 2007), phosphatase and tensin homolog (PTEN) (Zhao et al., 2005), the FP(B) prostanoid receptor (Fujino and Regan, 2001), integrin-linked kinase (Novak et al., 1998), nuclear hormone receptors (Mulholland et al., 2002; Saez et al., 2004; Mulholland et al., 2005; Schweizer et al., 2008) and oxidative stress (Mulholland et al., 2005; Almeida et al., 2007). It is noteworthy that aberrant activation of β-catenin is a crucial step in the pathogenesis of a wide variety of human cancers (Polakis, 2000).

Human ocular surface squamous neoplasia (OSSN) is the most common ocular surface pre-cancerous and cancerous lesion. It is also known by names such as conjunctival intraepithelial neoplasia, corneal intraepithelial neoplasia (CIN), or both together (CCIN) (Lee and Hirst, 1995). Clinically, OSSN manifests in different grades ranging from simple dysplasia to squamous cell carcinoma. Because of the high incidence of OSSN in the limbal area – where the corneal epithelial stem cells reside – the limbal transition zone theory (or stem cell theory) has been proposed for the development of CIN (Lee and Hirst, 1995). It has been suggested that the slow cycling limbal stem cells become hyper-proliferative by several factors, e.g. carcinogens, irradiation as well as the phorbolester and tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) (Tseng, 1989). Alterations in cell cycling within the limbal area can cause abnormal maturation of the conjunctival and corneal epithelium, and lead to the formation of CIN. In addition, human papilloma virus 16 (HPV16) and/or long-term UVB exposure are known to be the major risk factors associated with human OSSN (Scott et al., 2002; Kiire and Dhillon, 2006) but the actual molecular

pathway(s) linking these two known clinical findings remain unknown. We have recently developed a *Krt12*$^{rtTA/rtTA}$*/tet-O-FGF-7* double-transgenic mouse model in which overexpression of FGF-7 following induction by doxycycline (Dox) results in a tumor phenotype resembling OSSN (Chikama et al., 2008). In this tumor model, we found β-catenin nuclear translocalized in hyperplastic corneal epithelial cells upon Dox induction. These results implicated that β-catenin activation and nuclear translocation might be involved in the pathogenesis of human OSSN. In the present study, we conditionally expressed a *Ctnnb1* gain-of-function mutant in the differentiated corneal epithelial cells in vivo to test whether β-catenin activation per se can induce their hyperplastic or neoplastic transformation in vivo.

The gene-targeted *Ctnnb1*^{floxE3} mouse harbors two loxP sites flanking exon 3 of the *Ctnnb1* gene. Exon 3 encodes the crucial Ser/Thr residues for priming by casein kinase1 (CK1) (Ser45) and phosphorylation by GSK-3β (Ser33, Ser37 and Thr 41). Therefore, deletion of exon 3 results in the β-catenin mutant ΔE3β-catenin, which is resistant to phosphorylation and the subsequent degradation by proteosome, by mimicing β-catenin activation (Harada et al., 1999). We found that expression of ΔE3β-catenin in the differentiated corneal epithelial cells resulted in the hyperplastic transformation of corneal epithelial cells. Interestingly, nuclear β-catenin was also found in all the human OSSN samples examined. Thus, our findings might consolidate the relationship between β-catenin activation and the pathogenesis of OSSN.

Results

Conditional expression of ΔE3β-catenin in the corneal epithelium

Corneal epithelial homeostasis is essential to maintain a normal visual function. To investigate whether the persistent activation of β-catenin has any effect in the differentiated corneal epithelium, we employed Dox-inducible and Cre-mediated expression systems to drive the expression of *Ctnnb1* gain-of-function mutant (Harada et al., 1999) in differentiated – cytokeratin 12 (K12)-positive – corneal epithelial cells in vivo. *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ triple-transgenic mice were generated and treated with or without Dox from embryonic day (E) E14.5 to postnatal day (P) P21 given that the earliest time point of endogenous K12 expression is E14.5 (Liu et al., 1993). As expected, un-induced mice developed eyes that seemed normal (Fig. 1A). By contrast, the Dox-induced group exhibited severe corneal opacity with numerous epithelial nodules and engorged neovascularization (Fig. 1B). Western blotting analysis showed that these triple-transgenic mice expressed 94 kD wild-type β-catenin under normal circumstances (Fig. 1C, lane 1), but an extra 66-kD band representing ΔE3β-catenin mutant was detected in the Dox-treated corneal lysates (Fig. 1D, lane 2).

We then examined whether the ΔE3β-catenin executed its transcriptional activity in collaboration with Lef/Tcf using a TOP-Gal reporter mouse line (DasGupta and Fuchs, 1999). The X-Gal-stained mouse eyeballs revealed that little or no β-galactosidase activity was shown in un-induced *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$/TOP-Gal quadruple-transgenic mice (Fig. 2A,B), but many abnormal nodules with strong X-Gal-positive reactions (dark blue) appeared in the corneal surface of the Dox-induced quadruple-transgenic mice (Fig. 2C,D). These data demonstrate that the canonical Wnt/β-catenin signaling pathway is either inactive or suppressed in the normal cornea and limbus during fetal and neonatal stages. Also, the expression of ΔE3β-catenin mutant potentially triggers Tcf/Lef-dependent transcriptional activity,

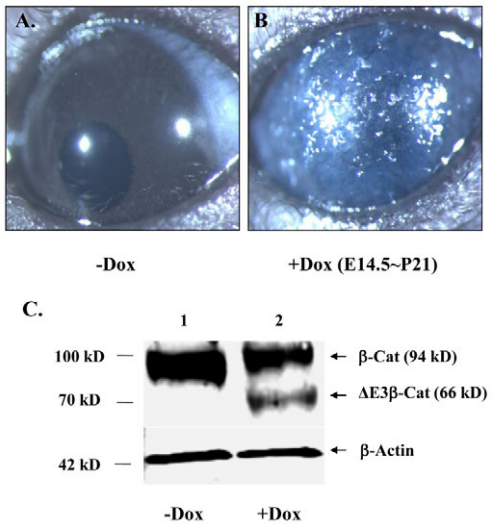

Fig. 1. Dox-inducible expression of ΔE3β-catenin in corneal epithelium. (**A**,**B**) When compared with non-induced age-matched mouse with normal looking corneal surface (A), *K12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxEx3/Wt}$ triple-transgenic mice treated with Dox from E14.5 through P21 exhibited corneal epithelial hyperplasia (B). (**C**) Western blotting revealed that wild-type β-catenin (94-kD band) appeared in both lanes; however, ΔE3β-catenin (66-kD band) was only detected in Dox-treated (lane 2) *K12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice. The 42-kD band corresponding to β-actin served as the loading control.

leading to the corneal nodule formation in differentiating and differentiated corneal epithelium.

To confirm that these epithelial nodules have Cre activity, we employed the global double-fluorescent Cre reporter mouse strain *ROSA*^{mTmG}, which expresses membrane-tagged red fluorescence before and membrane-tagged green fluorescence after Cre-mediated recombination in a wide variety of cell types (Muzumdar et al., 2007). Corneas from un-induced *K12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$*/ROSA*$^{mTmG/Wt}$ or *K12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{Wt/Wt}$*/ROSA*$^{mTmG/Wt}$ quadruple-transgenic mice, exhibited whole-body red fluorescence (data not shown). However, in both *Ctnnb1* wild-type and *Ctnnb1*$^{floxE3/Wt}$ mutant mice administrated Dox, red and green fluorescence patterns were mutually excluded by individual corneal epithelial cell (Fig. 2E-H). Moreover, as expected, no abnormality was found in the *Ctnnb1* wild-type cornea (Fig. 2E,F) but various sizes of abnormal corneal nodules showing green fluorescence appeared in the *Ctnnb1*^{floxE3}cornea (Fig. 2G,H).

Expression of ΔE3β-catenin per se caused hyperplastic transformation in the corneal epithelium

To investigate whether the pathological progression resulted from the expression of ΔE3β-catenin, *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ triple-transgenic mice were administered Dox through IP-injection of the pregnant dam and examined at different developmental stages from E14.5 to P21. Morphological and immunohistochemical studies revealed that neither significant alterations nor nuclear β-catenin was detected during E14.5-E16.5 (data not shown). However, at E17.5 the corneal epithelium began to show epithelial protrusions in mice expressing ΔE3β-catenin (Fig. 3D), whereas age-matched Dox-induced single-transgenic (not shown) or un-induced triple-transgenic embryos exhibited normal corneal epithelium with typical two-cell layers (Fig. 3A). Immunofluorescence staining revealed that β-catenin

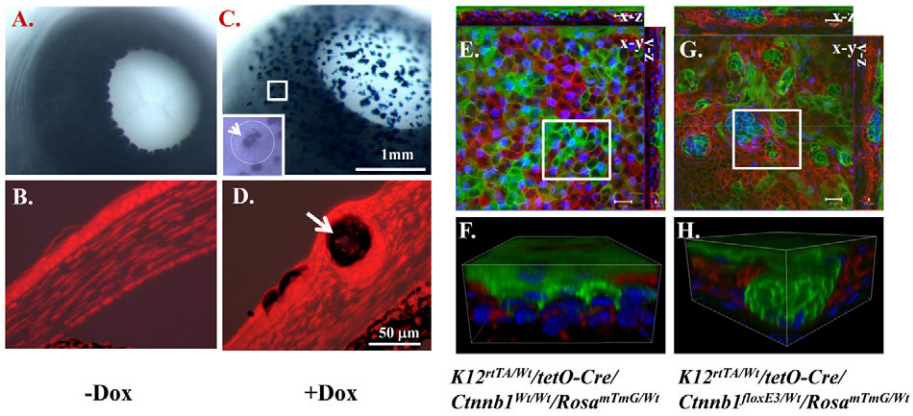

Fig. 2. Dox-inducible Cre-mediated expression of ΔE3β-catenin induced corneal epithelial nodules. (**A-D**) Whole-mount X-Gal stained eyes of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$/TOP-Gal quadruple-transgenic mouse administered without (A,B) or with (C,D) Dox from E0.5 to P10. Paraffin sections prepared from X-Gal-stained eyeballs were counter-stained with propidium iodide (red fluorescence in B,D). In non-induced eye, no X-Gal staining was observed, suggesting that Wnt/β-catenin signaling was repressed (A,B). By contrast, X-Gal-positive rough corneal surface (dark blue) appeared in Dox-induced eye (C,D). X-Gal-positive cells were tightly associated with hyperplastic transformation (arrow in D). (**E-H**) Confocal fluorescence micrographs showing that whole-mount corneas of the adult (6-weeks old) quadruple-transgenic mice *K12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*^{Wt}*/Rosa* $^{mTmG/Wt}$ (E,F) or *K12*$^{rtTA/Wt}$*/tetO Cre/Ctnnb1*^{floxE3}*/Rosa*$^{mTmG/Wt}$ (G,H) administered Dox chow for three days. Blue, green and red represent DAPI, EGFP and tomato red fluorescence, respectively. The EGFP (green fluorescence) expression indicated the Cre recombinase activity. Notice that all the epithelial nodules displayed green but not red fluorescence, suggesting that the Cre-mediated expression of ΔE3β-catenin resulted in the corneal epithelial nodules. Bars, 20 μm (E,G).

expression was restricted to the cell membrane of the corneal epithelium (Fig. 3B,B'); however, Dox-treated *Ctnnb1*$^{floxE3/Wt}$ mutants exhibited many epithelial cells with nuclear β-catenin (Fig. 3E,E'). In a consecutive section, we found that the expression of K12, normally expressed throughout the entire corneal epithelium (Fig. 3C,C'), was downregulated upon expression of ΔE3β-catenin (Fig. 3F,F'). Mice expressing ΔE3β-catenin until P2 displayed more-prominent hyperplastic nodules (compare Fig. 3G with J) than those found at E17.5 and, as before, cells within the nodules showed nuclear β-catenin staining (Fig. 3K,K') and lost K12 expression (Fig. 3L,L').

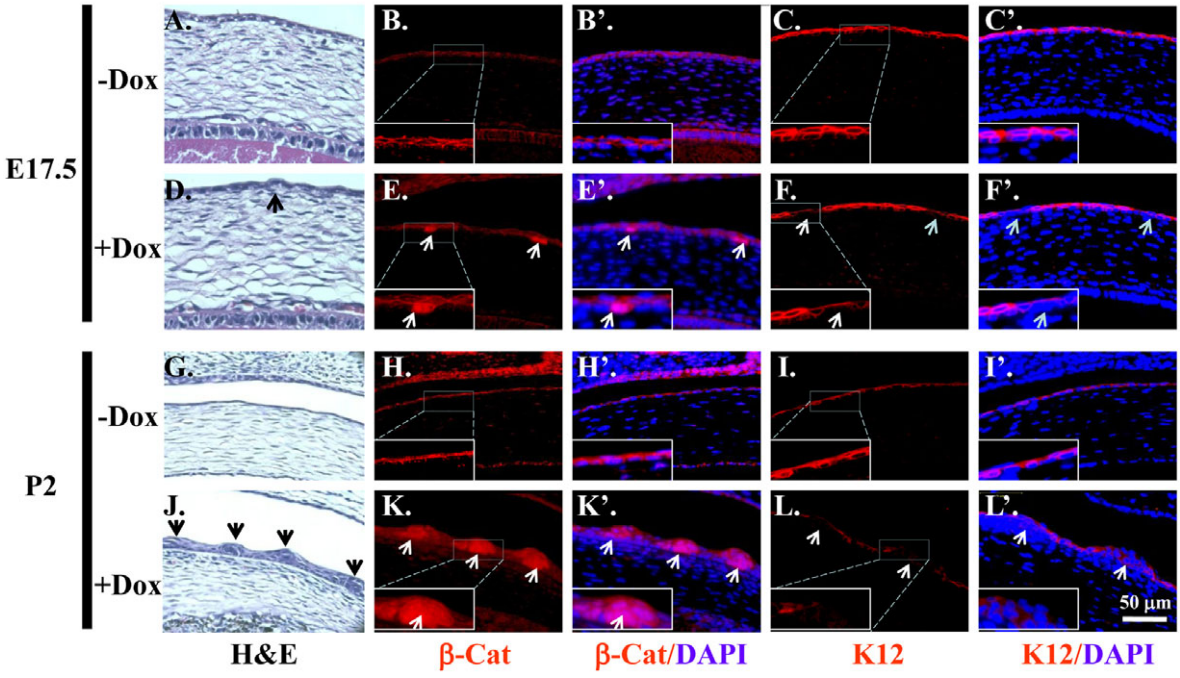

Fig. 3. Expression of ΔE3β-catenin initiates epithelial nodule formation and loss of K12 expression in corneal epithelium. (**A-L'**) Corneal sections from E17.5 (A-F') and P2 (G-L') of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ triple-transgenic mice induced with (+Dox) or without Dox (–Dox) were subjected to H&E staining (A,D,G,J) and immunofluorescence staining of β-catenin (B,B',E,E',H,H',K,K') and K12 (C,C',F,F',I,I',L,L'). Corneal epithelium exhibited no dramatic morphological alterations except few protrusions in those of Dox-treated mice (arrow in D) at E17.5. However, epithelial nodules were obviously found in Dox-treated mice at P2 (arrows in J). Dox-induced expression of ΔE3β-catenin was found in the nucleus (arrows in E,E',K,K') of the cells in which K12 expression was missing (arrows in F,F',L,L'). Insets are higher magnification of their corresponding panels.

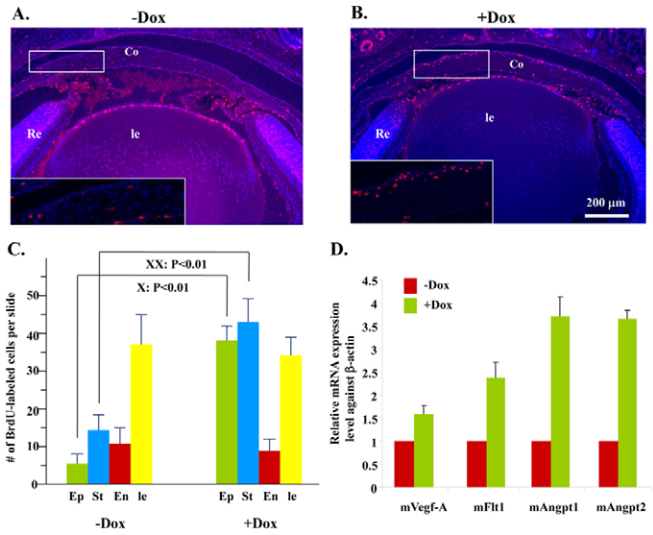

Fig. 4. Expression of ΔE3β-catenin increase DNA synthesis in corneal epithelium and stroma. (**A**,**B**) Immunofluorescence staining of BrdU of P2 corneas in *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ triple-transgenic mice induced without (A) or with Dox (B). Higher magnification shows that BrdU-labeled cells (red) are concentrated in the epithelial nodules (inset in B). Quantitative analysis of BrdU-labeled cells in different regions of the eye section. (**C**) Data were calculated from ten slides of each sample and Student's *t*-test was performed. (**D**) Quantitative RT-PCR analysis showed Dox-induced ΔE3β-catenin expression upregulate pro-angiogenic factors such as m*Flt1*, *mVegf*, *mAngiopoietin (Angpt)-1* and *Angpt-2* gene expressions. Co, cornea; ep, epithelium; st, stroma; en, endothelium; le, lens; Re, retina.

Consistent with the formation of hyperplastic epithelial nodules, expression of ΔE3β-catenin resulted in an eightfold and threefold increase in BrdU incorporation within the corneal epithelium and stroma, respectively (Fig. 4A-C). In addition, ΔE3β-catenin expression resulted in the upregulation of vascular endothelium growth factor-A (Vegf-A), its receptor Vegfr1 (Flt1), and angiopoietin (Angpt)-1 and Angpt-2 mRNA levels (Fig. 4D) – yet no obvious blood vessels were noticed in the cornea at this stage (Fig. 3J). These data suggest that expression of ΔE3β-catenin is responsible for the increase of corneal epithelial cell proliferation and the production of pro-angiogenic factors.

ΔE3β-catenin-upregulated expression of MMP-7 downregulated E-cadherin expression and disrupted the basement membrane

Corneal epithelial homeostasis was completely compromised, with profound angiogenesis and stromal invasion, in the Dox-treated *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice at P21 (Fig. 5E-H). It prompted us to investigate whether the proteins involved in epithelial invasion rather than the maintenance of corneal epithelial integrity are altered by ΔE3β-catenin. Immunofluorescence staining demonstrated a reduction in E-cadherin expression levels, particularly in the epithelial nodules (Fig. 5L,L') where anti-β-catenin staining showed strongly in the nuclei (Fig. 5K,K'). In addition, the major components of the basement membrane laminin-β1 and laminin-α1 (data not shown) were expressed in the epithelium during corneal morphogenesis and restricted to the basement membrane at P21 (Fig. 6A-C), but were drastically downregulated from E17.5 to P2 (Fig. 6D,E) and completely diminished at P21 (Fig. 6F) in mice expressing ΔE3β-catenin. Laminin has been known to be a proteolytic substrate for metalloproteases (MMPs) (Siu and Cheng, 2004), among which MMP-7 (also known as *MMP7*, matrilysin) is a direct downstream gene target of activated β-catenin (Gustavson et al., 2004). Indeed, MMP-7 was not detected in a normal corneal epithelium (Fig. 6G,G'), but aberrantly expressed in the ΔE3β-catenin mutant corneal epithelium (Fig. 6H,H'). These data implicate that ΔE3β-catenin directly downregulates E-cadherin gene expression and simultaneously upregulate expression of *MMP-7* leading to the degradation of laminins and the subsequent disruption of the basement membrane.

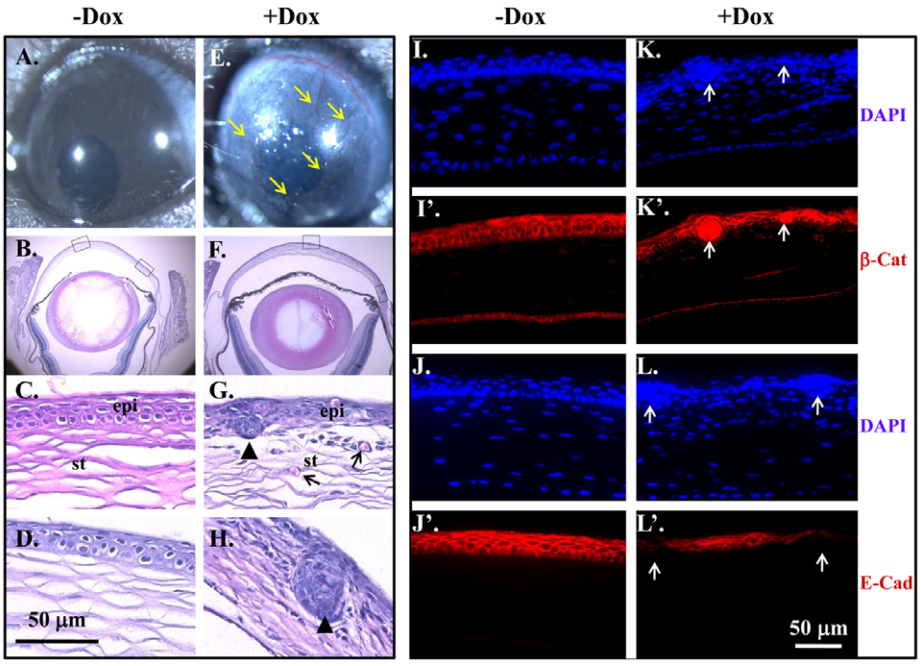

Fig. 5. Expression of ΔE3β-catenin induced corneal epithelial hyperplastic nodules and downregulated E-cadherin. (**A-H**) Stereo micrographs of ocular surface in *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mouse at P21 induced without (A) or with (E) Dox since E14.5. It was obvious that ΔE3β-catenin expression led to corneal opacity and profound neovascularization (arrows in E). H&E staining showed that non-induced eye developed into a well-organized stratified corneal epithelium (B,C,D). However, in Dox-treated eye, corneal epithelial cells arranged into epithelial nodules instead of stratified epithelium (arrowhead in G,H), thereby corneal integrity was compromised concomitant with angiogenesis (arrows in G). (**I-L'**) Corneal sections from P21 of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice induced without Dox (I,I',J,J') or with (K,K',L,L') were subjected to immunofluorescence staining of β-catenin (I,I',K,K') and E-cadherin (J,J',L,L'). Note that ΔE3β-catenin was accumulated in the nucleus (arrows in K') and E-cadherin expression was reduced in the epithelial nodules (arrows in L'). ep, epithelium; st, stroma.

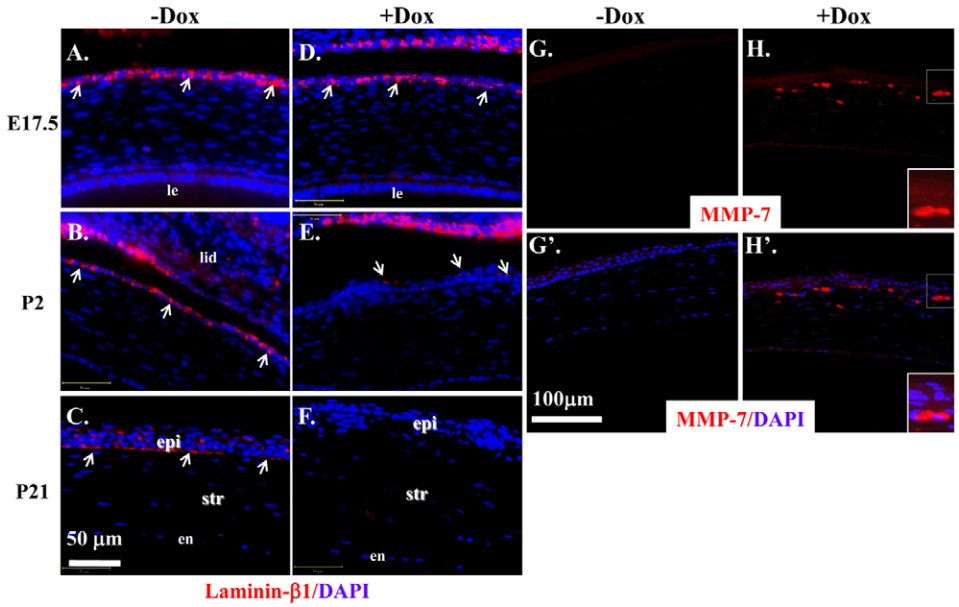

Fig. 6. Expression of ΔE3β-catenin disrupted basement membrane formation concomitantly with upregulated MMP-7. (**A-F**) Corneal sections from E17.5 (A,D), P2 (B,E), and P21(C,F) of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ triple-transgenic mice induced without Dox (–Dox) or with (+Dox) were subjected to immunofluorescence staining of laminin-β1. In non-induced mice, laminin-β1 expression was detected in basolateral region at E17.5 (A) and P2 (B) and more restricted to the basal region at P21(C), respectively, of the corneal epithelial cells. Note that, in Dox-treated cornea, the laminin-β1 expression pattern did not seem to be changed at E17.5 (D) but was drastically downregulated in P2 (E) and undetectable in P21 (F) (arrows in D,D',E,E'). (**G-H'**) Corneal sections from P21 of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice induced without Dox (G,G') or with (H,H') were subjected to immunofluorescence staining of MMP-7. Note that MMP-7 expression level was undetectable in the normal corneal epithelium (G,G') but upregulated by ΔE3β-catenin in epithelial cells (H,H'). epi, epithelium; str, stroma; en, endothelium.

Expression of ΔE3β-catenin enhanced cell proliferation and changed the differentiation status in differentiated corneal epithelium

To investigate the molecular and cellular mechanism by which the expression of ΔE3β-catenin had strong impact on proliferation and differentiation that might lead to disruption of corneal epithelial homeostasis, immunofluorescent staining revealed that both protein expression of PCNA and p63 was increased in ΔE3β-catenin expressing corneal epithelium (Fig. 7A-D). However, expression of ΔE3β-catenin resulted in the loss of Pax-6 (Fig. 7F) and K12 (Fig. 8B) expression in the hyperplastic epithelial nodules, indicating that the corneal differentiation phenotype was somehow lost. Moreover,

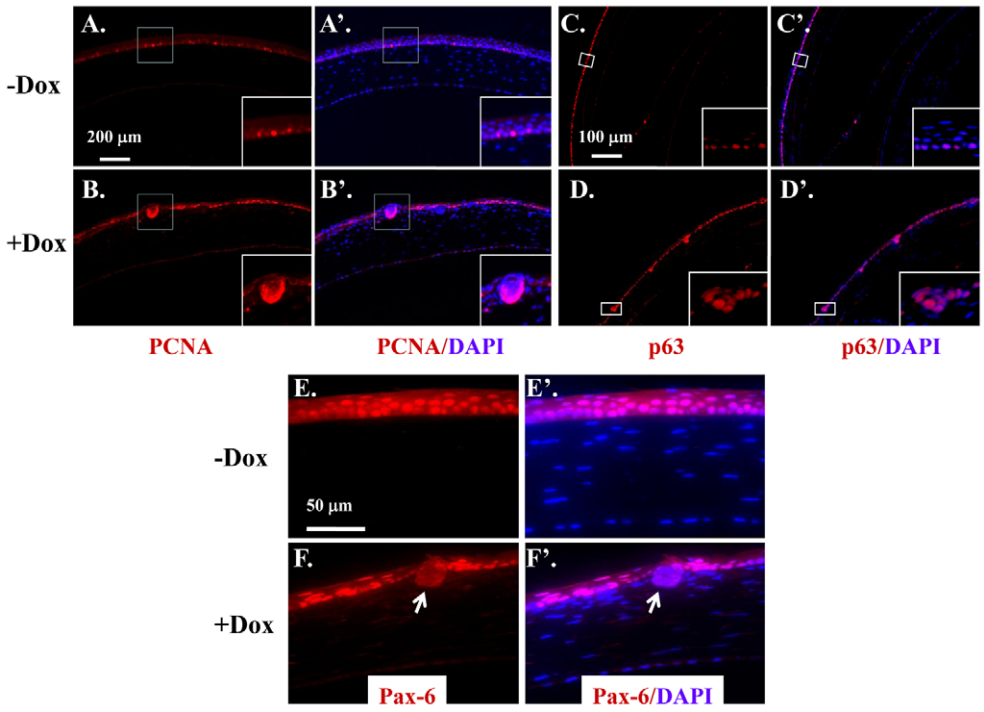

Fig. 7. Expression of ΔE3β-catenin in corneal epithelium increased cell proliferation, upregulated p63 expression but downregulated Pax-6. (**A-F'**) Corneal sections from P21 of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice induced without Dox (–Dox) or with (+Dox) were subjected to immunofluorescence staining of PCNA (A,A',B,B'), p63 (C,C',D,D'), and Pax-6 (E,E',F,F'), respectively. PCNA was highly expressed in the epithelial nodules of Dox-treated mice (insets of B,B'). Likewise, p63 expression was restricted to the epithelial basal cells (insets of C,C') in non-induced mice but was detected in the entire epithelial nodules of Dox-treated mice (insets of D,D'). Pax-6 was expressed in the full thickness of epithelium of non-induced mice (E,E'). Interestingly, Pax-6 expression in Dox-treated cornea was comparable with that in non-induced mice and diminished only in the epithelial nodules (arrows in F,F').

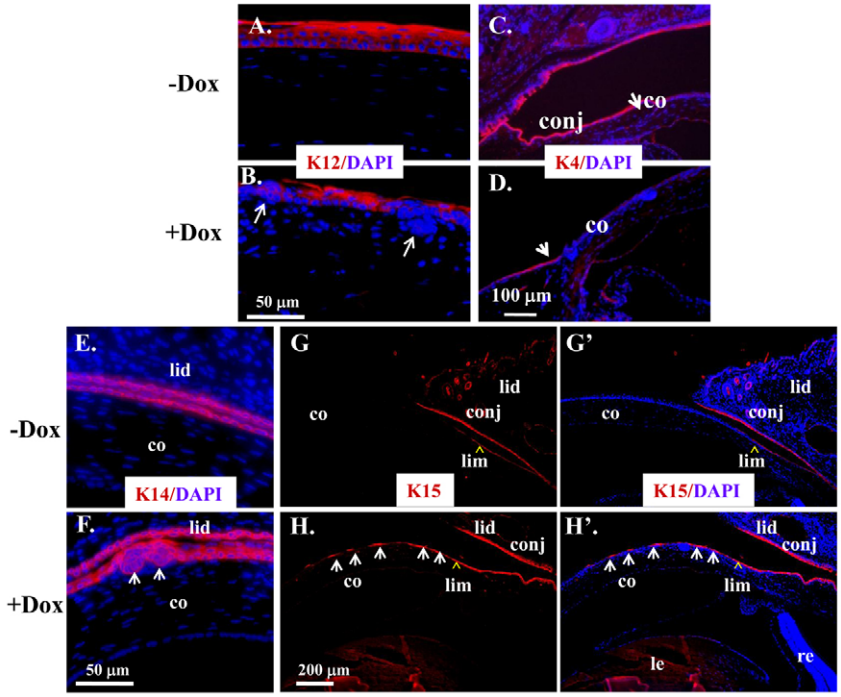

Fig. 8. Expression of ΔE3β-catenin altered keratin expression pattern in corneal epithelium. (**A-H'**) Corneal sections from P21 of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice induced without Dox (–Dox) or with (+Dox) were subjected to immunofluorescence staining of K12 (A,B) K4 (C,D), K14 (E,F), and K15 (G,G',H,H'). K12 was expressed in the full-thickness of epithelium (A) of non-induced mice. However, K12 expression was dramatically reduced in Dox-induced epithelial nodules (arrows in B). However, K4 expression pattern was not altered by ΔE3β-catenin (C,D), but K15 was expressed in limbal and conjunctival epithelia but not cornea of the non-induced mice (E,E'). Interestingly, ΔE3β-catenin expression showed extension of K15 expression to the central cornea (arrows in F,F'). Epithelial nodules were stained positive for K14 (arrows in H) and might assume epithelial progenitor cell phenotype.

the expression pattern of keratin 4 (K4; Fig. 8C,D) and Muc5A/C (data not shown) was not changed by the expression of ΔE3β-catenin. Likewise, no positive staining of keratin 10 (K10), vimentin or α-smooth muscle actin (α-SMA) was found in the epithelial nodules (data not shown), suggesting that ΔE3β-catenin expression did not induce transdifferentiation of corneal epithelium into conjunctival or epidermal epithelia or mesenchymal cells. Interestingly, these nodules were strongly positive for keratin 14 (K14; Fig. 8F), however, the expression of keratin 15 (K15), which is normally detected in the conjunctival and limbal epithelium (Yoshida et al., 2006), was aberrantly extended into the central corneal epithelium upon expression of ΔE3β-catenin (Fig. 8G,H). Collectively, these data implicate that expression of the ΔE3β-catenin mutant enhance cell proliferation and may induce 'dedifferentiation' of the corneal epithelium towards limbal epithelium, but not 'transdifferentiation' into conjunctival or epidermal epithelium or hair follicle (Pearton et al., 2005).

Expression of ΔE3β-catenin can lead to ocular surface neoplasia in adult mice

To investigate the effect of ΔE3β-catenin expression in the adult corneal epithelium, 6-week-old *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice were administered Dox by a one-time IP- injection followed by Dox chow ad libitum for 2 weeks. Littermates of the same genotype were fed with normal chow and served as a control. Immunofluorescence staining showed that nuclear β-catenin was detected 3 days after Dox induction (Fig. 9A,B). Interestingly, as compared with un-induced corneal epithelium, which was well-organized into four to six layers (Fig. 9A,A',A''), aberrant expression of ΔE3β-catenin caused mis-arrangement of the cells with many epithelial nodules (Fig. 9B,B',B''). More interestingly, all mice (n=4) that expressed ΔE3β-catenin developed corneal neoplasia at 14 days after Dox treatment with leukoplakic lesion and profound neovascularization, resembling to human OSSN (Fig. 9E-H).

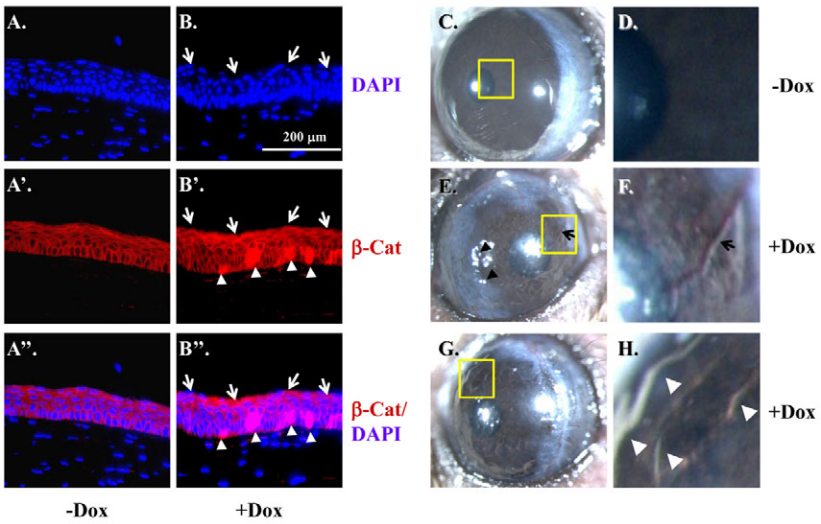

Fig. 9. Expression of ΔE3β-catenin alone can induce corneal epithelial neoplasia in adult mice. (**A-B''**) *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice induced without (–Dox) (A,A',A'') or with Dox (+Dox) (B,B',B'') for three days. Mouse eyes were enucleated and prepared for paraffin blocks. Corneal sections were subjected to immunofluorescence staining with anti-β-catenin antibody (red) and counterstained with DAPI (blue). Wild-type β-catenin was presented on the cell membrane of non-induced cornea (A,A',A''), whereas Dox-induced expression of ΔE3β-catenin was found in nuclei (arrowheads in B',B'') and was tightly associated with the mis-arrangement of the corneal epithelium to form corneal nodules (arrows in B,B',B''). (**C-H**) As compared with un-induced mice that exhibited transparent cornea (C,D), four out of four *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mice induced with Dox (from P21-P35) developed OSSN (E-H) with leukoplakic pannus-like lesions (arrowheads in E,H) in the cornea with neovascularization (arrows in E,F). D, F and H represented higher magnification images from C, E and G, respectively.

β-catenin nuclear translocation in human OSSN

The etiology and pathogenesis of human OSSN remain elusive. To investigate whether nuclear translocation of β-catenin has any association with human OSSN, we examined clinical specimens from the eye pathology laboratory and performed immunohistochemistry. Out of eight human OSSN specimens, all of them showed moderate or strong nuclear β-catenin staining, whereas a normal eye-bank cornea revealed β-catenin immunoreactivity only in the cell membrane (Fig. 10; supplementary material Table S1).

Discussion

In this study, we have provided strong evidence that a β-catenin gain-of-function mutant can disrupt corneal epithelial homeostasis and induce hyperplastic transformation in *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxEx3/Wt}$ tri-transgenic mice regardless the initial time of Dox-treatment during or following embryonic development. We have demonstrated that forced expression of stabilized ΔE3β-catenin in the differentiated corneal epithelium can cause tumorigenesis. The phenotypes are similar but not identical to those observed previously in Dox-treated *Krt12*$^{rtTA/rtTA}$*/tetO-FGF-7* mice, which also displayed hyperplastic nodules on the corneal surface (Chikama et al., 2008).

Repression of the Wnt/β-catenin signaling pathway has been implicated as an essential prerequisite for the differentiation of corneal epithelial progenitor cells into a non-keratinizing stratified epithelium during corneal morphogenesis (Mukhopadhyay et al., 2006; Gage et al., 2008). It has been previously shown that stabilized ΔE3β-catenin driven by the *Le-Cre* promoter suppresses surface ectoderm invagination for lens formation at ~E9.5 to ~E10.5. These data suggested that Wnt/β-catenin signaling is unfavorable during the early stages of ocular surface morphogenesis (Smith et al., 2005). The *K12*^{rtTA}*/tetO-Cre* driver mice allow us to assess the role of Wnt/β-catenin signaling in a corneal cell-type specific and Dox-inducible manner. In the present study, we demonstrated that persistent expression of ΔE3β-catenin driven by the endogenous *Ctnnb*1 enhancer-promoter had strong impact to the differentiated (Pax-6-positive and K12-positive) corneal epithelial cells. Therefore, the stringent control of Wnt/β-catenin signaling is crucial for the maintenance of homeostasis and integrity in differentiated corneal epithelium.

We have documented that endogenous K12 can be detected in the corneal epithelium by in-situ hybridization at E14.5 and by immunohistochemistry at E15.5 (Liu et al., 1993). Therefore, we expected the phenotypes caused by ΔE3β-catenin to be observed ~E14 to ~E15.5 if mice were exposured to Dox at the beginning of life. However, we have never detected nuclear β-catenin or noticed any significant morphological change before E17.5 in the *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ mouse embryos induced with Dox since E0.5. This could be attributed to the delay resulting from a series of cellular events including rtTA activation by Dox, transcriptional activation of *tetO-Cre*, generation of the *Ctnnb1*$^{\Delta E3}$ allele followed by its transcription and/or translation, and the accumulation of the ΔE3β-catenin protein. Indeed, we previously documented that it took 48 hours following induction to drive the expression of a *Cre*-reporter gene using the *K12*^{rtTA}*/tetO-Cre* driver mouse line (Chikama et al., 2005). Expression of ΔE3β-catenin had a tremendous impact on the corneal epithelium as early as at E17.5. Initially, only a few protrusions were displayed on the corneal epithelium at E17.5 but these quickly progressed into hyperplastic epithelial nodules at P2. It is noteworthy that expression of ΔE3β-catenin in the corneal epithelium also enhanced underneath stromal cell proliferation. This result strongly indicated that Wnt/β-catenin signaling elicited from the corneal epithelium triggered an epithelium-stromal mesenchyme interaction via a putative growth-promoting factor to enhance stromal cell proliferation. Although the identity of this growth promoting factor is unknown, FGF family members are probably involved because it has been documented that Wnt/β-catenin signaling upregulated FGFs including FGF-4, FGF-9, FGF-18 and FGF-20 in various cellular context (Kratochil et al., 2002; Hendrix et al., 2005; Shimokawa et al., 2003; Chamorro et al., 2006). Since these hyperplastic nodules appeared only in the epithelium before the opening of the eyelid and we did not observe F4/80-positive macrophage in any of these corneas (data not shown), this dramatic pathological progression is probably caused by the autonomous effect of ΔE3β-catenin. This argument is somewhat supported by our results in that the *Ctnnb1* gene is required for the Dox-induced corneal epithelial hyperplasia and/or neoplasia formation in the *Krt12*$^{rtTA/rtTA}$*/tetO-FGF-7* mice (Y.Z. and C.-Y.L., unpublished results)

Although we cannot exclude additional secondary or tertiary effects following ΔE3β-catenin expression that might involve subsequent pathological progression (Fig. 5E), it is possible that ΔE3β-catenin expression still causes directly and actively epithelial invasion into the corneal stroma, and neovascularization at P21. To explore the mechanism by which stabilized ΔE3β-catenin causes the aforementioned pathology in the cornea, we have tested the expression levels and/or patterns of some related genes such as E-cadherin (*Cdh1*), MMP-7 (*Mmp7*), and Vegf-A (*Vegfa*). For example, E-cadherin, known to be the Wnt/β-catenin target gene (Jamora et al., 2003; ten Berge et al., 2008), was drastically downregulated in the epithelial nodules (Fig. 5L,L'), suggesting that the adherent junction was compromised by the expression of ΔE3β-catenin. Since reduced

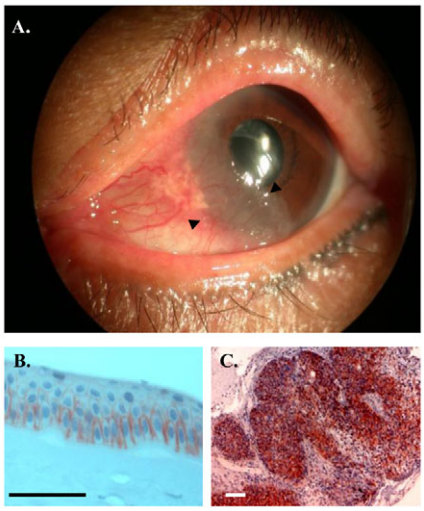

Fig. 10. Nuclear β-catenin may be associated with human OSSN.
(**A**) Photograph of the eye taken from an OSSN patient (see supplementary material Table S1, case 4), who presented a leukoplakic pannus-like lesion with engorged vessels involving the area from the five-o'clock to ten-o'clock position with a 4-mm extension into the corneal side.
(**B**,**C**) Immunohistochemical staining by using anti-β-catenin antibody shows that normal corneal epithelium contains membranous β-catenin (B). By contrast, human OSSN shows abundant nuclear β-catenin (C). Bars, 200 μm (B), 100 μm (C).

E-cadherin gene expression has been shown to be associated with epithelial tumor formation and invasion (Birchmeier, 2005), it is probable that the formation of the hyperproliferative epithelial nodules is directly associated with the downregulation of E-cadherin by ΔE3β-catenin. However, both Vegf-A and MMP-7 genes, which contain Lef/Tcf binding sites (Zhang et al., 2001; Crawford et al., 1999; Brabletz et al., 1999), were upregulated in the corneas of *Krt12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{floxE3/Wt}$ triple-heterozygous mice. It is obvious that upregulation of Vegf-A and other proangiogenic factors, such as Vegfr1, Angpt-1 and Angpt-2, through direct or indirect effects of ΔE3β-catenin expression resulted in profound corneal neovascularization. Likewise, ectopic upregulation of MMP-7 probably caused proteolytic degradation of laminins (Siu and Cheng, 2004; Gustavson et al., 2004) and disruption of basement membrane; therefore, the overall corneal homeostasis was compromised.

Both β-catenin and Lef/Tcf function as factors to sustain tissue progenitor cells in the adult (Lowry et al., 2005). Our immunofluorescence staining results showed that BrdU uptake (Fig. 4) aswell as expression of PCNA and p63 increased, but Pax-6 expression decreased (Fig. 7). This was consistent with the previous report stating that activation of Wnt/β-catenin signaling enhanced Ki67 expression and cell division, and induced corneal epithelial dedifferentiation (Pearton et al., 2005). Moreover, the cell-type-specific pattern of epithelial keratin expression showed downregulated expression of K12 (the corneal epithelial marker) and ectopic expression of K15 (the limbal marker) in the central cornea, but no changes in the expression patterns of K4 (the conjunctival marker) or K10 (the epidermal marker). Our results argue that the expression of ΔE3β-catenin in K12-positive corneal epithelial cells do not trigger their transdifferentiation into conjunctival or epidermal epithelium but rather induced dedifferentiation towards limbal epithelial progenitor cells, and that their further differentiating into corneal epithelium could be dependent in the presence of other growth and/or differentiation factors in the environment, such as FGF-7. This is probably why K12 expression in the FGF-7 model remained (Chikama et al., 2008) but was absent in β-catenin gain-of-function mice. Nevertheless, it requires further study to demonstrate whether these ΔE3β-catenin-expressing nodules, indeed, maintain the characteristic features of stem and/or progenitor cells and can differentiate into corneal epithelial cells in vitro and/or in vivo.

Whereas lack of the Wnt antagonist *Dkk2* resulted in the ectopic appearance of conjunctiva, epidermis and hair follicles in the corneal epithelium due to direct and/or indirect effect (Mukhopadhyay et al., 2006; Gage et al., 2008), we did not observe these phenotypes in our animal model, regardless of the initial time of Dox-treatment to induce expression of ΔE3β-catenin in K12-positive corneal epithelial cells. One of the possibilities is that Wnt/β-catenin activation in the *Dkk2* null-mutant affects corneal epithelial stem and/or progenitor cells, whereas only the K12-positive cells (including differentiating – or transient amplifying cells – and differentiated corneal epithelial cells) are influenced in our mouse model. Because *Dkk2* knockout was done much earlier and might have targeted the limbal stromal mesenchyme, one can appreciate why more plasticity into other cell lineages is possible. Moreover, it is possible that loss of *Dkk2* not only activates Wnt signaling, but also changes other signaling pathway(s) that are necessary for fating cornea into epidermis. Furthermore, activation of β-catenin per se is not sufficient to change the differentiation of corneal epithelium into epidermal or hair follicle. Other molecules involved in signaling pathways, such as noggin or the bone morphogenetic protein (BMP) antagonist, might also be needed to switch a dedifferentiated corneal epithelium into epidermal epithelium (Pearton et al., 2005).

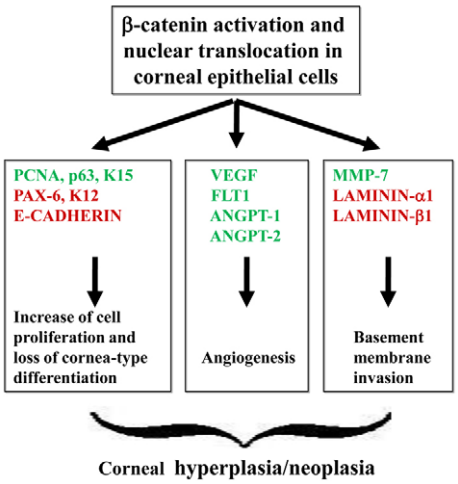

Fig. 11. Model of β-catenin nuclear translocation leading to corneal hyperplasia and/or neoplasia. Depicted is the activation of the β-catenin through aberrant expression of ΔE3β-catenin. Nuclear translocation of β-catenin results directly or indirectly in the up- or downregulation (green or red, respectively) of a wide variety of genes. This, in turn, leads to loss of corneal epithelial cell-type differentiation, which promotes cell proliferation, increases pro-angiogenic factors and disrupts basement membrane components, all of which eventually results in corneal hyperplasia and/or neoplasia.

It is of particular interest that, panni that have been surgically removed from human OSSN patients also exhibited nuclear translocation of β-catenin (Fig. 10). These findings implicate that β-catenin activation might be a common mechanism in tumorigenesis resulting from corneal progenitor cells that undergo oncogenic transformation as a result of insults, such as infection with the HPV16 and/or long-term UVB exposure (Scott et al., 2002; Kiire and Dhillon, 2006). In the *Ctnnb*1 gain-of-function mouse model, the generation of *Ctnnb1*$^{\Delta E3}$ allele is dependent upon Dox-inducible Cre activity, but the expression of ΔE3β-catenin is under transcriptional control by its own promoter and/or enhancers. In human cancers, exon 3 of the *CTNNB*1 gene is a mutational hot spot for the gain-of-function isoform (Moon et al., 2002). Since the *CTNNB*1 gene is transcriptionally active in the corneal epithelial cells, it is possible to activate the gain-of-function β-catenin isoform through the somatic mutation in corneal epithelium. Nevertheless, it requires further investigation to determine whether the exon 3 mutation of *CTNNB*1 is linked with the pathogenesis of human OSSN.

Altogether, our data, which rely on the Dox-inducible corneal specific *K12*^{rtTA}*/tetO-Cre* driver mice, suggest that the aberrant expression of a dominant stable ΔE3β-catenin mutant triggers a variety of cellular alterations including cell proliferation and differentiation by regulating the expression of related genes and, thus, disturbing corneal morphogenesis and homeostasis with the formation of nodules and neovascularization in the cornea. Tight regulation of β-catenin signaling to control gene expression is indispensable for cornea development and correct maintenance of its homeostasis, and may otherwise lead to tumorigenesis such as OSSN (Fig. 11). Thus, inhibition of β-catenin activation might be beneficial for treating this disease.

Materials and Methods

Human samples

Human OSSN samples and eye bank eye samples were obtained in accordance with the Institutional Review Board at Chang Gung Memorial Hospital (protocol #97-1256B to L.K.Y.) and all participants gave informed written consent.

Animals

Experimental animals were housed under pathogen-free conditions in accordance with institutional guidelines. Animal care and use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati.

Compound transgenic mice

Genetic modified mouse lines such as *Krt12*^{rtTA} (Chikama et al., 2005), *tetO-Cre* (Perl et al., 2002), *Ctnnb1*^{floxE3} (Harada et al., 1999), *TOP-Gal* (DasGupta and Fuchs, 1999) and *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato-EGFP)Luo}$/J (here referred to as *ROSA*^{mTmG}) mice (Muzumdar et al., 2007) have been previously described. Compound transgenic mice were generated by natural breeding of individual mouse lines, and the genotypes were identified by polymerase chain reaction (PCR) using oligonucleotide primers specific for each transgene. In all experiments, detection time of the vaginal plug was defined as E0.5 of embryonic development. To activate *tetO-Cre* expression, mice were intraperitoneally (IP) injected once with Dox (Sigma, 80 μg/g body weight) at a concentration of 10 mg/ml (Utomo et al., 1999) and fed Dox-chow (1 g/kg chow, Bioserv Corporation). Control animals were fed regular chow.

Immunohistochemical and western blotting analyses

The following antibodies were used: rabbit anti-K12 antibody (2 μg/ml) (Liu et al., 1994); rabbit anti-Pax-6 (1:100), rabbit anti-K15 (1:100), rabbit anti-K10 (1:200) (Covance Inc.); rabbit monoclonal anti-MMP-7 (1:100), mouse 4A4 anti-p63 (1:50); mouse anti-BrdU (1:100); mouse PC10 anti-PCNA (1:100) (Lab Vision, Corp.); mouse anti-β-catenin (1:50, BD Biosciences Pharmingen); rabbit anti-β-catenin (1:2000, Sigma); mouse 6B10 anti-K4 (1:100, Abcam, Inc.); Goat anti-laminin-α1 (1:100), anti-laminin-β1(1:100) (Santa Cruz Biotechnology, Inc.). Alexa-Fluor-555-conjugated donkey anti-goat-IgG or goat anti-rabbit IgG was used for immunofluorescent staining. For immunohistological analysis, excised eyes were fixed in 4% paraformaldehyde in PBS and paraffin embedded. Tissue sections (5 μm) were de-paraffinized and hydrated in a graded ethanol series. The primary antibody was incubated at the dilutions mentioned above in TBST/BSA (3%) at 4°C overnight. Non-specific antibody binding was removed by washing with TBST three times. Alexa-Fluor-555-conjugated secondary antibody was then incubated at room temperature for 1 hour. Sections for immunofluorescence analysis were mounted (SlowFade Light Antifade Kit; Molecular Probes, Inc.) in the presence of 4′,6′-diamidino-2-phenylindole (DAPI), and observed using a Nikon Eclipse E800 epifluorescent microscope and photographed using a SPOT RT digital camera system (Diagnostic Instruments, Inc.). For western blotting analysis**,** total cellular lysates from mouse corneas were prepared in lysis buffer [Tris-HCl, 50 mM, pH 7.4; NaCl, 250 mM; EDTA, 5 mM; NP-40, 0.1%; NaF, 25 mM; 1× protease inhibitor cocktail (Sigma P8340), and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10%). First antibody was incubated at dilutions as mentioned above in TBST/BSA (3%) at 4°C overnight. Alkaline phosphatase (AP)-conjugated secondary antibody was then incubated at room temperature for 1 hour. Positive signals were visualized by Western blue substrate for AP (Promega, Inc.).

Real-time RT-PCR

Total RNA (10 μg) was isolated from corneal epithelium, annealed to random primer and reverse transcribed with avian reverse transcriptase (RT) kits from Promega Inc. (Madison, WI) according to manufacturer's instructions. Single-strand cDNA was subjected to PCR using primer pairs. Real-time RT-PCR was performed by using the CFX96 real-time system equipped on a C1000^{TM} Thermal Cycler (Bio-Rad Laboratories Inc.). After the initial step at 95°C for 5 minutes, 40 cycles at 95°C 30 seconds, 62°C 30 seconds, 72°C 30 seconds. The cycle threshold values were used to calculate the normalized expression of mouse Vegf-A, Vegfr1 (Flt1), Angp-1, and Angpt-2 against β-actin using Q-Gene software. Primer sets and their PCR products for the quantitative RT-PCR are listed as follows: mVegf-A forward primer 5′-TTCCTACAGCACAGCAGATG-3′, mVegf-A reverse primer 5′-TTACACGTCT-GCGGATCTTG-3′, PCR product 122 base pairs (bps); mFlt-1 forward primer 5′-GTCTTGCCTTACGCGCTGCT-3′, mFlt-1 reverse primer 5′-AGAGTCTGG-CCTGCTTGCAT-3′, PCR product 121 bps; mAngpt-1 forward primer 5′-ATGC-GCTCTCATGCTAACAG-3′, mAngpt-1 reverse primer 5′-CCGCAGTGTAGAA-CATTCCA-3′, PCR product 80 bps; mAngpt-2 forward primer 5′-ACGAGGCGCA-TTCGCTGTAT-3′, mAngpt-2 reverse primer 5′-CTGGTTGGCTGATGCTACTT-3′, PCR product 117 bps; mβ-actin forward primer 5′-GCTCTGGCTCCTAGCACC-ATGA-3′, mβ-actin reverse primer 5′-CCTGCTTGCTGATCCACATCTG-3′, PCR product 127 bps.

β-galactosidase histochemical analysis

Embryos were stained as whole mounts overnight at room temperature in a solution of X-Gal (Sigma) at a final concentration of 0.4 mg/ml made from a 40 mg/ml stock in DMSO, with 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6{\cdot}6H_2O$, 2 mM $MgCl_2$ in PBS. After staining, tissues and embryos were rinsed with PBS and photographed as whole mounts. These samples were then embedded in paraffin as previously described. Paraffin sections were counterstained with hematoxylin, observed with a Nikon Eclipse E800 epifluorescent microscope and photographed with a SPOT RT digital camera system (Diagnostic Instruments, Inc.).

Confocal microscopic scanning

Adult (6-weeks old) quadruple-transgenic mice *K12*$^{rtTA/Wt}$*/tetO-Cre/Ctnnb1*$^{Wt/Wt}$*/ROSA*$^{mTmG/Wt}$ or *K12*$^{rtTA/Wt}$*/tetO Cre/Ctnnb1*$^{floxE3/Wt}$*/ROSA*$^{mTmG/Wt}$ administered Dox chow for 3 days. Mouse eyes were enucleated and corneas were dissected, counterstained with DAPI, mounted on slides. The *Z*-stack images were collected using the Zeiss LSM 510 META two-photon laser scanning confocal microscope (Carl Zeiss MicroImaging, Inc.). The fluorescent signal detection from tomato red was obtained by using the 543-nm laser line of the red helium-neon laser. Emitted light was detected by using a 565-615 nm filter. For the detection of EGFP, the 488-nm laser line of the argon laser was selected and emitted light was caught by a filter of 500-550 nm. DAPI signals were captured by using a model-locked MaiTai laser (Carl Zeiss). Three dimensional images were acquired by using the Axiovision AxioVs40 V 4.7.0.0 software (Carl Zeiss). Images were treated using Adobe Photoshop CS2 (Adobe Microsystems).

Statistical analysis

Two-tailed Student's *t*-test (Excel, Microsoft, Redmond, WA) was used in analyzing the number of BrdU-positive cells. All quantification data are presented as the mean ± s.d.

We thank the reviewers for their instructive comments for this manuscript. This work was supported by grants from NIH/NEI RO1 EY12486 (C.-Y.L.) and EY13755 (W.W-Y.K.), Research Prevent Blindness, Ohio Lions Foundation for Eye Research. Deposited in PMC for release after 12 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/8/1285/DC1

References

Almeida, M., Han, L., Martin-Millan, M., O'Brien, C. A. and Manolagas, S. C. (2007). Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J. Biol. Chem.* **282**, 27298-27305.

Brabletz, T., Jung, A., Dag, S., Hlubek, F. and Kirchner, T. (1999). beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am. J. Pathol.* **155**, 1033-1038.

Birchmeier, W. (2005). E-cadherin as a tumor (invasion) suppressor gene. *Bioassay* **17**, 97-99.

Chamorro, M. N., Schwartz, D. R., Vonica, A., Brivanlou, A. H., Cho, K. R. and Varmus, H. E. (2006). FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development**.** *Cancer Res.* **66**, 1354-1362.

Chikama, T., Hayashi, Y., Liu, C. Y., Terai, N., Terai, K., Kao, C. W., Wang, L., Hayashi, M., Nishida, T., Sanford, P. et al. (2005). Characterization of tetracycline-inducible bitransgenic Krt12$^{rtTA/+}$/tet-O-LacZ mice.*Invest. Ophthalmol. Vis. Sci.* **46**, 1966-1972.

Chikama, T., Liu, C. Y., Meij, J. T., Hayashi, Y., Wang, I. J., Yang, L., Nishida, T. and Kao, W. W. (2008). Excess FGF-7 in corneal epithelium causes corneal intraepithelial neoplasia in young mice and epithelium hyperplasia in adult mice. *Am. J. Pathol.* **172**, 638-649.

Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P. and Matrisian, L. M. (1999). The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene.* **18**, 2883-2891.

DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.

Eblaghie, M. C., Song, S. J., Kim, J. Y., Akita, K., Tickle, C. and Jung, H. S. (2004). Interactions between FGF and Wnt signals and Tbx3 gene expression in mammary gland initiation in mouse embryos. *J. Anat.* **205**, 1-13.

Fujino, H. and Regan, J. W. (2001). FP prostanoid receptor activation of a Tcf/beta-catenin signaling pathway. *J. Biol. Chem.* **276**, 12489-12492.

Gage, P. J., Qian, M., Wu, D. and Rosenberg, K. I. (2008). The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development. *Dev. Biol.* **317**, 310-324.

Gustavson, M. D., Crawford, H. C., Fingleton, B. and Matrisian, L. M. (2004). Tcf binding sequence and position determines beta-catenin and Lef-1 responsiveness of MMP-7 promoters. *Mol. Carcinog.* **41**, 125-139.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M. and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* **18**, 5931-5942.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

Hendrix, N. D., Wu, R., Kuick, R., Schwartz, D. R., Fearon, E. R. and Cho, K. R. (2005). Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas. *EMBO J.* **24**, 73-84.

Jamora, C., DasGupta, R., Kocieniewski, P. and Fuchs, E. (2003). Links between signal transduction, transcription and adhesion in epithelial bud development. *Nature* **422**, 317-322.

Katoh, M. and Katoh, M. (2007). WNT signaling pathway and stem cell signaling network. *Clin. Cancer Res.* **13**, 4042-4045.

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9**, 317-321.

Kiire, C. A. and Dhillon, B. (2006). The aetiology and associations of conjunctival intraepithelial neoplasia. *Br. J. Ophthalmol.* **90**, 109-113.

Kratochwil, K., Galceran, J., Tontsch, S., Roth, W. and Grosschedl, R. (2002). FGF4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Lef1$^{(-/-)}$ mice. *Genes Dev.* **16**, 3173-3185.

Lee, G. A. and Hirst, L. W. (1995). Ocular surface squamous neoplasia. *Surv. Ophthalmol.* **39**, 429-450.

Lin, S. Y., Xia, W., Wang, J. C., Kwong, K. Y., Spohn, B., Wen, Y., Pestell, R. G. and Hung, M. C. (2000). β-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. USA.* **97**, 4262-4266.

Liu, C. Y., Zhu, G., Westerhausen-Larson, A., Converse, R. L., Kao, C. W., Su, T. T. and Kao, W. W. (1993). Cornea-specific expression of K12 keratin during mouse development. *Curr. Eye Res.* **12**, 963-974.

Liu, C. Y., Zhu, G., Converse, R., Kao, C. W., Nakamura, H., Tseng, S. C., Mui, M. M., Seyer, J., Justice, M. J., Stech, M. E. et al. (1994). Characterization and chromosomal localization of the cornea-specific murine keratin gene Krt1.12. *J. Biol. Chem.* **269**, 24627-24636.

Lowry, W. E., Blanpain, C., Nowak, J. A., Guasch, G., Lewis, L. and Fuchs, E. (2005). Defining the impact of β-catenin/Tcf transactivation on epithelial stem cell. *Genes Dev.* **19**, 1596-1611.

Moon, R. T., Bowerman, B., Boutros, M. and Perrimon, N. (2002). The promise and perils of Wnt signaling through beta-catenin. *Science* **296**, 1644-1646.

Mukhopadhyay, M., Gorivodsky, M., Shtrom, S., Grinberg, A., Niehrs, C., Morasso, M. I. and Westphal, H. (2006). DKK2 plays an essential role in the corneal fate of the ocular surface epithelium. *Development* **33**, 2149-2154.

Mulholland, D. J., Cheng, H., Reid, K., Rennie, P. S. and Nelson, C. C. (2002). The androgen receptor can promote beta-catenin nuclear translocation independently of adenomatous polyposis coli. *J. Biol. Chem.* **277**, 17933-17943.

Mulholland, D. J., Dedhar, S., Coetzee, G. A. and Nelson, C. C. (2005). Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocr. Rev.* **26**, 898-915.

Musgrove, E. A. (2004). Wnt signalling via the epidermal growth factor receptor: a role in breast cancer? *Breast Cancer Res.* **6**, 65-68.

Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo. L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593-605.

Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483-1487.

Novak, A., Hsu, S. C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R., Roskelley, C., Grosschedl, R. and Dedhar, S. (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad. Sci. USA.* **95**, 4374-4379.

Orsulic, S., Huber, O., Aberle, H., Arnold, S. and Kemler, R. (1999). E-cadherin binding prevents β-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J. Cell Sci.* **112**, 1237-1245.

Pearton, D. J., Yang, Y. and Dhouailly, D. (2005). Trans-differentiation of corneal epithelium into epidermis occurs by means of a multistep process triggered by dermal developmental signals. *Proc. Natl. Acad. Sci. USA* **102**, 3714-3719.

Perl, A. K., Wert, S. E., Nagy, A., Lobe, C. G. and Whitsett, J. A. (2002). Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc. Natl. Acad. Sci. USA.* **99**, 10482-10487.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev.* **14**, 1837-1851.

Richard-Parpaillon, L., Héligon, C., Chesnel, F., Boujard, D. and Philpott, A. (2002). The IGF pathway regulates head formation by inhibiting wnt signaling in *Xenopus*. *Dev. Biol.* **244**, 407-417.

Saez, E., Rosenfeld, J., Livolsi, A., Olson, P., Lombardo, E., Nelson, M., Banayo, E., Cardiff, R. D., Izpisua-Belmonte, J. C. and Evans, R. M. (2004). PPARγ signaling exacerbates mammary gland tumor development. *Genes Dev.* **18**, 528-540.

Schweizer, L., Rizzo, C. A., Spires, T. E., Platero, J. S., Wu, Q., Lin, T. A., Gottardis, M. M. and Attar, R. M. (2008). The androgen receptor can signal through Wnt/beta-Catenin in prostate cancer cells as an adaptation mechanism to castration levels of androgens. *BMC Cell Biol.* **9**, 4.

Scott, I. U., Karp, C. L. and Nuove, G. J. (2002). Human papillomavirus 16 and 18 expression in conjunctival intraepithelial neoplasia. *Ophthalmology* **109**, 542-547.

Shimokawa, T., Furukawa, Y., Sakai, M., Li, M., Miwa, N., Lin, Y. M. and Nakamura, Y. (2003). Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex. *Cancer Res.* **63**, 6116-6120.

Siu, M. K. Y. and Cheng, C. Y. (2004). Interactions of proteases, protease inhibitors, and the β1 integrin/laminin γ3 protein complex in the regulation of ectoplasmic specialization dynamics in the rat testis. *Biol. Reprod.* **70**, 945-964.

Smith, A. N., Miller, L. A., Song, N., Taketo, M. M. and Lang, R. A. (2005). The duality of beta-catenin function: a requirement in lens morphogenesis and signaling suppression of lens fate in periocular ectoderm. *Dev. Biol.* **285**, 477-489.

ten Berge, D., Koole, W., Fuerer, C., Fish, M., Eroglu, E. and Nusse, R. (2008).Wnt signaling mediates self-organization and axis formation in embryoid bodies. *Cell Stem Cell* **3**, 508-518.

Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-426.

Tseng, S. C.-G. (1989). Concept and application of limbal stem cells. *Eye* **3**,141-157.

Utomo, A. R., Nikitin, A. Y. and Lee, W. H. (1999). Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat. Biotechnol.* **17**, 1091-1096.

Wahl, M. B., Deng, C., Lewandoski, M. and Pourquié, O. (2007). FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. *Development* **134**, 4033-4041.

Yoshida, S., Shimmura, S., Kawakita, T., Miyashita, H., Den, S., Shimazaki, J. and Tsubota, K. (2006). Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest. Ophthalmol. Vis. Sci.* **47**, 4780-4786.

Zhang, X., Gaspard, J. P. and Chung, D. C. (2001). Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res.* **61**, 6050-6054.

Zhao, H., Cui, Y., Dupont, J., Sun, H., Hennighausen, L. and Yakar, S. (2005). Overexpression of the tumor suppressor gene phosphatase and tensin homologue partially inhibits wnt-1-induced mammary tumorigenesis. *Cancer Res.* **65**, 6864-6873.

Zhu, W., Shiojima, I., Ito, Y., Li, Z., Ikeda, H., Yoshida, M., Naito, A. T., Nishi, J., Ueno, H., Umezawa, A. et al. (2008). IGFBP-4 is an inhibitor of canonical Wnt signaling required for cardiogenesis. *Nature* **454**, 345-349.