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Co-opted JNK/SAPK Signaling in Wnt/β-catenin–Induced Tumorigenesis[1]

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Abstract

Aberrant stimulation of the canonical Wnt pathway induces mammary tumorigenesis in mice. It has been well documented that two types of tumors, adenocarcinoma and adenocarcinoma with squamous metaplasia, develop in these mutants. However, the molecular mechanism underlying the induction of squamous transdifferentiation remains largely unknown. Here, we show that JNK/SAPK signaling plays an important role in Wnt-dependent mammary development and malignant transformation. The JNK/SAPK pathway is stimulated in pregnancy-mediated lobulo-alveolar morphogenesis, a process highly dependent on Wnt/β-catenin signaling. Strong elevations of JNK/SAPK signaling are associated with squamous metaplasia of the Wnt-induced adenocarcinoma. Reconstitution of β-catenin and JNK/SAPK signaling activities also promotes expression of the squamous cell marker in cultured epithelial cells. Furthermore, a synergistic activation of these two pathways can be identified in the malignant squamous cells of human endometrial and lung cancers. This is potentially a significant discovery in modern cancer therapy because of the effectiveness of an angiogenesis inhibitor, Avastin, for the treatment of adenocarcinoma, but not squamous cell carcinoma, in human lung cancers. Our finding may improve the usage of biomarkers to distinguish these two poorly differentiated tumor types, sharing similar histologic features.

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Introduction

Wnt signaling is important for diverse processes during embryonic, fetal, and postnatal development [1]. Deregulation of Wnt signaling has also been linked to pathogenesis of various diseases [2]. β-Catenin, a key component of adherens junction, plays an essential role in transduction of the canonical Wnt pathway [3]. The cellular level of β-catenin is controlled by a disruption complex, including Axin, APC, and GSK-3β [4]. In the absence of a Wnt signal, this complex binds to β-catenin, mediating its phosphorylation and degradation through the ubiquitin-mediated proteolysis system [5]. Wnt signals perturb the formation of the disruption complex by activating the upstream regulators, leading to nuclear accumulations of β-catenin [6]. The downstream signaling events are triggered by its interaction with transcription factors of the lymphoid enhancer factor/T cell factor (LEF/TCF) family to regulate target gene expression [7].

Wnt signaling is intimately involved in the development and neoplastic transformation of the mammary gland [8]. Several members of the Wnt family are expressed in the epithelium and stromal

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compartments at various stages of mammary development [9]. It has been well established that aberrant stimulation of the canonical Wnt pathway, such as transgenic expression of Wnt1, Wnt10b, results in mammary tumorigenesis in mice [10,11]. Expression of a degradation-deficient form of β-catenin leads to the formation of mammary tumors in mice [12]. Transgenic mice with elevated levels of the Wnt target cyclin D1 or c-myc also develop mammary tumors [13,14]. Furthermore, Wnt signals are required not only for induction but also for the inhibition of mammary development. The loss of Wnt4 impairs the early phases of pregnancy-dependent mammary morphogenesis [15]. In contrast, Wnt5a-null mammary tissue exhibits an accelerated cell growth and proliferation capacity [16]. This probably is due to the involvement of Wnt5a in an alternative pathway (see below). Reduction of Wnt/β-catenin signaling by high levels of Axin or a dominant-negative β-catenin interferes with alveolar morphogenesis at late pregnancy stages [17,18]. The loss of LEF-1 transcription factor in mice led to the lack of mammary bud formation at embryogenesis [19]. These studies imply that Wnt signaling is critical for mammary development in health and disease.

In addition to β-catenin, Wnt signals have been shown to transduce through alternative pathways [20,21]. Wnt5a, Wnt5b, and Wnt11 are representatives of noncanonical Wnt signaling through JNK/SAPK (Jun N-terminal kinase/stress–activated protein kinase), a cascade similar to the planer cell polarity pathway in *Drosophila*. Aberrant activation of Wnt/JNK pathway seems to develop more malignant phenotypes, such as abnormal tissue polarity, invasion, and metastasis [21]. Stimulation of Wnt5a has been associated with various types of human cancers, including gastric cancer, lung cancer, and melanoma. There are a few molecules playing an essential role in both canonical and noncanonical pathways, suggesting that the Wnt signal might diverge at these transducers. Both Dsh/Dvl and Axin have been shown to activate Wnt/JNK signaling cascade through domains distinct from those regulating β-catenin [22]. Axin induces JNK/SAPK activity through its MEKK1 binding and self-association domains [23]. These domains do not seem to be required for down-regulation of β-catenin in *Xenopus* axis determination [24]. In *Drosophila*, the DIX domain of Dsh is dispensable for the planer cell polarity pathway, whereas it is essential for β-catenin signaling [25]. The dual role of signal transducers raises the question of overlap between the Wnt pathways, as well as other signaling pathways. However, the significance of cross-signaling interaction in normal developmental processes and pathogenesis of human diseases remains largely elusive.

In this study, we investigated the potential role of JNK/SAPK signaling in Wnt-dependent mammary development and tumorigenesis in mice. The JNK and its related p38 MAP kinases were stimulated during pregnancy-mediated lobulo-alveolar morphogenesis, a process highly dependent on Wnt/β-catenin signaling. In the hyperplasia and adenocarcinoma caused by high levels of Wnt, JNK/SAPK signaling apparently was not induced. However, the Wnt/JNK pathway was strongly stimulated in squamous metaplasia, a process in which the presence of glandular epithelium is replaced by squamous epithelium, suggesting its involvement in the transdifferentiation of malignant cells. Reconstitution of β-catenin and JNK/SAPK signaling activities promoted expression of squamous cell markers in cultured epithelial cells. Furthermore, our finding led us to identify that these two pathways are synergistically activated in the malignant squamous cells of human endometrial and lung cancers. Our results suggest that JNK/SAPK signaling not only has a crucial function in squamous cell differentiation but also plays a co-opted role in the Wnt/β-catenin–mediated oncogenic transformation.

Materials and Methods

Animals and Human Samples

MMTV-Wnt1 mice, expressing Wnt1 under control of the MMTV promoter, were generated and described previously [26]. Briefly, mammary hyperplasia could be detected in all of the transgenic animals. Approximately 50% of the females developed mammary tumor at 3 to 6 months of age. Mice carrying the *MMTV-Wnt1* transgene were genotyped by polymerase chain reaction analysis using primers (5′-ggacttgcttctcttctcatagcc-3′ and 5′-ccacacaggcatagagtgtctgc-3′) to identify the transgene. The polymerase chain reaction was performed by denaturation at 94°C for 2 minutes and 34 cycles of amplification (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds), followed by a 2-minute extension at 72°C. Care and use of experimental animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester. The paraffin sections of human endometrial and lung cancers were obtained based on a protocol approved by University of Rochester Research Subjects Review Board. The identifiers of all patients have been removed to protect confidentiality. The study, approved by the program director at the National Cancer Institute, was judged "NO" for human subject research because we could not have an intervention with a living person and would not receive or record any identifiable information.

Cells and DNA

C57MG and C57MG-Wnt1 mammary epithelial cell lines were obtained from Arnold Levine [27]. Cells were cultured with DMEM containing 10% FCS, 100 U of penicillin, and 100 mg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C. The pcatC-Lef1 is a DNA plasmid containing the C-terminal activation domain of human β-catenin (amino acids 696-781) fused to the amino terminus of LEF-1 [28]. The pCMV-ΔMEKK1 contains a dominant/constitutively active form of MEKK1 under control of a CMV promoter [29]. The pCMV-SEK^{ED} is a plasmid permitting the expression of a partially active SEK1 [30]. Approximately 2×10^4 cells were plated 24 hours before transfection. Introduction of DNA plasmids (0.5 μg per plasmid) into the cultured cells was performed by Lipofectamine 2000–mediated transfection according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were harvested 24 hours after the transfection.

Histologic Diagnosis, Immunostaining, and Immunoblot

Samples were fixed, paraffin-embedded, sectioned, and stained with hematoxylin/eosin for histologic evaluation as described [31,32]. Tissue sections were subject to immunologic staining with avidin/biotinlylated enzyme complex or indirect fluorescent staining as described [17,33,34]. Briefly, samples were deparaffinized and hydrated, followed by antigen unmasking (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide. For the use of mouse primary antibodies, sections were incubated with mouse IgG blocking solution (Vector Laboratories). After addition of primary antibodies for 30 minutes at room temperature, sections were incubated with horseradish peroxidase–conjugated secondary antibodies for 10 minutes at

room temperature. The staining was then visualized by enzymatic color reaction according to the manufacturer's specification (Vector Laboratories). Images were analyzed using an Axio Observer microscope and a SPOT-RT camera (Carl Zeiss, Thornwood, NY). For immunoblot analysis, cells were lysed with M-PER reagent (78501; Pierce, Rockford, IL), containing protease inhibitor cocktail (diluted at 1:100, P8340; Sigma, St. Louis, MO), for 5 minutes with gentle shaking. Cell lysates were subject to immunoblot analysis as described [35,36]. Bound primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies, followed by enhanced chemical luminescence-mediated visualization (Amersham, Piscataway, NJ) and autoradiography. Mouse monoclonal antibodies actin (Lab Vision, Fremont, CA), ABC (Upstate, Billerica, MA), and HMW-Keratin (Lab Vision); rabbit polyclonal antibodies cyclin D1 (Lab Vision), β-catenin (Lab Vision), PJNK (Cell Signaling, Danvers, MA), Pp38 (Cell Signaling), PErk1/2 (Cell Signaling), PJun63 (Cell Signaling), and PJun73 (Cell Signaling) were used as primary antibodies.

Results

JNK/SAPK Signaling in Mammary Development

To investigate the involvement of JNK/SAPK signaling in mammary development, we examined the expression of phosphorylated forms of JNK/SAPK signaling molecules. Immunostaining of phosphorylated JNK (PJNK) did not show its presence in nulliparous glands at 3 months (Figure 1*A*). Because JNK/SAPK belongs to the mitogen-activated protein kinase (MAPK) family, we therefore tested the activity of the other two signaling cascades. Phosphorylation of a closely related p38 MAP kinase (Pp38), which is also activated by a variety of cellular stresses, was not detected (Figure 1*B*). However, the expression of PJNK and Pp38 was highly stimulated in mammary epithelial cells undergoing pregnancy-dependent alveolar development (Figure 1, *F* and *G*). In contrast, phosphorylated Erk1/2 (PErk1/2), a third member of the MAPK family, was present in both nulliparous and parous glands (Figure 1, *C* and *H*). The stimulation of PJNK and Pp38 were accompanied by enhanced phosphorylation of c-Jun at Ser63 (PJun63) and Ser73 (PJun73) in the luminal epithelia (Figure 1, *D*, *E*, *I*, and *J*). Although PJun73 could be found in few cells before the pregnancy-mediated differentiation of lobulo-alveoli, the results were consistent with a drastic stimulation of JNK/SAPK signaling, suggesting its involvement in mammary development.

Stimulation of JNK/SAPK Signaling in Squamous Metaplasia

We next determined whether JNK/SAPK signaling plays a role in Wnt-induced mammary tumorigenesis by examining MMTV-Wnt1 mice, in which Wnt1 is expressed constitutively under control of the mouse mammary tumor virus (MMTV) promoter. Consistent with the importance of β-catenin–mediated malignant transformation, β-catenin became nuclearly localized in the hyperplastic epithelia, before the onset of tumorigenesis, induced by MMTV-Wnt1 (Figure 2*G*). Meanwhile, only membrane staining of β-catenin was displayed in the control littermate (Figure 2*A*). However, JNK/SAPK signaling was not affected by the constitutive expression of Wnt1 in the hyperplastic stage (Figure 2). The phosphorylated JNK, p38, and c-Jun63 were not detected in both the MMTV-Wnt1 and the control mice (Figure 2, *B*, *C*, *E*, *H*, *I*, and *K*). The *MMTV-Wnt1* transgene also had no effects on the expression of PErk1/2 at a low level, and the expression of PJun73 was noticeable in few cells, in both the normal and hyperplastic mammary glands (Figure 2, *D*, *F*, *J*, and *L*). Overall, there were no significant differences in the expression of PJNK, Pp38, PErk1/2, PJun63, and PJun73 between the MMTV-Wnt1 and control mice. The stress-induced signaling, including that of JNK/SAPK and p38 MAPK, was not affected in hyperplasia induced by high levels of Wnt/β-catenin signaling.

In MMTV-Wnt1 mice, two types of mammary tumors, adenocarcinoma (AC) and adenocarcinoma with squamous metaplasia (SM), were identified usually after 3 months (Figure 3, *A* and *I*). Immunostaining of high–molecular weight keratin (HMW-K), a marker for squamous cells, revealed a relatively high percentage of the Wnt-induced tumors undergoing squamous differentiation (62.5%, n = 8; Figure 3, *H* and *P*). We then examined the activity of Wnt/β-catenin and JNK/SAPK signaling in AC and SM. Consistent with

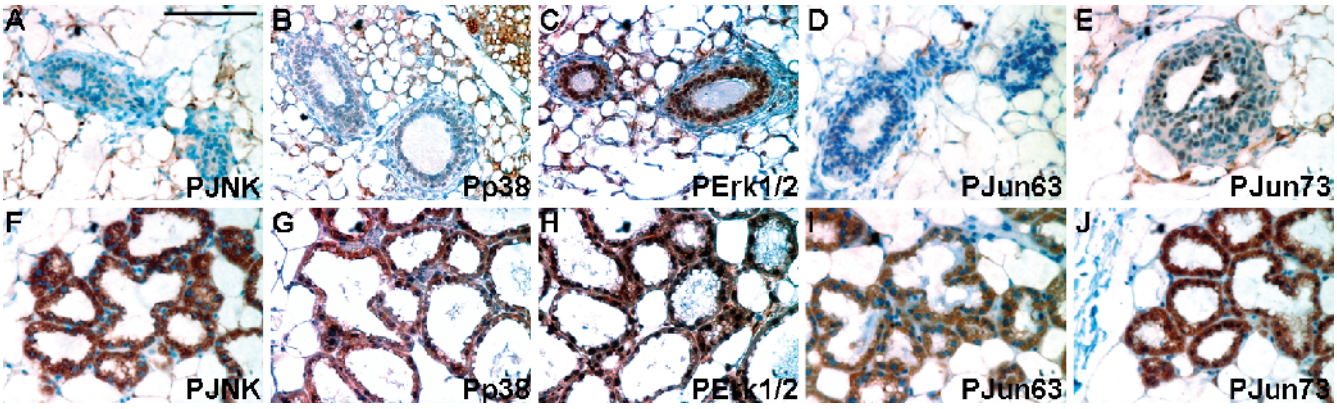

Figure 1. The involvement of JNK/SAPK signaling in mammary gland development. Sections of mouse virgin gland (A–E) and mammary gland undergoing pregnancy-dependent morphogenesis (F–J) were analyzed by immunochemical staining for phosphorylated JNK (PJNK; A, F), phosphorylated p38 (Pp38; B, G), and phosphorylated Erk1/2 (PErk1/2; C, H), phosphorylated c-Jun at Ser63 (PJun63; D, I) and phosphorylated c-Jun at Ser73 (PJun73; E, J). In the virgin gland, mammary epithelia showed that PErk1/2 is highly expressed and PJun73 is positive for a few cells. In contrast, all JNK/SAPK signaling molecules were strongly activated during pregnancy-mediated mammary development. The immunochemistry (brown)–stained sections were counterstained with hematoxylin (blue). The experiments were performed in three different wild type and transgenic animals (n = 3). Scale bar, 100 μm (A–J).

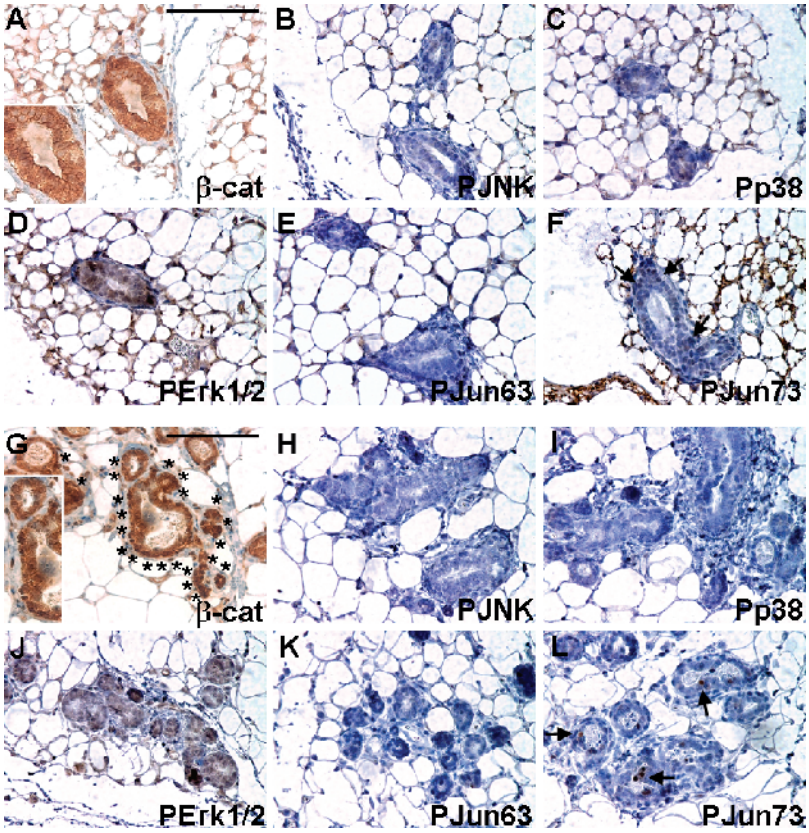

Figure 2. JNK/SAPK signaling is not induced in mammary hyperplasia caused by elevated levels of Wnt/β-catenin signaling. Sections of the wild type (A–F) and hyperplastic (G–L) mammary glands caused by high levels of Wnt signaling were analyzed by immunochemistry for the expression of β-catenin (β-cat; A, G), PJNK (B, H), Pp38 (C, I), PErk1/2 (D, J), PJun63 (E, K), and PJun73 (F, L). The insets in A and G display magnified images revealing differential localization of β-catenin. The immunochemistry (brown)–stained sections were counterstained with hematoxylin (blue). The experiments were performed in four different wild type and transgenic animals ($n = 4$). Scale bars, 100 μm (A–I).

previous findings, the expressions of β-catenin and its direct downstream gene *cyclin D1* [37,38] were elevated in both AC and SM (Figure 3, *B*, *C*, *J* and *K*; $n = 8$), supporting the notion that transgenic expression of a stabilized form of β-catenin or cyclin D1 in the mouse mammary epithelia developed both types of tumor [13,39]. Squamous differentiation is often observed in the Wnt-induced tumorigenesis.

To further elucidate the mechanism underlying Wnt-mediated oncogenic transformation, we studied whether the activation of JNK/SAPK signaling is associated with this process. Similar to what was observed in the Wnt-induced hyperplasia, the expression of PJNK, Pp38 and PJun73 also was not detected in AC (Figure 3, *D*, *E*, and *G*). PErk1/2 was detected in both AC and SM (Figure 3, *F* and *N*). However, PJNK, Pp38, and PJun73 were highly stimulated in SM (Figure 3, *L*, *M*, and *O*; $n = 5$). These data demonstrated that stress signaling was dramatically enhanced in the Wnt-induced malignant cells undergoing squamous differentiation. This raises the possibility that synergistic activation of these two signaling cascades might be involved in the metaplastic transformation.

Molecular Mechanism of JNK/SAPK Activation

To examine whether activation of Wnt/β-catenin signaling has any effects on JNK/SAPK signaling, a dominant-negative form of Axin (dnAxin) and a fusion protein of β-catenin and LEF1 (catCLEF1) were used to induce this signaling pathway. The catCLEF1 protein is a chimera, which links the C-terminal activation domain of human β-catenin to the N-terminus of LEF-1 and constitutively induces transcription of downstream genes [28]. In cells expressing catCLEF1, we detected a higher–molecular weight protein, presumably the chimera, using an antibody recognizing a nonphosphorylated/stabilized form of β-catenin (Figure 4*A*). This was accompanied by a slight elevation of cyclin D1, but no alteration in the expression of PJNK, Pp38, PJun63, and PJun73 was observed in these catCLEF1-expressing cells (Figure 4*A*). We previously demonstrated that expression of dnAxin, which lacks the RGS/APC–binding domain, is able to induce axis duplication through stimulation of Wnt/β-catenin signaling [40]. A previous report also showed that deletion of the APC-binding domain of Axin did not interfere with its ability to activate JNK/SAPK in a c-Jun kinase assay *in vitro* [23]. Consistent with the previous finding, we detected an increased level of the stabilized β-catenin (sβ-cat) when dnAxin was expressed (Figure 4*A*). This also led to a slight enhancement of the cyclin D1 expression (Figure 4*A*). We next examined if Axin could stimulate JNK/SAPK signaling because it was suggested to diverge from the Wnt/β-catenin pathway at this level [23]. Although Pp38 showed a significant elevation, the expression of dnAxin was insufficient to induce the expression of PJNK, PJun63, and PJun73 (Figure 4*A*). The lack of JNK and c-Jun stimulation was not due to the deletion of the RGS/APC-binding domain because similar results were detected using a full-length Axin (data not shown). The results imply that the signaling cascade induced by Axin alone may not be sufficient to promote JNK/SAPK activation in transfected cells.

We next investigated at which molecular level the signaling activation is required to stimulate both JNK and p38. Dominant forms of MEKK1 (ΔMEKK1) and SEK1 (SEK^{ED}) were used to activate the downstream signaling cascade. MEKK1 is an MAPKKK which turns on JNK through activation of SEK1 (a MAPKK also known as MKK4) [41,42]. The expression of dominant SEK1 had no significant effects on the phosphorylation of JNK, and c-Jun at Ser63 and Ser73, but slightly enhanced the phosphorylation of p38 (data not shown). However, the phosphorylation of p38, JNK, and c-Jun at Ser63 and Ser73 was strongly stimulated by the dominant MEKK1 (Figure 4*A*). Consistent with the immunostaining results, Erk1/2 apparently was not involved in this signaling pathway (data not shown). These data suggest that stimulation of the JNK/SAPK pathway, which is critical for mammary development and tumorigenesis, needs to be activated at the level of, or upstream of MEKK1.

On the basis of prior reports, this could be achieved by activation of Axin. However, we failed to detect stimulation of JNK/SAPK signaling by high levels of Axin. The result is contradictory to the previous observations, suggesting that activation of JNK/SAPK signaling is at the level upstream of Axin. Alternatively, a second signal parallel to the Wnt pathway may be required. We then examined if overexpression of Wnt is sufficient to stimulate JNK/SAPK signaling. In cells transfected by Wnt1, we detected similar results to those observed in the dnAxin-expressing cells (Figure 4*B*). High levels of Wnt1 failed to induce the JNK/SAPK activity, supporting that a second signal parallel to the Wnt pathway is necessary for the JNK/SAPK induction. Similar signaling effects were also detected in cells treated with LiCl to induce Wnt/β-catenin signaling (Figure 4*B*). Although these data imply our model of parallel signaling, we do not rule out the possibility that this second pathway is converged at other Wnt signaling molecules.

Induction of Squamous Differentiation by Wnt/β-catenin and JNK/SAPK Pathways

On the basis of the above discovery, we hypothesized that simultaneous stimulation of Wnt/β-catenin and stress signaling promotes squamous differentiation of epithelial cells. We therefore used a cell culture system, which is more amenable to reconstituting the molecular events. First, the C57MG mammary epithelial cell line was examined for the status of Wnt/β-catenin and JNK/SAPK signaling. The JNK/SAPK signaling activity was apparently detected in C57MG cells as PJun73 was detected in every single cell whereas PJun63 was positive in only a few cells (Figure 5, *A* and *B*). We noticed that phosphorylation at Ser63 seems to be more tightly

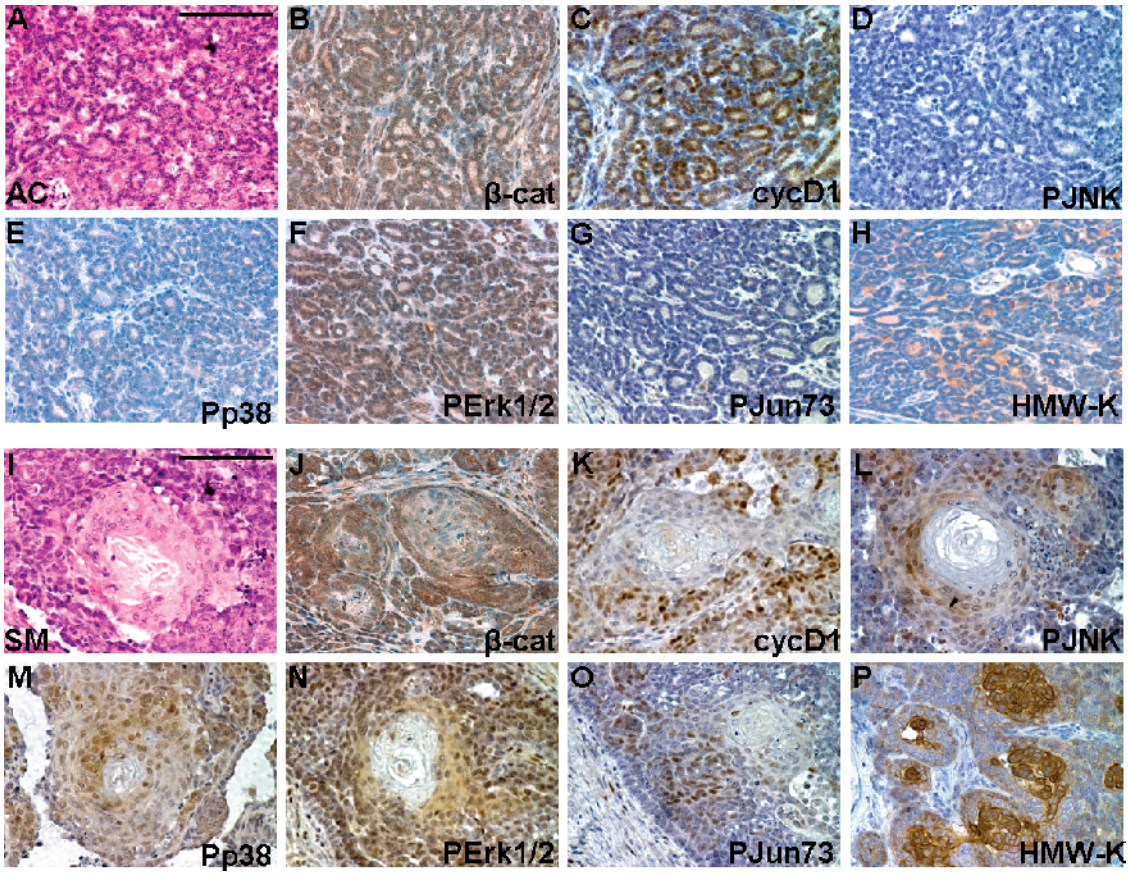

Figure 3. Stimulation of JNK/SAPK signaling is linked to the Wnt/β-catenin–induced squamous metaplasia in mice. Sections of the mammary tumors, adenocarcinoma (AC; A–H) and adenocarcinoma with squamous metaplasia (SM; I–P), induced by Wnt1 were analyzed by histology (A, I) and immunochemistry (B–H and J–P) for the expression of β-cat (B, J), cyclin D1 (cycD1; C, K), PJNK (D, L), Pp38 (E, M), and PErk1/2 (F, N), PJun73 (G, O), and high–molecule weight keratin (HMW-K; H, P). The immunochemistry (brown)–stained sections were counterstained with hematoxylin (blue). Wnt signaling is stimulated in both AC and SM because β-catenin is nuclearly localized and cyclin D1 is highly expressed in malignant cells (B–C and J–K). In contrast, JNK/SAPK signaling is exclusively elevated in SM (D–G and L–O). Scale bars, 100 μm (A–P).

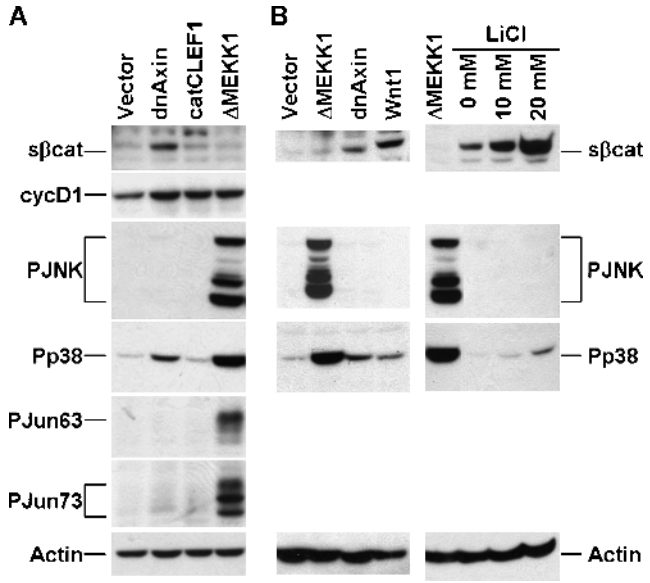

Figure 4. Stimulation of Wnt/β-catenin signaling has no direct effects on JNK/SAPK activation. (A) Expression of dnAxin or catCLEF1 stabilizes β-catenin and enhances the expression of its target cyclin D1 but has no significant effects on JNK/SAPK signaling. Phosphorylation of p38, JNK, and c-Jun at Ser63 and Ser73 is stimulated by a constitutively active form of MEKK1 (ΔMEKK1). (B) Expression of Wnt1 or stimulation of Wnt signaling by LiCl induces β-catenin and p38 but not JNK, similar to the effect caused by dnAxin. Immunoblot analyses of a stabilized form β-catenin (sβcat), cycD1, PJNK, Pp38, PJun63, and PJun73 were performed with lysates of 293T cells transfected with an empty vector, a plasmid expressing dnAxin, catCLEF1, or ΔMEKK1, or treated with LiCl. The expression of actin was analyzed as a loading control.

controlled, similar to those observed in mammary development (Figure 1) and hyperplasia (Figure 2). Wnt/β-catenin was not stimulated because there was very little, if any, accumulation of β-catenin found in these cells (Figure 5, *C* and *D*). In addition, the expression of a squamous cell marker HMW-K was not detectable (Figure 5*E*). The preexisting JNK/SAPK signaling activation at certain levels makes C57MG a good system to test our theory. We then examined the C57MGWnt1 cell line, a derivative of C57MG with constitutive expression of Wnt1 [27]. C57MGWnt1 cells expressed PJun63 and PJun73 at levels comparable to those in the parental cell line (Figure 5, *F* and *G*). Due to constitutive expression of Wnt1, strong nuclear accumulations of β-catenin were clearly exhibited in these cells (Figure 5, *H* and *I*). Therefore, in addition to JNK/SAPK, Wnt/β-catenin signaling is also elevated in C57MGWnt1 cells. A significant portion of C57MGWnt1 cells expressed HMW-K, a well-characterized feature of squamous differentiation (Figure 5*J*).

To further confirm that squamous differentiation is promoted by a synergistic activation of both pathways, we transiently expressed catCLEF1 and ΔMEKK1 to induce Wnt/β-catenin and JNK/SAPK signaling, respectively. The catCLEF1 protein constitutively induces transcription of the Wnt downstream genes [28] whereas ΔMEKK1 constitutively activates JNK/SAPK signaling [29]. High levels of HMW-K were detected in the C57MG cells expressing catCLEF1 and ΔMEKK1, indicating squamous differentiation (Figure 5, *K* and *N*). Expression of either ΔMEKK1 (Figure 5*L*) or catCLEF1 (Figure 5*M*) slightly enhances the expression of HMW-K. The Wnt and JNK/SAPK pathways act synergistically to induce squamous differentiation. Next, we examine if inhibition of JNK signaling prevents squamous differentiation. The addition of JNK inhibitor had no significant effect on the expression of HMW-K in C57MGWnt1 cells (Figure 5, *O* and *P*), suggesting that activation of JNK/SAPK is not the limiting factor. It is conceivable that squamous differentiation is caused by the activation of signaling events downstream of JNK/SAPK in these cells. However, the induction of HMW-K by coexpression of catCLEF1 and ΔMEKK1 is prevented by JNK inhibitor in C57MG cells (Figure 5, *Q* and *R*). These data suggest a crucial role of the JNK/SAPK pathway in squamous metaplasia of the Wnt-induced malignancy.

Activation of JNK/SAPK and Wnt/β-Catenin in Human Endometrial and Lung Cancers

Squamous metaplasia rarely develops in human breast cancers but is often found in other types of tumors including endometrial and lung cancers. Activation of Wnt/β-catenin signaling has been linked in the development of these cancers [43–48]. Therefore, we tested if our finding has a general implication in development of human cancers. We first screened samples of human endometrioid adenocarcinoma with squamous metaplasia (Figure 6*A*), expressing HMW-K (Figure 6*B*), for the activation of Wnt/β-catenin signaling. Immunostaining of β-catenin and its transcriptional target cyclin D1 was performed to determine if the canonical Wnt pathway was stimulated. We found a 100% correlation of nuclear accumulations of β-catenin with cyclin D1 in tumors with SM (Figure 6, *C* and *D*; $n = 8$). Importantly, PJNK, Pp38, PErk1/2, PJun63, and PJun73 were strongly expressed only in the cells undergoing squamous differentiation (Figure 6, *E–I*; $n = 3$). Activation of JNK/SAPK and p38 coincided with the expression of the squamous cell marker HMW-K (Figure 6*B*; $n = 3$). No expression of these proteins was detected in AC ($n = 7$; data not shown). We next tested our hypothesis using human lung cancer samples. Squamous cell carcinoma of the lung expressed high levels of HMW-K (Figure 6, *J* and *K*). The accumulation of β-catenin was again correlated perfectly with the expression of cyclin D1 (Figure 6, *L* and *M*). High levels of PJNK, Pp38, PErk1/2, PJun63, and PJun73 were also observed in the malignant squamous cells (Figure 6, *N–R*; $n = 7$). On the basis of these observations, it is possible that stimulation of JNK/SAPK signaling might play a role in the Wnt-mediated squamous neoplasm of human cancers.

Discussion

The present study shows that stress signaling is stimulated during normal development as well as neoplastic transformation of the mammary gland. The JNK/SAPK and p38 MAPK cascades are strongly activated in the lobulo-alveolar cells during pregnancy-mediated mammary development. This developmental process is highly dependent on the canonical Wnt pathway as we and others demonstrated previously [12,17,18]. As an alternative pathway of Wnt, JNK/SAPK might coordinate with the canonical pathway to modulate alveolar morphogenesis. Whereas β-catenin signaling is known to expand the cell population, a second pathway might be important for the differentiation process. It has been well recognized that activation of the canonical Wnt pathway is tightly associated with the malignant squamous phenotype [10,13,39,49]. However, the molecular mechanism underlying the squamous metaplasia mediated by high levels of Wnt/β-catenin signaling remains elusive. We have identified JNK/SAPK as a candidate pathway for the secondary signaling cascade

involved in squamous differentiation. In the Wnt-induced tumorigenesis, JNK/SAPK signaling is silent in hyperplasia and adenocarcinoma but is highly elevated in cells undergoing squamous differentiation. Reconstitution of these molecular events by synergistic activation of β-catenin and JNK/SAPK signaling promotes squamous differentiation in C57MG mammary epithelial cells, suggesting a co-opted role of the JNK/SAPK signaling cascade in Wnt-mediated oncogenic transformation.

We have extended our findings into the biology of human cancers by showing that coordinated stimulation of these two pathways is correlated with development of the malignant squamous cell type in human endometrial and lung cancers. In addition, the JNK/SAPK signaling molecules may serve as biomarkers for identifying the malignant squamous cell types in human cancers. This is of important clinical significance with the advancement in modern cancer therapy. In the past, it was sufficient to divide human lung cancers into small cell and non–small cell types with the latter comprised mostly of adenocarcinoma and squamous carcinoma. That was the era when the therapeutic choice for small cell carcinoma was chemoradiation, whereas adenocarcinoma and squamous carcinoma were similarly treated with surgery. A new angiogenesis inhibitor, Avastin, is effective only for adenocarcinoma of the lung but has the potential

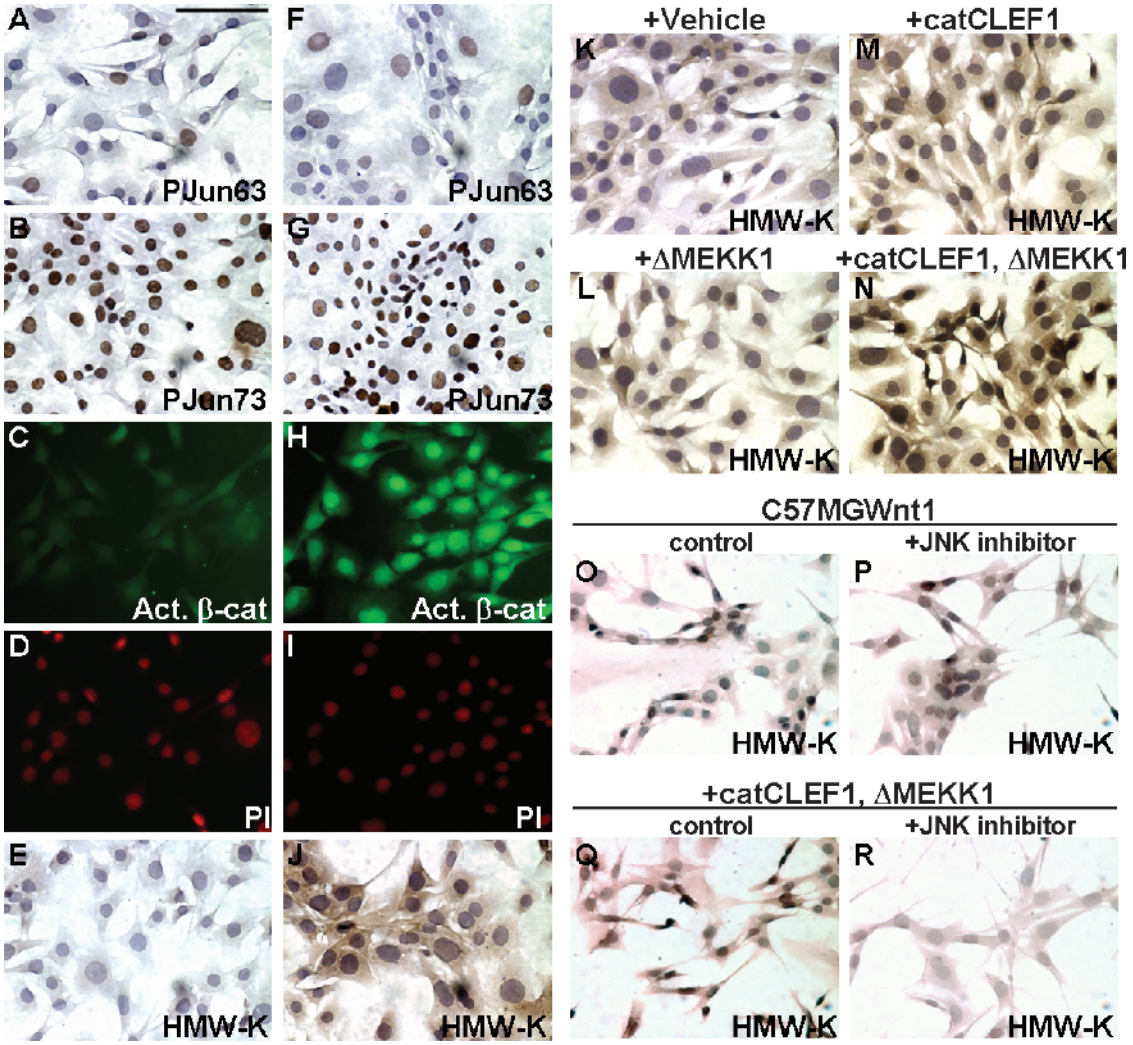

Figure 5. Squamous cell differentiation is promoted by synergistic activation of Wnt/β-catenin and JNK/SAPK. Mammary epithelial cells C57MG (A–E) and C57MGWnt1 (F–J), a derivative of C57MG constitutively expressing Wnt1, were immunochemically stained with specific antibodies for the expression of PJun63 (A, F), PJun73 (B, G), and HMW-K (E, J). The immunochemistry (brown)–stained cells were counterstained with hematoxylin (blue). Immunostaining of C57MG and C57MGWnt1 cells with an antibody recognizing only activated β-catenin (Act. β-cat; C, H) was counterstained by propidium iodide (D, I). Note that the C57MG cell line contains certain levels of JNK/SAPK activity, leading to the phosphorylation of c-Jun at Ser63 and Ser73 (A–B and F–G). However, no squamous differentiation was evident because HMW-K is negative (E). Constitutive expression of Wnt1 induced β-catenin signaling as it accumulated in the nucleus (H), which, together with the existing JNK/SAPK signaling, promotes squamous differentiation (J). Synergistic activation of Wnt/β-catenin and JNK/SAPK signaling by transient expression of a β-catenin-LEF1 fusion protein (catC-Lef1) and a dominant MEKK1 (ΔMEKK1), respectively, strongly promotes squamous differentiation in mammary epithelial cells (N). The control transfected with vehicle only showed no expression of squamous cell marker HMW-K (K). Expression of either ΔMEKK1 (L) or catC-Lef1 (M) slightly enhances the HMW-K expression. (O, P) The presence of JNK inhibitor has no effect on the expression of HMW-K in C57MGWnt1 cells. In C57MG cells, the induction of HMW-K by transfection of catC-Lef1 and ΔMEKK1 is prohibited by JNK inhibitor (Q, R). Scale bar, 100 μm (A–R).

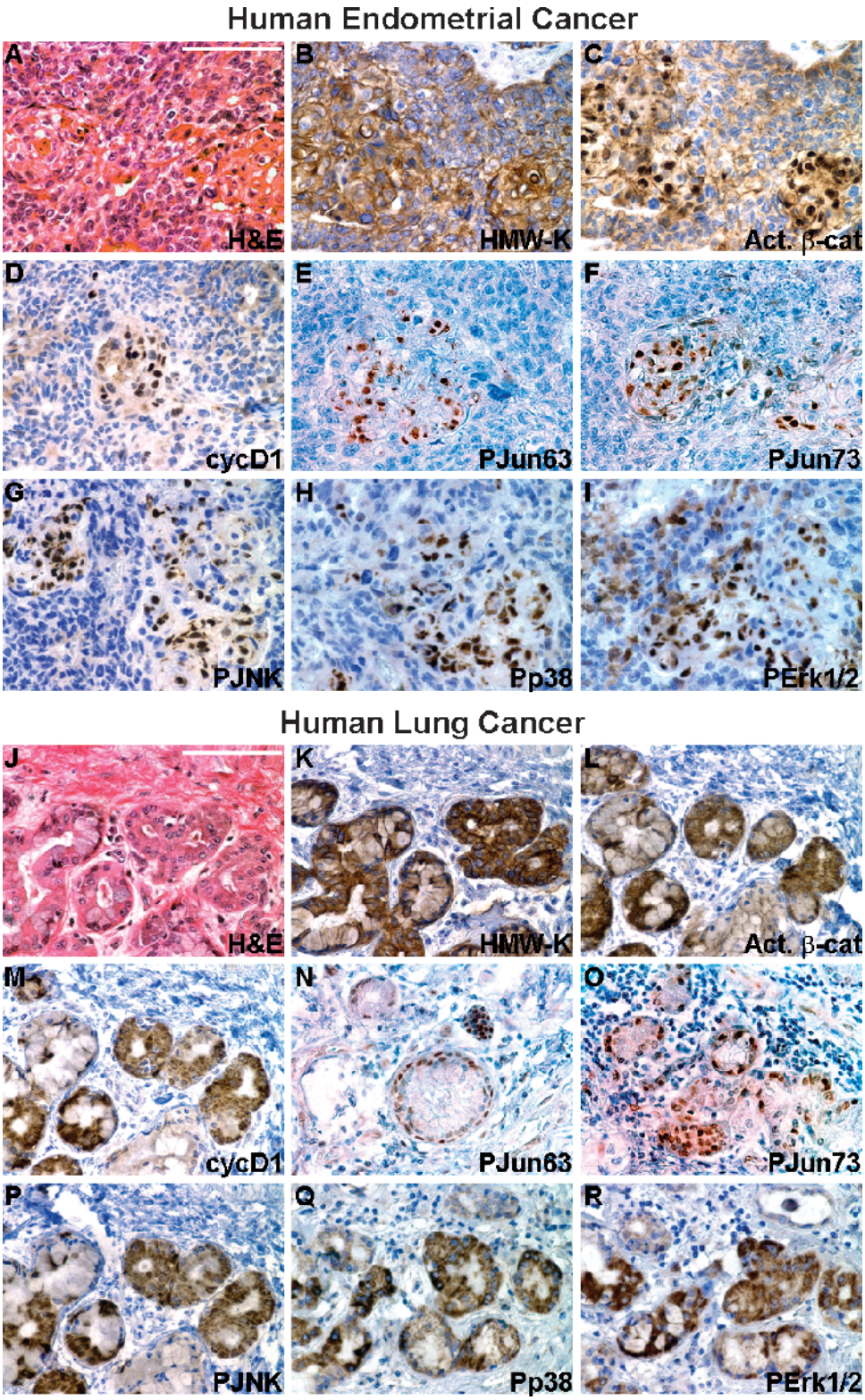

Figure 6. Co-opted JNK/SAPK signaling in the Wnt/β-catenin–mediated malignant transformation of human cancers. Sections of human endometrial cancer exhibiting adenocarcinoma with squamous metaplasia (A–I) and human lung squamous cell carcinoma (J–R) were analyzed by histologic diagnosis (A, J) and immunochemistry (B–I and K–R) for the expression of HMW-K (B, K), activated β-catenin (Act. β-cat; C, L), cycD1 (D, M), PJun63 (E, N), PJun73 (F, O), PJNK (G, P), Pp38 (H, Q), and PErk1/2 (I, R). The immunochemistry (brown)–stained sections were counterstained with hematoxylin (blue). Scale bars, 100 μm (A–R).

to cause fetal pulmonary hemorrhage in patients with squamous cell carcinoma. Since the advent of this drug, it has become critical to separate these two tumor types. However, adenocarcinoma and squamous cell carcinoma, sharing similar histologic features, can be difficult to distinguish when they are poorly differentiated. It would be interesting to determine whether the JNK/SAPK signaling molecules can be used as additional markers for differential diagnosis. The study may also shed light on the molecular pathogenesis of these two tumor types in lung.

A previous report indicated that Wnt5a up-regulated in human oral cancers is a potential marker of the malignant phenotype of squamous cell carcinoma [50]. Because Wnt5a is a member of noncanonical Wnt, the data support the notion of co-opted JNK/SAPK signaling in Wnt-induced tumorigenesis. Activation of the JNK/SAPK pathway was shown to mediate the expression of a squamous cell marker SPRR1B in respiratory injuries caused by environmental pollutants [51,52]. In a rat model of tobacco smoke–induced cell proliferation and squamous metaplasia, the JNK/SAPK signaling cascade and cyclin D1 expression were also stimulated [53]. Indeed, Wnt/β-catenin signaling was shown to be activated in cigarette smoke–induced lung tumorigenesis, further supporting our hypothesis [54]. In addition, targeted disruption of Wnt7b in mice caused lung hypoplasia due to defects in mesenchymal proliferation [55]. Wnt/β-catenin signaling is also required for branching morphogenesis and cell type specification of embryonic lung development [56–58]. It has been suggested that Wnt/JNK signaling could diverge from the canonical pathway at the level of Axin, which binds to MEKK1 directly [23]. However, we failed to detect stimulation of PJNK, PJun63, and PJun73 by Axin or dnAxin that contains all the necessary domains required to activate JNK/SAPK as reported previously [23]. Recent reports showed that ectopic expression of Axin1 modulates the JNK/SAPK activity affecting embryonic dorsoventral patterning [59] and that the Axin-dependent JNK/SAPK activation might contribute to the dominant-negative phenotypes of the $Axin^{Fu}$ allele [60]. Nevertheless, there is still a lack of *in vivo* evidence to support the notion that Axin is essential for JNK/SAPK activation. We also did not find any alterations of the JNK/SAPK pathway in the Axin2-null mutants during skull morphogenesis (Liu and Hsu, unpublished observations). Whether the loss of JNK/SAPK activation contributed to the phenotypic defects caused by the disruption of Axin1 [40] or Axin2 [33] remains obscure. The present study suggests that a stress-activated pathway parallel to Wnt/β-catenin is necessary for the stimulation of JNK/SAPK signaling in squamous metaplasia. However, we are not able to rule out the possibility that this signaling converged at some point with the Wnt pathway, or modulated its signaling, to induce squamous differentiation. The signaling event might be modulated at the level upstream of Axin, e.g., Dsh/Dvl [25,61]. Diversin, shown to function as a molecular switch to suppress Wnt/β-catenin while promoting Wnt/JNK, is another candidate [62]. Future investigation focusing on the cooperation of the Wnt/β-catenin and Wnt/JNK signaling cascades promises important insight into the mechanism underlying normal development and pathogenesis of human diseases.

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