Oncogene (2007) 26, 4571–4579

www.nature.com/onc

ORIGINAL ARTICLE

A hidden oncogenic positive feedback loop caused by crosstalk between Wnt and ERK Pathways

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The Wnt and the extracellular signal regulated-kinase (ERK) pathways are both involved in the pathogenesis of various kinds of cancers. Recently, the existence of crosstalk between Wnt and ERK pathways was reported. Gathering all reported results, we have discovered a positive feedback loop embedded in the crosstalk between the Wnt and ERK pathways. We have developed a plausible model that represents the role of this hidden positive feedback loop in the Wnt/ERK pathway crosstalk based on the integration of experimental reports and employing established basic mathematical models of each pathway. Our analysis shows that the positive feedback loop can generate bistability in both the Wnt and ERK signaling pathways, and this prediction was further validated by experiments. In particular, using the commonly accepted assumption that mutations in signaling proteins contribute to cancerogenesis, we have found two conditions through which mutations could evoke an irreversible response leading to a sustained activation of both pathways. One condition is enhanced production of β-catenin, the other is a reduction of the velocity of MAP kinase phosphatase(s). This enables that high activities of Wnt and ERK pathways are maintained even without a persistent extracellular signal. Thus, our study adds a novel aspect to the molecular mechanisms of carcinogenesis by showing that mutational changes in individual proteins can cause fundamental functional changes well beyond the pathway they function in by a positive feedback loop embedded in crosstalk. Thus, crosstalk between signaling pathways provides a vehicle through which mutations of individual components can affect properties of the system at a larger scale.
Oncogene (2007) **26,** 4571–4579; doi:10.1038/sj.onc.1210230; published online 22 January 2007

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Received 3 March 2006; revised 1 November 2006; accepted 22 November 2006; published online 22 January 2007

Keywords: signaling pathways; crosstalk; positive feedback loop; Wnt pathway; ERK pathway; colorectal cancer

Introduction

In general, normal cells maintain a homeostatic balance between proliferation and apoptosis by properly responding to extracellular signaling molecules (e.g. growth factors and hormones). This balance is broken down in cancer cells, which can sustain proliferation and escape apoptosis in the absence of persistent extracellular stimulation. The molecular basis for this dysregulation usually is due to mutations in signaling proteins, which disable repressors and constitutively activate enhancers of cell proliferation and survival, thereby allowing cancers to grow unrestricted and largely independent of external cues.

The Wnt signaling pathway is one of the well-known oncogenic pathways, which is of particular importance for colorectal cancer. It is named after the Wnt family proteins, which are a family of glycoprotein growth factors that control cellular growth, differentiation and tumorigenesis. In normal cells Wnt proteins stimulate adequate cellular proliferation through upregulation of the β-catenin/TCF (T-cell factor) complex which functions as nuclear transcription factor. Physiologically, the Wnt pathway is especially important for the regeneration of the intestinal epithelium by controlling the proliferation of the intestinal stem cells at the bottom of crypt (Sancho *et al*., 2003; Reya and Clevers, 2005). The pathway is normally controlled by regulation of the β-catenin protein levels (Behrens and Lustig, 2004; Sancho *et al*., 2004).

A protein complex comprising axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK-3β) proteins binds β-catenin to promote phosphorylation of β-catenin by GSK-3β, which leads to the degradation of β-catenin. Wnt induces the inhibition of GSK-3β, thereby elevating β-catenin levels and accumulation of β-catenin in the nucleus, where it associates with TCF to form a transcription factor that can drive proliferation and – when constitutively active – tumourigenesis (Behrens, 2005). While the physiological Wnt

signal is reversible colorectal cancer cells commonly have a constitutively high β-catenin level caused by inactivating mutations in APC or less frequently by mutations of the GSK-3β phosphorylation sites in β-catenin, leading to aberrant accumulation of β-catenin and constitutive formation of the β-catenin/TCF transcription factor.

The ERK pathway is also being recognized as a major cellular proliferation signaling pathway that physiologically mediates proliferation in response to extracellular mitogens and growth factors. However, this pathway is found constitutively activated in approximately 30% of all human cancers including colorectal cancer (Kolch *et al*., 2002; Fang and Richardson, 2005). Physiologically the pathway is activated by cell surface receptors, whereby the activation through the epidermal growth factor receptor (EGFR) is most thoroughly understood (Kolch, 2005). The activated EGFR recruits the Grb2/SOS protein complex to the cell membrane, where it activates Ras proteins. Active Ras initiates the activation of Raf kinases, which phosphorylate and activate MEK, which in turn phosphorylates and activate ERK, thus mediating the signal flow through the Raf-1$\rightarrow$MEK$\rightarrow$ERK cascade. Double phosphorylated ERK (ERKpp) can translocate into the nucleus to phosphorylate other signaling molecules and transcription factors that participate in regulation of proliferation. Like the Wnt pathway, the ERK pathway is also known to play a role in colorectal cancer since mutations in Ras and B-Raf are found in ca. 50 and 15% of colorectal cancers, respectively (Downward, 2003).

Recently, several researchers have identified crosstalk between the Wnt and ERK pathways. The experimental evidence is as follows: (1) Wnt signal activates Ras (Yun *et al*., 2005); (2) β-catenin/TCF activates Raf-1 through a yet unknown mechanism, here tentatively summarized as activator molecule X (Rottinger *et al*., 2004; Yun *et al*., 2005); (3) ERKpp phosphorylates GSK-3β and primes it for inhibition (Almeida *et al*., 2005; Ding *et al*., 2005). By integrating these experimental evidences into a pathway model (Figure 1), we infer that a hidden positive feedback loop exists between the Wnt and ERK signaling pathways (Figure 2). This positive feedback may have its own regulatory role in the Wnt/ERK pathways that was not explicitly realized by the previous findings. In fact, several kinds of genetic mutations were observed in humans or animal models, and we consider that most of those mutations can play a role in enhancing the Wnt/ERK positive feedback (see Table 1 for summary). This suggests that the positive feedback in the Wnt/ERK pathway might causally participate in the pathogenesis of colorectal cancer. In this paper, we analyse the role of this positive feedback and show that it might crucially contribute to cellular transformation.

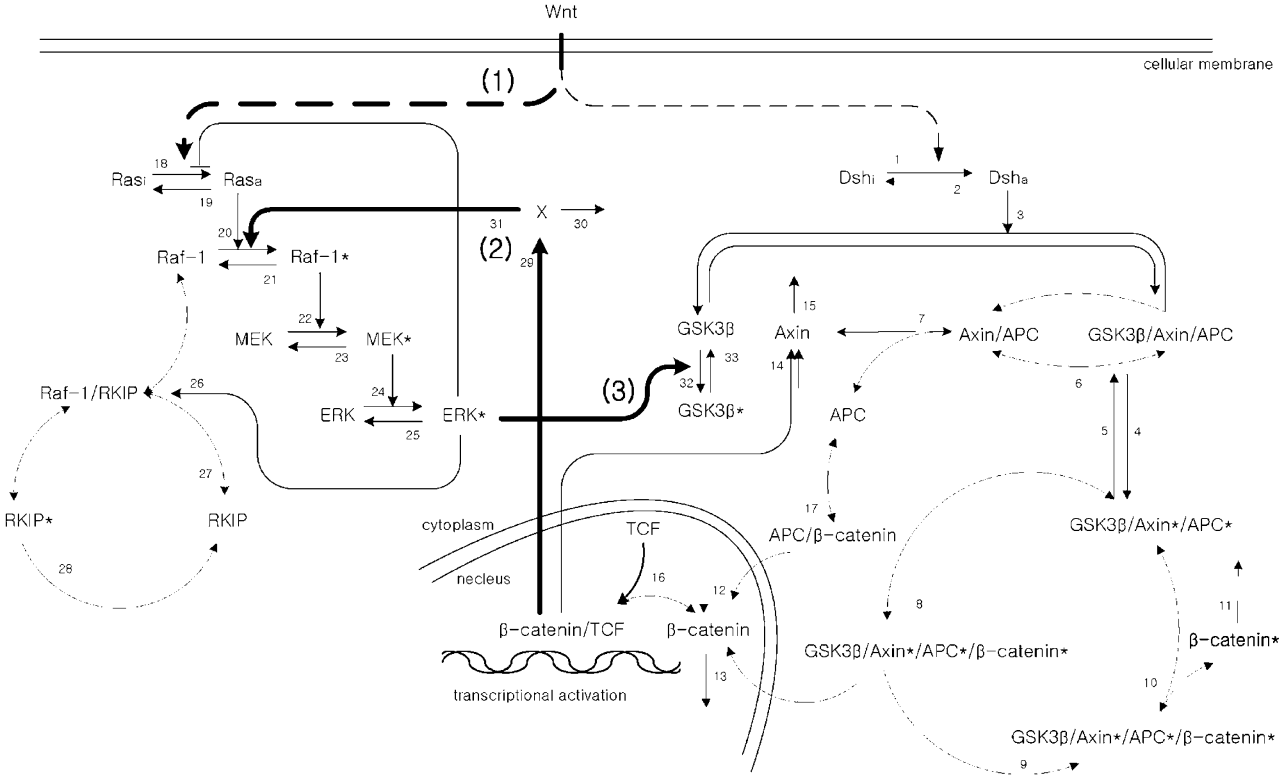

Figure 1 A diagram showing the Wnt and ERK pathways. Interactions between the pathways are indicated by bold arrows: (1) denotes the activation of Ras by the Wnt signal; (2) indicates the activation of Raf-1 by the unknown molecule X which is upregulated by the transcriptional activation of β-catenin/TCF; and (3) denotes the phosphorylation and subsequent inhibition of GSK3β by the activated ERK. Note that (2) and (3) form a positive feedback loop between the Wnt and ERK pathways. (X*, Xi and Xa indicate the phosphorylated state, the inactivated state and the activated state, respectively.)

Results

Effect of the Wnt/ERK crosstalks on β-catenin/TCF levels and ERK activation: comparison of dynamics according to the variation of effective parameters and its experimental validation

The existing models did not consider any crosstalk between the ERK and the Wnt pathways, and hence they were regarded as independent of each other. However, by including crosstalk into the pathway topologies, it becomes possible to describe the effects of activated ERK on the regulation of β-catenin/TCF levels as well as the effects of upregulated β-catenin/TCF on ERK activity. There are reports that crosstalk appears when the Wnt and ERK signaling pathways are activated (Ding *et al*., 2005; Yun *et al*., 2005). From these reports it seems plausible that the crosstalk also may influence the dynamic behavior of these pathways. Therefore, we designed a mathematical model of the ERK and Wnt pathways as described in the Materials and methods section, and used this model to simulate the effects of constitutive activation of the ERK or Wnt pathways. Levels of ERKpp and the β-catenin/TCF transcription factor were used as outputs of the respective pathways. Constitutive activation was mimicked by (a) assuming an enhanced expression of β-catenin, (b) using inhibitors of ERK phosphatases (okadaic acid; OA), (c) inhibitors of GSK-3β (SB216763; SB) or (d) a combination of OA + SB. These conditions functionally correspond to the constitutive activation of the Wnt pathway (conditions (a) and (c)), ERK pathway (condition (b)) or both (condition (d)). As these conditions are usually achieved by mutations we refer to them as 'mutated systems' in the computational simulations. Simulating these four 'mutated' conditions (Figure 3), we found that the positive feedback loop formed by the crosstalks can induce a state transition from an inactive state to an activated state. Importantly, all these conditions do not only switch both the Wnt and the ERK pathways into a constitutively activated state, but also maintain it. Not unexpected, the direct overexpression of β-catenin had the biggest effect on β-catenin/TCF levels, while indirect perturbations that induce β-catenin stabilization still switched the behavior, but with comparatively longer delays and smaller effects. ERKpp levels were switched on by all conditions to similar extents, with the fastest kinetics provided by the simultaneous inhibition of GSK-3β and ERK phosphatases.

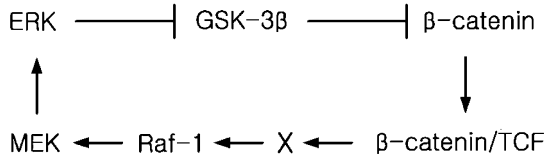

Figure 2 A diagram summarizing the hidden positive feedback mechanism between the Wnt and ERK pathways. Arrows denote activation and the blunted line indicates inhibition. The activated ERK induces GSK-3β inhibition, which stops β-catenin degradation leading to its accumulation. The upregulated β-catenin translocates into the nucleus and forms a complex with TCF. The β-catenin/TCF complex enhances the expression or activation of the unknown crosstalk molecule X. Then, X activates ERK via stimulation of Raf-1 and MEK.

In order to validate these simulation results, we tested salient predictions by biochemical experiments in cells. While no specific pharmacological inhibitors for MAP kinase phosphatases (MKPs) are currently available, the tumor promoter OA increases ERK phosphorylation by inhibiting protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2A) which are considered major ERK phosphatases and MEK phosphatases (Amaral *et al*., 1993; Casillas *et al*., 1993). OA was administered in increasing doses for 240 min, which is the predicted time point around which both ERK and β-catenin signaling should switch into the constitutively activated stated when ERK phosphatase activity or β-catenin levels are altered (Figure 3). The results are shown in Figure 4. OA readily increased the endogenous ERK phosphorylation at already a low dose (1 nM) while the ERK phosphorylation did not increase any more for higher dose (e.g., 100 nM) of OA. This is because once the system has undergone a state transition triggered by the low does of OA (1 nM) then any further stimulation cannot cause a further change (Figure 4). Similarly, the transfection of myc-tagged β-catenin increased total β-catenin protein levels and induced robust ERK phosphorylation validating the prediction that elevation of β-catenin levels lead to ERK activation and constitutive accumulation of ERKpp. The OA induced increase in ERK phosphorylation was completely abolished by treatment with the MEK inhibitor U0126 (UO) showing that ERK activation by phosphatase inhibition requires the MEK pathway. The GSK-3β inhibitor SB216763 (SB) also induced a weak but reproducible phosphorylation of ERK, which was blocked by UO consistent with the prediction that activation of β-catenin signaling by GSK-3β inhibition causes activation of ERK using the classical pathway via MEK. Under all conditions, SB administration increased the levels of endogenous β-catenin protein expression, as also predicted by the mathematical model. Furthermore, OA and SB induced

Table 1 Mutations found in colorectal cancer

Conditions	*Role*	*References*
APC mutation	Increase β-catenin levels	Harada *et al*. (1999)
β-Catenin mutation	Increase signaling through the Tcf/Lef factors	Mirabelli-Primdahl *et al*. (1999)
PPP2RB1 gene mutation	Decrease the PP2A (protein phosphatase 2A) activity	Takagi *et al*. (2000)
β-Catenin overexpression	Increase β-catenin levels	Kim *et al*. (2005)
B-Raf mutation	Increase the activity of the ERK pathway	Aust *et al*. (2005)

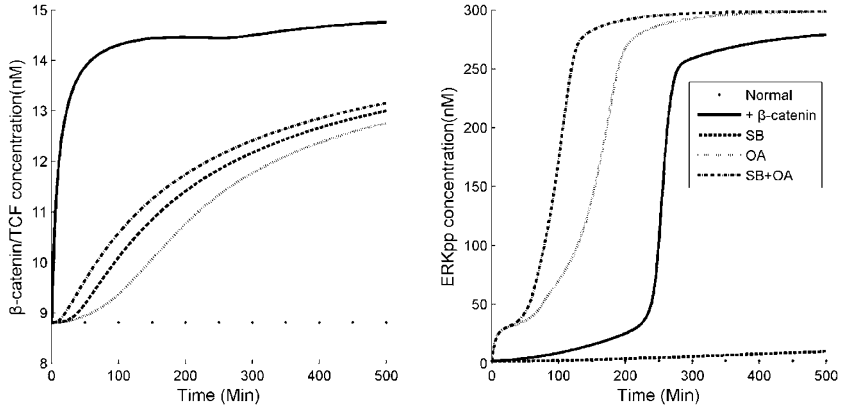

Figure 3 The effect of parameter perturbations on β-catenin and ERKpp levels. Simulation of β-catenin/TCF and ERKpp levels under normal and mutated conditions. 'Normal': normal system. '$+\beta$-catenin': 20 times higher β-catenin synthetic rate (v12) than the normal system. 'SB': 1/100 of the GSK-3β phosphatase reaction velocity (V_{max7}) than the normal system. 'OA': half of the MEK phosphatase reaction velocity (V_{max4}) and ERK phosphatase reaction velocity (V_{max5}) than the normal system. 'OA + SB': 'OA' and 'SB' at the same time.

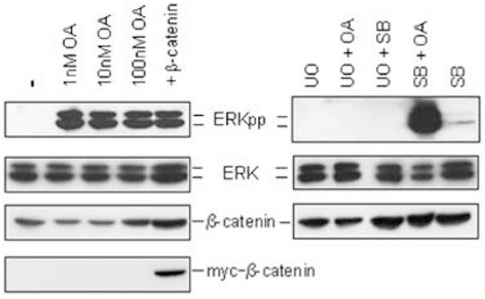

Figure 4 The effect of pharmacological perturbations on β-catenin and ERKpp levels. Cells were treated with drugs as indicated for 4 h before harvest and analysed by Western blotting with the indicated antibodies. In one lane wild-type myc-tagged β-catenin was transfected and detected with an anti-myc-tag antibody. OA, okadaic acid (phosphatase inhibitor); UO, U0126 (MEK inhibitor); SB, SB 216763 (GSK-3β inhibitor).

a very high level ERK phosphorylation showing that the combined inhibition of PP2A and stabilization of β-catenin acted synergistically to activate ERK. Again this is qualitatively consistent with the mathematical prediction (Figures 3 and 4).

In order to investigate the effects of these perturbations on β-catenin signaling in more detail, we measured the transcriptional output of β-catenin signaling. For this purpose, we used TOPFLASH and FOPFLASH reporter constructs, which contain wild-type or mutated (as control) promoter-binding sites for the TCF/β-catenin transcription factor, respectively (Figure 5). OA induced the transcriptional activation by TCF/β-catenin at 1–100 nM dose in a β-catenin-dependent manner (Figure 5a). It has to be noted that the transcriptional activations may not seem very big, but are well in the range of biologically significant effects when using standard TOPFLASH reporters (Kim *et al*., 2000; Esufali and Bapat, 2004). Taken together, these results suggest that the inhibition of ERK/MEK phosphatases and elevation of β-catenin levels cooperate consistent with the predictions shown in Figure 3. The sensitivity of the system to β-catenin levels was further

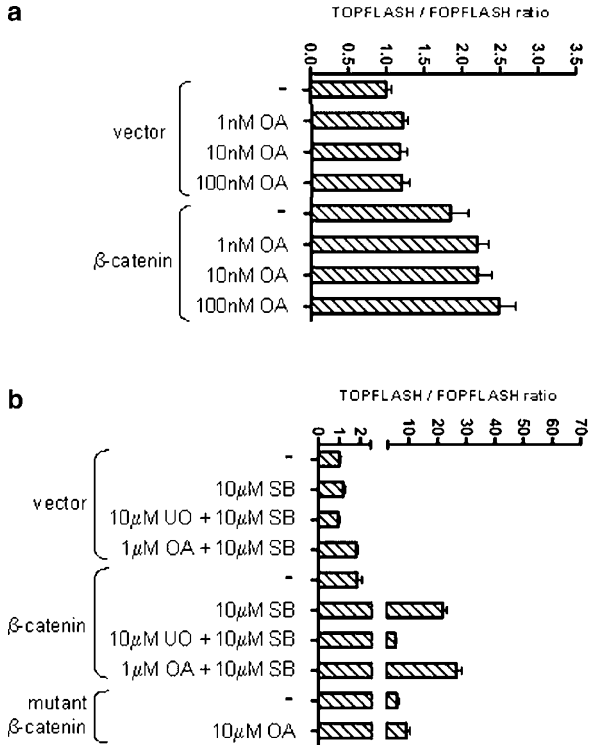

Figure 5 TOPFLASH /FOPFLASH reporter gene assay for β-catenin transcriptional activity. Cells were transfected with reporter gene constructs and assayed as described in Materials and methods. (**a**) Effects of phosphatase (OA) and MEK (UO) inhibition. (**b**) Effects of GSK-3β inhibition (SB) in conjunction with phosphatase (OA) and MEK (UO) inhibition. OA, okadaic acid (phosphatase inhibitor); UO, U0126 (MEK inhibitor); SB, SB 216763 (GSK-3β inhibitor).

explored using perturbation experiments in cells transfected with β-catenin (Figure 5b). In empty vector transfected cells the inhibitors had only very small effects: the GSK-3β inhibitor SB slightly enhanced TCF/β-catenin induced transcription, while the MEK inhibitor UO slightly reduced it. OA plus SB showed a small but significant further enhancement. Transfection of

β-catenin resulted in a similar approximately twofold increase of reporter gene transcription. The inhibitors now had strong effects when β-catenin was transfected, suggesting that the availability of β-catenin is a limiting factor for transcriptional output, and that the cooperation between the ERK and Wnt pathways is dependent on β-catenin levels. SB induced a very robust activation of the transcriptional output, which was reduced by UO revealing that ERK activity contributes to TCF/β-catenin-mediated transcription. OA only slightly enhanced the SB-stimulated transcription. This indicates that ERK activation contributes to GSK-3β inhibition, and hence has little further effect when GSK-3β is already inhibited pharmacologically by SB. These results are also consistent with the biochemical data shown in Figure 4. The direct inhibition of GSK-3β by SB increases β-catenin levels under all conditions as assayed by Western blotting (Figure 4) and also increases the transcriptional output (Figure 5b). ERK phosphorylation and transcriptional activity diverge, as the combination of OA and SB only very slightly increased transcriptional output, while resulting in a dramatic increase in ERK phosphorylation. This is due to the different endpoints of measurement. The massive induction of ERK phosphorylation by OA is observable by Western blotting (Figure 4), but its contribution to transcription induced by GSK-3β inhibition is abrogated when GSK-3β is inhibited directly by SB. We also transfected a mutant β-catenin, where the GSK-3β phosphorylation sites are eliminated resulting in the stabilization of the protein (Aberle *et al*., 1997). As expected, this β-catenin mutant robustly activated transcription. Importantly, mutantβ-catenin was only minimally responsive to ERK activation by OA showing that ERK exerts its effect on the Wnt pathway by inhibiting GSK-3β mediated β-catenin phosphorylation. Thus, the results of the interference experiments shown in Figures 4 and 5 are entirely consistent with the predictions of the mathematical model shown in Figure 3.

Synergistic effect of Wnt signaling with mutations

Normally a signaling pathway system is only activated in response to an appropriate input signal, typically an extracellular growth factor, and this activation declines when the signal is removed in order to maintain the homeostasis in cellular systems. This also holds true in our model for the Wnt and ERK pathways. The pathway outputs, that is the upregulated β-catenin/TCF complex and the ERKpp, return to their basal levels after the Wnt signal is removed ($W=0$) (Figure 6, Normal). However, if the β-catenin synthetic rate is elevated above normal (twofold of normal in the simulation) or if the MEK/ERK phosphatase activities are reduced (three-fourths of normal in the simulation), the pathway outputs show completely different behavior compared to the normal system although the 'mutations' are much smaller than those in Figure 3. When $W=0$, such minor mutations maintain the inactivated state of the signaling system (0–500 min). However, once β-catenin/TCF and ERKpp are upregulated for the Wnt signal $W=1$ (500–1000 min), the system does not return to its inactivate state even after the Wnt signal disappears (1000–1500 min) (Figure 6). This implies that the system has changed its steady state to activated, and that the changed state is maintained even after the external stimulation (i.e., the Wnt signal) has ceased.

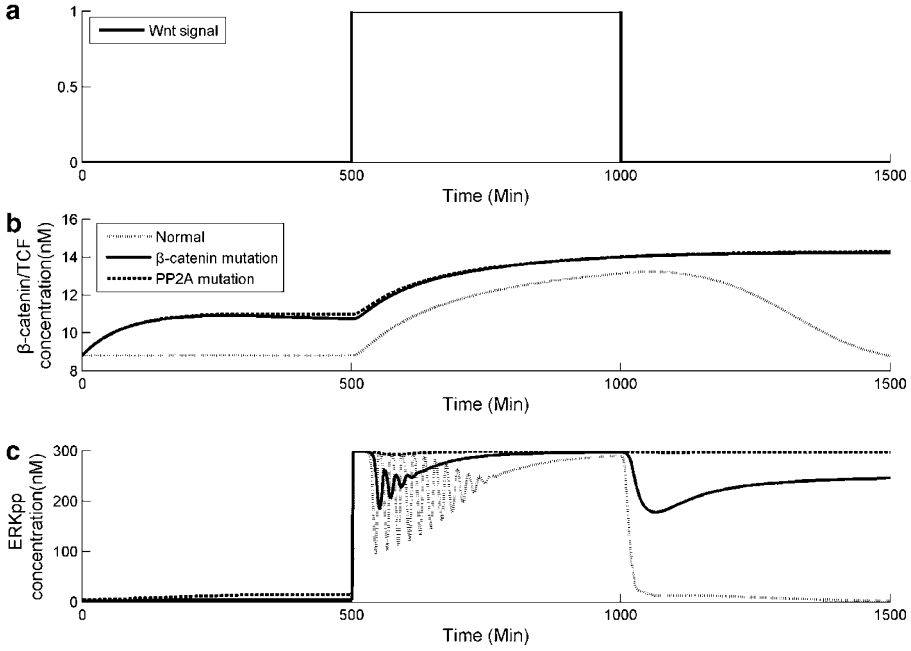

Figure 6 The simulation results under conditions of normal and mutated Wnt signaling. (**a**) Duration of the Wnt signal used for the simulation. (**b**) β-catenin/TCF and (**c**) ERKpp concentrations in each system. 'Normal' denotes the normal system, 'PP2A mutation' denotes three-fourths of the PP2A activity than the normal system, and 'β-catenin mutation' denotes twofold of the β-catenin synthetic rate than the normal system.

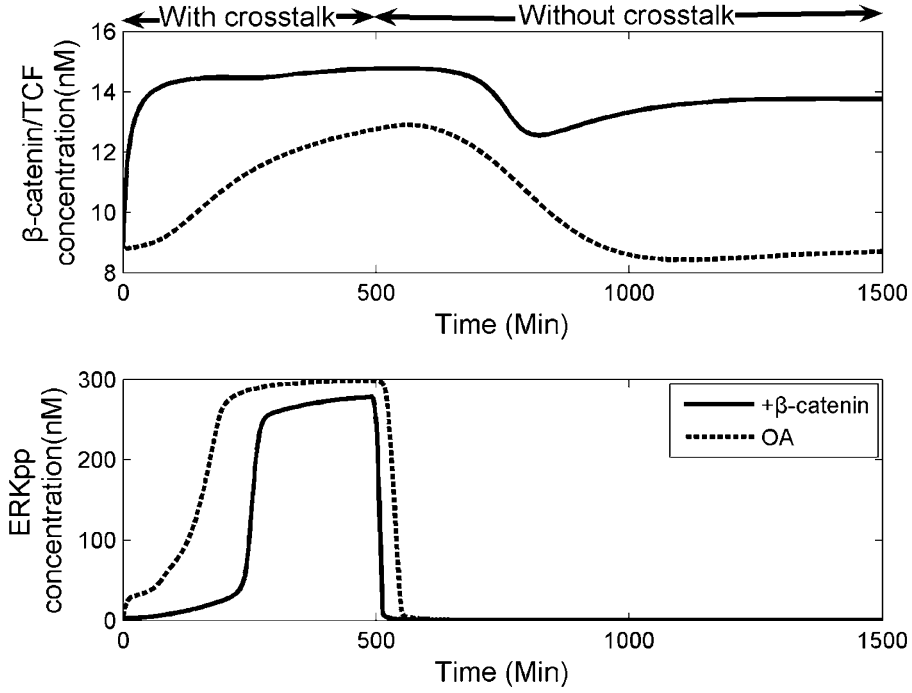

Figure 7 Comparison of the Wnt and ERK signaling dynamics with and without crosstalk. '$+\beta$-catenin': twenty times of the β-catenin synthetic rate (v12) than the normal system. 'OA': half of the MEK phosphatase activity (V_{max4}) and ERK phosphatase activity (V_{max5}) than the normal system. 'With crosstalk': the normal synthetic rate of the unknown molecule X (0–500 min). 'Without crosstalk': zero synthetic rate of the unknown molecule X (500–1500 min).

As the positive feedback makes the system bistable, the up-regulated crosstalk molecule X produced by β-catenin/TCF and activated ERKpp seems to sustain this highly activated status. The threshold of the state transition is set by the level of molecule X. This level is determined by the crosstalk and also indirectly influenced by the level of RKIP (Raf-1 kinase inhibitor protein). By inhibiting MEK activation (Yeung *et al*., 1999), RKIP also can function as a suppressor of the Wnt/ERK positive feedback. If the molecule X is not synthesized by β-catenin/TCF, the state transition cannot occur, and even will return to the inactivated state due to the breakdown of the Wnt/ERK positive feedback (Figure 7). On the other hand, if the level of RKIP is decreased then the threshold for the Wnt and ERK pathways to change their steady states is lowered, although this is less effective than molecule X (data not shown). In summary, our data suggest that the positive feedback between the Wnt and ERK pathways induces a state transition from an inactive to a constitutively activated state that may provide a chronic stimulus for cell proliferation that is maintained even in the absence of extracellular growth factor stimulation (this is synergistically enhanced by Wnt signaling), and hence may contribute to the pathogenesis of cancer.

Discussion

Most studies on tumorigenesis have focused on the role of abnormal activities of single pathways resulting from mutations of individual signaling proteins. However, it increasingly transpires that the signaling systems feature extensive crosstalk, and thereby form a much more complex network which is not easily understandable by an experimental approach alone. Here, we have revealed a crosstalk between the Wnt and ERK pathways that has the properties of a positive feedback loop and can induce state changes leading to permanent activation.

Positive feedback loops in signaling pathways are well known for playing important roles in determining the system dynamics by inducing switch-like or irreversible behaviors (Ferrell, 2002). The positive feedback loop embedded in the crosstalk between the Wnt and ERK pathways also has a similar role. It induces maximal and sustained ERK activity and thereby upregulates β-catenin/TCF. Under physiological circumstances such a switch-like behavior can contribute to filter true signals from noise and maintain response fidelity to for example growth and differentiation factors in noisy environments. This is of particular importance in highly mitotic tissues such as the colon epithelium, which needs to be constantly replenished by a finely coordinated balance between cell proliferation and differentiation. If this balance is disturbed epithelial hyperplasia can result. Progression into overt cancer can occur by the accumulation of further changes including changes in the expression or mutations in oncogenes and tumor suppressor genes (Kinzler and Vogelstein, 1996). This progression sequence reveals that malignant transformation of human cells requires multiple cooperating changes. While it is clear that the accumulation of changes is critical for the development of a cancer, it is less clear how these changes interact to cause malignant transformation.

In this paper, we have identified a positive feedback loop embedded in the Wnt/ERK pathways. Through computational modeling and simulations followed by further experimental validations, we have inferred that the positive feedback may have a critical contribution to carcinogenesis by leading to bistability of the system, which is not achievable by analysing a single oncogenic pathway alone. Our study shows that the positive feedback induces a state transition from an inactivated status to a persistently activated, that is biologically proliferative status. Remarkably, our results show that small changes in the concentrations of β-catenin or MKP activities that exhibit almost normal behaviors without stimulus can lead to the persistent and irreversible activation of both pathways, and act synergistically with Wnt signaling. Thus, we speculate that due to this crosstalk, cells with minor mutations or aberrations can become committed to continuous proliferation independently of the further supply of extracellular signals. In this context, it has been recently shown that a constitutional 50% decrease in expression of just one APC gene allele can promote the development of familial adenomatous polyposis coli (Yan *et al*., 2002). In a similar vein, the expression of MKP-1, a MAPK phosphatase, has been reported to be reduced during colorectal cancer progression (Loda *et al*., 1996). Likewise, the expression of RKIP, which antagonizes the positive feedback, has recently been reported to be silenced by promoter methylation in hyperplasic polyposis coli (Minoo *et al*., 2006). Thus, aberrations that affect the key players of the positive feedback loop and that are consistent with our two model mutation scenarios have been described.

Currently, most of the rationally developed cancer drug treatment strategies aim at the selective inhibition of individual target proteins thought to be required for cancer cell proliferation and survival. Unfortunately, in most cases this strategy has been without convincing clinical effects, and one usually has to resort to combination therapy where such compounds then show efficacy. However, the high costs of clinical trials and ethical considerations severely curtail the systematic testing of potentially synergistic drug combinations. Hence, as the selection of drug combinations is largely done empirically, there is a clear need for guidance in selecting drug combinations. We believe that in part such guidance can come from the computational modeling of signaling pathways affected in cancer cells. Our study also suggests a new drug target point in that the oncogenic status can be returned to normal status by inhibiting the positive feedback in the mutated Wnt/ERK pathways. Although such inhibition might disturb other normal functions, this study provides a new possibility of cancer treatment.

Materials and methods

Mathematical model

The original quantitative model for the Wnt pathway was derived from a Xenopus egg extract system (Lee *et al*., 2003). We have employed this as a basic model and further modified it by including a negative feedback loop that induces Axin from β-catenin/TCF to better represent the mechanism of a human eukaryotic cell (Lustig *et al*., 2002). The resulting model describes the conserved core mechanism of the Wnt pathway including the β-catenin destruction cycle (Cho *et al*., 2006). Moreover, there have been many studies about the MAPK pathway (Kolch *et al*., 2005; Wolkenhauer *et al*., 2005). Based on these reports, we have employed the ERK pathway model by including the Ras/Raf-1/MEK/ERK cascade and RKIP that plays as a signaling switch (Cho *et al*., 2003).

We have combined three experimental findings that are recently reported in the Wnt and ERK signaling pathway models (Figure 1). These are as follows: first, Wnt family signaling molecules bind to the receptor protein called Frizzled, and they can activate Ras on their own (Yun *et al*., 2005), or via the activation of ErbB1 (Civenni *et al*., 2003). Secondly, the upregulated β-catenin/TCF induces the expression or activation of an activator molecule X which activates Raf-1 (Rottinger *et al*., 2004; Yun *et al*., 2005). Thirdly, ERKpp phosphorylates GSK-3β, which promotes its subsequent inactivation by the RSK kinase (Almeida *et al*., 2005; Ding *et al*., 2005).

All of the mathematical models are constructed by using ordinary differential equations (ODEs) (see Supplementary Information for detailed modeling and parameter values used for simulation). According to the assumptions of previous models, the total concentrations of Ras, Raf-1, MEK, ERK, RKIP, Dishevelled (Dsh), APC, TCF, GSK-3β and all phosphatases are assumed to be constant over the whole simulation periods. In addition, steady-states of other molecules are determined by parameters. All the simulations have been performed after 100 000 min with $W=0$ in order to begin from the steady-state value of each molecular concentration. Since the recently reported experiments did not provide any kinetic details on the discovered mechanisms, we have followed general kinetic rules by employing mass action equations (basic chemical reaction kinetics) for the reaction of self-degradation of X (v31); Michaelis–Menten equations (enzyme kinetics) for the reaction of Raf-1 activation by molecule X (v30), GSK-3β phosphorylation by ERKpp (v32) and GSK-3β phosphatase activity (v33); Hill equations (transcriptional activation kinetics) for the reaction of transcriptional synthesis of activator X by β-catenin/TCF (v29). We have set the nominal parameter values of the crosstalk-related reactions in a way that the overall behaviors are consistent with the experimental results. It is widely known that β-catenin is highly upregulated and Ras/Raf-1/MEK/ERK cascade leads to an activated status in colorectal cancer. Hence, we assume that the β-catenin synthetic rate (v12) increases in the Wnt pathway and the maximal velocity of the MEK phosphatase (V_{max4}) and ERK phosphatase (V_{max5}) decreases in the ERK pathway, respectively, to reflect the behavior of mutated systems. All these parameters were selected from two published models (Cho *et al*., 2006, 2003). From additional simulations over a range of parameters, we have confirmed that any small change of the parameters does not affect our hypothesis (data not shown). To further validate our hypothesis, we have also conducted experiments (see below).

Cell Lines and Inhibitors

HEK 293 cells (ATCC CRL-1573) were cultured in DMEM (Invitrogen, Paisley, UK), supplemented with 10% fetal bovine serum (Autogen Bioclear, Calne, UK). UO126

(Promega, Southampton, UK), SB216763 (Calbiochem, Nottingham, UK) and Okadaic acid (Sigma, Gillingham, UK) solutions were freshly prepared for every assay.

TOPFLASH/FOPFLASH Reporter Assay
HEK-293 cells (250 000) were transfected with 2 μg of pTOPFLASH (TCF/β-catenin reporter) or pFOPFLASH (mutant promoter control) (Upstate, Chandler's Ford, UK), 1ng of pSV40-Renilla (Promega), and the other indicated constructs using an Amaxa Nucleofector (Amaxa, Cologne, Germany) according to the manufacturer's instructions. Plasmids for myc tagged mouse β-catenin and stabilized mouse β-catenin, where the GSK-3β phosphorylation sites are mutated, were kind gifts by Rolf Kemler (Aberle *et al*., 1997). Cells (5000) per 96-well were seeded in triplicates on a white luminescence 96-well plate (Nunc). At 48 h after transfection, the respective inhibitors were added for 4 h. Subsequently, both Firefly and Renilla luciferase activity were measured with a luminometer (Safire2, Tecan, Reading, UK) using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency by Renilla luciferase activity. Relative TCF/β-catenin activity is defined as the ratio of pTOPFLASH to pFOPFLASH. The ratio for control is defined as 1.

Immunoblotting experiments
HEK-293 cells (250 000) were transfected with 2 μg of mouse *β-catenin* using an Amaxa Nucleofector (Amaxa) according to the manufacturer's instructions. The transfected cells were seeded in a six-well plate (Nunc). At 48 h after transfection, the respective inhibitors cells were added for 4 h before the cells were harvested. Cells were lysed in ice-cold lysis buffer (10 mM HEPES, pH 7.8/1.5 mM $MgCl_2$/10 mM KCl/0.1% NP-40) supplemented with standard protease inhibitors (Sigma), incubated on ice for 10 min, and sonicated 3 × for 5 s. After centrifugation for 15 min at 10 000 *g*, the supernatant was taken and protein concentrations were determined by using the BCA protein assay kit (Pierce, Perbio Science UK Ltd., Cramlington, UK). Aliquots (20 μg) of protein per sample were separated by sodium dodecyl sulfate (SDS)–gel electrophoresis and transferred to Hybond-PVDF membrane (GE Healthcare, Amersham, UK) followed by immunoblotting and ECL detection. The following antibodies were used: mouse anti-Myc 4A6 (1:1000, Cancer Research, UK), mouse anti-ERKpp (1:5000; Sigma), mouse anti-ERK (1:2000; Sigma), horseradish peroxidase-conjugated anti-mouse IgG (1:7500; New England Biolabs, Hitchin, UK).

Abbreviations

APC, adenomatous polyposis coli; Dsh, dishevelled; EGFR, epidermal growth factor receptor; ERK, extracellular signal related-kinase; GSK-3β, glycogen synthase kinase 3β; MKP, MAP kinase phosphatase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RKIP, Raf-1 kinase inhibitor protein; TCF, T-cell factor.

Acknowledgements

This work was supported by a Grant 2005-B0000002 from the Korea Bio-Hub Program of Korea Ministry of Commerce, Industry & Energy, by a grant from the Korea Ministry of Science and Technology (Korean Systems Biology Research Grant, M10503010001-05N030100111), by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG05-0204-3-0), Republic of Korea, and by the European Union FP6 project 'Computational Systems Biology of Cell Signalling' (LSHG-CT-2004-512060) and Cancer Research UK. D Kim and K-H Cho were supported by the second stage Brain Korea 21 Project in 2006.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).