Cellular Signalling 20 (2008) 1697–1704

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Review

Wnt and beyond Wnt: Multiple mechanisms control the transcriptional property of β-catenin

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ARTICLE INFO

Article history:
Received 26 March 2008
Accepted 23 April 2008
Available online 8 May 2008

Keywords:
Wnt
cat/TCF
Insulin
IGF-1
FOXO
mTOR

ABSTRACT

The bipartite transcription factor β-catenin/TCF (cat/TCF) has been recognized as the major effector of the Wnt signaling pathway for more than a decade, and its over-activation has been associated with malignancy such as colon and breast cancer. Extensive examination in different cell lineages has shown that the activity of cat/TCF can be stimulated by mechanisms other than via the Wnt glycoproteins, including the stimulation of β-cat nuclear translocation and enhanced binding of cat/TCF to the Wnt target gene promoters by insulin and insulin-like growth factor-1 (IGF-1). In addition, the heterotrimeric G proteins of the G_{12} subfamily can interact with the cytoplasmic domain of cadherins, resulting in the release of the transcriptional activator β-cat. Furthermore, certain peptide hormones may stimulate cat/TCF-mediated gene transcription via activation of their corresponding G-protein coupled receptors. Recently, the serine/threonine kinase GSK-3 has been recognized to coordinate with AMP activated protein kinase (AMPK) in phosphorylation and activation of TSC2, the major component of the tumor suppressor complex TSC1/2. Thus, Wnt activation can stimulate protein translation via GSK-3 and TSC1/2 inactivation, followed by mTOR activation. Finally, β-cat also functions as a pivotal molecule in defense against oxidative stress via serving as a partner of forkhead box O (FOXO) transcription factors. Thus, FOXO proteins, which mainly mediate aging and stress signaling, and TCF factors, which mainly mediate developmental and proliferation signaling, compete for a limited pool of free β-cat. Insulin and growth factors, on the other hand, control the balance between TCF- and FOXO-mediated gene transcription via phosphorylation and nuclear exclusion of FOXO proteins. These observations provide new insight to understand how Wnt, insulin/growth factors, and FOXOs are involved in versatile physiological events and the development and progression of various human diseases.

Contents

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doi:10.1016/j.cellsig.2008.04.014

1. Introduction

Fifteen years ago, Rubinfeld et al. identified a physical interaction between the 92-kD protein β-catenin (β-cat) and the adenomatous polyposis coli (APC) tumor suppressor [1]. Later, Munemitsu et al. demonstrated that the expression of wild-type APC, but not a truncated mutant, caused a pronounced reduction in total β-cat levels in the colon cancer cell line SW480, and the reduction was due to an enhanced rate of β-cat degradation [2]. Subsequent studies in *Xenopus*, *Drosophila* and mammalian cells defined the role of the bipartite transcription factor β-catenin/TCF (cat/TCF) as the major effector of the canonical Wnt signaling pathway [3–6]. Therefore, free cytoplasmic β-cat level has been often utilized as one of the important indicators of the Wnt activity [7–16].

Extensive examination in different organisms and cell lineages over the past decade has illustrated the complexity of the Wnt signaling pathway and versatile role of β-cat as a transcription factor. Many studies have shown that the activity of cat/TCF can be activated by factors other than the Wnt glycoproteins, including insulin, insulin-like growth factor-1 (IGF-1), other hormonal factors and the lipid metabolite lysophosphatidic acid (LPA) [17,18]. In addition to β-cat stabilization, inhibition of the serine/threonine kinase GSK-3 in response to Wnt activation will lead to the inactivation of the tumor suppressor complex TSC1/2, followed by activation of the mTOR signaling pathway [19]. Thus, Wnt pathway activation can stimulate not only gene transcription via the bipartite transcription factor cat/TCF, but also protein synthesis via mTOR activation [19,20]. It is well known that insulin, IGF-1, and certain other mitogens are also able to activate mTOR via PI3K and PKB activation. Because insulin and IGF-1 can also stimulate cat/TCF-mediated gene transcription in many cell lineages, the cross-talk between Wnt and insulin/IGF-1 signaling pathways allows the activation of each of these to stimulate the expression of their target genes at both the transcriptional and translational levels [17]. More importantly, recent studies have revealed that in both *C. elegans* and mammals, the stress sensor and mediator, forkhead box O (FOXO) proteins, compete with TCF factors for a limited pool of β-cat. Since insulin, IGF-1 and many other growth factors antagonize the function of FOXOs via a phosphorylation-mediated nuclear exclusion process [21], the potential physiological and pathological role of insulin/IGF-1 signaling in diverting β-cat from FOXO- to TCF-mediated transcription should be appreciated.

In this review article, we will focus first on the stimulation of cat/TCF activity by factors other than the Wnt glycoproteins. We will then discuss the discovery showing that GSK-3 coordinates with AMPK in phosphorylation and inactivation of TSC1/2 complex, which is a major inhibitor of mTOR. Third, we will summarize the studies indicating that the stress sensor and mediator FOXO proteins are able to compete with TCF factors for β-cat and how their function is negatively regulated by insulin and growth factors. These discoveries and observations bring us a new picture of how Wnt, insulin and growth factors, and FOXOs may interact in the pathogenesis of many human diseases, including diabetes, cancer, aging, and osteoporosis. For cross-talk between Wnt and other signaling pathways, such as Hodgehog, Notch, and nuclear receptors (NRs), the reader is referred to excellent review articles elsewhere [22–25].

2. Stimulation of cat/TCF transcriptional activity by factors other than Wnt glycoproteins

2.1. The canonical Wnt signaling pathway and β-catenin

The Wnt signaling pathway was initially characterized during investigation of colon cancer and in embryonic development studies of *Drosophila*, *Xenopus* and other organisms. Aberrant activation of Wnt signaling may lead to the development of colorectal tumors [26]. The major effector of the Wnt pathway is the bipartite transcription factor cat/TCF, formed by free β-cat and a TCF factor (TCF-1, TCF-3, TCF-4 and LEF-1). Among them, TCF-4 is the major partner of β-cat in intestinal cells [26,27]. Under non-stimulating conditions, the concentration of free β-cat is tightly controlled by a proteasome-mediated degradation process, with the participation of the tumor suppressor APC, Axin, glycogen synthase kinase-3 (GSK-3), and casein kinase-1α (CK-1α) [28,29]. Wnt glycoproteins exert their effect via the seven-transmembrane domain Frizzled receptors (Fz) and LRP5/6 co-receptors. Following receptor binding, Wnt signals are transmitted by the association between Wnt receptors and Dishevelled (Dvl), an event that triggers the disruption of the complex containing APC, Axin, GSK-3 and β-cat, preventing phosphorylation-dependent degradation of β-cat. The serine/threonine kinase GSK-3, therefore, functions as one of the important negative modulators of Wnt signaling. The enzymatic activity of GSK-3 can also be inactivated by lithium, and the inactivation will lead to free cytoplasmic β-cat accumulation [9]. Since lithium mimics the function of Wnt activation, it has been broadly utilized in studying the functional outcome of Wnt signaling activation and target gene expression [9,30].

2.2. Growth factors and stimulation of cat/TCF-mediated transcriptional activity

2.2.1. Insulin and IGF-1

Type II diabetes mellitus (T2D) is one of the leading causes of mortality in developed countries and its onset is preceded by the development of insulin resistance and compensatory hyperinsulinemia. There are numerous known risk factors for T2D, including high fat diet and sedentary life style [31–33]. Significantly, colorectal cancer shares many of the risk factors with T2D [33–36], and epidemiological studies have indicated that subjects with T2D have an increased risk of the development of colorectal and breast cancer [33,35–40]. In addition, *in vivo* studies have demonstrated colorectal tumor formation in T2D animal models [35]. Furthermore, acute insulin treatment in rodents leads to increased intestinal crypt cell proliferation and expression of proto-oncogenes [36,41,42], suggesting that hyperinsulinemia may contribute to the development of colorectal cancer. This raises the question of whether insulin mediates these effects via a stimulation of Wnt responsive or cat/TCF-mediated gene transcription.

GSK-3 can also be phosphorylated at Ser21/9 residues by PKB/Akt via insulin or IGF-1 treatment. Ding et al., however, showed that a 2-h insulin treatment, possibly through PKB activation, led to stimulated phosphorylation of GSK-β at Ser9 and inactivated GSK-3β enzymatic activity in a number of cancer cell lines and in fibroblasts [7]. However, free cytoplasmic β-cat levels were not altered in their studies [7]. In

contrast, both Wnt molecules and lithium induced free cytoplasmic β-cat accumulation but did not affect the phosphorylation status of GSK-3β [7]. Based on these observations, Ding et al. suggested that insulin and the Wnt signals regulate GSK-3β through different mechanisms, thereby leading to distinct downstream events and that the phosphorylation of GSK-3β at Ser9 is not sufficient to induce free β-cat accumulation [7]. On the other hand, a few studies have shown that in a number of cancer cell lines, insulin and/or IGF-1 could activate the expression of a Wnt target gene or a Wnt (cat/TCF) responsive reporter gene system [43–45]. For example, Desbois-Mouthon et al. have shown that in the HepG2 cell line, both insulin and IGF-1 stimulated the transcription of a cat/TCF-binding site reporter gene, and increased cytoplasmic levels of β-cat [43]. Based on studies using chemical inhibitors and dominant negative molecules, Desbois-Mouthon et al. suggested that both the PI3K/PKB/GSK-3 and Ras signaling pathways were involved in mediating the stimulatory effect of insulin and IGF-1 [43]. Verras and Sun reported a stimulatory effect of IGF-1 on both nuclear and cytoplasmic β-cat levels in prostate cancer cells [44]. Similarly, in early melanoma cells, Satyamoorthy et al. found that IGF-1 stimulated the phosphorylation of Erk1/2, activated Akt, inhibited its downstream effector GSK3, and stabilized β-cat [45]. It was unclear from this study whether IGF-1 stabilized β-cat via Akt/GSK-3 activation or Erk activation.

In intestinal endocrine L cells, the proglucagon gene (glu) is a downstream target of the Wnt signaling pathway [27,30]. This gene encodes the important incretin hormone GLP-1. We observed that glu promoter can be activated by lithium or a constitutively active β-cat (the S33Y mutant, [46]). Lithium also stimulated endogenous glu mRNA and GLP-1 production in both a cultured endocrine L cell line and primary intestinal endocrine cells [46]. We then demonstrated that the activation was mediated by binding of cat/TCF-4 to a TCF-binding motif within the G2 enhancer element of the glu gene promoter. Expression of dominant negative TCF-4 not only abolished lithium stimulated glu mRNA expression and GLP-1 production, it also significantly attenuated basal glu mRNA expression [27]. More recently, we examined the effect of insulin and IGF-1 on glu mRNA expression and GLP-1 production in both *in vitro* and *in vivo* settings and observed that insulin utilizes the same *cis*-element (TCF-binding site within the G2 enhancer element) and *trans*-elements (cat/TCF-4) that are employed by Wnt signaling in stimulating glu expression [18]. "Knocking-down" of the expression of β-cat or introducing a dominant negative TCF-4 blocked the stimulatory effect of insulin. It appears that the stimulation was not mediated by PKB activation because the effect of insulin on glu promoter expression could be blocked by PI3K inhibition, but not by PKB inhibition. Furthermore, it appears that GSK-3 phosphorylation was also not required. Although insulin effectively stimulated GSK-3 phosphorylation in both intestinal endocrine L cells and in "control" pancreatic α cells, insulin repressed glu mRNA expression in the pancreatic α cells [18]. In addition, in L cells insulin increased nuclear β-cat content and binding of both β-cat and TCF-4 to the glu promoter G2 enhancer element, detected by quantitative chromatin immunoprecipitation (ChIP) assay. Finally, we found that in a hyperinsulinemic mouse model, glu mRNA and GLP-1 production were significantly higher than that in sex and age-matched controls, suggesting that a high level of circulating insulin can increase GLP-1 production [18].

Insulin is able to stimulate the expression of a number of proto-oncogenes, including c-Myc and cyclin D1, which are also suggested downstream targets of Wnt signaling [47,48]. We found that insulin stimulates c-Myc expression at both the translational and transcriptional levels [17]. The stimulatory effect on translation was sensitive to mTOR inhibition and was dependent on PKB activity. The stimulatory effect at the transcriptional level, however, was not dependent on PKB, although it required PI3K activation. Furthermore, insulin enhanced β-cat nuclear translocation and the binding of β-cat to two TCF-binding motifs within the human c-Myc gene promoter, detected by quantitative ChIP. This enhancement was also blocked by PI3K inactivation, but not by PKB or mTOR inhibition [17]. These observations indicate that, at least in the intestinal cells, insulin signaling interacts with the Wnt pathway by increasing nuclear β-cat content and binding of cat/TCF to Wnt target gene promoters.

Our observations also indicate that the cross-talk between the insulin and Wnt pathways is not mediated by PKB-GSK-3, although PI3K activation is involved. PKB-independent PI3K signaling has not been recognized until recently. p21-activated kinase 1 (PAK-1) has been implicated as an important mediator of mitogenic factors. Thus, Zhang et al. found that in bronchial epithelial cells, cigarette smoke-stimulated EGF receptor activated the expression of the proto-oncogene FRA-1 via the PI3K-(PAK-1)-(Raf)-MEK-ERK signaling cascade, without involving PKB [49]. In addition, IGF-1 was shown to regulate the activity of a transcription factor, namely RUNX2, through a PI3K-dependent but PKB-independent signaling pathway [50]. Of relevance, both FRA-1 and RUNX2 are known downstream targets of the Wnt signaling pathway [51,52].

If GSK-3 is not involved, by what mechanism does insulin increase the content of nuclear β-cat? Using mass spectrometry, Tian et al. have shown that β-cat interacts with a member of the 14-3-3 protein family, 14-3-3ζ [34]14-3-3ζ was shown to enhance β-cat dependent transcription by maintaining a high level of free β-cat [34]. Thus, one interesting possibility to examine will be whether 14-3-3ζ binding stabilizes β-cat and/or enhances its nuclear translocation, and whether these putative actions lead to enhanced binding of cat/TCF to the Wnt target gene promoters. Very recently, Gherzi et al. reported that the half life of β-cat mRNA is prolonged not only by Wnt but also by PI3K-Akt signaling, adding further complexity to the potential mechanisms by which insulin/IGF-1 could interact with Wnt signaling [53]. Interestingly, the authors showed that Akt phosphorylated the mRNA decay-promoting factor KSRP at a unique serine residue, induced its association with 14-3-3, which prevented KSRP interaction with the exoribonucleolytic exosome complex. Therefore, 14-3-3 could be involved in two events: stabilization of β-cat protein as well as that of β-cat mRNA.

2.2.2. Platelet-derived growth factor (PDGF)

Recently, PDGF treatment was also shown, by Yang et al., to stimulate β-cat nuclear translocation [54,55]. The nuclear p68 RNA helicases (p68) are a member of the DEAD (Asp-Glu-Ala-Asp) box family of RNA helicase [56]. p68 and another member of the family, p72, may also function as transcriptional co-regulators of MyoD, p53, CBP, and p300 [57–60]. p68 can be phosphorylated at Y593 in response to PDGF treatment, via the non-receptor tyrosine kinase c-Ab1 in the human colon cancer cell line HT-29 [54]. Ectopic expression of Y593-phosphorylated p68 stimulated β-cat nuclear translocation, apparently achieved by blocking the phosphorylation of β-cat by GSK-3 and displacing Axin from β-cat [54]. This PDGF-mediated p68 phosphorylation could be essential for PDGF-stimulated epithelial–mesenchymal transition (EMT) [54]. Subsequently, Yang et al. further demonstrated that Y593-phosphorylated p68 promoted cell proliferation by activating the transcription of cyclin D1 and c-Myc [55], two known downstream targets of Wnt signaling. Shin et al. reported that the expression of p68 and p72 was strongly increased during the polyp>adenoma>adenocarcinoma transition in the colon [61]. Both p68 and p72 were also found to be over-expressed in a number of colon cancer cell lines and physically interacted with β-cat, detected by coimmunoprecipitation. Furthermore, either p68 or p72 was found to augment β-cat or S33Y-β-cat stimulated expression of TOPFlash, a cat/TCF-responsive artificial reporter gene system. Finally, knocking-down p68 and p72 with a retrovirus-mediated short hairpin RNA (shRNA) system resulted in reduced expression of c-Myc, cyclin D1, c-Jun, and Fra-1 [61]. Therefore, in response to PDGF–PDGF receptor (PDGFR) activation, p68 may affect the β-cat signaling pathway in two

ways in cancer cells. In the cytoplasm, it protects β-cat from GSK-3 phosphorylation and proteasome-mediated degradation. In the nucleus, it augments β-cat mediated transcriptional activation [61].

2.3. G-protein coupled receptors and stimulation of cat/TCF transcriptional activity

2.3.1. Cyclic AMP (cAMP) and PKA mediated GSK-3 and β-cat phosphorylation

Fang et al. reported that Ser21/9 of GSK-3 are also physiological targets of PKA, and PKA phosphorylated GSK-3 shows attenuated enzymatic activity [62,63]. Li et al. demonstrated such phosphorylation in neuronal cells, and suggested that phosphorylation of GSK-3 by PKA may contribute to the inhibition of neuronal cell apoptosis by the second messenger cAMP [64]. These authors did not examine the effect of cAMP or PKA on β-cat levels or cat/TCF-mediated gene transcription. Hino et al., however, found that several activators of PKA, including prostaglandin E(1), isoproterenol, and dibutyryl cAMP increased cytoplasmic and nuclear β-cat levels, and stimulated TCF-dependent transcription through β-cat [65]. They demonstrated that although PKA did not interact with any of the components of the destructive complex of β-cat (APC, Axin, GSK-3 and CK-1), it directly phosphorylated β-cat at Ser675, which appeared to stabilize β-cat, preventing its ubiquitination-mediated degradation [65].

2.3.2. Enhanced dissociation of β-cat from cadherins by G_{12} activation

As discussed above, free β-cat serves as the partner of TCF factors in regulating gene transcription. The majority of β-cat, however, is associated with cadherins to form the adhesion structures of cells [66]. G proteins are classified according to their α-subunits into four subfamilies, Gs, Gi, Gq, and G_{12}. $G\alpha_{12}$ and $G\alpha_{13}$, two members of the G_{12} subfamily, are involved in cellular processes including cytoskeletal rearrangement, cell proliferation and oncogenic transformation [67–69]. Meigs et al. observed a specific interaction between G_{12} and the cytoplasmic tails of members of the cadherin family. The interaction resulted in the dissociation of β-cat and cadherin molecules. In the functional-APC-lacking colon cancer cell line SW480, expression of a mutant constitutively active $G\alpha_{12}$ or $G\alpha_{13}$ caused an increase in cat/TCF-mediated transcriptional activation [69].

2.3.3. Prostaglandin E2 (PGE2) and LPA stimulated cat/TCF transcriptional activity

Both prostaglandin E2 (PGE2) and the lipid metabolite lysophosphatidic acid (LPA) play critical roles in tumorigenesis [70]. The FP(B) prostanoid receptor is a GPCR that couples to Gq, which was shown by Fujino and Regan to activate the cat/TCF-mediated transcriptional response [71]. The EP(2) and EP(4) prostanoid receptors, however, couple to Gs [66]. Fujino et al. found that these two GPCRs also activated cat/TCF-mediated gene transcription in response to PGE2 treatment [70]. Their further investigations indicated that the activation by the EP(2) receptors occurred primarily through a PKA-dependent pathway, whereas the EP(4) receptors activated cat/Tcf signaling mainly through a PI3K-dependent pathway [70]. LPA exerts its stimulatory effect on cancer cell proliferation via binding to three known GPCRs, LPA(1), LPA(2) and LPA(3), which couple to Gs [70]. Yang et al. reported that LPA activated the major signaling events in the β-cat pathway, including GSK-3 phosphorylation, β-cat nuclear translocation, and cat/TCF-mediated transcriptional activation [72].

2.3.4. The cross-talk between Frizzled receptors and GPCRs

Frizzled proteins are seven-transmembrane receptors which bind Wnt ligands. These receptors share structural homology with those of the GPCR family. Liu et al. generated a chimeric receptor with the ligand-binding and transmembrane segments from β2-adrenergic receptor (β2AR) fused to the cytoplasmic domains of Frizzled-1. When this chimeric receptor was expressed in mouse F9 cells, the β-adrenergic agonist, isoproterenol, stabilized β-cat and stimulated the expression of a β-cat-sensitive promoter [73]. Indeed, Malbon has suggested that Frizzled receptors are new members of the GPCR family [73–75]. More detailed information on cross-talk between Frizzled receptors and GPCRs is reviewed by Force and colleagues [76,77].

3. The cross-talk between Wnt and mTOR signaling pathways

3.1. Regulation of mTOR by insulin and growth factors

In order to respond to diverse environmental cues, such as pH, osmolarity, and the availability of nutrients (especially glucose), eukaryotic cells have evolved effective mechanisms to maintain homeostasis [20]. The serine/threonine kinase mTOR is a key component of the mTORC1 complex, which consists of mTOR, Raptor, and mLST8 (Fig. 1). The mTORC1 complex is a major regulator of ribosome biogenesis and protein synthesis [78], and it is sensitive to inhibition by rapamycin. mTOR is able to phosphorylate proteins including 4E-BP1 and S6K1, which are involved in the biosynthesis of ribosomes and the initiation process of protein translation. As shown in Fig. 1, the function of mTOR is tightly regulated by Rheb, a small G protein and Ras homolog. Rheb is in turn regulated by the TSC1/2 tumor suppressor complex, which has Rheb GAP activity. The activity of TSC1/2, however, is determined by its phosphorylation status, involving various protein kinases that mediate the effects of nutrients, energy status, growth factors, and other environmental cues. Tuberous sclerosis is an autosomal-dominant disease that is caused by mutations in either the TSC1 or TSC2 genes. This disease is characterized by the formation of benign tumors in various tissues such as lungs, kidneys, heart and brain [79]. TSC2 is a protein of approximately 180 kD in size, and it can be phosphorylated at multiple positions by various protein kinases. Depending on the sites that have been phosphorylated, the GAP activity of TSC2 toward Rheb can be either inhibited or activated. Thus, inhibition of TSC2 GAP activity maintains the active form, i.e., RhebGTP which stimulates the mTORC1 complex (Fig. 1).

It is well known that insulin and many growth factors/mitogens, through binding of their receptor tyrosine kinases (RTKs) and activation of the downstream effector Alt/PKB, inhibit TSC2 activity, resulting in positive signaling to mTOR. This is achieved by attenuating the conversion of RhebGTP to RhebGDP. PKB is able to phosphorylate

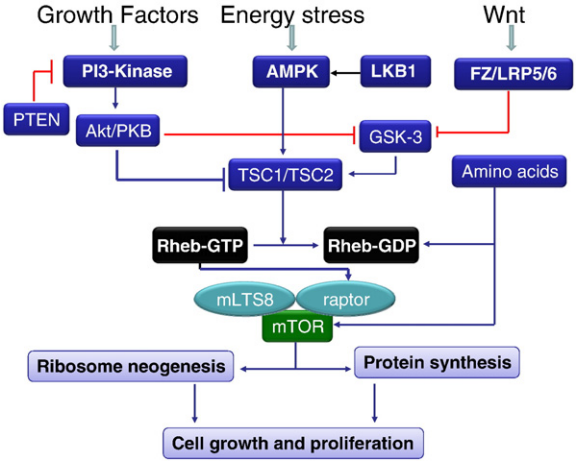

Fig. 1. Cross-talk between Wnt and mTOR. In response to energy stress, AMPK and GSK-3 phosphorylate and thereby stimulate the tumor suppressor TSC2 in a coordinated manner, resulting in the inactivation of mTOR and reduced protein synthesis [19]. Wnt activation is, therefore able to inhibit TSC1/2 complex and stimulate mTOR via GSK-3 inactivation.

TSC2 at Ser939, Ser 981, and Thr1462. Shaw and Cantley found that mutations at Ser939 and Thr1462 residues blocked the ability of PKB to activate S6K1 in response to various growth factors [78]. Furthermore, Cai et al. have shown that phosphorylation of TSC2 at Ser939 and Ser981 by PKB generates binding sites for interaction with the 14-3-3 protein [80]. Binding of TSC2 by 14-3-3 will trigger its interaction with Dishevelled (Dvl), preventing its interaction with TSC1 [80,81]. In contrast, during energetic stress and the increase of AMP/ATP ratio, the tumor suppressor LKB1 will phosphorylate AMP activated kinase (AMPK), which was shown by Inoki et al., to phosphorylate TSC2 at Ser1345, promoting its GAP activity toward Rheb, resulting in Rheb inactivation and mTOR inhibition [82].

3.2. TSC2 integrates energy and Wnt signals via coordinated phosphorylation by AMPK and GSK3

More recently, Inoki et al. observed that Wnt stimulation could also activate mTOR, and the activation was dependent on GSK-3 but not β-cat [19]. They then observed that in TSC2, four potential residues, Thr1329, Ser1333, Ser1337, and Ser1341, matched GSK3 phosphorylation site consensus sequence. Because GSK3 recognizes Ser or Thr residues separated by three residues, and often requires priming phosphorylation three residues C-terminal to the phosphorylation site [83,84], Inoki et al. hypothesized that AMPK primes TSC2 at Ser1345 for subsequent phosphorylation by GSK3 at the above potential residues. Their further experiments demonstrated that, indeed, mutating Ser1345 in TSC2 blocked TSC2 phosphorylation by GSK-3; and GSK-3 was able to phosphorylate Ser1337 and Ser1341 [19]. These observations established a link between nutrient-growth factor-mTOR and the Wnt signaling pathway. In studying the functional role of GSK-3 in mTOR inhibition, Inoki et al. demonstrated that over-expression of a constitutively active GSK-3β in cells growing with serum was sufficient to block S6K1 activation, suggesting that GSK inactivation is required for mTOR activation (Fig. 1). In addition, these authors further demonstrated that either knock-down or chemical inhibition of GSK-3 was sufficient to stimulate mTOR/S6K1 [19].

The data presented by Inoki et al. suggested that Wnt activation not only activates gene transcription via the formation of the bipartite transcription factor cat/TCF, but also protein synthesis via mTOR activation [19]. Our studies on the cross-talk between insulin/IGF-1 and Wnt pathways in non-endocrine intestinal cells suggested that insulin can activate c-Myc proto-oncogene expression via both mTOR activation (at the level of protein synthesis) and the increase of nuclear β-cat and binding of cat/TCF to the c-Myc gene promoter (at the level of transcription) [17]. In intestinal endocrine cells, insulin was also shown to stimulate the binding of cat/TCF-4 to the glu gene promoter, detected by quantitative ChIP assay, associated with increased promoter activity [18]. Therefore, both Wnt and insulin (and possibly IGF-1 and other mitogens) regulate target gene expression at both the transcriptional and translational levels (Fig. 2).

There are a number of questions in this area which remain to be addressed. First, it is essential to identify the signaling component(s) that mediates the cross-talk between the insulin and Wnt signaling pathways in view of the data that PKB is not involved in the transcriptional responses. Second, if both insulin and Wnt are able to stimulate common gene transcription and translation targets via these networks, one might also expect to see the existence of interacting or "cross-talking" negative feedback systems. Finally, additional animal models and clinical studies are needed to explore the physiological and pathophysiological roles of this cross-talk. From a physiological point of view, we suggest that eukaryotic cells have evolved to allow more than one signaling system to utilize common effectors to efficiently regulate gene expression at both transcriptional and translational levels. From a pathological point of view, these findings enhance our appreciation of the multiple mechanisms of Wnt pathway activation and the complex and varied influences on cancer

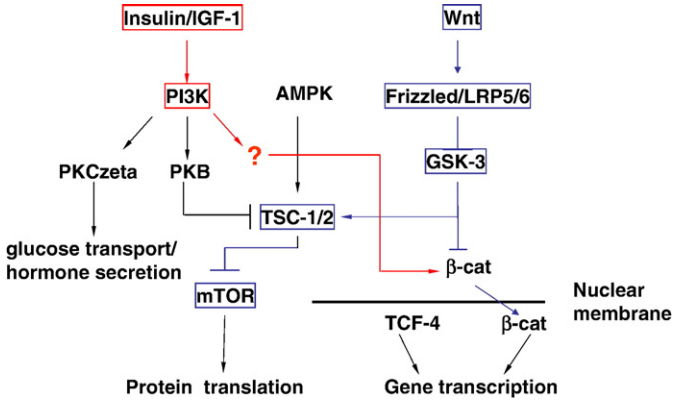

Fig. 2. Both Wnt and insulin/IGF-1 are able to stimulate gene expression at the transcriptional and translational levels. Wnt activation stimulates cat/TCF-mediated gene transcription via increasing the formation of the bipartite transcription factor cat/TCF. It also stimulates mTOR and therefore protein synthesis via the inactivation of GSK-3 and the TSC1/2 complex. Insulin/growth factors stimulate mTOR via the activation of PI3K-PKB signaling. It also stimulates β-cat nuclear translocation and the binding of cat/TCF to the Wnt target gene promoters via a PKB-independent mechanism.

development. As suggested by Choo et al., the work by Inoki et al. provides a novel therapeutic target for Wnt signaling pathway dependent cancers [19,20]. Therefore, "rapamycin" may prove to be efficacious for the treatment of tumors that are "addicted" to the Wnt signaling pathway [20]. In addition, T2D is characterized by hyperinsulinemia and insulin resistance and high fat diet feeding has been shown to stimulate GLP-1 production [85]. If insulin is indeed important for the homeostasis of GLP-1 production, one might speculate that although hyperinsulinemia in T2D subjects should have some "beneficial effect" by enhancing GLP-1 production, this could be partially attenuated due to insulin resistance. Indeed, in 12-week old hyperinsulinemic and insulin resistant MKR mice, we observed a more than fivefold increase in glu mRNA expression, but only a 46% increase of GLP-1 production [18]. We propose that insulin resistance may lead to a signaling shift from PKB>mTOR to a pathway which favors the formation of cat/TCF to stimulate glu transcription, potentially as a compensatory mechanism. In the long run, insulin resistance does not allow adequate compensation to meet GLP-1 and insulin requirement [86]. On the other hand, the "cost" of the compensation is the increased activity of the Wnt pathway, which may be responsible for the increased risk of developing colorectal tumors in T2D patients.

4. FOXO proteins and the Wnt signaling pathway

4.1. Function of FOXO proteins is inactivated by insulin/growth factors and stimulated by stress and aging

In the last few years, extensive investigations have led to the discovery of the insulin-FOXO protein signaling cascade [21,87–89]. This regulatory system controls metabolic homeostasis and other important physiological and pathophysiological events. FOXOs are members of a subfamily of the forkhead transcription factors and their functions are evidently negatively regulated by insulin-PI3K-PKB signaling [21]. In the absence of insulin or growth factors, FOXOs are mainly located within nuclei and up-regulate a set of target genes, thereby promoting cell cycle arrest, stress resistance, and apoptosis. In the presence of insulin or growth factors, FOXOs are exported into the cytoplasm, with the participation of the chaperone protein 14-3-3. This nuclear exclusion event is mainly mediated via the phosphorylation of FOXOs by PKB and serum- and glucocorticoid-regulated protein kinase (SGK) [21]. In this way, FOXOs function to control cell and organismal growth, development, metabolism and possibly, longevity in response to insulin, IGF-1, and many other growth factors. In contrast to insulin signaling, low levels of oxidative stress generated

by hydrogen peroxide (H_2O_2) treatment may induce the activation of FOXO proteins, demonstrated first by Essers et al. with FOXO4 as the example [90]. It appears that this is due to the activation of the small GTPase Ral, which will result in a JNK-dependent phosphorylation of FOXO4 at Thr447 and Thr451, followed by nuclear translocation of FOXO4, along with increased transcriptional activity [90]. The serine/threonine kinase MstI resides predominantly in the cytoplasm. Recently, Lehtinen et al. reported that MstI is able to mediate the effect of H_2O_2 on the nuclear translocation of FOXO3. Mst1 was shown to phosphorylate FOXO3 at four conserved sites within the forkhead domain that disrupted its interaction with protein 14-3-3, promoted FOXO nuclear translocation, and thereby induced cell death in neurons [91]. Jang et al., however, found that Akt was able to phosphorylate MstI, and this phosphorylation strongly inhibited the kinase activity of MstI on FOXO3, preventing its nuclear translocation and capacity to induce apoptosis [92]. Finally aging may lead to increased reactive oxygen species (ROS), resulting in enhanced FOXO activity [93].

4.2. FOXO proteins interact with β-cat

In 2005, Essers et al. reported an evolutionarily conserved interaction between β-cat and FOXO proteins [94]. In mammalian cells, a yeast two hybrid screen detected an interaction between β-cat and FOXO1 and FOXO3. The interaction required armadillo repeats 1 to 8 of the β-cat molecule and the C-terminal half of a FOXO protein [94]. In contrast, FOXO did not bind to the armadillo repeats of either APC1 or APC2, suggesting that the interaction between FOXO and β-cat is a specific event. Furthermore, Essers et al. detected an interaction between hemagglutinin (HA) tagged FOXO4 and FLAG tagged β-cat by coimmunoprecipitation. The gene bar-1 encodes the *C. elegans* homolog of β-cat [95], while the FOXO homologue in this organism is DAF16 [96]. The interaction between BAR-1 and DAF16 was also detected in coimmunoprecipitation experiments [94]. In mammalian cells, binding of β-cat to FOXO enhances the transcriptional activity of FOXO. In *C. elegans*, the loss of BAR-1 reduces the activity of DAF-16 in dauer formation and life span [94]. Furthermore, the association between β-cat and FOXO was enhanced in cells that are exposed to oxidative stress [94].

4.3. FOXO proteins compete with TCF factors for a limited pool of free β-cat in response to stress

Extensive *in vitro*, *in vivo*, and clinical investigations have shown that oxidative stress could play a pivotal pathogenic role in skeletal involution, independent of aging [93,97–100]. Importantly, recent studies have further suggested a pathophysiological role for the interaction between FOXO proteins and β-cat in bone diseases: the reduction of cat/TCF-mediated gene expression. Such a role may also be important in the pathogenesis of other oxidative stress-mediated and age-dependent diseases, including diabetes, metabolic syndrome and cardiovascular disease [93].

A few studies have shown that osteoporosis occurs in mouse models of premature aging [97,101–103]. For example, mice that express truncated/activated p53 display an early onset of phenotypes associated with aging, including reduced longevity and osteoporosis [102]. The gene XPD encodes a DNA helicase that functions in both DNA repair and transcription. Mutations of this gene are associated with the human disorder trichothiodystrophy (TTD). De Boer et al. found that mice with a mutation in XPD show many symptoms of premature aging, including osteoporosis and reduced life span [103]. More recently, Almeida et al. found that sex steroid sufficient male and female mice lose bone mass and strength progressively during the ages of 4 to 31 months, and this was associated with enhanced osteoblast and osteocyte apoptosis, reduced osteoblast number and bone formation rate, and elevated levels of ROS [97]. These observations indicate that osteoporosis can occur in a steroid-hormone-deficiency independent manner. Furthermore, Almeida et al. have shown that in the C57BL/6 mice, organismal aging is associated with reduced expression of Wnt target gene mRNAs, including that of Axin2 and Opg, and increased expression of the FOXO target gene Gadd45 [98]. H_2O_2 treatment in an uncommitted mesenchymal cell line C2C12, however, increased FOXO-mediated transcription and attenuated both basal as well as Wnt3a-stimulated levels of Axin2 and other Wnt target gene expression. Opposite effects of H_2O_2 on FOXO- and TCF-mediated transcription were then confirmed by measuring the activity of FOXO-Luciferase (FOXO-LUC) and TCF-LUC to different dosages of H_2O_2. These authors confirmed that FOXO-mediated transcription required the activation of Wnt signaling and increased β-cat levels. β-cat over-expression attenuated the repressive effect of H_2O_2 on TCF-mediated transcription, and the ROS and FOXO suppression of osteoblast differentiation [98]. These observations suggest that, at least in osteoblastic cells, increased ROS due to aging and other disorders attenuates the Wnt signaling pathway through FOXO activation, occurring by the diversion of the limited pool of β-cat from TCF factors to FOXO-mediated gene transcription [93]. More recently, the concept that the interaction of FOXO with β-cat inhibits cat/TCF activity was further confirmed by Hoogeboom et al. They demonstrated that siRNA mediated knock-down of FOXO reverts the loss of β-cat binding to TCF after cellular oxidative stress [104].

5. Summary and final remarks

Extensive advances have been made over the past few years in understanding the complexity of Wnt signaling and the cross-talk between Wnt and other signaling pathways. We have learned that in addition to Wnt ligands, other factors including growth factors, hormones, and lipid metabolites, such as LPA, are also able to stimulate cat/TCF activity and Wnt target gene expression by several different mechanisms. We have also learned that both Wnt and insulin/IGF-1 are able to regulate gene transcription via the bipartite transcription factor cat/TCF and protein synthesis via mTOR activation. Obviously, β-cat is "not just for frizzleds anymore" [75]. Furthermore, the function of β-cat is bi-directional. This pivotal molecule regulates many physiological and pathological events via controlling cell cycle progression and cell growth. When teamed up with TCF factors, β-cat activates Wnt target gene expression and stimulates these two processes. In contrast, when it is teamed up with FOXOs, it stimulates FOXO target gene expression and represses these two processes

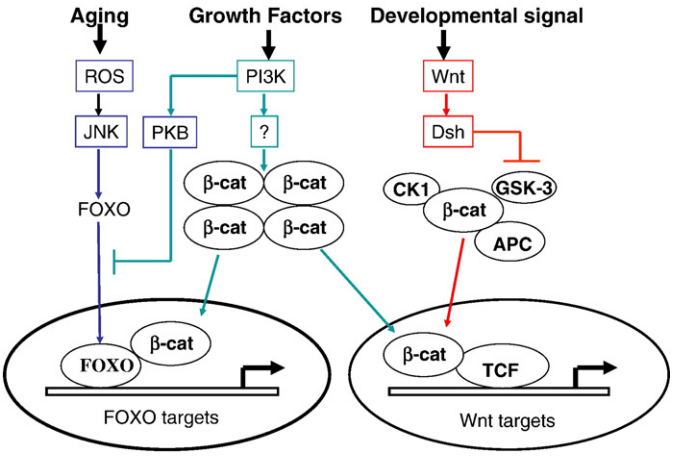

Fig. 3. Insulin/IGF-1 controls the balance between FOXO- and TCF-mediated gene expressions. FOXOs and TCF factors compete for a limited pool of β-cat. During aging and oxidative stress, the production of ROS leads to increased FOXO-mediated gene transcription and reduced TCF-mediated gene transcription. Insulin/growth factors help to restore the balance by two means. First, they stimulate nuclear exclusion of FOXOs via PKB mediated phosphorylation. Second, they enhance nuclear content of β-cat and the binding of cat/TCF to the Wnt target gene promoters.

(Fig. 3). Because FOXOs and TCF factors compete for a limited pool of β-cat, during aging and under oxidative stress, the balance will be upset, moving towards the side of FOXOs. Insulin and growth factors, however, may be able to restore the balance by both PKB-dependent and PKB-independent mechanisms. First, PI3K-PKB activation will lead to the phosphorylation and nuclear exclusion of FOXO proteins [21,88]. Second, PI3K activation may lead to stimulated β-cat nuclear translocation and the binding of cat/TCF to the Wnt target gene promoters, in a PKB-independent manner [17,18]. Therefore, further exploration of the molecular mechanisms underlying the cross-talk between insulin and Wnt signaling pathways will advance our knowledge of the pathogenesis of not only cancer and diabetes, but also of many other metabolic and age-dependent diseases, including osteoporosis and cardiovascular disease. The exploration may also lead to the discovery of novel drug target for these aging-driven diseases.

Acknowledgements

This work was supported by an operating grant of the Canadian Institutes of Health Research (CIHR) to TJ (68991) and an operating grant of CIHR to IGF (38009). JS is a recipient of Banting and Best Diabetes Centre Graduate Student Scholarship. We thank Dr. Qinghua Wang and Adam Sirek for their valuable comments and suggestions.

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