Molecular and Cellular Pathobiology

Wnt/Snail Signaling Regulates Cytochrome c Oxidase and Glucose Metabolism

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Abstract

Wnt signaling plays a critical role in embryonic development, and its deregulation is closely linked to the occurrence of a number of malignant tumors, including breast and colon cancer. The pathway also induces Snail-dependent epithelial-to-mesenchymal transition (EMT), which is responsible for tumor invasion and metastasis. In this study, we show that Wnt suppresses mitochondrial respiration and cytochrome C oxidase (COX) activity by inhibiting the expression of 3 COX subunits, namely, COXVIc, COXVIIa, and COXVIIc. We found that Wnt induced a glycolytic switch via increased glucose consumption and lactate production, with induction of pyruvate carboxylase (PC), a key enzyme of anaplerosis. In addition, Wnt-induced mitochondrial repression and glycolytic switching occurred through the canonical β-catenin/T-cell receptor 4/Snail pathway. Short hairpin RNA–mediated knockdown of E-cadherin, a regulator of EMT, repressed mitochondrial respiration and induced a glycolytic switch via Snail activation, indicating that EMT may contribute to Wnt/Snail regulation of mitochondrial respiration and glucose metabolism. Together, our findings provide a new function for Wnt/Snail signaling in the regulation of mitochondrial respiration (via COX gene expression) and glucose metabolism (via PC gene expression) in tumor growth and progression. Cancer Res; 72(14); 3607–17. ©2012 AACR.

Introduction

Wnt signaling plays an important role in multiple embryonic developmental processes, and its aberrant activation occurs in association with the development and progression of many human cancers, including breast and colon cancer (1–3). β-Catenin plays a key role in the canonical Wnt pathway (1, 2). In the absence of Wnt, cytosolic β-catenin is associated with a destruction complex containing axis inhibition protein 1/2 (Axin1/2), adenomatous polyposis coli (APC), and glycogen synthase kinase 3b (GSK3b). It is phosphorylated by GSK3b and is rapidly degraded by the ubiquitin-proteasome system. When Wnt binds to the Fzd receptor, the β-catenin destruction complex is disassembled, resulting in stabilization

of β-catenin. The stabilized β-catenin is translocated to the nucleus and forms a complex with the transcription factors T-cell factor (TCF)/lymphoid enhancer factor to induce the expression of Wnt/β-catenin target genes such as c-Myc and cyclin D. Wnt signaling activates an epithelial-to-mesenchymal transition (EMT) program that is linked to invasion and metastasis of tumor cells (4–7). Aixin2, one of the Wnt target genes, plays an important role in this event. Aixin2 acts as a nucleoeytoplasmic chaperone for GSK3b (6). GSK3b can phosphorylate the EMT-inducing zinc finger transcription factor, Snail, at consensus motifs (Ser96 or Ser104/Ser107), thereby causing its degradation by the ubiquitin-proteasome system (7, 8). GSK3b localizes to both the cytosolic and nuclear compartments. If GSK3b is moved to the cytosolic space by Aixin2, Snail protein stability in the nucleus is increased and represses the expression of E-cadherin and other epithelial markers while activating the expression of mesenchymal markers, thereby inducing EMT (6).

Mitochondria play a central role in several cellular functions including ATP production, metabolism, and apoptosis. Dysregulation of mitochondrial functions has been implicated in a number of human disorders including cancer development. In tumors, mitochondrial function is downregulated by mutations in mitochondrial or nuclear DNA–encoding mitochondrial proteins (9, 10) and/or by the transcription factors that regulate mitochondrial proteins encoded by nuclear genes (11, 12). Mitochondrial dysfunction favors the shift to glycolytic metabolism, referred to as "the Warburg effect," a phenomenon whereby cancer cells rely mainly on aerobic glycolysis to generate ATP even in the presence of O2 (11–14). Because of
the low efficiency of glycolysis in generating ATP, tumor cells with a glycolytic switch increase glucose uptake for ATP production, resulting in high lactate production and the acidification of the microenvironment, which facilitates tumor cell migration and invasion. In addition, such alteration in glucose metabolism confers survival advantages to tumor cells by providing precursors for the synthesis of nucleotides, lipids, and amino acids, which are required for tumor cell growth (14, 15). To support cell proliferation, tumor cells also activate a process termed anaerobiosis, which replenishes the precursors that are extracted from the tricarboxylic acid (TCA) cycle by cataplerosis (16). Two enzymes, pyruvate carboxylase (PC) that converts pyruvate to oxaloacetate and glutaminase that converts glutamine to glutamate, play a critical role(s) in anaerobiosis (16–21). Thus, a glycolytic switch with higher glucose uptake correlates with more aggressive phenotypes and poorer clinical outcomes; however, the mechanism underlying mitochondrial dysregulation and glycolytic switch remains largely unknown.

Herein, we show that Wnt/Snail signaling suppresses mitochondrial respiration and cytochrome c oxidase (COX) activity, with induction of the glycolytic switch and PC expression, providing a new function for Wnt/Snail signaling in tumor growth and progression.

Materials and Methods

Cell lines

MCF-7, MDA-MB-231, Madin Darby Canine Kidney (MDCK), and L cells were obtained from the American Type Culture Collection (ATCC; authenticated by short tandem repeat profiling). The cell lines were passaged 2 times per week after the receipt and low-passage cultures (passage 5–25) were used for the experiments. Wnt3a-secreting L cells and MCF-7 #5 cells were provided by Dr. Min DS (Pusan National University, Pusan, Korea) and by Dr. Yook Ji (Yonsei University, Seoul, South Korea), respectively. The cells were routinely tested negative for mycoplasma using the Mycoplasma PCR Detection Kit (iNtRON Biotechnology). Detailed information for cell culture is provided in the Supplementary Methods.

Transfection and short hairpin RNA interference

The expression vectors pCR3.1-Snail-Flg, pCR3.1-S96A-Snail-Flg, pCR3.1-S104, 107A-Snail-Flg, and pCDNA3.1-Wnt1 (provided by Dr. Yook Ji, Yonsei University) were transfected into MCF-7 cells using jetPEI (Polyplus-transfection). pSUPER vectors expressing short hairpin RNA (shRNA) for control, Snail, β-catenin, TCF4, Axin1, Axin2, PC, and E-cadherin were generated (Supplementary Table S1) and transfected as described previously (22).

Proteomics

MCF-7 #5 cells were treated with doxycycline and analyzed by quantitative differential proteome analysis using 2-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF/MS; Yonsei Proteome Research Center, Seoul, Korea).

Assays for mitochondrial respiration, COX activity, glucose consumption, lactate production, and ATP production

Mitochondrial respiration and COX activity were measured as described previously (23; for detailed information, see Supplementary Methods). Glucose, lactate, and intracellular ATP levels were determined using a Glucose Oxidation Assay Kit (Sigma), a colorimetric- and fluorescence-based Lactate Assay Kit (BioVision), and an ATP Bioluminescence Assay Kit (Roche), respectively. The level of ATP produced by aerobic respiration and glycolysis was determined by measuring lactate production and oxygen consumption (24).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were conducted using a ChIP Assay Kit (Millipore). Rabbit IgG or rabbit anti-Snail (Santa Cruz Biotechnology, Inc.) were used to immunoprecipitate DNA-containing complexes. ChIP-enriched DNA was analyzed by PCR using primers (Supplementary Table S2) complementary to the promoter regions containing E-box.

Results

Wnt/Snail signaling inhibits mitochondrial respiration

In this study, we examined whether Wnt regulates mitochondrial respiration in breast cancer MCF-7 cells. Wnt3a-conditioned medium (CM, obtained from Wnt3a-secreting L cells) induced EMT, the morphologic changes to mesenchymal cells, including loss of intercellular adhesion and formation of a spindle-like cell shape and pseudopodia (Supplementary Fig. S1A), with downregulation of E-cadherin, one of the hallmarks of EMT (Supplementary Table S3). Wnt3a CM markedly decreased mitochondrial respiration in a time-dependent manner with inhibition to 77% of control at 7 days (Fig. 1A). Recombinant Wnt3a (rWnt3a) protein also decreased mitochondrial O2 consumption in MCF-7 cells (Fig. 1B). Similar results were obtained in MCF-7 cells transfected with Wnt1, another Wnt molecule (Fig. 1C).

As shown by other investigators (6), Wnt3a CM induced nuclear accumulation of Snail proteins in MCF-7 cells (Fig. 1D). The increased nuclear Snail levels were detected at 12 hours and persisted for up to 7 days. Interestingly, Wnt3a-induced Snail proteins migrated as a closely spaced doublet (~30 kDa) in SDS-PAGE, possibly due to a posttranslational modification such as phosphorylation. A similar result with 2 to 3 bands was obtained in Wnt1-transfected cells (Fig. 1E). We investigated whether Snail is implicated in Wnt-induced mitochondrial repression. To this end, we used 2 different Snail shRNAs, which could effectively reduce Snail levels (Fig. 1F; Supplementary Table S1). In addition to suppressing Wnt3a/Wnt1-induced EMT (data not shown) and E-cadherin downregulation (Supplementary Table S3), Snail shRNA prevented Wnt3a- (Fig. 1F) and Wnt1- (Fig. 1G) induced repression of mitochondrial respiration. Wnt is known to induce nuclear Snail levels via the canonical pathway, which includes β-catenin, TCF4, and Axin1/2 (6). As shown in Fig. 1, knockdown of either β-catenin, TCF4, or Axin1/2 (Fig. 1H) prevented Wnt3a-induced nuclear accumulation of Snail (Fig. 1I), confirming the
canonical Wnt pathway–dependent Snail induction. shRNA for β-catenin, TCF4, Axin1, and Axin2 suppressed Wnt3a-induced inhibition of mitochondrial respiration (Fig. II), with inhibition of Wnt3a-induced E-cadherin downregulation (Supplementary Table S3).

We examined whether Snail affects mitochondrial function. To this end, we first used Snail tetracycline-inducible MCF-7 #5 cells (6). Upon Snail induction by doxycycline in MCF-7 #5 cells (Fig. 2A and B), the cells exhibited EMT (Supplementary Fig. S1B), with reduced expression of E-cadherin (Supplementary Table S3). Increased Snail expression significantly suppressed mitochondrial respiration with inhibition to 70% of control at 3 to 7 days (Fig. 2C). Furthermore, we transiently transfected MCF-7 cells with pCR3.1-Snail-Flag, and phosphorylation-resistant mutant Snail vectors, pCR3.1-S96A-Snail-Flag and pCR3.1-S104, 107A-Snail-Flag (Fig. 2D). Both wild-type and mutant Snail proteins were detected until 5 days after transfection (Fig. 2E). Although wild-type Snail is less stable than Snail mutant proteins (7), its steady-state levels persisted for 5 days because it was overexpressed. Transient transfection with Snail expression vectors significantly decreased O2 consumption in MCF-7 cells (Fig. 2F). Wild-type Snail could repress mitochondrial respiration to a similar extent as stable mutants.

Similarly, wild-type Snail could induce EMT similar to that observed in mutant Snail-overexpressing MCF-7 cells (Supplementary Fig. S1C), as shown by other investigators (7). In contrast, Snail shRNA increased O2 consumption in MCF-7 (Fig. 1F and G) and MDA-MB-231 cells (Fig. 2G). Taken together, our results indicate that Wnt represses mitochondrial respiration through the canonical β-catenin/TCF4/Snail pathway.

We examined the effects of Wnt signaling on mitochondrial mass using Green Fluorescent MitoTracker Green FM. Although Wnt3a and Wnt1 inhibited mitochondrial respiration, they did not exert any significant inhibitory effects on mitochondrial mass (Supplementary Fig. S2), indicating that Wnt may suppress mitochondrial respiration without affecting mitochondrial biogenesis thereby resulting in the accumulation of nonfunctional mitochondria in the cells.

**Wnt/Snail signaling inhibits COX activity**

Changes in the activity of COX, the terminal enzyme of the mitochondrial respiratory chain, are closely associated with dysregulation of mitochondrial respiratory activity. Therefore, we examined the effects of Wnt on COX activity. Wnt3a seemed to inhibit COX activity in MCF-7 cells in a time-dependent manner.
Wnt1 also significantly decreased COX activity in MCF-7 cells (Fig. 3B). Snail shRNA prevented Wnt3a- (Fig. 3C) and Wnt1- (Fig. 3D) induced repression of COX activity. In addition, increased Snail expression significantly reduced COX enzymatic activity with inhibition to 61% of control at 4 days (Fig. 3E). In contrast, Snail shRNA increased COX activity in MCF-7 (Fig. 3C and D) and MDA-MB-231 cells (Fig. 3F). These results indicate that Wnt/Snail signaling represses mitochondrial respiration by inhibiting COX activity.

**Wnt/Snail signaling represses the expression of COXVIc, COXVIIa, and COXVIIc**

Eukaryotic COX is a multimeric protein complex in the inner mitochondrial membrane that is composed of 13 different subunits encoded by 3 mitochondrial genes and 10 nuclear genes, and its assembly is regulated by the sequential action of several nucleus-encoded assembly factors (Supplementary Table S3; 25–28). Therefore, we investigated the effects of Wnt and Snail on the gene expression of COX subunits and assembly factors using real-time PCR to identify the target genes. We screened the genes whose levels are altered to less than 70% of control (Supplementary Table S3). We found that Wnt1, Wnt3a, and Snail commonly decreased mRNA levels of COXVIc, COXVIIa, and COXVIIc, although some other genes, such as COXVIIb, COX18, and SCO2 are differentially regulated depending on stimuli (Supplementary Table S3). Wnt3a-mediated decrease in the mRNA levels of COXVIc, COXVIIa, and COXVIIc was prevented by shRNA for Snail, β-catenin, and TCF4 (Supplementary Table S3). Western blot analysis showed that COXVIc, COXVIIa, and COXVIIc protein levels, but not those of other proteins, were reduced in Wnt1-transfected, Wnt3atreated, or Snail-overexpressing MCF-7 cells (Fig. 3B). Snail shRNA prevented Wnt3a- (Fig. 3C) and Wnt1- (Fig. 3D) induced repression of COX activity. In addition, increased Snail expression significantly reduced COX enzymatic activity with inhibition to 61% of control at 4 days (Fig. 3E). In contrast, Snail shRNA increased COX activity in MCF-7 (Fig. 3C and D) and MDA-MB-231 cells (Fig. 3F). These results indicate that Wnt/Snail signaling represses mitochondrial respiration by inhibiting COX activity.

We conducted a ChIP assay to examine in vivo binding of Snail to the promoter of COXVIc, COXVIIa, and COXVIIc. Several Snail-binding sites (E-box) were found in the promoters of these genes (Fig. 4B; Supplementary Table S4). The E-cadherin promoter region containing E-box and the COXVIIa and SCO2 exonic regions (Fig. 4B) were used as a positive and negative control, respectively. Wnt3a enhanced the binding of Snail to the E-box sites of the COXVIc, COXVIIa, and COXVIIc promoters, but not to the E-box of the COXVIIb promoter (Fig. 4C). Similar binding was detected in Snail-overexpressing cells (Fig. 4C). Although SCO2 protein levels were not significantly changed by Wnt3a treatment and Snail overexpression (Fig. 4A), Wnt3a induced the binding of Snail to the E-box sites of the SCO2 promoter (Fig. 4C).

In Wnt3a-treated cells, in vivo binding of Snail to the promoters of COXVIc, COXVIIa, and COXVIIc, and E-cadherin was detected at 12 hours and persisted for 7 days, showing the similar kinetics of Snail induction, the time to steady-state levels, and E-box DNA binding. These results explain that the decreased mitochondrial activity by Wnt3a persists 7 days after treatment (Supplementary Fig. S4A). In the case of Snail transfection, E-box DNA binding by Snail mutants was detected at 1 to 5 days (Supplementary Fig. S4B); however, E-box DNA binding by wild-type Snail was detected at 1 to 3 days, but not at 5 days (even though increased Snail levels were observed at this time point; Fig. 2E; SupplementaryFig. S4B).
These results indicate that Snail binding to E-box for 3 days is sufficient to inhibit mitochondrial respiration and to induce EMT.

**Wnt/Snail signaling induces the glycolytic switch**

Mitochondrial dysfunction favors the emergence of the glycolytic phenotype (12–14). Thus, we investigated whether Wnt/Snail signaling induces a glycolytic switch. Wnt3a (Fig. 5A), Wnt1 (Fig. 5B), and Snail expression either by doxycycline treatment in MCF-7 #5 cells (Fig. 5C) or by transfection of S104,107A mutant Snail in MCF-7 cells (Fig. 5D) significantly increased glucose consumption and lactate production without affecting total ATP production. Wnt and Snail increased the ratio of ATP produced by glycolysis versus ATP produced by aerobic respiration (Fig. 5A–D). Snail shRNA caused a decrease in glucose consumption and lactate production in MDA-MB-231 cells with reduced glycolysis-dependent ATP production (Fig. 5E). These results indicate that Wnt/Snail signaling induces a glycolytic switch.

**Wnt/Snail signaling induces PC**

To identify Snail-responsive targets that are linked to the glycolytic switch, we conducted quantitative differential proteome analysis. Comparative analysis of 2-DE showed that of the 1,103 proteins analyzed, 19 were upregulated by more than 3-fold and 12 were downregulated by more than 3-fold. One of the Snail-inducible proteins was PC (Fig. 6A), a mitochondrial enzyme that replenishes oxaloacetate to the TCA cycle by anaplerosis (16–18); its level was increased 5.5-fold by Snail. Real-time PCR and Western blot analysis show that PC levels are increased by Snail and Wnt (Fig. 6B–D). Furthermore, Wnt3a-mediated increase in PC levels was prevented by shRNAs for Snail, β-catenin, and TCF4 (Supplementary Fig. S5A), indicating the regulation of PC by the canonical Wnt/β-catenin/TCF4/Snail pathway. To further examine whether PC gene expression is regulated by Snail, we conducted a ChIP assay. Three E-box sequences were found in the PC promoter (Fig. 6E; Supplementary Table S4). As shown in Fig. 6F, however, the binding of Snail to the E-box sites of the PC promoters...
was not detected in Snail-overexpressing or Wnt3a-treated MCF-7 cells. We examined the role(s) of PC in the growth of Wnt3a-treated MCF-7 cells. PC shRNA seemed to increase the cell doubling time for Wnt3a-treated MCF-7 cells as well as for control MCF-7 cells (Supplementary Table S5), indicating that PC plays an important role in tumor cell growth. Finally, we examined whether the PC induction relates to the inhibition of mitochondrial respiration and COX and the glycolytic switch by the Wnt pathway. PC supplies oxaloacetate to the TCA cycle, thereby maintaining its activity and subsequently mitochondrial respiration, with an inhibitory effect on lactate production (16–18). In line with this, we observed that shRNA-mediated PC loss (Supplementary Fig. S5B) suppressed mitochondrial respiration/COX activity and induced a glycolytic switch with increased lactate production (Supplementary Fig. S5C) while it did not exert any significant effects on Wnt3a-induced regulation of mitochondrial respiration and glucose metabolism.

**EMT is linked to mitochondrial respiration and the glycolytic switch**

Because the EMT inducer Snail regulates mitochondrial respiration and the glycolytic switch, we examined whether EMT itself is linked to the regulation of mitochondrial respiration and the glycolytic switch. E-cadherin shRNA has been shown to induce EMT (29). We found that in addition to...
inducing EMT (Supplementary Fig. S6A), E-cadherin shRNA repressed mitochondrial respiration and COX activity and induced the glycolytic switch (Fig. 7A). We examined whether Snail is involved in E-cadherin loss–induced mitochondrial repression and glycolytic switch. E-cadherin shRNA seemed to increase Snail mRNA and protein levels (Supplementary Fig. S6B, Fig. 7B), indicating that E-cadherin, a Snail-repressible gene, could affect Snail levels, forming a negative-feedback loop between E-cadherin and Snail levels; the molecular basis for E-cadherin loss–triggered Snail induction is under investigation. We further found that Snail shRNA prevented E-cadherin loss–induced EMT (Supplementary Fig. S6A) and vimentin (a mesenchymal marker) induction (Fig. 7C). Because downregulation of cell adhesion molecules, other than E-cadherin, is required for full EMT, we suspect that Snail activated by E-cadherin shRNA may downregulate other cell adhesion molecules for full EMT to occur. Snail shRNA also prevented E-cadherin loss–induced mitochondrial respiration and COX activity.

Figure 5. Induction of the glycolytic switch by Wnt/Snail signaling. A–E, glucose consumption, lactate production, and total ATP concentration were measured in MCF-7 cells treated with Wnt3a CM (A) or transfected with Wnt1 (B), in MCF-7 #5 cells treated with DOX (C), in MCF-7 cells transfected with Snail S104, 107A mutant (D), and in MDA-MB-231 cells transfected with Snail shRNA (E). *, P < 0.05; **, P < 0.01 versus control (untreated, mock, and control shRNA). The level of ATP produced by aerobic respiration (black bars) and glycolysis (gray bars) was determined.
repression and glycolytic switch (Fig. 7A). We examined whether E-cadherin shRNA affects the expression of COX subunits and PC. Real-time PCR (Supplementary Fig. S6B) and Western blot analysis (Fig. 7C, Supplementary Fig. S6C) showed that E-cadherin shRNA reduced the expression of COXVIc and COXVIIa, without affecting the expression of COXVIIc and PC. In addition, Snail shRNA prevented E-cadherin loss–induced downregulation of COXVIc and COXVIIa (Fig. 7C, Supplementary Fig. S6B). The ChIP assay showed that E-cadherin shRNA enhanced the binding of Snail to the E-box sites of the COXVIc and COXVIIa promoters but not to those of the COXVIIc promoters (Fig. 7D). Thus, under this particular condition, Snail could bind to COXVIc and COXVIIa promoters but not to the promoter of COXVIIc; a more detailed mechanism for Snail’s differential binding to the promoters remains to be elucidated. These results show that EMT influences mitochondrial function and glucose metabolism via Snail activation, suggesting that EMT may contribute to Wnt/Snail regulation of mitochondria/metabolism (Supplementary Fig. S7).

Discussion

Wnt/Snail signaling is known to induce EMT, an essential process for tumor invasion and metastasis (4–7). In this study, we show a new function of the Wnt/Snail signaling in suppressing mitochondrial respiration and COX activity in MCF-7 and MDA-MB-231 cells (Figs. 1–3). Similar results were observed in MDCK cells (data not shown). COX regulation occurred through inhibition of the expression of COXVIc, COXVIIa, and COXVIIc. In addition, Wnt/Snail signaling induced a glycolytic switch (Fig. 5). Recently, β-catenin, a downstream mediator of the canonical Wnt pathway, has been shown to downregulate 2 mitochondrial ATPase subunits (ATP5a1 and ATP5b; 30), and...
to induce the expression of genes involved in glucose/glutamine metabolism, including lactate dehydrogenase (LDH; 30), glucose-6-phosphatase, phosphoenolpyruvate carboxykinase (31), glutamine synthetase, ornithine aminotransferase, and glutamate transporter GLT-1 (32). Thus, Wnt signaling seems to affect mitochondrial respiration and glucose/glutamine metabolism by β-catenin- and Snail-dependent mechanisms.

Wnt/Snail signaling seems to regulate PC, a major anaplerotic enzyme (Fig. 6). PC protein is known to be activated or highly expressed in human lung tumors (33) and in hepatic tumors in rats (34). We observed that PC shRNA suppressed mitochondrial respiration/COX activity, and induced the glycolytic switch with lactate production (Supplementary Fig. S5C). Similar enhanced lactate production was observed in cells treated with PC shRNA (35). Although Wnt induces PC, it increased lactate production possibly due to an increase in LDH levels through β-catenin, which is activated in response to Wnt (30). Thus, pyruvate seems to be converted either to lactate by LDH or to oxaloacetate by PC (Supplementary Fig. S7). Although PC could positively regulate the TCA cycle and mitochondrial respiration, mitochondrial function is inhibited by the Wnt/β-catenin/Snail pathway; thus, only the TCA cycle may be active. The active TCA cycle could supply substrates into biosynthetic pathways for tumor cell growth (Supplementary Fig. S7). However, because Wnt3a (Supplementary Table S5) and Snail (36) inhibit cell growth with EMT induction in MCF-7 cells, PC may be implicated in other aspects of cell regulation than cell proliferation. Besides supplying substrates into biosynthetic pathways for cell proliferation (16), PC-mediated anaplerosis is also important for other biologic processes; for example, PC is necessary for glucose-induced insulin secretion by providing oxaloacetate to form malate in islets and for de novo synthesis of glutamate, an important excitatory neurotransmitter in astrocytes (18). At present, the precise role(s) of PC in Wnt3a-induced cell regulation remains to be characterized.

In most cases, Snail acts as a repressor; however, it also acts as a transcriptional activator (37). Because Snail binds to the E-box in the COXIVc, COXVIIa, and COXVIIc promoter regions (Fig. 4C), these genes are likely to be directly regulated by Snail. In the case of PC, although E-boxes were found in the PC gene promoter, Snail did not bind to the promoter (Fig. 6F). Thus, other Snail-binding elements may be responsible for PC upregulation; otherwise, Snail may induce PC expression through

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Figure 7. Inhibition of mitochondrial respiration and induction of the glycolytic switch by E-cadherin knockdown. A, MCF-7 cells were cotransfected with E-cadherin shRNA and Snail shRNA and analyzed for mitochondrial respiration, COX activity, glucose consumption, lactate production, and total ATP concentration. **, P < 0.05; ***, P < 0.01 versus control shRNA; #, P < 0.01 versus E-cadherin shRNA alone. The level of ATP produced by aerobic respiration (black bars) and glycolysis (gray bars) was determined. B and C, MCF-7 cells were cotransfected with E-cadherin shRNA and Snail shRNA. The nuclear fractions (B) or whole-cell lysates (C) were analyzed by Western blotting using the indicated antibodies. D, MCF-7 cells were transfected with E-cadherin shRNA. ChIP assays were conducted using rabbit IgG or rabbit anti-Snail and ChIP-enriched DNA was analyzed by PCR using primers for the indicated genes.
an indirect effect via other transcription factors and/or coactivators/corepressors. Tumor cells with dysregulated mitochondria and the glycolytic switch have been shown to exert apoptotic resistance (38) and exhibit necrosis that could promote tumor progression and aggressiveness upon metabolic stress (39, 40). Wnt (41) and Snail (36, 42, 43) are known to inhibit apoptosis. Furthermore, Snail regulates necrosis in response to metabolic stress (22). Thus, mitochondrial repression by Wnt/Snail may contribute to its known antiapoptotic and pro necrotic activities.

Snail levels are regulated by cytokines such as TGF-β and transcription factors such as hypoxia-inducible factor-1, NF-κB, and p53 (44–46). Therefore, these signals may regulate mitochondrial respiration and glucose metabolism via Snail activation. In fact, TGF-β has been shown to inhibit mitochondrial respiration (23). In this study, we also showed that E-cadherin shRNA, acting as an EMT inducer, repressed mitochondrial respiration and COX activity and induced the glycolytic switch (Fig. 7A), indicating that EMT may contribute to the regulation of mitochondrial function and metabolism by Wnt/Snail and possibly other EMT inducers. Interestingly, Snail was found to be implicated in E-cadherin loss–induced cell regulation (Fig. 7). Recently, Onder and colleagues (2008) showed that E-cadherin loss induces EMT via activation of β-catenin and an EMT inducer, Twist (29); thus, E-cadherin loss seems to activate multiple factors, including Snail, β-catenin, and Twist, to elicit diverse biologic responses including EMT and regulation of mitochondria/metabolism. EMT has been shown to induce generation of cancer stem cells (47–49). Snail is also known to induce acquisition of a stem cell–like phenotype in ovarian cancer cells (43). In addition, human embryonic stem cells exhibit a high glycolytic rate similar to cancer cells (50). Thus, Snail-induced mitochondrial repression and glycolytic switch may be associated with alterations in mitochondrial function and metabolism of embryonic and cancer stem cells.

In summary, we show a new function of the Wnt/Snail signal pathway in the regulation of mitochondrial function and glucose metabolism. These findings provide a novel mechanistic insight into the mechanism of Wnt/Snail-mediated tumor progression and aggressiveness and how the Wnt/Snail signal pathway causes a survival advantage and withstands therapeutic agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Grant Support
This work was supported by the Bio-Scientific Research Grant funded by the Pusan National University (PNU, Bio-Scientific Research Grant; PNU-2008-101-103). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

Received January 4, 2012; revised April 20, 2012; accepted May 8, 2012; published OnlineFirst May 25, 2012.

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