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# Wnt6 induces the specification and epithelialization of F9 embryonal carcinoma cells to primitive endoderm

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**Abstract**

Epithelial-to-mesenchymal transitions (EMTs) play key roles in the normal development of an organism as well as its demise following the metastasis of a malignant tumour. An EMT during early mouse development results in the differentiation of primitive endoderm into the parietal endoderm that forms part of the parietal yolk sac. In the embryo, primitive endoderm develops from cells in the inner cell mass, but the signals that instruct these cells to become specified and adopt an epithelial fate are poorly understood. The mouse F9 teratocarcinoma cell line, a model that can recapitulate the in vivo primitive to parietal endoderm EMT, has been used extensively to elucidate the signalling cascades involved in extraembryonic endoderm differentiation. Here, we identified *Wnt6* as a gene up-regulated in F9 cells in response to RA and show that Wnt6 expressing cells or cells exposed to Wnt6 conditioned media form primitive endoderm. Wnt6 induction of primitive endoderm is accompanied by β-catenin and Snail1 translocation to the nucleus and the appearance of cytokeratin intermediate filaments. Attenuating glycogen synthase kinase 3 activity using LiCl gave similar results, but the fact that cells de-differentiate when LiCl is removed reveals that other signalling pathways are required to maintain cells as primitive endoderm. Finally, Wnt6-induced primitive endodermal cells were tested to determine their competency to complete the EMT and differentiate into parietal endoderm. Towards that end, results show that up-regulating protein kinase A activity is sufficient to induce markers of parietal endoderm. Together, these findings indicate that undifferentiated F9 cells are responsive to canonical Wnt signalling, which negatively regulates glycogen synthase kinase 3 activity leading to the epithelialization and specification of primitive endoderm competent to receive additional signals required for EMT. Considering the ability of F9 cells to mimic an in vivo EMT, the identification of this Wnt6–β-catenin–Snail signalling cascade has broad implications for understanding EMT mechanisms in embryogenesis and metastasis.


*Keywords:* Wnt6; Snail; β-catenin; F9 cells; EC cells; Primitive endoderm; EMT

## 1. Introduction

The formation of parietal endoderm from primitive endoderm is one of the earliest epithelial–mesenchymal transitions (EMTs) in mouse development. The F9 teratocarcinoma cell line has been used extensively as a cell autonomous model to study retinoic acid (RA) signalling, and following RA treatment the pluripotent stem cells differentiate into primitive endoderm [1]. When these cells are subsequently treated with dibutyryl cyclic AMP ($Bt_2$-cAMP), they complete the EMT and form parietal endoderm [2,3]. Through the use of exogenous agents, many signalling pathways and their associated proteins have been found to participate in the F9 differentiative process, including the MAP kinase pathway [4], G-proteins [5–9], cytoskeletal and ERM proteins [10–12], and numerous transcription factors [13–17]. β-catenin is one protein that interacts with the T-cell factor–lymphoid enhancer factor during F9 primitive endoderm differentiation [15]. We have extended these results to show that although β-catenin is necessary for F9 primitive endoderm differentiation, it alone is not sufficient to carry out this process to completion [9]. Our previous study reported that LiCl induced primitive endoderm differentiation, but when removed the cells

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reverted back to the undifferentiated state. Lithium directly inhibits glycogen synthase kinase 3 (GSK-3) activity [18], which influences the inactivation of glycogen synthase, and also the ability to target β-catenin to the proteasome for degradation [19]. This regulation of GSK-3 activity by insulin, growth factors and Wnt signalling pathways, is of paramount importance because it influences a host of biological responses including glycogen metabolism, protein synthesis, apoptosis, cell proliferation, migration and differentiation and the establishment of the embryonic body plan [20].

Wnts are secreted glycoproteins that stimulate a number of signal transduction pathways in cells of the embryo and adult of both vertebrates and invertebrates [21]. Nineteen distinct *Wnt* genes in the vertebrate genome encode ligands that signal through members of the Frizzled serpentine receptor family and low-density lipoprotein-receptor related protein co-receptors [reviewed in [21,22]]. In the vertebrate canonical-β-catenin pathway, Wnt bound to Frizzled and low-density lipoprotein-receptor related protein 5 or 6 employs Dishevelled and G-proteins to dismantle a multimeric protein complex that includes Axin, adenomatous polyposis coli tumour suppressor protein, casein kinase 1-α, GSK-3β and β-catenin [23]. Without this complex, β-catenin accumulates in the cytosol and eventually translocates to the nucleus where it interacts with members of the T-cell factor–lymphoid enhancer factor family to regulate the expression of a number of target genes. Wnt signalling in the non-canonical planar cell polarity pathway also plays a key role orchestrating cell movements during gastrulation [24,25]. In this case, Dishevelled signals downstream to Rho and its effector Rho-kinase or through Rac/CDC42 to c-Jun N-terminal kinase to modulate cytoskeletal dynamics [reviewed in [26]].

Wnt signalling positively and negatively regulates the differentiation of F9 cells into primitive endoderm [5–7,15,27]. Differentiation involves a highly coordinated molecular event that requires, in part, β-catenin–T-cell factor–lymphoid enhancer factor gene activation for primitive endoderm formation [15], followed by an up-regulation of the transcriptional repressor Snail1 for parietal endoderm [28,29]. Snail, a highly unstable protein with a half-life of approximately 25 min [30], represses several genes involved in cell adhesion including the cadherins [31], as well as genes encoding cell–cell communication proteins like connexins [29]. Snail1 is regulated by GSK-3β, which dictates its sub-cellular localization and subsequent degradation by the proteasome [32]. Thus, when GSK-3 is inactive, Snail1 levels increase and in turn, its ability to negatively regulate genes promotes EMT [33].

To better understand the signalling pathways governing EMT associated with extraembryonic endoderm differentiation, and extending from our previous results that canonical–β-catenin signalling plays a role during this differentiation, we focused on identifying the Wnt(s) up-regulated in response to RA treatment. An RT-PCR approach using degenerate oligodeoxynucleotide primers to clone *Wnt* genes [34] identified a cDNA with sequence homology to *Wnt6*. Wnt6 is an attractive endogenous candidate involved in primitive/parietal endoderm differentiation for several reasons: 1) it signals through β-catenin [35]; 2) its expression coincides with EMTs in chick [36]; 3) it is expressed at high levels in the inner cell mass of the developing mouse [37]; and 4) within the conserved region of the rat *Wnt6* and human *WNT6* promoters there exists a GATA binding site [38] with a signature recognized by GATA-6 [39], a key player in the differentiation of extraembryonic endoderm and whose gene is regulated by RA [40,41]. With this evidence we hypothesized that Wnt6 signalling through β-catenin accompanies the retinoic acid-induced differentiation of F9 cells to primitive endoderm. Furthermore, this signalling is expected to inactivate GSK-3, causing levels of the Snail1 protein to increase and eventually translocate to the nucleus where it acts to regulate the genes that impede EMT. Since *Wnt6* was found to be up-regulated in response to RA, we decided to test its effect on differentiation in the absence of RA. A mouse *Wnt6* cDNA was cloned and expressed in F9 cells or expressed in F9 cells or COS-7 cells and the conditioned media applied to undifferentiated F9 cells. Results show that transient over-expression of *Wnt6* in F9 cells is capable of inducing primitive endoderm with β-catenin and Snail1 translocating to the nucleus. LiCl treatment gave similar results, but cells de-differentiate when LiCl was removed. Furthermore, Wnt6-induced primitive endoderm cells remain competent to respond to additional cues and by artificially activating protein kinase A, differentiate to parietal endoderm. Thus, undifferentiated F9 cells respond to canonical Wnt6–β-catenin signalling, become specified to be epithelial in nature and will become determined to form primitive endoderm, competent to complete EMT if the appropriate signal(s) are present.

## 2. Materials and methods

### 2.1. Cell culturing and protein extractions

Mouse F9 teratocarcinoma cells (ATCC) were cultured on 0.1% gelatin coated 35 mm plates for protein isolation, or gelatin coated cover slips for immunofluorescence. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (PS) antibiotic (Gibco). When cells were approximately 40% confluent, they were treated with either $10^{-7}$ M retinoic acid (RA all-*trans*; Sigma), or $10^{-7}$ M RA plus 1 mM dibutyryl cAMP ($Bt_2$-cAMP; Sigma). Control and treated cells, incubated at 37 °C and 5% $CO_2$, were collected 24 h after the initial treatment and every 24 h thereafter. For protein isolation, cells were lysed in 1 ml of 20% sodium dodecyl sulfate (SDS) loading buffer containing 10 μl of 100 mM phenylmethylsulfonyl fluoride (Sigma). Extracts were collected and centrifuged at 15,000 ×*g* for 15 min at room temperature and the supernatants stored at −20 °C.

### 2.2. Immunoblot analysis

Protein concentration from control and treated cells was determined using the Bradford assay (Bio-Rad). Twenty micrograms of protein was separated on 10% SDS polyacrylamide gels. Following electrophoresis, gels were equilibrated in 20 mM Tris–acetate, 0.1% SDS and 0.1 mM DTT and the proteins transferred electrophoretically to nitrocellulose transfer membrane (Biotrace; Pall Corp), using methanol as the buffer. Membranes were probed with antibodies and the signals detected using the West Pico Western Blot Chemiluminescent Detection System (Pierce). The primary antibodies were directed against Thrombomodulin (TM) (1:1000; Santa Cruz), GAPDH (1:1000; Santa Cruz) and TROMA-1 (1:50; Developmental Studies Hybridoma Bank). Horseradish peroxidase-conjugated goat anti-rabbit, anti-rat, or rabbit anti-goat secondary antibodies (Pierce) were used at a dilution of 1:1000.