ELSEVIER

Mechanisms of Development 91 (2000) 249–258

MOD

www.elsevier.com/locate/modo

Brachyury is a target gene of the Wnt/β-catenin signaling pathway

Sebastian J. Arnold, Jörg Stappert, Andreas Bauer, Andreas Kispert, Bernhard G. Herrmann, Rolf Kemler*

Max-Planck Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany

Received 11 October 1999; received in revised form 15 November 1999; accepted 16 November 1999

Abstract

To identify target genes of the Wnt/β-catenin signaling pathway in early mouse embryonic development we have established a co-culture system consisting of NIH3T3 fibroblasts expressing different Wnts as feeder layer cells and embryonic stem (ES) cells expressing a green fluorescent protein (GFP) reporter gene transcriptionally regulated by the TCF/β-catenin complex. ES cells specifically respond to Wnt signal as monitored by GFP expression. In GFP-positive ES cells we observe expression of *Brachyury*. Two TCF binding sites located in a 500 bp *Brachyury* promoter fragment bind the LEF-1/β-catenin complex and respond specifically to β-catenin-dependent transactivation. From these results we conclude that *Brachyury* is a target gene for Wnt/β-catenin signaling. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Mouse embryonic development; Mesoderm formation; Embryonic stem cells; Wnt/β-catenin signaling pathway; Brachyury

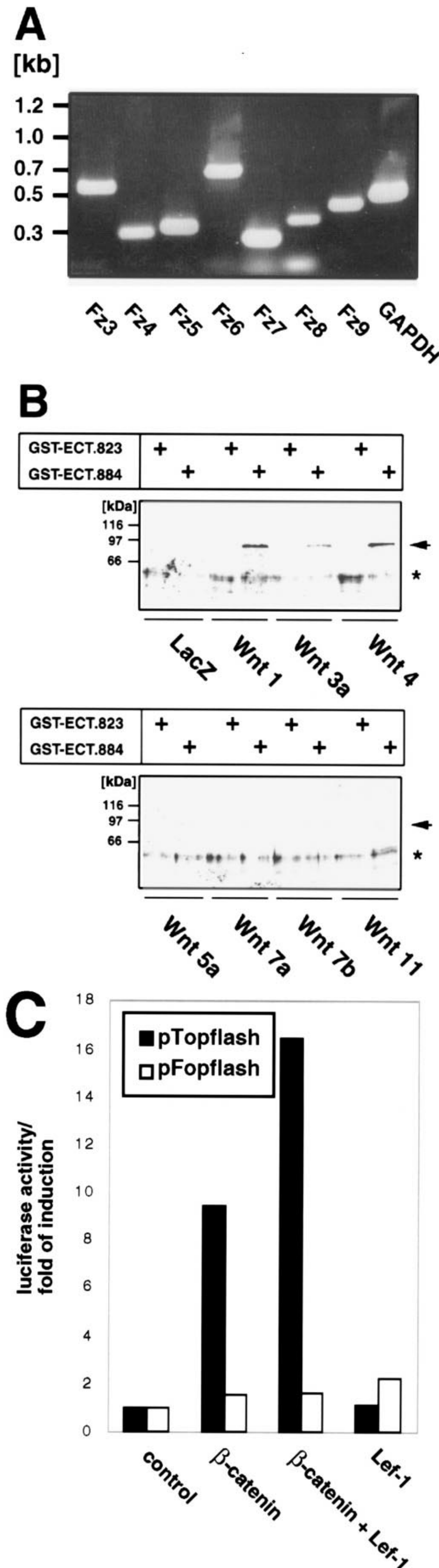
1. Introduction

Genetic analysis in *Drosophila* has already identified the major components of the Wingless (Wg) signal transduction pathway, which determines cell fate decision during embryonic development (for review, Cadigan and Nusse, 1997; Peifer, 1997). More recently, additional components of this pathway have been described in vertebrate cells, demonstrating that this signaling pathway is evolutionarily highly conserved and controls important cellular processes such as embryonic induction, cell fate specification, and the generation of cell polarity (for review, Gumbiner, 1998; Moon and Kimelman, 1998; Gradl et al., 1999a). Structural and functional homologues of the pathway exist throughout the animal kingdom but have been best characterized in *Drosophila* and vertebrates: The Wg/Wnt secreted glycoproteins bind to the Frizzled (fz) membrane receptors, which interact with dishevelled, leading to an inactivation of the shaggy/GSK3β kinase. The inactivation of the kinase results in a cytoplasmic stabilization of Armadillo/β-catenin, due to hypo-phosphorylation which enables binding of Armadillo/β-catenin to pangolin/TCF/LEF-1 transcription factors and a nuclear translocation of this protein complex that ultimately controls target gene expression. β-catenin is a pivotal component of the Wnt/Wg signaling pathway since its cytoplasmic turnover is tightly regulated (Yost et al., 1996; Aberle et al., 1997; Orford et al., 1997; Jiang and Struhl, 1998). Degradation of β-catenin requires, besides GSK3β, multimeric protein interactions involving adenomatous polyposis coli (APC) and Axin/Conductin. β-Catenin is also a major component of the cellular adhesion machinery, since it binds to the intracellular domain of cadherins and through its association with α-catenin links cadherins to the actin cytoskeleton (Kemler, 1993; Ben-Ze'ev and Geiger, 1998). Thus β-catenin can form complexes with various binding partners present in different cell compartments, i.e. the plasma membrane, the cytoplasm and the nucleus. The cell has developed elaborate ways to assure the specific function of β-catenin in each compartment. One aspect of this regulation is inherent in the structural organization of β-catenin itself, since the key protein interactions with either E-cadherin, APC or TCF/LEF-1 are at overlapping sites and thus mutually exclusive (Hulsken et al., 1994; Rubinfeld et al., 1995; Orsulic et al., 1999). Moreover, there appears to be a hierarchy for β-catenin in binding to these partners, with binding to E-cadherin being dominant over that to APC and TCF/LEF-1, while TCF/LEF-1

* Corresponding author. Tel.: +49-761-510-8471; fax: +49-761-510-8474.

E-mail address: kemler@immunbio.mpg.de (R. Kemler)

0925-4773/00/$ - see front matter © 2000 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0925-4773(99)00309-3



can bind to β-catenin which has escaped the APC/Axin/GSK3β degradation machinery (Orsulic et al., 1999). An interaction of the cytoplasmic, stabilized β-catenin with TCF/LEF-1 and its nuclear localization is, under physiological conditions, the result of a Wnt signal. Intriguingly, the Wnt signaling cascade becomes activated in several types of tumor cells. For example the proto-oncogene Wnt-1 promotes mammary gland hyperplasia in mice (Nusse and Varmus, 1982). Unphysiologically stabilized cytoplasmic β-catenin has been observed in colon carcinomas, due either to gain-of-function mutations in β-catenin itself or to mutations in its binding partners E-cadherin and APC (Munemitsu et al., 1995; Robbins et al., 1996; Korinek et al., 1997; Rubinfeld et al., 1997). In the nucleus TCF/LEF-1 binds to specific nucleotide sequences and together with β-catenin confers transactivation activity, and this complex is thus believed to regulate the expression of target genes. Several potential target genes have recently been reported in development, e.g. in *Drosophila ultrabithorax* (Riese et al., 1997), in *Xenopus siamois* (Brannon et al., 1997), *Twin* (*Xtwin*; Laurent et al., 1997), the secreted protein *nodal-related 3* (*Xnr3*; McKendry et al., 1997), engrailed-2 (McGrew et al., 1999) and *fibronectin* (Gradl et al., 1999b) as well as in colon cancer, e.g. *c-myc* and *cyclin D1* (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999).

We are interested in identifying Wnt target genes during early mouse development. In a working model we had previously proposed that Wnt signaling might play an important role in the epithelial-mesenchymal transition in gastrulation stage embryos (Huber et al., 1996a). To monitor Wnt-dependent gene expression we have exploited mouse embryonic stem (ES) cells and established a co-culture system with NIH3T3 cells expressing different Wnts. Here we provide evidence that the mesoderm-specific gene *Brachyury* is regulated by the Wnt signaling pathway in ES cells.

Fig. 1. Characterization of Wnt signaling components in ES cells. (A) ES cells were tested for their competence to receive Wnt signal by RT-PCR analysis with primer pairs specific for different Frizzleds. Frizzled 3 to 9 are expressed on the mRNA level. (B) Affinity precipitation with recombinant fusion protein GST-ECT.884 containing the entire cytoplasmic region of E-cadherin or with GST-ECT.823 lacking the β-catenin-binding site as a control and cell lysates from ES cells stimulated by different Wnt-expressing NIH3T3 cells. GST-ECT.884 efficiently precipitated β-catenin from ES cells stimulated by Wnt1, 3a and 4. Arrow indicates the position of β-catenin; asterisk denotes a contamination due to secondary antibody in western blots. (C) ES cells respond to β-catenin/LEF-1 transactivation. Cells were co-transfected with pTOPFLASH and control pFOPFLASH reporter constructs and β-catenin and/or LEF-1 cDNAs in combination and analyzed for luciferase activity. Transfection with *lacZ* cDNA was included to correct variation in transfection efficiencies. pTOPFLASH contains three TCF/LEF-1 binding sites driving the luciferase reporter; the inactive mutant form of this vector (pFOPFLASH) was included for a control. β-catenin already led to an induction of activity, which was further increased by the addition of LEF-1.

2. Results and discussion

2.1. ES cells respond to Wnt signal

In our attempts to establish a cell system appropriate for the identification of Wnt-dependent target genes expressed during early mouse embryonic development we have concentrated on embryonic stem (ES) cells. We first investigated whether ES cells express the relevant components required for the transduction of Wnt signal. Indeed several Wnt receptors were found to be expressed in ES cells as monitored by RT-PCR analysis, including Frizzled (fz) 3 to 9 (Fig. 1A). These results suggested that ES cells can respond to Wnt signal when co-cultured on fibroblasts which express different Wnts. And in fact, a free cytoplasmic pool of β-catenin was observed in ES cells co-cultured on Wnt-expressing NIH3T3 fibroblasts (Fig. 1B). For these studies, we used fusion proteins containing the glutathione-*S*-transferase (GST) tag and the full-length cytoplasmic domain of E-cadherin (GST-ECT.884), or, as a control, a truncated version lacking the β-catenin-binding site (GST-ECT.823) in binding assays with cell lysates from ES cells stimulated by different Wnt-expressing NIH3T3 cells. Interestingly, not all Wnts were able to induce a cytoplasmic pool of β-catenin in ES cells (Fig. 1B). Stimulation by Wnt1, 3a and 4 resulted in a clearly detectable free cytoplasmic pool of β-catenin, while others (Wnt5a, 7b) only occasionally induced a small amount of cytoplasmic β-catenin. Among those which could induce cytoplasmic β-catenin was Wnt4, which is believed to belong to the subgroup of Wnt molecules not signaling via β-catenin. It is likely that Wnt4 uses one of the several Frizzled receptors under these experimental conditions (Fig. 1A). It has been reported previously that ES cells express TCF3 and 4, the downstream components of the Wnt signaling pathway which are required for the nuclear function of β-catenin (Korinek et al., 1998). To test the ability of ES cells to respond to a Wnt signal, ES cells were transiently transfected with the TCF/β-catenin-dependent luciferase reporter plasmid pTOPFLASH; pFOPLASH served as a control. These two plasmids contain promoters with three copies of an optimal or in the control a mutant version of a TCF-response motif upstream of a minimal c-*fos* promoter driving luciferase expression (Korinek et al., 1997). Transient transfection of the pTOPFLASH reporter plasmid together with a β-catenin cDNA encoding a stabilized mutant form of the protein (S33A) (Aberle et al., 1997) led to high luciferase activity which was further increased by the co-transfection of LEF-1 (Fig. 1C). A *lacZ* expression construct was included in all transfection reactions so that β-galactosidase (β-gal) activity could be used to normalize luciferase activity (not shown). All these results indicated that ES cells would be suitable to study the response to Wnt signaling.

Next we tested whether ES cells respond to Wnt signals expressed by NIH3T3 fibroblasts stably transfected with different Wnt cDNAs (Kispert et al., 1998). The expression efficiency of Wnts in the fibroblasts was controlled by northern blot and RT-PCR analysis (not shown). To monitor the Wnt responsiveness of ES cells under such culture conditions, a green fluorescent protein reporter construct (pTOP-FLASH-GFP) was stably introduced into ES cells. The conceptual idea behind this approach had two aspects. First, ES cells which received a Wnt signal should express GFP and thus could be isolated by fluorescence-activated cell sorting (FACS). Second, we assumed that in GFP-positive ES cells endogenous target genes would also be activated due to Wnt signal. As can be seen in Fig. 2, ES cells indeed respond to Wnt signaling as monitored by GFP expression. However, ES cells did not respond to all Wnts; co-culture of ES cells on NIH3T3 cells expressing Wnt1, 3a and 4 clearly led to an expression of GFP, while co-culture on fibroblasts expressing Wnt5a, 7a, 7b and 11 had no effect on GFP expression (not shown), similar to co-culture with NIH3T3 cells expressing *lacZ*.

These results clearly demonstrated that ES cells differentially respond to Wnt signals.

2.2. The β-catenin/LEF-1 complex binds and activates the *Brachyury* promoter fragment

A first hint that *Brachyury* might be a potential target

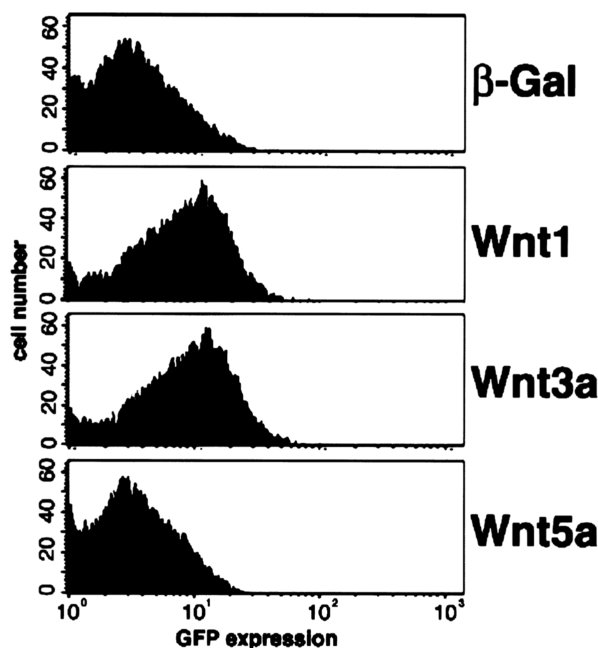


Fig. 2. ES cells respond to Wnt signaling. A co-culture system was established with NIH3T3 fibroblasts expressing different Wnts and β-galactosidase (β-Gal) as feeder layer cells. Stably transfected ES cells expressing TOPFLASH-GFP were co-cultured on Wnt-expressing fibroblasts for 24 h and cells were subjected to FACS analysis. Co-culture of NIH3T3 cells transfected with *lacZ* and Wnt5a led only to a basal expression of GFP. Similar results were obtained with fibroblasts expressing Wnt7a, 7b, 11 (not shown). An induction of GFP-expression was observed in ES cells co-cultured on fibroblasts expressing Wnt1, 3a (4, not shown).

gene for Wnt signaling came from our studies with cells genetically altered for E-cadherin expression (Huber et al., 1996b). We noticed that ES cells negative for E-cadherin (E-cad −/−) mimic part of the Wnt signaling pathway, particularly that they expressed LEF-1 and exhibited nuclear localization of β-catenin (Huber et al., 1996b; Orsulic et al., 1999). Moreover, E-cad −/− cells expressed *Brachyury* as shown in northern blot analysis (Larue et al., 1996). Remarkably, re-introducing E-cadherin into E-cad −/− ES cells caused β-catenin to be complexed at the cell membrane, resulting in a down-regulation of both LEF-1 and *Brachyury* expression. These results suggested some link between the expression of LEF-1 and *Brachyury* and the localization of β-catenin in different cell compartments. This stimulated us to make a sequence analysis of the *Brachyury* promoter for potential TCF/LEF-1 binding sites matching the consensus sequence WWCAAWGG. Two potential TCF/LEF-1 binding sites were identified 191 (TCF-site I) and 273 (TCF-site II) bp upstream from the transcriptional start site of *Brachyury* (Fig. 3A, boxed sequences). To directly assay for binding of LEF-1 and β-catenin to these sites, electrophoretic gel-mobility shift analysis was performed with recombinant LEF-1 and β-catenin produced in *E. coli* (Fig. 3B). LEF-1 bound specifically to the promoter fragment of *Brachyury*, and co-incubation with β-catenin produced a LEF-1/β-catenin complex that interacts with DNA. The size of this complex was further increased by the addition of monoclonal anti-β-catenin antibody. As expected, no binding of β-catenin/LEF-1 was observed when TCF binding sites I and II in the promoter were mutated (Fig. 3B). These experiments show that there is specific binding of the β-catenin/LEF-1 complex to either or both of the two TCF recognition sites in the *Brachyury* promoter. To demonstrate that these two TCF-sites can confer transactivation activity mediated by β-catenin, luficerase reporter assays were performed in ES cells. Wild-type (wt) and mutated (TCF I^m and II^m) *Brachyury* promoter-luciferase constructs were co-transfected with the S33A β-catenin mutant (Fig. 3C). S33A β-catenin caused a more than 10x fold increase of the reporter activity from the wild-type promoter. However, mutations in either TCF I or TCF II considerably reduced β-catenin-dependent activation. Mutations in both TCF sites at once resulted in a further reduction of β-catenin-dependent luciferase activity to almost basal level (Fig. 3C). The addition of LEF-1 in these reporter assays gave only a marginal increase of activation (not shown), most likely due to the fact that ES cells express the TCF/LEF-1 family members TCF3 and 4 endogenously (Korinek et al., 1998). These experiments strongly suggested that *Brachyury* is regulated by β-catenin/LEF-1 and may thus be a target gene of the Wnt-signaling pathway.

2.3. Wnt signal induces Brachyury expression

We next examined whether *Brachyury* expression can also be detected in ES cells co-cultured on Wnt-expressing feeder cells. For this, ES cells were co-cultured on Wnt-expressing NIH3T3 cells for 24 h and subjected to FACS cell sorting. The GFP-positive ES cells were analyzed for *Brachyury* expression by RT-PCR (Fig. 4A).

Indeed, expression of *Brachyury* was induced in GFP-positive ES cells by Wnt1, 3a and 4, but not by Wnt5a, 7a, 7b and 11. These results are remarkable in several respects. Most importantly, endogenous *Brachyury* expression can be induced in ES cells after co-culture on Wnt-expressing fibroblasts. Yet, not all Wnts appear to induce *Brachyury* expression. It is very unlikely that *Brachyury* expression is due to some unspecific mitogenic stimulus since not all Wnts were able to induce *Brachyury* expression. To demonstrate unambiguously the Wnt-induced expression of *Brachyury*, northern blot analysis were performed on mRNA from ES cells co-cultured on either Wnt3a- or β-gal-expressing NIH3T3 cells (Fig. 4B). *Brachyury* mRNA was only detected in cells exposed to Wnt3a signal. The finding that *Brachyury* expression is a direct consequence of the Wnt signaling pathway was further supported by experiments in which the pathway was activated by the stable β-catenin mutant S33A, acting downstream of Wnt. ES cells were transiently transfected with a vector coding either GFP and myc-tagged S33A-β-catenin or with a vector coding only GFP as a control. GFP-positive cells of both transfections were sorted by FACS. As can been seen in an immunoblot with anti-myc antibodies (Fig. 4C left panel), β-catenin is only detectable in cells expressing the mutant S33A form and only in these cells was *Brachyury* expression detected by RT-PCR analysis (Fig. 4C, right panel). These experiments clearly demonstrate that *Brachyury* is a target gene of the Wnt signaling pathway.

The *Brachyury* gene was originally identified by positional cloning in the mouse (Herrmann et al., 1990) and is the founder member of a family of transcription factors called the T-box gene family (Papaioannou and Silver, 1998; Smith, 1999). *Brachyury* homologues have been isolated in various species and the expression pattern is highly conserved among different vertebrates. In the mouse, *Brachyury* expression starts at the onset of gastrulation. It is restricted to the nascent and migrating mesoderm, the node, the notochordal plate and the notochord (Wilkinson et al., 1990).

The regulation of *Brachyury* expression has largely been studied in *Xenopus* and mouse. It has been shown that mesoderm-inducing signals of the fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) families can induce the expression of *Xbra*, the *Xenopus Brachyury* gene (Latinkic et al., 1997). A 381 bp fragment 5′ of the *Xbra*2 transcription start site is sufficient to confer responsiveness to FGF and activin. In analyzing different portions of the *Brachyury* promoter region in the mouse, it became apparent that several regulatory sequences in the promoter are required for activation of *Brachyury* in the primitive

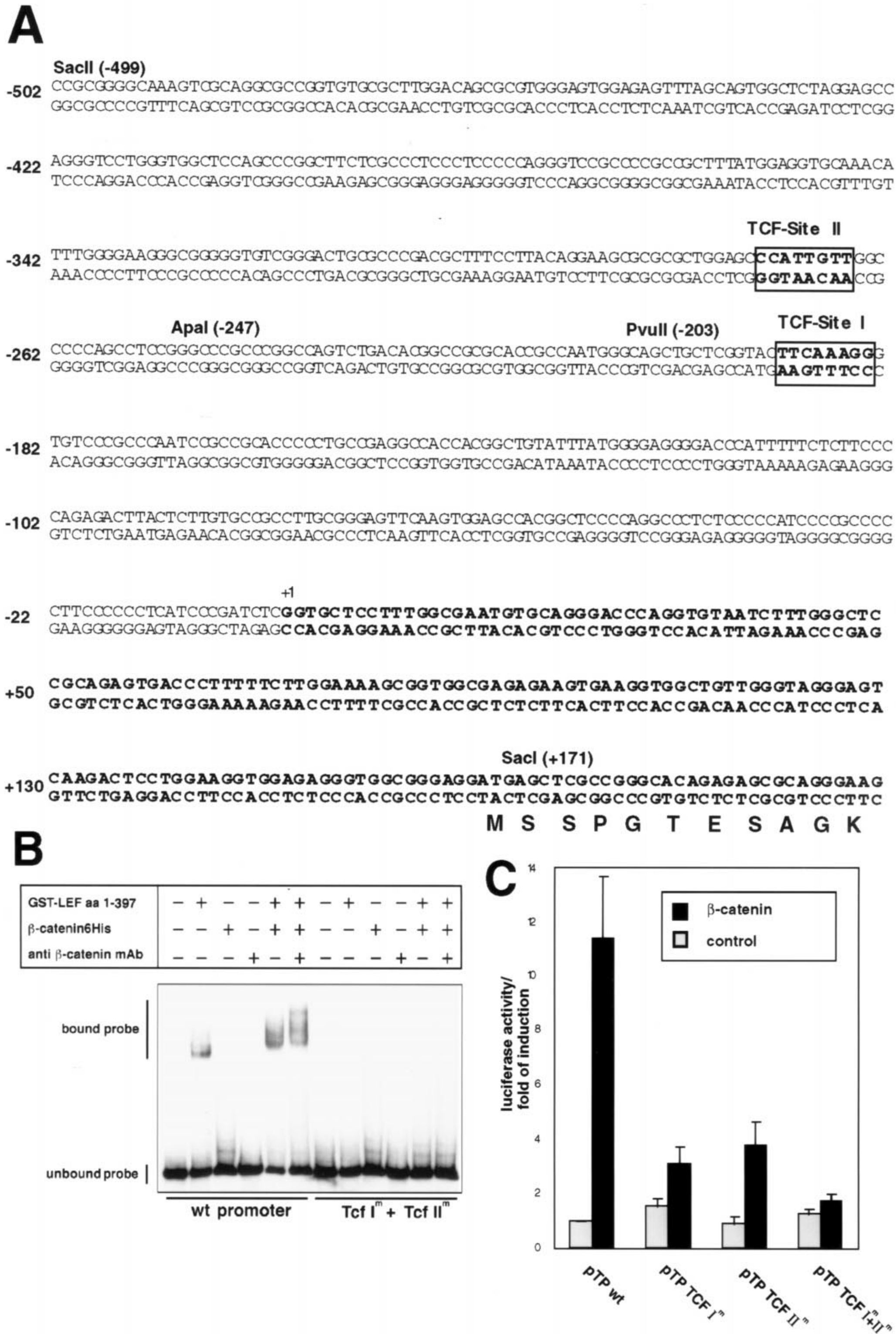


Fig. 3. The 500 bp promoter fragment of *Brachyury* contains two functional TCF motifs. (A) Nucleotide sequence of the 500 bp promoter fragment with the boxed TCF binding sites indicated (TCF-site I and II). The transcriptional and translational start sites of the *Brachyury* mRNA are depicted and the transcribed region is in bold letters. (B) Electrophoretic gel-mobility shifts with the *Brachyury* promoter fragment showing binding of recombinant LEF-1 and β-catenin to the wild-type (wt) promoter fragment but not to the promoter with mutated TCF motifs (TCF I^m and II^m). (C) Luciferase reporter assays with the 500 bp promoter fragment of *Brachyury* in ES cells. The wild-type promoter fragment (pTP wt) is induced approximately 11-fold upon co-transfection with the stabilized form of β-catenin S33A. Mutations in either TCF binding site in the promoter (TCF I^m or TCF II^m) resulted in a reduction of β-catenin-dependent transactivation. Mutations in both TCF binding sites gave an even stronger reduction of activity (TCF I^m + II^m).

streak, the node, or the notochord (Clements et al., 1996). A 500 bp promoter fragment 5′ of the mouse *Brachyury* transcriptional start site is sufficient to drive the expression of the *lacZ* reporter gene in the primitive streak but is not sufficient to confer expression of the transgene in the head process and notochord (Clements et al., 1996). From these results it was concluded that the 500 bp promoter harbors the transcriptional control elements that mediate the response to mesoderm-inducing signals. We show here that the 500 bp promoter fragment is regulated by β-catenin/LEF-1 and that Wnt signaling induces expression of *Brachyury*. Thus, alongside the already mentioned regulation by FGF and activin, we add a new control mechanism which regulates the expression of *Brachyury*. It is likely that Wnt, FGF, and TGF-like signaling pathways act in concert to control *Brachyury* expression. Such a cooperation of different signaling pathways in regulating gene expression in development is likely to be of general importance (Moon et al., 1997; Torres et al., 1999). It has been reported that in *Xenopus* the expression of Siamois is regulated by the cooperation of the Wnt and the SMAD2 pathways (Crease et al., 1998). Interestingly, two potential TCF binding sites can also be found in the *Xenopus Xbra* promoter region at comparable intervals and distances from the transcriptional start (Latinkic et al., 1997). This similarity suggests that Wnt signaling controls *Brachyury* expression in *Xenopus* as well as in mouse.

In the mouse, among the known Wnt genes, Wnt3 is the most likely candidate to regulate *Brachyury* expression during early mesoderm formation. Recently it has been shown by gene targeting that Wnt3 is required for embryonic axis formation (Liu et al., 1999). Embryos lacking Wnt3 do not form a primitive streak, mesoderm or node, and expression of *Brachyury* is abolished in these embryos. In later stages of development, Wnt3a must also be considered as a regulator of *Brachyury* expression, i.e. in the paraxial mesoderm. Experimental gene targeting of Wnt3a affects dorsal mesoderm development with a primary defect in developing somites and *Brachyury* expression is greatly reduced and altered (Takada et al., 1994). A Wnt3a-like mutant phenotype was also observed in LEF-1/TCF1 double mutant embryos (Galceran et al., 1999). From the results reported here and the gene targeting of Wnt3 and Wnt3a

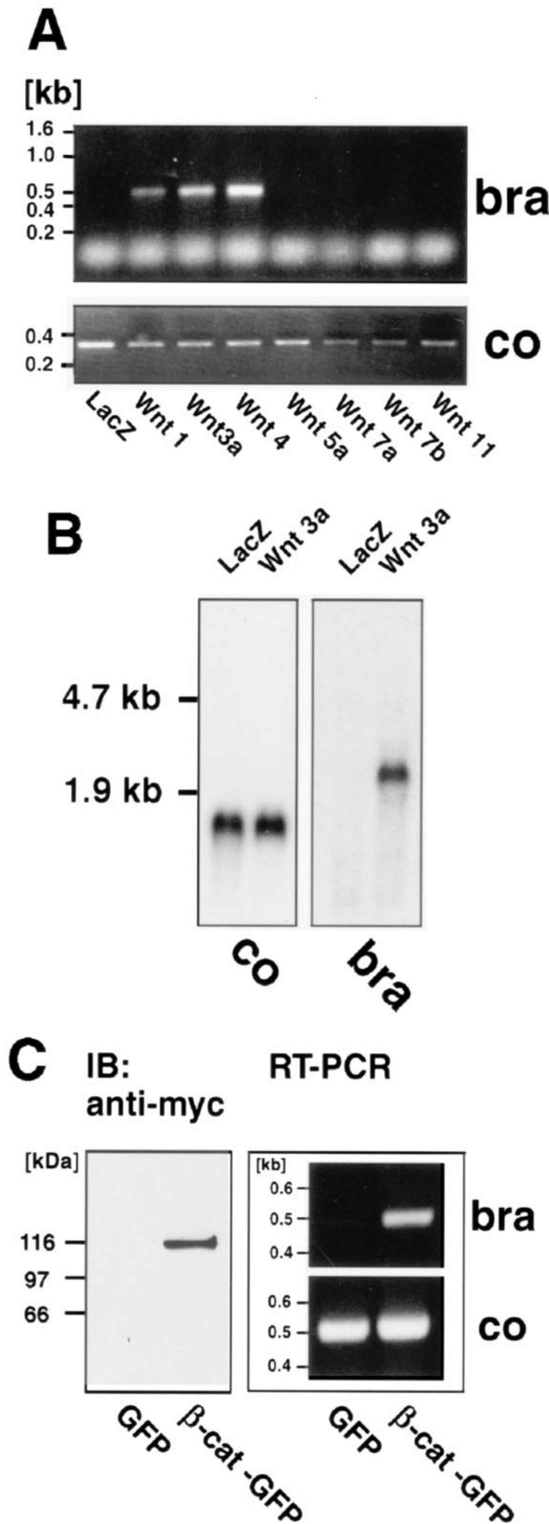


Fig. 4. Induction of *Brachyury* mRNA by Wnt/β-catenin signaling. (A) GFP-positive ES cells obtained after co-culture on Wnt-expressing NIH3T3 cells and isolated by FACS were subjected to RT-PCR analysis with primer pairs for *Brachyury* and for HPRT as a control. *Brachyury* is expressed only in ES cells which received either Wnt1, 3a or 4 signals but not in ES cells stimulated by Wnt5a, 7a, 7b and 11 nor by control co-cultures on fibroblasts transfected with *lacZ*. (B) Poly(A)$^+$ RNA (2 μg per lane) from ES cells co-cultured on NIH3T3 cells transfected with either Wnt3a or *lacZ* cDNA was probed for the expression of *Brachyury* by northern blot analysis. GAPDH expression was included for a control. (C) β-catenin induces expression of *Brachyury* in ES cells. Cells were transiently transfected with an expression cassette containing *gfp* and the myc-tagged S33A β-catenin cDNA (pCS2+ MMBCS33A6mt-GFP) or with *gfp* cDNA (pCS2+-GFP) as a control and GFP-positive ES cells were isolated by FACS after 36 h. GFP-positive cells were subjected to immunoblot analysis with anti-myc to detect myc-β-catenin protein (left panels) or RT-PCR analysis to monitor *Brachyury* expression (right panels). GAPDH was included for a control. The expression of *Brachyury* was detected only in ES cells which are positive for myc-β-catenin. bra, *Brachyury*; co, control HPRT/GAPDH.

we propose that Wnt3 is the signaling component regulating early streak expression of *Brachyury*, while Wnt3a controls the expression in the paraxial mesoderm and tailbud (Galceran et al., 1999). Although Wnt3 was not included in our ES cell co-culture experiments, its closest homologue Wnt3a can induce *Brachyury* expression, suggesting that ES cells may also be able to respond to Wnt3.

The ES cell co-culture system reported here seems well suited to identify more mesoderm-specific genes which are induced by Wnt signal. We have also observed induction of several other early mesodermal marker genes in our co-culture system. These genes are currently being investigated as potential targets of Wnt signaling.

3. Materials and methods

3.1. Cell culture

Mouse embryonic stem (ES) cells were grown on gelatinized tissue culture dishes in 60% buffalo rat liver (BRL) cell conditioned medium (Smith and Hooper, 1987).

NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle's (DMEM)-medium supplemented with 10% fetal calf serum (FCS).

For co-culturing of ES cells with NIH3T3 fibroblasts, the fibroblasts were plated 1 day before co-cultivation at a density to reach 80% confluence on the day of co-cultivation. The DMEM medium was replaced by BRL-medium two hours before adding the ES cells directly onto the fibroblasts. After 24 h of co-culturing, the cells were prepared for RT-PCR, transactivation assays, or flow cytometry. ES cells containing the pTOPFLASH-EGFP reporter plasmid were transfected by a modified calcium phosphate precipitation method (Chen and Okayama, 1987) and selected with 500 μg/ml G418. EGFP (*E*nhanced *G*reen *F*luorescent *P*rotein) expression of G418-resistant clones was tested by culturing the cells in the presence of 20 mM LiCl (Hedgepeth et al., 1997). Expression of EGFP (referred to throughout as simply GFP) was monitored by visualizing fluorescence under an Axioskop microscope (Zeiss, Jena).

3.2. Plasmids and constructs

The *Brachyury*-promoter luciferase reporter construct (pTPwt: *T*-*P*romoter in p*GL3*Basic) was generated by cloning a 618 bp promoter fragment of the mouse *Brachyury* gene, corresponding to region −484 to +134 of the 5′region of *Brachyury* (Clements et al., 1996), into the promoterless luciferase reporter plasmid pGl-3Basic (Promega).

Mutations of the TCF-sites were generated within the pTPuWt plasmid by PCR-based site-directed mutagenesis as described (Mikaelian and Sergeant, 1992). For this, the following primers were used:

- TCF-site I: 5′-ATTGGGCGGGACAGTCGACGAAG-TACCGAGCA-3′,
- TCF-site II: 5′-CGCGCTGGAGCCACTAGTTGGCC-CCCAGCC-3′,
- pGL3RV-primer3 sense: 5′-CTAGCAAAATAGGCT-GTCCC-3′,
- mutagenesis primer GL3: 5′-AAATCCAACAGTAC-CGGAATGCCAAGC-3′

to give the vectors, pTPTCF I^m, pTPTCF II^m and pTPTCF $I^m + II^m$.

To construct the reporter plasmid pTOPFLASH-GFP, the promoter/enhancer region of the expression vector pEGFP-N3 (Clontech) was removed by *Xho*I and *Ase*I cleavage, and replaced by a *Sal*I-fragment of the TCF-activatable plasmid pTOPFLASH, containing three TCF-binding elements in front of the c-*fos* minimal promoter (Korinek et al., 1997) (kindly provided by H. Clevers, Utrecht/Netherlands).

The expression vector pCS2+ MMBCS33A6mt-GFP with cDNAs for both the mus musculus β-catenin mutant form S33A (Aberle et al., 1997), C-terminally fused to six *myc t*ags and GFP was constructed as follows: the GFP cassette of the vector pEGFP-N3, including the promoter and a polyA-signal, was removed by cleaving pEGFP-N3 with *Ase*I and *Afl*II. The sites were made blunt with Klenow fragment and the cassette was inserted into pCS2+ MMBCS33A6mt cleaved with *Not*I, after making blunt with Klenow fragment prior to ligation. The cDNA of β-catenin was C-terminally fused to six *myc* tags by cleaving the vector pCS2+ S33A (Aberle et al., 1997) with *Bam*HI, generating a fragment containing the whole cDNA of mutated β-catenin. This fragment was than cloned into the *Bam*HI-cleaved pCS2+ 6mt.

pCS2+ 6mt-GFP was cloned as described above, but it does not contain the cDNA for β-catenin S33A. For the reporter transactivation assays we used pCS2+ S33A, in which the six *myc* tags were removed by cloning the *Bam*HI β-catenin fragment of pCS2+ MMBCS33A6mt into the pCS2+ -vector.

3.3. Reporter assays

ES-cells were plated at a density of 4×10^5 per 35 mm tissue culture dish one day before transfection. Transfection was done by calcium phosphate precipitation with 1.5 μg of the following plasmids: pCMVβGal, which was used for standardization of transfection efficiency and pCS2+ or pCS2+ S33A. β-galactosidase and luciferase assays were performed as described (Orsulic et al., 1999) on an EG&G Berthold Autolumat LB953.

All measurements were repeated five times independently and luciferase activity was normalized to the β-galactosidase activity as an internal tranfection control.

3.4. Flow cytometry

GFP expression of ES cells, stably transfected with the reporter plasmid pTOPFLASH-GFP and stimulated by the co-culture system, was analyzed on a Beckton-Dickinson

FASCScan. Separation of GFP-positive ES cells was done on a Beckton-Dickinson FACStar^{Plus}.

3.5. RT-PCR

Total RNA was purified using the RNeasy-kit (Qiagen) following the manufacturer's instructions. Before first-strand cDNA synthesis, 1 μg of total RNA was treated with DNAseI (Gibco, BRL) and reverse transcription was done with the Superscript™ Preamplification System (Gibco BRL) using oligo(dT) as primer.

One-tenth of the cDNA was used for a semi-quantitative PCR on a Perkin Elmer 2400 thermal cycler in a reaction volume of 25 μl (cycle conditions: denaturation 95°C, 30 s; annealing 55°C, 30 s; extension 72°C, 30 s; 28 cycles for *Brachyury*). For the amplification of *Brachyury* the following primers were used:

- *Brachyury* sense: 5′-TGCTGCCTGTGAGTCATAC-3′,
- *Brachyury* antisense 5′-ACAAGAGGCTGTAGAACA-TG-3′.

RT-PCR on Frizzled receptors was done using 1/10 of cDNA from ES cells in a reaction volume of 25 μl with primers specific for the different frizzled genes (cycle conditions: denaturation 95°C, 15 s; annealing 54°C, 30 s; extension 72°C, 45 s; 32 cycles). To verify that equal amounts of cDNA pools were used for RT-PCR, 1/10 of each cDNA was also amplified with HPRT- or GAPDH-specific primers using the same conditions.

3.6. Northern blot hybridization

Total RNA was isolated from cells grown in co-culture for 24 h as described (Chomczynski and Sacchi, 1987). From total RNA, poly(A)$^+$ RNA was isolated using an mRNA purification kit (Pharmacia). Two micrograms of purified poly(A)$^+$ mRNA was blotted and hybridized to a full-length *Brachyury* cDNA probe. The probe was radioactively labeled with [α-^{32}P]dCTP using the Megaprime™ DNA labeling system (Amersham Pharmacia Biotech). Washing procedures were done as recommended by the manufacturer (Amersham Pharmacia Biotech). As a control for RNA loading the blot was also hybridized to a 500 bp PCR-product of the GAPDH gene. The following primers were used for amplification:

- GAPDH sense: 5′-ACCACAGTCCATGCCATCAC-3′
- GAPDH antisense: 5′-TCCACCACCCTGTTGCTGT-A-3′

3.7. Antibodies and Western blot analysis

For western blot analysis 1×10^5 GFP-positive cells, sorted by FACStar^{Plus}, were lysed in 50 μl CSK buffer (10 mM PIPES (pH 6.8); 150 mM NaCl; 3 mM $MgCl_2$; 300 mM sucrose; 0.5% Triton X-100; 10 μg/ml phenylmethylsulfonyl fluoride; Complete™ (Boehringer Mannheim). Samples were separated by 10% SDS-PAGE as described (Ozawa et al., 1989). The *myc*-tagged version of β-catenin was detected with the anti-c-myc(9E10) mAb (Santa Cruz Biotechnology, Santa Cruz, CA). Purified GST-tagged cytoplasmic domain of mouse E-cadherin (GST-ECT) was used for affinity precipitation of β-catenin from whole cell lysates as described (Aberle et al., 1997).

3.8. Electrophoretic mobility shift

For in vitro binding assays, a 186 bp *Brachyury* promoter region (corresponding to nt positions −322 to −136) comprising wt or mutated TCF binding sites respectively, were amplified with the following primers: 5′-GGGTC-CCGGGACTGCGCCCGACGC-3′ and 5′-CCCAGAATT-CCAGCCGTGGTGGCC-3′ (cycle conditions: denaturation 95°C, 30 s; annealing 55°C, 30 s; extension 72°C, 30 s; 30 cycles (The resulting fragments were cleaved with *Xma*I and *Eco*RI and cloned into pBluescript SK (Stratagene). From each of these the 186 bp fragment was excised with *Xma*I and *Eco*RI and the resulting 5′overhang was radioactively labeled with [α-^{32}P]dCTP. The radioactive probe was purified over a NICK™ Column (Pharmacia). Recombinant proteins GST-LEF-1 and β-catenin-His_6 were prepared as described (Huber et al., 1996a).

Labeled promoter fragments (1×10^4 counts/min) were incubated in the presence of 20 mM HEPES (pH 8.0); 50 mM EDTA; 5 mM $MgCl_2$; 10% glycerol; 1 mM DTT, with 1 μg poly(d(I-C)) in a total volume of 10 μl, together with protein (100–200 ng), for 20 min on ice. Electrophoresis was performed through 4% native polyacrylamide gels in 0.25× TBE at room temperature.

Acknowledgements

We are grateful to H. Clevers for the pTOPFLASH and pFOPFLASH plasmids, and to R. Rupp for the pCS2+ vector. We thank Rosemary Schneider for typing, and Dr. Randy Cassada for critically reading the manuscript. This work was supported by the German-Israeli Foundation for Scientific Research and Development (GIF).

References

Aberle, H., Bauer, A., Stappert, J., Kispert, A., Kemler, R., 1997. beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. 16, 3797–3804.

Ben-Ze'ev, A., Geiger, B., 1998. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr. Opin. Cell Biol. 10, 629–639.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R.T., Kimelman, D., 1997. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev. 11, 2359–2370.

Cadigan, K.M., Nusse, R., 1997. Wnt signaling: a common theme in animal development. Genes Dev. 11, 3286–3305.

Chen, C., Okayama, H., 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7, 2725–2745.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Clements, D., Taylor, H.C., Herrmann, B.G., Stott, D., 1996. Distinct regulatory control of the Brachyury gene in axial and non-axial mesoderm suggests separation of mesoderm lineages early in mouse gastrulation. Mech. Dev. 56, 139–149.

Crease, D.J., Dyson, S., Gurdon, J.B., 1998. Cooperation between the activin and Wnt pathways in the spatial control of organizer gene expression. Proc. Natl. Acad. Sci. USA 8, 4398–4403.

Galceran, J., Fariñas, I., Depew, M.J., Clevers, H., Grosschedl, R., 1999. Wnt3a−/−-like phenotype and limb deficiency in Lef1(−/−)Tcf1 (−/−) mice. Genes Dev 13, 709–717.

Gradl, D., Kühl, M., Wedlich, D., 1999a. Keeping a close eye on Wnt-1/wg signaling in Xenopus. Mech. Dev. 86, 3–15.

Gradl, D., Kühl, M., Wedlich, D., 1999b. The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. Mol. Cell. Biol. 19, 5576–5587.

Gumbiner, B.M., 1998. Propagation and localization of Wnt signaling. Curr. Opin. Genet. Dev. 8, 430–435.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Identification of c-MYC as a target of the APC pathway. Science 281, 1509–1512.

Hedgepeth, C.M., Conrad, L.J., Zhang, J., Huang, H.C., Lee, V.M., Klein, P.S., 1997. Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. Dev. Biol. 185, 82–91.

Herrmann, B.G., Labeit, S., Poustka, A., King, T.R., Lehrach, H., 1990. Cloning of the T gene required in mesoderm formation in the mouse. Nature 343, 617–622.

Huber, O., Bierkamp, C., Kemler, R., 1996a. Cadherins and catenins in development. Mech. Dev. 8, 685–691.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B.G., Kemler, R., 1996b. Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech. Dev. 59, 3–10.

Hulsken, J., Birchmeier, W., Behrens, J., 1994. E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. J. Cell Biol. 127, 2061–2069.

Jiang, J., Struhl, G., 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature 391, 493–496.

Kemler, R., 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet. 9, 317–321.

Kispert, A., Vainio, S., McMahon, A.P., 1998. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. Development 125, 4225–4234.

Korinek, V., Barker, N., Morin, P.J., Vanwichen, D., Deweger, R., Kinzler, K.W., Vogelstein, B., Clevers, H., 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in Apc(−/−) colon carcinoma. Science 275, 1784–1787.

Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O., Clevers, H., 1998. Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. Mol. Cell. Biol. 18, 1248–1256.

Larue, L., Antos, C., Butz, S., Huber, O., Delmas, V., Dominis, M., Kemler, R., 1996. A role for cadherins in tissue formation. Development 122, 3185–3194.

Latinkic, B.V., Umbhauer, M., Neal, K.A., Lerchner, W., Smith, J.C., Cunliffe, V., 1997. The Xenopus Brachyury promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins. Genes Dev 11, 3265–3276.

Laurent, M.N., Blitz, I.L., Hashimoto, C., Rothbacher, U., Cho, K.W., 1997. The Xenopus homeobox gene twin mediates Wnt induction of goosecoid in establishment of Spemann's organizer. Development 124, 4905–4916.

Liu, P., Wakamiya, M., Shea, M.J., Albrecht, U., Behringer, R.R., Bradley, A., 1999. Requirement for Wnt3 in vertebrate axis formation. Nat. Genet. 22, 361–365.

McGrew, L.L., Takemura, K.-I., Bates, R., Moon, R.T., 1999. Direct regulation of the *Xenopus engrailed*-2 promoter by the Wnt signaling pathway, and a molecular screen for Wnt-responsive genes, confirm a role for Wnt signaling during neural patterning in *Xenopus*. Mech. Dev. 87, 21–31.

McKendry, R., Hsu, S.C., Harland, R.M., Grosschedl, R., 1997. LEF-1/TCF proteins mediate wnt-inducible transcription from the Xenopus nodal-related 3 promoter. Dev. Biol. 192, 420–431.

Mikaelian, I., Sergeant, A., 1992. A general and fast method to generate multiple site directed mutations. Nucleic Acids Res. 20, 376.

Moon, R.T., Kimelman, D., 1998. From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in Xenopus. BioEssays 20, 536–545.

Moon, R.T., Brown, J.D., Torres, M., 1997. WNTs modulate cell fate and behavior during vertebrate development. Trends Genet. 13, 157–162.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., Polakis, P., 1995. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. USA 92, 3046–3050.

Nusse, R., Varmus, H.E., 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31, 99–109.

Orford, K., Crockett, C., Jensen, J.P., Weissman, A.M., Byers, S.W., 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J. Biol. Chem. 272, 24735–24738.

Orsulic, S., Huber, O., Aberle, H., Arnold, S., Kemler, R., 1999. E-Cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. J. Cell Sci. 112, 1237–1245.

Ozawa, M., Baribault, H., Kemler, R., 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J. 8, 1711–1717.

Papaioannou, V.E., Silver, L.M., 1998. The T-box gene family. BioEssays 20, 9–19.

Peifer, M., 1997. Cancer – beta-catenin as oncogene – the smoking gun. Science 275, 1752–1753.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.C., Grosschedl, R., Bienz, M., 1997. LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. Cell 88, 777–787.

Robbins, P.F., El-Gamil, M., Li, Y.F., Kawakami, Y., Loftus, D., Appella, E., Rosenberg, S.A., 1996. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J. Exp. Med. 183, 1185–1192.

Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S., Polakis, P., 1995. The APC protein and E-cadherin form similar but independent complexes with alpha-catenin, beta-catenin, and plakoglobin. J. Biol. Chem. 270, 5549–5555.

Rubinfeld, B., Robbins, P., Elgamil, M., Albert, I., Porfiri, E., Polakis, P., 1997. Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science 275, 1790–1792.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., Ben-Ze'ev, A., 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA 96, 5522–5527.

Smith, A.G., Hooper, M.L., 1987. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev. Biol. 121, 1–9.

Smith, J., 1999. T-box genes: what they do and how they do it. Trends Genet. 15, 154–158.

Takada, S., Stark, K.L., Shea, M.J., Vassileva, G., McMahon, J.A., McMa-

hon, A.P., 1994. Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. 8, 174–189.

Tetsu, O., McCormick, F., 1999. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422–426.

Torres, M.A., Eldar-Finkelman, H., Krebs, E.G., Moon, R.T., 1999. Regulation of ribosomal S6 protein kinase-p90(rsk), glycogen synthase kinase 3, and beta-catenin in early Xenopus development. Mol. Cell. Biol. 19, 1427–1437.

Wilkinson, D.G., Bhatt, S., Herrmann, B.G., 1990. Expression pattern of the mouse T gene and its role in mesoderm formation. Nature 343, 657–659.

Yost, C., Torres, M., Miller, R.R., Huang, E., Kimelman, D., Moon, R.T., 1996. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in xenopus embryos by glycogen synthase kinase 3. Genes Dev. 10, 1443–1454.