Wnt Target Genes Identified by DNA Microarrays in Immature CD34+ Thymocytes Regulate Proliferation and Cell Adhesion[1]

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The thymus is seeded by very small numbers of progenitor cells that undergo massive proliferation before differentiation and rearrangement of *TCR* genes occurs. Various signals mediate proliferation and differentiation of these cells, including Wnt signals. Wnt signals induce the interaction of the cytoplasmic cofactor β-catenin with nuclear T cell factor (TCF) transcription factors. We identified target genes of the Wnt/β-catenin/TCF pathway in the most immature (CD4−CD8−CD34+) thymocytes using Affymetrix DNA microarrays in combination with three different functional assays for in vitro induction of Wnt signaling. A relatively small number (∼30) of genes changed expression, including several proliferation-inducing transcription factors such as c-*fos* and c-*jun*, protein phosphatases, and adhesion molecules, but no genes involved in differentiation to mature T cell stages. The adhesion molecules likely confine the proliferating immature thymocytes to the appropriate anatomical sites in the thymus. For several of these target genes, we validated that they are true Wnt/β-catenin/TCF target genes using real-time quantitative PCR and reporter gene assays. The same core set of genes was repressed in Tcf-1-null mice, explaining the block in early thymocyte development in these mice. In conclusion, Wnt signals mediate proliferation and cell adhesion, but not differentiation of the immature thymic progenitor pool. *The Journal of Immunology,* 2004, 172: 1099–1108.

The Wnt protein family consists of 18 secreted factors that regulate cell fate, polarity, and cell growth in receptive cells (1). Many of the *Wnt* genes in the mouse have been investigated by targeted mutation, leading to very specific developmental defects (2). In addition, deregulated Wnt signaling is involved in oncogenesis of several different types of human cancers, including tumors of the colon, kidney, liver, prostate, and ovarium (3). Current models of Wnt signaling assume that secreted Wnt proteins bind to receptors of the Frizzled family on the cell surface. Via several different proteins, the signal is transduced to cytoplasmic β-catenin, which then enters the nucleus and forms a complex with members of the T cell factor (TCF)[3] family of transcription factors to activate transcription of Wnt target genes (4).

The founding members of the TCF family are Tcf-1 and lymphoid enhancing factor-1 (Lef-1) (5). In adult mammals, Tcf-1 is expressed uniquely in T lymphocytes, whereas Lef-1 is expressed at lower levels in T cells, as well as in B cells and other cell types (6). The functions of both of these genes have been investigated by targeted mutation experiments. Tcf-1-null mice have an incomplete, but marked block in thymocyte development (7), and fail to generate de novo thymocytes after transplantation of hemopoietic precursors in irradiated hosts (8). Two different Tcf-1 mutant mice have been generated (7). In Tcf-1(V) mutant mice, a low level of a truncated yet functional Tcf-1 is still expressed, whereas the Tcf-1(VII) mutation abolishes the DNA binding activity of Tcf-1 completely, and these mice are regarded as true Tcf-1-null mutants. Young Tcf-1(VII)−/− mice have an incomplete block at the CD4−CD8− double-negative (DN) and a more prominent block at the immature single-positive (ISP) stages of thymocyte differentiation, whereas older mice display a complete block at the DN1 stage of development. Lef-1-null mice lack hair, teeth, mammary glands, and trigeminal nuclei, and die around birth (9), but have normal thymocyte development. Lef-1 can partially compensate for the Tcf-1 defect, especially in the neonatal and fetal thymus. Although Tcf-1(V) mutant mice are viable and have modestly impaired thymocyte development, Lef-1/Tcf-1(V) double-mutant mice display a complete block in thymocyte development at the ISP stage (10). Lef-1/Tcf-1(VII) double-mutant mice die at day 10 of embryogenesis (11) with dramatic developmental defects affecting the limb buds and neural tube. Interestingly, the phenotypic abnormalities of these double-mutant mice are highly reminiscent of those in Wnt3A mutant mice (11).

Both Tcf-1 and Lef-1 function as nuclear effectors of Wnt signals (12, 13). Both nuclear proteins are activated by binding to β-catenin, which supplies a powerful transactivation domain to the DNA binding activity of TCFs. β-Catenin is subject to complex regulation via several key components of the Wnt pathway, including the tumor suppressor genes *Axin* and *adenomous polyposis coli* and the negative regulatory protein kinase glycogen synthase

[3] Abbreviations used in this paper: TCF, T cell factor; Lef-1, lymphoid enhancing factor-1; DN, double negative; DP, double positive; ISP, immature single positive; GSK-3β, glycogen synthase kinase-3β; HSC, hemopoietic stem cell; CHX, cycloheximide; SAM, significance analysis of microarrays; RQ-PCR, real-time quantitative PCR; CRP, cysteine-rich protein; GILZ, glucocorticoid-induced lymphoid Zinc finger; PTPase, protein tyrosine phosphatase; ECM, extracellular matrix; hsp70, heat shock protein 70; NRPB, nuclear restricted protein brain; LMO, Lim domain only; MMP7, matrix metalloproteinase 7.

kinase-3β (GSK-3β) (1) (see Fig. 1). In absence of Wnt signaling, GSK-3β is actively inducing phosphorylation of β-catenin and its subsequent degradation via the ubiquitin/proteasome pathway. In the absence of β-catenin in the nucleus, TCFs may recruit members of the transducin-like enhancer of split/Grg family, which act as global repressors of transcription (14). Upon Wnt signaling, GSK-3β is inactivated, leading to accumulation of dephosphorylated β-catenin. Lithium can strongly inhibit GSK-3β activity and can therefore be used to mimic Wnt signaling (15). Lithium treatment can activate transcription of *TCF* reporter genes in fibroblasts and epithelial lines (15) and, to a lesser extent, in T cells (16).

Wnt signaling has been shown to occur in hemopoietic stem cells (HSC) and developing T and B cells (17–20). Several reports indicate that Wnts can act in synergy with cytokines as hemopoietic growth factors. For instance, Wnts induce expansion of murine fetal liver stem cells and induce proliferation of human CD34$^+$ HSC (17). In addition, Reya et al. (18) demonstrated that Wnts can act as growth factors for pro-B cells. We have shown that Wnt signaling induces proliferation of fetal DN thymocytes (19). Moreover, retroviral expression of soluble Wnt receptor mutants that block Wnt signaling impairs thymocyte development, indicating that Wnt signaling is required for normal T cell development (19). Finally, Wnt signaling is important in the regulation of survival of double-positive (DP) thymocytes (20).

To further understand the role of Wnt signaling in T cell development, it is imperative to identify the target genes of the β-catenin/TCF transcription factor complex in thymocytes. The recent development of DNA microarrays allows the rapid and unbiased identification of genes expressed under different conditions. We have chosen to purify human CD34$^+$ DN thymocytes instead of murine early DN thymocytes for several reasons. First, many more thymocytes can be obtained from human thymi than from the mouse. Given that the most immature DN thymocytes (DN1 and DN2) comprise only a small ($\sim$1%) subset of adult thymocytes, the choice of human thymocytes allows more readily the isolation of sufficient amounts of RNA for use on DNA microarrays. Second, the currently available microarrays for humans generally contain more genes than the murine chips. Third, human immature thymocytes can easily be purified based on the presence of the CD34 stem/progenitor cell marker on their cell surface. In this study, we have used Affymetrix (Santa Clara, CA) microarrays to identify the transcriptional response of the Wnt/β-catenin/TCF pathway in the most immature thymocytes.

Materials and Methods

Isolation of immature human thymocytes

Thymic tissue was obtained from children undergoing cardiac surgery who did not have any immunological abnormalities. All tissues were obtained according to the guidelines of the Medical Ethical Committee (Institutional Review Board) of Erasmus MC. Thymocytes were isolated by mechanical disrupture of the tissue and squeezing through a mesh. Thymocytes were frozen in liquid nitrogen in RPMI 1640 medium containing 10% FCS and 10% DMSO. Thymocytes from 5–10 donors were pooled to reduce individual variation. After thawing, pooling, and Ficoll density centrifugation, CD34$^+$ thymocytes were purified by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), according to standard protocols. The degree of purification was assessed by flow cytometry.

Isolation of murine thymocytes

Thymi were removed from 7-wk-old C57BL/6 and Tcf-1(VII)-null mice (backcrossed to C57BL/6). Cell suspensions were made and stained with Abs specific for mouse CD4, CD8, and CD3. DN and ISP subpopulations were sorted on a FACS Digital Vantage (DiVa) cell sorter (BD Biosciences, San Jose, CA) to >98% purity and immediately used for RNA isolation.

Methods for in vitro induction of the Wnt/β-catenin/TCF pathway

Treatment with pharmacological agents. CD34$^+$ thymocytes (10 $\times$ 10^6) were treated with 20 mM LiCl or KCl as control for the indicated length of time. In some experiments, cells were also treated with the protein synthesis inhibitor cycloheximide (CHX; 1 μg/ml). These treatments were done in the presence of 10 ng/ml human IL-7 to make the results comparable to the retroviral transduction data. LiCl-induced Wnt signaling was assessed by staining of dephosphorylated β-catenin (21).

Retroviral transduction. The retroviral plasmids LZRS-S33-β-catenin-IRES-Lyt2a and LZRS-IRES-Lyt2a (as control) were constructed and transfected into Phoenix amphotropic packaging cell lines using Fugene-6 (Roche Molecular Biochemicals, Branchbury, NY). Stable high-titer producer clones were selected with puromycin (1 μg/ml). CD34$^+$ thymocytes (30–50 $\times$ 10^6) were prestimulated with IL-7 (10 ng/ml) for 1 day, and subsequently transduced using Retronectin-coated petri dishes (Takara, Shiga, Japan) and recombinant retrovirus containing supernatant for 2 days, with daily replenishing of retroviral supernatant. The efficiency of the retroviral transduction was evaluated by flow cytometry. Cells were stained with Abs against human CD34 and mouse CD8 (Lyt2a) and analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences).

Stimulation with Wnt3A-containing supernatant. For stimulation with Wnt3A, cells were incubated with conditioned medium from L cells stably transfected with a pCDNA-Wnt3A construct. The supernatant was filtered through a 0.22-μm filter and incubated with immature thymocytes for 4 h, using supernatant from parental L cells as negative control.

Induction of Wnt signaling in CD34$^+$ thymocytes by Wnt3A was assessed by Western blotting. In short, immature thymocytes were isolated, treated with Wnt3A or control supernatant, and lysed in boiling 2$\times$ SDS sample buffer as described (21). Lysates were run on a 10% SDS-PAGE gel, blotted, and stained with an Ab against the activated form of β-catenin (anti-ABC) (21, 22) or a pan-β-catenin Ab (Transduction Labs, Lexington, KY) and visualized by ECL (Amersham, Little Chalfont, U.K.). Treatment with LiCl or KCl was used as extra positive or negative control for detection of activated β-catenin

Microarray analysis

RNA was isolated using RNeasy columns as described by the manufacturer (Qiagen, Hilden, Germany). The integrity of the RNA was tested on 1% formaldehyde-containing agarose gels. Five to 8 μg of RNA was used to generate double-stranded cDNA using SuperScript reverse transcriptase and a T7-oligo(dT) primer. The resulting cDNA was used in an in vitro cRNA reaction using T7 RNA polymerase and biotinylated ribonucleotides using an ENZO (Farmingdale, NY) kit. The biotinylated cRNA was cleaned up using RNeasy spin columns (Qiagen) and quantified by spectophotometric methods. An adjusted cRNA yield was calculated to reflect carryover of unlabeled total RNA. Fragmentation of 20 μg of cRNA was performed at 95°C for 35 min. Five micrograms of cRNA were hybridized to a Test3 microarray (Affymetrix) to check the quality of the procedure. Ten micrograms of fragmented cRNA was subsequently hybridized for 16 h to U95Av2 microarrays (Affymetrix) at 45°C. After washing and staining, the arrays were scanned in a Hewlett-Packard/Affymetrix scanner at 570 nm. For all experiments reported here, the 5'/3' ratios of GAPDH were 1.5 or less (usually 0.9–1.1). In comparison experiments, care was taken that the scaling factor, noise, and presence calls were comparable.

Bioinformatics

Data reported here are extracted from a total of 16 different U95Av2 GeneChips used under the various conditions described. The scanned images were analyzed using Affymetrix Microarray Suite 4.2 software, using the KCl-treated samples or the Lyt2a-transduced sample or the sample treated with L cell supernatant as baseline. Further analysis was done using MicroDB and desktop Mining Tool 3.0 software. Statistical significance of the overnight LiCl treatment was tested using the freeware program significance analysis of microarrays (SAM), version 1.12. The false discovery rate was 9% for the SAM analysis. Hierarchical clustering was done on the three time points analyzed using GeneLinker Gold 2.0 (Molecular Mining, Cambridge, MA).

Transcription factor binding sites in promoter sequences were identified using Alibaba 2.1 and Transfac 4.0 (gene regulation web site, http://www.gene-regulation.com).

TaqMan-based real-time quantitative PCR (RQ-PCR)

The mRNA expression of several target genes was tested using TaqMan-based RQ-PCR. The isolated RNA was treated with 1 U of DNase I, and subsequently, 1 μg of RNA was transcribed to cDNA with avian myeloblastosis virus-reverse transcriptase (5 U), oligo(dT), and random hexanucleotide primers. A 1/20 cDNA mixture was used for RQ-PCR for each primer/probe set and performed in duplicate. The cDNA was normalized to GAPDH (Applied Biosystems, Foster City, CA). The RQ-PCR mixture of 25 μl contained TaqMan Universal MasterMix, 900 nM primers, and 100 nM probe, and was run on the PRISM 7700 sequence detection system containing a 96-well thermal cycler (Applied Biosystems). All primer and probe sequences were designed with the Primer Express, version 1.5, software (Applied Biosystems). The primer and probe sequences used were as follows: CFOS (forward), 5′-AACCTGTCAAGAGCATCAGCAG-3′, CFOS (reverse), 5′-GAGCGGGCTGTCTCAGAGC-3′, CFOS (probe), 5′-AGCTGAAGACCGAGCCCTTTGATGACTT-3′; CL100 (forward), 5′-GCAGAGGCGAAGCATCATCT-3′, CL100 (reverse), 5′-CAGCACCT GGGACTCAAACTG-3′, and CL100 (probe), 5′-CCAACTTCAGCTT CATGGGCCAGC-3′; GILZ (forward), 5′-GCGTGAGAACACCCTGTT GA-3′, GILZ (reverse), 5′-GGCTCAGACAGGACTGGAACTT-3′, GILZ (probe), 5′-ACCCTGGCAAGCCCAGAGCAGCT-3′; Brachyury (forward), 5′-TCCTTCCTGCTGGACTTTGTG-3′, Brachyury (reverse), 5′-CATTCCCCGTTCGCGTACT-3′, Brachyury (probe), 5′-CGGCGGATA ACCACCGCTGG-3′; and Fibronectin (forward), 5′-TGAGGAACATGG TTTTAGGCG-3′, Fibronectin (reverse), 5′-CTTGGCCTATGCCTTATG GG-3′, Fibronectin (probe), 5′-CACCGCCCACAACGGCCAC-3′. For murine genes, we used the following: CL100 (forward), 5′-TGTTGGAT TGTCGCTCCTTCT-3′, CL100 (reverse), 5-TGAAGCGCACGTTCATC GAG-3′, and CL100 (probe), 5′-TCAACGCCGGCCACATCGC-3′; c-*fos* (forward), 5′-GGAGGTCTGCCTGAGGCTTC-3′, c-*fos* (reverse), 5′-CACGTTGCTGATGCTCTTGAC-3′, c-*fos* (probe), 5′-CAACGACCCT GAGCCCAAGCCAT-3′; and c-*jun* (forward), 5′-GACGGACCGTTC TATGACTGC-3′, c-*jun* (reverse), 5′-GGAGGAACGAGGCGTTGA-3′, c-*jun* (probe) 5′-TGGAAACGACCTTCTACGACGATGCC-3′.

Reporter gene assays

293T cells were transfected with the luciferase reporter genes TATA-TOP (TCF-optimal), TATA-FOP (mutated constructs), and natural promoter constructs from *Wnt* target genes, together with a pCI-S33-β-catenin expression construct or a pCI-neo negative control construct. For natural promoters, we constructed pGL3-fibonectin by cloning the fibronectin 1.7-kb promoter from FN-CAT (kindly provided by Dr. T. Gorogh (University of Kiel, Kiel, Germany)) into the pGL3 basic luciferase vector (Promega, Madison, WI). The brachyury-promoter-luc was generated by cloning the brachyury promoter from brachy-LacZ into pGL3-basic-luciferase (brachyury promoter was kindly provided by Dr. B. Herrmann (Max-Planck Institut für Immunbiologic, Freiburg, Germany)). The Fos-luc reporter construct was kindly provided by Dr. T. Moeroey (University of Essen, Essen, Germany), and the β_7 integrin promoter-luc by Dr. G. Krissansen (University of Auckland, Auckland, New Zealand). Luciferase assays were essentially done as described previously (19).

Results

The experimental system

It is well established that recombinant Wnt proteins are difficult to produce in soluble form in vitro, with the notable exception of murine Wnt3A, which can be made in biologically active form by transfected L cells, a system that very recently has become available (http://www.stanford.edu/~rnusse/assays/wntproteins.html). Therefore, in almost all studies, researchers have relied on pharmacological or genetic ways to induce Wnt signaling in vitro. In this study, we have used the following three functional assays to induce Wnt signaling (see Fig. 1): 1) treatment with LiCl, which inhibits GSK-3β, a key step in activating the Wnt pathway; 2) retroviral expression of an activated form of β-catenin; and 3) supernatant from L cells transfected with Wnt3A expression vector. Treatment with LiCl has the advantage of its rapid action, allowing time course investigations of Wnt signaling, but lacks some specificity, because GSK-3β is also involved in regulation of Rel/NF-AT transcription factors (23, 24). The retroviral system is highly specific, but is not suited for time course experiments, because of the nature of the transduction process.
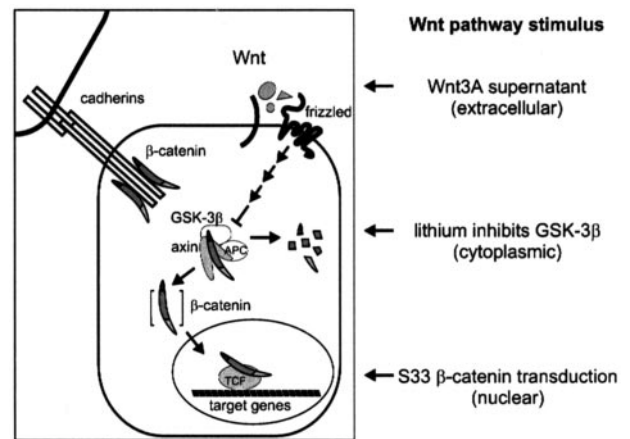
FIGURE 1. Simplified overview of Wnt signaling and stimuli used in this study. The Wnt pathway can be activated at multiple levels, either by stimulation of a Wnt ligand binding to a Frizzled receptor, by pharmacological inhibition of the GSK-3β kinase, or by providing the activated form of β-catenin. All three ways lead to the formation of an active β-catenin-Tcf transcription factor complex. The target genes of this pathway in the thymus were unknown until this study.

To isolate sufficiently high numbers of CD4$^-$CD8$^-$ immature thymocytes that can be manipulated to induce Wnt signaling and yield sufficient RNA for microarray experiments, we isolated human CD34$^+$ thymocytes. CD34$^+$ immature thymocytes were isolated by magnetic beads to >96% purity (Fig. 2*A*) and treated with LiCl and KCl for various lengths of time. LiCl is a well-known inducer of Wnt signaling, but its induction of TCF-dependent transcription (the ultimate nuclear consequence of the Wnt cascade) is not as strong in mature T lymphocytes as in other cell types (e.g., fibroblasts, epithelial cells (25)), but using sensitive reporter assays, a 5- to 10-fold induction of TCF-dependent transcription can
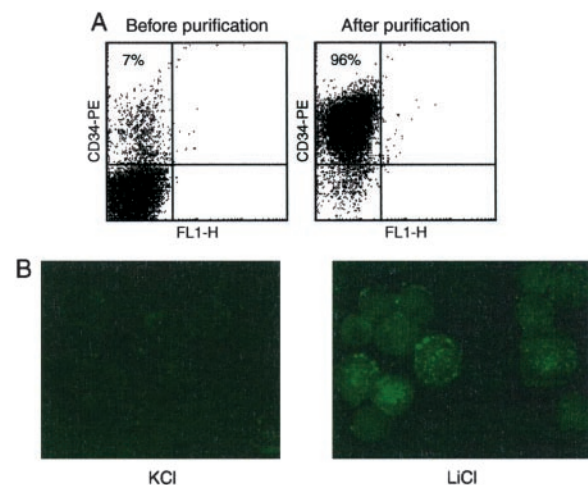
FIGURE 2. Lithium activates Wnt signaling in CD34$^+$ immature thymocytes. *A*, Purification of immature CD34$^+$ thymocytes. Thymocytes were purified by magnetic-bead isolation using the CD34 marker. In fresh thymocytes, this CD34$^+$ population constitutes ~1% of the cells, but in frozen and subsequently thawed thymocytes, 3–7%, because of the selective loss of DP thymocytes after freeze/thawing. After MACS purification, cells were >96% CD34$^+$. *B*, Lithium activates Wnt signaling in CD34$^+$ immature thymocytes. Purified CD34$^+$ thymocytes were treated for 6 h with 20 mM LiCl or KCl. Cytospins were made and analyzed by immunofluorescence using an Ab specific for dephosphorylated β-catenin, followed by an FITC-labeled secondary Ab. The clear increase in nuclear β-catenin indicates that Wnt signaling has been induced in these cells.

be observed in Jurkat T cells (data not shown). Therefore, we first evaluated whether LiCl could induce Wnt signaling in primary prothymocytes. These primary cells are not transfectable with reporter gene plasmids, and therefore, we used a recently developed Ab that specifically recognizes the activated, dephosphorylated form of β-catenin (21, 22). LiCl indeed induced an increase in the amount of dephosphorylated β-catenin (Fig. 2B).

Wnt signaling induced by lithium treatment

We isolated RNA from CD34^{+} thymocytes treated for 3, 6, or 18 h with 20 mM LiCl, using KCl as control treatment. Microarray analysis on Affymetrix U95 GeneChips (containing 12,600 genes) revealed that ~15 genes were up-regulated and ~10 were down-regulated >3-fold at the 3- and 6-h time points (Table I). During the shorter time points (3 and 6 h), CHX was added to inhibit de novo protein synthesis, thereby allowing identification of primary target genes. For the 18-h time point, this was not possible, due to cytotoxicity of longer CHX treatment. Most genes were slightly more up-regulated after 6 h compared with 3-h treatment. The up-regulated genes included proliferation-inducing transcription factors such as c-*fos*, *fosB*, and other nuclear proteins (AHNAK, glucocorticoid-induced lymphoid Zinc finger (GILZ), and cysteine-rich protein (CRP)1 and CRP2), cell cycle regulators (checkpoint suppressor 1, cyclin-dependent kinase 15 kinase, cdc-like kinase 2, and end-binding protein 3, which binds mitotic spindles), and signaling molecules such as the CL100 dual-specificity phosphatase. Down-regulated genes also concerned nuclear targets such as B cell translocation gene 1, breast cancer gene 2, nuclear pore complex proteins (Nup88), c-myb, and signaling molecules such as protein tyrosine phosphatase (PTPase) 2A. In addition, the proapoptotic gene *TRAIL* was down-regulated. Several other genes, including the known Wnt target gene in other cell types and the T-box transcription factor *Brachyury*, also were induced, but at a low level (2- to 2.5-fold).

To identify those genes that were statistically significantly changed, we performed the 18-h treatment with biological triplicates (six microarrays). SAM is a statistical test specifically developed for identification of such genes (26). A tuning parameter δ can be set corresponding to the number of false-positive genes included in the set of genes identified as significantly changing in expression. We chose to have less than one false-positive gene included and performed SAM analysis on the three 18-h treatment experiments. In doing so, we identified nine transcripts that significantly changed, all up-regulated after LiCl treatment (see Table II). Four of these genes were also found at the earlier time points, suggesting that they are direct target genes. These are the *CRP1* and *CRP2* genes, which are highly conserved nuclear proteins with a LIM/double-Zn finger motif, the large (700-kDa) nuclear protein AHNAK (meaning "giant" in Hebrew), and the antiapoptotic, lymphoid-specific Zn finger protein GILZ. The other genes are apparently induced with slower kinetics or may not be direct target genes.

Another way to identify relevant genes in the wealth of microarray data is clustering analysis. We performed hierarchical clustering on the three time points to identify clusters of genes that behaved similarly in terms of induction or repression (Fig. 3A). In general, similar sets of genes were found as those described in Tables I and II. As expected, the c-*fos* and *fosB* genes were most highly induced after 3 and 6 h, but not after 18 h. To further validate the differential expression of the identified genes, we performed TaqMan-based RQ-PCR analysis for several transcripts. In general, these data correlated well with the microarray findings (Fig. 3B) for both the earlier and later time points.

Wnt signaling induced by retroviral transduction with constitutively active β-catenin

As a second treatment to induce Wnt signaling in CD34^{+} immature thymocytes, we transduced the cells with either a mouse CD8 (Lyt2a) marker virus or a retrovirus encoding a constitutively active form of β-catenin linked via an IRES sequence to the *Lyt2a* marker gene. The S33-β-catenin mutant is a specific activator of Wnt signaling and provides a strong transactivation domain for its nuclear partner Tcf-1, although not being under negative control of

Table I. *Induced and repressed genes in CD34^{+} thymocytes after treatment with LiCl[a]*

	Fold Change (3 h)	Fold Change (6 h)
Annotation induced genes		
CDK15 kinase	4.3	1.3
CL100 phosphatase	3.2	2.0
EGR	1.7	3.0
HrpA0	4.4	3.0
hyp protein with Ankyrin repeats	1.6	3.2
HRV	2.7	3.4
α-tubulin	2.6	3.4
ADP ribosylation factor-like protein	4.6	3.6
GILZ	2.2	3.9
fosB	5.7	5.4
c-fos	6.8	4.5
EB3 protein	4.7	6.3
CRP	3.9	10.1
AHNAK	5.7	13.2
Checkpoint suppressor	4.0	13.8
Annotation repressed genes		
diacylglycerol kinase	−2.3	−3
ISGF-3	−1.9	−3
CD8 β-chain	−4.6	−3.1
ganglioside expression factor 2-like	−1.7	−3.2
PP2A	−4.5	−3.3
RNA polymerase II largest subunit	−2.6	−3.4
phosphodiesterase	−2.5	−3.6
TRAIL	−2.1	−3.6
SLP-76	−2.1	−3.2
CD53	−1.8	−4.1
BTG nuclear protein	−3.8	−4.6
Nup88	−1.8	−4.8

[a] CD34^{+} thymocytes were treated with LiCl or KCl for 3 or 6 h after which RNA was isolated and probed on U95A GeneChips. Only genes that were expressed and showed reliable up-regulation or down-regulation of 3-fold or more at at least one time point are shown.

Table II. *SAM analysis of triplicate microarrays[a]*

| Annotation	Average Fold Change (LiCl/KCl)
CRP1	9.1
AHNAK	6.5
Fizzy-related cell cycle regulator	5.3
GILZ	6.2
Cysteine protease	4.7
Cellular fibronectin	5.5
S100 calcium binding protein	5.3
CRP2	6.5
Fibronectin, alternative splice	4.0

[a] Eighteen-hour treatment of CD34^{+} thymocytes with LiCl and KCl. Three independent experiments were performed (six U95A microarrays). Average fold change in LiCl-treated CD34^{+} thymocytes as compared to control (KCl treated).
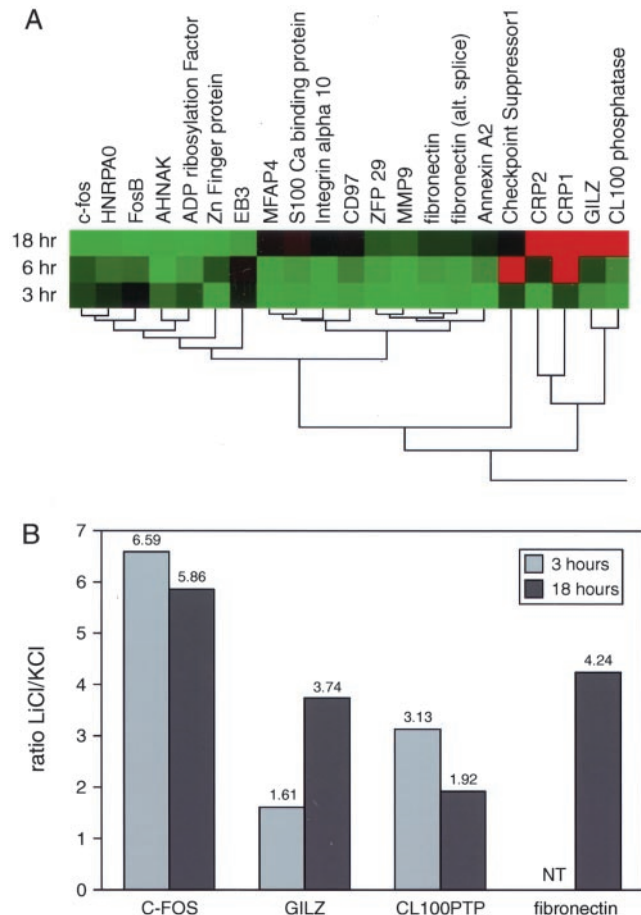
FIGURE 3. Target genes of the Wnt/β-catenin/TCF pathway induced by LiCl. *A*, Cluster analysis of Wnt target genes at 3, 6, and 18 h after stimulation with LiCl. The data from the genes found to be differentially induced or repressed with Microarray Suite software were used to perform hierarchical clustering using GeneLinker Gold program. Although this cluster analysis does not indicate statistical significance, similar sets of genes were found as by SAM analysis. Red indicates induced genes, green indicates repressed genes, and genes in black do not significantly change. *B*, Independent validation of Wnt target genes by TaqMan RQ-PCR. RNA was extracted from KCl- and LiCl-treated immature thymocytes. RQ-PCR was done for c-fos, GILZ, CL100 phosphatase, and fibronectin. Data shown are the average of triplicate measurements of one experiment of three done. NT, Not tested (because fibronectin is not induced at earlier time points).

GSK-3β, because one of the serine residues phosphorylated by GSK-3β (S33) is mutated.

Immature CD34$^+$ thymocytes were transduced with the *IRES-Lyt2a* marker gene or S33-β-catenin-IRES-Lyt2a with very similar efficiencies (Fig. 4*A*), making them comparable for microarray analysis. After transduction, RNA was isolated and used for microarray analysis. Similar to the experiments described above, only a limited number of genes ($n = 14$) was found to be differentially regulated between the two treatments (Fig. 4*B*). Wnt target genes identified after transduction with S33-β-catenin include c-*fos*, *fosB*, the *CL100 dual-specificity phosphatase*, and β_7 and *p50,95 integrins*, which were also found in the lithium experiments, as well as *JWA* and *nuclear restricted protein brain* (*NRPB*). Down-regulated were the structural proteins laminin and the proapoptotic gene *Human Requiem* (*HREQ*) (both low, 2-fold). Repeat experiments revealed similar clusters of genes, although the fold induction or repression varied. We reasoned that this could be caused by
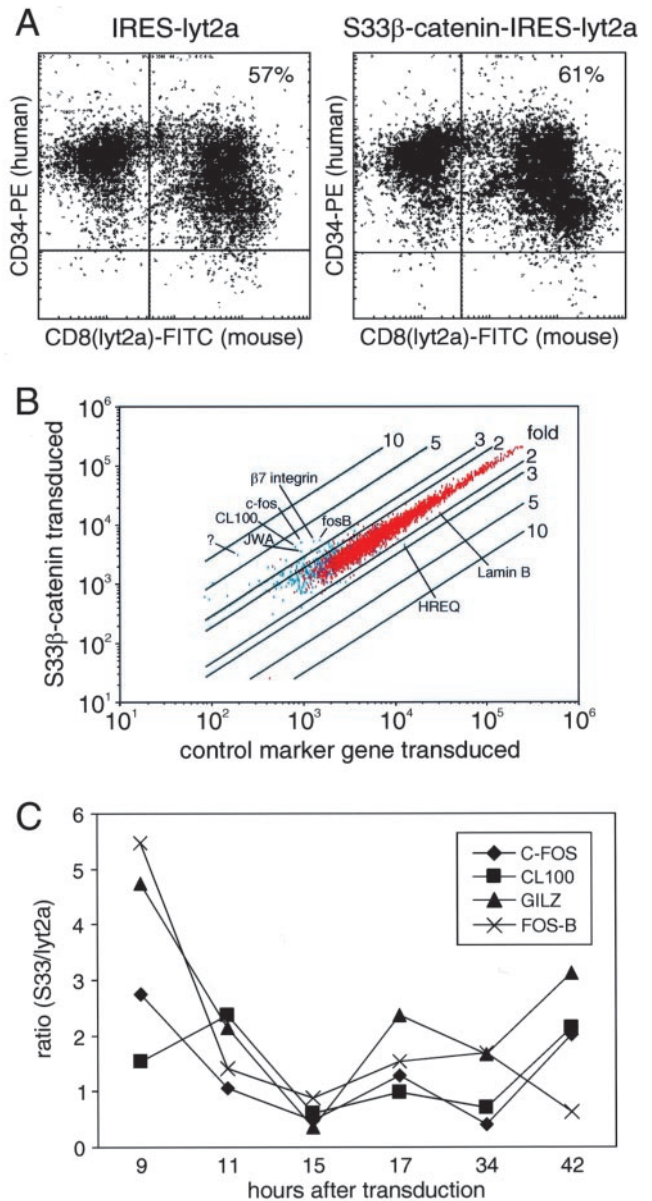
FIGURE 4. Wnt target genes identified after transduction of CD34$^+$ immature thymocytes with a retrovirus encoding constitutively active β-catenin. *A*, Transduction of CD34$^+$ cells with a retrovirus encoding the marker gene *Lyt2a* (murine CD8), or the *S33-β-catenin-IRES-Lyt2a* gene. Cells were transduced in the presence of IL-7 for 2 days and stained with Abs against CD34 and murine CD8. *B*, Genes induced or repressed by induction of Wnt signaling as identified by Affymetrix microarray analysis of Lyt2a- vs S33-β-catenin-IRES-Lyt2a-transduced immature thymocytes. Shown is a dot plot for all genes expressed after 2 days of transduction. Gene expression level is indicated in arbitrary fluorescence units. Genes that do not change in expression (the vast majority) follow the line under a 45° angle. The other lines indicate 2-, 3-, 5-, and 10-fold changes in gene expression. *C*, TaqMan RQ-PCR validation of differential expression of Wnt target genes after induction of Wnt signaling by transduction of S33-β-catenin. RNA extracted from Lyt2a- or S33-β-catenin-IRES-Lyt2a-transduced cells was used for quantitative PCR analysis using *GAPDH* as control gene. Results are shown as a ratio of S33-β-catenin vs Lyt2a signals. A time course was done at various time points (9–48 h) after initiation of transduction. Shown are data for c-*fos*, *fosB*, *CL100 phosphatase*, and *GILZ*.

differences in kinetics in the retroviral transduction experiments. The complexities of transduction experiments, including integration, expression of β-catenin, and induction of *Wnt/TCF* target

genes, do not allow direct assessment of the kinetics of induction and consequently may cause variations in the target genes detected. Therefore, we performed a time course experiment for many time points after transduction of immature thymocytes with S33-β-catenin using RQ-PCR as the readout for several Wnt target genes (Fig. 4C). These kinetic experiments revealed a rapid induction of c-*fos* and *GILZ* (presumably by transcription of β-catenin from the unintegrated proviral DNA) followed by a lag time and a 2- to 2.5-fold induction at later time points (presumably expression of β-catenin from the integrated retroviral long terminal repeat). Therefore, this induction of Wnt target genes in the time course experiments confirms the microarray experiments.

Wnt signaling induced by stimulation with supernatant from Wnt3A transfectants

During the course of our studies, L cells transfected with murine Wnt3A were shown to produce biologically active Wnt3A. For two reasons, this system may also be able to induce Wnt signaling in human CD34$^+$ immature thymocytes. First, murine Wnt3A and human Wnt3 are highly homologous (85% amino acid identity), and second, Wnt3 (and Wnt3A) are expressed in human and mouse thymus, although at much lower levels than Wnt1, Wnt4, and some other Wnt family members (F. Weerkamp, M. R. M. Baert, B. A. E. Naber, E. F. E. de Haas, E. E. L. Koster, J. J. M. van Dongen, and F. J. T. Staal, manuscript in preparation).

Immature thymocytes were incubated with supernatant from Wnt3A-transfected L cells and from parental L cells (negative control) and analyzed on Western blot using the above-described Ab against the active (dephosphorylated) form of β-catenin. Wnt3A-containing supernatant was able to induce active β-catenin in immature thymocytes, indicating that Wnt signaling was active in these cells (Fig. 5). We next used similar conditions for microarray experiments. Genes that were induced or repressed >2.5-fold are listed in Table III. The genes for Fos and Jun and several other transcription factors were induced, such as Lim domain only (LMO)2, NRPB, and cAMP responsive element modulator, and at lower levels of significance, CL100 phosphatase, β$_7$ integrin, and AHNAK. Cytochrome P450, which was modestly induced after β-catenin transduction, was more clearly induced after Wnt3A stimulation. Interestingly, the most highly induced gene after Wnt3A stimulation encodes an ubiquitin ligase, known to be involved in degradation of β-catenin, thereby providing a negative feedback on Wnt signaling. Among the down-regulated genes, several proteases that modify extracellular matrix (ECM) components stand out (matrix metalloproteinase 7 (MMP7), tryptase1), potentially influencing cell adhesion to ECM components.
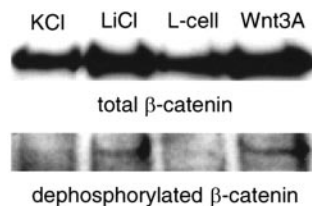
FIGURE 5. Murine Wnt3A activates Wnt signaling in immature CD34$^+$ thymocytes. CD34$^+$ DN thymocytes were isolated and incubated with supernatant from parental L cells or L cells stably transfected with a Wnt3A expression construct. As a positive control, purified CD34$^+$ thymocytes were also treated for 6 h with 20 mM LiCl or KCl. Cells were lysed in boiling SDS sample buffer and analyzed by Western blotting with anti-ABC, an Ab specific for the activated form of β-catenin (*bottom*) and with an Ab recognizing the total β-catenin pool (*top*). The expected increase in total β-catenin levels after LiCl treatment was also seen after stimulation with Wnt3A.

Table III. *Stimulation of CD34$^+$ immature thymocytes with Wnt3A^a*

	Fold Change (Wnt3A vs L Cell)
Annotation induced genes	
Ubiquitin ligase E2	9
GPNMB glycoprotein	8
c-*jun*	5.7
CYBB	4.6
LMO2	4.3
HRY	3.7
ENC1 nuclear matrix protein	2.8
EST (?)	2.6
MIR-7	2.6
Dual-specificity PTPase 6	2.6
CREM	2.6
c-*fos*	2.5
TSH-R	2.5
cytP450	2.5
IFI30	2.5
TIEG	2.5
Annotation repressed genes	
NK4	−5.8
MMP7 matrilysin	−4.8
PDCL	−4.3
IL1β	−3.8
tryptase1	−3.1
TNFRSF4	−2.9
GRO1	−2.9
MAP4K2	−2.5

^{a} Induced and repressed genes (>2.5-fold) after stimulation with parental L cell supernatant vs Wnt3A-containing supernatant for 4 h. A 2.5-fold threshold is set, because this is the lowest difference that can be reliably validated by RQ-PCR.

We again validated the microarray results using RQ-PCR for the Wnt target genes c-*fos*, *CL100 phosphatase*, and β$_7$ *integrin*. For unknown reasons, the differential expression as detected by RQ-PCR was significantly higher than detected by microarray, namely, c-*fos*, 5-fold; *CL100 phosphatase*, 4.8-fold; and β$_7$ *integrin*, 2.2-fold (data not shown), more in line with the fold induction in the retroviral transduction experiments.

TCF-reporter gene assays

To validate the identification of target genes of the Wnt/β-catenin/TCF pathway, we performed two different types of experiments on selected target genes. First, we tested the promoters of several genes identified by different treatments (*fibronectin*, c-*fos*, *brachyury*, and β$_7$ *integrin*) for induction of TCF-dependent transcription in transient transfection assays with luciferase reporters. Second, we checked expression of core target genes in thymocyte subsets of Tcf-1(VII)$^{-/-}$ mice.

Reporter gene experiments reveal much smaller differences with natural promoters (containing binding sites for many transcription factors in addition to the one being tested) than with artificial promoters consisting of multimerized binding sites for the tested transcription factors. Nevertheless, we could demonstrate a small induction of promoter activity for fibronectin, brachyury, and β$_7$ integrin (Fig. 6), whereas for fos, the effect was minimal, probably because our 600-bp fos promoter construct contained only suboptimal TCF binding sites. Most likely other promoter/enhancer sites around the c-*fos* gene contain additional TCF sites. Functional identification of such sites is a daunting task, given that enhancers can lie >50 kb up- and downstream of their target genes. Our reporter assays are consistent with the microarray and RQ-PCR data in identification of Wnt target genes, but show a smaller induction. For instance, although the induction of fibronectin by LiCl
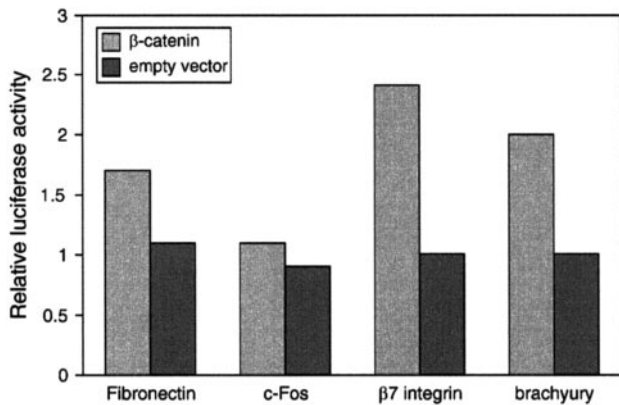
FIGURE 6. TCF- reporter gene analysis of several Wnt target genes. Promoters of c-*fos*, *fibronectin*, and *brachyury* were cloned in front of a luciferase reporter gene and used in transient transfection assays together with cotransfection of S33-β-catenin or vector control. For reference, the optimal Tcf reporter construct (TOP), consisting of multimerized binding sites for TCF, was included as well (>50-fold activation; not shown). The brachyury and β_7 *integrin* promoter constructs contain one optimal Tcf binding site, the fibronectin promoter contains two, whereas the c-*fos* promoter used contains only two suboptimal sites. Data shown are the average of duplicate measurements of one experiment of three done. The luciferase values obtained with vector control were set as 1.0.

was ~4-fold as assessed by microarray, the induction of fibronectin promoter activity in the luciferase reporter gene assay was barely 2-fold. A likely explanation for this difference is that the fibronectin reporter construct lacks important TCF sites that are activated by Wnt signals in vivo. Similarly low induction of the fibronectin promoter by Wnt signals has recently been reported for *Xenopus* fibronectin (27). Our reporter gene data also show that genes identified by only one stimulus, such as *brachyury* and *fibronectin*, behave as target genes in isolated reporter gene assays, whereas in functional assays they may not be bona fide Wnt targets in prothymocytes because of the lack of induction by Wnt3A or S33-β-catenin. Thus, we regard c-*fos* as a true *Wnt* target gene, because it was found in all three conditions to induce Wnt signaling (including those under cyclohexamide treatment), even though in the reporter gene assays with the short c-*fos* promoter, we could not demonstrate induction by Wnt signals. In contrast, genes such as *fibronectin* or *brachyury* were only found after lithium treatment and are Wnt targets in other cell types, but probably not in thymocytes.

We checked the expression of the core set of seven Wnt target genes by RQ-PCR throughout T cell development (see Table IV). Interestingly, many of these genes were expressed at the highest level in CD34$^+$ thymocytes (c-*fos*, *CL100 PTPase*, *heat shock protein 70* (hsp70)), suggesting that their expression is significantly regulated by Wnt signals. Undoubtedly, other pathways also

regulate expression of Wnt target genes, in particular those found to be highly expressed at later stages of development (c-*jun*, *fosB*).

Expression of Wnt target genes in Tcf-1-null mice (Tcf-1(VII)$^{-/-}$)

It is likely that important Wnt target genes are evolutionarily conserved in the same types of cells. Therefore, we checked the expression of three prominent target genes, c-*fos*, c-*jun*, and *CL100 phosphatase* in mice that lack the Tcf-1 transcription factor (hereafter referred to as Tcf-1(VII)$^{-/-}$). These mice are not able to induce Wnt target genes because of the lack of nuclear integration of Wnt signals. Young Tcf-1(VII)$^{-/-}$ (<12 wk) mice have an incomplete block in T cell development at the ISP stage, later (~6 mo of age) followed by a more complete block at the DN stage. We used thymi from 7-wk-old wild-type control mice and Tcf-1(VII)$^{-/-}$ mice, sorted the DN and ISP cell populations, and checked the levels of Wnt target genes by RQ-PCR (Fig. 7A). Although the levels of c-*fos*, c-*jun*, and CL100 phosphatase were not dramatically different in the DN stage (where cells still develop normally in Tcf-1(VII)$^{-/-}$ mice of this age), the levels of c-*fos* and c-*jun* were dramatically reduced in ISP cells (Fig. 7B). In addition, the levels of CL100 phosphatase were 3-fold reduced. These experiments underscore that these genes function as Wnt target genes and confirm the microarray experiments.

Discussion

Target genes of the Wnt/β-catenin/TCF pathway in thymocytes have remained elusive. In this study, we have investigated the target genes induced by Wnt signals that activate the β-catenin/TCF complex in CD34$^+$ DN thymocytes (prothymocytes) using three different functional assays to activate Wnt signaling in vitro. Remarkably, no markers of T cell differentiation were induced; we instead found that markers of proliferation and cell-cell adhesion were up-regulated. This is consistent with the notion that Wnt proteins can provide proliferative signals for stem cells in various tissues, such as crypt cells in the colon, HSC, pro-B cells, and pro-T cells.

In general, a relatively small number of *Wnt* target genes (in total, ~30) was found. LiCl, although widely used as a pharmacological agent to mimic Wnt signaling, is somewhat nonspecific, as is reflected by the much larger number of genes found after LiCl treatment than with the other two stimuli (Fig. 8). By definition, genes identified by transduction with S33-β-catenin or Wnt3A stimulation are Wnt target genes. A core set of genes is induced by both specific stimuli: c-*fos*, *fosB*, *CL100 phosphatase*, *integrin p150,95*, c-*jun*, *hsp70*, and *NRPB*; most of these are induced by LiCl as well. Because the three functional stimuli differ in modes of action, signal strengths, and kinetics of induction, it is not surprising that also different genes were found, although the functional clusters were highly comparable. The identified target genes can be clustered into several groups: genes encoding transcription

Table IV. *Expression of core Wnt target genes during thymocyte development as measured by RQ-PCR[a]*

Stage	c-*fos*	*fosB*	c-*jun*	*CL100 PTPase*	*NRPB*	*p150,95*	*hsp70*
CD34$^+$CD1a$^-$	92	1	1	466	<1	<1	522
CD34$^+$CD1a$^+$	93	0.4	10	367	<1	<1	561
DN (CD34$^-$)	19	6	22	49	<1	<1	11
DP	30	9	32	64	<1	<1	15
CD4 SP	55	11	16	37	<1	<1	4
CD8 SP	80	35	26	110	<1	<1	5

[a] Data are presented as 1000 times the level of a particular transcript compared with the level of *GAPDH* as housekeeping gene (i.e., 1000 corresponds to the level of GAPDH in a particular subset). Subpopulations were sorted from the human thymus to >95% purity.
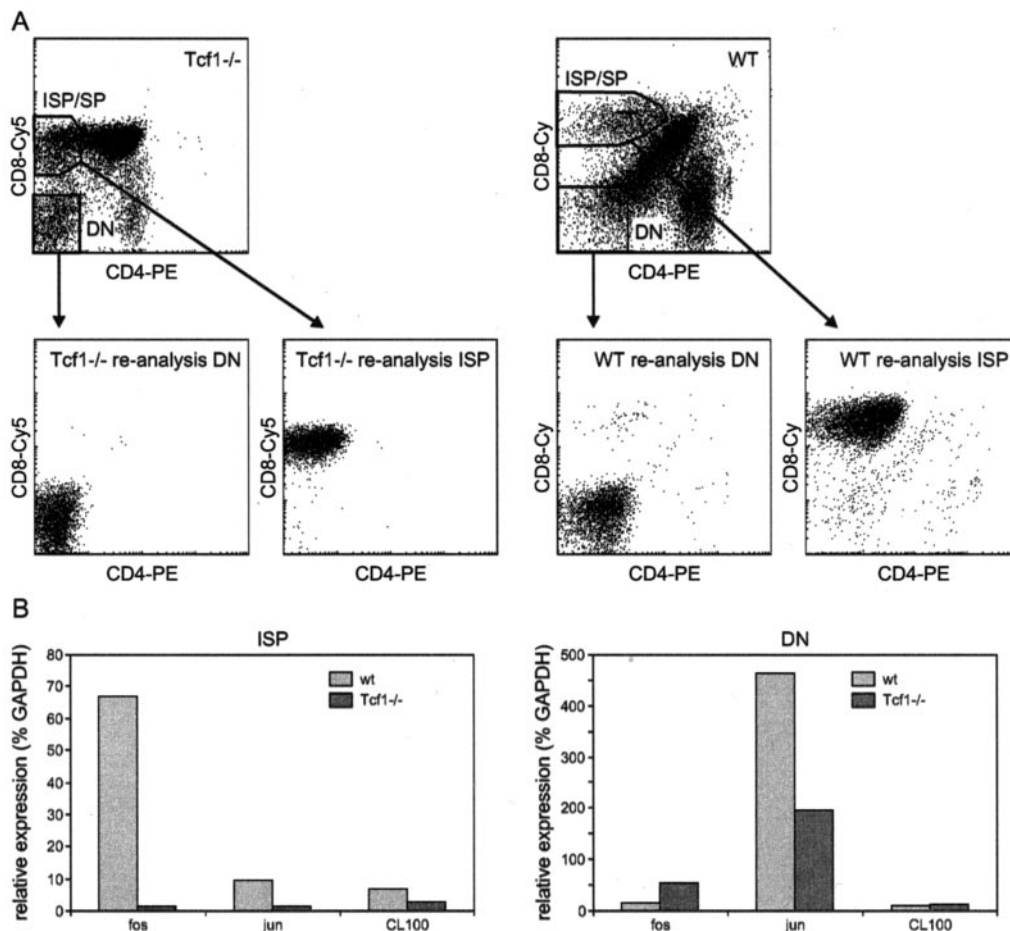
FIGURE 7. Expression of *Wnt* target genes is decreased in Tcf-1(VII)$^{-/-}$ mice compared with wild-type mice. Thymi from wild-type and Tcf-1(VII)$^{-/-}$ mice were sorted in DN and ISP populations based on staining with CD4, CD8, and CD3. DN cells were defined as CD4$^-$CD8$^-$CD3$^-$, and ISP cells were defined as CD8$^+$CD3$^-$. Starting populations and reanalysis of sorted populations are shown in CD4, CD8 FACS plots under *A*. All sorted populations were >98% pure and CD3$^-$ (not shown). RNA was extracted and the expression of c-*fos*, c-*jun*, and CL100 phosphatase was analyzed in DN and ISP cells by RQ-PCR, with GAPDH as control (*B*). Values are given as percentage of GAPDH × 1000.

factors and other nuclear proteins (c-*fos*, *fosB*, *AHNAK*, *GILZ*, *NRPB*, *LMO*), together forming a cluster of genes involved in cell proliferation; signaling molecules in particular phosphatases (*CL100 phosphatase*); molecules involved in apoptosis (*GILZ*, *TRAIL*); cell adhesion molecules (*fibronectin*, *laminin*, *p150,95*, *β$_7$ integrin*); and ECM modifiers (*MMP7*, *tryptase1*).

Some genes found only after lithium treatment may be true Wnt targets in thymocytes as well, because they have been identified as Wnt targets in other species, including the T-box transcription factors brachyury in murine ES cells (28) and cellular fibronectin in *Xenopus* (27). Promoters of these genes are responsive to β-catenin (see Fig. 5). However, because they were not significantly induced by S33β-catenin transduction nor Wnt3A stimulation, we have excluded them in the cluster of 31 target genes.

It should be noted that a number of known Wnt target genes in other tissues are apparently not induced by Wnt signaling in CD34$^+$ thymocytes. Of note, *CyclinD1* and c-*myc* expression were not changed, even though both genes are represented on the arrays used. In addition, expression of antiapoptotic gene *Bcl-x* did not change, even though this gene has been proposed to play role in TCF-induced protection against apoptosis in DP thymocytes (20). Apparently, Wnt signaling differs between immature and more mature stages of T cell differentiation. However, we cannot exclude the possibility that Wnt signaling in vivo can provide differentiation signals to prothymocytes, or that the in vitro functional assays

used in this study induce differentiation in a small subset of cells that cannot be detected by microarray analysis.

The lack of known T cell differentiation genes among the identified target genes seems at variance with data from Gounari et al. (29), who showed that transgenic mice overexpressing constitutively active β-catenin seem to bypass signals transmitted via the pre-TCR and differentiate into DP cells. However, in vivo differentiation experiments cannot evaluate the effects of constitutively active β-catenin per differentiation stage. For example, the pre-TCR provides proliferative signals as well. These proliferative signals superimposed on other differentiation signals given in the context of the thymus might be sufficient to induce differentiation. However, the DP and SP cells generated by somatic activation of β-catenin lack rearranged TCRβ chains and do not survive properly (29), indicating that the proliferative signals provided by ectopic activation of Wnt signaling do not mimic normal proliferation and differentiation signals emanating from the pre-TCR. In conclusion, our results support the idea that Wnt signals induce proliferation, but not differentiation of the earliest progenitor cell compartment in the thymus. This is consistent with a recent report by Sen and colleagues (30) showing a proliferative defect in thymocytes of Wnt4/Wnt1-null mice.

c-*fos* is one of the target genes induced the strongest (>5-fold) and found in all three types of experiments, whereas c-*jun* is found in the Wnt3A and S33 β-catenin experiments. Both c-*fos* and c-*jun*
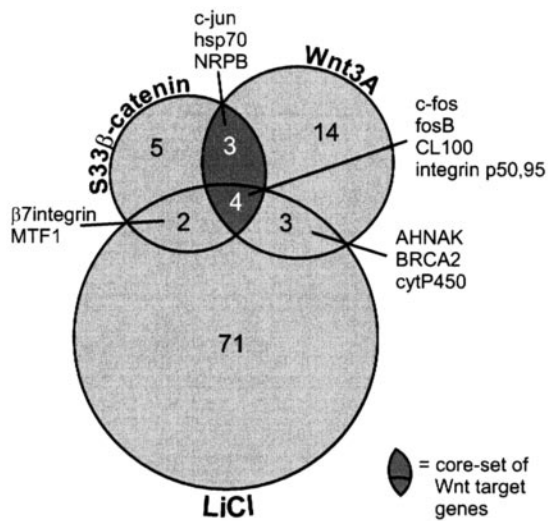
FIGURE 8. Venn diagram of Wnt target genes in prothymocytes identified by three different functional stimulations. The area of each circle is proportional to the number of target genes identified by each stimulus. Note that not all genes induced by LiCl are bona fide Wnt targets. A core set of 7 genes is identified among the 31 Wnt target genes defined.

are well-known transcription factors that mediate cell cycle progression and proliferation (31, 32). c-*fos*-null mice show a reduced cellularity in the thymus (33), without any strong blocks at a particular stage, consistent with a proliferative defect at various stages of development and somewhat reminiscent of thymi from young Tcf-1(VII)$^{-/-}$ mice. FOS family members (fos, fosB, fra1) form the so-called AP1 complexes by heterodimerization with JUN family members (jun, junB, junD). Because redundancy between these factors (e.g., between fos and fosB) can affect the consequence of gene-targeting experiments, dominant-negative molecules may better reveal the importance of fos/jun in biological processes. Mice with a dominant-negative c-*jun* have been made (34) and display a defect in cell cycle progression during the transition of DN to DP thymic development, indicating that fos/jun transcription factors promote proliferation at DN and ISP stages. These are the same stages of development affected in Tcf-1(VII)$^{-/-}$ mice. Given that fos/jun transcription factors are strongly decreased in expression in those thymic subpopulations that are affected in Tcf-1(VII)$^{-/-}$ mice (Fig. 7), lack of Wnt-induced fos/jun-mediated proliferation explains the defect in Tcf-1(VII)$^{-/-}$ mice. Thus, our findings provide a molecular mechanism for the developmental defect in Tcf-1(VII)$^{-/-}$ mice.

Interestingly, in a differential screen between genes expressed in CD34$^+$CD38$^-$ HSC vs CD34$^+$CD38$^+$ progenitor cells in the human bone marrow, Eaves and colleagues (35) have identified *Fos* and *Jun* as genes highly expressed in HSC (CD34$^+$CD38$^-$) compared with more mature progenitor cells (CD34$^+$CD38$^+$), consistent with a role of these factors in proliferation of stem cells.

Among the several signaling molecules found, CL100 dual-specificity phosphatase was highly induced in all three types of experiments. This gene encodes a mitogen-activated protein kinase 3 phosphatase implicated in cell proliferation (36, 37). Interestingly, this gene is preferentially expressed in CD34$^+$CD38$^-$ HSC compared with CD34$^+$CD38$^+$ cells (38), similar to c-*fos* (see above), suggesting that Wnt signaling in early progenitor cells in the thymus induces similar target genes as those expressed in HSC.

Several genes involved in cell-cell adhesion, including *fibronectin* and *integrins β7* and *p150,95*, were found as target genes as well. These proteins are involved in compartmentalization and cell

migration within the cortical region of the thymus and perhaps in attracting common lymphoid progenitors to the thymus. Petrie and coworkers (39) have found that DN1 cells are confined to the perimedullary cortex, whereas DN2 and DN3 cells are found in the outer cortical regions. At the transition of DN1 to DN3 cell (CD34$^+$ pro-T cell to pre-TCR$^+$ T cell), thymocytes up-regulate β_7 integrin expression (β_7 heterodimerized with α_4 or α_E integrins) and become more adhesive to fibronectin. Thus, Wnt signals may assure a proper migration of precursors along this stromal matrix or keep cells localized to a certain region of the cortex where cells receive proliferative signals before pre-TCR expression and TCRβ selection.

Based on these findings, we propose that Wnt-induced β-catenin/TCF target genes in immature thymocytes are not directly involved in inducing differentiation, but instead provide proliferative signals (for instance, via c-*fos* and c-*jun*) to induce cell division in the precursor cell compartment of the thymus (Fig. 9). In fact, because only very few progenitor cells from bone marrow or fetal liver seed the thymus, a vigorous proliferation of the most immature cells in the thymus is required. This has been recognized for over two decades by various groups and is still studied (40–42). The nature of this proliferative signal is provided by cytokines such as IL-7 and stem cell factor, but apparently also by Wnt proteins (19). Additionally, Wnt signals can be involved in the migration of immature thymocytes through thymic cortex or in confinement of precursor cells to those cortical regions where proliferation can occur (Fig. 9). This may help preventing *TCR* gene rearrangement at proliferative stages of development or occurrence of selection processes at the corticomedullary junction at inappropriate stages of development. Apparently, the Wnt/β-catenin/TCF pathway provides both proliferative signals for immature thymocytes to maintain the thymic precursor cell compartment and adhesion signals that assure that T cell development proceeds at the appropriate anatomical sites in the thymus.
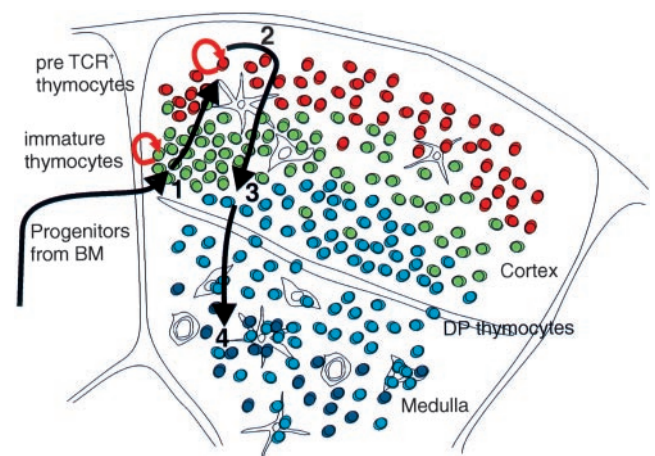
FIGURE 9. Model: Wnt signals induce proliferation and cell adhesion of immature CD34$^+$ thymocytes. HSC or common lymphoid progenitors are seeding the thymus from the bone marrow and undergo proliferation, partially driven by Wnt signals, but also by cytokines such as IL-7 and stem cell factor (1). Cells migrate to the outer cortex via different integrins and ECM components, some of which are Wnt targets (β_7 integrin, fibronectin). After this proliferative stage, rearrangement of TCRβ occurs, leading to expression of the pre-TCR (β/pTα) on the cell membrane. This generates another stage of proliferation (2) where Wnt signaling may play a role after which cells start expressing CD4 and CD8 to become DP thymocytes (3), followed by rearrangement of TCRα and positive and negative selection at the corticomedullary junction (3). Mature SP thymocytes subsequently end up in the medulla (4).

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