Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue

Huiling Liang,[1] Qin Chen,[4] Andrew H. Coles,[1] Stephen J. Anderson,[5,8] German Pihan,[2] Allan Bradley,[6] Rachel Gerstein,[3] Roland Jurecic,[7] and Stephen N. Jones[1,*]

[1]Department of Cell Biology
[2]Department of Pathology
[3]Department of Molecular Genetics and Microbiology
University of Massachusetts Medical School, Worcester, Massachusetts 01655
[4]Division of Cancer Biology, Serono Reproductive Biology Institute, Rockland, Massachusetts 02370
[5]Becton Dickinson Immunocytometry Systems, San Jose, California 95131
[6]Departments of Molecular and Human Genetics, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030
[7]Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Florida 33136
[8]Present address: Lexicon Genetics, The Woodlands, Texas 77381.
*Correspondence: stephen.jones@umassmed.edu

Summary

Wnt5a is a member of the Wnt family of secreted glycoproteins that play essential organizing roles in development. Similar to other Wnt members, Wnt5a can upregulate cell proliferation and has been proposed to have oncogenic function. Here we report that Wnt5a signals through the noncanonical Wnt/Ca^{++} pathway to suppress cyclin D1 expression and negatively regulate B cell proliferation in a cell-autonomous manner. Wnt5a hemizygous mice develop myeloid leukemias and B cell lymphomas that are clonal in origin and display loss of Wnt5a function in tumor tissues. Furthermore, analysis of human primary leukemias reveals deletion of the WNT5A gene and/or loss of WNT5A expression in a majority of the patient samples. These results demonstrate that Wnt5a suppresses hematopoietic malignancies.

Introduction

Wnt proteins are a large family of cysteine-rich, secreted glycoprotein signaling molecules. At least 19 Wnt members have been identified in mammals to date. The members exhibit unique expression patterns and distinct functions in development, although some overlap is observed (Bouillet et al., 1996; Stark et al., 1994; Takada et al., 1994; van Gijn et al., 2002; Wolda et al., 1993; Yamaguchi et al., 1999). Wnt1, the initial member of the family, was discovered as a protooncogene that induced mammary tumor development when overexpressed in mice (Nusse and Varmus, 1982). In addition to Wnt1, other Wnt family members have been found to have transforming abilities and have been classified based upon their ability to induce transformation of the mouse mammary epithelial cell line, C57MG. Wnt1, 3A, and 7A are members of the highly transforming class, whereas Wnt2, 5b, and 7b also induce cell transformation but with a lower frequency. In contrast, Wnt4, 5a, and 6 fail to induce cell transformation in these assays (Wong et al., 1994).

In some contexts, Wnt5a can act similar to other Wnt family members and bind with Frizzled (Fz) receptors located on the cell surface and activates cytosolic Dishevelled (Dsh) proteins, leading to stabilization of β-catenin (He et al., 1997). Accumulation of β-catenin results in its nuclear translocation and activation of Lef /Tcf transcription factors (Behrens et al., 1996; Molenaar et al., 1996; Toyofuku et al., 2000). In addition to utilizing the canonical Wnt signaling pathway, studies of convergent extension movements of *Xenopus* embryos and in zebrafish suggested that Wnt5a also signals through a noncanonical Ca^{++} pathway (Kuhl et al., 2000; Wallingford et al. 2001; Yamanaka et al., 2002; Myers et al. 2002). Binding of Wnt5a to Fz will, in some context, signal through heterotrimeric G proteins to activate PLC, inducing phospholipid turnover in endoplasmic reticulum membranes and triggering the release of intracellular Ca^{++} (Slusarski et al., 1997a, 1997b), which activates protein kinase C (PKC) (Sheldahl et al., 1999) and calmodulin-dependent protein kinase II (CamKII) (Kuhl et al., 2000), leading to the block of

SIGNIFICANCE

Wnt5a is a member of the Wnt family of secreted signaling molecules important in development and in cancer. Wnt5a has been previously shown to have growth-enhancing or oncogenic potential: Wnt5a upregulates proliferation of progenitor cells common to many structures in mice, Wnta5a expression is upregulated in a variety of human primary tumor samples, and WNT5A facilitates cell invasion in human metastatic melanoma. Here we demonstrate that Wnt5a negatively regulates B cell proliferation in a cell-autonomous manner. Deletion of the Wnt5a gene, loss of Wnt5a expression, and increased cyclin D1 expression is observed in mouse and human B cell lymphomas and myeloid leukemias. These results demonstrate that a member of the Wnt family of protooncogenes can function as a tumor suppressor.

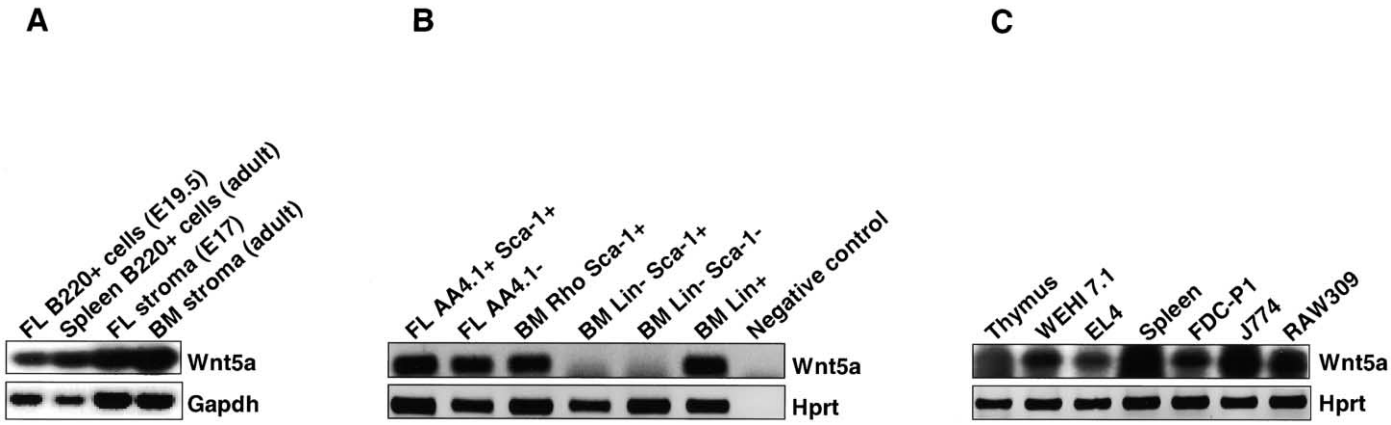

Figure 1. *Wnt5a* expression in hematopoietic tissues and cells

Total RNA was extracted from indicated samples and analyzed for *Wnt5a* expression by RT-PCR. The amplified products were detected by Southern analysis using a *Wnt5a*-specific oligo probe. RT-PCR analysis of mouse Gapdh or Hprt expression was performed as a positive control.
A: *Wnt5a* expression in $B220^+$ cells and in cultured primary stromal cells.
B: Expression of *Wnt5a* in mouse hemotopoietic progenitor and mature cells.
C: Wnt5a expression in adult hemotopoietic tissues and T cell lymphoma cell lines: WEHI 7.1 and EL4; myeloid progenitor cell line: FDC-P1; and myeloid-macrophage cell lines: J774 and RAW309.

β-catenin signaling cascade (Torres et al., 1996; Kuhl et al., 2001; Ishitani et al., 2003) and to the activation of calcium-sensitive transcription factor NFAT (Saneyoshi et al., 2002).

Functional analysis of many of the Wnt genes has been performed via gene targeting experiments in mouse embryonic stem cells. Many of the resulting mice display development defects in tissue patterning or morphogenesis, indicating that the Wnt family members play critical roles in mammalian development (Wodarz and Nusse, 1998; Yamaguchi, 2001). We have previously generated Wnt5a-deficient mice through gene targeting in mouse ES cells and found that Wnt5a was essential for proper development (Yamaguchi et al., 1999). Wnt5a null mice exhibit perinatal lethality and fail to extend multiple structures that outgrow from the primary body axis. Interestingly, this defect in morphogenesis was not due to loss of tissue identity, but rather to a reduced rate of cell proliferation in tissue underlying the outgrowing structures. These findings suggest that Wnt5a is capable of inducing cell proliferation. Several other lines of evidence further suggest that Wnt5a has growth-enhancing or oncogenic properties. Addition of exogenous Wnt5a to the media of cultured cells stimulated hematopoietic stem cell proliferation, including lymphoid and myeloid progenitor cells, and intraperitoneal injection of Wnt5a into mice has been found recently to augment the repopulating capacity of transplanted human primitive hematopoietic stem cells (Murdoch et al., 2003). Furthermore, upregulation of WNT5A has been observed in human primary breast cancer, gastric cancer, lung cancer, prostate cancer, and melanoma (Austin et al., 1997; Iozzo et al., 1995; Lejeune et al., 1995; Saitoh and Katoh, 2001; Van Den Berg et al., 1998), and WNT5A has been reported to facilitate cell invasion in human metastatic melanoma (Weeraratna et al., 2002).

Wnt5a heterozygous mice are normal, viable, and fertile (Yamaguchi et al., 1999). Surprisingly, some of the $Wn5a^{+/-}$ mice developed B cell lymphomas. This prompted us to examine the role of Wnt5a in B cell proliferation and differentiation. Here we report that Wnt5a is widely expressed in murine hematopoietic tissues and that Wnt5a signals in a cell-autonomous manner via the noncanonical Wnt/Ca^{++} pathway to inhibit B cell proliferation by negatively regulating the response of B cells to IL-7 and suppressing cyclin D1 expression. Furthermore, a subset of Wnt5a heterozygous mice develop either spontaneous B cell lymphoma that is clonal in origin or chronic myeloid leukemia, and tumors arising in the Wnt5a heterozygous mice display loss of Wnt5a function. Finally, analysis of primary tumors isolated from patients with acute lymphocytic leukemia and acute myeloid leukemia reveals loss of *WNT5A* and *WNT5A* gene expression in a majority of these human samples. These results indicate that Wnt5a functions in a cell-autonomous manner to negatively regulate B cell proliferation and suppresses hematopoietic malignancies.

Results

Wnt5a expression in hematopoietic tissues

To determine the pattern of Wnt5a expression in mouse hematopoietic tissues, RT-PCR was performed. Wnt5a is expressed in splenic B cells, fetal liver B cell progenitors, and in primary cultures of fetal liver and bone marrow stromal cells (Figure 1A). During fetal hematopoiesis, Wnt5a is transcribed in cell populations enriched for fetal liver hematopoietic stem cells (HSC) ($Sca\text{-}1^+/c\text{-}kit^+/AA4.1^+/Lin^-$ cells) and progenitor cells ($AA4.1^-$ cells) (Figure 1B). Wnt5a is also expressed in adult bone marrow (BM) HSC ($Rho\text{-}123^{low}/Sca\text{-}1^+/c\text{-}kit^+/Lin^-$) that is highly enriched for HSC activity (Jordan et al., 1995; Petrenko et al., 1999; Phillips et al., 2000). Interestingly, Wnt5a transcripts are not observed in more heterogeneous populations of HSC ($Lin^-/Sca\text{-}1^+$ BM cells) or progenitors ($Lin^-/Sca\text{-}1^-$ cells). Wnt5a expression is upregulated in committed progenitors and mature blood cell types (Lin^+ bone marrow cells). Analysis of a panel of mouse lineage-specific hematopoietic cell lines demonstrates

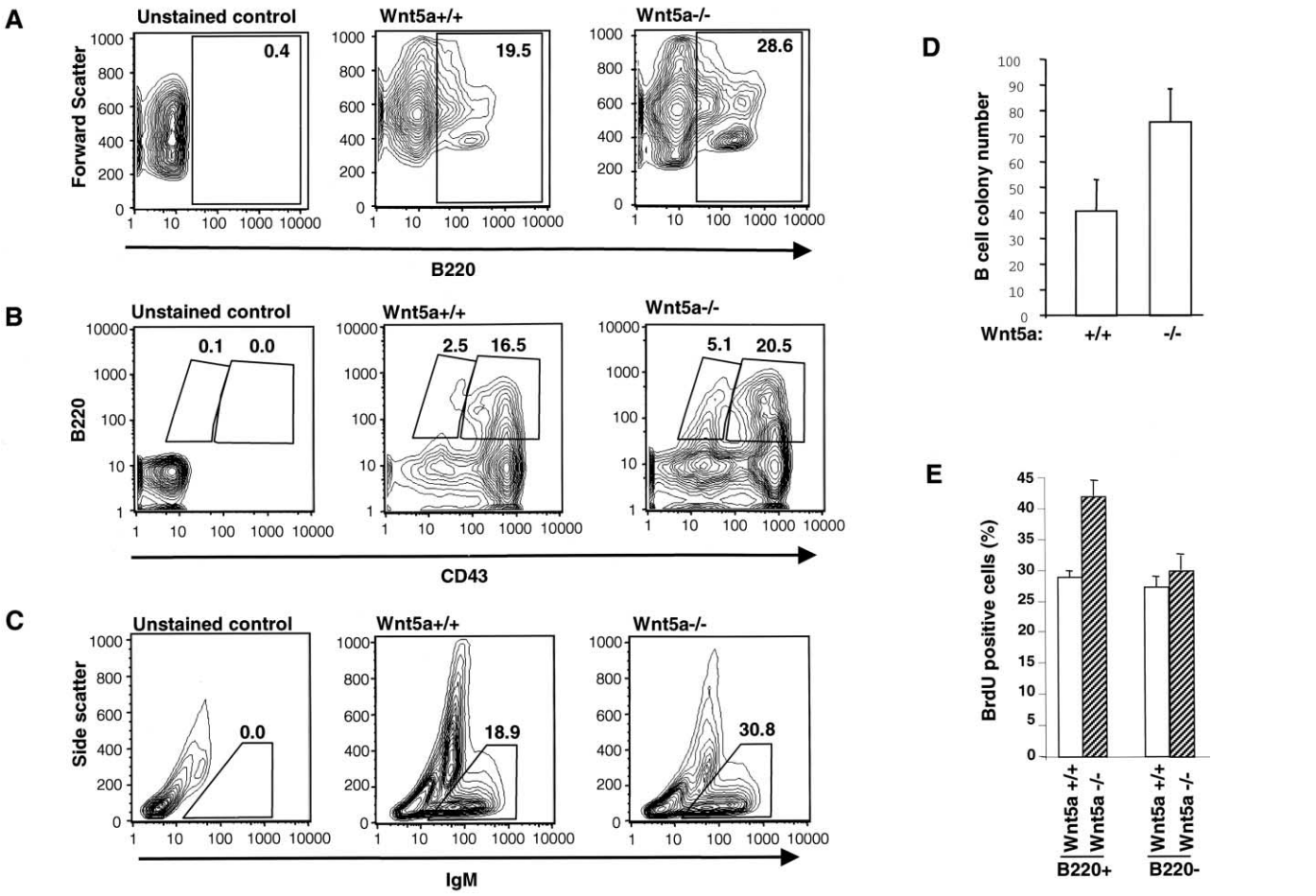

Figure 2. Expansion and hyperproliferation of B lymphocytes in Wnt5a null mice

A, **B**, and **C:** Flow cytometry analysis of antibody-stained E19 *Wnt5a*$^{+/+}$ and $^{-/-}$ FL cells.

D: Methylcellulose colony-forming assay for B cells using wild-type and Wnt5a null FL cells. The results are given as the number of colonies formed per 10^5 cells.

E: BrdU incorporation assays. B220-positive and -negative FL cells, labeled in vivo with BrdU, were stained with anti-BrdU-FITC and analyzed by flow cytometry. Error bars represent means ± SD (**D** and **E**).

that Wnt5a is ubiquitously expressed in different stages of B cell, T cell, and myeloid cell development (Figure 1C). These results demonstrate that Wnt5a is expressed widely in hematopoietic tissues.

Absence of Wnt5a increases B cell proliferation in a cell-autonomous manner

Due to the perinatal lethality of Wnt5a null mice, examination of the Wnt5a effects on B cells was restricted to fetal liver (FL), which supports B cell development from embryonic day 13 (E13) to birth. E19 FL cells were isolated and stained with anti-B220 antibody, a B cell surface marker, and analyzed by flow cytometry. Wnt5a null mice display a 35% increase in the proportion of B lineage cells (Figure 2A) and a 55% increase in the absolute numbers of B cells. To examine if absence of Wnt5a alters B cell differentiation, staining with combinations of antibodies against B220, CD43, and IgM was performed. Increased numbers of pro-B cells (B220$^+$/CD43$^+$) and pre-B cells (B220$^+$/CD43$^-$) were present in Wnt5a null FL (Figure 2B). Because immature B cells (IgM$^+$) are very rare in E19 fetal liver, we assayed for this population in Wnt5a null and wild-type FL by in vitro culture of FL in the presence of IL-7. After 8 days in culture, Wnt5a null FL produced 1.7 times as many immature B cells as did wild-type FL (Figure 2C). Thus, absence of Wnt5a does not block B cell development from pro-B to immature B stage.

To confirm the increase in progenitor B cells in Wnt5a null FL, colony-forming assays were performed using FL cells harvested from wild-type or Wnt5a null embryos at E19. Nearly twice as many colonies were detected withWnt5a null FL than with wild-type FL when grown in methylcellulose cultures supplemented with IL-7, which facilitates growth of pro-B and pre-B cell colonies (StemCell Technologies) (Figure 2D). In addition to forming more colonies, FL cells from Wnt5a null mice also formed larger sized colonies (data not shown), suggesting that Wnt5a null B cells proliferate faster than wild-type B cells in these assays.

To determine whether the augmented B cell numbers in Wnt5a null FL are due to higher rates of cell proliferation or due to increased resistance of Wnt5a null cells to apoptosis, BrdU

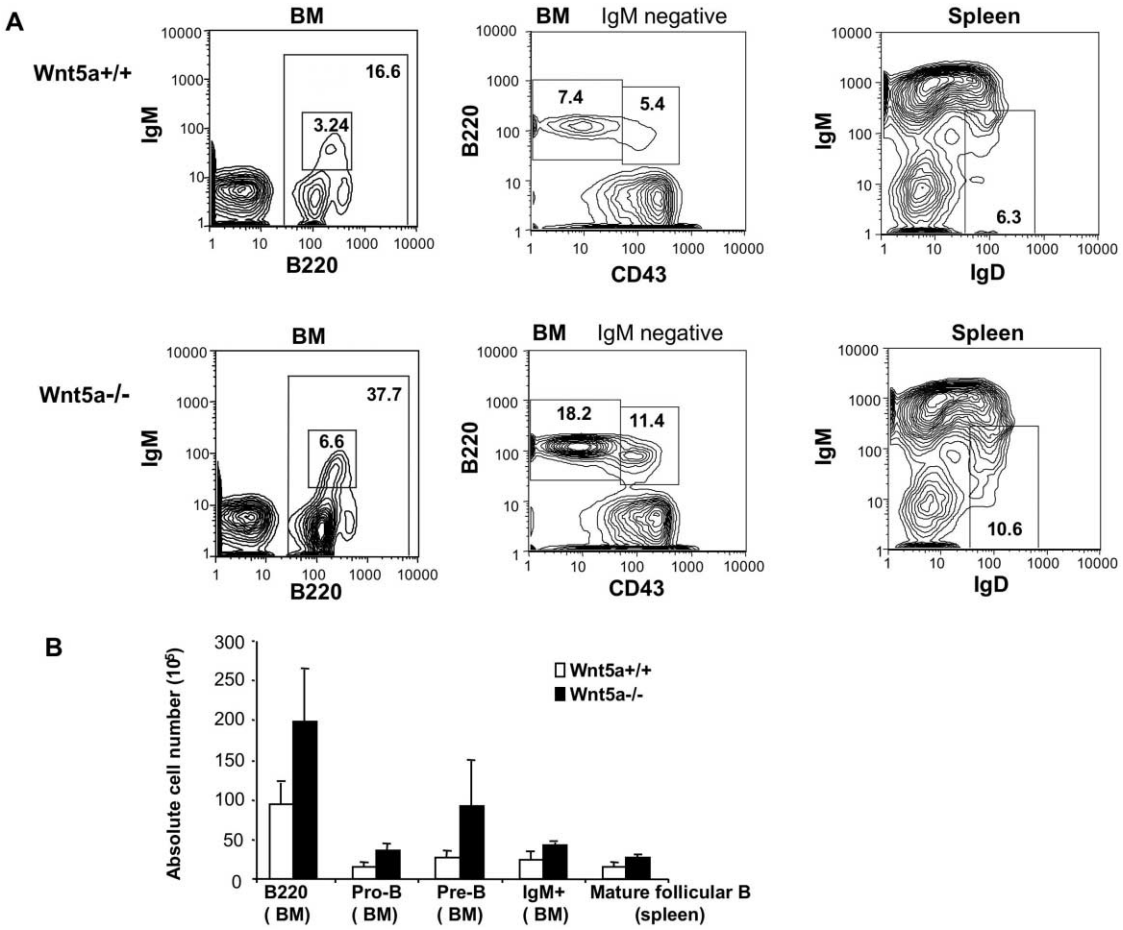

Figure 3. Wnt5a regulates B cell proliferation in a cell-autonomous manner

Tissues from mice transplanted with wt or Wn5a$^{-/-}$ FL were harvested, stained with antibodies as indicated, and analyzed by flow cytometry.
A: Representative result of flow cytometric analysis.
B: Absolute numbers of different B cell populations.

incorporation assays and TUNEL (terminal deoxynuclotidyl transferase-mediated dUTP nick end-labeling) assays were performed on FL cells. Wnt5a null mice contain 50% more BrdU- and B220-positive cells than wild-type mice (Figure 2E), indicating that Wnt5a negatively regulates B cell proliferation. The difference in cell proliferation rates between Wnt5a null and wild-type mice is less profound in the B220-negative population. No difference was found in the number of TUNEL-positive cells in E19 wild-type and Wnt5a mutant B220^{+} cells (data not shown). Taken together, we concluded that increase of the B lineage cell compartment in Wnt5a null FL results from increased proliferation of B cells and not from alteration in the resistance of B cells to apoptosis.

To determine whether Wnt5a inhibits B cell proliferation in a cell-autonomous manner, we performed adoptive transfer experiments. Wild-type or Wnt5a null FL cells were transplanted into lethally irradiated Rag1$^{-/-}$ mice. Six weeks post-transplant, bone marrow, spleen, and peripheral blood cells were harvested and examined by flow cytometry. Both the percentage and the absolute numbers of pro-B (B220^{+}/CD43^{+}), pre-B (B220^{+}/CD43^{-}/IgM^{-}), IgM^{+} B cells (B220^{+}/IgM^{+}) in bone marrow and mature follicular B cells (IgM^{low}/IgD^{high}) in the spleen were greater in mice transplanted with Wnt5a null FL cells than those rescued with wild-type FL (Figure 3). Increased numbers of mature follicular B cells (IgM^{low}/IgD^{high}) were also observed in the peripheral blood of recipients transplanted with Wnt5a null FL (data not shown). These findings indicate that the increased rate of B cell proliferation observed in Wnt5a-deficient mice is due to loss of a cell-intrinsic signal that cannot be complemented by physiologic levels of Wnt5a present in serum or in the stroma.

Wnt5a inhibits the response of B cells to IL-7

B cell progenitors are critically dependent on the cytokine interleukin-7 (IL-7) for growth (Fry and Mackall, 2002), and the increase in Wnt5a null B cell numbers and size of the colonies observed in the colony-forming assays suggests that hyperproliferation in the absence of Wnt5a could result from alterations in the responsiveness of the B cells to IL-7. To test this possibility, [^{3}H] thymidine uptake assays were performed on FACS-sorted FL B cells cultured in the presences or absence of IL-7 (Figure 4A). Wild-type and mutant B cells have similar [^{3}H] thymidine uptake in the absence of IL-7. However, Wnt5a null B cells display an increased rate of [^{3}H] thymidine uptake in the presence of IL-7 relative to wild-type samples, suggesting that

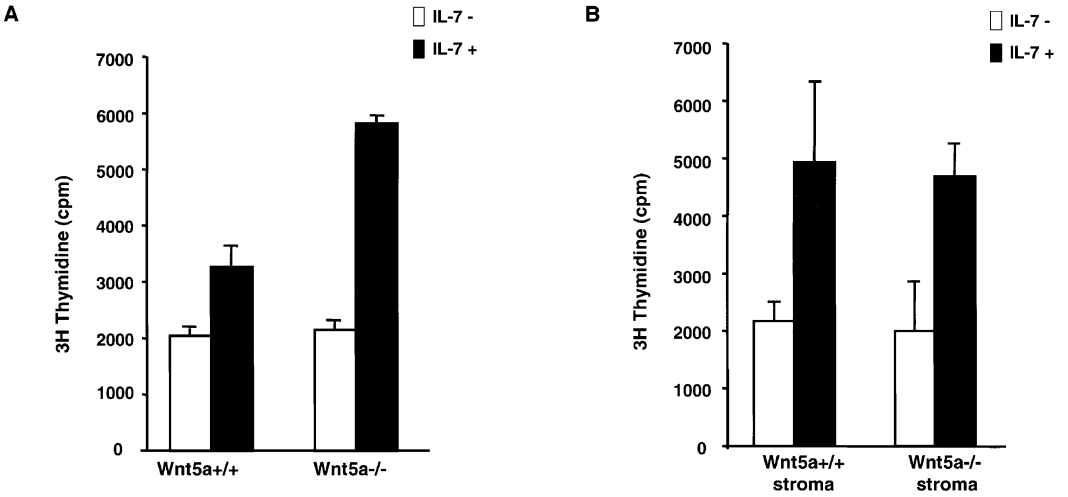

Figure 4. Absence of Wnt5a increases B200$^+$ cell responsiveness to IL-7 in a cell-transic manner

A: [^{3}H] thymidine uptake assay was performed on cultured B220-positive cells, sorted from wild-type and Wnt5a null FL, in the presence or the absence of IL-7.

B: The sorted Wnt5a null FL B220$^+$ cells were cultured on stromal layers derived from wild-type or Wnt5a null FL in the presence or the absence of IL-7, and [^{3}H] thymidine uptake assay was performed as in Figure 4A. Thymidine uptake of FL B220$^+$ cells was adjusted by subtracting H3 uptake of stromal cells. Error bars represent means ± SD (**A** and **B**).

Wnt5a alters the responsiveness of B cells to IL-7. We also assessed the proliferation rate of Wnt5a null B220$^+$ cells when cultured on stromal cell feeder layers derived from either wild-type FL or Wnt5a null FL. There was no difference in the response of Wnt5a null B220$^+$ cells to IL-7 stimulation when plated on stromal layers that contained or lacked Wnt5a (Figure 4B). The inability of the wild-type stromal cells to compensate dysfunction of Wnt5a in the B cells indicates that Wnt5a-mediated suppression of IL-7 stimulation is cell autonomous and is consistent with the results of the FL adoptive transfer experiment.

Wnt5a signals through the noncanonical Wnt/Ca^{++} pathway and downregulates cyclin D1 expression

Wnt5a has been proposed to activate both the canonical Wnt/β-catenin pathway (He et al., 1997; Toyofuku et al., 2000) and Wnt/Ca^{++} pathway (Figure 5A), and activation of the Wnt/Ca^{++} pathway has been suggested to block the Wnt/β-catenin signaling cascade (Torres et al., 1996; Kuhl et al., 2001; Ishitani et al., 2003). To dissect the mechanistic pathway utilized by Wnt5a in regulating B cell proliferation, we analyzed β-catenin levels in wild-type and Wnt5a null FL. No difference was detected in β-catenin levels in whole-cell or in nuclear extracts between the two genotypes, suggesting that Wnt5a was neither activating nor inhibiting the canonical Wnt/β-catenin pathway in FL (Figure 5B). Therefore, we examined the Wnt/Ca^{++} pathway. CamKII activity assays showed that Wnt5a null FL cells possess 40% less activity than wild-type FL cells (Figure 5C), even though Western blot analysis revealed equal amounts of total CamKII in both samples (data not shown). Immunoblotting with antibodies against phosphorylated PKC demonstrated a reduction in activated PKC in both Wnt5a null FL and in Wnt5a null B220$^+$ cells sorted from E18 FL (Figure 5D). In addition, PKC has been found to activate the c-*jun* amino-teminal kinase (JNK) (Mitsutake et al., 2001; Saneyoshi et al., 2002). Although total JNK levels were equal in Wnt5a wild-type and null fetal liver, activated JNK was decreased in the absence of Wnt5a (Figure 5E), further suggesting that absence of Wnt5a leads to a reduction in PKC activity. These results indicate that Wnt5a is signaling via the Wnt/Ca^{++} pathway to activate CamKII and PKC. Cyclin D1 and c-*myc* are cell cycle regulatory genes whose expression was previously reported to be upregulated in response to Wnt1-mediated activation of the β-catenin pathway. We examined the effect of Wnt5a upon regulation of these two genes using real-time PCR (Figure 6A). The threshold cycle numbers (C_T) for cyclin D1 in wild-type FL was 28.5 versus 26.8 for Wnt5 null FL. Control experiments utilizing cyclin D1 cDNA as input template confirmed that a two cycle difference in threshold values equates to a 4-fold difference in template concentration, indicating that cyclin D1 expression in Wnt5 null FL was increased 4-fold relative to wild-type levels. The C_T for c-*myc* in *Wnt5a* wild-type and null cells was 26.3 and 26.5, respectively, suggesting no significant difference in c-*myc* expression levels in the presence or absence of Wnt5a.

To further confirm the ability of Wnt5a to downregulate cyclin D1 and inhibit cell proliferation, we transduced Wnt5a into two independent mouse B cell lines. 7C6 cells and 1–8 cells are Abelson leukemia virus-transformed B cells lines that lack Wnt5a expression. Mouse Wnt5a cDNA was cloned into an MSCV2.2IRES-GFP vector, and MSC-Wnt5a-GFP or MSC-GFP (empty vector) DNA was electroporated into 7C6 cells and 1–8 cells. Transduced cells were recovered by FACS and analyzed for proliferation and for expression of Wnt5a and cyclin D1. Transduction of Wnt5a into 7C6 or 1–8 cells increased the level of phosphorylated PKC and decreased cyclin D1 levels, indicating that Wnt5a expression activates the noncanonical signaling pathway and inhibits cyclin D1 (Figure 6B). BrdU staining of the transduced cells revealed that expression of Wnt5a reduced proliferation in both B cell lines (Figure 6C). These data are consistent with our in vivo results indicating that Wnt5a negatively regulates B cell proliferation in a cell-autonomous manner

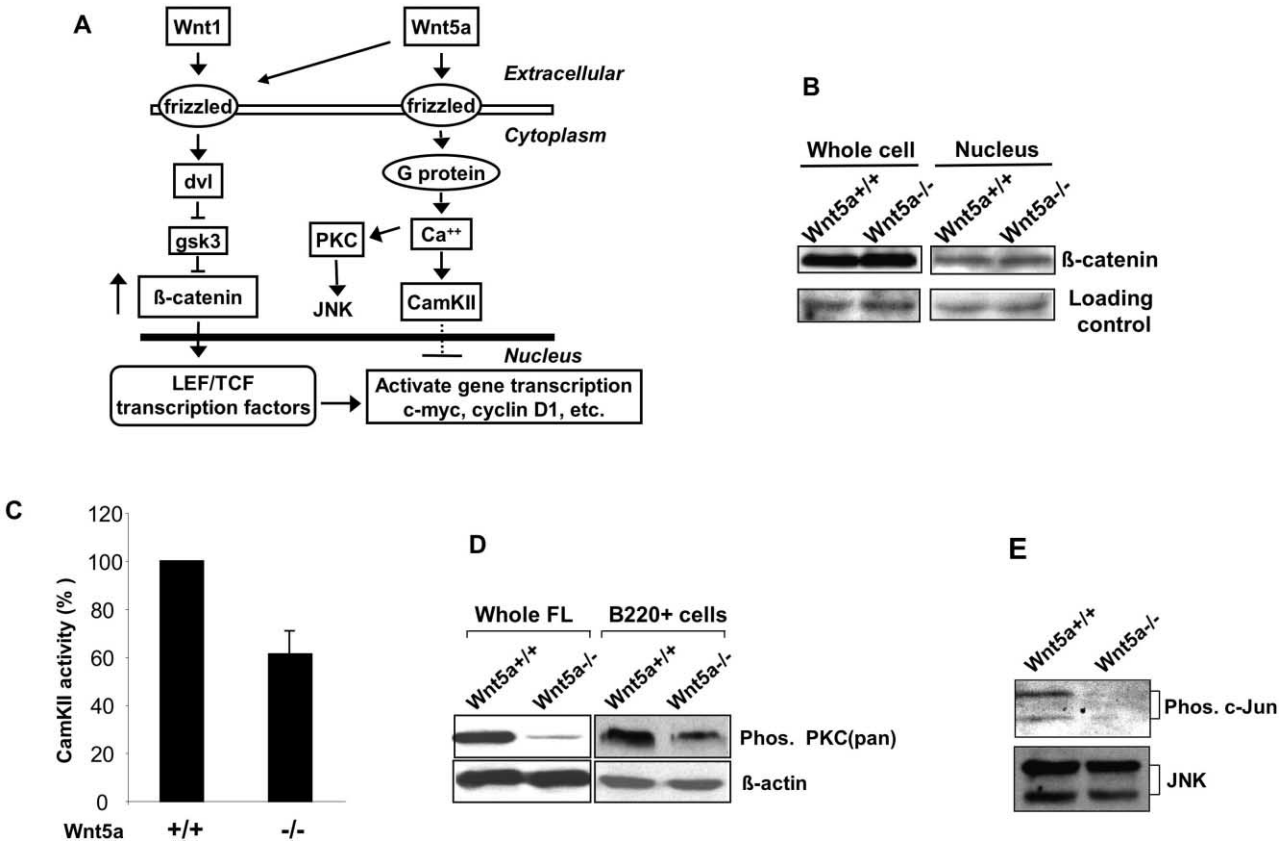

Figure 5. Wnt5a signals through Ca^{++} pathway and not the β-catenin pathway in FL

A: Schematic presentation of Wnt5a signaling pathways.
B: Whole-cell and nuclear β-catenin levels in wild-type and Wnt5a null FL. Antibodies against mouse β-tubulin and Ini1 are used as protein loading controls for whole-cell and nuclear extracts, respectively.
C: CamKII activity assays. Wild-type FL CamKII activity is presented as 100% and mutant FL is given as CamKII percentage relative to wild-type. Error bars represent mean ± SD.
D: Western blot on E19 FL to detect phosphorylated PKC. Antibodies against phosphorylated PKC (Pan) or β-actin (for verification of equal loading) were used.
E: JKN kinase activity was performed using immunoprecipitates of JNK. The immunocomplexes were assayed for JNK activity using an antibody against phosphorylated c-*jun*. Total JNK was detected by Western blot analysis using antibodies against JNK.

by activating the Wnt/Ca^{++} pathway and inhibiting cyclin D1 expression.

Wnt5a heterozygous mice develop B cell lymphoma and chronic myeloid leukemia with loss of heterozygosity for Wnt5a function

To explore the role of Wnt5a in tumorigenesis, we performed tumor assays on cohorts of *Wnt5a* heterozygous mice and on genetically matched, wild-type controls. Seventeen percent of inbred 129 *Wnt5a* heterozygous mice and twenty-five percent of C57Bl/6X129 (mixed background) *Wnt5a* heterozygous mice developed spontaneous B cell lymphoma or chronic myeloid leukemia by 24 months of age (Figure 7A). In contrast, age- and strain-matched wild-type control mice did not present with tumors during this timeframe. Affected *Wnt5a* heterozygous mice presented with spleenomegaly and enlarged liver and lymph glands. Moribund mice were sacrificed and analysis of the tumor tissues was performed using physical staining and by antibody staining to confirm the tumor types (Figure 7B). Thirty-eight percent of the observed tumors were B cell lymphomas as determined by $B220^+$ staining, with the remainder staining negative for $B220^+$, positive for chloroacetate esterase, and classified as myeloid leukemias. Five representative B cell lymphomas were further analyzed to determine if the lymphomas were clonal in origin. PCR assays performed using DNA isolated from tumor tissues revealed specific rearrangements of the VDJ region of the immunoglobulin heavy chain locus in each tumor, indicating that the B cell lymphomas were clonal in origin (Figure 7C). Analysis of DNA isolated from representative B cell lymphomas revealed loss or reduction of the Wnt5a wild-type allele, indicating loss of heterozygousity (LOH) for Wnt5a in the tumor tissue (Figure 7D). To confirm loss of Wnt5a function in the tumors, select tumors were isolated from representative mice and analyzed for the presence of Wnt5a protein by Western blot. Wnt5a was found to be absent in four of four tumor samples (Figure 7E). We also observed increased cyclin D1 levels in these tumor samples (Figure 7E). The loss of functional Wnt5a in the tumors characterizes Wnt5a as a tumor suppressor in B cell lymphoma and myeloid leukemia.

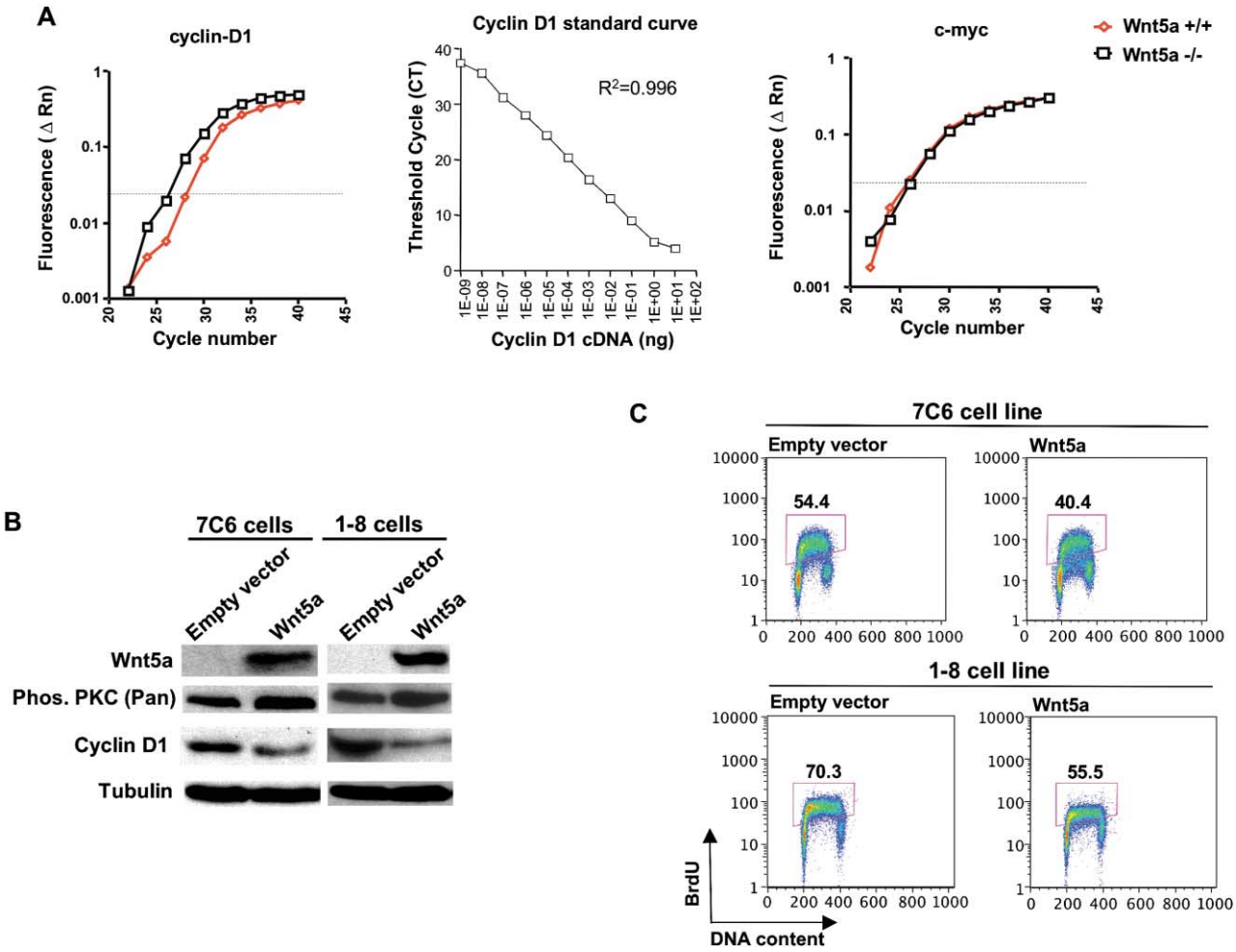

Figure 6. Wnt5a suppresses cyclin D1 expression

A: Real-time PCR on total RNA extracted from E19 wild-type or Wnt5a null FL. The threshold cycle number (C_T) for each sample was calculated with a fluorescence threshold (*Rn*) of 0.03. Dashed line indicates the fluorescence threshold (*Rn*). Control real-time PCR using cyclin D1 cDNA as a standard is also shown.

B: Transduction of Wnt5a into 7C6 or 1–8 mouse B cell lines activates the Wnt/Ca^+ pathway and suppresses cyclin D1 levels.

C: Cell cycle analysis of asynchronously dividing B cells transduced with Wnt5a or empty vector. Transduction of Wnt5a into the transformed, Wnt5a null B cell lines inhibits cell proliferation as determined by BrdU uptake (percentage of cells in S phase given).

Loss of WNT5A expression in primary human leukemia tissue

To examine whether WNT5A might also function as a tumor suppressor in human malignancies, primary tumor tissues from ten human acute lymphoblastic leukemias (ALL, $CD19^+$, or $CD4^+$) and ten acute myeloid leukemias (AML, $CD13^+/CD33^+$) were examined for *WNT5A* expression by RT-PCR and Southern analysis. *WNT5A* expression has been reported previously in human B cell lines and myeloid cell lines and was readily detected in normal human peripheral white blood cells as well as in normal human $CD34^+$ bone marrow cells (Figure 8A). However, eight ALL samples (pre-B cell ALL, $CD19^+$) lacked *WNT5A* transcripts, whereas two ALL samples (one T cell ALL, $CD4^+$ and $CD19^-$ and one mixed preB/myeloid cell ALL, $CD19^+/CD33^+$) retained WNT5A expression. All of the myeloid leukemia samples displayed either greatly reduced levels or complete absence of *WNT5A* transcripts. Therefore, loss of *WNT5A* expression correlates with tumorigenesis in human hematopoietic tissues. Eight AML samples and eight B cell ALL samples were analyzed further for presence of the WNT5A gene. Southern analysis of genomic DNA purified from the tumor tissues was performed using a probe against WNT5A exon 5 coding sequences. Several of the AML samples and half of the B cell ALL samples displayed either loss or a large reduction in Wnt5a gene dosage (Figure 8B). Examination of cyclin D1 expression levels in the eight primary ALL samples lacking WNT5A expression and in the primary AML samples using real-time PCR revealed a 4.7-fold increase (on average) in cyclin D1 expression relative to normal control samples (Table 1).

Discussion

Previous studies have reported that Wnt5a induces cell proliferation, and Wnt5a is upregulated in a variety of human cancers (Austin et al., 1997; Iozzo et al., 1995; Lejeune et al., 1995; Saitoh and Katoh, 2001; Van Den Berg et al., 1998). In addition,

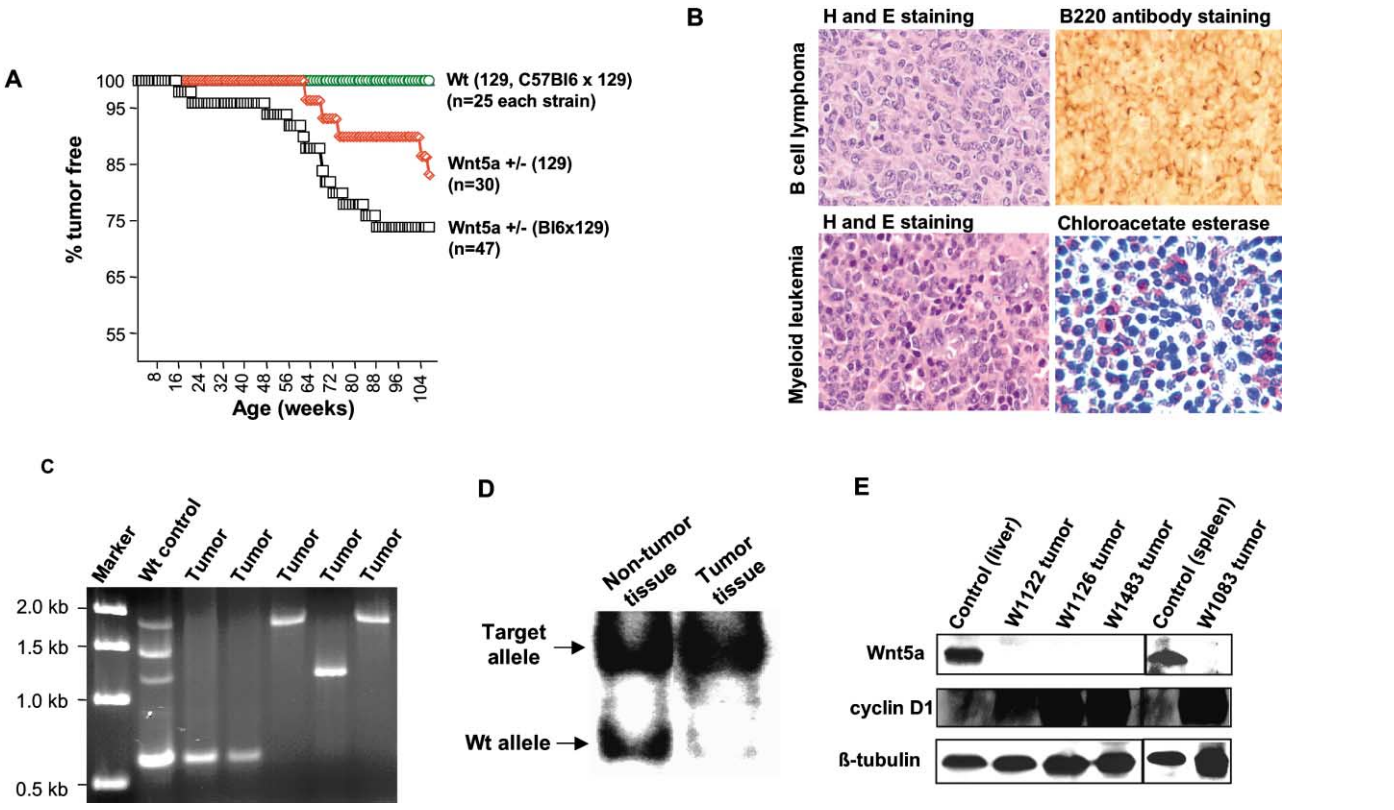

Figure 7. Wnt5a deficiency induces mouse tumor formation

A: Tumorigenesis in *Wnt5a* heterozygous mice. Wild-type 129 and 129xC57Bl/6 strain mice (circle) displayed no tumor formation during the time course of the assay. *Wnt5a* heterozygous mice on a pure 129 strain background (triangle) or on a mixed 129xC57Bl/6 background (rectangle) presented with spontaneous tumor formation. n = number of mice tested.

B: *Wnt5a*$^{+/-}$ mice develop B cell lymphoma or chronic myeloid leukemia. Representative tumor sections harvested from *Wnt5a*$^{+/-}$ mice. Serial sections were stained with H&E or with B220 antibody. CML samples were confirmed by chloroacetate staining.

C: Ethidium bromide stained agarose gel of PCR results using five representative B cell mouse tumors with wild-type spleen as control.

D: Southern analysis of genomic DNA isolated from representative mouse B cell lymphoma demonstrating LOH for the wild-type Wnt5a allele.

E: Loss of Wnt5a correlates with tumor formation and increased cyclin D1. Representative tumor samples isolated from liver (W1122, W1126, W1483) or spleen (W1083) and control tissue (*Wnt5a*$^{+/-}$ non-tumor liver and spleen) were immunoblotted with anti-Wnt5a or cyclin D1 antibodies or anti-β-tubulin as loading control.

exogenous expression of human Wnt5a has been recently found to induce melanoma progression and metastasis (Weeraratna et al., 2002), further underscoring the oncogenic potential of Wnt5a. Using genetically defined cells and mice, we now demonstrate that Wnt5a negatively regulates progenitor B cell proliferation and responsiveness to IL-7. The more rapid proliferation of Wnt5a null cells in methylcellulose cultures, the increased thymidine uptake exhibited by Wnt5a null cells in liquid culture, and the increased numbers of B cells in the Rag1$^{-/-}$ mice transplanted with Wnt5a null FL indicate that Wnt5a-mediated regulation of B cell proliferation is cell intrinsic. In addition, Wnt5a null fetal liver cells respond similarly to IL-7 when grown on stromal cells derived from Wnt5a null or wild-type mice, further confirming that Wnt5a functions in a cell-autonomous manner to regulate B cell proliferation. Lastly, reintroduction of Wnt5a into Wnt5a-deficient B cell lines downregulated proliferation of the transduced cells. The ability of Wnt5a to regulate cell growth clearly is cell context dependent, as other tissues in the Wnt5a null mice show reduced cell proliferation (Yamaguchi et al., 1999), and mouse embryonic fibroblasts derived from Wnt5a null mice proliferate more slowly than those derived from wild-type littermates (data not shown).

Loss of Wnt5a leads to B cell lymphomas and myeloid leukemias in Wnt5a heterozygous mice. Although onset of tumorigenesis is quite rapid in some mice, the incomplete penetrance of the tumor phenotype suggests that multiple molecular alterations are likely needed in the Wnt5a null cells in order for tumorigenesis to occur. The reduction or absence of human Wnt5a expression in human primary tumor samples suggests that loss of Wnt5a is a frequent event in these hematopoietic tumors. Loss of Wnt5a gene dosage observed in several of the primary tumors is also compelling, as presumably the cells were not heterozygous prior to the onset of the disease. Thus, absence of WNT5A in these tumor tissues required the deletion of both alleles. Absence of WNT5A expression was also observed in four B cell ALL samples that retained *WNT5A* coding sequences, suggesting that loss of WNT5A in these tumors might be due to other mutations that either destabilize the RNA or silence the *WNT5A* promoter. WNT5A expression was completely undetectable in eight acute B cell leukemias but was

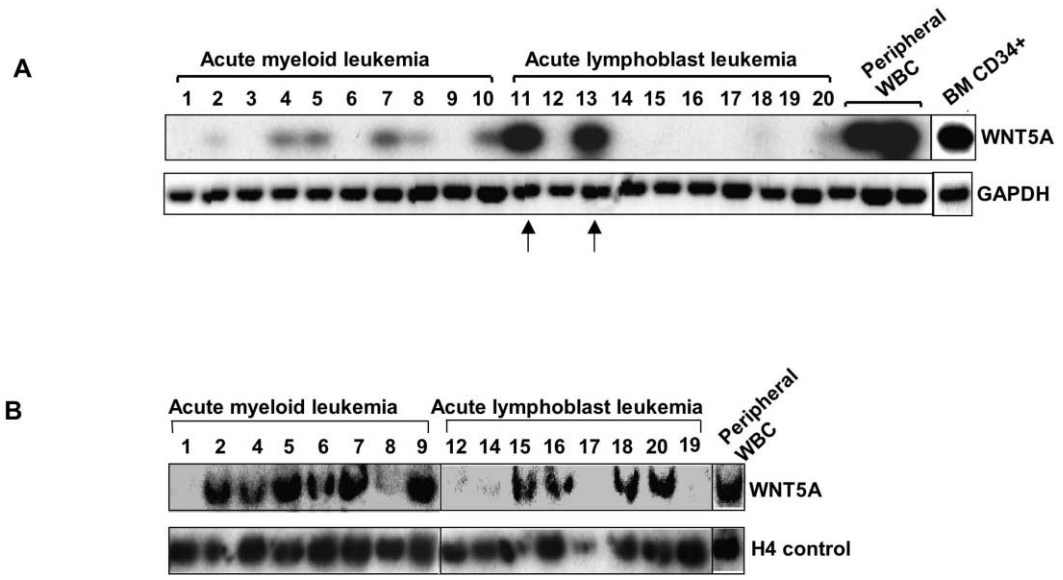

Figure 8. Loss of WNT5A and increased cyclin D1 in human cancer tissues

A: RNA samples extracted from patients and from healthy individuals were examined for *WNT5A* expression by RT-PCR and Southern blot hybridization. Arrows indicate T cell or mixed myeloid/B cell lymphocytic leukemia sample.

B: Southern analysis of genomic DNA isolated from the patient primary tumors using WNT5A or human histone H4 cDNA as probes. Loss of *WNT5A* is observed in tumor samples 1, 8, 12, 14, 17, and 19.

observed in one acute T cell leukemia and one mixed preB/myeloid cell leukemia. Although Wnt5a is widely expressed in hematopoietic development, the absence of T cell leukemia in the Wnt5a het mice may therefore reflect differences in the role of Wnt5a in regulating B cell and T cell growth. The increase in cyclin D1 expression in both mouse and human tumors lacking Wnt5a corroborates the results of the fetal liver experiments and transduction data, indicating that Wnt5a negatively regulates cell proliferation and tumorigenesis by inhibiting cyclin D1 expression in hematopoietic tissues.

Table 1. Cyclin D1 expression levels of human cancer samples

Patients	Leukemia type	Cyclin D1 cycles[a]	GAPDH cycles[a]	Normalized cyclin D1 cycles[b]	Fold increase in cyclin D1 expression[c]
Control	Normal	23.05	17.06	23.05	—
P1	AML	23.47	17.13	23.37	−0.6
P2	AML	22.41	16.78	22.78	0.5
P3	AML	19.83	17.45	19.38	7.3
P4	AML	19.37	16.61	19.89	6.3
P5	AML	19.38	16.80	19.67	6.7
P6	AML	20.46	17.22	20.26	5.5
P7	AML	19.14	17.08	19.11	7.8
P8	AML	20.21	17.17	20.08	5.9
P9	AML	20.01	17.03	20.04	6.0
P10	AML	21.25	17.18	21.10	3.8
P12	PreB-ALL	21.07	16.75	21.45	3.1
P14	PreB-ALL	20.41	17.17	20.27	5.5
P15	PreB-ALL	21.14	17.27	20.88	4.3
P16	PreB-ALL	19.82	17.2	19.65	6.7
P17	PreB-ALL	21.25	16.95	21.38	3.3
P18	preB-ALL	21.05	16.91	21.23	3.6
P19	PreB-ALL	19.71	16.64	20.20	5.6
P20	PreB-ALL	22.07	17.46	21.56	2.9

[a]The threshold cycle number for each sample was calculated using the Applied Biosystems ABI prism 7700 sequence detection system software at a fluorescence threshold (Rn) of 0.011.
[b]Threshold cycle numbers of the tumor samples were normalized for GAPDH.
[c]The increase in cyclin D1 expression was calculated by multiplying by two the difference between the normalized threshold cycle numbers of the tumor sample and of control.

Development studies and cell differentiation studies using various model systems have revealed that Wnt5a can signal through both Wnt/β-catenin pathway and Wnt/Ca^{++} pathway to regulate cell adhesion, mobility, proliferation, and differentiation (He et al., 1997; Kuhl et al., 2000, 2001; Sheldahl et al., 1999; Toyofuku et al., 2000), and it has been proposed that the signaling pathway induced by Wnt5a may depend upon the Frz receptor type that binds Wnt5a (Holmen et al., 2002). Signaling by Wnt5a via the Wnt/Ca^{++} pathway results in the activation of PKC and CamKII and downregulation of Wnt/β-catenin-responsive gene transcription (Kuhl et al., 2001). To determine which pathway mediates Wnt5a signaling in B cell development, we analyzed activation of these pathways in Wnt5a null and wild-type fetal liver tissue. Whole-cell and nuclear β-catenin level remained unchanged in the presence or absence of Wnt5a, while activity of CamKII and PKC was decreased in Wnt5a null cells. Although expression of c-*myc* was unchanged in the absence of Wnt5a, expression of cyclin D1 was increased in Wnt5a null cells. These results indicate that Wnt5a signals via the Wnt/Ca^{++} pathway to inhibit cyclin D1 expression. Because β-catenin levels and c-*myc* expression levels were unchanged in the presence or absence of Wnt5a, it is unlikely that Wnt5a inhibits the Wnt/β-catenin signaling cascade in these cells.

It is unclear at present how Wnt5a signaling via the Ca^{++} pathway inhibits cyclin D1 expression and B cell proliferation. A recent study in PKC∂ knockout mice found that deletion of PKC∂ led to increased B cell proliferation and autoimmunity

(Miyamoto et al., 2002). Since total phosphorylated PKC was found to be lower in the Wnt5a null cells in our study, this PKC isoform is also likely to be downregulated in these cells. Furthermore, several lines of evidences indicate that CamKII activation exerts a dominant inhibitory effect on gene transcription by phosphorylating CREB, the cAMP response element binding protein (Sun et al., 1994; Wu and McMurray, 2001). Cyclin D1, unlike c-Myc, contains a cAMP-responsive element in its promoter region, and it is possible that the increased cyclin D1 mRNA levels seen in Wnt5a null cells are results from attenuation of CamKII activity. Thus, Wnt5a-mediated activation of both CamKII and PKC may function to inhibit B cell proliferation.

In summary, our studies reveal that Wnt5a negatively regulated B cell proliferation and response to IL-7 through the Wnt/Ca^{++} signaling pathway. Loss of *Wnt5a* expression in both mice and human tumors indicates that Wnt5a suppresses tumorigenesis in these hematopoietic tissues. To our knowledge, this is the first demonstration that any member of the Wnt family of protooncogenes can function as a tumor suppressor. Our conclusion is supported in part by previous experiments in which ectopic expression of WNT5A in a transformed uroepithelial cell line showed inhibition of cell growth (Olson et al., 1997) and transfection of antisense WNT5A into C57MG mammary epithelial cells induced a transformed cell morphology (Olson and Gibo, 1998). Wnt5a signaling could be a potential therapeutic target for the treatment of acute lymphocytic leukemia and myeloid leukemia. Further studies on the role of Wnt5a in myeloid cell proliferation and differentiation, as well as characterization of additional functional roles for Wnt5a in hematopoietic development, are presently ongoing.

Experimental procedures

Generation of Wnt5a null embryos

Mice bearing a functionally inactivated allele of Wnt5a were generated previously (Yamaguchi et al., 1999). In order to maintain an inbred genetic background in these studies, germline-transmitting Wnt5a chimeric mice were bred with $129Sv^{Brd}$ mice to establish 129-strain mice bearing the Wnt5a null allele. Embryos were harvested from intercrosses of these Wnt5a heterozygous mice, and Wnt5a null mice were initially identified by morphology. The genotypes of all samples were subsequently confirmed by PCR.

Analysis of RNA by RT-PCR and Southern blots

Total RNA was prepared using TRIzol (Invitrogen). Reverse transcription with polymerase chain reaction (RT-PCR) assay was carried out using SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen). Primer sequences (from 5′ to 3′) are human WNT5A, forward: ctacgagagtgctcgcatcct catg, reverse: cattgcgcacgcagtagtcag; mouse Wnt5a, forward: tcgggactgg ttgtgggg, reverse: agctcgcagccgtccatc; human GAPDH, forward: cagcct caagatcatcagca, reverse: tgagcttgacaaagtggtcg; mouse Gapdh, forward: caccatggagaaggccgggg; reverse: gacggacacattgggggtag. The RT-PCR cycling parameters are 48°C for 30 s; 94°C for 2 min; then followed by 26 cycles (for GAPDH) or 42 cycles (for human and mouse Wnt5a) at 94°C for 30 s, 55°C (for human WNT5A), or 60°C (for mouse Wnt5a and GAPDH of both mouse and human) for 30 s and 72°C for 1 min with a final extension of 72°C for 10 min after the last cycle. Following RT-PCR and gel electrophoresis, Southern analysis was performed to confirm the identity of the Wnt5a PCR fragments.

Stromal cell primary culture

Cells prepared from E17 FL of wild-type and Wnt5a null mice and from 6-week-old wild-type mouse bone marrow were cultured as described previously (Whitlock et al., 1984).

Flow cytometry

Single-cell suspensions were prepared from E19 FL and stained with anti-B220-APC (Caltag Laboratory), -CD43-PE (PharMingen), IgM-FITC (PharMingen). The analysis was performed with FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software (Tree Star, Inc.).

Fluorescence-activated cell sorting (FACS)

Single-cell suspensions were prepared from FL, bone marrow, and spleen of 6- to 8-week-old mice and were antibody stained and sorted using FACSVantage (Becton Dickinson).

Colony forming assays

Cells were prepared from E19.5 $Wnt5a^{+/+}$ and $^{-/-}$ FL, and 9×10^4 cells were plated in methylcellulose media M3630 supplemented with IL-7, according to the manufacturer's instructions (StemCell Technologies). Colonies (>50 cells) were counted 7 days later using an inverted microscope at 40× magnification.

BrdU analysis

Mice were injected intraperitoneally with 50 μg BrdU/gram body weight on the 18th day of pregnancy. Embryos were harvested 1 hr after injection, and the fetal liver removed and stained with anti-B220-APC and sorted. Cells were subsequently stained with anti-Brdu-FITC (Becton Dickinson) according to supplier's instruction and analyzed by flow cytometry as above.

B cell proliferation assays

Sorted 2×10^5 B220-positive cells were plated into wells of 96-well plates with 200 μl of RPMI supplemented with 10% FCS, 2 mM L-glutanine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM 2-mercaptoethanol (GIBCO-BRL) in the presence or absence of IL-7 (0.5 ng/ml, StemCell Technologies). Following 48 hr of culture at 37°C and 10% CO_2, cells were pulsed with [^{3}H] thymidine (1 μCi per well) for 16 hr prior to harvest. Incorporated radioactivity was quantified with a Liquid Scintillation and Luminescence Counter (Perkin Elmer Wallac Inc.).

Transduction of B cells

Mouse Wnt5a cDNA (Upstate Biotechnologies) was inserted into the pMSCV2.2 IRES-GFP vector. The 7C6 and 1–8 mouse B cell lines were cultured in RPMI supplemented with 10% FCS, 2 mM L-glutanine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM 2-mercaptoethanol (GIBCO-BRL). Control vector or Wnt5a expression vector was transduced into cells using a Gene Pulser II (BioRad) electroporator at 275 V, 950 μF, and GFP-positive cells were sorted by FACS. Transduced cells were harvested for Western blot analysis as well as propidium iodide and BrdU staining at 48 to 72 hr post-electroporation.

Adoptive transplantation of fetal livers

$Rag1^{-/-}$ mice (C57BL/6 strain, from Jackson Laboratory) were used as recipients and received 900 cGy γ-irradiation (^{137}Cs source, dose rate 81 cGy/min) 4 hr before cell transfer. 5×10^6 cells prepared from E16 FL were injected intravenously into the recipient mice. Bone marrow, spleen, and peripheral blood cells were harvested for flow cytometry analysis at 6 weeks post-transplantation.

Kinase activity assays

Fetal liver was lysed by sonication in 50 mM Hepes, 270 mM KCl, 0.1 mM. EDTA, 0.1 mM EGTA, 10% glycerol, and 1% NP40. Protein was recovered by centrifugation and concentrations were determined using BCA Protein Assays (Pierce). CamKII activity was measured by using a CaM Kinase II Assay Kit (Upstate) and 100 μg protein extract according to supplier's instruction. Incorporated radioactivity was monitored with Multi-Purpose Scintillation Counter (Beckman Instruments, Inc.). JNK kinase assays were performed using immunoprecipitates of JNK as previously described (Raingeaud et al., 1995), with phosphorylation of c-Jun detected using phospho-c-Jun antibody (Ser63) (Cell Signaling Technology).

Western blotting

Cells or tissues were lysed with 1% NP40, 0.5% sodium deoxylate, 0.1% SDS, and protease inhibitor cocktail tables (Complete) in PBS. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred

to nitrocellulose membrane (BIO-RAD). Immunoblots were probed with primary antibodies against Wnt5a (R&D System), CamKII, cyclin D1 (Santa Cruz Biotechnology, Inc.), JNK (Cell signaling), phosphorylated-PKC-pan (Cell Signaling), or β-catenin-Exon 3 (Calbiochem), then with anti-IgG-HRP antibodies, and visualized using ECL (Amersham Pharmacia Biotech) according to the supplier's instructions.

Quantitative real-time PCR (QRT-PCR)

Relative quantities of mRNA expression were analyzed using QRT-PCR (Applied Biosystems ABI Prism 7700 Sequence Detection System, Applied Biosystems). One-step QRT-PCR reactions were performed in the presence of SYBR green. The primer sequences (5′ to 3′) were as follows: mouse cyclin D1 forward: gcgtaccctgacaccaatct, reverse: cacaacttctcggcagtcaa; mouse c-*myc* forward: tgcgacgaggaagagaattt, reverse: aaccgctccacata cagtcc; human cyclin D1 forward: cccgcacgatttcattgaac, reverse: gcggattg gaaatgaacttcac; The PCR cycling parameters were the same as used in the RT-PCR described previously. The specificity of amplification was confirmed by electrophoresis of the products on an agarose gel.

Tumorigenesis studies

Cohorts of *Wnt5a* wild-type or heterozygous mice on either a 129SVbrd or 129 × C57Bl/6 genetic background were monitored for spontaneous tumor formation. Moribund mice were sacrificed for necropsy, select tissues harvested for DNA, RNA, and protein, and the remaining tissue fixed in 10% phosphate-buffered formalin and paraffin embedded. Sections were prepared and stained with hematoxylin/eosin and examined microscopically. In some cases, sections were histochemically stained with B220 antibody or with chloroacetate esterase to confirm diagnosis of B cell lymphoma or myeloid leukemia, respectively. Human peripheral blood and bone marrow were obtained from patients diagnosed with either acute lymphoblastic leukemia or acute myeloid leukemia. Healthy, noncancerous individuals' peripheral blood and bone marrow ($CD34^+$) cells were used as controls. White blood cells were isolated by gradient centrifugation of the whole blood over Histopaque-1077 (Sigma). RT-PCR and Southern blot to verify the PCR product specificity were carried out as described above. LOH of Wnt5a in mouse tumor sample was detected by Southern blot as previously described (Yamaguchi et al., 1999). For human samples, genomic DNA was isolated from the primary tumor samples and digested with HindIII. A DNA fragment of WNT5A exon 5 coding sequences was generated by PCR using primers 5′-ccagcatcacatcacaacacgg-3′ (sense) and 5′-atttgggcagcagggaggaaag-3′ (antisense) and used as probe for the Southern analysis. A human histone H4 gene probe was used as a loading control in these experiments.

Clonal analysis of mouse B cell tumors

Analysis of the clonality of mouse B cell lymphomas was perfomed as described previously (Rolink et al., 1993). Genomic DNA from representative tumor samples was isolated and used as template for PCR reactions using primer pair (5′ of D_H) 5′-acaagcttcaaagcacaatgcctggct-3′ and (3′ of J_{H4}) 5′-gggtctagactctcagccggctccctcaggg-3′. PCR amplification of wild-type mouse spleen yielded a 1.7 Kb $D_H J_{H1}$ fragment, a 1.45 Kb $D_H J_{H2}$ fragment, a 1.1 Kb $D_H J_{H3}$ fragment, and a 0.6 Kb $D_H J_{H4}$ fragment.

Quantitative real-time PCR (QRT-PCR)

Relative quantities of mRNA expression were analyzed using QRT-PCR (Applied Biosystems ABI Prism 7700 Sequence Detection System, Applied Biosystems). One-step QRT-PCR reactions were performed in the presence of SYBR green. The primer sequences (5′ to 3′) were as follows: mouse cyclin D1 forward: gcgtaccctgacaccaatct, reverse: cacaacttctcggcagtcaa; mouse c-*myc* forward: tgcgacgaggaagagaattt, reverse: aaccgctccacata cagtcc; human cyclin D1 fowward: cccgcacgatttcattgaac, reverse: gcggattg gaaatgaacttcac; The PCR cycling parameters were the same as used in the RT-PCR described previously. The specificity of amplification was confirmed by electrophoresis of the products on an agarose gel.

Acknowledgments

We thank Marcia Woda and members of the UMASS Medical School FACS Core for their help, Jianhua Liu and Janice Wallace for technical assistance with the experiments, Gary Stein for the human histone H4 probe, and Roger Davis, Michelle Kelliher, Richard Goldsby, and Barbara Osborne for helpful comments on this manuscript. Core facilities used to perform some of these experiments were supported by Program Project Grant 5P30DK32520 from the National Institute of Diabetes and Digestive and Kidney Diseases. This research was supported by NIH grant CA77735 to S.N.J.

Received: March 19, 2003
Revised: July 30, 2003
Published: November 24, 2003

References

Austin, T.W., Solar, G.P., Ziegler, F.C., Liem, L., and Matthews, W. (1997). A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood *89*, 3624–3635.

Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature *382*, 638–642.

Bouillet, P., Oulad-Abdelghani, M., Ward, S.J., Bronner, S., Chambon, P., and Dolle, P. (1996). A new mouse member of the Wnt gene family, mWnt-8, is expressed during early embryogenesis and is ectopically induced by retinoic acid. Mech. Dev. *58*, 141–152.

Fry, T.J., and Mackall, C.L. (2002). Interleukin-7: from bench to clinic. Blood *99*, 3892–3904.

He, X., Saint-Jeannet, J.P., Wang, Y., Nathans, J., Dawid, I., and Varmus, H. (1997). A member of the Frizzled protein family mediating axis induction by Wnt-5A. Science *275*, 1652–1654.

Holmen, S.L., Salic, A., Zylstra, C.R., Kirschner, M.W., and Williams, B.O. (2002). A novel set of wnt-frizzled fusion proteins identifies receptor components that activate beta-catenin-dependent signalling. J. Biol. Chem. *277*, 34727–34735.

Iozzo, R.V., Eichstetter, I., and Danielson, K.G. (1995). Aberrant expression of the growth factor Wnt-5A in human malignancy. Cancer Res. *55*, 3495–3499.

Ishitani, T., Kishida, S., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R.T., Ninomiya-Tsuji, J., and Matsumoto, K. (2003). The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signalling. Mol. Cell. Biol. *1*, 131–139.

Jordan, C.T., Astle, C.M., Zawadzki, J., Mackarehtschian, K., Lemischka, I.R., and Harrison, D.E. (1995). Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. Exp. Hematol. *23*, 1011–1015.

Kuhl, M., Sheldahl, L.C., Malbon, C.C., and Moon, R.T. (2000). Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. J. Biol. Chem. *275*, 12701–12711.

Kuhl, M., Geis, K., Sheldahl, L.C., Pukrop, T., Moon, R.T., and Wedlich, D. (2001). Antagonistic regulation of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signalling. Mech. Dev. *106*, 61–76.

Lejeune, S., Huguet, E.L., Hamby, A., Poulsom, R., and Harris, A.L. (1995). Wnt5a cloning, expression, and up-regulation in human primary breast cancers. Clin. Cancer Res. *1*, 215–222.

Mitsutake, N., Namba, H., Shklyaev, S.S., Tsukazaki, T., Ohtsuru, A., Ohba, M., Kuroki, T., Ayabe, H., and Yamashita, S. (2001). PKC delta mediates ionizing radiation-induced activation of c-Jun NH(2)-terminal kinase through MKK7 in human thyroid cells. Oncogene *20*, 989–996.

Miyamoto, A., Nakayama, K., Imaki, H., Hirose, S., Jiang, Y., Abe, M., Tsukiyama, T., Nagahama, H., Ohno, S., Hatakeyama, S., and Nakayama, K.I. (2002). Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta. Nature *416*, 865–869.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996).

XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell *86*, 391–399.

Murdoch, B., Chadwick, K., Martin, M., Shojaei, F., Shah, K.V., Gallacher, L., Moon, R.T., and Bhatia, M. (2003). Wnt-5a augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. Proc. Natl. Acad. Sci. USA *100*, 3422–3427.

Myers, D.C., Sepich, D.S., and Solnica-Krezel, L. (2002). Bmp activity gradient regulates convergent extension during zebrafish gastrulation. Dev. Biol. *243*, 81–98.

Nusse, R., and 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.

Olson, D.J., and Gibo, D.M. (1998). Antisense wnt-5a mimics wnt-1-mediated C57MG mammary epithelial cell transformation. Exp. Cell Res. *241*, 134–141.

Olson, D.J., Gibo, D.M., Saggers, G., Debinski, W., and Kumar, R. (1997). Reversion of uroepithelial cell tumorigenesis by the ectopic expression of human wnt-5a. Cell Growth Differ. *8*, 417–423.

Petrenko, O., Beavis, A., Klaine, M., Kittappa, R., Godin, I., and Lemischka, I.R. (1999). The molecular characterization of the fetal stem cell marker AA4. Immunity *10*, 691–700.

Phillips, R.L., Ernst, R.E., Brunk, B., Ivanova, N., Mahan, M.A., Deanehan, J.K., Moore, K.A., Overton, G.C., and Lemischka, I.R. (2000). The genetic program of hematopoietic stem cells. Science *288*, 1635–1640.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., and Davis, R.J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. *270*, 7420–7426.

Rolink, A., Haasner, D., Nishikawa, S.-I., and Melchers, F. (1993). Changes in frequencies of clonable pre B cells during life in different lymphoid organs of mice. Blood *81*, 2290–2300.

Saitoh, T., and Katoh, M. (2001). Molecular cloning and characterization of human WNT5B on chromosome 12p13.3 region. Int. J. Oncol. *19*, 347–351.

Saneyoshi, T., Kume, S., Amasaki, Y., and Mikoshiba, K. (2002). The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. Nature *417*, 295–299.

Sheldahl, L.C., Park, M., Malbon, C.C., and Moon, R.T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Curr. Biol. *9*, 695–698.

Slusarski, D.C., Yang-Snyder, J., Busa, W.B., and Moon, R.T. (1997a). Modulation of embryonic intracellular Ca2+ signalling by Wnt-5A. Dev. Biol. *182*, 114–120.

Slusarski, D.C., Corces, V.G., and Moon, R.T. (1997b). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. Nature *390*, 410–413.

Stark, K., Vainio, S., Vassileva, G., and McMahon, A.P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature *372*, 679–683.

Sun, P., Enslen, H., Myung, P.S., and Maurer, R.A. (1994). Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. *8*, 2527–2539.

Takada, S., Stark, K.L., Shea, M.J., Vassileva, G., McMahon, J.A., and McMahon, A.P. (1994). Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. *8*, 174–189.

Torres, M.A., Yang-Snyder, J.A., Purcell, S.M., DeMarais, A.A., McGrew, L.L., and Moon, R.T. (1996). Activities of the Wnt-1 class of secreted signalling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. J. Cell Biol. *133*, 1123–1137.

Toyofuku, T., Hong, Z., Kuzuya, T., Tada, M., and Hori, M. (2000). Wnt/frizzled-2 signalling induces aggregation and adhesion among cardiac myocytes by increased cadherin-beta-catenin complex. J. Cell Biol. *150*, 225–241.

Van Den Berg, D.J., Sharma, A.K., Bruno, E., and Hoffman, R. (1998). Role of members of the Wnt gene family in human hematopoiesis. Blood *92*, 3189–3202.

van Gijn, M.E., Daemen, M.J., Smits, J.F., and Blankesteijn, W.M. (2002). The wnt-frizzled cascade in cardiovascular disease. Cardiovasc. Res. *55*, 16–24.

Wallingford, J.B., Vogeli, K.M., and Harland, R.M. (2001). Regulation of convergent extension in Xenopus by Wnt5a and Frizzled-8 is independent of the canonical Wnt pathway. Int. J. Dev. Biol. *45*, 225–227.

Weeraratna, A.T., Jiang, Y., Hostetter, G., Rosenblatt, K., Duray, P., Bittner, M., and Trent, J.M. (2002). Wnt5a signalling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell *1*, 279–288.

Whitlock, C.A., Robertson, D., and Witte, O.N. (1984). Murine B cell lymphopoiesis in long term culture. J. Immunol. Methods *67*, 353–369.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signalling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.

Wolda, S.L., Moody, C.J., and Moon, R.T. (1993). Overlapping expression of Xwnt-3A and Xwnt-1 in neural tissue of Xenopus laevis embryos. Dev. Biol. *155*, 46–57.

Wong, G.T., Gavin, B.J., and McMahon, A.P. (1994). Differential transformation of mammary epithelial cells by Wnt genes. Mol. Cell. Biol. *14*, 6278–6286.

Wu, X., and McMurray, C.T. (2001). Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein. J. Biol. Chem. *276*, 1735–1741.

Yamaguchi, T.P. (2001). Heads or tails: Wnts and anterior-posterior patterning. Curr. Biol. *11*, R713–R724.

Yamaguchi, T.P., Bradley, A., McMahon, A.P., and Jones, S. (1999). A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development *126*, 1211–1223.

Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S., and Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. EMBO Rep. *3*, 69–75.