Functional Analysis of Tcl1 Using Tcl1-Deficient Mouse Embryonic Stem Cells

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Abstract

Tcl1 is highly expressed in embryonic stem (ES) cells, but its expression rapidly decreases following differentiation. To assess Tcl1’s roles in ES cells, we generated Tcl1-deficient and -overexpressing mouse ES cell lines. We found that Tcl1 was neither essential nor sufficient for maintaining the undifferentiated state. Tcl1 is reported to activate Akt and to enhance cell proliferation. We found that Tcl1 expression levels correlated positively with the proliferation rate and negatively with the apoptosis of ES cells, but did not affect Akt phosphorylation. On the other hand, the phosphorylation level of β-catenin decreased in response to Tcl1 overexpression. We measured the β-catenin activity using the TOPflash reporter assay, and found that wild-type ES cells had low activity, which Tcl1 overexpression enhanced 1.8-fold. When the canonical Wnt signaling is activated by β-catenin stabilization, it reportedly helps maintain ES cells in the undifferentiated state. We then performed DNA microarray analyses between the Tcl1-deficient and -expressing ES cells. The results revealed that Tcl1 expression downregulated a distinct group of genes, including Ndp52, whose expression is very high in blastocysts but reduced in the primitive ectoderm. Based on these results, we discuss the possible roles of Tcl1 in ES cells.

Introduction

To elucidate the key molecules involved in the pluripotency of mouse embryonic stem (ES) cells, we compared expressed sequence tag (EST) counts between embryonic stem (ES) cells and somatic tissues using digital differential display [http://www.ncbi.nlm.nih.gov/Unigene/info_ddd.html] [1]. The T-cell lymphoma breakpoint 1 gene, Tcl1, was one of the genes we identified using this method. This gene is expressed at high levels in ES cells. The normal expression of Tcl1 in mice is restricted to early embryogenesis [2], fetal tissues (liver, thymus, bone marrow, and yolk sac) [3], developing lymphocytes [4], and adult testis [5], suggesting that it functions in stem cells and progenitor cells. The human ortholog, TCL1A, is responsible for T-cell tumors caused by chromosomal rearrangements involving 14q32 [6]. Thus, Tcl1 may have a positive role in cell proliferation and/or survival, an idea that is supported by the occurrence of T-cell leukemia in mice carrying a TCL1 transgene under control of the lck promoter [7]. On the other hand, an analysis of Tcl1-null mutant mice indicated that Tcl1 is important for the development of preimplantation embryos; a lack of maternally derived Tcl1 impairs the embryo’s ability to undergo normal cleavage and develop to the morula stage, especially in vitro [2].

Glover et al. [8] identified genes whose expression changes when ES cells are induced to differentiate. Tcl1 is one of seven genes that showed a rapid decrease in expression concurrent with a decrease in the frequency of undifferentiated cells. Genetic manipulations that affect the undifferentiated state of ES cells are often reported to downregulate Tcl1 together with pluripotency-related genes, such as Dppa3, Klf4, and Zfp42 [9,10]. Matoba et al. [11] identified Tcl1 as a downstream target of Oct3/4 using the ZHBTc4 ES cell line, in which the expression of Oct3/4 (encoded by Pou5f1) can be downregulated by tetracycline [12]. They showed that Oct3/4 binds to the promoter region of the Tcl1 gene to activate its transcription, and, using ES cells in which Tcl1 was knocked down by shRNA, they showed that Tcl1 is involved in regulating proliferation, but not differentiation. However, the effect of complete loss of the Tcl1 gene on the state of ES cells has not been reported. In the present study, we generated Tcl1-deficient and -overexpressing ES cell lines and compared the undifferentiated phenotypes and gene expression patterns between them.

Results

Generation of -deficient ES Cells

We first examined the Tcl1 expression during ES cell differentiation into trophectoderm using the ZHBTc4 ES cell line, in which the expression of Oct3/4 can be downregulated by tetracycline [10]. As shown in Figure S1, Tcl1 expression decreased with similar kinetics as Fgf4 and Zfp42 [10,11]. Matoba et al. [11] that Tcl1 is a downstream target of Oct3/4.

To examine Tcl1’s function, we generated Tcl1-deficient ES cells. We used a gene-targeting vector in which parts of Tcl1 exons 2 and 3 were replaced by the PGK-puro cassette (Figure 1A) and
obtained several Tcl1+/− clones. Two of these clones were subjected to a high concentration of puromycin, to select for Tcl1+/− clones (Figure 1B). We chose Tcl1+/− clone #2, derived from one of the Tcl1+/− cell clones, and Tcl1+/− clones #4 and #5, derived from the other clone, for further analysis. Since uniparental disomy accounts for most of the loss of heterozygosity in ES cells [14], it was important to confirm that the phenotypes seen in the Tcl1+/− clones were due to Tcl1 deficiency. We first performed karyotype analysis for these Tcl1+/− clones #2, #4, and #5. More than 60% of the cells from each clone were shown to be karyotypically normal (6/7, 6/7, and 6/10, respectively). We then rescued the Tcl1 expression in these three Tcl1+/− ES cell clones by introducing a CAG promoter-driven expression vector containing the Tcl1 cDNA (CAG-Tcl1). The resulting transfectant clones, Tcl1−/− (CAG-Tcl1) #1 and #3, and Tcl1−/− (CAG-EGFP) #5 were derived from Tcl1+/− (KO) #4.

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Neither the Tcl1−/− ES cells nor the Tcl1−/− (CAG-Tcl1) ES cells showed any apparent changes in cell or colony morphology compared with wild-type ES cells (data not shown). Quantitative real time PCR analysis was performed for wild-type, Tcl1−/−, Tcl1−/− (CAG-Tcl1) #11 and #14 ES cells. The result showed an increase in Nanog, Zfp42 (Rex1), Fgf5, and T (Bra) expression in the Tcl1−/− ES cells, but the expression of Oct3/4 and Gata4 was not considerably affected by the Tcl1 expression (Figure 2 and S2). We also induced the differentiation of these cells by embryoid body (EB) formation and examined the expression of these genes, but did not observe any considerable differences among them (Figure 2).


**Effects of Tcl1 Deficiency on the Proliferation, Apoptosis, and Differentiation of ES Cells**

Tcl1 is known to augment Akt function through direct interaction [15]. We next asked whether Tcl1 affected Akt function in our ES cell lines. Akt has three isoforms with similar functions and is activated by various growth factors through its translocation to the cell membrane. This translocation is dependent on phosphatidylinositol 3-kinase (PI3K), which phosphorylates D3 phosphoinositide bound to Akt’s pleckstrin homology domain. At the cell membrane, Akt is activated by phosphorylation at Thr308 (in the case of Akt1) by PDK1 and at Ser473. Activated Akt promotes cell proliferation and survival by inhibiting G1 arrest and proapoptotic factors [16,17]. Therefore, we analyzed the cell growth of the wild-type, Tcl1−/−, and Tcl1−/− (CAG-Tcl1) ES cells by MTT assay. As shown in Figure 3A, Tcl1 deficiency reduced the cell proliferation by approximately 30%, while Tcl1 overexpression clearly reverses this effect. These effects were observed using two independent Tcl1−/− clones (#4 and #5) and their stable transfectant clones (#1, #3, #11, and #14).

We also examined the effects of Tcl1 deficiency on apoptosis by immunostaining cleaved caspase 3 (Figure 3B). The Tcl1−/− ES clones (#4 and #5) showed 1.8- and 1.5-fold increase in percent cleaved caspase 3-positive cells, respectively, compared with wild-type ES cells. Thus, Tcl1 overexpression in these Tcl1−/− ES cells clearly reduced the frequency of apoptotic cells, which was even lower than that in wild-type ES cells.

We next tested the in vivo growth capacity of these cells by performing teratoma formation assays (Figure 3C). The Tcl1−/− ES cells formed smaller tumors than did wild-type ES cells, consistent with their slower growth rate. To our surprise, however, the Tcl1−/− (CAG-Tcl1) ES cells produced only barely recognizable tumors. Considering that the Tcl1−/− (CAG-Tcl1) ES cells showed a similar or even better proliferation rate in vitro than did wild-type ES cells, this result might have been owing to an effect of Tcl1 overexpression on the differentiation capacity or status of the ES cells.

Thus, we examined the teratomas derived from the Tcl1−/− and Tcl1−/− (CAG-Tcl1) ES cells histologically. Within individual tumors, neural rosettes (ectoderm), epidermis (ectoderm), cartilage (mesoderm), adipose tissue (mesoderm), and gut-like epithelium (endoderm) were found, indicative of the differentiation into cells fated for each of the three germ layers (Figure 3D).

**Effect of Tcl1 on Wnt-β-catenin Signaling in ES Cells**

To gain further insight into Tcl1’s function, we looked for signaling pathways that might be affected by it. Akt is known to phosphorylate GSK3β in insulin signaling [16], and GSK3β also serves as a component of the canonical Wnt pathway involving β-catenin. In addition, other reports have indicated a functional link between Akt and β-catenin [18,19]. Since the Wnt pathway has been implicated in maintaining the undifferentiated state of ES cells, we explored whether Tcl1 acts as a bridge between the Akt signaling and Wnt pathways. In canonical Wnt signaling, briefly, when extracellular Wnt is absent, β-catenin is phosphorylated by casein kinase I and GSK3β. Phosphorylated β-catenin is recognized by E3 ligase and targeted for degradation. Upon Wnt-signaling activation, the phosphorylation of β-catenin is inhibited, and the accumulated β-catenin translocates to the nucleus, where it drives the expression of target genes through an association with Tcf/Lef [20].

To examine the effect of Tcl1 on Wnt-β-catenin signaling, we performed western blot analyses to determine the phosphorylation levels of Akt, GSK3, and β-catenin (Figure 4A). Interestingly, the phosphorylation of β-catenin was dramatically reduced by Tcl1 overexpression, but the total amount of β-catenin appeared unchanged, indicating there was a reverse correlation between the Tcl1 expression and β-catenin phosphorylation level. At the same time, the Akt and GSK3 phosphorylation levels did not correlate with the β-catenin phosphorylation level, implying that Tcl1 was closely, perhaps directly, involved in regulating β-catenin. We next examined whether Tcl1 overexpression led to an increase in nonphosphorylated β-catenin in the nuclear fraction, and found that the nonphosphorylated active β-catenin levels were enhanced in the nuclei (Figure 4B).

To measure the canonical Wnt signaling directly, we used a Tcf1/β-catenin reporter system, the TOPflash assay [21]. The reporter activity was not affected by the Tcl1 knockout, but Tcl1 overexpression enhanced it approximately 1.8-fold compared with wild-type ES cells (Figure 4C). These results suggested that β-catenin signaling is normally repressed in ES cells and is enhanced by Tcl1 overexpression. We further examined the expression levels of well-known canonical Wnt target genes, such as c-myc, axin2, Lef1, and Dll1 [http://www.stanford.edu/˜rnusse/pathways/targets.html]. Since Tcl1 positively regulates Wnt signaling, the expression levels of these genes might be lower in Tcl1−/− ES cells and higher in Tcl1−/− (CAG-Tcl1) cells than in wild-type ES cells. However, our quantitative RT-PCR results showed that the Tcl1 expression level did not significantly affect these Wnt target genes (data not shown). The only exception was Gbx2, a recently identified Wnt-β-catenin signaling target [22], which showed elevated expression in the Tcl1-overexpressing ES cells (see below).

**Effects of Tcl1 Deficiency on the Gene Expression Pattern of ES Cells**

To our knowledge, the Wnt targets in ES cells have not been systematically investigated, and there are reports of Tcl1/Lef-independent targets in some other cell types. To find genes that respond to Tcl1 overexpression in ES cells possibly through Wnt/β-catenin signaling, we compared the gene expression profiles by dual-channel DNA microarray analysis between Tcl1−/− #4 and Tcl1−/− (CAG-Tcl1) #1, between Tcl1−/− (CAG-EGFP) #6 and Tcl1−/− (CAG-Tcl1) #4, between Tcl1−/− #4 and wild-type ES cells, and between Tcl1−/− #5 and wild-type ES cells. The genes whose expression levels were consistently affected by Tcl1 expression by more than 1.7 fold were listed in Table 1. We found 16 genes (Pom, Ndp52, Tnem64, Dpa23, Tset1, Fbox15, Ephx2, Miana, Zfp42, Jam2, Meca1, Tcfp211, Pox1, Pox2, Myl7, and Plac8) that were consistently downregulated by Tcl1 expression. On the other hand, only two genes, Gbs2 and Fndc4, showed elevated expression in Tcl1-expressing cells compared with Tcl1-deficient cells and the elevation of their expression by Tcl1 was 2.7 and 2.2 fold, respectively. Quantitative RT-PCR analysis was performed for these affected genes, using Tcl1−/− #5, Tcl1−/− (CAG-Tcl1) #11, Tcl1−/− (CAG-Tcl1) #14, and wild-type ES cells. As shown in Figure 5A, the expression of Gbs2 and Fndc5 was significantly upregulated by Tcl1 overexpression, although the
expression levels of the Gbx2 gene were not considerably different between Tcl1^−/− (KO) and wild-type ES cells. All of the 16 genes were shown to be downregulated by Tcl1 expression in agreement with the DNA microarray data (Figure 5B; see Figure 2 for Zfp42).

Discussion

In the current study, we sought to elucidate the molecular pathways in which Tcl1 is involved, and the physiological role of Tcl1 in ES cell management. One well-documented function of Tcl1 is to bind Akt and increase its kinase activity [23]. Important roles of Akt and/or its upstream signal molecule, PI3K, in the self-renewal of ES cells have been reported [24–27]. Matoba et al. [11] showed that Tcl1 downregulation leads to a reduction in Akt phosphorylation in ES cells. Ema et al. [28] showed that Krüppel-like factor 5 (Klf5) is essential for the normal self-renewal of mouse ES cells using Klf5-knockout ES cells, and that Tcl1 is downregulated in the Klf5-knockout ES cells. They also showed that the Akt phosphorylation is reduced in these ES cells. These reports support the idea that Tcl1 regulates ES-cell proliferation via Akt phosphorylation. In fact, we observed that Tcl1 overexpression in ES cells clearly increased the cell proliferation and reduced the frequency of apoptotic cells as shown in Figure 3. However, these effects of Tcl1 could not be accounted for by an increase of Akt phosphorylation. Our data showed that Tcl1 expression was not correlated with the global phosphorylation levels of Akt and GSK3β (Figure 4A). Although we do not know the reason for this discrepancy, it should be noted that high-level Tcl1 expression does not necessarily lead to Akt phosphorylation, because Akt
phosphorylation is undetectable in seminomas and CD4⁺CD56⁺ blastic tumors of dendritic cell origin, in which Tcl1 is highly expressed [2,29]. In any case, we believe that our Tcl1-deficient and overexpressing ES cells are ideal tools for identifying Tcl1 targets in ES cells.

Interestingly, our data showed that Tcl1 expression was correlated with the phosphorylation level of β-catenin (Figure 4A) in ES cells. Tcl1 may regulate β-catenin through a hitherto unknown pathway related or unrelated to Akt or GSK3β. β-catenin plays a major role in the canonical Wnt pathway. It was reported that Wnt-pathway activation by 6-bromoindirubin-3’-oxime (BIO), a specific pharmacological inhibitor of GSK-3, helps maintain the undifferentiated phenotype in human and mouse ES cells [30]. However, Wnt’s precise role in mouse ES cells has been debated [31–33].

Stimulation of the canonical Wnt pathway through the binding of Wnt ligand to its receptor causes a repression of GSK3β activity, which further inhibits β-catenin degradation, resulting in β-catenin’s recruitment to the cell membrane to associate with E-cadherin or in its localization to the nucleus. Nuclear β-catenin associates with Tcl1 and activates its transcriptional activator function. It was reported that e-myc, a downstream β-catenin target, is involved in maintaining the undifferentiated state [34]. However, it is not known to what degree such transcriptional activation contributes to the maintenance of pluripotency in ES cells. In addition, β-catenin binds to Tcf3 and inhibits its repressor function. Because Tcfβ interacts with core pluripotency-associated transcription factors, such as Oct3/4, to repress their transcriptional activity, its binding to β-catenin may stabilize pluripotency [31]. It is possible that different threshold levels of these two action modes operate in ES cells, in which a low level of Wnt signaling is sufficient to direct a derepression of gene expression through the inhibition of Tcf3 repressor function, whereas high levels are required for the activation through Tcf1. The overexpression of Tcl1 led to a nearly complete loss of β-catenin phosphorylation, but not to a parallel enhancement of Tcf/Lef reporter activity and activation of target genes. The canonical Wnt activity might be actively repressed or maintained in a narrow range in ES cells. In addition, the nuclear partners of β-catenin in stem cells might be different from those in well-studied somatic cells.

As a negative modulator of β-catenin phosphorylation, Tcl1 may well be involved in anterior-posterior determination and gastrulation development, especially considering that many reports point to the importance of β-catenin at this stage [35–37]. The poor teratoma formation elicited by the Tcl1⁻/⁻(CAG-Tcl1) ES cells may reflect lineage restriction or premature stem cell/progenitor loss, as was shown to occur during the epithelial-mesenchymal transition in mouse embryos expressing dominant stable β-catenin [35]. Further examination of this issue will require a longer incubation of EBs and...
were consistently downregulated by expression could be classified into four categories (Table S1): (1) gene expression of ES cells. These genes downregulated by seemed to exert repressive rather than stimulative effects on the Tcl1

**Exp. 1:**
- **Genes upregulated in Tcl1-expressing ES cells**
  - **Gene Name** | **Systematic Name** | Exp. 1** | Exp. 2*** | Exp. 3****
  - Plac8 | NM_139198 | 2.8 | 2.5 | 1.9
  - Ephx2 | NM_007940 | 4.5 | 2.5 | 2.2
  - Mlana | AK029028 | 2.8 | 3.1 | 2.6
  - Zfp42 (Rhox5) | NM_008818 | 2.9 | 3.2 | 2.3
  - Ndp52 (Calcoca2) | AK010816 | 13.4 | 6.5 | 2.9
  - Tmem64 | NM_181401 | 5.1 | 8.4 | 1.7
  - Dppa3 (Stella) | NM_139218 | 6.3 | 5.1 | 1.8
  - Tcstv1 | NM_018756 | 3.5 | 3.5 | 4.2
  - Fbxo15 | AF176530 | 2.7 | 4.7 | 2.6
  - Tcfcp2l1 (CRTR-1) | NM_018756 | 3.5 | 3.5 | 4.2

**Exp. 2:**
- **Genes downregulated in Tcl1-deficient ES cells**
  - **Gene Name** | **Systematic Name** | Exp. 1** | Exp. 2*** | Exp. 3****
  - Plac8 | NM_139198 | 2.8 | 2.5 | 1.9
  - Ephx2 | NM_007940 | 4.5 | 2.5 | 2.2
  - Mlana | AK029028 | 2.8 | 3.1 | 2.6
  - Zfp42 (Rhox5) | NM_008818 | 2.9 | 3.2 | 2.3
  - Ndp52 (Calcoca2) | AK010816 | 13.4 | 6.5 | 2.9
  - Tmem64 | NM_181401 | 5.1 | 8.4 | 1.7
  - Dppa3 (Stella) | NM_139218 | 6.3 | 5.1 | 1.8
  - Tcstv1 | NM_018756 | 3.5 | 3.5 | 4.2
  - Fbxo15 | AF176530 | 2.7 | 4.7 | 2.6
  - Tcfcp2l1 (CRTR-1) | NM_018756 | 3.5 | 3.5 | 4.2

**Exp. 3:**
- **Genes downregulated in Tcl1-expressing ES cells**
  - **Gene Name** | **Systematic Name** | Exp. 1** | Exp. 2*** | Exp. 3****
  - Plac8 | NM_139198 | 2.8 | 2.5 | 1.9
  - Ephx2 | NM_007940 | 4.5 | 2.5 | 2.2
  - Mlana | AK029028 | 2.8 | 3.1 | 2.6
  - Zfp42 (Rhox5) | NM_008818 | 2.9 | 3.2 | 2.3
  - Ndp52 (Calcoca2) | AK010816 | 13.4 | 6.5 | 2.9
  - Tmem64 | NM_181401 | 5.1 | 8.4 | 1.7
  - Dppa3 (Stella) | NM_139218 | 6.3 | 5.1 | 1.8
  - Tcstv1 | NM_018756 | 3.5 | 3.5 | 4.2
  - Fbxo15 | AF176530 | 2.7 | 4.7 | 2.6
  - Tcfcp2l1 (CRTR-1) | NM_018756 | 3.5 | 3.5 | 4.2

*Genes listed are those which showed more than 2.0-fold differences in Exp. 1 and 2 and more than 1.7-fold differences in Exp. 3 between Tcl1-expressing and deficient ES cells.

Tcl1 affects the gene expression in ES cells via an unidentified pathway. Tcl1 expression is reported to be fairly high in embryos. The expression is diminished during the transition from ICM to primitive ectoderm. However, the period is yet to be investigated, but major roles of Tcl1 may be repression of the trophectoderm fate and promotion of the transition from ICM to primitive ectoderm. The fact that the genes involved in placental function and the genes whose expression diminishes during the transition from ICM to primitive ectoderm were downregulated in Tcl1-expressing ES cells may be consistent with this notion. Interestingly, the latter genes included *Dppa3*, which is considered a defining marker of the mouse ES cell state and is not expressed in epiblast stem cells [38]. Its expression was reported to be heterogeneous in mouse ES cell cultures. It is thought that the *Dppa3*-negative mouse ES cells are more 'epiblast-like' but have not been stably committed to this transition since they readily revert to *Dppa3*-positive [39]. Thus, Tcl1 may be involved in the the metastability and plasticity of ES cells.

**Materials and Methods**

**Ethics Statement**

Experiments involving animals were carried out in accordance with institutional guidelines under protocols (No. 21-089-4) approved by the Animal Care and Use Committee of the Osaka University Graduate School of Medicine.

**ES Cell Culture and Differentiation**

The murine ES cell line, E14tg2a ES cells, was maintained without feeder cells in Glasgow Minimum Essential Medium (GMEM) (Cat.#G6148; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum, 100 μM 2-mercaptoethanol, 1% non-essential amino acids (Cat.#11140-050; Life Technologies, Carlsbad, CA), 1% sodium pyruvate (Cat.#11360-070; Life Technologies), and leukemia inhibitory factor (LIF) (Cat.#129-05601; Wako Pure Chem., Osaka, Japan) on gelatin-coated dishes [12]. All assays were carried out before passage 30. For EB formation, approximately 3.0×10⁶ trypsinized cells were seeded into a 10-cm² bacterial grade dish, and the culture medium was changed every day starting on day 2.

**Targeted Disruption of the Tcl1 gene**

The targeting vector contained two genomic fragments that had been amplified by long PCR using genomic DNA isolated from E14 ES cells as a template. The oligonucleotides used for PCR were: left arm forward, 5'-GGCGAGATTATATAGATGTCCTCACCCTGATGAC-3' (S抓获), left arm reverse, 5'-GCCGATATCGACCGATACTGCA-3' (S抓获), right arm forward, 5'-ATAGAGGGCGGCCGCAAGTGAAATAACCCA-3' (N抓获), right arm reverse, 5'-TTTAGGGGGCGCTTGGATCTTCTTGTTCCTC-3' (N抓获). One fragment (left arm) was 4.8-kb long and contained exon 1 and part of exon 2; the other (right arm) was 4.3-kb long and extended from within exon 3 to 2.8-kb downstream of exon 4. To assemble the targeting vector, the XhoI-NcoI fragment containing the MC1 promoter-driven diphtheria toxin A gene was excised from pMC1-DTA-pA[41] and subcloned into pBluescript II in which the SalI site had been modified to a S抓获 site. The amplified left arm was digested with S抓获 and EcoRV, ligated to a DNA fragment containing a phosphoglucerase kinase (Pkg) promoter-driven puromycin acetyl transferase gene cassette, and subsequently introduced into the Tcl1-N抓获 site of the above vector, which harbored a diphtheria toxin A cassette. Finally, the N抓获-digested right arm was cloned into the N抓获 site of the targeting vector. The targeting vector was linearized by S抓获 digestion and introduced into E14 ES cells by electroporation. Two days later, positive selection was started with 1.5 μg/ml puromycin (Cat.#A11138-03; Life Technologies). Clones resistant to puromycin were screened for homologous recombination.
by long and accurate PCR using primers P1, 5'-TCAGCCCATCTTGGCACATCTGGCAGATT-3' and P2, 5'-TACTTC\ CATTTGTCACGTCCTGCACGACG-3'. Two of the resulting Tcl1+/2 clones were subjected to a high concentration of puromycin to obtain Tcl1+/2 colonies. The PCR primers used to detect the loss of heterozygosity were H1, 5'-AGGAGCCTGATGATGGTGC-3' and H2, 5'-GGTCTGGGTTATTCATCGTT-3'. Twenty-eight of 32 clones resistant to the high concentration of puromycin (20–30 μg/ml) were found to be Tcl1+/2.

Generation of Tcl1+/2(CAG-Tcl1) ES Cells

The Tcl1 ORF was amplified by PCR using Pfu polymerase (Cat#11708; Life Technologies) with the following primers: 5'-CGGAATTCATGGCTACCCAGCGGGCACAG-3' (EcoRI tailed) and 5'-CGGAATTCGGTCTGGGTTATTCATCGTTGAC-3' (EcoRI tailed). The product was digested with EcoRI and cloned into the multiple cloning site of pCAG-IZ [42]. The entire expression cassette was excised with SalI and BamHI and inserted between two tandem repeats of loxP in a pBS246 derivative [43] lacking the EcoRI site. Then, Tcl1+/2 ES cells were transfected with the linearized vector by electroporation. After the transfection, ES cells were selected in the presence of 20 μg/ml Zeocin (Cat#R250-01; Life Technologies) for 7 days.

As a control, Tcl1−/− ES cells were stably transfected with an EGFP (enhanced green fluorescence protein) expression plasmid (CAG-EGFP), resulting in Tcl1−/−(CAG-EGFP).

Teratoma Formation

For the teratoma-formation assay, 1.0 × 10^6 cells in 75 μl PBS were injected subcutaneously into histocompatible F1 adult mice (C57BL/6J × 129/Ola). Four weeks later, the mice were sacrificed, and the tumors were weighed. The tumors were fixed in 20% formaldehyde and processed for paraffin embedding. Sections of paraffin-embedded tumors (5-μm thick) were deparaffinized, stained with hematoxylin-eosin, dehydrated, and examined with a microscope.

Western Blot Analysis

The total protein was extracted from ES cells. Nuclear and cytoplasmic protein fractions were prepared from ES cells using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Cat#78833; Thermo Fisher Scientific, Rockford, IL). Cell lysates or fractions were subjected to SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane (Cat#IPVH09120; Merck Millipore, Billerica, MA). The primary antibodies used were: rabbit anti-Akt (Cat#CST9272; Cell Signaling, Beverly, MA), rabbit anti-phos-
pho-Akt (Ser473) (Cat#CST9271; Cell Signaling), rabbit anti-phospho-GSK3β (Ser21/9) (Cat#CST9331; Cell Signaling), rabbit anti-phospho-β-catenin (Thr41/Ser45/Cat#CST9365; Cell Signaling), rabbit anti-phospho-β-catenin (Ser37/33/Thr41) (Cat#CST9361; Cell Signaling), rabbit anti-Tcl1 (Cat#CST4042; Cell Signaling), mouse monoclonal anti-β-catenin (Cat#610153; BD Biosciences, Franklin Lakes, NJ), mouse monoclonal antibody recognizing active β-catenin that is unphosphorylated at Ser37 and Thr41 (mAb 8E7) (Cat#05-665; Merck Millipore), mouse monoclonal anti-HSP90 (Cat#ADI-SPA-630; Enzo Life Sciences, Farmingdale, NY), mouse monoclonal anti-Oct4/3/Cat#sc-5279; Santa Cruz Biotechnol., Santa Cruz, CA), and horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-β-actin (Cat#ab20272; Abcam, Cambridge, MA). Goat anti-rabbit IgG (Cat#D0448; DakoCytomation, Houston, TX) and goat anti-mouse IgG (Cat#7072; New England BioLabs, Ipswich, MA) antibodies, both conjugated to HRP, were used as the secondary antibodies at a 1:1000 dilution, and blots were developed using the ECL Western Blotting Detection System, using the SYBR Green PCR Core Reagents (Cat#4913850; Roche, Mannheim, Germany) (primer sequences are shown in Table S3). PCR was performed with an initial step of 10 sec or 10 min at 95°C followed by 40 cycles of 5 sec at 95°C and 31 sec at 60°C. The expression levels of targeted genes were normalized to that of β-actin. Statistical analysis was performed by Student’s t-test.

Real Time PCR Analysis
Real time PCR was performed on an ABI Prism 7300 Sequence Detection System, using the SYBR Green PCR Core Reagents (Cat#4304886; Applied Biosystems, Foster City, CA) or FastStart Universal SYBR Green Master (Cat#4913850; Roche, Mannheim, Germany) (primer sequences are shown in Table S3). The expression levels of targeted genes were normalized to that of β-actin. Statistical analysis was performed by Student’s t-test.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] Assays
ES cells were seeded in 96-well plates and incubated for 24 or 48 hours, after which MTT (Cat#M5655; Sigma, St. Louis, MO) reagent was added to the wells. After 4 hours, when purple precipitates were visible under a microscope, detergent reagent was added to the wells, and the cells were incubated overnight. A microplate reader (Bio-Rad, Hercules, CA) was used to determine the optical density of each well at 550 nm.

Apoptosis Study
Apoptosis was assessed by cleaved caspase 3 staining (Cat#CST9661; Cell Signaling) and quantifying the percentage of positive nuclei among DAPI (4', 6-diamidino-2-phenylindole) -positive nuclei.

Statistical Analysis
Statistical manipulations were performed using the open-source statistical environment R (http://www.r-project.org). The P-values for the teratoma weights were calculated with exact Wilcoxon rank sum tests in the exacRankTests package, then adjusted with Holm’s method. The P values for the TOPflash assay were calculated likewise. Quantitative PCR results are presented as the mean ± SEM. Statistical analyses were carried out by Student’s t-test. A value of P<0.05 was considered statistically significant.

GEO Accession Number
The microarray data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE4801.

Supporting Information
Figure S1 Tcl1 expression rapidly declines after Oct3/4 suppression. ZHBt4 ES cells lack both alleles of the Pou3f1 gene, and contain an Oct3/4 transgene whose expression is suppressed by tetracycline [12]. RNA was extracted before and 24, 48, 72, and 96 hours after tetracycline was added to the ZHBt4 cell culture. The Tcl1, Fgf4, Pou3f1 (Oct3/4), and Gapdh gene expressions were analyzed by reverse transcription polymerase chain reaction (RT-PCR). (TIF)

Figure S2 RT-PCR analysis of Tcl1-deficient and -overexpressing ES cells. The expression of stem cell and differentiation markers was examined by RT-PCR in wild-type (WT), Tcl1−/− (KO) #4 and #5, and Tcl1−/− (CAG-Tcl1) #1 and #3 ES cells grown in LIF+ culture. Tcl1−/− (CAG-Tcl1) #1 and #3 were derived from Tcl1−/− (KO) #4. The expression of T (Bachyny) was enhanced in the Tcl1−/− ES cells grown in LIF+ culture. (TIF)
Table S1 Annotations for genes of interest in the microarray analysis.

Table S2 Primers and cycles for semi-quantitative RT-PCR.

Table S3 Primers used for real time PCR.

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Author Contributions

Conceived and designed the experiments: TM SM JM. Performed the experiments: TM SM MA TT FT. Analyzed the data: TM SM MA TT JM. Wrote the paper: TM SM JM.