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The Wilms' tumor gene *WT1* is a common marker of progenitor cells in fetal liver

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Abstract

It is well known that the Wilms' tumor gene *WT1* plays an important role in cell proliferation and differentiation, and in organ development. In this study, to examine the role of the *WT1* gene in lineage determination, fetal liver cells from LacZ-transgenic mice, in which *WT1* expression was marked by the expression of the *LacZ* gene driven by WT1 promoter, were FACS-sorted according to *LacZ* expression of high ($LacZ^{++}$) or undetectable ($LacZ^{-}$) levels, which paralleled endogenous *WT1* expression levels. $LacZ^{++}$ fetal liver cells were enriched by hepatocyte and endothelial progenitor cells. These results indicated that *WT1* expression is a common marker of both hepatocyte and endothelial progenitors. These results also implied a role of the *WT1* gene in lineage determination.

Keywords: Wilms' tumor gene; WT1; LacZ; Fetal liver cells

The Wilms' tumor gene *WT1* was identified as a gene responsible for a pediatric renal cancer, Wilms' tumor [1,2]. The *WT1* gene encodes a transcription factor with zinc finger motifs and regulates the expression of many genes associated with growth, differentiation, and apoptosis [3]. The *WT1* gene has an important role in mRNA splicing [4]. Analysis of mice carrying the disrupted *WT1* gene demonstrated a crucial role of the *WT1* gene in early urogenital development [5].

WT1 is expressed in several kinds of cancers. Sugiyama and co-workers [6,7] and others [8–11] had previously reported that high expression of the wild-type *WT1* is over-expressed gene in almost all leukemia samples regardless of disease types. Furthermore, *WT1* was over-expressed in many kinds of solid tumors, such as malignant mesothelioma [12], renal cell carcinoma [13], lung [14], breast [15], thyroid [16], and colon cancers [17], head and neck squamous cell carcinoma [18], brain tumor [19], and sarcoma of bone and soft tissues [20].

In contrast, *WT1* expression level is very low but not zero in many of the normal tissues [3], although high level of *WT1* expression is detected in some-specific tissues such as kidney, testis, and ovary [3]. We previously analyzed *WT1* expression in normal hematopoietic cells. Interestingly, *WT1* expression was restricted to $CD34^{+}$ hematopoietic stem or progenitor cells [21–25].

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Moreover, we analyzed *WT1* expression in normal hematopoietic progenitor cells at a single cell-level, and elucidated that only 1.2% of $CD34^+$ hematopoietic progenitor cells expressed *WT1* [26]. The low levels of *WT1* expression in normal hematopoietic cells reflected this infrequent subpopulation of progenitor cells. These findings suggested the possibilities that *WT1* might also be expressed in progenitors of other tissues. In fact, there are some reports showing that the *WT1* gene is expressed in some tissue-specific stem or progenitor cells, such as small hepatocyte-like progenitor cells [27], retinal neuroblast [28], and epicardially derived progenitors of smooth muscle cells and endothelial cells of developing heart [29]. Fetal liver is an appropriate organ for examination of whether *WT1* is expressed in progenitor cells of several tissues, since it contains progenitors of endothelial cells, hepatocytes, or hematopoietic cells.

In this study, we examine whether or not *WT1* is specifically expressed in progenitors in fetal liver cells of WT470LZ transgenic (WT470LZ-Tg) mice, in which the expression of the *WT1* gene is marked by β-galactosidase activity [30], and show that both hepatocyte and endothelial progenitor cells are enriched in highly *LacZ*-expressing (*WT1*-expressing) fetal liver cells.

Materials and methods

WT470LZ transgenic mouse. The WT470LZ transgenic (WT470LZ-Tg) mice were generated by Moore et al. [30]. In these transgenic mice, a 470-kb yeast artificial chromosome (YAC) spanning the WT1 locus was used as a transgene. To follow *WT1* expression, a *LacZ* reporter gene was introduced into the exon 1 of the *WT1* gene in the YAC by homologous recombination in yeast. WT470LZ vector was obtained by this recombination. WT470LZ-Tg mice were produced by pronuclear microinjection of WT470LZ vector. The mice used in this study were handled in compliance with the Guide for the Care and Use of Experimental animals published by the Institute of Experimental Animal Sciences Osaka University Medical School (IEXAS).

β-Galactosidase assays and antibody staining. Fetal liver cell suspensions were prepared and cells were labelled with fluorescein di-β-D-galactoside (FDG; Molecular Probes, Eugene, Oregon, USA), as previously described [31]. The existence of the *LacZ* transgene was checked by X-gal staining of fetal liver cells and confirmed by PCR. Briefly, fetal liver cells from the WT470LZ-Tg mice embryos were suspended in FDG staining medium [potassium phosphate-buffered balanced salt solution (PBS) supplemented with 4% fetal bovine serum (FBS), 10 mM Hepes, pH 7.2] and hypotonic loading was performed by diluting the cell suspension at 1:1 with warmed 2 mM FDG and by the following incubation at 37 °C for 2 min. The uptake of FDG was stopped by the addition of ice-cold FDG staining medium. After FDG staining, fetal liver cell suspensions were incubated on ice for 30 min with a combination of phycoerythrin (PE)-conjugated anti-CD49f (Becton–Dickinson (BD) Pharmingen, San Diego, CA), anti-Flk-1 (eBioscience, San Diego, CA), biotinylated anti-c-kit, anti-CD45, and anti-TER119 antibodies (BD Pharmingen, San Diego, CA). After two washes with staining medium (PBS supplemented with 1% FBS, 1 mM EDTA), cells were incubated with allophycocyanin (APC)-conjugated streptavidin (BD Pharmingen, San Diego, CA) on ice for 30 min. Finally, cells were washed twice and resuspended in staining medium. Before analysis and sorting, propidium iodide (PI, 1.5 μM) or Hoechst33258 (Sigma, St. Louis, MO, 5 μM) was added to stain dead cells and viable cells were analyzed and sorted on a FACS Vantage SE (BD Immunocytometry Systems, San Jose, CA). Background levels of $LacZ^{++}$ cell fractions were determined from unstained fetal liver cells of WT470LZ-Tg mice and FDG-stained fetal liver cells of wild-type mice, respectively, as previously reported [32,33].

Real-time RT-PCR. The expression of WT1 and β-actin mRNA in FACS-sorted fetal liver cells was measured by real-time RT-PCR. Sorted cells were lysed in Isogen (Nippon Gene, Toyama, Japan) and total RNA was extracted according to the manufacturer's instruction. Then, it was converted into cDNA with MMLV reverse transcriptase (Promega, Madison, WI). Real-time PCR and subsequent calculations were performed on ABI Prism 7700 Sequence Detection System (Perkin–Elmer Applied Biosystems (PE), Foster City, CA), as described elsewhere [34]. The standard curves were drawn using serial dilution of the WT1 and β-actin cDNA, and the *WT1* expression levels were shown as copy numbers of the WT1 mRNA relative to those of β-actin mRNA. All experiments were performed in duplicate and the average values were shown.

Hepatic colony-forming units in culture assay. Hepatic colony-forming units in culture (H-CFU-C) assay was performed according to the previous report [35]. FACS-sorted cells were plated in the fresh standard medium containing human recombinant HGF (50 ng/ml), and EGF (20 ng/ml) at a density of 5.0×10^3 cells/dish on laminin-coated 6-well plates (BD Discovery Labware, Bedford, MA) and incubated at 37 °C in humidified 5% CO_2 air. After the cultures for five days, colonies containing more than 100 cells were counted as H-CFU-C colonies. To confirm that the colonies were derived from hepatocyte progenitors, immunocytochemistry was performed, as previously reported [35]. Colony cells were fixed with 4% paraformaldhyde (PFA) in PBS and blocking procedure was performed with 10 mg/ml bovine serum albumin (BSA) in PBS. Colony cells were stained with an anti-albumin antibody (rabbit polyclonal IgG, Intercell technologies, Hopewell, NJ) and visualized with a Rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology, San Jose, CA). To determine background levels, colonies were stained with rabbit IgG isotype control (BD Pharmingen, San Diego, CA).

Endothelial progenitor cell culture assay. The frequencies of endothelial progenitor cells (EPC) were analyzed using EPC culture system, as previously reported [36]. FACS-sorted fetal liver cells were plated in EBM-2 Bullet Kit medium (Clonetics, San Diego, CA) supplemented with 5% FBS on human fibronectin-coated plastic plates (BD Discovery Labware, Bedford, MA) at a density of 1.5×10^4 cells/dish and incubated at 37 °C in humidified 5% CO_2 air. After the cultures for four days, to confirm that the colonies were derived from endothelial progenitors, immunocytochemistry was performed, as previously reported [37–39]. Colony cells were fixed with 4% PFA in PBS and permeabilized with 0.1% Triton X-100 in PBS. Blocking procedure was performed with 10 mg/ml BSA in PBS. Colony cells were stained with an anti-VE-cadherin antibody (F8, mouse monoclonal IgG_1, Santa Cruz Biotechnology, San Jose, CA) and visualized with the indirect immuno-alkaline phosphatase method. Colonies containing more than 100 cells were counted as EPC colonies. To determine background levels, colonies were stained with mouse IgG_1 isotype control (BD Pharmingen, San Jose, CA).

Results

Murine fetal liver cells can be separated into two distinct populations by LacZ expression levels

WT1-expressing cells in WT470LZ-Tg mice were identified by their β-galactosidase activities and FACS-