**FULL PAPER** Internal Medicine

**Quantitative Analysis of mRNA Transcripts of Hox, SHH, PTCH, Wnt, and Fzd Genes in Canine Hematopoietic Progenitor Cells and Various in vitro Colonies Differentiated from the Cells**

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**ABSTRACT.** Homeobox (Hox), Sonic hedgehog (SHH), and Wingless-type MMTV integration site family (Wnt) are known to modulate the self-renewal and expansion of hematopoietic progenitor/stem cells in humans and mice. Frizzled (Fzd) and Patched1 (PTCH1) represent the receptors of Wnt and SHH, respectively. In this study, the amounts of mRNA transcripts of the genes associated with the self-renewal of hematopoietic stem cells, HoxB3, HoxA10, Wnt5a, Wnt2b, Fzd1, Fzd6, SHH, and PTCH1, were measured in canine unfractionated bone marrow cells, CD34-enriched cells, and various colony-forming units in culture (CFU-C). Partial cDNA sequences of these 9 canine genes were determined in this study. Quantitative real-time polymerase chain reaction was employed to indicate their relative amounts of mRNA transcripts. Amounts of mRNA transcripts of HoxB3, HoxA10, PTCH1, and Wnt5a genes in canine CD34-enriched cell fraction were significantly larger than those in the CD34-depleted cell fraction. Amounts of mRNA transcripts of HoxB3, HoxA10, PTCH1, Wnt5a, and Wnt2b genes in various CFU-C cells were significantly smaller than those in the seeded CD34-enriched cell fraction. These results suggested important roles of the products of these genes in self-renewal, expansion, and survival of hematopoietic progenitor cells in dogs as shown in humans and rodents.

**KEY WORDS:** canine, CD34, Hox, SHH, Wnt.

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Hematopoietic stem cells (HSCs) possess a capacity of self-renewal and the ability to give rise to all types of blood cells. This tremendous potential for reconstituting the hematopoietic system has allowed the development of the transplantation of the HSCs as a clinical strategy in humans [14, 16, 39, 41, 44]. Conventional transplants leave engraftment to natural interactions between the graft and the host, requiring considerably large amounts of graft tissue. Technologies that efficiently expand the HSCs *ex vivo* would free the limitations resulting from graft shortages. Obtaining a sufficient amount of bone marrow graft consumes both time and effort. Since the *ex vivo* expansion of HSCs remains difficult, these highly potent therapeutic options have not been applied in clinical practice so far. Although the clinical applications of bone marrow transplantation combined with intensified chemotherapy have recently been initiated in veterinary medicine [11], identical problems exist in this field as well.

In humans and mice, many efforts to expand HSCs have been made using various cytokine cocktails, resulting in some remarkable results [51]. Key cytokines used today are stem cell factor (SCF), FLT-ligand, interleukin (IL)-3, IL-6, and thrombopoietin (TPO) [5, 6, 37, 46, 51]. However, methods that use these cytokines require a large amount of expensive recombinant proteins. More importantly, a number of recombinant cytokines that are widely available are limited to humans and mice. The activities of cytokines, particularly that of IL-3, are known to be species-specific, and their full effects can only be expected with recombinants of the same species [8, 17, 35]. Therefore, currently, it is very difficult to obtain large amounts of hematopoietic cytokines in order to expand HSCs in dogs.

Some of the recent studies on HSC focused on the endogenous transcription factors and exogenous signaling molecules. Representatives of the former group are Homeobox (Hox) and BMI1 polycomb ring finger oncogene (BMI-1), whereas those of the latter group are Wingless-type MMTV integration site family (Wnt), Sonic hedgehog (SHH) protein, bone morphogenetic protein (BMP), and Notch ligands [46]. In particular, Hox, Wnt, and SHH are known to play important roles in the self-renewal of the HSCs [3, 19, 21, 40, 46, 53].

Hox, SHH, and Wnt have been reported to be important regulators during development [22, 27, 46, 47, 53, 55]. There are 39 Hox genes known today, and they are classified into 4 groups, from A to D [21, 46]. In particular, HoxB3, B4, and A10 are known to be expressed in human CD34+ cells [20, 42, 49]. Hematopoietic progenitors retrovirally transduced with HoxB4 have demonstrated up to a 1000-fold expansion of HSCs relative to control cells transduced with control vectors [1]. Moreover, the transplantation of...
these HoxB4-transduced HSCs was shown to be repeatable without leukemia development [1, 43]. HoxB3, its gene located upstream of HoxB4 gene, is suggested to play a similar role as HoxB4 [4, 21, 42, 49]. Further, HoxA10 has been suggested to have a major influence on the lineage commitments of the hematopoietic cells [3, 46].  

SHH is a member of the “hedgehog” family of proteins. Of the 5 members of the family, SHH has been most intensively studied in relation to the self-renewal of HSCs [3, 46]. Patched (PTCH) and Smoothed (SMOH) are known as receptors of SHH, and PTCH inhibits SMOH activation in a normal state [31, 32, 55]. When SHH binds to PTCH, a repressor against SMOH is released, leading to the activation of the downstream Gli family transcription factors, which results in the activation of target genes [3, 9]. SHH has been shown to induce HSC proliferation via BMP signals in humans [3].  

Wnt is a group of secreted glycoproteins, and 19 families of Wnt genes are known in humans and mice so far [34, 45, 47]. Similar to the hedgehog family, Wnt acts via autocrine and paracrine pathways. Frizzled (Fzd) proteins act as Wnt receptors. Of the 19 molecules of the family, Wnt2b, 5A, and 10B are known to promote the proliferation of murine HSCs [2, 30, 52].  

Much evidence supports the possibility that these molecules–Hox, SHH, and Wnt–are associated with one another and work together to maintain normal hematopoiesis [10, 40]. Unlike cytokines, Hox, SHH, and Wnt are highly conserved among species from flies to humans [15, 24, 46, 47, 54, 55]. However, studies regarding the self-renewal and expansion of the HSC are extremely limited in dogs.   

The final goal of this study is to utilize these novel factors for clinical application, particularly for HSC transplantation in dogs. Knowing the profiles of the transcripts from these molecules in HSCs in dogs was considered to be the first essential step. In this study, the amounts of mRNA transcripts derived from selected Hox, SHH, and Wnt genes were measured in bone marrow-derived mononuclear cells (BMMCs), CD34-enriched and CD34-depleted fractions, bone marrow-derived stromal cells, and various CFU-Cs in dogs.  

MATERIALS AND METHODS  

Animals and cells: Bone marrow cells were aspirated from 4 healthy Beagle dogs under general anesthesia. These dogs were 1–5 years old; 1 dog was male and 3 dogs were female. Sodium citrate was used as an anticoagulant for harvesting the bone marrow cells. This study was conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.  

Isolation of BMMCs and CD34-enriched cell fraction: BMMCs were isolated by performing density gradient centrifugation of the aspirated bone marrow cells with Ficoll-Hypaque Plus (GE Healthcare UK Ltd., Buckinghamshire, UK). The CD34-enriched cell fraction was isolated from the BMMCs by a magnetic-activated cell separation system (MACS) (Miltenyi Biotec GmbH, Gladbach, Germany) according to the manufacturer’s instructions with some modifications. Mouse anti-canine CD34 monoclonal antibody, clone 1H6 (BD Biosciences, San Diego, CA.), and rat anti-mouse IgG microbeads (Miltenyi Biotec) were used for the selection of the CD34-enriched cell fraction. Beside the MACS separation of CD34-enriched cell fraction, a CD34-depleted cell fraction was also collected. For flow cytometry (FACSCalibur™; BD Biosciences), the cells were treated with biotin-conjugated mouse anti-canine CD34 monoclonal antibody and streptavidin-PerCP-Cy 5.5 conjugate to detect canine CD34+ cells. Biotin-conjugated mouse IgG1 (BD Biosciences) was used as an isotype control. Data analysis was carried out with CellQuest™ (BD Biosciences). The freshly isolated BMMCs, CD34-enriched cell fraction, and CD34-depleted cell fraction were washed, flash-frozen in liquid nitrogen, and stored at −80°C until use.  

Stromal cell primary culture: Stromal cells were cultured according to a method previously reported [33]. Briefly, BMMCs were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 20% horse serum (Gibco, Invitrogen, Carlsbad, CA) and 10−7 M of hydrocortisone (Solu-Cortef®; Pfizer Japan Inc., Tokyo, Japan). After cultivation for 2–3 weeks, a mixture of adherent cells with a fibroblastic and macrophage-like appearance was obtained. After removing the culture medium containing nonadherent cells, the adherent stromal cells were harvested by digestion with 0.5% trypsin-EDTA (Gibco).  

CFU-C assay: CFU-Cs were obtained by culturing the CD34-enriched cell fraction in methylcellulose CFU assay. Prior to the experiment, phytohemagglutinin (PHA-P)-activated lymphocyte conditioned medium (PHA-LCM; PHA-P, Sigma-Aldrich) was prepared. Canine peripheral blood mononuclear cells (2 × 106 cells/ml) were cultured in IMDM supplemented with 15% fetal bovine serum (FBS) and 10 μg of PHA-P per ml for 1 week. The culture supernatant was collected, clarified by centrifugation, and then stored at −80°C until use. The CD34-enriched cell fraction (1.14 × 10^6 cells) suspended in 250 μl PHA-LCM was added to 2.5 ml of methylcellulose medium with human recombinant cytokine supplements (SCF, 50 ng/ml; GM-CSF, 20 ng/ml; IL-3, 20 ng/ml; IL-6, 20 ng/ml; G-CSF, 20 ng/ml; and EPO, 3 U/ml) (Methocult GF+ 4435; Stem Cell Technologies Inc., Vancouver, Canada). Then, 2.2 ml of the cell suspension was dropped into 35-mm plates (Sumitomo Bakelite Co., Tokyo, Japan), and the plates were incubated at 37°C in a humidified atmosphere of 5% CO2 for 2 weeks. Colonies (number of cells > 50) were morphologically differentiated and counted under an inverted microscope. Each colony type was confirmed by Wright-Giemsa stain. Under an inverted microscope, the colonies were carefully picked up into RNase-free phosphate-buffered saline (PBS) (Sigma-Aldrich). To obtain a sufficient number of cells for an adequate amount of RNA, approximately 5 colonies of the same
type were collected into a single tube. Harvested stromal cells and CFU-Cs were washed, flash-frozen in liquid nitrogen, and kept at –80°C until use.

**RNA extraction and cDNA synthesis:** Total RNA samples were extracted from the BMMCs, CD34-enriched cell fraction, CD34-depleted cell fraction, bone marrow stromal cells, and CFU-Cs by using the Illustra RNAspin Mini RNA isolation kit (GE Healthcare UK Ltd., Buckinghamshire, UK) in combination with DNase treatment. cDNAs were synthesized with the Superscript™ III First-Strand Synthesis System (Invitrogen) according to the manufacturers’ instructions and then kept at –20°C until use. Elimination of genomic DNA was confirmed by analyzing samples without reverse transcriptase treatment.

**Cloning of the target genes:** Canine homologues of 9 genes—SHH, PTCH1, Wnt5a, Wnt2b, Fzd1, Fzd6, HoxB3, HoxB4, and HoxA10—were analyzed. The sequence of cDNA of the canine hypoxanthine phosphoribosyl transferase (HPRT) gene—which was already registered (Genbank accession number, AY283372)—was used as internal control. All of the genes except HPRT were partially cloned in this study to determine the target sequences for quantitative real-time polymerase chain reaction (RT-PCR). Various canine tissues were used as templates; they were as follows: uterus (HoxB4, SHH), spleen (HoxB3), BMMCs (PTCH1, Wnt5a, Fzd1, and Fzd6), bone marrow stromal cells (Wnt2B), and small intestine (HoxA10). The polymerases used for the cloning were ExTaq® Hot Start Version (TaKaRa Bio, Otsu, Japan) and LA Taq™ (TaKaRa Bio). Primer pairs were designed using Primer3 software (http://frodo.wi.mit.edu/). The primer pairs used for cloning each gene, their melting temperatures (Tm), sizes of the cloned sequences, and their Genbank accession numbers are listed in Table 1. Conditions for the thermal cycler (Takara PCR Thermal Cycler Dice® Gradient; TaKaRa Bio) were as follows: denaturing at 95°C for 30 sec, followed by amplification with 45 cycles of 98°C for 10 sec, each Tm for 30 sec and 72°C for 1 min, then final extension at 72°C for 7 min. PCR products were electrophoresed in 2% agarose, and the single bands were directly ligated into pGEM®-T Easy plasmid vector (Promega Corporation, Madison, WI), while nonspecific bands were extracted from the gel using QIAEXII (QIAGEN, Valencia, CA) prior to ligation. Sequencing analyses of these PCR products were carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and Genetic Analyzer 3130/3130x1 (Applied Biosystems). Cloned sequences were searched for the dog genome [23] using NCBI BLAST software.

**Quantitative RT-PCR:** Primer pairs for RT-PCR were designed based on the partially cloned sequences of the 9 genes. All the primers except those of the HPRT gene [36] were designed in this study (Table 2). For RT-PCR, the SYBR green system (SYBR® Premix Ex Taq™; TaKaRa) was employed to detect double-stranded nucleic acids formed during the PCR cycles. Conditions of the thermal cycler for real-time PCR (Thermal Cycler Dice® Real Time System; TaKaRa) were as follows: incubation at 95°C for 10 sec; followed by 45 cycles of 95°C for 5 sec and 60°C for 30 sec during which the fluorescence data were collected; followed by the last step to determine dissociation at 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec. Dissociation curves and data from polyacrylamide gel electrophoresis (data not shown) of the products were used to confirm that there was a single product for each gene. The threshold cycle (Ct) was determined by the second derivative method, and the amount of cDNA derived from the mRNA transcript of each gene was calculated as a relative value of the amount of HPRT cDNA, with a software attached to the thermal cycler (TP800) (TaKaRa).

**Statistical analysis:** All statistical analyses were performed using JMP computer software (SAS Institute Inc., Cary, NC). Wilcoxon rank sum test was used for comparison of data between CD34-enriched and CD34-depleted cell fractions. Dunnott’s test was employed for comparison of data between CD34-enriched cell fraction and various CFU-Cs.

**RESULTS**

Sequences of partially cloned genes were all confirmed by BLAST search, and they were registered in the Genbank with the accession numbers that are listed in Table 1.

The percentage of CD34+ cells in the unfractionated BMMCs was 1.37 ± 0.73%. The percentages of CD34+ cells were 82.2 ± 5.0% and 0.87 ± 0.43% in the CD34-enriched and CD34-depleted cell fractions, respectively. Amounts of mRNA transcripts of the genes examined were shown as relative values in comparison to that of the HPRT gene. The amounts of mRNA transcripts of HoxB4, HoxB3, HoxA10, SHH, PTCH1, Wnt5a, Wnt2b, Fzd1, and Fzd6 genes in the CD34-enriched cell fraction were compared to those in the CD34-depleted cell fraction (Fig. 1). Of the 9 genes examined, the amounts of mRNA of HoxB3, HoxA10, PTCH1, and Wnt5a genes in the CD34-enriched cell fraction were significantly larger than those in the CD34-depleted cell fraction. Notably, the amount of mRNA transcript of HoxA10 gene in the CD34-depleted cell population was less than 10⁻¹-fold quantity of the transcript in the CD34-enriched cell population. The amount of HoxA10 mRNA transcript in unfractionated BMMCs was significantly smaller than that of the CD34-enriched cell fraction but was within the detectable range. Unlike the HoxA10 gene, the amounts of mRNA transcripts of the other 8 genes were similar between the unfractionated BMMCs and the CD34-depleted cell fraction.

Figure 2 shows the amounts of mRNA transcripts of HoxB4, HoxB3, HoxA10, SHH, PTCH1, Wnt5a, Wnt2b, Fzd1, and Fzd6 genes in the CD34-enriched cell fraction and CFU-Cs derived from the culture of the CD34-enriched cells. The amounts of mRNA transcripts of HoxB4 gene in CFU-granulocyte/erythroid/monocyte/megakaryocyte (CFU-GEMM), CFU-granulocyte/monocyte (CFU-GM), CFU-granulocyte (CFU-G), and burst-forming-unit-
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The amounts of mRNA transcripts of \textit{HoxB3}, \textit{HoxA10}, \textit{SHH}, \textit{PTCH1}, \textit{Wnt5a}, and \textit{Wnt2b} genes were significantly smaller in all of the CFU-Cs examined than those in the CD34-enriched cell fractions. The amounts of mRNA transcripts of \textit{Wnt5a} and \textit{Wnt2b} genes in all of the CFU-Cs examined were smaller than those in the CD34-enriched cell fractions. The amounts of mRNA transcripts of \textit{Fzd6} genes showed no statistical differences between any of the cell populations.

In stromal cells derived from BMMCs, the amounts of mRNA transcripts of the \textit{HoxB3}, \textit{HoxB4}, \textit{HoxA10}, \textit{PTCH1}, \textit{Wnt5a} and \textit{Wnt2b} genes were significantly smaller than those of the CD34-enriched cell fractions. Compared to the fresh BMMCs, the amounts of mRNA transcript of the examined genes except \textit{Fzd6} in the stromal cells were smaller, yet without statistical differences.

### DISCUSSION

In this study, the amounts of mRNA transcripts of \textit{HoxB3}, \textit{HoxB4}, \textit{HoxA10}, \textit{SHH}, \textit{PTCH1}, \textit{Wnt5a} and \textit{Wnt2b} genes were quantitatively analyzed in canine bone marrow cells: BMMCs, CD34-enriched cell fraction, CD34-depleted cell fraction, stromal cells, and CFU-Cs. The three \textit{Hox} genes, \textit{SHH} gene, and two \textit{Wnt} genes were selected based on the previous studies in humans and rodents in which their important roles in self-renewal, maintenance, and survival of HSCs had been reported [3, 19, 21, 40, 46, 53]. \textit{PTCH1} and \textit{Fzd6} genes were analyzed because they code the receptors of \textit{SHH} and \textit{Wnt}, respectively [32, 47].

The amounts of mRNA transcripts of three \textit{Hox} genes shown to be larger in canine CD34-enriched cell fraction. Similarly, the amounts of mRNA transcripts of \textit{HoxB3} and \textit{HoxA10} genes in all of the CFU-Cs examined were significantly smaller than those in the seeded CD34-enriched cell fractions. The amounts of mRNA transcripts of \textit{SHH} gene in CFU-GEMM and CFU-GM were smaller than that in the CD34-enriched cell fraction. The results observed in the \textit{HoxB3}, \textit{HoxA10}, and \textit{PTCH1} genes, the amounts of mRNA transcripts of \textit{Wnt5a} and \textit{Wnt2b} genes in all of the CFU-Cs examined were significantly smaller than those in the CD34-enriched cell fractions. The amounts of mRNA transcripts of the \textit{Fzd1} and \textit{Fzd6} genes showed no statistical differences between any of the cell populations.

In stromal cells derived from BMMCs, the amounts of mRNA transcripts of the \textit{HoxB3}, \textit{HoxB4}, \textit{HoxA10}, \textit{PTCH1}, \textit{Wnt5a} and \textit{Wnt2b} genes were significantly smaller than those of the CD34-enriched cell fractions. Compared to the fresh BMMCs, the amounts of mRNA transcript of the examined genes except \textit{Fzd6} in the stromal cells were smaller, yet without statistical differences.

### DISCUSSION

In this study, the amounts of mRNA transcripts of \textit{HoxB3}, \textit{HoxB4}, \textit{HoxA10}, \textit{SHH}, \textit{PTCH1}, \textit{Wnt5a} and \textit{Wnt2b} genes were quantitatively analyzed in canine bone marrow cells: BMMCs, CD34-enriched cell fraction, CD34-depleted cell fraction, stromal cells, and CFU-Cs. The three \textit{Hox} genes, \textit{SHH} gene, and two \textit{Wnt} genes were selected based on the previous studies in humans and rodents in which their important roles in self-renewal, maintenance, and survival of HSCs had been reported [3, 19, 21, 40, 46, 53]. \textit{PTCH1} and \textit{Fzd6} genes were analyzed because they code the receptors of \textit{SHH} and \textit{Wnt}, respectively [32, 47].

The amounts of mRNA transcripts of three \textit{Hox} genes shown to be larger in canine CD34-enriched cell fraction than the differentiated CFU-Cs in this study. The \textit{HoxB4} gene was initially the top candidate strongly expressed in the CD34-enriched cell fraction; however, no significant difference in the amount of its mRNA transcript was observed between the CD34-enriched and CD34-depleted fractions in this study. Instead, the amounts of mRNA transcripts of \textit{HoxB3} and \textit{HoxA10} genes were significantly larger in the CD34-enriched cell fraction than in unfractiuated BMMCs, CD34-depleted cells, stromal cells, and CFU-Cs. Relatively large amounts of the mRNA tran-

### Table 1. Primer pairs used for cloning and Genbank accession numbers and the sizes of the cloned sequences

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### Table 2. Primer pairs used for the real-time PCR

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scripts of these three \( Hox \) genes in the CD34-enriched cell fraction are suggestive of their important roles in the hematopoietic progenitors as described in human and rodents. Moreover, with respect to \( HoxB3 \) and \( HoxB4 \), which are genes adjacent to the same chromosome, similar results were obtained in this study. In a study on human cells, \( Hox \) gene expressions were shown to be similar between genes close to each other by position rather than those between genes with paralogous relationships [50]. Although paralogs were not analyzed in this study, the very similar pattern observed in canine \( HoxB3 \) and \( HoxB4 \) might reflect this consistency. In other human studies, the expressions of various \( Hox \) genes were analyzed in hematopoietic progenitors [13, 42]. In that study, the order of the expression level was \( HoxA \), \( HoxB \), and \( HoxC \); further, no \( HoxD \) expression was detected [13, 21, 42]. In this study, the amount of mRNA transcript of \( HoxA10 \) gene was comparable to those of \( HoxB3 \) and \( HoxB4 \). The most distinct difference between the CD34-enriched and CD34-depleted cell fractions was observed in the amount of the \( HoxA10 \) gene transcript in the present study. In human cells, \( HoxA10 \) expression is known to be strictly limited to early-stage progenitors and is downregulated in differentiated cells such as neutrophils and monocytes [20]. Moreover, high \( HoxA10 \) expression was reported in human CD34+ cells together with a marked reduction in CD34- cells [20, 42]. The current results obtained in canine cells were consistent with the results in these reports. Although the amounts of \( SHH \) gene transcript in BMMCs, CD34-enriched cell fraction, CD34-depleted cell fraction, CFU-M, CFU-G, and BFU-E were above the lower detection limit, the amounts were generally small in this study. In previous studies, \( SHH \) treatment induced HSC proliferation in human cells [3]; however, its expression was low or nondetectable in adult tissues whereas it was relatively high in fetal endodermal tissues [26]. A relatively small amount of mRNA transcript of \( SHH \) gene detected in canine CD34-enriched cell fraction in this study was consistent with the previous results shown in humans, which is suggestive of a similar phenomenon in both humans and dogs. Another possibility is that \( SHH \) may be produced and secreted at a distant site and is received by \( PTCH1 \)-expressing bone marrow cells. This was suggested because the amount of \( PTCH1 \) mRNA transcript was significantly larger in the CD34-enriched cell fraction than any of the other cell groups in this study. In humans, \( PTCH1 \) is expressed in normal bone marrow cells, while it is not detectable in differentiated peripheral blood mononuclear cells [10].

The amount of \( Wnt5a \) mRNA transcript in the CD34-enriched cell fraction was significantly larger than that in all of the other cells groups; this was similar to the results with respect to \( HoxB3 \), \( HoxA10 \), and \( PTCH1 \) genes. On the other hand, the amount of \( Wnt2b \) transcript in the CD34-enriched cell fraction was significantly larger than that in CFU-GEMM, CFU-GM, CFU-M, CFU-G, BFU-E, and stromal cells. These results were partly consistent with previous studies; both \( Wnt5a \) and \( Wnt2b \) genes were expressed in human bone marrow CD34+ cells, both adult and fetal, as well as in bone marrow stromal cells [19, 52]. When the genes of Wnt receptors were analyzed, the results on the \( Fzd1 \) and \( Fzd6 \) genes were different from those on most of other genes examined in this study. The amounts of mRNA transcripts of \( Fzd1 \) and \( Fzd6 \) genes were different from those on most of other genes examined in this study. The amounts of \( Fzd6 \) transcript in CFU-Cs were similar to that in the primary CD34-enriched cell fraction. Continuous expression of \( Fzd6 \) through the lineage commission suggests a certain relationship of the Wnt signals with lineage differentiations in dogs.
In dogs, CD34 is currently the most commonly used marker to detect or isolate early hematopoietic progenitors [28, 29]. While the amounts of mRNA transcripts of all genes examined except for \textit{SHH} gene were larger in the CD34-enriched cell fraction than those in the CD34-depleted cell fraction, significant differences were observed in the \textit{HoxB3, HoxA10, PTCH1}, and \textit{Wnt5a} genes. Yet, the differences between these 2 populations were not as large as
expected prior to this study; particularly in the amount of HoxB4 transcript. Previous studies in normal human bone marrow cells showed that the expression levels of HoxB3, HoxB4, and HoxA10 were significantly higher, at least by 10-fold, in CD34-enriched cell fractions compared to CD34-depleted cell fractions [18, 20, 42]. One of the possible explanations is the limitation of MACS sorting. The purity of CD34-enriched cell faction was 82.2 ± 5.0%, and the percentage of CD34+ cells in the CD34-depleted cell fraction was 0.87 ± 0.43%, nearly the same as BMMCs. This indicates that the CD34-enriched fraction contained approximately 80 times more CD34+ cells than the CD34-depleted cell fraction; however, the differences in the amounts of mRNA transcripts did not exceed 10-fold. In humans and mice, CD34 is no longer the landmark for stem cells; CD34 is known to be expressed in multiple progenitors including the stem cells, and combined markers are available for the isolation of more primitive HSCs particularly in mice and humans. In addition, HoxB4 is known to be expressed later than HoxB3 within the primitive hematopoietic progenitors [12]. If this fact was also true for dogs, the CD34-enriched cell fraction used in this study may have been a population that had not fully expressed HoxB4. Nevertheless, CD34 is a marker of primitive hematopoietic progenitors, and current results suggest that recently recognized factors such as Hox, SHH, and Wnt may play some roles particularly in these cells.

In the present study, the mRNA transcripts of 9 kinds of genes possibly associated with the self-renewal of hematopoietic stem cells were analyzed for their amounts relative to that of an internal reference (housekeeper) gene, HPRT, in canine bone marrow-derived cells. Since HPRT gene has been used as a suitable internal reference gene for the quantitative assay of the mRNA transcripts of Hox family and other related genes [7, 25, 48] in human and murine bone marrow-derived cells, we chose HPRT gene as a normalizer in this study. Moreover, it has been shown that HPRT gene can be one of the most stable internal control genes for the quantitative RT-PCR assay for the canine bone marrow samples [36]. As indicated in the same recent report, use of multiple internal control genes was recommended for the quantitative RT-PCR analysis for tissues from dogs as well as humans and rodents [36]. In the present study, the amounts of transcripts of various genes were estimated as relative values in comparison to the amount of transcript of a single internal reference gene, HPRT, in canine bone marrow-derived cells. Although HPRT gene can be considered as a suitable internal reference gene for the quantitative RT-PCR in bone marrow cells, further strict consideration to use multiple internal references genes would be needed.

In the future, we must endeavor to utilize these promising factors in expanding HSCs for transplantation and/or tissue engineering as well as use them as growth factors, which may eventually substitute cytokines. Investigations are underway in humans in various fields spanning HSC expansion to regenerative medicine. For these goals to be achieved in dogs, functional researches must also be conducted using canine cells. There has been a report of retroviral induction of the human HoxB4 gene into a canine CD34+ cell resulting in immortalization of the cells [56]. The report also showed different outcomes with the cells from different species such as humans, suggesting species compatibilities between the transgene and the cells. Other molecules such as SHH and Wnt in dogs should be investigated in the future. However, the fact that they are highly conserved between species promises a potential use for such technologies in the future of veterinary medicine.

REFERENCES


