RESEARCH ARTICLE

Real-time Expression of Stemness and Self-Renewal Genes in Canine Hematopoietic Progenitor Cells

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Abstract

Blood cells are responsible for constant maintenance and immune protection of every cell type of the body and this relentless and brutal work requires cells that have the greatest powers of self-renewal and are designated as hematopoietic progenitor cells (HPCs). Peripheral blood stem cells in circulation have become the most common source of hematopoietic stem cells intended for transplantation after minimal manipulation. 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. Unlike cytokines, Hox, SHH, and Wnt are highly conserved among species from flies to humans, but studies regarding the self-renewal and expansion of the HSC are extremely limited in dogs.

Keywords: Blood, Canine, Gene expression, Self-renewal.

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Introduction

Hematopoietic stem progenitor cells (HSPCs) are immature blood cells capable of differentiating into all types of cells found in the peripheral blood (Bruno *et al*., 1999 and Hartnett *et al*. 2002). Verfaillie, (2002) reported on the efficacy of hematopoietic progenitor cells of mobilized peripheral blood from patients who were treated using chemotherapy, which could be used for transplantation. Dogs are a source of well-characterized homologs of human genetic diseases (Patterson, 2000). This helps in a clear understanding of genetic diseases in large animal systems and the correlation of results with human genetic diseases. *Hox* gene involvement in stem cell differentiation, and others suggested that their role based on observed effects of *Hox* genes *in vivo* or *in vitro* tissue development (Barber *et al*. 2010). It was reported that the *Hox A* cluster played a role in hematopoiesis (Mulgrew *et al*. 2014), but the expression of *HoxB4* which directs transition from the embryonic to the adult hematopoietic program was shown to be a major factor (Daley *et al*. 2009). *Wnt* is a group of secreted glycoproteins, and 19 families of *Wnt* genes are known in human and mice so far (Nusse *et al*. 1982; Taghon *et al*. 2002 and Staal *et al*., 2005). *Wnt* acts via autocrine and paracrine pathways. Frizzled proteins act as *Wnt* receptors. Of the 19 molecules of the family, *Wnt2b, 5A*, and *B10* are known to promote the proliferation of murine HSCs (Austin *et al*., 1997 Berg *et al*., 1999 and Murdoch *et al*., 2003). Sato *et al*. (1987) and Borrow *et al*. (1996) reported that they have indicated the involvement of *Hox* genes in leukemogenesis, underlining their role in normal hematopoiesis. Hematopoietic progenitors retrovirally transuded with *HoxB4* have demonstrated up to a 1000 fold expansion of HSCs relative to control cells transduced with control vectors (Antonchuk *et al*., 2002).

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Ethical Committee permission

The research work has been carried out as per the approval of the Institutional ethical committee for stem cell research and therapy N0.05/ICSCRT/2017 dated 11.12.2017 of Chairman, ICSCRT AND Dean, Faculty of Basic Sciences, Madras Veterinary College, Chennai-7

Materials and Methods

Isolation of canine peripheral blood mononuclear cells

Blood sample was diluted with phosphate-buffered saline (PBS) in 1:2 ratio in 15mL centrifuge tubes. The diluted blood sample was laid very slowly in 1:2 ratio on lymphocytes separation medium (LSM). The cells were centrifuged at 400xg for 30 minutes at room temperature. The mononuclear cells (lymphocytes and monocytes) appeared as a hazy layer

was carefully aspirated by a sterile pipette into another sterile tube. The cells were washed with PBS and 2% FBS buffer solution at 1500 rpm for ten minutes. The supernatant was discarded, and the pellet was resuspended with one ml of RBC lysis buffer for 2-3 minutes over ice. It was centrifuged at 2500 rpm at ten minutes. The supernatant was discarded, and the pellet was reconstituted in one mL of culture medium.

Enrichment of progenitor cells

Enrichment of cells was done as per the manufactures instruction of Easy Sep™ (Stem Cell Technologies) with minor modifications to suit our needs.

Culture of isolated enriched cells

Isolated enriched cells were cultured in T25 culture flask at a cell concentration of 1x10cells/mL in maintenance medium. The culture flasks were allowed to incubate at 37at 5% CO_2 level without any disturbance for one to two days. Then the suspension cells were transferred into six-well plate with fresh growth medium. The cultures were allowed to grow for 7-14 days with the change of medium at two days intervals.

RNA isolation and cDNA synthesis

RNA isolation of cultured progenitor cells performed by the Trizol method. 100µl of cells were taken, and 1mlLof TRIZOL RS reagent was added the content was mixed thoroughly. This mixture was incubated for 20 minutes in ice. After incubation, 200µl of chloroform was added and allowed to stand for 5 minutes; the contents were mixed by vortexing for 15 sec. The content was centrifuged at 12,000 rpm for 20 minutes at 4. The upper aqueous layer was separated, and the RNA was precipitated by adding an equal volume of Isopropanal. This mixture was centrifuged at 12,000 rpm for 20 minutes at 4 to pellet the RNA. The supernatant was then discarded, and the pellet was washed twice with 75% ethanol by centrifuging at 7500 rpm for 5minutes at 4, and the RNA pellet was air-dried. The RNA pellet was resuspended in 10µL of DNase-RNase free water. The concentration of RNA was estimated at 260/280 A^{θ} using spectrophotometer (Eppendrof, Biophotometer Plus).

cDNA synthesis from isolated RNA

cDNA synthesis (Reverse Transcription) was done using random hexamers with initial concentration of 500ng of total RNA sample using the High Capacity cDNA synthesis kit (Applied Biosystem, USA) following manufacturer's instructions of 20µl final volume (Table 1) and (Table2).

Gene expression analysis by qRT-PCR

Quantitative real-time PCR was performed using the SYBER green (TaKaRa) reagent on a Biorad CFX connects real-time PCR system. Data were analyzed using Biorad CFX Manager Software Version 2.1. Gene expression was normalized according to the expression of the housekeeping gene -actin. *Hox b3* and *Wnt 2b* was targeted to analyze the expression in isolated and cultured progenitor cells (Table 3). In the negative control reactions, instead of cDNA, 1µl of nuclease-free water was added. Condition of thermal cycler for Real-Time PCR were as follows; incubation at 95 for 10 secs; followed by 45 cycles of 95 for 5 sec an 60 for 30 sec during which the fluorescence data were collected; followed by the last step determine dissociation at 95 for 15 sec, 60 for 30 sec, and 95 for 15 sec. The real-time data were recorded with the cyclic condition, including the melt curve.

Table 1: Component volume (µl) for cDNA synthesis

10X RT Bbuffer	2.0
25X dNTP mix (100mM)	0.8
10X RT random primers	2.0
Mulitiscribe™ reverse transcriptase	1.0
Nuclease-free water	4.2
Total per reaction	10

Results and Discussion

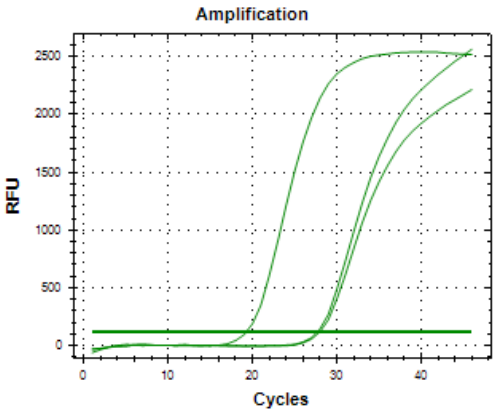
In the present study, isolation of $CD34^+$ positive cells was carried out using a rapid, easy SepMat protocol. Canine PBMCs were fractionated according to density and were then separated by $CD34^+$ selection. Real-time expression of *Wnt 2b, HoxB3,* and *HoxB4* in canine hematopoietic progenitor cells was observed. The amplification curve of β-actin showed that the amplification starts at nearly 15^{th} cycle, and it has amplification upto30 cycles. The amplification curve of *Wnt2b* showed that the amplification started at18^{th} cycle it has amplification up to30 cycles. *HoxB3* amplification starts at the 17^{th} cycle, and it has amplification up to 32 cycles. *HoxB4* shows that the amplification starts at the 12^{th} cycle and it has amplification up to 30 cycles. The optimum annealing temperature was found to be 55˚C which has been checked in gradient PCR with a range of 50 ˚C to 65˚ C. The quantitative expression of *HoxB3, HoxB4* and *Wnt2b* gene were observed in hematopoietic progenitor cultured cells showed as $2^{-\Delta\Delta ct}$ value were *Wnt2b* 0.02, *HoxB4* 0.16, *HoxB3* 0.21 respectively. The amplification was approximately 1.5 folds in *HoxB3 1.2 folds in HoxB4*, whereas the amplification of *Wnt2b* was less than one fold when compared with references β-actin gene **(**Figure1).

Table 2: Cycling Condition for cDNA synthesis

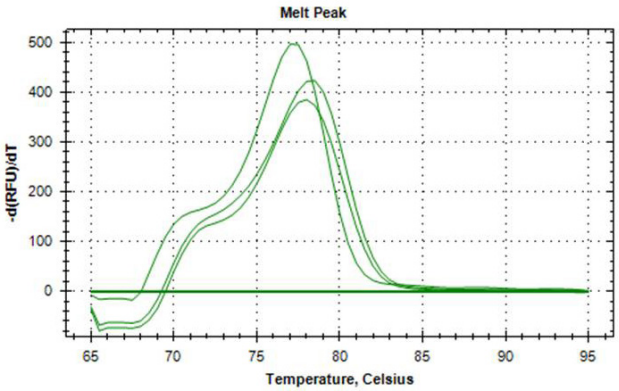
	Step 1	*Step 2*	*Step 3*	*Step4*
Temperature	25	37	85	4
Time	10 minutes	120 minutes	5 minutes	

Table 3: Component volume (µl) for Real Time PCR

Component volume (µl)	
SYBER green master mix (2x)	10.0µl
Forward primer (10pmol/µl)	1.0 µl
Reverse primer (10pmol/µl)	1.0 µl
cDNA (from 100 ng of RNA)	2.0 µl
Nuclease free water	6.0 µl
Total reaction volume	20 µl



1. (a). Melt curve analysis of specific genes for self-renewal in CD 34^{+}cells



1. (b). Amplification of specific genes for self-renewal in CD 34+ cells

Figure1: Quantitative real-time expression of *Wnt2b, HoxB3*and *HoxB4* in CD34^{+} cells

Conclusion

Real time-PCR was performed to analyze the expression of genes specific for self-renewal such as *HoxB3, HoxB4*, and *Wnt2b*. The amplification was approximately 1.5 folds in *HoxB3,* 1.2 folds in*HoxB4*, whereas the amplification of *Wnt2b*was less than one fold, in the isolated hematopoietic cells.

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