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

S. Nandhini¹, Mangala Gowri A.¹†, G.R. Baranidharan², T.V. Meenambigai³

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.

Agricultural Science Digest (2019)

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 8B 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 transduced with HoxB4 have demonstrated up to a 1000 fold expansion of HSCs relative to control cells transduced with control vectors (Antonchuk et al., 2002).

Source of support: Acknowledgement to the DBT, GoI as the research work was supported by the DBT project on Foldscopes as user-friendly demonstration tool on bone marrow stem cell model

Conflict of interest: None

How to cite this article: Nandhini, S., Mangala, G.A., Baranidharan, G.R. and Meenambigai, T.V. (2019). Real-time expression of stemness and self-renewal genes in Canine Hematopoietic Progenitor Cells. Agricultural Science Digest, 39(3): 254-256

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 manufacturers 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 1x10^6 cells/mL in maintenance medium. The culture flasks were allowed to incubate at 37° at 5% CO₂ 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 1ml of 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 5 minutes 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_260 using spectrophotometer (Eppendorf, 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 sec; 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.

**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 15th cycle, and it has amplification upto30 cycles. The amplification curve of Wnt2b showed that the amplification started at16th cycle it has amplification up to 30 cycles. HoxB3 amplification starts at the 17th cycle, and it has amplification up to 32 cycles. HoxB4 shows that the amplification starts at the 12th 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^ΔΔct 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 1:** Component 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 sec; 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.

**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 15th cycle, and it has amplification upto30 cycles. The amplification curve of Wnt2b showed that the amplification started at16th cycle it has amplification up to 30 cycles. HoxB3 amplification starts at the 17th cycle, and it has amplification up to 32 cycles. HoxB4 shows that the amplification starts at the 12th 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^ΔΔct 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 1: Component 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 sec; 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.

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

### Table 2: Cycling Condition for cDNA synthesis

| Step | Time | Temperature |
|------|------|-------------|
| Step 1 | 10 minutes | 95°C |
| Step 2 | 120 minutes | 55°C |
| Step 3 | 5 minutes | 95°C |
| Step 4 | 30 minutes | 60°C |

### 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 |
Real-time expression of stemness and self-renewal genes in Canine Hematopoietic Progenitor 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.

**Reference**

Antonchuk, J., Sauvageau, G., Humohries, R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells. Exvivo. Cell 109: 39-45.

Austin, T.W., Solar, G.P., Ziegler, F.C., Liem L., Mathews, W. (1997). A role for the Wnt gene family in hematopoiesis: expansion of multi lineage progenitor cells. Blood, 89:3624-3635.

Barber, B.A. and Rastegar, M. (2010). Epigenetic control of Hox genes during neurogenesis, development, and disease. Ann. Anat. 192: 261–274.

Berg, V.B., Sharma, A.K., Bruno, E., Hoffman, R. (1998). Role of members of the Wnt gene family in human hematopoiesis. Blood, 92: 3189-3202.

Borrow, J., Shearman, A.M., Stanton, V.P., Becher, R., Collins, T., Williams, A.J., Dube, I., Katz, F., Kwong, Y.L., Morris, C., Ohyashiki, K., Toyama, K. (1996). The t(7;11)(p15;p15) translocation in acute myeloid leukemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nat Genet,12: 159–167.

Bruno, B., Nash, R.A., Wallace, P.M., Gass, M.J., Thompson, J., Storb, R., Mc Sweeney, P.A. (1999). CD34+ selected bone marrow grafts are radioprotective and establish mixed chimerism in dogs given high dose total body irradiation. Transplantation, 68: 338-344.

Daley, G.W. (2009). Hematopoietic Stem Cells. Academic Press, pp: 211–217.

Hartnett, B.J., D. Yao, S.E. Suter, N.M. Ellinwood, P.S. Henthorn, P.E. Moore, P.A.

McSweeney, R.A. Nash, J.D. Brown, K.I. Weinberg and P.J. Felsburg, 2002. Transplantation of X-linked severe combined immune deficient dogs with CD34+ bone marrow cells. Biol. Blood Marrow Transpl. 8: 188-197.

Mulgrew, N.M., Kettyle, L.M., Ramsey, J.M., Cull, S., Smyth, L.J., Mervyn, D.M., Bjl, J.J., Thompson, A. (2014). c-Met inhibition in a HOXA9/Meis1 model of CN-AML. Dev Dyn. 243: 172–181.

Murdoch, B., Chadwick, K., Martin, M., Shojaei, F., Shah, K., Gallacher, L., Moon, R.T., Bhatia, M. (2003). Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells. In vitro. Proc. Natl. Acad. Sci. U.S.A. 100: 3422-3437.

Nusse, R. and H.E.Varmus. (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.

Patterson, D. (2000). Companion animal medicine in the age of medical genetics. J.Vet. Intern. Med. 14: 1-9.

Sato, Y., Abe, S., Mise, K., Sasaki, M., Kamada, N., Kouda, K., Musashi, M., HoriKoshi, Y. Minami,Y. (1987). Reciprocal translocation involving the short arms of chromosomes 7 and 11, t(7p;11p+), associated with myeloid leukemia with maturation. Blood; 70: 1654–1658.

Staal, F.J. and H.C. Clevers. (2005). Wnt signaling and hematopoiesis: a Wnt-Wnt situation. Nat.Rev.Immunol. 5: 21-30.

Taghon, T., Stolz, F., De Smedt, M., Cnockaert, M., Verhasselt, B., Plum, J., Leclercq, G. (2002). HoxA10 regulated hematopoietic lineage commitment: evidence for a monocytespecific transcription factor. Blood, 99: 1197-1204.

Verfallie, C.M., (2002). Hematopoietic stem cells for transplantation. Nature Immunology, vol 3, no4,pp: 314-317.