CHAPTER V

DISCUSSION AND CONCLUSION

CD 45^+ cells human lymphocytes are nuclear hematocytes, which can engrafte in the peripheral blood of sheep by In utero transplantation. Despite the wide use of different techniques for investigation of donor cells engraftment, lack of standardization of methods and detection was a limitation of the assay. [81,82] In this *in vitro* study, artificially prepared mixtures on the human and sheep lymphocytes were analyzed to establish the connection of Flow Cytometry and FISH examination on the human and sheep chimeric. There was evidence that donor cells engraftment in the viable chimeric lambs after birth could be detected by Flow Cytometry but not confirmed by FISH analysis. [76] Both Flow Cytometry and FISH assays were rapid and sensitive methods. Other studies found that Flow Cytometry and FISH could readily detect levels of human lymphocytes comprise about 0.1% to 1 % of the total artificial of mixtures human and sheep lymphocytes. [76,83] In an attempt to increase the sensitivity of detecting human lymphocytes at levels below 1% in mixed experiments. Many studies had shown that the absolute number of circulating CD 45^+ human lymphocytes could be accurately measured when the concentration of human lymphocytes was higher could than 1 % because of measurement of lower concentration would be difficult to detect and interpret. In addition, the dilution experiments of lower concentration of human lymphocytes had yield a higher CV percentage. [76,83,84,85] These results were comparable with our study. The evidences were shown in Table 6 for Flow Cytometry analysis and Table 7 for FISH analysis, the mean of CV human lymphocytes at all dilutions 0.01%-1% in mixed experiments of FISH analysis was less than Flow Cytometry analysis, indicated that our FISH analysis demonstrated smaller variations than Flow Cytometry analysis.

Concerning the sensitivity of the different cell types to be detected by Flow Cytometry analysis which may depend on the cell population and the antibodies used. There was unambiguous and correct retrieval of the lowest number of added different cell types equal the limit of identification and has been reported to be as low as 1 in 10^5 of prostate cancer [86] , 1 in 10^6 of stem cells[87] and 1 in 10^7 of breast cancer.[88] Fuchimoto et al [89] recently reported the detection of donor cells in pigs after kidney transplantation reported a frequency of donor – derived cells in the range of 0.5% using a detection limit, which was identified by Flow Cytometry .

Although the sensitivity of Flow Cytometry measurement is very high due to the advanced laser and fluorescence technology of the machine and the hydrodynamic focusing of the single cells.[90] There are studies that demonstrate artificially prepared mixtures of human and sheep stromal cells can detected levels of human cells 0.1% and failed to detected a small percentage of stromal human cells. [91] In our study of human lymphocytes with Flow Cytometry that strategies set the gate on only the lymphocytes region. In order to achieve a limit of sensitivity detection of 0.01% to 1%human lymphocytes in mixed human/sheep lymphocytes with approximately a higher of CV percentage. Although the results represented in Flow Cytometry analysis are the actual measurements of lower concentration of 0.01% - 0.02% human lymphocytes are uninterpret due to that were not different from those obtained in negative control. Moreover, False positive signals of Flow Cytometry analysis can be caused by nonspecific signals that occur contaminating free fluorescence dye in the antibodies preparation especially the amount of derbies in a cell preparation.

The results from this study, a comparison of the actual measurement of the percent human lymphocytes (CD 45^+ cells) taken from artificially mixtures human and sheep lymphocytes at various dilutions 1:100 (1%), 1:500(0.2%), 1:1,000 (0.1%), 1:5,000 (0.02%) and 1:10,000 (0.01%), has demonstrated the percent human lymphocytes at dilutions 0.1% - 1% of Flow Cytometry is lower than FISH analysis without statistical significance. This reason may be explained that the concentration of human lymphocyte at 0.1% - 1% was higher ,thus the actual measurement of the

percent human lymphocytes was not different from both method. However, the results of measurement CD 45^{+} cells at ratio 0.01%- 0.02% by Flow Cytometry was difference significance those obtained in FISH analysis. The actual measured number of human lymphocytes by Flow Cytometry analysis was tended to be lower than FISH analysis and the values of CV was tended to be larger, indicating that the use of Flow Cytometry in measuring a low concentration of human lymphocyte is less reproducible than FISH analysis.

FISH analysis , alternative methods to document the presence of human cells in the mixed experiments were used to confirm the positive cells. This work demonstrated that the feasibility of FISH analysis could be performed on the same mixed dilution between human and sheep lymphocytes with good image contrast. Because of human lymphocyte morphologically and genetically differed from sheep lymphocytes, thus it facilitated both manual and automated image analysis when performed with lower concentration. There were false positive rate of 0.03% in lymphocytes by FISH analysis. Cottret et al [92] , propose that FISH technique and immunophenotying on smears are not sensitive enough to detect a low number of lymphocytes. They developed a quantitative technique for processing samples in two steps : Flow Cytometry Sorting followed by FISH analysis. We planned to test these techniques but this could not be done due to the laser electronic problem of our FACSort .

This approach was applied to detect (*in vivo* experiments)donor cells (human lymphocytes) engraftment in fetal sheep after in utero transplantation. The results of percent donor cells engraftment in two lambs born (no. 40A3 and 41J2) was found to be low than 1% of human lymphocytes in peripheral blood (PB).The percentage of donor cells engraftment of both lambs in the PB at 2 stages : 10 days and 1 month showed higher decrease with longer intervals by Flow Cytometry analysis. In contrast, FISH analysis did not show any evidence of human lymphocytes engraftment. These results agreed with several reports which had been shown lower levels of engraftment ranging from 0% to 1.2% by karyotyping at about chimeric

lambs age of 2 weeks after birth [76] and Zanjani et al found that cells expressing of CD 45^{+} antigen in PB of these two lambs at 3 weeks were less than 0.5% of human cells detected by Flow Cytometry analysis.

In our results the percentage of donor cells engraftment in newborn lambs was below 1%, as this frequency of donor cells engraftment is called microchimerism, defined by a rather low amounts of donor cells in the organism of recipients after transplantation and its contribution to tolerance induction. [93] Thus, the interpretation of a positive event as being truly positive or nonspecific was not different detected by Flow Cytometry analysis. The reason of low engraftment by Flow Cytometry may be due to barriers in xenogeneic transplantation. Other reports suggested that the fetal hematopoietic compartment might not be suitable for homing and engraftment of human donor cells.[94] The cotransplantation of donor – specific with stromal cells had shown significantly increased in short and long term donor cell engraftment in the sheep model.[95] The analytic approach in this study might be possible in such research to detect significant amount of donor cells but might not be appropriate or practically feasible in rare event analysis. However, further development of Flow Cytometry and FISH methods are recommended for application in clinical transplantation engraftment analysis. Another possible application would include the research for microchimerism in patients with hematologic diseases after hematopoietic stem cells transplantation.

In conclusion, Flow Cytometry and FISH methods were designed to provide measurement of human CD 45 + cells concentration in artificially mixtures of human/sheep lymphocytes (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000). Our results had shown that FISH analysis produce less variation in the concentration of human cells in mixed experiment than Flow Cytometry analysis. This technique could possibly be applied to detect donor cells engraftment in sheep chimeras after in utero transplantation. The level of donor cells engraftment or microchimerism was lower than 1% at 10 days and 1 month of age of newborn lambs.stem cell in utero.Exp Hematol.1999;27:1569-1575.