CHAPTER IV

RESULTS

1. Characteristic of CB samples, and $CD133^+$-enriched cells

Four CB samples were used for $CD133^+$ cell separation. The collection volume, MNC number, enriched $CD133^+$ cells number and purity of $CD133^+$ fraction of each CB sample display in Table4.

Table4. The collection volume, MNC number, enriched $CD133^+$ cells number and purity of $CD133^+$ fraction.

CB sample	Collection volume (ml.)	No. of MNCs ($x10^6$) (cells)	No. of $CD133^+$-enriched cells ($x10^5$) (cells)	$CD133^+$ cell purity (%)
1	80.0	67.0	5.6	97.29
2	65.0	40.0	3.8	89.12
3	90.0	88.0	7.5	89.63
4	115.0	182.0	12.3	93.11
Mean	87.5	94.3	7.3	92.29

The mean collection volume was 87.5 ± 10.51 ml. and enriched MNCs ranged from $40x10^6$ to $182x10^6$ (mean $94.3x10^6$ ± 3.09) cells. The total number of $CD133^+$ cells separated by MiniMacs ranged from $3.8x10^5$ to $1.23x10^6$ (mean $7.3x10^5$ ± 1.83). The mean purity of $CD133^+$-enriched cells, following CD133 column separation, was 92.29% ± 1.89% (range 89.12% - 97.29%).

2. **Evaluation of ex vivo expansion of total cell**

One to three hundred thousand human CB $CD133^+$-enriched cells were expanded for 28 days in four different conditions: (1) MSC+IL-1α, (2) MSC, (3) MSC+FL+TPO, and (4) FL+TPO to evaluate their proliferative potential. To determine whether MSCs were capable of supporting the ex vivo proliferation and differentiation of HSC, confluent monolayer MSC of passage 3-5 were used to culture human CB $CD133^+$ cells. Cells not adhering and adhering weakly to MSC were collected by gentle pipetting for analysis.

Culture of CB on MSCs without added cytokines (condition2) resulted in the mean of total nucleated cells after 14 days of culture was 3.18 ± 0.10 times (n=3) the initial input number. In contrast, in the presence of cytokine, the addition of IL-1α (condition1) or FL+TPO (condition3) further enhanced the expansion of total nucleated cells and progenitors cells as well as CFC-C formation. Representative photomicrographs of MSCs and growing hematopoietic cells in culture are shown in figure 4A-E. Figure 5. shows the fold increase in total number of cells with the time in culture obtained when CB $CD133^+$ cells were cultured (See table5).

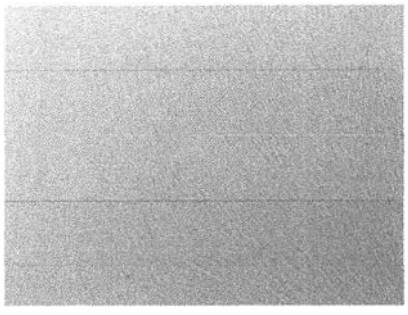


Fig.4A BM derived-MSCs

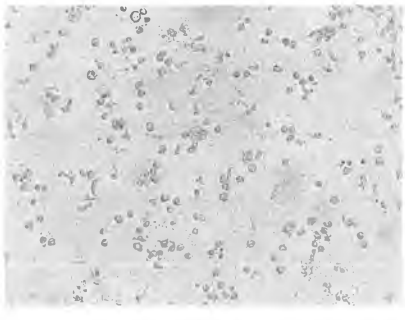


Fig.4B MSC+IL-1α
(condition1)

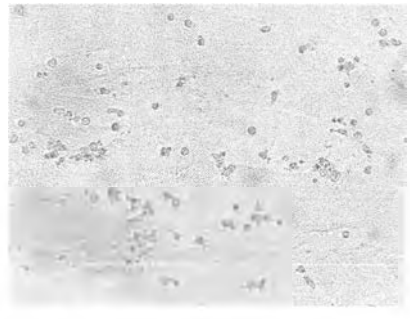


Fig.4C MSC
(condition2)

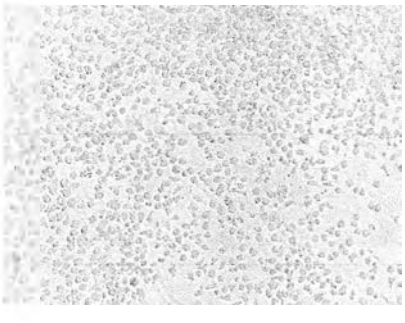


Fig.4D MSC+FL+TPO
(condition3)

Fig.4E FL+TPO
(condition4)

Figure4 Representative photomicrographs of MSCs and growing hematopoietic cells in culture. (A) BM derived-MSCs, (B) MSC+IL-1α, (C) MSC, (D) MSC+FL+TPO and (E) FL+TPO.

Table5 Fold increase of total cell number

condition	Days						
	4	8	12	16	20	24	28
MSC+IL-1α	5.35 ± 0.37	40.98 ± 2.35	178.73 ± 23.41	225.97 ± 18.80	179.38 ± 29.64	129.81 ± 23.49	100.54 ± 15.67
MSC+FL+TPO	9.99 ± 1.75[a]	58.13 ± 4.66[a]	281.87 ± 30.64[a]	302.32 ± 28.53	280.17 ± 32.93	204.93 ± 31.54	184.67 ± 27.81[a]
FL+TPO	3.06 ± 0.62[b]	26.36 ± 3.58[b]	74.23 ± 13.52[ab]	109.79 ± 20.50[ab]	72.25 ± 16.44[b]	47.28 ± 7.71[b]	37.07 ± 3.84[b]

[a] condition 3,4 different from condition 1

[b] condition 4 different from condition 3

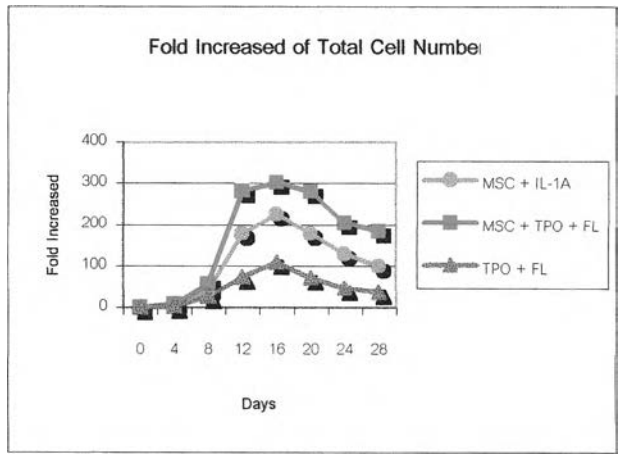


Figure 5. Total cell expansion of $CD133^{+}$-enriched cells culture in three conditions. Cell number for MSC+IL-1α cultured system (blue line,), in the MSC+FL+TPO culture system (red line,) and in the FL+TPO without MSC culture system (green line,) are expressed as mean fold expansion ± SEM. CB cells (0.8-2.85 x 10^{5}) were cultured for 28 days (n=4).

The maximal expansion seen with the CB $CD133^+$-enriched cell cocultured with MSC supplemented with FL and TPO was observed at day 16 with a mean 302-fold increase (range 222-fold to 357-fold).The output of total cells at day 16 of culture with MSC treated IL-1α and culture in FL and TPO without MSC was 226- and 110-fold, respectively, the input value. For 16 culture days using IL-1α or FL and TPO with the MSCs feeder layer in culture condition 1 and 3 significantly higher than that obtain in MSC-free cultured at day12 and day16 of culture (Table5)

3. Evaluation of ex vivo expansion of $CD133^+CD34^+$, $CD34^+CD38^-$ and $CD34^+$ cells

The maximal expansion of $CD133^+CD34^+$ cells, $CD34^+CD38^-$ cells, and $CD34^+$ cells in condition1 and 3 was observed at day 12 of cultures while the maximal expansion of those cells in condition4 was observed at day16 of cultures. For example, $CD133^+CD34^+$ cell numbers counted after MACS system with CD133 antibody were increased up to 12.52-, 33.28- and 7.25-fold in culture condition 1, 3 and 4 respectively. $CD34^+CD38^-$ cell number counted were increased up to 10.13-, 46.23- and 20.16-fold in culture condition1, 3 and 4 respectively. $CD34^+$ cell number counted were increased up to 35.37-, 93.77- and 25.27-fold in culture condition1, 3 and 4 respectively

Fold increase output in MSC-based system was significantly higher than that obtain in MSC-free cultured at day 12 of culture ($p<0.05$) due to the significantly higher of total cell fold increase. Table6-8 show percentage and fold increase of $CD133^+/CD34^+$ antigen.

Table6 Percentage and fold increase of $CD133^{+}/CD34^{+}$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	92.29 ± 1.89	40.49 ± 7.50	16.88 ± 4.92	6.91 ± 1.56	1.89 ± 0.35	1.08 ± 0.47	0.66 ± 0.14	2.41 ± 0.55
MSC+FL+TPO	92.29 ± 1.89	43.77 ± 1.86	15.73 ± 0.61	10.86 ± 0.64	4.55 ± 0.80[a]	1.86 ± 0.68	2.26 ± 0.48	1.23 ± 0.42
FL+TPO	92.29 ± 1.89	53.51 ± 3.60	12.54 ± 0.85	6.84 ± 0.48	6.25 ± 0.64[a]	4.02 ± 1.15	2.36 ± 0.60	1.35 ± 0.22
condition	Fold increase							
	0	4	8	12	16	20	24	28
MSC+IL-1α	1.00 ± 0.00	2.27 ± 0.32	7.24 ± 2.14	12.52 ± 2.71	4.48 ± 0.62	1.67 ± 0.55	0.90 ± 0.27	2.80 ± 0.90
MSC+FL+TPO	1.00 ± 0.00	4.66 ± 0.65[a]	9.83 ± 0.45	33.28 ± 4.17[a]	14.56 ± 2.42[a]	5.66 ± 2.16	5.21 ± 1.62[a]	2.73 ± 0.99
FL+TPO	1.00 ± 0.00	1.75 ± 0.36[b]	3.63 ± 0.62[b]	5.44 ± 0.94[b]	7.25 ± 1.16[b]	2.92 ± 0.93	1.10 ± 0.27[b]	0.89 ± 0.31

Table7 Percentage of $CD34^{+}CD38^{-}$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	93.15 ± 1.22	63.19 ± 7.41	19.83 ± 8.36	5.91 ± 1.71	1.33 ± 0.26	0.52 ± 0.15	0.20 ± 0.07	1.16 ± 0.55
MSC+FL+TPO	93.15 ± 1.22	68.14 ± 3.71	28.82 ± 3.28	15.07 ± 1.82	7.62 ± 1.95	5.00 ± 1.92	1.28 ± 0.33	1.72 ± 1.05
FL+TPO	93.15 ± 1.22	82.73 ± 1.71	49.10 ± 7.26[a]	22.27 ± 10.73	17.39 ± 8.82	13.05 ± 6.58	3.35 ± 1.12[a]	2.22 ± 0.85
condition	Fold increase							
	0	4	8	12	16	20	24	28
MSC+IL-1α	1.00 ± 0.00	3.57 ± 0.31	8.82 ± 3.63	10.13 ± 1.42	3.16 ± 0.61	0.90 ± 0.26	0.24 ± 0.09	1.15 ± 0.65
MSC+FL+TPO	1.00 ± 0.00	7.13 ± 0.89[a]	18.29 ± 3.33	46.23 ± 7.68[a]	25.03 ± 6.77	15.73 ± 6.05	2.79 ± 0.84	3.24 ± 2.20
FL+TPO	1.00 ± 0.00	2.72 ± 0.55[a]	13.75 ± 2.17	17.29 ± 8.12[b]	20.13 ± 9.89	11.45 ± 6.94	1.71 ± 0.70	0.89 ± 0.38

Table8 Percentage of $CD34^+$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	93.15 ± 1.22	82.72 ± 3.87	54.91 ± 5.36	18.67 ± 2.33	5.77 ± 0.62	2.09 ± 0.60	1.31 ± 0.26	5.88 ± 1.53
MSC+FL+TPO	93.15 ± 1.22	90.54 ± 0.94	52.73 ± 4.56	30.34 ± 5.81	13.76 ± 3.31	13.80 ± 4.76	4.15 ± 0.97	5.82 ± 1.83
FL+TPO	93.15 ± 1.22	95.12 ± 0.34[a]	56.12 ± 8.88	25.97 ± 10.97	22.30 ± 9.18	16.68 ± 6.91	5.99 ± 1.68	6.26 ± 2.39
condition	Fold increase							
	0	4	8	12	16	20	24	28
MSC+IL-1α	1.00 ± 0.00	4.73 ± 0.32	24.22 ± 2.78	35.37 ± 5.87	14.28 ± 2.36	4.01 ± 1.39	1.78 ± 0.48	6.44 ± 2.24
MSC+FL+TPO	1.00 ± 0.00	9.71 ± 1.67[a]	32.56 ± 2.71	93.77 ± 21.43[a]	45.06 ± 11.57	44.05 ± 15.67	8.90 ± 2.69[a]	11.59 ± 4.15
FL+TPO	1.00 ± 0.00	3.14 ± 0.65[b]	15.79 ± 2.72[b]	19.99 ± 8.29[b]	25.27 ± 10.30	14.29 ± 7.47	2.91 ± 0.95	2.61 ± 1.22

[a] condition 3,4 different from condition 1

[b] condition 4 different from condition 3

As can be seen in Fig.6A-C, an increase in absolute number of $CD133^+$ $CD34^+$ cells, $CD34^+CD38^-$ cells and $CD34^+$ cells was observed with the time in culture despite the decreases in percentage of these same cell population in all conditions.

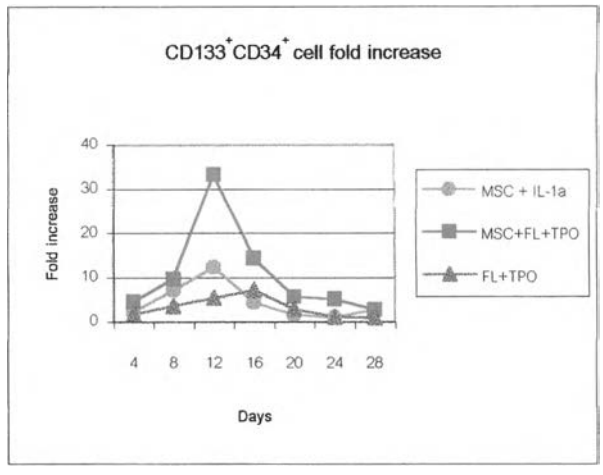


Fig.6A Fold increase of $CD133^{+}CD34^{+}$cell

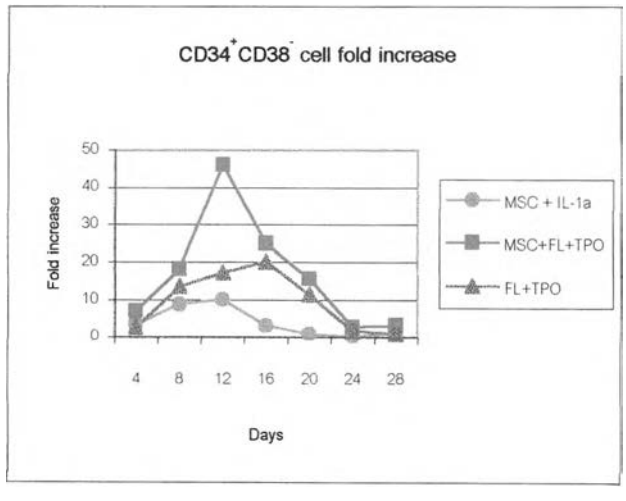


Fig.6B Fold increase of $CD34^{+}CD38^{-}$ cell

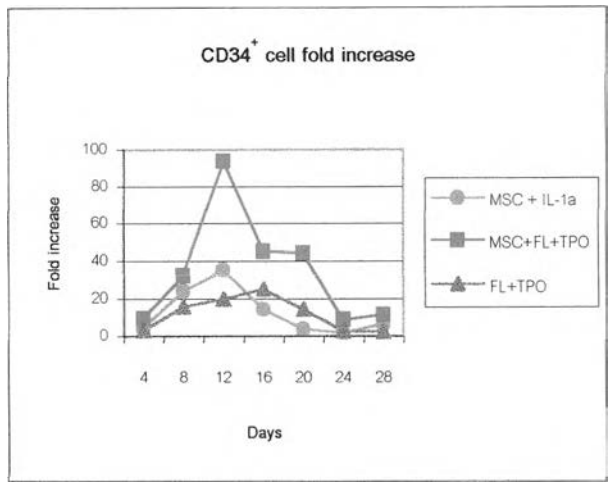


Fig.6C Fold increase of $CD34^+$ cell

4. Immunophenotypic characterization of $CD133^+$-enriched cells and effect of culture conditions on differentiation potential of expanded cells

The coexpression of CD34 and other CD antigens on $CD133^+$-enriched CB cells was analyzed. The results of differentiative potential of the expanded CB cell populations are presented in Table9-12.

Figure7A shows that almost all the lymphocyte gating region was CD133 cells and coexpress with $CD34^+$, $CD38^{dim}$, HLA-DR^+, $CD33^+$ and $CD45^{dim}$, whereas negative for CD7, CD15, CD19, CD41 and GlyA.These results suggest that $CD133^+CD34^-$ cells are enriched in primitive hematopoietic cells and myeloid progenitors.

In 3 of the 4 CB sample, almost all $CD133^+$ cells were positive for the HLA-DR antigen and a representative two-color flow cytometric analysis after $CD133^+$-positive selection is shown in figure 4. In only 1 of 4 CB samples, almost all $CD133^+$ cells were negative for the HLA-DR antigen (Fig.7B). We did not include this case for HLA-DR interpretation

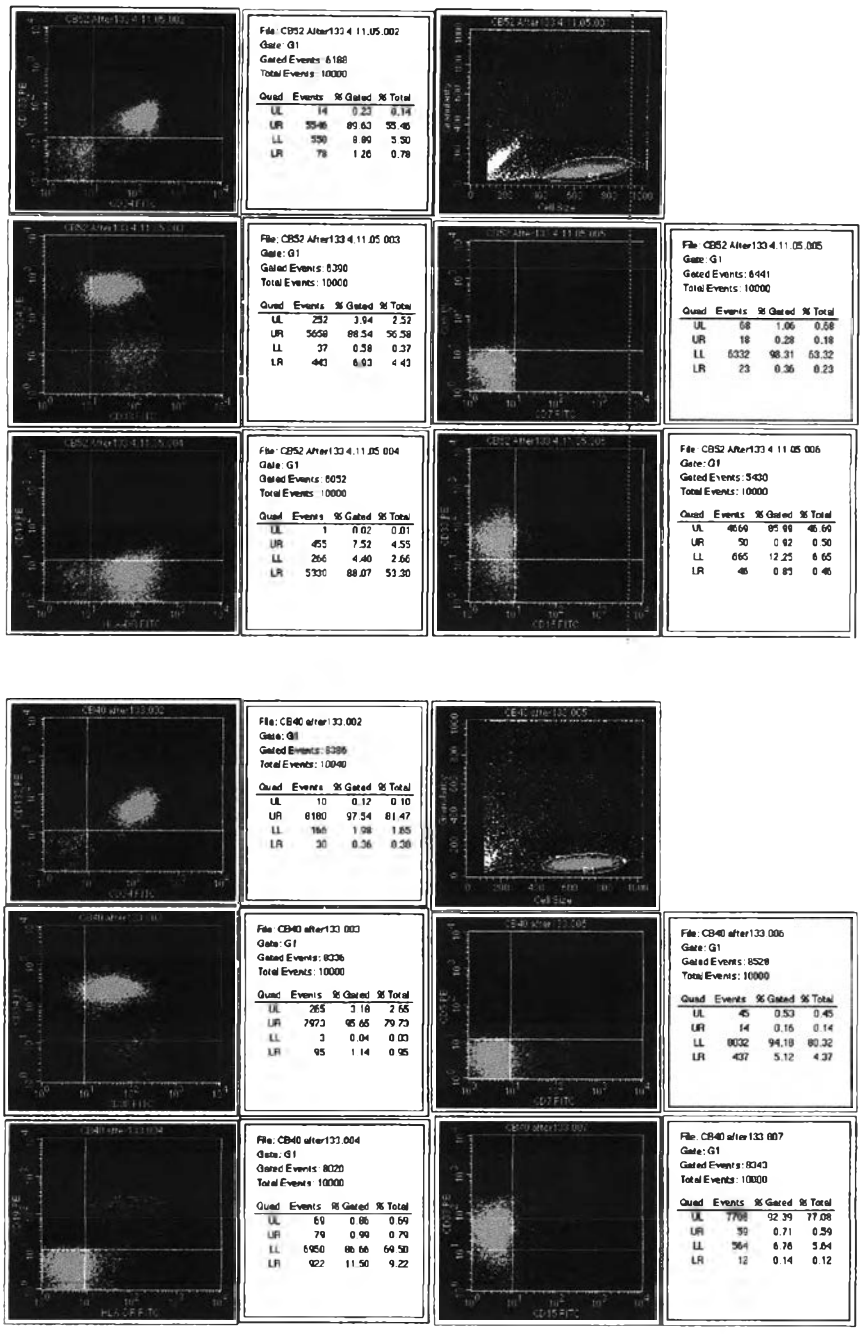


Figure7 Coexpression of CD34 and other CD surface antigens on MACS-selected $CD133^{+}$ cells. Cells were analyzed on a flow cytometer using the Cell Quest software program. (A) CB sample contain $CD133^{+}$ $HLA\text{-}DR^{+}$ cells. (B) CB sample contain $CD133^{+}$ $HLA\text{-}DR^{-}$ cells

It was found that MSC treated with IL-1α condition induced more $CD133^+$ cell to proliferate and differentiate. On the other hand, MSC treated with FL+TPO and FL+TPO condition provoked slower $CD133^+$ cell proliferation but maintained a higher proportion of the earliest HSC, i.e. $CD15^+$ (% of total viable $CD15^+$ cell population after 20 day in culture with MSC+IL-1α: 88.60 ± 2.20 or MSC+FL+TPO: 70.68 ± 3.14 or FL+TPO: 57.85 ± 5.76).Table5-8 show percentage of $HLA\text{-}DR^+$, $CD7^+$, $CD15^+$, and $CD33^+$ antigen respectively.

Table9 Percentage of $HLA\text{-}DR^+$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	94.26 ± 2.65	89.56 ± 0.58	60.17 ± 15.56	56.59 ± 11.50	27.75 ± 3.75	36.82 ± 4.72	25.52 ± 6.18	47.82 ± 4.25
MSC+FL+TPO	94.26 ± 2.65	86.77 ± 5.21	69.23 ± 7.51	68.74 ± 6.68	39.73 ± 6.39	46.78 ± 2.76	46.69 ± 7.91	37.68 ± 5.43
FL+TPO	94.26 ± 2.65	93.87 ± 1.76	84.30 ± 4.84	80.06 ± 3.35	67.70 ± 2.68[ab]	57.60 ± 6.68	53.17 ± 7.10	65.86 ± 14.50

Table10 Percentage of $CD7^+$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	0.00 ± 0.00	12.44 ± 6.36	32.90 ± 4.80	58.88 ± 1.72	50.48 ± 12.70	48.95 ± 8.23	39.18 ± 10.16	35.07 ± 11.28
MSC+FL+TPO	0.00 ± 0.00	8.55 ± 1.60	26.73 ± 3.70	36.23 ± 4.57^a	27.72 ± 3.47	40.74 ± 2.74	48.44 ± 6.28	30.61 ± 11.12
FL+TPO	0.00 ± 0.00	2.45 ± 0.99	4.55 ± 1.23^{ab}	6.38 ± 0.99^{ab}	12.29 ± 1.36^a	15.05 ± 2.29^{ab}	24.87 ± 6.39	30.87 ± 8.48

Table 11 Percentage of $CD15^+$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	0.00 ± 0.00	26.34 ± 5.61	44.84 ± 8.85	71.13 ± 1.68	82.35 ± 2.67	88.60 ± 2.20	92.38 ± 2.24	85.22 ± 7.99
MSC+FL+TPO	0.00 ± 0.00	29.78 ± 4.32	47.16 ± 3.16	49.04 ± 3.24^a	56.88 ± 4.11^a	70.68 ± 3.14^a	79.46 ± 6.62	77.83 ± 4.05
FL+TPO	0.00 ± 0.00	20.36 ± 1.90	28.81 ± 2.35	32.64 ± 3.17^{ab}	41.18 ± 3.93^{ab}	57.85 ± 5.76^a	67.31 ± 3.18^a	67.92 ± 8.46

Table12 Percentage of $CD33^+$ antigen

condition	Percentage							
	0	4	8	12	16	20	24	28
MSC+IL-1α	87.21 ± 2.04	75.57 ± 13.34	88.06 ± 6.45	95.27 ± 1.88	93.93 ± 1.01	92.67 ± 1.47	84.13 ± 9.04	91.71 ± 2.59
MSC+FL+TPO	87.21 ± 2.04	99.54 ± 0.16	97.79 ± 0.88	94.07 ± 2.07	79.04 ± 4.34[a]	89.23 ± 1.37	82.31 ± 4.72	93.28 ± 3.77
FL+TPO	87.21 ± 2.04	99.61 ± 0.16	99.21 ± 0.35	95.99 ± 0.65	86.08 ± 2.44	88.44 ± 3.72	81.97 ± 6.82	97.40 ± 0.91

[a] condition 3,4 different from condition 1

[b] condition 4 different from condition 3

Flow cytometric analysis of MSC cocultures expanded CB cells (condtition 1 and 3) revealed that culures were primarily composed of cells expressing a myeloid phenotype: $CD33^+$, $CD15^+$. A limited expansion of $CD7^+$ and $CD15^+$ cells was observed. The percentage of these marker achieved in the absence of MSC (condition 4) was considerably lower than that obtained in the coculture system. No $CD5^+$, $CD19^+$, $CD41^+$ or $GlyA^+$ cell populations were detected in all conditions.

Of importance was a significant increase in the percentage of $CD7^+$ cells with time in cultures grown on MSC layers. By day 20, the percentage of $CD7^+$ cells was 40% - 48%, showing that in our culture system we were also able to expand cells with an early lymphocytic phenotype.

Result of $CD15^+$ together with $CD7^+$ perecetage show that expanded cells in MSC-independent (condition4) produced significantly higher level of progenitor cells when compared with $CD133^+$ cell culture in MSC-dependent condition(condition1 and 3).

5. **Clonogenic potential of the expanded cells**

In order to assess the effects of expansion on the clonogenic potential of $CD133^+$-enriched cells, cells were harvested from the cultured at different time points and $2x10^3$ cells (day0) or $5x10^3$ (day8) or $1x10^4$ (day16) or $1.5x10^4$ (day28) were plated in methycellulose as described in the Material and Methods section. Figure8 present the CFC of CB cells cultured.

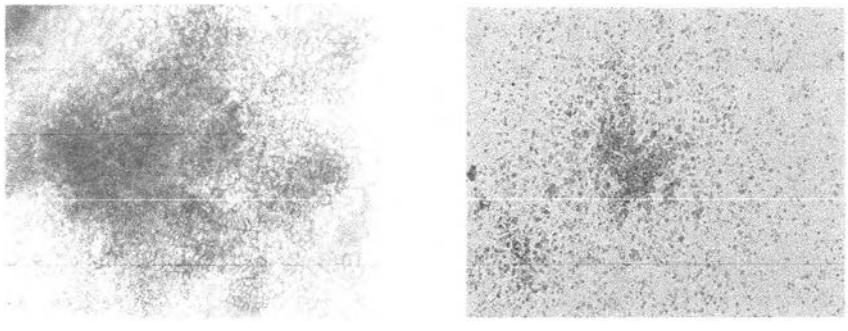


Fig8A CFU-GEMM Fig8A CFU-GM

Figure8 Photomicrograph of cultured CB CFC. (A) CFU-GEMM and (B) CFU-GM

$CD133^+$-enriched cells generated $4.19x10^2 \pm 57.27$ CFU-GM colonies per 10^4 cells before culture initiation. As a more primitive progenitor cells, the frequency of CFU-GEMM was $3.97x10^{-3} \pm 80.02$ colonies per 10^4 cells before culture initiation. In all conditions, both CFU-GM and CFU-GEMM colonies dramatically increased with culture days until day8 of culture and then decreased with prolonged culture days. Table13-14 displays the clonogenic potential with time in culture of CB cells cultured.

Table13 CFU-GM number per 10^4 cell and fold increase in culture of CB cells

condition	Percentage			
	0	8	16	24
MSC+IL-1α	419.25 ± 57.27	741.00 ± 75.50	315.53 ± 14.82	378.00 ± 67.87
MSC+FL+TPO	419.25 ± 57.27	704.00 ± 84.87	319.78 ± 13.87	379.75 ± 62.24
FL+TPO	419.25 ± 57.27	610.80 ± 181.11	371.73 ± 16.55	391.75 ± 53.75
condition	Fold increase			
	0	8	16	24
MSC+IL-1α	1.00 ± 0.00	78.83 ± 17.24	1,878.51 ± 405.40	139.55 ± 39.08
MSC+FL+TPO	1.00 ± 0.00	104.47 ± 21.16	2,503.69 ± 478.30	211.29 ± 56.22
FL+TPO	1.00 ± 0.00	52.58 ± 5.32	1,090.22 ± 143.61	49.27 ± 12.35

Table14 CFU-GEMM number per 10^4 cell and fold increase in culture of CB cells

condition	Percentage			
	0	8	16	24
MSC+IL-1α	396.50 ± 80.02	470.43 ± 27.16	680.75 ± 27.38	109.00 ± 18.18
MSC+FL+TPO	396.50 ± 80.02	501.80 ± 29.70	553.25 ± 73.46	186.25 ± 41.46
FL+TPO	396.50 ± 80.02	541.70 ± 13.74	670.50 ± 71.69	205.00 ± 33.63
condition	Fold increase			
	0	8	16	24
MSC+IL-1α	1.00 ± 0.00	540.40 ± 107.30	433.21 ± 95.42	41.51 ± 13.85
MSC+FL+TPO	1.00 ± 0.00	862.33 ± 234.82	501.25 ± 176.11	102.53 ± 25.43
FL+TPO	1.00 ± 0.00	431.68 ± 126.69	248.23 ± 121.27	27.68 ± 8.12[b]

[a] condition 3,4 different from condition 1

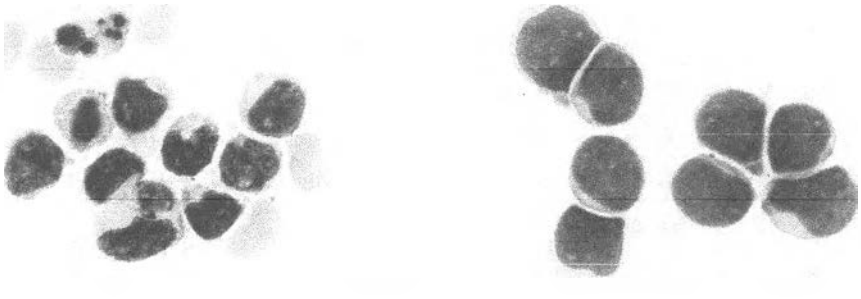
[b] condition 4 different from condition 3

The total fold increase in clonogenic potential of CFU-GM at day 16 was 1,878.51-fold ± 405.40-fold for $CD133^+$ culture with MSC+IL-1α, 2,503.69-fold ± 478.30-fold for culture with MSC+FL+TPO and 1,090.22-fold ± 143.61-fold for the cultures in absence of MSC.

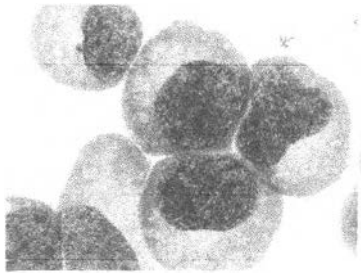
The total fold increase in clonogenic potential of CFU-GEMM at day 8 was 540.40-fold ± 107.30-fold for $CD133^+$ culture with MSC+IL-1α, 862.33-fold ± 234.82-fold for culture with MSC+FL+TPO and 431.68-fold ± 126.69-fold for the cultures in absence of MSC, showing that culture in different condition did not result in significanty differentent of the fold increase of both CFU-GM and CFU-GEMM.

6. Morphology study

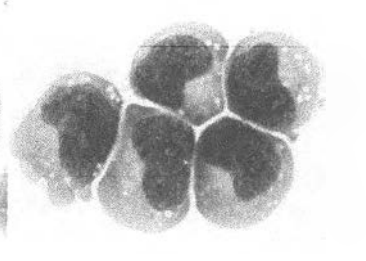
The result correlate with the result of percentage of $CD15^+$ *antigen by flow* cytometric analysis that MSC-based condition induce $CD133^+$ cells to differentiate to $CD15^+$ cells more rapidly than in MSC-free condition.The morphology of expanded cells in condition 1 and 3 bacome to granularity cells more rapidly than in condition 4. Figure9A-W show the Giemsa staining of MNCs, $CD133^+$-enriched cells and expanded cells.



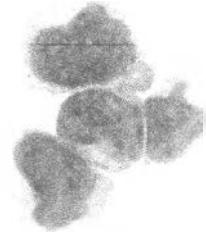
(A) MNC (B) CB $CD133^+$-enriched cell



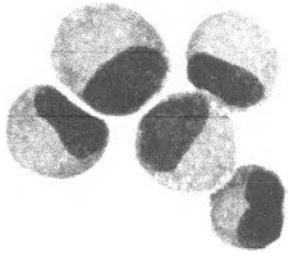
(C) Day4 condition1



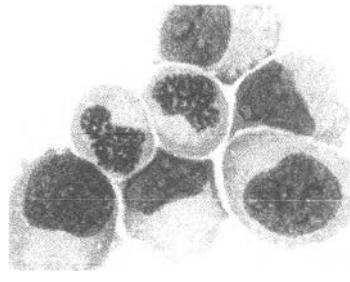
(D) Day4 condition3



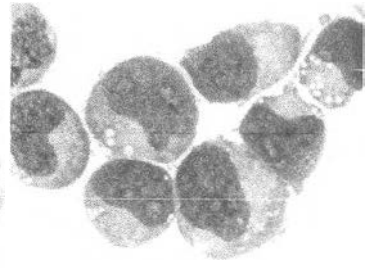
(E) Day4 condition4



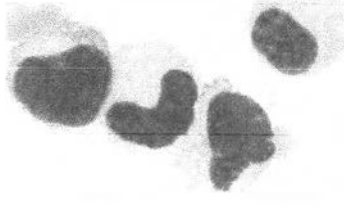
(F) Day8 condition1



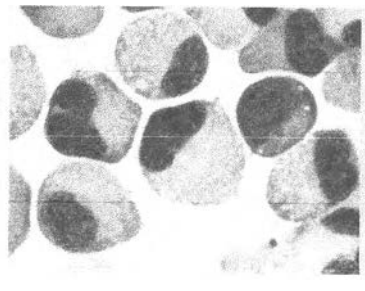
(G) Day8 condition3



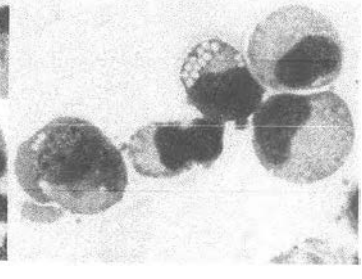
(H) Day8 condition4



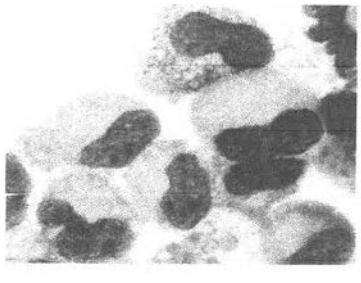
(I) Day12 condition1



(J) Day12 condition3

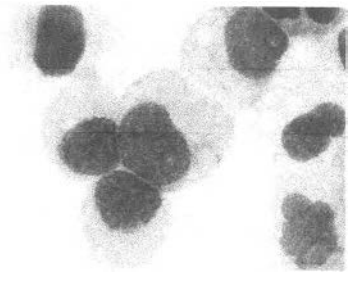


(K) Day12 condition4

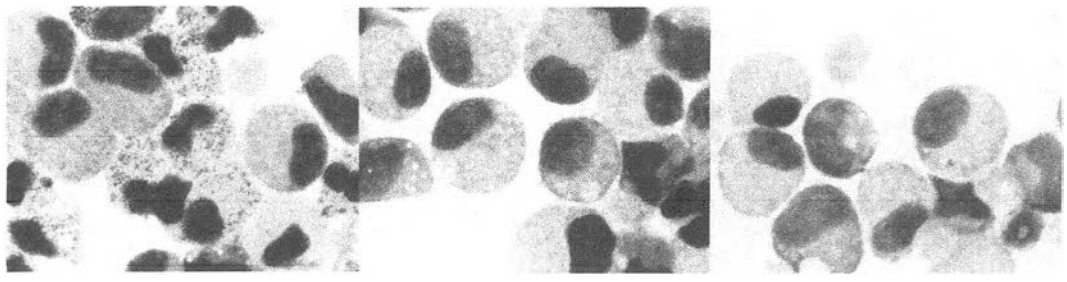


(L) Day16 condition1

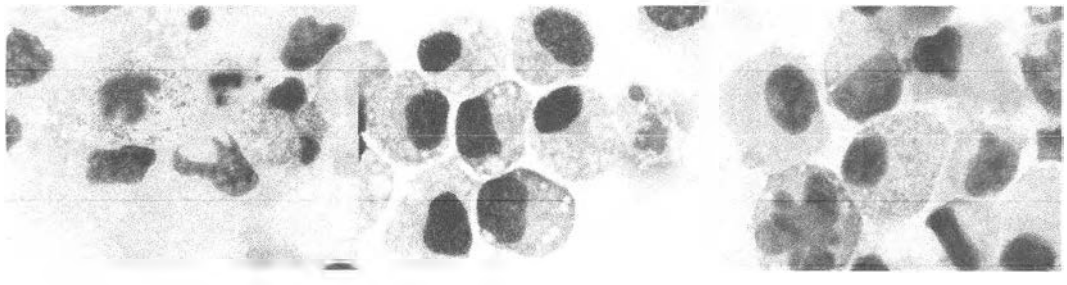
(M) Day16 condition3



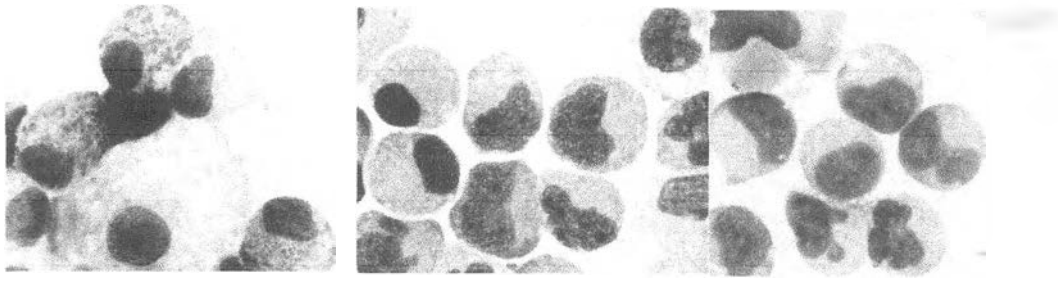
(N) Day16 condition4



(O) Day20 condition1 (P) Day20 condition3 (Q) Day20 condition4



(R) Day24 condition1 (S) Day24 condition3 (T) Day24 condition4



(U) Day24 condition1 (V) Day24 condition3 (W) Day24 condition4

Figure9A-W Giemsa staining of MNCs, $CD133^{+}$-enriched cells and expanded cells