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Co-culture of stromal and erythroleukemia cells in a perfused hollow fiber bioreactor system as an *in vitro* bone marrow model for myeloid leukemia

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Abstract

We have developed a hematopoietic co-culture system using the hollow fiber bioreactor (HFBR) as a potential *in vitro* bone marrow model for evaluating leukemia. Supporting stroma using HS-5 cells was established in HFBR system and the current bioprocess configuration yielded an average glucose consumption of 640 mg/day with an average continous protein production of 56 mg/mL over 28 days. Co-culture with erythroleukemia K562 cells was used as a model for myelo-leukemic cell proliferation and differentiation. Two distinct localizations of K562 cells (loosely adhered and adherent cells) were identified and characterized after two weeks. The HFBR co-culture resulted in greater leukemic cell expansion (3130 folds vs. 43 folds) compared to a standard tissue culture polystyrene (TCP) culture. Majority of expanded cells (68%) in HFBR culture were the adherent population, highlighting the importance of cell-cell contact for myelo-leukemic proliferation. Differentiation tendencies in TCP favored maturation toward monocyte and erythrocyte lineages but maintained a pool of myeloid progenitors. In contrast, HFBR co-culture exhibited greater lineage diversity, stimulating monocytic and megakaryocytic differentiation while inhibiting erythroid maturation. With the extensive stromal expansion capacity on surfaces of hollow fibers, the bioreactor system is able to achieve high cell densities and 3D cell-cell contacts mimicking the bone marrow microenvironment. The proposed *in vitro* system represents a dynamic and highly scalable 3D co-culture platform for the study of cell-stroma dependent hematopoietic/leukemic cell functions and *ex vivo* expansion.

Key Words: Hollow fiber bioreactor, leukemia, hematopoietic, co-culture, stromal cell

Introduction

Extensive research has been pursued to understand hematopoiesis and the etiology of leukemia. Hematopoiesis, the process of blood formation, requires an intricate balance in hematopoietic stem cell (HSC) self-renewal and differentiation which critically depends on its specific microenvironment called the stem cell niche (Iwasaki and Suda 2010; Li and Li 2006; Schofield 1978; Wilson and Trumpp 2006). Failure in homeostasis results in leukemia where hematopoietic progenitors no longer differentiate and acquire instead an uninhibited capacity to proliferate. Though hematopoietic mulfunction is often a consequence of genetic abberation, alternations in the hematopoietic microenvironment further contributes to the progression and/or sustenance of many hematological diseases.

The importance of the microenvironment for HSC function was revealed when a mutation in the gene encoding membrane-bound stem cell factor (SCF) present in a *Sl/Sl*^{d} mice (steel-Dickie mice), altered the HSC niche which eventually resulted in bone marrow failure and HSC maintenance *in vivo* (Barker 1994; McCulloch et al. 1965). This study suggests that regulatory signals involve not only direct cell-cell contact, typically between stem cells and niche cells, but also indirect interactions mediated by the extracellular matrix (ECM) or cytokines (Torok-Storb et al. 1999). Besides functional hematopoietic cells residing in the bone marrow, the niche also consists of stromal cells which synthesize growth, differentiating, and inhibiting factors, along with ECM components to provide cues and signals needed for the maintenance and differentiation of hematopoietic stem and progenitor cells (Bramono et al. 2010; Breems et al. 1998; Jing et al. 2010; Naughton 2003; Thalmeier et al. 1994; Wagner et al. 2007; Zuckerman 1984).

Significance of supporting stromal cells in maintaining long-term hematopoietic cultures *in vitro* was first demonstrated in a feeder-layer-based co-culture technique by Dexter (Dexter et al. 1977). The co-culture was estalished by inoculating mouse bone marrow

cells onto a confluent irradiated stromal cell layer which will support hematopoietic cell culture. This technique proved to be superior in maintaining HSC stemness as opposed to cells cultured in the absence of a feeder layer or additional cytokines which terminally differentiated within the first two weeks of culture (Brandt et al. 1990; Naughton 2003; Verfaillie 1992) as a consequence of *in vitro* spontaneous differentiation (Sorrentino 2004). However, static co-culture systems often suffer from decreased viability at 8 to 12 weeks and reduced cell proliferation over time (Toogood et al. 1980).

Culture improvements such as the utilty of perfusion can be made to optimize conditions for furthering cellular growth. For large scale expansion, bioprocess stability and scalability become yet more critical factors. This area has consequently led to the development of bioreactor systems in various configurations; perfusion chambers, stirred reactors, packed and fluidized bed reactors have been explored for *ex vivo* expansion (Cabral 2001; Cabrita et al. 2003). Many scientists believed that the best approach in understanding hematopoietic biology and designing an ideal *in vitro* system for hematopoiesis is to replicate the bone marrow microenvironment. To this end, our goal is to develop an *in vitro* culture system that can replicate key aspects of the cell-stroma microenvrionemnt found in the bone marrow. The bone marrow, as depicted in Figure 1A, consists of a vast vasculature of medullary sinuses surrounded by a packed density of hematopoietic and supporting cells. One method which we believe holds much promise in replicating cellular densities and fluid dynamicity inside the bone marrow is the hollow fiber bioreactor (HFBR). The discovery of HFBR system addressed many drawbacks in conventional static culture systems such as constraints in growth space and non-homogenous distribution of dissolved oxygen, pH, glucose and cytokines (Collins et al. 1998). The HFBR is a continuous perfusion system that ensures a constant media exchange and optimized oxygen delivery (Figure 1B). With a high cell surface to volume ratio and three-dimensional (3D) structural contact, it can model *in*

vivo tissue conditions and replicate cell-stroma and ECM interactions found in the stem cell niche (Figure 1B). Furthermore, this system facilitates bioprocess scalability replacing the need for hundreds of tissue culture flasks while concentrating the secreted products by hundred times or more (Cadwell 2004; Cadwell and Whitford 2009).

In this study, a continuous co-culture system for leukemic cells using the HFBR is described. A supporting stroma culture using HS-5 stromal cells was first established and maintained for up to 28 days; the stroma culture was characterized by evaluating the kinetics of glucose uptake and protein production. Subsequently, the leukemic model was developed using K562 erythroleukemia cells for chronic myeloid leukemia (CML). To assess bioprocess efficacy of the HFBR as a large-scale co-culture system for hematopoietic/leukemic cells, the culture was evaluated for its ability to support and expand cells *in vitro* and induce hematopoietic differentiation.

Materials and Methods

Cell and culture resources

An immortalized human bone marrow stromal cell line, HS-5 (ATCC) and human erythroleukemia cell line, K562 (ATCC) were used in this study. Cells were routinely propagated in tissue culture polystyrene (TCP)-flasks in complete medium at 37°C in a humidified incubator with 5% CO_2. Cells were passaged every 2-3 days on standard 2D TCP-flask cultures. Dulbecco's modified eagle medium (DMEM) (Invitrogen) and Iscove's modified dulbecco's medium (IMDM) (Invitrogen) were used for HS-5 and K562 cell culture respectively. Both medium containing 4.5 g/L glucose were supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories), 1.5 g/L sodium bicarbonate (Sigma) and 1% penicillin/streptomycin (A&E Scientific) to constitute the complete medium.

Hollow Fiber Bioreactor (HFBR) System

The hollow fiber bioreactor (medium sized cartridge C2011) used for this study was purchased from FiberCell Systems, USA. The hollow fibers are made of polysulfone with a diameter of approximately 200 µm and have a molecular weight cut off (MWCO) of 20 kDa at 50%, retaining approximately half of a secreted protein of 20 kDa within the extracapillary space (ECS). Fibers in the bioreactor create a total surface area of 2100 cm^2 within an ECS volume of 15 mL.

Inoculation and cell culture

Prior to cell inoculation, the HFBR system was circulated twice with phosphate buffed saline (PBS; Sigma Aldrich), once with DMEM medium (Invitrogen) and finally once with complete DMEM medium. Each cycle lasted for at least 24 h. To establish a stroma culture, a total of 1 x 10^8 HS-5 cells were resuspended in 15 mL of DMEM complete medium. The cells were inoculated using a syringe through the left side port with both central end ports closed. To ensure a uniform distribution of cells in the ECS, cells were flushed back and forth 3 to 4 times through the side ports. Excess medium in the syringes were forced across the fiber membrane into the reservoir by reverse filtration. The culture was initiated with 100 mL of complete medium in the reservoir and a media replacement was performed every two days by removing 75% of the waste medium. The reservoir volume was gradually increased from 100 ml to 400 ml (Figure 2A). Media replacement strategy was performed according to manufacturer's guidelines while concurrently monitoring glucose concentration in the reservoir. During the first two weeks of culture, reservior media was replaced every two days, subsequently this was performed everyday (Figure 2B). Medium in the ECS is also regularly replaced as needed. For TCP stroma culture, 1.5 x 10^6 cells were innoculated into a standard T75 flask and a change of media was performed every two days using a total volume of 15 mL as per standard practise.

To establish a co-culture in the HFBR, stroma culture was established and maintained in DMEM complete medium for five days prior to innoculation of K562 cells (5 x 10^5 cells) and the reservior medium was switched to IMDM complete media. Full ECS medium replacement was done on day 4 of co-culture onwards. Spent ECS medium containing loose cells were centrifuged and inoculated back into the ECS. The K562 co-culture was maintained for 14 days. Static TCP suspension culture (control) and co-culture were established and used as a comparison.

Glucose and Protein Assays

Media samples of 100 µL were taken during each medium replacement and measured for glucose and protein concentrations. Glucose concentration in culture media was determined with a glucose assay reagent (Sigma Aldrich) via phosphorylation of glucose to glucose-6-phosphate, followed by its oxidation to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) over a 15 min incubation period. This oxidation causes an equimolar reduction of NAD to NADH, resulting in an increase in absorbance at 340 nm which is directly proportional to glucose concentration. Glucose consumption was calculated based on the difference in glucose levels to a reading taken from the previous day.

Concentration of solubilized protein was assessed using a dye-binding assay (Bio-Rad), causing a differential color change of the dye in response to various protein concentrations. Subsequent to a 5 min incubation period, the colored reaction solution was measured spectrophotometrically at a wavelength of 595 nm. Relative protein concentrations were determined based on bovine serum albumin (BSA) standard curve. Protein production was calculated based on the difference in protein levels to a previous day reading.

Cell Harvesting

Cells were dislodged from the fibers using StemPro Accutase cell dissociation reagent (Invitrogen) with 30 min incubation at 37°C for each cycle and were harvested by flushing

through the ECS using syringes. Two harvest cycles were performed for each experiment. For HFBR co-culture, cells removed from bioreactor with and without the presence of Accutase were termed as adherent and loose cells respectively. To separate K562 cells from HS-5 cells, cell suspension were reseeded into plates and left in the incubator for 1 h. Suspension K562 cells were then collected for analysis. Growth and viability of cells were assessed by the enumeration of live cells performed via trypan blue exclusion and manual counting using a hemocytometer.

Flow Cytometry

To monitor cell differentiation, harvested K562 cells (1 x 10^6) were incubated for 30 min in 4°C with a FITC-, PE- or PerCP-conjugated antibodies against Gly-A (Becton Diskinson), CD71 (Becton Diskinson), CD13 (Becton Diskinson), CD45 (Becton Diskinson) and CD41 (Santa Cruz). To exclude non-specific binding of antibodies, FITC-conjugated mouse IgG_{2b} isotype control (Santa Cruz), and PE- and PerCP-conjugated mouse IgG_1 isotype control (Santa Cruz), PE-conjugated IgG_3 isotype control (Santa Cruz) were used. Cells were washed twice with PBS, resuspended in buffer containing PBS, 2% FBS and 0.1% sodium azide (Sigma-Aldrich) and analyzed on a flow cytometer (Beckman Coulter).

Wright-Giemsa Staining

For hematological identification, K562 cells were cytospined on clean glass slides at 100 x *g* for 3 min, stained with Wright-Giemsa (Merck Chemicals) and then visualized under a light microscope. Hematopoietic cell populations were distinguished and identified via their morphological appearances.

Data Analysis

Results obtained from flow cytometry data and cell fold expansion were analyzed and represented as the mean ± standard deviation of triplicates. Statistical analysis was performed

using paired and unpaired Student's T-test for evaluating surface marker expression levels before and after 14 day cultures, and between different experimental groups. Observed differences were regarded as statistically significant if the calculated two-sided *P* value was less than 0.05.

Results and Discussion

Process characteristics of hollow fiber stromal culture

Growth kinetics of HS-5 cells

Growth kinetics of HS-5 cells on TCP culture were per normal growth *in vitro* with a steady exponential increase during the first 16 days of culture before reaching a plateau when cells are space-inhibited for further expansion. The calculated average population doubling time was 2.86 days and the total harvested cell number was 4.5 x 10^7 cells (Table I). Growth kinetics of HS-5 cells in HFBR cannot be readily characterized however, under the same culture volume provided in the ECS (15 mL), final cell harvest in the HFBR system achieved a significantly higher total cell number with a minimum harvest of 4.4 x 10^8 cells. Initial seeding density in the HFBR is also significantly higher (1.0 x 10^8 versus 1.5 x 10^6 in TCP). This is due to the high surface area to volume ratio (2100 cm^2 in a 15 mL reactor) compared to conventional TCP which enables HFBR to support large cell number expansion. However, a complete harvest was not readily achievable due to the high packing density of cells and fibers. Use of collagenase improved cell dissociation but we believe that some cells remain trapped between fibers. Besides achieving a large cell harvest, good cell viability (> 95%) was accomplished at the end of 28 day culture.

Glucose consumption

No significant changes in glucose levels of the TCP culture was observed during the first two days but an exponential decrease began after day 2 and a final concentration plateau was

reached on day 10. The final glucose concentration maintained in TCP culture was 0.95 ± 0.08 mg/ml (Figure 3A). In the HFBR, a more gradual decrease in glucose levels was achieved and reached steady state in 4 days (Figure 3A). The final concentration at bioprocess stability was 1.5 ± 0.3 mg/ml (from day 4 to 20). In comparison, average physiological blood glucose level is around 1 g/L glucose and typical cell culture formulations contain glucose of 1 g/L up to 10 g/L. Average glucose consumption in TCP culture was 45 mg/day while those in the HFBR was 640 ± 220 mg/day. Daily glucose consumption in our HFBR system is considered to be under "good" conditions of a healthy cell culture per manufacturer's recommendation (< 1000 mg/day). The focus on bioprocess monitoring should be emphasized to ensure a healthy cell culture in the bioreactor (Lim et al. 2007) as high glucose consumption induces high lactate production; the exposure to high amounts of lactate has been reported to inhibit cell growth (Strand et al. 1984). The advantage of perfusion in the HFBR system allows one to tune process operations in line with the needs to the culture and ensures an overall superior bioprocesss stability than flask cultures (von Woedtke et al. 2002).

Protein production

During the first 8 days of culture in TCP, protein production averaged at 9.6 mg/day but decreased after day 8 to a stable production of approximately 4.5 ± 2.4 mg/day (Figure 4A). For the HFBR culture, protein production began to steady after day 6 but ranged variably from 20 to 100 mg/day over a 3-week culture. Production of protein in the HFBR appears to be highly affected by the feeding rate and can therefore be tuned according to culture requirements. The average amount of protein generated in the ECS after glucose levels has stablized was 56.3 ± 23.3 mg. The total amount of protein produced in the HFBR system was on average 12.5-fold greater than that of TCP culture.

Protein measurements taken in the reservoir indicated negligible traces thus no net production of protein was found from the reservoir media (results not shown). Previous research has shown that polysulfone fibers which is a hydrophobic material, readily adsorbs proteins (Norde 1986; Patkar et al. 1993); this will therefore affect to some extent the recovery of secreted proteins. Pre-coating polysulfone fibers with another protein such as bovine serum albumin (BSA) or κ-caesin have shown to solve this problem (Arnebrant and Nylander 1986; Patkar et al. 1993). The low molecular weight cut-off (MWCO-20) permits only small molecules < 20 kDa to diffuse freely across the membrane. Larger proteins (> 20 kDa) produced by stromal cells in the ECS will therefore not readily diffuse through the polysulfone membrane; these include growth factors such as IL-6 and LIF. Our protein gel (Figure 4B) revealed that most of the proteins produced from HS-5 stromal cells and collected in TCP or ECS were above 20 kDa. Apparent bands between 28-36 kDa observed in protein samples from both cultures are likely growth factors typically produced by HS-5 such as IL-1α and IL-1β at around 31 kDa, M-CSF at 36.4 kDa, and glycosylated LIF which could range from 32-62 kDa. Strong bands at around 28 kDa and weak bands below 28 kDa are likely growth factors IL-11 or IL-6. Interlekin-11, a multi-functional cytokine which plays a key role in megakaryocyte maturation, has a predicted molecular weight of 19.3 kDa but under reducing conditions, appear on SDS gel at 24-28 kDa. Interleukin-6 which is a prototypic pleiotrophic cytokine is often variably glycosylated and will show as multiple bands between 22-27 kDa. Our observations suggest that exogenous growth factor production is preserved in the HFBR as produced in normal TCP cultures.

Co-culture of erythroleukemia cells

In vitro proliferation of human K562 erythroleukemia cells in TCP was first evaluated in the presence of HS-5 stromal cells and in a stroma-free culture (control). Maximum growth of K562 cells in TCP on stroma supported versus stroma-free culture were 43-fold and 52-fold

respectively but this comparison showed no statistical difference (Figure 5A). Proteins secreted from stromal cells, both soluble and ECM proteins, may induce enhancing or inhibitory effects on hematopoietic and leukemia cells. In one study, conditioned media collected from HS-5 stromal culture imposed inhibitory effects on proliferation of K562 cells *in vitro* (Lee et al. 2008) while in another study, a direct contact culture improved short-term proliferation and viability of primary AML cells *in vitro* by protection from apoptosis (Garrido et al. 2001). The competing effects between secreted soluble factors and stromal contact could have resulted in a net no-change effect of myeloid leukemia cell proliferation in our TCP experiments. Stromal cell numbers remained the same after 14 days of co-culture thus the ratio of stroma to leukemic cell contact becomes limiting as K562 cell number increases. This limitation is a main disadvantage of 2D TCP cultures.

In contrast, co-culture established in HFBR supported a prolonged continuous growth of K562 cells *in vitro*, reaching a fold increase of 3130 (initial inoculum of 5 x 10^5 cells) after 2 weeks in culture compared to 43 folds in the TCP co-culture system (Figure 5B). The average population doubling time of K562 cells cultured in the HFBR system was estimated at 29 hr, which corresponds well to the normal doubling time of K562 cells of 24-30 hr (Drexler 2000) while that observed in TCP which also averaged at 30-31 hr lasted only one week. Very little/slow growth was observed on week 2, as K562 cells cease to proliferate on TCP *in vitro*. This demonstrates an advantage of the HFBR system in sustaining normal and healthy cell division continuously throughout a 2-week culture period whereas in TCP culture, it is limited by an availability of space and sub-optimal physiological conditions slowed growth rates and resulted in an eventual cease of growth. Furthermore, low MWCO fibers allow the retention of many key proteins within the ECS (where K562 cells reisde) while maintaining a continual exchange of nutrients and metabolites; this ensures a well-balanced physiological culture environment. We believe that the retention of key soluble

proteins and growth factors within the ECS significantly improved the cultivation of K562 cells *in vitro* whereas in TCP culture, these viable proteins are continuously being removed during each change of culture media. Greater stromal contact is another reason in supporting sustained cell proliferation.

Harvested K562 cells from HFBR system were classified into two populations: loose and adherent. Loose cells were loosely adhering onto the HFBR co-culture which can be removed by flushing medium in a continuous repeated motion through the side ports of the cartridge. The adherent population were cells that would only detach by the action of accutase. In this study, adherent K562 cells made up 68% of the total cell harvest accounting for over 2000-fold increase in cell numbers thus suggesting that stromal cell surface is the predominant site of K562 cell proliferation. Our observations are therefore in congruent with the findings of others and support the notion of cell-stromal contact enhanced leukemic survival and proliferation. Due to the large surface area to volume ratio provided in the HFBR system, cell-stromal contact is maximized. This provides not only fertile grounds for a long-term stable co-culture but also the opportunity for interrogation of stromal effects due to its biomimicry in cell densities and 3D cell-cell contacts found in the bone marrow. A manual evaluation by trypan blue exclusion showed high cell viability of > 90% in both TCP and HFBR co-cultures (Figure 5C).

Morphological observations

The cytoplasm and progressive condensation of nuclear chromatin were used as morphological evidence of cytoplasmic and nuclear maturation respectively. Early myeloid progenitors (myelocytes and myeloblasts) make up the majority of K562 cell population, and this was indicative of the K562 cell population pre-culture (Figure 6A). These progenitors have a diameter of about 15 to 20 µm with a round/ovoid nucleus and a small cytoplasm to nucleus ratio. Erythroid progenitors, namely proerythroblasts and orthochromatic

erythroblasts (labeled 3 in Figures 6A-D), tend to have denser nuclei structure, indicating chromatin condensation. They also tend to have more rounded nucleus with a dark to light blue cytoplasm. Monocytes can vary in appearances and sizes which can range from 20 to 40 µm, and often have a lobed nucleus. The appearance of monocytes was greater after 14 days in co-culture (Figure 6B-D). Neutrophilic granulocytes tend to have segmented nucleus (Figure 6B). Megakaryocytes and its early progenitors which are generally larger cells with a variable size range of 30 to 100 µm appeared more prominent in HFBR cultures (Figures 6C and D). They also have multiple nuclei and a cytoplasm full of granulation. The morphological analysis reflect the presence of monocyte, erythrocyte and megakaryocyte lineages, signifying the differentiating capacity of K562 cell line to a diverse myeloid population achieved in the HFBR system *in vitro*.

Immunophenotypic analysis

Previous studies have shown that K562 cells can exhibit both erythroid and megakaryocyte markers in absence of any inducing agent (Rowley et al. 1992) and this is parallel to our results shown in Figure 7. Surface marker expression of K562 cells at the start of a culture, day 0, showed high percentages of Gly-A, CD71 and CD41 positive cells. Direct contact culture in TCP and HFBR displayed no considerable difference in the percentage of Gly-A (late erythroid marker) positive cells but a significant increase in CD13 positive cells was observed (Figure 7B and 7C). An increase in CD13 positive cells which was not observed in the suspension culture suggests that stroma-supported cultures favor the expansion of myeloid progenitors. This could be an attritube from growth factors produced by HS-5 stromal cells, such as IL-1, IL-6, G-CSF and LIF, which showed to induce differentiation to myeloid and macrophage/monocyte lineages (Cohen et al. 1992; Metcalf 1989; Yamamoto-Yamaguchi et al. 1989). Besides the effects of endogenously-produced factors, emerging evidence showed that cell-cell interactions, playing an essential role in HSC regulation of

normal hematopoiesis, may be as equally important for stromal cell-mediated inhibition in leukemic cell differentiation (Weber and Tykocinski 1994). A difference in CD13 expression between the loose and adherent cell population (38.09% and 99.86% respectively) found in HFBR culture (Figure 7C) lean toward this hypothesis. Loosely adherent cells which do not benefit from direct cell-cell contact with HS-5 stromal cells tend toward a more mature cell population which have likely lost their adhesive properties. Thus a low percentage of CD13 surface expression in loosely adherent cells is preserved; their profile resembles suspension culture in TCP control (27.63%) (Figure 7B) but loosely adherent cells show slightly greater numbers of CD13 positive cells due to the presence of exogenously secreted growth factors supporting myeloid proliferation. Direct contact culture in TCP displayed two populations of CD13 positive cells at low and mid-level expressions, this corresponds to loosely-adherent and adherent populations observed in the HFBR culture. Bone marrow stromal cells in a direct contact culture have so far shown no inhibitory effects on the differentiation potential of K562 cells in the presence of a stimulatory factor.

Direct contact culture on TCP did not have an effect on erythroid and megakaryocytic differentiation as both TCP cultures (stroma-free versus stroma-supported) showed similar trends in CD41, CD71 and Gly-A expressions (Figure 7). A closer analysis in the sub-populations of erythroid lineages revealed that erythroid maturation is mildly induced on TCP cultures leading to a decrease in Gly-A^+/$CD71^+$ (CFU-E and pronormoblasts) by ~20% and a corresponding increase ~25% in Gly-A^+/$CD71^-$ (recticulocyte or mature erythroid cells) population. In contrast, co-culture established in the HFBR did not induce erythroid maturation i.e. the same percentages of CFU-E and pronormoblasts (Gly-A^+/$CD71^+$) at ~90% were maintained as those observed at the start of the culture (Day 0) with very few mature erythroid cells (Gly-A^+/$CD71^-$) present. Overall, co-cultures in both TCP and HFBR favored

monocytic progenitor cell proliferation and supported the survival of both erythroid and megakaryocytic cell lineages.

Conclusion

This study premiers the utility of a HFBR modeled as a bone marrow mimetic *in vitro* system for leukemic cell proliferation and differentiation. Our unique HFBR co-culture approach enables the extension of the well-established Dexter's culture in process scalability and biomimicry. Localization of leukemic cells to stromal cells which is significantly enhanced in the HFBR resulted in sustained cell growth over a two week culture as opposed to a TCP culture where cell growth ceased after one week. Our data further suggests that the stromal cell surface remains the predominant site of K562 cell proliferation along with diversity in multi-lineage myeloid differentiation. With the advantage of achieving high cell densities and 3D cellular contact, the HFBR system recreates a better mimicry of the cell-stroma microenvironment which can provide significant improvements to *in vitro* studies of stroma-dependent leukemic and hematopoietic cell functions.

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Table I. Comparison of HS-5 stromal cell growth in TCP and HFBR systems.

System	Surface Area (cm^2)	Total cells inoculated ($\times 10^6$)	Inoculum density ($cells/cm^2$) ($\times 10^4$)	Growth period (days)	Total cells harvested ($\times 10^6$)	Final cell density ($cells/cm^2$) ($\times 10^4$)
TCP	75	1.5	2	28	45.14	60.2
HFBR	2100	100	4.76	28	442.24	21.1

Figure 1. Schematic diagrams of the human bone marrow vasculature (A) and the co-culture technology used in the hollow fiber bioreactor system (B). Stromal cells acting as feeder layers grow in the extracapillary space outside the fiber wall. Culture medium flows inside the fiber and due to the semipermeable nature of the fiber, nutrients and metabolic waste are allowed to diffuse across the fiber.

Figure 2. Feeding strategies implemented for HFBR and standard TCP cultures reflecting the total volume of media (A) and frequency of media change (B). Total volume of media used in HFBR starts at 100 mL and increased to 400 mL as cell number increases. The frequency of media chance in TCP was every two days and that in the HFBR was either every two days or everyday.

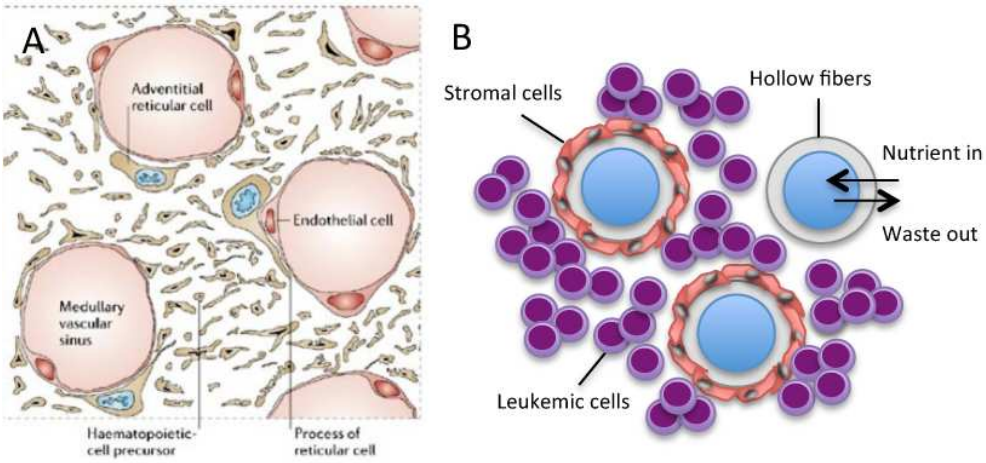
Figure 3. Comparison of glucose concentration (A) and glucose consumption (B) in medium of TCP and reservoir and ECS of hollow fiber system. A steady decline in glucose concentration was seen in TCP culture. Similar trend was also observed in HFBR culture until day 22. Glucose concentration in reservoir and ECS was relatively comparable. Cells in HFBR system consumed more glucose as compared to TCP.

Figure 4. Total protein production in TCP and HFBR culture (A). Protein production started to decrease after 10 days of TCP culture whereas in HFBR culture, production of protein changes depending on its feeding rate. Gel electrophoresis of protein samples (B). Lanes 1-3 from TCP on days 8, 14 and 21; Lanes 4-6 from HFBR on days 8, 14 and 21.

Figure 5. Total cell expansion and cell viability of K562 cells co-cultured in the presence of HS-5 stromal cells. Cells were co-cultured in TCP and HFBR systems for 14 days; harvested K562 cells were compared with control i.e. suspension culture in the absence of stromal cells in TCP system (A). Expansion in HFBR is significantly higher than both TCP cultures (* for p-value < 0.05). Harvested cells in HFBR co-culture were sorted into two populations: loose and adherent (B). Adherent cells showed significantly higher proliferation than loosely adherent cells (* for p-value < 0.05). Cell viability for control and TCP were determined using trypan blue exclusion (C).

Figure 6. Morphological appearances of K562 cells at the start of the culture – Day 0 (A), 14 days in 2D co-culture (B), adherent population in HFBR culture after 14 days (C) and the loose population in HFBR culture after 14 days (D). Distinct populations of myelocytes [1], myeloblasts [2], erythroblasts [3], monocytes [4], neutrophilic granulocyte [5] and megakaryocytes [6] were identified in these images. Scale bar is 50 µm.

Figure 7. Surface marker expression of cells expanded in TCP and HFBR co-culture. Plots from FACS analysis of K562 cells with monoclonal antibodies directed against various hematopoietic cell surface markers (blue peaks for day 0; orange peaks for day 14) (A). An isotype control was included in each experiment to identify background fluorescence (red peaks). Number of cells analysed (events) was plotted on the y-axis; intensity of staining was plotted on the x-axis. Percentage of cells expressing each marker in TCP culture (B). Percentage of cells expressing each marker in HFBR culture (C).



Bone Marrow vs Hollow Fiber
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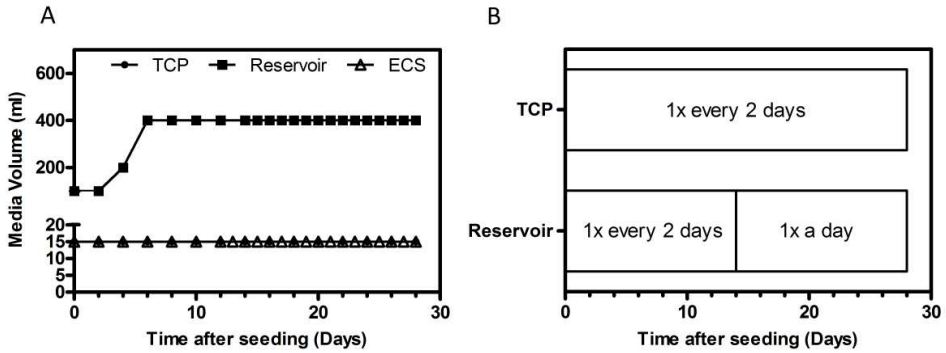

Feeding strategy
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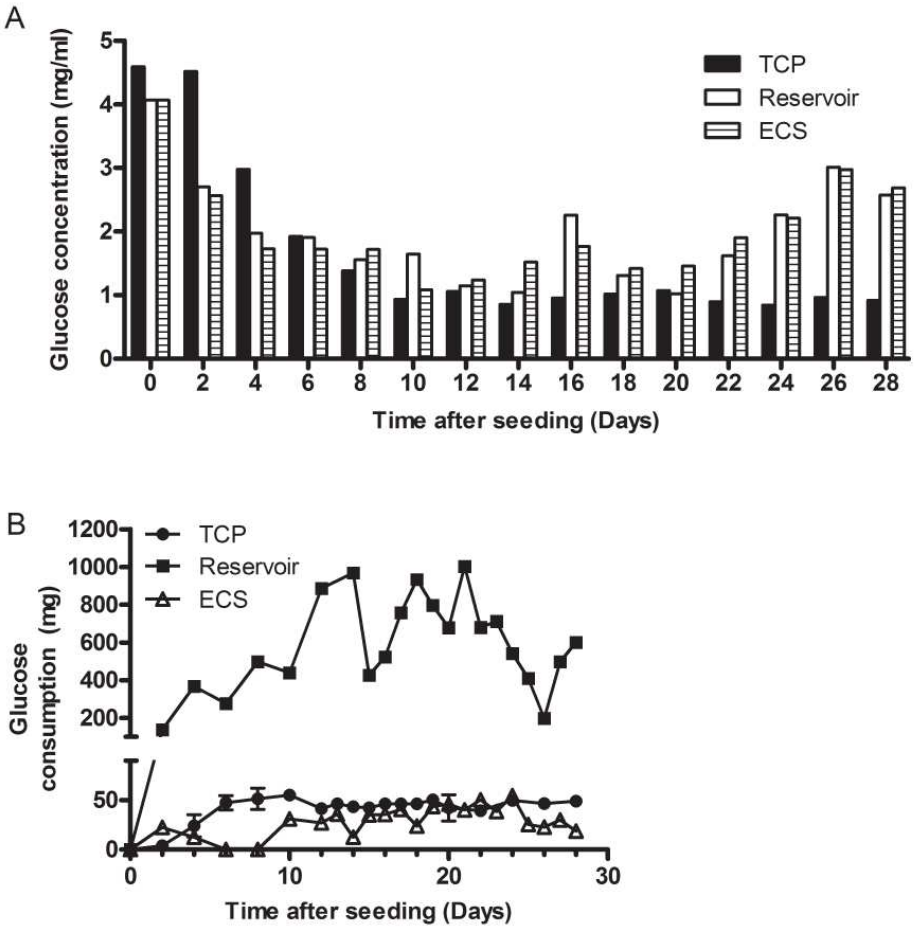

Glucose monitoring
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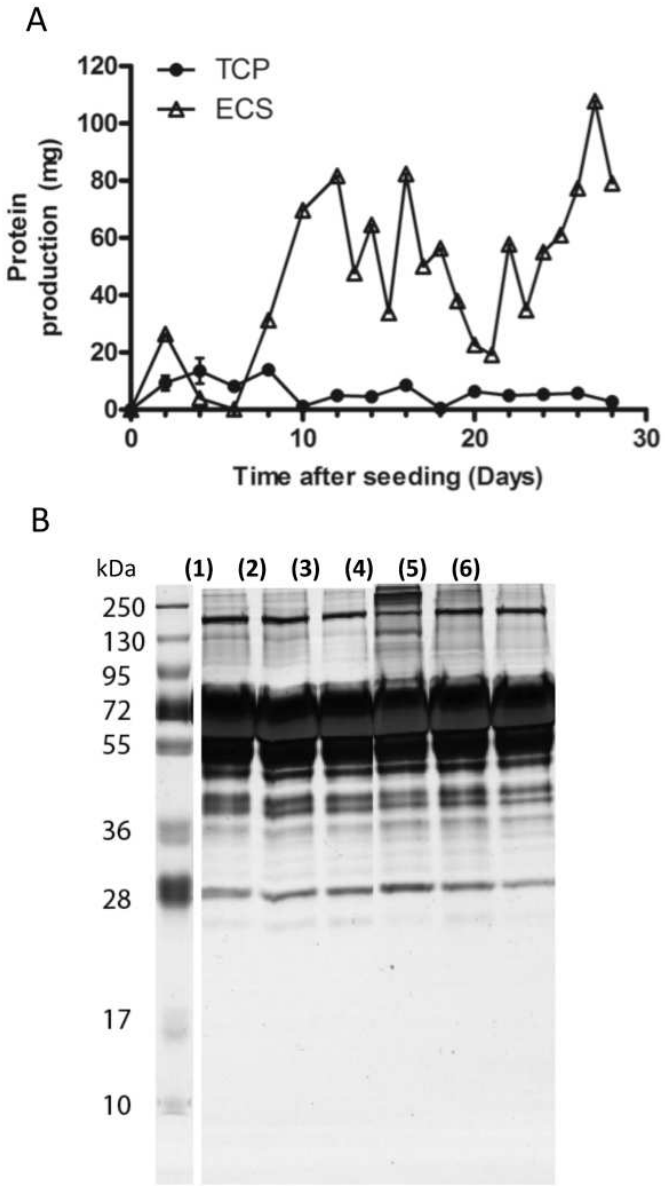

Protein production
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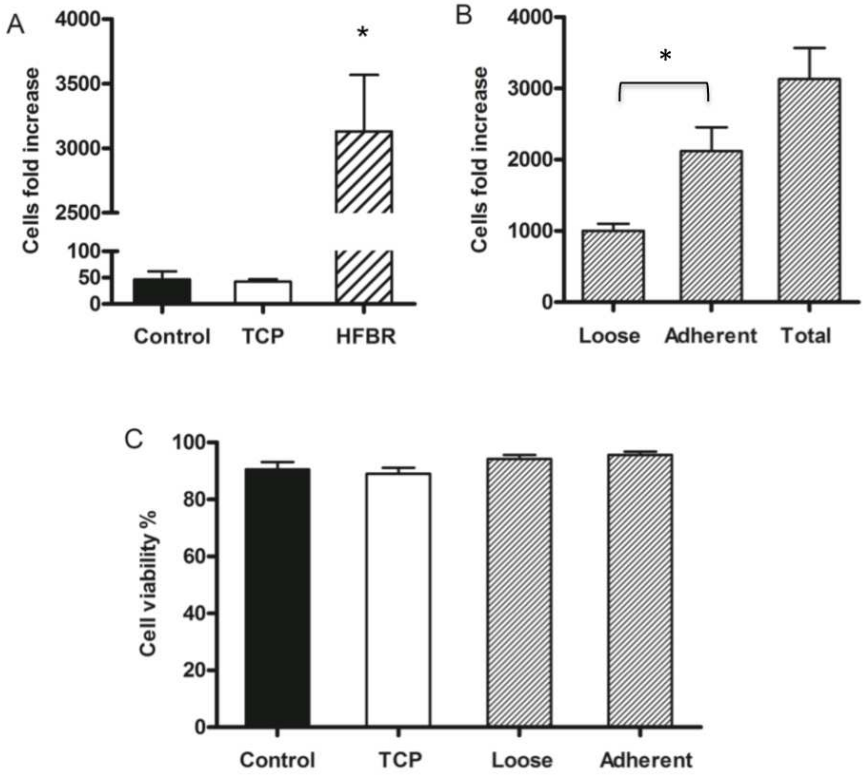

Cell growth
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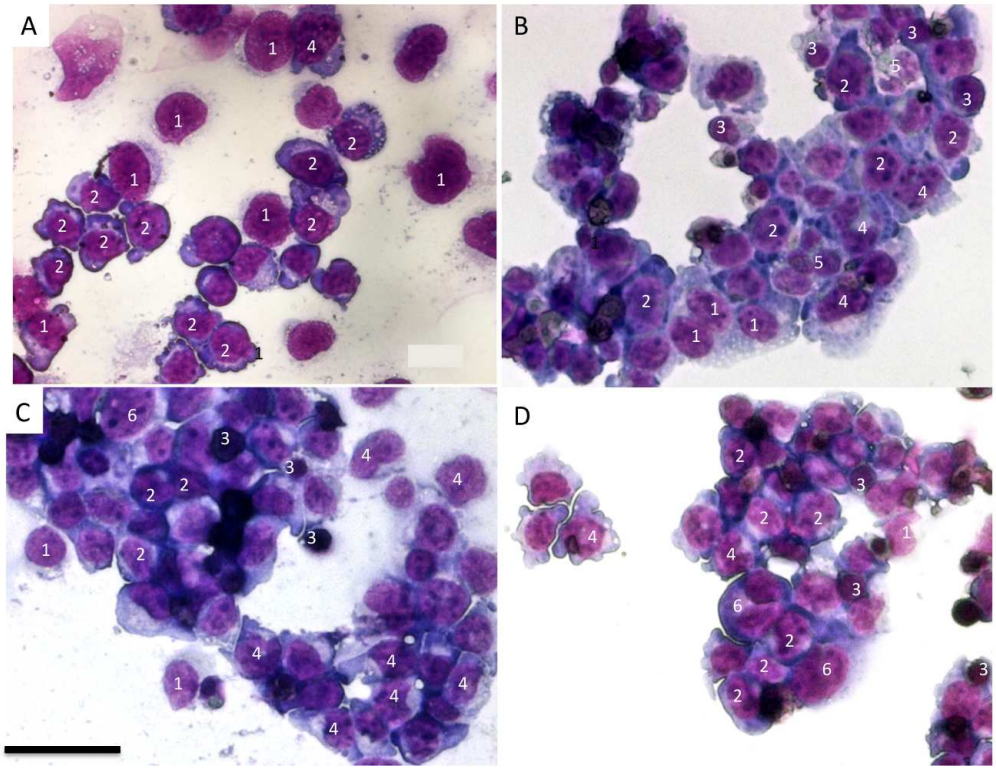

Cell morphology
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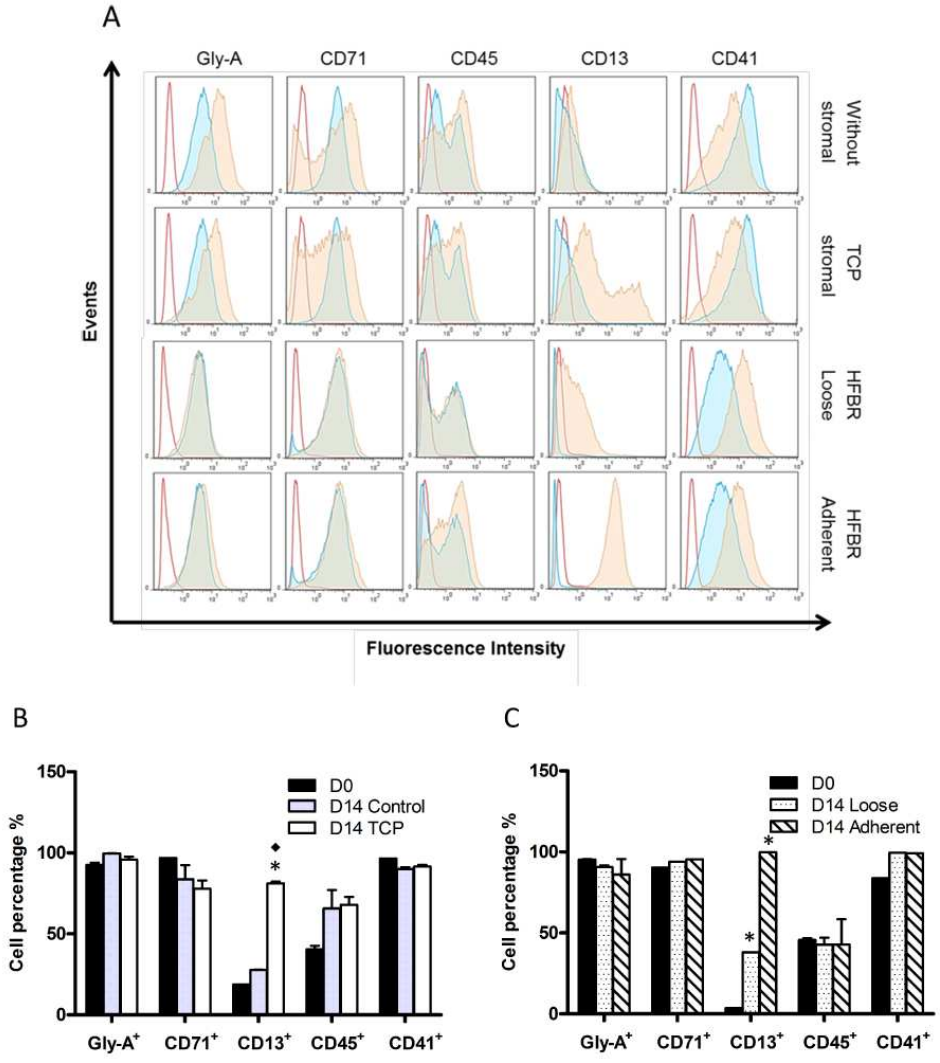

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