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A revisionist history of adult marrow stem cell biology or ‘they forgot about the discard’

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Abstract

The adult marrow hematopoietic stem cell biology has largely been based on studies of highly purified stem cells. This is unfortunate because during the stem cell purification the great bulk of stem cells are discarded. These cells are actively proliferating. The final purified stem cell is dormant and not representative of the whole stem cell compartment. Thus, a large number of studies on the cellular characteristics, regulators and molecular details of stem cells have been carried on out of non-represented cells. Niche studies have largely pursued using these purified stem cells and these are largely un-interpretable. Other considerations include the distinction between baseline and transplant stem cells and the modulation of stem cell phenotype by extracellular vesicles, to cite a non-inclusive list. Work needs to proceed on characterizing the true stem cell population.

In the storied fifth game of the 1987 basketball playoffs, the Pistons went down to the wire with the Celtics. With a 1-point lead and seconds left, Isaiah Thomas inbounded the ball, Larry Bird intercepted the inbound pass, flipped it to DJ for the winning basket. Danny Ainge when asked his take on the game, replied with a huge smile on his face, ‘They forgot about Larry’. The story below outlines how the whole stem cell research enterprise ‘forgot about the marrow discard’. This is a long, exciting and somewhat frightening tale about how research can go astray; beautiful technologically impressive research all based on a false premise. That is that one had to purify marrow stem cells to homogeneity in order to appropriately study them. Thus the field and its leaders focused on purification approaches, followed by elegant studies on molecular mechanisms, pathways and cellular regulators. This has involved virtually everyone in the field, including ourselves. The general mindset of the field is illustrated by these quotes from a recent review by Rossi *et al.*¹ ‘Separating HSCs from differentiated cells that reside in the bone marrow has been the focus of intense investigation for years’ and ‘Identifying the rare cell population, on which the hematopoietic homeostasis is elegantly built, represents therefore one of the major challenges in the field’. We will develop the story below, showing that marrow cells discarded during the purification procedure are actively cycling and always changing on a cell cycle-related continuum and represent the vast majority of marrow hematopoietic stem cells, while the purified

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CONFLICT OF INTEREST

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population that is quiescent and has characteristic surface markers is not a representative stem cell population. The vast bulk of studies on stem cell phenotype and regulation have been carried out on this non-representative population of purified marrow cells.

A GOOD BEGINNING

In 1961, Till and McCulloch² reported on an approach that appeared to assay the pluripotent marrow stem cell. Measuring radiation sensitivity and infusing murine marrow cells, they noted lumps on the spleen 9–10 days after cell infusion. These lumps turned out to be clonal units consisting of erythrocytes, granulocytes, megakaryocytes and probably lymphocytes. This was the original marrow stem cell assay, which has come on hard times because of a lack of correlation with subsequently purified stem cells. The cell was termed colony-forming unit spleen (CFU-S) and is probably an excellent stem cell assay, despite disparagement of many. Extensive work on this assay showed that CFU-S was clonal and that self-renewal was completely heterogeneous. In a classic paper, Till *et al.*³ described the stochastic nature of the CFU-S. They compared radioisotopes to CFU-S; the radioisotope population decay rate was stable with precise half-lives but the decay rates of individual isotopes were very heterogeneous. The stem cells appeared to behave like radioisotopes. This should have been a warning to future stem cell investigators seeking to purify to homogeneity the marrow renewal stem cell; the quest should have been after the appropriate population, not the individual stem cell. The CFU-S was characterized as to differentiation potential, proliferation potential, environmental influences and proliferative state. In general, it was described as being relatively dormant or not in active cycle. However, this conclusion was poorly supported by extant data, which showed a wide variation in cycle status between different laboratories.

FILLING IN THE MODEL

Bradley and Metcalf,⁴ and Pluznik and Sachs⁵ published critical manuscripts describing cloning of marrow cells in semi-solid cultures; the granulocyte-macrophage colony-forming cells or progenitors. These cells appeared more differentiated than CFU-S and were in a higher proliferative state. There followed a steady stream of descriptions of *in vitro* assays for progenitors with different lineage potentials; erythroid,⁶ megakaryocyte,⁷ mast cell,⁸ eosinophil,⁹ T cell¹⁰ and B cell,¹¹ and then more primitive progenitors termed blast forming units and progenitors with multilineage potentials.^{12,13} Eventually progenitors with virtually every possible mix of hematopoietic cells were described with multilineage ‘primitive’ progenitors responding to multiple cytokines and with more restricted progenitors responding to fewer cytokines and finally with unilineage progenitors responding to single cytokines. The definition of these progenitors led to the characterization and eventual cloning of many growth factors, further defining the field.

As noted in an editorial in *Experimental Hematology* in 1991¹⁴ ‘Dr. Ogawa came along and messed everything up’. He described a bewildering array of different colony types,^{13,15} and ‘perhaps the most devastating Ogawaian data are the daughter cell experiments, which indicate that within one cell cycle transit totally different lineages may be pursued by two daughter cells derived from a blast colony cell. This discovery was akin to exploding a bomb

in the center of all hierarchical models'. This work should have been a clear warning to the evolving stem cell purificationists, espousing hierarchical models of stem cell differentiation, but the implications of these studies were not perceived by most, and, despite these observations, very tidy hierarchical models were proposed, which were enthusiastically accepted by the stem cell community. In these very satisfying models, a primitive stem cell, which was considered dormant or noncycling, gave rise to a wide variety of progenitors with progressively increasing differentiated characteristics and decreasing lineage potential. This was, of course a classic hierarchical model of stem cell differentiation. A discerning comment at the end of 'Blueness'¹⁴ was, 'Could much of what we are looking at relate to one essential cell in various functional states'. Oh Yea.

TECHNOLOGICAL BRILLIANCE WITH A NARROW SCOPE

The report in the New York Times Sunday supplement startled the field. Hematopoietic stem cell researchers purified the marrow stem cell, the cell responsible for hematopoietic renewal in irradiated mice.¹⁶ There was some grumbling about a failure to recognize previous work, but this was soon forgotten. There was a truly impressive stream of publications defining an elegant stem cell system characterized by a variety of lineage-specific cell surface epitopes.^{17–19} The development of the hematopoietic stem cell field is illustrated in Figure 1 and the resultant hierarchical model in Figure 2. This was so inside the box, elegant and reasonable, that it has been universally accepted as true dogma. The demonstration of a hierarchy of marrow cells with progressively diminishing differentiative potential, relatively high proliferative rates and selectively responsive to different growth factors was a very satisfactory hierarchy. There were progenitors for virtually all differentiated hematopoietic cell types, with the more primitive progenitors requiring more cytokines and the more differentiated fewer to only one cytokine. These described progenitor classes were in fact the basis for the characterization of a number of the hematopoietic cytokines. With the cloning of the cytokines and the purification of different cell classes, the hematopoietic stem cell system appeared to be almost completely characterized, and the goal was now for a formal definition of the master pluripotent marrow renewal stem cell. There was extensive work along this line, and with the description of the lineage negative/c-Kit+/Sca-1+ cell, the so-called LSK cell, many felt the goal had been reached, although latter work added CD150 (Slam) positivity as a further refinement of the separations.²⁰ It was this purified cell, the LSK or LSK-slam cell, which was studied in a vast myriad of elegant studies to define their metabolic pathways, genetic characteristics and general regulatory pathways. Wnt, beta-catenin, Notch receptors and ligands, cell fate determinants in embryonic development, were extensively studied with contradictory results.^{21–25} For example, Notch signaling has been shown to be involved in expansion of long-term repopulating cells and progenitor populations, differentiation decisions, and maintenance of hematopoietic stem cell quiescence and self-renewal.^{21,22} However, other studies suggested that loss of Notch function had minimal effect on hematopoietic stem cell maintenance and was not required for self-renewal.²³ Similarly, befuddling results for the importance of Wnt signaling have been produced, with some studies showing Wnt signaling to be important for the maintenance of hematopoietic stem cells and B-catenin activation to promote *in vitro* HSC expansion, and maintain immature HSC state, with other studies indicating that B-catenin

constitutive activation induces cell cycle entry, exhaustion and reduced multilineage reconstitution by HSCs.^{24,25} In addition to Wnt and Notch, a large number of other putative stem cell regulators were described in studies on these purified cells. Virtually all these studies were on highly purified stem cells. Therefore, although the differences in experimental approach are likely to blame, in part, for such discrepancies, as it turns out, most of these studies are likely not interpretable, because the cell under study, that is, the highly purified stem cell, is likely not representative of the true marrow stem cell.

THE CONTINUUM THEORY OF STEM CELL BIOLOGY

During this time our group had been developing an alternative model of hematopoiesis and stem cell biology. This was generally disregarded or simply ignored. This story began around 1996. We were investigating engraftment of marrow cells into non-treated mice and were especially interested in enhancing this engraftment as a basis for possible gene therapy approaches. Accordingly, we incubated murine marrow cells with the cytokines IL-6, IL-11, IL-3 and steel factor and then engrafted them into nonirradiated mice hoping to see increased engraftment. A sullen post-doc came back with the accusatory statement ‘Your experiment did not work, it was a bad experiment, there was no increase, they didn’t engraft’. My response was ‘Did the controls engraft?’ ‘Yes’ from Stefan ‘Well isn’t that interesting?’ The final answer was in these initial experiments^{26,27} although it has taken over 20 years to finally determine the true state of affairs. These experiments showed that with cytokine exposure, engraftment was lost and then regained—not a hierarchical setting. Mapping through cell cycle showed that engraftment was lost at the S/G2 interface and regained in the next cycle. These changes were reproduced in a total of seven experiments. There followed a large series of experiments using whole-murine marrow or highly purified lineage negative/rhodamine low/Hoechst low stem cells or lineage negative/Sca-1-positive stem cells with either IL-3, IL-6, IL-11 and steel factor or thrombopoietin, steel factor and FLT3 ligand cytokine combinations and following the cells through synchronized cell cycle. The synchrony was tight²⁸ but results of differentiation remained heterogeneous.²⁹ Various biologic readouts were determined. Cell cycle-related fluctuations in phenotype, in general reversible, were seen with differentiation into megakaryocytes and granulocytes³⁰ homing to marrow,³¹ progenitor fluctuations,³² and genetic conversions of marrow cells with lung-derived extracellular vesicles.³³ The phenotype of the marrow stem cells thus fluctuated reversibly with cell cycle transit. This suggested a continuum model of stem cell biology (Figure 3), in which the nature of the marrow stem cell was continually changing tied to cell cycle phase.³⁴

FOCUS ON CELL CYCLE CHARACTERISTICS OF MARROW LONG-TERM MULTILINEAGE REPOPULATING CELLS IN IRRADIATED MICE; THE GOLD STANDARD

Passegué *et al.*³⁵ reported an elegant study on the cell cycle status of the lineage negative/Sca-1+/c-Kit+/FLK-2 – hematopoietic stem cell; the long-term reconstituting hematopoietic stem cell (LT-HSC). They isolated this rare cell and then used the supravital dyes Pyronin (RNA) and Hoechst 33342 (DNA) to stain these cells and separate them into GO (very low

Pyronin 2NDNA), G1 (intermediate Pyronin 2NDNA) and S/G2/M (increasing DNA content). These cells were then competitively engrafted into lethally irradiated mice. They found that all engraftment capacity resided in the G0 population. This immediately concerned us, since it suggested that many of our findings might be *in vitro* artifacts. However, we noted that no one had evaluated the cycle status of repopulating cells in whole unseparated marrow. We also referred back to some of our own work on purified lineage negative/rhodamine low/Hoechst low stem cells, in which we had determined that well over 90% of stem cell capacity was lost or discarded with the separation.³⁶ The following experiments established the cycling status of stem cells in whole marrow. We harvested whole marrow from B6.SJL (CD45.1) mice and then carried out the Pyronin/Hoechst staining as described by Passegué *et al.*³⁵ or Hoechst staining alone and engrafted G0, G1 or S/G2/M or G0/G1 and S/G2/M B6.SJL whole-marrow cells competed with C57BL/6J marrow into lethally irradiated C57BL/6J hosts and determined blood and marrow chimerism out to 12 months. There was significant engraftment within the S/G2/M fraction, a sharp contrast to results seen with cell cycle sub-fractionated LT-HSC.³⁷ There was consistently 50% or more engraftment from the cycling population. This was an instantaneous look at cell cycle. To overcome the objections of impurity of FACS separated populations, we evaluated an alternative approach to cell cycle analysis; tritiated thymidine suicide.³⁷ In this approach, high specific activity tritiated thymidine is incubated with marrow cells for 30 min; this incorporates into newly synthesized DNA and will kill the incorporating cell. The path length of this beta emitter assures against innocent bystander effects, but in any case these were ruled out by separate mixing experiments. The uptake of radioactive tritium is blocked by incubation with a large excess of 'cold' thymidine. Cells incubated in hot radioactive thymidine and control cells incubated either in cold thymidine or media alone are then engrafted with competitor cells into lethally irradiated mice and the engraftment outcomes determined. The extent of decrease in engraftment with the tritiated thymidine-treated marrow cells indicates the percent of cells in S phase during the 30 min incubation. In these experiments there was a >70% kill (and sometimes a 100% kill) of engraftable cells in the cells treated with tritiated thymidine. These experiments strongly suggested that virtually all the long-term repopulating marrow stem cells were in cell cycle. We employed BrdU *in vivo* labeling to determine the flux of stem cell through cell cycle.³⁷ BrdU is incorporated into DNA during S phase and its presence can be determined by antibody staining. Thus, *in vivo* over time, if BrdU is constantly present, the number of antibody-labeled cells at any one time point will represent cells that have traversed through cell cycle and S phase during the period of study. Mice were given BrdU both intraperitoneally and orally over a 48 h period and lineage negative C-kit+Sca-1+ marrow cells were isolated at different time points. These are the cells, which had previously been established to be in G0, that is, noncycling. By 48 h, over 70% and in some experiments up to 86% of the LT-HSC were labeled with BrdU, thus they had transited cycle over a 48-h time frame. Separate experiments showed that BrdU was not inducing cells into cycle. These data rigorously established that virtually all long-term hematopoietic repopulating stem cells are actively transiting cell cycle. In this case they will continually be changing their characteristics including surface protein epitopes.

These data indicate that stem cells are on a continuum of change linked to cell cycle and thus purification by a specific set of surface markers is problematic. Our continuing studies show that the bulk of long-term repopulating marrow stem cells are in the lineage positive and the 'non stem cell' lineage negative marrow cells.³⁷

This opens wonderful possibilities for new investigators in the field as they attempt to define the true stem cell population. Thoughts of immortal strand come to mind.³⁸

Previous works on heterogeneity of stem cells at specific points in cell cycle are notable with regard to the continuum theory. Lineage negative/rhodamine low/Hoechst low marrow cells are separated on basis of quiescence,^{28,39} and three of four have repopulation potential and up to 90% form high-proliferative potential colonies in presence of six to seven growth factors. These cells maintain their synchrony out to six population doublings.²⁸ When cultured in TPO, FLT3L and steel factor up to 98% were in S phase at 40 h.²⁹ When these cells were isolated on a single-cell basis, as they transited cell cycle and then grown *in vitro* in the presence of different cytokine cocktails, they showed total heterogeneity as to colony morphology, colony cell numbers and differentiation phenotype. This adds further support to the continuum theory of stem cell biology. Other work has also indicated the presence of significant heterogeneity in different stem cell populations.^{40,41} An assumption in these results is that surface epitopes must be continually changing. There are listed a large number of specific surface markers purporting to define different hematopoietic stem/progenitor cells, in large part derived from the work of Drs Weissman, Morrison and colleagues.^{42,43}

The markers are listed in Table 1. Our continuum model indicates that these are markers which shift reversibly with cell cycle transit and do not define specific cell populations. Preliminary work in our laboratory has shown cycle-related fluctuation of C-kit and Sca-1 indicating a flux of markers with cycle transit. A related question is whether these epitopes consistently determine 'stemness'. Our results with the discarded populations³⁷ as well as the work of many others showing that conventional stem cell markers can fluctuate with developmental stage and activation state,⁴⁴⁻⁴⁶ indicate that these epitopes do not unfailingly determine 'stemness'.

A CONSIDERATION OF HIM OR NICHES

HIM is what Trentin⁴⁷ referred to as hematopoietic inductive microenvironment or HIM. Lord *et al.*⁴⁸ described the spatial distribution of CFU-s and CFU-c in normal mouse femur with localization toward the endosteum and Schofield⁴⁹ formulated a niche hypothesis for stem cells. Early studies on the microenvironment were in fact informative. It was shown that microenvironment was a critical factor influencing CFU-S differentiation. Here about half the colonies were on the surface of the spleen and 80% of these were erythroid or mixed.⁵⁰ Erythroid colonies were not found in the empty lymphoid follicles and granulocytic colonies were found along the trabeculae of the spleen or in sub capsular sheets. Megakaryocyte colonies were usually beneath the capsule.⁵¹ When colonies of engrafted marrow were examined in marrow tissue they were predominantly granulocytic.⁵¹⁻⁵⁴ When irradiated marrow stroma was implanted into spleens of lethally irradiated mice and a marrow cell suspension infused colonies that formed in the marrow

stroma were mostly granulocytic and in the spleen stroma erythroid.⁵² SL/SLd mutant mice were shown to have a normal stem cell compartment but defective stroma, which could be normalized by engraftment of stroma, but not marrow stem cells.⁵⁵ Further evidence of the role of stromal elements was evidenced by patterns of hematopoiesis seen when mice were exposed to granulopoietic stresses; the marrow becomes granulocytic and the spleen erythropoietic.^{56,57} Two *in vitro* culture systems appeared to monitor either a myeloid or lymphoid microenvironment.^{58,59} In murine Dexter cultures long term support of variety of stem/progenitor cells, granulocytes, macrophages and megakaryocytes was seen in the obligate presence of an adherent stromal layer.⁶⁰ The latter consists of 70–80% macrophages and the rest mostly adventitial reticular preadipocytic fibroblasts. Some have reported the presence of a minority population of endothelial cells. Altering culture conditions results in the Whitlock-Witte culture system, which predominantly supports B lymphopoiesis.⁶⁰ The stromal cells were predominantly adventitial reticular stromal cells. Thus there is little question that certain stromal cells in *in vitro* or *in vivo* locations preferentially support different types of marrow stem cell differentiation.

There has ensued a plethora of studies utilizing purified stem cells designed to define the niche or niches. We were early in this game reporting that lineage negative/rhodamine low and Hoechst low stem cells when infused into non-irradiated hosts localized near the bone surfaces.⁶¹ Many reports followed. There have been concerted efforts to characterize the marrow hematopoietic stem cell niche. Initial focus has been on osteoblast and endothelial cells. Lord proposed that endosteal cells provided the niche and Nilsson *et al.*⁶¹ show a localization of highly purified lineage negative/rhodamine low/Hoechst low stem cells to endosteal area in mice not exposed to cytotoxic treatments. It has been proposed that the osteoblast niche selectively supported quiescent cells,^{62,63} while the endothelial niche supported proliferating stem cells.^{64,65} Further work has indicated that key events may occur around sinusoidal perivascular niche that may involve endothelial cells, pericytes, CXCL abundant reticular cells, sinusoidal-megakaryocyte cells, neural crest cells, osteoblasts, osteoclasts, T cells, mesenchymal stem cells, macrophages, non-myelinating Schwann cells and preadipocytic fibroblasts.^{66–72} A large number of putative regulators of the niche have also been described, including CXCL12,⁷³ E-selectin,⁷⁴ VEGFR 2,⁷⁵ N-cadherin⁷⁶ and osteopontin⁷⁷ and membrane bound steel factor.⁷⁸ Although these entities have various effects on hematopoietic stem cells their true role as niche maintenance factors is uncertain, except for that of membrane bound SCF. Extensive studies in the SL/SLd mouse model have shown a critical role for this factor in microenvironmental support of hematopoiesis, although whether it functions in specific niches remains unclear. There have also been suggestions that hypoxia may be important in microenvironmental support function.⁷⁹ As noted above early studies clearly demonstrated that physical location in marrow had distinct effects on differentiation into granulocytes or erythroid cells. Macrophages were also implicated as supportive cells for erythropoiesis with clear descriptions of erythroid nests made up of macrophages and erythroid cells.⁸⁰ Dexter stromal cells (macrophages and adventitial reticular cells) clearly gave stem cell support and influenced differentiation into macrophages, granulocytes and megakaryocytes, while Whitlock–Witte stromal cells (predominantly adventitial reticular cells) supported B lymphopoiesis. Thus microenvironmental direction of hematopoietic differentiation clearly exists, but there are

several problems with the described definition of stem cell supportive niches, eventually appearing to define endothelial and osteoblastic niches with different characteristics. More recent work indicates that it is questionable whether these localizations were really valid, but this is probably not pertinent, since it would appear that these studies with purified stem cells, including our own, are suspect because the cells used may not be representative of the true marrow stem cell population. Other intriguing work in non irradiated normal mice has indicated that stromal 'spaces' are not limiting and that, in fact virtually all infused marrow cells, including stem cells home to marrow in normal mice; the final outcomes are determined simply by competition between infused and host stem cells.⁸¹ This is clearly an important area, but also needs reconsideration. There are likely multiple changing niches and niche/stem/progenitor interactions that continually vary on their own continuum (Figures 4 and 5).

OTHER MARROW STEM CELLS

We have above considered predominantly one class of primitive marrow renewing stem cell, the LT-HSC. Stem cells have been divided into totipotent, pluripotent, multipotent and monopotent stem cells, with totipotent giving rise to both embryo and placenta, pluripotent giving rise to all three germ layers of the embryo, multipotent giving rise to cells from one or two of the germ cell layers and monopotent tissue committed cells giving rise to cells of one lineage.⁸² This latter class of stem cells has been described for hematopoietic, epidermal, intestinal, neural, liver or skeletal muscle cells. Pluripotent cells include embryonic stem cells and induced pluripotent stem cells. A number of non-hematopoietic stem cells have been isolated from marrow; these include mesenchymal stem cells, multipotent adult progenitors cells,⁸³ marrow-isolated adult multilineage inducible cells⁸⁴ and unrestricted somatic stem cells found in cord blood.⁸⁵ We suggest that all these entities are on the stem cell continuum described above, although this has not been approached experimentally. A very different special marrow-derived stem cell has been described; the very small embryonic-like stem cell.⁸⁶ This is a very small cell, probably gated out in most FACS separations, and characterized as CD133+CXCR4+CD34+ SSEA-4+ in humans and Sca-1+ CXCR4+ SSEA-1+ Lin – and CD45 – in mice. These very primitive cells may be a precursor of all the others. Another consideration in stem cell biology is the 'immortal strand' hypothesis. It was proposed that as the stem cell divides it selectively retains those sister chromatids containing the older template DNA strands in the daughter destined to be the renewed stem cell.⁸⁷ Original observations were in embryonic fibroblasts and intestinal epithelium.^{88,89} This has been quite controversial⁹⁰ but recent evidence in support of this hypothesis was detected in neurosphere cultures, immortalized mouse tumor cells and muscle stem cells.^{91–93} This needs to be readdressed in hematopoietic stem cells.

Beautiful work from Sun *et al.*⁹⁴ promises to extend our understanding of *in vivo* hematopoiesis. These workers, utilizing transposon-tagging to clonally mark cells, concluded that 'a large number of long-lived progenitors rather than classically defined hematopoietic stem cells are the main drivers of steady-state hematopoiesis during most of adulthood'. Thus one can begin to envision two modes of hematopoiesis; transplant and steady state.

CONFOUNDING VARIABLES

There are many confounding variables in marrow stem cell research and here we limit ourselves to normal marrow biology. The disease states present an altogether different problem. Much of the basic work on stem cell biology has involved extracting marrow cells and then isolating them. Just the extraction procedure itself is a huge intervention, potentially precluding interpretable data. Marrow stem cells live *in vivo* surrounded in close proximity to a wide variety of hematopoietic and non-hematopoietic marrow cells. These influences are lost with marrow cell extraction. Also lost are the impact of position, pressure and pulsatile influences.

EXTRACELLULAR VESICLES

There is the growing realization that extracellular vesicles may represent a whole new aspect of regulatory influences on marrow stem cells. This is a rapidly expanding field of research with recent work highlighting the functional effects of vesicles on many different cell systems.^{95,96} Initially, vesicles from erythrocyte and platelets were studied. They were considered to be ‘cell junk’, but were tested for vaccine activity. Many types of vesicles were described, but a general consensus felt that there were two main types exosomes and microvesicles. Exosomes, 50–100 nm diameter particles, were felt to derive from multivesicular bodies, while larger microvesicles, 100–1000 nm, were felt to derive from budding of the cell membrane. Most recent focus has been on the capacity of cell-derived vesicles to alter the cell phenotype and fate of different target cell populations. Distinguishing these entities gave rise to heated arguments but finally it appeared more reasonable to use the general term extracellular vesicles and then define the originating and target cell in functional experiments and the specific context of the experiments. Studies of the effects of extracellular vesicles on hematopoiesis and hematopoietic stem cells are in their infancy. We have recently studied the capacity of exosomes, microvesicles and a mixture of the two to repair radiation damage to murine marrow. We found that mesenchymal stem cell-derived vesicles could reverse *in vitro* damage to murine marrow progenitor/stem cells and partially restore stem cell function *in vivo* after 500 cGy.⁹⁷ In these same studies, we demonstrated significant stimulatory effects of MSC-derived vesicles *in vitro* on normal murine hematopoiesis and progenitors. Furthermore, it has been shown that modulation of recipient cell phenotype by extracellular vesicles is influenced by the cell cycle phase of the target cell.³³ Vesicles essentially provide a movable microenvironment whose effects may be dependent on cell cycle phase of the recipient cells. Such effects expand tremendously the potential for stem cell modulation.

CIRCADIAN RHYTHMS

Another important variable, which is often not considered in hematopoietic stem cell studies, is circadian rhythm, even though there is a background literature showing impressive effects of circadian rhythm on both cell cycle progression and hematopoiesis. A circadian rhythm was demonstrated for progenitor/stem cells⁹⁸ and these rhythms showed a cell cycle component.⁹⁹ In addition, a number of studies have shown circadian influence on HSC

trafficking and proliferation,¹⁰⁰ again, highlighting the importance of continuing to explore the influence of circadian rhythm on HSC function.

These are difficult and challenging problems, but approachable with the right mix of perseverance and humility. As we have attempted to illustrate here, an over reliance on sophisticated technology does not necessarily move us forward. However the appropriate utilization of our evolving technologies holds great promise to what is still a wide open field.

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Figure 1.

This illustrates the progression of studies in marrow hematopoietic stem cell biology from the CFU-s assay of Till and McCulloch to progenitor assays first described by Bradley and Metcalf, and Pluznik and Sachs, to the antibody-based FACS separations for purified stem cells.

Figure 2.

This presents a classical hierarchical model of hematopoiesis. A primitive stem cell with extensive potential but dormant and undifferentiated then differentiates into a series of progenitors with increasing differentiation specification and proliferative potential to finally give rise to differentiated functional end cells.

Figure 3.

The continuum model is a model of continual, gradual, change over time, with the key aspect being reversibility. A major unanswered question is when does irreversibility occur and what triggers it? This figure shows aspect of change occurring over time related to cell cycle, but as we will deal with below, there is more than cell cycle alone to consider here.

Figure 4.

A niche model. Micro environmental influences on hematopoiesis are manifold. The cycling and ever-changing hematopoietic stem cell interfacing with vascular and endosteal niches and include the influences of cytokines, other hematopoietic cells and neural cells.

Figure 5.

An expanded view of the stem cell continuum. This indicates the continual change of cell phenotype with cell cycle progression interfaced with other influences including circadian rhythms, extracellular vesicles, environmental niches, cytokines, neural stimuli and adhesion events.

Table 1

Stem cell progenitor markers

LT-HSC: CD34 ⁻ , CD38 ⁻ , SCA-1 ⁺ , Thy1.1 ^{-/lo} , C-kit ⁺ , Lin negative ^a , CD135 ⁻ , CD150 ⁺
ST-HSC: CD34 ⁺ , CD38 ⁺ , SCA-1 ⁺ , Th1.1 ^{-/lo} , C-kit ⁺ , Lin negative, CD135-CD150+Mac-11o
Early MPP: CD34 ⁺ , SCA-1 ⁺ , Thy1.1 ⁻ , C-kit ⁺ , Lin ⁻ , CD135 ⁺ , CD150 ⁻ , Mac-11o, CD41o
Late MPP: CD34 ⁺ , SCA-1 ⁺ , Thy1.1 ⁻ , c-kit ⁺ , lin ⁻ , CD135 high, CD150 ⁻ , Mac-11o, CD41o

Abbreviations: early MPP, early multipotent progenitor; late MPP, late multipotent progenitor; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell.

^aLin negative (Lineage negative): depleted of 8220, Mac-1, Ter119, GR-1, and CD3, CD4 and CDS cells.