Evolution of normal and neoplastic tissue stem cells: progress after Robert Hooke

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The appearance of stem cells coincides with the transition from single-celled organisms to metazoans. Stem cells are capable of self-renewal as well as differentiation. Each tissue is maintained by self-renewing tissue-specific stem cells. The accumulation of mutations that lead to preleukaemia are in the blood-forming stem cell, while the transition to leukaemia stem cells occurs in the clone at a progenitor stage. All leukaemia and cancer cells escape being removed by scavenger macrophages by expressing the ‘don’t eat me’ signal CD47. Blocking antibodies to CD47 are therapeutics for all cancers, and are currently being tested in clinical trials in the US and UK.

1. Introduction

Stem cells are now defined as cells, at the single-cell level, that both self-renew and differentiate to produce all cells of a particular tissue or organ or organism [1]. In vertebrate metazoans, the zygote goes through a set of divisions to form the blastula stage, called the blastocyst, before it implants into the uterus (in mammals) or in the early stages of development within the egg in non-mammals. After the blastocyst stage, the formation of the three major ‘germlayers’ of mesoderm, ectoderm and endoderm, plus the early germline progenitor cells, develop following gastrulation, and development progresses in the embryo to the neurula stage, which coincides with the beginning of formation of the adult type organs and tissues; this constitutes the transition from embryo to fetus. However, these early embryonic progenitors (e.g. the early endoderm, mesoderm and ectoderm germ layer cells) do not persist past development and there are no remnants of these cell types that exist in adults; they do not self-renew and are not stem cells. Thus stem cells are an invention of fetal development and its processes of tissue formation and organogenesis. Developmental and stem cell biologists have created laboratory artefacts of pluripotent stem cells in culture from pluripotent inner cell mass populations within the blastocyst, and these are useful for biological and developmental studies [2], but the existence of pluripotent stem cells in vivo has been claimed [3–10], but not verified by careful research [11,12]. Metazoan stem cells are like many single-celled organisms such as dicyostelium [13] and sporulating bacteria insofar as they can self-renew and differentiate, and it is conceivable that genetic programs for self-renewal versus differentiation will be shared, at least in part, in all living organisms.

Given that natural selection operates on units of organization, not just single genes within the units, it is appropriate to consider if not only individual metazoan organisms, and groups of organisms (such as individuals in a colonial organism [14], but also stem cell lineages could be units in natural selection [1]. That is the topic of this treatise.

2. Stem cell competitions

Colonial organisms such as the urochordate Botryllus schlosseri montereyi undergo life histories wherein the usual chordate stages of zygote → blastula → gastrula → neurula → fetus → new-born are followed by migration of the ‘tadpole’ new-born to a subtidal surface, and thence metamorphosis to an invertebrate stage via programmed cell death (PCD) and programmed cell removal (PrCR) of the chordate features of notochord, neural tube, segmented musculature, tail, etc. (figure 1) [14,15]. Within the tunic surrounding the metamorphosed ‘oozoid’,
cells within the oozoid bud through the body wall to begin a two-week cycle of organogenesis and growth and form identical progeny called blastozooids; their development includes generation of a gastrointestinal system, gill slits, gonads and a two chambered heart with an intracorporeal blood vasculature connected to an extracorporeal vasculature in the tunic; and many diverse organs and blood cell types (figure 1). At the end of three weeks, the individuals die via PCD and PrCR, with linkage between death of the old and budding of the new [19]. None of the steps of organogenesis come from an embryonic set of events, and so this is akin to tissue and organ regeneration, although it occurs in new buds rather than repairing ageing resident organs [20]. The genome of the colony, therefore, outlives the lives of any of the individuals in the colony. In this way, as in other ways [20] the colony is a unit of natural selection, as is the tadpole that made it.

How does organogenesis occur in these animals, and what is the impact of their colonial organization on stem cell participation? The principal cells in the nascent bud are a mixture of germline and somatic stem cells [20,22]. Do these stem cells circulate or are they sessile? A peculiar feature of these colonial tunicates is that they are able to undergo allorecognition in the wild [23–25]. When two zooids or colonies abut on the same subtidal surface they extend blunt-ended ampullae of the blood vessels into the tunic of the other colony, and within a day this results in vascular anastomoses or rapid rejection. Fusion or rejection is controlled by a single, highly polymorphic locus (perhaps hundreds of alleles [24]) called Botryllus histocompatibility factor (BHF) [26]. Sharing a single allele at this locus allows anastomosis [24], usually between kin, and this results in the formation of natural chimeras [27]. In my laboratory, we have shown that these are somatic chimeras beginning with the next budding cycle [22], and more remarkably, itinerant germline stem cells not only can inhabit the testis or ovary of the anastomosed partner, but that heritable germline stem cell competitions usually result in all gonads of all individuals in the colony pair carrying only the germline of the ‘winner’ genotype [22,27]. This establishes a relatively common circumstance in the laboratory and in the wild that sibling oozoids give rise to anastomosed natural parasites wherein one animal’s body harbours a sibling’s germline [22,27]. The BHF-based immune rejection prevents both vascular anastomoses and chimera formations [26]. Therefore, the potential of germline stem cell competitions prevented by immune allorecognition effectively limits germline stem cell predation to kin, usually siblings, and provides a basis for maintaining diversity of this species [22,24]. Over 30 years ago, Buss...
[13] proposed that highly polymorphic histocompatibility genes derived from competitions for ‘germline’ niches, using as an example the formation of a ‘metazoan’ dictyostelium as single amoeba coalesce to form a fruiting body colony wherein the stalk is topped by a spore-containing tissue, the spores (or cheaters) being the ‘germline’ equivalent. For us this represents two important aspects of metazoan evolution—stem cell competitions and mechanisms such as alloimmunity to limit it to the extent that homogenization by successful germline predation does not occur [22,24].

It is within this framework that my lab re-approached the potential diversity of mouse germline [28] and somatic haematopoietic stem cells (HSCs) [29,30], and discovered stem cell competitions in both. In the germline of mice, four cells that form the germline [28] expand outside the embryo proper, then migrate to the incipient gonads of the genital ridge [31]. Although each developing seminiferous tubule contains a mixture of distinct immigrant germline stem cells, at a stage that just precedes the first meiotic division, the great majority of these cells undergo PCD (and presumably PrCR), and the non-culled stem cells give rise to groups of adjacent seminiferous tubules containing the clonal spermatogenic progeny from a single clonal survivor [28]. I proposed this could occur following endogenous transposon movements that would be natural mutagens, and that a process of competition leads to survivors versus those that are culled [28].

What about somatic stem cells? Prospective isolation of a somatic stem cell, the HSC, was first accomplished in 1988 [32], when it seemed clear that such cells should make up a homogeneous population of cells, each of which is capable of many cycles of self-renewal leading to production of all blood cell types for the life of the individual [32]. However, early studies on the differences between young mouse and old mouse HSCs revealed differences in outcome, analysing first their potency in transplanting lifelong haematopoiesis, their capability to home to haematopoietic sites on transplantation, and their burst size of production of more HSCs relative to the number of cells in the blood forming organ, bone marrow [30,33–35]. Cell intrinsic differences were found between old and young stem cells as a population [30]. Individual young mouse HSCs produced a balanced outcome of lymphoid committed progenitors (CLP) and myeloid committed progenitors (CMP) (figure 2), while old mouse [33] and old human [37] bone marrows were dominated by myeloid biased HSCs. This also proved to be cell intrinsic [30,37]. When a cell surface marker was found that distinguished myeloid biased from balanced HSCs, it became clear that what was changing between young and old was the frequency of predetermined HSCs that were mainly in the balanced or the myeloid biased category [30]. This could be explained by the maturation of young to old by epigenetic transitions, or by clonal competitions between HSC subsets predetermined to be one or the other type. Current evidence from ours and many other laboratories is for the clonal diversity hypothesis [30,38–41], but crucial in situ lineage tracing of a single HSC from young to old is not yet feasible.

3. Stem cell competitions in the organismal evolution of cancer stem cells

Cancers are made up of cells derived from tissues and organs which contain normal stem cells, progenitors and lineage committed cells, e.g. the blood system (figure 2). Having nearly the complete roadmap of quantal transitions from HSC through multipotent progenitors lacking self-renewal (short term-HSC and multipotent progenitors (MPP) [42]) in mice, to CMP in mice [43] and humans [44], and CLP [45], and downstream from them ever more committed progenitors (e.g. granulocyte–macrophage progenitor (GMP) and megakaryocyte/erythroid progenitor (MEP) [43,46]), allowed me and my colleagues to look at mouse and human myelogenous leukaemias to attempt to understand where in the lineage tree the leukaemias emerged. The starting point was producing a strain of mice wherein PCD via two pathways was blocked in haematopoietic cells [47]. About 15% of the mice developed acute myelogenous leukaemia (AML), and their leukaemias could be transferred to other mice only with GMP cells. My colleagues and I could infer that the progression to leukaemia required at least five to seven rare events, either genetic or epigenetic. Most of these events could not confer self-renewal, and so must have occurred in self-renewing cells to persist sufficiently to form a clone that was leukaemic [48]. In 2000, we analysed AML patients’ bone marrows taken from a repository at Hiroshima Hospital. The patients analysed were known to have a chromosomal translocation (8:21) to form a fusion gene, aml 1:eto, which occurs in many patients with AML. Because we could isolate HSCs from MPP, from CMP and from GMP, we found the cells in the leukaemia that would make leukaemic cells in vitro [49] and would transfer the leukaemias to immunodeficient mice [50]. These leukaemia stem cells (LSC) were at the MPP stage, and neither HSCs nor myeloid lineage large (blast) cells from them transferred the leukaemia [50,51]. The particular breakpoint of the translocation produced a clonal tracer for that leukaemia, and in every case, the MPP–LSC translocation was found in otherwise normal HSCs in the same patient, and at a frequency that could only be explained by expansion from a single cell to hundreds of thousands of ‘preleukaemia’ cells [49]. This clearly implied that normal HSCs harboured the initiating event and were expanding, but that more events would be required before the preleukaemic and marked HSCs could give rise to the leukaemic MPP–LSC [48]. What we did not realize is that the preleukaemic stem cells were expanding at the expense of normal HSCs, not adding to normal HSCs.

This latter point became clear when my laboratory studied several cases of blood diseases, which can progress to AML. Chronic myelogenous leukaemia (CML) is the precursor to blast crisis acute leukaemia, which is usually in the myeloid lineage (MBC). Fialkow [52] had shown that in the chronic phase, the myeloproliferative clone was not only limited to the increased frequency granulocyte lineage, but also dominated progeny of erythroid, monocytic, megakaryocytic and B lymphoid cells. Nowell et al. [53] and Rowley et al. [54] showed later that CML resulted from a prototypic chromosomal translocation, 9:22, to produce a bcr:abl fusion oncogene. My colleagues and I showed that the translocation was in HSCs, and that the bcr:abl HSC clone expanded at the expense of normal HSCs, without measurably increasing the total frequency of HSC phenotype cells [55,56]. The disease appeared to be a strong myeloid bias of the bcr:abl HSCs rather than an uncontrolled self-renewal of these HSCs [51]. The transition of CML to MBC CML led to dramatically expanded GMP stage cells which could transfer the tumour in vivo and give leukaemic colonies in vitro [51]. The GMP LSC had activated the wnt/β-catenin pathway....
Figure 2. Haematopoietic lineage tree. Schematic of cells at successive differentiation stages with distinctive immunophenotypes (marker profiles) in mouse and human. The haematopoietic stem cell (HSC) is capable of self-renewal and long-term reconstitution of the entire haematopoietic system; the multipotent progenitors are capable of minimal or no self-renewal, resulting in transient reconstitution of multiple lineages. CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; MkP, megakaryocyte progenitor; EP, erythroid progenitor; GP, granulocyte progenitor; MacP, macrophage progenitor; Pro-DC, dendritic cell progenitor; Pro-B, B lymphocyte progenitor; Pro-T, T lymphocyte progenitor; Pro-NK, natural killer cell progenitor. Adapted from [36]. (Online version in colour.)
function. These migrations can occur within an organ, but in the case of blood and blood forming cells, migrations are required between organs via the blood vasculature. My laboratory and others, for example, have defined several homing receptors, chemokine receptors, cell surface adhesins and endothelial addressins to allow various naive, activated and memory T and B lymphocytes to give site-appropriate and whole body protection against various microbial pathogens, toxins and even emergent malignant cells [65–67]. Malignant lymphomas can use these homing receptors for site-specific metastases [68–70], and tumours such as mouse melanomas must lose homophilic adhesive molecules to home to distant bone marrow and haematopoietic sites via heterophilic binding with sinusoidal endothelial cell VCAM-1 via heterophilic adhesion [72,73], and is used by migrating mouse bone marrow HSCs and early progenitors to home to distant bone marrow and haematopoietic sites via heterophilic binding with sinusoidal endothelial cell VCAM-1 [74–76]. Presumably, this circulation capacity of HSCs and progenitors allows niches that lost their resident haematopoietic cells via cell death or migration. AML LSC probably use homing receptors such as α4β1 to metastasize first to other haematopoietic sites before their malignant expansion overwhelms the capacity of marrow, liver and spleen to keep them. (Botryllus stem cells also must use site-appropriate homing receptors, adhesive proteins and chemokine receptors for their migration from one individual to a bud in multi-individual colonies.)

Within tissues, especially surrounding vascular traffic zones such as the bone marrow sinuses, are macrophages, themselves probably highly diverse, which detect and phagocytose not only microbial pathogens but also damaged, dying and potentially premalignant and malignant cells. Macrophages are first seeded from yolk sac haematopoiesis in the embryo to fetal transition [77,78], and that subset, like early subsets of T cells [79], innate lymphocytic subsets [80,81], and antigen-presenting cells such as Langerhans cells [81,82] reside next to epithelial organ cells and proliferate pari passu with these developing tissues. Other macrophages in the post fetal period are derived continuously from HSCs and myeloid progenitor derived blood monocytes [83]. Successful migration of cells past macrophages occurs if the cells do not express ‘eat me’ markers on their surface, or if they express dominant ‘don’t eat me’ markers, to counteract the ‘eat me’ signals [84]. For example, granulocytes in mice have a 1–2 day lifespan, and this is controlled by both PCD and PrCR [85]. New-born granulocytes express no ‘eat me’ signals, but 12–24 h later, normally just after the initiation of PCD but before the cells burst, they express ‘eat me’ signals that signal macrophages to phagocytose them [85]. Even granulocytes deprived of PCD by enforced expression of the anti-PCD molecule bc2 express the ‘eat me’ signal at the time their dying counterparts without bc2 express it, and homeostasis is assured by PrCR by macrophages with receptors for the ‘eat me’ signals [85]. I proposed that events leading to cell death trigger the independent pathways of PCD and PrCR [48,84,85]. These events could be intrinsic clocks, like in neutrophils, DNA damage, mutations, unfolded protein response, inflammation, intracellular infection, over-proliferation and at least some mutations in the progression to cancer [84]. When my colleagues and I compared the gene expression profiles of LSC and their normal haematopoietic counterparts in mouse [47,86] and human [50], one of the highly overexpressed genes was CD47. Oldenborg et al. [87] had shown in 2000 that CD47 was the predominant determinant of mouse red blood cell intravascular lifespan. They further showed that it was a ‘don’t eat me’ signal for macrophages, acting as a ligand for macrophage SIRPα, a receptor tyrosine phosphatase acting via its cytoplasmic ITIM motif to activate SHP1 to block phagocytosis [87]. Towards the end of the red cell life, the balance of ‘eat me’ to CD47 signals allows phagocytosis in the marrow, splenic and liver sinuses by macrophages (for review, see Chao et al. [84]). We showed that CD47 was expressed at low levels in quiescent bone

Figure 3. Single-cell analysis determines the sequence of mutations acquired in preleukaemic HSCs in acute myeloid leukaemia. Each row depicts the proposed clonal evolution of leukaemia in each of three patients. Adapted from [59]. (Online version in colour.)
isolated human fetal brain CNS stem cells transplant into immune-deficient mouse brains successfully, become restricted to the CNS zones of the subventricular zones of the lateral ventricle and the dentate gyrus (DG) of the hippocampus [61], where for the life of the host show site-appropriate neurogenesis, self-renewal, migration and differentiation to progeny cells at distant sites [61,110]. This continued cycle of proliferation, self-renewal, migration and differentiation probably occurs at lower rates than haematopoiesis or intestinal cell turnover, but is similarly susceptible to mutations and epigenetic change. It is clear from the discussion of progression to leukemic malignancy above that the same will apply to neurogenesis, e.g. for the development of glioblastoma, and that should be testable when downstream oligolineage progenitors in neural lineages get to the point of having prospectively isolated intermediates from human or mouse neuronal stem cells. A curiosity is the many cases of neurodegenerative disease that derive from germline-inherited mutations, but do not manifest as disease until far later. Perhaps other functions of the brain that have both structural and even ‘mind’ functions should similarly be open to mutations and/or heritable epigenetic changes that alter functions such as migration, with consequences that result in disease. Kemperman, Gage and co-workers [111,112] opened the possibility that hippocampal DG neurogenesis plays a role in the mind functions of types of short-term memory. Dendritic and axonal connections that result from DG neurogenesis probably mediate the rapid acquisition of short-term memory, and insofar as that type of memory gets transferred via circuitry to cortical foci, feedback and feed-forward events could be involved.

Two examples of diseases of the mind could be relevant: in senile dementias such as Alzheimer’s disease (AD), the first signs are loss of short-term memory [113]. AD phenotype mice develop amyloid plaques [114], thought by many to be the toxic cause of loss of hippocampal neurogenesis [115]. However, transplantation of normal human fetal CNS stem cell derived neurephore cells into the DG region of AD immune-deficient mice shows healthy neurogenesis in apoposition to plaques, implying that cell intrinsic toxicity rather than β-amyloid induced toxicity is important (figure 4; N Uchida, A Tsukamoto-Weissman, IL Weissman, G Carlson 2003 unpublished data). This could result from germline heritable mutations as initiating events, only to become pathogenic later in life with the acquisition of dominant CNS SC mutations or epigenetic changes. The second example is schizophrenia. My colleagues and I have written elsewhere [116] that the initial sign of this disease is usually reported as auditory hallucinations, or ‘voices in the brain’. PET scans of patients with this disease show focal prefrontal or temporal active foci of increased glucose uptake in concert with the voices. Spontaneous discharges from focal areas of brain can result in epileptic seizures when in the motor cortex. We proposed that this could begin with hippocampal neurogenesis, and result from unregulated circuitry, e.g. in areas not surrounded by adult Gaba-ergic inhibitory neurons. The manifestations of voices, later attributed to persona, could be the result of disease progression. The finding that a group of patients from a Scottish family had heritable disease linked to the gene disc-1 allowed Ming, Song et al. [117] to test the role of disc-1 in hippocampal neurogenesis. New-born DG neurons with disc-1 knockdown mis-migrated to regions in and around the DG lacking surrounding neurons, including Gaba-ergic neurons. Because these CNS stem cell clones are derived over a lifetime.

marrow resident HSCs, but that it was upregulated upon natural or induced (e.g. by Cytoxan and G-CSF [88–90]) mobilization of HSCs [91]. HSCs and progenitors achieve high levels of CD47 just prior to entry to the bloodstream, and these levels are maintained as they migrate to splenic, liver and marrow sinusoids, cross the perivascular fields of macrophages, only to return to low levels when the itinerant cells reach bone marrow niches [91]. Both human mobilized HSCs and umbilical cord blood HSCs are CD47hi [91], but not as high as AML cells, including but not limited to LSC [91,92]. In fact, all human cancers tested upregulate CD47 [92–98]. Antibodies or engineered proteins that block CD47-SIRPα interactions lead to cancer/leukemia phagocytosis by macrophages in vitro, and alone or in combination with other anti-tumour monoclonal antibodies of the IgG1 isotype that binds to the phagocytic high affinity FcR on macrophages lead to human tumour xenographs in immune deficient NSG mice to be cleared, or slowed in growth [92–97,99], and eliminates human tumour metastases [96,100]. A blocking antibody to CD47, Hu5F9-G4, has been humanized [101] and is currently in early phase clinical trials for all solid tumours at Stanford, and AML patients to begin autumn 2015 in the UK.

If CD47 is the ‘don’t eat me’ signal, is there an ‘eat me’ signal that counteracts? In human AML, non-Hodgkin lymphoma and ovanar cancers, the dominant ‘eat me’ signal is cell-surface calreticulin [102]. Calreticulin is an endoplasmic reticulum (ER) chaperone for glycoproteins undergoing maturation of glycans in the ER before transfer to the Golgi [84,103]. It appears on the cell surface of damaged cells, cells dying of ‘immunogenic cell death’ via certain anti-cancer agents [104,105]. How does calreticulin get to the cell surface? In damaged cells [105], at least some tumour cells [102] and in activated macrophages [103,106] the innate immune system toll-like receptors 3, 4 and 7 when stimulated activate Bruton’s tyrosine kinase, which in turn directly or indirectly phosphorylates calreticulin, leading to cleavage of the protein, leaving in the ER the KDEL retention signal, and the truncated calreticulin is secreted [103]. The secreted calreticulin has binding sites for the phagocytic macrophage receptor CD91/LRP1, and for targets on cancer cells; calreticulin is the link between macrophage and cancer cell that leads to tumour phagocytosis [103].

An exemplar of stem cell competition, blood disease and cancer is the case of human MDS [55]. Aged patients with MDS come to the clinic with anaemia and/or thrombocytopenia and/or neutropenia, and their bone marrow looks dysplastic. My laboratory has shown that the blood abnormality in human MDS appears to be because of two factors: clonal HSCs with an initiating event out compete normal hemopoiesis, and clones dominate marrow niches [55]. These competitive MDS HSCs produce oligolineage progenitors such as GMP, and these CD34+38 progenitors become calreticulin+ (CD47lo) and are phagocytosed in a calreticulin-dependent manner [55]. Thus, MDS HSC clones dominate the marrow niches, but cannot efficiently make blood. These MDS clones often progress to AML, and the AML are CD47+ [55].

Can this scenario of stem cell competition, clonal acquisition of mutations or epigenetic events, and enabling or disabling the PCD/PrCR axis occur in other tissue stem cells, and play a role in disease?

Mouse and human neurogenesis occurs throughout postnatal life by subsets of CNS stem cells [107–109]. Prospectively
from self-renewing cells, it is conceivable that genetic or somatic variations in a dominant CNS SC clone could eventually connect with a circuitry transferred to the part of the brain responsible for generating voices, or stories. This kind of hypothesis leads to the potential for basic and clinical experiments to connect the biology of neurogenesis and the properties described above of stem cells to the development of these kinds of diseases.

In conclusion, with the appearance of metazoans and the tissue and organ segregation of functions, a class of cells, stem cells, retain the property of self-renewal to maintain homeostasis. The stem cell functions of self-renewal, migration and regulated differentiation to non-self-renewing progeny have resulted in diversity of tissue stem cells within an individual in order for clonal selection to allow maximal fitness of tissue homeostasis throughout the changing internal events in long-lived organisms. Clonal selection also operates in the stem cell competitions between tissue stem cells within an organ, and on germline stem cells, which can be units of natural selection between individuals in a species. Selection can be based on the variations also occurring with mutations or unscheduled epigenetic alterations, and the progeny that are more competitive are on a path to cancer and/or tissue or organ degenerative diseases, in part controlled by the events of PCD and PrCR. Studying the biology, developmental biology and stem cell biology of stem cell systems may seem like opening the pages of a book already read, but can also offer clues for disease pathogenesis and functions of the brain.

**Competing interests.** I declare I have no competing interests.

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**Endnote**

1There are colonies of pelagic tunicates which have taken shape like the finger of a glove. Each member of the colony is an individual animal, but the colony is another individual animal, not like the sum of its individuals... So a man of individualistic reason, if he must ask, 'Which is the animal?' must abandon his particular kind of reason and say, 'Why, it's two animals and they aren't alike any more than the cells of my body are like me. I am much more than the sum of my cells, and, for all I know, they are much more than the division of me' [21].

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