IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Review Article
December 2020 Vol.:20, Issue:1

Zebrafish Specification in Stem Cell Research and Detail Study

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Suvarna Bhadane*, Janhavi Lavande, Maria Khan, Nisrullah Khan, Smita Takarkhede

Ideal College of Pharmacy and Research, Bhal, Kalyan – 421306, Dist – Thane, Maharashtra, India

Submitted: 12 November 2020
Revised: 02 December 2020
Accepted: 22 December 2020

Keywords: Zebrafish, Haematopoiesis, Stem Cell Therapy, Pluripotency, Cytogenesis

ABSTRACT

In recent years stem cell therapy treatment has gotten a very promising and progressed logical examination theme. The improvement of treatment techniques has evoked extraordinary desires. Zebrafish (Danio rerio) has gotten an astounding model to examine the improvement of hematopoietic undifferentiated organisms (HSCs). All vertebrates including zebrafish have crude and authoritative influxes of hematopoiesis, yet self-restoring pluripotent HSCs areas it was created by the authoritative wave. Hematopoietic stem cells (HSCs) maintain the entire blood system throughout life and are utilized in therapeutic approaches for blood diseases. Prospective isolation of highly purified HSCs is crucial to understand the molecular mechanisms underlying the regulation of HSCs. Methodologies like Cytology, gamma radiation, genomic DNA extraction PCR analysis, adult zebrafish characterization, Isolation of cd41-gfp^{+} cells by flow cytometry, Cell collection, Thin-section electron microscopy are used for detailed study and enhance overview on stem cells of zebrafish. This summary is summed up by difficulties that undifferentiated stem cell therapy must use to be acknowledged around the world.

1. INTRODUCTION:

1.1 What Is a Stem Cell?

Stem cells are defined functionally as cells that can self-renew as well as the ability to generate differentiated cells. More explicitly, stem cells can generate daughter cells identical to their mother (self-renewal) as well as produce progeny with more restricted potential (differentiated cells). This simple and broad definition may be satisfactory for embryonic or fetal stem cells that do not perdure for the lifetime of an organism. But this definition breaks down in trying to discriminate between transient adult progenitor cells that have a reduced capacity for self-renewal and adult stem cells. (1) It is therefore important when describing adult stem cells to further restrict this definition to cells that self-renew throughout the life span of the animal. Another parameter that should be considered is potency: Does the stem cell generate multiple differentiated cell types (multipotent), or is it only capable of producing one type of differentiated cell (unipotent)? Thus, a more complete description of a stem cell includes consideration of replication capacity, clonality, and potency. (2)

A] Self Renewal:

Stem cell literature is replete with terms such as “immortal,” “unlimited,” “continuous,” and “capable of extensive proliferation,” all used to describe the cell’s replicative capacity. These rather extreme and vague terms are not very helpful, as it can be noted that experiments designed to test the “immortality” of a stem cell would by necessity outlast authors and readers alike. Most somatic cells cultured in vitro display a finite number of (less than 80) population doublings before replicative arrest or senescence, and this can be contrasted with the seemingly unlimited proliferative capacity of stem cells in culture. Therefore, it is reasonable to say that a cell that can undergo more than twice this number of population doublings (160) without oncogenic transformation can be termed “capable of extensive proliferation.” In a few cases, these criteria have been met, most notably with embryonic stem (ES) cells derived from either humans or mice as well as with adult neural stem cells (NSCs). An incomplete understanding of the factors required for self-renewal *ex vivo* for many adult stem cells precludes establishing similar proliferative limits *in vitro*. In some cases, a rigorous assessment of the capacity for self-renewal of certain adult stem cells can be obtained by single-cell or serial transfer into acceptable hosts, an excellent example of which is adult hematopoietic stem cells (HSCs). Adult stem cells are probably still best defined *in vivo*, where they must display sufficient proliferative capacity to last the lifetime of the

animal. Terms such as “immortal” and “unlimited” are probably best used sparingly if at all. (2)

B] Clonality:

A second parameter, perhaps the most important, is the idea that stem cells are clonogenic entities: single cells with the capacity to create more stem cells. This issue has been exhaustively dealt with elsewhere and is essential for any definitive characterization of self-renewal, potential, and lineage. Method for tracing the lineage of stem cells are described in subsequent chapters. Although the clonal “gold standard” is well understood, there remain several confusing practical issues. For instance, what constitutes a cell line? The lowest standard would include any population of cells that can be grown in culture, frozen, thawed, and subsequently repassage *in vitro*. A higher standard would be a clonal or homogenous population of cells with these characteristics, but it must be recognized that cellular preparations that do not derive from a single cell may be a mixed population containing stem cells and a separate population of “supportive” cells required for the propagation of the purported stem cells. Hence, any reference to a stem cell line should be made with an explanation of their derivation. For example, it can be misleading to report on stem cells or “stem cell lines” from tissue if they are cellular preparations containing a mixed population, possibly contaminated by stem cells from another tissue. (2)

C] Potency:

The issue of potency may be the most contentious part of a widely accepted definition for stem cells. A multipotent stem cell sits atop a lineage hierarchy and can generate multiple types of differentiated cells, the latter being cells with distinct morphologies and gene expression patterns. At the same time, many would argue that a self-renewing cell that can only produce one type of differentiated descendant is nonetheless a stem cell. A case can be made, for clarity, that a unipotent cell is probably best described as a progenitor. Progenitors are typically the descendants of stem cells, only they are more constrained in their differentiation potential or capacity for self-renewal and are often more limited in both senses. (2)

In conclusion, a working definition of a stem cell is a clonal, self-renewing entity that is multipotent and thus can generate several differentiated cell types. Admittedly, this definition

is not applicable in all instances and is best used as a guide to help describe cellular attributes. (2)

1.2 Where Does Stem Cell Come From?

The origin or lineage of stem cells is well understood for ES cells; their origin in adults is less clear and in some cases controversial. It may be significant that ES cells originate before germ layer commitment, raising the intriguing possibility that this may be a mechanism for the development of multipotent stem cells, including some adult stem cells. (3) The paucity of information on the developmental origins of adult stems cells leaves open the possibility that they too escape lineage restriction in the early embryo and subsequently colonize specialized niches, which function to both maintain their potency as well as restrict their lineage potential. (4) Alternatively, the more widely believed, though still unsubstantiated, model for the origin of adult stem cells assumes that they are derived after somatic lineage specification, whereupon multipotent stem cells–progenitors arise and colonize their respective cellular niches. In this section, I briefly summarize the origin of stem cells from the early embryo and explain what is known about the ontogeny of adult stem cells focusing attention on HSCs and NSCs.(2)

1.3 How Are Stem Cells Identified, Isolated, and Characterized?

How stem cells are identified, isolated, and characterized are the key methodological questions in stem cell biology, so much so that subsequent chapters are devoted to addressing these problems in detail. (5) Here, I briefly outline standards and criteria that may be employed when approaching the challenge of identifying, isolating, and characterizing a stem cell. (2)

A] Embryonic Stem Cells:

The basic characteristics of an ES cell include self-renewal, multilineage differentiation in vitro and in vivo, clonogenicity, a normal karyotype, extensive proliferation in vitro under well-defined culture conditions, and the ability to be frozen and thawed. In animal species, in vivo differentiation can be assessed rigorously by the ability of ES cells to contribute to all somatic lineages and produce germ line chimerism. These criteria are not appropriate for human ES cells; consequently, these cells must generate embryoid bodies and teratomas containing differentiated cells of all three germ layers. (2)

B] Adult Stem Cells:

The basic characteristics of an adult stem cell are a single cell (clonal) that self-renews and generates differentiated cells. The most rigorous assessment of these characteristics is to prospectively purify a population of cells (usually by cell surface markers), transplant a single cell into an acceptable host without any intervening *in vitro* culture, and observe self-renewal and tissue, organ, or lineage reconstitution. Admittedly, this type of *in vivo* reconstitution assay is not well defined for many types of adult stem cells. Thus, it is important to arrive at an accurate functional definition for cells whose developmental potential is assessed in vitro only. Above all, clonal assays should be the standard by which fetal and adult stem cells are evaluated because this assay removes doubts about contamination with other cell types. (2)

2. Molecular Bases of Pluripotency

Early mammalian embryogenesis is characterized by a gradual restriction in the developmental potential of the cells that constitute the embryo. The zygote and single blastomeres from a 2–4 cell morula are totipotent. As the embryo continues to cleave, the blastomeres lose the potential to differentiate into all lineages. The blastocyst is the first landmark of the embryo in which lineage restriction is apparent. At this stage, the outer cells of the embryo compact into the trophectoderm, from which the placenta will derive. The inner cells, termed inner cell mass (ICM), will give rise to all cell lineages of the embryo proper but cannot contribute to the trophoblast and thus are considered pluripotent. Once isolated and cultured *in vitro* under permissive conditions, the ICM may be propagated as an embryonic stem (ES) cell line. These cells are the in vitro substitutes for embryos in the search for the genetic switches and molecular mechanisms required to ensure pluripotency. Mutations affecting the ability of ES cells to self-renew or differentiate and contribute to distinct cell lineages provide the necessary tools to unravel the molecular network underlying pluripotency. (6) In an attempt to define the molecular basis underlying pluripotency, we will focus on four main areas: 1. Influence of extracellular factors on pluripotency and self-renewal (ligands, cytokines, receptors). 2. Signaling pathways activated in pluripotent cells. 3. Gene transcriptional programs operating in pluripotent cells (mainly Oct4 and its target genes). 4. Gene function during development of the early mammalian embryo. (2)

2.1 The Stem Cell Environment: Cytokines and Pluripotency

The stem cell niche: In many cases, the culture of stem cells has been complicated by the lack of knowledge of their cellular environment or niche. Hematopoietic stem cells, for example, were identified more than 40 years ago, in 1961, but conditions still have to be established to ensure their maintenance in vitro. Besides, they might require complicated three-dimensional arrangements of specific stromal cells to proliferate. The ES cell niche: In contrast, ES cells are relatively easy to be technically handled. The establishment of the first murine embryonic stem lines was achieved by culturing early embryos on a layer of mitotically inactivated Zebrafish fibroblasts. Without such a "feeder" layer, cultured embryonic cells would not remain pluripotent, suggesting that fibroblasts either promote self-renewal or suppress differentiation or both. LIF and other cytokines: Fibroblasts maintain pluripotency of ES cells by secreting a factor, which was identified as leukemia inhibiting factor (LIF), also known as differentiation inhibiting activity (DIA). LIF is a member of the interleukin 6 family of cytokines, including IL-6, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and corticotrophin-1 (CT-1). The IL-6 family cytokines are structurally and functionally related. They act on a variety of cells (i.e., they are pleiotropic) and can mediate proliferation or differentiation or both according to the target cell types. For example, LIF, OSM, and IL-6 are competent to induce myeloma growth and to inhibit macrophage differentiation of M1 cells. The redundancy in biological function is due mainly to the structural similarity of the receptor complex involved in signal transduction redundant cytokine functions and development: The absence of a developmental phenotype in IL-6-, LIF-, and CTNF-null mice confirms that IL-6-related cytokines are indeed functionally redundant. However, LIF mutant females are infertile, as the interaction between embryo and a uterine wall (decidual reaction) strictly depends on a surge of estrogen on the fourth day of gestation, which coincides also with a surge in LIF production by the uterus. As a consequence, LIF -/- females fail to support embryo implantation, LIF -/- embryos can implant and develop to term in a normal uterus. ES self-renewal dependence on cytokine supply can be attributed to several factors. LIF may influence the rate of cell proliferation or cell cycle progression and act on the stem cell phenotype by activating a signaling cascade that operates on the up-or down-regulation of genes that are exclusively expressed in "pluripotent" or differentiated cells, respectively. Analysis of the ES expression profile does not favor either one of the two above-mentioned explanations, since LIF withdrawal triggers the disappearance and appearance of pluripotent and differentiated markers, respectively, within 24 h. (7) A complete and systematic analysis

of the target genes lying downstream of the LIF-induced signaling pathways is necessary to clarify the cytokine modus operandi on the ES cell phenotype. (2)

2.2 A Genetic Model for Molecular Control of Pluripotency (8)

Oct4 cannot be considered to be a master gene for pluripotency since it cannot prevent the differentiation of ES cells upon LIF withdrawal. This finding implies that Oct4 and LIF probably activate two different pathways of gene activation, with the second relying on STAT3. The fact that both Oct4 upregulation and LIF withdrawal lead to the same pattern of ES cell differentiation can be explained by assuming a cross-talk between the two pathways. Hitoshi Niwa has suggested a model of the known molecular mechanisms controlling ES cell phenotype, which is outlined below:

Oct4 target genes can be subdivided into three categories:

A. Those activated by Oct4 and Sox2 (FGF4, UTF).

B. Those repressed by Oct4 (hCG a, b).

C. Those activated by Oct4, but also repressed by a squelching mechanism when Oct4 is overexpressed (Rex1). This last group of genes is considered to comprise the cross-talk junction between the Oct4 and the LIF-STAT3 signaling pathway, as they should be co-activated by Oct4 and unidentified X factors which lie downstream the STAT3 activation cascade. (2)

STAT3 is hypothesized to activate ES "state" genes or to suppress endodermal/mesodermal genes or both. As described above, activated STAT3 and subtle changes in the Oct4 expression are compatible with the maintenance of a pluripotent ES cell fate. To achieve a pluripotent status, group A and C genes need to be activated and group B genes need to be silenced by Oct4. Group B genes are activated only when Oct4 expression falls below the 50% threshold and are specific for the trophectoderm lineages. A 50% increase in the expression of Oct4, or LIF withdrawal, induces down-regulation of group C genes, either by squelching of the X-co-activators, lying downstream the STAT3 pathway, or by down-regulation of STAT3-induced transcriptional program, leading to differentiation into mesoderm/endoderm. The validity of this model is supported by the existence of the E1A-like activities postulated to exist in ES cells, which may likely represent the mentioned co-activators X. Nanog would be incorporated within this model as an essential determinant of

pluripotency, which induces ES state gene activation and/or repression of visceral/parietal state genes. Identification of Oct4 co-activators and Nanog/STAT3 target genes is required to enrich and validate the described transcriptional network. In vivo Oct4 is essential for the specification of a pluripotent ICM. Nanog and Oct4 would be critical for the maintenance of the epiblast during the formation of the hypoblast. Postimplantation maintenance of the epiblast would be dependent on Oct4, Sox2, and FoxD3. Plenty of questions regarding the mechanisms of pluripotency remain unanswered: how are the Oct4, STAT3, Nanog transcriptional pathways regulated, and how do they crosstalk? Are any other genes regulating pluripotency? And, in particular, is there anyone master gene controlling pluripotency? The Discovery of such a master gene(s) would constitute the panacea of modern human regenerative medicine, as it would obviate the need for human cloning with all its genetic implications and ethical considerations. However, it appears that pluripotency is most likely achieved through the combination of properly sequenced processes that control chromatin accessibility, chromatin modifications, activation, and repression of specific genes. This is further complicated by potential sensitivity to subtle changes in gene expression levels. (2)

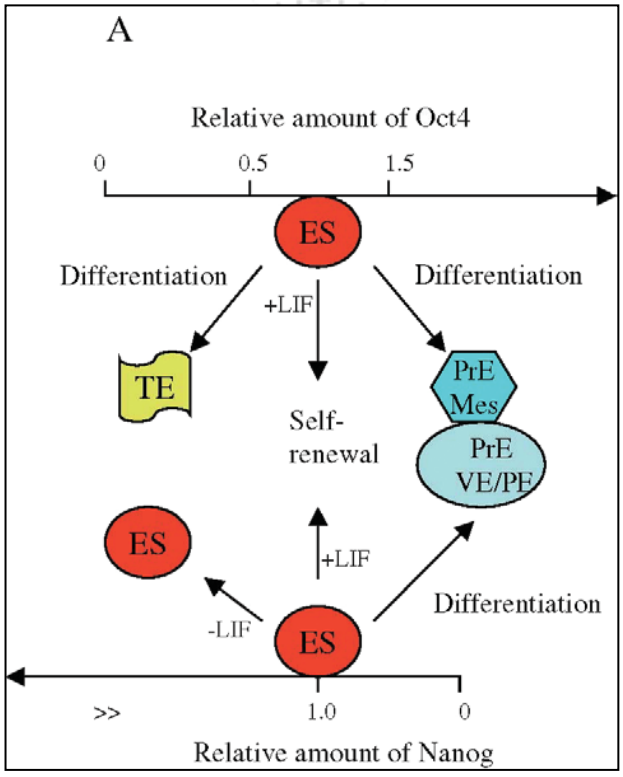

Figure. No 1 A. Model relative to the integrated roles played by Oct4, Nanog, and LIF on ES cells fate specification according to the variable concentration of Oct4 and Nanog.

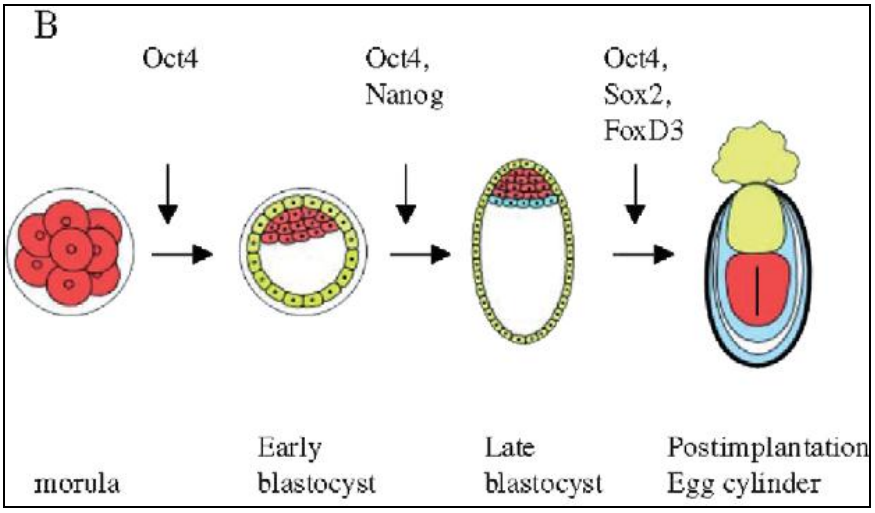

Figure 1 B. Model relative to the role played by Oct4, Nanog, Sox2, and FoxD3 during early Zebrafish development. (2)

2.3 Mechanisms of Stem Cell Self-Renewal

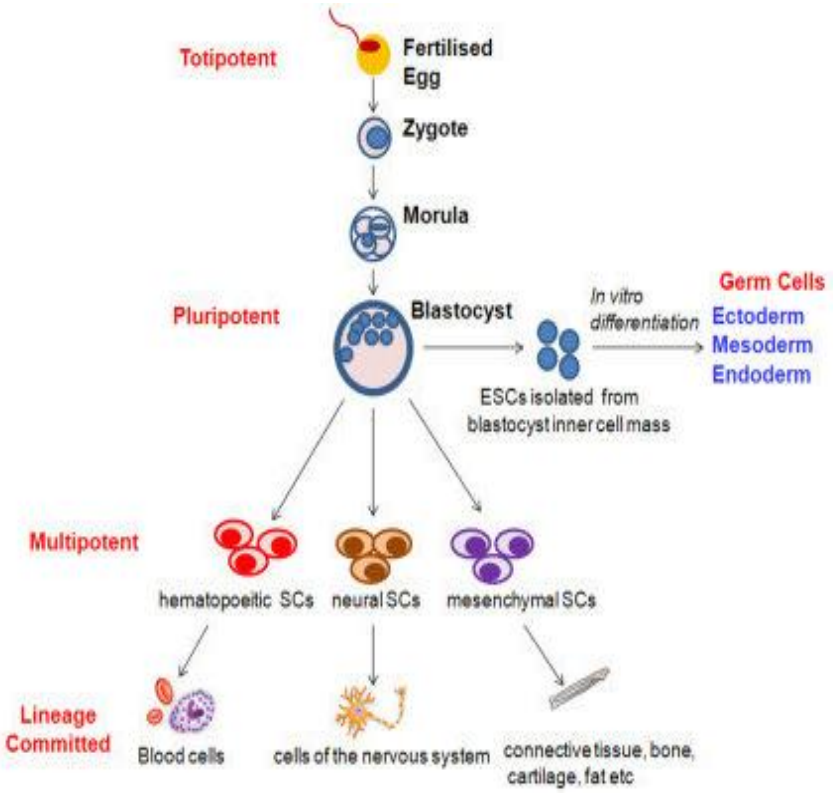

Figure. No 2: Schematic diagram illustrating the stem cell hierarchy.

3. Cell Cycle Regulators in Stem Cells

Adult stem cells have defined therapeutic roles evident in clinical bone marrow transplantation. The promise of broader therapeutic use for adult stem cells has been fueled

by the recent controversial finding that cells derived from one tissue type may display phenotypic characteristics of other tissue types under appropriate environmental cues. The therapeutic efficacy of stem cells in part depends on their proliferation; therefore, strategies to manipulate them require an understanding of their cell cycle control. A significant hurdle restricting the broader use of adult stem cells is their limited number and differentiation in response to proliferative stimuli, thus compromising ex vivo expansion efforts. (9) Cell cycle regulators play key roles in this process. In this chapter, we do not intend to detail the biochemical pathways of general cell cycle regulation because they were largely obtained from other model systems and have been extensively reviewed elsewhere. Instead, we focus on the distinct cell cycle kinetics in stem cell populations and its molecular base exemplified by the defining roles of the CKIs in murine HSCs. Admittedly, those studies do not give the whole picture concerning how the cell cycle in stem cells is controlled. Nevertheless, they underscore the importance of further investigation of other cell cycle regulators in stem cell biology and offer new paradigms for therapeutic manipulations of stem cells. (2)

4. Cell Cycle Kinetics of Stem Cells

A] ***In Vivo***

As largely modeled in the hematopoietic system, maintenance of mature cell production requires a cytokine-responsive progenitor cell pool with prodigious proliferative capacity and a much smaller population of stem cells intermittently giving rise to daughter cells, some of which constitute the proliferative progenitor compartment. Under activating conditions such as after transplantation, an increase in stem cell divisions takes place as evidenced by the depletion of cycling cells using the S-phase toxin (5-fluorouracil [5-FU] or hydroxyurea). However, relative quiescence or slow cycling in the stem cell pool appears to be essential to prevent premature depletion under conditions of physiologic stress over the lifetime of the organism. Therefore, the highly regulated proliferation of HSCs occurs at a very limited rate under homeostatic conditions. Stem cell proliferation has been directly measured by bromodeoxyuridine (BrdU)-labeling experiments and cell cycle lengths have been estimated at approximately 30 days in small rodents, or only about 8% of the cells cycling daily. Similar analyses using population kinetics have estimated that stem cells replicate once per 10 weeks in cats. In higher-order primates, the frequency of cell division in the stem cell pool has been estimated to occur once per year. However, it is still not clear whether the relative quiescence reflects a complete cell cycle arrest of most cells in the stem cell compartment,

termed the clonal succession model, or a very prolonged G1 or G2 phase of cycling stem cells. Although the retrovirus-based clonal marking studies indicated a dormancy of most stem cells at a given time, which supports the clonal succession model, this view has been challenged by the competitive repopulation model and by BrdU incorporation in defined stem cell pools. (2)

B] ***Ex Vivo***

The relative quiescence of stem cells may prevent their premature exhaustion, but it is problematic in the context of the in vitro expansion necessary for transplantation and gene therapy. Methods for inducing stem cell proliferation have long been sought as a means to expand the population of cells capable of repopulating the marrow of ablated hosts and to render stem cells transducable with virus-based gene transfer vectors. Although great effort has been made to directly expand stem cells using different combinations of hematopoietic growth factors (cytokine cocktails), few culture systems have been applied in the clinical setting at least in part because of the lack of proof that any of the culture conditions support the expansion of long-term repopulating HSCs in humans. Gene-marking studies in large animals, including primate and human, indicate poor transduction in the stem cell compartment during long-term engraftment. These cytokine-based efforts to expand stem cells have often resulted in increased cell numbers but at the expense of multipotentiality and homing ability. Although data suggest that under some specific conditions murine HSCs may divide in vitro, net expansion is achieved in a limited fashion and is always associated with, and often dominated by cellular differentiation. Recent studies on the potent effect of Notch ligands and wnt proteins on stem cell expansion in vitro are promising. However, whether such "successful" protocols can be adapted to clinically useful human HSC expansion remains to be determined. (2)

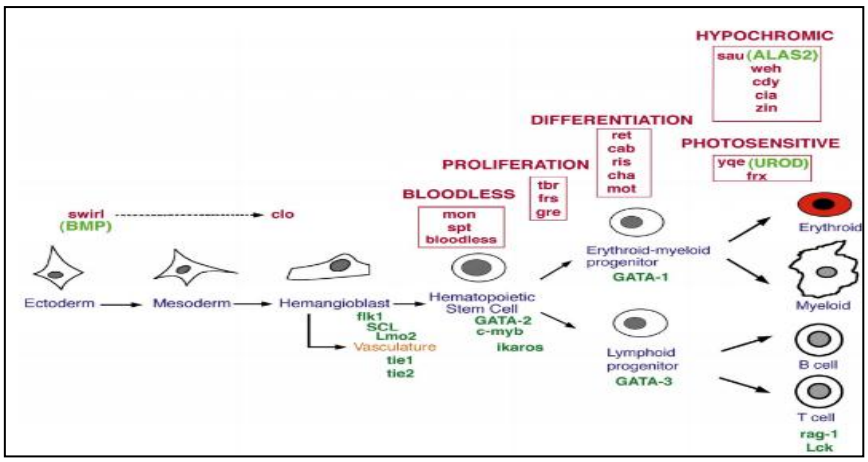

Figure no.3: The hematopoietic program invertebrates. (10)

5. Hematopoietic System

An HSC is generally defined as a cell that can:

(1) Affect the long-term reconstitution of all hematopoietic lineages;

(2) Be serially transplanted into naive recipients and recapitulate hematopoiesis;

(3) Reconstitute hematopoiesis after infusion of as little as a single cell into an irradiated host. [3]

5.1 Sites of Initiation of Primitive and Definitive Hematopoiesis and Vasculogenesis:

A] **Yolk sac development:**

The YS forms during gastrulation which begin in the Zebrafish embryo on day 6.5 (E6.5). Mesodermal cells destined for extraembryonic sites exit the posterior primitive streak and subdivide the embryo into three separate cavities by the neural plate stage (E7.5). The central cavity, the exocoelom, becomes completely lined with mesoderm and, where this is adjacent to the visceral endoderm, visceral YS forms. Between E7 and E7.5, mesodermal cells in the visceral YS proliferate and form mesodermal cell masses that are the precursor of the blood islands. Central cells accumulate hemoglobin, while the outer cells flatten and form endothelium. Lineage tracing experiments show that the hematopoietic mesoderm arises from posterior primitive streak mesoderm. Tissue recombination studies in the chick embryo also indicated that YS hematopoiesis requires diffusible signals from extraembryonic endoderm (hypoblast) that are analogous to the visceral endoderm of the Zebrafish. Indian hedgehog (Ihh) and smoothened (Smo), a receptor component essential for all Hedgehog signaling, are

required for yolk sac development. It appears to be the endodermal signal inducing hematopoietic and vascular specification of YS mesoderm, acting via induction of bone morphogenic protein-4 (BMP-4) in mesoderm that in turn induces hemato-vascular development. (2)

The onset of Primitive and Definitive Hematopoiesis in the Yolk Sac. The YS is the site of both primitive erythropoiesis and macrophage production and also of definitive multilineage, and myeloid and erythroid lineage-restricted, progenitor cell production. The wave of primitive erythropoiesis begins in the YS at E7.5, with nucleated red cells producing embryonic globin predominating in the circulation through E14.5 and with eventual replacement by fetal liver-derived red blood cells (RBC) expressing adult globins by E15.5–16.6. In humans, b-like embryonic globin (hemoglobin e) is expressed first in embryonic nucleated RBCs in YS blood islands. Subsequently, fetal globins (hemoglobin Ag and Gg) are expressed in definitive RBCs developing in the fetal liver. Finally, adult d- and b-globins are expressed around the time of birth within bone marrow-derived RBCs. The zinc-finger transcription factor, Erythroid Kruppel-like Factor (EKLF), plays a role in coordinating erythroid cell proliferation and hemoglobinization, participating in the switch from embryonic to fetal or fetal to adult b-globin expression. (2)

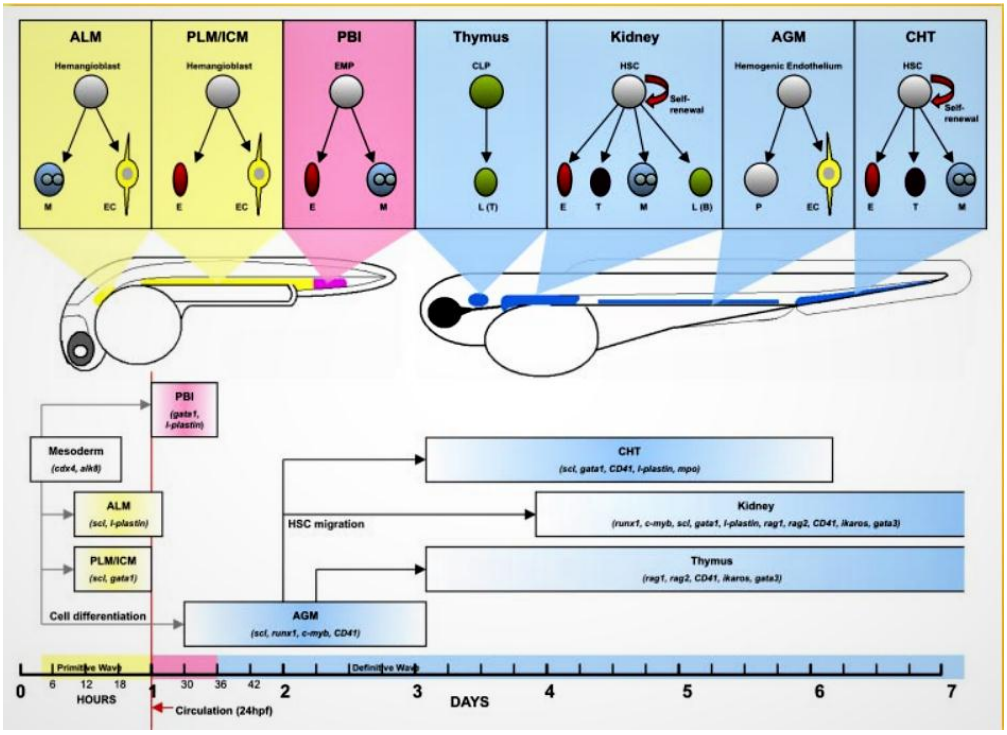

Figure No. 4: Spatial and temporal representation of hematopoiesis in the zebrafish.

B] Ontogeny of the vasculature:

Morphogenesis of blood vessels is defined by a sequential pattern of gene expression in which SCL/Tal1 and Flk1 are expressed first at the angioblast stage, followed by PECAM, CD34, VE-cadherin, and later Tie2, while SCL expression is down-regulated in the endothelium of mature vessels. As somatogenesis begins, vascular development is asymmetric, centered in YS blood islands, and in the embryo proper where angioblasts begin to coalesce into the aorta. By the murine 3 somite stage, vascular development has spread through the YS and extended aortic tubes are visible. Beginning at the 4 somite stage, erythroblasts disperse through the distal yolk sac and a few erythroblasts appear in the head and tail region of the embryo where the embryonic and YS vasculature meet. The circulation onset is defined as erythroblast movement within vessels and is initiated at the murine 4–6 somite stage during E8.25.(2)

C] Macrophage and microglial ontogeny:

In murine ontogeny, primitive macrophage progenitors appear in the proximal region of the egg cylinder associated with the expression of SCL/tal-1 and GATA-1 at E7. They increase until the early somite stage (E8.25), and then they decline sharply to undetectable levels by the 20 somite stage. Definitive lineage multipotent precursors with macrophage potential (HPP-CFC) as well as more restricted definitive CFU-GM and CFU-M appear at E8.25. Separation of YS and embryo at 8.25 (before the circulation) showed that HPP-CFC was found exclusively in YS. Once the circulation is established, HPP-CFC can be found in the circulation, indicating migration into the embryo. In avian studies, chick-quail chimeras established as early as E2.5 show numerous YS-derived cells, including macrophages, within the embryo vasculature and mesenchyme. At late E2, numerous scattered CD45+ cells appear in YS and the blood, exiting from the circulation through the endothelium and rapidly invading the whole embryo. These cells express high levels of CD45 and correspond exactly to cells identified as monocyte-macrophages.YS derived macrophages showing marked acid phosphatase activity and phagocytic capacity appear within the neural tube, liver anlage, and nephric rudiments, beginning even before the circulation is established. In the Zebrafish, the macrophage progenitors appearing in the YS at E7.5–8 migrate into the mesenchyme surrounding the brain rudiment. Initial migration occurs before circulation is established and is interstitial, but after E8.2 seeding occurs via the vasculature. These YS-derived macrophages continue to proliferate and generate the brain microglia, which ultimately

comprises 10% of the brain. The primitive generation YS-derived macrophages develop at E7–8 in the absence of a monocyte or promonocyte intermediary stage and differ from adult macrophages in the pattern of enzymes produced. Peroxidasepositive promonocytes of the definitive lineage appear in YS at E10.(2)

6. Isolation and characterization of hematopoietic stem cells

Central to the development of methods for stem cell isolation is the availability of quantitative techniques for the assessment of stem cell function. In this regard, any approach to identification and isolation of adult stem cells can be no better than the assay used to detect function. This is a critical issue with blood stem cells because the robust nature of hematopoiesis requires a massive expansion of very few stem cells to provide a continual source of replacements for mature cells that die every day. (5) The degree of expansion that occurs from stem cells to progenitor cells to mature cells is vast enough to prevent the absolute distinction between the most primitive stem cells and the differentiating progeny of these cells. Methods for hematopoietic stem cell (HSC) isolation described here include approaches that minimize co-isolation of non-stem cells, while also providing techniques to isolate populations of progenitor cells possessing remarkable proliferative potential in the absence of stem cell activity. A comparison of primitive HSCs with early progenitor cells provides interesting insights into the early stages of hematopoietic development. (2)

7. Zebra Fish and Stem Cell Research

7.1 Morphological And Embryonic Attributes:

Zebrafish are relatively small (3 to 4 cm) and reach sexual maturity at 2 to 3 months. The fish mate year-round, with females mating weekly. (11) Because of the small size of the fish, facilities can maintain thousands of individuals in a compact laboratory environment. Few, if any, vertebrate model organisms allow for such population size or ease of care. Furthermore, females lay between 100 and 200 eggs per mating, permitting large-scale genetic screens as well as Mendelian approaches and analysis. Of particular importance to the study of early blood development and hematopoietic stem cells is the unique embryonic morphology of the zebrafish. (12) Zebrafish embryos develop externally from the one-cell stage and are transparent, permitting embryonic development to be readily viewed under a dissecting microscope. Circulation begins by 24 hours postfertilization (HPF), and the number and morphology of blood cells may be identified under a microscope. (2)

Hematopoiesis at different sites is depicted in the top panel. The primitive wave of hematopoiesis (in yellow) consists of presumptive hemangioblasts that give rise to myeloid (M) and endothelial cells (EC) in the anterior lateral mesoderm (ALM) and to erythroid (E) and ECs in the posterior lateral mesoderm (PLM), which later becomes the intermediate cell mass (ICM). The first hematopoietic progenitor cells with multilineage potential are found in the posterior blood island (PBI); these erythromyeloid progenitors (EMPs) give rise to erythroid and myeloid cells in a transient definitive wave (in pink). The definitive wave of hematopoiesis (in blue) that contains long-term self-renewing hematopoietic stem cells (HSCs) originates in the aorta-gonad-mesonephros (AGM). The hemogenic endothelium in the AGM differentiates into ECs and hematopoietic progenitor cells (P) that migrate to and colonize other sites of definitive hematopoiesis. The AGM progenitor cells seed the caudal hematopoietic tissue (CHT) and kidney and proliferate to a population of HSCs with self-renewal potential. Based on the expression patterns of blood-specific markers, erythroid, myeloid, and thromboid (T) lineages are found in the CHT. Kidney marrow contains all the different blood lineages, including the lymphoid B cell (L(B)) lineage. (13) The common lymphoid progenitors (CLP) mature into lymphoid T cells (L(T)) in the thymus. The locations of different sites of hematopoiesis are depicted in a 24 h post-fertilization (HPF) embryo (left) and a 72 HPF larva (right). The timeline for the expression of blood-specific markers at different sites is shown in the bottom panel. Important blood-specific transcription factors and markers are listed in each box. Mesoderm cells (white box and gray arrows) differentiate into four independent populations of blood progenitor cells in the ALM, PLM/ICM, PBI, and AGM. Blood circulation begins around 24 HPF (red line and arrow). The AGM progenitors begin to enter the circulation around 33 HPF, and by 48 HPF, the CHT and kidney are simultaneously seeded by these progenitors (black arrows). The CLPs from the AGM begins to migrate to the thymus around 54 HPF. The CHT serves as an intermediate site of definitive blood development, then from the larval stage and into adulthood, the kidney is the primary site of hematopoiesis. The thymus is the site of maturation of lymphoid T cells, however, the CLPs, like other blood progenitors in the adult, originates in the kidney. ALM, anterior lateral mesoderm; PLM, posterior lateral mesoderm; ICM, intermediate cell mass; PBI, posterior blood island; AGM, aorta-gonad-mesonephros; CHT, caudal hematopoietic tissue; HSC, hematopoietic stem cell; M, myeloid; EC, endothelial cell; E, erythroid; EMP, erythromyeloid progenitor; CLP, common lymphoid progenitor; L(T), lymphoid T cell; L(B), lymphoid B cell; T, thromboid; P, hematopoietic progenitor cell; HPF, hour post-fertilization. (14)

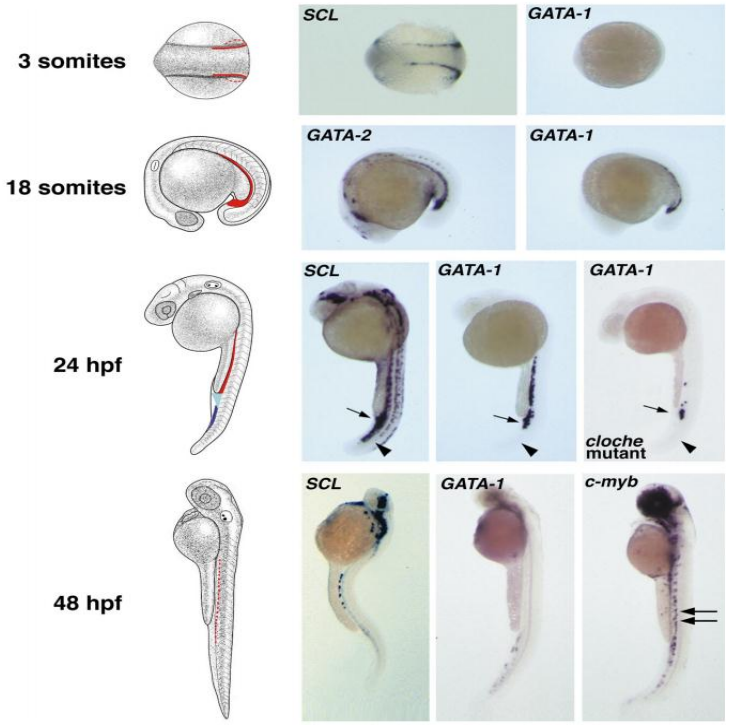

Figure No. 5: The expression pattern of hematopoietic factors during zebrafish embryogenesis.

Whole-embryo in situ hybridization analysis showing the localization of hematopoietic factors in zebrafish embryos at 3 somites, 18 somites, 24 h, or 48 h postfertilization (HPF). In the schematic panels (left), colored regions mark the anatomic areas involved in embryonic hematopoiesis. Early markers such as SCL are expressed in two stripes of lateral plate mesoderm by the 3-somite stage. GATA-1 expression, not detectable at this stage, begins at approximately 5 somites. Between 18 somites and 24 HPF, the stripes converge and fuse to form the ICM (red, anterior ICM; light blue, posterior ICM; dark blue, ventral tail region). Unlike SCL, which is expressed throughout the ICM and in the ventral tail, GATA-1 expression is limited to the anterior and posterior ICM (arrows, posterior ICM; arrowheads, ventral tail putative hematopoietic region). (8) In cloche mutant embryos, GATA-1 persists only in the posterior ICM. By 48 HPF, SCL and GATA-1 expression in the blood is decreased, though SCL is highly expressed in the brain. c-myb is expressed in the ventral wall of the dorsal aorta (double arrow). 3 somites, dorsal view; 18 somites, anterior is to the left, dorsal is up; 24 and 48 HPF, anterior is up, dorsal is to the right. (10)

7.2 Blood formation in the zebrafish:

The hematopoietic program in vertebrates begins with mesodermal patterning along the dorsal-ventral axis. During the process of mesoderm patterning, a subset of cells gives rise to a putative population termed hemangioblasts. These cells are hypothesized to be a common precursor of blood and vascular lineages due to their close physical association and common gene expression during early development. Unlike many other vertebrates that form primitive HSCs in extraembryonic yolk sac blood islands, the initial site of blood formation in zebrafish is the inner cell mass (ICM), an intraembryonic tissue. The zebrafish ICM tissue is analogous to the extraembryonic mammalian blood islands in its role as the primary site of primitive erythropoiesis. (13)

As in all vertebrates, a shift in hematopoietic sites occurs in the zebrafish. Cells within the ICM are hypothesized to populate the dorsal mesentery as well as the ventral wall of the dorsal aorta. This region is thought to be the zebrafish equivalent of the aorta–gonad–mesonephros region (AGM), an intraembryonic site believed to specify definitive HSCs invertebrate organisms. These HSCs are then thought to colonize the developing kidney where blood formation will continue throughout the juvenile and adult life of the fish. In contrast, the primary sites of definitive hematopoiesis in mammals are the fetal liver and the adult bone marrow. Despite some differences (for example, fish erythrocytes remain nucleated), zebrafish blood is remarkably similar to mammalian blood, with all lineages—erythroid, myeloid, lymphoid, and thrombocytic—represented. Thus, the fish is a capable and applicable model organism for the study of hematopoiesis. The identification and isolation of zebrafish homologs of factors deemed critical for normal hematopoiesis and vasculogenesis, such as the transcription factors scl, lmo2, GATA1, GATA-2, and c-myb, have permitted investigations into many aspects of blood and vascular tissue formation. RNA in situ hybridization studies using whole zebrafish embryos provide gene expression patterns that may be used to investigate the timing and anatomy of hematopoiesis and vasculogenesis. The first appearance of vascular and blood markers is seen at the three-somite stage (11 HPF) with the expression of GATA-2 and scl in two stripes of lateral plate mesoderm. Some cells within these stripes will become blood in the fish. By the 18-somite stage (18 HPF), these expression stripes have fused to form the tubular ICM structure. The ICM is fully formed by 23 HPF, just before the commencement of circulation. The presence of c-myb expressing cells located in the ventral wall of the dorsal aorta at 36 HPF suggests the presence of

definitive HSCs in the zebrafish. Finally, by 4 days postfertilization (dpf), HSCs have migrated to the developing kidney. (13)

Although the discrete steps necessary for the induction of hematopoietic precursors into differentiated blood lineages, as well as the program directing the different hematopoietic waves, are still unclear, the zebrafish is a powerful model organism with which to begin to unravel the story. The process of differentiation of hematopoietic stem cells into the erythroid, myeloid, and lymphoid lineages is the result of a highly conserved gene program. It is also clear that the zebrafish, like other vertebrates, possess discrete waves of hematopoietic events characterized by the expression of specific and recognized transcription factors. As will be discussed below, zebrafish have played an important role in uncovering novel genes involved in these programs via large-scale genetic screens. (13)

7.3 Genetic screens in zebrafish:

The first large-scale genetic screens in a vertebrate organism were conducted in zebrafish in Boston, Massachusetts, and Tübingen, Germany, in the mid-1990s. The power of screens in zebrafish is the ability to uncover novel genes involved in specific developmental processes in an unbiased manner. (2)

An important aspect of the zebrafish is the ease with which mutations may be generated in the fish. The chemical ethylnitrosourea (ENU), which causes point mutations throughout much of the genome, is a popular and effective mutagen for zebrafish. However, many other chemicals and avenues are available to achieve mutations in the fish, all achieving slightly different results. Gamma and X rays may be utilized to create large genomic deletions and translocation events, as well as point mutations. In a newer approach, insertional mutagenesis has been employed in zebrafish. Mutations are achieved by injecting retroviruses into the 1000–2000 cell stage embryo. Although the degree of mutations achieved by this approach is significantly lower than ENU mutagenesis, the ease of cloning the mutated gene makes it an exciting resource for the field. (2)

7.4 Cell sorting and transplantation:

New techniques have recently been carried out in zebrafish by David Traver and Leonard Zon, allowing differentiated blood cell populations to be sorted via flow cytometry. Samples have been collected from the kidney, spleen, and blood and analyzed by their light scatter

characteristics. Kidney tissue in the zebrafish, the site of adult definitive hematopoiesis, reveals distinct scatter populations when examined via this method. (2)

Cell sorting may be used to characterize the blood mutants obtained through genetic screens in the fish. The light scatters populations of mutant fish may be compared to those of wild-type fish, enabling the quantification of population deficits or increases in specific blood lineages in the mutant fish. Many primitive blood mutants exhibit a homozygous lethal phenotype analyzing adult homozygous mutants impossible. The analysis of blood lineages of adult heterozygous fish carrying only one copy of the mutant allele, however, can be revealed using this technique. This technique has exposed several cases of haploinsufficiency, suggesting that many genes required for embryonic and primitive hematopoiesis are additionally important in the adult fish. (2)

8. Biology of the stem cell and the relationship of primitive and definitive hematopoiesis

Blood formation depends on the prior events of mesodermal patterning and the generation of hemangioblasts. Mutations affecting these prior events will thus have effects on hematopoiesis, as is the case with the mutations spt and clo. In contrast, a mutation specifically interfering with the generation or maintenance of primitive or definitive hematopoietic stem cells would be expected to exhibit defects in blood but not vasculature or other mesoderm-derived structures. Zebrafish candidates for such a stem cell mutation include bloodless (which has also been called sort-of bloodless) and moonshine (mon). bloodless embryos are bloodless at the onset of circulation. The phenotype of bloodless is dominant with incomplete penetrance, and some heterozygous mutant animals recover blood production and can be raised to adulthood. GATA-1 expression in the anterior ICM of bloodless animals is severely diminished, while expression of GATA-2, SCL, and Lmo2 in the ventral tail is preserved (S. J. Pratt, A. Oates, E. Liao, N. Trede, and L. Zon, unpublished results). moonshine (also isolated as the bloodless mutant vampire) is a recessive mutation with eight identified alleles, seven of which are embryonic lethal as homozygotes. The strongest alleles are bloodless at the onset of circulation; others have a maximum of 50–100 circulating cells by day 4. GATA-1 expression is also affected and correlates with the severity of the phenotype, with the most severe alleles lacking detectable GATA-1. Rare homozygous mutant animals that survive to adulthood exhibit defects in erythroid differentiation (D. G. Ransom and L. I. Zon, unpublished data). Thus, mon likely represents a defect in both embryonic and adult erythropoiesis. mon mutants also exhibit increased

proliferation of iridophores (a neural crest derivative) in the posterior tail. This combination of neural crest and hematopoietic defects is reminiscent of the phenotype of mice bearing mutations in the c-kit (stem cell factor) gene; however, linkage analysis excludes zebrafish SCF/c-kit as the mon mutation. Do the two posterior regions, the posterior ICM, and the ventral tail, represent novel sites of hematopoiesis, perhaps stem-cell compartments? The posterior ICM expresses all of the blood-related markers tested to date; however, the cells in this region enter circulation slightly later than those in the anterior ICM. In clo mutants, a small amount of SCL, GATA-1, c-myb, and flk1 expression persists in the posterior ICM, again suggesting these cells may differ from those in the anterior ICM. The ventral tail, which does not express GATA-1, expresses other blood and vascular markers both early (18–24 hpf) and late (4 dpf) in embryogenesis. c-myb, which in mice is required for definitive but not primitive hematopoiesis, is coexpressed at high levels along with SCL in the ventral tail at 4 dpf. Thompson and Ransom et al. examined b316 embryos, which bear a deletion that includes the zebrafish c-myb gene, and found normal levels of GATA-1 expression. Thus, as in mice, c-myb is dispensable for primitive erythropoiesis. (The b316 deletion is lethal after 24 hpf, so no effect on definitive hematopoiesis could be determined.) It is not yet established that the ventral tail region is, in fact, truly hematopoietic. Interestingly, GATA-1, normally absent from the tail, is expressed in the ventral tail of anemic sauternes embryos at 3 dpf. This suggests that these cells have at least the potential to undergo hematopoietic differentiation in response to embryonic anemia. The nature of these posterior cells, and their relation to the ICM and aorta–gonads–mesonephros regions, will be clarified by cell-lineage and transplantation experiments. [2]

9. Methodology in zebrafish

9.1 Gamma irradiation:

Recipient zebrafish were anesthetized with 0.01% tricaine, then irradiated using a 137Cesium source (Gammacell 1000; Best Theratronics). Irradiation was conducted 24-72 hours before transplantation. (15)

9.2 Cell collection:

Donor's kidneys were dissected from CD41-GFP zebrafish as previously described.19 Wild-type AB zebrafish whole kidney marrow (WKM) was harvested and admixed with flow-sorted CD41-GFPlo cells in some of the transplant experiments. Citrate phosphate dextrose

solution (CPD) was used as an anticoagulant. WKM cells were processed by successive filtration, centrifugation, and washing in 0.9×PBS supplemented with 5% FCS and CPD.(15)

9.3 Isolation of cd41-gfp^{+} cells by flow cytometry:

Propidium iodide (PI; Sigma-Aldrich) was added to single-cell suspensions of WKM at a final concentration of 1 g/mL. FACS analysis and sorting were performed based on PI exclusion, and forward and side light scattering using a FACSVantage flow cytometer (BD Biosciences) at the FACS Core Facility, Children's Hospital Boston. Cells were kept on ice after sorting and before transplantation. Peripheral blood and kidney samples from transplanted fish were isolated, washed, and resuspended, as described in "Cell collection," and analyzed by flow cytometry. Controls included cells collected from wild-type non-transplanted and wild-type fish that had been transplanted but had not been engrafted. (15)

9.4 Analysis of Hoechst staining by flow cytometry:

WKM cells were obtained from 15-40 Tg(CD41: GFP) zebrafish, as previously described.20 Erythrocytes were depleted by briefly pipetting the WKM cells in 1-5 mL of sterile water, followed by immediate addition of 2 volumes of 2× cold 0.9× PBS/2% FBS. Before staining, cells were resuspended at a concentration of 106/mL. Hoechst 33342 dye (Sigma-Aldrich) was added at a final concentration of 1, 3, 5, 7.5, and 10 g/ mL.21,22 All samples were incubated for 60, 90, and 120 minutes at 28°C or 37°C in a water bath, protected from light. In parallel, 1×106 cells were stained with Hoechst 33342 along with 50M verapamil (Sigma-Aldrich). PI was added to all samples at 2 g/mL final concentration to exclude nonviable cells. SP cells were detected in a FACSVantage flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software Version 7.6. (15)

9.5 Adult zebrafish characterization

Anesthetized fish were placed on a sponge, with the heart exposed by lightly squeezing the animal near the pectoral fins. Donor cells were transplanted by intracardiac injection with a 10-L syringe (Hamilton Company) equipped with a 31-gauge needle. Initially, 16 000 flow-purified GFP^{lo} or GFP^{hi} cells were transplanted along with 0.8 × 106 unsorted wild-types WKM "carrier" cells. Subsequent transplantation of flow-sorted GFP^{lo} cells was carried out after 25 or 35 Gy of GFP^{+} cells in transplant recipients was taken as evidence of engraftment. (15) irradiation with from 10 to 9000 CD41-GFPlo cells per fish. Each of the surviving fish

was anesthetized with 0.01% tricaine 30 days after the transplantation and examined under an inverted fluorescent microscope (Leica DM-IRE2) for the presence of circulating GFP^+ cells.

9.6 Genomic DNA extraction and PCR analysis:

Genomic DNA was extracted from peripheral blood, spleen, and whole kidney marrow as well as from flow-sorted cell populations using the REDExtract-N-Amp Tissue and Blood PCR Kit (Sigma-Aldrich). The primers used were specific for the CD41-GFP transgene as previously reported.15 Amplification conditions were 94°C for 3 minutes followed by 32 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplification products were examined by electrophoresis on 0.8% agarose gel and stained with ethidium bromide. All samples were analyzed with negative control (wild-type AB fish), a positive control (CD41-GFP fish), and samples from irradiated transplanted fish that had not been engrafted. We also tested all samples with primers for the zebrafish elongation factor 1 α (EF1α) gene. The EF1α primers were: (1) forward primer: CGGTGACAACATGCTGGAGG; (2) reverse primer: ACCAGTCTCCACACGACCCA. The percentage of chimerism was determined by quantitative real-time PCR using an ABI PRISM 7700 Real-Time PCR System (Applied Biosystems). The SYBR Green ER Supermix reaction mixtures contained diluted genomic DNA, 2× SYBR Green ER Supermix, 7.5 mmol/liter of each gene-specific primer, and nuclease-free water to a final volume of 20 L. PCR thermal-cycle conditions were as follows: an initial step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 30 seconds. The specificity of the PCR products was controlled by melting curve analysis. The comparative threshold cycle (CT) method and internal control (EF1α) were used to normalize donor-specific gene expression. (15)

9.7 Cytology:

For cytologic studies, 0.5~1 × 105 cells were centrifuged onto glass slides using the Shandon Cytospin 3 centrifuge (GMI Inc). Sorted GFP^+ cells were kept in 0.9× PBS with 5% FCS, centrifuged at 200g onto poly-Lysine–coated coverslips placed at the bottom of the wells of a 48-well microtiter plate. The coverslips were immediately transferred onto a microscope slide that allowed the cells to be kept in media while being observed under an inverted fluorescent microscope (Leica DM-IRE2). (15)

9.8 Thin-section electron microscopy:

Cells were fixed with 1.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, for 8 hours. Cells were dehydrated through a series of alcohols, infiltrated with propylene oxide, and embedded in epoxy resin in an inverted beam capsule. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Tecnai G2 Spirit BioTWIN transmission electron microscope (Dymek Company) at an accelerating voltage of 80 kV. Images were recorded with an AMT 2k CCD camera (Advanced Microscopy Techniques). (15)

10. Advancements and future of zebrafish

The value of es and ESL cells as resources for both basic and applied research is now acknowledged almost universally. Present barriers to exploitation of their full potential in both areas are considered in the next sections of this chapter, together with possible ways of addressing these. Fundamental to progress is gaining a better understanding of both the nature and the basic biology of these cells. (16)

The study of HSCs in zebrafish has shed new light on many aspects of vertebrate HSC development. The molecular properties of many transcription factors and regulatory pathways in HSCs have been defined and redefined by zebrafish studies. The zebrafish has become an excellent model system to study HSCs because of the many technological advancements and innovations that can be applied to the HSC system. A breakthrough in zebrafish research came with large-scale forward-genetic mutagenesis screens. Mutations are introduced in the fish germline using potent chemical mutagens, such as ENU (N-ethyl-N-nitrosourea), or retroviral vectors that are injected into blastula-stage embryos to induce insertional mutations. The same scale of the genetic screen is difficult to conduct in other vertebrate model systems due to the high cost, space limitations, and maintenance issues. Blood mutants from first zebrafish mutagenesis screens, such as cloche and spadetail, carry mutations in genes involved in HSC development and have become invaluable resources for the HSC studies. Many novel genes that are important in hematopoiesis have been positionally cloned using these blood mutants, and their biochemical pathways have been investigated. (14)

Secondly, many innovative reverse-genetic approaches to study HSCs have been developed in the zebrafish. Producing transgenic zebrafish lines to express a gene of interest under tissue-restricted gene promoters or inducible promoters has been one of the most common

ways to manipulate and study a specific gene. DNA constructs containing fluorescent protein-coding genes (e.g., GFP or DsRed) driven by any number of defined tissue-specific promoters, can be introduced to follow the temporal and spatial expression of a gene or cell type. These tools have been used to follow the migration routes of HSCs during development. Heat or chemically inducible conditional Cre/loxregulated transgenic zebrafish lines are also being generated to study the effects of over-expression of a gene in a specific tissue at a given time point and to establish a system for long-term lineage analysis. Gene knockdown of an mRNA transcript by anti-sense morpholino is widely used in the zebrafish. Morpholinos persist in the embryos for 1–5 days after injection into one-cell embryos and inhibit proper gene translation by targeting either the translation initiation site or RNA splice sites. Although morpholinos can only be used to evaluate gene function during the first few days of development, they often closely recapitulate mutant phenotypes and can be applied rapidly if a specific genetic mutation is not available. Morpholinos have been successfully applied to the study of the blood system as evidenced by the loss of primitive and definitive hematopoietic cell lineages in scl morphants. (14)

Recently, a new permanent gene knockout technology has been developed in zebrafish using engineered zinc-finger nucleases (ZFNs). Engineered ZFNs introduce heritable mutations in the genome, and the creation of knockout fish lines for HSC related genes has become possible with this technology. The conditional knockout technology that is widely available in mouse is not yet available in the zebrafish, but will undoubtedly become possible in the future. (14)

Target induced local lesions in genomes (TILLING) is another reverse-genetic approach to produce mutants in specific genes. Standard chemical mutagenesis, as used in the forward-genetic screens, is used in TILLING to produce many random mutations in the germline of treated animals. The genome from F1 progeny are sequenced for mutations in a gene of interest, and a mutant line for a specific gene is created by incrossing the progeny of the F1 fish carrying the mutation in the gene. In TILLING screens, each gene of interest can have a series of mutations ranging from null to hypomorphic alleles; thus, an allelic series of mutations that modify gene function by different degrees can be studied. Many gene knockout zebrafish lines have been created using this approach, and many more will be identified using next-generation sequencing technologies. The zebrafish community is currently organizing a TILLING project to generate knockout lines for all essential zebrafish

genes. More genes will likely be discovered that play important roles in hematopoiesis and HSC biology. (14)

Recently, the transplantation of whole kidney marrow or HSC transplantation has been developed in zebrafish. The whole kidney marrow of the donor fish is isolated, different blood cell lineages are sorted, and the HSCs are transplanted into the circulation of irradiated recipient fish via intracardiac or retroorbital injection. The survival and engraftment rates of the transplanted fish are carefully monitored to analyze the homing and transplantability of the HSCs. More recently, with the improvement of the zebrafish genome assembly, major histocompatibility complex (MHC) loci of the zebrafish have been identified and are matched between the donors and the recipients in the HSC transplantation experiments.(14)

Chemical screens have also been conducted in zebrafish to find novel drugs that can affect HSC formation or blood development. Many zebrafish HSC or blood mutants mirror human diseases and can be used as disease models to seek more effective treatment for human patients. Due to the small size of zebrafish, and their ability to absorb chemicals from the surrounding water, chemical screens can be conducted in a high-throughput manner. PGE2 as a regulator of HSC number was first discovered in a zebrafish chemical screen, and post-chemotherapy or irradiation patients taking derivative of PGE2 is the potential to accelerate recovery of their hematopoietic system. Many more chemical screens are ongoing in zebrafish, and we can expect that other compounds will be found for the treatment of blood diseases.(14)

Lastly, a double pigmentation mutant named Casper has recently been generated that is transparent as an embryo as well as an adult. Creating transgenic lines in Casper background, and conducting HSC transplantation assays using Casper, bring the zebrafish to a new level of manipulating and studying HSCs. With the use of the Casper zebrafish, stem cell engraftment, homing assays, and monitoring the effectiveness of chemical treatment on HSCs can be conducted in vivo. All of these technological advancements in zebrafish have been a major driving force in vertebrate HSC research. As more mutant lines are made, more pathways are mapped, and more potent chemicals are discovered, HSC research in zebrafish will help us to understand the genetic network or molecular aspects controlling the development and function of stem cells in all vertebrates and contribute to the treatment of human hematopoietic diseases.(14)

REFERENCES

1. What is a stem cell? | Facts | yourgenome.org. [cited 2020 Dec 4]. Available from: https://www.yourgenome.org/facts/what-is-a-stem-cell
2. Lanza RP, editor. Essentials of stem cell biology. Amsterdam ; Boston: Elsevier/Academic Press; 2006. 548 p.
3. Why use the zebrafish in research?. yourgenome. [cited 2020 Dec 4]. Available from: /facts/why-use-the-zebrafish-in-research
4. What Are Stem Cells? - Health Encyclopedia - University of Rochester Medical Center. [cited 2020 Dec 4]. Available from: https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=160&contentid=38
5. Biehl JK, Russell B. Introduction to Stem Cell Therapy. :6.
6. Ogawa M, LaRue AC, Mehrotra M. Hematopoietic stem cells are pluripotent and not just "hematopoietic." Blood Cells, Molecules, and Diseases. 2013 Jun;51(1):3–8.
7. Kobayashi I. Enrichment of hematopoietic stem/progenitor cells in the zebrafish kidney. :11.
8. Fan L, Collodi P. Isolation and Culture of Zebra Fish ES Cells. In: Handbook of Stem Cells. Elsevier; 2004 [cited 2020 Sep 7]. p. 493–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780124366435500560
9. Importance of Stem Cells | Stem Cells | University of Nebraska Medical Center. [cited 2020 Dec4]. Available from: https://www.unmc.edu/stemcells/educational-resources/importance.html
10. Figure no - 3 Amatruda JF, Zon LI. Dissecting Hematopoiesis and Disease Using the Zebrafish. 1999;15.
11. Newport C. Abstract concept learning in fish. Current Opinion in Behavioral Sciences. 2021;7.
12. Pugach EK, Finley KR, Zon LI. Zebrafish and Stem Cell Research. :8.
13. Finley KR, Zon LI. Zebra Fish and Stem Cell Research. :7.
14. Chen AT, Zon LI. Zebrafish blood stem cells. J Cell Biochem. 2009 Sep 1;108(1):35–42.
15. Ma D, Zhang J, Lin H, Italiano J, Handin RI. The identification and characterization of zebrafish hematopoietic stem cells. 2011;118(2):10.
16. Zakrzewski W, Dobrzyński M, Szymonowicz M, Rybak Z. Stem cells: past, present, and future. Stem Cell Res Ther. 2019 Dec;10(1):68.

	***Author Name –Mrs. Suvarna Bhadane*** *Author Affiliation: University of Mumbai* *Author Address/Institute Address :* IDEAL COLLEGE OF PHARMACY AND RESEARCH, BHAL, KALYAN - 421306, DIST – THANE MAHARASHTRA, INDIA
	***Author Name: Ms. Janhavi Lavande*** *Author Affiliation: University of Mumbai* *Author Address/Institute Address:* IDEAL COLLEGE OF PHARMACY AND RESEARCH, BHAL, KALYAN - 421306, DIST – THANE MAHARASHTRA, INDIA
	***Author Name: Ms. Maria Khan*** *Author Affiliation: University of Mumbai* *Author Address/InstituteAddress:* IDEAL COLLEGE OF PHARMACY AND RESEARCH, BHAL, KALYAN - 421306, DIST – THANE MAHARASHTRA, INDIA
	***Author Name: Nishrullah Khan*** *Author Affiliation; University of Mumbai* *Author Address/Institute Address:* IDEAL COLLEGE OF PHARMACY AND RESEARCH, BHAL, KALYAN - 421306, DIST – THANE MAHARASHTRA, INDIA
	***Author Name: Dr. Smita Takarkhede*** *Author Affiliation : University of Mumbai* *Author Address/InstituteAddress :* IDEAL COLLEGE OF PHARMACY AND RESEARCH, BHAL, KALYAN - 421306, DIST – THANE MAHARASHTRA, INDIA