Regulatory role of fibroblast growth factors on hematopoietic stem cells
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CHAPTER 1

Fibroblast growth factors as regulators of stem cell self renewal and aging

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Abstract

Organ and tissue dysfunction which is readily observable during aging results from a loss of cellular homeostasis and reduced stem cell self renewal. Over the past 10 years, studies have been aimed at delineating growth factors that will sustain and promote the self renewal potential of stem cells and support the expansion of primitive stem cells in vitro and in vivo. Recently, strong evidence is emerging indicating that fibroblast growth factors (FGFs) play a crucial role in stem cell maintenance. FGFs belong to a family of polypeptide growth factors that are involved in multiple functions including cell proliferation, differentiation, survival and motility. In this review, we discuss the regulatory role of FGFs on hematopoietic stem cells (HSCs), neural stem cells (NSCs) and embryonic stem (ES) cells in maintaining stem cell self renewal. These findings are useful and important to further our knowledge in stem cell biology and for therapeutic approaches.
Introduction

FGFs are a family of polypeptide growth factors that are involved in embryonic development and adult tissue homeostasis. Twenty-two FGF genes have been identified in the genome of humans and mice. During embryogenesis, FGFs (FGF-7, -8 and -10) govern the development of parenchymal organs, such as the lung and limb. In adults, FGFs are important in maintaining general tissue homeostasis, including functions such as tissue repair and regeneration, metabolism and angiogenesis.

Stem cells are active during embryonic development and in most adult tissues, playing an important role in normal homeostasis and tissue integrity. In adult tissues, stem cells have been identified or isolated from an ever increasing array of tissues, such as bone marrow, skin, intestinal epithelium, myocardium and brain. The unique ability of tissue specific stem cells to self renew, differentiate and proliferate is critically important to an organism during development and to maintain tissue homeostasis. Stem cell self renewal ensures that a potentially unlimited supply of stem cells exists, irrespective of demands put on the system by repetitive cell turnover during aging or stress.

However, unlimited stem cell self renewal may not exist, as stem cells are exposed to both intrinsic and extrinsic effectors of damage. For example, the skin will age more rapidly for an individual who has more exposure to ultraviolet rays than one who does not. The liver of an alcoholic would have aged more rapidly than a teetotaler and chemotherapeutic drugs used to treat cancer will have deleterious effects on HSCs. Evidently, exposure to environmental or genetic factors induces differentiation and apoptosis or inhibits the asymmetrical self renewal division of stem cells. Tissues in which stem cell self renewal is inhibited would not be able to replenish the differentiated cells, thereby impending function and integrity. Thus, this suggests that tissue aging results from stem cell aging which in turn results from a decrease in stem cell self renewal capacity. Consequently, it has become increasingly important to study regulators which will enhance stem cell self renewal during aging. Here, we review recent advances in our understanding of the effects of FGFs on enhanced self renewal and maintenance of stem cells. Our focus will be on HSC, NSCs and ES cells as FGFs are crucial growth factors for all three stem cell systems. In addition, these three stem cell species are by far the best characterized stem cells in in vitro and in vivo.
vivo models. Finally, these stem cells appear to be of vital relevance in a variety of age-related diseases, ranging from hematopoietic disorders, cancer, cardiovascular disease and neurodegenerative disorders.

**Fibroblast Growth Factors**

To date, 22 FGFs have been identified. They range in molecular weight from 17 to 34 kDa and share 13-71% amino acid identity in vertebrates (reviewed by Ornitz and Itoh, 2001\(^1\)). FGFs mediate their biological responses by binding with high affinity to cell surface tyrosine kinase FGF receptors (FGFR). Four functional FGFR genes (*Fgfr1*-*Fgfr4*) have been identified in vertebrates\(^1\). Following receptor binding, FGFs induce dimerization and phosphorylation of specific cytoplasmic tyrosine residues. The phosphorylation of FGFRs triggers the activation of downstream cytoplasmic signal transduction pathways\(^2\text{"3}\). Furthermore, FGFs interact with low affinity to heparan sulfate proteoglycans (HSPGs)\(^2\text{"4}\), which acts to stabilize FGFs and prevent thermal denaturation and proteolysis. In addition HSPGs are required for FGFs to activate FGFRs effectively (reviewed by Powers et al. 2000; Dailey et al. 2005 \(^6\text{"25}\)).

The expression of all known FGFs in an array of mouse and human tissues and organs, was analyzed by researchers at the Genomics Institute of the Novartis Research Foundation (GNF) and is publicly available in the SymAtlas database (http://symatlas.gnf.org/SymAtlas/)\(^2\text{"6}\). Figure 1.1 illustrates a selection of those FGFs with the most variable expression levels across various tissues and organs. Most FGFs are ubiquitously expressed. However, some FGFs are selectively expressed in tissues and organs such as spleen (FGF-2), blastocysts (FGF-4), kidney (FGF-1), lung (FGF-1), dorsal root ganglion (FGF-1 and -13), embryo (FGF-5 and -15), salivary gland (FGF-23), pancreas (FGF-4 and -18) and pituitary (FGF-14). The complex expression of FGFs suggests that these growth factors play an important role in maintaining tissue homeostasis in many tissues and organs.
Figure 1.1: Expression levels of FGFs in an array of tissues and organs obtained from SymAtlas database. Only FGFs showing significant expression are shown in this figure. Further information regarding the remaining FGFs can be found at http://symstlas.gnf.org/SymAtlas. Horizontal scales are linear, ranging from zero to the final marked value.
Effects of FGFs on stem cells

Hematopoietic Stem Cells

HSCs are pluripotent cells, possessing high proliferative and self renewal potential. They ultimately regenerate and maintain all blood cell types of both the lymphoid and myeloid lineages, producing billions of new circulating but short-lived cells each day. In the early 1960s, Till and McCulloch introduced the first quantitative in vivo assay for stem cells in the bone marrow. Ever since, researchers have focused on soluble or cell-bound growth factors that promote HSC self-renewal, maintenance, survival and proliferation.

Hematopoiesis involves interactions between HSCs and stroma to provide a favorable microenvironment for the development of progenitors. Stromal cells are an important source of cytokines that regulate proliferation, differentiation and maturation of progenitor cells. As hematopoietic regulatory factors, FGFs can potentially exert their effects at two critical aspects of hematopoiesis: the HSC proper and/or maintenance of stem cell supporting stromal cells.

Initial studies demonstrated that FGF-1 induced granulopoiesis and megakaryocytopoiesis. Recent studies have shown that both FGF-1 and FGF-2 can sustain the proliferation of hematopoietic progenitor cells, maintaining their primitive phenotype. FGF-1 was shown to be involved in the expansion of multilineage, serially transplantable long-term repopulating HSCs. Most noticeably, we reported that the culturing of unfractionated mouse bone marrow in serum-free media supplemented only with FGF-1 resulted in a significant and sustained expansion of cells with both lymphoid and myeloid repopulating capacity. Additionally, we recently reported that culturing bone marrow stem cells in the combination of FGF-1 and FGF-2 for at least five weeks, maintained long-term repopulation (LTR). Using the FGF culture system as a tool, Crcareva et al. demonstrated that FGF-1 expanded bone marrow cells could be utilized for gene delivery to promote radioprotection and increase long-term BM reconstitution. FGF-1 has also been used in combination with stem cell factor (SCF), thrombopoietin (TPO) and insulin-like growth factor 2 (IGF2) to culture BM HSCs for 10 days. Competitive repopulation analysis revealed a ~20-fold increase in long-term HSCs.

FGF-2 has been known to enhance the colony stimulating activity of IL-3, erythropoietin (Epo) and granulocyte macrophage colony stimulating factor (GM-
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CSF) on hematopoietic progenitor cells derived from normal human adult peripheral blood and murine bone marrow in vitro. In addition, FGF-2 appears to have a stimulatory role on murine pluripotent hematopoietic cells in a spleen colony-forming unit (CFU-S) assay. Murine non-adherent bone marrow cells were infused into lethally irradiated mice to reconstitute hematopoiesis in vivo. Preincubation of the marrow cells with FGF-2 lead to an increase in the number of day 9 and day 12 CFU-S. Several reports exist demonstrating a role of other FGFs in maintaining hematopoiesis and HSCs. For example, FGF-4 alters the hematopoietic potential of human long-term bone marrow cultures by increasing the number of progenitors of the cultures.

The stimulation of both early and late hematopoiesis suggests the presence of FGFRs on both pluripotent and lineage committed progenitors. Fgfr1 and Fgfr2 expression was detected in murine unfractionated bone marrow cells, and in several purified mature peripheral cell populations (megakaryocytes and platelets, macrophages, granulocytes, T and B lymphocytes). Previous studies from our group have shown that Fgfr1, -3 and -4 can be detected on a primitive mouse Lin-Sca-1+c-Kit+ HSC population.

Unlike systemically acting growth factors, such as Epo and granulocyte colony stimulating factor (G-CSF), FGFs probably function locally in a paracrine or autocrine manner. For example, it is clear that FGF-2 plays a regulatory role in early and late hematopoiesis possibly by interacting directly with HSCs. However, as FGF-2 lacks signal sequences it is a poorly secreted protein suggesting that it must be produced locally by surrounding cells. Thus it is of importance to know which cells produce the growth factor in situ as these cells may specify stem cell self renewal. Hematopoietic stem cells are in intimate contact with the bone microenvironment (niche) and cell-cell contact appears to be responsible for the proliferative capacity of HSCs. Within the niche, the bone marrow extracellular matrix is postulated to serve as a reservoir of growth factors and hematopoietic cytokines. Since FGF-2 was found to be present in the bone marrow extracellular matrix, the most obvious source of FGF-2 is the stromal fibroblasts, which are able to both produce and respond to FGF-2. FGF-2 was reported to be a potent mitogen for bone marrow stromal cells in vitro. Non-adherent hematopoietic progenitors can grow and differentiate over a pre-established stromal cell layer in long-term bone marrow cultures. Under these conditions, FGF-2 stimulated myelopoiesis by increasing the number of non-adherent
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myeloid precursors\(^4\). Clearly, FGFs, in particular FGF-2 affects both stroma and hematopoietic progenitor cells, indicating that it is involved in both hematopoiesis and maintenance of the microenvironment.

It is therefore highly probable that besides acting on the stem cell itself, FGFs may interact with stromal niche cells (Figure 1.2\(^29,49,50\)). Both mechanisms may induce the release of other molecules important for the regulation of cell proliferation. Taken together, these results indicate that in vitro FGFs play a variety of regulatory roles which prolong homeostatic tissue renewal. The relevance of these in vitro data for in vivo stem cell behavior remains to be explored. Most notably, it would be highly relevant to assess how activities of FGFs provide a buffer against age-related cell stress that may occur.

**Figure 1.2:** Mechanism of action of FGFs on HSCs. FGFs may interact directly with the HSC promoting self renewal and/or interact with progenitor cells, releasing autocrine growth factors or other molecules important for cell proliferation. FGFs may also interact directly with stromal cells within the stem cell niche, releasing secondary growth factors necessary for the expansion of HSCs. Both methods are plausible as receptors for FGFs have been found on HSCs and stromal cells. Red diamonds represent the FGF ligand, green rectangles represent FGFR and question marks indicate that the existence of FGF/FGFR complex remains speculative.
Neural stem cells

Neurogenesis occurs throughout vertebrate life in the subgranular zone of the hippocampal dentate gyrus and in the telencephalic subventricular zone (SVZ). Although neurogenesis persists in the adult, its rate declines with age in rats\textsuperscript{51}, mice\textsuperscript{52}, monkeys\textsuperscript{53} and humans\textsuperscript{54,55}. The functional consequence of age-associated reduction in neurogenesis is not clear. However, restoring neurogenesis may be a strategy for preventing neural stem cell aging.

Like many other stem cells, neural stem cells (NSCs) possess three cardinal properties: self renewal, extensive proliferation and the ability to generate functional end-stage cells such as neurons, glial cells and oligodendrocytes (see review by Gage, 2000; Alvarez-Buylla et al., 2001; Weiss et al., 1996; Seaberg et al., 2003 and Rao, 1999\textsuperscript{56-60}). NSCs appear to be present in the SVZ in all vertebrate species tested. They can be selectively cultured from the central nervous system using only two growth factors; epidermal growth factor (EGF) and FGF-2\textsuperscript{18,61,62}. For example, FGF-2 and EGF have been used alone and in combination to isolate and maintain stem cells of the adult SVZ, the spinal cord, adult striatum and hippocampus\textsuperscript{18,63-69}.

In the presence of EGF and/or FGF-2, cultured NSCs in suspension give rise to clonally expanded aggregates called neurospheres. Cultured neurospheres will generate neurons and glia when plated without growth factor on adhesive substrates\textsuperscript{18}. Neurospheres have been derived from adult striatum, hippocampus, mesencephalon, SVZ and spinal cord\textsuperscript{18,67,68,70,71}. It is important to realize that not all the NSC progeny in neurospheres are stem cells. Rather, a heterogenous population of cells exists in which only 10\% to 50\% of the progeny retain stem cell features and the remaining cells undergo spontaneous differentiation. Quite similar to the situation in hematopoiesis, this brings up the concept of a stem cell niche. The cluster of a heterogeneous population of cells may aid the survival of NSCs in vitro or enable growth factors such as FGF-2 to act on differentiated/accessory cells, resulting in the release of additional growth factors which regulate NSC self renewal and proliferation. Following in vitro culturing differentiating/differentiated cells rapidly die and only surviving NSCs that retain long-term, self-renewal capacity produce new neurospheres\textsuperscript{72}. Neurospheres may be cultured for extended periods of time, representing a renewable source of NSCs that may facilitate neurogenesis. It is therefore a promising cellular source for biotherapies of neurodegenerative diseases.
There is also ample evidence that FGFs are relevant for in vivo neurogenesis. The intraventricular delivery of FGF-2 increased cell proliferation within the adult SVZ\textsuperscript{73,74}, however it no longer promoted the generation of pyramidal neurons in the cerebral cortex\textsuperscript{75}. Further studies report that subcutaneous injections of FGF-2 enhanced dentate neurogenesis in both neonatal and adult brain\textsuperscript{76,77}. Intracerebroventricular infusion of FGF-2 has been shown to upregulate dentate neurogenesis in the aged brain\textsuperscript{78}. FGF-2 knock-out mice are viable, fertile and phenotypically indistinguishable from wild-type. However they do display a reduction in neuronal density in the motor cortex, neuronal deficiency in the cervical spinal cord and ectopic neurons in the hippocampal commissure\textsuperscript{79,80}. With these phenotypes, it is not surprising that FGF-2 plays such a crucial role in neurogenesis and NSCs regulation. Mice deficient for FGF-4\textsuperscript{81}, FGF-8\textsuperscript{82-85}, FGF-9\textsuperscript{86} and FGF-10\textsuperscript{87} are lethal. Taken together, it is evident that FGF-2 plays a key role in regulating the proliferation, differentiation and survival of NSCs in vitro and in vivo. Despite a reduction in basal neurogenesis in dentate gyrus and SVZ the aged mouse retains the ability to respond to neurogenesis-stimulating effects of growth factors, such as FGF-2\textsuperscript{78}. These observations suggest that a decrease in levels of stem/progenitor cell proliferation factors, such as FGF-2, in the microenvironment of the subgranular zone are one of the potential causes of age-related decreases in neurogenesis. Increasing the concentration of FGF-2 and perhaps other growth factors may decrease the process of stem cell aging and enhance neurogenesis.

\textit{Embryonic stem cells}

Embryonic stem (ES) cells are derived from totipotent cells of the inner cell mass of the early mammalian embryo and are capable of seemingly unlimited and undifferentiated proliferation in vitro\textsuperscript{88,89}. For unknown reasons they are refractory to the senescence program that halts proliferation in adult cells. Potentially, the high levels of telomerase in ES cells contribute to this characteristic indefinite self renewal potential. As a result, ES cells have much greater developmental potential than adult stem cells. ES cell lines have been derived from mouse (mES) cells and human (hES). The factors and culture condition that regulate and mediate self-renewal of mouse and hES cells appear to be different, although their isolation conditions are similar. Similar to mES cells, hES cells were initially isolated by culturing inner cell mass cells on fibroblast feeder layers in medium containing serum\textsuperscript{90,91}. In contrast to mES
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cells\textsuperscript{92,93}, leukemia inhibiting factor (LIF) does not sustain and support hES cells\textsuperscript{90,94}. It is now widely accepted that the exogenous addition of FGF-2 to hES cells promote hES cell self-renewal and the capacity to differentiate into a large number of somatic cell types\textsuperscript{95,96}. The addition of FGF-2 to medium containing a commercially available serum replacement enables the clonal culture of hES cells on fibroblasts\textsuperscript{95}. Recently, it was reported that high doses of FGF-2 are adequate to maintain hES cells over 30 passages under feeder-free and serum-free growth conditions\textsuperscript{97}. Wang et al. showed that incubation of conditioned media from feeders with a neutralizing antibody against FGF-2 abrogates the capacity of conditioned media to support hES cells, suggesting that indeed FGF-2 is an essential factor produced by feeder cells\textsuperscript{97}. The recent observation that high dose FGF-2 (40ng/ml) can sustain hES cells has been independently reported, thereby corroborating this important insight\textsuperscript{98,99}. It has recently been demonstrated that elevated levels of FGF-2, FGF-11, FGF-13 and all four FGFRs are expressed in undifferentiated hES cells\textsuperscript{100-105}. Correlating with reported data, SymAtlas database analysis (Figure 1) demonstrates that most FGFs (15 out of 22) are expressed in blastocysts and fertilized egg of the mouse genome. The mechanism of action for FGF-2 in maintaining self-renewal of hES cells is still not understood. Because expression of all four FGFRs was observed in cultured hES cells\textsuperscript{106,107}, one can speculate that FGF-2 may stimulate undifferentiated hES cell proliferation directly. Alternatively, FGF-2 may block the differentiation of hES cells, as FGF-2 has been shown to inhibit maturation of oligodendrocyte precursors\textsuperscript{108,109}. Together, it is now clear that FGF signaling is unconditionally required for the sustained self-renewal and pluripotency of hES cells. This knowledge may serve as a base for future developments in using FGFs to culture undifferentiated hES cells for cell-based therapies to counteract the process of age-related diseases.

**Concluding Remarks and Future Perspectives**

The behavior of stem cells is carefully regulated to meet the demands of normal homeostasis of the organism, ensuring that a balance between proliferation, survival and differentiation exists. For many tissues, this balance is impeded during aging. Data presented in this review describe the role of FGFs and their receptors in promoting self-renewal, maintenance and proliferation of HSCs, ES cells and NSCs. Many studies have shown striking similarities with respect to FGF biology shared
between these three stem cell species. However, the molecular mechanisms by which the effects of FGFs occur in all three types of stem cells remains unknown. Although most stem cell studies that have been carried out were restricted to the use of FGF-2. In total, 22 FGFs exist (not including spliced forms). Why has FGF-2 historically been the most commonly used growth factor? Given their pleiotropic effects and sequence similarities it seems reasonable to argue that at least some other members of the FGF family will possess interesting stem cell stimulating activity. Clearly the next challenge will be to examine the effects of the remaining FGFs in well characterized stem cell systems. Only then would we be able to begin to understand the complete role of FGFs in regulating stem cell self renewal behavior and its impact on cellular aging, tissue aging and indeed organismal aging.
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