Department of Stem Cell Regulation (Amgen)

Our research interest is to characterize functional molecules of stem cells, particularly 1) signaling molecules that regulate proliferation and differentiation of stem cells, and 2) genetic manipulation of stem cells by appropriate gene transfer system. For this purpose, we are focusing on; 1) the mechanism of self-renewal in embryonic stem cells, 2) ex vivo expansion of hematopoietic stem cells using chimeric cytokine receptor transgenic mice, 3) identification of master regulator(s) for pronephric tubule formation using animal caps of Xenopus embryos, and 4) identification of self-renewal molecule(s) for neural stem cells.

Functional cells of blood, immune system, nervous system and skin have a relatively short life compared to an individual. To supply these cells, functional cells are maintained and generated from stem cells of various lineage during embryonic development or adult, resulting in the formation, maintenance, repair and regeneration of tissues and organs. Stem cells are defined by virtue of their functional attributes as (a) undifferentiated cells (i.e. lacking certain tissue specific differentiation markers), (b) capable of proliferation, (c) able to self-maintain the population (self-renewal), (d) able to produce a large number of differentiated, functional progeny, (e) able to regenerate the tissue after injury, and (f) flexible use of these options.

A major impetus here has been the desire to identify these stem cells in tissues so that strategies for targeting these permanent lineage ancestor cells for gene therapy may be developed. Also there has been an increased interest in manipulating the tissue stem cells in various clinical situations including, for example, the normal tissue stem cells during cancer therapy. It is still not possible to identify stem cells in most tissues by either their morphological characteristics or by the use of a specific marker. Numerous attempts have been made to find stem cell specific markers, but the problem here may be that what characterizes these cells is more likely to be the absence of specific features than the presence of something that can be identified by a marker or probe. So far, there are no established stem cell lines and self-renewal factors for stem cells, except embryonic stem (ES) cells and leukemia inhibitory factor (LIF), respectively. Therefore, identification, establishment and in vitro expansion of stem cells is essential not only for analyzing their self-renewal mechanism, but also for a variety of clinical applications, such as bone marrow transplantation, tissue regeneration, and gene therapy.

We have been investigating (1) the mechanism of self-renewal in ES cells using chimeric receptor approach, (2) ex vivo expansion of hematopoietic stem cells (HSC) using chimeric receptor transgenic mice, (3) involvement of STAT3 in ventralization of Xenopus embryos and identification of master regulator(s) for pronephric tubule formation using animal caps of Xenopus embryos, and (4) identification and establishment of neural stem cells from mouse fetal brain.

1. Analyses of self-renewal and differentiation in embryonic stem cells and myeloid leukemic cell lines

a. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells

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Embryonic stem (ES) cells can be maintained in an undifferentiated state in the presence of leukemia inhibitory factor (LIF). LIF acts through a receptor complex composed of a low affinity LIF receptor (LIFRβ) and gp130. We reported that the intracellular domain of gp130 plays an important role in self-renewal of ES cells. In the present study, we examined the signaling pathway through which gp130 contributes to the self-renewal of ES cells. Mutational analysis of the cytoplasmic domain of gp130 revealed that the tyrosine residue of gp130 responsible for STAT3 activation is necessary for self-renewal of ES cells, while that required for SHP2 and MAP kinase activation was dispensable. Next, we constructed a fusion protein composed of the entire coding region of STAT3 and the ligand binding domain of the estrogen receptor. This construction (STAT3ER) induced expression of junB (one of the targets of STAT3) in ES cells in the presence of the synthetic ligand 4-hydroxytamoxifen (4HT), thereby indicating that STAT3ER is a conditionally active form. ES cells transfected with STAT3ER cultured in the presence of 4HT maintained an undifferentiated state. Taken together, these results strongly suggest that STAT3 activation is required and sufficient to maintain the undifferentiated state of ES cells.

b. Cytoplasmic domains of the leukemia inhibitory factor receptor required for STAT3 activation, inducing differentiation, and growth arrest of myeloid leukemic cells

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Leukemia inhibitory factor (LIF) induces growth arrest and macrophage differentiation of mouse myeloid leukemic cells through the functional LIF receptor (LIFR), which comprises a heterodimeric complex of the LIFR subunit and gp130. To identify the regions within the cytoplasmic domain of LIFR that generate the signals for growth arrest, macrophage differentiation and STAT3 activation independently of gp130, we constructed chimeric receptors by linking the transmembrane and intracellular regions of mouse LIFR to the extracellular domains of the human granulocyte macrophage colony-stimulating factor receptor (hGM-CSFR) α and βc chains. Using the full-length cytoplasmic domain and mutants with progressive C-terminal truncations or point mutations, we show that the two membrane-distal tyrosines with the YXXQ motif of LIFR are critical not only for STAT3 activation but also for growth arrest and differentiation of WEHI-3B D+ cells. A truncated STAT3 which acts in a dominant negative manner was introduced into WEHI-3B D+ cells expressing GM-CSFRα / LIFR and GM-CSFRβc / LIFR. These cells were not induced to differentiate by hGM-CSF. The results indicate that STAT3 plays essential roles in the signals for growth arrest and differentiation mediated through LIFR.

2. Chimeric cytokine receptor can transduce expansion signals in interleukin 6 receptor α (IL-6Rα)-, IL-11Rα- and gp130-low to -negative primitive hematopoietic progenitors

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We generated transgenic mice expressing chimeric receptors which comprise extracellular domains of the human granulocyte / macrophage colony-stimulating factor (hGM-CSF) receptor and transmembrane and cytoplasmic domains of the mouse LIF receptor. In suspension cultures of lineage-negative (Lin^-), 5-fluorouracil-resistant bone marrow cells of the transgenic mice, a combination of hGM-CSF and stem cell factor (SCF) induced exponential expansions of mixed colony-forming unit (CFU-Mix). The combination of hGM-CSF and SCF was effective on enriched, $Lin^-Sca\text{-}1^+c\text{-}kit^+$ progenitors, and increased either CFU-Mix or cobblestone area forming cells. In case of stimulation with hGM-CSF + SCF, interleukin-6 (IL-6) + SCF, or IL-11 + SCF, the most efficient expansion was achieved with hGM-CSF + SCF. When $Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^-$, further enriched progenitors were clone-sorted and individually incubated in the presence of SCF, hGM-CSF stimulated a larger number of cells than did IL-6, IL-6 and soluble IL-6 receptor (sIL-6R), or IL-11. These data suggest the presence of IL-6Rα-, IL-11Rα-, and gp130-low to -negative primitive hematopoietic progenitors. Such primitive progenitors are equipped with signal transduction molecules and can expand when the hGM-CSF-mLIF chimeric receptors are genetically introduced into the cells and stimulated with hGM-CSF in the presence of SCF.

3. Organogenesis using Xenopus embryos

a. Activation of Stat3 by cytokine receptor gp130 ventralizes Xenopus embryos independent of BMP-4

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Stat3 is one of main signaling components of cytokine receptors, including gp130. Here we show that activation of cytokine receptor gp130 resulted in a dramatic ventralization of Xenopus embryos and that the ventralization correlated well with Stat3 activation potential of the receptor. This finding led to identification of Xenopus Stat3 (Xstat3), which showed a 95% homology to murine and human counterparts, at the amino acid level, and was expressed from the one-cell stage throughout development. The mechanism of gp130/XStat3-mediated ventralization proved to be independent of BMP-4. gp130/Xstat3 stimulation inhibited Smad2-induced ectopic axis formation in embryos and Smad2-dependent luciferase activity. A dominant-negative Stat3, in contrast, dorsalized Xenopus embryos, resulting in ectopic axis formation. We propose that Stat3-mediated signaling has the capacity to modify dorsoventral patterning in the early development of Xenopus.

b. Molecular cloning of a novel Xenopus spalt gene (Xsal-3)

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The sal (spalt) gene family is characterized by unique double zinc finger motifs and is conserved among various species from Drosophila to humans. Here we report a new Xenopus member of this family, Xsal-3. It is 38% homologous at the amino acid level to the previously reported Xenopus homologue of the spalt gene, Xsal-1. Alternatively spliced Xsal-3 transcripts give rise to RNAs coding either two or three double zinc fingers, and the longer form is expressed maternally. Xsal-3 is expressed in the neural tube, the mandibular, hyoid, and branchial arch, and the pronephric duct, which is different from the expression pattern of Xsal-1. These findings suggest that Xsal-3 may have distinct roles in early Xenopus development.

c. Cloning and expression pattern of a Xenopus pronephros-specific gene, XSMP-30

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The first step in kidney development is the formation of the pronephros which is derived from mesoderm. Xenopus is an appropriate model to study this process since the pronephros can be efficiently induced in animal cap explants by treatment with activin and retinoic acid (RA). Using this in vitro system, we isolated a Xenopus homologue of SMP-30 (Senescence marker protein-30), which is a Ca^{2+}-binding protein that is highly conserved in vertebrates. This gene, termed XSMP-30, was found to be selectively expressed in pronephric tubules from the late tadpole stage, by whole mount in situ hybridization. Furthermore XSMP-30 was expressed in animal caps treated with both activin and RA, a condition in which the pronephros is formed in vitro. These data indicate that XSMP-30 is a specific marker for the pronephros.

4. Neural stem cells

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Recent studies on neuroepithelial precursor cells isolated from many regions of the embryonic or adult brain show that these cells have many common characteristics of stem cells: (1) they can proliferate and self-renew in vitro in response to mitogens such as EGF or bFGF, (2) they are multipotent, able to produce neurons and glia, and (3) they can differentiate into neural subtypes appropriate to a new host region after transplantation. Although there are many reports with biological aspects of neural stem cells, little is known about the molecular mechanism for self-renewal or differentiation of neural stem cells. Moreover, no convenient markers that recognize cell surface antigens of neural stem cells results in the difficulty of characterization of neural stem cells. Based on these backgrounds, we intend to characterize the cell surface or soluble factor molecules derived from neural stem cells, using the following three strategies: (a) characterization of sphere formation enhancing factor, secreted into conditioned medium, (b) preparation of neural stem cell-specific monoclonal antibodies, and (c) identification of neural stem cell specific molecules containing signal sequence.

a. For characterizing the factor(s) that enhances neural sphere formation, we have already established assay system, using transwell culture apparatus. This system allowed us to identify the presence of sphere formation enhancing activity in conditioned medium derived from primary neurosphere culture. This activity could be recovered from lyophilized sample. Further characterization revealed that the fraction below MW. 3,500 retained this activity. Treatment with heat or several enzymes such as trypsin, proteinase and lipase fluctuated the background level, therefore the susceptibilities towards these treatments were obscure. Analysis using SMART system is in plan.

b. We intend to make monoclonal antibodies that recognize neural stem cells. At present, anti-nestin or anti-musashi antibody is used for characterizing neural stem cells. However, each of these antibodies recognize the antigen within cells, resulting in unsuccessful of analyzing living cells. Therefore, We intend to make monoclonal antibodies that recognize surface antigens of neural stem cells. For this pur-

pose, complete Freund's adjuvant was injected in advance of immunization using whole cells. 3 independent clones, which recognize adherent neural stem cells maintained in the presence of bFGF, were identified. 2 clones out of 3 showed characteristic staining pattern of embryonic brain section, in brief stained cells along the ventricle and within cortical regions. These antibodies stained small portion of dissociated sphere cells. These positive cells had a tendency to proliferate in the presence of EGF. Interestingly, 1 clone of these two had a functional activity, which enhanced sphere formation efficiency in the presence of EGF. The characterization of these antibodies is under investigation.

c. Characterization of secreted or cell surface molecules from neural stem cells was tried. For this purpose, signal sequence trap methodology is useful. The combination of retroviral vector system and constitutively active MPL (1 amino acid replacement within transmembrane domain results in a constitutively active MPL) proved to be an efficient assay system using Ba/F3 cells. At first, 140 clones were analyzed. 86 clones had signal sequences, in which various kinds of receptor molecules, factor molecules and cell adhesion molecules were included. 23 clones without homology to defined molecules were identified. Characterization of these clones is under investigation.

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