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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 tubules 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 either by 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 Jak/STAT pathway in ventralization of *Xenopus* embryos and identification of master regulator(s) for pronephric tubules 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. A selective switch-on system for self-renewal of embryonic stem cells using chimeric cytokine receptors

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Propagation of embryonic stem (ES) cells with an undifferentiated pluripotential phenotype depends on leukemia inhibitory factor (LIF). The LIF receptor complex is composed of a heterodimer of LIF receptor β (LIFRβ) and gp130. To activate LIFR signaling pathways independently from endogenous ones, we constructed chimeric receptors by linking the extracellular domain of human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α or β (hGMRα or β) to the transmembrane and cytoplasmic regions of either mouse LIFRβ or gp130. The chimeric receptors, hGMRα/mLIFR + hGMRβ/mgp130 or hGMRα/mgp130 + hGMRβ/mgp130, but not hGMRα/mLIFR + hGMRβ/mLIFR, preserved the self-renewal activity in A3 ES cells. All of these chimeric receptors were phosphorylated after hGM-CSF stimulation, without phosphorylation of endogenous gp130. Phosphorylation of the signal transducer and activator of transcription 3 (STAT3) through chimeric receptors correlated with the undifferentiated phenotype. Therefore, these chimeric receptors prove useful to analyze mechanisms of the self-renewal of ES cells.

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

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We have previously 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 acitivation is necessary for self-renewal of ES cells, while that required for SHP2 and MAP kinase activation was dispensible. 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 synthetic ligand 4-hydroxytamoxyfen (4HT), thereby indicating that STAT3ER is a conditionally active form. ES cells transfected with STAT3ER cultured in the presence of 4HT, maintained the undifferentiated state. Taken together, these results strongly suggest that STAT3 activation is required and sufficient to maintain the undifferentiated state of ES cells.

c. Cytoplasmic domains of the

leukemia inhibitory factor receptor required for STAT3 activation, and 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-CSFR α/LIFR and GM-CSFR βc/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. Controlled expansion of primitive hematopoietic progenitors through chimeric granulocyte-macrophage colony-stimulating factor(GM-CSF) -leukemia inhibitory factor (LIF) receptor expressed in transgenic mice

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The LIF receptor consists of the LIFR b chain and gp130, a signal transducing receptor component which is known to be essential for hematopoiesis. To examine roles of LIFR signaling in expansion of primitive hematopoietic progenitors, we generated transgenic mice expressing chimeric receptors which comprise extracellular domains of the human GM-CSF receptor and transmembrane and cytoplasmic domains of the mouse LIF receptor. The chimeric receptor transgenic mice were phenotypically normal, but when treated *in vivo* with exogenous hGM-CSF, there was a marked increase in CFU-Mix, which gave rise to multilineage hematopoietic colonies in methylcellulose culture, in either bone marrow (BM) or spleen. *In vitro*, a combination of hGM-CSF and stem cell factor (SCF) induced exponential expansions of CFU-Mix from the lineage-negative (Lin-), 5-FU-resistant BM cells of the chimeric receptor transgenic mice in a serum-free culture. The combination of hGM-CSF and SCF was also effective on enriched, Lin- Sca-1+ *c-kit*+ BM cells of the chimeric receptor transgenic mice, and increased either CFU-Mix or cobblestone area forming cells (CAFC) with high hematopoietic activities. In addition, CFU-Mix within the AGM

(aorta-gonads- mesonephros)region of the chimeric receptor transgenic mouse embryos also increased in response to hGM-CSF. These data suggest that the expansion of hematopoietic cells of the chimeric receptor transgenic mice is controllable by exogenous hGM-CSF, both *in vivo* and *in vitro*. Such evidence means that the hGM-CSFR-mLIFR chimeric receptor may function as a molecular switch for expansion of primitive hematopoietic progenitors.

3. Organogenesis using *Xenopus* embryos

a. Cytokine receptor gp130 ventralizes *Xenopus* embryos through Stat3 and antagonizes Smad2 and Xwnt-8

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gp130 is a receptor subunit shared by multiple cytokines. To investigate the role of this receptor in early development, we introduced into *Xenopus* embryos chimeric cytokine receptors containing the intracellular region of gp130. Activation of the receptors resulted in a dramatic ventralization of embryos. It also ventralized dorsal marginal zone explants and activin-treated animal caps. Mutation analysis of the chimeric receptors revealed that the ventralization correlated well with Stat3 activation. This led to identification of *Xenopus* Stat3, 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. A dominant-negative *Xenopus* Stat3 inhibited the gp130-mediated ventralizing effect. Furthermore gp130 signaling inhibited the activin pathway downstream of Smad2 and also antagonized the Xwnt-8 pathway. This ventralization mechanism proved to be independent of BMP-4. These data suggest that a cytokine signaling utilizing Stat3 is conserved among various species and functions to modify dorsoventral patterning in early development.

b. Identification of a *Xenopus* pronephros-specific gene

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Animal caps of *Xenopus* embryos, which are treated with activin and retinoic acid, give rise to pronephric tubules. We have used a cDNA library subtraction method to identify molecular markers of pronephros and eventually essential molecules for pronephros development. We recently cloned one gene (Senescence Marker Protein-30 (SMP30) homologue), which was specifically expressed in pronephros from st. 30 throughout early development. Animal caps treated with activin and retinoic acid, but not those treated with activin alone or retinoic acid alone, expressed this gene, indicating that it serves as a good marker for pronephros formation, though functions of this gene remain unknown. We have isolated an upstream genomic region of this gene and are planning to use it for luciferase assays both in animal caps and in whole embryos, to identify essential regions for gene expression in pronephros.

4. Self-renewal of neural stem cells induced by activation of leukemia inhibitory factor receptor (LIFR)

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The mammalian multi-potential neural stem cells have long been thought to exist only during embryogenesis. However, recent evidence suggests that the adult brain does indeed contain multi-potential neural stem cells. These stem cells can proliferate in the presence of EGF and be passaged continuously. It is well known that several signal transduction pathways are activated after the stimulation of EGF receptor, i.e. Ras-MAPK, Jak/STAT, PI3 kinase, PLC−g et al. Although numerous experiments on EGF signal transduction have been performed, there is no report which signal is involved in the self-renewal of neural stem cells. STAT3 is well known to be activated by EGF as well as LIF-related cytokines. LIF-related cytokines stimulated astrocyte differentiation and the direct involvement of STAT3 activation is revealed. However, EGF does not induce astrocyte differentiation, but rather maintains the self-renewal even in the activation of STAT3. These opposite phenomena might be derived from different culture conditions (EGF:suspension culture, LIF:adherent culture). Therefore, we examined the effect of LIFR signaling on the self-renewal of neural stem cells in suspension culture. To activate LIFR at will, striatal neural stem cells from transgenic mouse embryo, which expresses chimeric receptors consisting of human GM-CSFR extracellular domain and mouse LIFPb/gp130 intracellular domain, were prepared. hGM-CSF stimulated the self-renewal of neural stem cells, but not so strongly as EGF. The formed neurospheres differentiated into neurons, astrocytes and oligodendrocytes after the removal of hGM-CSF. These data imply that neural stem cells can be maintatined in the presence of LIF signaling as well as EGF. Further analysis is under way.

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