Cancer Science

MEETING REPORT

Cancer Stem Cells

The Sixteenth International Symposium of the Hiroshima Cancer Seminar, October 2006

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Introduction

The Sixteenth International Symposium of the Hiroshima Cancer Seminar (HCS) Foundation was held on 22 October 2006 at the International Conference Center, Hiroshima. The symposium consisted of 10 special lectures and 23 free paper presentations for poster session; about 230 people were present and actively discussed on Cancer Stem Cells. Prior to this symposium, an Open Lecture to the public by HCS and Japan Society for Dying with Dignity was held on 21 October. Shigehito Yamawaki (Hiroshima University, Hiroshima) and Kazuko Hamanaka (Hamanaka Dermatological Clinic, Hiroshima) spoke about Psychooncology and Breast Cancer to more than 260 people.

At the beginning, Eiichi Tahara (Hiroshima Cancer Seminar Foundation), Chairman of the Organizing Committee of the Sixteenth International Symposium, and Chairman of the HCS Foundation, gave an opening address. Tahara introduced a brief background and the purpose of this series of symposia. Since the establishment of the HCS Foundation in 1992, annual international symposia are organized to create an opportunity for basic scientists and clinical researchers to exchange ideas for cancer research, cancer prevention and cancer therapy. This year, the organizing committee planed to explore the important issue of " Cancer Stem Cells ". Stem cells have a critical role not only in the generation of new population of normal cells but also in the development of tumors. The balance between self-renewal and differentiation is strictly regulated to maintain normal stem cell pool and to generate the required supply of fully differentiated cells. Recent evidences suggest that a subset of cancer cells within the tumor, so-called cancer stem cells, may drive the growth and progression of the tumor. Eradication of cancer stem cells may be essential to a cure of cancer. Advances in our knowledge that regulate proliferation, self-renewal, survival and differentiation of cancer stem cell and normal stem cell may shed light on mechanism that lead to cancer and improve cancer treatment. The participants will be able to profit by exchanging and learning ideas from the informative presentations and discussions, and will contribute to understanding of stem cell in relation with cancer development and treatment.

Special lectures on Cancer Stem Cells

Elsa Quintana (University of Michigan, USA) opened the symposium by describing stem cell self-renewal and cancer cell proliferation. Recent advances have highlighted extensive phenotypic and functional similarities between normal stem cells and cancer stem

cells. This raises the question of whether it will be possible to develop therapies that eliminate cancer stem cells without eliminating normal stem cells. To address this question, the function of the *Pten* tumor suppressor was examined. Conditional deletion of *Pten* in adult hematopoietic cells led to myeloproliferative disease within days and transplantable leukemias within weeks. *Pten* deletion also promoted hematopoietic stem cell (HSC) proliferation. However, this cell-autonomously led to HSC depletion, preventing these cells from stably reconstituting irradiated mice. In contrast to leukemia-initiating cells, HSCs were therefore unable to maintain themselves without *Pten*. These effects of *Pten* deficiency were largely mediated by mTor as they could be inhibited by rapamycin. Rapamycin not only depleted leukemia-initiating cells but also restored normal HSC function. Mechanistic differences between the maintenance of normal stem cells and cancer stem cells can thus be targeted to eliminate cancer stem cells without damaging normal stem cells. Her research group is now extending these studies in a variety of directions, testing the cancer stem cell model in various nervous system malignancies, examining the utility of rapamycin in other cancers, and considering clinical trials to test the effectiveness of rapamycin in patients with leukemia.

Shin-Ichi Nishikawa (RIKEN Center for Developmental Biology, Kobe) described niche for quiescent cells. Niche has become the most important issue in stem cell biology, but it is still a hypothetical notion that cannot be defined in better way than the microenvironment supporting stem cell systems. However, the notion of ”niche” per se is used in more restricted meaning than that of microenvironment, which stands for the special environment involved in the maintenance of most immature stem cells. There may be two types for niche role, one is to maintain self-renewal and the other is to maintain quiescence. The molecular mechanisms that induce and maintain the quiescent stem cell should be interesting. It is believed that the best model for addressing this issue would be melanocytes, because quiescent stem cells are distinguished from proliferating pool by their location. For last 5 years, Nishikawa’s group have developed methods to isolate quiescent stem cells, defined the molecular difference between quiescent stem cell and proliferating pool, and evaluated function of some of molecules in development and maintenance of melanocyte stem cells. The previous studies were first summarized in this symposium. Quiescent stem cells are induced by multiple steps, suggesting an enormous complexity for the niche. In vivo analysis, however, has difficulty in determining which molecules are indeed sufficient for inducing quiescent stem cells. Hence, a method to culture proliferating melanocytes has

been developed. Using this new culture system, it has started survey for molecules that convert the proliferating pool into the quiescent pool. The latest results on candidate molecules that induce quiescent stem cells were presented. As quiescence is also an important factor that confer the resistance of cancer to treatment, Nishikawa also discuss about the potential contribution of their melanocyte study to the understanding of cancer stem cells.

Lopa Mishra (Georgetown University, USA) gave a talk on TGF-β signaling in stem cells and gastrointestinal cancers. TGF-β family signaling is markedly prominent at the interface between development and cancer in gut epithelial cells. The TGF-β family proteins play key roles in the self-renewal and maintenance of stem cells in their undifferentiated state, whereas changes in TGF-β family signals drive the selection of defined differentiation pathways and their progression of differentiation. When deregulated, changes in TGF-β family signaling may contribute to impaired differentiation and allow for the development of cancers, thus linking the differentiation of stem cells with suppression of carcinogenesis. Several TGF-β signaling components are bona fide tumor suppressors with the ability to constrain cell growth and inhibit cancer development at its early stages. Inactivation of at least one of these components (such as the TGF-β receptors, Smad2, or Smad4, and adaptors such as ELF) occurs in almost all gastrointestinal tumors. For instance, S*mad4*$^{+/-}$ mice develop gastric tumors and intercrossing of the *Smad4*$^{+/-}$ genotype into mice with a mutation in the adenomatous polyposis coli tumor suppressor APC$^{\bullet 716}$ results in the development of larger and more invasive colorectal tumors than those observed in the presence of the two *Smad4* alleles. Moreover, intercrosses between S*mad4*$^{+/-}$ mice and *elf*$^{+/-}$ mice result in the development of gastric cancer in 90% of offspring. Interestingly, a third of *elf*$^{+/-}$ mice develop hepatocellular cancer spontaneously, but not gastric cancer, suggesting that a full dose of Smad4 is sufficient to suppress gastric cancer formation. BMP signaling also plays an active role in the stem cell compartments of the colon, presumably by suppressing the effects of Wnt signaling and consequently limiting stem cell renewal. Mutations in the BMP receptor BMPR1A, and Smad4 contribute to juvenile intestinal polyposis and Cowden disease, respectively. Furthermore, inactivation of the gene for one of the type I BMP receptors in mice allows for an expansion of the stem and progenitor cell populations, eventually leading to intestinal polyposis resembling the human juvenile polyposis syndrome. TGF-β signaling appears to be important for the transition of stem

cells to a progenitor and fully differentiated phenotype in the gastrointestinal system. Accordingly, Smad 2, 3, 4, adaptors and stem cell proteins regulated by TGF-β may be a pivotal for gastrointestinal epithelial cell differentiation. The absence of this drive to normal epithelial differentiation favors formation of human gastrointestinal carcinoma.

Yoshiaki Ito (National University of Singapore, Singapore,) reported that *RUNX3* links Wnt and TGF-β signaling pathways in gastrointestinal epithelium and referred possible roles of *RUNX* genes in cancer stem cells. The continued growth and propagation of cancer cells is considered to be dependent on a small subpopulation called cancer stem cells. Cancer stem cells have been studied most intensely in hematopoietic cells and leukemia stem cells (LSC) share the main characteristics of normal hematopoietic stem cells (HSC), namely the self-renewing capacity and multipotency in differentiation, although the latter characteristic is often aberrant in LSC. The *RUNX1/AML1* gene encodes a transcription factor essential for the generation of HSC and is frequently targeted in human leukemia. In human *RUNX1*-related leukemias, the RAS pathway is often concurrently mutated, but the mechanism of the synergism remains elusive. Ito's group recently found that inactivation of *Runx1* in mouse bone marrow cells results in an increase in the stem/progenitor cell fraction due to suppression of apoptosis and elevated expression of the polycomb gene *Bmi-1*, which is important for stem cell self-renewal. Introduction of oncogenic *N-RAS* into wild-type cells, in contrast, reduced the stem/progenitor cell fraction due to senescence, apoptosis or differentiation. Such detrimental events occurred presumably due to the cellular failsafe program, although hyperproliferation was induced initially by an oncogenic stimulus. *Runx1* insufficiency appears to attenuate such failsafe mechanism, particularly in the stem/progenitor cells, thereby supporting the clonal maintenance of leukemia-initiating cells expressing an activated oncogene. RUNX3 is related to RUNX1 sharing the highly conserved Runt domain. Unlike *RUNX1*, *RUNX3* is ubiquitously expressed in many different types of cells including the epithelial cells of gastrointestinal tract. RUNX3 is a strong candidate for a gastric cancer tumor suppressor functioning downstream of TGF-β pathway. *RUNX3* is inactivated very frequently in gastric cancer by epigenetic silencing of the gene and the protein mislocalization. Moreover, the inactivation was also reported in a wide range of other cancer types including colorectal cancer. Our recent study revealed that intestinal epithelial cells of *Runx3*-null mice show the high proliferation capacity with up-regulated β-catenin/TCF4 activity. RUNX3 was found to negatively regulate the transcriptional activity of β-catenin/TCF4 by inhibiting its DNA binding activity through the formation of a

ternary complex with β-catenin and TCF4. In the mouse model, adenomatous polyps were formed at similar frequency in *Runx3*$^{+/-}$ and *Apc*$^{min/+}$ intestine, and in the compound mice having *Apc*$^{min/+}$ and *Runx3*$^{+/-}$, adenocarcinomas were induced. It is suggested that the ternary complex composed of RUNX3, β-catenin, and TCF4 is a nodal point to integrate Wnt and TGF-β signalings to regulate intestinal epithelial cells in a complementary fashion. Since Wnt signal is considered to be essential for the maintenance of intestinal stem cells, it is important to study to see if molecular and biological interaction between β-catenin/TCFs and RUNX3 is taking place in intestinal stem cells.

Freddy Radtke (Swiss Institute for Experimental Cancer Research, Switzerland) described Notch signaling in self-renewing tissues and cancer. Notch proteins are large single transmembrane receptors, which regulate many cell fate decisions, and differentiation processes during fetal and post-natal developmental. In addition, aberrant Notch signaling has been associated with an oncogenic role in tumorigenesis. Genetic approaches are needed to aim at establishing the role of Notch receptors and/or ligands in self-renewing systems such as the hematopoietic system, the skin or the gut. However, it is not possible by conventional gene targeting as the embryos die during gestation. Therefore, the Cre-loxP system was used to study the physiological role of multiple components of the Notch pathway, in the epidermis of the skin as well as in the intestine of adult mice by an inducible loss of function approach. The role of Notch1 signaling in mammalian skin is not well understood and is mainly based on in vitro studies suggesting that Notch signaling induces differentiation in mammalian skin. Unexpectedly, ablation of the *Notch1* gene results first in epidermal hyperplasia followed by the development of skin tumors and facilitated chemical induced skin carcinogenesis, in part explained by reduced p21$^{WAF1/Cip1}$ levels. Moreover, Notch1 deficiency in the skin results in derepressed β-catenin and shh signaling which cumulates in the development of basal cell carcinoma like tumors over time. Thus *Notch1* functions as a tumor suppressor gene in mammalian skin. Inducible inactivation of the transcription factor RBP-J (CSL) which mediates Notch signaling of all Notch receptors in the intestine results in the conversion of undifferentiated proliferative crypt cells into post-mitotic goblet cells suggesting that Notch signaling is essential for the maintenance of the progenitor/stem cell compartment of the gut. Thus Notch functions are pleiotropic and context dependent. Notch can function as: a lineage specifier, oncogene, tumor suppressor and stem cell gate-keeper.

Toru Kondo (RIKEN Center for Developmental Biology, Kobe) introduced stem cell-like cells in cancer cell lines. Both stem cells and cancer cells are thought to be capable of unlimited proliferation. Moreover, many tumors and cancer cell lines express stem cell markers, including CD133, CD24, CD44, and ATP-binding cassette transporters, by which the cells pump out a specific fluorescence dyes, such as Hoechst33342, as well as anti-cancer drugs. Therefore, it is possible that cancer cells resemble stem cells or cancers contain stem cell-like cells. Using the flow-cytometry-based side population (SP) analysis that identifies a kind of stem cells, Kondo's group have succeeded to find SP cells in rat C6 glioma, rat B104 neuroblastoma, human MCF7 breast cancer, and other cancer cell lines. The purified C6 SP cells, but not the other cells, self-renewed and formed tumors, which contain both neuronal marker and glial marker positive cells, when transplanted *in vivo*, suggesting that C6 SP cells have characteristics of both neural stem cell and cancer. By analyzing the expression profile in both C6 SP and non-SP cells, several factors were identified, which are dominantly expressed in C6 SP cells, and it was found that one of such factors is crucial for the proliferation of C6 cells. Taken together, these findings suggest that SP cells in cancers might be a crucial target for the curable cancer therapy.

Kyung-Sun Kang (Seoul National University, Republic of Korea) described cancer stem cell in human breast. Human breast composed of secretory alveoli connected by a system of branching duct embedded in connective tissue. These lobulo-alveolar structures were composed of three cell lineages: myoepithelial cells forming the basal layer of ducts and alveoli, ductal epithelial cells that line the lumen of ducts, and alveolar epithelial cells that synthesize milk proteins. These cells proliferate extensively and differentiate during each pregnancy and lactation and undergo apoptosis during mammary involution. Like other organs, human mammary epithelium may be also derived from stem cell component. Studies on properties of human mammary stem cells have been greatly aided by parallel studies on rodent mammary stem cells. Common adult stem cell properties studied on bone marrow, skin, and brain were also applied to study on mammary stem cells. Recently, in vitro culture system were established that allow for propagation of primary human mammary epithelial stem cells and progenitor cells in an undifferentiated state, based on their ability to proliferate in suspension as spherical structures, which termed mammospheres. It was described that mammospheres are composed of stem cells and progenitor cells capable of self-renewal and multilineage differentiation and also showed their cell surface marker change when stem/progenitor cells differentiate. Stem cell self-renewal must be regulated to avoid either

stem cell loss or hyper proliferation. Misregulation of self-renewal mechanism might cause carcinogenesis. This theory was called 'stem cell theory' in carcinogenesis for a long time. Recent paper by Al-Hajj and colleagues has provided evidence for the existence of stem cells for breast cancer. They demonstrated that small part of cells in breast tumors has the capacity to proliferate extensively and form new tumors.

Masaki Mori (Kyushu University, Beppu) gave a talk on cancer stem cell-like cells in digestive organ. A subset of stem cells, termed side population (SP) cells, has been identified and characterized in several mammalian tissues and cell lines. However, SP cells have never been identified or isolated from gastrointestinal cancers. To isolate SP cells from various human gastrointestinal system cancer cell lines, flow cytometry and the DNA-binding dye Hoechst 33342 were used. Fifteen of 16 cancer cell lines from the gastrointestinal system contained 0.3-2.2% SP cells. Next, differentially expressed genes between SP and non-SP cells of hepatoma HuH7 were analyzed using an oligonucleotide microarray. The expression of *GATA6*, which is associated with embryonic development and hepatocytic differentiation, was significantly up-regulated in HuH7 SP cells. The expression of ABCG2, ABCB1 and *CEACAM6*, which are associated with chemoresistance, were also significantly increased in the SP cells. In addition, some epithelial markers and mesenchymal markers were overexpressed in SP cells. RT-PCR and immunocytochemical staining validated these results, and suggested a multi-lineage potential for HuH7 SP cells. In hepatoma HuH7 and colorectal SW480 cell lines, SP cells showed evidence for self-renewal, generating both SP and non-SP cells. Finally, chemoresistance to anticancer agents, including Doxorubicin, 5-fluorouracil and Gemcitabine, were compared between HuH7 SP and non-SP cells using an ATP bioluminescence assay. The HuH7 SP cells expressed a higher resistance to Doxorubicin, 5-fluorouracil and gemcitabine compared to non-SP cells. These findings demonstrate that cancers of the gastrointestinal system do contain SP cells that show some characteristics of so-called stem cells.

Atsushi Hirao (Kanazawa University, Kanazawa) described regulation of stem cell self-renewal by tumor-related genes. Recently, it has been shown that key molecules for tumor development play critical roles in regulation of stem cell self-renewal. Hirao's group demonstrated that ATM, which is a cell cycle checkpoint molecule in response to DNA damage, has an essential role in the self-renewal capacity of the hematopoietic stem cells (HSCs). $Atm^{-/-}$ mice over the age of 24 weeks showed progressive bone marrow failure due to a defect in HSC function that was associated with up-regulation of tumor suppressor genes,

$p16^{Ink4a}$ and $p19^{Arf}$, in response to elevated reactive oxygen species (ROS). Treatment of anti-oxidative agents restored the reconstitutive capacity of $Atm^{-/-}$ HSCs. These data demonstrate that the self-renewal capacity of HSC depends on Atm-mediated inhibition of oxidative stress. In normal HSCs, increasing levels of ROS limits the lifespan *in vivo*. Elevation of ROS induced HSC-specific phosphorylation of p38 MAPK accompanied by the up-regulation of $p16^{Ink4a}$ and $p19^{Arf}$ during serial bone marrow transplantation. Inhibition of p38 MAPK rescued ROS-induced defects in HSC repopulating capacity, indicating that the ROS-p38 MAPK pathway contributes to exhaustion of the stem cell population. Prolonged treatment with an anti-oxidant or a p38 MAPK inhibitor extended the lifespan of HSCs in serial transplantation, indicating that inactivation of p38 MAPK protects HSCs against loss of self-renewal capacity. Furthermore, Atm-mediated ROS regulation is shown to be essential for proper DNA recombination, preventing immunodeficiency and lymphomagenesis. Thus, regulation of ROS level is critical for both tumor development and stem cell self-renewal.

Hideki Taniguchi (Yokohama City University, Yokohama) described clonal identification multipotent stem in the normal liver and hepatocellular carcinoma using flowcytometric cell sorting. Using flowcytometry combined with in vitro and in vivo single-cell-based assays, hepatic stem cells were prospectively identified with multi-lineage differentiation potential and self-renewing capability in the developing mouse liver. c-Met+ CD49f+/low CD29+ c-Kit- CD45- TER119- cells in fetal liver could be clonally propagated in culture, where they continuously produced hepatocytes and cholangiocytes as descendants while maintaining primitive stem cells. When cells that expanded in vitro were transplanted into recipient animals, they morphologically and functionally differentiated into hepatocytes and cholangiocytes, with reconstitution of hepatocyte and bile duct structures. Furthermore, these cells differentiated into pancreatic ductal and acinar cells or intestinal epithelial cells when transplanted into pancreas or duodenal wall. These data indicate that self-renewing multipotent stem cells are retained in mid-gestational developing liver. Clonally isolated stem cells could be used to reveal the mechanism of cell differentiation in embryonic gut endoderm, and also could provide new insight into therapies for diseases of the digestive system. On the other hand, recent advances in stem cell biology enable us to identify cancer stem cells in solid tumors as well as putative stem cells in normal solid organs. In this study, side population (SP) cell analysis and sorting to established hepatocellular carcinoma (HCC) cell lines were applied in order to detect subpopulations which function as cancer stem cells and to elucidate their roles in tumorigenesis. SP cells demonstrated high proliferative

potential and anti-apoptotic properties compared with those of Non-SP cells. Immunocytochemistry examination revealed that SP fractions contain a large number of cells presenting characteristics of both hepatocyte and cholangiocyte lineages. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) xenograft transplant experiments revealed that only $1x10^3$ SP cells were sufficient for tumor formation, while an injection of $1x10^6$ Non-SP cells did not initiate tumors. Microarray analysis discriminated a differential gene expression profile between SP and Non-SP cells, and several so-called "stemness genes" were up-regulated in SP cells in HCC cells. In conclusion, Taniguchi proposed that the minority detected as SP cells in HCC cells possess extreme tumorigenic potential and constitute heterogeneity in the cancer stem cell system characterized by distinct hierarchy.

Active discussion was made in the poster session with 23 presentations concerning cancer chemotherapy. The papers entitled "Establishment of a labeling system for hematopoietic stem cells by Nucleostemin-EGFP transgenic mice" by Kazuhito Naka (Center for Cancer and Stem Cell Research Institute, Kanazawa University, Kanazawa), and "Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion" by Manabu Kurayoshi (Hiroshima University, Hiroshima) were awarded the best poster prize.

Freddy Radtke made closing remarks. We need to clarify the commonness and difference between the normal stem cells and cancer stem cells. For this purpose, the technology is necessary to separate cancer stem cells alone. It is extremely important to clarify the mechanism of the cancer stem cell for the resistance to chemotherapy in order to control cancer.