Eighth Japanese-German Workshop on Molecular and Cellular Aspects of Carcinogenesis¹

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Initiated in 1987, the biennial Japanese-German Workshops of the Essen series on “Molecular and Cellular Aspects of Carcinogenesis” are organized traditionally at the Institute of Cell Biology (Cancer Research) [IFZ], University of Essen Medical School and West German Cancer Center Essen, Germany. This 8th Workshop was generously supported by the International Committee for the Cancer Research Program of the Ministry of Education, Culture, Sports, Science & Technology and the International Cooperation Program for 2nd-Term Comprehensive 10-Year Strategy for Cancer Control of the Ministry of Health, Labor & Welfare on the Japanese side, and by the Federal Ministry of Education and Research (BMBF), the Deutsche Forschungsgemeinschaft (DFG) and the Alfried Krupp von Bohlen und Halbach-Stiftung, Essen, in Germany. Additional support from numerous other sponsors in Germany and Japan is gratefully acknowledged. The names and affiliations of Workshop participants are listed at the end of this report.

Presentations at this Workshop focused on DNA damage and repair, and their interconnections with cell cycle control, the ubiquitin system, and apoptosis; the genetics and biochemistry of carcinogenesis and its suppression; intra- and intercellular molecular signaling; telomerase functions in ageing and cancer; and therapeutic and preventive approaches based on molecular cell and cancer biology. Two *Special Lectures* open to a larger audience addressed the role of tight junctions in epithelial cell polarization/growth (S. Tsukita, Kyoto) and the molecular functions of β -catenin in development and cancer (W. Birchmeier, Berlin, Germany).

DNA Damage, DNA Repair, and the Cell Cycle

Seven human genes encoding mismatch repair (MR) proteins have been identified to date: hMSH2, hMSH3, hMSH6, hMLH1, hMLH3, hPMS1, and hPMS2. J. Jiricny (Zurich, Switzerland) addressed the functions of these proteins in postreplicative MR, in concert with PCNA, RFC, polymerase- δ , RPA, a DNA-helicase, and 5'-3' as well as 3'-5' exonuclease(s). He described the heterodimeric mismatch recognition factors hMSH2/hMSH6 (hMutS α) and

hMSH2/hMSH3 (hMutS β), their ability to complement extracts of MR-deficient cell lines, their enzymatic activity, and their mode of action. hMutS α and hMutS β interact with PCNA *via* the hMSH6 and hMSH3 subunits, respectively. Aside from the ATPase activity of the hMLH1/hPMS2 (hMutL α) heterodimer, higher order protein/protein interactions, particularly those of hMutL α with hMutS α bound to mismatch-containing DNA, are currently under study. MR gene variants are utilized that carry mutations affecting the nucleotide cofactor or DNA binding sites or mutations found in kindreds with the cancer predisposition syndrome, hereditary non-polyposis colon cancer.

Ionizing radiation (IR) induces a broad spectrum of different DNA lesions. Double-strand breaks (DSB) are probably the most critical IR-induced lesions due to their potential to cause cell death, mutations, chromosomal aberrations, and malignant transformation. The rejoining of IR-induced DSB in the DNA of eukaryotic cells follows biphasic kinetics, with a fast component (t_{50} , 5–30 min) attributed to DNA-PK dependent nonhomologous endjoining (NHEJ) and a slow, as yet uncharacterized component (t_{50} , 1–20 h). G. Iliakis (Essen, Germany) has carried out genetic studies in DT40 chicken cells and a series of mutants defective in homologous recombination (HR), to examine whether HR contributes to DSB rejoining. DT40 cells rejoined DSB with t_{50} values of 13 min and 4.5 h, respectively, and contributions by the fast (78%) and slow (22%) components were similar to those of other vertebrate cells with 10³-fold lower HR activity. After deletion of *RAD51B*, *RAD52*, and *RAD54*, and in a conditional knock-out *RAD51* mutant, both the rejoining kinetics and the contribution of the slow component remained unchanged. *Ku70*^{-/-} DT40 cells, and a *Ku70*^{-/-}/*RAD54*^{-/-} double mutant, exhibited a reduced contribution of the fast component; however, the slow component (t_{50} , 18.4 h) still enabled the rejoining of ~63% of DSB. Thus, large variations in HR do not alter the kinetics of DNA DSB rejoining and fail to modify the contribution of the slow component in a manner compatible with a dependence on HR. Unlike yeast cells, vertebrate cells thus appear to be “hard-wired” in the utilization of NHEJ as the main pathway for rejoining IR-induced DSB; HR repair may contribute at a stage subsequent to the initial rejoining. Compared with the NHEJ operating on “clean” DSB induced by restriction endonucleases (RE), little is known

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about the joining of "dirty" DSB induced by IR. This is mainly due to the random IR energy deposition along a given DNA sequence. The positions of the resulting DNA lesions are thus unknown. Therefore, P. Pfeiffer (Essen, Germany) used short triplex-forming oligonucleotides (TFO) containing a single ^{125}I -labeled nucleotide and a plasmid with an appropriate target sequence that becomes linearized by the ^{125}I -induced site-specific DSB. TFO-linearized DNA was incubated with cell-free extracts from CHO-K1 cells, and the efficiency and quality of NHEJ were assessed in comparison with the rejoining of RE-induced DSB. IR-induced DSB were indeed rejoined in the presence of the extracts, albeit with ~20-fold lower efficiency. Sequence analyses showed a highly heterogeneous spectrum of junctions, most of which contained deletions in the range of 1–30 bp, similar to the mutational spectra observed after exposure to IR *in vivo*.

Poly(ADP-ribose) polymerase (Parp) family proteins, including Parp-1, tankyrase, Parp-2, Parp-3, and VPARP, catalyze poly(ADP-ribosylation) reactions using NAD as a substrate. Parp-1 is activated by its binding to DNA strand breaks (SB), and is involved in DNA SB repair, base excision repair, DNA recombination, and cell death, through NAD depletion under oxidative stress. M. Masutani (Tokyo) has studied the role of Parp-1 in cell differentiation and carcinogenesis using *Parp-1*^{-/-} mice (disruption of *Parp-1* exon 1). Compared with their *Parp-1*^{+/-} counterparts, *Parp-1*^{-/-} mice exposed to N-nitrosobis(2-hydroxypropyl)amine showed a markedly higher incidence of liver hemangiomas. Liver hemangiosarcomas exclusively developed in *Parp-1*^{-/-} animals. Similarly, *Parp-1*^{-/-} mice developed azoxymethane-induced colon tumors and hyperplastic liver nodules at higher frequency. The effect of *Parp-1* deficiency on cell differentiation was studied during germ cell tumor development. Tumors derived from *Parp-1*^{-/-} embryonic stem (ES) cells implanted s.c. into nude mice contained trophoblast giant cells (TGC) with single or multiple megalonuclei, reminiscent of the syncytiotrophoblastic giant cells (STGC) of human germ cell tumors. TGC were present in the intratumoral blood pools. Placental lactogen I- and Tpbp/4311-positive TGC precursors (spongiorhoblasts) were identified in the *Parp-1*^{-/-} tumors by *in situ* hybridization, indicating that Parp-1 deficiency can direct ES cell differentiation into the trophoblast lineage. Parp-1 localizes in both the nuclei and the centrosomes, which have a regulatory role for DNA ploidy. Extensive hyperploidy was observed in *Parp-1*^{-/-} mouse embryo fibroblasts. Thus, Parp-1 deficiency may cause abnormal centrosome function and contribute to the development of TGC from spongiorhoblasts through endoreduplication. A. Bürkle (Newcastle upon Tyne, U.K.) investigated interrelationships between poly(ADP-ribosylation), genomic instability, and mammalian longevity, using a stably transfected hamster cell clone with con-

ditional Parp-1 overexpression. These cells overaccumulated poly(ADP-ribose) by several-fold, and strongly suppressed sister-chromatid exchange (SCE) and micronucleus formation induced by almost non-cytotoxic concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The cytotoxicity of MNNG was not suppressed (but rather slightly increased) over a wide range of concentrations. Viewed together with the potentiation of genomic instability by reduced Parp-1 activity, these data indicate that Parp-1 is a negative regulator of DNA damage-induced SCE and micronucleus formation. Parp-1 may thus mediate the tuning of genomic instability (as provoked by endogenous and exogenous DNA damage) to a level appropriate for the longevity potential of a given organism or species. Interestingly, L-selegiline, a neuroprotective anti-Parkinson drug with a lifespan-extending effect in laboratory animals, augments IR-induced poly(ADP-ribose) formation in intact cells *in vitro*.

Besides its well-known function in selective intracellular proteolysis, the ubiquitin system plays an important role in DNA repair. RAD6, a ubiquitin-conjugating enzyme (UBC) conserved in yeast and mammals, is involved in the control of postreplication repair (PRR). PRR is of particular importance for the distinction between mutagenic and error-free reversal of DNA damage. RAD6 pathway subbranches are dependent on the ubiquitin-conjugating activity of RAD6, but the relationships between many of the individual factors are still poorly defined. A second UBC, the heteromeric UBC13/MMS2 complex, has recently been implicated in an error-free PRR branch. H. D. Ulrich (Marburg, Germany) presented genetic and biochemical data defining the position of MMS2 and UBC13 within the RAD6 epistasis group in *Saccharomyces cerevisiae*. The chromatin-associated RING finger proteins RAD18 and RAD5 play a central role in mediating physical contacts between members of the PRR pathway, involving the UBC13/MMS2 complex. RAD5 recruits this complex to DNA by means of its RING finger domain. Its association with RAD18 brings UBC13/MMS2 into contact with the RAD6/RAD18 complex. Interaction between the two RING finger proteins thus promotes the formation of a heteromeric complex in which the two distinct ubiquitin-conjugating activities of RAD6 and UBC13/MMS2 can be coordinated. Genetic analyses of *rad5*, *ubc13*, and *mms2*, mutants with respect to UV sensitivity, DSB repair, cell cycle dependence, and interactions with other DNA repair proteins (e.g., the helicase SRS2), confirmed that *UBC13* and *MMS2* activity in repair depends on the function of *RAD5* throughout the cell cycle. This ensemble of repair factors is particularly important for cell survival during and after DNA replication. The RAD6 pathway may mediate repair not primarily by direct reversal of DNA damage, but rather through the channelling of DNA lesions into different repair pathways.

In support of this notion, *rad5* and *ubc13* mutants are defective in DSB repair by HR instead of favoring the NHEJ pathway. On the other hand, deletion of *SRS2* suppresses the UV sensitivity of these mutants in a manner dependent on functional recombination. UBC13/MMS2 may thus closely cooperate with RAD5, suggesting a regulatory mechanism for the activity of this repair pathway. As described by S. Jentsch (Martinsried, Germany), membrane fluidity in yeast is largely controlled by the OLE pathway regulating the abundance of the ER-bound enzyme $\Delta 9$ fatty acid desaturase OLE1. This pathway is turned on by the activation of two homologous transcription factors driving OLE1 transcription, namely the nuclear factor NF- κ B homologs SPT23 and MGA2, both of which are synthesized as inactive, ER-membrane bound precursors. In cells with low levels of unsaturated fatty acids, SPT23 is processed at the ER by RUP (regulated ubiquitin/proteasome-dependent processing), yielding the active transcription factor p90. Subsequently p90 is mobilized for nuclear targeting by the chaperone-like CDC48^{UFD1/NPL4} machinery. The OLE pathway is turned off by several mechanisms, including RUP repression, SPT23p90 degradation, and OLE1 mRNA decay.

A crucial role of the ubiquitin-proteasome system lies in the regulation of cell cycle progression. The multisubunit anaphase-promoting complex/cyclosome (APC) operates as a ubiquitin ligase specific for various cell cycle proteins (cyclin B, cyclin A, mitotic kinases, anaphase inhibitors, spindle-associated proteins, inhibitors of DNA replication). In yeast and mammalian cells, APC activity is tightly regulated, being high from late mitosis until late G₁, but low in S, G₂, and early mitosis. H. Saya (Kumamoto) referred to genetic and biochemical analyses in yeast, *Drosophila*, and *Xenopus*, revealing that APC function is regulated in a substrate-specific manner by two types of WD-40 repeat-containing proteins, Cdc20 (fizzy[fzy]/p55CDC) and Cdh1 (Hct1/srw1/fizzy-related [fzr]). In budding yeast, Cdc20 appears to be essential for both sister-chromatid separation and proteolysis of mitotic cyclin clb2, suggesting that Cdc20-activated APC is required for anaphase initiation as well as for the exit from mitosis. Cdh1 is believed to maintain APC activity from the end of mitosis to the end of G₁. In *Hct1* mutants, clb2 is highly stabilized in G₁. In *Drosophila*, the loss of *fzr* causes the unscheduled accumulation of mitotic cyclins in G₁ following an extra division cycle in the epidermis. This suggests that the Cdh1-dependent APC activity targets mitotic cyclins for proteolysis from the end of mitosis to G₁, but is dispensable for metaphase/anaphase transition and the exit from mitosis. Clones of chicken DT40 cells with disrupted *Cdh1* loci were generated to investigate the function of Cdh1 in vertebrate cells. Cdh1 proved to be dispensable for viability and cell cycle progression. Similar to yeast and *Drosophila*, however, loss of *Cdh1* caused

the unscheduled accumulation of mitotic cyclins in G₁, resulting in the abrogation of G₁ arrest after exposure to the p27^{kip1} inducer rapamycin. Furthermore, *Cdh1*^{-/-} cells fail to maintain DNA damage-induced G₂ arrest, and Cdh1-APC is activated by X-ray-induced DNA damage. Activation of Cdh1-APC thus plays a critical role in both Cdk inhibitor-dependent G₁ arrest and DNA damage-induced G₂ arrest.

Genetics and Biochemistry of Carcinogenesis and Tumor Suppression

Loss of heterozygosity (LOH) on the long arm of chromosome 11 has been reported in human non-small cell lung cancer (NSCLC). This region may contain multiple tumor suppressor genes, including the recently identified PPP2R1B gene at 11q23-24. Y. Murakami (Tokyo) has combined LOH analyses with functional complementation of A549 human NSCLC cells by yeast artificial chromosomes (YAC) from chromosome 11 to localize tumor suppressor activity to a region of 700 kb on 11q23.2. Most of this activity is associated with a 100 kb segment (transfer of fragmented YAC) containing a single gene, *TSLC1*, whose expression is reduced or lacking in A549 cells and other NSCLC, hepatocellular carcinoma (HCC), and pancreatic cancer (Pac) cell lines. Restoration of *TSLC1* expression to normal or higher levels abrogated tumor formation by A549 cells in nude mice. *TSLC1* expression or suppression was strongly correlated with the promoter methylation state. Promoter hypermethylation, and in a few cases inactivating mutations of *TSLC1*, were observed in most of the primary NSCLC, HCC, and Pac tumors with LOH for 11q23.2. Thus, hypermethylation of the *TSLC1* promoter is a likely candidate for the "second hit" in NSCLC with LOH. *TSLC1* encodes a plasma membrane glycoprotein exhibiting significant homology with proteins of the immunoglobulin (Ig) superfamily (including NCAM). A possible involvement of the TSLC1 protein in epithelial cell adhesion was discussed. R. Küppers (Cologne, Germany) discussed the role of somatic hypermutation and other Ig-remodeling processes in the development of B-cell lymphomas. Reciprocal chromosomal translocations involving the Ig loci are hallmarks of mature B-cell lymphomas and usually result in dysregulated expression of proto-oncogenes brought under the control of the Ig enhancers. Mechanisms that lead to Ig gene remodeling include V(D)J recombination, isotype switching, and somatic hypermutation, all of which generate DNA DSB. Isotype switching and somatic hypermutation occur in germinal center (GC) B-cells. The origin of a large number of B-cell lymphomas from these cells is, therefore, likely to be associated with their aberrant hypermutation and isotype switching activity. Somatic hypermutation may also be involved in B-cell lymphomagenesis through the targeting of non-Ig genes. Thus, the *BCL-6*

and the *Fas* (*CD95*) genes acquire somatic mutations in a considerable fraction of normal GC B-cells. The potential proto-oncogene *BCL-6* is frequently translocated and mutated in B-cell lymphomas, and *Fas* mutants may rescue lymphoma cells from Fas-mediated apoptosis. In a search for other mutant genes, 4 proto-oncogenes were identified that were specifically mutated in a large fraction of diffuse large cell lymphomas, but not in normal GC B-cells or other types of B-cell lymphomas. Aberrant hypermutation may therefore be a major contributor to lymphomagenesis in diffuse large cell lymphomas, thus defining a novel type of genomic instability. In children, rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma, whereas medulloblastoma, a highly malignant tumor of the cerebellum, accounts for ~20% of brain tumors. In Gorlin syndrome, both RMS and medulloblastoma are associated with a deficiency in the tumor suppressor gene *Patched*. Targeted disruption of both *Ptch* alleles in the mouse results in embryonic lethality, while heterozygous *Ptch*^{+/-} mice survive and display many of the features characteristic of this syndrome, including medulloblastomas and RMS. H. Hahn (Neuherberg, Germany) used *Ptch*^{+/-} mice as an animal model of human RMS. Wild-type *Ptch* mRNA was found in all tumors analyzed, but subsequent LOH and sequence analyses failed to identify any mutation. Deletion of one copy of *Ptch* may thus be sufficient to trigger RMS development, probably in concert with genetic events at other loci. To identify candidate genes other than *Ptch*, gene expression profiles (430 cDNA clones implicated in cancer, cell cycle, genomic stability, cholesterol homeostasis, and embryogenesis) of RMS were analyzed in comparison with normal muscle tissue. The results showed differential activation of the p53 and P13' kinase signaling pathways, suggesting that deregulated *Ptch* function might contribute to tumor cell survival by modulating apoptotic signals.

There is growing evidence that programmed cell death is also important in the pathogenesis of a variety of infectious diseases. Unravelling the molecular mechanisms modulating apoptosis in bacteria may therefore provide cell biologists with elegant tools to further dissect apoptotic pathways. O. Steinbach (Konstanz, Germany) described his studies on *Chlamydia pneumoniae* aimed at the development of new generation antibiotics directed against these gram-negative bacteria, which require eukaryotic host cells for their intracellular replication. With the use of suppressive subtractive hybridization (SSH) and high density DNA arrays, about 2000 deregulated genes were identified in *Chlamydia*-infected human cells. Interesting candidate genes are associated with signal transduction, the cell cycle, metabolism, transcription, and stress response (e.g., apoptosis). Approximately 10% of the sequences are novel, i.e., unknown or without functional assignment. During the early infection phase, certain

anti-apoptotic factors are expressed by the host cells, and apoptosis induced by various agents is completely blocked during this phase. This inhibition is removed towards the end of the infection cycle. Some of the deregulated genes are targets of NF- κ B, which translocates to the nucleus after the cellular entry of the pathogen. The inhibition of apoptosis can be released by transient application of NF- κ B inhibitors or by blocking protein neosynthesis. Efforts are under way to isolate potential chlamydial antiapoptotic factors, which might be pharmaceutically tractable targets. These studies underscore the necessary interaction of massive parallel high-throughput analysis, complex data mining, database enquiries, biological expertise, and appropriate validation experiments, in preclinical research and development.

M. Yamamoto (Tsukuba) has investigated the regulation by Nrf2 and Keap1 of phase II drug-metabolizing enzyme genes in relation to carcinogenesis. Transcriptional activation of genes encoding phase II enzymes and anti-oxidative stress proteins is mediated by the electrophile (EpRE) and antioxidant (ARE) responsive elements. Based on the observation that the ARE consensus sequence has a high similarity to the NF-E2 binding sequence (a cis-acting erythroid gene regulatory element), BHA-inducible expression of several detoxifying enzyme genes was examined in *nrf2* knock-out mice. The induction of phase II and other drug metabolizing enzyme genes was significantly affected, demonstrating that Nrf2 regulates the inducible expression of these genes. Moreover, Nrf2 regulates the expression of anti-oxidative stress enzyme and protein genes in mice and appears to be essential for the coordinated induction of cellular defence enzymes and proteins under ARE regulation. *Nrf2*-deficient mice are quite susceptible to carcinogens, and antioxidants fail to prevent carcinogenesis in these animals. A new protein, Keap1, was identified that suppresses Nrf2 activity by specific binding to its N-terminal Neh2 regulatory domain. Nrf2 is liberated from Keap1 by electrophilic agents/phase II inducers, suggesting that the Nrf2/Keap1 system acts as a sensor for xenobiotics and oxidative stress. The finding that the ARE response is defective in *nrf2* knock-out mice, together with the observations on Nrf2 activation and its repression by Keap1, significantly advances our knowledge regarding the induction of phase II and antioxidant enzyme genes.

H. M. Rabes (Munich, Germany) reported on additional variants and novel types of *RET* gene rearrangements identified in human papillary thyroid carcinomas (PTC) that had developed after the Chernobyl reactor accident. A number of hitherto unknown *RET* fusions were found. In PTC5, *RET* is fused to the 5' part of *GOLGA5*, a Golgi integral membrane protein (chromosomal location: 14q). PTC6 shows a fusion of *RET* to *HITF*, a transcriptional coactivator of a nuclear receptor (chromosome 7q32). In

PTC7, the *RET*-fused gene consists of a homologue to the *HTIF* gene (chromosome 1p13). In PTC8, a fusion of *RET* to a gene of unknown function was found at 18q21-22. Other authors described 4 additional fusion genes: RI α , the catalytic domain of cAMP-dependent protein kinase A (17q23); ELKS (12p13); PCM-1 (8p21-22), and kinectin (14q22.1), a cytoplasm-oriented vesicle membrane-anchored protein. These rare *RET*-fused genes result from interchromosomal translocations, in contrast to the predominant *RET* fusions (PTC1 and PTC3), where all of the participant genes reside on chromosome 10, with *RET* at 10q11.2, *ELE1* (*GOLGA5*) also at 10q11.2, and *H4* at 10q21. The latter fusions are rearranged by balanced intra-chromosomal inversions (apparently a predominant effect of thyrocyte irradiation), with subsequent selection and further progression of cells carrying this genetic aberration. Recent studies of *ELE1/RET* rearrangements have disclosed a breakpoint distribution mainly in *RET* exon 11 without major intron clustering. Direct and inverted repeats at, or close to, breakpoints, and a lack of major deletions or insertions, suggested microhomology-mediated DNA endjoining of DNA breaks as the preferred rearrangement and fusion mechanism. A common principle becomes evident when the variants and novel types of *RET* rearrangements are compared: in all cases, the receptor tyrosine kinase domain of *RET* is preserved, but devoid of the transmembrane and extracellular parts that are essential for the stringent control of *RET* activity under normal conditions. Interaction of the *RET* ligand GDNF, GDNF-R α , and the extracellular ligand-binding domain of *RET* is a prerequisite for *RET* activation. Instead, the fused parts of the fusion genes, all of which appear to be ubiquitously expressed, contain coiled-coil domains with putative dimerization potential. Dimerization may lead to constitutive, ligand-dependent activation of the *RET* tyrosine kinase and its autophosphorylation. Ectopic activation of *RET* (normally inactive in thyrocytes) then unleashes the clonogenic potential of the affected cell(s), with an augmented risk of expressing a malignant phenotype.

There are ~1.2 million carriers of the human T-cell leukemia virus type I (HTLV-I) in Japan, where the incidence of HTLV-I associated adult T-cell leukemia is the highest in the world. K. Tajima (Nagoya), with his colleague S. Sonoda (Kagoshima), have compared the geographical distributions of HTLV-I and HTLV type II (HTLV-II) carriers in South America. While the former are limited to the northern and southern Andes, the latter are widely spread among South American natives from the Caribbean to South Patagonia. The origins of HTLV-I/II in this region are probably linked to the history of human dispersal, with geographical clustering due to the natural transmission routes of mother-to-child and man-to-woman. To test for a common origin of HTLV-I in Asia and the Andes, HTLV-I

proviral DNA from Andean mummies of about 1500 years old was analyzed. Two out of 104 mummy bone marrow specimens yielded a band of 110 base pair (bp) DNA of the β -globin gene, and 1 out of 2 gave bands of 159 bp and 157 bp of the HTLV-I-pX and -LTR genes. The nucleotide sequences of the ancient HTLV-I-pX and -LTR clones were similar to those of contemporary Andeans and Japanese (with sequence microheterogeneity in some of the mummy DNA clones). These results provided the first evidence that HTLV-I had been carried to the Andes by ancient mongoloids before the colonial era. Because of the similarity of the genetic background between southern Japanese and Andeans in South America, it now seems plausible that people carrying HTLV-I arrived in the Andes through a transpacific route after the migration of Asian HTLV-II carriers to the South American continent.

Intracellular and Intercellular Signaling

Newly clarified regulatory mechanisms of β -catenin signaling were discussed by A. Kikuchi (Hiroshima). Originally identified as a protein interacting with cadherin, β -catenin links cadherin to α -catenin which, in turn, controls intercellular communication. β -Catenin exhibits signaling functions as a component of the Wnt signaling pathway, which also involves two recently discovered molecules, Axin and Idax. Axin is an Axin binding protein stimulating the degradation of β -catenin in mammalian cells and inducing ventralization in *Xenopus* embryos. Furthermore, Axin shows catalytic activity to remove SUMO-1 from sumoylated proteins. A mutant lacking this activity was impaired in its capacity to down-regulate β -catenin and to induce ventralization, suggesting that desumoylation is involved in the downregulation of β -catenin. Idax, a Dvl-binding protein, inhibits the interaction of Dvl with Axin, thereby suppressing the Wnt-dependent accumulation of β -catenin in mammalian cells. Moreover, Idax inhibits Wnt- and Dvl-dependent, but not β -catenin-dependent, axis duplication in *Xenopus* embryos. Axin and Idax are, therefore, important components of the Wnt signaling pathway and may represent putative tumor suppressor gene products.

TGF- β s are multifunctional peptides regulating all proliferation and differentiation and binding to cognate type II and type I serine/threonine kinase receptors which mediate intracellular signals through Smad proteins. K. Miyazono (Tokyo) reported on the inhibitory mechanisms of I-Smads (inhibitory Smads) on TGF- β signaling. Smad7 is induced by TGF- β , stably interacts with activated TGF- β type I receptor (T β R-I), and interferes with the signaling by receptor-regulated Smads (R-Smads). Smurf1, a HECT-type E3 ubiquitin ligase, interacts with Smad7, inducing Smad7 ubiquitination and its translocation into the cytoplasm. Moreover, Smurf1 associates with T β R-I through Smad7, with subsequent enhancement of the turn-

over of T β R-I and Smad7. Smad7 thus induces the degradation of T β R-I through the recruitment of an E3 ligase to the cell surface receptor. I-Smads have conserved carboxy-terminal Mad homology 2 (MH2) domains, whereas the amino acid sequences of their amino-terminal regions (N-domains) are highly divergent from those of other Smads. Of the two mammalian I-Smads, both Smad6 and Smad7 inhibit signaling by BMPs; however, Smad7 is more effective than Smad6 in the inhibition of TGF- β signaling. Deletion mutants and chimeras of Smad6 and Smad7 were used to show that the MH2 domains are responsible for the inhibition of TGF- β and BMP signaling by I-Smads. However, the N-domain of Smad7 is specifically involved in the inhibition of TGF- β signaling, possibly through the association with the MH2 domain and T β R-I. *Smad4*, first identified as *DPC4* (deleted in pancreatic carcinoma, locus 4), is a tumor suppressor gene that is functionally inactivated in ~50% of pancreatic adenocarcinomas, one-third of metastatic colorectal carcinomas, and in smaller subsets of other types of tumors. The function of Smad4 as a tumor suppressor has largely been attributed to its role as a transcriptional mediator of the tumor suppressive effects of TGF- β . However, as pointed out by I. Schwarte-Waldhoff (Bochum, Germany), the role of TGF- β in carcinogenesis is a complex one. TGF- β may behave as a potent tumor suppressor at early stages of the process, but act as a tumor promoter later on. In either case, it is not yet known whether and how Smad4 is involved. Second, Smad signaling is not restricted to TGF- β cytokines, but is also regulated through cross-talk with other signaling cascades. Moreover, Smads cooperate functionally with a variety of transcription factors, coactivators and repressors. Therefore, Smad4 may function as a versatile transcriptional comodulator in a context-dependent manner. Models based on a signaling network rather than on a single linear pathway may thus be required to understand the tumor suppressor function of Smad4. When Smad4 was stably restored in Smad4-deficient human carcinoma cells, tumor suppression and the restoration of TGF- β induced growth-inhibitory response were found to be uncoupled. Reexpression of Smad4 in nude mouse xenografts of human colon and pancreatic carcinoma cells at (sub-)physiological levels was sufficient to suppress tumorigenicity. Yet, the sensitivity of these cells to TGF- β was not restored *in vitro*, indicating that loss of Smad4 and the acquisition of TGF- β resistance may be independent events in carcinogenesis. Smad4 induced a complex reprogramming of expression profiles and affected cellular functions associated with angiogenesis, invasion, and metastasis. In Hs766T pancreatic adenocarcinoma cells, Smad4 decreased the expression of vascular endothelial growth factor (VEGF) and increased the levels of the angiogenesis inhibitor thrombospondin-1, causing the cells to switch from a potentially angiogenic to an anti-angiogenic

state *in vitro* and *in vivo*. In SW480 colon carcinoma cells, Smad4 reexpression was associated with striking alterations of cell morphology towards an epithelioid phenotype. Moreover, Smad4 induced the expression of E-cadherin and P-cadherin, restoring intercellular adhesion. Loss of Smad4 at the genetic level may, therefore, be a late step in human carcinogenesis, associated with the acquisition of angiogenic, invasive, and metastatic potential.

Oncogenic Ras signaling profoundly affects gene transcription and represents a major mechanism in cancer initiation and progression. Several regulators of gene activity, including Ets-domain transcription factor Elk1, serum responsive factor (SRF), the leucine zipper protein Jun, activation transcription factor 2 (ATF2), and NF- κ B, are stimulated by signaling pathways downstream of Ras. R. Schäfer (Berlin, Germany) has undertaken a large-scale assessment of transcriptional changes associated with permanent Ras signaling in rat fibroblasts. Very recently, a partially overlapping set of genes was identified that were transcriptionally altered upon the transition of normal ovarian epithelial cells to their *KRAS*-transformed derivatives (A2/5). Gene expression profiles of non-tumorigenic 208F rat fibroblasts were contrasted with those of a *HRAS*-transformed derivative (FE-8), using subtractive suppression hybridization (SSH). More than 1200 gene fragments were recovered from forward and reverse subtraction libraries, and sequenced. A total of 823 individual cDNA fragments and differential expression of 393 genes and expressed sequence tags (EST) were recorded. FE-8 cells expressed elevated levels of a number of genes mediating invasion and metastasis. However, there was also gene repression: Several targets coding for anti-proliferative, anti-invasive, and anti-angiogenic cell properties were simultaneously down-regulated. Moreover, differentially expressed genes were recovered that were involved in diverse signal transduction processes, mitogenesis, survival, protein processing, cytoskeletal organization, stress responses, oxidative phosphorylation, glycolytic energy generation, fatty acid oxidation, transport, and cytotoxic drug processing. Of the entire transcriptome (5000–15 000 different mRNAs), 2.6–7.8% were transcriptionally altered in the *HRAS*-transformed tumorigenic fibroblasts relative to their normal counterparts. Although the gene expression profiles provided correlative rather than causal information, the specific set of genes identified by SSH can be further used to investigate functional relationships between oncogenic signaling molecules and their targets. A MEK inhibition study performed in parallel suggests that there are functional modules comprising single branches of signal transduction pathways and specific transcriptional targets. Since the blocking of the Raf/MEK/ERK pathway resulted in reversion of the transformed phenotype, the number of targets potentially

responsible for cancer cell properties may be narrowed down to a limited set of genes (<100) that need to be assayed functionally. Overall, the application of SSH has permitted the efficient identification of Ras target genes with known function as well as the unbiased isolation of novel sequences not represented among preselected genes available on DNA microarrays. The expression profiling approach may also help define novel targets for therapeutic drugs.

Since the discovery by Y. Nishizuka, that phorbol ester tumor promoters activate protein kinase C (PKC), much attention has been paid to the cellular functions regulated by this enzyme. T. Kuroki (Gifu), with M. Kashiwagi (Tokyo), focused on PKC η , which is highly expressed in epithelial tissues—especially in squamous epithelia—in close association with terminal differentiation. When differentiation was induced, PKC η was found to bind to the cdk2/cyclin E/p21 complex and to inhibit cdk2 kinase activity, leading to G₁ arrest. P21 was identified as a substrate of PKC η in this complex. Serine 146 is phosphorylated by PKC η , while PKC δ phosphorylates serines 146 and 153 located in the PCNA binding and nuclear localization domains, resulting in the functional impairment of these domains. A new isoform of PKC, PKC θ II, is generated from the *PKC θ* gene by alternative splicing. *PKC θ II* was found to be expressed exclusively in the seminiferous tubules of mouse testis in an age-dependent manner with sexual maturation. Consistent with its molecular structure, PKC θ II is constitutively active and may play an important role in spermatogenesis or some related testicular function.

The impairment of cell-cell communication *via* gap junctions is an important issue in relation to carcinogenesis. In rodent liver, gap junction channels between hepatocytes are composed of connexin (Cx) 32 and 26 proteins. K. Willecke (Bonn, Germany) has deleted the *Cx32* coding region in mice. These animals show a strongly decreased release of glucose from glycogen upon the electrical stimulation of sympathetic nerves, and an increased incidence of spontaneous and *N,N*-diethylnitrosamine-induced liver tumors, but a lack of phenobarbital-mediated promotability of hepatocarcinogenesis. In contrast, *Cx26*-deficient mice die around embryonic day 9.5. Therefore, mice were generated in which the *Cx26* coding region is flanked by loxP recognition sites of Cre recombinase, and crossed with mice expressing the *Cre* gene under the control of the albumin promoter and α -fetoprotein enhancer (provided by G. Schütz, Heidelberg, Germany). The resulting animals do not express any *Cx26* in the liver and were crossed with *Cx32*^{-/-} mice to generate doubly homozygous connexin-deficient animals. The hepatocytes of these mice were totally uncoupled, as ascertained by microinjection of Alexa Fluor 488 in precision-cut liver slices. First analyses showed a strongly increased BrdU labeling index,

indicative of hepatocyte hyperproliferation, suggesting an augmented probability of hepatocarcinogenesis compared with the *Cx32*^{-/-} animals. As in most choriocarcinoma (CC) cell lines, gap junction channels of invasive trophoblast cells in first trimester placenta are formed by *Cx40* proteins. E. Winterhager (Essen, Germany) demonstrated that the non-coupled CC cell line Jeg-3 switches to a lower proliferation rate and increased β -hCG secretion upon the restoration of cell-cell communication by transfection with *Cx26* and *Cx43*, but not *Cx40*. Upon implantation into nude mice, however, these clones ceased to express the exogenous connexin at the transcript and protein levels. Application of a tet-on vector system and a system using a eukaryotic cell-specific α -hCG promoter, led to stable, homogeneous clones expressing *Cx43* or *Cx40*. These cells were well-coupled (FACS analyses using calcein-loaded donor cells and Dil-labeled acceptor cells). The basal activity of the tet-on system was very low, and a marked upregulation of *Cx43* and *Cx40* transcripts was observed upon doxycycline treatment. In nude mice, Jeg-3 cells transfected with *Cx43* under the control of the α -hCG as well as the tet promoter exhibited a reduced tumor growth rate relative to vector control cells. Application of either vector system resulted in the differentiation of *Cx43*-transfected CC-cells into giant cells. In contrast, neither a significant reduction of tumor growth rate nor the induction of differentiation was seen in *Cx40* transfectants. A functional *Cx43* channel can thus counterbalance tumor growth by enabling the terminal differentiation of malignant trophoblast cells into non-proliferative giant cells.

Telomerase Function in Ageing and Cancer

Normal cells stop dividing after a finite number of cell divisions, a condition called senescence and supposed to be one of the defence mechanisms against cancer development. It has been widely accepted that cellular senescence is mainly caused by telomere length attrition due to the inability of conventional DNA polymerase to replicate the end of a linear DNA molecule (the “end replication problem”). However, no *in vitro* system for analysis of the molecular mechanisms underlying telomere shortening has been reported as yet, and the extent of telomere shortening is not precisely known. F. Ishikawa (Yokohama) reported on a novel *in vitro* replication system for linear DNA molecules based on SV40 DNA that will provide a valuable tool for future detailed analyses of telomere replication. While it had previously been suggested that linear DNA molecules are poor substrates for SV40-based *in vitro* replication, an optimized condition was found that permits efficient linear DNA replication. By coupling both ends of the linear DNA template to beads, the product could be examined after a single round of replication using the original template. The results indicate that DNA ends become shortened by 70 bp per cell division on average.

Stabilization of chromosome ends by the ribonucleoprotein complex telomerase is believed to be a requirement for the unlimited proliferation of cancer cells. Thus, the induction of telomerase has been reported to cause cell immortalization while retaining an otherwise normal genotype, suggesting that (i) telomerase activity may be associated with longevity, and (ii) telomerase inhibition might force cancer cells into a suicidal mode of proliferation. Earlier observations by P. Boukamp (Heidelberg, Germany) showed that the adult epidermis, a highly proliferative steady-state system, is one of the few tissues that constitutively express telomerase. Yet epidermal cells senesce and ultimately die, similar to telomerase-negative dermal fibroblasts. Under standard conditions, telomere loss in human skin keratinocytes is similar to that seen in telomerase-negative fibroblasts. Even epidermal stem cells apparently lose telomeric sequences, suggesting that telomerase *per se* is unable to counteract telomere erosion. However, telomerase activity is up-regulated in immortal cells, correlating with stable telomere length. Further increasing telomerase activity by introducing exogenous *c-myc* or *hTERT* cDNA resulted in tightly controlled telomere elongation, indicating that additional, telomerase-independent factors are involved in telomere length regulation. In the epidermis, telomerase activity is up-regulated when slowly cycling stem cells acquire the state of actively proliferating "transit amplifying cells." Preliminary evidence suggests that *c-myc*, known to positively regulate *hTERT* transcription, is involved in this pathway. Introduction of *hTERT* into telomerase-negative fibroblasts clearly increased their rate of proliferation, favoring longevity. At variance with earlier reports, however, these cells did not remain genetically normal. The observed up-regulation of cell proliferation may thus not simply be promoted by telomerase, but at least require cooperative mechanisms effected, e.g., by other genetic changes associated with cell immortalization.

Telomerase also represents an interesting target molecule for anti-cancer therapeutics. A novel, potent, and highly selective telomerase inhibitor, telomestatin, was isolated from *Streptomyces anulatus* 3533-SV4 and structurally analyzed by K. Shin-ya (Tokyo). Telomestatin inhibits telomerase activity with an IC_{50} value of 5 nM, with only slight inhibitory activity for polymerases and reverse transcriptases (IC_{50} values >10 μ M). The new inhibitor caused pronounced telomere shortening in human cancer cell lines, at a rate higher than expected from the number of population doublings in cells with senescence-like changes. Telomestatin-induced telomerase shortening and senescence correlated with the abrogation of tumorigenicity. At concentrations effective in cancer cells the drug was inactive in normal cells. Cell cycle arrest or apoptosis was also induced in cancer cells in which telomere maintenance depends on the ALT (alternative lengthening

of telomeres) system. Since telomestatin may act on telomere structure rather than on the telomerase itself, it could possibly be used in the treatment of both telomerase- and ALT-dependent cancers.

Therapeutic and Preventive Approaches Based on Molecular Cell Biology

The identification of BCRP/MXR/ABCP as a transporter of topoisomerase I (topo I) inhibitors with an indolocarbazole structure (compounds 1 and 2) was reported by S. Nishimura (Tsukuba). Compound 1 is now in phase II clinical trial. Cytotoxicity and incorporation of the drug into cancer cells are well correlated. Resistant cells exhibited an effective efflux pump. Oligonucleotide microarray technology was used to detect the transporter molecule responsible for the efflux of compound 1, and the *BCRP* gene was successfully identified among 34 020 genes. This is one of the rare cases where microarray technology has been applied to identify a target gene. Sensitive PC-13 cells transfected with a *BCRP* expression vector displayed resistance to compound 1. Interestingly, the transfectants were not resistant to mitoxantrone or topotecan, both of which had previously been identified as BCRP substrates. The *BCRP* gene from these clones as well as from normal human tissues contained AGG (Arg) in codon 482, whereas the same *BCRP* codon of the clone previously reported to be resistant to mitoxantrone or topotecan was ACG (Thr). This suggests that amino acid substitution had taken place at this position of *BCRP* in the resistant cells. *BCRP* expression levels were very low or undetectable in >50% of human cancer specimens analyzed by real-time RT-PCR, while *BCRP* expression was detected in various normal tissues. *BCRP* expression analysis may thus be a useful predictor of target cell sensitivity to indolocarbazole anticancer agents.

Strategies to increase the efficiency and selectivity of cancer radioimmunotherapy include the application of "pretargeting" procedures. K. Krüger (Essen, Germany) reported on the use of bispecific antibodies and low molecular weight haptens (LMWH), permitting the slow biodistribution phase of the antibody to be decoupled from the onset of therapeutic activity. A modular affinity system based on monoclonal antibody (Mab) EM-6-47 is applied for the two-step delivery of functional molecules to the target cells. Mab EM-6-47 binds to 3- and 3,8-substituted adenines with high affinity (K_d >10⁹ liter/mol), without cross-reacting with naturally occurring purine derivatives. This Mab serves as the hapten-specific fusion partner to produce bispecific Mabs (bs-Mabs) recognizing a target cell antigen and a LMWH as a carrier molecule for, e.g., radionuclides. Either the C-8 or the N-3 position of adenines can be used for conjugation with effector molecules; the remaining position may be substituted with different moieties in order to modulate the pharmacokinetics

of the hapten. Conjugates of different 3- and 3,8-substituted adenines to the chelates DOTA and DTPA were efficiently labeled with ^{111}In or ^{90}Y , leaving their binding to Mab EM-6-47 almost unaffected. The *in vivo* potential of this system was first ascertained in a animal model. Mab EM-6-47-coated polystyrene beads were implanted i.m. into the hind legs of rats, followed by injection of ^{111}In -labeled radiohaptens, and confirmation by γ -imaging of the selective transport of the radiohapten to the prelocalized Mab (bead:blood "targeting ratio" at 4 h after radiohapten administration, ~500:1). The system was then tested in human tumor xenografts (LS 174T colon carcinoma) in nude mice. First, bs-Mab CEA511-6-47 was applied, recognizing both the tumor-associated CEA antigen and the radiohapten. The ^{111}In -labeled radiohapten was injected 24–72 h later. The tumor:blood ratio was maximal at 4 h after radiohapten injection. This modular system is currently being further optimized and represents a powerful tool for pretargeting strategies relying on bs-Mabs and LMWH. Cellular antigens can be targeted by fusing the appropriate Mabs with the hapten-specific Mab EM-6-47, and tailor-made 3-substituted adenines may be labeled with diagnostic or therapeutic radionuclides or other functional molecules.

Both the induction of cytotoxic DNA damage and the capacity of target cells for DNA repair critically affect the efficacy of DNA-reactive anticancer agents. J. Thomale (Essen, Germany) reported on a set of novel Mabs generated for the quantitation of different platinum (Pt)-DNA adducts at the single-cell level and in specific gene sequences. The kinetics of formation and removal of defined Pt-adducts in the nuclear DNA, e.g., of gastric cancer cells from patients undergoing Pt-chemotherapy, exhibited distinct inter-cell and inter-individual differences, and are to be correlated with the clinical outcome. Nucleotide excision repair (NER) is considered an important pathway for the processing of Pt-DNA lesions. In joint studies with K. Tanaka (Osaka), the acute toxicity of Pt was found to be far higher in NER-deficient mice lacking a functional XPA protein compared with their wild-type counterparts. This hypersensitivity correlated with a much higher accumulation of Pt-adducts in the cellular DNA of NER-deficient mice within the first 12 h after Pt administration. However, both $\text{XPA}^{+/+}$ and $\text{XPA}^{-/-}$ mice were still able to remove a significant fraction (30–50%) of Pt-adducts from the nuclear DNA of various cell types. XPA-dependent NER thus appears to act predominantly on the monovalent Pt-DNA adducts initially formed during Pt-DNA interaction. Once cross-linking to the neighboring base has taken place, other modes of DNA repair seem to become involved. Gene sequence-specific repair analyses may be used to address the question of why almost half of the G-G intrastrand cross-links formed in the nuclear DNA of mouse cells apparently remain inaccessible to repair.

Cyclooxygenase-2 (COX-2), the product of the immediate-early gene *Ptgs-2*, is both an endogenous tumor promoter and a target for cancer chemoprevention. As pointed out by F. Marks (Heidelberg, Germany), this cancer chemopreventive effect of nonsteroidal anti-inflammatory drugs (NSAID), e.g. Aspirin, has been firmly established for human colorectal cancer, and most likely results from the inhibition of COX-2. In animal models, the development of a wide variety of epithelial cancers has been found to be NSAID-susceptible. In a key reaction of prostanoïd biosynthesis, cyclooxygenases catalyze the oxygenation of arachidonic acid to prostaglandin endoperoxide. Overproduction of prostaglandins is thought to enhance tumorigenesis by inhibiting terminal differentiation and apoptosis, by stimulating cell proliferation and angiogenesis, and by suppressing the immune system. COX-2 is strongly but reversibly expressed in most tissues only when a higher supply of prostanoids is required (e.g., upon hormonal stimulation, environmental stress, or during inflammation). The *Ptgs-2* promoter contains numerous elements responsive to a wide variety of exogenous and endogenous stimuli. *Ptgs-2* is targeted by the three known MAP-kinase cascades, by the NF- κ B cascade, and by other signal transducing pathways, explaining the extraordinary range of stimulatory effects. Aberrant COX-2 expression seems to be a feature of many neoplastic tissues and preneoplastic conditions. Constitutive overexpression of COX-2 in tumors appears to be due to autocrine dysregulation mediated by growth factors and prostaglandins, or as a consequence of mutational proto-oncogene activation or tumor suppressor gene inactivation. Studies with COX-2-deficient animals and the powerful cancer-preventive effects of specific COX-2 inhibitors strongly support the relationship between COX-2 expression and cancer development. The effects of aberrant constitutive COX-2 expression on normal and pathological skin development were studied in transgenic mice carrying the complete *Ptgs-2* gene under the control of the keratin-5 promoter (selectively expressed in the basal cells of stratified epithelia). The epidermis of the heterozygous transgenics displayed a dysplastic morphology, hyperplasia and hyperkeratosis, loss of cell polarity, the occurrence of proliferative cells in suprabasal cell layers, endophytic papillary growth into the underlying dermis, and an almost 3-fold higher density of blood capillaries relative to wild-type animals. These preneoplastic changes are also seen in the course of two-stage skin carcinogenesis, and could be completely reversed by treatment with a specific COX-2 inhibitor starting immediately after birth. When dimethylbenz(a)anthracene is applied as the initiator, wild-type mice develop skin papillomas and carcinomas only when subsequently exposed to the tumor promoter TPA for several weeks. In contrast, TPA treatment was not required for tumor development in the transgenics, i.e., COX-2

overexpression compensated for the effect of TPA. Whether chemoprevention by COX-2 inhibition will remain restricted to individuals/populations at high risk for developing particular types of cancer, or become more generally applicable, will largely depend on the management of side-effects expected to occur upon long-term inhibition of COX-2. Because lipoxygenase-catalyzed arachidonic acid metabolism may also play an important role in tumorigenesis, future cancer chemoprevention may combine the use of specific drugs inhibiting cyclooxygenases, lipoxygenases, and perhaps arachidonic acid-releasing phospholipases, admixed with less specific antioxidants and radical scavengers, and accompanied by the appropriate adjustment of life-style.

Like the previous seven Japanese-German Workshops of the Essen series, this conference convened outstanding cancer researchers from both countries for in-depth scientific exchange and the initiation of collaborative projects in an informal and friendly seminar atmosphere. Twelve Japanese and eleven German speakers, one speaker from Switzerland, and nine invited discussants, reported on topical studies on the molecular cell biology and genetics of experimental and human cancer, and on new approaches towards improved cancer diagnostics and therapy. The Workshop participants were impressed by rapid advances in the characterization of cancer-relevant genes and proteins and their involvement in different pathways and effector mechanisms. It was equally clear, however, that the multiplicity and complexity of regulatory networks in mammalian cells still represent major barriers to our understanding of the process(es) of carcinogenesis and our capability to prevent or cure human cancers.

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