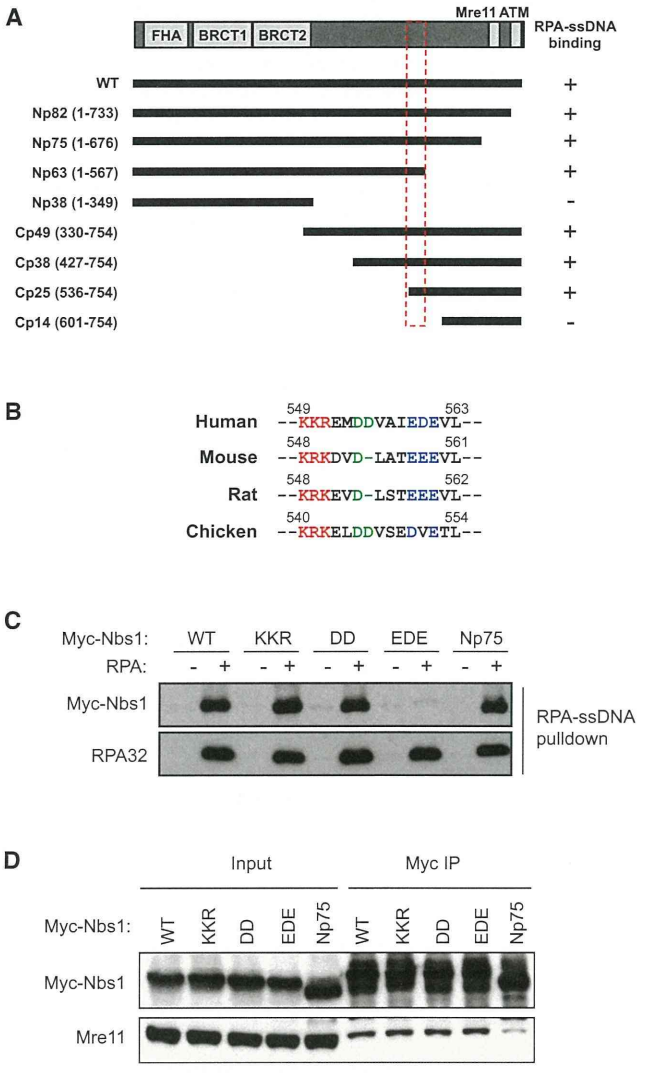

Figure 6. Mapping the RPA-ssDNA-Interacting Domain of Nbs1

(A) Schematic of the Nbs1 fragments used in this study and their ability to bind RPA-ssDNA.

(B) The RPA-ssDNA-binding domain of human Nbs1 is aligned to the corresponding regions in the Nbs1 of other higher vertebrates. The conserved charged amino acids are highlighted in colors.

(C) The conserved charged amino acids in the RPA-ssDNA-binding domain of Nbs1 were mutated in myc-tagged, full-length Nbs1 (KKR to EEG, DD to AA, and EDE to AAA). These Nbs1 mutants were transiently expressed in 293T cells, and their ability to bind RPA-ssDNA was tested in cell extracts using biotinylated ssDNA coated with RPA. WT Nbs1 and an Nbs1 mutant lacking the Mre11- and ATM-binding domains (Np75) were also tested as controls.

(D) Myc-tagged, full-length Nbs1 (WT) and its mutant derivatives (KKR, DD, EDE, and Np75) were transiently expressed in 293T cells. The myc-tagged Nbs1 proteins were immunoprecipitated with myc antibody, and the coprecipitated Mre11 was analyzed by western blot using Mre11 antibody.

See also Figure S3.

How do Rad17 and Nbs1 regulate the activation of ATR during resection? As resection is initiated at DSBs and ssDNA/dsDNA junctions are generated, the Rad17-RFC complex recognizes these junctions and loads 9-1-1 complexes onto dsDNA (Figure 7D). This Rad17- and ssDNA/dsDNA-junction-mediated process promotes ATR activation and Chk1 phosphorylation around the ssDNA/dsDNA junctions. In addition, a fraction of RPA adjacent to ssDNA/dsDNA junctions is phosphorylated by ATR in a Rad17-dependent manner during this early phase of resection. As resection continues, the ssDNA is gradually lengthened, and increasing amounts of RPA and ATR-ATRIP are placed on the ssDNA distal to ssDNA/dsDNA junctions (Figure 7D). In the late phase of resection, a fraction of RPA and ATR-ATRIP on ssDNA becomes "out of reach" for the Rad17 and 9-1-1 complexes at ssDNA/dsDNA junctions. In this situation, the MRN complex may directly recognize RPA-ssDNA via Nbs1, and activate ATR-ATRIP by recruiting TopBP1 (Yoo et al., 2009). Consistent with this model, substantial RPA32 Ser33 phosphorylation is driven by resection and is dependent upon both Nbs1 and TopBP1 in vivo and in vitro. Furthermore, the direct interaction between Nbs1 and RPA-ssDNA is needed for efficient RPA32 Ser33 phosphorylation both in vivo and in vitro. Since the Nbs1-mediated mode of ATR activation is independent of Rad17, which interacts with Claspin to promote Chk1 activation (Wang et al., 2006), this mode of ATR activation is specifically directed to RPA but not Chk1.

When and where does Nbs1 activate ATR in cells? In laser microirradiated cells, a fraction of Nbs1 precisely colocalizes with RPA in the ssDNA subcompartments at DSBs (Bekker-Jensen et al., 2006). In the context of DNA replication, Mre11 partially colocalizes with PCNA during S phase (Maser et al., 2001). Although RPA32 Ser33 phosphorylation is rapidly induced by CPT, it does not occur robustly in hydroxyurea (HU)-treated cells until DSBs become detectable in these cells (Figures 1A and S1F; Sakasai et al., 2006). This observation, together with the dependency of RPA32 Ser33 phosphorylation on resection, suggests that RPA32 Ser33 phosphorylation occurs primarily at replication-associated DSBs. Indeed, in previous studies, the phosphorylation of RPA32 was implicated in the progression and repair of stressed replication forks (Liu et al., 2012; Shi et al., 2010; Vassin et al., 2009). We found that when the interaction between Nbs1 and RPA-ssDNA was disrupted, the recovery of collapsed replication forks was compromised (Figure 7C). These findings suggest that the Nbs1-mediated mode of ATR activation may be particularly important for the repair of replication-associated DSBs. Consistent with this idea, the MRN complex was shown to colocalize with RPA at collapsed forks (Manthey et al., 2007). Furthermore, the MRN complex is required for preventing accumulation of DSBs during replication (Costanzo et al., 2001). In addition to the mechanism that we propose, recent studies suggested that Mre11 degrades stalled replication forks in the absence of BRCA1/2 and Fanconi anemia proteins, and promotes ATR activation via a nuclease-dependent mechanism at stalled forks (Lee and Dunphy, 2013; Schlacher et al., 2011, 2012). Another recent study showed that the *Xenopus* MRN complex recognizes the ssDNA/dsDNA junctions in synthetic DNA templates and recruits TopBP1 to DNA (Duursma et al., 2013). We note that the EDE motif of human Nbs1 is not conserved in *Xenopus* Nbs1, suggesting that the role of MRN in ATR activation may have expanded during evolution. The MRN complex may regulate the ATR response and protect replication forks through multiple mechanisms.

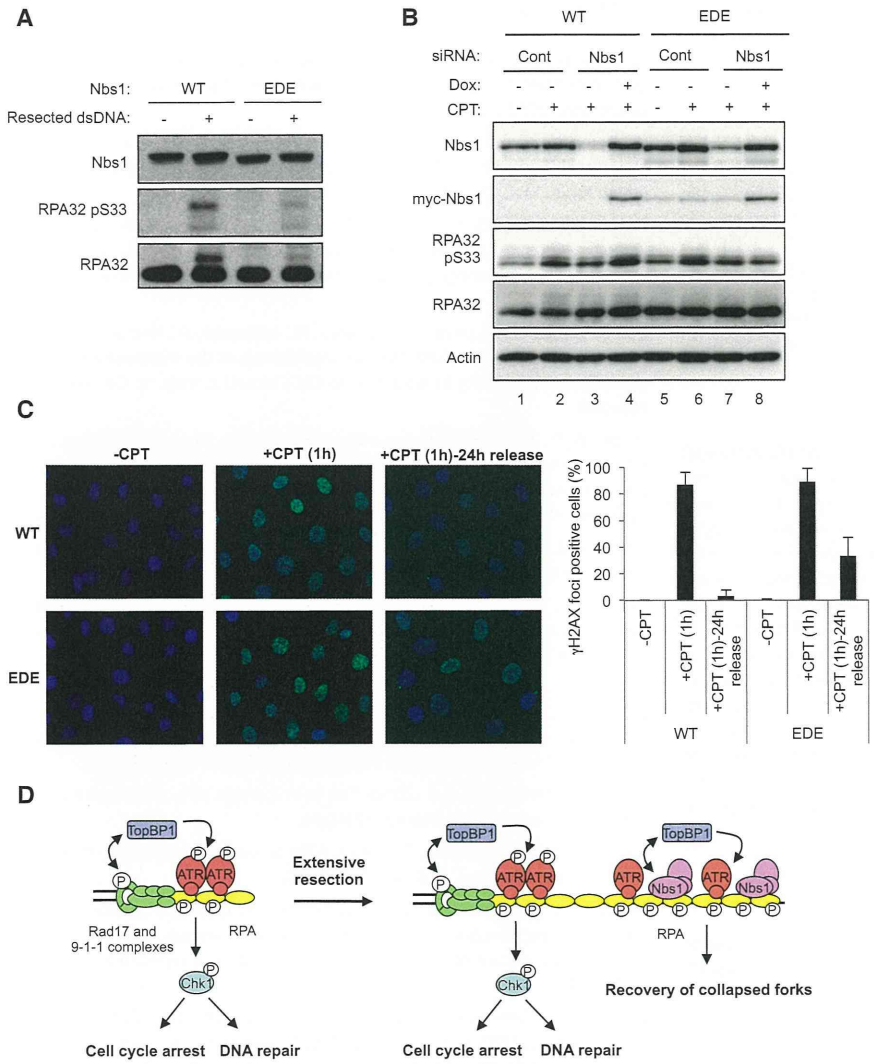

Figure 7. The Interaction of Nbs1 and RPA Is Required for Nbs1-Mediated RPA32 Phosphorylation and Recovery of Collapsed Replication Forks

(A) U2OS-derivative cell lines were established to express myc-tagged Nbs1^{WT} and Nbs1^{EDE} conditionally. As indicated, cells were treated with 1 μg/ml doxycycline (Dox) to induce myc-tagged Nbs1 proteins, and transfected with siNbs1-2 to knock down endogenous Nbs1. Nuclear extracts were prepared from cells, and the ability of resected dsDNA to induce RPA32 Ser33 phosphorylation was analyzed with phosphospecific antibody.

(B) Myc-tagged Nbs1^{WT} and Nbs1^{EDE} were used to replace endogenous Nbs1 as in (A). Cells were synchronized in S phase as described in Experimental Procedures and subsequently treated with CPT (1 μM) for 45 min or mock treated. The levels of the indicated proteins and the phosphorylation of RPA32 Ser33 were analyzed by western blot.

(C) Myc-tagged Nbs1^{WT} and Nbs1^{EDE} were used to replace endogenous Nbs1 as in (A). Cells were synchronized in S phase, treated with CPT (1 μM) for 1 hr or mock treated, and then released into CPT-free medium. After 24 hr, cells were immunostained with antibody to γH2AX and the fractions of cells with γH2AX foci were quantified. Error bars: SDs from three independent experiments (n = 3).

(D) A model that depicts the two distinct modes of ATR activation at resected DSBs.

It is important to note that although the phosphorylation of RPA32 Ser33 is a marker for Nbs1-mediated ATR activation, ATR may have additional substrates when it is activated in this mode. Many of the known or putative substrates of ATR, including a number of DNA repair proteins, interact with RPA and may accumulate on RPA-ssDNA during resection (Matsuoka et al., 2007). The Nbs1-mediated mode of ATR activation may contribute to the phosphorylation of these ATR substrates and to DNA repair, particularly during the late phase of the DNA damage response. Together, Rad17 and Nbs1 may orchestrate the phosphorylation of two distinct sets of ATR substrates in two sequential phases of the DNA damage response. Whereas Rad17 may promote DNA damage signaling, cell-cycle arrest, and the early events of DNA repair through Chk1 phosphorylation, Nbs1 may promote the late events of DNA repair through progressive phosphorylation of ATR substrates on RPA-ssDNA. The coordination of these two distinct mechanisms of ATR activation may be important for the full function of ATR in the DNA damage response.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment

HeLa, U2OS, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NBS-ILB1 cells were cultured in DMEM supplemented with 15% FBS. NBS1-ILB1 cells stably expressing the full-length Nbs1 transgene were maintained in the same medium containing 500 μg/ml G418. For RNAi, cells were transfected with 40 nM siRNA using RNAi MAX transfection reagent (Invitrogen). The sequences of the siRNAs used in this study are listed in the Extended Experimental Procedures. Plasmid transfection of 293T cells was performed with Lipofectamine 2000 (Invitrogen) and the transfected cells were analyzed after 48 hr. U2OS derivative cell lines stably expressing myc-Nbs1 or mutants were cultured in DMEM with 10% FBS and 100 μg/mL Zeocin (Invitrogen). Expression of myc-Nbs1 was induced with 1 μg/mL doxycycline (Sigma). To synchronize cells in S phase, cells were treated with 2.5 mM thymidine (Sigma) for 20 hr, washed thoroughly with PBS, and released into thymidine-free medium for 3 hr. To assess the recovery of collapsed replication forks, synchronized S phase cells were treated with CPT for 1 hr and then released into CPT-free medium. Kinase inhibitors VE-821, KU55933, and NU7026 were used at 10 μM. CPT was used at 1 μM.

Antibodies

The phosphospecific antibodies to ATR pT1989 were previously described (Liu et al., 2011). We obtained ATR, Nbs1, Mre11, Rad50, TopBP1, phospho-Rad17 (Ser645), and phospho-RPA32 (Ser33 and Ser4/Ser8) antibodies from Bethyl; Rad17 and Chk1 antibodies from Santa Cruz; phospho-Chk1 (Ser345), phospho-Chk2 (Thr68), and phospho-p53 (Ser15) antibodies from Cell Signaling; phospho-H2AX (Ser139) antibody from Millipore; phospho-ATM (Ser1981) antibody from Epitomics; RPA32 antibody from Thermo

Scientific; and Myc antibody from Medical & Biological Laboratories. Phospho-DNA-PKcs (Ser2056) and CtIP antibodies were kindly provided by Drs. B. Chen and R. Baer, respectively.

Extract-Based ATR Activation Assay

An extract-based ATR activation assay was performed as described previously (Shiotani and Zou, 2011). To analyze ssDNA-induced ATR activation, nuclear extracts were pretreated with 10 μM of KU-55933 and NU7026 for 15 min on ice to inhibit ATM and DNA-PKcs, and supplemented with the reaction buffer (buffer R), which brought the final buffer compositions to 10 mM HEPES (pH 7.6), 50 mM KCl, 0.1 mM $MgCl_2$, 1 mM phenylmethanesulfonylfluoride, 0.5 mM dithiothreitol, 1 mM ATP, 10 μg/ml creatine kinase, and 5 mM phosphocreatine. ssDNA of various lengths was incubated in the extracts for 30 min at 37°C. The sequences of the DNA oligonucleotides used in this study are listed in the Extended Experimental Procedures.

RPA-ssDNA Binding Assay

Biotinylated ssDNA (5′TGCAGCTGGCACGACAGGTTTTAATGAATCGGCCA-ACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCT-biotin-3′) was attached to streptavidin-coated magnetic beads in binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10% glycerol, 0.01% NP-40, and 10 μg/ml bovine serum albumin) followed by incubation with or without purified RPA at room temperature for 30 min. To analyze the binding of various proteins to RPA-ssDNA, we incubated RPA-ssDNA with purified MRN, Nbs1, or cell lysates in the binding buffer. To map the RPA-ssDNA-interacting domain of Nbs1, we used lysates prepared from cells expressing various myc-Nbs1 mutants. After a 30 min incubation at room temperature, beads were retrieved and washed twice with the binding buffer. The proteins bound to beads were denatured in the SDS sample buffer, separated on SDS-PAGE, and analyzed by western blot. Recombinant RPA complex was purified from *Escherichia coli* as previously described (Henricksen et al., 1994). The MRN complex and Nbs1 were purified from insect cells infected with baculoviruses expressing Mre11, Rad50, and Nbs1 (Paull and Gellert, 1999).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.018.

LICENSING INFORMATION

ACKNOWLEDGMENTS

We thank Dr. K. Cimprich for communicating previously unpublished results (Duursma et al., 2013); Drs. S. Elledge, A. Nussenzweig, T. Paull, X. Wu, B. Chen, R. Baer, P. Concannon, and K. Komatsu for reagents; and members of the Zou lab for helpful discussions. P.H. was supported by a postdoctoral fellowship from the Swedish Research Council. A.M. is supported by an FRSQ fellowship. L.Z. is a Jim and Ann Orr Massachusetts General Hospital Research Scholar. This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to B.S. (KAKENHI 24800044) and H.T. (KAKENHI 23300363), and grants from the NIH (GM076388), the ACS (RSG-08-297), and the Federal Share of MGH Proton Program to L.Z.

Received: October 16, 2012
Revised: April 5, 2013
Accepted: April 19, 2013
Published: May 16, 2013

REFERENCES

Anantha, R.W., Vassin, V.M., and Borowiec, J.A. (2007). Sequential and synergistic modification of human RPA stimulates chromosomal DNA repair. J. Biol. Chem. *282*, 35910–35923.

Avemann, K., Knippers, R., Koller, T., and Sogo, J.M. (1988). Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. Mol. Cell. Biol. *8*, 3026–3034.

Barlow, J.H., Faryabi, R.B., Callén, E., Wong, N., Malhowski, A., Chen, H.T., Gutierrez-Cruz, G., Sun, H.W., McKinnon, P., Wright, G., et al. (2013). Identification of early replicating fragile sites that contribute to genome instability. Cell *152*, 620–632.

Bekker-Jensen, S., Lukas, C., Kitagawa, R., Melander, F., Kastan, M.B., Bartek, J., and Lukas, J. (2006). Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J. Cell Biol. *173*, 195–206.

Block, W.D., Yu, Y., and Lees-Miller, S.P. (2004). Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases (PIKKs) are required for DNA damage-induced phosphorylation of the 32 kDa subunit of replication protein A at threonine 21. Nucleic Acids Res. *32*, 997–1005.

Brown, E.J., and Baltimore, D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev. *14*, 397–402.

Byun, T.S., Pacek, M., Yee, M.C., Walter, J.C., and Cimprich, K.A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev. *19*, 1040–1052.

Carney, J.P., Maser, R.S., Olivares, H., Davis, E.M., Le Beau, M., Yates, J.R., 3rd, Hays, L., Morgan, W.F., and Petrini, J.H. (1998). The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell *93*, 477–486.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Mol. Cell *40*, 179–204.

Cimprich, K.A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. Nat. Rev. Mol. Cell Biol. *9*, 616–627.

Costanzo, V., Robertson, K., Bibikova, M., Kim, E., Grieco, D., Gottesman, M., Carroll, D., and Gautier, J. (2001). Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. Mol. Cell *8*, 137–147.

Costanzo, V., Shechter, D., Lupardus, P.J., Cimprich, K.A., Gottesman, M., and Gautier, J. (2003). An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. Mol. Cell *11*, 203–213.

Cotta-Ramusino, C., McDonald, E.R., 3rd, Hurov, K., Sowa, M.E., Harper, J.W., and Elledge, S.J. (2011). A DNA damage response screen identifies RHINO, a 9-1-1 and TopBP1 interacting protein required for ATR signaling. Science *332*, 1313–1317.

Delacroix, S., Wagner, J.M., Kobayashi, M., Yamamoto, K., and Karnitz, L.M. (2007). The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. Genes Dev. *21*, 1472–1477.

Difilippantonio, S., Celeste, A., Fernandez-Capetillo, O., Chen, H.T., Reina San Martin, B., Van Laethem, F., Yang, Y.P., Petukhova, G.V., Eckhaus, M., Feigenbaum, L., et al. (2005). Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. Nat. Cell Biol. *7*, 675–685.

Duursma, A.M., Driscoll, R., Elias, J.E., and Cimprich, K.A. (2013). A Role for the MRN Complex in ATR Activation via TOPBP1 Recruitment. Mol. Cell *50*, 116–122.

Ellison, V., and Stillman, B. (2003). Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5′ recessed DNA. PLoS Biol. *1*, E33.

Flynn, R.L., and Zou, L. (2011). ATR: a master conductor of cellular responses to DNA replication stress. Trends Biochem. Sci. *36*, 133–140.

Guo, Z., and Dunphy, W.G. (2000). Response of Xenopus Cds1 in cell-free extracts to DNA templates with double-stranded ends. Mol. Biol. Cell *11*, 1535–1546.

Henricksen, L.A., Umbricht, C.B., and Wold, M.S. (1994). Recombinant replication protein A: expression, complex formation, and functional characterization. J. Biol. Chem. *269*, 11121–11132.

Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G.C., Lukas, J., and Jackson, S.P. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat. Cell Biol. *8*, 37–45.

Kousholt, A.N., Fugger, K., Hoffmann, S., Larsen, B.D., Menzel, T., Sartori, A.A., and Sørensen, C.S. (2012). CtIP-dependent DNA resection is required for DNA damage checkpoint maintenance but not initiation. J. Cell Biol. *197*, 869–876.

Kumagai, A., Lee, J., Yoo, H.Y., and Dunphy, W.G. (2006). TopBP1 activates the ATR-ATRIP complex. Cell *124*, 943–955.

Lee, J., and Dunphy, W.G. (2010). Rad17 plays a central role in establishment of the interaction between TopBP1 and the Rad9-Hus1-Rad1 complex at stalled replication forks. Mol. Biol. Cell *21*, 926–935.

Lee, J., and Dunphy, W.G. (2013). The Mre11-Rad50-Nbs1 (MRN) complex has a specific role in the activation of Chk1 in response to stalled replication forks. Mol. Biol. Cell. Published online March 6, 2013. http://dx.doi.org/10.1091/mbc.E13-01-0025.

Lee, J., Kumagai, A., and Dunphy, W.G. (2007). The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. J. Biol. Chem. *282*, 28036–28044.

Liaw, H., Lee, D., and Myung, K. (2011). DNA-PK-dependent RPA2 hyperphosphorylation facilitates DNA repair and suppresses sister chromatid exchange. PLoS ONE *6*, e21424.

Lin, S.J., Wardlaw, C.P., Morishita, T., Miyabe, I., Chahwan, C., Caspari, T., Schmidt, U., Carr, A.M., and Garcia, V. (2012). The Rad4(TopBP1) ATR-activation domain functions in G1/S phase in a chromatin-dependent manner. PLoS Genet. *8*, e1002801.

Liu, S., Bekker-Jensen, S., Mailand, N., Lukas, C., Bartek, J., and Lukas, J. (2006). Claspin operates downstream of TopBP1 to direct ATR signaling towards Chk1 activation. Mol. Cell. Biol. *26*, 6056–6064.

Liu, S., Shiotani, B., Lahiri, M., Maréchal, A., Tse, A., Leung, C.C., Glover, J.N., Yang, X.H., and Zou, L. (2011). ATR autophosphorylation as a molecular switch for checkpoint activation. Mol. Cell *43*, 192–202.

Liu, S., Opiyo, S.O., Manthey, K., Glanzer, J.G., Ashley, A.K., Amerin, C., Troksa, K., Shrivastav, M., Nickoloff, J.A., and Oakley, G.G. (2012). Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. Nucleic Acids Res. *40*, 10780–10794.

MacDougall, C.A., Byun, T.S., Van, C., Yee, M.C., and Cimprich, K.A. (2007). The structural determinants of checkpoint activation. Genes Dev. *21*, 898–903.

Manthey, K.C., Opiyo, S., Glanzer, J.G., Dimitrova, D., Elliott, J., and Oakley, G.G. (2007). NBS1 mediates ATR-dependent RPA hyperphosphorylation following replication-fork stall and collapse. J. Cell Sci. *120*, 4221–4229.

Maser, R.S., Mirzoeva, O.K., Wells, J., Olivares, H., Williams, B.R., Zinkel, R.A., Farnham, P.J., and Petrini, J.H. (2001). Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. Mol. Cell. Biol. *21*, 6006–6016.

Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., et al. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science *316*, 1160–1166.

Mordes, D.A., Glick, G.G., Zhao, R., and Cortez, D. (2008). TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. Genes Dev. *22*, 1478–1489.

Murga, M., Bunting, S., Montaña, M.F., Soria, R., Mulero, F., Cañamero, M., Lee, Y., McKinnon, P.J., Nussenzweig, A., and Fernandez-Capetillo, O. (2009). A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nat. Genet. *41*, 891–898.

Myers, J.S., and Cortez, D. (2006). Rapid activation of ATR by ionizing radiation requires ATM and Mre11. J. Biol. Chem. *281*, 9346–9350.

Navadgi-Patil, V.M., and Burgers, P.M. (2009). The unstructured C-terminal tail of the 9-1-1 clamp subunit Ddc1 activates Mec1/ATR via two distinct mechanisms. Mol. Cell *36*, 743–753.

Oakley, G.G., Tillison, K., Opiyo, S.A., Glanzer, J.G., Horn, J.M., and Patrick, S.M. (2009). Physical interaction between replication protein A (RPA) and MRN: involvement of RPA2 phosphorylation and the N-terminus of RPA1. Biochemistry *48*, 7473–7481.

Olson, E., Nievera, C.J., Lee, A.Y., Chen, L., and Wu, X. (2007a). The Mre11-Rad50-Nbs1 complex acts both upstream and downstream of ataxia telangiectasia mutated and Rad3-related protein (ATR) to regulate the S-phase checkpoint following UV treatment. J. Biol. Chem. *282*, 22939–22952.

Olson, E., Nievera, C.J., Liu, E., Lee, A.Y., Chen, L., and Wu, X. (2007b). The Mre11 complex mediates the S-phase checkpoint through an interaction with replication protein A. Mol. Cell. Biol. *27*, 6053–6067.

Paull, T.T., and Gellert, M. (1999). Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. Genes Dev. *13*, 1276–1288.

Peterson, S.E., Li, Y., Wu-Baer, F., Chait, B.T., Baer, R., Yan, H., Gottesman, M.E., and Gautier, J. (2013). Activation of DSB processing requires phosphorylation of CtIP by ATR. Mol. Cell *49*, 657–667.

Reaper, P.M., Griffiths, M.R., Long, J.M., Charrier, J.D., Maccormick, S., Charlton, P.A., Golec, J.M., and Pollard, J.R. (2011). Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. Nat. Chem. Biol. *7*, 428–430.

Sacho, E.J., and Maizels, N. (2011). DNA repair factor MRE11/RAD50 cleaves 3′-phosphotyrosyl bonds and resects DNA to repair damage caused by topoisomerase 1 poisons. J. Biol. Chem. *286*, 44945–44951.

Sakasai, R., Shinohe, K., Ichijima, Y., Okita, N., Shibata, A., Asahina, K., and Teraoka, H. (2006). Differential involvement of phosphatidylinositol 3-kinase-related protein kinases in hyperphosphorylation of replication protein A2 in response to replication-mediated DNA double-strand breaks. Genes Cells *11*, 237–246.

Sartori, A.A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S.P. (2007). Human CtIP promotes DNA end resection. Nature *450*, 509–514.

Schlacher, K., Christ, N., Siaud, N., Egashira, A., Wu, H., and Jasin, M. (2011). Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. Cell *145*, 529–542.

Schlacher, K., Wu, H., and Jasin, M. (2012). A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. Cancer Cell *22*, 106–116.

Shi, W., Feng, Z., Zhang, J., Gonzalez-Suarez, I., Vanderwaal, R.P., Wu, X., Powell, S.N., Roti Roti, J.L., Gonzalo, S., and Zhang, J. (2010). The role of RPA2 phosphorylation in homologous recombination in response to replication arrest. Carcinogenesis *31*, 994–1002.

Shiotani, B., and Zou, L. (2009). Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. Mol. Cell *33*, 547–558.

Shiotani, B., and Zou, L. (2011). A human cell extract-based assay for the activation of ATM and ATR checkpoint kinases. Methods Mol. Biol. *782*, 181–191.

Stewart, G.S., Maser, R.S., Stankovic, T., Bressan, D.A., Kaplan, M.I., Jaspers, N.G., Raams, A., Byrd, P.J., Petrini, J.H., and Taylor, A.M. (1999). The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. Cell *99*, 577–587.

Stiff, T., Reis, C., Alderton, G.K., Woodbine, L., O'Driscoll, M., and Jeggo, P.A. (2005). Nbs1 is required for ATR-dependent phosphorylation events. EMBO J. *24*, 199–208.

Stracker, T.H., and Petrini, J.H. (2011). The MRE11 complex: starting from the ends. Nat. Rev. Mol. Cell Biol. *12*, 90–103.

Symington, L.S., and Gautier, J. (2011). Double-strand break end resection and repair pathway choice. Annu. Rev. Genet. *45*, 247–271.

Toledo, L.I., Murga, M., Zur, R., Soria, R., Rodriguez, A., Martinez, S., Oyarzabal, J., Pastor, J., Bischoff, J.R., and Fernandez-Capetillo, O. (2011). A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. Nat. Struct. Mol. Biol. *18*, 721–727.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003). Requirement of the MRN complex for ATM activation by DNA damage. EMBO J. *22*, 5612–5621.

Van, C., Yan, S., Michael, W.M., Waga, S., and Cimprich, K.A. (2010). Continued primer synthesis at stalled replication forks contributes to checkpoint activation. J. Cell Biol. *189*, 233–246.

Vassin, V.M., Anantha, R.W., Sokolova, E., Kanner, S., and Borowiec, J.A. (2009). Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. J. Cell Sci. *122*, 4070–4080.

Wang, X., Zou, L., Lu, T., Bao, S., Hurov, K.E., Hittelman, W.N., Elledge, S.J., and Li, L. (2006). Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. Mol. Cell *23*, 331–341.

Wang, J., Gong, Z., and Chen, J. (2011). MDC1 collaborates with TopBP1 in DNA replication checkpoint control. J. Cell Biol. *193*, 267–273.

Yamane, K., Chen, J., and Kinsella, T.J. (2003). Both DNA topoisomerase II-binding protein 1 and BRCA1 regulate the G2-M cell cycle checkpoint. Cancer Res. *63*, 3049–3053.

Yan, S., and Michael, W.M. (2009). TopBP1 and DNA polymerase-alpha directly recruit the 9-1-1 complex to stalled DNA replication forks. J. Cell Biol. *184*, 793–804.

Yoo, H.Y., Kumagai, A., Shevchenko, A., Shevchenko, A., and Dunphy, W.G. (2009). The Mre11-Rad50-Nbs1 complex mediates activation of TopBP1 by ATM. Mol. Biol. Cell *20*, 2351–2360.

Zhong, H., Bryson, A., Eckersdorff, M., and Ferguson, D.O. (2005). Rad50 depletion impacts upon ATR-dependent DNA damage responses. Hum. Mol. Genet. *14*, 2685–2693.

Zou, L., and Elledge, S.J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542–1548.

Zou, L., Cortez, D., and Elledge, S.J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev. *16*, 198–208.

Zou, L., Liu, D., and Elledge, S.J. (2003). Replication protein A-mediated recruitment and activation of Rad17 complexes. Proc. Natl. Acad. Sci. USA *100*, 13827–13832.

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2013;43(5)579–582
doi:10.1093/jjco/hyt037
Advance Access Publication 13 March 2013

Meeting Report

MicroRNAs in Cancer: The 22nd Hiroshima Cancer Seminar/The 4th Japanese Association for RNA Interference Joint International Symposium, 30 August 2012, Grand Prince Hotel Hiroshima

Hidetoshi Tahara[1], Mark A. Kay[2], Wataru Yasui[3] and Eiichi Tahara[4]

[1]Department of Cellular and Molecular Biology, Hiroshima University Graduate School of Biomedical Science, Hiroshima, Japan, [2]Department of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA, USA, [3]Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Science, and [4]Hiroshima Cancer Seminar Foundation, Hiroshima, Japan

*For reprints and all correspondence: Eiichi Tahara, Hiroshima Cancer Seminar Foundation, 3-8-6 Sendamachi, Nakaku-ku, Hiroshima 730-0052, Japan. E-mail: etahara@h-gan.com

Received December 26, 2012; accepted February 15, 2013

The joint international symposium of the 22nd Hiroshima Cancer Seminar and the 4th Japanese Association for RNA Interference focused on a pivotal role of microRNAs in carcinogenesis, progression and therapy of human cancer. Mammalian immune regulator MCPIP1 (Zc3h12a) RNase acts as a novel suppressor of microRNA activity and biogenesis, suggesting the involvement of MCPIP1 in the alteration of microRNA biogenesis in tumorigenesis. Gene set enrichment analysis and functional assignment of microRNAs via enrichment analysis enable the prediction of microRNA activities from mRNA expression data by combining rank-based enrichment analysis and weighted evaluation of microRNA–mRNA interactions. MiR-124 and miR-203 function as tumor-suppressor microRNAs silenced by DNA methylation in hepatocellular carcinoma. Stella-induced DNA hypomethylation would confer the pathogenic function of DNA hypomethylation in cancer. Senescence-associated microRNA, miR-22, suppresses tumor growth and metastasis *in vivo* in a murine breast cancer model, and exosomal senescence-associated microRNA may affect the tumor microenvironment. The therapeutic potential of microRNAs for preventing and treating lung cancer using the $Kras^{LSL\text{-}G12D/+}$;$p53^{LSL\text{-}R172H/+}$mouse model suggests that miR-34 may be useful in sensitizing tumors to other conventional therapeutics. MiR-1 and miR-133a cluster may function as tumor suppressors regulating novel pathways in human cancers. The down-regulation of miR-148a is implicated in invasion of gastric cancer, while high miR-21 expression in colorectal cancer is associated with poor survival. Neutral sphingomyelinase 2 regulates exosomal microRNA secretion and promotes angiogenesis within the tumor microenvironment as well as metastasis; in particular, the exosomal miR-210 secretion by neutral sphingomyelinase 2 confers the formation of the tumor vessel network.

Key words: microRNAs – biomarkers – carcinogenesis – progression – DNA methylation – tumor microenvironment – exosome

The 22nd International Symposium of Hiroshima Cancer Seminar (HCS) in conjunction with the 4th Japanese Association for RNA Interference was held on 30 August 2012 at Grand Prince Hotel Hiroshima. The symposium composed of nine invited speakers, had ~200 participants and made active discussion on a pivotal role of microRNAs (miRNAs) in carcinogenesis, progression and early detection as well as therapeutic strategies of human cancers. Invited speakers include Hiroshi I. Suzuki (University of Tokyo, Tokyo), Johji Inazawa (Tokyo Medical and Dental University,

Tokyo), Toru Nakano (Osaka University, Osaka), Mark A.K. (Stanford University), H.T.(Hiroshima University, Hiroshima), Andrea L. Kasinski (Yale University, USA) and Naohiko Seki (Chiba University, Chiba), Naohide Oue (Hiroshima University, Hiroshima) and Nobuyoshi Kosaka (National Cancer Center Research Institute, Tokyo).

OPENING ADDRESS

Eiichi Tahara (HCS Foundation), Chairman of the Organizing Committee and HCS Foundation, gave an opening address. E.T. mentioned a brief background and the purpose of this series of 1-day's symposia annually organized since the establishment of the HCS Foundation in 1992. This year, the organizing committee focused on the importance of miRNA in the development, tumor microenvironment, diagnostic and therapeutic potential of human cancers. E.T. also introduced the biogenesis of miRNA and its dysfunction in gastric cancer induced by *Helicobacter pylori*. In addition, he described a complex interplay between miRNAs and gene regulation systems implicated in the invasion, lymphocyte activation, cancer stem cells and angiogenesis in tumor microenvironment.

SPECIAL LECTURES ON miRNAS IN CACRINOGENESIS,

Progression and Therapy of Human Cancers

The aim of the symposium was to understand a pivotal role of miRNAs in carcinogenesis, progression and therapy of human cancers.

Hiroshi I. Suzuki opened the symposium by describing that mammalian immune regulator MCPIP1 (Zc3h12a) RNase acts as a novel suppressor of miRNA activity and biogenesis (1). MCPIP1 counteracts Dicer and suppresses miRNA biosynthesis through the cleavage of the terminal loops of precursor miRNAs (pre-miRNAs). Transcriptome analysis showed a potential antagonistic relationship between MCPIP1 and Dicer in human cancer, suggesting the involvement of MCPIP1 in the alteration of miRNA biogenesis in carcinogenesis. Regarding the non-cell-autonomous roles of miRNAs in cancer progression processes, microRNA-135b (miR-135b) participates in nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-driven oncogenicity and plays a non-cell-autonomous role in the pathogenesis of malignant lymphoma subtype. The term of non-cell-autonomous cancer progression processes means the cancer progression processes modulated independently by the regulation of cell-autonomous function of cancer cells themselves.

Interestingly, miR-135b suppressed Th2 master regulators, GATA3 and STAT6, and rendered Th17-like immunophenotype to anaplastic large cell lymphoma (ALCL) cells, thereby promoting paracrine inflammatory response and tumor angiogenesis. These results collectively illuminated unique contribution of oncogenic kinase-linked miRNA to tumorigenesis through the modulation of tumor immunophenotype and microenvironment. In addition, a novel approach for the dissection of mRNA–miRNA network in various cancers was presented. Previous studies have shown that mammalian miRNAs decrease the levels of many target mRNAs and reduce protein production predominantly by the destabilization of target mRNAs. However, it has not yet been fully assessed whether this scheme is widely applicable to more realistic conditions. Excitingly, gene set enrichment analysis and functional assignment of microRNAs via enrichment analysis (GFA) enables the prediction of miRNA activities from mRNA expression data by combining rank-based enrichment analysis and weighted evaluation of miRNA–mRNA interactions. This cooperative approach delineated a better widespread correlation between predicted miRNA activities and miRNA expression levels in cancer transcriptomes, thereby providing proof-of-concept of the mRNA-destabilization scenario. Moreover, the inference of miRNA activity by GFA could be utilized for the selection of prognostic miRNAs in the development of cancer survival prediction models. These results will provide the molecular basis for the development of diagnostic and therapeutic strategies based on small RNA biology.

Johji Inazawa reported tumor-suppressor miRNAs (TS-miRNAs) silenced by DNA methylation using three different approaches as follows:(i) DNA methylation-based screening by combined bisulfate restriction analysis and bisulfate sequencing, (ii) expression-based approach with quantitative PCR assays of 148 miRNAs in a panel of cancer cell lines and the control and (iii) function-based screening with a cell proliferation assay for synthetic miRNA precursor libraries and a series of sequential analyses of DNA methylation and expression. Through the methylation-based screening, miR-124 and miR-203 were identified as TS-miRNAs in hepatocellular carcinoma (HCC). MiR-124 and miR-203 showed frequent tumor-specific methylation, and their expression status was inversely correlated with methylation status. The ectopic expression of miR-124 or miR-203 in HCC cells lacking their expression inhibited cell growth, with the direct down-regulation of possible targets, *CDK6*, *vimentin (VIM)*, *SMYD3* and *IQGAP1* or *ABCE1*, respectively. In addition, epigenetic silencing of miR-137 and miR-193 might play a pivotal role in oral carcinogenesis. Moreover, using the function-based approach, epigenetically silenced TS-miR, miR-218, was found in oral squamous cell carcinoma (2) and miR-152 in endometrial cancer, respectively. Interestingly, those two miRNAs can inactivate rapamycin-insensitive companion of MTOR as their target, inducing the activation of a TOR-Akt signaling pathway. Restoring the function of TS-miR(s) by miRNA replacement may be a promising therapy in cancers.

Toru Nakano described Stella-mediated DNA hypomethylation in transformation.

Stella, also known as PGC7 and Dppa3, is a protein essential for early embryogenesis, as Stella binds to the chromatin containing di-methylated histone H3 lysine 9 (H3K9me2) and controls the subcellular localization of Tet3, which is a critical enzyme for active DNA demethylation in early embryos (3). To examine the molecular function of Stella in more detail, the gene was introduced into NIH3T3 cells. Although it was expected that Stella would have protected the DNA methylation in the somatic cells as well as in the early embryos, global DNA hypomethylation took place oppositely. This hypomethylation is presumably due to the binding of Stella to NP95, a hemi-methylated DNA-binding protein essential for the maintenance of DNA methylation. In addition, quite unexpectedly, the transformation of 3T3 cells and the enhancement of metastatic ability of B16 melanoma cells were brought about by Stella. The experimental system of Stella-induced DNA hypomethylation would be an excellent model to cast new insights onto the pathogenic function of DNA hypomethylation in cancer.

M.A.K. introduced parameters influencing the generation and loading of siRNAs from transcriptionally derived duplex RNAs. In order to optimize the shRNA activity, he studied the parameters that affect miRNA/shRNA processing and duplex RNA loading into RNA-induced silencing complex (RISC) in mammalian cells. The mammalian RISC contains a single-stranded RNA derived from a duplex miRNA/siRNA and one-of-four Argonaute (Ago) proteins. Ago loading is the process of duplex RNA association followed by the removal of the inactive passenger strand RNA. He established that shRNAs are loaded into mammalian Agos in two stepwise processes, physical association and activation. Although RNA duplexes processed from shRNAs bind to Agos in cells with similar affinity, the degree by which the complexes are activated (coupled with the removal of the passenger strand) correlates with the thermodynamic instability of RNA duplexes rather than the structure of the RNA, as was previously demonstrated in *Drosophila* (4). He has begun to further dissect the process of RISC loading by studying the function of the evolutionary conserved PAZ (Piwi/Agos/Zwille) domain of Ago proteins. These genetic, cellular and biochemical studies establish that this domain plays an important role in RISC maturation. Taken together these results provide insights into new shRNA designs for RNA interference-based therapeutics.

Hidetoshi Tahara talked on senescent-associated miRNAs and extracellular vesicles in aging and cancer. Cellular senescence confers an important mechanism of tumor suppression, and thus dysregulation of senescence-associated miRNAs (SA-miRNAs) may promote tumor formation *in vivo*. Among identified putative several SA-miRNAs, miR-22 is significantly up-regulated in senescent fibroblasts and down-regulated in various cancer cell lines (5). Importantly, miR-22-induced cellular senescence accompanied by enlarged morphology, senescent-associated β-Gal activity (SA-β-Gal) and senescent-associated heterochromatin foci formation. Surprisingly, therapeutic miR-22 delivery significantly suppresses tumor growth and metastasis *in vivo* in a murine breast cancer model. In fact, miR-22 down-regulates several putative target genes such as CDK6, SP-1 and SIRT1 by 3′UTR assay and western blotting. Interestingly, while these three putative target genes are involved in senescent phenotype, miR-22 activates senescence-associated secretory phenotype (SASP), which regulates chemokine and inflammatory cytokine signals in senescent cells. Recently, extracellular vesicles such as exosome-like vesicles, which are 50–100 nm in size including miRNA, also secrete from mammalian cells. These exosome-like vesicles can interact with neighboring cells as well as other cells in tissue through communication with body fluids such as blood. Although SASP is well characterized in cellular senescence, little is known about exosome in cellular senescence. Therefore, he examined exosome secretion and exosome-miRNA profiling using miRNA array analysis and then found the significant increased secretion of exosomes in senescent fibroblast cells. Interestingly, little secretion of extracellular exosomes containing SA-miRNAs such as miR-22, miR-30 and miR-34a is found in senescent cells, whereas significant secretion of exosomes is found in senescent cells. In addition, these senescent-associated exosomes may also influence the tumor microenvironment. Taken together, these results indicate that the alterations of intracellular senescence-associated miRNA expression accompanied with the alteration of secretory factors, including exosomes and SASP during cellular senescence, are key regulators of aging, and may affect tumor microenvironments.

Andrea L. Kasinski presented the therapeutic potential of miRNAs for preventing and treating lung cancer using the $Kras^{LSL\text{-}G12D/+};p53^{LSL\text{-}R172H/+}$ mouse model, which is characterized by one of the most accurate representations of human lung cancer generated thus far. In the model, both *Kras* and *p53* transgenes are induced spatially and temporally following recombination when exposed to cre-recombinase, which was delivered intratracheally to the lung using lentiviral-cre. Following transgene activation, adenocarcinomas were evident as early as 10 weeks with severe lung inflammation presenting at 22 weeks. Epithelial cells from these tumors were generated and shown to be capable of supporting growth in soft agar assays, invading based on transwell migration assays and forming palpable tumors in nude mice. The levels of miR-34 were evaluated in both the cell lines and lung tumor tissue obtained from *Kras;p53* mice. While the three miR-34 family members showed some variability in their expression levels, globally total precursor and mature miR-34 levels were decreased substantially while oncogenic miRNAs such as miR-21 and miR-155 were elevated. To identify whether miR-34 replacement might be a viable option *in vivo*, miR-34a was first introduced into the *Kras;p53* mutant cells using replication incompetent miR-34a expressing lentiviral particles, which upon integrating into the genome of transduced cells results in sustained high pre-miR-34a expression. When compared with control-transfected cells, miR-34-treated lines were reduced in their

proliferation, ability to form colonies in two dimensions, capability to recover from a scratch wound and invasion potential. Molecularly, the miR-34 targets, Bcl-2 and Met were down-regulated following transient transfection with miR-34a. Based on these promising results, two series of *in vivo* experiments were presented. First, the contribution of miR-34 to prevent tumor formation in $Kras^{LSL\text{-}G12D/+}$; $p53^{LSL\text{-}R172H/+}$ mice was evaluated. Animals that were treated with lentivirus of miR-34 at the same time as cre-induced recombination of transgenes showed little-to-no evidence of tumorigenesis 19 weeks post-transgene activation/treatment, while control animals had multiple nodules that represented ~8% of the total lung area. The second series of *in vivo* experiments evaluated the ability of miR-34 to function as a treatment for preformed tumors using lentivirus delivery. Although miR-34 was unable to reduce the size of the preformed tumors in these animals, it did prevent further tumor growth. These data support the use of miR-34 as a tumor preventive mechanism and suggest that miR-34 may be useful in sensitizing tumors to other conventional therapeutics (6).

Naohiko Seki introduced the expression profiles of tumor suppressive miRNAs in several types of human cancers including head neck cancer, lung cancer, bladder cancer, renal cell cancer and prostate cancer. Excitingly, the expression levels of miR-1 and miR-133a were significantly down-regulated in various cancers (7). In addition, miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1 are clustered on different chromosomal regions in the human genome, 20q13.33 and 18q11.2, respectively. Restoration of miR-1 or miR-133a in cancer cells revealed significant inhibition of proliferation, migration and invasion, suggesting that both the miRNAs may function as tumor suppressors regulating several oncogenic pathways in human cancers.

Naohide Oue addressed on alterations of miRNA expression in gastrointestinal cancer. miRNA expression was analyzed in 20 gastric cancer (GC) cases by miRNA-PCR array. By comparing miRNA expression profiles, the down-regulation of miR-148a frequently took place in GC. Array analysis revealed that the expression of several invasion-associated genes, such as MMP1 and MIA, was repressed in miR-148a-transfected GC cells, and the invasion ability of miR-148a-transfected GC cells was 40% less than that of the negative control siRNA-transfected GC cells. In looking at whether the expression levels of miR-21 can predict the prognosis for patients with colorectal cancer (CRC), high miR-21 expression in tumors was associated with poor survival, independent of clinical covariates, including TNM staging. In patients who received adjuvant chemotherapy, high miR-21 expression was significantly associated with poor therapeutic outcomes. These results indicate that miRNAs play important roles in cancer cell invasion and provide a great deal of information on biomarkers to identify patients with poor prognosis.

Nobutoshi Kosaka described exosomal miRNA as a novel humoral factor for cancer development. Recent evidence indicates that exosomal miRNAs play critical roles in mediating cell–cell communication, specifically between immune cells, endothelial cells and cancer cells. He recently found that miRNAs are released through neutral sphingomyelinase 2 (nSMase2)-regulated secretary machinery and that these secretory minas by exosome are transferable and functional in recipient cells. The established breast cancer cell lines stably overexpressing siRNA specific for mouse nSMase2 showed that preventing nSMase2 expression abrogated the metastatic ability of cancer cells to target lung tissues, whereas administration of exosomes isolated from metastatic cancer cells rescued this phenomenon. Interestingly, the number of endothelial cells observed in inoculated tumors was proportional to the expression level of nSMase2 in cancer cells. In fact, exosomes derived from a metastatic cancer cell line enhanced the capillary formation of endothelial cells *in vitro*, which provides evidence of a permissive niche for outgoing tumor cells. In addition, the expression profile of exosomal miRNAs obtained from metastatic cancer cells demonstrated that a set of angiogenic miRNAs were highly concentrated in these exosomes. One of them, miR-210, was up-regulated under the hypoxic condition, leading to the enrichment of miR-210 in exosome. This miR-210-enriched exosome enhanced the migration and capillary formation through the suppression of specific target gene, which resulted in enhanced angiogenesis, suggesting that exosomal miRNAs secretion confers the formation of tumor vessel network, thereby promoting the metastasis. These findings provide the evidence that the intercellular network between cancer cells and their environmental cells via secretory miRNAs exported by exosome is essential for cancer development and metastasis (8).

Conflict of interest statement

None declared.

References

1. Suzuki HI, Arase M, Matsuyama H, et al. MCPIP1 ribonuclease antagonizes dicer and terminates microRNA biogenesis through precursor microRNA degradation. *Mol Cell* 2011;44:424–36.
2. Uesugi A, Kozaki K, Tsuruta T, et al. The tumor suppressive microRNA miR-218 targets the mTOR component Roctor and inhibits AKT phosphorylation in oral cancer. *Cancer Res* 2011;71:5765–78.
3. Nakamura T, Liu YU, Nakshima H, et al. PGC7 binds histone H3K9me2 to protect against conversion of 5MeC to 5HmC in early embryos. *Nature* 2012;486:415–9.
4. Gu S, Jin J, Zhang Y, et al. Thermodynamic stability of small hairpin RNAs highly influences the loading process of different mammalian Argonautes. *Proc Natl Acad Sci USA* 2011;108:9208–13.
5. Xu D, Takeshita F, Hino Y, et al. MiR-22 represses cancer progression by including cellular senescence. *J Cell Biol* 2011;191:409–42.
6. Kasinski AL, Slack FJ. MiRNA-34 prevents cancer initiation and progression in a therapeutically resistant K-ras and p53-induced mouse model of lung adenocarcinoma. *Cancer Res* 2012;72:5576–87.
7. Kojima S, Chiyomaru T, Kawakami K, et al. Tumour suppressors miR-1 and miR-133a target the oncogenic function of purine nucleoside phosphorylase (PNP) in prostae cancer. *Br J Cancer* 2012;106:405–10.
8. Kosaka N, Iguchi H, Yoshioka Y, Hagiwara K, Takeshita F, Ochiya T. Competitive interaction of cancer cells and normal cells via microRNAs. *J Biol Chem* 2012:287:1397–405.

Hepatology Research

Hepatology Research 2013 doi: 10.1111/hepr.12177

Original Article

Validation and limitation of age–period–cohort model in simulating mortality due to hepatocellular carcinoma from 1940 to 2010 in Japan

Tomoyuki Akita,[1] Masayuki Ohisa,[1] Yuki Kimura,[1] Mayumi Fujimoto,[1] Yuzo Miyakawa[2] and Junko Tanaka[1]

[1]*Department of Epidemiology, Infectious Disease Control and Prevention, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, and* [2]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Aim: We aimed to simulate the mortality due to hepatocellular carcinoma (HCC) by the age–period–cohort (APC) model with use of sex- and age-specific mortality data, for the purpose of validating the utility and assessing the limitation of this model.

Methods: Age-specific mortality due to HCC was gleaned from people aged 20–84 years during 1940 through 2010 in Japan.

Results: The APC model had a high performance in reproducing HCC mortality (modified determination coefficient $R^2_{COR} \geq 0.99$). Risk of HCC increased with age in both sexes, while risk of period barely changed in both sexes. The birth cohort factor in the APC model in males highlighted the maximum point within birth years 1931–1935. The observed HCC mortality in 2010 in males (19 444) was lower than the predicted, and corresponded to 72.3% of the predicted 26 883.4, and in all age groups by 5-year increments (55.6–90.9%). In females, the observed mortality was lower than that predicted in those aged 64 years or less, but not in those aged 65 years or more.

Conclusion: We applied the APC model to predict HCC mortality rate, and it reproduced the observed mortality rate faithfully. However, in the recent past, the observed morality rate in males was only 72.3% that of the predicted. Such differences would be attributed to combined effects of medical interventions, such as antiviral treatments and screening for hepatitis viruses implemented in the early 1990s in Japan.

Key words: age–period–cohort model, epidemiology, hepatitis B virus, hepatitis C virus, hepatocellular carcinoma

INTRODUCTION

MALIGNANT NEOPLASM REMAINS the most common cause of death in Japan. Mortality caused by liver cancer in males started to increase in 1975, peaked at around 2000, and has been slightly decreasing in recent years. By contrast, the mortality due to liver cancer in females is still increasing slightly. At present, liver cancer remains the fourth leading cause of death among malignant neoplasms in Japan, and 32 765 people died of it in 2010.[1]

The age–period–cohort (APC) model[2] is based on epidemiological experiences in which incidence or mortality is influenced by three major factors: (i) age factor; (ii) period factor; and (iii) birth cohort factor. Age factor reflects the risk of aging, while period factor mirrors the common risk posed on constituent members during a given period, regardless of age. The birth cohort factor reflects the risk of historical background of medical policies, such as treatments, vaccinations, health insurance and screenings, as well as environment shared by the birth cohort. The APC model is used increasingly frequently for analyzing temporal age-specific incidence or mortality data in late years. For example, Pham *et al.*[3] analyzed the mortality due to chronic obstructive pulmonary disease in Japan using

Correspondence: Professor Junko Tanaka, Department of Epidemiology, Infectious Disease Control and Prevention, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Email: jun-tanaka@hiroshima-u.ac.jp
Financial disclosure: None to declare.
Conflict of interest: None.
Received 30 April 2013; revision 23 May 2013; accepted 29 May 2013.