AUTOIMMUNE DISEASES

Intriguingly, a positive link between L1-RTP and the development of autoimmune diseases was proposed recently (Crow, 2010). Originally, heterozygous mutations of *Trex1*, a gene that encodes the 3′ repair exonuclease 1, were detected in familial chilblain lupus and Aicardi–Goutières syndrome (Rice et al., 2007). Interestingly, *Trex1*-deficient mice provided evidence that Trex1 functions as a negative regulator of the interferon-regulatory DNA response (Stetson et al., 2008). Without Trex1 function, mice die due to severe autoimmunity. However, such lethal effects of Trex1 deficiency were attenuated in mice with *IRF3*, *IFNαR1*, and *RAG2* gene deficiencies (Stetson et al., 2008). Moreover, single-stranded DNA derived from endogenous retroelements accumulated in *Trex1*-deficient cells, and *Trex1* overexpression blocked RTP of L1 (Stetson et al., 2008). Consistent with these experimental data, Trex1 activity was reduced in the synovial fibroblasts of rheumatoid arthritis patients (Neidhart et al., 2010). These observations suggest that RTP-induced production of DNA derived from endogenous retroelements is a potential molecular mechanism for the development of autoimmune disorders.

FUTURE PERSPECTIVES

L1-RTP depends on three steps: transcription, RT, and integration. It is known that the expression of L1 is regulated by the methylation of CpG islands in the 5′ UTR region (Hata and Sakaki, 1997; Woodcock et al., 1997; Muotri et al., 2010). Moreover, the 5′ UTR of L1 contains a ubiquitously active antisense promoter that encodes siRNAs that effectively suppress L1-RTP (Yang and Kazazian, 2006). Although these reports indicate that L1 activity is regulated at the transcriptional level, our observations and those of others indicate that L1-RTP is also regulated post-transcriptionally. Environmental compounds induce the chromatin recruitment of ORF1 via bHLH/PAS proteins, suggesting that the functional coupling of L1-RTP and bHLH/PAS proteins is another pivotal step in the regulation of L1-RTP.

Since bHLH/PAS proteins are transcription factors that strictly recognize *cis* elements, it is plausible that the induction of L1-RTP in the genome depends on the selection of bHLH/PAS proteins by the individual compounds. DMBA-induced L1-RTP depends on both AhR and ARNT1, and it is likely that L1-RTP is induced in the vicinity of the XRE (**Figure 1**). Further study is required to identify a target locus for the induction of L1-RTP, which would provide novel information regarding the biological relevance of L1-RTP in somatic cells.

It is interesting to note that *Candida albicans* possesses a non-LTR-type retroelement with a structure similar to that of human L1 (Dong et al., 2009). This was shown to be functional in *Saccharomyces cerevisiae*, indicating that an endogenous retroelement similar to human L1 is functional in lower eukaryotes. Conversely, *AhR* homologs are present in the genomes of lower eukaryotes such as *D. melanogaster* and *Caenorhabditis elegans* (Hahn, 2002). Combined with our observation that L1-RTP and bHLH/PAS are linked functionally, these results suggest that genome shuffling by bHLH/PAS-dependent L1-RTP may facilitate adaptation to environmental changes. It is tempting to speculate that bHLH/PAS molecules recognize environmental pollutants and promote genome shuffling by RTP to generate novel cellular properties that can overcome changes in the environment (**Figure 2**). Such property of L1 might contribute to revolution.

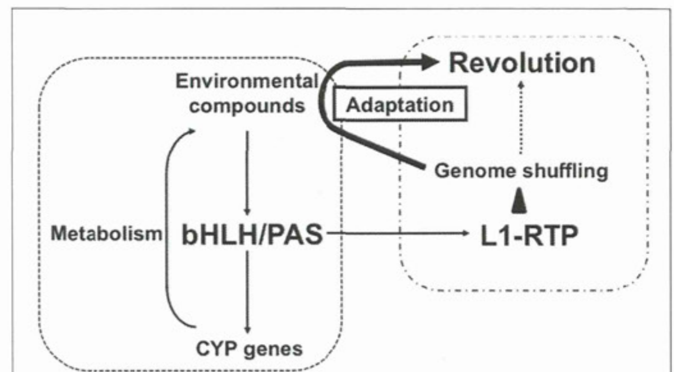

FIGURE 2 | Possible functional link between bHLH/PAS proteins and L1-RTP in environmental adaptation and evolution. As a well-characterized biological function, bHLH/PAS proteins of AhR and ARNT1 induce gene expression of *CYP* genes in response to various compounds. The induced CYP proteins metabolize the environmental compounds and detoxify them. By contrast, L1-RTP induces genome shuffling, and random mutagenesis increases the chance of the emergence of living organisms that possess novel characteristics. As shown here, bHLH/PAS proteins are involved in the induction of L1-RTP, indicating the functional link between these two well-conserved cellular activities. The novel properties can overcome the disadvantageous effects of environmental compounds, enabling living organisms to survive under the selective pressure in the altered environment and contributing to evolution.

After the divergence of humans and chimpanzees about 6 myrs ago, both L1s continued to propagate, and Ta-1d emerged as the major subfamily in *H. sapiens* (Boissinot et al., 2000). Together with our observation that L1-RTP is induced in somatic cells by environmental factors, this affords the opportunity to speculate about novel aspects of L1 biology. Since human- and chimpanzee-specific L1 copy numbers are similar (Lee et al., 2007), it is likely that L1-RTP in the germ cells of both species is strictly regulated. However, the observation that *H. sapiens* has more copies of active L1 than do chimpanzees suggests that "hot" L1 offers an unidentified advantage to human activity. Interestingly, L1-RTP was induced in the dentate gyrus of the hippocampus when mice performed voluntary exercise (Muotri et al., 2009), and some data suggest that L1 copy numbers are increased in the human hippocampus (Coufal et al., 2011). One plausible direction for future research on L1 would be to focus on its role in the central nervous system (Hancks and Kazazian, 2012).

ACKNOWLEDGMENTS

This work was supported in parts by a Grant-in-Aid for Research from the Ministry of Health, Labour, and Welfare of Japan (09156296), a research grant for the Log-range Research Initiative (LRI) from Japan Chemical Industry Association (JCIA) and a Grant-in-Aid from the Tokyo Biochemical Research Foundation. Mr. Noriyuki Okudaira is an applicant supported by the Grant-in-Aid from the Tokyo Biochemical Research Foundation.

REFERENCES

Abel, E. L., Angel, J. M., Riggs, P. K., Langfield, L., Lo, H. H., Person, M. D., Awasthi, Y. C., Wang, L. E., Strom, S. S., Wei, Q., and DiGiovanni, J. (2010). Evidence that Gsta4 modifies susceptibility to skin tumor development in mice and humans. *J. Natl. Cancer Inst.* 102, 1663–1675.

Angel, J. M., Beltrán, L., Minda, K., Rupp, T., and DiGiovanni, J. (1997). Association of a murine chromosome 9 locus (Psl1) with susceptibility to mouse skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Mol. Carcinog.* 20, 162–167.

Babushok, D. V., and Kazazian, H. H. Jr. (2007). Progress in understanding the biology of the human mutagen LINE-1. *Hum. Mutat.* 28, 527–539.

Baillie, J. K., Barnett, M. W., Upton, K. R., Gerhardt, D. J., Richmond, T. A., De Sapio, F., Brennan, P. M., Rizzu, P., Smith, S., Fell, M., Talbot, R. T., Gustincich, S., Freeman, T. C., Mattick, J. S., Hume, D. A., Heutink, P., Carninci, P., Jeddeloh, J. A., and Faulkner, G. J. (2011). Somatic retrotransposition alters the genetic landscape of the human brain. *Nature* 479, 534–537.

Balmain, A., Ramsden, M., Bowden, G. T., and Smith, J. (1984). Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. *Nature* 307, 658–660.

Bannert, N., and Kurth, R. (2004). Retroelements and the human genome: new perspectives on an old relation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14572–14579.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C. L., Dyrskjot, L., Ørntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T. D., Bartek, J., and Gorgoulis, V. G. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444, 633–637.

Beischlag, T. V., Luis Morales, J., Hollingshead, B. D., and Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. *Crit. Rev. Eukaryot. Gene Expr.* 18, 207–250.

Belancio, V. P., Roy-Engel, A. M., and Deininger, P. L. (2010). All y'all need to know 'bout retroelements in cancer. *Semin. Cancer Biol.* 20, 200–210.

Boissinot, S., Chevret, P., and Furano, A. V. (2000). L1 (LINE-1) retrotransposon evolution and amplification in recent human history. *Mol. Biol. Evol.* 17, 915–928.

Brouha, B., Schustak, J., Badge, R. M., Lutz-Prigge, S., Farley, A. H., Moran, J. V., and Kazazian, H. H. Jr. (2003). Hot L1s account for the bulk of retrotransposition in the human population. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5280–5285.

Cargnello, M., and Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* 75, 50–83.

Chiu, Y. L., and Greene, W. C. (2008). The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annu. Rev. Immunol.* 26, 317–353.

Coufal, N. G., Garcia-Perez, J. L., Peng, G. E., Marchetto, M. C., Muotri, A. R., Mu, Y., Carson, C. T., Macia, A., Moran, J. V., and Gage, F. H. (2011). Ataxia telangiectasia mutated (ATM) modulates long interspersed element-1 (L1) retrotransposition in human neural stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20382–20387.

Crow, M. K. (2010). Long interspersed nuclear elements (LINE-1): potential triggers of systemic autoimmune disease. *Autoimmunity* 43, 7–16.

Dewannieux, M., Esnault, C., and Heidmann, T. (2003). LINE-mediated retrotransposition of marked Alu sequences. *Nat. Genet.* 35, 41–48.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Nuciforo, P. G., Bensimon, A., Maestro, R., Pelicci, P. G., and d'Adda di Fagagna, F. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444, 638–642.

Dong, C., Poulter, R. T., and Han, J. S. (2009). LINE-like retrotransposition in *Saccharomyces cerevisiae*. *Genetics* 181, 301–311.

Eguchi, H., Ikuta, T., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1997). A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. *J. Biol. Chem.* 272, 17640–17647.

El-Sawy, M., Kale, S. P., Dugan, C., Nguyen, T. Q., Belancio, V., Bruch, H., Roy-Engel, A. M., and Deininger, P. L. (2005). Nickel stimulates L1 retrotransposition by a post-transcriptional mechanism. *J. Mol. Biol.* 354, 246–257.

Farkash, E. A., Kao, G. D., Horman, S. R., and Prak, E. T. (2006). Gamma radiation increases endonuclease-dependent L1 retrotransposition in a cultured cell assay. *Nucleic Acids Res.* 34, 1196–1204.

Feng, Q., Moran, J. V., Kazazian, H. H. Jr., and Boeke, J. D. (1996). Human L1 retrotransposon encodes a conserved endonuclease required for retrotransposition. *Cell* 87, 905–916.

Gasior, S. L., Wakeman, T. P., Xu, B., and Deininger, P. L. (2006). The human LINE-1 retrotransposon creates DNA double-strand breaks. *J. Mol. Biol.* 357, 1383–1393.

Gebow, D., Miselis, N., and Liber, H. L. (2000). Homologous and nonhomologous recombination resulting in deletion: effects of p53 status, microhomology, and repetitive DNA length and orientation. *Mol. Cell Biol.* 20, 4028–4035.

Georgiou, I., Noutsopoulos, D., Dimitriadou, E., Markopoulos, G., Apergi, A., Lazaros, L., Vaxevanoglou, T., Pantos, K., Syrrou, M., and Tzavaras, T. (2009). Retrotransposon RNA expression and evidence for retrotransposition events in human oocytes. *Hum. Mol. Genet.* 18, 1221–1228.

Gilbert, N., Luts, S., Morrisj, T. A., and Moran, J. V. (2005). Multiple fates of L1 retrotransposition intermideaites in cultured human cells. *Mol. Cell Biol.* 25, 7780–7795.

Gilbert, N., Lutz-Prigge, S., and Moran, J. V. (2002). Genomic deletions created upon LINE-1 retrotransposition. *Cell* 110, 315–325.

Goodier, J. L., and Kazazian, H. H. Jr. (2008). Retrotransposons revisited: the restraint and rehabilitation of parasites. *Cell* 135, 23–35.

Goodier, J. L., Zhang, L., Vetter, M. R., and Kazazian, H. H. Jr. (2007). LINE-1 ORF1 protein localizes in stress granules with other RNA-binding proteins, including components of RNA interference RNA-induced silencing complex. *Mol. Cell Biol.* 27, 6469–6483.

Hancks, D. C., and Kazazian, H. H. Jr. (2012). Active human retrotransposons: variation and disease. *Curr. Opin. Genet. Dev.* 22, 1–13.

Hanel, W., and Moll, U. M. (2012). Links between mutant p53 and genomic instability. *J. Cell Biochem.* 113, 433–439.

Hahn, M. E. (2002). Aryl hydrocarbon receptors: diversity and evolution. *Chem. Biol. Interact.* 141, 131–160.

Hata, K., and Sakaki, Y. (1997). Identification of critical CpG sites for repression of L1 transcription by DNA methylation. *Gene* 189, 227–234.

Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* 252, 954–958.

Hohjoh, H., and Singer, M. F. (1996). Cytoplasmic ribonucleoprotein complexes containing human LINE-1 protein and RNA. *EMBO J.* 15, 630–639.

Ishizaka, Y., Okudaira, N., and Okamura, T. (2012). Regulation of retrotransposition of long interspersed element-1 by mitogen-activated protein kinases. In Protein kinase (in press).

Iskow, R. C., McCabe, M. T., Mills, R. E., Torene, S., Pittard, W. S., Neuwald, A. F., Van Meir, E. G., Vertino, P. M., and Devine, S. E. (2010). Natural mutagenesis of human genomes by endogenous retrotransposons. *Cell* 141, 1253–1261.

Jurka, J. (1997). Sequence patterns indicate an enzymatic involvement in integration of mammalian retroposons. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1872–1877.

Kale, S. P., Moore, L., Deininger, P. L., and Roy-Engel, A. M. (2005). Heavy metals stimulate human LINE-1 retrotransposition. *Int. J. Environ. Res. Public Health* 2, 14–23.

Kano, H., Godoy, I., Courtney, C., Vetter, M. R., Gerton, G. L., Ostertag, E. M., and Kazazian, H. H. Jr. (2009). L1 retrotransposition occurs mainly in embryogenesis and creates somatic mosaicism. *Genes Dev.* 23, 1303–1312.

Kazazian, H. H. Jr. (2004). Mobile elements: drivers of genome evolution. *Science* 303, 1626–1632.

Kazazian, H. H. Jr, Wong, C., Youssoufian, H., Scott, A. F., Phillips, D. G., and Antonarakis, S. E. (1988). Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. *Nature* 332, 164–166.

Konkel, M. K., and Batzer, M. A. (2010). A mobile threat to genome stability: the impact of non-LTR retrotransposons upon the human genome. *Semin. Cancer Biol.* 20, 211–221.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R.,

McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian. A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R. A., Muzny, D. M., Scherer, S. E., Bouck, J. B., Sodergren, E. J., Worley, K. C., Rives, C. M., Gorrell, J. H., Metzker, M. L., Naylor, S. L., Kucherlapati, R. S., Nelson, D. L., Weinstock, G. M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D. R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H. M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R. W., Federspiel, N. A., Abola, A. P., Proctor, M. J., Myers, R. M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D. R., Olson, M. V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G. A., Athanasiou, M., Schultz, R., Roe, B. A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W. R., de la Bastide, M., Dedhia, N., Blöcker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J. A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D. G., Burge, C. B., Cerutti, L., Chen, H. C., Church, D., Clamp, M., Copley, R. R., Doerks, T., Eddy, S. R., Eichler, E. E., Furey, T. S., Galagan, J., Gilbert, J. G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L. S., Jones, T. A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W. J., Kitts, P., Koonin, E. V., Korf, I., Kulp, D., Lancet, D., Lowe, T. M., McLysaght, A., Mikkelsen, T., Moran, J. V., Mulder, N., Pollara, V. J., Ponting, C. P., Schuler, G., Schultz, J., Slater, G., Smit, A. F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y. I., Wolfe, K. H., Yang, S. P., Yeh, R. F., Collins, F., Guyer, M. S., Peterson, J., Felsenfeld, A., Wetterstrand, K. A., Patrinos, A., Morgan, M. J., de Jong, P., Catanese, J. J., Osoegawa, K., Shizuya, H., Choi, S., and Chen, Y. J.; International Human Genome Sequencing Consortium. (2001). Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

Lee, J., Cordaux, R., Han, K., Wang, J., Hedges, D. J., Liang, P., and Batzer, M. A. (2007). Different evolutionary fates of recently integrated human and chimpanzee LINE-1 retrotransposons. *Gene* 390, 18–27.

Martin, S. L., Cruceanu, M., Branciforte, D., Wai-Lun Li, P., Kwok, S. C., Hodges, R. S., and Williams, M. C. (2005). LINE-1 retrotransposition requires the nucleic acid chaperone activity of the ORF1 protein. *J. Mol. Biol.* 348,549–561.

Mathias, S. L., Scott, A. F., Kazazian, H. H. Jr., Boeke, J. D., and Gabriel, A. (1991). Reverse transcriptase encoded by a human transposable element. *Science* 254, 1808–1810.

Mazoyer, S. (2005). Genomic rearrangements in the BRCA1 and BRCA2 genes. *Hum. Mutat.* 25, 415–422.

McIntosh, B. E., Hogenesch, J. B., and Bradfield, C. A. (2010). Mammalian Per-Arnt-Sim proteins in environmental adaptation. *Annu. Rev. Physiol.* 72, 625–645.

Miki, Y., Nishisho, I., Horii, A., Miyoshi, Y., Utsunomiya, J., Kinzler, K. W., Vogelstein, B., and Nakamura, Y. (1992). Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. *Cancer Res.* 52, 643–645.

Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T. N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., and Fujii-Kuriyama, Y. (1997). Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* 2, 645–654.

Morisada, N., Rendtorff, N. D., Nozu, K., Morishita, T., Miyakawa, T., Matsumoto, T., Hisano, S., Iijima, K., Tranebjaerg, L., Shirahata, A., Matsuo, M., and Kusuhara, K. (2010). Branchio-oto-renal syndrome caused by partial EYA1 deletion due to LINE-1 insertion. *Pediatr. Nephrol.* 25, 1343–1348.

Morse, B., Rotherg, P. G., South, V. J., Spandorfer, J. M., and Astrin, S. M. (1988). Insertional mutagenesis of the myc locus by a LINE-1 sequence in a human breast carcinoma. *Nature* 333, 87–90.

Muotri, A. R., Chu, V. T., Marchetto, M. C., Deng, W., Moran, J. V., and Gage, F. H. (2005). Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. *Nature* 435, 903–910.

Muotri, A. R., Marchetto, M. C., Coufal, N. G., Oefner, R., Yeo, G., Nakashima, K., and Gage, F. H. (2010). L1 retrotransposition in neurons is modulated by MeCP2. *Nature* 468, 443–446.

Muotri, A. R., Zhao, C., Marchetto, M. C., and Gage, F. H. (2009). Environmental influence on L1 retrotransposons in the adult hippocampus. *Hippocampus* 19, 1002–1007.

Neidhart, M., Karouzakis, E., Schumann, G. G., Gay, R. E., and Gay, S. (2010). Trex-1 deficiency in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum.* 62, 2673–2679.

Nelson, M. A., Futscher, B. W., Kinsella, T., Wymer, J., and Bowden, G. T. (1992). Detection of mutant Ha-ras genes in chemically initiated mouse skin epidermis before the development of benign tumors. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6398–6402.

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., and Kato, S. (2003). Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* 423, 545–550.

Okudaira, N., Iijima, K., Koyama, T., Minemoto, Y., Kano, S., Mimori, A., and Ishizaka, Y. (2010). Induction of long interspersed nucleotide element-1 (L1) retrotransposition by 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan photoproduct. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18487–18492.

Okudaira, N., Goto, M., Yanobu-Takanashi, R., Tamura, M., An, A., Abe, Y., Kano, S., Hagiwara, S., Ishizaka, Y., and Okamura, T. (2011). Involvement of retrotransposition of long interspersed nucleotide element-1 in skin tumorigenesis induced by 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate. *Cancer Sci.* 102, 2000–2006.

O'Neil, J., Tchinda, J., Gutierrez, A., Moreau, L., Maser, R. S., Wong, K. K., Li, W., McKenna, K., Liu, X. S., Feng, B., Neuberg, D., Silverman, L., DeAngelo, D. J., Kutok, J. L., Rothstein, R., DePinho, R. A., Chin, L., Lee, C., and Look, A. T. (2007). Alu elements mediate MYB gene tandem duplication in human T-ALL. *J. Exp. Med.* 204, 3059–3066.

Probst, M. R., Reisz-Porszasz, S., Agbunag, R. V., Ong, M. S., and Hankinson, O. (1993). Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. *Mol. Pharmacol.* 44, 511–518.

Raiz, J., Damert, A., Chira, S., Held, U., Klawitter, S., Hamdorf, M., Löwer, J., Strätling, W. H., Löwer, R., and Schumann, G. G. (2012). The non-autonomous retrotransposon SVA is trans-mobilized by the human LINE-1 protein machinery. *Nucleic Acids Res.* 40, 1666–1683.

Rice, G., Newman, W. G., Dean, J., Patrick, T., Parmar, R., Flintoff, K., Robins, P., Harvey, S., Hollis, T., O'Hara, A., Herrick, A. L., Bowden, A. P., Perrino, F. W., Lindahl, T., and Barnes, D. E., and Crow, Y. J. (2007). Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. *Am. J. Hum. Genet.* 80, 811–815.

Stetson, D. B., Ko, J. S., Heidmann, T., and Medzhitov, R. (2008). Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134, 587–598.

Stribinskis, V., and Ramos, K. S. (2006). Activation of human long interspersed nuclear element 1 retrotransposition by benzo(a)pyrene, an ubiquitous environmental carcinogen. *Cancer Res.* 66, 2616–2620.

Strout, M. P., Marcucci, G., Bloomfield, C. D., and Caligiuri, M. A. (1998). The partial tandem duplication of ALL1 (MLL) is consistently generated by Alu-mediated homologous recombination in acute myeloid leukemia. *Proc. Natl. Acad. Sci. U.S.A.* 95, 2390–2395.

Symer, D. E., Connelly, C., Szak, S. T., Caputo, E. M., Cost, G. J., Parmigiani, G., and Boeke, J. D. (2002). Human l1 retrotransposition is associated with genetic instability in vivo. *Cell* 110, 327–338.

Ting, D. T., Lipson, D., Paul, S., Brannigan, B. W., Akhavanfard, S.,

Coffman, E. J., Contino, G., Deshpande, V., Iafrate, A. J., Letovsky, S., Rivera, M. N., Bardeesy, N., Maheswaran, S., and Haber, D. A. (2011). Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. *Science* 331, 593–596.

van den Hurk, J. A., Meij, I. C., Seleme, M. C., Kano, H., Nikopoulos, K, Hoefsloot, L. H., Sistermans, E. A., de Wijs, I. J., Mukhopadhyay, A., Plomp, A. S., de Jong, P. T., Kazazian, H. H., and Cremers, F. P. (2007). L1 retrotransposition can occur early in human embryonic development. *Hum. Mol. Genet.* 16, 1587–1592.

Vitullo, P., Sciamanna, I., Baiocchi, M., Sinibaldi-Vallebona, P., and Spadafora, C. (2012). LINE-1 retrotransposon copies are amplified during murine early embryo development. *Mol. Reprod. Dev.* 79, 118–127.

Wallace, N., Wagstaff, B. J., Deininger, P. L., and Roy-Engel, A. M. (2008). LINE-1 ORF1 protein enhances Alu SINE retrotransposition. *Gene* 419, 1–6.

Wei, W., Gilbert, N., Ooi, S. L., Lawler, J. F., Ostertag, E. M., Kazazian, H. H., Boeke, J. D., and Moran, J. V. (2001). Human L1 retrotransposition: cis preference versus trans complementation. *Mol. Cell Biol.* 21, 1429–1439.

Wimmer, K., Callens, T., Wernstedt, A., and Messiaen, L. (2011). The NF1 gene contains hotspots for L1 endonuclease-dependent de novo insertion. *PLoS Genet.* 7, e1002371.

Wincent, E., Amini, N., Luecke, S., Glatt, H., Bergman, J., Crescenzi, C., Rannug, A., and Rannug, U. (2009). The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P4501 substrate 6-formylindolo[3,2-b]carbazole is present in humans. *J. Biol. Chem.* 284, 2690–2696.

Woodcock, D. M., Lawler, C. B., Linsenmeyer, M. E., Doherty, J. P., and Warren, W. D. (1997). Asymmetric methylation in the hypermethylated CpG promoter region of the human L1 retrotransposon. *J. Biol. Chem.* 272, 7810–7816.

Yang, N., and Kazazian, H. H. Jr. (2006). L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. *Nat. Struct. Mol. Biol.* 13, 763–771.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 April 2012; paper pending published: 20 April 2012; accepted: 10 May 2012; published online: 31 May 2012.

Citation: Ishizaka Y, Okudaira N, Tamura M, Iijima K, Shimura M, Goto M and Okamura T (2012) Modes of retrotransposition of long interspersed element-1 by environmental factors. Front. Microbio. ***3****:191. doi: 10.3389/fmicb.2012.00191*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

Copyright © 2012 Ishizaka, Okudaira, Tamura, Iijima, Shimura, Goto and Okamura. This is an open-access article distributed under the terms of the Creative Commons Attribution Non Commercial License, which permits noncommercial use, distribution, and reproduction in other forums, provided the original authors and source are credited.

Cancer Science
The official journal of the Japanese Cancer Association

Involvement of retrotransposition of long interspersed nucleotide element-1 in skin tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate

Noriyuki Okudaira,[1,2] Motohito Goto,[3] Rieko Yanobu-Takanashi,[3] Masato Tamura,[1] Akihiro An,[1] Yukiko Abe,[1,7] Shigeyuki Kano,[2,4] Shotaro Hagiwara,[5] Yukihito Ishizaka[1,6] and Tadashi Okamura[3]

[1]Department of Intractable Diseases, National Center for Global Health and Medicine, Shinjuku-ku, Tokyo; [2]Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba; [3]Division of Animal Model, Department of Infections Diseases, [4]Department of Tropical Medicine and Malaria, [5]Division of Hematology, Department of Internal Medicine, National Center for Global Health and Medicine, Shinjuku-ku, Tokyo, Japan

(Received April 21, 2011/Revised June 10, 2011; July 19, 2011/Accepted August 1, 2011/Accepted manuscript online August 9, 2011/Article first published online September 19, 2011)

Tumor development induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a well-characterized model of multistep carcinogenesis. DMBA mutates the *Ha-ras* gene, whereas TPA promotes the growth of transformed cells by activating cellular signaling molecules. It remains to be clarified how repeated TPA treatment endows transformed cells with autonomous cell growth. Long interspersed nucleotide element-1 (L1) is an endogenous retroelement, and 80–100 copies of L1 function as autonomous mobile elements. Although the L1 retrotransposition (RTP) has been found in various human tumors, implying the possible mobility of L1 during carcinogenesis, little is known about how L1-RTP arises in tumor cells, owing to a lack of experimental models. To dissect the mechanism of L1-RTP during carcinogenesis, we established a line of transgenic mice carrying human L1 and enhanced green fluorescent protein (hL1-EGFP mice) and subjected them to DMBA/TPA-induced skin tumorigenesis. Of 15 skin tumors examined, 13 were positive for L1-RTP; L1-RTP was not detected in normal skin tissues adjacent to the tumors. Moreover, nine L1-RTP-positive tumors were positive for activated *Ha-ras*, and immunohistochemical analysis revealed cells positive for both L1-RTP and phosphorylated Stat3, a marker of tumor cells. Additional *in vivo* experiments suggested that L1-RTP occurred during tumor promotion by TPA. This is the first report on the involvement of L1-RTP in chemical carcinogenesis. We propose hL1-EGFP mice as a versatile system for investigating the mode of L1-RTP in tumor development and discuss the possible role of L1-RTP in tumorigenesis. (*Cancer Sci* 2011; 102: 2000–2006)

Long interspersed nucleotide element-1 (L1) is the most abundant transposable element, comprising approximately 17% of the human genome.[1,2] A single human cell contains more than 5×10^5 copies of L1, and 80–100 copies are competent for retrotransposition (L1-RTP).[3] Long interspersed nucleotide element-1 has been identified in the c-*myc* and *APC* genes in breast carcinoma and colon carcinoma, respectively.[4,5] Recently, L1 insertion was found in the pericentric region of various human tumors, and it was suggested that the newly inserted L1 altered global gene expression in the tumor cells by modulating RNA expression from satellite repeats.[6] Moreover, L1-RTP induces a variety of genetic alterations, including gene deletions, inversions, and insertions,[7,8] further supporting the involvement of L1-RTP in carcinogenesis. However, the mechanism of L1-RTP induction during carcinogenesis is yet to be elucidated, owing to the lack of animal models in which the mobility of L1 could be monitored *in vivo*.

The development of skin tumors in response to treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a well-characterized model of multistep carcinogenesis.[9] A single dose of DMBA mutates the *Ha-ras* gene at codon 61 (A to T).[10] Repeated treatment with TPA supports the growth of transformed cells by activating inflammatory signaling molecules,[11] including epidermal growth factor receptor (EGFR),[12] ERK,[13] and signal transducer and activator of transcription 3 (Stat3).[14] It is well established that tumor susceptibility differs among mouse strains; for example, DBA/2 mice are sensitive to TPA-induced tumor promotion, whereas C57BL/6 mice are resistant.[15–19] Backcross experiments have identified several loci of TPA-susceptible genes,[19] but how TPA treatment enables transformed cells to grow autonomously is as yet unknown.

To identify factors that modulate L1-RTP during tumor promotion, we established a line of transgenic mice carrying human L1 as a transgene (hL1-EGFP mice) and subjected the mice to DMBA/TPA-induced tumorigenesis. Intriguingly, L1-RTP was frequently detected in skin tumors, but not in normal skin tissues adjacent to the tumors. The results of biochemical analyses and *in vivo* experiments involving brief exposure to the compounds suggested that L1-RTP occurred during tumor promotion directed by repeated TPA treatment. This is the first report on the induction of L1-RTP during chemical carcinogenesis. We conclude this report with a discussion of the possible role of L1-RTP in TPA-induced tumor promotion.

Materials and Methods

Cell lines and chemicals. HuH-7 cells (RCB1366; Riken BioResource Center Cell Bank, Tsukuba, Japan) were cultured in DMEM supplemented with 10% FCS (Sigma, St. Louis, MO, USA). The transfection efficiency of this cell line was approximately 70%, as assessed by transient expression of plasmid DNA encoding EGFP and subsequent FACS analysis, carried out on day 2 post-transfection using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). We purchased TPA, DMBA, and the kinase inhibitors SB202190,[20] SP600125,[21] PD98,059,[22] and CL387,785[23] from Sigma.

[6]To whom correspondence should be addressed. E-mail: zakay@ri.ncgm.go.jp
[7]Present address: Section of Animal Research, Center of Disease Biology and Integrative Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.

doi: 10.1111/j.1349-7006.2011.02060.x
© 2011 Japanese Cancer Association

Protease inhibitors were from Roche Diagnostics (Basel, Switzerland).

L1-RTP assays. Colony and PCR-based assays were carried out using pCEP4/L1*mneo*I/ColE1 (pL1-Neo^{R})[24] and pER06R,[25] as described previously[26] (described in detail in Data S1). Both constructs contained human L1 with a CMV promoter (Fig. 1A). In the colony assay, each sample was assayed in quadruplicate, and the mean number of colonies and SD were calculated. Statistical analysis was carried out using the Mann–Whitney *U*-test. Each experiment was repeated more than twice. In the PCR-based assay, the relative intensity was calculated based on the signal intensities of amplified DNA derived from *EGFP* and *β-actin*.

Downregulation of endogenous protein expression by siRNAs. Three different siRNAs, synthesized by Applied Biosystems (Foster City, CA, USA), were prepared for each gene, and their functions were evaluated. The nucleotide sequences of the siRNAs specific for the aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator 1 (*ARNT1*) are given Table S1. In each experiment, two independent siRNAs that targeted different sequences were used. Silencer Negative Control siRNA #2 (Cat# AM4637; Applied Biosystems, Foster City, CA, USA) was used as a control.

Production of hL1-RTP-transgenic mice. An 8.8-kb *Not*I-*Apa*I fragment of pEF06R was microinjected into the pronuclei of fertilized eggs of BDF1 × C57BL/6 mice (Japan SLC, Hamamatsu, Japan), as described.[27] The injected DNA Japan fragment possessed the 5′-untranslated region of hL1, allowing expression of the L1 transgene under the control of its own promoter (Fig. 1A, arrow). Among 16 founder mice, #4 and #67 were selected as suitable strains (the procedures are described in detail in Data S1) and were backcrossed to C57BL/6 mice. Then N3 generations were subjected to further experiments.

DMBA/TPA-induced skin carcinogenesis. Seven mice (two from strain #4 and five from #67) were subjected to DMBA/TPA-induced skin carcinogenesis. After 7 days of treatment with DMBA (400 nmol in 200 μL acetone), treatment with TPA (17 nmol in 200 μL acetone) was initiated.[18] TPA was applied topically twice per week until tumors developed. We did not observe definite induction of L1-mRNA in skin tissues treated with TPA (R. Yanobu-Takanashi, data not shown). Tumor tissues and skin tissues were analyzed using a PCR-based assay to detect L1-RTP. The status of the *Ha-ras* gene was examined by PCR amplification followed by sequence analysis.[28] Histopathological analysis of skin tumors was carried out after staining with H&E (Biopathology Institute, Ohita, Japan).

Immunohistochemical analysis. When L1-RTP occurred, the cells became EGFP-positive (Fig. 1A, Data S1). Therefore, to characterize cells positive for L1-RTP, immunohistochemical analysis was carried out using an anti-EGFP antibody (αEGFP; Abcam, Cambridge, England, UK). An antibody specific for phosphorylated Stat3 (pStat3) (Cell Signaling Technology, Beverly, MA, USA) was used to identify tumor cells. The procedures are described in detail in the Data S1.

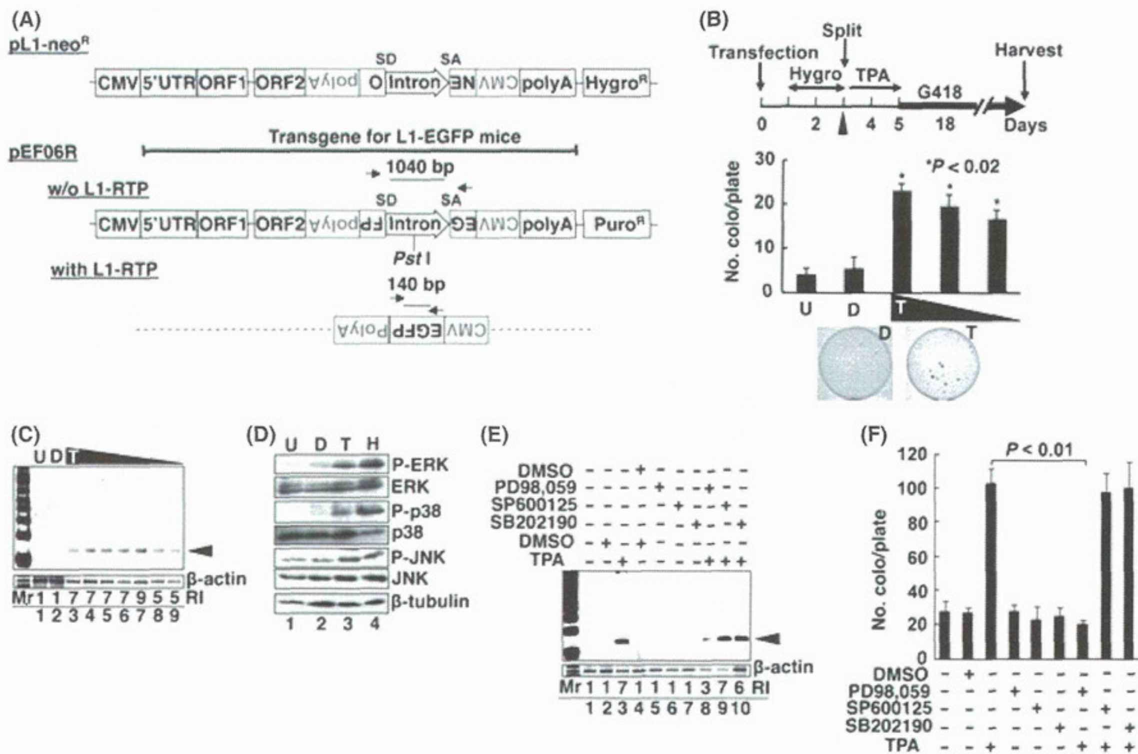

Fig. 1. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces long interspersed nucleotide element-1 retrotransposition (L1-RTP). (A) Schematics showing the constructs for detecting L1-RTP and the rationale for the PCR-based assay. In the PCR-based assay, a 1040-bp band was amplified without (w/o) L1-RTP, and a 140-bp band was amplified with L1-RTP. Arrows indicate the positions of the PCR-based assay primers. EGFP, enhanced green fluorescent protein; ORF, open reading frame; SA, splicing acceptor; SD, splicing donor; UTR, untranslated region. (B) Schematic of the protocol and colony assay results. TPA (10, 100, or 200 nM) was applied to approximately 1×10^5 cells, and G418 selection was carried out. Photographic images show G418-resistant colonies obtained with DMSO (D) or 200 nM TPA (T). Mean number of colonies + SD is shown. colo, colonies; U, untreated. (C) PCR-based assay of TPA-induced L1-RTP. Relative intensity (RI) was calculated based on the signal intensity of the 140-bp band (arrowhead), using *β-actin* as an internal control. Mr, marker. Lane 1, untreated (U); lane 2, DMSO (D); lanes 3–9, 200, 100, 10, 1, 0.1, 0.01, and 0.001 nM TPA, respectively. (D) Phosphorylation of MAPK substrates in response to TPA treatment (T). Cell extracts were prepared 30 min after treatment with 200 nM TPA (lane 3) or 1 mM H_2O_2 (lane 4). H, hydrogen peroxide as positive control. (E,F) Effects of MAPK inhibitors on TPA-induced L1-RTP, as assessed by PCR-based (E) and colony assays (F). PD98,058, SB202190, and SP600125 were applied at concentrations of 20, 1, and 100 μM, respectively. Mean number of colonies ± SD is shown.

© 2011 Japanese Cancer Association

Results

We first observed that TPA induced L1-RTP (Fig. 1B, $P < 0.02$). As assessed by the number of G418-resistant colonies, the frequency of TPA-induced L1-RTP was 10^{-4}–10^{-5}. A PCR-based assay confirmed TPA-induced L1-RTP by detecting a 140-bp band generated as a result of L1-RTP (Fig. 1C; for rationale see Fig. 1A).[26] No obvious cytotoxicity was detected in cells treated with TPA at the concentrations used in the L1-RTP experiments (Fig. S1A). Western blot analysis indicated that TPA induced the phosphorylation of ERK, p38 MAPK, and JNK (Fig. 1D, lane 3). Interestingly, the ERK inhibitor PD98,059[22] considerably attenuated TPA-induced L1-RTP (Fig. 1E, lane 8). In contrast, TPA-induced L1-RTP was not blocked by SB202190 or SP600125, which are inhibitors of p38 and JNK,[20,21] respectively (Fig. 1E, lanes 9, 10). The effects of these inhibitors on L1-RTP were confirmed in a colony assay (Fig. 1F, $P < 0.01$).

Similar to TPA, DMBA induced L1-RTP (Fig. 2A) at doses that did not cause cytotoxicity (Fig. S1B). DMBA is a bulky compound that is presumed to bind to AhR, eliciting cellular signals in concert with ARNT1, as reported for TCDD (dioxin).[29] To test whether these molecules were involved in the induction of L1-RTP, the effects of *AhR*- and *ARNT1*-specific siRNAs were examined. Using a previously reported method,[26] we first confirmed that each siRNA reduced endogenous protein expression to <10% of the control level (Fig. 2B, upper panels). With the siRNAs, DMBA-induced L1-RTP was completely abrogated (Fig. 2B, right panel, lanes 9, 12). In contrast, TPA-induced L1-RTP was not blocked by the siRNAs (Fig. 2C, lanes 11, 15). None of the MAPK inhibitors examined inhibited DMBA-induced L1-RTP (Fig. 2D, lanes 9, 11, 13). Additionally, CL387,785, an inhibitor of EGFR,[23] suppressed TPA-induced L1-RTP, but not L1-RTP induced by DMBA (Fig. 2E). These data indicate DMBA and TPA induce L1-RTP through different mechanisms.

To confirm the involvement of L1-RTP in tumorigenesis *in vivo*, we generated transgenic mice carrying human L1 as a transgene (hL1-EGFP mice). To clarify the role of L1 during tumorigenesis, we thought it important to select hL1-EGFP mice that had low or no background L1-RTP, but that displayed vigorous L1-RTP activity in response to external stimuli (Fig. S2). Of 16 original transgenic lines, we selected #4 and #67 (Fig. 3A) for use in further experiments. L1 transgene copy numbers, as estimated by Southern blotting and quantitative PCR, were approximately 10 and 20 for lines #4 and #67, respectively (Fig. S3, Table S2).

We then examined L1-RTP in DMBA/TPA-induced skin tumors in experiments carried out according to the reported protocol (Fig. 3B).[18] hL1-EGFP mice subjected to DMBA/TPA-induced skin carcinogenesis consisted of theoretically 87.5% of C57BL/6 background, which is known as a resistant strain[15–18] (see Materials and Methods). We used relatively high doses of DMBA (400 nmol) and TPA (17 nmol twice weekly) in order to ensure a substantial tumor response in the C57BL/6 background. From seven hL1-EGFP mice, 15 tumors were obtained 35 weeks after the initiation of TPA treatment (Fig. 3B, arrowheads). At least one tumor was formed per mouse, and the tumor

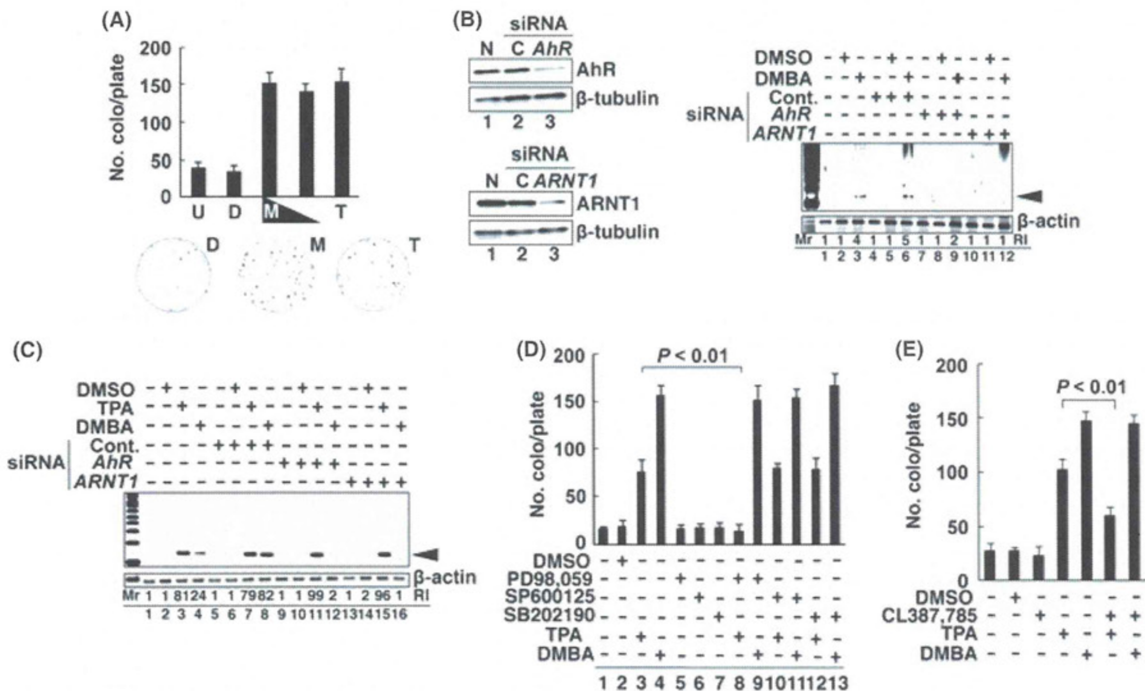

Fig. 2. 7,12-Dimethylbenz[*a*]anthracene (DMBA) induces long interspersed nucleotide element-1 retrotransposition (L1-RTP). (A) Colony assay of DMBA-induced L1-RTP. DMBA was used at concentrations of 1 and 10 nM. Photographic images show G418-resistant colonies obtained with DMSO (D) and 10 nM DMBA (M). Mean number of colonies (colo) + SD is shown. T, 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA); U, untreated. (B) Aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator (*ARNT1*) are both required for DMBA-induced L1-RTP. Upper panels, target siRNAs downregulated endogenous AhR and ARNT1 protein expression. Lane 1, control cells transfected with pEF06R (N); lane 2, cells cotransfected with control siRNA and pEF06R (C); lane 3, cells cotransfected with target siRNAs and pEF06R. Lower panel, PCR-based assay results. Data shown are representative of two independent siRNAs. Mr, marker; RI, relative intensity. (C) TPA-induced L1-RTP is not dependent on *AhR* or *ARNT1*. TPA-induced L1-RTP was examined under conditions of downregulated expression of endogenous *AhR* or *ARNT1*, as explained in (B). (D) Different cellular signaling pathways are involved in TPA- and DMBA-induced L1-RTP. A colony assay was carried out using a 1040-bp band amplified without (w/o) L1-RTP, and a 140-bp band amplified with L1-RTP. Mean number of colonies ± SD is shown. (E) Effect of epidermal growth factor receptor (EGFR) inhibition on TPA- and DMBA-induced L1-RTP. CL387,785 (100 nM), an inhibitor of EGFR, was added to the culture medium 60 min before the addition of 200 nM TPA or 1 nM DMBA. Mean number of colonies ± SD is shown. The effect of the inhibitor on TPA-induced L1-RTP was statistically significant ($P < 0.01$).

doi: 10.1111/j.1349-7006.2011.02060.x
© 2011 Japanese Cancer Association

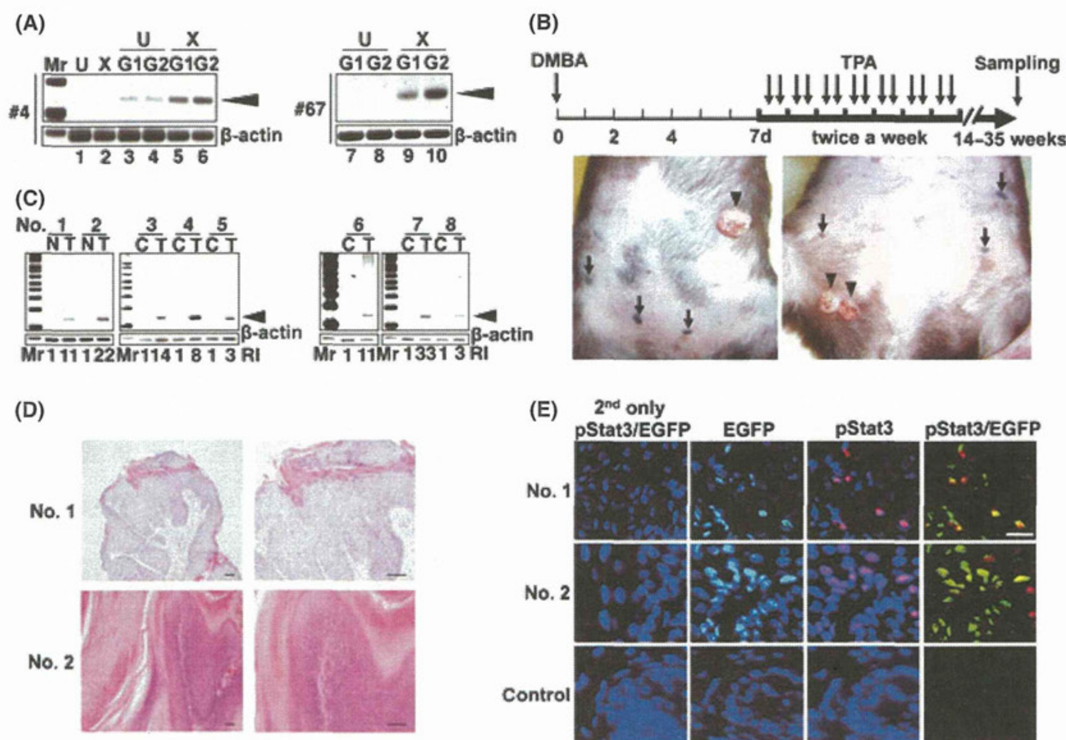

Fig. 3. Long interspersed nucleotide element-1 retrotransposition (L1-RTP) is induced in 7,12-dimethylbenz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate (DMBA/TPA)-induced skin tumors. (A) Generation of transgenic mice carrying human L1. L1-RTP induced in mouse embryonic fibroblasts of transgenic strains #4 and #67 after X-ray irradiation. Embryos were divided into two groups, and the pooled mouse embryonic fibroblasts (G1 and G2) were subjected to PCR-based assay. Mr, marker; U, untreated; X, X-ray treatment (4.5 Gy). (B) Experimental protocol and skin tumors. On day 7 after DMBA treatment, repeated treatment with TPA (twice a week) was begun. Skin tumors (arrowheads) and nevi (arrows) developed. (C) Induction of L1-RTP in skin tumors, but not normal skin or tail tissues. Each tumor (T) was enucleated and analyzed using the PCR-based assay. Control skin (N) and tail tissues (C) were also analyzed. RI, relative intensity. Tumors 1, 3, 5, and 6 were independent tumors derived from #4 mice; tumors 2, 4, 7, and 8 were obtained from #67 mice. (D) Histopathology of representative skin tumor lesions after staining with H&E. Upper panels, squamous cell papillomas formed in mouse strain #4 (No. 1). Lower panels, keratoacanthomas formed in #67 mice (No. 2). Tumor numbers correspond to those described in (C). Bar, 50 μm. (E) L1-RTP occurred in tumor cells. Immunohistochemical analyses were carried out using αEGFP (green) and αpStat3 (red) antibodies. Results for a squamous cell papilloma (No. 1, upper panels), a keratoacanthoma (No. 2, middle panels), and a control (lower panels) are shown. Left panels, results obtained using secondary antibody only. Nuclei were stained using Hoechst stain (blue). Bar, 20 μm.

multiplicity, which was calculated based on the numbers of subjected mice and tumors, was 2.3 ± 1.3 (tumors per mouse). In a study by Feith *et al.*, tumor incidence and multiplicity in C57BL/6 mice subjected to a similar protocol were approximately 95% and 6–7 per mouse, respectively,[18] suggesting that the hL1 transgene did not increase tumor susceptibility in DMBA/TPA-induced skin tumorigenesis.

Each tumor was enucleated, and the extracted DNA was subjected to a PCR-based assay for L1-RTP. As shown in Figure 3(C), L1-RTP was detected in 13 of the 15 independent tumors examined (see also Table 1). Histopathological analysis of seven samples positive for L1-RTP revealed that three were

Table 1. Summary of long interspersed nucleotide element-1 retrotransposition in skin tumors and nevi induced by 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate

Mouse lines	Tumors	Normal skin tissues	Nevi
#4	4/4†	0/6	0/1
#67	9/11	0/14	1/7
Total	13/15 (86.7)‡	0/20	1 (12.5)

†Positive number/number examined. ‡Number (%). $P < 0.001$, Fisher's exact test. Odds ratio (95% confidence interval) was 45.5.

squamous cell papillomas (Fig. 3D, upper panel, No. 1) and another three were keratoacanthomas (Fig. 3D, lower panel, No. 2). One sample was composed of inflammatory cells. Interestingly, we also observed nevi (melanocyte hyperplasia, Fig. 3B, arrows), as has been reported.[30] The PCR-based assay detected L1-RTP in one of eight nevi examined (Fig. S4). The difference in the frequency of L1-RTP between tumors (13/15) and nevi was remarkable ($P < 0.01$; Table 1).

The status of the *Ha-ras* gene in the tumors was also examined. Eleven of 13 tumors were positive for the A61T mutation.[28] Of these 11 tumors, nine were positive for L1-RTP. No mutations in codons 12 or 13 were detected. Activated *Ha-ras* was not detected in the skin tumor sample composed of inflammatory cells. To obtain direct evidence that L1-RTP was induced in tumor cells, immunohistochemical analyses were carried out for EGFP and pStat3, which were also detected in skin carcinogenesis.[14] As shown in Figure 3(E), EGFP and pStat3 were detected in the same tumor cells in a squamous cell papilloma (Fig. 3E, upper panels, No. 1) and a keratoacanthoma (Fig. 3E, middle panels, No. 2). In contrast, pStat3 was not detected in normal skin tissues (Fig. 3E, lower panels).

In the experimental protocol for DMBA/TPA-induced skin tumorigenesis,[9,18] DMBA was applied as a single dose, whereas TPA was applied repeatedly from day 7 after DMBA treatment. Given that both compounds can induce L1-RTP (Figs 1,2), we wanted to clarify which compound was responsible

© 2011 Japanese Cancer Association

for the induction of L1-RTP in the tumors. Cells were first treated with DMBA and subsequently transfected with pEF06R on day 7 (Fig. 4A, left panel). A PCR-based assay was carried out 2 days later. As shown in Figure 4(A), no induction of L1-RTP was observed in cells that had been treated with DMBA 7 days previously (right panel). Similar observations were made in *in vivo* experiments. We did not detect L1-RTP on day 7 after treatment with DMBA (Fig. 4B, lanes 7–9). In striking contrast, L1-RTP was detected after dual treatment with TPA (Fig. 4B, lanes 10–12). These data indicate that the effects of DMBA on the induction of L1-RTP had disappeared before the initial treatment with TPA, and that L1-RTP occurred in the tumors during repeated treatment with TPA.

Discussion

In the present work, the induction of L1-RTP was shown to accompany DMBA/TPA-induced skin tumorigenesis. Specifically, L1-RTP was detected in 13 of 15 skin tumors, but not in any of the 20 normal skin tissues examined. Of particular interest was the observation that, whereas both DMBA and TPA activated L1-RTP *in vitro*, the brief exposure of hL1-EGFP mice to these same compounds indicated that L1-RTP was induced by TPA *in vivo* (Fig. 4). Although several studies have identified L1 insertions in human tumors,[1,7,8] none have provided insight into the mode of L1-RTP. To the best of our knowledge, ours is the first report showing the induction of L1-RTP during the promotion step of carcinogenesis. Considering that TPA is known to induce cellular inflammation through a mechanism involving EGFR and ERK (Fig. 2D,E),[12,13] an eminently reasonable hypothesis of the mode of L1-RTP in human tumors is that environmental stimuli such as chronic inflammation can activate L1-RTP. Given that our hL1-EGFP mice had low background levels of spontaneous L1-RTP in mouse embryonic fibroblasts (Fig. 3A), but responded well to external stimuli at 4–5 weeks after birth, these mice offer a versatile system for elucidating the molecular mechanism of tumor promotion.

Immunohistochemical analysis revealed Stat3 phosphorylation in cells positive for EFGP (Fig. 3E), a marker of L1-RTP induction. It has been shown that Stat3 is involved in not only DMBA/TPA-induced skin carcinogenesis[14,31,32] but also in the induction of cellular transformation by oncogenes.[33,34] To exclude the possibility that L1-RTP was initiated secondarily to the activation of Stat3, L1-RTP was examined under the forced expression of a constitutively active form of Stat3 (Stat3-C).[35] A PCR-based assay for L1-RTP indicated that L1-RTP was not induced by Stat3-C (Fig. S5). Additionally, the forced expression of activated *Ha-ras* did not cause Stat3 to be phosphorylated (Fig. S6), as has been reported.[34] Together, these observations support the notion that L1-RTP is not induced as a consequence of cellular transformation. Instead, L1-RTP induction appears to be an upstream event of Stat3 activation. Importantly, it was shown that activation of the *Ha-ras* gene in normal cells activates a growth arrest signal that depends on ataxia telangiectasia mutated (ATM).[36,37] An additional genetic alteration is required for tumor development, otherwise ATM-dependent

Table 2. Summary of long interspersed nucleotide element-1 retrotransposition (L1-RTP) and activated *Ha-ras* in skin tumors and nevi induced by 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate

Mouse lines	Tumors				Nevi		
	No.	L1-RTP	*Ha-ras*†	L1-RTP + *Ha-ras*	No.	L1-RTP	*H-ras*
#4	4	4	3	3	1	0	0
#67	11	9	8	6	7	1	0
Total	15	13	11	9	8	1	0
		(86.7)‡	(73.3)	(60.0)		(12.5)	

†From A to T at the first letter of codon 61 of *Ha-ras*. ‡Frequency expressed as percentage.

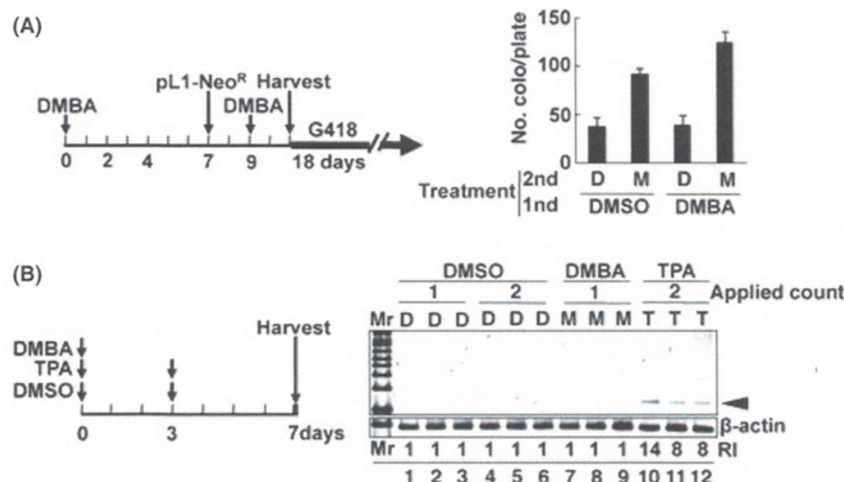

Fig. 4. Long interspersed nucleotide element-1 retrotransposition (L1-RTP) occurred in skin tumors during repeated treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). (A) The effects of 7,12-dimethylbenz[*a*]anthracene (DMBA) on the induction of L1-RTP were transient. Left panel, experimental protocol used to examine the effects of DMBA. Right panel, colony (colo) assay results. HuH-7 cells were exposed to 1 nM DMBA and cultured for a further 7 days. They were then transfected with pL1-Neo^R and divided into two aliquots. One aliquot was directly subjected to G418 selection without exposure to DMBA. As a positive control, the second aliquot was treated with the same concentration of DMBA then subjected to G418 selection. Mean number of colonies ± SD is shown. (B) Induction of L1-RTP by TPA in mice carrying human L1 and enhanced green fluorescent protein (hL1-EGFP mice). L1-RTP was assayed in hL1-EGFP mice that had been treated with TPA (T) or DMBA (M). Skin tissues of three different portions were harvested on day 2 after treatment with TPA or day 7 after treatment with DMBA. DNA extracted from each sample was subjected to PCR-based analysis. Relative intensity (RI) was calculated based on the signal intensity of the 140-bp band (arrowhead), using *β-actin* as an internal control. D, DMSO. Note: TPA treatment induced skin inflammation, in contrast to the reaction of skin tissues after repetitive treatment during chemical carcinogenesis.

doi: 10.1111/j.1349-7006.2011.02060.x
© 2011 Japanese Cancer Association

signaling would cause senescence rather than tumor growth. In the current study, the activation of *Ha-ras* was also detected in skin tumors (Table 2); thus, one plausible explanation is that the genetic alteration caused by TPA-induced L1-RTP attenuates ATM-dependent growth arrest by co-activating Stat3.

In vitro experiments revealed that DMBA and TPA induced L1-RTP by different mechanisms: DMBA-induced L1-RTP was dependent on AhR; TPA-induced L1-RTP was not. AhR functions as a receptor for environmental pollutants including dioxin,[38] and ligand-bound AhR is recruited to chromatin depending on the nuclear localization signals of ARNT1.[39] A heterodimer of AhR and ARNT1 then induces gene expression through xenobiotic responsive elements.[38] Notably, we observed that L1-RTP was induced by other genotoxic carcinogens, such as 3-methylcholanthrene and benzo[*a*]pyrene (B[*a*]P, with a similar dependency on AhR (Fig. S7). In contrast, we recently reported that 6-formylindolo(3,2-*b*)carbazole (FICZ), a photoproduct of tryptophan, induced L1-RTP, but in this case, L1-RTP was AhR-independent.[26] One possibility is that AhR mediates carcinogenic activity of the genotoxic compounds by inducing L1-RTP. To date, no reports on the carcinogenicity of FICZ are available. A dependency of carcinogen-induced L1-RTP on AhR is consistent with a previous report in which B[*a*]P-induced skin tumorigenesis was shown to require intact *AhR* alleles.[40]

An important next step will be to clarify the roles of TPA-induced L1-RTP. Recently, the glutathione S-transferase α4 (*Gstα4*) gene was identified as a candidate gene targeted by TPA.[41] *Gstα4* is located in a region of chromosome 9 that was identified by a genetic approach based on mouse strains with different susceptibilities to tumor promotion by TPA.[19] Notably, DMBA-induced tumor development is enhanced in mice lacking the *Gstα4* gene.[41] These observations suggest that the *Gstα4* gene is a candidate target for TPA-induced L1-RTP. Our PCR-based analyses, however, detected no apparent structural alterations in the *Gstα4* gene in DMBA/TPA-induced skin tumors (Fig. S8). Further analysis is required to identify the gene(s) responsible for tumor promotion by TPA, which in turn will provide insight into the functions of non-genotoxic carcinogens in tumor promotion.

Acknowledgments

We are grateful to Drs. Elena T. Luning Prak (University of Pennsylvania Medical Center, Philadelphia, PA, USA), Gilbert Nicolas (University of Michigan Medical School, Ann Arbor, MI, USA), and Jacqueline F. Bromberg (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) for providing us with pEF06R, pCEP4/L1*mneo*I/ColE1, and Stat3-C/pBabe, respectively. We thank Drs. Takayoshi Koyama and Hideaki Kawada and Miss Yukiko Shimizu (National Center for Global Health and Medicine, Tokyo, Japan) for technical assistance. We are also grateful to Drs. Tetsuya Mizoue and Yi Siyan (National Center for Global Health and Medicine) for assisting with statistical analysis of the experimental data. This work was supported in part by a Grant-in-Aid for Research from the Ministry of Health, Labour, and Welfare of Japan (09156296), a grant from the National Center for Global Health and Medicine (21A-104), and a grant from the Long-range Research Initiative from the Japan Chemical Industry Association. Mr. Noriyuki Okudaira was supported by a Grant-in-Aid from the Tokyo Biochemical Research Foundation.

Disclosure Statement

The authors have no conflicts of interest.

References

1 Goodier JL, Kazazian HH Jr. Retrotransposons revisited: the restraint and rehabilitation of parasites. *Cell* 2008; **135**: 23–35.

2 Bannert N, Kurth R. Retroelements and the human genome: new perspectives on an old relation. *Proc Natl Acad Sci USA* 2004; **101**: 14572–9.

3 Brouha B, Schustak J, Badge RM *et al.* Hot L1s account for the bulk of retrotransposition in the human population. *Proc Natl Acad Sci USA* 2003; **100**: 5280–5.

4 Morse B, Rotherg PG, Sourg VJ, Spandorfer JM, Astrin SM. Insertional mutagenesis of the myc locus by a LINE-1 sequence in a human breast carcinoma. *Nature* 1988; **333**: 87–90.

5 Miki Y, Nishio I, Horii A *et al.* Disruption of the APC gene by a retrotransposable insertion of L1 sequence in a colon cancer. *Cancer Res* 1992; **52**: 643–5.

6 Ting DT, Lipson D, Paul S *et al.* Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. *Science* 2011; **331**: 593–6.

7 Gilbert N, Lutz-Prigge S, Moran JV. Genomic deletions created upon LINE-1 retrotransposition. *Cell* 2002; **110**: 315–25.

8 Symer DE, Connelly C, Szak ST *et al.* Human l1 retrotransposition is associated with genetic instability in vivo. *Cell* 2002; **110**: 327–38.

9 Balmain A, Ramsden M, Bowden GT, Smith J. Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. *Nature* 1984; **307**: 658–60.

10 Nelson MA, Futscher BW, Kinsella T, Wymer J, Bowden GT. Detection of mutant Ha-ras genes in chemically initiated mouse skin epidermis before the development of benign tumors. *Proc Natl Acad Sci USA* 1992; **89**: 6398–402.

11 Chun KS, Kim SH, Song YS, Surh YJ. Celecoxib inhibits phorbol ester-induced expression of COX-2 and activation of AP-1 and p38 MAP kinase in mouse skin. *Carcinogenesis* 2004; **25**: 713–22.

12 Casanova ML, Larcher F, Casanova B *et al.* A critical role for ras-mediated, epidermal growth factor receptor-dependent angiogenesis in mouse skin carcinogenesis. *Cancer Res* 2002; **62**: 3402–7.

13 Bourcier C, Jacquel A, Hess J *et al.* p44 mitogen-activated protein kinase (extracellular signal-regulated kinase 1)-dependent signaling contributes to epithelial skin carcinogenesis. *Cancer Res* 2006; **66**: 2700–7.

14 Chan KS, Carbajal S, Kiguchi K *et al.* Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. *Cancer Res* 2004; **64**: 2382–9.

15 Naito M, Chenicek KJ, Naito Y, DiGiovanni J. Susceptibility to phorbol ester skin tumor promotion in (C57BL/6 × DBA/2) F1 mice is inherited as an incomplete dominant trait: evidence for multi-locus involvement. *Carcinogenesis* 1988; **9**: 639–45.

16 Reiners JJ Jr, Nesnow S, Slaga TJ. Murine susceptibility to two-stage skin carcinogenesis is influenced by the agent used for promotion. *Carcinogenesis* 1984; **5**: 301–7.

17 Angel JM, Beltrán L, Minda K, Rupp T, DiGiovanni J. Association of a murine chromosome 9 locus (Psl1) with susceptibility to mouse skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Mol Carcinog* 1997; **20**: 162–7.

18 Feith DJ, Shantz LM, Shoop PL *et al.* Mouse skin chemical carcinogenesis is inhibited by antizyme in promotion-sensitive and promotion-resistant genetic backgrounds. *Mol Carcinog* 2007; **46**: 453–65.

19 Angel JM, Caballero M, DiGiovanni J. Identification of novel genetic loci contributing to 12-O-tetradecanoylphorbol-13-acetate skin tumor promotion susceptibility in DBA/2 and C57BL/6 mice. *Cancer Res* 2003; **63**: 2747–51.

20 Hoshino S, Konishi M, Mori M *et al.* HIV-1 Vpr induces TLR4/MyD88-mediated IL-6 production and reactivates viral production from latency. *J Leukoc Biol* 2010; **87**: 1133–43.

21 Bennett BL, Sasaki DT, Murray BW *et al.* SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001; **98**: 13681–6.

22 Engedal N, Blomhoff HK. Combined action of ERK and NF kappa B mediates the protective effect of phorbol ester on Fas-induced apoptosis in Jurkat cells. *J Biol Chem* 2003; **278**: 10934–41.

23 Kobayashi S, Shimamura T, Monti S *et al.* Transcriptional profiling identifies cyclin D1 as a critical downstream effector of mutant epidermal growth factor receptor signaling. *Cancer Res* 2006; **66**: 11389–98.

24 Gilbert N, Lutz S, Morrish TA, Moran JV. Multiple fates of L1 retrotransposition intermediates in cultured human cells. *Mol Cell Biol* 2005; **25**: 7780–95.

25 Farkash EA, Kao GD, Horman SR, Prak ET. Gamma radiation increases endonuclease-dependent L1 retrotransposition in a cultured cell assay. *Nucleic Acids Res* 2006; **34**: 1196–204.

26 Okudaira N, Iijma K, Koyama T *et al.* Induction of long interspersed nucleotide element by 6-formylindolo[3,2-*b*]carbazole, a tryptophan photoproduct. *Proc Natl Acad Sci USA* 2010; **107**: 18487–92.

© 2011 Japanese Cancer Association

27 Okamura T, Miyoshi I, Takahashi K *et al.* Bilateral congenital cataracts result from a gain-of-function mutation in the gene for aquaporin-0 in mice. *Genomics* 2003; **81**: 361–8.
28 Brown K, Buchmann A, Balmain A. Carcinogen-induced mutations in the mouse c-Ha-ras gene provide evidence of multiple pathways for tumor progression. *Proc Natl Acad Sci USA* 1990; **87**: 538–42.
29 Denison MS, Pandini A, Nagy SR, Baldwin EP, Bonati L. Ligand binding and activation of the Ah receptor. *Chem Biol Interact* 2002; **141**: 3–24.
30 Husain Z, Pathak MA, Flotte T, Wick MM. Role of ultraviolet radiation in the induction of melanocytic tumors in hairless mice following 7,12-dimethylbenz(a)anthracene application and ultraviolet irradiation. *Cancer Res* 1991; **51**: 4964–70.
31 Chan KS, Sano S, Kataoka K *et al.* Forced expression of a constitutively active form of Stat3 in mouse epidermis enhances malignant progression of skin tumors induced by two-stage carcinogenesis. *Oncogene* 2008; **27**: 1087–94.
32 Chan KS, Sano S, Kiguchi K *et al.* Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. *J Clin Invest* 2004; **114**: 720–8.
33 Schlessinger K, Levy DE. Malignant Transformation but not normal cell growth depends on signal transducer and activator of transcription 3. *Cancer Res* 2005; **65**: 5828–34.
34 Gough DJ, Corlett A, Schlenssinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial stat3 supports ras-dependent oncogenic transformation. *Science* 2009; **324**: 1713–16.
35 Shen Y, Devgan G, Darnell JE Jr, Bromberg JF. Constitutively activated stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated stat1. *Proc Natl Acad Sci USA* 2001; **98**: 1543–8.
36 Bartkova J, Rezaei N, Liontos M *et al.* Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 2006; **444**: 633–7.
37 Di Micco R, Fumagalli M, Cicalese A *et al.* Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 2006; **444**: 638–42.
38 Beischlag TV, Luis Morales J, Hollingshead BD, Perdew GH. The aryl hydrocarbon receptor complex and the control of gene expression. *Crit Rev Eukaryot Gene Expr* 2008; **18**: 207–50.
39 Eguchi H, Ikuta T, Tachibana T, Yoneda Y, Kawajiri K. A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. *J Biol Chem* 1997; **272**: 17640–7.
40 Shimizu Y, Nakatsuru Y, Ichinose M *et al.* Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc Natl Acad Sci USA* 2000; **97**: 779–82.
41 Abel EL, Angel JM, Riggs PK *et al.* Evidence that Gsta4 modifies susceptibility to skin tumor development in mice and humans. *J Natl Cancer Inst* 2010; **102**: 1663–75.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. No cytotoxicity was detected in cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenz[*a*]anthracene (DMBA).

Fig. S2. Selection of human long interspersed nucleotide element-1–enhanced green fluorescent protein (hL1-EGFP) mouse strains suitable for carcinogenesis experiments.

Fig. S3. Southern blot analysis of copy numbers of transgenes in #4 and #67 in mice carrying human long interspersed nucleotide element-1 and enhanced green fluorescent protein (hL1-EGFP).

Fig. S4. Long interspersed nucleotide element-1 retrotransposition (L1-RTP) in nevi. Eight nevi samples were analyzed by PCR-based assay.

Fig. S5. Forced expression of constitutively activated form of Stat3 did not induce long interspersed nucleotide element-1 retrotransposition (L1-RTP).

Fig. S6. Forced expression of activated *H-ras* did not phosphorylate Stat3.

Fig. S7. Aryl hydrocarbon receptor (AhR) is required for long interspersed nucleotide element-1 retrotransposition (L1-RTP) by genotoxic carcinogens.

Fig. S8. No apparent abnormality of *Gsta4* gene in tumors induced by 7,12-dimethylbenz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate (DMBA/TPA).

Table S1. Nucleotide sequences of siRNAs targeting aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator 1 (*ARNT1*).

Table S2. Quantitative PCR of copy numbers of transgene in #4 and #67 in mice carrying human long interspersed nucleotide element-1 and enhanced green fluorescent protein (L1-EGFP).

Data S1. Full details of experimental methods used in this work.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi: 10.1111/j.1349-7006.2011.02060.x
© 2011 Japanese Cancer Association