research. Moreover, there is no systematic system for education about the theories and methods of behavioral genetics in the official curriculums of Japanese universities.

Recruiting twins into research programs presents a further difficulty. We do not have free access to official electronic databases of Japanese residents for scientific use, and conducting manual searches of the BRR is expensive. Compared with many Western countries, Japanese citizens tend to be less willing to participate in scientific research, particularly in psychology and social sciences. The overall average participation rate in our field is around 20% (Ogiwara, 2009; Shinogi, 2010), so data attrition is a serious problem.

Twin research is transitioning from traditional, quantitative-only methodology to the new integrated methodology of neurogenomics research. Recently, researchers from other fields such as economics, sociology, and even philosophy have become involved in twin studies in Japan. We believe that this promising trend will lead to a 'paradigm shift' in the human sciences in Japan.

Acknowledgments

The studies of the Keio Twin Research Center was supported by Grant-in-Aid for Scientific Research, Human Frontier Science Program, Brain Science and Education Program of RISTEX-JST, and Keio University. The authors thank Maki Koyama and Yoshiaki Someya for their contribution to MRI investigation. The authors also thank their technical assistants and twin families who contributed to their research activities.

References

Abidin, R. R. (1995). *Parenting stress index: Professional manual.* Odessa, FL: Psychological Assessment Resources.

Ahadi, S. A., Rothbart, M. K., & Ye, R. (1993). Children's temperament in the US and China: Similarities and differences. *European Journal of Personality, 7,* 359–377.

Ando, J., Nonaka, K., Ozaki, K., Sato, N., Fujisawa, K. K., Ozaki, K., ⋮⋮ Ooki, S. (2006). The Tokyo Twin Cohort Project: Overview and initial findings. *Twin Research and Human Genetics, 9,* 817–826.

Ando, J., Ono, Y., & Wright, M. (2001). Genetic structure of spatial and verbal working memory. *Behavior Genetics, 31,* 615–624.

Ando, J., Ono, Y., Yoshimura, K., Onoda, N., Shinohara, M., Kanba, S., & Asai, M. (2002). The genetic structure of Cloninger's seven-factor model of temperament and character in a Japanese sample. *Journal of Personality, 70,* 583–609.

Ando, J., Suzuki, A., Yamagata, S., Kijima, N., Maekawa, H., Ono, Y., & Jang, K. L. (2004). Genetic and environmental structure of Cloninger's temperament and character dimensions. *Journal of Personality Disorders, 18,* 379–393.

Anme, T. (1997). 'Evaluation of Environmental Stimulation (EES)' as predictive method related to decrease of physical 3 years later. *International Disability, 5,* 435–441.

Baron-Cohen, S., Allen, J., & Gillberg, C. (1992). Can autism be detected at 18 months? The needle, the haystack, and the CHAT. *British Journal of Psychiatry, 161,* 839–843.

Baron-Cohen, S., Hoekstra, R. A., Knickmeyer, R., & Wheelwright, S. (2006). The Autism Spectrum Quotient (AQ) — Adolescent version. *Journal of Autism Developmental Disorders, 36,* 343–350.

Bayley, N. (1993). *Bayley Scales of Infant Development: Administering and scoring manual.* New York: Psychological Corporation.

Bem, S. L. (1974). The measurement of psychological androgyny. *Journal of Consulting and Clinical Psychology, 42,* 155–162.

Birch, L. L., Fisher, J. O., Grimm-Thomas, K., Markey, C. N., Sawyer, R., & Johnson, S. L. (2001). Confirmatory factor analysis of the Child Feeding Questionnaire: A measure of parental attitudes, beliefs and practices about child feeding and obesity proneness. *Appetite, 36,* 201–210.

Bullinger, M., Mackensen, S., & Kirchberger, I. (1994). KINDL — ein Fragebogen zur gesundheitsbezogenen Lebensqualität von Kindern. *Zeitschrift für Gesundheitspsychologie, 2,* 64–67.

Byrne, B., Deleland, C., Fieldling-Barnsley, R., Quain, P., Samuelson, S., Hoien, T., ⋮⋮ Olson, R. K. (2002). Longitudinal twin study of early reading development in three countries: Preliminary results. *Annals of Dyslexia, 52,* 49–73.

Carver, C. S., & White, T. L. (1994). Behavioral inhibition, behavioral activation, and affective responses to impending reward and punishment: The BIS/BAS Scales. *Journal of Personality and Social Psychology, 67,* 319–333.

Cloninger, C. R., Svrakic, D. M., & Przybeck, T. R. (1993). A psychobiological model of temperament and character. *Archives of Genetic Psychiatry, 50,* 975–990.

Constantino, J. N., Cloninger, C. R., Clarke, A. R., & Hashemi, B. P. T. (2002). Application of the seven-factor model of personality to early childhood. *Psychiatry Research, 109,* 229–244.

Costa, P. T., Jr., & McCrae, R. R. (1992). *Revised NEO Personality Inventory (NEO-PI-R) and NEO Five-Factor Inventory (NEO-FFI) professional manual.* Odessa, FL: Psychological Assessment Resources.

Daniels, D., & Plomin, R. (1985). Differential experience of siblings in the same family. *Developmental Psychology, 21,* 747–760.

Deater-Deckard, K. (1996). *The Parent Feelings Questionnaire.* London: Institute of Psychiatry.

Deater-Deckard, K. (2000). Parenting and child behavioral adjustment in early childhood: A quantitative genetic approach to studying family processes and child development. *Child Development, 71,* 468–484.

DuPaul, G. J., Power, T. J., Anastopoulos, A. D., & Reid, R. (1998). *ADHD Rating Scale IV: Checklists, norms, and clinical interpretation.* New York: Guilford.

Ekehammar, B., Akrami, N., Hedlund, L. E., Yoshimura, K., Ono, Y., Ando, J., & Yamagata, S. (2010). The generality of personality heritability: Big-Five trait heritability predicts response time to trait items. *Journal of Individual Differences, 31,* 209–214.

Fenson, L., Dale, P. S., Reznick, J. S., Thal, D., Bates, E., Hartung, J. P., ⋮⋮⋮Reilly, J. S. (1993). *The MacArthur Communicative Development Inventories: User's guide and technical manual.* San Diego: Singular Publishing Group.

Fincham, F. D., & Bradbury, T. N. (1992). Assessing attributions in marriage: The Relationship Attribution Measure. *Journal of Personality and Social Psychology, 62,* 457–468.

Frankenburg, W. K., Dodds, J., Archer, P., Shapiro, H., & Bresnick, B. (1992). The Denver II: A major revision and restandardization of the Denver Developmental Screening Test. *Pediatrics, 89,* 91–97.

Fujisawa, K. K., & Ando, J. (2010). Behavior genetic analyses for cognitive development in early childhood: Comparisons between 42 and 60 months. *CARLS Series of Advanced Study of Logic and Sensibility, 3,* 121–130.

Fujisawa, K. K., & Ando, J. (2011). Genetic and environmental continuity and change of cognitive abilities between 42 and 60 months. *CARLS Series of Advanced Study of Logic and Sensibility, 4,* 153–163.

Fujisawa, K. K., Ozaki, K., Ozaki, K., Yamagata, S., Kawahashi, I., & Ando, J. (2012a). The genetic and environmental relationships between head circumference growth in the first year of life and sociocognitive development in the second year: A longitudinal twin study. *Developmental Science, 15,* 99–112.

Fujisawa, K. K., Wadsworth, S. J., Kakihana, S., Olson, R. K, DeFries, J. C., Byrne, B. & Ando, J. (2012b). *A multivariate twin study of early literacy in Japanese Kana.* Manuscript submitted for publication.

Fujisawa, K. K., Yamagata, S., Ozaki, K., & Ando, J. (2012c). Hyperactivity/inattention problems moderate environmental but not genetic mediation between negative parenting and conduct problems. *Journal of Abnormal Child Psychology, 40,* 189–200.

Garner, D. M., Olmsted, M. P., Bohr, Y., & Garfinkel, P. E. (1982). The Eating Attitudes Test: Psychometric features and clinical correlates. *Psychological Medicine, 12,* 871–878.

Gartstein, M. A., & Rothbart, M. K. (2003). Studying infant temperament via the Revised Infant Behavior Questionnaire. *Infant Behavior & Development, 26,* 64–86.

Golombok, S., & Rust, J. (1993). The Pre-School Activities Inventory: A standardized assessment of gender role in children. *Psychological Assessment, 5,* 131–136.

Goodman, R. (1999). The extended version of the Strengths and Difficulties Questionnaire as a guide to child psychiatric caseness and consequent burden. *Journal of Child Psychology and Psychiatry, 40,* 791–801.

Harada, Y., Saitoh, K., Iida, J., Sakuma, A., Iwasaka, H., Imai, J., ⋮⋮⋮Amano, N. (2004). The reliability and validity of the Oppositional Defiant Behavior Inventory. *European Child & Adolescent Psychiatry, 13,* 185–190.

Heath, A. C., Kessler, R. C., Neale, M. C., Hewitt, J. K., Eaves, L. J., & Kendler, K. S. (1993). Testing hypothesis about direction of causation using cross-sectional family data. *Behavior Genetics, 23,* 29–50.

Hiraishi, K., Ando, J., Ono, Y., & Hasegawa, T. (2008a). General trust and individual differences on the Wason selection task with sharing-rule. *Psychologia, 47,* 226–237.

Hiraishi, K., Sasaki, S., Shikishima, C., & Ando, J. (2012). The second to fourth digit ratio (2D:4D) in a Japanese twin sample: Heritability, prenatal hormone transfer and association with sexual orientation. *Archives of Sexual Behavior, 41,* 711–724.

Hiraishi, K., Yamagata, S., Shikishima, C., & Ando, J. (2008b). Maintenance of genetic variation in personality through control of mental mechanisms: A test of trust, extraversion, and agreeableness. *Evolution and Human Behavior, 29,* 79–85.

Horne, J. A., & Östberg, O. (1976). A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *International Journal of Chronobiology, 4,* 97–100.

Hur, Y. M., Kaprio, J., Iacono, W. G., Boomsma, D. I., McGue, M., Silventoinen, K., ⋮⋮⋮Mitchell, K. (2008). Genetic influences on the difference in variability of height, weight and body mass index between Caucasian and East Asian adolescent twins. *International Journal of Obesity, 32,* 1455–1467.

Jang, K. L., Hu, S., Livesley, W. J., Angleitner, A., Riemann, R., Ando, J., ⋮⋮⋮Hamer, D. H. (2001). The covariance structure of Neuroticism and Agreeableness: A twin and molecular genetic analysis of the role of the serotonin transporter gene. *Journal of Personality and Social Psychology, 81,* 295–304.

Jang, K. L., Livesley, W. J., Ando, J., Yamagata, S., Suzuki, A., Angleitner, A., ⋮⋮⋮Spinath, F. M. (2006). Behavioral genetics of the higher order factors of the Big Five. *Personality and Individual Differences, 41,* 261–272.

Kakihana, S., Ando, J., Koyama, M., Iitaka, S., & Sugawara, I. (2009). Cognitive factors relating to the development early literacy in the Kana syllabary. *Japanese Journal of Educational Psychology, 57,* 295–308.

Kamakura, T., Ando, J., & Ono, Y. (2001). Genetic and environmental influences on self-esteem in a Japanese twin sample. *Twin Research and Human Genetics, 4,* 439–442.

Kamakura, T., Ando, J., & Ono, Y. (2003). A twin study of genetic and environmental influences on psychological traits of eating disorders in a Japanese female sample. *Twin Research and Human Genetics, 6,* 292–296.

Kamakura, T., Ando, J., & Ono, Y. (2007). Genetic and environmental effects of stability and change in self-esteem during adolescence. *Personality and Individual Differences, 42,* 181–190.

Kaufman, A. S., & Kaufman, N. L. (1983). *K-ABC interpretative manual.* Circle Pines, MN: American Guidance Service.

Kitamura, T. (1993). Hospital anxiety and depression scale. *Seishinka Shindangaku, 4,* 371–373.

Klein, F., Sepekoff, B., & Wolf, T. J. (1985). Sexual orientation: A multi-variable dynamic process. *Journal of Homosexuality, 11,* 35–49.

Locke, H. J., & Wallace, K. M. (1959). Short marital adjustment and prediction tests: Their reliability and validity. *Marriage and Family Living, 21,* 251–255.

Lynn, R., Hampson, S., & Bingham, R. (1987). Japanese, British and American adolescents compared for Spearman's and for the verbal, numerical and visuospatial abilities. *Psychologia, 30*, 137–144.

McCrae, R. R., Yamagata, S., Jang, K. L., Riemann, R., Ando, J., Ono, Y., ⋮⋮ .Spinath, F. M. (2008). Substance and artifact in the higher-order factors of the Big Five. *Journal of Personality and Social Psychology, 95*, 442–455.

Müller, M. E. (1994). A questionnaire to measure mother-to-infant attachment. *Journal of Nursing Measurement, 2*, 129–141.

Mundy, P., Delgado, C., Block, J., Venezia, M., Hogan, A., & Seibert, J. (2003). *A manual for the Abridged Early Social Communication SCALES (ESCS)*. Miami, FL: University of Miami.

Murayama, K., Elliot, A. J., & Yamagata, S. (2011). Separation of performance-approach and performance-avoidance achievement goals: A broader analysis. *Journal of Educational Psychology, 103*, 238–256.

Nakagawa, A., & Sukigawa, M. (2005). How are cultural differences in the interpretation of infant behavior reflected in the Japanese Revised Infant Behavior Questionnaire? *Japanese Journal of Educational Psychology, 53*, 491–503.

Nozaki, M., Fujisawa, K. K., Ando, J., & Hasegawa, T. (In press). The effects of sibling relationships on social adjustment among Japanese twins compared with singletons. *Twin Research and Human Genetics.*

Ogiwara, N. (2009). Recent public polls [Research Services Report, 621, 1–5].

Olson, D. H. (1985). *FACES III (Family Adaptation and Cohesion Scales)*. St. Paul, MN: University of Minnesota.

Ono, Y., Ando, J., Onoda, N., Yoshimura, K., Kanba, S., Hirano, M., & Asai, M. (2000). Genetic structure of the five-factor model of personality in a Japanese twin population. *Keio Journal of Medicine, 49*, 152–158.

Ono, Y., Ando, J., Onoda, N., Yoshimura, K., Momose, T., Hirano, M., & Kanba, S. (2002). Dimensions of temperament as vulnerability factors in depression. *Molecular Psychiatry, 7*, 948–953.

Ooki, S., & Asaka, A. (2004). Zygosity diagnosis in young twins by questionnaire for twins' mothers and twins' self-reports. *Twin Research, 7*, 5–12.

Ooki, S., Yamada, K., Asaka, A., & Hayakawa, K. (1990). Zygosity diagnosis of twins by questionnaire. *Acta Geneticae Medicae et Gemellogiae, 39*, 190–115.

Osaka, R., & Umemoto, A. (1973). *Shintei Kyodai Nx15- dai nihan [Kyoto University new Nx15- intelligence test, the second version]*. Tokyo: Taisei shuppan.

Ozaki, K. (2008). Twin analysis on paired comparison data. *Behavior Genetics, 38*, 212–222.

Ozaki, K., & Ando, J. (2009). Direction of causation between shared and non-shared environmental factors. *Behavior Genetics, 39*, 321–336.

Ozaki, K., Toyoda, H., Iwama, N., Kubo, S., & Ando, J. (2011). Using non-normal SEM to resolve the ACDE model in the classical twin design. *Behavior Genetics, 41*, 329–339.

Parker, G., Tupling, H., & Brown, L. B. (1979). A parental bonding instrument. *British Journal of Medical Psychology, 52*, 1–10.

Putman, S. P., Jones, L. B., & Rothbart, M. K. (2002). *The Early Childhood Behavior Questionnaire: Development, psychometrics, factor structure, and relations with behavior problems.* Poster presented at the Biennial Meetings of the International Conference on Infant Studies, Toronto, Canada.

Robins, D., Fein, D., Barton, M., & Green, J. (2001). The Modified Checklist for Autism in Toddlers: An initial study investigating the early detection of autism and pervasive developmental disorders. *Journal of Autism and Developmental Disorders, 31*, 131–144.

Rosenberg, M. (1965). *Society and the adolescent self-image.* Princeton, NJ: Princeton University Press.

Rothbart, M. K., Ahadi, S. A., & Evans, D. E. (2000). Temperament and personality: Origins and outcomes. *Journal of Personality and Social Psychology, 78*, 122–135.

Rush, A. J., Trivedi, M. H., Ibrahim, H. M., Carmody, T. J., Arnow, B., Klein, D. N., ⋮⋮Keller, M. B. (2003). The 16-item Quick Inventory of Depressive Symptomatology (QIDS) Clinician Rating (QIDS-C) and Self-Report (QIDS-SR): A psychometric evaluation in patients with chronic major depression. *Biological Psychiatry, 54*, 573–583.

Rushton, J. P., Bons, T. A., Ando, J., Hur, Y.-M., Irwing, P., Vernon, P. A., ⋮⋮. Barbaranelli, C. (2009). A general factor of personality from multitrait–multimethod data and cross–national twins. *Twin Research and Human Genetics, 12*, 356–365.

Sadeh, A. (2004). A Brief Screening Questionnaire for Infant Sleep Problems: Validation and findings for an internet sample. *Pediatrics, 113*, 570–577.

Sasaki, S., Yamagata, S., Shikishima, C., Ozaki, K., & Ando, J. (2009). Gender differences in genetic and environmental etiology of gender role personality (BSRI). *Japanese Journal of Psychology, 80*, 330–338.

Sell, H., & Nagpal, R. (1992). *The subjective well being inventory* (Regional Health Paper, SEARO, 24). New Delhi: WHO.

Shikishima, C., & Ando, J. (2004). Transmission of social attitudes in a family: A behavioral genetic approach. *Japanese Journal of Family Sociology, 16*, 12–20.

Shikishima, C., Ando, J., Ono, Y., Toda, T., & Yoshimura, K. (2006). Registry of adolescent and young adult twins in the Tokyo area. *Twin Research and Human Genetics, 9*, 811–816.

Shikishima, C., Ando, J., Yamagata, S., Ozaki, K., Takahashi, Y., & Nonaka, K. (2008). Familial transmission of authoritarian conservatism: Genetic inheritance or cultural transmission? *Sociological Theory and Methods, 23*, 105–126.

Shikishima, C., Hiraishi, K., & Ando, J. (2006). Genetic and environmental influences on general trust: A test of a theory of trust with behavioral genetic and evolutionary psychological approaches. *Japanese Journal of Social Psychology, 22*, 48–57.

Shikishima, C., Hiraishi, K., Yamagata, S., & Ando, J (2011a). Factors affecting the formtion of empathy: A gene-environment interaction model. *Japanese Journal of Social Psychology, 26*, 188–201.

Shikishima, C., Hiraishi, K., Yamagata, S., Neiderhiser, J. M., & Ando, J. (In press). Culture moderates the genetic and environmental etiologies of parenting: A cultural behavior genetic approach. *Social Psychological and Personality Science.*

Shikishima, C., Hiraishi, K., Yamagata, S., Sugimoto, Y., Takemura, R., Ozaki, K., ⋮⋮ Ando, J. (2009). Is g an entity? A Japanese twin study using syllogisms and intelligence tests. *Intelligence, 37,* 256–267.

Shikishima, C., Ozaki, K., Ando, J., Toda, T., & Yoshimura, K. (2007). *Zygosity and physical/psychological similarities of twins.* Paper presented to the 21st Annual Meeting of Japan Society for Twin Studies, National Institute of Public Health, Saitama, Japan.

Shikishima, C., Yamagata, S., Hiraishi, K., Sugimoto, Y., Murayama, K., & Ando, J. (2011b). A simple syllogism-solving test: Empirical findings and implications for g research. *Intelligence, 39,* 89–99.

Shinogi, M. (2010). Changes in response rates of social research. *Advances in Social Research, 5,* 5–15.

Spielberger, C. D., Gorsuch, R., & Lushene, P. R. (1970). *Manual for the State-Trait Anxiety Inventory (form X).* Palo Alto, CA: Consulting Psychologists Press.

Stunkard, A. J., & Messick, S. (1985). The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. *Journal of Psychosomatic Research, 29,* 71–83.

Suzuki, K., Ando, J., & Sato, N. (2009). Genetic effects on infant handedness under spatial constraint conditions. *Developmental Psychobiology, 51,* 605–615.

Suzuki, K., Shikishima, C., & Ando, J. (2011). Genetic and environmental sex differences in mental rotation ability: A Japanese twin study. *Twin Research and Human Genetics, 14,* 437–443.

Takahashi, Y., Yamagata, S., Kijima, N., Shigemasu, K., Ono, Y., & Ando, J. (2007). Continuity and change in behavioral inhibition and activation systems: A longitudinal behavioral genetic study. *Personality and Individual Differences, 43,* 1616–1625.

Torrubia, R., Avila, C., Molto, J., & Caseras, X. (2001). The Sensitivity to Punishment and Sensitivity to Reward Questionnaire (SPSRQ) as a measure of Gray's anxiety and impulsivity dimensions. *Personality and Individual Differences, 31,* 837–862.

Uchida, A., Bribiescas, R. G., Ellison, P. T., Kanamori, M., Ando, J., Hirose, N., & Ono, Y. (2006). Age related variation of salivary testosterone values in healthy Japanese males. *The Aging Male, 9,* 207–213.

Vandenberg, S. G., & Kuse, A. R. (1978). Mental rotations, a group test of three-dimensional spatial visualization. *Perceptual and Motor Skills, 47,* 599–604.

Volling, B. L., & Blandon, A. Y. (2005). Positive indicators of sibling relationship quality: The Sibling Inventory of Behavior. In K. Anderson Moore & L. H. Lippman (Eds.), *What do children need to flourish? Conceptualizing and measuring indicators of positive development* (pp. 203–218). New York: Springer Science and Business Media.

Wright, J., De Geus, E., Ando, J., Luciano, M., Psothuma, D., Ono, Y., ⋮⋮ Boomsma, D. (2001). Genetics of cognition: Outline of a collaborative twin study. *Twin Research and Human Genetics, 4,* 48–56.

Yamagata, S., Suzuki, A., Ando, J., Ono, Y., Kijima, N., Yoshimura, K., ⋮⋮ Jang, K. L. (2006). Is the genetic structure of human personality universal? A cross-cultural twin study from North America, Europe, and Asia. *Journal of Personality and Social Psychology, 90,* 987–998.

Yamagata, S., Takahashi, Y., Kijima, N., Maekawa, H., Ono, Y., & Ando, J. (2005a). Genetic and environmental etiology of effortful control. *Twin Research and Human Genetics, 8,* 300–306.

Yamagata, S., Takahashi, Y., Ozaki, K., Fujisawa, K. K., Nonaka, K., & Ando, J. (In press). Bidirectional influences between maternal parenting and children's peer problems: A longitudinal monozygotic twin difference study. *Developmental Science.*

Yamagata, S., Takahashi, Y., Shigemasu, K., Ono, Y., & Kijima, N. (2005b). Development and validation of the Japanese version of Effortful Control scale for adults. *Japanese Journal of Personality Psychology, 14,* 30–41.

Yoshimura, K., Nakamura, K., Ono, Y., Sakurai, A., Saito, N., Mitani, M., ⋮⋮ Asai, M. (1998). Reliability and validity of the Japanese version of the NEO Five-Factor Inventory (NEO-FFI): A population-based survey in Aomori prefecture. *The Japanese Journal of Stress Sciences, 13,* 45–53.

Yu, C. C., Furukawa, M., Kobayashi, K., Shikishima, C., Cha, P. C., ⋮⋮ Toda, T. (2012). Genome-wide DNA methylation and gene expression analyses of monozygotic twins discordant for intelligence levels. *PLoS ONE, 7,* e47081.

Zigmond, A. S., & Snaith, R. P. (1983). The hospital anxiety and depression scale. *Acta Psychiatrica Scandinavia, 67,* 361–370.

Zung, W. W. K. (1965). A self-rating depression scale. *Archives of Genetic Psychiatry, 12,* 63–70.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 36, pp. 30823–30832, August 31, 2012

Human Natural Killer-1 Sulfotransferase (HNK-1ST)-induced Sulfate Transfer Regulates Laminin-binding Glycans on α-Dystroglycan*[S]

Received for publication, March 6, 2012, and in revised form, July 13, 2012 Published, JBC Papers in Press, July 16, 2012, DOI 10.1074/jbc.M112.363036

Naoki Nakagawa[‡], Hiroshi Manya[§], Tatsushi Toda[¶], Tamao Endo[§], and Shogo Oka[‡1]

From the [‡]Department of Biological Chemistry, Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, the [§]Molecular Glycobiology, Research Team for Mechanism of Aging, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo 173-0015, and the [¶]Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

Background: α-Dystroglycan undergoes extensive glycosylation required for the interaction between α-dystroglycan and its ligands such as laminin.
Results: HNK-1ST suppressed the glycosylation and reduced the ligand binding activity of α-dystroglycan.
Conclusion: The sulfotransferase activity of HNK-1ST is essential for the modulation of α-dystroglycan.
Significance: This study identifies a novel role for HNK-1ST as a regulator of the functional glycans on α-dystroglycan other than HNK-1 biosynthesis.

Retinoic acid (RA) is a well established anti-tumor agent inducing differentiation in various cancer cells. Recently, a robust up-regulation of human natural killer-1 sulfotransferase (HNK-1ST) was found in several subsets of melanoma cells during RA-mediated differentiation. However, the molecular mechanism underlying the tumor suppression mediated by HNK-1ST remains unclear. Here, we show that HNK-1ST changed the glycosylation state and reduced the ligand binding activity of α-dystroglycan (α-DG) in RA-treated S91 melanoma cells, which contributed to an attenuation of cell migration. Knockdown of HNK-1ST restored the glycosylation of α-DG and the migration of RA-treated S91 cells, indicating that HNK-1ST functions through glycans on α-DG. Using CHO-K1 cells, we provide direct evidence that HNK-1ST but not other homologous sulfotransferases (C4ST1 and GalNAc4ST1) suppresses the glycosylation of α-DG. The activity-abolished mutant of HNK-1ST did not show the α-DG-modulating function, indicating that the sulfotransferase activity of HNK-1ST is essential. Finally, the HNK-1ST-dependent incorporation of [^{35}S]sulfate groups was detected on α-DG. These findings suggest a novel role for HNK-1ST as a tumor suppressor controlling the functional glycans on α-DG and the importance of sulfate transfer in the glycosylation of α-DG.

Invasiveness is a hallmark of malignant tumors. In the initial phase of invasion, cell-cell and/or cell-extracellular matrix interactions are crucial (1). The external region of a cell membrane, known as the glycocalyx, is dominated by glycosylated molecules, which have important roles in these interactions (2). Therefore, the aberrant expression of various genes involved in glycan synthesis or degradation, which causes compositional changes of the glycocalyx, is frequently associated with malignant transformation (3–5). Recently, Zhao *et al.* (6) reported the expression of human natural killer-1 sulfotransferase (HNK-1ST) to be strongly up-regulated in several subsets of murine and human melanoma cells during retinoic acid (RA)[2]-mediated differentiation. The expression of HNK-1ST is activated via an RA receptor-γ pathway, and the invasiveness of melanoma cells is suppressed along with HNK-1ST induction (6). HNK-1ST is a sulfotransferase involved in the biosynthesis of the HNK-1 carbohydrate, a neural glyco-epitope exhibiting abundant expression during brain development (7). Although HNK-1ST has the potential to control the cell surface expression of the HNK-1 carbohydrate, it is not clear how HNK-1ST is associated with the tumor-suppressive function.

We have demonstrated that the HNK-1 carbohydrate is required for the structural and functional development of the mammalian nervous system, such as the maturation of dendritic spines and the acquisition of synaptic plasticity, respectively (8–10). The HNK-1 carbohydrate has a unique structural feature, *i.e.* a sulfated glucuronic acid is attached to the nonreducing terminal of an *N*-acetyllactosamine residue (11, 12). Because the *N*-acetyllactosamine structure is commonly found in various glycoproteins and glycolipids, two glucuronyltransferases (GlcAT-P and GlcAT-S) and a sulfotransferase (HNK-1ST) had been cloned and characterized as key enzymes for the biosynthesis (7). GlcAT-P or GlcAT-S and HNK-1ST interact closely as a functional complex, cooperatively synthesizing the HNK-1 carbohydrate (13). However, although GlcAT-P and GlcAT-S show a highly restricted tissue distribution (14, 15),

* This work was supported in part by Grant-in-aid for Challenging Exploratory Research 23659153 (to S. O.) from Japan Society for the Promotion of Science and for Scientific Research on Innovative Areas and Grant 23110006 (to S. O.) from Ministry of Education, Culture, Sports, Science and Technology.
[S] This article contains supplemental Figs. S1–S5.
[1] To whom correspondence should be addressed: Kawahara-cho 53, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan. Tel./Fax: 81-75-751-3959; E-mail: shogo@hs.med.kyoto-u.ac.jp.

[2] The abbreviations used are: RA, retinoic acid; α-DG, α-dystroglycan; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; CMD, congenital muscular dystrophy; EGFP, enhanced GFP.

HNK-1ST is more ubiquitous and exists in several tissues where neither GlcAT-P nor GlcAT-S is observed, including skeletal muscle, heart, spleen, and reproductive organs (16, 17). These findings suggest that HNK-1ST has another function, which might underlie the RA-mediated melanoma differentiation.

α-Dystroglycan (α-DG) is a ubiquitously expressed peripheral membrane glycoprotein, which serves as a receptor for extracellular matrix components, including laminin, agrin, and perlecan (18, 19). α-DG is anchored on the plasma membrane by β-DG, which interacts with cytoskeletal proteins, together comprising the DG complex that provides physical links between the cell and basal lamina (18–20). α-DG undergoes extensive glycosylation in a tissue-specific manner (18, 19, 21), and the attached glycan acts as a critical mediator of the interaction between α-DG and its ligands (22, 23). Although the precise structure of the glycan important for the function of α-DG has not completely been determined, aberrant glycosylation of α-DG has already been identified in the pathogenesis of several types of congenital muscular dystrophy (CMD) accompanied by brain and eye malformations (24, 25). In CMD patients, mutations in six known or putative glycosyltransferase genes involved in the biosynthesis of *O*-mannosyl glycan, including protein *O*-mannosyltransferase 1 (*POMT1*), *POMT2*, protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (*POMGnT1*), *fukutin*, fukutin-related protein (*FKRP*), and like-acetylglucosaminyltransferase (*LARGE*), have been found (26–31). These observations indicate that *O*-mannosyl modification is essential for the functional glycosylation of α-DG. Furthermore, altered glycosylation of α-DG is also implicated in epithelium-derived cancer progression, demonstrating the involvement of α-DG in tumorigenic phenotypes (32, 33).

In this report, we show that HNK-1ST induced in RA-treated S91 melanoma cells suppressed the glycosylation and ligand binding activity of α-DG. The functional loss of α-DG resulted in a reduction in cell migration. Using CHO-K1 cells, we provide direct evidence that HNK-1ST actually has the ability to inhibit the synthesis of glycans on α-DG, suppressing the interaction between α-DG and laminin. Furthermore, the HNK-1ST-dependent incorporation of sulfate groups was detected on α-DG. These results suggest a novel tumor-suppressive role for HNK-1ST, which acts as a functional regulator of α-DG via sulfate transfer.

EXPERIMENTAL PROCEDURES

cDNA Construction—For α-DG-Fc, the coding sequence of human α-DG was amplified by PCR with primers ACGATCGATGCCACCATGAGGATGTCTGT (with ClaI site) and CCGACTAGTACTCACCGCCCCGGGTGATATTCTGCA (with SpeI site), using pcDNA3.1 containing human *DAG1* cDNA as a template, and subcloned into pEF-Fc. Expression plasmids for FLAG-GlcAT-P, pIRES-GlcAT-P/HNK-1ST (pIRES-P/ST), HNK-1ST-EGFP, C4ST1-EGFP, and GalNAc4ST1-EGFP were described previously (13). For LARGE-myc, the full-length cDNA of mouse *LARGE* was amplified from pBC SK$^+$ containing mouse *LARGE* cDNA, which was provided by Kazusa DNA Research Institute, using primers TCTGAGAGGATGCTGGGAAT and AAAGGGCCCCTGTTGTTCTCAGCTGTGAG (skipping stop codon, with ApaI site). After the resulting fragment had been digested with ApaI, it was ligated to pcDNA3.1/myc-His B (Invitrogen), which had been digested with EcoRV and ApaI. Construction of R189A-EGFP, the plasmid encoding the R189A mutant of HNK-1ST, was described previously (34).

Cell Culture and Transfection—CHO-K1 cells were maintained in α-minimum essential medium with 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. S91 murine melanoma cells (a gift from Dr. A. Kurosaka, Kyoto Sangyo University), also known as M3, were maintained in Dulbecco's modified Eagle's medium with 10% FBS in 5% CO_2 at 37 °C. For cDNA transfection, cells were grown overnight and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To obtain the extracellularly secreted proteins, the culture medium was replaced with serum-free OPTI-MEM I (Invitrogen) after 5 h of incubation.

RA Treatment—RA (all-*trans*-retinoic acid, Sigma) was dissolved in DMSO, kept as a 10 mM stock solution at −20 °C, and then diluted to the final concentration in the growth medium before being added to cells. Cells were harvested after 16 h for the RT-PCR analysis or after 48 h for Western blotting and the migration assay.

siRNA-mediated Knockdown—siRNA oligonucleotides specific for mouse *HNK-1ST* and a negative control siRNA were obtained from Qiagen. Oligonucleotide sequences used were as follows: si-ST1 5′-GGAUGGGUAUAGUGCCAAATT-3′ and 5′-UUUGGCACUAUACCCAUCCGG-3′ and si-ST2 5′-CAGAUUUCUUGCUAAAUUATT-3′ and 5′-UAAUUUAGCAAGAAAUCUGGT-3′. siRNA oligonucleotides were transiently transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The culture medium was replaced with growth medium after 5 h of incubation.

Purification of Recombinant α-DG-Fc—The culture medium and cells were collected at 48 h post-transfection. The cells were lysed with Tris-buffered saline (pH 7.4) containing 1% Triton X-100 and protease inhibitor mixture (Nakalai Tesque), and then the cell extracts were obtained by centrifugation. The culture medium and the cell extracts were incubated with protein G-Sepharose (GE Healthcare) for 2 h at 4 °C. The beads were washed extensively with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, and the bound proteins were eluted by boiling in Laemmli sample buffer (LSB).

Western Blotting and Laminin Overlay Assay—Proteins solubilized in LSB were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. Protein bands were detected with Super Signal West Pico chemiluminescence reagent (Thermo Scientific) using an LAS-3000 Luminoimage Analyzer (FUJIFILM). The following primary antibodies were used: HNK-1 mAb (a hybridoma cell line was purchased from American Type Culture Collection); M6749 mAb (against a nonsulfated form of the HNK-1 carbohydrate, a gift from Dr. H. Tanaka, Kumamoto University); GP2 pAb (a rabbit anti-GlcAT-P pAb raised against the catalytic region of the recombinant human GlcAT-P); anti-Fc pAb (Jackson ImmunoResearch); anti-EGFP mAb (Clontech); anti-Myc mAb and IIH6 mAb (Millipore); anti-laminin pAb and anti-

FLAG pAb (Sigma); anti-β-DG mAb (Novocastra); α-DG core pAb (goat polyclonal antibody against the C-terminal domain of the α-DG polypeptide) (35); and anti-GAPDH mAb (Calbiochem). For the laminin overlay assay, nitrocellulose membranes with transferred proteins were blocked with laminin-binding buffer (LBB: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) containing 5% nonfat dry milk. After being washed in LBB, the membranes were incubated with 1 μg/ml laminin-1 (Sigma) diluted with LBB containing 3% bovine serum albumin (BSA) for 90 min at room temperature. Bound laminin-1 was detected using anti-laminin antibody by immunoblotting as described above. For the quantification of the intensity of the protein bands, densitometric analyses were performed using image analysis software ImageGauge (FUJIFILM).

Biotinylation of Cell Surface Proteins—Cells cultured in 60-mm dishes were washed twice with ice-cold PBS and incubated with 1 mg/ml EZ-link Sulfo-NHS-SS-biotin (Thermo Scientific) in PBS for 30 min at 4 °C. Cells were washed twice with PBS and lysed with Tris-buffered saline (pH 7.4) containing 1% Triton X-100 and protease inhibitor mixture, and then the cell extracts were obtained by centrifugation. The biotinylated proteins were precipitated with immobilized streptavidin (Thermo Scientific) and analyzed by Western blotting as described above.

Radioactive Metabolic Labeling—CHO cells were grown overnight in 25-cm^2 cell culture flasks under normal conditions. At 5 h post-transfection, the culture medium was replaced with sulfate-free M8028 minimum Eagle's medium (Sigma), and incubation was continued for 1 h. The cells were then labeled with 30 μCi/ml [^{35}S]sodium sulfate (ARC Inc.). The culture medium was collected after two nights of labeling and incubated with protein G-Sepharose for 2 h. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then subjected to autoradiography or Western blotting using anti-Fc pAb.

RNA Isolation and RT-PCR—S91 cells were harvested and homogenized in TRIzol (Invitrogen) and extracted with chloroform and isopropyl alcohol. The RNA was dissolved in RNase-free water. One microgram of total RNA was digested with DNaseI and then converted to cDNA using SuperScriptII (Invitrogen). The following primer pairs were used for detecting the mRNA expression: HNK-1ST, GACCCGGGGGATCCAGTTTGAAGAT and GTCTCGTTTGCTGATGCCCAGGAAG; α-DG, GCCAGATTCGCCCAACACTGACAAT and CCACCCAGGCATCTACCCTGTCAAT; LARGE, GTCAGATGCAGAAGCCCAGCAGTTC and TGGGGAAAGAGAGTCTGTAGCGCAG; LARGE2, CGAGAGCTGCTCACTCTGAT and GGCATCCAAAGAGCTCTCTT; POMGnT1, TCGTGGGACGAAAAGGAGGTCC and TGGGCCGGTTCCCTGCAATG; POMT1, TTGCCCGCATCACCCAAGGC and GGCTGCGACATCGTGCGTGTT; POMT2, TTGCTGGCTACCTGAGCGGG and AGGGGGCAGAGAAAGGCCTGTT; and GAPDH, GGAAGGGCTCATGACCACAGTCCAT and CATACTTGGCAGGTTTCTCCAGGCG. Quantitative PCR was performed with a Chromo 4 Real Time System (Bio-Rad) using SYBR Green I. Each sample was run in triplicate, and GAPDH mRNA was amplified from the same sample to normalize the expression level.

Migration Assays—Cell migration was assayed using 24-well transwell plates (8 μm pore size) (BD Biosciences) according to the manufacturer's manual. Prior to the assay, S91 cells were treated with 1 μM RA or 0.1% DMSO for 48 h, and the insert membranes were coated with laminin-1 (10 μg/ml) (BD Biosciences). The 1×10^5 S91 cells suspended in serum-free DMEM were seeded into the upper chamber, and DMEM supplemented with 10% FCS was placed in the lower chamber. 1 μM RA or 0.1% DMSO was added to both chambers during the migration period. After 24 h, the inserts were fixed in ice-cold methanol, and nonmigrating cells were removed from the upper surface of the membrane. The inserts were stained in 0.1% Toluidine Blue O solution for 15 min and washed two times with PBS. The membranes were cut and mounted on glass slides. Five randomly selected fields per membrane were counted, and the average was shown as the migrated cell number.

Immunofluorescence—Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and then incubated with primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies. For permeabilization of the plasma membrane, cells were incubated with PBS containing 3% BSA and 0.1% Triton X-100 for 15 min after the fixation. Immunofluorescent images were acquired by a Fluoview laser confocal microscope system (Olympus). The following antibodies were used: IIH6 mAb (Millipore); anti-HIS pAb (Santa Cruz Biotechnology); GM130 mAb (BD Biosciences); and anti-β-DG mAb (Novocastra).

Statistics—Statistical significance was determined by a two-tailed Student's *t* test for comparisons between two groups and by an analysis of variance with Ryan's test for comparisons among multiple groups.

RESULTS

Specific Induction of HNK-1ST Expression and Alternate Glycosylation of α-DG in RA-treated Melanoma Cells—To investigate whether HNK-1ST governs tumor-related phenotypes of melanoma cells via production of the HNK-1 carbohydrate or not, we evaluated the mRNA expression of the enzymes synthesizing HNK-1 in S91 murine melanoma cells, which undergo RA-mediated differentiation (6, 36). Following treatment with 1 μM RA, a marked increase of HNK-1ST mRNA was detected using RT-PCR (Fig. 1, *A* and *B*), consistent with a previous report (6). However, neither of the glucuronyltransferases (GlcAT-P and GlcAT-S) responsible for producing HNK-1 was observed (Fig. 1*A*), suggesting the absence of the HNK-1 carbohydrate in S91 cells. To confirm this notion, we examined the expression of the HNK-1 carbohydrate in S91 cells treated with DMSO or RA using an HNK-1 monoclonal antibody (mAb). As expected, HNK-1 carbohydrate was not detected even in RA-treated cells (Fig. 1*C*). As we reported previously that the HNK-1 mAb specifically recognizes sulfated form of HNK-1 epitope but not nonsulfated one (17), we tried to examine whether the sulfated form of HNK-1 epitope was expressed in S91 cells by the transfection of GlcAT-P. As expected, HNK-1 immunoreactivity was detected by GlcAT-P transfection, sug-

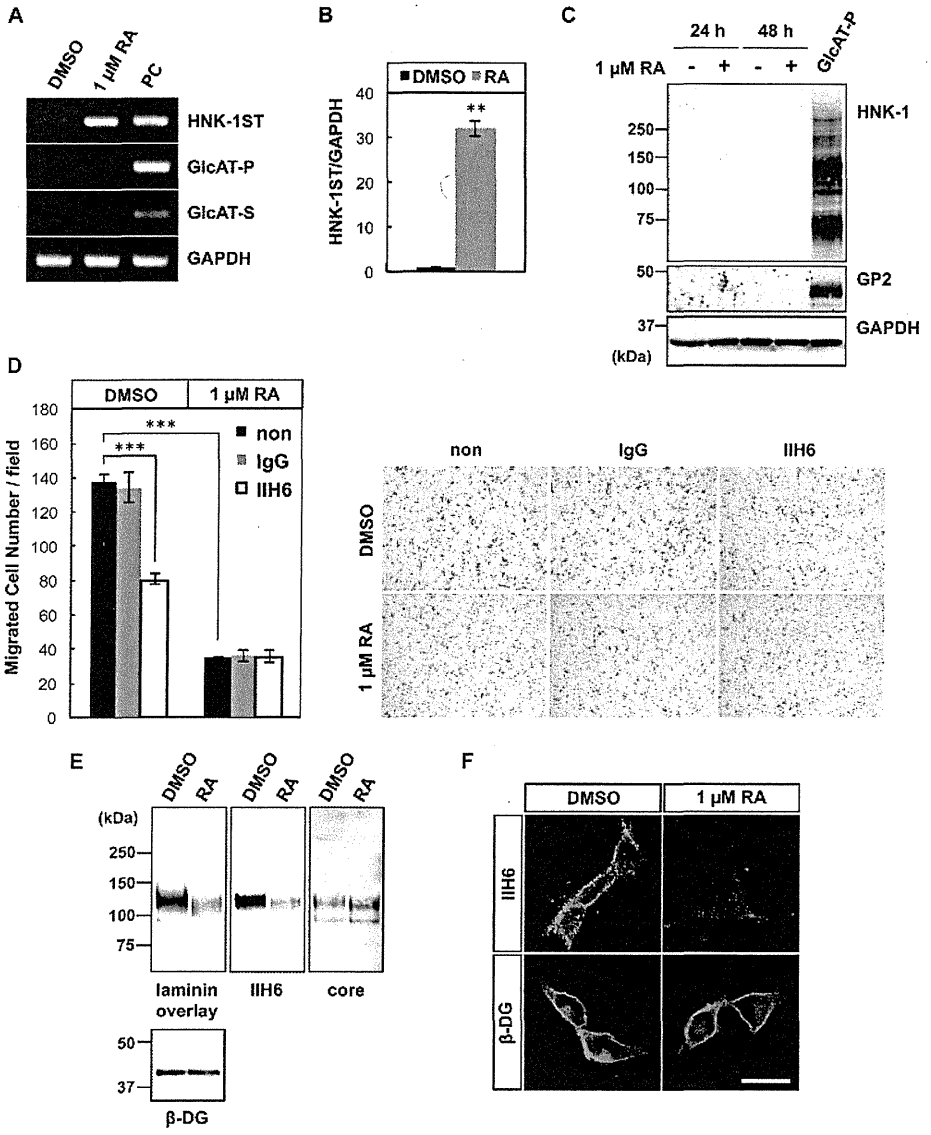

FIGURE 1. **Altered glycosylation of α-DG associated with HNK-1ST induction in RA-treated melanoma cells.** *A*, RT-PCR was performed using mRNA extracted from S91 cells treated with DMSO or 1 μM RA for 16 h. mRNA prepared from 2-week-old mouse brain was used as a positive control (*PC*). *B*, amount of HNK-1ST mRNA was quantified by quantitative RT-PCR, normalized to that of GAPDH mRNA, and shown as HNK-1ST/GAPDH. The value for DMSO-treated cells was set at 1. The *graphs* indicate the mean ± S.E. for three independent experiments. **, $p < 0.01$. *C*, S91 cells were treated with DMSO or 1 μM RA for the periods indicated. The cells were lysed, and an equal amount of protein (50 μg) from each sample was analyzed by Western blotting with HNK-1 mAb, GP2 (anti-GlcAT-P) pAb, and anti-GAPDH mAb. As a positive control for the HNK-1 mAb, GlcAT-P cDNA was transiently transfected into S91 cells (GlcAT-P). *D*, migration assay was carried out using transwell chambers with insert membranes coated with 10 μg/ml laminin-1. The migration of S91 cells pretreated with DMSO or 1 μM RA for 48 h was assessed (*non*). For antibody treatment assays, IIH6 mAb or normal mouse IgG was added in the upper chamber (IIH6 and IgG, respectively). The *graphs* indicate the mean ± S.E. for three independent experiments (*left panel*). ***, $p < 0.001$. The representative toluidine blue staining images of the insert membranes are shown (*right panel*). *E* and *F*, effect of RA on α-DG was investigated using S91 cells treated with DMSO or 1 μM RA for 48 h. *E*, cell surface proteins were biotinylated, pulled down by streptavidin-agarose beads, and analyzed by laminin overlay assay or Western blotting with IIH6 mAb, anti-β-DG mAb, and α-DG core pAb. *F*, cells were immunostained by IIH6 or anti-β-DG mAb. *Scale bar*, 20 μm.

gesting that a functional HNK-1ST was expressed in the cells (Fig. 1*C*). These results indicate that HNK-1ST acts solely as a tumor suppressor and its function is independent of HNK-1 biosynthesis.

It has been demonstrated that α-DG, especially the glycan attached to it, is involved in tumor invasiveness in various cancer types such as breast, prostate, and lung carcinomas (32, 33). Because HNK-1ST associates with the modification of glycans, up-regulation of HNK-1ST expression might affect the glycosylation in cells. Hence, we speculated that RA treatment causes a compositional change in the glycan on α-DG, leading to a suppression of melanoma invasiveness. To test this possibility, we explored the involvement of α-DG in the migratory behavior of S91 cells using a transwell migration assay with laminin-coated membranes. The RA-treated cells showed significantly decreased migration (Fig. 1*D*), which confirmed the anti-tumor effect of RA. Intriguingly, on the addition of an IIH6 mAb, which recognizes the laminin-binding glycan on α-DG and can disturb the α-DG-ligand interaction (23), S91 cells showed substantially reduced migration (Fig. 1*D*). Moreover, RA treatment

eliminated the susceptibility to the IIH6 mAb (Fig. 1*D*). These findings suggest that the glycan recognized by IIH6 on α-DG positively regulates the motility of control S91 cells, and the reduced motility in RA-treated cells is due to the glycosylation state of α-DG. Then, we employed biochemical analyses to clarify the functional alteration of α-DG caused by RA. α-DG was enriched from RA-treated cells using cell surface biotinylation and subjected to a laminin overlay assay and immunoblotting with the IIH6 mAb. RA-treated S91 cells exhibited considerably decreased laminin binding activity of α-DG (to 43.8%) and drastically reduced IIH6 immunoreactivity (to 14.5%) (Fig. 1*E* and supplemental Fig. S1*A*). The expression of α-DG core protein and β-DG was unaltered by RA (Fig. 1*E*), indicating that the treatment resulted in a change in the glycosylation of α-DG but not in the cell surface abundance of α-DG itself. Immunofluorescence analyses also demonstrated RA-dependent disappearance of the IIH6 epitope, which intrinsically localized on the plasma membrane of S91 cells (Fig. 1*F*). Although the precise glycan structure recognized by the IIH6 mAb is still unknown, LARGE, a putative glycosyltransferase, is one of the most potent inducers of the IIH6-positive laminin-binding glycan on α-DG (37, 38). The IIH6-positive laminin-binding glycan on α-DG was induced by the transfection of LARGE in S91 cells (supplemental Fig. S2, *A* and *B*). However, RA treatment eliminated the generation of the IIH6 epitope even in cells overexpressing LARGE (supplemental Fig. S2, *A* and *B*). Taken together, these results revealed that RA had a strong effect inducing a functional change of α-DG by altering its glycosylation, which contributed at least in part to the RA-mediated suppression of cell motility.

Involvement of HNK-1ST in Functional Glycan Synthesis on α-DG and Cell Migration—In DMSO- and RA-treated S91 cells, the expression patterns of α-DG and various glycosyltransferases involved in the synthesis of laminin-binding glycan and IIH6 epitope were unchanged (Fig. 2*A*). Therefore, we sought the role of HNK-1ST, which showed dynamic induction by RA, as a key determinant controlling α-DG glycosylation. To examine whether the RA-dependent regulation of α-DG was mediated by HNK-1ST, we performed knockdown analyses of HNK-1ST using siRNA. Two different siRNAs against *HNK-1ST* (si-ST1 and -2) were used. Western blot analysis showed that both si-ST1 and -2 substantially restored the laminin binding activity (from 46.4 to 77 and 60.3%) and IIH6 epitope of α-DG (from 19.1 to 60 and 42.7%) in RA-treated S91 cells, compared with the control siRNA (si-Cont) (Fig. 2*B* and supplemental Fig. S1*B*). Then we assessed the knockdown efficacy in siRNA-transfected cells by quantitative RT-PCR. Compared with the RA-treated control, the amount of HNK-1ST mRNA was reduced to 38.9 and 47.1% in si-ST1- and -2-transfected cells, respectively (Fig. 2*C*). In contrast, forced expression of EGFP-tagged HNK-1ST effectively reduced the laminin binding activity of α-DG and IIH6 epitope production regardless of LARGE overexpression (Fig. 2*D*). Collectively, these analyses provide direct evidence that HNK-1ST negatively regulates the glycosylation of α-DG, which is a novel role for HNK-1ST as a functional regulator of α-DG. Furthermore, we analyzed the effect of down-regulation of HNK-1ST on the migration of S91 cells. Using the transwell assay, both si-ST1 and -2 were found to partially ameliorate the migration of RA-treated S91 cells (Fig. 2*E*). si-ST1 induced a much more effective recovery of migration than si-ST2, which was well correlated with the amount of IIH6 epitope shown in Fig. 2*B*, indicating significant involvement of this glyco-epitope in the migration of S91 cells.

Expression of HNK-1ST Abrogates LARGE-dependent Glycosylation on α-DG—HNK-1ST was found to have the potential to suppress the glycosylation by LARGE, prompting us to further investigate the functional interaction between HNK-1ST and LARGE in the glycosylation of α-DG. We generated an expression plasmid encoding α-DG fused to a human IgG Fc fragment (α-DG-Fc), which would be secreted into the culture medium. In addition to α-DG-Fc, LARGE-myc and HNK-1ST-EGFP were simultaneously transfected into CHO-K1 cells. α-DG-Fc was pulled down from the culture medium and analyzed by Western blotting. The extensive glycosylation induced by LARGE was detected by laminin overlay assay and immunoblotting with IIH6 mAb, as a broad and high molecular band (Fig. 3*A*). However, when α-DG-Fc was co-transfected with HNK-1ST-EGFP, there was a remarkable decrease in the laminin binding activity and almost complete loss of IIH6 immunoreactivity, despite the comparable expression of LARGE-myc (Fig. 3, *A* and *B*). The results obtained from this simple expression system clearly demonstrated that HNK-1ST actually inhibits the formation of the glycan on α-DG. Furthermore, to explore whether a similar effect could be found with other homologous sulfotransferases, we co-transfected LARGE-myc and C4ST1-EGFP or GalNAc4ST1-EGFP, both of which belong to the HNK-1ST family (39, 40). LARGE-dependent glycosylation of α-DG was not suppressed by either C4ST1 or GalNAc4ST1 (Fig. 4, *A* and *B*), indicating that the α-DG-modulating function is specific to HNK-1ST.

Interaction between α-DG and LARGE Is Unaltered in the Presence of HNK-1ST—To investigate the molecular basis underlying the inhibitory effect of HNK-1ST on the glycosylation of α-DG, we tested the following two possibilities: that HNK-1ST, causing steric hindrance, prevents glycosyltransferases from approaching α-DG, and that HNK-1ST acts as a sulfotransferase to suppress the glycosylation of α-DG. First, we analyzed whether the interaction between α-DG and LARGE is attenuated in the presence of HNK-1ST because the interaction is a crucial step in the LARGE-dependent glycosylation of α-DG (41). We observed no significant change in the interaction between α-DG-Fc and LARGE-myc, regardless of HNK-1ST-EGFP expression (supplemental Fig. S3*A*), indicating that HNK-1ST does not cause steric hindrance. In addition, we confirmed that the subcellular localization of LARGE-myc in the Golgi apparatus (42) was unaltered by co-expression with HNK-1ST-EGFP (supplemental Fig. S3*B*).

Sulfotransferase Activity Is Prerequisite for HNK-1ST to Modulate α-DG Glycosylation—Next, we generated R189A-EGFP, a plasmid encoding a form of HNK-1ST that harbors a mutation of Arg^{189} to Ala, exhibiting almost no enzymatic activity due to impaired binding to the donor substrate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (34, 43). R189A-EGFP did not synthesize the HNK-1 carbohydrate when co-transfected with GlcAT-P, which confirmed the disappearance of its sulfotrans-

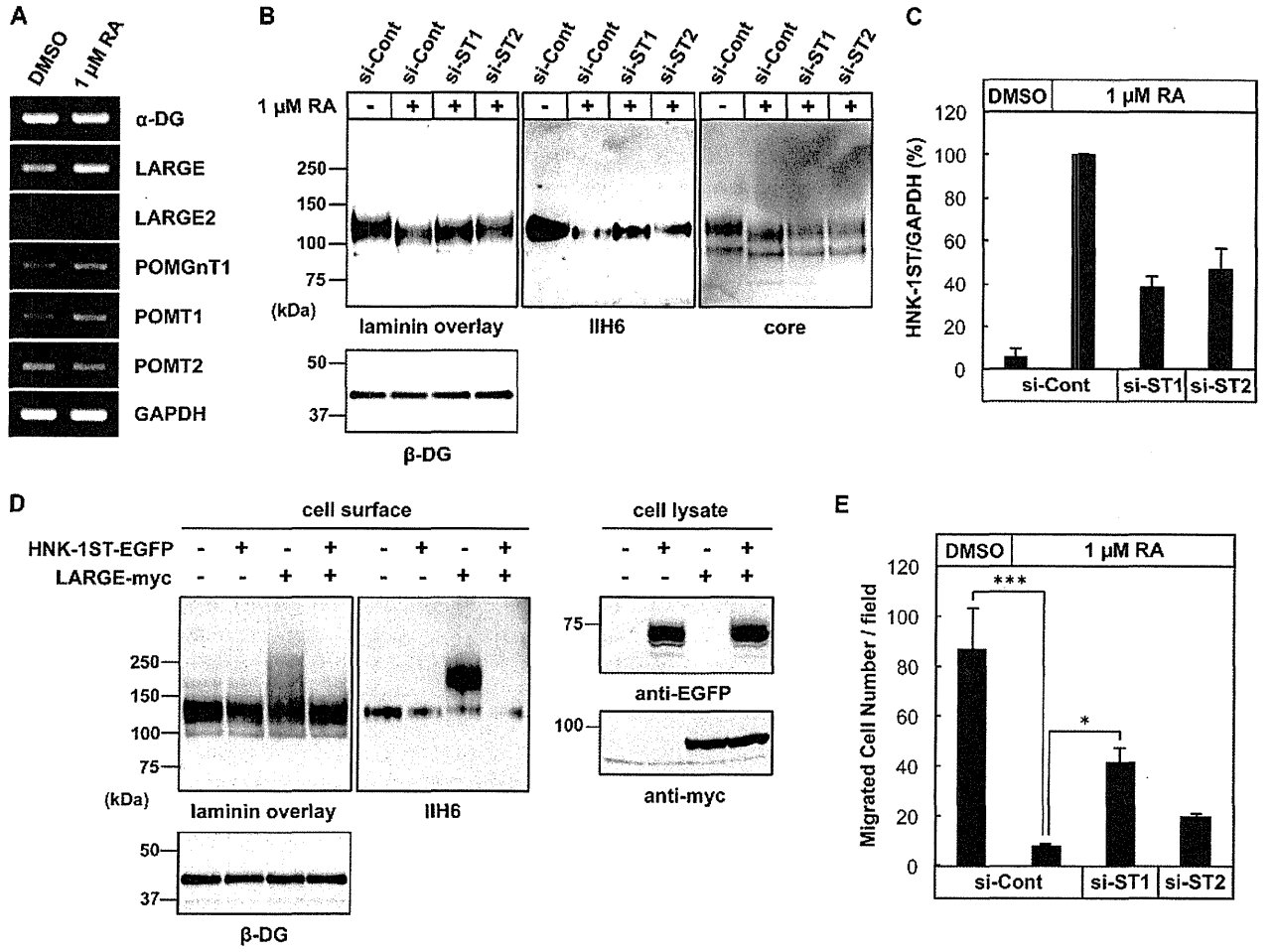

FIGURE 2. **Effect of HNK-1ST knockdown on the glycosylation of α-DG and cell motility.** *A*, S91 cells were treated with DMSO or 1 μM RA for 16 h. mRNA was extracted and subjected to RT-PCR analyses using primer sets as indicated. *B*, S91 cells were transfected with siRNA and treated with DMSO (−) or 1 μM RA (+) for 48 h. Then cell surface proteins were biotinylated, pulled down by streptavidin-agarose beads, and analyzed by laminin overlay assay or Western blotting with IIH6 mAb, anti-β-DG mAb, and α-DG core pAb. *C*, S91 cells were transfected with siRNA and treated with DMSO (−) or 1 μM RA (+) for 16 h. The amount of HNK-1ST mRNA was evaluated by quantitative RT-PCR, normalized to that of GAPDH mRNA, and shown as HNK-1ST/GAPDH. The value for RA-treated and si-Cont-transfected cells was set at 100. The graphs indicate the mean ± S.E. for three independent experiments. *D*, HNK-1ST-EGFP and LARGE-myc were transiently co-expressed in S91 cells as indicated. The cell surface proteins were biotinylated, pulled down, and analyzed by laminin overlay assay or Western blotting with IIH6 and anti-β-DG mAb (cell surface). Cell lysates were analyzed by Western blotting using anti-Myc and anti-EGFP mAbs to assess the expression of LARGE-myc and HNK-1ST-EGFP (cell lysate). *E*, S91 cells were transfected with siRNA, treated with DMSO or 1 μM RA for 48 h, and then subjected to the transwell migration assay. The insert membranes were coated with 10 μg/ml laminin-1. The graphs indicate the mean ± S.E. for three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

ferase activity (supplemental Fig. S4*A*). While showing no enzymatic activity, R189A-EGFP properly localized in the Golgi apparatus (supplemental Fig. S4*B*). Then we utilized the mutant to determine the requirement of the sulfotransferase activity of HNK-1ST in the modulation of α-DG glycosylation. Judging from the laminin overlay assay and immunoblotting with IIH6 mAb, R189A-EGFP did not suppress the LARGE-dependent modification (Fig. 5, *A* and *B*), indicating that sulfotransferase activity is essential for HNK-1ST to regulate the glycosylation of α-DG. To further confirm this evidence, we carried out an inhibition assay for PAPS production using sodium chlorate ($NaClO_3$). $NaClO_3$ is a specific inhibitor of ATP sulfurylase, an enzyme responsible for the production of PAPS in cells, resulting in depression of the intracellular sulfation (44). Treatment with 50 mM $NaClO_3$ obviously suppressed HNK-1 carbohydrate synthesis, showing that sulfate transfer is effectively abrogated in CHO-K1 cells (supplemental Fig. S5). As expected, $NaClO_3$ treatment considerably restored the LARGE-dependent glycosylation in HNK-1ST-EGFP-expressing cells (Fig. 5, *C* and *D*). Taken together, these results provide strong evidence that the sulfate transfer induced by HNK-1ST plays a regulatory role in the formation of functional glycans on α-DG.

α-DG Undergoes Sulfate Transfer by HNK-1ST—Considering that HNK-1ST also suppressed the laminin binding activity of α-DG in the absence of LARGE (Fig. 6*A*), we assumed that α-DG is the target of sulfation by HNK-1ST, rather than LARGE. Hence, to verify the incorporation of the sulfate moiety into α-DG, we labeled CHO-K1 cells with radioactive [^{35}S]-sodium sulfate. The HNK-1ST-dependent incorporation of sulfate into α-DG-Fc was detected by autoradiography (Fig. 6*B*), suggesting that a sulfated glycan is generated by HNK-1ST on α-DG, which might have a crucial effect on the formation of functional glycans on α-DG.

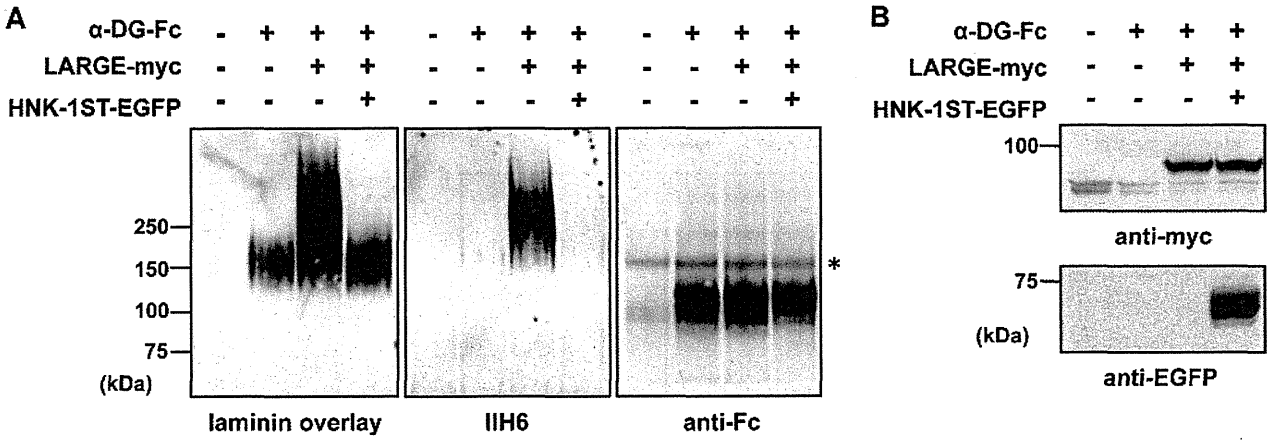

FIGURE 3. **Influence of HNK-1ST on the glycosylation and function of α-DG.** *A*, α-DG-Fc, LARGE-myc, and HNK-1ST-EGFP were transiently co-expressed in CHO-K1 cells as shown. α-DG-Fc was pulled down from the culture medium and assayed for laminin binding activity by the ligand overlay assay and for glycosylation by Western blotting with IIH6 mAb. Anti-Fc pAb was used to confirm equal protein loading. * indicates nonspecific bands. *B*, CHO-K1 cell lysates were analyzed by Western blotting using anti-Myc and anti-EGFP mAbs to assess the expression of LARGE-myc and HNK-1ST-EGFP.

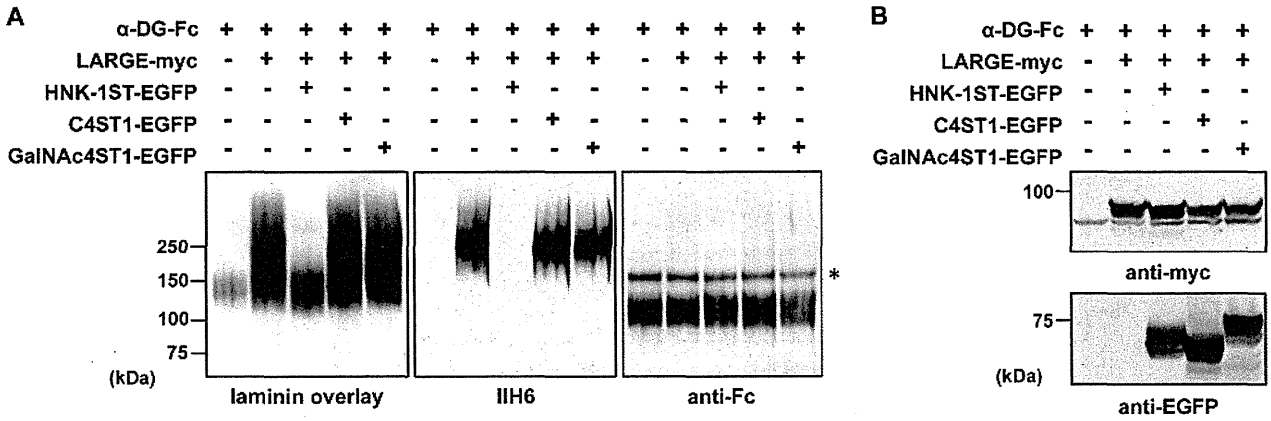

FIGURE 4. **Effect of other sulfotransferases on the glycosylation of α-DG.** *A*, CHO-K1 cells were co-transfected with α-DG-Fc, LARGE-myc, and various EGFP-fused sulfotransferases belonging to the HNK-1ST family as indicated. α-DG-Fc was pulled down from the culture medium and analyzed by laminin overlay assay and Western blotting with IIH6 mAb and anti-Fc pAb. * indicates nonspecific bands. *B*, expression of LARGE-myc and sulfotransferases was confirmed by Western blotting of cell lysates using anti-Myc mAb and anti-EGFP mAb.

DISCUSSION

Melanoma is one of the most malignant tumors, showing high metastatic ability and a rapid progression, which leads to a poor prognosis. Expression of the HNK-1 epitope is found in both primary and metastatic lesions in cases of melanoma (45, 46) and correlates with metastatic behavior (46). In addition, the HNK-1 carbohydrate positively affects the invasive and adhesive functions of melanoma cells, demonstrating the relationship between HNK-1 expression and the aggressiveness of melanomas (47). Meanwhile, HNK-1ST, one of the enzymes producing the HNK-1 carbohydrate, was identified as a candidate suppressor for melanoma invasiveness by Zhao *et al.* (6). Apparent confounding issues are that HNK-1ST functions as a tumor suppressor, although the resulting product, the HNK-1 epitope, promotes metastasis. However, it should be noted that whereas Zhao *et al.* (6) reported that HNK-1ST functions as a tumor suppressor, they failed to detect the HNK-1 epitope in 56 primary and 20 metastatic melanomas. This means that HNK-1ST might regulate invasiveness through an HNK-1 epitope-independent pathway. As a possible solution to this problem, our findings revealed that α-DG-dependent migration is another mechanism of metastasis independent of the HNK-1 epitope. Furthermore, we disclosed here a novel role of HNK-1ST, the functional regulation of α-DG via post-translational modification. This distinct function of HNK-1ST does not require GlcAT-P and GlcAT-S (Fig. 3), which accounts for the absence of the HNK-1 epitope despite the expression of HNK-1ST. Moreover, HNK-1 is not constantly expressed in melanoma lesions or cell lines (45–47), indicating that there are at least two subpopulations of melanomas, *i.e.* HNK-1-positive and -negative. Hence, α-DG-dependent migration controlled by HNK-1ST might predominate in HNK-1-negative melanomas. The unique glycan structure expressed on α-DG has been shown to have a close relationship to tumor-related phenotypes such as invasiveness (32, 33). Previous studies reported that the IIH6 mAb-reactive glycan of α-DG had a suppressive effect on tumor invasion in cases of breast, prostate, and lung carcinoma (32, 33), although we obtained the opposite results using melanoma cells (Figs. 1 and 2), suggesting that the role of α-DG varies depending on the type of cancer. Therefore, we found that

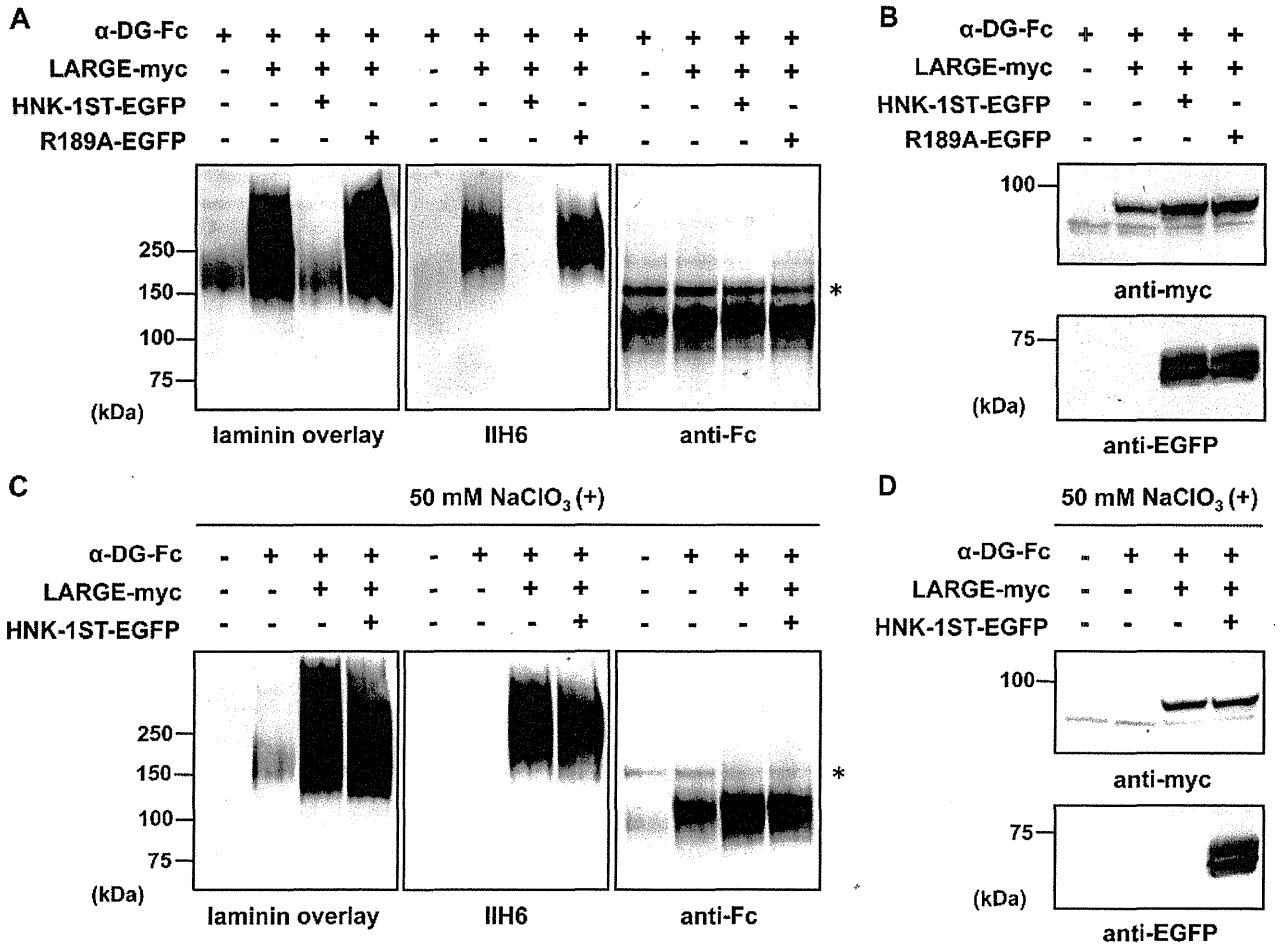

FIGURE 5. **Importance of sulfotransferase activity of HNK-1ST to the α-DG-modulating function.** *A* and *B*, requirement of the sulfotransferase activity was investigated using an activity-abolished mutant of HNK-1ST (R189A-EGFP). *A*, α-DG-Fc, LARGE-myc, and wild-type or R189A HNK-1ST-EGFP were transiently co-expressed in CHO-K1 cells as shown. α-DG-Fc was precipitated from the culture medium and analyzed by laminin overlay assay and Western blotting with IIH6 mAb and anti-Fc pAb. * indicates nonspecific bands. *B*, cell lysates were subjected to Western blotting using anti-Myc and anti-EGFP mAbs to assess the expression of LARGE-myc and wild-type or R189A HNK-1ST-EGFP. *C* and *D*, importance of the sulfotransferase activity was examined by PAPS inhibition experiments using sodium chlorate ($NaClO_3$). *C*, CHO-K1 cells were transiently transfected with α-DG-Fc, LARGE-myc, and HNK-1ST-EGFP in combination as indicated and then treated with $NaClO_3$ for 48 h. α-DG-Fc was precipitated from the culture medium and analyzed by laminin overlay assay and Western blotting with IIH6 mAb and anti-Fc pAb. * indicates nonspecific bands. *D*, expression of LARGE-myc and HNK-1ST-EGFP was confirmed by Western blotting of CHO-K1 cell lysates using anti-Myc and anti-EGFP mAbs.

HNK-1ST has a potential role modulating invasiveness by controlling the glycosylation of α-DG, leading to tumor suppression in melanoma cases.

Of particular interest was that overexpression or RA-mediated up-regulation of HNK-1ST did not completely abolish the laminin binding activity of α-DG in S91 and CHO-K1 cells, although IIH6 immunoreactivity disappeared in the same samples (Figs. 1–5). This suggests that sulfation by HNK-1ST evokes inhibitory effects predominantly on the IIH6 mAb-reactive glycan among the heterogeneous carbohydrate structures of α-DG, resulting in substantial laminin binding activity of α-DG remaining. The IIH6-reactive epitope and laminin-binding glycan are known to somewhat overlap (23). However, whether these two moieties are identical or not is still unclear despite a number of structural analyses on the glycosylation of α-DG (48–50). Chiba *et al.* (51) reported that a unique *O*-mannosyltetrasaccharide on α-DG has the ability to bind to laminin. More recently, a novel phosphate-containing glycan was identified on α-DG (52). The phosphate is attached to the 6-*O*-position of *O*-linked mannose, and post-phosphoryl glycosylation mediated by LARGE is essential for α-DG-ligand interaction and IIH6 epitope production (52). We demonstrated that LARGE could not generate the IIH6 epitope on α-DG in the presence of HNK-1ST (Figs. 3–5) and HNK-1ST indeed transferred a sulfate group onto α-DG (Fig. 6*B*), suggesting that HNK-1ST inhibits the LARGE-dependent post-phosphoryl modification of α-DG by sulfate transfer. Therefore, identification of the specific site of α-DG sulfated by HNK-1ST and the structure of the resulting sulfated glycan might be important for elucidating LARGE-dependent glycosylation.

During the preparation of this manuscript, Campbell and co-workers (53) reported that LARGE could act as a bifunctional glycosyltransferase with both xylosyl- and glucuronyltransferase activities and could generate a linear polysaccharide structure composed of repeating disaccharide units (-3-xylose-α1,3-GlcAβ1-) on α-DG. HNK-1ST has the ability to transfer a

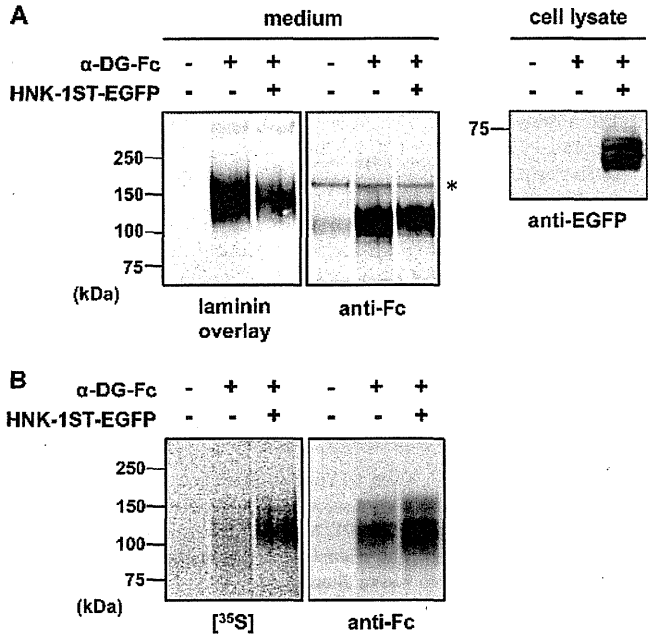

FIGURE 6. **HNK-1ST-mediated incorporation of sulfate into α-DG.** *A*, α-DG-Fc and HNK-1ST-EGFP were transiently expressed in CHO-K1 cells as shown. α-DG-Fc was pulled down from the cultured medium and analyzed by laminin overlay assay and Western blotting with anti-Fc pAb (*medium*). The cell lysates were subjected to Western blotting using anti-EGFP mAb to assess the expression of HNK-1ST-EGFP (*cell lysate*). * indicates nonspecific bands. *B*, CHO-K1 cells transiently expressing α-DG-Fc with (+) or without (−) HNK-1ST-EGFP were labeled with radioactive [^{35}S]sodium sulfate. α-DG-Fc was pulled down from the culture medium, separated by SDS-PAGE, and subjected to autoradiography and Western blotting with anti-Fc.

sulfate group to the C-3 position of terminal GlcA, where xylose is transferred; therefore, it makes sense that HNK-1ST inhibits IIH6-reactive glycan produced by LARGE. Our data presented in this study are highly important for understanding dystroglycan function via its glycosylation.

In mammals, an apparent molecular mass of α-DG varies from highly limited (about 120 kDa, *e.g.* brain) to rather broad (120–200 kDa, *e.g.* muscle) due to its glycosylation in a tissue-dependent manner (18, 19, 21). In contrast, in experiments using cell lines, forced expression of LARGE always yields an extensively glycosylated α-DG that appears as a band of ≥200 kDa on SDS-PAGE (Figs. 2–5) (37, 38, 41), implying the presence of an unidentified machinery that negatively regulates the glycosylation of α-DG *in vivo*. Hence, we propose HNK-1ST to be one such suppressive factor for α-DG function, acting as a "molecular brake" to generate properly glycosylated α-DG. In this regard, future studies might identify a pathogenic mutation of *HNK-1ST* in CMD patients, which causes hyperactivation of HNK-1ST resulting in hypoglycosylation of α-DG. Investigating the α-DG-modulating function of HNK-1ST could be a powerful means of uncovering the regulatory system of α-DG glycosylation, contributing to the development of therapeutic strategies for glycosylation-defective CMDs.

REFERENCES

1. Friedl, P., and Alexander, S. (2011) Cancer invasion and the microenvironment. Plasticity and reciprocity. *Cell* **147,** 992–1009
2. Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126,** 855–867
3. Fuster, M. M., and Esko, J. D. (2005) The sweet and sour of cancer. Glycans as novel therapeutic targets. *Nat. Rev. Cancer* **5,** 526–542
4. Meany, D. L., and Chan, D. W. (2011) Aberrant glycosylation associated with enzymes as cancer biomarkers. *Clin. Proteomics* **8,** 7
5. Hakomori, S. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res.* **56,** 5309–5318
6. Zhao, X., Graves, C., Ames, S. J., Fisher, D. E., and Spanjaard, R. A. (2009) Mechanism of regulation and suppression of melanoma invasiveness by novel retinoic acid receptor-γ target gene carbohydrate sulfotransferase 10. *Cancer Res.* **69,** 5218–5225
7. Morita, I., Kizuka, Y., Kakuda, S., and Oka, S. (2008) Expression and function of the HNK-1 carbohydrate. *J. Biochem.* **143,** 719–724
8. Morita, I., Kakuda, S., Takeuchi, Y., Kawasaki, T., and Oka, S. (2009) HNK-1 (human natural killer-1) glyco-epitope is essential for normal spine morphogenesis in developing hippocampal neurons. *Neuroscience* **164,** 1685–1694
9. Morita, I., Kakuda, S., Takeuchi, Y., Itoh, S., Kawasaki, N., Kizuka, Y., Kawasaki, T., and Oka, S. (2009) HNK-1 glyco-epitope regulates the stability of the glutamate receptor subunit GluR2 on the neuronal cell surface. *J. Biol. Chem.* **284,** 30209–30217
10. Yamamoto, S., Oka, S., Inoue, M., Shimuta, M., Manabe, T., Takahashi, H., Miyamoto, M., Asano, M., Sakagami, J., Sudo, K., Iwakura, Y., Ono, K., and Kawasaki, T. (2002) Mice deficient in nervous system-specific carbohydrate epitope HNK-1 exhibit impaired synaptic plasticity and spatial learning. *J. Biol. Chem.* **277,** 27227–27231
11. Chou, D. K., Ilyas, A. A., Evans, J. E., Costello, C., Quarles, R. H., and Jungalwala, F. B. (1986) Structure of sulfated glucuronyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. *J. Biol. Chem.* **261,** 11717–11725
12. Ariga, T., Kohriyama, T., Freddo, L., Latov, N., Saito, M., Kon, K., Ando, S., Suzuki, M., Hemling, M. E., and Rinehart, K. L., Jr. (1987) Characterization of sulfated glucuronic acid containing glycolipids reacting with IgM M-proteins in patients with neuropathy. *J. Biol. Chem.* **262,** 848–853
13. Kizuka, Y., Matsui, T., Takematsu, H., Kozutsumi, Y., Kawasaki, T., and Oka, S. (2006) Physical and functional association of glucuronyltransferases and sulfotransferase involved in HNK-1 biosynthesis. *J. Biol. Chem.* **281,** 13644–13651
14. Terayama, K., Oka, S., Seiki, T., Miki, Y., Nakamura, A., Kozutsumi, Y., Takio, K., and Kawasaki, T. (1997) Cloning and functional expression of a novel glucuronyltransferase involved in the biosynthesis of the carbohydrate epitope HNK-1. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 6093–6098
15. Seiki, T., Oka, S., Terayama, K., Imiya, K., and Kawasaki, T. (1999) Molecular cloning and expression of a second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope. *Biochem. Biophys. Res. Commun.* **255,** 182–187
16. Ong, E., Yeh, J. C., Ding, Y., Hindsgaul, O., and Fukuda, M. (1998) Expression cloning of a human sulfotransferase that directs the synthesis of the HNK-1 glycan on the neural cell adhesion molecule and glycolipids. *J. Biol. Chem.* **273,** 5190–5195
17. Tagawa, H., Kizuka, Y., Ikeda, T., Itoh, S., Kawasaki, N., Kurihara, H., Onozato, M. L., Tojo, A., Sakai, T., Kawasaki, T., and Oka, S. (2005) A nonsulfated form of the HNK-1 carbohydrate is expressed in mouse kidney. *J. Biol. Chem.* **280,** 23876–23883
18. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355,** 696–702
19. Barresi, R., and Campbell, K. P. (2006) Dystroglycan. From biosynthesis to pathogenesis of human disease. *J. Cell Sci.* **119,** 199–207
20. Han, R., Kanagawa, M., Yoshida-Moriguchi, T., Rader, E. P., Ng, R. A., Michele, D. E., Muirhead, D. E., Kunz, S., Moore, S. A., Iannaccone, S. T., Miyake, K., McNeil, P. L., Mayer, U., Oldstone, M. B., Faulkner, J. A., and Campbell, K. P. (2009) Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of α-dystroglycan. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12573–12579
21. Ervasti, J. M., Burwell, A. L., and Geissler, A. L. (1997) Tissue-specific heterogeneity in α-dystroglycan sialoglycosylation. Skeletal muscle α-dystroglycan is a latent receptor for *Vicia villosa* agglutinin b4 masked by

sialic acid modification. *J. Biol. Chem.* **272,** 22315–22321
22. Michele, D. E., and Campbell, K. P. (2003) Dystrophin-glycoprotein complex. Post-translational processing and dystroglycan function. *J. Biol. Chem.* **278,** 15457–15460
23. Ervasti, J. M., and Campbell, K. P. (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122,** 809–823
24. Michele, D. E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R. D., Satz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H., Straub, V., Mathews, K. D., Moore, S. A., and Campbell, K. P. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418,** 417–422
25. Muntoni, F., Torelli, S., and Brockington, M. (2008) Muscular dystrophies due to glycosylation defects. *Neurotherapeutics* **5,** 627–632
26. Beltrán-Valero de Bernabé, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H., and Brunner, H. G. (2002) Mutations in the *O*-mannosyltransferase gene *POMT1* give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am. J. Hum. Genet.* **71,** 1033–1043
27. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabé, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., Walsh, C. A., Barth, P. G., Brunner, H. G., and van Bokhoven, H. (2005) POMT2 mutations cause α-dystroglycan hypoglycosylation and Walker-Warburg syndrome. *J. Med. Genet.* **42,** 907–912
28. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell* **1,** 717–724
29. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., Nonaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K., and Toda, T. (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* **394,** 388–392
30. Brockington, M., Blake, D. J., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estournet, B., Romero, N. B., Mercuri, E., Voit, T., Sewry, C. A., Guicheney, P., and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin α2 deficiency and abnormal glycosylation of α-dystroglycan. *Am. J. Hum. Genet.* **69,** 1198–1209
31. Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R. K., Voit, T., Merlini, L., Sewry, C. A., Brown, S. C., and Muntoni, F. (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. *Hum. Mol. Genet.* **12,** 2853–2861
32. Bao, X., Kobayashi, M., Hatakeyama, S., Angata, K., Gullberg, D., Nakayama, J., Fukuda, M. N., and Fukuda, M. (2009) Tumor suppressor function of laminin-binding α-dystroglycan requires a distinct β3-*N*-acetylglucosaminyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12109–12114
33. de Bernabé, D. B., Inamori, K., Yoshida-Moriguchi, T., Weydert, C. J., Harper, H. A., Willer, T., Henry, M. D., and Campbell, K. P. (2009) Loss of α-dystroglycan laminin binding in epithelium-derived cancers is caused by silencing of LARGE. *J. Biol. Chem.* **284,** 11279–11284
34. Nakagawa, N., Izumikawa, T., Kitagawa, H., and Oka, S. (2011) Sulfation of glucuronic acid in the linkage tetrasaccharide by HNK-1 sulfotransferase is an inhibitory signal for the expression of a chondroitin sulfate chain on thrombomodulin. *Biochem. Biophys. Res. Commun.* **415,** 109–113
35. Kanagawa, M., Nishimoto, A., Chiyonobu, T., Takeda, S., Miyagoe-Suzuki, Y., Wang, F., Fujikake, N., Taniguchi, M., Lu, Z., Tachikawa, M., Nagai, Y., Tashiro, F., Miyazaki, J., Tajima, Y., Takeda, S., Endo, T., Kobayashi, K., Campbell, K. P., and Toda, T. (2009) Residual laminin binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy. *Hum. Mol. Genet.* **18,** 621–631
36. Spanjaard, R. A., Ikeda, M., Lee, P. J., Charpentier, B., Chin, W. W., and Eberlein, T. J. (1997) Specific activation of retinoic acid receptors (RARs) and retinoid X receptors reveals a unique role for RARγ in induction of differentiation and apoptosis of S91 melanoma cells. *J. Biol. Chem.* **272,** 18990–18999
37. Barresi, R., Michele, D. E., Kanagawa, M., Harper, H. A., Dovico, S. A., Satz, J. S., Moore, S. A., Zhang, W., Schachter, H., Dumanski, J. P., Cohn, R. D., Nishino, I., and Campbell, K. P. (2004) LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. *Nat. Med.* **10,** 696–703
38. Patnaik, S. K., and Stanley, P. (2005) Mouse large can modify complex *N*- and mucin *O*-glycans on α-dystroglycan to induce laminin binding. *J. Biol. Chem.* **280,** 20851–20859
39. Yamauchi, S., Mita, S., Matsubara, T., Fukuta, M., Habuchi, H., Kimata, K., and Habuchi, O. (2000) Molecular cloning and expression of chondroitin 4-sulfotransferase. *J. Biol. Chem.* **275,** 8975–8981
40. Okuda, T., Mita, S., Yamauchi, S., Fukuta, M., Nakano, H., Sawada, T., and Habuchi, O. (2000) Molecular cloning and characterization of GalNAc 4-sulfotransferase expressed in human pituitary gland. *J. Biol. Chem.* **275,** 40605–40613
41. Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y. M., Muschler, J., Dumanski, J. P., Michele, D. E., Oldstone, M. B., and Campbell, K. P. (2004) Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell* **117,** 953–964
42. Brockington, M., Torelli, S., Prandini, P., Boito, C., Dolatshad, N. F., Longman, C., Brown, S. C., and Muntoni, F. (2005) Localization and functional analysis of the LARGE family of glycosyltransferases. Significance for muscular dystrophy. *Hum. Mol. Genet.* **14,** 657–665
43. Ong, E., Yeh, J. C., Ding, Y., Hindsgaul, O., Pedersen, L. C., Negishi, M., and Fukuda, M. (1999) Structure and function of HNK-1 sulfotransferase. Identification of donor and acceptor binding sites by site-directed mutagenesis. *J. Biol. Chem.* **274,** 25608–25612
44. Girard, J. P., Baekkevold, E. S., and Amalric, F. (1998) Sulfation in high endothelial venules. Cloning and expression of the human PAPS synthetase. *FASEB J.* **12,** 603–612
45. Mooy, C. M., Luyten, G. P., de Jong, P. T., Jensen, O. A., Luider, T. M., van der Ham, F., and Bosman, F. T. (1995) Neural cell adhesion molecule distribution in primary and metastatic uveal melanoma. *Hum. Pathol.* **26,** 1185–1190
46. Thies, A., Schachner, M., Berger, J., Moll, I., Schulze, H. J., Brunner, G., and Schumacher, U. (2004) The developmentally regulated neural crest-associated glycotope HNK-1 predicts metastasis in cutaneous malignant melanoma. *J. Pathol.* **203,** 933–939
47. Casado, J. G., Delgado, E., Patsavoudi, E., Durán, E., Sanchez-Correa, B., Morgado, S., Solana, R., and Tarazona, R. (2008) Functional implications of HNK-1 expression on invasive behavior of melanoma cells. *Tumour Biol.* **29,** 304–310
48. Stalnaker, S. H., Hashmi, S., Lim, J. M., Aoki, K., Porterfield, M., Gutierrez-Sanchez, G., Wheeler, J., Ervasti, J. M., Bergmann, C., Tiemeyer, M., and Wells, L. (2010) Site mapping and characterization of *O*-glycan structures on α-dystroglycan isolated from rabbit skeletal muscle. *J. Biol. Chem.* **285,** 24882–24891
49. Nilsson, J., Nilsson, J., Larson, G., and Grahn, A. (2010) Characterization of site-specific *O*-glycan structures within the mucin-like domain of α-dystroglycan from human skeletal muscle. *Glycobiology* **20,** 1160–1169
50. Stalnaker, S. H., Aoki, K., Lim, J. M., Porterfield, M., Liu, M., Satz, J. S., Buskirk, S., Xiong, Y., Zhang, P., Campbell, K. P., Hu, H., Live, D., Tiemeyer, M., and Wells, L. (2011) Glycomic analyses of mouse models of congenital muscular dystrophy. *J. Biol. Chem.* **286,** 21180–21190
51. Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin. *J. Biol. Chem.* **272,** 2156–2162
52. Yoshida-Moriguchi, T., Yu, L., Stalnaker, S. H., Davis, S., Kunz, S., Madson, M., Oldstone, M. B., Schachter, H., Wells, L., and Campbell, K. P. (2010) *O*-Mannosyl phosphorylation of α-dystroglycan is required for laminin binding. *Science* **327,** 88–92
53. Inamori, K., Yoshida-Moriguchi, T., Hara, Y., Anderson, M. E., Yu, L., and Campbell, K. P. (2012) Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. *Science* **335,** 93–96

Biochemical and Biophysical Research Communications 424 (2012) 354–357

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Detection of the dystroglycanopathy protein, fukutin, using a new panel of site-specific monoclonal antibodies

Tracy A. Lynch [a,1,2], Le Thanh Lam [a,2], Nguyen thi Man [a,2], Kazuhiro Kobayashi [c,2], Tatsushi Toda [c], Glenn E. Morris [a,b,*]

[a] Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry, SY10 7AG, UK
[b] Institute for Science and Technology in Medicine, Keele University, UK
[c] Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

ARTICLE INFO

Article history:
Received 25 June 2012
Available online 6 July 2012

Keywords:
Fukutin
Fukuyama muscular dystrophy
Monoclonal antibody
Dystroglycan
Dystroglycanopathy
Golgi
Phage-displayed peptide library
Epitope mapping

ABSTRACT

Mutations in the gene encoding fukutin protein cause Fukuyama muscular dystrophy, a severe congenital disorder that occurs mainly in Japan. A major consequence of the mutation is reduced glycosylation of alpha-dystroglycan, which is also a feature of other forms of congenital and limb-girdle muscular dystrophy. Immunodetection of endogenous fukutin in cells and tissues has been difficult and this has hampered progress in understanding fukutin function and disease pathogenesis. Using a new panel of monoclonal antibodies which bind to different defined sites on the fukutin molecule, we now show that fukutin has the predicted size for a protein without extensive glycosylation and is present at the Golgi apparatus at very low levels. These antibodies should enable more rapid future progress in understanding the molecular function of fukutin.

1. Introduction

Fukuyama-type congenital muscular dystrophy (FCMD: MIM 253800), one of the most prevalent autosomal recessive disorders in the Japanese population, was originally described clinically as a muscular dystrophy combined with cortical dysgenesis (micropolygyria) and ocular abnormalities [1]. The gene responsible for FCMD was identified on chromosome 9q31 by linkage analysis and positional cloning and was named fukutin [2,3]. A 3-kb retrotransposal insertion in the 3' non-coding region of the fukutin gene is the most common mutation in Japan, but other fukutin mutations occur outside Japan and cause various phenotypes, including Walker–Warburg syndrome (WWS: MIM 236670) and limb-girdle muscular dystrophy (LGMD) [4–6]. It is clear, therefore, that partial or complete loss of fukutin function can give rise to a wide spectrum of phenotypes with different severities.

Mutations in fukutin cause abnormal glycosylation of cell surface α-dystroglycan which in turn reduces its laminin-binding activity [7], but a direct catalytic function for fukutin has not been established. Transfected fukutin is targeted to the Golgi apparatus, where glycosylation events usually occur, by an amino-terminal transmembrane domain. Fukutin also binds directly to the enzyme POMGnT1 (*O*-mannose-β-1,2-*N*-acetylglucosaminyltransferase1) and the transmembrane domain is required for this interaction [8]. This suggests that fukutin mutations may affect αDG glycosylation by their influence on POMGnT1 [8], mutations in which are responsible for a related dystroglycanopathy, muscle-eye-brain disease (MEB: MIM 253280) [9]. Both POMGnT1 and the POMT1/2 complex have glycosyltransferase activities directly involved in synthesis of *O*-mannosyl sugar chains on α-DG [9,10]. Yoshida-Moriguchi and colleagues [11] reported that a phosphodiester-linked moiety on *O*-mannose of α-DG is defective in fukutin-deficient and other dystroglycanopathies and that this specific modification is necessary for laminin binding activity. The enzyme that carries out this modification has not been identified. An indirect role for fukutin in α-DG glycosylation remains a possibility. Thus, Tachikawa et al. [12] found that four pathogenic missense mutants of fukutin caused mis-localization to the endoplasmic reticulum (ER) instead of the Golgi, rather than having a direct effect on α-DG glycosylation. These mutants were able to localize correctly when mis-folding was inhibited. Zebrafish studies [13] have suggested that fukutin may also have a role in maintaining the Unfolded Protein Response (UPR) and this may contribute to the unique clinical features of Fukuyama MD.

* Corresponding author at: Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry, SY10 7AG, UK. Fax: +44 1691 404170.
E-mail address: glenn.morris@rjah.nhs.uk (G.E. Morris).
[1] Present address: Diagnostic Innovations Ltd., St. Asaph Business Park, St. Asaph, UK.
[2] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.147

2. Materials and methods

A fukutin cDNA fragment (bases 60–1,494) containing the open reading frame was subcloned by PCR into the vector pEGFP-N1 (Clontech) and into pET vectors for bacterial expression [3]. Subfragments were cloned into pGEX vectors for bacterial expression as GST-fusion proteins. Monoclonal antibody production [14] and epitope mapping with phage-displayed peptide libraries [15] were performed as previously described. HeLa cells were grown as monolayers on tissue culture plastic Petri dishes or glass coverslips in DMEM with 10% fetal bovine serum. Immunoprecipitation and western blotting were performed as previously described [16]. Secondary antibodies were from DAKOpatts, Copenhagen.

3. Results

Balb/c mice were immunized either with full-length recombinant human fukutin or with a mixture of 4 GST-fusion proteins containing fukutin fragments (amino-acids 26–58, 177–220, 233–268 and 415–461). Sera from 3 out of 8 mice recognized a 54 kDa band on western blots of human muscle (Fig. 1). This is the predicted size for the unmodified amino-acid sequence of fukutin. However, experience with rabbit and goat antisera against fukutin (our unpublished data) showed that protein bands of this size on western blots are not always authentic fukutin. To select mice for hybridoma fusions, we therefore used an additional criterion: recognition of over-expressed GFP-fukutin at the Golgi apparatus (Fig. 2) to show that their sera contained anti-fukutin antibodies.

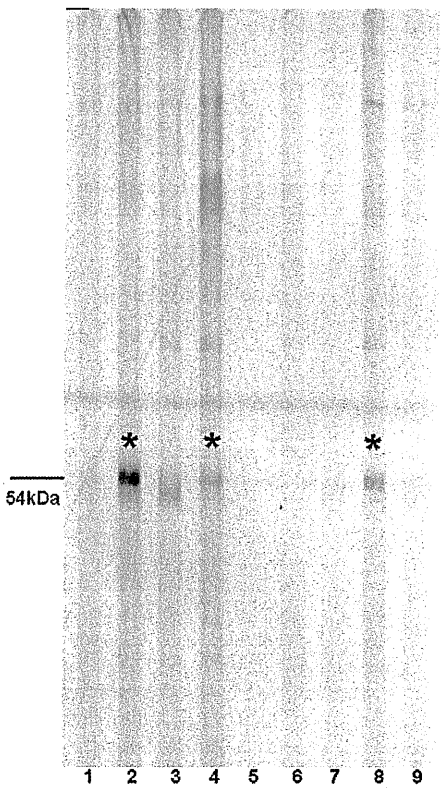

Fig. 1. Three mouse antisera recognize a 54 kDa protein in human muscle. An extract of normal human muscle was subjected to SDS–PAGE on a 3–12.5% gradient gel as a strip and antisera (0.07 ml of 1/100 in PBS) were applied in the lanes of a miniblotter. The position of a 54 kD marker is indicated and the three positive antisera are marked with an astérix. The lanes are as follows: (lanes 1–4), four mice immunized with mixture of GST-fukutin fragments, mice "*GST1 to 4*"; (lane 5) PBS control, (lane 6) normal mouse serum control, (lanes 7–9) three mice immunized with full-length fukutin, mice "*pETF1 to 3*". *GST2* and *pETF2* were used for hybridoma fusions (see Table 1).

Four hybridoma fusions were performed using spleens from three mice (*pETF2, G70 and J41*) that responded to full-length protein and one mouse (*GST2*) that responded to the fragment mixture. Twelve mAbs that recognized recombinant fukutin in both ELISA and western blot are shown in Table 1; all except one were from the immunizations with full-length protein. None of the mAbs recognized endogenous 54 kDa fukutin on western blots. Seven of them did, however, recognize over-expressed GFP-fukutin at the Golgi apparatus (Table 1).

Because it seemed likely that endogenous fukutin was present at too low concentration in muscle for detection, we concentrated fukutin from HeLa cells by immunoprecipitation with a goat polyclonal antibody [16] and tested 14 mAbs by western blotting of this highly-enriched extract. Eleven of them reacted well with a single 54 kDa protein band, while three reacted only very weakly or not at all (Fig. 3). All mAbs were used at a concentration of about 1 μg/ml and immuno-precipitations using pre-immune serum as negative control showed that only the 54 kDa band is fukutin-specific (Fig. 3).

Epitope mapping of the binding sites on fukutin for the mAb panel was performed using phage-displayed random 15-mer peptide libraries [15]. In this method, only the amino-acids within the 15-mer peptide which are important for mAb binding match with the target sequence. Reactivity with phage-displayed peptides revealed six mapping groups, plus MANFU5 which would not react with any phage colonies (possibly a conformational epitope). Groups 1 (amino-acids 223–231: MANFU4), 2 (amino-acids 452–461: MANFU11 and 12) and 3 (amino-acids 182–187: MANFU7-10 inclusive) were mapped with confidence by matching 3 or 4 different peptides from the random library to the fukutin sequence (Fig. 4). Group 4 (MANFU2 and 3) reacted with a single peptide only, but with four sequential amino-acids matched (PHSR: amino-acids 243–246), this was unlikely to have occurred by chance. It was possible to place other mAbs into Groups 1, 5 or 6 by their reactivity with different phage peptides, even though it was not possible to match the phage peptides with the fukutin sequence in these cases, possibly because the epitope is conformation-dependent.

4. Discussion

The results are consistent with the view that fukutin is a very low abundance protein, required for glycosylation of α-dystroglycan at the Golgi, though not substantially-glycosylated itself. It

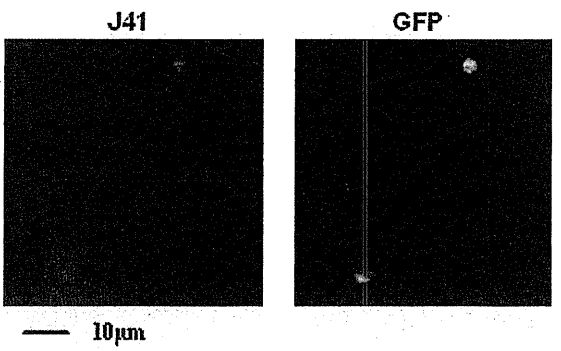

Fig. 2. Antibody staining co-localizes with GFP at the Golgi of GFP-fukutin-transfected HeLa cells. A HeLa cell-line expressing GFP-fukutin was grown on coverslips. After fixing with acetone-methanol, the cells were incubated with mouse antibody against fukutin (mouse number J41) followed by TRITC anti-(mouse Ig).

Table 1
Twelve monoclonal antibodies against fukutin. Column 1: Antibody name and clone number; column 2: intensity of western blot staining in Fig. 3; Column 3: name given to the mouse used for hybridoma production, *GST2* (mix) was immunized with the mixture of four fukutin fragments and the other three mice (*pETF2*, *G70* and *J41*) with full-length fukutin; Column 4: phage mapping: the epitope group allocated and amino-acid sequence of the epitope from Fig. 4; Column 5: HeLa-GFP IMF: reactivity at the Golgi in the *IMF* (immunofluorescence microscopy) experiment shown in Fig. 2, rated as either "*good*" (similar to Fig. 2) or "*poor*".

Antibody	Western Blot	Origin (name of mouse)	Phage mapping	HeLa-GFP IMF
MANFU1 7A2	++	GST2 (mix)	Group 6, clone	Poor
MANFU2 10F9	++	PETF2	Group 4 mapped AA243–246	Poor
MANFU3 4E6	+++	PETF2	Group 4 mapped AA243–246	Good
MANFU4 3C7	++	PETF2	Group 1, clone	Good
MANFU5 7H2	++	PETF2	No clones	Good
MANFU6 4H8	(w+)	PETF2	Group 5, clone	Poor
MANFU7 1B5	++	G70	Group 3 mapped AA180–187	Poor
MANFU8 2F5	++	G70	Group 3 mapped AA180–187	Good
MANFU9 4E12	++	G70	Group 3 mapped AA180–187	Good
MANFU10 5H10	++	G70	Group 3 mapped AA180–187	Poor
MANFU11 3E4	++	J41	Group 2 mapped AA452–461	Good
MANFU12 6B5	+++	J41	Group 2 mapped AA452–461	Good

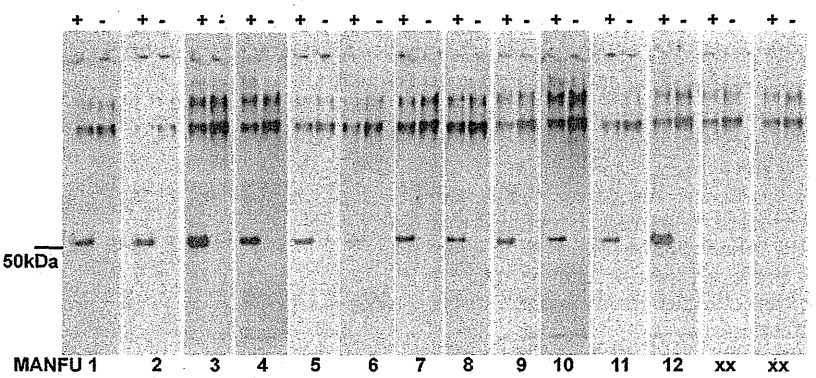

Fig. 3. Twelve mAbs recognize endogenous fukutin from HeLa cells after enrichment by immunoprecitation. HeLa cell extracts were immunoprecipitated with either a goat anti-fukutin serum (+) or with pre-immune control serum (−) and these were loaded in pairs for SDS–PAGE and western blotting with 14 different hybridoma culture supernatants adjusted to 1 μg/ml mouse Ig concn. Each lane of the gel contained all the protein immunoprecipitated from two 100 mm culture dishes of HeLa monolayers. The supernatants were MANFU1 to 12 (see Table 1) plus two negative hybridoma supernatants from the same fusions (XX). The fukutin band runs at the expected size, just above the 50 kDa Mr. marker.

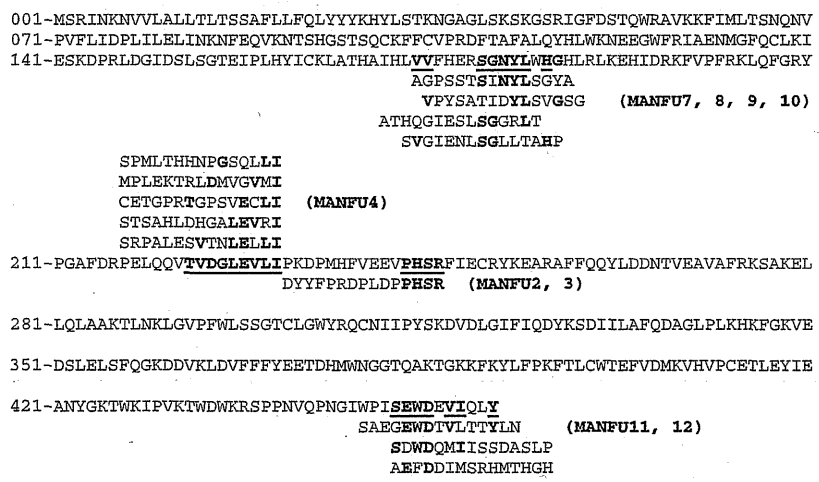

Fig. 4. Epitope mapping of eight mAbs to specific binding sites on fukutin. The mAbs were attached to Petri dishes coated with anti-(mouse Ig) and used to select phage displaying random 15-mer peptides on their surface [15]. After determining the mAb specificity of each selected phage clone, the DNA was sequenced to determine the amino-acid sequence of the peptide displayed. The complete 461aa sequence of fukutin is shown, together with the 15-mer phage peptide sequences that matched it. In each case, the peptides were recognized specifically by the named mAbs only and not by other mAbs in the panel. Where several peptides are shown alongside several mAbs (e.g. MANFU7-10), ALL the mAbs recognized ALL the peptides.

may have an enzymatic function, but the alternative possibility that it has an essential supporting role in dystroglycan glycosylation by modifying the localization or activity of other proteins has not been ruled out.

Detection of endogenous fukutin by mAbs required pre-enrichment of cell extracts (Fig 3), but the 54 kD band was confidently identifiable as authentic fukutin because it was recognized by mAbs against five different fukutin epitopes. Only monoclonal antibodies against different epitopes on fukutin make it possible to distinguish with confidence between endogenous fukutin and a more abundant cross-reacting protein on western blots, as we have shown for another low-abundance antigen, DMPK [17]. Although polyclonal mouse antisera were able to detect a 54 kD protein on western blots of total cell extracts (Fig. 1), we are unable to prove that this is authentic fukutin.

The localization of over-expressed fukutin very specifically to the Golgi (Fig. 2) and its detection at this site by mAbs against five different fukutin epitopes (Table 1) suggests that low endogenous protein levels can explain the failure of mAbs to detect fukutin in untransfected cells. The alternative possibility that epitope masking at the Golgi prevents antibody access to endogenous fukutin appears less likely when mAbs against five different fukutin epitopes give the same result.

We have shown that at least six or seven different epitopes are recognized by the panel of 12 mAbs (Table 1) and four regions of the fukutin amino-acid sequence have been shown to contain binding sites for 9 mAbs using phage-displayed peptide libraries (Fig. 4). The two mAbs against the extreme C-terminus of fukutin may be especially valuable for further functional studies. The remaining three mAbs may recognize conformational epitopes that either select no clones from the peptide library (MANFU5) or select clones that do not match the linear sequence of fukutin (MANFU1 and 6). Table 1 shows that epitope diversity was increased by using spleens from four different mice.

The mAbs described in this study are freely available for academic research from the MDA Monoclonal Antibody Resource (www.glennmorris.org/mabs.htm).

Acknowledgments

Monoclonal antibody production was supported by a Grant from the Muscular Dystrophy Campaign UK (GEM) and epitope mapping was supported by a grant from the Muscular Dystrophy Association USA (GEM).

References

[1] Y. Fukuyama, M. Osawa, M., H. Suzuki, Congenital progressive muscular dystrophy of the Fukuyama type – clinical, genetic and pathological considerations, Brain Dev. 3 (1981) 1–29.
[2] T. Toda, M. Segawa, Y. Nomura, I. Nonaka, et al., Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31-33, Nat. Genet. 5 (1993) 283–286.
[3] K. Kobayashi, Y. Nakahori, M. Miyake, K. Matsumura, et al., An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy, Nature 394 (1998) 388–392.
[4] D. Beltrán-Valero de Bernabé, H. van Bokhoven, E. van Beusekom, W. van den Akker, et al., J. Med. Genet. 40 (2003) 845–848.
[5] C. Godfrey, D. Escolar, M. Brockington, E.M. Clement, et al., Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy, Ann. Neurol. 60 (2006) 603–610.
[6] C. Godfrey, E. Clement, R. Mein, M. Brockington, et al., Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan, Brain 130 (2007) 2725–2735.
[7] D.E. Michele, R. Barresi, M. Kanagawa, F. Saito, et al., Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies, Nature 418 (2002) 417–422.
[8] H. Xiong, K. Kobayashi, M. Tachikawa, H. Manya, et al., Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of alpha-dystroglycan, Biochem. Biophys. Res. Commun. 350 (2006) 935–941.
[9] A. Yoshida, K. Kobayashi, H. Manya, K. Taniguchi, et al., Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1, Dev. Cell 1 (2001) 717–724.
[10] H. Manya, A. Chiba, A. Yoshida, X. Wang, et al., Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 500–505.
[11] T. Yoshida-Moriguchi, L. Yu, S.H. Stalnaker, S. Davis, et al., O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding, Science 327 (2010) 88–92.
[12] M. Tachikawa, M. Kanagawa, C.C. Yu, K. Kobayashi, T. Toda, Mislocalization of Fukutin protein by disease-causing missense mutations can be rescued with treatments directed at folding amelioration, J. Biol. Chem. 287 (2012) 8398–8406.
[13] Y.Y. Lin, R.J. White, S. Torelli, S. Cirak, F. Muntoni, D.L. Stemple, Zebrafish Fukutin family proteins link the unfolded protein response with dystroglycanopathies, Hum. Mol. Genet. 20 (2011) 1763–1775.
[14] Thi Man Nguyen, G.E. Morris, A rapid method for generating large numbers of high-affinity monoclonal antibodies from a single mouse, in: J.M. Walker (Ed.), The Protein Protocols Handbook, third ed., Humana Press, Totowa, NJ, 2009, pp. 1961–1974.
[15] A. Pereboev, G.E. Morris, Reiterative screening of phage display peptide libraries for epitope mapping, in: G.E. Morris (Ed.), Epitope Mapping Protocols, Meth. Mol. Biol., vol. 66, Humana Press, Totowa, NJ, 1996, pp. 195–206.
[16] M. Taniguchi-Ikeda, K. Kobayashi, M. Kanagawa M, et al., Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy, Nature 478 (2011) 127–131.
[17] Le Thanh Lam, Y.C.N. Pham, Thi Man Nguyen, G.E. Morris, Characterization of a monoclonal antibody panel shows that the myotonic dystrophy protein kinase, DMPK, is expressed almost exclusively in muscle and heart, Hum. Mol. Genet. 9 (2000) 2167–2173.

Journal of Human Genetics (2012) 57, 515–522

www.nature.com/jhg

ORIGINAL ARTICLE

Screening of genes involved in chromosome segregation during meiosis I: *in vitro* gene transfer to mouse fetal oocytes

Makiko Tsutsumi[1], Hiroe Kowa-Sugiyama[1], Hasbaira Bolor[1], Hiroshi Kogo[1], Hidehito Inagaki[1], Tamae Ohye[1], Kouji Yamada[1], Mariko Taniguchi-Ikeda[2], Tatsushi Toda[2] and Hiroki Kurahashi[1]

The events that take place during the prophase of meiosis I are essential for the correct segregation of homologous chromosomes. Defects in these processes likely contribute to infertility or recurrent pregnancy loss in humans. To screen for candidate genes for reproductive failure due to meiotic defects, we have analyzed the gene expression patterns in fetal, neonatal and adult gonads of both male and female mice by microarray and thereby identified 241 genes that are expressed specifically during prophase of meiosis I. Combined with our previous data obtained from developing spermatocytes, a total of 99 genes were identified that are upregulated in early prophase I. We confirmed the meiotic prophase I-specific expression of these genes using qRT-PCR. To further screen this panel for candidate genes that fulfill important roles in homologous pairing, synapsis and recombination, we established a gene transfer system for prophase I oocytes in combination with *in vitro* organ culture of ovaries, and successfully determined the localization of the selected genes. This gene set can thus serve as a resource for targeted sequence analysis via next-generation sequencing to identify the genes associated with human reproduction failure due to meiotic defects.
Journal of Human Genetics (2012) **57**, 515–522; doi:10.1038/jhg.2012.61; published online 31 May 2012

Keywords: infertility; meiosis; microarray

INTRODUCTION

Infertility is a serious clinical condition for couples who desire to have children. It is estimated that ~15% of couples attempting conception suffer from some form of infertility, and that in ~30% of these cases there is no definitive cause.[1] Although some infertile couples are successfully treated with assisted reproductive technology, some couples can go through years of infertility treatment without success. Another serious problem in reproduction is recurrent pregnancy loss (RPL), which affects ~5% of couples.[2] The cause of RPL cannot be determined in up to 50% of the cases.[3,4] As multiple factors underlie the etiology of infertility and RPL, these disorders are believed to be polygenic, whereas single-gene abnormalities have been demonstrated to be a cause of reproductive defects in animal models.[5] The heterogeneous and complex characteristics of RPL have made the causes challenging to elucidate. Moreover, it is difficult to identify the responsible gene(s) using pedigree analysis because the genetic factors that cause a susceptibility to infertility will be poorly transmitted through the germline.

Mutations or polymorphisms in genes associated with meiosis are potentially good candidates as genetic causes of infertility because they would create no other defects other than reproductive defects. Approaches involving the sequencing of candidate genes have enabled the identification of a few meiotic genes that are causal for infertility or RPL.[6–9] Further analyses of these candidate genes will be necessary to better understand the genetic basis of human infertility and RPL. It is likely also that many other genes involved in meiosis in mammals, and that that remain to be identified, have a role in these disorders.

Unlike mitotic division, homologous chromosomes segregate from each other during meiosis I. The formation of DNA double-strand breaks, subsequent repair by homologous recombination and maintenance of the resultant chiasma between homologous chromosomes until anaphase are essential for correct segregation. Studies in mouse models have revealed that impairment of these processes during early meiosis results in apoptosis or aneuploidy.[10] The extensive apoptosis of gametes leads to infertility, whereas aneuploidy in gametes, whether the abnormality is derived from a homologous or sister chromosome segregation error, contributes to RPL or to the birth of offspring with congenital defects. Interestingly, males and females differ in terms of meiotic defect outcomes. In males, infertility due to extensive apoptosis is the most predominant outcome, whereas segregation

[1]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan and [2]Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Hyogo, Japan
Correspondence: Dr H Kurahashi, Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan.
E-mail: kura@fujita-hu.ac.jp
Received 8 March 2012; revised 24 April 2012; accepted 2 May 2012; published online 31 May 2012

errors in maternal chromosomes often results in aneuploid oocytes.[11] It has been reasonably hypothesized that this sexual dimorphism might be due to a prolonged meiotic prophase I in females or due to differences in checkpoint robustness.[12,13] However, despite its importance to understanding the differences in the molecular mechanisms underlying correct segregation between males and females, there is little currently known about oogenesis because of the limited availability of oocytes.

To further investigate the etiology of infertility and RPL in humans, and the mechanisms of chromosome segregation in mammals, we have previously performed expression profiling analysis of mouse spermatocytes.[14] We focused on screening for genes upregulated in the transition to leptotene/zygotene spermatocytes from spermatogonia to identify the factors controlling the correct segregation of chromosomes during meiosis I. We thereby identified a total of 726 genes, MLZ (male leptotene and zygotene)-001-726. However, this MLZ gene panel actually included many spermatogenesis-specific genes, which hindered further analyses. In our current study, the gene expression profiles of both male and female gonads undergoing meiosis I were examined by microarray analysis to exclude the effects of male-specific genes in the MLZ gene set. This enabled an enrichment of meiotic genes and prevented the exclusion of female factors.

MATERIALS AND METHODS

Microarray analysis

All animal experiments were approved by the Animal Care and Use Committee at Fujita Health University. Total RNA was extracted from male and female gonads of C57BL6 mice at 15.5 days postcoitum (dpc), neonatal day 1 and 10 weeks postpartum. Microarray experiments were performed as described previously,[14] except that the GeneChip Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA, USA) was used in this study. Microarray data analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA), Genespring software, version 11 (Silicon Genetics, Redwood City, CA, USA) and Affymetrix Microarray Suite, Version 5 (MAS5, Affymetrix). All data were subjected to per chip and per gene normalization, and then used for further analysis. Annotations of all filtered transcripts were updated using Affymetrix NetAffx (http://www.netaffx.com), based on the July 2011 annotation update. Microarray data were deposited in the GEO database according to the MIAME Guidelines and assigned the accession number GSE35734.

Real-time RT-PCR

First-strand cDNAs were synthesized as described previously.[14] Real-time RT-PCR assays were also performed as described previously.[15] The primer sets used in these experiments are listed in Supplementary Table 1.

Expression of epitope-tagged proteins

The entire coding region of each selected candidate gene was obtained by RT-PCR and individually cloned into the pFLAG-CMV2 vector (Sigma-Aldrich, St Louis, MO, USA). Plasmids were dissolved in electroporation buffer (125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM glucose and 20 mM Hepes, pH 7.4)[16] at a concentration of 3.5 $\mu g\,\mu l^{-1}$. Mouse ovaries with attached mesonephroses at 13.5 dpc were removed. After injection of the tissues with plasmids suspended in L-15 medium, the ovaries were electroporated as described previously[17] with some modifications. Briefly, after injection, ovaries with mesonephroses were placed between electrodes (CUY501G2, Nepa Gene, Chiba, Japan) with a small volume of electroporation buffer. A 45-V, 50-ms rectangular pulse was charged 5 times at 100-ms intervals using an electroporator (CUY21SC, Nepa Gene). The tissues were then cultured for 2 days as described previously[17] except that Transwell membrane inserts (polycarbonate membrane, 24-mm diameter, 3.0-μm pore size, Corning, Corning, NY, USA) and minimum essential medium-alpha medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, and supplemented with penicillin and streptomycin were used. For expression in mouse spermatocytes, the plasmids were introduced into the testis by *in vivo* electroporation as described previously,[9] and these tissues were dissected out for immunostaining 2 days later.

Immunofluorescence microscopy

Testis frozen sections were fixed in acetone for 15 min and then washed three times in phosphate-buffered saline (PBS). After blocking with 10% normal donkey serum in PBS for 30 min at room temperature, primary antibodies were applied as described below. Squashed oocytes were prepared as described previously.[18,19] Slides were incubated with blocking buffer (5% normal donkey serum and 5% normal goat serum in PBS), and then incubated with primary antibodies diluted in PBS at 4 °C overnight at the following dilutions: rabbit anti-FLAG (D-8, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000; guinea-pig anti-SYCP3 antiserum,[15] 1:5000. The slides were then washed three times in PBS for 15 min, and incubated with the following secondary antibodies to enable detection by fluorescence microscopy: Alexa 594-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated goat anti-guinea-pig IgG (Invitrogen), or aminomethylcoumarin acetate-conjugated donkey anti-guinea-pig IgG (Jackson ImmunoReasearch, West Grove, PA, USA). The secondary antibodies were diluted in PBS and incubated at room temperature for 30 min. After again washing three times in PBS, slides were mounted in Mowiol/DABCO (Sigma-Aldrich) with or without DAPI (4′-6-diamidino-2-phenylindole) (0.5 $\mu g\,ml^{-1}$) and observed under a fluorescence microscope (Axio Imager M1, Carl Zeiss, Jena, Germany). Images were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

RESULTS

Enrichment of a candidate gene set for meiosis I prophase in mouse gonads

The gene expression profiles of gonads from a 15.5-dpc fetal male (FM), 15.5-dpc fetal female (FF), neonatal day 1 male (NM), neonatal day 1 female (NF), 10-week-old male (AM) and 10-week-old female (AF) mice were examined by microarray analysis. To screen for meiotic prophase I-related genes, we performed filtering as described in Figure 1a. First, we eliminated male-specific genes using flag calls as follows. Genes with a 'present' or 'marginal' call in either FF or NF were selected, which would be expected to include genes expressed in female meiosis I but no male-specific genes. Likewise genes with a 'present' or 'marginal' call in AM were selected to eliminate female-specific genes. These genes were further filtered by comparing the expression levels in AM with those in FM, NM and AF, followed by filtering of genes for which the expression levels in FF were more than threefold higher than those of AF. Thus, we extracted a total of 267 genes that were upregulated in both male and female gonads undergoing meiotic prophase I. After correcting for redundancy, 241 non-redundant genes were identified and these are listed in Supplementary Table 2. Within this panel, there were 99 matches for genes in the MLZ gene set (Figure 1b), and these are listed in Supplementary Table 3.

The composition of the final 99 selected genes is summarized in Table 1. Among these gene candidates, 76 are named and the remaining 23 are previously uncharacterized. Among the named genes, 17 genes (~22%) have been reported to show meiosis-preferred expression, with only six genes known to display testis-preferred expression. On the other hand, among the 578 named genes in the MLZ gene set identified previously, 101 genes have been demonstrated to show testis-preferred expression.[14] This indicates that the male-specific genes had mostly been excluded from the MLZ gene set in this study. Significantly, most of the genes known to function in meiotic prophase I were included in our final 99 gene panel, as shown in Table 2. These data suggest that the MLZ gene set had been successfully enriched with genes involved in the prophase of meiosis I. However, three genes, *Mei1*, *Msh5* and *Rec8*, in spite of their