twin projects have been independently conducting various psychological, behavioral, neurophysiological, and molecular genetic studies for several years, and have involved a range of funding sources and research teams. The early work of the KTS and ToTCoP was reported by Shikishima et al. (2006) and Ando et al. (2006), respectively. The KoTReC has also collected three anonymous one-shot twin data sets, one of which uses a cross-sectional design.

The current article provides a brief outline of the current status and main findings at the KoTReC.

Purpose of Twin Studies

Twin studies typically have three main aims: to obtain relevant information *of* twins, *by* twins, and *for* twins. Studies *of* twins are designed to collect information about factors differentiating twins from singletons, such as the development of linguistic abilities and sibling relationships. Studies *by* twins are typically behavioral genetic studies in which genetically and environmentally systematic information of twins are utilized as a biometrical method. This second type of research is the focus of the KoTReC. Studies *for* twins are focused on providing evidence-based support for nursing and educating twins, mainly in infancy and childhood, by producing relevant information about the causes of parenting stress and environmental effects on infant growth and development.

The age ranges of the two cohorts in the KTS and ToTCoP are different, and the aims of the projects also differ. In the KTS, which includes participants between 15 and 40 years of age, almost all the research is conducted with a behavioral genetic focus (i.e., a *by*-twins study), including psychological, psychiatric, sociological, socio-economic, neurological, and molecular genetic characteristics. On the other hand, the ToTCoP, which examines twin participants from birth to 6 years of age, includes all three of the main aims of twin studies. For studies *of* twins, a set of singleton data that is comparable to twin data were obtained for several important variables.

Recruitment of Twin Participants

The strategy to recruit twins and their families in both the KTS and ToTCoP is to send letters to twin families identified by the Basic Resident Register (BRR; nation-wide census). The BRR is a quasi-complete (i.e., complete at a specific time in a specific area) residential record of each municipal area, which contains each resident's name, gender, residential address, and date of birth. This information gathering is authorized by each municipal area's regulations, and data are available at city halls. Twins or higher multiples can be identified as individuals who share the same date of birth and address. With this method, it is difficult to recruit newborn and adult twins because it takes several months for newborns to be registered on the BRR and because most adult twins live apart. Because these data are not obtained electronically, but rather by printed documents with a substantial cost, well-trained staff are required to identify multiple births and transfer the information manually. This strategy has a number of methodological shortcomings (see Ando et al., 2006), but it is the only way to obtain 'population-based', rather than hospital-based or twin support group-based, twin data in Japan.

These research projects included three residential twin data collection periods from the BRR (1998–2002, 2003–2004, 2009), and cover the Tokyo metropolis and the neighboring prefectures (Kanagawa, Chiba, and Saitama). The 1998–2002 data contain approximately 10,000 pairs, which substantially overlap with the 2003–2004 data set, which contains 46,000 pairs. In addition, data from around 1,000 pairs of twins under 3 years of age were added in 2009. Currently, approximately 48,000 sets of multiple birth families are registered at our center.

Additional recruitment of twin child cohorts was conducted by voluntary participation through a poster campaign in public healthcare centers in the target areas and magazine advertisements in publications distributed nation-wide.

Zygosity Diagnosis and DNA Data

In order to identify twins' zygosity, the KTS project mainly used a three-item questionnaire administered to twins themselves (Ooki et al., 1990), whereas the ToTCoP administered the questionnaire to parents (Ooki & Asaka, 2004). These questionnaires asked for judgments about the twins' physical similarities, and experiences of being mistaken for each other. The items in the ToTCoP questionnaire (and the KTS questionnaire) were as follows: 'Were your twin children (you and your co-twin) as alike as two peas in a pod?' 'Were your twin children (you and your co-twin) mixed up (as children)?' and 'If so, by whom were your twin children (you) mixed up?' This questionnaire has been found to have almost 95% accuracy by comparison with genetic markers (Ooki & Asaka, 2004).

DNA samples were collected from approximately 600 pairs of adult twins (KTS) by analyzing blood (approximately 240 pairs in 1998, partially replicated in 1999), buccal smear (approximately 200 pairs in 2005), nail or hair roots (approximately 100 pairs in 2010), and saliva (approximately 60 pairs in 2011; Table 1). These DNA data were also used to identify zygosity. Agreement rate between the DNA-based diagnoses and the questionnaire-based diagnoses was 93.0% (94.3% for monozygotic (MZ) and 87.5% for dizygotic (DZ); a preliminary result was reported by Shikishima et al., 2007).

In the following sections, the research design and major findings of each of the sub-projects at the KoTReC are introduced.

TABLE 1

Data Collection History of the KTS (KTP)

			N	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Publications
Entry	Twin	Wave 1	315 pairs	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 2	45 pairs		→	→	→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 3	354 pairs			→	→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 4	312 pairs				→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 5	672 pairs										→	→	→	→	→	→	
		Wave 6	548 pairs													→	→	→	
	Parent of	Wave 1 & 2	196 Mothers					X									→	→	
			173 Fathers					X											
		Wave 6	600 Mothers																
Survey			On campus	X	X	X	X	X	X	X	X	X	X			X	X	X	
			On mail				X	X		X	X	X				X	X	X	
			Online												X	X	X		
Biology	DNA[a]			Bl	Bl						Bc	Bl				N & H/Bl		S/Bl	
			N of pairs	238	(70)						218	7				101/11		59/6	
	Hormone (teststerone)											X							Uchida, et al, 2006
Cognition	Nx_full										X	X				X	X		Shikishima et al., 2009
	Nx_sub			X	X			X		X	X								Ando et al., 2001
	BAROCO_full										X	X				X			Shikishima et al., 2009
	BAROCO Short															X	X		Shikishima, et al., 2011a
	WAIS					X	X	X	X										
	ERP/EEG					X	X	X	X										
	Reaction time					X													
	Inspection time					X													
	Working memory			X															Ando et al., 2001
	3D *mental rotation*			X	X			X		X	X								Suzuki et al., 2011
Decision making	Economic game[b]											PG			PG	PG	D/U		
	Time preference														X		X		
	Allais paradox															X	X		
	Ellsberg paradox															X	X		
Personality	NEO-PI-R			X	X		X	X											Ono et al., 2000/ Yamagata et al., 2006/ Jang et al., 2001/Jang et al., 2008 / McCrae et al., 2008/Ekehammar et al., 2010
	NEO-FFI										X		X				X	X	
	TCI				X		X	X							X	X		X	Ando et al., 2004/ Rushton et al., 2009
	BIS/BAS								X			X					X		Takahashi et al., 2007
	SPSRQ									X									
	EC								X										Yamagata et al., 2005a

TABLE 1

Continued.

		N	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Publications
Mental health	HADS		X			X	X					X						Ono et al., 2002
	SUBI			X		X	X								X		X	
	AQ						X	X										
	STAI							X										
	SDS							X										
	QIDS-SR									X								
	QLS									X								
Attitude	Social attitude						X	X							X			Shikishima et al., 2006
	Voting behavior													X	X			
	RSES			X		X	X	X								X	X	Kamakura et al., 2001/Kamakura et al., 2007/Shikishima & Ando, 2004
	General trust			X		X	X									X	X	Shikishima et al., 2006/Hiraishi et al., 2008b
	Empathy			X		X	X											Shikishima et al., 2011b
	Authoritarianism						X	X							X			Shikishima et al., 2008
Gender	PSAI									X								
	Klein Grid									X								Hiraishi et al., 2011
	BSRI			X		X	X											Sasaki et al., 2008
Eating	EAT					X	X											
	EDI								X	X								Kamakura et al., 2003
	TFEQ-R21									X								
Physical	Smoking/drinking			X		X	X									X		
	Height/weight			X		X	X		X	X						X		Hur et al., 2008
	2D4D								X	X								Hiraishi et al., 2011
	Laterality									X						X		
Environment	SIDE						X	X										
	School background															X		
	PBI			X		X	X											Shikishima et al., in press
	FACESIII						X	X							X		X	Shikishima & Ando, 2004
Parental data	FACESIII						X											Shikishima & Ando, 2004
	PBI						X											
	RSES						X											
	SUBI						X											
	HADS						X											
	TCI																X	

Note: [a]Bl = blood; S = saliva; Bc = buccal smear; N = nail; H = hair root.

[b]PG = public game; D = dictator game; U = ultimatum game.

Abbreviation of instruments not introduced in the text.

SPSRQ (The Sensitivity to Punishment and Sensitivity to Reward Questionnaire; Torrubia et al., 2001); EC (Japanese version of Effortful Control Scale; Yamagata et al., 2005b); Klein Grid (Klein et al., 1985); RSES (Rosenberg Self-Esteem Scale; Rosenberg, 1965); PSAI (Pre-School Activities Inventory; Golombok & Rust, 1993); BSRI (Bem Sex Role Inventory; Bem, 1974); EAT (Eating Attitude Test; Garner et al.,1982); TFEQ-R21 (Three Factor Eating Questionnaire; Stunkard & Messick, 1985); SIDE (The Sibling Inventory of Differential Experience; Daniels & Plomin, 1985); PBI (Parental Bonding Instrument; Parker et al., 1979); FACESIII (Family Adaptability and Cohesion Scale; Olson, 1985).

The KTS

The KTS, originally named the Keio Twin Project (Shikishima et al., 2006), was established in 1998 to conduct behavioral genetic studies in adolescence and early adulthood. Twins entering the study in 1998 were aged between 15 and 30 years of age, and new participants within the same age range were added subsequently. Table 1 shows the major variables and survey administration year by year. As shown in the table, there were six entry time points in 1998, 1999, 2001, 2002, 2007, and 2011, totaling more than 2,000 twin pair data sets, some of which include their parents' data.

The variables investigated include cognition (general and specific cognitive abilities), decision-making tasks, personality traits (two-, five-, and seven-factor models), mental health, attitude and gender, eating, physical traits, and family and school environment.

Cognition and Decision Making

Cognition has been an important phenotype of interest in the history of behavioral genetics. At the KoTReC, the Kyodai Nx15- (Lynn et al., 1987; Osaka & Umemoto, 1973, Shikishima et al., 2009) is used as a full-scale intelligence test to measure individual difference of general cognitive ability in adolescence and adulthood. The Kyodai Nx15- is the most systematic group intelligence test available for this age range in Japan, and consists of 12 sub-scales covering verbal and spatial aspects of reasoning, memory, and processing speed. In situations where the full-scale version is too long to be administered (i.e., in an experimental session with many variables), a four-subscale version with two verbal and two spatial sub-tests is used.

Overall, our results indicate that the cognitive domain is a unitary feature of its genetic structure. Ando et al. (2001) reported that different aspects of working memory, storage, and executive functions of verbal and spatial modalities are mediated by a single latent genetic factor that also explains general cognitive ability, measured by the sub-scale version of the Kyodai Nx-15. Shikishima and colleagues developed a syllogistic reasoning test called BAROCO (Shikishima et al., 2009), named from a mnemonic word to memorize a syllogism form in classical logic, with 100 items, and reported that its genetic component completely overlapped with those of the Kyodai Nx-15. Based on these findings, a shortened five-item version, the BAROCO Short, was developed and validated (Shikishima et al., 2011a).

Researchers in our project recently began investigating the possibility of a 'general intelligence gene' by comparing epigenetic differences of discordant identical twin siblings (Yu et al., 2012).

Gender differences in spatial ability were independently investigated using a mental rotation task (Suzuki et al., 2011; Vandenberg & Kuse, 1978). A sex limitation analysis revealed that there were no gender-specific genetic factors affecting this trait, but that the additive genetic influence was greater in males.

Endophenotypes of cognitive abilities, event-related potential (ERP) indices in a working memory task and electroencephalography under resting conditions with eyes open and closed were measured individually for approximately 150 pairs of twins, together with the full-scale Wechsler Adult Intelligence Scale and specific cognitive abilities, simple reaction time and inspection time, in an international collaborative study (Wright et al., 2001). These data, and data from another endophenotype (structural brain imaging examined using magnetic resonance imaging), will be analyzed and published in the near future.

We recently began to conduct behavioral genetic studies of 'decision-making' tasks, such as economic games (a public goods task, and the dictator and ultimatum games), time preferences, and Allais and Ellsberg paradoxes, which are commonly used tasks in behavioral economics. Collaborative studies with economists are also underway at the center.

Personality and Mental Health

Personality traits have been another important research focus in behavioral genetics, and studies in our project have investigated the genetic structure of personality and related phenotypes. Ono and colleagues reported the results of a univariate genetic analysis of the five-factor model of personality using the NEO Personality Inventory Revised Test (NEO-PI-R, Costa & McCrae, 1992; Yoshimura et al., 1998). The results clearly replicated a very robust finding of this field that there are substantial genetic and non-shared environmental influences on personality traits (Ono et al., 2000). Yamagata conducted an international comparative study of the five-factor model by conducting genetic factor analysis based upon 30 sub-scales of the NEO-PI-R, revealing that the genetic structure is strikingly congruent among Japan, Germany, and Canada (Yamagata et al., 2006). Using the same data set, Jang reported genetic comorbidity between Neuroticism and Agreeableness, and their molecular bases (Jang et al., 2001), and proposed a two-higher-order-genetic-factor structure of the Big Five factors (Jang et al., 2006). Furthermore, McCrae, who originally developed the NEO-PI-R, reported that these higher-order genetic factors contained artifacts as well as substance effects (McCrae et al., 2008). Conversely, Rushton proposed a single general personality factor model and reported its genetic validity using our NEO-PI-R and the Temperament and Character Inventory (TCI) data (Rushton et al., 2009).

The TCI was developed by Cloninger, based upon his theory of personality development (Cloninger et al., 1993), which proposes that four temperamental traits (Novelty Seeking, Harm Avoidance, Reward Dependence, and Persistence) are driven by genetic neurotransmission-related factors, whereas three character traits (Self-Directedness, Cooperativeness, and Self-Transcendence) are determined

by post-natal experience. A study in our project attempted to verify this theory, revealing that Novelty Seeking, Harm Avoidance, and Reward Dependence are genetically independent, as Cloninger et al.'s (1993) theory predicts, but persistence and the three character traits exhibited genetic overlap with the three temperamental traits (Ando et al., 2002). In addition, we found that one facet of Novelty Seeking (Exploratory Excitement) is strongly genetically correlated with Harm Avoidance, so should be rearranged by changing combination of facets to make scales genetically consistent (Ando et al., 2004). Yamagata and colleagues (2005) applied the same methodology to examine the genetic structure of Effortful Control (Rothbart et al., 2000) and confirmed its genetic coherence, supporting the validity of the theory.

Ono and colleagues investigated the genetic and environmental overlap between temperamental TCI traits and depressive symptoms measured by the Hospital Anxiety Depression Scale (Kitamura, 1993; Zigmond & Snaith, 1983), suggesting that there are no independent 'depression-specific genes', but that depressive symptoms are dependent on genetic factors involved in normal temperamental dimensions under specific unique environments (Ono et al., 2002). The twin studies at KoTReC are not hospital-based studies, and no medically diagnosed participants have been identified. However, the data from several scales related to mental health and psychiatry, including the Subjective Well-Being Inventory (SUBI; Sell & Nagpal, 1992), the Autism-Spectrum Questionnaire (AQ; Baron-Cohen et al., 2006), the State and Trait Anxiety Inventory (STAI; Spielberger et al., 1970), the Zung Self-Rating Depression Scale (SDS), the Quick Inventory of Depressive Symptomatology (QIDS-SR), and the Quality of Life Scale (QLS; Rush et al., 2003), are available for our normal twin samples. In addition, a univariate genetic analysis of Eating Disorder Inventory (EDI) data in this sample revealed substantial shared environmental influences on four of five sub-scales of the EDI (Kamakura et al., 2003).

Because the KTS is designed in a longitudinal fashion as shown in Table 1, several cognitive and personality phenotypes were measured at different time points for the same individuals. Developmental changes and the stability of the Behavioral Inhibition System (BIS) and Behavioral Activation System (BAS; Carver & White, 1994) — two measures of temperament based on Gray's reinforcement sensitivity theory — have been investigated (Takahashi et al., 2007). The results indicated that genetic influences contribute only to continuity, whereas environmental influences contribute to both continuity and change in the two traits, and that the degree of genetic influences does not differ across time.

Attitudes

Results similar to those reported by Takahashi et al.'s (2007) BIS/BAS longitudinal study were reported for the self-esteem scale (Kamakura et al., 2007). Developmental stability was affected by genetic and non-shared environmental factors, whereas developmental changes were affected by non-shared environmental factors. However, the degree of genetic influence increased during adolescence and young adulthood.

Self-esteem is a personality trait, and can be considered as a type of attitude. Our twin studies have involved a number of measures of attitudes other than self-esteem, such as general trust, voting behavior, empathy, and authoritarianism (Table 1). Shikishima reported a series of behavioral genetic studies on attitude variables traditionally thought to be transmitted through the family environment. The results revealed a substantial genetic influence on authoritarianism (Shikishima et al., 2008) and trust (Shikishima et al., 2006), with no significant effect of shared environmental factors. However, significant environment × environment interactions were found, indicating that shared family environmental factors significantly affected empathy for individuals exhibiting high or very low parental warmth (Shikishima et al., 2011b). A study using direction of causation (DOC) analysis (Heath et al., 1993), an application of behavioral genetic methodology, revealed that the level of general trust can be predicted by personality factors (Extraversion and Agreeableness; Hiraishi et al., 2008a), indicating that humans adaptively control the activation of domain-specific mental mechanisms in accord with domain-general genetic traits like personality.

Other Variables

As shown in Table 1, a large number of variables have been investigated in previous studies, some of which have been published. These variables include eating disorder symptoms (EDI; Kamakura et al., 2003), gender role personality factors (Sasaki et al., 2009), testosterone (Uchida et al., 2006), the relationship between second to fourth finger ratio (2D4D) and sexual orientation (Hiraishi et al., 2012), and parenting (Shikishima et al., in press).

In 2002, 2010, and 2011, parents of the twin participants in our studies provided information about several additional variables. Since 2009, the Web interface of our project (http://www.futago-labo.net/ in Japanese only) has been available to supplement some experimental and questionnaire data.

The ToTCoP

ToTCoP was established to conduct a longitudinal cohort twin study starting from 2003 (Ando et al., 2006) and continues to conduct studies *of*, *by*, and *for* twins from infancy. This project consists of four data sources; (1) questionnaires (Table 2), (2) cognitive and social investigations in the home (Table 3), (3) cognitive, linguistic, and social investigations in university-based laboratories (Table 4), and (4) brain

TABLE 2

Timeline of Investigation Tools in Questionnaire-Based Research

	Month	Entry	9 months	12 months	15 months	18 months	24–30 months	36 months	42 months	48 months	Preschool	1st grade
		1,639	142	256	376	458	249	585	675	475	392	303
Children's characteristics	Body sizes	x	x	x	x	x	x	x		x		
	Stressful life events		x	x	x		x	x		x		
	Zygosity				x		x	x				
	Laterality		x	x	x		x	x				
	Motor development		DenverII				DenverII					
	Temperament			IBQ-R R-ITQ			ECBQ		CBQ		BIS/BAS	BIS/BAS
	Developmental disorder-related symptoms/social behavior	Excerpt from M-CHAT	M-CHAT		Yale Screener	M-CHAT	Yale Screener				ADHD school refusal behavior (original)	ADHD school refusal behavior (original)
	Sleeping behavior	x			BISQ		BISQMEQ		BISQ	MEQ	MEQ	MEQ
	Problem behavior							SDQ		SDQ	SDQ ODBI KINDL	SDQ ODBI KINDL
	Nutrition	x	x	CFQ		x			x			
Parenting behavior/ environment	Attachment		MAI		MAI							
	Cultural/ educational environment					x			x		x (original)	x (original)
	Parental behavior		x		x				x		x (original)	x (original)
	Home environment		EES			EES					EES (6 items)	EES (6 items)
	Twin situation		x		x		x			x	SIB (18 items)	
Parenting stress	Depressive symptom			SDS		SDS		SDS		SDS		
	Parental stress		PSI		PSI						PSI	PSI
	Social support		x	x	x		x	x		x	x(1 item	x(1 item
	Marital status										RAM Short Marital-Adjustment Scale	RAM Short Marital-Adjustment Scale

Note: ADHD-RS-IV = ADHD Rating Scale — IV, (DuPaul et al., 1998); BISQ = Brief Infant Sleep Questionnaire, (Sadeh, 2004); BIS/BAS = Behavioral Inhibition and Activation Systems Scales (Carver & White, 1994); CBQ = Children's Behavior Questionnaire (Ahadi et al., 1993); CFQ = Child Feeding Questionnaire (Birch, 2001), Denver II (Frankenburg et al., 1992); ECBQ = Early Childhood Behavior Questionnaire (Putman et al., 2002); EES = Evaluation of Environmental Stimulation (Anme, 1997); MAI = Maternal Attachment Inventory (Müller, 1994); IBQ-R = Infant Behavior Questionnaire-Revised (Gartstein & Rothbart, 2003; Nakagawa & Sukigawa, 2005); MEQ = Morningness-Eveningness Questionnaire (Horne & Östberg, 1976); M-CHAT = Modified Checklist for Autism in Toddlers (Baron-Cohen et al., 1992; Robins et al., 2001); ODBI = Oppositional Defiant Behavior Inventory (Harada et al., 2004); PTCI = Preschool Temperament & Character Inventory (Constantino et al., 2002); KINDL = Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents (Bullinger et al., 1994); RAM = Relationship Attribution Measure (Fincham & Bradbury, 1992); SDQ = Strength and Difficulty Questionnaire (Goodman, 1999); SDS = Self-rated Depression Scale (Zung, 1965); SIB = *The Sibling Inventory of Behavior* (Volling & Blandon, 2005); *Short Marital-Adjustment Scale* (Locke & Wallace, 1959).

TABLE 3
Timeline of Home Assessment

Age *N* (pairs)		12 months 127	18 months 236	24 months 277	36 months 279	48 months 188
	Cognitive ability	Bayley II	Bayley II	Bayley II	K-ABC	K-ABC
	Vocabulary	CDI	CDI	CDI		
	Socio-cognitive ability	ESCS	ESCS			
				ToM	ToM	ToM
				EF	EF	EF
Observation	Parent–child relationship	x	x	x	x	x
	Twin sibling relationship				x	x
Questionnaire	Parenting stress	PSI	PSI	PSI	PSI	PSI
	Parenting behavior	x	x	x	x	x
	Depressive symptom			SDS		
	Marital relation				Marital love	Marital love
	Mom's personality				NEO-FFI	
	Problem behavior					SDQ

Note: Bayley = Bayley Scales of Infant Development (Bayley, 1993); ESCS = Early Social Communication Scales (Mundy et al., 2003); K-ABC = Kaufman Assessment Battery for Children (Kaufman & Kaufman, 1983); CDI = MacArthur Communicative Developmental Inventories (Fenson et al., 1993); PSI = Parenting Stress Inventory (Abidin et al., 1995) ; Marital love (Locke & Wallace, 1959).

TABLE 4
Timeline of Laboratory Assessment

Age *N* (pairs)		42 months 245	60 months 175
	Cognitive ability	K-ABC	K-ABC
	Reading	x	x
	Socio-cognitive ability	EF	
		ToM	
Observation	Parent–child relationship	x	x
	Twin sibling relationship	x	x
	Social communication (peer)	x	x
Questionnaire	Parenting Stress	PSI	PSI
	Parenting Behavior	x	x
			PFQ
			PDI
	Marital relation	Marital love	Marital love
	Mom's personality	NEO-FFI	
	Problem behavior	SDQ	SDQ
	Temperament		CBQ-VSF
	Twin sibling relationship		SIB
		MISR	MISR

Note: PFQ = Parental Feelings Questionnaire (Deater-Deckard, 1996; Deater-Deckard, 2000); PDI = Parental Discipline Interview, (Deater-Deckard, 2000); SIB = Sibling Inventory of Behavior (Volling & Blandon, 2005); MISR = Maternal Interview of Sibling Relationships (Stocker et al., 1989).

activity and motor skill experiments in university-based laboratories.

Questionnaire-Based Research

Table 2 shows the timeline of the questionnaire investigation tools used for each specific time point from infancy to childhood when twins enter elementary school. The variables in these questionnaires are related to children's characteristics and parents' characteristics, and both types of questions are given to both mothers and fathers until participants are 36 months old. The versions for fathers are partially shortened or different from the versions for mothers, which contain additional items regarding parenting stress. When participants are aged 42 months or older, the questionnaires are administered only to twins' mothers, because asking twins' fathers to answer questionnaires tended to lower the total response rate, and the reliability of fathers' evaluations of twins' behavior was low.

As Table 2 indicates, the number of participating twin families (over 1,600) was relatively large at the first session, constituting approximately 55% of the total twin births in the target area. Although we observed a high degree of data attrition, we retained substantial numbers of twin pairs that could be investigated longitudinally. For example, Fujisawa and colleagues investigated the relationship between head circumference growth from birth to 10 months of age, and socio-cognitive ability at 19 months. Although no significant phenotypic correlation was found between them, significant genetic and shared environmental correlations in opposite directions (i.e., genetically negative and environmentally positive) were reported (Fujisawa et al., 2012a). In addition, Yamagata examined the longitudinal association between authoritative parenting and children's peer problems at 42 and 48 months using a longitudinal MZ twin difference design. They reported that when genetic and family environmental covariates were controlled, authoritative parenting and children's peer problems concurrently influenced each other, peer problems increased authoritative parenting, and authoritative parenting decreased peer problems, canceling each other out (Yamagata et al., in press).

For preschool and first grade elementary school children, additional twin families were recruited. The main research target of these two age groups is social adaptation to changes in educational environmental conditions from preschool to elementary school. To tap these

TABLE 5
The Variables List of the CROSS Study

		Early childhood	Middle childhood	Late childhood		Adolescence		Adult
		3–5 years	7–9 years	10–12 years		13–18 years		19–26 years
	Mailed	3,291	3,196	3,396		5,279		5,095
	Returned (entry)	859	857	740		960		697
	Response rate	26.10%	26.80%	21.80%		18.90%		13.70%
	Informant	Parent	Parent	Child	Parent	Child	Parent	Child
Family and parents	Family structure	X	X		X		X	X
	Age, sex, zygosity, and sib order	X	X	X	X	X	X	X
	Twinship							X
	Sharing toys, room, clothes, etc.	X	X	X	X	X	X	
	Assisted reproductive technology	X	X		X		X	
	Maternal smoking/drinking in pregnancy				X		X	
	Sib interaction	X	X					
	Child-rearing attitude				X		X	X
	Join twin-mother organization?	X	X					
	Same class in nursery school?	X	X		X		X	
Academic-related variables	Income							X
	Name of schools	X	X	X	X	X	X	X
	Academic achievement		X	X	X	X	X	
	Time spent for academic learning		X	X	X	X	X	X
Academic-related variables	Academic motivation					X		
	Learning strategy					X		
	Contingent cognition of effortful results					X		
	Attribution of failure					X		
	Cognition of competition					X		
	Helplessness					X		
In classroom and out of classroom	Intrinsic/extrinsic motivation			X		X		
	Academic autonomy			X		X		
	Goal structure			X		X		
	Self-efficacy			X		X		
	Class mate			X		X		
	Teacher's explanation length			X		X		
	Skill for integrated learning			X		X		
	Class size			X		X		
	Classroom teacher			X		X		
	After-school activities			X		X		
	Academic rank of high school					X		X
	What is society/learning			X		X		X
	Club activity							X
	Family cultural environment		X	X		X		X
	Learning out of school	X	X	X		X		X
Mental health	Depression							X
	QOL							X
	Loneliness				X			X
	Adaptation to school			X		X		
	Suicide					X		X
	CFS			X				
	Problematic behavior	X	X	X		X		
	Gender orientation/identification	X	X	X	X	X	X	X
Personality and social attitude	Personality		X	X		X		X
	Language development	X						
	Correctivism					X		X
	Authoritarianism					X	X	X
	Value					X		
	Hobby							X
	Job supervisor							X
Body and physics	Height/weight	X	X	X		X		X
	Head/chest circumference	X						X
	Eye sight			X		X		X
	Sleeping	X	X	X		X		X
	Blood pressure/ fever					X		X
	Mense					X		
	Allergy	X	X		X			X
	Health			X		X		X
	Liability					X		
	Decade teeth			X				X
	Athletic ability			X		X		

TABLE 5
Continued.

		Early childhood	Middle childhood	Late childhood		Adolescence		Adult
		3–5 years	7–9 years	10–12 years		13–18 years		19–26 years
	Mailed	3,291	3,196	3,396		5,279		5,095
	Returned (entry)	859	857	740		960		697
	Response rate	26.10%	26.80%	21.80%		18.90%		13.70%
	Informant	Parent	Parent	Child	Parent	Child	Parent	Child
	Exercises	X	X					X
	Smoking/drinking							X
	Birth height/weight	X	X		X		X	
	Nutrition							X
	Eating/walking speed	X	X	X		X		X
	Ideal weight					X		
	Nutrition	X	X	X		X		X
Home environment	Home hygienic status	X	X		X		X	X
	Housing condition	X	X		X		X	X
	N of books	X	X		X		X	X
	Reading	X						
	School commuting			X		X		
	Coming home time							X
	Family cohesion					X	X	X
	Child rearing attitude	X	X		X		X	X
	Parenting		X				X	
	Parental intervention				X		X	
	Media exposure	X	X	X	X	X	X	X
	Cell phone			X		X		X
	TV game	X						
Parent	Parental job	X	X		X		X	X
	Parental educational history	X	X		X		X	X
	Parental income	X	X		X		X	X
	Sleeping time	X	X					
	Religiousness						X	
	Life-long education				X		X	
	Life events			X		X		X
	Parental personality	X	X		X		X	
	Rearing burden	X						
	Social support	X						

time-specific features of environmental change, we conducted preliminary but relatively large-scale studies with approximately 1,000 non-twin individuals to develop appropriate items.

Performance-Based Research at Home and in the University Lab

Two independent performance-based studies (with some overlapping twin pairs) are currently underway, as shown in Table 3 (assessment and observation at home) and Table 4 (assessment in the university laboratory). Both studies involve individual cognitive ability tests (Bayley II for younger and Kaufman Assessment Battery for Children (K-ABC) for older twin children), theory of mind and executive function tasks, questionnaires, and observation of dyadic and triadic interactions between twin siblings and among twin siblings and parents.

One of the main purposes of our studies is to investigate the development of pre-reading skills and the relationship with cognitive abilities during early childhood. The Japanese *kana* writing system is different from alphabetic systems such as English. Our experiments are designed to be comparable with English language experiments, such as Byrne et al.'s (2002) study. We developed a Japanese version of a test battery to measure pre-reading skills such as phonological awareness, non-word repetition, receptive vocabulary, and visual perceptual skills (Kakihana et al., 2009). Preliminary results revealed a significant influence of shared environmental factors on kana pre-reading skills, and no significant effect of genetic influence (Fujisawa et al., 2012b). However, we found that genetic factors had significant and stable effects on cognitive abilities (Fujisawa & Ando, 2010, 2011).

As mentioned above, studies *of* twins typically have another important aim. As such, we compared twin siblings with non-twin siblings to investigate the relationship between sibling relationships and social adjustment among children. We found that the effects of sibling relationships on pro-social behaviors and conduct problems were stronger for twin siblings than for non-twin siblings, and positive relationships between siblings increased peer problems only among MZ twins; this is the opposite effect compared with that reported among DZ twins and non-twin siblings (Nozaki et al., in press).

TABLE 6
Items of Two Anonymous High School Twin Studies

Category		2009	2010
Entry		Anonymous Junior/senior high 570 families (1,062 twins, 553 mothers, & 459 fathers)	Anonymous Senior high 424 families (751 twins, 402 mothers, & 318 fathers)
Physical	Height/weight	X	
Academic	App/av motivation	X	
	Sense of belonging to school	X	X
Cognition	BAROCO Short	X	X
Social attitude	Social attitude	X	
	Party identification	X	
	Attitude to political issues	X	
	RSES	X	X
	Authoritarianism	X	X
Gender	Gender identity	X	
Environment	Class atmosphere	X	X
Environment	Parent party identification	X	
Environment	FACESIII	X	
Parental data	FACESIII	X	
Parental data	BAROCO Short	X	X

Brain Activity and Motor Skills

The stimulation of brain function by social stimuli such as mothers' vocalizations in infancy and early childhood twins was investigated using ERPs and near infrared spectroscopy at 6, 9, 18, and 36 months, and data from a total of 161 pairs of twins are currently being analyzed. Development of laterality, especially handedness, has also been investigated. The results of these studies indicate a non-additive genetic influence on handedness, suggesting that spatial constraint is a crucial factor for the expression of genetic effects on handedness in infants (Suzuki et al., 2009).

Three Independent Anonymous Twin Studies

Longitudinal studies place a heavy burden on participants, sometimes resulting in severe data attrition. To obtain large samples to verify specific research questions, the KoTReC conducted three independent 'anonymous' twin studies (i.e., twins who received questionnaire mails do not have to inform their names to the KoTReC, which lets them know that they are not followed longitudinally and reduces their burdens to collaborate in our research), a large-scale cross-sectional twin study (CROSS) and two high school twin studies.

The CROSS was conducted in 2007 with over 4,000 pairs of twins and their parents, with an age range of 3 to 26 years old. There were five age categories: early childhood from 3 to 5 years old, middle childhood from 6 to 9 years, late childhood from 10 to 12 years, adolescence from 12 to 18 years, and adulthood from 19 to 26 years.

The design and sample size of this study is shown in Table 5. As shown in the table, the item questions in the CROSS were not based upon standardized, well-organized, or internationally used psychological scales like those in our cohort studies. Rather, the CROSS used independent measures focusing on specific questions, even though some were related and can be grouped in categories such as academic performance and parental stress. For example, Strengths and Difficulties Questionnaire (SDQ; Goodman, 1999) data were used to examine genetic and environmental influences on the relationship between negative parenting and conduct problems of children in terms of attention deficit hyperactivity disorder status (Fujisawa et al., 2012c).

Two high school twin studies (Table 6) were conducted to investigate the genetic and environmental relationships between educational attainment, cognitive ability, and family social environment. Murayama and colleagues (2011) applied academic motivation data to verify the performance-approach and performance-avoidance achievement goal theories (Murayama et al., 2011).

Ozaki (2008) challenged methodological limitations using paired comparison analysis applied to biometric modeling (Ozaki, 2008), non-normal structural equation modeling with higher order moments applied to DOC (Ozaki & Ando, 2009), and estimation of four parameters (additive genetic, non-additive genetic, shared, and non-shared environmental factors) at the same time (Ozaki et al., 2011).

Future Perspectives

The KoTReC has collected the largest active twin sample in Japan, with a total of approximately 9,000 twin pairs from infancy to young adulthood. Some of these data (approximately 2,000 pairs) are longitudinal, and data collection is ongoing. This is the largest Japanese twin research database ever developed. However, many aspects of the database are incomplete. We have not yet established a complete DNA sample from all twin participants in our project because of budget limitations, which have also led to difficulties in long-term planning and administration of well-organized

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.

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

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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),

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[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-

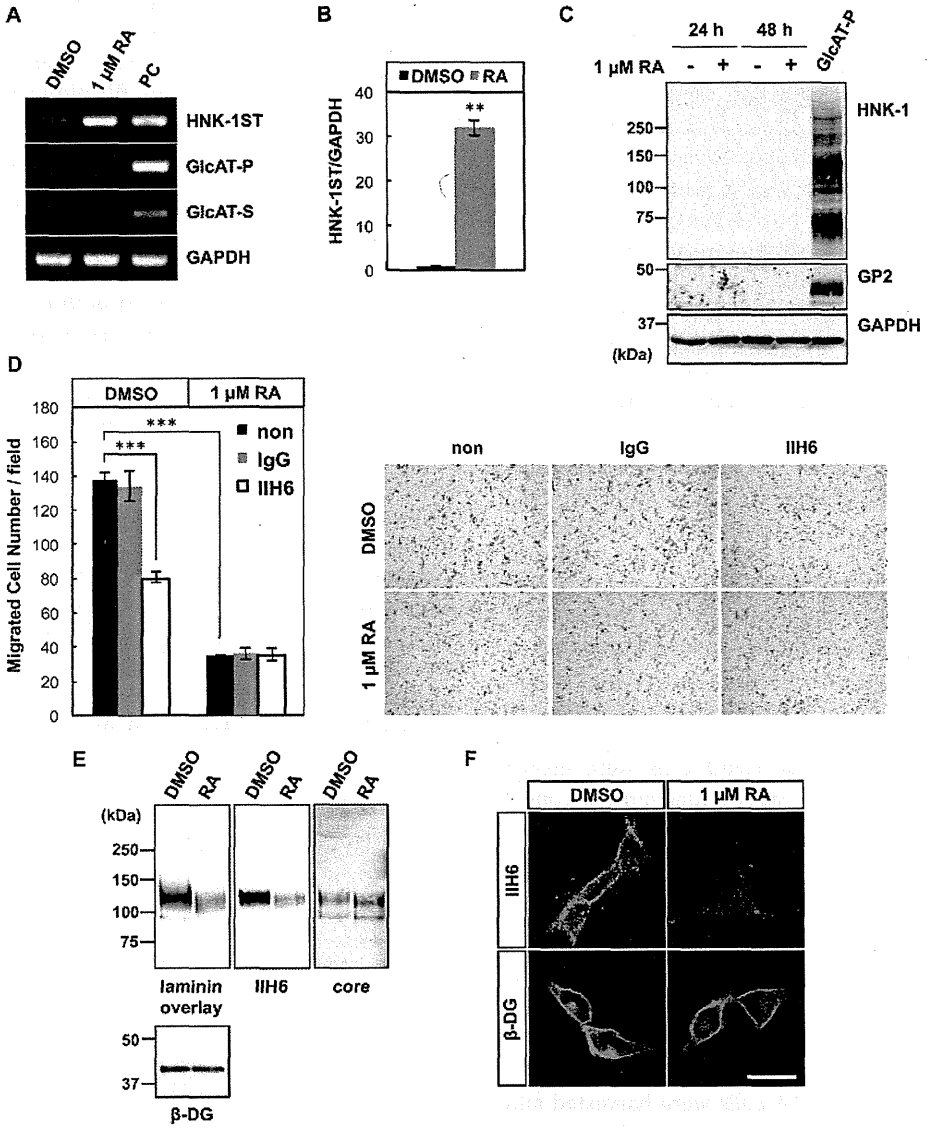

FIGURE 1. **Altered glycosylation of α-DG associated with HNK-1ST induction in RA-treated melanoma cells.** *A*, RT-PCR was performed using mRNA extracted from S91 cells treated with DMSO or 1 μM RA for 16 h. mRNA prepared from 2-week-old mouse brain was used as a positive control (*PC*). *B*, amount 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-

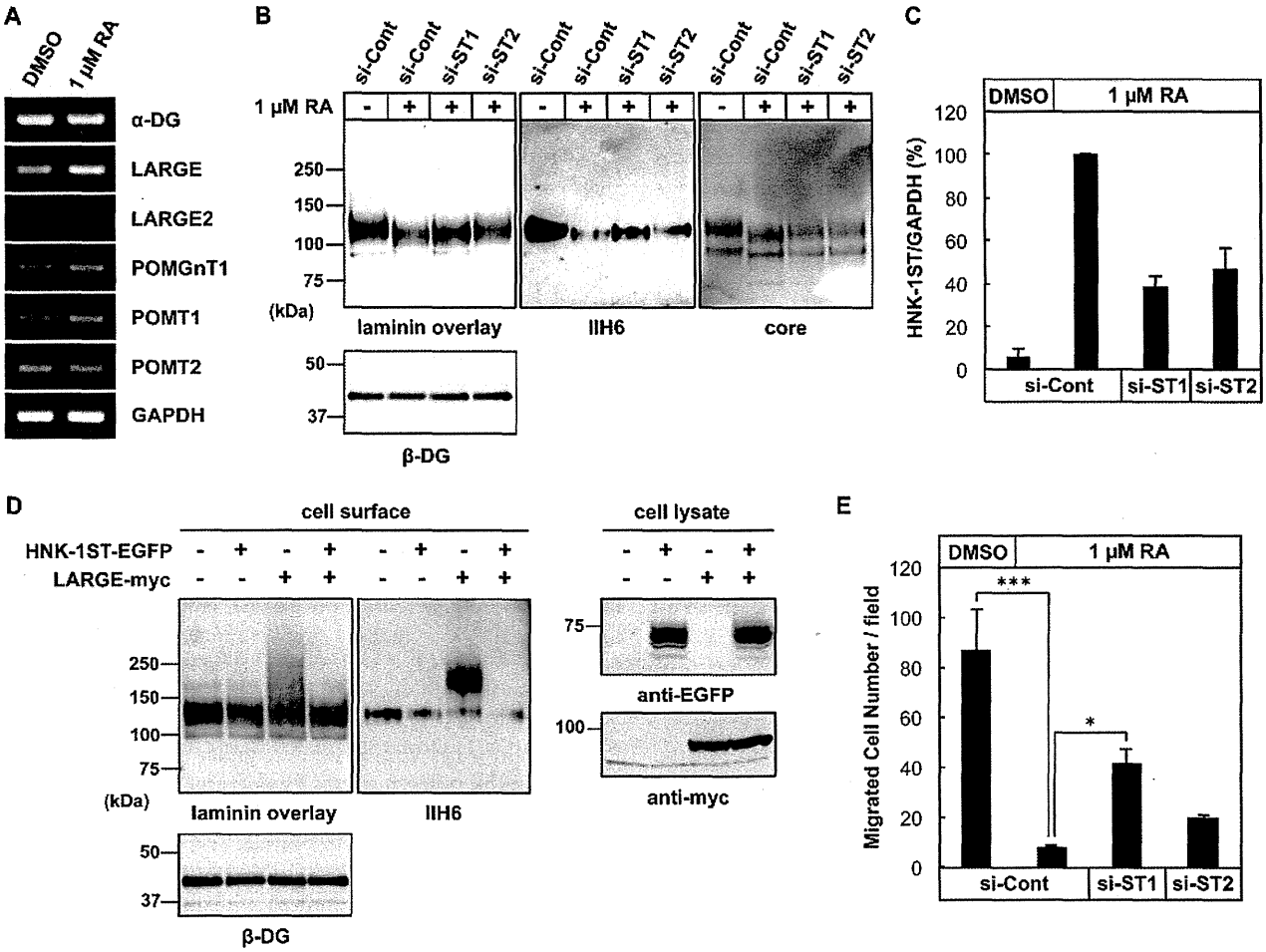

FIGURE 2. **Effect of HNK-1ST knockdown on the glycosylation of α-DG and cell motility.** *A*, S91 cells were treated with DMSO or 1 μM RA for 16 h. mRNA was extracted and subjected to RT-PCR analyses using primer sets as indicated. *B*, S91 cells were transfected with siRNA and treated with DMSO (−) 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.