TABLE 1. Experimental HCV infections performed in this study

Tupaia no.	Inoculum		Biopsy/sacrifice[b]
	Type	Quantity (GE/tupaia)[a]	
Group I[c]			
Tup.4	RCV	1×10^7	84, 94/144 wk p.i.
Tup.5	HCR6	6×10^5	95, 105/155 wk p.i.
Tup.6	HCR6	6×10^5	95, 105/155 wk p.i.
Tup.8	RCV	1×10^7	84, 94/144 wk p.i.
Group II[d]			
Tup.9	Tup.5 (5 wk p.i.)	1×10^2	NT
Tup.10	Tup.5 (5 wk p.i.)	1×10^2	NT
Tup.11	Tup.8 (10 wk p.i.)	1×10^2	NT
Tup.12	Tup.8 (10 wk p.i.)	1×10^2	NT
Tup.13	Tup.4 (8 wk p.i.)	1×10^2	NT
Tup.14	Tup.4 (8 wk p.i.)	1×10^2	NT
Group III[e]			
Tup.15	None		92/100 wk
Tup.17	None		92/100 wk
Tup.38	None		242 wk
Tup.39	None		242 wk

[a] Viral RNA GE/tupaia was estimated by Quantitative real-time RT-PCR (GE, genome equivalents; sensitivity > 10 GE/ml serum).

[b] Liver biopsy was performed at indicated time-point. p.i., postinoculation; NT, not tested.

[c] Group I, primary infection experiment in which 1-year-old animals were inoculated with two different types of inocula.

[d] Group II, reinfection experiment, where HCV RNA-positive sera from Group I experimental infections were passaged to naive animals.

[e] GroupIII, no-infection control.

ined. In the present study, we describe the clinical development and pathology of HCV-infected tupaias over an approximately 3-year time course.

MATERIALS AND METHODS

Animals. Table 1 summarizes the tupaias used in this study. Tupaias born in laboratory captivity were obtained from the Laboratory Animal Center at the Kunming Institute of Zoology (Chinese Academy of Sciences). Tupaias were imported with permission from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (7), quarantined for medical inspection, and housed individually in standard rat cages supplied with filtered air. The animals were fed a daily regimen of eggs, fruit, and the CMS-1 commercial diet for marmosets (CLEA, Japan). Their appetites and feces were carefully monitored. Animal care and experimental handling conformed to study guidelines established by the Subcommittee on Laboratory Animal Care at the Tokyo Metropolitan Institute of Science.

Patient serum used for animal infection. HCV genotype 1b serum, designated HCR6, was obtained from a patient with chronic active hepatitis C. The infectious titer of HCR6 was determined in chimpanzee and Molt4 cells and denoted plasma K (HCR6) by Shimizu et al. (24). The HCR6 serum exhibited a PCR titer of 6×10^6 genome equivalents/ml and an infectious titer of 3.7×10^4 50% chimpanzee infectious doses/ml. Serum aliquots were frozen at −80°C until they were used.

Virion reconstitution of cloned HCV. As described previously, pHCR6 (genotype 1b; 9,611 nucleotides; GenBank accession no. AY045720) is a plasmid carrying HCV genomic cDNA cloned from HCR6 serum (30). pHCR6Rz was designed for precisely trimmed RNA expression, with the entire genomic region of pHCR6Rz recloned under the control of the T7 promoter and the 5′ and 3′ distal ends flanked by hammerhead- and hepatitis D virus ribozyme-encoding sequences, respectively (22, 25).

For molecular reconstitution of HCV particles, pHCR6Rz was transfected into IMY-N9 cells as described previously (12). Briefly, semiconfluent IMY-N9 cells in 100-mm plastic dishes were transfected with 15 μg of plasmid using 40 μl of cationic lipids (DMRIE-C reagent; Life Technology) in accordance with the manufacturer's instructions. Five hours after transfection, the cells were infected with AdexCAT7 (2) (kindly provided by Y. Matsuura) at a multiplicity of infection of 20. After infection, the culture medium was replaced with Hepato-STIM (Becton Dickinson). The culture supernatants were collected at 24 h postinfection and stored at −80°C.

Virus inoculation and collection of serum samples. Animals were infected at 6 months of age. The anesthetic agent, ketamine hydrochloride, was administered intramuscularly at 50 mg/kg body weight prior to virus inoculation and bleeding of the tupaias. The inocula were introduced intravenously at 6×10^5 genome equivalents/animal for patient serum HCR6 and 1×10^7 genome equivalents/animal for reconstituted virions derived from the pHCR6Rz inoculation. Blood samples were drawn from infected and control animals pre- and postinfection. Briefly, the animals were bled weekly for 20 weeks and biweekly thereafter. At each time point, 0.5 ml of blood was drawn from the thigh vein; the sera were separated, aliquoted, and stored for subsequent assays.

Reinfection experiments were performed by transmission of HCV RNA-positive serum from group I (Table 1) to naive animals.

Serum alanine aminotransferase (ALT) concentrations were determined using a Transnase Nissui kit (Nissui Pharmaceutical Co.), standardized, and displayed as IU/liter.

RNA isolation and quantitative RTD-PCR assay for HCV RNA. Serum samples (100 μl) were tested for circulating HCV RNA in vivo using quantitative real-time detection (RTD)-PCR (TaqMan). RNA was extracted from the sera and livers of sacrificed animals using the acid guanidium-phenol chloroform method with tRNA as a carrier (3). Two tupaias (Tup.5 and Tup.6) were inoculated with patient serum HCR6. Another two animals (Tup.4 and Tup.8) were inoculated with reconstituted viral particles (RCV). Tup.15 served as a mock-infected control. Liver specimens (3- to 4-mm^2 blocks) from these tupaias were homogenized with 1.5 ml of 5 M guanidine thiocyanate using a polytron-type homogenizer (Ultra-Turrax T25; IKA Laborteehnik, Staufen, Germany). RNA was then reextracted with 4 M guanidine thiocyanate.

RNA samples were subjected to RTD-PCR on an ABI 7700 sequence detector (Applied Biosystems) as described previously (26). The extracted RNA was dissolved in 200 μl of diethyl pyrocarbonate-treated water containing 10 mM dithiothreitol and 200 units/ml RNase inhibitor in a siliconized tube. RTD-PCR was performed using 1 μg of total RNA, one set of PCR primers, and a probe for a location within the 5′ noncoding region using the EZ r*Tth* RNA PCR kit (Perkin Elmer) and the ABI Prism 7700 sequence detector system. A standard curve was constructed using a 10-fold dilution series of in vitro-transcribed and previously titrated synthetic HCV RNA.

Consequently, the quantities represented by genome equivalents correspond to an absolute standard curve (26). All quantitative RTD-PCR assays were performed using duplicate samples, with both negative control serum and HCV-positive serum included. The control sera were diluted before use and were estimated to contain low copy numbers of HCV RNA (100 genome equivalents/ml serum). Samples were deemed positive for HCV RNA if both duplicates yielded PCR-amplified product. Averages of the two estimated values are shown in the figures.

Histological analysis. Tissue samples were carefully collected from anesthetized animals by abdominal incision, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Silver and Sudan IV (Wako Pure Chemical Industries, Ltd.) staining were also carried out to visualize fiber generation and lipid degeneration, respectively. All histological staining was performed in accordance with conventional procedures. The histological status was determined using the modified hepatitis activity index scoring system, which grades necrosis and inflammation on a scale of 0 to 18 (periportal inflammation and necrosis, 0 to 10; lobular inflammation and necrosis, 0 to 4; portal inflammation, 0 to 4) (11). Fibrosis was scored using the Ishak fibrosis scale of 0 to 6 (0, no fibrosis; 1 or 2, portal fibrosis; 3 or 4, bridging fibrosis; and 5 or 6, cirrhosis). The values in each group (Table 2) represent the averages of the scores in five visual fields.

Statistical analysis. The statistical significance of differences between controls and HCV-infected animals was analyzed with the nonparametric Mann-Whitney U test. All comparisons were two tailed. The statistical analysis was conducted with SPSS 12.0 software (SPSS Inc., Chicago, IL).

RESULTS

Inoculation of HCV causes acute hepatitis and transient viremia in tupaias. To begin this study, two distinct but related inocula were chosen for infection of tupaias. Serum from a chronic hepatitis patient (designated HCR6) was chosen for its

TABLE 2. Grading: necroinflammatory scores and fibrosis

Group	Inoculum	Tupaia no.	Grade				Total	Avg	SD	Staging
			A	B	C	D				
94 wk p.i. (biopsy)										
I	HCR 6	Tup.5	0	0	0	0	0	1.3	1.5	0
		Tup.6	1	0	1	0	2			0
	RCV	Tup.4	0	0	0	0	0			0
		Tup.8	0	0	0	3	3			6
	Control	Tup.15	0	0	0	0	0	0	0	0
III		Tup.17	0	0	0	0	0			0
		Tup.38								
		Tup.39								
144 wk p.i. (sacrifice)										
I	HCR 6	Tup.5	1	0	2	3	6	5.5	3.7	0
		Tup.6	3	0	4	3	10			1
	RCV	Tup.4	0	0	0	1	1			0
		Tup.8	1	0	1	3	5			6
	Control	Tup.15						0	0	
III		Tup.17								
		Tup.38	0	0	0	0	0			0
		Tup.39	0	0	0	0	0			0

defined genotype (genotype 1b), and genetic heterogeneity was ascertained by the process of cloning consensus cDNA. The infectivity of this serum was also experimentally defined in chimpanzees; a 50% chimpanzee infectious dose was estimated at 3.7×10^4 50% chimpanzee infectious doses/ml. Furthermore, the consensus genomic sequence of HCV was cloned from the serum (pHCR6; 9,611 bases; GenBank AY045702.1). For the second inoculum (referred to as RCV), clonal viral particles were reconstituted as described in Materials and Methods. This inoculum was expected to be free of neutralizing antibodies and thus was considered potentially more infectious than patient sera. In the case of RCV infection, genetic diversification of viral RNA, also known as quasispecies, can be regarded as a direct indication of de novo synthesis of progenitor virus in vivo.

Either patient serum or cDNA-derived RCV was inoculated into tupaias (Table 1, group I). Two animals (one female and one male) were tested against each inoculum. Age-matched animals were bred as infection-free controls.

All experimental infections are described in Materials and Methods and Table 1. Prior to experimental infection, the normal serum ALT level in tupaias was measured at 22.3 IU/liter ($n = 23$).

Inoculation with patient serum HCR6 caused rapid fluctuations in the serum ALT concentrations, from two- to fivefold, in both inoculated tupaias, suggesting acute hepatitis in vivo (Fig. 1A and B). Correlative quantitative RTD-PCR revealed HCV viremia soon after serum inoculation in Tup.5, which continued to show transient viremia long term. The appearance of viremia sometimes coincided with a steep elevation in the serum ALT (Fig. 1A). Conversely, HCV RNA was not detected in the serum of Tup.6 up to 60 weeks postinoculation and only twice thereafter. Acute-phase ALT elevations (3 to 4 weeks postinoculation) in Tup.6 might represent tight control of HCV infection by the host immune system (Fig. 1B).

Distinct results were obtained for the two animals (Tup.4 and Tup.8) inoculated with RCV. Both animals displayed sustained viremia up to 10 weeks postinoculation (Fig. 1C and D), indicating persistent HCV infection and inability to eradicate the virus. Viremia was detected intermittently throughout the course of infection, sometimes accompanying the elevation of serum ALT. Humoral immune responses in Tup.5 and Tup.6 (see Fig. S1A in the supplemental material) and Tup.4 and Tup.6 (see Fig. S1B in the supplemental material) were indicated.

We performed RTD-PCR to confirm whether HCV could replicate in the tupaias' livers (Tup.4, Tup.5, Tup.6, and Tup.8) and obtained the following results (Fig. 1E): 310 ± 117 copies/μg total RNA in Tup.5, 80 ± 11 copies/μg in Tup.6, 199 ± 77 copies/μg in Tup.4, and 292 ± 48 copies/μg in Tup.8. In contrast, HCV RNA was not detected in the liver of the mock-infected animal (Tup.15).

HCV RNA was also not detected in samples from either preinoculation or age-matched, infection-free control tupaias (Table 1, group III), nor were significant elevations in serum ALT observed for any of the three infection-free controls (data not shown).

HCV causes chronic hepatitis in tupaia liver, leading to fibrosis and cirrhosis. Serum ALT and circulating HCV RNA levels in primary infected tupaias (Table 1, group I) were monitored for 3 years postinoculation. As described above, the magnitudes of serum ALT fluctuations varied substantially among infected animals (Fig. 1A, B, C, and D). Tupaia livers were examined for histological lesions in order to elucidate if HCV caused chronic hepatitis. Liver biopsies via abdominal incisions were performed at 2 years postinoculation. All animals were sacrificed at 3 years postinoculation (4.5 years for uninfected animals). H&E staining of liver specimens from HCV-infected tupaias showed infiltrating lymphocytes within sinusoids and around portal areas, indicating chronic hepatitis in the tupaia livers (Fig. 2B, D, and H). Infiltrating lymphocytes were also observed in limiting plates, indicating ongoing inflammation (Fig. 2G and H). Furthermore, a comparison of liver samples at 2 and 3 years postinoculation revealed that the

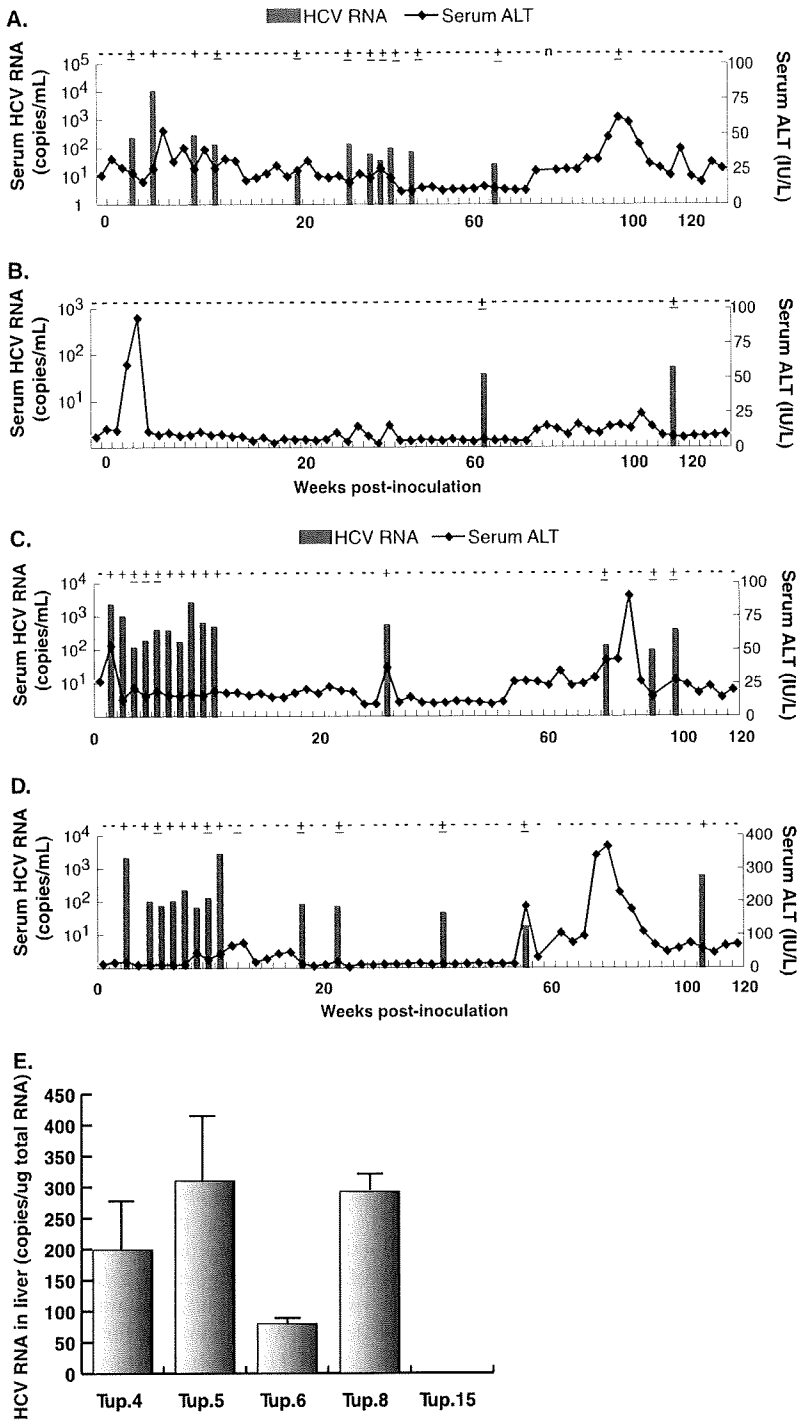

FIG. 1. Course of infection with patient serum HCR6 and RCV. (A) The results of quantitative RTD-PCR for HCV RNA and serum ALT concentrations were combined and plotted to show the course of infection in Tup.5. The bars and the ordinates on the left represent HCV RNA as genome equivalents/ml of serum. The curved line and the ordinates on the right represent serum ALT concentrations as IU/liter serum. (B) Serum HCV RNA and ALT concentrations for infection of Tup.6. (C) The graph for Tup.4. (D) The graph for Tup.8. The vertical axis for serum ALT in this graph is scaled differently from the others because of significant ALT elevation. (E) Quantification of HCV RNA in tupaia liver. HCV RNA in hepatocytes from tupaia (Tup.4, Tup.5, Tup.6, Tup.8, and Tup.15) livers was isolated 172 weeks after HCV infection and quantified by RTD-PCR. As few as 10 copies of the genome were detected, and the quantification range was between 10^1 and 10^8 copies (26).

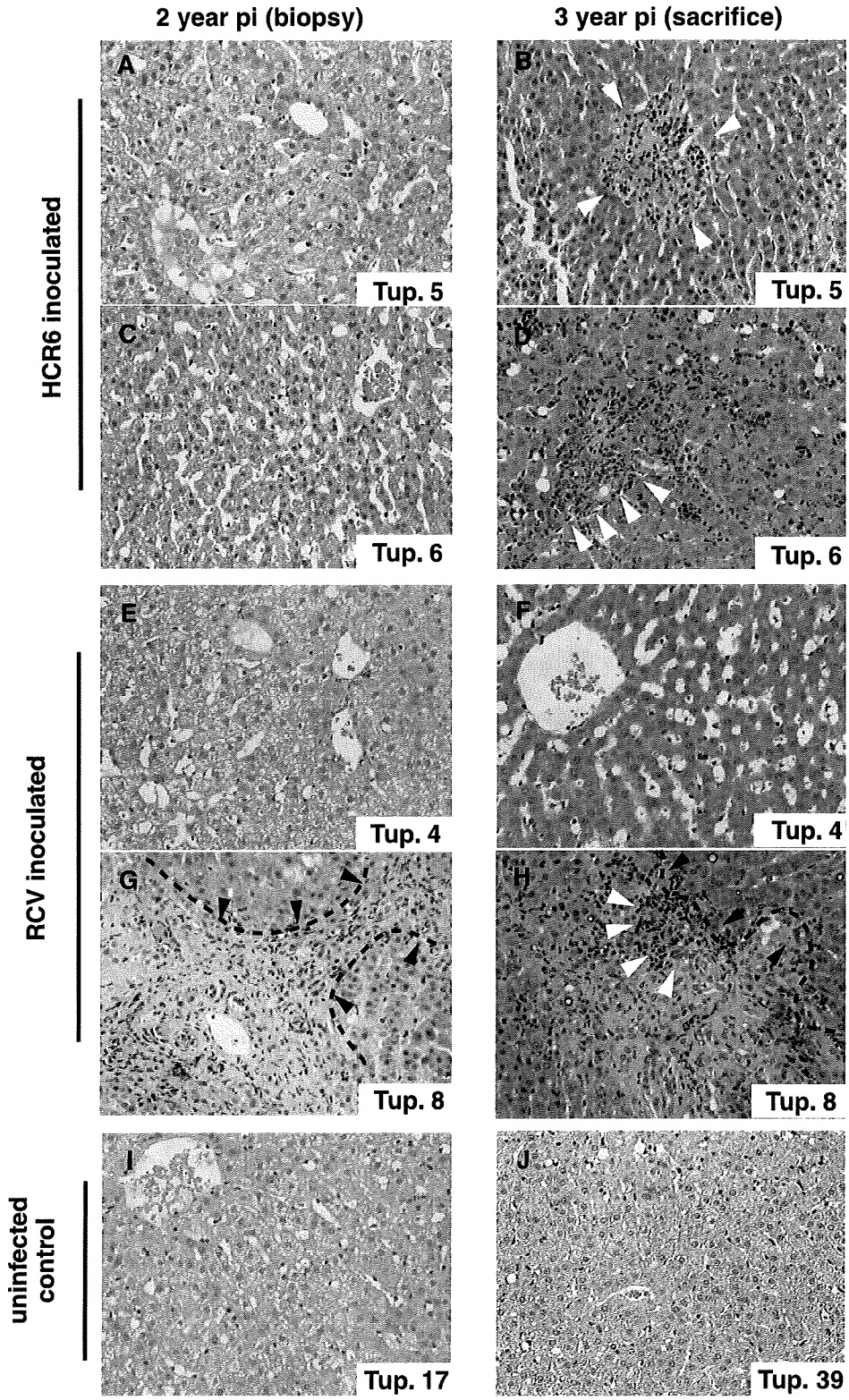

FIG. 2. Micrographs of liver specimens stained with H&E. Liver tissue from HCR6-inoculated tupaias (A to D) and RCV-inoculated tupaias (E to H) was obtained at 2 and 3 years postinoculation (pi). (I and J) Liver specimens from uninfected animals age matched to each inoculated animal were also obtained. The HCV-infected tupaia livers harbored infiltrating lymphocytes (white arrowheads) and fibrosis (broken lines and black arrowheads), which indicate chronic hepatitis.

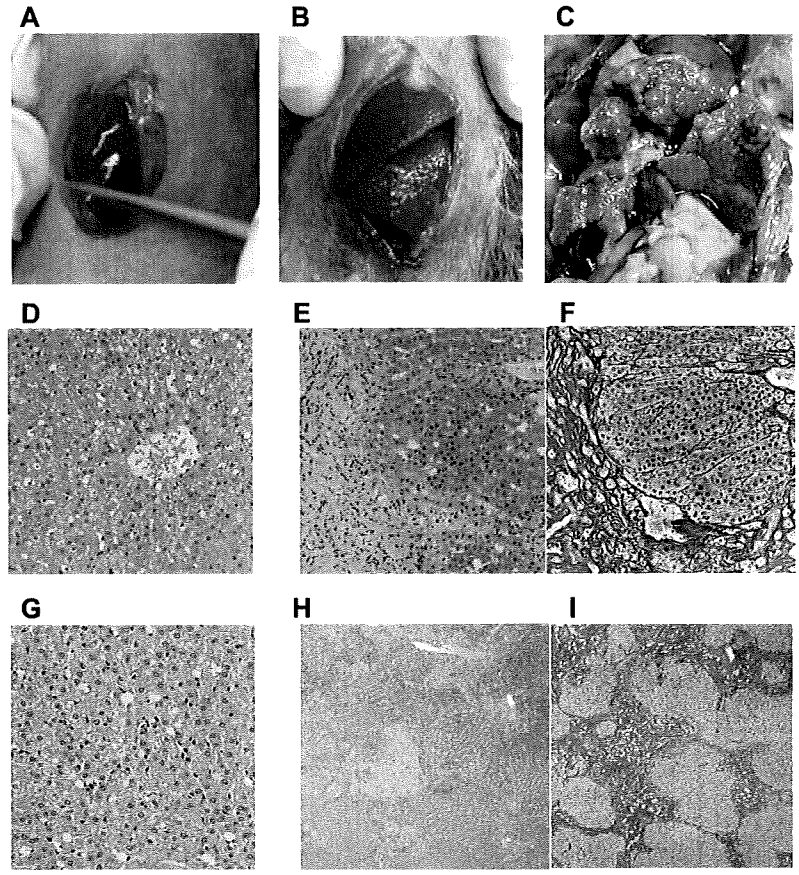

FIG. 3. Macro- and microscopic features of tupaia liver. (A) Infection-free control tupaia (Tup.15; 92 weeks). (B) RCV-infected animal displaying liver cirrhosis (Tup.8; 84 weeks postinoculation). (C) RCV-infected animal with massive surface nodules (Tup.8; 144 weeks postinoculation). (D and G) H&E staining of the uninfected Tup.15 at 92 weeks (D) and the uninfected Tup.39 at 242 weeks (G). (E, F, H, and I) H&E and silver staining of Tup.8 at 84 weeks postinoculation (E and F) or at 144 weeks postinoculation (H and I).

hepatitis had worsened with time in all HCV-infected tupaias (Fig. 2A to H and Table 2).

Fibrosis and cirrhosis were also examined. Mild fibrosis was seen in Tup.6, while severe fibrosis was seen in Tup.8. Cirrhosis was histologically investigated in all animals (Table 2). There was no significant difference between groups I and III at 94 weeks postinfection ($P = 0.194$), but at 144 weeks postinfection, a slight difference was observed ($P = 0.059$; SPSS 12.0). Macroscopic observation of the liver biopsy specimens (taken 2 years postinoculation) indicated liver cirrhosis in Tup.8 (Fig. 3B) compared with Tup.15 (uninfected control) (Fig. 3A), while silver staining of histology samples revealed fibrosis and cirrhotic nodules (Fig. 3E and F). Macroscopic observation upon sacrifice (3 years postinoculation) indicated that liver cirrhosis in Tup.8 had worsened (Fig. 3C). In contrast, age-matched infection-free negative control tupaias displayed none of these pathologies (Fig. 3A, D, and G).

Progressive lipid degeneration was noted in infected tupaias throughout the course of infection (Fig. 4). In particular, Tup.5 displayed microvesicular lipid droplets in the first biopsy specimens (at 2 years), which developed into macrovesicular droplets and foamy degeneration in biopsy specimens at 3 years (Fig. 4C and D). Liver specimens from other infected animals displayed intracellular micro- and macrovesicular lipid droplets in hepatocytes at 3 years postinoculation (Fig. 4F, H, and J). These anomalies were not present in liver specimens from infection-free control animals (Fig. 4A and B).

Transmission of viral-RNA-positive serum to naive animals reproduces acute hepatitis and viremia. To confirm virion regeneration in vivo, and to exclude the possibility of false-positive serum HCV RNA results due to amplification of the original inocula, HCV RNA-positive sera from primary inoculated tupaias were used to inoculate naive tupaias. Three different sera were tested in this passage experiment, with two naive tupaias used as recipient animals for each trial (see Materials and Methods) (Table 1, group II).

In the first reinfection experiment, serum from Tup.5 (originally infected with patient serum HCR6) was collected at 5 weeks postinoculation and used to infect two naive animals. The recipient animals showed intermittent viremia over the subsequent 3 months (Fig. 5A). In the second and third cases of reinfection, sera from Tup.8 at 10 weeks postinoculation and from Tup.4 at 8 weeks postinoculation also induced viremia in the naive inoculated animals, similar to the first reinfection experiment (Fig. 5B and C). Furthermore, the PCR titers of the recipient tupaias were significantly greater than the inoc-

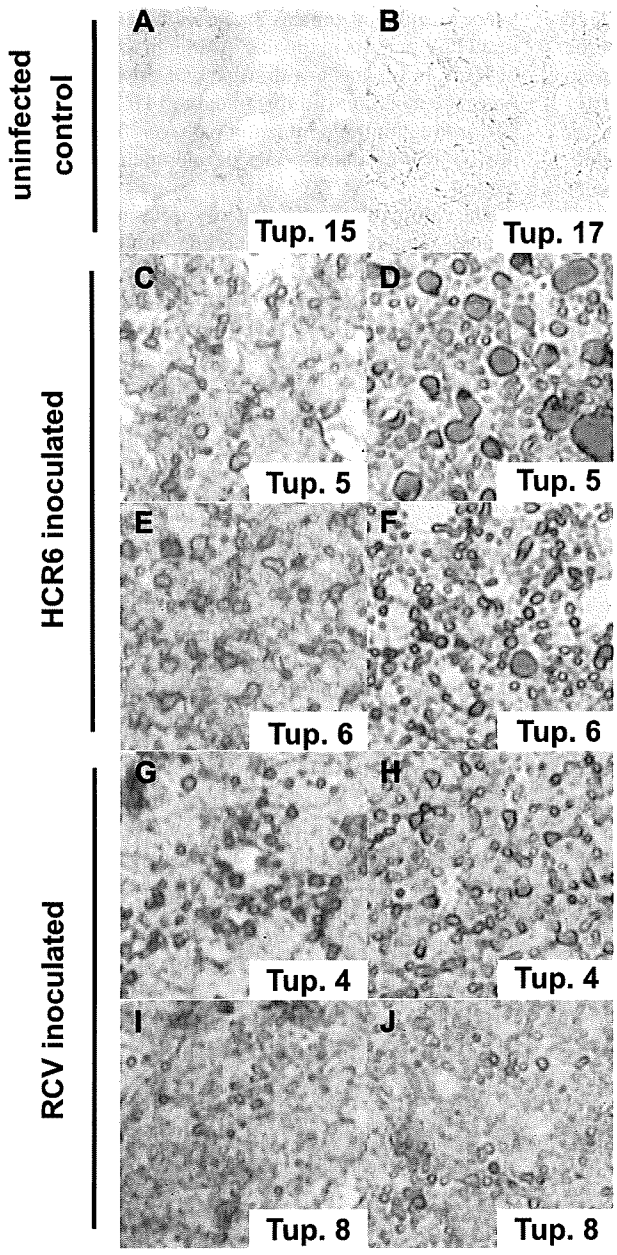

FIG. 4. Sudan IV-stained liver specimens exhibiting fatty liver degeneration. Cryosections of liver stained by Sudan IV as described in Materials and Methods show fatty liver degeneration. The left and right columns display biopsy specimens of infected animals (2 years postinoculation) and animals sacrificed at 3 years postinfection, respectively. (A and B) Uninfected controls at 2 years (Table 1 shows sample timing). (C to F) Patient serum HCR6-infected animals. (G to J) RCV-infected animals.

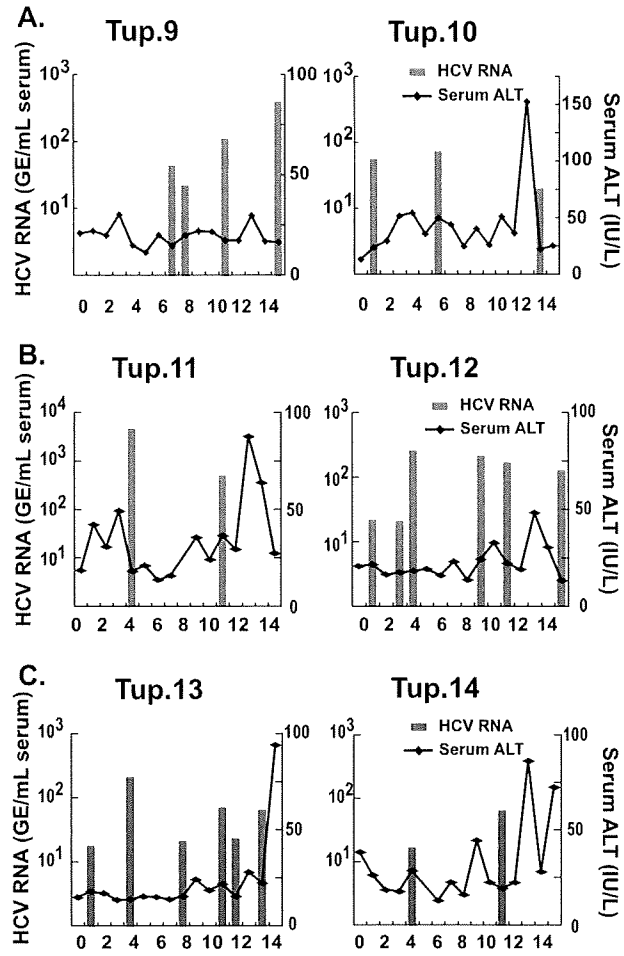

FIG. 5. Results of a reinfection experiment. (A) Quantitative RTD-PCR for HCV RNA and serum ALT levels are shown. Two naive animals were inoculated with tupaia serum (using serum taken at 5 weeks postinoculation from Tup.5, originally inoculated with patient serum HCR6) containing 100 genome equivalents (GE)/ml and were monitored for 15 weeks postinoculation (Table 1). (B) Tupaia serum (taken at 10 weeks postinoculation from Tup.8, originally inoculated with RCV) that was positive for HCV RNA was passaged into two naive animals. The animals were inoculated with tupaia serum at 100 GE/animal and monitored for 15 weeks postinoculation. (C) Tupaia serum (taken at 8 weeks postinoculation from Tup.4, originally inoculated with RCV) that was positive for HCV RNA was passaged into naive animals. The animals were inoculated with serum at 100 GE/animal and monitored for 20 weeks postinoculation.

ulation titers (10^2 genome equivalents/animal) (Table 1). For Tup.11, serum from 4 weeks postinoculation contained almost 10^4 genome equivalents/ml of HCV RNA (Fig. 5B). In addition, significant increases in serum ALT accompanied detection of serum HCV RNA. These results indicate that HCV RNA-positive sera from group I actually contained infectious virion particles. They also suggest that reconstituted HCV particles made from cDNA are infectious in tupaias.

We amplified a portion of the NS5A sequence, which is known as the interferon sensitivity determining region, by reverse transcription-PCR as described in the supplemental material. Each PCR product was subcloned and sequenced to compare the encoded amino acid sequences. For the purposes of this study, animals were inoculated with a molecular clonal virus consisting of a unique viral sequence of cDNA. The interferon sensitivity determining region sequences recovered from an animal infected with clonal inoculum (Tup.8 at 103 weeks postinoculation) were found to be heterogeneous, with

a few amino acid substitutions (K2212M for 2/10 cases, L2232P for 1/10 cases, and L2253S for 6/10 cases) (see Fig. S2E in the supplemental material). Interestingly, the codon for amino acid 2224 encodes valine, but it was found to be variant for alanine and valine in sequences from the original patient serum (HCR6). Tupaias infected with patient serum also exhibited variability at position 2224; valine occupancy was rare, as was seen in the original HCR6 population (see Fig. S2B and C in the supplemental material). On the other hand, this position was occupied solely by valine for sequences recovered from Tup.8 (see Fig. S2E in the supplemental material), indicating that genetic variations shown for Tup.8 originated from the pHCR6 cDNA sequence. Taken together, quasispecies detection of circulating virus represents further evidence demonstrating intrinsic replication of HCV in tupaias despite low levels and infrequent detection of viremia.

DISCUSSION

In the present study, we described persistent HCV infection in tupaias. Long-term follow-up was performed and revealed histological progression of HCV-related liver disorders in infected tupaias, including steatosis, fibrosis, and cirrhosis, in addition to acute and chronic hepatitis. HCV genomic RNA was detected in animal sera intermittently throughout the entire course of infection. However, HCV RNA was detected in the liver upon sacrifice (3 years postinoculation). Furthermore, HCV RNA in serum contained genomic variants that had diverged from the inoculated virus (see Fig. S1 and S2 in the supplemental material). These data strongly indicate an established persistent infection in the tupaias studied. All animals exhibited HCV viremia soon after inoculation, yet the viremia was intermittent and accompanied by relatively low RTD-PCR titers compared with equivalent human and chimpanzee infections. The discrepancy between humans and tupaias might be due to host-dependent differences in replication efficiency. Over the course of HCV infection in these tupaias, serum ALT profiles indicated repeated liver injury, probably due to host immune responses mediated by agents such as cytotoxic T lymphocytes rather than direct viral cytopathic effects.

In cases of tupaia infection, experimental inoculations rarely led to sustained viremia, which for most human cases lasts for the entire course of infection. Even the course of infection appeared transient and self-resolved. It seems likely that HCV replication is less compatible with the tupaia host environment. This possibility was substantiated by a previous report by Xu et al. (34), where tissue-cultured virions of cloned genotype 1b, referred to as HCVcc in the paper, could not cause chronic infection with sustained viremia in tupaias. Although HCVcc actually infected most of the inoculated tupaias (83%; 10/12), chronic infection was seen for only a fraction of them (20%; 2/10). In this study, we also tried to detect a humoral response to HCV core antigen. We found that tupaia sera were HCV positive for antibodies only at occasional time points, observable as intermittent steep responses (data not shown). Overall, sustained seroconversion was not seen in this study, probably because HCV propagation in vivo was so limited or well controlled by host immunity. Given that models of HCV propagation are severely limited, the most important and interesting finding of this study is the successful detection of HCV RNA in livers of infected tupaias 3 years after inoculation, indicating that HCV persists in tupaias. Although the limited propagation of HCV in tupaias is a drawback of this model at the present time, the isolation of tupaia-adapted HCV may be feasible by performing multiple infection passages. This possibility is supported by both quasispecies development and successful reinfection.

The chimpanzee is the animal species most closely related to humans, and as a model, it has contributed significantly to our understanding of HCV infection and pathogenesis. However, reproducing HCV pathogenesis in humans or chimpanzees can take as long as 10 to 20 years. The chronically infected tupaias in the present study developed complicated liver disorders in a much shorter time. Using tupaias, with their relatively short life span (3 to 5 years in the laboratory), as a model of HCV infection, we can evaluate HCV pathogenesis and correlate senescence and duration of infection.

The recent development of a primary human hepatocyte xenograft-uPA/SCID mouse model opened up opportunities to test putative antivirals against HCV replication in vivo (10, 17). In this innovative model, human hepatocytes, which are transplanted into the lobe of a mouse liver, can support HCV replication effectively. As a result, the level of circulating HCV RNA is comparable to that of a human patient. However, this mouse model is immunodeficient, and thus, it lacks the interplay between host immunity and viral infection. Therefore, it does not provide a suitable platform for characterizing immune responses to HCV infection.

HCV infection in tupaias represents an important model of HCV infection, particularly for the study of key determinants controlling virus propagation in vivo. The pathogenesis of HCV infection can be substantially different among humans, chimpanzees, and tupaias, and the mechanisms governing these differences are of great interest. Comparative studies of HCV infection in these different species will help us to understand the basic mechanisms of persistent infection.

ACKNOWLEDGMENTS

We thank Masahiro Shuda for helpful assistance and Etsuko Endo for creating the figures. We also thank the staffs of the Departments of Microbiology and Cell Biology and Mitsugu Takahashi for breeding the tupaias.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency of Japan; and the Ministry of Health, Labor and Welfare of Japan.

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DEVELOPMENTAL DYNAMICS 238:265–276, 2009

SPECIAL ISSUE REVIEWS-A PEER REVIEWED FORUM

Pleiotropic Function of FGF-4: Its Role in Development and Stem Cells

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Fibroblast growth factors (FGFs) were initially recognized as fibroblast-specific growth factor, and it is now apparent that these growth factors regulate multiple biological functions. The diversity of FGFs function is paralleled by the emerging diversity of interactions between FGF ligands and their receptors. FGF-4 is a member of the FGF superfamily and is a mitogen exhibiting strong action on numerous different cell types. It plays a role in various stages of development and morphogenesis, as well as in a variety of biological processes. Recent studies reveal the molecular mechanisms of FGF-4 gene regulation in mammalian cells, which is involved in the developmental process. Furthermore, FGF-4 also acts on the regulation of proliferation and differentiation in embryonic stem cells and tissue stem cells. In this review, we focus on the diverse biological functions of FGF-4 in the developmental process and also discuss its putative roles in stem cell biology. ***Developmental Dynamics 238:265–276, 2009.*** **© 2008 Wiley-Liss, Inc.**

Key words: FGF-4; embryogenesis; stem cell

Accepted 5 July 2008

INTRODUCTION

The fibroblast growth factor-4 (FGF-4) gene was identified as *HST-1* gene from human stomach cancers and Kaposi's sarcoma by a NIH3T3 transforming assay (Sakamoto et al., 1986; Delli Bovi et al., 1987). FGF-4 is also known as kFGF and HBGF-4. The deduced amino acid sequence of the FGF-4 is 43%, 38%, and 40%, identical, respectively, to human fibroblast growth factor-2 (FGF-2), human fibroblast growth factor-1 (FGF-1), and mouse fibroblast growth factor-3 (FGF-3) protein. The *FGF-4*, *FGF-3*, and *FGF-19* genes are located on human chromosome region 11q13 as clustered FGFs (Fig. 1) (Wada et al., 1988; Huebner et al., 1988; Katoh, 2002; Itoh and Ornitz, 2008). FGF-4 has a classic signal peptide sequence, whereas FGF-1 and FGF-2 have neither this sequence nor internal hydrophobic domains. This indicates that, unlike FGF-1 and FGF-2, the FGF-4 protein is cleaved after a signal peptide, glycosylated, and efficiently secreted as a mature protein of 176 amino acids excluding the 30 amino acid residue of a signal peptide (Fig. 1) (Yoshida et al., 1987; Taira et al., 1987). Inhibition of glycosylation impaired secretion, and the stability of the secreted FGF-4 were greatly enhanced by the presence of heparin in a cultured medium (Delli-Bovi et al., 1988; Miyagawa et al., 1988). However, unglycosylated FGF-4 was cleaved into two NH2-terminally truncated peptides of 13 and 15 kDa, which appeared to be more biologically active than the wild-type protein (Fuller-Pace et al., 1991; Bellosta et al., 1993). These two proteins also showed higher heparin binding affinity than that of wild-type FGF-4. Although it is not obvious whether cleavage of FGF-4 to generate these two proteins occurs in vivo, this could represent a novel mechanism of modulation of FGF-4 mediated biological activity.

FGFs constitute a large family of multifunctional, heparin-binding proteins that show diverse patterns of interaction with a family of receptors (FGFR-1 to -4) that are subject to alternative splicing. FGFR binding specificity is a vital mechanism in the regulation of FGF signaling and is achieved through usage of two alternative exons, IIIc and IIIb, for the second half of immunoglobulin-like domain 3 (D3) in FGFRs. While FGF-4 binds and activates the IIIc splice forms of FGFR1 to -3 at comparable

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DOI 10.1002/dvdy.21699
Published online 12 September 2008 in Wiley InterScience (www.interscience.wiley.com).

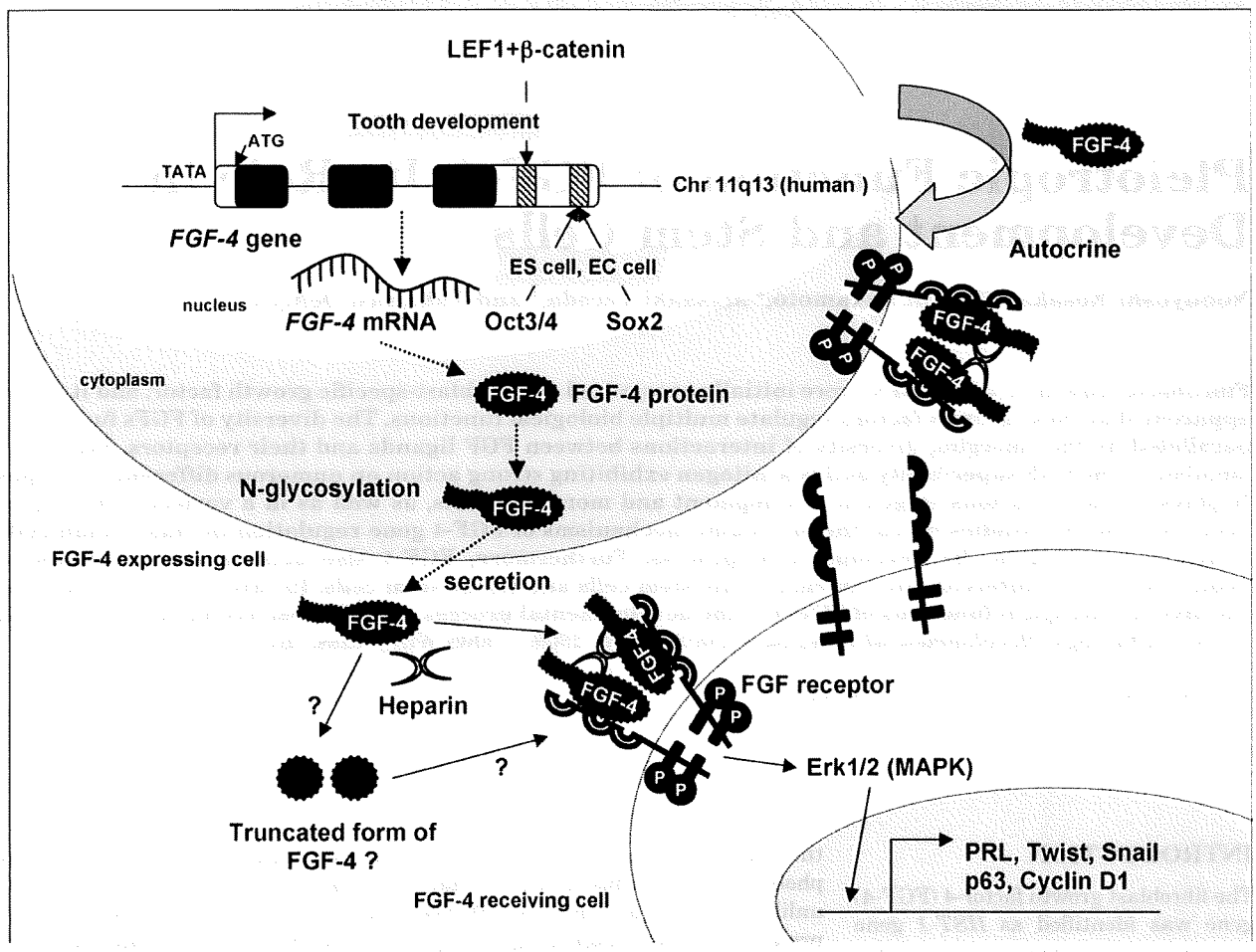

Fig. 1. Regulation and action of fibroblast growth factor-4 (FGF-4). The *FGF-4* gene is encoded by three exons (solid box). The distal enhancer is located in the untranslated region of the third exon (diagonal stripe). Initial binding of SOX-2 and Oct-3/4 to their cognate sequences in the FGF-4 distal enhancer enhances the transcription of *FGF-4* gene in embryonal carcinoma cells (EC cells) or embryonic stem cells (ES cells; Lamb and Rizzino, 1998). Intraepithelial Wnt10a and/or Wnt10b signals mediate, through LEF1 and β-catenin, the transcriptional activation of the *Fgf-4* gene in the epithelium (Kratochwil et al., 2002). FGF-4 protein is glycosylated, cleaved after a signal peptide (Yoshida et al., 1987; Taira et al., 1987). Stability of the secreted FGF-4 was enhanced by the presence of heparin (Delli-Bovi et al., 1988; Miyagawa et al., 1988). However, the unglycosylated FGF-4 was immediately cleaved into two NH2-terminally truncated peptides of 13 and 15 kDa, which appeared to be more biologically active than the wild-type protein (Fuller-Pace et al., 1991; Bellosta et al., 1993). These two proteins also showed higher heparin binding affinity than that of wild-type FGF-4. FGF-4 binds the IIIc splice forms of FGFR-1 to -3 (Ornitz et al., 1996). FGF-4 induced the phosphorylation of Erk1/2 not only in ES cell (Kunath et al., 2007), TS cell (Yang et al., 2006) and mesenchymal cells (Bobick et al., 2007) during embryogenesis, but also in Sertoli cells and germ cells (Hirai et al., 2004). FGF-4 induces the expression of prolactin (PRL) in primary rat pituitary cell (Shimon et al., 1996), and of Twist and Snail in the mesenchyme (Rice et al., 2005).

levels, it shows little activity toward the IIIb splice forms of FGFR-1 to -3 (Fig. 1; Ornitz et al., 1996; Itoh and Ornitz, 2004).

The *FGF-4* gene was originally discovered by assaying human tumor DNA for dominantly transforming oncogenes. Several groups reported the expression of FGF-4 in testicular germ cell tumors. Yoshida et al. reported the expression of FGF-4 in a human teratoma cell line and in five of nine surgically resected human testicular germ cell tumors including seminomas and embryonal carcinomas (Yoshida et al., 1988a). Strohmeyer et al. showed 70 testicular germ cell tumors analyzed at the DNA and RNA levels for the *c-kit*, *FGF-4*, and *FGF-3* oncogenes. There are significant differences in oncogene expression between seminomas and nonseminomas, with *c-kit* being expressed in 24 of 30 (80%) seminomas but in only 3 of 40 (7%) nonseminomatous tumors, and *FGF-4* being expressed in 24 of 38 (63%) nonseminomas but only 1 of 24 (4%) of seminomas, demonstrating an inverse relationship in the expression pattern of these 2 oncogenes in human testicular germ cell tumors. A significant association between tumor stage and *FGF-4* expression in the nonseminoma group was found. No gross alterations in the *c-kit*, *FGF-4*, and *FGF-3* loci were found in the DNA (Strohmeyer et al., 1991). Another group reported that the human teratocarcinoma cell line Tera 2 could be induced to differentiate in vitro after exposure to retinoic acid (Schofield et al., 1991). While the *FGF-4* oncogene is expressed in undifferentiated cells, the addition of retinoic acid rapidly downregulates the expression of this gene.

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Furthermore, FGF-4 is expressed in approximately one-third of primary human germ cell tumors. Through an immunohistochemical study, FGF-8, FGF-4, and FGFR1 are found to be expressed predominantly in nonseminomatous and highly proliferative testicular germ cell tumors (Suzuki et al., 2001). These observations indicate that FGF-4 is a potential target for the treatment of human testicular tumors. Minakuchi et al. reported that human FGF-4 small interfering RNA (siRNA) inhibited the cell growth of a human testicular tumor cell line (Minakuchi et al., 2004), which showed high levels of *FGF-4* mRNA expression in vitro and in vivo (Yoshida et al., 1988a). Data from this report show that FGF-4 siRNA transfer might be a significant novel method for inhibition of tumor growth in vivo.

Several reports showed the genomic amplification of *FGF-4* and *FGF-3* in a tumor. Kiuru-Kuhlefelt et al. reported that the DNA copy number and the expression of *FGF-4* were up-regulated in Kaposi's sarcoma (Kiuru-Kuhlefelt et al., 2000). Tsuda et al. showed that coamplification of the *FGF-4* and *FGF-3* genes was observed in esophageal carcinomas, primary tumor tissues, and metastatic tumors (Tsuda et al., 1989). The coamplification of the *FGF-4* and *FGF-3* genes had a tendency to correlate with the clinical stage. Theillet et al. reported that 1 of 13 melanomas (8%), 3 of 43 bladder tumors (7%), and 41 of 238 breast carcinomas (17%) contained amplified *FGF-4* and *FGF-3* (Theillet et al., 1989). Amplification of the chromosomal locus of the *FGF-4* and *FGF-3* genes might participate in carcinogenesis, in progression, and particularly in the metastasis of carcinomas. However, the role of FGF-4 in oncogenesis and metastasis still needs further investigation.

FUNCTION AND REGULATION OF FGF-4 IN EMBRYOGENESIS

Fgf-4 is expressed in preimplantation mouse blastocysts and is present in inner cell mass (ICM; Niswander and Martin, 1992; Rappolee et al., 1994). Mouse embryos expressed *Fgf-4* mRNA from the 1-cell stage. And *Fgf-4* mRNA is found as a maternal transcript, and is expressed at the blastocyst stage. In 1995, Feldman et al. showed an essential role of Fgf-4 in embryogenesis (Feldman et al., 1995). Inactivation of the *Fgf-4* gene in mice results in embryonic lethality after implantation. In contrast, proliferation of the ICM is rescued by addition of FGF-4 protein in *Fgf-4* null embryos. Drucker and Goldfarb (1993) also showed that the expression of murine *Fgf-4* was detected in primitive streak (E7.5–E8.5), paraxial presomitic mesoderm in the trunk, primitive neuroectoderm, pharyngeal pouch endoderm, branchial arch ectoderm, limb apical ectoderm, and skeletal myoblast groups. During the early stages of gastrulation, expression becomes restricted to the primitive streak (Niswander and Martin, 1992). Then the expression of *Fgf-4* is detected in the tail bud. Furthermore, *Fgf-4* mRNA is detected after the three primary germ layers are established and organogenesis begins. Of interest, FGF-4 can induce mesoderm formation in isolated *Xenopus laevis* animal pole explants and stimulate DNA synthesis in mammalian fibroblasts (Paterno et al., 1989). Embryonic FGF (eFGF), which is the amphibian orthologue of FGF-4, has mesoderm-inducing activity (Isaacs et al., 1992). *eFGF* mRNA is expressed maternally and zygotically with a peak during the gastrula stage. The zygotic expression is restricted in the posterior of the body axis and later in the tail bud. These results suggest that the FGF-4 has multiple roles during vertebrate embryogenesis.

Fgf-4 mRNA was detected in the Days 11 and 12 embryo, where it is localized to the apical ectodermal ridge (AER) of the limb bud (Suzuki et al., 1992). This structure is well known for its role in promoting distal outgrowth of the developing limb bud. The expression of *Fgf-4* mRNA is detected in both fore- and hindlimbs. FGF-4 stimulates proliferation of cells in the distal mesenchyme and maintains a signal from the posterior to the distal (Niswander et al., 1993; Niswander and Martin, 1993). Ochiya et al. established an organ culture system to allow mouse limb bud at 9.5–10 days postcoitus (dpc) embryo to differentiate into a limb at 12.5 dpc embryo (Ochiya et al., 1995). Exposure of embryonal limb bud explants to antisense oligodeoxynucleotides of *Fgf-4* blocks limb development. These results suggest that Fgf-4 plays the major function of the AER. However, Moon et al. showed *Fgf-4* conditional mutants have normal forelimbs (Moon et al., 2000). Furthermore, *Fgf-4* is not required for normal limb development or *Shh* expression in the zone of polarizing activity. Although *Fgf-4* expression is increased in the AER of *Fgf-8* conditional mutant forelimbs, *Shh* expression is decreased at E11.5 in *Fgf-8* conditional mutants (Moon and Capecchi, 2000). Inactivation of Fgf-8 in early limb ectoderm causes a reduction in limb-bud size, a delay in Shh expression, and misregulation of Fgf-4 expression (Lewandoski et al., 2000). Moreover, activation of *Fgf-4* in an *Fgf-8*-null limb bud causes polysyndactyly, but it rescues all the skeletal defects that result from loss of Fgf-8 function (Lu et al., 2006). These results indicate that FGF-4 and FGF-8 coordinately contribute to limb development. However, Fgf-8, rather than Fgf-4, is required to maintain *Shh* expression in the AER. Sun et al. suggest the hypothesis that there is a positive feedback mechanism between SHH from the ZPA and FGF-4 from the AER (Sun et al., 2000).

In mouse development, FGF-4 inhibits apoptosis in the dental mesenchyme when applied locally (Vaahtokari et al., 1996). Lymphoid enhancer factor (LEF1), a nuclear mediator of Wnt signaling, is a critical epithelial survival factor during tooth morphogenesis. The *Fgf-4* gene acts as a direct transcriptional target for LEF1 and shows that FGF-4 can rescue the developmental arrest of Lef1 (−/−) tooth germs (Fig. 1; Kratochwil et al., 2002). Taken together, these data indicate that FGF-4 can account for the function of LEF1 in tooth development, allowing for a communication between epithelium and mesenchyme.

FGF-2, FGF-1, and FGF-4 are exclusively detected in the endoderm at stage 5 and later in the myocardium of chick embryo, appearing as punctate cytoplasmic deposits (Zhu et al., 1996). Expression of all FGFs peaks at stages 18–24, decreasing thereafter in parallel with reduced myocardial cell proliferation. FGF-4 supports the proliferation and differentiation of precardiac myoblasts in vitro. Sugi et al.

reported that FGF-4 was expressed in cushion mesenchymal cells in the chick (Sugi et al., 2003). Addition of FGF-4 induces proliferation of cushion mesenchymal in vitro and in vivo. These findings support the hypothesis that FGF-4 is involved in the regulation of early heart development through paracrine and autocrine mechanisms.

FGF-4-mediated signaling is needed for establishing gut tube domains in chick embryos (Dessimoz et al., 2006). It is expressed in gastrulation and somite stage embryos in the close of the posterior endoderm. Exposure of the endoderm to FGF-4 at the gastrula stage reveals that it promotes a posterior gut cell fate and represses anterior endoderm cell fate. Additionally, FGF-4 represses the anterior endoderm markers and inhibits foregut morphogenesis. Disruption of FGF signaling demonstrates that FGF signaling is necessary for establishing midgut gene expression.

Fraidenraich et al. showed that a 3′-untranslated region enhancer region of *Fgf-4* gene is a target for the myogenic bHLH transcription factors MYF5 and MYOD in the myotomes and AER (Fraidenraich et al., 2000). Furthermore, Iwahori et al. found a minimal *Fgf-4* enhancer, which is a binding site for the GATA family of transcription factors in the myotomes (Iwahori et al., 2004). Fisher et al. showed regulation of the myogenic regulatory factor XmyoD in the skeletal muscle lineage of *Xenopus laevis*. The signalling molecule eFGF can directly induce the expression of XmyoD in myogenic cells (Fisher et al., 2002).

Epithelial–mesenchymal transitions (EMT) are important for morphogenesis during embryonic development (Thiery, 2002). FGFR1 promotes the EMT and morphogenesis of mesoderm at the primitive streak by controlling Snail and E-cadherin expression (Ciruna and Rossant, 2001). Snail and Twist are key inducers of EMT that represses E-cadherin expression (Batlle et al., 2000; Cano et al., 2000). Furthermore, Twist and Snail contribute to metastasis by promoting an EMT (Cano et al., 2000; Yang et al., 2004). During vertebrate gastrulation, EMT is necessary for migration of mesoderm from the primitive streak. To regulate mesoderm migration, Fgf signaling is required for expression of Snail (Zohn et al., 2006). Exogenous FGF-4 in tooth and FGF-2 in palate induce the expression of Twist and Snail in isolated mesenchymal explants (Rice et al., 2005). Moreover, in the Twist (−/−) forelimb bud, *Fgf-4* is not expressed (O'Rourke et al., 2002). β-Catenin has been shown to activate LEF-1 transcription during EMT induced in vitro by c-Fos. LEF-1 can induce EMT directly when its transcription activity is activated by stable nuclear β-catenin (Kim et al., 2002). These data suggest novel insights into the molecular basis and requirement of FGF-4 in the process of EMT.

Recently, Sweetman et al. found a restricted expression of microRNAs (miRNAs) in developing somites, in particular the developing myotome (Sweetman et al., 2006). The miRNAs are recently discovered short, noncoding RNAs, which regulate gene expression in metazoans. FGF-4–mediated signaling negatively regulates the initiation of miRNA expression during somite development. From this view, miRNAs is a potential regulator of the FGF-4 mediated developmental mechanism. As stated above, FGF-4 has a multiple function in vertebrate development. This newly discovered molecular mechanism involving miRNAs spotlights the complicated dynamics in development. The importance of FGF-4 in embryogenesis has been shown in many organisms. Further investigations to clarify the complex molecular mechanisms for FGF-4 will provide the answer for its function in stem cell and oncogenesis.

ROLE OF FGF-4 IN EMBRYONIC STEM CELLS AND TROPHOBLAST STEM CELLS

Expression of FGF-4 is restricted to undifferentiated embryonic stem (ES) cells and embryonal carcinoma (EC) cell lines such as F9 and P19, but not in differentiated cells (Yoshida et al., 1988b; Velcich et al. 1989; Hebert et al., 1990; Schoorlemmer and Kruijer, 1991). Stem cell specific expression of FGF-4 is controlled by a distally localized enhancer. This enhancer contains a consensus octamer binding site that controls positive regulation in EC and ES cells. The Sox2/Oct-3/4 complex, which is vital for normal pluripotent cell development and maintenance, can bind on *FGF-4* enhancer DNA sequences and promote transcriptional activation of FGF-4 (Fig. 2; Yuan et al., 1995). Oct-3/4 mRNA is expressed in both human and mouse oocytes and blastocysts (Schöler et al., 1989; Curatola and Basilico, 1990; Schöler, 1991). In the preimplantation stages of mouse development, expression patterns of Oct-3/4 are consistent with expression patterns of *Fgf-4* (Schöler, 1991; Niswander and Martin, 1992). However, in postimplantation mouse embryos, *Fgf-4* and *Oct-3/4* are expressed in distinct regions as well as in overlapping regions (Rosner et al., 1990; Niswander and Martin, 1992). This suggests that Oct-3/4 is an important regulator of Fgf-4 expression in preimplantation mammalian embryos. The *Fgf-4* null ES cells do not require Fgf-4 to proliferate in vitro, and addition of FGF-4 has little effect on their growth (Wilder et al., 1997). Furthermore, Fgf-4 null ES cells can differentiate in vitro after addition of retinoic acid. However, the survival of the differentiated ES cells is impaired. Importantly, the addition of FGF-4 to the culture medium increases the number of differentiated cells derived from the *Fgf-4* null ES cells, especially cells with many of the properties of parietal extraembryonic endoderm. Certain lineages formed in vitro are affected by the inactivation of the *Fgf-4* gene, in particular specific cells that form during the initial stage of ES cell differentiation. Kunath et al. reported that the function of FGF-4 in mouse undifferentiated ES cell was the activation of the Erk1/2 signaling cascade (Kunath et al., 2007). Inhibition of FGF or Erk activity does not disturb the expansion of undifferentiated ES cells. Instead, such treatments restrict the ability of ES cells to commit to differentiation. Disruption of Fgf-4 signaling impairs neural and mesodermal induction in ES cell. Moreover, Erk2-null ES cells fail to differentiate into either neural or mesodermal lineage, and maintain expression of pluripotency markers Oct-3/4, Nanog and Rex1. These findings indicate that FGF-4-Erk1/2 is important for neural and mesodermal commitment in ES cells. Recently, Ying et al. reported that blockage of FGF-4-ERK signaling by small molecule in-

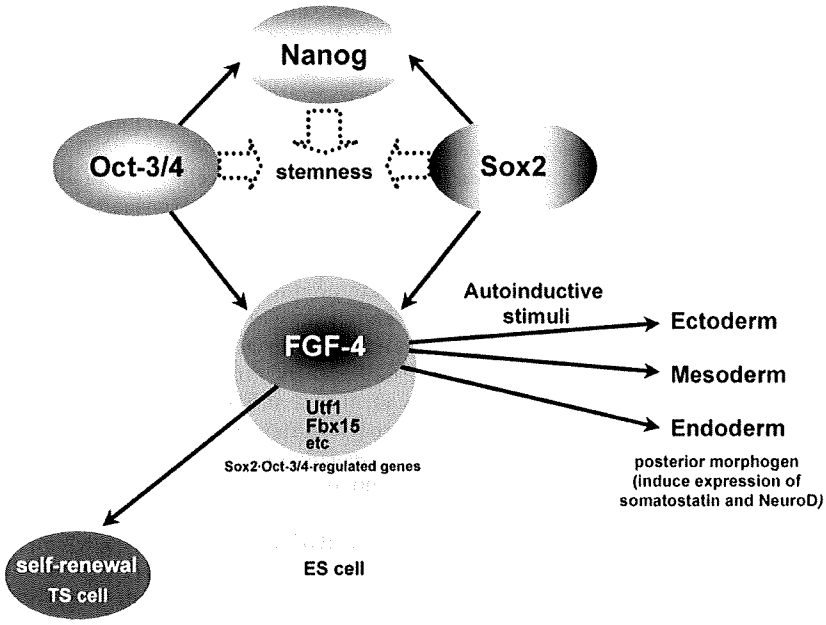

Fig. 2. Regulation and function of fibroblast growth factor-4 (FGF-4) in ES cell. The POU family transcription factor Oct-3/4 is a pivotal regulator of pluripotency in embryonic stem (ES) cells (Nichols et al., 1998). Sox2 is known to co-operate with Oct-3/4 in activating Oct-3/4 target genes (Yuan et al., 1995). ES-specific enhancers that contain binding sites for Oct-3/4 and Sox2 have been identified in several genes, including Fgf-4, Utf1 (Nishimoto et al., 1999), Fbx15 (Tokuzawa et al., 2003), and Nanog (Kuroda et al., 2005; Rodda et al., 2005). Nanog have been identified as transcription factors essential for maintaining pluripotency of ES cells in mice (Niwa, 2007). Absence of Fgf-4, progression of ES cells to either neural or mesodermal lineage commitment is arrested (Kunath et al., 2007). FGF-4 is expressed by the primitive streak and induces posterior endoderm markers (Wells and Melton, 2000). Trophoblast stem (TS) cell lines can be derived from both blastocysts and E6.5 extraembryonic ectoderm by culturing in the presence of FGF-4 (Tanaka et al., 1998). By removing FGF-4 and embryonic fibroblast conditioned medium, TS cells differentiate into various trophoblast subtypes in culture.

hibitors can maintain ES cell self-renewal (Ying et al., 2008). They used two major inhibitors for FGF receptor tyrosine kinases (SU5402) and ERK cascade (PD184352). By using a low dose of these two small-molecule inhibitors together, undifferentiated ES cell expand through multiple passages. Mayshar et al. reported that microarray analysis identified FGF-4 as a candidate for autocrine signaling in human embryonic stem (hES) cells (Mayshar et al., 2008). They indicated that FGF-4 was produced by multiple undifferentiated hES cells lines. Interestingly, undifferentiated hES cells lines also produce a FGF-4 splice isoform (FGF-4si) that codes for the amino-terminal half of FGF-4. Although FGF-4 supports the undifferentiated growth of hES cells, FGF-4si counteracts its effect by disrupting FGF-4-induced Erk1/2 phosphorylation. Expression of *FGF-4* and *FGF-4si* is detected in hES cells and early differentiated cells. Although the expression of *FGF-4* terminates in mature differentiated cells, mature differentiated hES cells maintain the expression of FGF-4si. Taken together, these reports suggest that FGF-4 is an autoinductive signal that stimulates differentiation of ES cells. Although the existence of *FGF-4si* in mouse ES cell is still unknown, it is important to show *FGF-4si* function there. Hence, it appears that if the expression profiles of *FGF-4si* are different between human and mouse ES cells, they may reflect the different characters of these cells.

Before implantation in the uterus, mammalian embryos produce trophoblast stem cells that are maintained in the extraembryonic ectoderm to develop the fetal region of the placenta. Oct-3/4 regulates autocrine growth factor signaling in ES cell precursors of trophectoderm (Nichols et al., 1998). Oct-3/4 autonomously induces expression of Fgf-4, which helps block differentiation of trophectoderm from stem cells. As shown before, the activity of Oct-3/4 is essential for the identity of the pluripotential cell population in the mammalian embryo. A culture of mouse blastocysts or early postimplantation trophoblasts in the presence of FGF-4 enables the isolation of trophoblast stem (TS) cell lines (Fig. 2; Tanaka et al., 1998). Furthermore, FGF-4 and TGF-β maintain long-term continuous TS cell proliferation (Erlebacher et al., 2004). Constitutive FGF-4 signaling in TS cells inhibits the ability of TGF-β to block c-myc expression. Furthermore, TGF-β-related protein Nodal induces FGF-4 expression in epiblast. Then Nodal and FGF-4 act directly on extraembryonic ectoderm to inhibit differentiation of trophoblast stem cells (Guzman-Ayala et al., 2004). Yang et al. reported that Shp2 was required for FGF-4-induced activation of the Src/Ras/Erk pathway (Yang et al., 2006). Depletion of the pro-apoptotic protein Bim blocks apoptosis and significantly restores Shp2 null TS cell proliferation. These results indicate that TS cells survive by FGF-4 through the Shp2/Src/Ras/Erk pathway.

Taken together, FGF-4 acts to maintain the pluripotency of ES cells and promote self-renewal of TS cells.

ROLE OF FGF-4 IN ADULT TISSUE STEM CELL

Yamamoto et al. performed a highly sensitive RT-PCR analysis to elucidate the expression of the *Fgf-4* gene in adult mice tissues (Yamamoto et al., 2000). *Fgf-4* gene expression is predominantly detected in the nervous system, intestines, and testis of normal adult mice, and is weakly recognized in other tissues such as the spleen, bone marrow, kidney, lung, eyeball, and tongue. In situ hybridization revealed cell type-specific *Fgf-4* gene expression: Purkinje cells in the cerebellum and Sertoli cells in the testis.

In our current experiments, we show that FGF-4 stimulates neural progenitor cell proliferation and induces neuronal differentiation in neurospheres, which are heterogeneous and composed of a mixed population of progenitors and stem cells (Kosaka et al., 2006). In situ hybridization re-

veals that the expression of *Fgf-4* mRNA is highly restricted in the subventricular zone, rostral migratory stream, and subgranular region of dentate gyrus, regions where adult neurogenesis is continuously occurring (van Praag et al., 2002). It was previously reported that FGF-4 acted as a mitogen for neural progenitor cells isolated from the fetal and adult rat central nervous system (Ray et al., 1997). Furthermore, the addition of FGF-4 increases the number of neural precursor cells that are generated from ES cells (Ying et al., 2003). Ye et al. have shown that FGF-4, which was expressed in the primitive streak, induced 5-hydroxytryptamine neurons in the ventral midbrain (Ye et al., 1998). In this regard, Shimozaki et al. reported that up-regulation of Oct-3/4 in ES cells led to neuroectoderm formation and neuronal differentiation (Shimozaki et al., 2003). Furthermore, Sox2 is expressed in the neural tube from the early stage of its formation (Zappone et al., 2000). The expression levels of Oct-3/4 are critical for their varied functions. A less than twofold increase from the normal expression level causes ES cell differentiation into ectoderm and mesoderm, whereas a reduction to less than 50% leads to their dedifferentiation into trophectoderm (Niwa et al., 2000). Oct-3/4 and Sox2 are known to cooperate in activating the transcription of *Fgf-4*, indicating a role for FGF-4 in neuronal differentiation.

Administration of adenoviruses carrying the *Fgf-4* gene or recombinant FGF-4 protein results in an increase in the platelet count. The number of megakaryocytes in the bone marrow and spleen of the animals with FGF-4 is increased compared with the control animals (Sakamoto et al., 1994). Furthermore, FGF-4 increases the count of large megakaryocytes in bone marrow, which specifically recover platelet counts in thrombocytopenic mice (Konishi et al., 1995). An in vitro study demonstrated that FGF-4 promoted megakaryocyte maturation, inducing increases in DNA ploidy, cytoplasmic and membrane maturation, and platelet-like particle release in human megakaryocytic Dami cells (Konishi et al., 1996). Moreover, FGF-4 acts on megakaryocytic cells to induce secretion of IL-6 and TNF-α, and increases adhesion of megakaryocytic cells to human endothelial cells through very late antigen-4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1) molecules. FGF-4 stimulates the proliferation of megakaryocyte progenitors not alone but synergistically with IL-3 and with thrombopoietin. These results are also demonstrated in an in vivo study (Avecilla et al., 2004). Avecilla et al. reported that FGF-4 and SDF-1 enhanced vascular cell adhesion molecule-1 (VCAM-1)- and VLA-4-mediated localization of CXCR4-positive megakaryocyte progenitors to the vascular niche, promoting survival, maturation and platelet release in thrombocytopenic, TPO-deficient or Mpl-deficient mice. Of interest, the addition of FGF-4 to human long-term bone marrow cultures increased both the cell density of the stromal layer and the number of hematopoietic progenitor cells (Quito et al., 1996). FGF-4 supportively contributes to the development of stromal cells both from leukemic and nonleukemic marrow cells (Koh et al., 2002). These observations indicate that FGF-4 stimulates hematopoietic progenitor cell expansion through stromal cell development, although a direct effect on hematopoietic stem or progenitor cells cannot be ruled out.

The *Fgf-4* gene expresses in the testis of normal adult mice (Yamamoto et al., 2000), which suggests its possible role in spermatogenesis. Conditional transgene expression of Fgf-4 demonstrated that the specific gain of function of the *Fgf-4* gene in the testis resulted in markedly enhanced spermatogenesis (Yamamoto et al., 2002). Transgenic mice overexpressing Fgf-4 in the testis were exposed to Adriamycin, an anticancer drug causing testicular toxicity. Enhanced expression of Fgf-4 in the testis recovered the adriamycin-induced testicular damage. Furthermore, FGF-4 can act as a physiological anti-apoptotic factor for male germ cells in stimulating lactate production of Sertoli cells (Hirai et al., 2004). Apoptosis plays an important role in controlling the number of male germ cells during testicular development and spermatogenesis. Testes of adult male mice that received an adenovirus carrying human FGF-4 or a control adenovirus were exposed to mild hyperthermia, which causes germ cell apoptosis. FGF-4 significantly reduces the apoptotic death of germ cells and prevents testicular weight loss and sperm count reduction. *Fgf-4* present in mice testes is up-regulated in vivo when the testes are exposed to mild hyperthermia, and endogenous *Fgf-4* mRNA expression in Sertoli cells is also induced when the cells are exposed to mild hyperthermia in vitro. On the other hand, upon FGF-4 stimulation, lactate production from Sertoli cells was induced, which is an indispensable nutrient for germ cell survival.

Recently many reports demonstrated that FGF-4 could induce hepatocyte differentiation from ES cell or mesenchymal stem cell (Banas et al., 2007b). For instance, ES cells treated with a combination of FGF-1, FGF-4, and HGF induced an increase in hepatocytic cell numbers (Teratani et al., 2005). Multipotent adult progenitor cells (MAPCs) from bone marrow can differentiate into most mesodermal cells and neuroectodermal cells in vitro and into all embryonic lineages in vivo (Reyes et al., 2002). MAPCs not only differentiate into mesenchymal cell types, but also into endothelium, as well as cells with neuroectodermal phenotype and function. Schwartz et al. showed that MAPCs from mice, rats, or humans treated in vitro with FGF-4 and HGF not only express hepatocyte markers but also have functional characteristics consistent with hepatocyte metabolic activities (Schwartz et al., 2002). On the other hand, adipose tissue-derived mesenchymal stem cells after incubation with HGF, FGF-1, and FGF-4, specifically the CD105 positive fraction of adipose tissue-derived mesenchymal stem cells, exhibited high hepatic differentiation ability in an adherent monoculture condition (Banas et al., 2007a). Furthermore, Fgf-4, whose expression levels are undetectable in normal liver, is up-regulated in the regenerating liver after CCl_4-treatment (Teratani et al., 2005). In mouse embryogenesis, hepatocytes differentiate from the endoderm during embryonic development. Fgf-4 expressed in primitive streak-mesoderm can induce the expression of NeuroD and somatostatin in endoderm and differentiation of endoderm (Wells and Melton, 2000). Furthermore, FGF-4 induces expression of these genes in a concentration-dependent manner, implicating it as a posterior

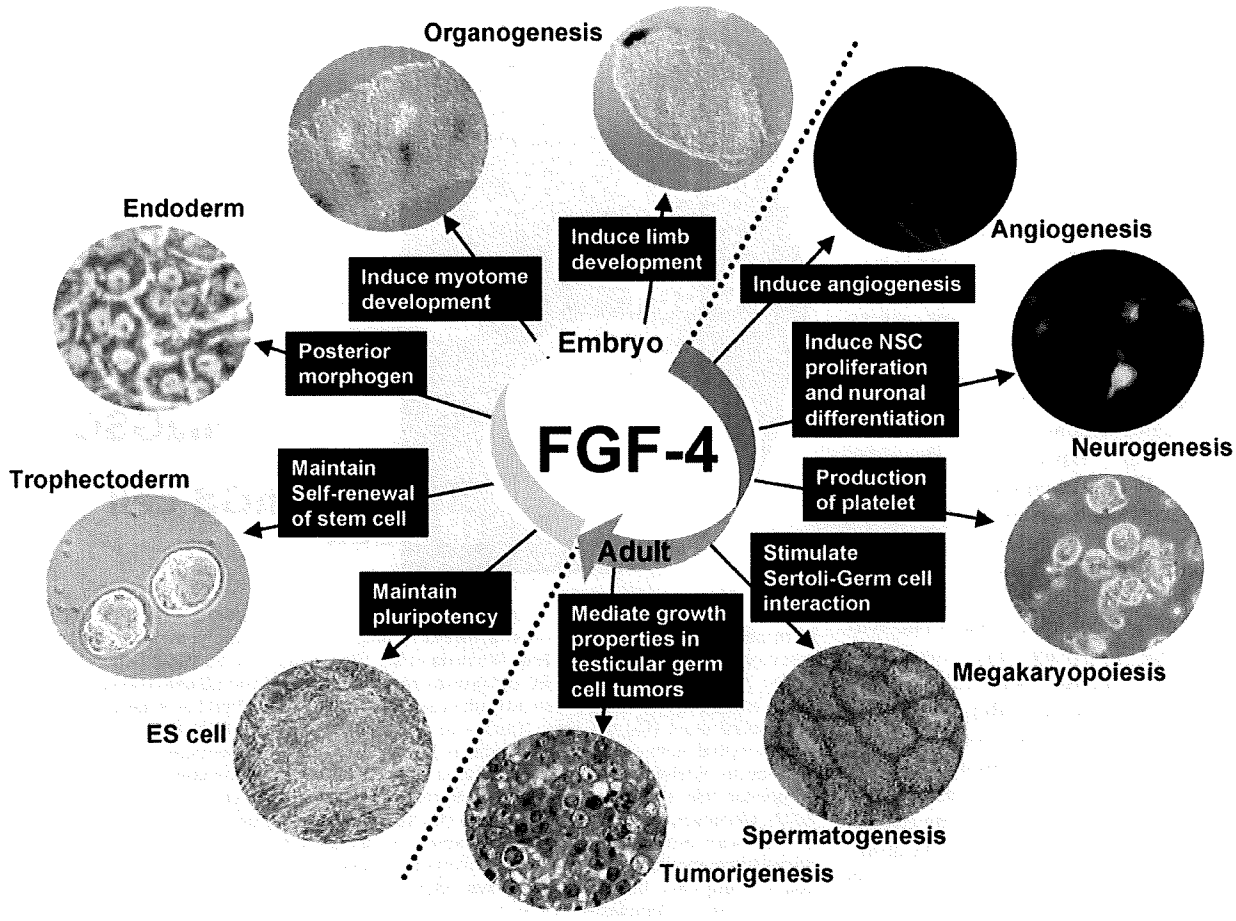

Fig. 3. Diverse functions of fibroblast growth factor-4 (FGF-4) during embryo and adult stages. FGF-4 has pleiotropic roles in many cell types and tissues; it is a mitogenic, angiogenic and survival factor, which is involved in cell proliferation and differentiation and in a variety of development processes.

morphogen. These reports indicate that FGF-4 stimulation promotes hepatocyte differentiation in ES cells, tissue-derived mesenchymal stem cells and embryogenesis. Hence, it might be interesting to study the role of FGF-4 in the differentiation of hepatic stem cells for a further understanding of the molecular mechanism of hepatogenesis.

In mouse small intestine, induction of endogenous *Fgf-4* expression was detected when mice were exposed to irradiation (Sasaki et al., 2004). Expression of *Fgf-4* is found in the epithelial cell of the villi and crypt cells. Pretreatment of FGF-4 causes an increase in the number of surviving crypt cells, and suppresses the radiation-induced apoptosis of the crypt cells. Moreover, exogenous FGF-4 enhanced epithelial cell migration and proliferation in an in vitro model. FGF-4 also has an angiogenic activity in vivo as well as in vitro (Yoshida et al., 1994). The NIH3T3 transformant transfected with the FGF-4 appeared to develop a highly vascularized tumor on nude mice. Taken together, these reports indicate that FGF-4 is a pleiotropic factor inducing many cellular functions, including angiogenesis, neurogenesis, spermatogenesis, and megakaryopoiesis (Fig. 3). However, regulation of FGF-4 and the FGF-4–evoked intracellular signaling in these cellular functions is still unclear. Verifying these mechanisms should lead to a clarification of FGF-4 controlled homeostasis in organisms.

PERSPECTIVE

Studies in the past 20 years clarified the essential role of FGF-4 as a cytokine to regulate embryogenesis. In addition, several tumors are shown to be caused by amplification of FGF-4. In this review, based on experimental observations, we have presented our views on how the FGF-4 was able to regulate stem cell fate including embryonic and somatic stem cells (Fig. 3). Finally, in this article, we wanted to emphasize the importance of FGF-4 in regulating stem cells and cancer stem cells (Fig. 4).

Most tissues and organs contain minor populations of stem cells and progenitor cells. These cells are necessary in the developing fetus for the generation of tissues and organs and later in the adult for ongoing tissue maintenance and regeneration after injury. Cancer stem cells are cancer cells that originate from the transformation of normal stem cells (Reya et al., 2001).

The most important property of a stem cell is its ability to self-renew. Both normal stem cells and cancer stem cells share various markers of "stemness."

The signaling pathways of Notch, Wnt, and Shh have recently been implicated in stem cell self-renewal. Notch activation promotes hematopoietic stem cell (HSC) self-renewal (Varnum-Finney et al., 2000; Karanu et al., 2000). The *Serrate-1* gene, which encodes transmembrane ligands to Notch receptors, is induced in dental mesenchyme by Fgf-4 during tooth development (Mitsiadis et al., 1997). RA induces *Fgf-4* expression in the ridge and FGF-4 subsequently activates *Shh* expression (Niswander et al., 1994). Moreover, Fgf-4 and Shh can mediate signaling from the ridge and posterior mesenchyme, respectively (Yang and Niswander, 1995). The Wnt signalling pathway has also been shown to regulate both self-renewal and oncogenesis in different organs. Wnts are involved in morphogenesis and patterning, and their proliferation-promoting roles are critical for stem cell maintenance and the expansion of progenitor pools (Willert et al., 2003; Hirabayashi et al., 2004). Overexpression of activated β-catenin (a downstream activator of the Wnt signalling pathway) in long-term cultures of HSCs expands the pool of HSCs. Cultured human keratinocytes with a higher proliferative potential have increased levels of β-catenin compared with keratinocytes with a lower proliferative capacity. However, the molecular mechanisms by which Wnt signalling and FGF-4 influence stem cells remain to be elucidated. For instance, the concerted action of FGFs and Wnts is believed to be important for inducing neural fate decisions (McGrew et al., 1997). Of interest, it was reported that the *Fgf-4* gene is a direct transcriptional target for LEF1, a nuclear mediator of Wnt signaling by association with its co-activator β-catenin. Loss of Lef1 results in inhibited tooth development at the late bud stage and LEF1 is required for a Wnt signaling to a cascade of FGF signaling activities to mediate the epithelial-mesenchymal interaction during tooth morphogenesis (Sasaki et al., 2005). However, exogenous FGF-4 can rescue the developmental arrest of *Lef1−/−* tooth germs (Kratochwil et al., 2002). It seems likely that FGF-4 regulates stem cells and cancer stem cells in conjunction with other molecules known to be important in this process. It will also be important to determine whether the Wnt, Notch, Shh, and FGF-4 pathways interact to regulate stem and progenitor cell self-renewal. If these signalings are dysregulated, these pathways could contribute to oncogenesis. There are a few reports that described the identification of FGF-4 induced gene expression. Guthridge et al. isolated the 21 cDNA of late-induced genes from a FGF-4 transformed NIH3T3 cell line. This study indicated that FGF-4 induced a wide range of genes including cyclin D1, HSP-90, LAMP-1 and p63 (Fig. 1; Guthridge et al., 1996). Overexpression of cyclin D1 is known to correlate with the early onset of cancer and tumor progression (Fu et al., 2004). Moreover, p63 is an essential regulator of stem-cell maintenance in stratified epithelial tissues (McKeon, 2004). Thus, these results suggested the indirect association of FGF-4 with stem cells and cancer development.

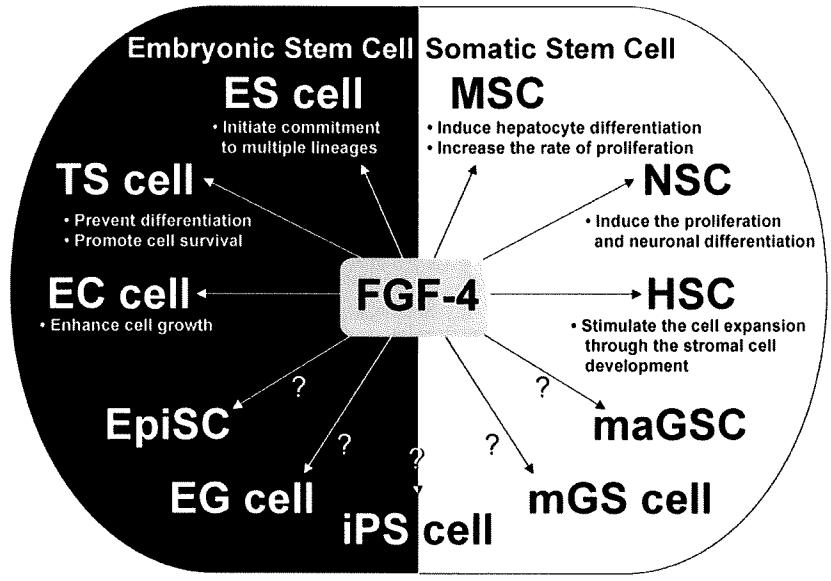

Fig. 4. Schematic description of the relationship between fibroblast growth factor-4 (FGF-4) and stem cells. Expression of *FGF-4* is restricted to undifferentiated embryonic stem (ES) cells and embryonal carcinoma (EC) cell lines (Yoshida et al., 1988; Velcich et al., 1989; Schoorlemmer and Kruijer, 1991). FGF-4 enhances EC cell growth (Maerz et al., 1998) and ES cells lacking FGF-4 resist neural and mesodermal induction (Kunath et al., 2007). FGF-4 permitted the isolation of permanent trophoblast stem (TS) cell lines (Tanaka et al., 1998). Exogenous FGF-4 increases the rate of mesenchymal stem cell (MSC) proliferation (Farré et al., 2007). Furthermore, FGF-4 could induce hepatocyte differentiation from MSC (Banas et al., 2007). FGF-4 stimulates hematopoietic stem/progenitor cells expansion through the stromal cell development (Quito et al., 1996; Koh et al., 2002). Moreover, FGF-4 stimulates neural stem/progenitor cell proliferation and induces neuronal differentiation (Kosaka et al., 2006). The expression of *FGF-4* in induced pluripotent stem (iPS) cell derived from mouse and human was confirmed (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). There have been no reports that address the expression of *FGF-4* in other stem cells, including EpiSC (epiblast-derived stem cells), EG (embryonic germ) cells, GCS, and maGSC (multipotent adult germline stem cells). However, FGF-4 gene is not expressed in mGS (multipotent germline stem) cells (Imamura et al., 2006).

Recently, pluripotent stem cells—which are called induced pluripotent stem (iPS) cells, produced from adult mouse and human fibroblasts by introducing four factors, Oct-3/4, Sox2, c-Myc, and Klf4—were established (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In iPS cells, expression of FGF-4 was confirmed as the marker for a pluripotent stem cell. However there are no reports that describe the function of FGF-4 in iPS cells. It is very interesting why these artificially induced pluripotent stem cells express FGF-4, which is the autoinductive stimuli in ES cells. There are several pluripotent stem cells that have been established other than iPS

cells. For example, FGF-2, in the presence of stem cell factor (SCF) and LIF, stimulates long-term proliferation of primordial germ cells (PGCs; Matsui et al., 1992; Resnick et al., 1992). These embryonic germ (EG) cells resemble embryonic stem cells. Moreover, two groups have reported a new stem cell line, derived from the epiblast, a tissue of the postimplantation embryo that generates the embryo (Tesar et al., 2007; Brons et al., 2007). These cells, which are referred to as EpiSCs (postimplantation epiblast-derived stem cells), express Oct-3/4, Nanog and Sox-2. Kanatsu-Shinohara et al. established ES-like cells, called mGS (multipotent germline stem), from neonatal mouse testis (Kanatsu-Shinohara et al., 2004). These ES-like cells are phenotypically similar to ES and EG cells. Furthermore, Guan et al. showed the isolation of spermatogonial stem cells (SSCs) from adult mouse testis (Guan et al., 2006). These isolated SSCs acquire embryonic stem cell properties. These cells are called multipotent adult germline stem cells (maGSCs). There is no strong evidence supporting a relationship between FGF-4 and these stem cells. However, the impact of FGF-4 on ES cell and TS cell suggests that FGF-4 has a function in regulating the stemness of these newly established stem cells.

The diverse function of FGF-4 is partly regulated by (1) specificity of ligand–receptor interactions in FGFR signaling and (2) by transcriptional factor (i.e., Oct-3/4 and Sox2). FGF signaling uses receptor tyrosine kinases that form high-affinity complexes with FGFs and heparan sulfate proteoglycans at the cell surface. Whereas FGF-2 binds heparan sulfate ubiquitously, FGF-4 exhibits a restricted pattern, failing to bind heparan sulfate in the heart and blood vessels and failing to activate signaling in embryonic day-18 mouse aortic endothelial cells. This suggests that FGF-4 seeks a specific heparan sulfate sulfation pattern, distinct from that of FGF-2, which is not expressed in most vascular tissues. This in turn suggests that FGF and FGFR recognition of specific heparan sulfate sulfation patterns is critical for the activation of FGF signaling, and that synthesis of these patterns is regulated during embryonic development (Allen et al., 2001). Urakawa et al. reported that a previously undescribed receptor conversion by Klotho, a senescence-related molecule, generates the FGF-23 receptor (Urakawa et al., 2006). They showed that the Klotho and FGFR1 (IIIc) reconstitute the FGF-23 receptor. These findings suggest the existence of a novel mechanism of interactions between FGF and FGFRs. Because mitogenic activity of FGF-4 and FGF-2 is similar, unidentified mechanisms for intracellular signaling regulate the different biological activity of FGF-4 and FGF-2. West et al. reported that neuropilin-1 (Npn-1), which is a receptor for semaphorin in the nervous system and interacts with the heparin binding isoforms of VEGF in endothelium, interacts with FGF-4 (West et al., 2005). This result raises the possibility that Npn-1 modulates FGF-4 activity. FGF-4 regulates various cell functions at a local level. In this regard, because the expression of FGF-4 is very low in a somatic organ, the gene could be regulated by transcription factors other than Oct-3/4 and Sox2.

Compelling insights into the molecular framework by which FGF-4 promotes several biological processes have been discovered, and further functional analysis based on well-defined models and more elaborate system models defining genetic responses of stem cells will contribute to the understanding of the novel function of FGF-4 in controlling the fate of embryonic and somatic stem cells.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control; Health Science Research grants for Research on the Human Genome and Gene Therapy from the Ministry of Health, Labour, and Welfare of Japan; a grant-in-aid for Scientific Research on Priority Areas (Cancer) from the Ministry of Education, Culture, Sports, Science, and Technology; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio); Japan Society for the Promotion of Science to N.K. supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

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