Liver Receptor Homolog-1 Regulates the Transcription of Steroidogenic Enzymes and Induces the Differentiation of Mesenchymal Stem Cells into Steroidogenic Cells

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Steroidogenic factor-1 (SF-1, also known as Ad4BP) has been demonstrated to be a primary transcriptional regulator of steroidogenic-related genes. However, mRNA for liver receptor homolog-1 (LRH-1), which together with SF-1, belongs to the NR5A nuclear receptor family, is expressed at much higher levels than SF-1 mRNA in the human gonad. In our previous studies, we demonstrated that SF-1 induced the differentiation of bone marrow-derived mesenchymal stem cells (MSCs) into steroidogenic cells such as Leydig or adrenocortical cells. The introduction of LRH-1 into human MSCs (hMSCs) with the aid of cAMP also induced the expression of steroidogenic enzymes, including CYP17, and their differentiation into steroid hormone-producing cells. Promoter analysis, EMSA, and chromatin immunoprecipitation assay using LRH-1-transduced hMSCs indicated that three LRH-1 binding sites were responsible for CYP17 transactivation. Immunohistochemical studies showed that LRH-1 protein was expressed in human Leydig cells. The CYP17 promoter region was highly methylated in hMSCs, whereas it was demethylated by the introduction of LRH-1 and cAMP treatment. These results indicate that LRH-1 could represent another key regulator of the steroidogenic lineage in MSCs and play a vital role in steroid hormone production in human Leydig cells. (*Endocrinology* 150: 3885–3893, 2009)

S teroidogenic factor-1 (SF-1; also known as Ad4BP) and liver receptor homolog (LRH)-1 belong to the NR5A subfamily of nuclear receptors (1). They function as monomers to regulate genes by binding to similar response elements (2–4).

SF-1 is essential for normal adrenal and gonadal development, and SF-1 knockout mice exhibit adrenal and gonadal agenesis and impaired gonadotropin expression, resulting in postnatal death due to severe adrenal insufficiency (5–8). SF-1 is expressed in the adrenal cortex, testicular Leydig and Sertoli cells, ovarian theca and granulosa cells, pituitary gonadotropes, hypothalamus, and spleen (7, 8). It regulates the cell-specific expression of a variety of different genes involved in steroidogenesis, including a number of steroid hydroxylases (2, 3). With the aid of cAMP, it can induce the differentiation of bone marrow-derived mesenchymal stem cells (MSCs) into

steroidogenic cells such as testicular Leydig cells and adrenocortical cells (9, 10).

LRH-1, however, is highly expressed in tissues of endodermal origin, such as liver and intestine (11, 12). It is essential for Oct4 (an essential gene for the maintenance of inner cell mass and pluripotency of embryonic stem cells) expression at the epiblast stage, and its disruption causes early embryonic death (13, 14). In the adult, LRH-1 functions in the control of cholesterol and bile acid homeostasis by regulating the transcription of a number of genes including CYP7A1 and CYP8A1 in coordination with two other nuclear receptors, the farnesoid X receptor and the short heterodimer partner (SHP) (15, 16), although some controversial results have recently been obtained in tissue-specific knockout mice, in which LRH-1 was not required for the expression of the Cyp7a1 gene (17, 18). LRH-1 has also been re-

ported to be involved in steroidogenesis in the gonad and intestine (19–25).

In this study, we investigated the role of some nuclear receptors in the differentiation of MSCs into steroidogenic cells. The introduction of LRH-1 and cAMP treatment resulted in the differentiation of human MSCs (hMSCs) into steroidogenic cells, as in the case of SF-1. Consistent with the fact that LRH-1 protein is expressed in human testicular Leydig cells, these cells expressed the CYP17 gene and produced testosterone. Our results demonstrate that LRH-1 could represent another key regulator to induce the differentiation of the stem cells into steroidogenic cells.

Materials and Methods

Cell culture, transfection, and luciferase assay

hMSCs (26, 27) and Phoenix cells were cultured in DMEM with 10% fetal calf serum. hMSCs and Phoenix cells were transfected using FuGENE 6.0 (Roche Molecular Biochemicals, Mannheim, Germany), Lipofectamine Plus (Invitrogen, Carlsbad, CA), and calcium coprecipitation. Luciferase assays were performed as described previously (10). Each data point represents the mean of at least four independent experiments.

Plasmids

The CYP17 promoter was amplified by PCR and cloned into the pGL3 Basic vector (Promega Corp., Madison, WI). Mutations of the SF-1/LRH-1 binding sites in the CYP17 promoter were created by PCR using the QuikChange site-directed mutagenesis kit (Stratagene La Jolla, CA). Each cDNA containing the entire coding region for SF-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1), NUR77, LRH-1, and blasticidin S deaminase was generated by PCR and subcloned into pcDNA 3 (Invitrogen), pQCXIP, and pQCXIX vectors (Takara Bio Inc., Shiga, Japan).

RT-PCR

Total RNA from the cultured cells was extracted using Trizol reagent (Invitrogen). RT-PCR was performed as described previously (9). The products were subjected to electrophoresis in a 1.5% agarose gel, and the resulting bands were visualized by staining with ethidium bromide. The primers used for PCR were as follows: human LRH-1, forward, agctagaagctgtaagggccgac, reverse, ttttagcctggacttgagcctca. The primers used for other genes have been described previously (9).

Retrovirus preparation and infection

The Phoenix packaging cells were transiently transfected with the retroviral plasmids using the Lipofectamine Plus reagent (Invitrogen) or by calcium phosphate coprecipitation. The supernatant was concentrated by centrifugation. The virus solution was stored at −80 C until use. MSCs were infected with the retrovirus in the presence of 8 μg/ml Polybrene (Sigma, St. Louis, MO) for 48 h. The cells were then replated and selected using puromycin or blasticidin S.

Immunohistochemistry

Immunohistochemistry was performed as described previously (10, 28). Human samples were purchased from Abcam PLC (Cambridge, UK). Sections were subjected to the antigen retrieval technique using HistoVT One (Nacalai Tesque, Kyoto, Japan) and treated with anti-LRH-1 (kindly donated by Dr. Luc Bèlanger, Laval University, Quèbec, Canada), anti-Ad4BP/SF-1 (kindly donated by Dr. Ken-ichirou Moro-hashi, University of Kyushu, Fukuoka, Japan), anti-CYP17 (kindly donated by Dr. Alan Conley, University of California School of Veterinary

Medicine, Davis, CA), and anti-HSD3B1 (kindly donated by Dr. J. Ian Mason, University Department of Reproductive and Developmental Sciences, Royal Infirmary of Edinburgh National Health Service Trust, Edinburgh, UK) antibodies. They were then developed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

EMSAs

EMSAs were performed as described previously (29). Nuclear extracts were prepared from LRH-1-transduced hMSCs cultured with or without cAMP. Double-stranded DNA fragments were prepared by annealing complementary oligonucleotides as described previously (30, 31). A nuclear extract (10 μg proteins) was incubated with ^{32}P-labeled oligonucleotides and unlabeled polydeoxyinosinic deoxycytidylic acid. In the competition experiments, a 200-fold molar excess of unlabeled competitor DNAs was added. A supershift assay was performed by preincubating the nuclear extracts with anti-LRH-1 antiserum (kindly provided by Dr. Luc Bèlanger, Laval University, Quèbec, Canada). After the binding reaction, the mixture was subjected to 4.5% or 6% PAGE, and the gel was then dried and autoradiographed.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using Dynabeads Protein G (VERITAS Corp., Tokyo, Japan), according to the manufacturer's instructions. Briefly, LRH-1-transduced hMSCs cultured with or without cAMP were cross-linked with 1% formaldehyde, rinsed with PBS, and resuspended in sodium dodecyl sulfate lysis buffer. Cell lysates were sonicated and immunoprecipitated with normal IgG or antibody-conjugated Dynabeads Protein G. Immunoprecipitated complexes were eluted with elution buffer. The cross-links were then reversed, and DNA fragments were purified for PCR analysis using the following primers: CYP17 proximal, forward, cttgtgaccctctgaatctgt, reverse, gcaggcaagatagacagcagtgg; CYP17 distal, forward, aacaaaggtgtgtatgagag, reverse, acacctcctacgatcaggtaaa. The products were subjected to electrophoresis in a 1.5% agarose gel, and the resulting bands were visualized by staining with ethidium bromide.

Bisulfite sequencing

Bisulfite treatment was performed using the MethylEasy DNA bisulfite modification kit (Human Genetic Signatures Pty. Ltd., Macquarie Park, Australia) according to the manufacturer's instructions. PCR primers were as follows: CYP11A1 gene, forward, tgggcccctcatttccccca, reverse, cggggactgctaggatgact; CYP17 gene, forward, gttgatggcatttgatcaa, reverse, ctggagcccagccaggcctg. Amplified products were cloned into pGEM-T Easy Vector (Promega, Bethesda, MD). Randomly selected clones were sequenced using the T7 forward and SP6 reverse primers for each gene.

Results

Differentiation of MSCs into steroidogenic cells by introduction of LRH-1

To compare the roles of the nuclear receptors reportedly involved in steroidogenesis and the development of steroidogenic organs on the induction of steroidogenic cells, hMSCs were transduced with SF-1 (6, 7), DAX-1 (32), NUR77 (33), and LRH-1 (19–25) by retrovirus-mediated transfection. Transduction of SF-1 or LRH-1 into hMSCs induced the production of progesterone at low levels (Table 1). As we reported previously (9), cAMP treatment further induced various steroidogenic enzymes including P450 side-chain cleavage (P450scc), CYP17, CYP19, and CYP11B1 (Fig. 1). Steroidogenic acute regulator (StAR) and ACTH receptor mRNAs were also induced in these

TABLE 1. Production of steroid hormones by MSCs stably expressing GFP, SF-1, and LRH-1 in the presence (+) or absence (−) of 8br-cAMP (ng/ml)

Cell (cAMP)	Progesterone	Testosterone	Estradiol	Cortisol	Aldosterone (ng/ml)
GFP-hMSC (−)	N.D.	N.D.	N.D.	N.D.	N.D.
GFP-hMSC(+)	N.D.	N.D.	N.D.	N.D.	N.D.
SF-1-hMSC (−)	27.5 ± 4.62	0.1 ± 0.11	N.D.	0.21 ± 0.08	N.D.
SF-1-hMSC (+)	887.4 ± 198.8	0.9 ± 1.29	0.21 ± 0.05	0.88 ± 0.17	0.78 ± 0.09
LRH-1-hMSC (−)	54.3 ± 16.0	0.25 ± 0.11	N.D.	0.36 ± 0.06	N.D.
LRH-1-hMSC (+)	987.0 ± 131.6	1.4 ± 1.29	0.11 ± 0.01	1.1 ± 0.22	0.81 ± 0.12

Data are means and sem values of at least triplicate assays. N.D., No detectable values.
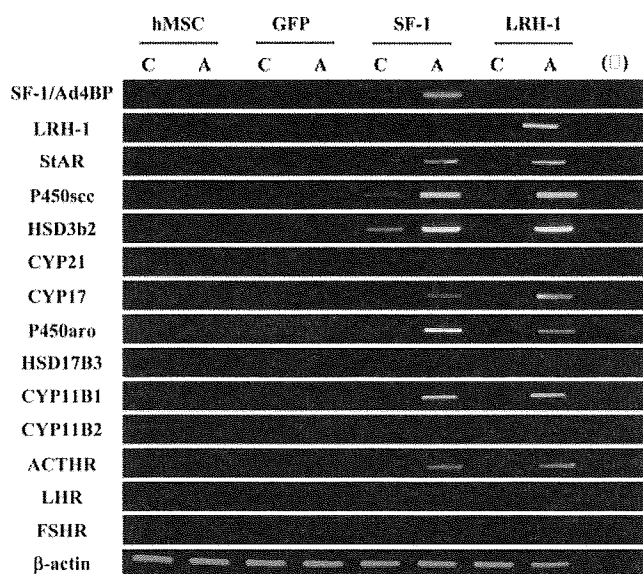
cells, and expression of the SF-1, as well as the LRH-1 gene, was increased by cAMP treatment. In addition, cAMP treatment markedly increased the production of various steroid hormones. SF-1 mRNA was never induced in LRH-1-transduced cells. In contrast, green fluorescent protein (GFP)-, DAX-1-, and NUR77-transduced hMSCs failed to express steroidogenic enzymes (Fig. 1 and supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) or produce any steroid hormones, even after cAMP treatment, as in control, untransduced cells. These results demonstrate that LRH-1, like SF-1, could act as a key regulator of the steroidogenic lineage in MSCs.

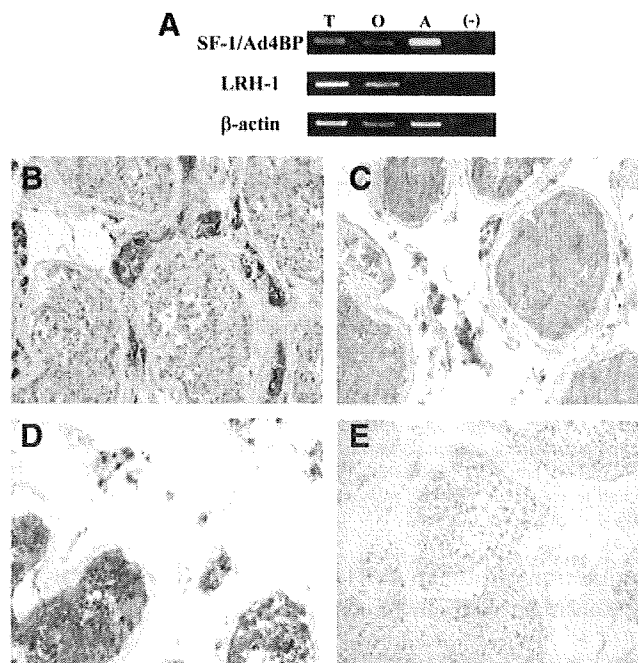## The expression of NR5A in steroidogenic organs

We investigated the expression of SF-1 and LRH-1 mRNA in human primary steroidogenic tissues using RT-PCR (Fig. 2A). In accordance with previous reports, SF-1 was abundantly expressed in both the adrenals and gonads. LRH-1 was barely detectable in the adrenals but was expressed at higher levels than SF-1 in the gonads. These results indicate that LRH-1 is important for steroidogenesis in the gonads. In the human ovary, LRH-1 has been reported to be expressed in luteal granulosa cells and involved in the production of pro-

gesterone (34). LRH-1-transduced hMSCs expressed CYP17 and produced testosterone after cAMP treatment, and it is therefore probable that LRH-1 is also involved in human testicular androgen production.

To investigate the localization of the NR5A proteins in the testis, immunohistochemical studies were performed on human testicular sections. CYP17 (Fig. 2B) and 3β-hydroxysteroid dehydrogenase type II (HSD3B2; data not shown) proteins were localized in the interstitial Leydig cells. SF-1 staining was mainly detected in the nucleus of Leydig cells (Fig. 2C). LRH-1 proteins, however, were detected in both the seminiferous tubules (spermatocytes) and the interstitial Leydig cells (Fig. 2D). These results suggest that, like SF-1, LRH-1 can also regulate androgen production in human testicular Leydig cells via transcriptional control of steroidogenic enzymes, including CYP17 and HSD3B2.

FIG. 2. The expression and localization of NR5A in human gonads and adrenal glands. A, mRNA levels of each gene were analyzed by RT-PCR. Lanes T, O, and A represent a testis, an ovary, and an adrenal, respectively. B–E, Localization of NR5A protein in human testis. Shown are positive staining for CYP17 (B), SF-1/Ad4BP in Leydig cells (C), and LRH-1 in spermatogenic cells (spermatocytes/spermatids stage) and Leydig cells (D). E, Absence of any staining in a control section incubated with nonimmune serum.

FIG. 1. Induction of steroidogenic genes and pituitary hormone receptors in hMSCs by SF-1 and LRH-1. hMSCs were transduced with GFP, SF-1, or LRH-1 by retrovirus-mediated transfection. RT-PCR analysis of each gene in each clone cultured with (A) or without (C) 8-bromoadenosine-cAMP (1 mm) for 2 d.
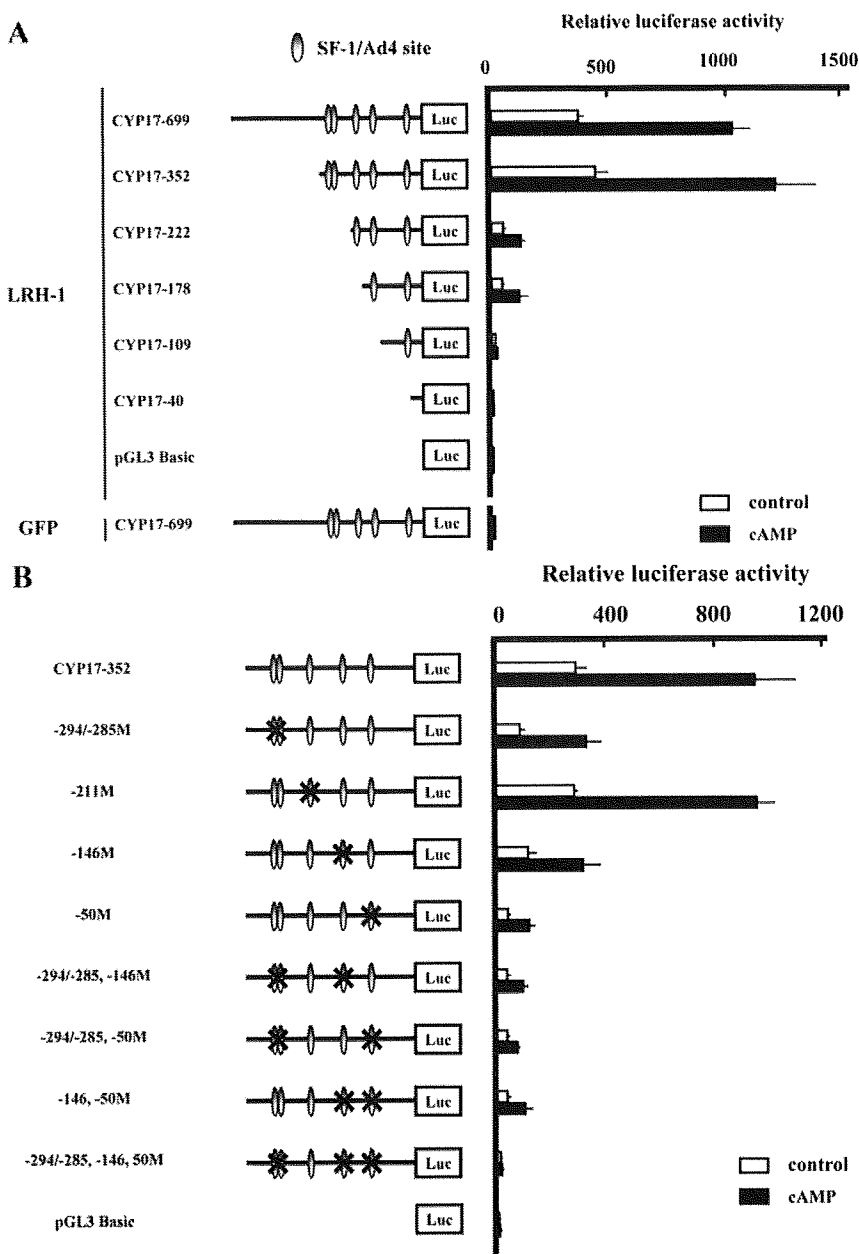
FIG. 3. Analysis of CYP17 promoter region in LRH-1-transduced hMSCs. A, 5′-deletion analysis of the human CYP17 promoter region. Progressive deletions of CYP17 promoter are schematically illustrated in the *left panel*. Each vector was transfected by lipofection into MSCs. Cells without and those treated with 1 mM cAMP were incubated for 24 h before preparing the extracts for luciferase (Luc) assays. Luciferase activities were measured and relative activities are shown. Data are the mean ± SEM values of at least four assays. B, Effects of mutation in the Ad4 site within the promoter region of the CYP17 gene. The mutant promoter constructs used are drawn schematically. Each vector was transfected by lipofection into MSCs. Cells without and those treated with 1 mM cAMP were incubated for 24 h before preparing the extracts for luciferase assays. Luciferase activities were measured and relative activities are shown. Data are the mean ± SEM values of at least four assays.

Regulation of human CYP17 gene expression by LRH-1

Consistent with our results, Volle *et al.* (20) reported that LRH-1^{+/−} mice showed lower plasma testosterone concentrations than LRH-1^{+/+} mice. However, even though the testicular mRNA expression of various steroidogenic enzymes was lower in LRH-1^{+/−} mice, there were no differences between LRH-1^{+/+} and LRH-1^{+/−} mice in their expression of CYP17. These results differed markedly from our result in hMSCs, in which CYP17 mRNA was induced in LRH-1 transduced cells (Fig. 1).

To determine whether the human CYP17 gene is a direct target of LRH-1, reporter assays and EMSAs were performed using LRH-1-transduced hMSCs. The CYP17 promoter region was transiently transfected into LRH-1-transduced hMSCs, which were then treated with cAMP for 24 h (Fig. 3A). CYP17 promoter activity was induced by about 300-fold compared with transfection of pGL3-Basic vector into LRH-1-transduced hMSCs. The promoter activity was also 100-fold higher than in GFP-infected or parental hMSCs. cAMP treatment further induced the promoter activity only in LRH-1-transduced cells. Transient transfection with an LRH-1 expression vector in parental hMSCs also induced CYP17 promoter activity in a dose-dependent manner (supplemental Fig. 2). These results indicate that the CYP17 promoter region contains LRH-1-dependent regulatory element(s).

To define the sequences required for promoter activity in LRH-1-transduced cells, deletion and mutation analyses were performed (Fig. 3B). The highest activity was shown by a construct that was truncated to nucleotide −352 (corresponding to the number of bases from the transcription start site). Promoter activity was markedly reduced by truncation of the upstream region to −221 and further to −108. Basal promoter activity was almost completely abolished by truncation to −40. cAMP responsiveness was also abolished in this construct.

Previous studies demonstrated that multiple Ad4/SF-1-binding sites were important for the activation of the human CYP17 gene (30, 31). Mutation of any one of three sites (−294/−285, −146, and −50) in the −352 fragment markedly reduced the promoter activity, whereas mutation of −211/−204 Ad4/SF-1 binding site had no effect (see Fig. 5). The results were consistent with those from deletion analysis. Constructs carrying mutations at two of the three sites showed further reductions in activity. In addition, simultaneous mutation of all three sites almost abrogated the basal and cAMP-induced responsiveness. It also abolished the promoter activation induced by transient expression of LRH-1 in parental cells (supplemental Fig. 2). Collectively, these results confirmed that three Ad4/SF-1/LRH-1 binding sites were important for controlling CYP17 gene expression in LRH-1-transduced hMSCs.

To investigate whether LRH-1 was able to bind to these sites, nuclear extracts from LRH-1-transduced hMSCs cultured with
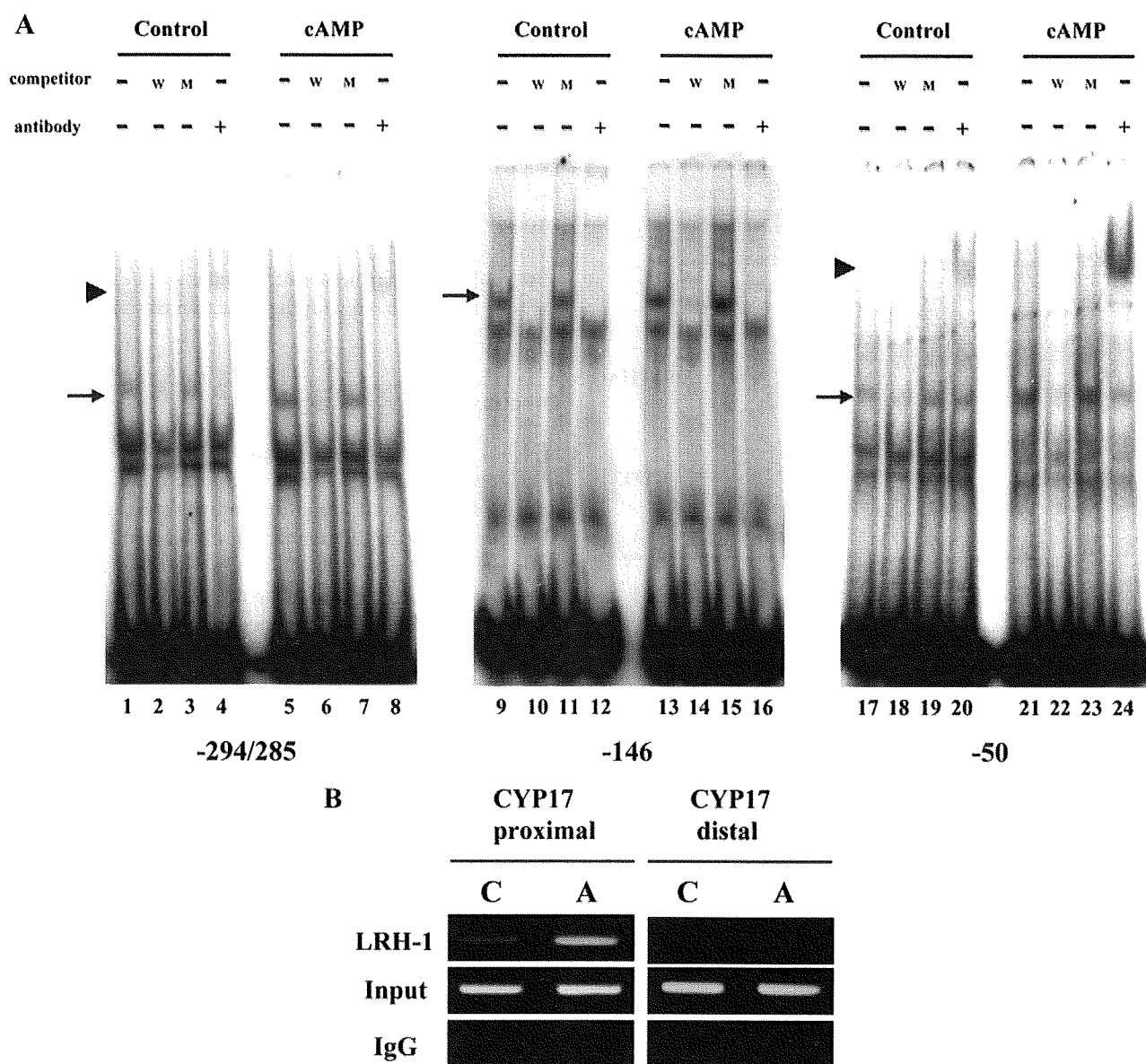
FIG. 4. A, EMSA analyses using Ad4/SF-1-binding sites of CYP17 promoter. Each end-labeled oligonucleotide was incubated with 10 μg of nuclear extracts from cAMP-treated (cAMP; lanes 5–8, 13–16, and 21–24) or cAMP-untreated (control; lanes 1–4, 9–12, and 17–20) LRH-1-transduced hMSCs and resolved by electrophoresis on a nondenaturing 4% (−294/−285 and −50) or 6% (−146) polyacrylamide gel. Wild-type (W; lanes 2, 6, 10, 14, 18, and 22) or mutant (M; lanes 3, 7, 11, 15, 19, and 23) unlabeled oligonucleotides (200-fold molar excess) were used as competitor DNAs. Where indicated (+), antiserum-specific for LRH-1 (lanes 4, 8, 12, 16, 20, 24) was included in the binding reaction. *Arrows* indicate the specific DNA-protein complexes. *Arrowheads* indicate the supershifted complexes. B, ChIP assays were carried out using immunoprecipitated chromatin with IgG or anti-LRH from cAMP-treated (A) or cAMP-untreated (C) LRH-1-transduced hMSCs. Recovered chromatin was subjected to PCR analysis using primers encompassing the LRH-1-binding sites (CYP17 proximal) or approximately 5 kb upstream of the proximal promoter region (CYP17 distal). Data shown are representative of three independent experiments.

or without cAMP were prepared for use in gel mobility shift assays. As shown in Fig. 4, a probe containing each Ad4/SF-1/LRH-1 binding site formed shifted complexes (*arrows*, lanes 1, 5, 9, 13, 17, and 21) that were competed for by each unlabeled oligonucleotide containing homologous sequences (lanes 2, 6, 10, 14, 18, and 22) but not by unlabeled nucleotides containing the mutated sequences that were used for site-directed mutagenesis (lanes 3, 7, 11, 15, 19, and 23). Preincubation of the nuclear extracts with a polyclonal antiserum against LRH-1 shifted (*arrowhead*, lanes 4, 8, 20, and 24) or abolished (lanes 12 and 16) the complex. The intensities of the complex bands were increased after cAMP treatment. ChIP analysis also revealed that the in-

duction of the CYP17 gene by LRH-1 was mediated through direct binding to its promoter region (Fig. 4B). Collectively, these results demonstrated that the CYP17 gene could be a direct target of LRH-1 in human steroidogenic cells.

DNA in the CYP17 promoter is demethylated by cAMP treatment in LRH-1-transduced hMSCs

As we previously reported in adult Leydig-like cells derived from SF-1-transfected murine MSCs (9), there was a time lag associated with the induction of steroidogenic enzymes by camp treatment in LRH-1-transduced hMSCs. P450scc and HSD3B2 mRNAs were rapidly induced within 6–12 h (Fig. 5A). This
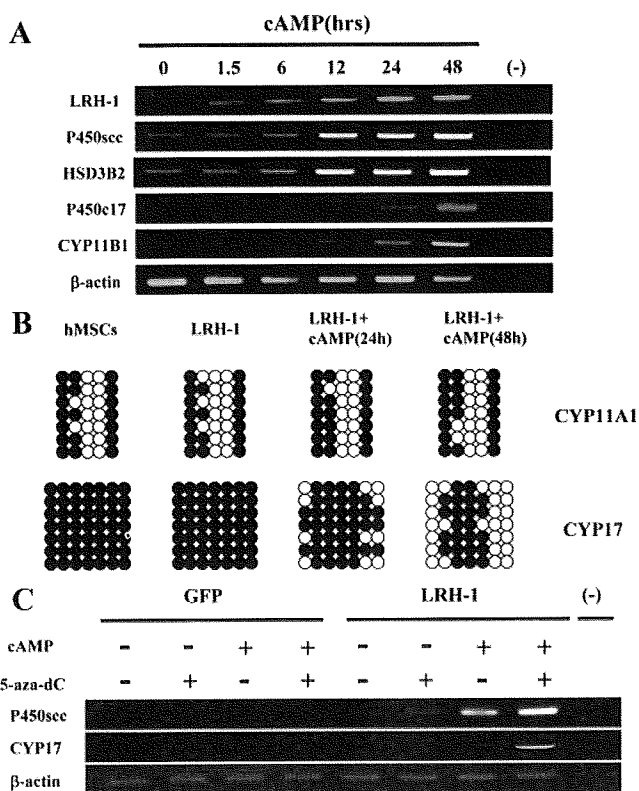
FIG. 5. A, Time-dependent induction of steroidogenic genes by cAMP in hMSCs. LRH-1-transduced hMSCs were cultured and treated with 8-bromo-cAMP (1 mM) for the indicated times. mRNA levels of each gene were analyzed by RT-PCR. B, Analyses of the promoter region of steroidogenic enzymes in nonsteroidogenic hMSCs and steroidogenic hMSCs. Bisulfite genomic sequencing of the promoter region of CYP11A1 and CYP17 in parental hMSCs and LRH-1-transduced hMSCs. LRH-1-transduced cells were treated with 8-bromoadenosine-cAMP (1 mM) for indicated times. *Open* and *closed circles* indicate unmethylated or methylated CpG, respectively. C, Effects of 5aza-dC on the expression of steroidogenic genes. Shown are RT-PCR analyses of each gene in cells cultured with or without cAMP (1 mM) for 18 h after the treatment with 5-aza-dC (25 μM) for 3 d.

pattern is very similar to that of LRH-1 mRNA induction. CYP17 and CYP11B1, however, were induced only after 24–48 h, much later than the induction of LRH-1.

In addition to transcriptional regulation by transcription factors, epigenetic modifications also contribute to the regulation of gene expression. Among them, DNA methylation at cytosine residues of the dinucleotide sequence CpG induces gene silencing and is essential for differentiation and development (35, 36). To compare the DNA methylation status of the steroidogenic genes induced at different times, bisulfite sequencing analysis was performed on the CYP11A1 (the gene encoding P450scc) and CYP17 promoter regions in hMSCs (Fig. 5B). CYP11A1 promoter regions were comparatively hypomethylated in parental hMSCs and GFP-transduced cells (data not shown). This condition was almost completely unchanged by LRH-1 transduction and cAMP treatment. In contrast, CYP17 promoter regions were completely methylated in parental hMSCs. Transduction by LRH-1 had no effect, as in the case of the CYP11A1 promoter. However, cAMP treatment mosaically demethylated the CYP17 promoter regions at 24 h and demethylation was increased by 48 h. These methylation patterns of the CYP11A1 and CYP17 promoters closely paralleled the induction patterns of both genes

by cAMP. To clarify the potential role of DNA methylation in the expression of steroidogenic enzymes in hMSCs, LRH-1-transduced cells were treated with the demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC) (supplemental Fig. 3 and Fig. 5C). Although 5-aza-dC was unable to induce CYP11A1 and CYP17 genes, it augmented the induction of both genes by cAMP, with the effect on CYP17 being particularly marked. These results strongly suggest that the status of DNA methylation in the promoter regions could be important for regulating the expression of steroidogenic enzymes in MSCs and might be responsible for the time lag associated with the induction of some genes.

Discussion

SF-1 has been clearly demonstrated to be a master regulator of steroidogenic organs. SF-1 knockout mice show agenesis of the primary steroidogenic organs, the adrenals and gonads (5–8). SF-1 can induce the differentiation of nonsteroidogenic MSCs into steroidogenic cells (9, 10, 37, 38). In this study, we demonstrated that LRH-1 could also act as a key regulator of the steroidogenic lineage. It was abundant in the gonads and induced the differentiation of MSCs into steroidogenic cells without SF-1. Consistent with our results, it has been reported that LRH-1 and SF-1 have similar roles in steroidogenesis in some cells (20, 39). The analysis of LRH-1 actions *in vivo* has been hampered by the embryonic lethality of LRH-1 knockout mice, although steroidogenesis in heterozygous Lrh-1^{+/−} mice was abnormal in various organs such as the testis, luteinized ovary, and intestine (20, 22–24). Moreover, it was recently reported that ovarian progesterone production was reduced in mice lacking LRH-1 in the granulosa cells (19). It is therefore possible that LRH-1, as well as SF-1, is important for adult steroidogenesis *in vivo*. In addition to detailed analysis of steroidogenesis in the adult, the generation and characterization of mice deficient for LRH-1 in their steroidogenic organs will also help to elucidate its involvement in the development of steroidogenic organs. In fact, granulosa cell-specific knockout model demonstrate a broader role for LRH-1 beyond steroidogenesis in these cells, such as the failure of luteinization (19).

LRH-1-transduced cells abundantly expressed CYP17 and produced testosterone with the aid of cAMP. In human steroidogenic organs, LRH-1 and CYP17 were colocalized in testicular Leydig cells. It is therefore possible that LRH-1 is involved in testicular androgen production. In accordance with our results, Volle *et al.* (20) reported that LRH-1^{+/−} mice showed lower plasma testosterone concentrations than LRH-1^{+/+} mice. However, even though the testicular mRNA levels of various steroidogenic genes such as Star, Cyp11a1, and Hsd3b1 were lower in LRH-1^{+/−} mice, there were no differences in the expression of the Cyp17 gene between LRH-1^{+/+} and LRH-1^{+/−} mice. Deletion of SHP, a transcriptional repressor of LRH-1, however, resulted in increased testosterone production due to enhanced expression of StAR, Cyp11a1, and Hsd3b1. In these mice, the testicular expression of Cyp17 was unaffected. The authors therefore concluded that expression of the Cyp17 gene might not be controlled by the LRH-1/SHP pathway. Our study, however,

clearly demonstrated that LRH-1 directly regulated the transcription of the CYP17 gene via three binding sites located in its promoter region. There are several possible explanations for the discrepancies between these results: it is possible that the regulation of CYP17/Cyp17 gene transcription by the NR5A family varies between species, and Miller and his colleagues (40) noted species-specific variation in the transcriptional regulatory strategies of the CYP17 gene. Observations made in one species cannot therefore reliably be applied to the CYP17 gene in another species, despite the high-sequence similarity of their promoter regions. Another possibility is that although LRH-1 could regulate the transcription of the Cyp17 gene in mouse Leydig cells, haploinsufficiency might be compensated for by other factors, such as SF-1. It is also possible that it could be compensated for by factor(s) derived from other cells by the reduction of LRH-1. Studies in a Leydig cell-specific knockout model would resolve these issues and clarify the role of LRH-1 in androgen production in adult Leydig cells. On the other hand, LRH-1 is unlikely to be involved in steroidogenesis in the fetal testis because it was not detectable in the interstitial space in fetuses or neonates, when the fetal Leydig cells are responsible for the production of testicular androgens (data not shown). In addition, our stem cell model used in this study could also contribute to the investigation of LRH-1 function in steroidogenesis because hMSCs differentiated into SF-1-deficient steroidogenic cells.

Its expression in spermatogenic cells indicates that, in addition to its role in androgen synthesis, LRH-1 could also play a vital role in human spermatogenesis. Expression of LRH-1 has also been reported in both Leydig cells and spermatogenic cells in rats (41). Its localization was similar to the spatial pattern of expression of the aromatase gene in the testis, leading to the suggestion that LRH-1 might regulate the expression of the aromatase gene in not only Leydig cells but also spermatogenic cells and so be involved in intratesticular estrogen signaling. This hypothesis is consistent with the results in MSCs (Fig. 1), in which aromatase was induced by the introduction of LRH-1, aided by cAMP signaling. On the other hand, ovarian aromatase expression was increased in granulosa cell-specific knockout. The results of ChIP analysis indicated that ovarian aromatase expression was not regulated by LRH-1. Hence, the regulation of aromatase gene expression might be complex. In addition to aromatase, LRH-1 could also regulate other genes associated with spermatogenesis in spermatogenic cells.

In addition to steroidogenic enzymes, SF-1 and LRH-1 mRNAs were also induced by cAMP treatment in their respectively transduced cells. Hormone/cAMP regulation of SF-1 and LRH-1 remains a debatable subject because results have varied, depending on the cells used. Similar phenomena to those seen in this study were observed in relatively undifferentiated adrenocortical cell lines stimulated with ACTH (42). However, it has been shown in Leydig or adrenocortical cell lines that SF-1 mRNA levels were largely unaffected by LH/human chorionic gonadotropin, ACTH, or agents that stimulated cAMP production (43–45). Although the reasons for this apparent discrepancy remain unclear, the expression of SF-1 in granulosa cells derived from diethylstilbestrol-primed rats may indicate the complex pattern needed to explain the differences between cells (29). In this model, SF-1 mRNA and protein levels remained almost constant for at least 4 h after FSH treatment but then increased markedly from 12 to 24 h and up to 48 h. This pattern parallels that of the induction of steroidogenic genes and steroidogenesis (luteinization). Once luteinized, SF-1 levels in granulosa cells were unaffected by human chorionic gonadotropin or cAMP treatment, even though progesterone production was increased by these treatments (46). Thus, SF-1 or LRH-1 might be up-regulated during the differentiation of steroidogenic cells by a pituitary hormone/cAMP pathway. However, their expressions should be unaffected after differentiation has occurred. In support of this hypothesis, SF-1 proteins were also induced in nonsteroidogenic Leydig stem cells when they were cultured in medium containing LH and differentiated into testosterone-producing cells (47). As described above, SF-1 was not responsive to the LH/cAMP pathway in differentiated Leydig cells (43–45). Although the precise mechanism underlying the up-regulation of SF-1 and LRH-1 mRNA by cAMP signaling is not clear, mRNA stabilization of both genes is likely to be responsible for this up-regulation; mRNA levels of other genes (GFP, DAX-1, and NUR77) transduced into hMSCs using the same retrovirus vector (same promoter) were not affected by cAMP treatment. Further studies are necessary to determine the mechanisms of NR5A-regulated expression.

Bisulfite sequencing analysis demonstrated that the CYP17 promoter was completely methylated in hMSCs and was demethylated by LRH-1 transduction and cAMP treatment. In contrast to the CYP17 gene, the CYP11A1 promoter region was relatively hypomethylated and was unaffected by LRH-1 and cAMP. It has been shown that methylation of the CpG site in the promoter region was involved in the silencing of tissue-specific genes (48, 49). Consistent with this fact, the methylation patterns of the CYP11A1 and CYP17 promoters closely paralleled the expression patterns of both genes. Low levels of P450scc mRNA were expressed in LRH-1-transduced cells before cAMP treatment, whereas CYP17 mRNAs were expressed after 24 h and only after cAMP treatment. Treatment with the demethylating agent 5-aza-dC up-regulated CYP17 mRNA. These results suggest that DNA methylation is an important determinant of the timing of induction of steroidogenic genes in MSC-derived steroidogenic cells. Similar results were obtained from SF-1-transduced MSCs (our unpublished results). Although the mechanisms of demethylation of the CYP17 promoter region by NR5A family and cAMP were not determined, it has been reported that cyclical changes in the methylation status of promoter CpG islands, mediated by DNA methyltransferases, occurred in genes regulated by the estrogen receptor and its ligand (50, 51). A similar phenomenon might occur in genes regulated by LRH-1 and SF-1, which is also a nuclear receptor. Further studies are necessary to elucidate the mechanisms involved in the regulation of the methylation states of steroidogenic genes in MSCs.

In summary, we have determined that LRH-1, as well as SF-1, could act as a key regulator controlling the differentiation of some stem cells into steroidogenic cells. It is possible that the function of SF-1 in primary steroidogenesis could be replaced by LRH-1. In support of this hypothesis, Ingraham and her colleagues (52) showed, using SF-1 haploinsufficient mice, that the

full SF-1 gene dosage was necessary for early adrenal develop-
ment but not in the adult adrenal, in which compensatory mech-
anisms restored near normal function. In addition, Morohashi
and his colleagues (8, 53), using transgenic mice, reported that
differential gene dosage effects of SF-1/Ad4BP existed between
the gonads and adrenals, even though they had a common de-
velopmental origin. Our results strongly suggest that LRH-1 is
involved in these phenomena and can compensate for SF-1 func-
tion in these models. Future studies aimed at simultaneously
investigating the functions of both SF-1 and LRH-1 will be es-
sential for developing a more precise understanding of the reg-
ulation of primary steroidogenesis.

Acknowledgments

References

1. Krylova IN, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, Juzumiene
D, Bynum JM, Madauss K, Montana V, Lebedeva L, Suzawa M, Williams JD,
Williams SP, Guy RK, Thornton JW, Fletterick RJ, Willson TM, Ingraham HA
2005 Structural analyses reveal phosphatidyl inositols as ligands for the NR5
orphan receptors SF-1 and LRH-1. Cell 120:343–355
2. Lala DS, Rice DA, Parker KL 1992 Steroidogenic factor I, a key regulator of
steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor
I. Mol Endocrinol 6:1249–1258
3. Morohashi K, Honda S, Inomata Y, Handa H, Omura T 1992 A common
trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic
P-450s. J Biol Chem 267:17913–17919
4. Galarneau L, Paré JF, Allard D, Hamel D, Levesque L, Tugwood JD, Green S,
Bélanger L 1996 The α1-fetoprotein locus is activated by a nuclear receptor of
the Drosophila FTZ-F1 family. Mol Cell Biol 16:3853–3865
5. Luo X, Ikeda Y, Parker KL 1994 A cell-specific nuclear receptor is essential for
adrenal and gonadal development and sexual differentiation. Cell 77:481–490
6. Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte
LM, Simburger K, Milbrandt J 1995 Mice deficient in the orphan receptor ste-
roidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-
cleavage enzyme in the placenta and have normal embryonic serum levels of cor-
ticosteroids. Proc Natl Acad Sci USA 92:10939–10943
7. Parker KL, Schimmer BP 1997 Steroidogenic factor 1: a key determinant of
endocrine development and function. Endocr Rev 18:361–377
8. Morohashi Ki 1999 Gonadal and extragonadal functions of Ad4BP/SF-1: de-
velopmental aspects. Trends Endocrinol Metab 10:169–173
9. Yazawa T, Mizutani T, Yamada K, Kawata H, Sekiguchi T, Yoshino M,
Kajitani T, Shou Z, Umezawa A, Miyamoto K 2006 Differentiation of adult
stem cells derived from bone marrow stroma into Leydig or adrenocortical
cells. Endocrinology 147:4104–4111
10. Yazawa T, Uesaka M, Inaoka Y, Mizutani T, Sekiguchi T, Kajitani T, Kitano
T, Umezawa A, Miyamoto K 2008 Cyp11b1 is induced in the murine gonad
by luteinizing hormone/ human chorionic gonadotropin and involved in the
production of 11-ketotestosterone, a major fish androgen; conservation and
evolution of androgen metabolic pathway. Endocrinology 149:1786–1792
11. Fayard E, Auwerx J, Schoonjans K 2004 LRH-1: an orphan nuclear receptor
involved in development, metabolism and steroidogenesis. Trends Cell Biol
14:250–260
12. Lee YK, Moore DD 2008 Liver receptor homolog-1, an emerging metabolic
modulator. Front Biosci 13:5950–5958
13. Gu P, Goodwin B, Chung AC, Xu X, Wheeler DA, Price RR, Galardi C, Peng
L, Latour AM, Koller BH, Gossen J, Kliewer SA, Cooney AJ 2005 Orphan
nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast
stage of embryonic development. Mol Cell Biol 25:3492–3505
14. Labelle-Dumais C, Jacob-Wagner M, Paré JF, Bélanger L, Dufort D 2006
Nuclear receptor NR5A2 is required for proper primitive streak morphogen-
esis. Dev Dyn 235:3359–3369
15. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf
DJ 2000 Molecular basis for feedback regulation of bile acid synthesis by nuclear
receptors. Mol Cell 6:507–515
16. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi
C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA
2000 A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1
represses bile acid biosynthesis. Mol Cell 6:517–526
17. Lee YK, Schmidt DR, Cummins CL, Choi M, Peng L, Zhang Y, Goodwin B,
Hammer RE, Mangelsdorf DJ, Kliewer SA 2008 Liver receptor homolog-1
regulates bile acid homeostasis but is not essential for feedback regulation of
bile acid synthesis. Mol Endocrinol 22:1345–1356
18. Mataki C, Magnier BC, Houten SM, Annicotte JS, Argmann C, Thomas C,
Overmars H, Kulik W, Metzger D, Auwerx J, Schoonjans K 2007 Compro-
mised intestinal lipid absorption in mice with a liver-specific deficiency of liver
receptor homolog 1. Mol Cell Biol 27:8330–8339
19. Duggavathi R, Volle DH, Mataki C, Antal MC, Messaddeq N, Auwerx J,
Murphy BD, Schoonjans K 2008 Liver receptor homolog 1 is essential for
ovulation. Genes Dev 22:1871–1876
20. Volle DH, Duggavathi R, Magnier BC, Houten SM, Cummins CL, Lobaccaro
JM, Verhoeven G, Schoonjans K, Auwerx J 2007 The small heterodimer part-
ner is a gonadal gatekeeper of sexual maturation in male mice. Genes Dev
21:303–315
21. Saxena D, Escamilla-Hernandez R, Little-Ihrig L, Zeleznik AJ 2007 Liver
receptor homolog-1 and steroidogenic factor-1 have similar actions on rat
granulosa cell steroidogenesis. Endocrinology 148:726–734
22. Labelle-Dumais C, Paré JF, Bélanger L, Farookhi R, Dufort D 2007 Impaired
progesterone production in Nr5a2$^{+/-}$ mice leads to a reduction in female
reproductive function. Biol Reprod 77:217–225
23. Mueller M, Cima I, Noti M, Fuhrer A, Jakob S, Dubuquoy L, Schoonjans K,
Brunner T 2006 The nuclear receptor LRH-1 critically regulates extra-adrenal
glucocorticoid synthesis in the intestine. J Exp Med 203:2057–2062
24. Coste A, Dubuquoy L, Barnouin R, Annicotte JS, Magnier B, Notti M,
Corazza N, Antal MC, Metzger D, Desreumaux P, Brunner T, Auwerx J,
Schoonjans K 2007 LRH-1-mediated glucocorticoid synthesis in enterocytes
protects against inflammatory bowel disease. Proc Natl Acad Sci USA 104:
13098–13103
25. Mueller M, Atanasov A, Cima I, Corazza N, Schoonjans K, Brunner T 2007
Differential regulation of glucocorticoid synthesis in murine intestinal epithe-
lial versus adrenocortical cell lines. Endocrinology 148:1445–1453
26. Mori T, Kiyono T, Imabayashi H, Takeda Y, Tsuchiya K, Miyoshi S, Makino
H, Matsumoto K, Saito H, Ogawa S, Sakamoto M, Hata J, Umezawa A 2005
Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life
span of bone marrow stromal cells from an elderly donor without affecting
their neurogenic potential. Mol Cell Biol 25:5183–5195
27. Okamoto T, Aoyama T, Nakayama T, Nakamata T, Hosaka T, Nishijo K,
Nakamura T, Kiyono T, Toguchida J 2002 Clonal heterogeneity in differen-
tiation potential of immortalized human mesenchymal stem cells. Biochem
Biophys Res Commun 295:354–361
28. Yazawa T, Nakayama Y, Fujimoto K, Matsuda Y, Abe K, Kitano T, Abé S,
Yamamoto T 2003 Abnormal spermatogenesis at low temperatures in the
Japanese red-bellied newt, *Cynops pyrrhogaster*: possible biological signifi-
cance of the cessation of spermatocytogenesis. Mol Reprod Dev 66:60–66
29. Yazawa T, Mizutani T, Yamada K, Kawata H, Sekiguchi T, Yoshino M,
Kajitani T, Shou Z, Miyamoto K 2003 Involvement of cyclic adenosine 5′-
monophosphate response element-binding protein, steroidogenic factor 1, and
Dax-1 in the regulation of gonadotropin-inducible ovarian transcription fac-
tor 1 gene expression by follicle-stimulating hormone in ovarian granulosa
cells. Endocrinology 144:1920–1930
30. Hanley NA, Rainey WE, Wilson DI, Ball SG, Parker KL 2001 Expression
profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential
interactions in gene regulation. Mol Endocrinol 15:57–68
31. Sewer MB, Nguyen VQ, Huang CJ, Tucker PW, Kagawa N, Waterman MR
2002 Transcriptional activation of human CYP17 in H295R adrenocortical

cells depends on complex formation among p54(nrb)/NonO, protein-associated splicing factor, and SF-1, a complex that also participates in repression of transcription. Endocrinology 143:1280–1290

32. Lalli E, Sassone-Corsi P 2003 DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. Mol Endocrinol 17:1445–1453

33. Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL 1993 The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. Mol Cell Biol 13:861–868

34. Peng N, Kim JW, Rainey WE, Carr BR, Attia GR 2003 The role of the orphan nuclear receptor, liver receptor homologue-1, in the regulation of human corpus luteum 3β-hydroxysteroid dehydrogenase type II. J Clin Endocrinol Metab 88:6020–6028

35. Li E, Bestor TH, Jaenisch R 1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926

36. Ng RK, Gurdon JB 2008 Epigenetic inheritance of cell differentiation status. Cell Cycle 7:1173–1177

37. Gondo S, Yanase T, Okabe T, Tanaka T, Morinaga H, Nomura M, Goto K, Nawata H 2004 SF-1/Ad4BP transforms primary long-term cultured bone marrow cells into ACTH-responsive steroidogenic cells. Genes Cells 9:1239–1247

38. Tanaka T, Gondo S, Okabe T, Ohe K, Shirohzu H, Morinaga H, Nomura M, Tani K, Takayanagi R, Nawata H, Yanase T 2007 Steroidogenic factor 1/adrenal 4 binding protein transforms human bone marrow mesenchymal cells into steroidogenic cells. J Mol Endocrinol 39:343–350

39. Wang ZN, Bassett M, Rainey WE 2001 Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11β-hydroxylase. J Mol Endocrinol 27:255–258

40. Lin CJ, Martens JW, Miller WL 2001 NF-1C, Sp1, and Sp3 are essential for transcription of the human gene for P450c17 (steroid 17α-hydroxylase/17,20 lyase) in human adrenal NCI-H295A cells. Mol Endocrinol 15:1277–1293

41. Pezzi V, Sirianni R, Chimento A, Maggiolini M, Bourguiba S, Delalande C, Carreau S, Andò S, Simpson ER, Clyne CD 2004 Differential expression of steroidogenic factor-1/adrenal 4 binding protein and liver receptor homolog-1 (LRH-1)/fetoprotein transcription factor in the rat testis: LRH-1 as a potential regulator of testicular aromatase expression. Endocrinology 145:2186–2196

42. Ragazzon B, Lefrançois-Martinez AM, Val P, Sahut-Barnola I, Tournaire C, Chambon C, Gachancard-Bouya JL, Begue RJ, Veyssière G, Martinez A 2006 Adrenocorticotropin-dependent changes in SF-1/DAX-1 ratio influence steroidogenic genes expression in a novel model of glucocorticoid-producing adrenocortical cell lines derived from targeted tumorigenesis. Endocrinology 147:1805–1818

43. Zhang P, Mellon SH 1996 The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3′,5′-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17α-hydroxylase/c17–20 lyase). Mol Endocrinol 10:147–158

44. Jo Y, Stocco DM 2004 Regulation of steroidogenesis and steroidogenic acute regulatory protein in R2C cells by DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1). Endocrinology 145:5629–5637

45. Chau YM, Crawford PA, Woodson KG, Polish JA, Olson LM, Sadovsky Y 1997 Role of steroidogenic-factor 1 in basal and 3′,5′-cyclic adenosine monophosphate-mediated regulation of cytochrome P450 side-chain cleavage enzyme in the mouse. Biol Reprod 57:765–771

46. Mamluk R, Greber Y, Meidan R 1999 Hormonal regulation of messenger ribonucleic acid expression for steroidogenic factor-1, steroidogenic acute regulatory protein, and cytochrome P450 side-chain cleavage in bovine luteal cells. Biol Reprod 60:628–634

47. Ge RS, Dong Q, Sottas CM, Papadopoulos V, Zirkin BR, Hardy MP 2006 In search of rat stem Leydig cells: identification, isolation, and lineage-specific development. Proc Natl Acad Sci USA 103:2719–2724

48. Eden S, Cedar H 1994 Role of DNA methylation in the regulation of transcription. Curr Opin Genet Dev 4:255–259

49. Xue Q, Lin Z, Yin P, Milad MP, Cheng YH, Confino E, Reierstad S, Bulun SE 2007 Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5′ CpG island in endometriosis. J Clin Endocrinol Metab 92:3261–3267

50. Métivier R, Gallais R, Tiffoche C, Le Péron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, Benes V, Jeltsch A, Gannon F, Salbert G 2008 Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50

51. Kangaspeska S, Stride B, Métivier R, Polycarpou-Schwarz M, Ibberson D, Carmouche RP, Benes V, Gannon F, Reid G 2008 Transient cyclical methylation of promoter DNA. Nature 452:112–115

52. Bland ML, Fowkes RC, Ingraham HA 2004 Differential requirement for steroidogenic factor-1 gene dosage in adrenal development versus endocrine function. Mol Endocrinol 18:941–952

53. Fatchiyah, Zubair M, Shima Y, Oka S, Ishihara S, Fukui-Katoh Y, Morohashi K 2006 Differential gene dosage effects of Ad4BP/SF-1 on target tissue development. Biochem Biophys Res Commun 341:1036–1045

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ORIGINAL ARTICLE

Living Donor Liver Transplantation for Glycogen Storage Disease Type Ib

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Glycogen storage disease type 1b (GSD-1b) is due to an autosomal recessive inborn error of carbohydrate metabolism caused by defects in glucose-6-phosphatase translocase. Patients with GSD-1b have severe hypoglycemia with several clinical manifestations of hepatomegaly, obesity, a doll-like face, and neutropenia. Liver transplantation has been indicated for severe glucose intolerance. This study retrospectively reviewed 4 children with a diagnosis of GSD-1b who underwent living-donor liver transplantation (LDLT). Between November 2005 and June 2008, 96 children underwent LDLT with overall patient and graft survival of 92.3%. Of these, 4 (4.2%) were indicated for GSD-1b. All patients are doing well with an excellent quality of life because of the stabilization of glucose intolerance, decreased hospital admission, and normalized neutrophil count. LDLT appears to be a feasible option and is associated with a better quality of life for patients with GSD-1b. Long-term observation may be necessary to collect sufficient data to confirm the efficacy of this treatment modality. *Liver Transpl 15:1867-1871, 2009.* © 2009 AASLD.

Glycogen storage disease type 1 (GSD-1) is an autosomal recessive inborn error of carbohydrate metabolism caused by defects of the glucose-6-phosphatase (G6Pase) complex, which is encoded on chromosome 11q23.[1] GSD-1 affects approximately 1 in 100,000 live births.[2] Two major types of the disease have been reported: GSD-1a, which is caused by a deficiency of microsomal G6Pase, and GSD-1b, which is caused by a glucose-6-phosphate translocase (G6PT) deficiency.[3] A patient with GSD-1 presents severe hypoglycemia, hepatomegaly, kidney enlargement, truncal obesity, a rounded doll-like face, wasted muscles, and a bleeding tendency.[4] GSD-1b shows the added features of neutropenia and neutrophil dysfunction.[5]

Major progress has been made in patient survival and the prevention of neurological sequelae secondary to hypoglycemia in affected children with early diagnosis and meticulous treatment. The aim of treatment is to prevent hypoglycemia and neutropenia, which predispose the patients to developing both neurological sequelae and severe infections. The usual medical treatment for this disease consists of frequent meals, continuous nocturnal gastric drip feeding, the administration of uncooked cornstarch for hypoglycemia, and the regular administration of recombinant human granulocyte colony stimulating factor (G-CSF) for neutropenia.[6,7] This treatment, however, is not always sufficient for avoiding hypoglycemia and recurrent infections. Moreover, such treatments may not prevent poor metabolic control and/or growth retardation.

Liver transplantation (LT) may offer a complete cure for genetically acquired errors of metabolism in the liver.[8] The main rationale of LT for congenital metabolic disorders is to supply the missing enzymes by sacrificing the native liver, which is an otherwise normally functioning entity. Recent case studies have reported

| | | | | | | Blood-Type | Graft | |
Case	Age	Sex	Height/Weight (SD)	Mutation	Donor	Combination	Type	Complication
1	8 years 4 months	Female	115.2 cm (−2.2)/ 26.3 kg (−0.1)	W118R	Father	Identical	LLS	Biliary stricture
2	3 years 6 months	Female	94.1 cm (−0.4)/ 16.2 kg (+1.2)	W118R	Father	Identical	LLS	None
3	3 years 11 months	Female	95.2 cm (−0.9)/ 16.9 kg (+0.9)	W118R/IVS1 +1G>A	Father	Incompatible	LLS	Tacrolimus encephalopathy
4	1 year 1 month	Male	71.2 cm (−2.4)/ 10.1 kg (−0.2)	G339D/IVS1 +1G>A	Mother	Incompatible	LLS	None

TABLE 1. Living Donor Liver Transplantation for Glycogen Storage Type 1b

Abbreviations: LLS, left lateral segment; SD, standard deviation.

the benefits of LT in GSD-1b, demonstrating that correcting the hepatic enzyme deficiency by LT leads to clinical improvements, including a normal feeding regimen and fewer intercurrent infections.[9] The present report describes the recent experience with living-donor liver transplantation (LDLT) in 4 GSD-1b patients.

PATIENTS AND METHODS

Between November 2005 and June 2008, 96 children underwent LDLT. Of these, 4 (4.2%) were indicated for GSD-1b. The diagnosis of GSD-1b was made by a mutation analysis of the glucose-6-phosphate (G6P) transporter gene in all case. Medical records were reviewed for the following: the personal and family history, physical findings, laboratory data, histological reports, operation records, and special findings obtained by cardiologists, nephrologists, gastroenterologists, and radiologists. A developmental delay was measured with the developmental quotient (DQ), which is a norm used to express aspects of a child's development similar to the intelligence quotient.[10]

Eight donor candidates were evaluated by standard liver function tests, blood group combination, anatomical variation, graft size matching, and mutation analysis. The mutation analysis revealed heterozygotes among all of the potential donors; however, laboratory data showed normal liver function and blood sugar and serum insulin levels. Table 1 shows profiles of the recipients and donors involved in this study.

All patients underwent LDLT by a standard procedure.[11] Gross hepatomegaly was observed in all cases. No venovenous bypass was used because total clamping of the inferior vena cava could be avoided in all cases. Immunosuppression was administered with tacrolimus and low-dose steroids. Tacrolimus administration was started on the day before transplantation. The target whole blood trough level of tacrolimus was 10 to 12 ng/mL for the first 2 weeks, approximately 10 ng/mL for the following 2 weeks, and 8 to 10 ng/mL thereafter. Treatment with steroids was initiated at the time of graft reperfusion at a dose of 10 mg/kg; the dose was tapered from 1 to 0.3 mg/kg/day during the first month, and the treatment was withdrawn within the first 3 months. Patient 3, who received an ABO-incom-

patible transplant, underwent preoperative plasma exchange in order to reduce the anti-ABH antibody titer, and prostaglandin E1, nafamostat mesilate, and steroids were administered postoperatively via a portal vein catheter.[12] Patient 4 was less than 2 years old and, with an ABO-incompatible transplant, had no preoperative treatment for ABO incompatibility.[13] This study was approved by the institutional review board, and informed consent was obtained in all the cases.

RESULTS

The patients presented with hepatomegaly (cases 1-4), hypoglycemia (cases 1-4), neutropenia (cases 1-4), recurrent respiratory (cases 1 and 3)/skin (cases 1-3) infections, sinusitis (cases 2-4), and failure to thrive (cases 1 and 4). Recurrent infection caused 20, 12, 40, and 8 casualty hospital attendances per year in the 4 patients, respectively. No patient demonstrated coagulopathy, inflammatory bowel disease, or renal insufficiency. Case 1 had experienced frequent hypoglycemic comas and had low mental development. The pretransplant DQs were 32, 92, 62, and 90, respectively. Antiepileptics were administered preoperatively in cases 1 and 3. The frequent daytime administration of uncooked cornstarch, continuous nighttime feeding (case 1), and 24-hour continuous nasogastric tube feeding (cases 2-4) did not provide satisfactory metabolic control. G-CSF was initiated for severe neutropenia 2 to 3 times per week at 5 years, 3 years, 6 months, and 8 months of age, respectively. Aspiration cytology of the bone marrow before transplantation showed hypercellular marrow in all cases. The indications for LDLT were poor metabolic control (cases 1-4) and poor growth (cases 1 and 4).

LDLT was performed at 8.3, 3.5, 3.8, and 1.1 years of age, respectively. The duration and blood loss of the recipient operation ranged from 436 to 557 minutes and from 365 to 1330 g, respectively. Cold and warm ischemic times ranged from 24 to 53 minutes and from 22 to 41 minutes, respectively. The histopathological examination of the explanted livers revealed gross hepatomegaly with microvesicular steatosis and mild fibrosis. The explanted native livers weighed 1399, 1065, 903, and 1200 g, that is, 219.3%, 237.0%, 195.7%, and

	Case 1		Case 2		Case 3		Case 4		Reference
	Before	After	Before	After	Before	After	Before	After	Range
Neutrophil count (c/mm^3)	329	640	170	1095	86	880	138	1289	1500–5000
G-CSF	On	Off	On	Off	On	Off	On	Off	
Platelet count (10^4/μL)	66.3	20.0	61.0	44.2	73.1	40.8	56.2	42.3	15.0–35.0
ALT (IU/L)	21	21	26	10	16	25	31	14	4–24
Glucose (mg/dL)	81	100	93	102	66	110	91	89	70–109
Total protein (g/dL)	8.4	7.1	8.0	7.0	7.3	6.4	7.4	6.1	6.4–8.1
Uric acid (mg/dL)	5.7	2.7	5.8	3.2	5.8	3.5	11.8	3.8	2.3–5.7
Triglycerides (mg/dL)	275	150	697	159	97	132	676	66	30–218
Cholesterol (mg/dL)	212	155	192	150	136	138	179	115	111–222
Lactate (mmol/L)	6.90	1.00	5.70	0.70	2.70	0.90	6.00	0.80	0.44–1.78

TABLE 2. Laboratory Data Before and After Liver Transplantation

Abbreviations: ALT, alanine aminotransferase; G-CSF, granulocyte stimulating factor.

398.5% of the estimated standard liver volume, respectively. No malignant lesion or solid occupied lesion was identified in any of the patients. Histological examinations of the grafts from the heterozygous donors showed 5%, 10%, 10%, and 30% microvesicular steatosis, respectively. All the donors were discharged from the hospital within 9 days after the operation, and they are currently doing well without any complications. The post-LDLT course was uneventful in case 2. Case 1 underwent duct-to-duct biliary reconstruction and showed a biliary stricture, which was successfully managed with radiological intervention. Case 3 experienced tacrolimus-related leukoencephalopathy on postoperative day 6, which was successfully managed with cyclosporine conversion. Cases 1 and 4 showed methicillin-resistant *Staphylococcus aureus* catheter sepsis on postoperative days 6 and 5, respectively. Cases 1 and 3 had cytomegalovirus infections on postoperative days 42 and 38, respectively, which were successfully treated with the administration of ganciclovir. All the children are doing well, with normal graft function at follow-up 3.5, 2.5, 1.5, and 1.0 years after LDLT, respectively (Table 2). There has been marked improvement in the patients' quality of life. All the patients achieved resolution of their metabolic derangement, including correction of hypoglycemia on a normal feeding regimen without a nasogastric tube. The posttransplant DQs were 36, 95, 75, and 95, respectively, which were not significantly different from the pretransplant DQs. The neutropenia improved during the follow-up period without the use of G-CSF; despite this, the absolute neutrophil count remained over 500 per cubic millimeter after LDLT in all cases. The patients have experienced no significant infectious episodes necessitating hospitalization after the successful LDLT.

DISCUSSION

The aim of this study was to evaluate the outcome of patients who underwent LT for GSD-1b. As a result of early diagnosis and a radical treatment with nocturnal nasogastric feeding and uncooked cornstarch, the prognosis of patients with GSD-1b has improved dra-

matically. After the initiation of a radical dietary treatment, however, the development of neurological impairment as a result of metabolic derangement has been reported in 40% of GSD-1b patients.[6] Three patients in the current series (75%) showed neurological disability at the time of LT. Early LT might be recommended from this point of view because it could reduce the magnitude of the progressive neurological disability resulting from poor metabolic control.

There have been 13 cases of LT for GSD-1b reported in the literature, including the present cases (Table 3). The indications for LT were poor metabolic control in 13 patients and poor growth in 4 patients. The median age of the recipients was 8.3 years (range, 1.1-34 years). Twelve of the 13 patients (92.3%) were alive with excellent graft function at the time of publication. If a patient's quality of life is impaired by the strict feeding schedule to avoid hypoglycemia and by recurrent infections, the patient should be listed for LT.

Improvements in neutropenia after LT were reported in 6 cases (46.2%), 5 of whom received a graft from a parental living donor. Inflammatory bowel disease was not seen in these 6 cases, and this is at variance with previously reported series in which it was associated with neutropenia.[6] Because no donor-derived leukocytes could be detected in the recipient's peripheral blood mononuclear cells by analysis for XY chromosomes in the 4 present cases (data not shown), it appears that the donor-derived leukocytes were not cotransplanted or migrated with the liver graft in the recipient's bone marrow and did not restore its cellularity.[19] G6P is transported to the endoplasmic reticulum by G6PT.[20] Recently, the stoichiometry and topological relationship between the catalytic subunits of G6P and G6PT revealed that the complex forming between G6PT and glucose-6-phosphate catalytic subunit 1/2 (G6PC1/2) appears to maintain normoglycemia and that G6PC3 is needed for neutrophil viability; this suggests an important role for glucose in the homeostasis of human neutrophils.[21] An impairment of this function may cause glucose and neutrophil abnormalities. Neutrophils have been reported to express the

	Age				Improved	Follow-Up	
Case	(Years)	Sex	Donor	Indications	Neutropenia	(Years)	Reference
				TABLE 3. Worldwide Experience in Liver Transplantation for Glycogen Storage Type 1b			
1	7	Female	Deceased	PMC	No	2	13
2	7.4	Female	Deceased	PMC	No	6.2	14
3	13.8	Female	Deceased	PMC	No	4.4	14
4	8	Male	Deceased	PG, PMC	No	4	15
5	11.1	Male	Deceased	PG, PMC	No	—	15
6	32	Male	Deceased	PMC	Yes	4	16
7	34	Female	Deceased*	PMC	No	0.7	17
8	13.2	Male	Living	PMC	—	$0.1^\dagger$	18
9	18	Male	Living	PMC	Yes	4	19
10	8.3	Female	Living	PG, PMC	Yes	3.5	This report
11	3.5	Female	Living	PMC	Yes	2.5	This report
12	3.8	Female	Living	PMC	Yes	1.5	This report
13	1.1	Male	Living	PG, PMC	Yes	1	This report

Abbreviations: PG, poor growth; PMC, poor metabolic control.
*Staged kidney and liver transplantation.
†The patient died from systemic candidiasis.

ubiquitously expressed G6PT and G6Pase-beta, which together transport G6P into the endoplasmic reticulum lumen and hydrolyze it into glucose. G6PT-deficient neutrophils, which are unable to produce glucose, may have endoplasmic reticulum stress and increased apoptosis.[22] Overall, a hypothesis explaining why neutropenia improves after transplantation is still not very clear here.

Although LT remains an option in patients with GSD-1b, it does not necessarily address neutropenia, and it thus remains an open question whether LT improves neutropenia in patients with GSD-1b. The mechanisms of G6PT and G6PC3 expression and endoplasmic reticulum stress and the role of G6P-beta are now under investigation in order to clarify the exact role of improved neutropenia in this study population.

In summary, LDLT appears to be a feasible option and is associated with a better quality of life for patients with GSD-1b. Long-term observation may, however, be necessary to obtain sufficient data and establish a clear protocol for this treatment modality.

REFERENCES

1. Cori GT, Cori CT. Glucose-6-phosphatase in the liver in glycogen storage disease. J Biol Chem 1992;199:661-667.
2. Chen YT, Burchell A. Glycogen storage disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic Basis of Inherited Disease. 8th ed. New York, NY: McGraw-Hill; 2001:1521-1551.
3. Kure S, Suzuki Y, Matsubara Y, Sakamoto O, Shintaku H, Issiki G, et al. Molecular analysis of glycogen storage disease type 1b: identification of a prevalent mutation among Japanese patients and assignment of putative glucose-6-phosphate translocase gene to chromosome 11. Biochem Biophys Res Commun 1998;248:426-431.
4. Fernandes J, Smit GPA. The glycogen storage diseases. In: Fernandes J, Saudubray JM, Berghe G, eds. Inborn Metabolic Disease. 3rd ed. New York, NY: Springer; 2000:85-101.
5. Gitzelmann R, Bosshard NU. Defective neutrophil and monocyte functions in glycogen storage disease type 1b: a literature review. Eur J Pediatr 1993;152:S33-S38.
6. Rake JP, Visser G, Labrune P, Leonard JV, Ullrich K, Smit GPA. Glycogen storage disease type 1: diagnosis, management, clinical course and outcome. Results of the European Study on Glycogen Storage Disease Type 1 (ESGSD1). Eur J Pediatr 2002;161:S20-S30.
7. Lachaux A, Boillot O, Stamm D, Canterino I, Dumontet C, Regnier F, et al. Treatment with lenograstim (glycosylated recombinant human granulocyte colony-stimulating factor) and orthotopic liver transplantation for glycogen storage disease type 1b. J Pediatr 1993;123:1005-1008.
8. Todo S, Starzl TE, Tzakis A, Benkow KJ, Kalousek F, Saheki T, et al. Orthotopic liver transplantation for urea cycle enzyme deficiency. Hepatology 1992;15:419-422.
9. Bhattacharya K, Heaton N, Rela M, Walter JH, Lee PJ. The benefits of liver transplantation in glycogenosis type 1b. J Inherit Metab Dis 2004;27:539-540.
10. Hudon L, Moise KJ, Hegemier SE, Hill RM, Moise AA, Smith EO, Carpenter RJ. Long-term neurodevelopmental outcome after intrauterine transfusion for the treatment of fetal hemolytic disease. Am J Obstet Gynecol 1998;179:858-863.
11. Tanaka K, Uemoto S, Tokunaga Y, Fujita S, Sano K, Nishizawa T, et al. Surgical techniques and innovations in living related liver transplantation. Ann Surg 1993;217:81-92.
12. Egawa H, Teramukai S, Haga H, Tanabe M, Fukushima M, Shimazu M. Present status of ABO-incompatible living donor liver transplantation in Japan. Hepatology 2008;47:143-152.
13. Tanaka A, Tanaka K, Kitai T, Yanabu N, Tokunaga A, Sato B, et al. Living related liver transplantation across ABO blood groups. Transplantation 1994;58:548-553.
14. Matern D, Starzl TE, Arnaout W, Barnard J, Bynon JS, Dhawan A, et al. Liver transplantation for glycogen storage disease types I, III and IV. Eur J Pediatr 1999;158(suppl 2):S43-S48.
15. Bhattacharya N, Heaton N, Rela M, Walter JH, Lee PJ. The benefits of liver transplantation in glycogenosis type Ib. J Inherit Metab Dis 2004;27:539-540.
16. Olmos MA, Sanroman LA, Vaquero MP, Perez M, Barcena R, Vicente E, et al. Liver transplantation for type Ib glyco-

genesis with reversal of cyclic neutropenia. Clin Nutr 2001;20:375-377.

17. Martin AP, Bartels M, Schreiber S, Buehrdel P, Hauss J, Fangman J. Successful staged kidney and liver transplantation for glycogen storage disease type 1b: a case report. Transplant Proc 2006;38:3615-3619.

18. Morioka D, Kasahara M, Takada Y, Garbanzo JP, Yoshizawa A, Sakamoto S, et al. Living donor liver transplantation for pediatric patients with inheritable metabolic disorders. Am J Transplant 2005;5:2754-2763.

19. Adachi M, Shinkai M, Ohhama Y, Tachibana K, Kuratsuji T, Saji H, Maruya E. Improved neutrophil function in a glycogen storage disease type 1b patient after liver transplantation. Eur J Pediatr 2004;5:202-206.

20. Kure S, Hou DC, Suzuki Y, Yamagishi A, Hiratsuka M, Fukuda T, et al. Glycogen storage disease type Ib without neutropenia. J Pediatr 2000;137:253-256.

21. Boztug K, Appaswamy G, Ashikov A, Schaffer A, Salzer U, Diestelhorst J, et al. A syndrome with congenital neutropenia and mutation in G6PC3. N Engl J Med 2009;360:32-43.

22. Kim SY, Jun HS, Mead PA, Mansfield BC, Chou JY. Neutrophil stress and apoptosis underline myeloid dysfunction in glycogen storage disease type 1b. Blood 2008;111: 5704-5711.

IMAGES IN LIVER TRANSPLANTATION

Living Donor Liver Transplantation for Congenital Absence of the Portal Vein with Situs Inversus

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Congenital absence of the portal vein (CAPV) is a rare disorder that may lead to the development of hepatic neoplasms, hepatic encephalopathy, pulmonary hypertension (PH), and hepatopulmonary syndrome (HPS). The portal vein develops by selective involution of the vitelline venous system, and associated abnormalities may result in CAPV. Some patients with CAPV are diagnosed at the time of neonatal screening for hypergalactosemia.[1] The etiology of PH/HPS is an imbalance of vasoconstrictors and vasodilators, either produced or metabolized by the liver, that affect the pulmonary arterioles and capillaries by a portosystemic shunt.[2] A patient whose portal vein is patent and perfuses the liver as well as a shunt vessel (type 2) can be treated by shunt ligation (or graded shunt embolization) without portal hypertension being induced. However, if the liver is not perfused with portal blood and the entire splanchnic blood supply flows through a shunt vessel into a systemic vein (type 1), then either surgical or radiological intervention of the portal vein might be contraindicated. Liver transplantation (LT) is indicated as a curative operation for CAPV in patients with uncontrollable hepatic encephalopathy and PH/HPS. Situs inversus (SI) is also a rare congenital anomaly with a frequency of 0.002% to 0.1%.[3] SI occurs in association with polysplenia syndrome and midgut malrotation, a preduodenal portal vein, an aberrant hepatic arterial supply, and absence of the inferior vena cava

(IVC). Consideration, therefore, has to be given to additional vascular reconstruction at LT for CAPV with SI.

A 16-month-old Asian girl weighing 9.5 kg who presented with hyperammonemia (serum NH_3, 150 μmol/L) was admitted to the hospital. The patient was diagnosed to have hypergalactosemia by neonatal metabolic screening. At the time of assessment, a laboratory evaluation showed the following: serum bilirubin, 0.34 mg/dL; aspartate aminotransferase, 72 IU/L; gamma glutamyl transferase, 21 IU/L; albumin, 2.8 g/dL; total protein, 4.8 g/dL; total biliary acid, 126.6 μmol/L; and prothrombin time international normalized ratio, 1.21. Further imaging studies revealed CAPV with SI, polysplenia, and absence of the retrohepatic IVC (Fig. 1). The results of a chest and cardiac examination were unremarkable. Because of recurrent hyperammonemia and progressive hyperintensity in the globus pallidus on magnetic resonance imaging, despite medical treatment and protein restriction, the patient underwent living donor LT.

The donor was her 27-year-old mother, who had the identical blood type. The liver graft, a reduced left lateral sector weighing 154 g and thus representing 1.62% of the graft-to-recipient weight ratio, was procured. The recipient laparotomy showed CAPV, SI, polysplenia, midgut malrotation, and absence of the retrohepatic IVC. The hepatic veins drained directly into the right atrium (Fig. 2A). The right renal vein was patent and
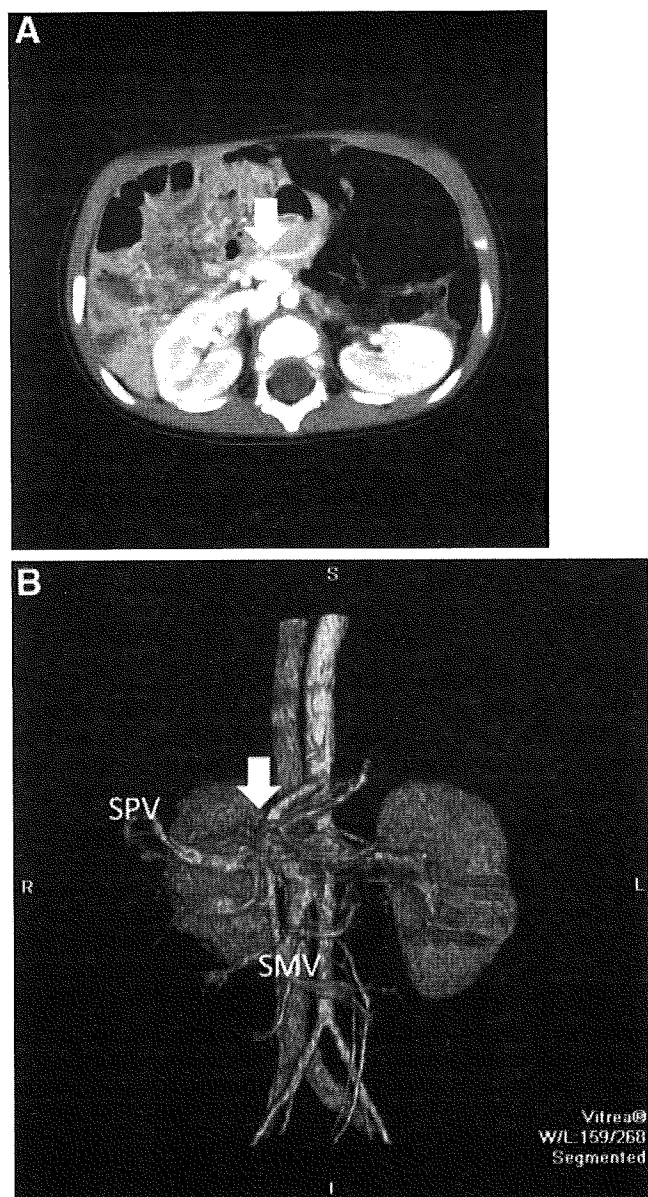
Figure 1. Preoperative computed tomography. The conduit (arrow) of the superior mesenteric vein (SMV) and splenic vein (SPV) drained into the right renal vein without entering the hepatic hilum.

Figure 2. (A) An intraoperative view shows the absence of the retrohepatic inferior vena cava (white arrow) and that the hepatic veins drained directly into the right atrium (asterisk). Note the Arantius ligament (black arrow) and absence of portal branches in the hepatic hilum. (B) A histological examination showed a normal bile duct and hepatic artery and an atrophic portal vein in the portal tract.

drained through the retroperitoneal channels into the azygous system. The recipient hepatectomy was uncomplicated. A histological examination of the 200 g of explanted symmetric native liver, which was 61.6% of the estimated standard liver volume, showed atrophic portal veins visible in the portal tracts (Fig. 2B). The conduit of the superior mesenteric vein and splenic vein drained into the right renal vein without entering the hepatic hilum (Fig. 3A). After the hepatic vein reconstruction to the right atrium, the shunt vessel was divided at its junction with the right renal vein. The stump of the shunt vessels, measuring 3.0 cm in length, was turned upward behind the pancreas and anastomosed directly to the graft portal vein in an end-to-end fashion (Fig. 3B). During the clamping of the portal vein, the portal vein pressure increased from 12 to 32 mm Hg. Mesenteric venous congestion developed, but it was tolerable over the 21 minutes of warm ischemic time. Biliary reconstruction was carried out with a Roux-en-Y choledochojejunostomy. The operation lasted 8 hours 36 minutes, and the blood loss was 520 mL. The postoperative course was uneventful, and the patient was discharged on postoperative day 39. During the 9-month follow-up, the patient did well with normal liver function and normal magnetic resonance imaging findings without hyperammonemia.

The type 1 anomaly seen in the present case is often associated with aberrant malformations, such as biliary atresia, liver tumors, cardiac anomalies, and polysplenia. The present case had 3 additional conditions not reported so far: SI, polysplenia, and absence of the retrohepatic IVC, which are otherwise technically demanding if LT is indicated. The indications for LT in
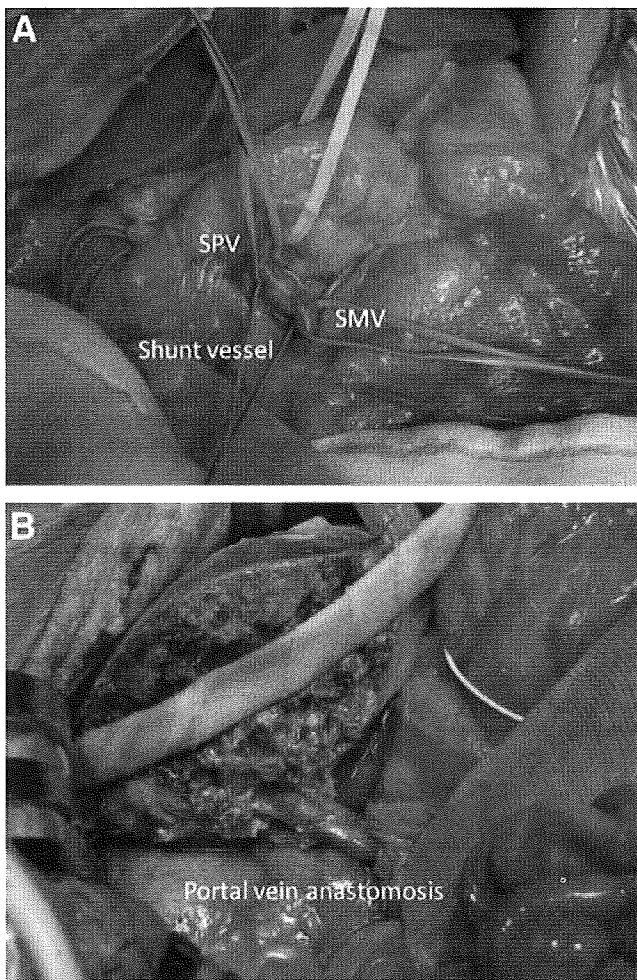
Figure 3. (A) An intraoperative view shows that the shunt vessel, conduit of the superior mesenteric vein (SMV) and splenic vein (SPV), drained into the right renal vein. (B) The stump of the shunt vessel was turned upward behind the pancreas and anastomosed directly to the graft portal vein in an end-to-end fashion.

patients with CAPV have not yet been established. Ten cases of CAPV treated with LT have been reported.[1,4-13] The indications for LT were liver cirrhosis secondary to biliary atresia in 5, an unresectable liver tumor in 3, hyperammonemia in 3, PH in 2, HPS in 1, and hematochezia in 1, and they were resistant to conventional medical treatment. PH was not seen in the present patient; the pathophysiology of PH in CAPV is demonstrated as thromboembolic pulmonary arterial hypertension, and this state can be cured if the shunt vessel can be closed. Recently, preemptive LT for a CAPV patient was performed because LT is the only therapeutic option to prevent regression of progressive PH.[14] In HPS, there is an imbalance between vasodilator and vasoconstrictor substances that have activity in the pulmonary circulation. Pulmonary vasodilation may occur if portocaval shunting impairs the metabolism of

vasoactive substances by the liver. Emre et al.[13] reported that HPS could be treated by the routing of the portal flow into the liver with auxiliary LT because it confirms that a major cause of HPS is impaired hepatic clearance of vasoactive substances.

Early LT should therefore be indicated in symptomatic CAPV patients, even in those without impaired liver function, before advanced PH/HPS and/or irreversible brain damage due to hyperammonemia.

REFERENCES

1. Howard ER, Davenport M. Congenital extrahepatic portocaval shunts. The Aberranthy malformation. J Pediatr Surg 1997;32:494-497.
2. Hoeper MM, Krowka MJ, Strassburg CP. Portopulmonary hypertension and hepatopulmonary syndrome. Lancet 2004;363:1461-1468.
3. Blegen HM. Surgery in situs inversus. Ann Surg 1949;129: 244-259.
4. Barton JW, Keller MS. Liver transplantation for hepatoblastoma in a child with congenital absence of the portal vein. Pediatr Radiol 1989;20:113-114.
5. Woodle ES, Thistlethtwaite JR, Emond JC, Whitington PF, Vogelbach P, Yousefzadeh DK, Broelsch CE. Successful hepatic transplantation in congenital absence of portal vein. Surgery 1990;107:475-479.
6. Morgan G, Superina R. Congenital absence of the portal vein: two cases and proposed classification system for portasystemic vascular anomalies. J Pediatr Surg 1994; 29:1239-1241.
7. Taoube KA, Alonso CJL, Yandza T, St. Vil D, Blanchard H. Congenital absence of portal vein in a girl with biliary atresia treated with liver transplant. Cir Pediatr 1999;12: 38-41.
8. Shinkai M, Ohhama Y, Nishi T, Yamamoo H, Fujita S, Take H, et al. Congenital absence of portal vein and role of liver transplantation in children. J Pediatr Surg 2001;36:1026-1031.
9. Charre L, Roggan F, Lemaire J, Mathijs J, Goffete P, Danse E, Lerut J. Hematochezia and congenital extrahepatic portocaval shunt with absent portal vein: successful treatment by liver transplantation. Transplantation 2004;78: 1404-1406.
10. Ohnishi Y, Ueda M, Doi H, Kasahara M, Haga H, Kamei H, et al. Successful liver transplantation for congenital absence of the portal vein complicated by intrapulmonary shunt and brain abscess. J Pediatr Surg 2005;40:1-3.
11. Takeichi T, Okajima H, Suda H, Hayashida S, Iwasaki H, Ramires MZ, et al. Living domino liver transplantation in an adult with congenital absence of portal vein. Liver Transpl 2005;11:1285-1288.
12. Soejima Y, Taguchi T, Ogita K, Taketomi A, Yoshizumi T, Uchiyama H, et al. Auxiliary partial orthotopic living donor liver transplantation for a child with congenital absence of portal vein. Liver Transpl 2006;12:845-849.
13. Emre S, Arnon R, Cohen E, Morotti RA, Vaysman D, Shneider BL. Resolution of hepatopulmonary syndrome after auxiliary partial orthotopic liver transplantation in Abernethy malformation. A case report. Liver Transpl 2007;13:1662-1668.
14. Ohno T, Muneuchi J, Ihara K, Yuge T, Kanaya Y, Yamaki S, Hara T. Pulmonary hypertension in patients with congenital portosystemic venous shunt: a previously unrecognized association. Pediatrics 2008;121:892-899.

BMC Research Notes

Data Note

Collection of *Macaca fascicularis* cDNAs derived from bone marrow, kidney, liver, pancreas, spleen, and thymus

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Abstract

Background: Consolidating transcriptome data of non-human primates is essential to annotate primate genome sequences, and will facilitate research using non-human primates in the genomic era. *Macaca fascicularis* is a macaque monkey that is commonly used for biomedical and ecological research.

Findings: We constructed cDNA libraries of *Macaca fascicularis*, derived from tissues obtained from bone marrow, liver, pancreas, spleen, and thymus of a young male, and kidney of a young female. In total, 5'-end sequences of 56,856 clones were determined. Including the previously established cDNA libraries from brain and testis, we have isolated 112,587 cDNAs of *Macaca fascicularis*, which correspond to 56% of the curated human reference genes.

Conclusion: These sequences were deposited in the public sequence database as well as in-house macaque genome database http://genebank.nibio.go.jp/qfbase/. These data will become valuable resources for identifying functional parts of the genome of macaque monkeys in future studies.

Findings

Macaca fascicularis (cynomolgus, crab-eating, or long-tail macaque) is one of the most popular primate species used in biomedical research, and is closely related to *Macaca mulatta* (rhesus macaque). The draft sequence of the *Macaca mulatta* genome, which has an evolutionary important position, was published in 2007 [1].

Transcriptiome data broadens the application of genome sequences. Compared with several millions of human transcript sequences, macaque transcriptome data has only been analyzed in a limited numbers of studies [2-6]. A complete list of macaque genes will be beneficial for performing genetic studies using macaques in the future. We aim to elucidate all the macaque transcripts that cor-

respond to human genes, which have been widely accepted as reference sequences, such as the RefSeq sequences [7].

We have published expressed sequence tag (EST) and full-length sequences, which were obtained from cDNA libraries of brain and testis of *Macaca fascicularis*, using a variety of research subjects [5,8-13]. Here, we present 5'-EST sequences from six other tissues of *Macaca fascicularis*. Bone marrow, liver, pancreas, spleen, and thymus from a 4-year-old male Malaysian *Macaca fascicularis*, and kidney from a 3-year-old female Philippine *Macaca fascicularis* were harvested. These animals are bred and reared in the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation (Ibaraki, Japan). The tissues were harvested in the P2 facility in TPRC, in accordance with the guidelines of the Laboratory Biosafety Manual, World Health Organization. The libraries for kidney (QreA and QreB) and liver (QlvC) were constructed using the vector-capping method [14], and those for bone marrow (QbmA), pancreas (QpaA), spleen (QspA), and thymus (QthA) were constructed using the oligo-capping method [15]. The sequences of 5'-EST were determined by Sanger sequencing using an ABI 3730 sequencer, and all vector sequences were filtered out [5]. Nucleotide calls with a quality value (QV) of less than 15 were masked as ambiguous. After the masking, the sequences were trimmed, such that they did not contain more than four ambiguous nucleotides in a 10-bp width window, and sequences shorter than 100 bp after the trimming were filtered out. After the trimming, the average sequence length was 886.9 bp.

In total, we obtained 56,856 EST sequences from the six tissues. The repeat sequences were masked by Repbase Update before the BLAST search [16]. The BLAST search (BLASTN) was performed with a cut-off value (*E*-value) of 1e-60 against human RefSeq data [7]. Since RefSeq sequences contain partially overlapped isoforms, we constructed non-redundant RefSeq sequences based on the Entrez Gene database [17]. Hereafter, we shall refer to the non-redundant RefSeq sequences as RefSeq genes. There were 23,236 RefSeq genes, including non-coding RNAs in the human genome at the time of investigation (Release 34) [7]. Out of the newly isolated 56,856 cDNA clones, 44,603 matched to 4940 human RefSeq genes. Of the 12,253 non-RefSeq clones, 40 consisted of repeat sequences, and the other 1631 did not show any homology to human transcript sequences in public databases using a lower cutoff value (1e-15). Meanwhile, 23,900 EST sequences were homologous to multiple RefSeq genes with the high cutoff value (1e-60). The average nucleotide sequence identity between the best BLAST hit pairs was 95.26%. The nucleotide sequence identity was slightly lower than that estimated using full-length cDNA

sequences of high quality [5], and supposed to reflect some sequencing errors in the EST sequences. In some cases, the nucleotide sequence identity between the best and second best hit pairs were very close, which was probably due to gene duplications specific in the human lineage. The difference in nucleotide sequence identities between the best and second best BLAST hits were less than 0.5% in 8996 ESTs. In such cases, the best hit orthologs would not be regarded as unique orthologs of humans and macaques. In Figure 1, we classify the macaque ESTs according to the number of BLAST hits to RefSeq genes. The average nucleotide sequence identities were ordered by the rank of BLAST hits. For example, the nucleotide sequence identity in the second bin represents the identity between the second best hit pairs.

In conjunction with the previously sequenced cDNA clones, we obtained 112,587 EST sequences corresponding to 8262 human RefSeq genes, which correspond to 36% of all human RefSeq genes. When we restricted the analysis of the human RefSeq genes in the manually curated status (Reviewed or Validated status) [7], 56% (6,177/11,080) of the human RefSeq genes were covered by the macaque transcriptome.

As shown in Table 1, the number of RefSeq genes that were represented in the libraries was different in different tissues. In order to measure the unbiased transcript redun-
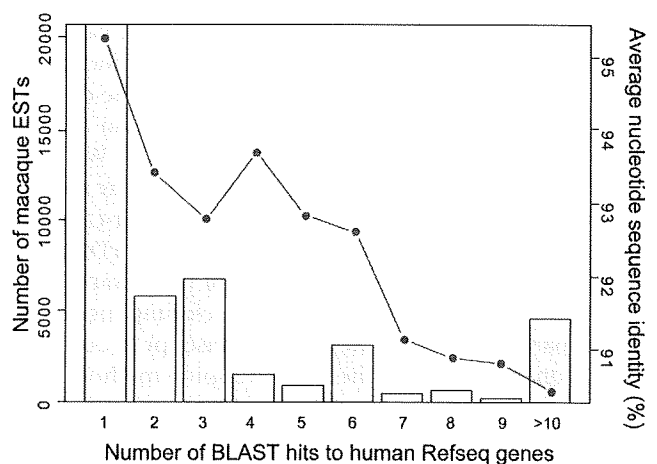
Figure I
Number of BLAST hits (cutoff: 1e-60) against the human RefSeq genes. The grey bars represent the number of macaque ESTs matched to the human RefSeq genes. ESTs matched more than nine RefSeq genes were combined into a single bin. The red circles and lines represent the average nucleotide sequence identity between the macaque ESTs and RefSeq genes, ordered by the rank of BLAST hits. For example, the sequence identity in the second bin represents the sequence identity between the second best hits.

Table 1: Summary of *Macaca fascicularis* cDNA libraries

Tissue	Total clones	Covered RefSeq[d]	non-RefSeq[e]	Redundancy[f]
Brain cortex[a, c]	28679	4035	10259	2.32
Brain stem[b, c]	5758	1591	2050	2.40
Cerebellum[c]	11003	2340	4179	2.32
Testis[c]	8551	1833	3300	2.36
Liver	9188	1360	3853	3.21
Kidney	9558	2495	2630	1.91
Bone marrow	9472	1366	1317	3.26
Spleen	9783	1556	1527	3.15
Thymus	9566	1295	1491	2.96
Pancreas	9289	534	1435	9.83
All	112587	8262	32269	2.14

[a]Brain cortex includes parietal lobe (Qnp), temporal lobe (Qtr), occipital lobe (Qor), and frontal lobe (Qfl).
[b]Brain stem includes medulla oblongata (Qmo) and the other part of brain stem (Qbs).
[c]These sequences were determined by the previous studies [8-10,12].
[d]Number of human RefSeq genes that have macaque homologs in each library.
[e]TheNumber of macaque cDNA clones that do not have human RefSeq homologs.
[f]Estimated from randomly chosen 1000 macaque transcripts, averaged over 1000 simulations.

dancy in each tissue, we estimated the redundancy of the human RefSeq homologs in 1000 macaque transcripts in each tissue. We randomized the transcript data and selected 1000 transcripts to enumerate the human RefSeq genes covered by the transcripts. The redundancy was given by the number of transcripts (1000) divided by the number of human RefSeq genes covered by the transcripts. This procedure was repeated 1000 times for each tissue, and the average redundancy was estimated. The results are shown in the last column of Table 1. Pancreas showed the highest redundancy; while brain and testis showed low redundancy, indicating that the gene expression complexity in brain and testis is higher than that in the other tissues, as suggested previously [18]. We also found that the kidney library (QreA) had very low redundancy. It was constructed using the vector-capping method, which does not amplify the template cDNA by PCR and may reduce the redundancy of the library [14]. In order to test the effectiveness of the cloning methods, we compared the redundancy of the transcript in our liver library constructed using the vector-capping method, and the previously reported liver library constructed using the oligo-capping method [6]. The redundancy in the vector-capped liver library was 3.21 (Table 1). In contrast, the redundancy in the oligo-capped liver library was 5.19 [6], which was significantly higher than that in the vector-capped library ($P < 0.001$, permutation test).

We have developed an in-house database for the genome data of *Macaca fascicularis* (QFbase: http://genebank.nibio.go.jp/qfbase/) [5]. The *Macaca fascicularis* cDNA sequences described in this report were annotated and added to this database. They were also mapped on the rhesus macaque genome sequence using the BLAT program [19]. The results can be viewed in the *Macaca fascic-*

ularis genome browser http://genebank.nibio.go.jp/cgi-bin/gbrowse/rheMac2/, which is implemented using GBrowse software [20]. The DDBJ/EMBL/Genbank accession numbers of these sequences are DC629777-DC639249 (bone marrow), DC639249-DC648806 (kidney), DC620589-DC629776 (liver), FS362802-FS372090 (pancreas), DC848487-DC858269 (spleen), and DK575154-DK584719 (thymus).

Availability and requirements
• **Project name**: *Macaca fascicularis* cDNA sequencing project

• **Project home page**: http://genebank.nibio.go.jp/qfbase/

• **Operating system(s)**: Platform independent

• **Programming language**: PERL

• **Other requirements**: Generic web browser

• **License**: GNU, GPL

• **Any restrictions to use by non-academics**: none

Abbreviations
EST: expressed sequence tag; QV: quality value;

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
NO, KT, JK, YK, KH, and IT contributed to the design of the research. NO analyzed the data. NO and KH wrote the