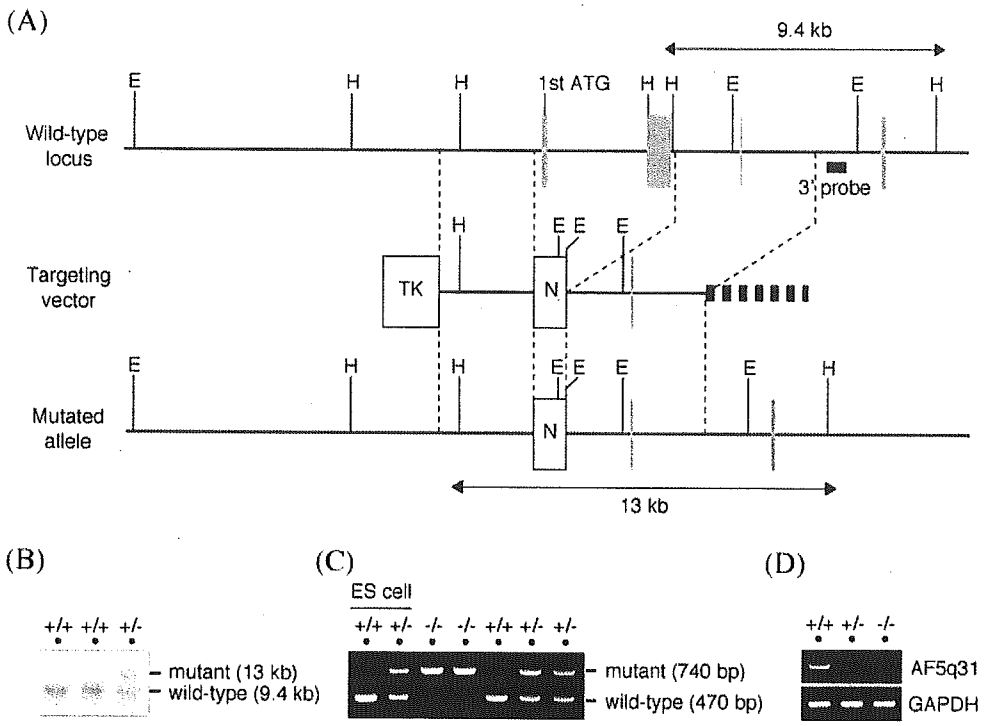

FIG. 2. Targeted disruption of the *AF5q31* gene. (A) Schematic representation of the wild-type allele of mouse *AF5q31* (top), the targeting vector (middle), and the mutant allele resulting from a homologous recombination (bottom). Filled boxes are exons, and open boxes are selection marker genes. H, HindIII restriction site; E, EcoRI restriction site; N, neomycin resistance gene cassette; TK, thymidine kinase gene cassette. (B) Southern blot analysis of HindIII-digested genomic DNAs (5 μg/lane) from ES clones with an external 3′ probe. The 9.4-kb and 13-kb bands represent the wild-type and targeted alleles, respectively. An external 3′ probe used to analyze is shown in panel A. (C) PCR-based genotype analysis of tail DNAs isolated from the pups of AF5q31$^{+/-}$ intercrosses. Three kinds of primers (see Materials and Methods) detected both the wild-type allele (470-bp band) and the targeted allele (740-bp band). As controls, parental and targeted ES cells were used. (D) RT-PCR analysis of total RNAs from AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ MEFs. The primers located on exons I and IV of the *AF5q31* gene were used. RT-PCR for GAPDH confirms equivalent amounts of RNAs used for the analysis.

RESULTS

High expression of *AF5q31* in testis. To explore the tissue distribution of *AF5q31*, Northern blot analysis was performed on various tissues of the adult mice. *AF5q31* was present at a high level in testis and low levels in several other tissues (Fig. 1). Rehybridizations were also carried out with *AF4*, *LAF4*, and *FMR2* cDNA probes. Expression of *AF4* was detected in the heart, kidney, thyroid, and salivary gland at relatively high levels and at low levels in the spleen, liver, and thymus, as reported elsewhere (4). *LAF4* transcript was expressed in the brain and weakly in the spleen and lung. Previously, mouse *LAF4* was shown to be expressed predominantly in the thymus and the spleen of adult mice (41); however, we could not reproduce these results. Almost no signal of *FMR2* expression, except in the testis, was consistent with the finding in the previous report that the expression of *FMR2* occurs on or around 7.0 dpc, reaches its highest level at 10.5 to 11.5 dpc, and is very slight in other stages (9). Compared with these expression profiles, *AF5q31* transcript in the testis was remarkably high.

Targeted disruption of *AF5q31*. To clarify the physiological role of AF5q31, AF5q31$^{-/-}$ mice were generated by gene targeting. Examination of the sequences in the databases revealed that the mouse *AF5q31* gene consists of at least 21 exons (coding exons II to XXI) within 70 kb of the genomic DNA. The region encoded by exons II and III carries the N-terminal homology domain and the partial transactivation domain conserved in AF5q31, AF4, LAF4, and FMR2, which consists of the N-terminal 25% of AF5q31 (2). A targeting vector was constructed by replacing exons II and III with the *neo* gene (Fig. 2A) and introduced into mouse ES cells. ES clones carrying the mutation were identified using Southern blots and an external 3′ probe (Fig. 2B). The blot rehybridized with a *neo* probe yielded only the 13-kbp band, and the EcoRI-digested genomic DNAs probed with an external 5′ probe

TABLE 1. Genotyping of staged embryos and newborn pups by AF5q31$^{+/-}$ intercrossing

Embryonic stage	No. of embryos				Total
	Progeny with the following genotypes:			Resorbed	
	+/+	+/−	−/−		
8.5 dpc	14	11	9 (1[a])		34
9.5 dpc	4 (1[a])	9	10		23
10.5 dpc	9 (1[a])	14 (1[a])	8 (4[a])	1	32
12.5 dpc	7	11	3	11	32
Newborn	44 (6[b])	115 (6[b])	24 (17[b])		183

[a] Number of growth-retarded embryos.
[b] Number of neonates dead within 24 h of birth.

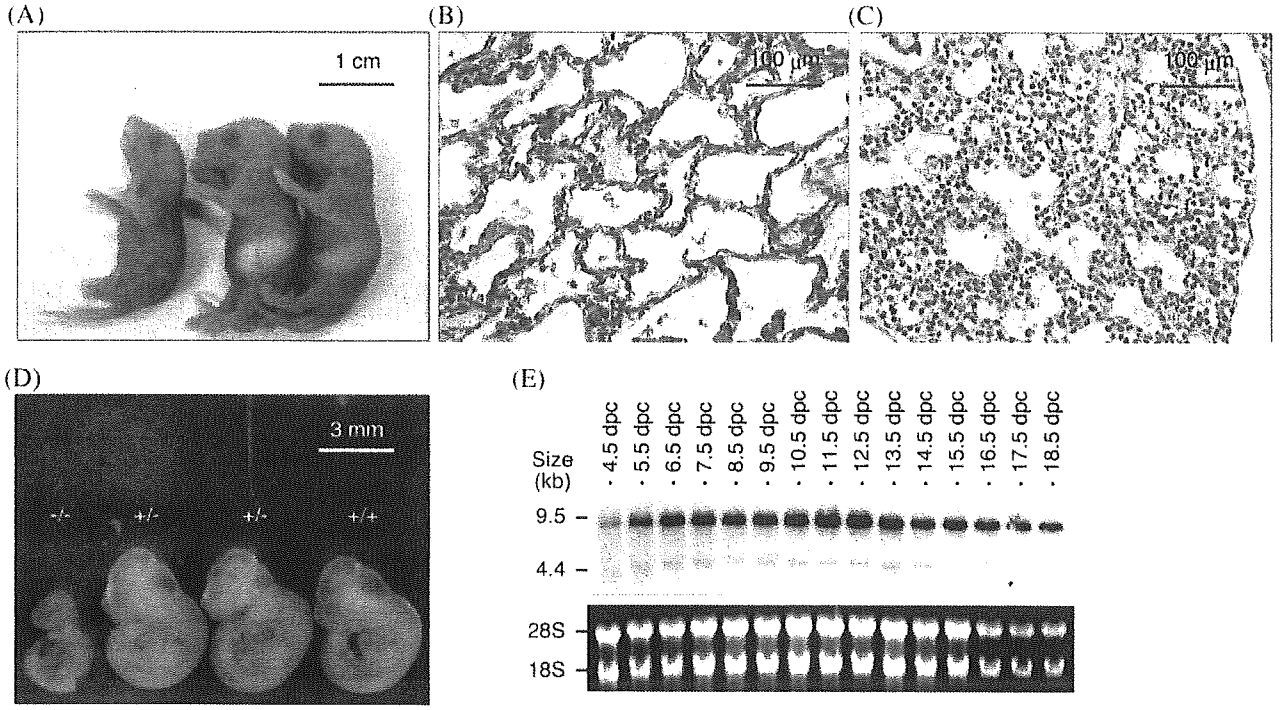

FIG. 3. Macroscopic and microscopic analyses of AF5q31-deficient mice at different ages and the expression profiles of *AF5q31* in the normal mouse embryos. (A) Gross morphology of neonatal littermates representing AF5q31$^{+/+}$ (right), AF5q31$^{+/-}$ (center), and AF5q31$^{-/-}$ (left). (B and C) Histological sections of the lung from AF5q31$^{+/+}$ (B) and AF5q31$^{-/-}$ (C) neonatal littermates stained with hematoxylin and eosin stain. (D) Gross morphology of the AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ embryos of a litter at 10.5 dpc. (E) Northern blot analysis of total RNAs (20 μg/lane) from each embryo stage of the wild-type mouse. The blot was hybridized to a radioactive *AF5q31* probe. As a loading control, 18S and 28S rRNAs in total RNA are demonstrated.

further corroborated appropriate homologous recombination (a 21-kbp band in the wild type and a 15-kbp band in the mutant) (data not shown). After injection of the ES clones into blastocysts, generation of the chimeric mice, and backcrossing of the chimeras, AF5q31$^{+/-}$ mice were obtained. Genotyping of the progenies from intercrosses of the heterozygotes by PCR revealed the presence of AF5q31$^{-/-}$ mice (Fig. 2C). To confirm the deletion in the *AF5q31* mRNA of the mutant mice, RNAs from the MEFs in AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ mice were analyzed by RT-PCR. When sequences from exons I and IV were used as primers, RT-PCR with RNAs from the AF5q31$^{+/+}$ and AF5q31$^{+/-}$ MEFs produced a band of 973 bp, whereas no bands were detected with RNA from the AF5q31$^{-/-}$ MEFs (Fig. 2D). This result indicated that the *AF5q31* mRNA in the mutant mice lacked the sequence for exons II and III.

AF5q31 is important for embryonic development. Genotype analysis of the neonates showed a decrease by 55% in the AF5q31$^{-/-}$ mice relative to the numbers of wild-type and heterozygous littermates, based on the Mendelian ratio, and 71% of the AF5q31$^{-/-}$ neonates died as early as 12 to 24 h postpartum (Table 1). It was noteworthy that neonates that would die had no milk spots and breathed abnormally (Fig. 3A). Precise histochemical analyses of the entire set of neonates revealed that the lethality of AF5q31$^{-/-}$ neonates was potentially caused at least in part by severely shrunken alveoli of the lung (Fig. 3C), compared with the lungs of wild-type mice (Fig. 3B).

When analyzed during gestation, AF5q31$^{-/-}$ mice accounted for 25% of all embryos at 10.5 dpc, demonstrating that disruption of the *AF5q31* gene does not affect the viability of embryos until this stage (Table 1). However, growth retardations, but no obvious malformations, were macroscopically observed in 50% of the mutant embryos at 10.5 dpc (Fig. 3D), and these embryos were likely to be absorbed at 12.5 dpc, indicating that up to 50% of the mutant embryos were lethal around these periods. The expression pattern during mouse development was examined to identify the correct time at which *AF5q31* expression occurs. Northern blot analysis on the RNAs from 4.5-dpc to 18.5-dpc mouse embryos revealed sustained expression of *AF5q31* throughout embryogenesis, and the expression reached its highest level at 10.5 to 12.5 dpc (Fig. 3E). Hence, *AF5q31* appears to be important for embryonic development in this period.

Failure of spermatogenesis in AF5q31$^{-/-}$ male mice. AF5q31$^{-/-}$ male and female mice that survived for >2 months (13% of the AF5q31$^{-/-}$ mice of the C57BL/6/129 background and none of the inbred 129 background so far) seemed normal in health and behavior, and no abnormalities in any organ or tissue examined were found (data not shown), except for the testis (see below). Interestingly AF5q31$^{-/-}$ males were infertile whereas AF5q31$^{-/-}$ females were fertile. Essentially, identical results were obtained in both mouse lines derived from two independent ES cell clones. AF5q31$^{+/-}$ male mice exhibited normal fertility. To evaluate fertility in 9-week-old AF5q31 mutant male mice, each of the AF5q31$^{+/+}$, AF5q31$^{+/-}$, and

TABLE 2. Fertility assessment

Mice	Avg. no. of litters			Vaginal plug[a]
	1	2	3	
AF5q31$^{+/+}$	10 ± 0	6.5 ± 0.5	8.5 ± 0.5	+
AF5q31$^{+/-}$	9 ± 1.0	7.5 ± 0.5	7 ± 0	+
AF5q31$^{-/-}$	0	0	0	±

[a] +, always gave vaginal plugs; ±, some gave vaginal plugs and some did not.

AF5q31$^{-/-}$ mice was mated with 8-week-old C57BL/6 female mice (10, 26). Although AF5q31$^{+/+}$ and AF5q31$^{+/-}$ male mice always gave vaginal plugs the morning after mating and impregnated their mates, some of the AF5q31$^{-/-}$ males failed to give vaginal plugs and all of the AF5q31$^{-/-}$ males could not impregnate their mates in three successive sets of 2-week pairings (Table 2). As a control, the same female mice (after 2 weeks of matings with AF5q31$^{-/-}$ male mice) were always impregnated after mating with C57BL/6 male mice.

Phenotype analysis showed that there was no detectable difference in the morphology of urogenital tracts between the wild-type and mutant mice (data not shown), albeit the sizes of the testes and epididymides in AF5q31$^{-/-}$ mice were significantly smaller and the body weights and the sizes of seminal vesicles were larger than those of AF5q31$^{+/+}$ and AF5q31$^{+/-}$ mice (Fig. 4A to E). Serum hormone assays showed that the levels of testosterone, LH, and FSH in AF5q31$^{-/-}$ mice were not significantly different from those in AF5q31$^{+/+}$ mice in statistical analyses (Fig. 4F to H). Also, mRNAs for the androgen receptor (*AR*), *LH-R*, and *FSH-R* were equally expressed in the testes of AF5q31$^{-/-}$ and control littermates (see Fig. 6D). Consistent with the finding that AF5q31$^{-/-}$ mice were infertile, their seminal fluids were devoid of mature spermatozoa and only debris was present, indicating an arrest of spermatogenesis (Fig. 4I). The spermatozoa of the AF5q31$^{+/-}$ males displayed normal motility with no evident morphological abnormalities (data not shown).

To verify the defect in the spermatogenesis of AF5q31$^{-/-}$ mice, testes were histologically analyzed. As expected, no sperm were found in the cauda epididymides of the AF5q31$^{-/-}$ mice, in contrast to those of the wild-type mice, which accounts for the infertility of the mutant mice (Fig. 5A and B). Detailed histological analysis revealed that round spermatids in the seminiferous tubules of the AF5q31$^{-/-}$ mice differentiated until at least step 11 but failed to undergo normal morphological change to elongated spermatids and to be released as spermatozoa within the germinal epithelium, while somatic Sertoli cells appeared morphologically normal (Fig. 5D, F, and H) and the morphology of seminiferous tubules in the mutant mice was indistinguishable from that of the wild-type mice. In contrast, most stages of the spermatogenic cycles in wild-type mice were represented (Fig. 5C, E, and G). Thus,

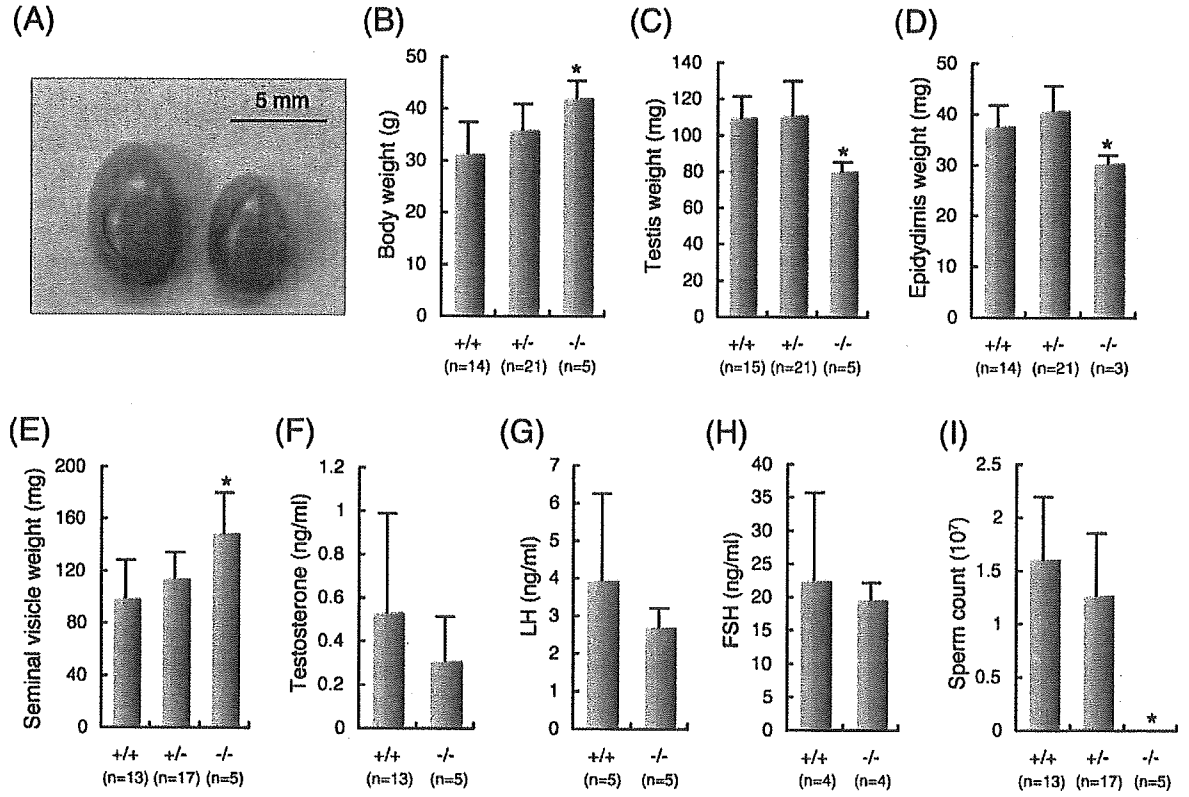

FIG. 4. Weights, hormone levels, and sperm counts in AF5q31$^{-/-}$ and control mice. (A) Testes from 24-week-old AF5q31$^{+/+}$ (left) and AF5q31$^{-/-}$ (right) male mice. (B to E) Weights of body and urogenital tracts of 12-week-old AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ male mice. (F to H) Serum testosterone, LH, and FSH levels in AF5q31$^{+/+}$ and AF5q31$^{-/-}$ male mice. (I) Numbers of sperm cells prepared from 12-week-old AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ male mice. The data are given as averages. Error bars represent standard errors. Statistical significance (*, $P < 0.01$) in each assay was assessed using Student's *t* test between the wild-type and AF5q31$^{-/-}$ mice.

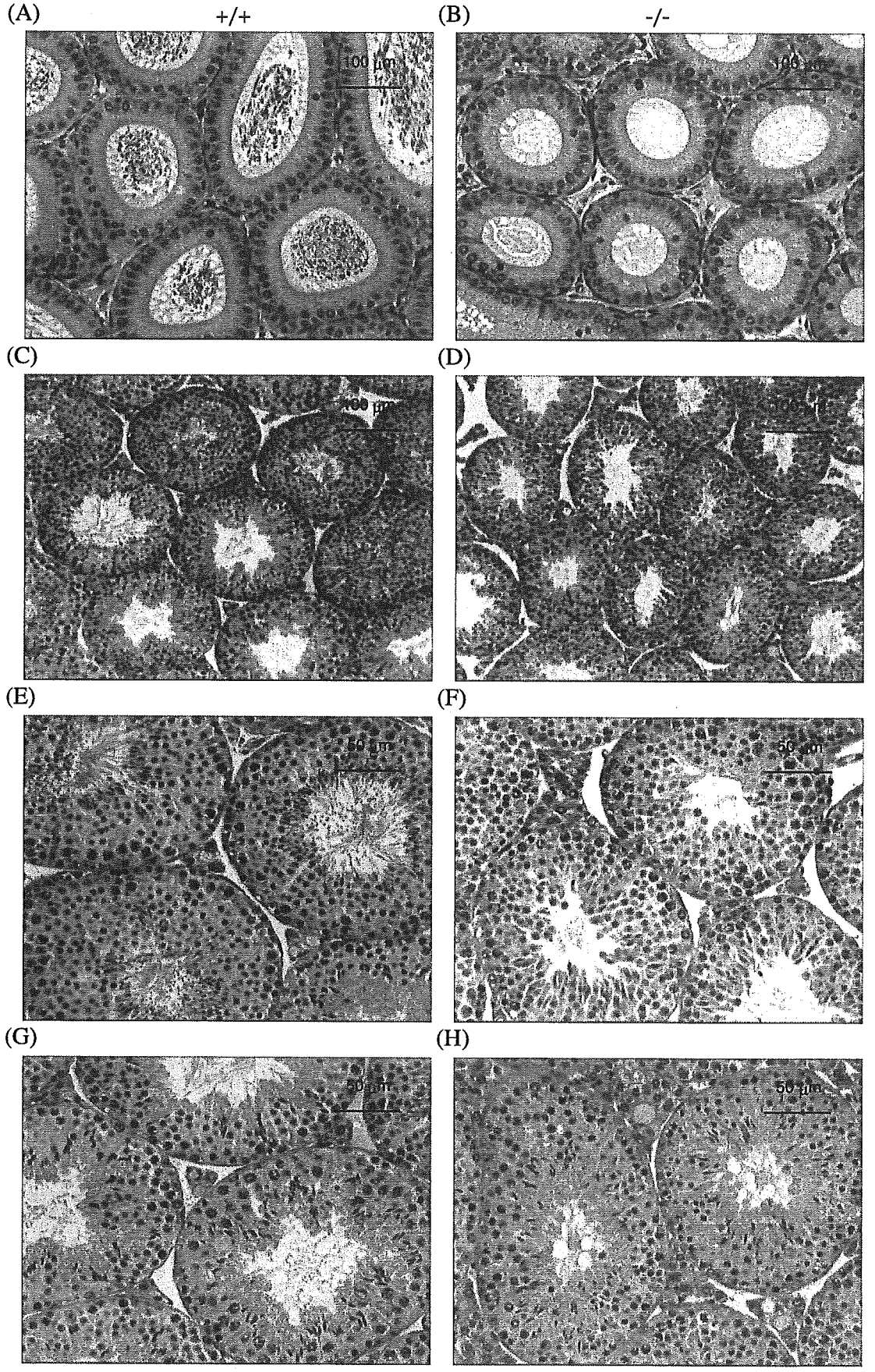

FIG. 5. Histology of epididymides and seminiferous tubules of AF5q31$^{+/+}$ and AF5q31$^{-/-}$ male mice. The epididymal (A and B) and testicular (C to H) sections from 24-week-old AF5q31$^{+/+}$ (A, C, E, and G) and AF5q31$^{-/-}$ (B, D, F, and H) male mice were stained with hematoxylin and eosin stain.

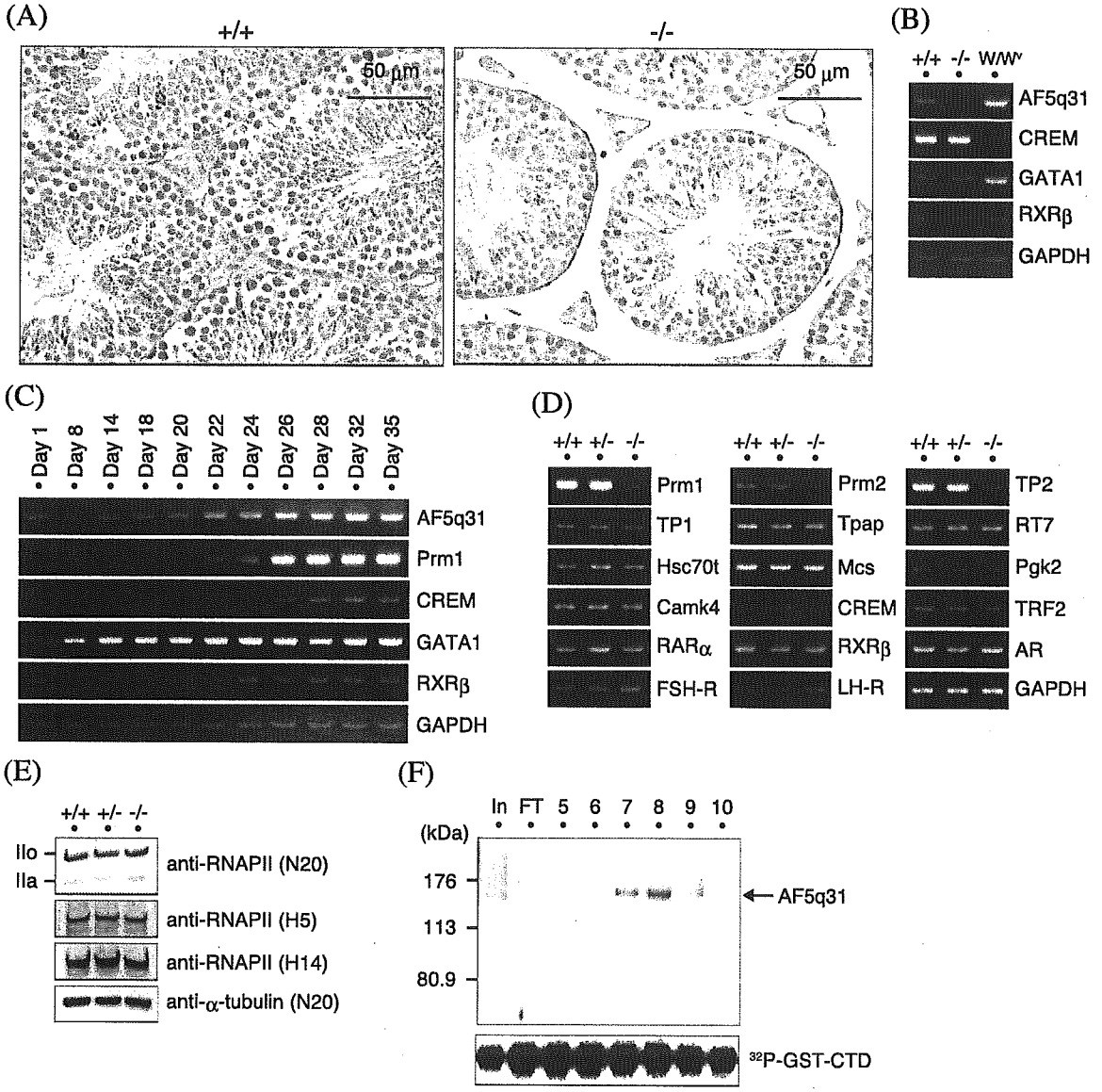

FIG. 6. Mechanism of defective spermatogenesis in AF5q31-deficient mice. (A) Expression of AF5q31 in testes. Immunohistochemical staining was performed with an anti-mAF5q31-E4 antibody on sections of the testes from 12-week-old AF5q31$^{+/+}$ and AF5q31$^{-/-}$ mice. Sections were counterstained with hematoxylin. Brown areas represent the positive signals. (B) RT-PCR analyses of *AF5q31* expression using total RNAs isolated from the testes of 12-week-old AF5q31$^{+/+}$ and AF5q31$^{-/-}$ male mice and 9-week-old W/W^{v} male mice. RT-PCR for GAPDH confirms the equivalent amounts of RNAs used for the analysis. (C) Expression of *AF5q31* during juvenile testis development in mice. RT-PCR analyses of *AF5q31* exons V to VIII and several marker genes in testis are demonstrated. RT-PCR for GAPDH confirms the equivalent amounts of RNAs used for the analysis. (D) Expression of spermatogenesis- and spermiogenesis-related genes in the testes of 12-week-old AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ male mice. RT-PCR for GAPDH confirms the equivalent amounts of RNAs used for the analysis. (E) RNAPII CTD phosphorylation in AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ MEFs. Whole-cell extracts (10 μg/lane) were immunoblotted with the indicated antibodies. As a control, anti-α-tubulin was used to monitor the loading amounts. (F) In vitro kinase assay of P-TEFb in the presence or absence of AF5q31. Chromatography of purified HA-AF5q31-Flag on a Mono Q column revealed the presence of full-length AF5q31 (140 kDa). Each fraction (4 μl) on the Mono Q column was analyzed by SDS-PAGE and silver staining (upper panel). The lane marked "In" represents a part of the material before loading the column, and the lane marked "FT" indicates the flowthrough of the Mono Q column. Equal aliquots from each fraction were added to the kinase reaction mixture containing P-TEFb and GST-CTD and resolved by SDS-PAGE. Phosphorylated GST-CTD was detected by autoradiography (lower panel).

spermatogenesis is arrested at the stage of spermiogenesis in AF5q31$^{-/-}$ mice.

Mechanism of infertility in AF5q31$^{-/-}$ mice. Immunohistochemical analysis on testes with a purified anti-AF5q31 antibody disclosed that AF5q31 was expressed preferentially in Sertoli cells, weakly in germ cells, and barely in Leydig cells (Fig. 6A). Consistent with this finding is that *AF5q31* expression in RT-PCR analysis was elevated in c-*kit* mutant W/W^{v} male mice which harbor greatly reduced numbers of germ cells (38), compared with that in the mice with the normal c-*kit* gene (Fig. 6B). This pattern in RT-PCR analysis is similar to that of *GATA1* which is expressed only in Sertoli cells in the testis (71)

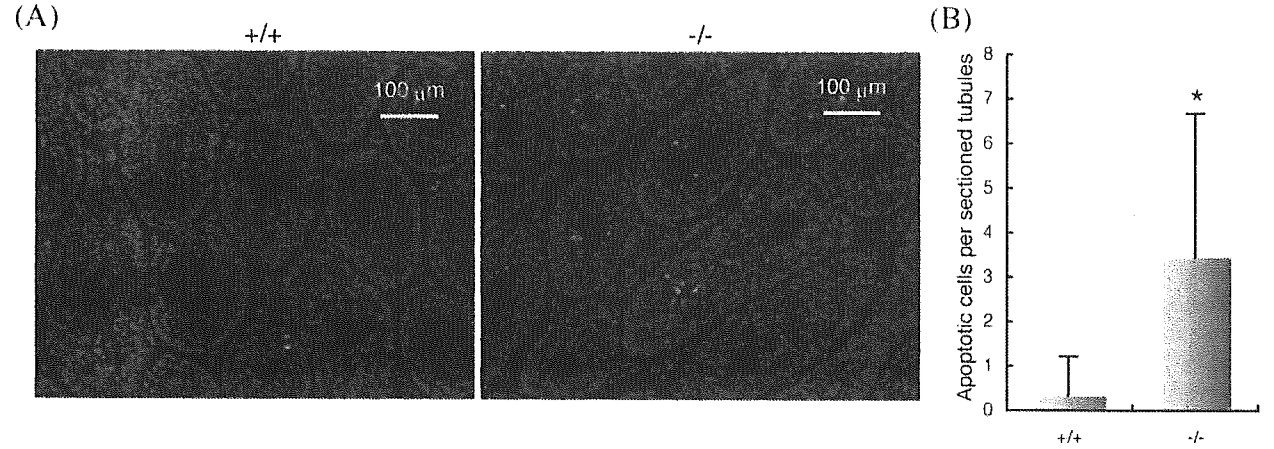

FIG. 7. Germ cell apoptosis in AF5q31$^{+/+}$ and AF5q31$^{-/-}$ mice. (A) Apoptotic cells detected by an in situ TUNEL assay in testis sections from 12-week-old AF5q31$^{+/+}$ (left) and AF5q31$^{-/-}$ (right) mice. TUNEL-positive cells were seen with fluorescein isothiocyanate (green). All the cells were visualized with DAPI (blue). (B) Quantification of apoptotic germ cells in the seminiferous tubules of 12-week-old AF5q31$^{+/+}$ and AF5q31$^{-/-}$ mice. In each testis, TUNEL-positive (apoptotic) nuclei in more than 100 randomly sectioned seminiferous tubules were counted and averaged. Error bars represent standard errors. Statistical significance (*, $P < 0.01$) was assessed by Student's t test.

and is the opposite of that of the cyclic AMP-responsive element modulator (*CREM*), which is exclusively expressed in postmeiotic germ cells in the testis (5, 51) (Fig. 6B). Furthermore, early expression of *AF5q31* during testis development also supports the preferential expression of *AF5q31* in Sertoli cells (Fig. 6C). As Sertoli cells are known to regulate spermatogenesis through the interactions with germ cells (23, 62), we determined if the transcription of some of spermatogenesis-related genes would be deregulated in AF5q31$^{-/-}$ mice by RT-PCR assays (Fig. 6D). Four genes which have critical roles in transcriptional regulation, *CREM*, TBP-related factor 2 (*TRF2*), retinoic acid receptor α (*RAR*α), and retinoid X receptor β (*RXR*β), were normally expressed in the testes of AF5q31$^{-/-}$ mice (5, 36, 40, 43, 51, 74). Furthermore, testis-specific cytoplasmic poly(A) polymerase (*Tpap*), sperm outer dense fiber protein (*RT7*), heat shock protein *Hsc70t*, mitochondria capsule selenoprotein (*Mcs*), and phosphoglycerate kinase-2 (*Pgk2*), which are known to be expressed in spermiogenesis, were not significantly changed, except for a slight decrease of *Mcs* in the mutant testes (35). After meiosis, histones are replaced by protamines (protamines 1 and 2 [*Prm1* and *Prm2*, respectively]) through transition proteins (transition proteins 1 and 2 [*TP1* and *TP2*, respectively]) in order to package the haploid genome within the sperm head in mammals (61). Intriguingly, expression levels of *TP2*, *Prm1*, and *Prm2* were drastically decreased and that of TP1 was slightly decreased in AF5q31$^{-/-}$ testes. But the expression levels of Ca^{2+}/calmodulin-dependent protein kinase IV (*Camk4*), which is expressed in spermatids and phosphorylates Prm2, did not differ among AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ mice (68, 69).

One report demonstrated that AF5q31 is associated with P-TEFb and may contribute to regulate RNAPII processivity by phosphorylation of the CTD (20). To monitor RNAPII phosphorylation in MEFs derived from AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ embryos, we did Western blotting with antibodies N20, H5, and H14 that recognize both the IIo and IIa RNAPII, Ser2, and Ser5 CTD phosphopeptides of RNAPII, respectively. Although the IIo form predominantly existed in MEFs, the proportion of the IIo to IIa form was not distinctly changed among AF5q31$^{+/+}$, AF5q31$^{+/-}$, and AF5q31$^{-/-}$ MEFs (Fig. 6E). The reason for this may relate to the compensation by other factors, including AF4, LAF4, and FMR2, in the absence of AF5q31. To assess the effect of AF5q31 on P-TEFb, an in vitro kinase assay was performed using reconstitution proteins. To obtain a sufficient quantity of AF5q31 for further biochemical studies, whole-cell lysates of Sf9 cells expressing epitope-tagged AF5q31 (N-terminal HA tag and C-terminal FLAG tag) were purified by immunoaffinity chromatography using anti-Flag and anti-HA antibody columns, successively. Epitope-tagged AF5q31 proteins were allowed to bind to a Mono Q column and were then eluted with a linear gradient from 200 mM to 400 mM KCl (Fig. 6F, upper panel). Fractions peaking from 320 to 380 mM KCl (fractions 7 to 9) were found to contain AF5q31. The activities of each eluate were compared by the CTD in vitro kinase assay (66, 67). However, the CTD phosphorylations corresponding to fractions 7 to 9 were not significantly changed from those corresponding to the other fractions (Fig. 6F, lower panel). These results suggested that AF5q31 regulates spermiogenesis through the modulation of tissue-specific gene expression in Sertoli cells rather than affecting general transcriptional machinery.

Germ cell apoptosis in AF5q31$^{-/-}$ mice. To further clarify why AF5q31$^{-/-}$ mice were infertile and azoospermatic, the frequency of apoptotic cells in testes was compared between AF5q31$^{+/+}$ and AF5q31$^{-/-}$ mice by using a TUNEL assay (Fig. 7A). This assay revealed a 6.5-fold increase in apoptotic germ cells in seminiferous tubules in 12-week-old mutant mice, yet these were barely detectable in wild-type littermates (Fig. 7B). Hence, AF5q31 appears to be essential in both the differentiation program and the survival of germ cells.

DISCUSSION

Incomplete penetrance of the embryonic and neonatal lethality observed in *AF5q31*-deficient mice indicates that the

loss of AF5q31 does not cause a complete and uniform block of embryogenesis at a given point but that AF5q31 possesses versatile roles during embryogenesis. Since *AF5q31* and *AF4* are widely expressed during embryogenesis and in the adult tissues of mice, it is possible that AF4 functionally compensates for the lack of AF5q31 in most tissues (4, 33). Presently, it is unclear why the embryonic and neonatal death occurs and whether the incomplete penetrance of this phenotype results from heterogeneity in the genetic background of the mutant mice.

Spermatogenesis is a multistep process from spermatogonia, which are the stem cells of the germ cell lineage, to spermatozoa (14). Sertoli cells play major roles in supporting spermatogenesis, which involves the complex interaction of germ cells and Sertoli cells within the seminiferous tubules (23, 62), and Leydig cells produce the testosterone. The expression of AF5q31 in Sertoli cells without the expression of other family genes in the testis suggests an indispensable role for AF5q31 in the testis. It should be kept in mind that serum levels of testosterone, LH, and FSH and expression levels of *AR*, *LH-R*, and *FSH-R* did not show any significant difference between the wild-type and AF5q31$^{-/-}$ mice. Thus, azoospermia in AF5q31$^{-/-}$ mice seems to be caused by functional defects in testicular somatic cells, particularly Sertoli cells. Several reports suggested that abnormal Sertoli cells were impaired regarding the ability to assist the normal maturation and release of spermatids in the deficient mice for the nuclear receptors and related cofactors such as *RARα*, *RXRβ*, *AR*, and *Cnot7* (10, 15, 30, 36, 40, 48). It is possible that AF5q31 functions as a coregulator of these transcription factors in spermatogenesis.

Human infertility affects 10 to 15% of couples, with an approximately equal contribution from both partners (16). In a large number of male infertility patients, the cause of the infertility might be related to disturbances in the replacement of histones by protamines during spermatogenesis. Previous reports stated that sperm from sterile males shows abnormal protein contents, with anomalously elevated levels of histones and/or an altered protamine 1/2 ratio (3, 11, 17). In mice and humans, genes encoding *Prm1*, *Prm2*, and *TP2* are clustered together on chromosome 16 (52). In addition, these three genes lie in the same orientation to one another and are coordinately expressed in a haploid-specific manner during spermatogenesis. Notwithstanding the subtle decrease of *TP1* expression, the levels of *TP2*, *Prm1*, and *Prm2* were dramatically reduced in AF5q31$^{-/-}$ mice. Previous studies demonstrated that the transcription of transition proteins and protamines initiates shortly after the completion of meiosis in round spermatids (after step 7 in spermiogenesis) and ceases in elongating spermatids (step 11) with a global repression of transcription (37, 42). In addition, the haplo-insufficient chimeras of *Prm1* and *Prm2* were infertile, displaying an abnormal nuclear condensation (12). Thus, the reduced levels of TP2, Prm1, and Prm2 may be the cause of spermiogenesis arrest in AF5q31$^{-/-}$ mice.

Selective decreases in the levels of mRNAs of *TP2*, *Prm1*, and *Prm2* among a set of postmeiotic genes in germ cells raise the possibility that AF5q31 also directly regulates the transcription of these genes. In fact, AF5q31 is weakly expressed in germ cells. It remains to be determined if Sertoli cells and germ cells are independently affected by the lack of AF5q31 or whether germ cells are secondarily affected, or both. Clarification of a potential role for AF5q31 in regulating the expression levels of *TP2*, *Prm1*, and *Prm2* may provide new insights into the mechanisms of human male infertility.

ALLs are characterized by the clonal proliferation, accumulation, and tissue infiltration of neoplastic cells (21). The majority of cases of ALL demonstrate abnormal karyotypes, either in chromosome number or as structural changes such as translocations, inversions, or deletions. As a consequence of translocations between chromosomes 5 and 11, the reciprocal fusion gene is generated and it encodes the MLL-AF5q31 fusion protein, which is expressed in the leukemic blasts (63). It is unknown whether the fusion protein can act as a dominant negative product on AF5q31 function in the leukemic blasts. However, the fact that AF5q31$^{-/-}$ mice did not show any hematological abnormalities suggests that the dominant negative effects of this fusion protein on AF5q31 in leukemogenesis are less likely. It is more likely that MLL-AF5q31 fusion leads to constitutive activation of the MLL target genes (1, 27). Clarification of the AF5q31-mediated gene regulation in testes will also help us to elucidate the molecular mechanism by which the fusion converts normal MLL into the leukemogenic form.

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Physiologic Assessment of Coronary Artery Stenosis without Stress Tests: Noninvasive Analysis of Phasic Flow Characteristics by Transthoracic Doppler Echocardiography

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We evaluated the significance of the diastolic-to-systolic blood flow velocity ratio (DSVR) determined by transthoracic Doppler echocardiography, for a physiologic assessment of the severity of coronary stenosis without stress tests, as compared with thallium 201 single photon emission computed tomography. In 95 patients undergoing thallium 201 single photon emission computed tomography for coronary artery disease, the flow velocity in the distal left anterior descending coronary artery was obtained with transthoracic Doppler echocardiography. The mean and peak DSVR values were calculated using mean and peak coronary flow velocity. DSVR was successfully measured for 82 patients (86.3%), including 33 patients with reversible perfusion defects in the left anterior descending coronary artery territories. For predicting reversible perfusion defects in thallium 201 single photon emission computed tomography, the best cut-off points were 1.5 for mean DSVR (sensitivity 81.8%, specificity 85.7%) and 1.6 for peak DSVR (sensitivity 75.7%, specificity 83.6%). Noninvasive measurement of DSVR with transthoracic Doppler echocardiography provides physiologic estimation of the left anterior descending coronary artery stenosis severity at high success rate, without stress tests. (J Am Soc Echocardiogr 2005;18:949-955.)

Coronary angiography is the currently accepted standard method for assessing coronary artery disease. However, several clinical reports have pointed out discrepancies between angiographic and physiologic estimates of coronary lesion severity.[1-3] Thus, some form of stress testing is often required to detect critical coronary stenosis.

Coronary blood flow velocity analyses have been proposed to allow additional evaluation of coronary artery stenosis, including the diastolic-to-systolic blood flow velocity ratio (DSVR), proximal/distal blood flow velocity ratio, and the coronary blood flow velocity reserve.[4-12] Despite the clinical usefulness of those parameters, they have been assessed mainly by invasive intracoronary Doppler guidewire and their clinical use is, therefore, generally restricted. It was recently reported that DSVR in the distal left anterior descending coronary artery (LAD), one of the coronary blood flow measurements available without stress tests, could be assessed by totally noninvasive transthoracic Doppler echocardiography (TTDE) with technologic advancement, and was useful for detecting angiographic severe stenosis in the LAD.[13-15] However, DSVR determined by TTDE has not been evaluated sufficiently to determine its usefulness for the physiologic assessment of the severity of coronary artery stenosis.

In this study, we evaluated the value of DSVR as determined by TTDE, for the physiologic assessment of the severity of coronary stenosis without stress tests, in comparison with exercise thallium 201 single photon emission computed tomography (Tl-SPECT). In addition, several recent reports have shown that contrast-enhanced Doppler echocardiography has a high success rate in the evaluation of coronary blood flow noninvasively.[16,17] We also evaluated the effect of contrast enhancement on improvement of measuring DSVR by high-frequency TTDE.

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METHODS

Patient Population

We prospectively enrolled 95 consecutive patients (mean age 63 ± 9 years; 78 men and 17 women) who had been admitted to our hospital for assessment of coronary artery disease. Exclusion criteria were previous myocardial infarction, previous cardiac operation, artificial pacemaker, nonsinus rhythm, significant valvular heart disease, angina at rest, chronic obstructive pulmonary disease, and congestive heart failure. All patients continued administration of anti-ischemic medication (nitrates, β-blockers, calcium antagonists) and antiplatelet agents (aspirin, 81 mg) on the day of the echocardiographic study. On 2-dimensional echocardiography, none had evidence of left ventricular wall-motion abnormality, left ventricular hypertrophy (wall thickness at end diastole >12 mm), or valvular heart disease. All patients underwent Tl-SPECT and coronary angiography within 1 week of the echocardiographic study. Informed consent was obtained from all patients included in the study. The protocol was approved by our hospital committee on medical ethics and clinical investigation.

TTDE

Echocardiography was performed with a digital ultrasound system (Sequoia 512, Siemens, Mountain View, Calif) using a high-frequency transducer (5-7 MHz). For color Doppler echocardiography, velocity was set in the range of ± 12.0 to ± 24.0 cm/s. Adequate filtering was used to minimize low-frequency wall-motion artifacts. Echocardiographic images were obtained from the acoustic window around the midclavicular line in the fourth and fifth intercostal spaces in the left lateral decubitus position. After the lower portion of the interventricular sulcus had been located in the long-axis cross section, the ultrasound beam was rotated laterally, visualizing the distal portion of LAD under color flow mapping guidance.[18] Color flow of LAD was visualized by high-frequency (3.5-MHz) color Doppler technique. Blood flow velocity was measured by pulsed wave Doppler (Doppler frequency 3.5 MHz) using a sample volume (2.0-3.0 mm) placed on the color signal in the distal LAD. We tried to align the ultrasound beam direction to the distal LAD flow in as parallel a manner as possible. In addition, it has been known that coronary blood flow can be accelerated locally at stenotic sites, and this accelerated coronary flow velocity could lead to the error in DSVR measurements. On the other hand, it has also been reported that localized aliasing, which reflects increased velocity over the velocity range, could be displayed at the stenotic sites in the coronary artery by color flow mapping,[19] even with nonsignificant stenosis (< 50%). Therefore, in the cases with localized color aliasing in LAD, we put the sample volume at the distal site to the aliasing, to avoid the error in DSVR measurements because of local accelerated coronary flow. All studies were continuously recorded on .5-in super-VHS videotape, and clips of stopped frames were also stored digitally on magneto-optical disks (230 MB) for offline analysis. Blood pressure and heart rate were recorded simultaneously.

Echocardiographic Contrast Enhancement

In cases where visualization of the color signals in LAD was unsuccessful or Doppler spectral tracing of velocity was not clear, an echocardiographic contrast agent (Levovist, Tanabe Seiyaku Inc, Osaka, Japan, and Schering, Berlin, Germany) was used to improve visualization of color Doppler signals and obtain clear spectral Doppler signals. On the basis of the results of previous studies,[16,17] contrast agent at 300 mg/mL was infused intravenously to a total volume of 7 mL at a rate of 1 mL/min by infusion pump during coronary blood flow velocity measurements. The infusion rate was adjusted in a range from 0.5 to 2.0 mL/min depending on the quality of the Doppler signal enhancement achieved.

Analysis of Coronary Flow Velocity Characteristics

An experienced operator with no knowledge of the results of Tl-SPECT and coronary angiography assessed DSVR. Measurements of blood flow velocity were performed offline using the integrated evaluation program in the ultrasound system. Four parameters were measured by tracing the contour of the Doppler velocity pattern: (1) mean systolic velocity; (2) mean diastolic velocity; (3) peak systolic velocity; and (4) peak diastolic velocity. Values for each parameter were obtained by averaging measurements from 3 consecutive cardiac cycles. Mean and peak DSVRs were computed as the ratios of diastolic-to-systolic mean and peak coronary flow velocities.

Exercise Tl-SPECT

Tl-SPECT was performed within 1 week of the echocardiographic studies by TTDE. All patients performed symptom-limited exercise on a bicycle ergometer in the sitting position. Nitrates, β-blockers, and calcium channel blockers were withheld on the morning of the test. Twelve-lead electrocardiograms and blood pressure measurements were obtained at baseline and every minute during exercise. The initial workload was 50 W, which was increased by 25 W every 2 minutes until an end point was reached. The end points included excessive fatigue, dyspnea, dizziness, angina, hypotension, diagnostic S-T segment depression (> 1.5 mm horizontal or downsloping or > 2.0 mm upsloping), or significant arrhythmia. At peak exercise a dose of 111 MBq of Tl-201 was injected intravenously. The initial images were obtained immediately after the termination of exercise, and delayed images were obtained 4 hours later.

Single-photon emission computed tomography was performed using a single-head gamma scintillation camera equipped with a low-energy, all-purpose, parallel-hole collimator. A total of 32 equidistant projections were acquired over 180 degrees from the right anterior oblique to the left posterior oblique view at 25 s/projection.

On the Tl-SPECT images, anteroseptal and apical segments were considered to be in the LAD territory. The images were analyzed individually by two experienced nuclear physicians who had no knowledge of the angiographic or echocardiographic data. Disagreements in interpretation were resolved by consensus of the two physicians. The patients were considered to have myocardial ischemia when Tl-SPECT revealed perfusion defects with redistribution on delayed imaging.

Coronary Angiography

Coronary angiography was performed in all patients using standard techniques within 1 week of echocardiographic studies by TTDE. Angiographic data were subsequently analyzed by an experienced investigator who had no knowledge of the echocardiographic or Tl-SPECT results. The severity of coronary stenosis was visually determined and expressed as the percent lumen diameter. Stenosis was considered significant if there was more than 50% diameter stenosis in at least one projection. Electrical calipers were used when necessary.

Statistical Analysis

On the basis of the Tl-SPECT data for the LAD territories, the study patients were classified into group A with abnormal perfusion and group B with normal perfusion. Parametric data were presented as mean ± SD. Categoric variables were compared using Fisher exact test. Mean and peak DSVR values for groups A and B were compared by unpaired *t* test. For all analyses, $P < .05$ was considered significant. For mean and peak DSVRs, sensitivity and specificity, as predictors of abnormal perfusion in Tl-SPECT, were determined for ratio values (in 0.1 increments) between 1.0 and 2.0, with determination of ratio cut-off values yielding the highest combined sensitivity and specificity values. The positive and negative predictive values of DSVR for determination of the presence of abnormal perfusion in Tl-SPECT were determined in the traditional manner.

RESULTS

Effect of Contrast Enhancement on Coronary Flow Velocity Measurements

Of the 95 study patients, adequate spectral Doppler recordings of coronary flow through both systole and diastole in distal LAD were obtained for 82 patients (86.3%), including 14 patients given an echocardiographic contrast agent to improve spectral Doppler signals. The use of contrast enhancement improved the feasibility of spectral Doppler recording with high-frequency TTDE in LAD from 71.6% to 86.3% (Figure 1). In the other 13 patients DSVR measurements could not be obtained because of obscure color Doppler signal (3 patients) or spectral Doppler recordings of only diastole (10 patients), even with contrast enhancement, and they were excluded from the study. Therefore, 82 patients comprised the study group in which we compared echocardiographic data with Tl SPECT results.

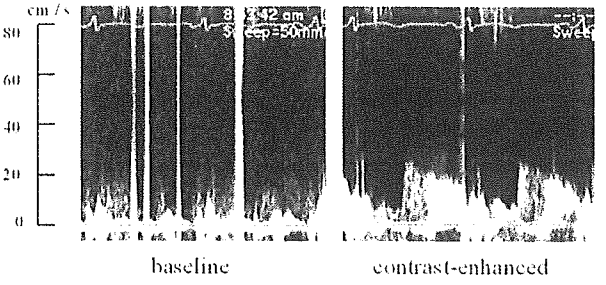

Figure 1 Use of contrast enhancement improved spectral Doppler signal recording in left anterior descending coronary artery (**B**) in case with poor Doppler signals (**A**).

Tl-SPECT

All patients in this study performed exercise tests until the end points. Of the 82 patients, 33 qualitatively exhibited abnormal perfusion in the LAD territories on Tl-SPECT and were classified into group A. The remaining patients (n = 49) had normal perfusion (n = 34) or abnormal perfusion in other territories (n = 15) and were, thus, classified into group B (49 patients). The peak heart rate and rate-pressure product were similar in groups A and B for all exercise tests. No significant difference was found in terms of age, sex, heart rate, and systemic blood pressure between groups A and B (Table 1).

Coronary Angiography

Coronary angiography demonstrated significant coronary stenosis from mid- to proximal LAD of all 33 patients of group A, and in 15 patients of group B. Of the 13 patients who could not undergo Doppler recording, two had total occlusion in the proximal LAD, with good collateral flow from the right coronary artery.

DSVR Measured by TTDE Versus Tl-SPECT

There were no significant differences in both mean and peak systolic flow velocities between groups A and B (Table 2). However, both mean and peak diastolic velocities were significantly smaller in group A than in group B (16.3 ± 5.2 vs 19.8 ± 6.7 cm/s and 20.7 ± 6.5 vs 25.0 ± 9.1 cm/s, $P < .05$, respectively) (Table 2). Thus, there were significant differences in both mean DSVR and peak DSVR between groups A and B (1.4 ± 0.4 vs 1.9 ± 0.6 and 1.4 ± 0.4 vs 2.0 ± 0.5, $P < .0001$, respectively) (Table 2; Figure 2).

For determination of physiologic stenosis in LAD by Tl-SPECT (abnormal perfusion), the sensitivity

Table 1 Clinical data

	Group A (N = 33)	Group B (N = 49)
Age, y	63 ± 8	62 ± 11
Sex, M/F	28/5	38/11
SBP, mm Hg	129 ± 25	123 ± 21
DBP, mm Hg	71 ± 13	69 ± 12
HR, beats/min	58 ± 11	61 ± 12

DBP, Diastolic blood pressure; *F*, female; *HR*, heart rate; *M*, male; *SBP*, systolic blood pressure.Data presented are mean value ± SD or No. of patients.

Table 2 Coronary flow velocity and diastolic-to-systolic flow velocity ratio

	Group A	Group B
MCFV, cm/s		
Systolic	11.7 ± 3.7	10.9 ± 3.9
Diastolic	16.3 ± 5.2	19.8 ± 6.7*
PCFV, cm/s		
Systolic	14.1 ± 4.5	13.4 ± 5.5
Diastolic	20.7 ± 6.5	25.0 ± 9.1*
DSVR		
Mean	1.4 ± 0.4	1.9 ± 0.6†
Peak	1.4 ± 0.4	2.0 ± 0.5†

DSVR, Diastolic-to-systolic flow velocity ratio; *MCFV*, mean coronary flow velocity; *PCFV*, peak coronary flow velocity.
*$P < .05$ vs. group A; †$P < .0001$ vs group A.

and specificity curves for the mean and peak DSVRs were obtained and shown in Figure 3. The best cut-off points were 1.5 for mean DSVR (sensitivity 81.8%, specificity 85.7%, positive predictive value 79.4%, negative predictive value 87.5%) and 1.6 for peak DSVR (sensitivity 75.7%, specificity 83.6%, positive predictive value 75.7%, negative predictive value 83.6%).

Observer Variability

Interobserver and intraobserver variabilities for measurement of Doppler velocity recording were 5.0% and 3.9%, respectively.

DISCUSSION

This study demonstrated that measurement of DSVR by TTDE permits noninvasive and physiologic assessment of the severity of LAD stenosis and prediction of myocardial ischemia in comparison with the value obtained by exercise myocardial perfusion imaging results. It also showed that the combination of contrast enhancement and high-frequency transducer has a high success rate in the noninvasive measurement of DSVR by TTDE.

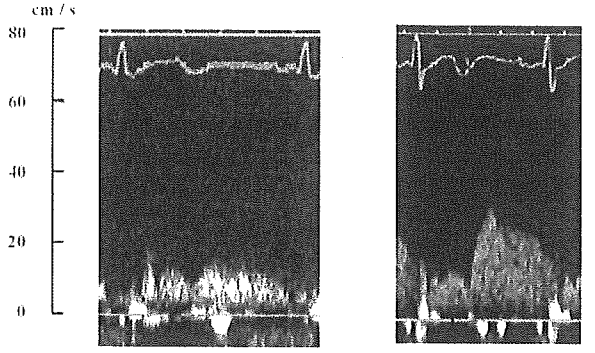

Figure 2 Examples of spectral Doppler signals for patients with abnormal (*left*) and normal (*right*) perfusion in left anterior descending coronary artery territories.

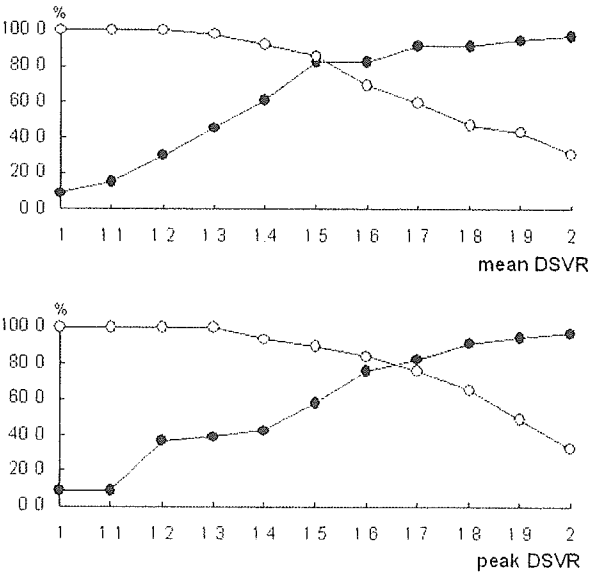

Figure 3 Sensitivity (*open circles*) and specificity (*closed circles*) as functional cut-off values over whole spectrum of mean (*top*) and peak (*bottom*) diastolic-to-systolic blood flow velocity ratio (DSVR). Best cut-off points were 1.5 for mean DSVR (sensitivity, 81.8%; specificity, 85.7%) and 1.6 for peak DSVR (sensitivity, 75.7%; specificity, 83.6%).

Physiologic Assessment of Coronary Stenosis by TTDE

Experimental studies have indicated that coronary blood flow at rest generally can be maintained in coronary stenosis with the severity of less than 85% by autoregulation mechanism.[20,21] However, coronary angiography has limited reliability in predicting the physiologic significance of coronary artery stenosis in the clinical stetting.[1-3] Even an anatomic quantitative intravascular ultrasound has been reported to lack power to predict the physiologic response of coronary stenosis.[22] Thus, clinical decisions concerning the presence of myocardial isch-

emia depended mainly on diagnostic information from stress tests such as the radionuclide stress test or stress echocardiography. This discrepancy between angiographic and physiologic estimates of coronary lesion severity led to research into the evaluation of coronary hemodynamics using the invasive Doppler guidewire.

This study is the first to report that the evaluation of noninvasive measurement of DSVR by TTDE may reveal the physiologic severity of coronary stenosis in the clinical setting, as confirmed by a previous report that the phasic coronary flow pattern was altered with increasing amounts of fixed partial obstruction, and DSVR was decreased as percent stenosis was increased in an experimental study.[4]

Our predictive value of 1.5 for mean DSVR (sensitivity 81.8% and specificity 85.7%) and 1.6 for peak DSVR (sensitivity 75.7% and specificity 83.6%) for physiologic significance of coronary stenosis are higher compared with a previous report in which DSVR by TTDE was compared with angiographic 85% stenosis (1.5 for mean DSVR, sensitivity 77.0% and specificity 77.9%; 1.6 for peak DSVR, sensitivity 79.0% and specificity 75.7%).[15] Despite autoregulation of coronary blood flow suggested by experimental studies,[20,21] a number of studies have shown altered poststenotic phasic coronary blood flow pattern,[4-15] sometimes even in moderate coronary artery stenosis less than 85% obstruction.[10,11,14] Furthermore, some have indicated that altered poststenotic phasic coronary blood flow patterns can reflect the results of some form of examinations for physiologic assessment of coronary stenosis.[10,11,14] These facts support the concept that angiography has limited reliability in predicting physiologic significance of coronary stenosis, and are consistent with our results.

Comparison with Previous Methods

Recently, we reported that measurement of coronary flow reserve by TTDE provides physiologic information on the severity of coronary stenosis equivalent to that obtained with Tl-SPECT with a high success rate (92%).[23] As a matter of fact, coronary flow reserve is a better predictor than DSVR measurement, as indicated in that previous report.[23] Furthermore, a higher success rate could be achieved in the measurement of coronary flow reserve than in that of DSVR because of the difficulty in recording systolic blood flow with TTDE,[16] as also shown in the current study where the recording of diastolic blood flow only was obtained in 10 of the patients. However, the fact that measurement of DSVR by TTDE does not require any pharmacologic stress, which is associated with atrioventricular block, systemic hypotension, and dyspnea,[24] represents an important advantage in addition to the fact that it is an absolutely noninvasive procedure. To our knowledge, no previous studies have reported any other modalities applicable in the clinical setting that allow functional estimates of the severity of coronary stenosis, without hemodynamic monitoring or stress test, besides our method.

Coronary Flow Velocity Measurements with Contrast-enhanced TTDE

In this study, we measured spectral Doppler recordings of coronary flow through both the systole and diastole in distal LAD with a high success rate (86.3%), sufficient for application in the clinical setting, using contrast-enhanced Doppler recording and a high-frequency transducer. Measurement of DSVR by TTDE is the only noninvasive method for assessing the severity of coronary stenosis, without any specific stress testing, available in the clinical setting thus far. However, its success rate was often not high enough to allow its clinical use. These days, technical advancements in Doppler echocardiography, including second-harmonic Doppler imaging and contrast-enhanced Doppler imaging, have enabled us to assess coronary blood flow noninvasively with a high success rate. In particular, contrast enhancement has proved to be useful in increasing the Doppler signal-to-noise ratio in coronary artery by increasing the amplitude of the signal[25,26] and in detecting color Doppler signals in LAD with second-harmonic technology.[16,17]

On the other hand, a high-frequency transducer also has been known to be useful for assessing diastolic coronary blood flow with a high success rate.[18,23,27] This study demonstrated that the combination of contrast enhancement and high-frequency transducer also allows the noninvasive assessment of coronary blood flow velocity through both systole and diastole by TTDE.

Study Limitations

First, there were still some cases in which it was difficult to obtain complete Doppler spectral envelopes throughout the entire cardiac cycle. However, as can be expected from recent studies, further technical advancements will result in even higher success rates for this noninvasive method of DSVR measurement in the near future.

In this study, the angle between the Doppler beam and the artery was quite large (> 30 degrees) in a certain number of cases, causing underestimation of the true flow velocity. However, for the purpose of DSVR evaluation, the absolute velocity value was not needed because DSVR is a quotient of two velocities.

We excluded several factors influencing DSVR measurement such as left ventricular hypertrophy, systemic hypertension, and myocardial infarction in this study. Other potential determinants of DSVR that were not measured and were not excluded in

this study may affect the physiologic estimates of the severity of LAD stenosis by our technique. Moreover, stenotic lesions other than the proximal LAD possibly affected our DSVR assessment. Further studies are needed that include patients with these variables.

Our method of physiologic assessment of coronary artery stenosis with TTDE is currently restricted to LAD for anatomic reasons. However, one randomized trial has shown that coronary artery bypass grafting improved survival of those with significant stenosis of the proximal portion of LAD in the 2- or 3-vessel coronary artery disease.[28] Accordingly, physiologic assessment of the severity of LAD stenosis can have a substantial clinical impact on the prognosis of patients with coronary artery disease.

Finally, we used exercise Tl-SPECT as a gold standard for assessing the physiologic severity of LAD stenosis. Indeed, there are not any reports that Tl-SPECT can reveal absolute physiologic severity of coronary stenosis. However, exercise TL-SPECT has been widely accepted as a method for physiologic estimates of the severity of coronary artery stenosis in routine clinical practice. Furthermore, many previous studies[10,23] have elucidated the usefulness of various parameters for the physiologic assessment of the severity of the coronary artery stenosis in comparison with exercise Tl-SPECT. Therefore, we believe that the use of exercise Tl-SPECT should be valid for our study design.

Clinical Implications

The fact that our procedure, compared with conventional methods, is totally noninvasive, inexpensive, and generally available, without any stress testing accompanied by the possibility of side effects, is considered an important advantage. It may provide additional information to physicians for making clinical decisions on the physiologic severity of coronary stenosis. It may also be useful for cases in which exercise stress testing cannot be performed.

Furthermore, stress echocardiography, including myocardial contrast echocardiography, for the diagnosis of coronary artery disease has recently gained increasing popularity. The assessment of DSVR during these stress echocardiograms is assumed to be available and must provide important diagnostic information for the physiologic estimate of the severity of LAD stenosis.

Conclusions

The measurement of DSVR by TTDE provides physiologic information concerning the severity of LAD stenosis without stress testing and the combination of contrast enhancement and high-frequency transducer allows high feasibility of this measurement with high success rate. This totally noninvasive method is free from the side effects of drugs and is useful for the clinical evaluation of myocardial ischemia.

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Promotion of Cardiac Regeneration by Cardiac Stem Cells

Toshio Nagai, Ichiro Shiojima, Katsuhisa Matsuura, Issei Komuro

Research on myocardial regeneration is an exciting and promising area, which challenges the dogma that the heart is a nonregenerating organ. Recently, several methods of stem cell therapy have been developed. One method is to transplant cells into the infarcted area of the myocardium. Currently, clinical trials of autologous skeletal myoblast transplantation into the failed heart are underway and have been reported to improve the cardiac function.[1] However, the mechanism of its efficacy is unknown, and there are some questions about the safety because myoblasts do not transdifferentiate into cardiomyocytes and may induce lethal arrhythmia.[1] In this point, embryonic stem (ES) cells that can differentiate into cardiomyocytes are thought to be more promising.[2] For patients experiencing extensive myocardial infarction or dilated cardiomyopathy, however, the effectiveness of cell transplantation is questionable. Bone marrow–derived cells have been reported to transdifferentiate into various types of cells in situ. Indeed, bone marrow–derived stem cells were reported to prevent left ventricular remodeling after myocardial infarction and improve cardiac function by their differentiation into cardiomyocytes.[3] However, recent accumulating evidence has indicated that very few bone marrow cells, if any, transdifferentiate into cardiomyocytes.[4–6] Cytokine therapy using G-CSF strongly prevents ventricular remodeling after myocardial infarction by antiapoptotic and angiogenic effects, but not by recruitment of bone marrow cells.[7]

Over the past few years, adult hearts have been reported to contain the cardiac stem/progenitor cells such as c-kit+,[8] Sca-1+,[9,10] isl-1+,[11] and side population cells.[12] Because these cells have the ability to proliferate and differentiate into cardiomyocytes in vitro and in vivo, they might have the potential to regenerate the injured heart. However, there is almost no regeneration in the human heart after myocardial infarction, suggesting that cardiac regeneration accomplished by proliferation, migration, and differentiation of the cardiac stem/progenitor cells, is inhibited in vivo.

In this issue of *Circulation Research*, Urbanek et al address the possibility of cardiac regeneration by inducing migration and protection of cardiac stem cells and early committed cells (CSCs–ECCs) in a rodent myocardial infarction model.[13] CSCs are defined by the expression of the stem cell-related antigens, c-kit, Sca-1, or MDR1. A fraction of CSCs, which expressed MEF2C (cardiac transcription factor), GATA6 (smooth muscle cell transcription factor), or Ets-1 (endothelial cell transcription factor) was named ECCs. Immunohistochemical analysis revealed that CSCs–ECCs express HGF receptor c-Met and IGF-1 receptor (IGF-R) and, under the treatment with their ligands, cultured CSCs–ECCs secreted HGF/IGF-1. When myocardial infarction was produced and human HGF/IGF-1 was locally injected, expression levels of murine mRNA and proteins for HGF/IGF-1 in infarcted tissue were increased. Activation of these growth factor signals was confirmed by phosphorylation of c-Met, IGF-R, and their downstream targets. Migration studies demonstrated that HGF promoted motogenic and invasive activity of CSCs–ECCs, whereas IGF-1 had little effect. Conversely, IGF-1 showed more antiapoptotic and proliferative effects on CSCs–ECCs compared with HGF.

Based on these in vitro findings, Urbanek et al examined whether HGF/IGF-1 stimulate migration, proliferation, and differentiation of CSCs–ECCs in the infarcted heart. They used highly sophisticated techniques to evaluate the migration of CSCs–ECCs. They found that cycling CSCs–ECCs exist in the atrioventricular groove so that retrovirus expressing enhanced green fluorescent protein (EGFP) was injected in this region. After EGFP was integrated into the CSCs–ECCs, myocardial infarction was made and subsequently HGF/IGF-1 were injected into the predicted pathway of migrating cells with gradient of their concentration. They examined ex vivo heart preparation by 2 photon microscopy and demonstrated migration of EGFP-positive cells toward the infarcted area through the interstitium of the heart. Furthermore immunohistochemical analysis showed that the locomotive EGFP-positive cells possess the characteristics of CSCs–ECCs. Consistent with their in vitro data, HGF but not IGF-1 had locomotive effects on CSCs–ECCs. Regenerated myocardium after the HGF/IGF-1 combined treatment was identified as BrdU positive cardiomyocytes and vessels. HGF/IGF-1 treatment increased the number of newly-formed cells, resulting in an increased volume of myocardium, improvement of cardiac function, and better survival. The BrdU-positive new myocytes isolated from regenerated myocardium are smaller than old cells, and these small cells exhibit better contractile function than cardiomyocytes isolated from spared myocardium.

The same group has reported that self-renewing, clonogenic, and multipotent cardiac stem cells exist in the various species, including human[8,14,15]. However, in vivo kinetics of cardiac stem cells had been unknown because of the difficulty

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of labeling and tracking of the cardiac stem cells in vivo. Urbanek et al overcame the difficulty by their extensive and skillful analyses and showed that exogenously-applied HGF induced migration of distant cardiac stem cells toward the infarcted area through the pathway defined by fibronectin. Despite the fact that many types of cytokines and growth factors, including HGF/IGF-1, were released in the ischemic myocardium, the resident stem cells cannot survive in infarcted myocardium, and the stem cells in the distant area do not take a part in regenerating heart tissue. The findings of this study suggest that the amount of the intrinsic factors are far less than the amount necessary to break the silence of the resident stem cells or protect them from apoptosis.

Although the study by Urbanek et al sheds light on intrinsic stem cell therapy by local administration of growth factors, it also raises several important questions that should be addressed by future studies. First, what determines the CSCs–ECCs quiescence in "cardiac niche"? In bone marrow niche, angiopoietin-1/Tie-2 signaling is critical for the maintenance of hematopoietic stem cell quiescence.[16] Although it is not clear whether the similar mechanism is present in the heart, it is possible that signals to maintain CSCs–ECCs quiescence are antagonized by HGF/IGF-1 signaling. CSCs–ECCs coexpress HGF/IGF-1 and c-Met/IGF-R, and there is a positive feedback loop of HGF/IGF-1 signaling in CSCs–ECCs. Therefore, CSCs–ECCs may exit from the quiescence when challenged by higher doses of HGF/IGF-1, and the activated state of CSCs–ECCs may be maintained by the positive feedback loop of HGF/IGF-1 signaling. Identification of the mechanism that maintains CSCs–ECCs quiescence will be of particular importance, because inhibition of CSCs–ECCs quiescence can be a novel strategy to promote myocardial regeneration by enhancing CSCs–ECCs proliferation. Second, how does HGF induce migration of CSCs–ECCs to the infarct area? Among several signaling molecules activated by HGF, PI3-kinase (PI3K) seems to be critical for HGF-mediated cell migration, because PI3K is required for HGF-induced lamellipodia formation and subsequent migration in MDCK and C2C12 cells.[17–19] Whether PI3K is also critical in CSCs–ECCs migration should be determined. In addition, it is possible that some negative regulators of CSCs–ECCs migration are expressed in the infarct area. Inhibition of such factors can be another strategy to promote myocardial regeneration. Third, what induces apoptosis of CSCs–ECCs in the infarct area? Small numbers of CSCs–ECCs migrate into infarct area without growth factor treatment. Understanding of the mechanism of the apoptosis would lead to more specific treatment by protecting stem cells. Finally, it is interesting to examine the effect of HGF/IGF-1 treatment in chronic heart failure models. In the study by Urbanek et al, animals were treated with growth factors 5 hours after coronary ligation. Whether administration of HGF/IGF-1 is also effective in the chronic stage after myocardial infarction, when ventricular remodeling is already established, should be investigated. Likewise, to determine whether growth factor treatment is also effective in dilated cardiomyopathy or other models of chronic heart failure with diffuse contractile dysfunction will be of great importance.

In summary, the study by Urbanek et al clearly demonstrates the therapeutic potential of CSCs–ECCs for myocardial regeneration. To address the questions raised by Urbanek et al would further advance our understanding of the stem cell system in the heart and provide important clues to the development of novel therapeutic strategies for heart diseases.

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KEY WORDS: cardiac stem cell ■ cardiomyocytes ■ regeneration ■ growth factor ■ myocardial infarction

Pulmonary Vein Morphology Before and After Segmental Isolation in Patients with Atrial Fibrillation

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UEDA, M., ET AL.: Pulmonary Vein Morphology Before and After Segmental Isolation in Patients with Atrial Fibrillation. ***Background:*** *The morphology of the pulmonary veins (PVs) before and after segmental isolation of the PVs has not been sufficiently characterized.*

Methods and Results: *Multi-slice computed tomography was performed before and 3 ± 1 months after ablation in 30 patients with atrial fibrillation who underwent PV isolation. Before ablation, PV narrowing (≥25% luminal reduction) was found in nine (8%) PVs. After ablation, de novo PV narrowing was found in 24 PVs (26%) and was detected only in the supero-inferior direction in 14 PVs (58%). The diameter reduction inside the PVs after ablation was greater in the supero-inferior direction (14 ± 12%) than in the antero-posterior direction (9 ± 13%; P < 0.0001). In the ablated PVs, the PV trunk was shorter than before ablation (P < 0.0001). The reduction in the diameters of both the PV ostium and the ablation site in the ablated PVs, as well as the diameter of the PV ostium in the nonablated PVs, correlated with the decrease in the left atrial diameter. Shortening of the PV trunk correlated with the severity of PV narrowing, but it was not related to the percent diameter reduction of the left atrium. PV narrowing before or after ablation did not result in any clinical consequences.*

Conclusions: *PV narrowing is present in about 10% of PVs before ablation. Asymmetric luminal reduction and longitudinal shrinkage of the PV trunk occur after ablation. Reverse remodeling of the PV and contraction of the PV wall may contribute to the reduction in the PV diameter. PV morphology should be assessed with multi-directional views to avoid missing heterogeneous legions. (PACE 2005; 28:944–953)*

atrial fibrillation, catheter ablation, radiology

Introduction

Segmental ostial ablation to isolate the pulmonary veins (PV isolation) has been demonstrated to be effective in curing atrial fibrillation (AF).[1,2] However, radiofrequency (RF) energy delivery in the PVs is associated with the development of PV stenosis.[3–6] The morphology of the PVs and anatomical alternation after RF ablation have been investigated using magnetic resonance imaging and computed tomography (CT).[4–7] However, a detailed and quantitative analysis of the PV morphology after ablation using multi-directional views has not been performed. Furthermore, the presence and incidence of PV narrowing before ablation or longitudinal PV shrinkage after ablation has not yet been examined. It is well known that AF can induce structural remodeling of the atria and cause atrial enlargement, which may be reversible if AF is converted and sinus rhythm maintained (reverse remodeling).[4,5] However, reverse remodeling of the PVs after ablation has not been sufficiently demonstated. The purpose of this study was to clarify those points.

Methods

Study Patients

This study included 30 patients with drug-resistant, paroxysmal AF (24 men, 6 women; mean age, 59 ± 8 years). The mean AF duration was 7 ± 7 years, and the mean number of symptomatic AF episodes per month was 20 ± 22. Echocardiography demonstrated a mean left ventricular ejection fraction of 0.63 ± 0.09. Two patients had coronary artery disease and the remaining 28 had no structural heart disease. In 10 patients (33%), linear ablation of the cavo-tricuspid isthmus was also performed for typical atrial flutter at the time of PV isolation. Multi-slice CT was performed within 1 week before and 3 ± 1 months after the PV isolation. At the time of acquisition of the CT before and after the PV isolation, all patients were in normal

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