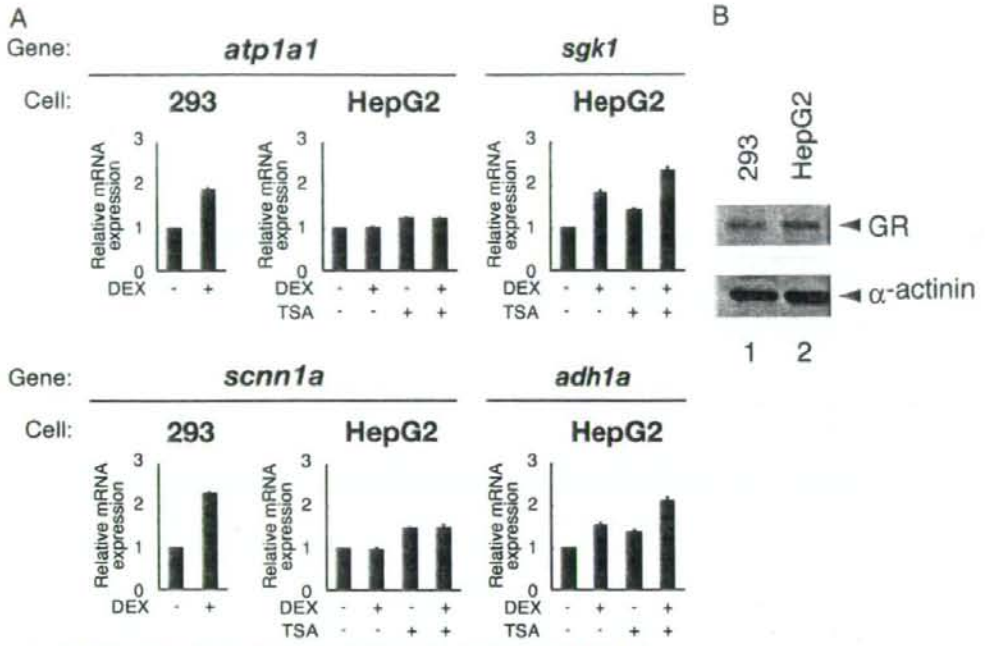

Fig. 1. DEX Resistance of *atp1a1* and *scnn1a* Not in 293 Cells but in HepG2 Cells

A, 293 cells and HepG2 cells were cultured in phenol red-free Opti-MEM I for 24 h and treated with or without 100 nM DEX for 6 h in the presence or absence of 100 nM TSA, as indicated. Total RNA was prepared and endogenous mRNA for Na^+, K^+-ATPase α1 (*atp1a1*), amiloride-sensitive Na^+ channel 1α (*scnn1a*), serum and glucocorticoid-regulated kinase 1 (*sgk1*), alcohol dehydrogenase 1A (*adh1a*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was measured with qRT-PCR. Samples were normalized to *gapdh* mRNA levels, and relative expression levels to vehicle-treated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. B, Cell lysates from 293 cells and HepG2 cells were subjected to Western blot analysis using indicated antibodies.

2.3-fold, respectively (Fig. 1A). In contrast, mRNA expression of neither *atp1a1* nor *scnn1a* was induced in the presence of 100 nM DEX in HepG2 cells (Fig. 1A). Our previous DNA microarray analysis also supported this notion (data not shown). Note that GR is comparably expressed in HepG2 cells and 293 cells (Fig. 1B), and that mRNA expression of *sgk1* and *adh1a*, both of which are known to be glucocorticoid target genes as well (20–23), are induced by 1.8-fold and 1.6-fold, respectively, after DEX treatment in HepG2 cells (Fig. 1A). It is indicated, therefore, that, despite the presence of functional GR, mRNA expression of a particular set of genes, *i.e. atp1a1* and *scnn1a*, shows a cell- or tissue-specific resistance to glucocorticoids. Treatment with a histone deacetylase inhibitor trichostatin A (TSA) increased both basal and induced mRNA levels of *sgk1* and *adh1a*, but, in the case of *atp1a1* and *scnn1a*, only basal mRNA levels were increased without restoration of responsiveness to DEX (Fig. 1A). Glucocorticoid insensitivity of *atp1a1* and *scnn1a* in HepG2 cells, thus, does not appear to be related to histone acetylation-dependent mechanisms.

Cell Type Difference in Hormone-Dependent GR Recruitment onto GRE in *atp1a1* Promoter

In GR-dependent transactivation, binding of liganded receptors onto target DNA is generally believed to be an essential trigger for transcription initiation and elongation (12–15). We, therefore, tested *in vivo* occupancy of *atp1a1* promoter by endogenous GR in comparison with *sgk1* promoter using chromatin immunoprecipitation (ChIP) assay, because both promoters are known to contain GRE (Fig. 2A) (49, 50). In HepG2 cells, when *sgk1* promoter was tested, GR was recruited onto the promoter in a time- and hormone-dependent manner. In contrast, *atp1a1* promoter did not recruit GR even in the presence of DEX (Fig. 2A). Next, to examine the relationship between GR recruitment and ongoing transcription, we compared GR binding and RNAPII binding on *atp1a1* promoter after DEX treatment. RNAPII binding, as well as that of GR, was not increased after DEX treatment in HepG2 cells. In clear contrast, RNAPII binding was enhanced after DEX treatment in concert with increase of GR binding in 293 cells (Fig. 2B). These results indicate that glucocorticoid

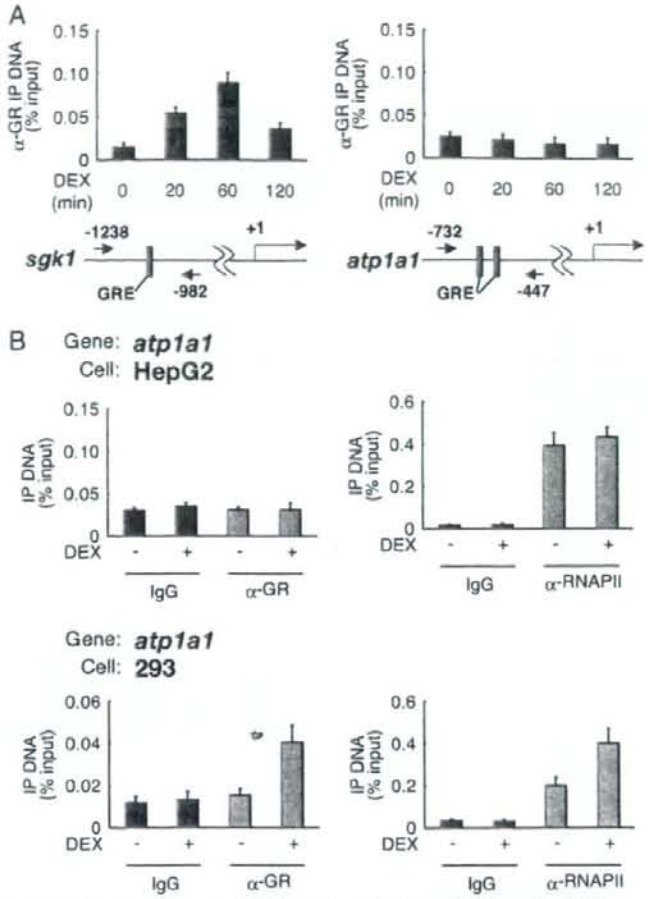

Fig. 2. Cell Type Difference in Hormone-Dependent GR Recruitment onto *atp1a1* Promoter GRE

A, HepG2 cells were cultured in phenol red-free Opti-MEM I for 24 h and treated with 1 μM DEX for the indicated time periods. ChIP assays were performed with anti-GR polyclonal antibodies, and recovered GRE-containing DNA fragments were measured with qRT-PCR. GREs in *sgk1* and *atp1a1* 5′-flanking regions are indicated as *gray boxes*. The positions of the primers are shown as *numbered arrows*. Values are expressed as percentage of immunoprecipitated DNA to input. *Error bars* represent SD values of at least three independent experiments. B, HepG2 cells and 293 cells were cultured as described in panel A and treated with 1 μM DEX for 60 min. ChIP assays were performed with the indicated antibodies and primer sets as described in *Materials and Methods*. IP, Immunoprecipitation.

resistance of *atp1a1* promoter may be due to cell type-dependent deficiency of GR recruitment despite the presence of the receptor.

Endogenous HEXIM1 Negatively Modulates Glucocorticoid-Mediated Transcriptional Activation

It was previously described that HEXIM1 mRNA is ubiquitously expressed but its expression levels are variable among human tissues (27). Protein expression levels of endogenous HEXIM1 also show a great diversity among tissues in mice; HEXIM1 expression levels appeared to be low in kidney compared with those in liver, brain, lung, spleen, and heart (Fig. 3A). These tissue-dependent differences of HEXIM1 expression levels were also observed in human tissue-derived cell lines, *i.e.* HepG2 cells abundantly express HEXIM1 compared with 293 cells (Fig. 3A). Together with the fact that certain GR target genes, *e.g. atp1a1* and *scnn1a*, are resistant to hormone treatment in HEXIM1-rich HepG2 cells, we hypothesized that HEXIM1 may participate in cell type-dependent hormone resistance at the level of transcriptional regulation of these genes.

Given this, we then addressed whether endogenous HEXIM1 contributes to glucocorticoid insensitivity of

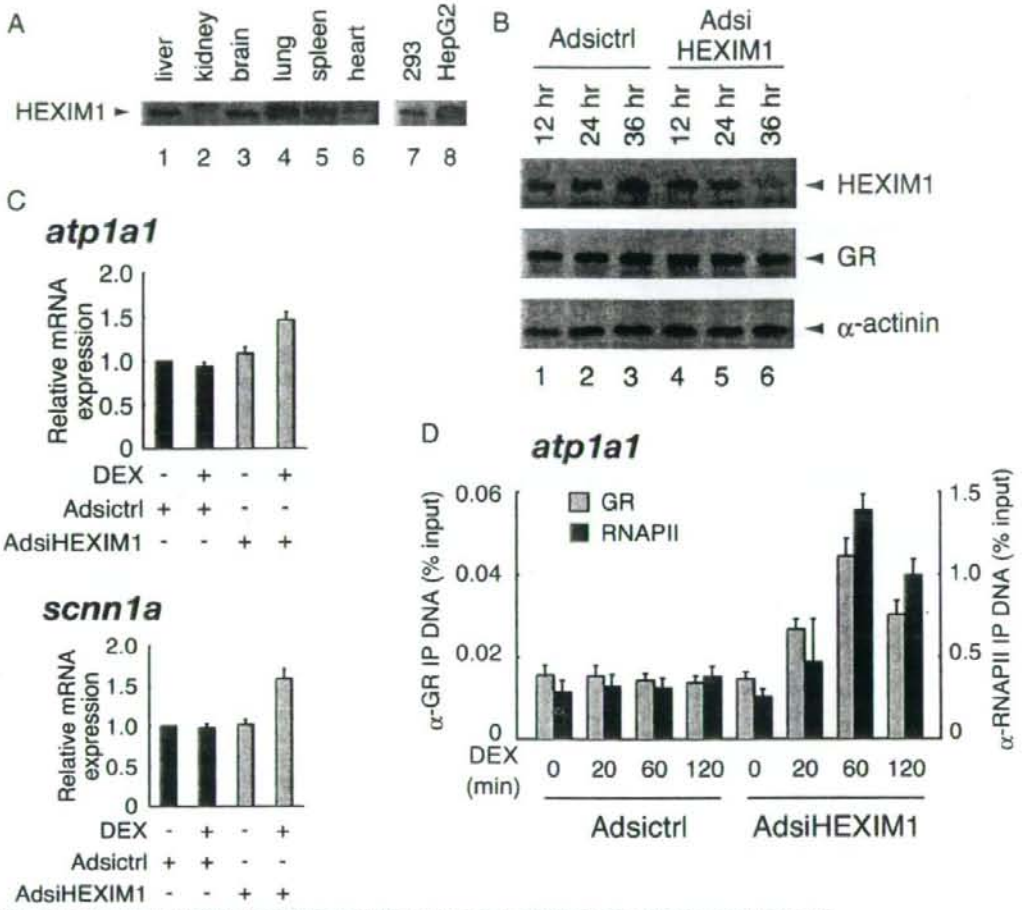

Fig. 3. Endogenous HEXIM1 Negatively Modulates Glucocorticoid-Mediated Transcriptional Activation

A, Cell lysates from various tissues from adult mice were subjected to Western blot analysis using indicated antibodies. B, HepG2 cells were infected with Adsictrl or AdsiHEXIM1 in phenol red-free Opti-MEM I at MOI of 100 for indicated time periods. Whole-cell extracts were prepared, and protein expression levels of endogenous HEXIM1, GR, and α-actinin were assessed in Western blotting. C, HepG2 cells were infected with the recombinant adenoviruses for 36 h as described in panel B and stimulated with 100 nM DEX for 6 h. Total RNA was prepared and mRNA for *atp1a1*, *scnn1a*, and *gapdh* was measured with qRT-PCR. Samples were normalized to *gapdh*, and relative expression levels to vehicle-treated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. D, HepG2 cells were infected with the recombinant adenoviruses for 36 h as described in panel B and stimulated with 1 μM DEX for indicated time periods. ChIP assays were performed with the indicated antibodies as described in *Materials and Methods*. IP, Immunoprecipitation.

atp1a1 and *scnn1a* in HepG2 cells. For that purpose, we constructed the recombinant adenoviruses expressing small interfering RNA (siRNA) against HEXIM1 named Adsi-HEXIM1, and unrelated siRNA named Adsictrl, as described in *Materials and Methods*. Figure 3B shows that infection of AdsiHEXIM1 diminished endogenous HEXIM1 protein expression down to less than 10% of the control without significant alteration of GR protein expression level. Again, mRNA expression of *atp1a1* and *scnn1a* was not significantly induced in the presence of 100 nM DEX in Adsictrl-infected HepG2 cells (Fig. 3C). Infection of AdsiHEXIM1 had little effect on basal levels of but significant effect on DEX-inducibility of *atp1a1* and *scnn1a* (Fig. 3C). Using *atp1a1* as a model, we then studied the influence of knockdown of endogenous HEXIM1 on hormone-dependent GR recruitment onto *atp1a1* promoter in ChIP analysis. As shown in Fig. 3D, GR was recruited in a time-dependent manner onto the promoter after DEX treatment in AdsiHEXIM1-infected cells. Moreover, RNAPII was also incorporated to the promoter in parallel with GR recruitment (Fig.

3D). Similar results were obtained when *scnn1a* promoter was used for ChIP assay (data not shown). These findings strongly support the notion that protein levels of endogenous HEXIM1 might determine GR recruitment onto these promoters and subsequent glucocorticoid-responsive transcription of *atp1a1* and *scnn1a* in HepG2 cells.

GR and HEXIM1 Stochastically Contribute to Hormone-Dependent Transcriptional Regulation of GR-Target Genes

To test relative contribution of GR and HEXIM1 in the expression of *atp1a1*, we overexpressed FLAG-GR alone or in combination with HEXIM1 in HepG2 cells

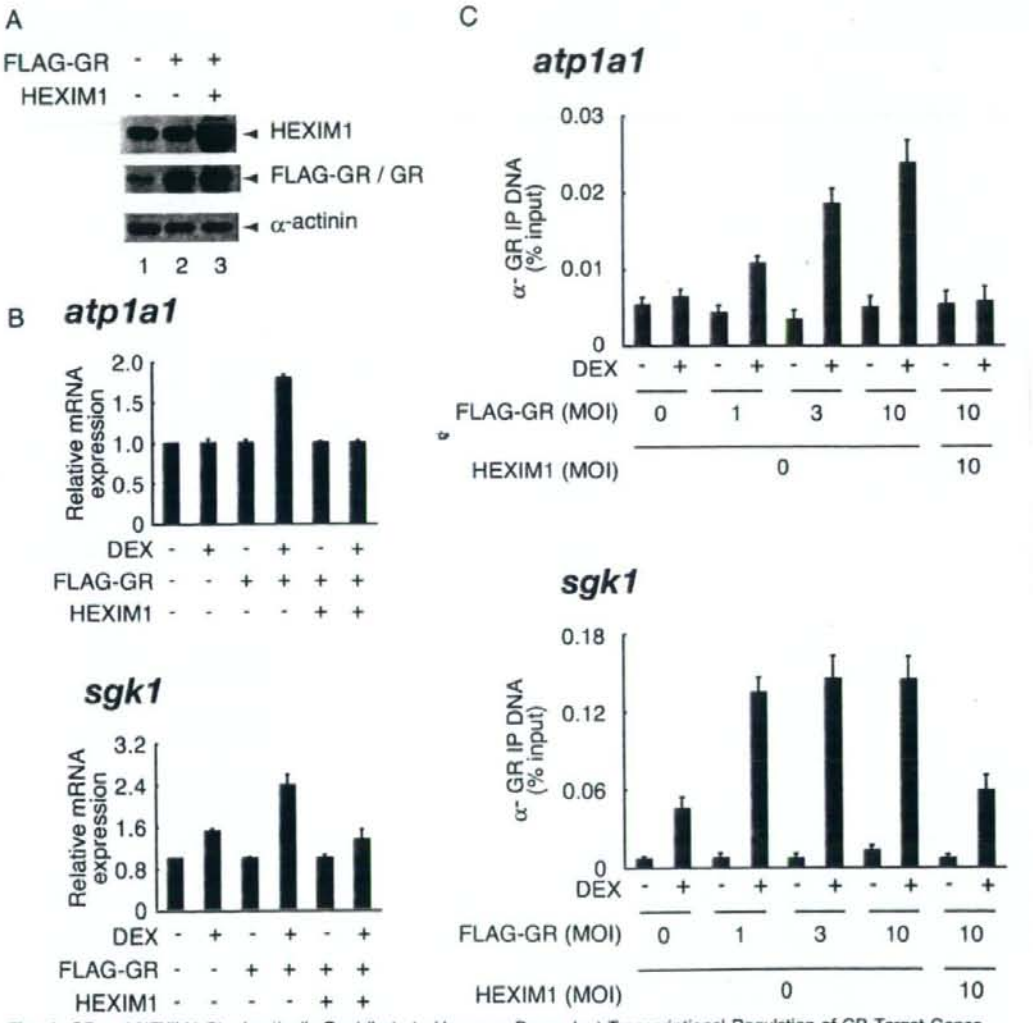

Fig. 4. GR and HEXIM1 Stochastically Contribute to Hormone-Dependent Transcriptional Regulation of GR-Target Genes

A, HepG2 cells were infected with the recombinant adenoviruses expressing FLAG-GR and HEXIM1 in phenol red-free Opti-MEM I at MOI of 5 for 24 h as indicated. Cells were lysed and subjected to Western blot analysis using the indicated antibodies. B, HepG2 cells were infected with the recombinant adenoviruses as described in panel A and treated with or without 100 nM DEX for 6 h. Total RNA was prepared, and mRNA for *atp1a1*, *sgk1*, and *gapdh* was measured with qRT-PCR. Samples were normalized to *gapdh*, and relative expression levels to vehicle-treated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. C, HepG2 cells were infected with the recombinant adenoviruses in phenol red-free Opti-MEM I for 24 h as indicated and treated with or without 1 μM DEX for 20 min. ChIP assays were performed with anti-GR antibodies as described in *Materials and Methods*. IP, Immunoprecipitation.

using recombinant adenoviruses. Western blots showed that the protein levels of expressed FLAG-GR and HEXIM1 were approximately 5-fold and 8-fold compared with those of endogenous GR and HEXIM1, respectively (Fig. 4A). In HepG2 cells, overexpression of FLAG-GR restored glucocorticoid responsiveness and resulted in DEX-dependent induction of *atp1a1* mRNA by 1.8-fold, which was again canceled by coexpression of HEXIM1 (Fig. 4B). We may propose, therefore, that high-level expression of HEXIM1 relative to GR confers tissue-specific glucocorticoid resistance of *atp1a1* in HepG2 cells. This is also the case in *scnn1a* mRNA expression (data not shown). Interestingly, mRNA expression of *sgk1* was also negatively affected by exogenous expression of HEXIM1 (Fig. 4B). Our ChIP assay revealed that GR overexpression restored hormone-dependent recruitment of GR to *atp1a1* promoter in a dose-dependent manner, which was again canceled by overexpression of HEXIM1 (Fig. 4C). In the case of *sgk1* promoter as well, overexpression of GR further increased hormone-dependent GR recruitment, which was antagonized by exogenous HEXIM1 (Fig. 4C). These findings strongly support the notion that GR and HEXIM1 stochastically contribute to hormone-dependent transcriptional regulation of both *atp1a1* and *sgk1*, and that *atp1a1* promoter is more susceptible to HEXIM1.

Knockdown of HEXIM1 Enhanced P-TEFb-Dependent Gene Expression

In addition to suppressing GR recruitment onto the target DNA, HEXIM1 is originally reported to inactivate kinase activity of P-TEFb, thereby suppressing transcription elongation (see *Introduction*). To investigate whether knockdown of HEXIM1 in our system affects P-TEFb-dependent mRNA expression as previously reported (51), we analyzed mRNA expression of several genes, expression of which has been reported to be critically regulated by P-TEFb at the step of transcription elongation.

mRNA level of *cyp1a1* was increased after stimulation with 1 h treatment of 10 nM 3-methylcholantrene (3MC) by 3.7-fold, probably via activation of AhR and subsequent recruitment of P-TEFb onto *cyp1a1* promoter (38), and AdsiHEXIM1 further enhanced this 3MC effect by 5.0-fold (Fig. 5A). IL-6-mediated expression of *junB* mRNA (14-fold), which is mediated by STAT3 (52), was also enhanced by AdsiHEXIM1 (22-fold). Finally, heat shock-mediated amplification of mRNA expression of *hspa1a* and *fos* (11-fold and 8.1-fold, respectively), which is mediated by HSF1 (53, 54), was further enhanced by

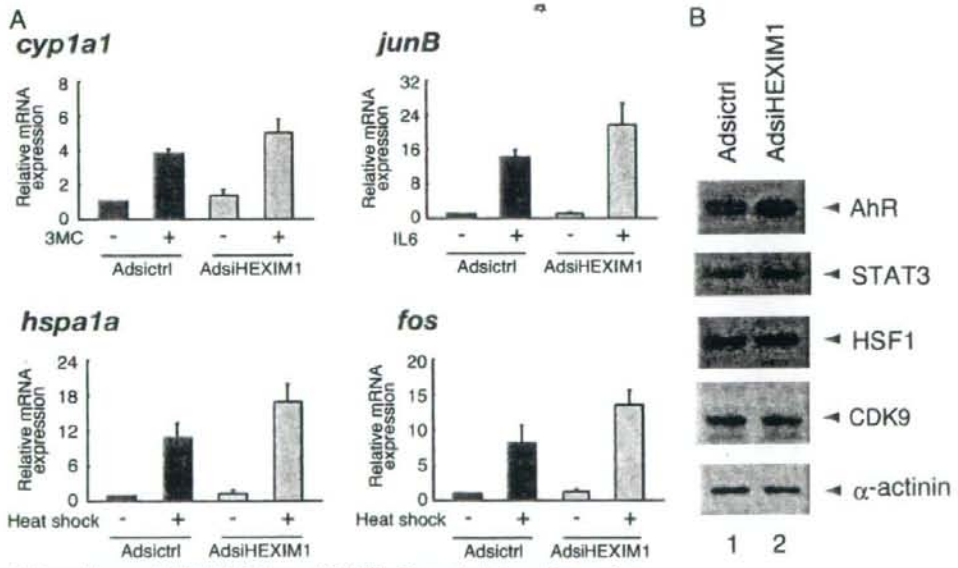

Fig. 5. Knockdown of HEXIM1 Enhanced P-TEFb-Dependent Gene Expression

A, HepG2 cells were infected with Adsictrl or AdsiHEXIM1 as described in Fig. 3B and stimulated with 10 nM 3MC, 100 ng/ml IL-6, or culture at 42 C (heat shock) for 1 h as indicated. Endogenous mRNA for cytochrome P450, family 1, subfamily A, polypeptide 1 (*cyp1a1*), JunB (*junB*), heat shock 70-kDa protein 1A (*hspa1a*), Fos (*fos*), and *gapdh* was measured with qRT-PCR. Samples were normalized to *gapdh*, and relative expression levels to the Adsictrl-infected and unstimulated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. B, HepG2 cells were infected with the recombinant adenoviruses and stimulated as described in panel A. Nuclear extracts were prepared, and protein expression levels of endogenous AhR, STAT3, HSF1, CDK9, and α-actinin were assessed by Western blotting.

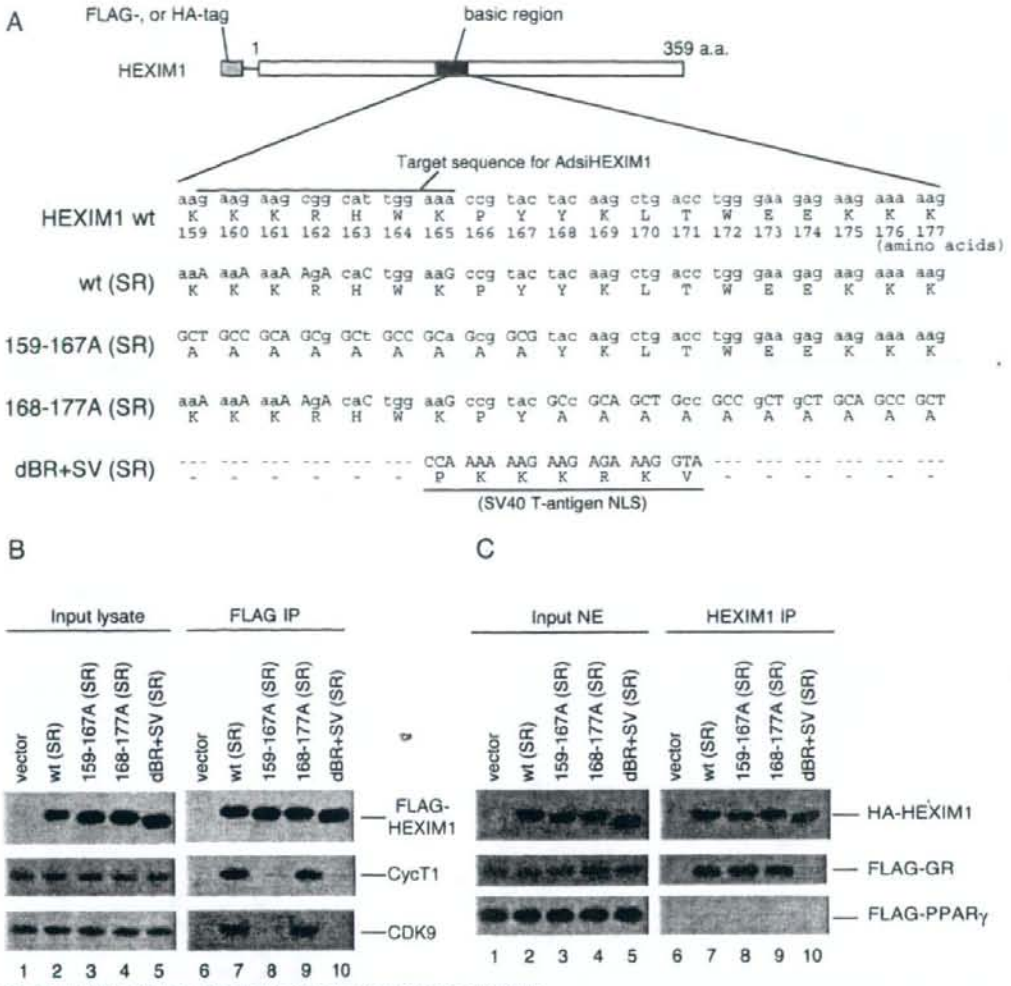

Fig. 6. P-TEFb-Binding and GR Binding Are Separable for HEXIM1

A, Schematic illustration of wild-type (wt) and mutant HEXIM1 used in this study. BR encompassing 150–177 amino acids are depicted as a *solid box*. *Numbers* depict positions of amino acids. Nucleotide and amino acid sequences in the BR are shown. Substitutions of nucleotides are shown in *uppercase letters*. wt (SR) and 168–177A (SR) have nucleotide substitutions in the target nucleotide sequence for AdsiHEXIM1 without affecting original amino acid sequence. 159–167A (SR) and dBR+SV (SR) are resistant to AdsiHEXIM1 by nature. B, HeLa cells were cotransfected with empty vector or expression plasmids for indicated FLAG-tagged mutant HEXIM1. Whole-cell lysates were prepared and subjected to FLAG-affinity purification as described in *Materials and Methods*. Western blot analysis of input lysates (lanes 1–5) and affinity-purified fractions (lanes 6–10) were performed using anti-FLAG peptide, anti-CycT1, and anti-CDK9 antibodies. C, COS7 cells were cotransfected with empty vector or expression plasmids for indicated HA-tagged mutant HEXIM1 along with either FLAG-tagged GR (*middle panel*) or FLAG-tagged PPAR γ (*lower panel*) expression plasmid. Cells were treated with 100 nM DEX (*middle panel*) or 100 nM TGZ for 2 h. Nuclear extracts were prepared and immunoprecipitated with anti-HEXIM1 antibodies. Western blot analysis of input extracts (lanes 1–5) and immunoprecipitated fractions (lanes 6–10) were performed using anti-HA peptide and anti-FLAG peptide antibodies. a.a., Amino acids; IP, immunoprecipitation; NE, nuclear extract; NLS, nuclear localization signal.

AdsiHEXIM1 (17-fold and 14-fold, respectively). Note that protein expression levels of AhR, STAT3, HSF1, and CDK9 were not significantly influenced by infection of AdsiHEXIM1 (Fig. 5B). We may conclude, therefore, that P-TEFb activity was enhanced in HEXIM1-knocked down cells and that the P-TEFb-dependent elongation process was up-regulated in certain genes.

P-TEFb Binding and GR Binding Are Separable for HEXIM1

To highlight the GR target gene-selective role of HEXIM1, we established an experimental system that enables us to clarify which function of HEXIM1 is important in regulation of GR-target gene expression, P-TEFb suppression or GR sequestration. In short, endogenous HEXIM1 was knocked down by infection of AdsiHEXIM1, and mutant HEXIM1, which lacks either P-TEFb-suppressing activity or direct interaction with GR, or both, was exogenously complemented. To obtain such a mutant HEXIM1, we focused on basic region (BR) of HEXIM1 and made alanine substitution and domain swap mutants, as schematically depicted in Fig. 6A, because we and others previously showed that BR is essential for nuclear localization, interaction with GR, and P-TEFb-inhibition (28, 30, 41, 55). Because siRNA against HEXIM1 in AdsiHEXIM1 was designed to target the region corresponding to amino acids 159–165 (Fig. 6A), the expression plasmids for siRNA-resistant wild-type (SR) and 168–177A (SR) were created with several nucleotide substitutions in HEXIM1 cDNA without affecting original amino acid sequence (Fig. 6A). In indirect immunofluorescence analysis, every mutant HEXIM1 protein was expressed in the nucleus in transfected cells (data not shown).

To verify the presence or absence of the interaction between P-TEFb and these mutant FLAG-tagged HEXIM1, we, after transfection of their expression plasmids into HeLa cells, immunoprecipitated cell lysate with anti-FLAG monoclonal antibody, and blots were probed with the antibodies against major P-TEFb subunits CycT1 and CDK9. As expected, substitution of BR to the nuclear localization signal from simian virus (SV) 40 large T antigen, resulting in dBR+SV (SR), completely abolished binding of CycT1 and CDK9 (Fig. 6B). Alanine substitution of amino acids 159–167, which was shown to disrupt the interaction with 7SK snRNA (55), diminished consecutive recruitment of CycT1 and CDK9 (Fig. 6B), as seen in dBR+SV (SR). On the other hand, alteration of amino acids 168–177 to alanines did not affect binding of CycT1 or CDK9 (Fig. 6B).

Using these HEXIM1 mutants, we also studied the physical interaction between HEXIM1 and GR. For this purpose, hemagglutinin (HA)-tagged wild-type (SR) and mutant HEXIM1 were expressed in COS7 cells along with either FLAG-tagged GR or FLAG-tagged peroxisome proliferator-activated receptor γ (PPARγ) as a control and immunoprecipitated with anti-HEXIM1 antibodies. As shown in Fig. 6C, GR bound not only wild-type (SR) but also HEXIM1 mutants with alanine substitution, but the swap mutant dBR+SV (SR) did not bind GR. These results may suggest that amino acids 159–177 of HEXIM1 are not critical for binding GR, but protein configuration of BR and its proximity is important for GR recognition. In contrast, PPARγ did not bind wild-type (SR) or any mutant HEXIM1 (Fig. 6C). To further confirm that these HEXIM1 mutants, especially 159–167A (SR), retain not P-TEFb-inhibition but GR suppression, we tested their

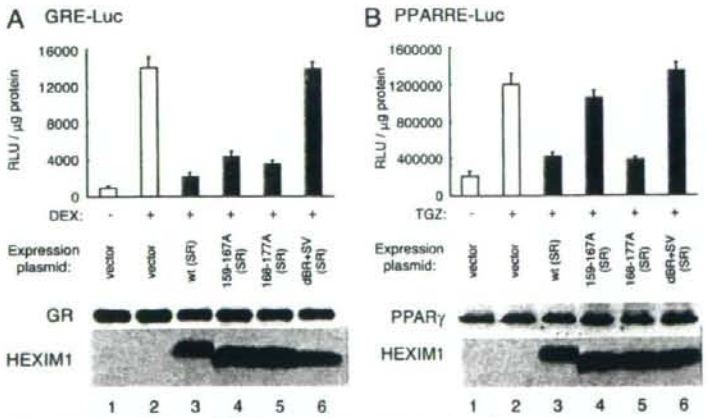

Fig. 7. Differential Functions of HEXIM1 on P-TEFb- and GR-Dependent Gene Expression

A, COS7 cells were cotransfected with empty vector or expression plasmids for the indicated HA-tagged mutant HEXIM1 along with GR expression plasmid and GRE reporter plasmid. Four hours later, media were replaced, further cultured for 20 h, and treated with vehicle or 100 nM DEX for 18 h as indicated. Cells were lysed and subjected to luciferase assay. Results are presented as relative light units (RLU) per microgram of protein in the lysates. *Error bars* represent SD values of at least three independent experiments. Protein expression levels of GR and HA-HEXIM1 were assessed in Western blotting. B, COS7 cells were cotransfected with empty vector or expression plasmids for indicated HA-tagged mutant HEXIM1 along with PPARγ expression plasmid and PPARRE reporter plasmid. Media were replaced 4 h later, further cultured for 20 h, and treated with vehicle or 100 nM TGZ for 18 h as indicated. Cells were lysed and subjected to luciferase assay. Results are presented as RLU per microgram of protein in the lysates. *Error bars* represent SD values of at least three independent experiments. Protein expression levels of PPARγ and HA-HEXIM1 were assessed by Western blotting.

functions in a GRE-luciferase reporter gene assay (Fig. 7A). PPARγ-dependent reporter gene assay served as a control (Fig. 7B), because neither wild-type (SR) nor any mutant HEXIM1 was capable of binding PPARγ (Fig. 6C). HEXIM1 dBR+SV (SR), which lacks binding activity to either P-TEFb or GR, did not significantly affect either reporter gene activity, as expected (Fig. 7, A and B, *top*). With respect to GR-driven reporter gene expression, any alanine-substituted HEXIM1 mutant suppressed ligand-dependent activation of the reporter gene as well as wild type (SR), indicating its functional interaction with GR (Fig. 7A, *top*). In clear contrast, PPARγ-mediated activation of the reporter gene was repressed solely by wild-type (SR) and 168–177A (SR) (Fig. 7B, *top*). In these experimental settings, protein expression of FLAG-tagged GR or FLAG-tagged PPARγ was not significantly affected by HEXIM1 mutants (Fig. 7, A and B, *bottom*). It is concluded, therefore, that these HEXIM1 mutants can serve an efficient tool for delineating mechanism of suppressing expression of particular genes by HEXIM1, *i.e.* P-TEFb suppression or GR binding.

P-TEFb Is Not Involved in HEXIM1-Mediated Suppression of Glucocorticoid Responsiveness

Finally, we differentially evaluated the importance of P-TEFb-suppressing and GR-binding activities of HEXIM1 in regulating glucocorticoid sensitivity of glucocorticoid-inducible mRNA expression of *atp1a1* and *scnn1a*. HeLa cells were transfected with the expression plasmids for HEXIM1 (SR) mutants, infected with AdsiHEXIM1, and treated with the cognate ligands, after which RNA was isolated for qRT-PCR analyses. In HEXIM1 knocked-down cells, mRNA expression of *cyp1a1*, which is known to be P-TEFb dependent (38), was stimulated by 10-fold in response to 6 h treatment with 10 nM 3MC (Fig. 8A). Adding back of wild-type (SR) HEXIM1 repressed induction of mRNA expression of *cyp1a1*, to 3.3-fold, suggesting that ectopically expressed HEXIM1 (SR) functionally suppressed P-TEFb activity (Fig. 8A). However, neither 159–167A (SR) nor dBR+SV (SR) repressed *cyp1a1* expression, confirming that the suppression of P-TEFb activity may be critical for the repression (Fig. 8A). In support of this, 168–177A (SR), which binds P-TEFb

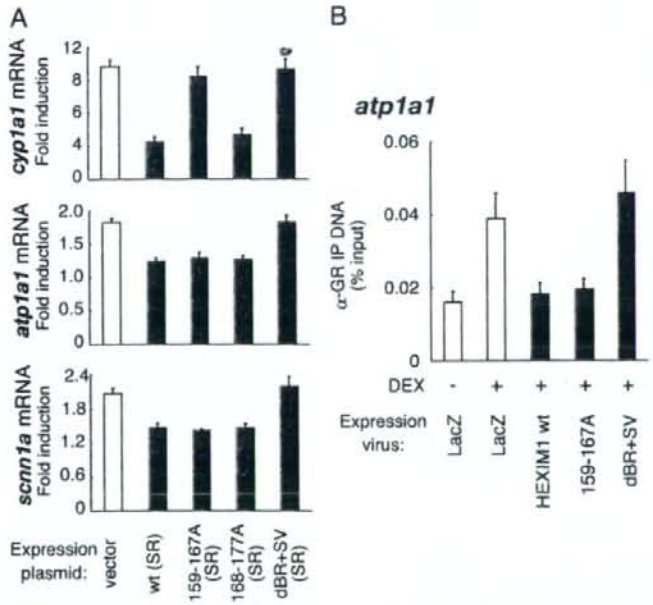

Fig. 8. P-TEFb Is Not Involved in HEXIM1-Mediated Suppression of Glucocorticoid Responsiveness

A, HeLa cells were transfected with 3 μg of empty vector or expression plasmids for the indicated HA-tagged mutant HEXIM1. Cells were infected 4 h later with AdsiHEXIM1 in phenol red-free Opti-MEM I at MOI of 100 for 36 h, and treated with vehicle or 10 nM 3MC (*top panel*), or 100 nM DEX (*middle* and *bottom panels*) for 6 h. Endogenous mRNA levels for *cyp1a1*, *atp1a1*, *scnn1a*, and *gapdh* were measured with qRT-PCR. Samples were normalized to *gapdh* mRNA, and mRNA induction levels by cognate ligands are shown in fold induction. *Error bars* represent SD values of at least three independent experiments. B, HepG2 cells were infected with FLAG-GR-expressing adenovirus (MOI of 50) along with LacZ- or mutant HEXIM1-expressing adenoviruses (MOI of 40) in phenol red-free Opti-MEM I for 24 h, and the cells were treated with 1 μM DEX for 20 min. ChIP assays were performed with polyclonal anti-GR antibodies as described in *Materials and Methods*. *Error bars* represent SD values of at least three independent experiments. IP, Immunoprecipitation.

(Figs. 6B and 7B), suppressed AhR-mediated transcription as well as wild type (SR) (Fig. 8A). These effects of wild-type (SR) and mutant HEXIM1 were also observed in the other P-TEFb-regulated genes depicted in Fig. 5A (data not shown). With respect to GR target genes, mRNA expression of *atp1a1* and *scnn1a* was stimulated by 1.8-fold and 2.1-fold, respectively, in response to 6 h treatment with 100 nM DEX in HEXIM1 knocked-down cells (Fig. 8A). Complementation of wild-type (SR) HEXIM1 or 168–177A (SR) significantly repressed induction of mRNA expression of *atp1a1* and *scnn1a* (Fig. 8A). In contrast to *cyp1a1*, 159–167A (SR) suppressed glucocorticoid-induced enhancement of mRNA expression of *atp1a1* and *scnn1a* comparable to that of wild type (SR) (Fig. 8A), indicating that P-TEFb-binding activity of HEXIM1 is dispensable but GR-binding activity is important for the suppression. Consistently, dBR+SV (SR), which does not bind GR, did not affect mRNA induction of *atp1a1* and *scnn1a* (Fig. 8A). The importance of GR binding of HEXIM1 was also confirmed in ChIP assay. Recombinant adenovirus-mediated expression of 159–167A in HepG2 cells suppressed DEX-dependent recruitment of FLAG-GR onto *atp1a1* promoter, whereas dBR+SV did not (Fig. 8B). Using *scnn1a*, we obtained identical results (data not shown). Taken together, we may conclude that direct interaction between GR and HEXIM1 is critical for HEXIM1-mediated glucocorticoid resistance of *atp1a1* and *scnn1a* in HepG2 cells.

DISCUSSION

As described in the introductory section, HEXIM1 is currently considered to be a multifunctional protein, acting at a specific stage of gene expression. In the present study, we intended to characterize endogenous HEXIM1 function for modulation of GR-mediated transcriptional regulation. For that purpose, we focused on *atp1a1* and *scnn1a*, because expression of these genes is resistant in HEXIM1-rich HepG2 cells to treatment with DEX (Fig. 1A). Treatment with histone deacetylase inhibitor did not result in liberation of these genes in HepG2 cells (Fig. 1A), suggesting that the observed DEX resistance is not due to irreversible alteration in higher order chromatin structure or histone acetylation-related chromatin packaging. In support of this, these genes retain responsiveness to other extracellular stimuli in liver and HepG2 cells (Refs. 10 and 11 and data not shown). We showed that, in HepG2 cells, knockdown of HEXIM1 by siRNA not only canceled the DEX resistance but also rather enhanced DEX-responsive mRNA expression of these genes (Fig. 3C). Moreover, our ChIP assay clearly demonstrated that siRNA-mediated knockdown of HEXIM1 restored hormone-dependent GR recruitment onto the promoters of those genes in parallel with corresponding increase in RNAPII binding (Fig. 3D). Such effect of reduction in endogenous HEXIM1 level was mimicked by exogenous overexpression of GR (Fig. 4, B and C), indicating that GR-HEXIM1 ratio could be a determinant of glucocorticoid resistance/sensitivity of those genes. As anticipated, overexpression of HEXIM1 turned those promoters more or less resistant to DEX (Fig. 4B).

Endogenous HEXIM1 seems to negatively modulate all GR target genes but not completely diminish DEX responsiveness of all of them in HepG2 cells (Fig. 4), indicating that efficiency of the suppression by HEXIM1 is dependent on gene context. Indeed, our previous DNA microarray analyses showed that the extent of reducing DEX responsiveness by overexpressed HEXIM1 was variable among different genes in HepG2 cells (30). It is also reported that GRE occupancy with GR in alveolar epithelial A549 cells is generally restricted to such genes that are actually regulated by glucocorticoids in those cells (21). This observation strongly supports the idea that gene-specific determination of GR recruitment to GRE is important in tissue-specific regulation of glucocorticoid-responsive gene expression at the level preceding transcription initiation. We recently demonstrated that HEXIM1 directly binds GR and that GR or other oxosteroid receptors are preferential partners of HEXIM1 (48). In this line, we might speculate that HEXIM1 squelches GR in the nucleus and inhibits its access to target gene promoter, and such negative effect of HEXIM1 is, more or less, shared by many genes. Some GR-target genes, including *atp1a1* and *scnn1a*, therefore, might be particularly susceptible to HEXIM1 and resistant to glucocorticoids in HEXIM1-rich cells, *i.e.* HepG2 cells. Certain promoters, *e.g. sgk1* promoter, allow hormone-dependent GR binding in HepG2 cells, strongly supporting the notion that promoter recruitment of GR is determined in a gene context-dependent manner as previously predicted in other GR-regulated genes (56).

We also revealed that P-TEFb-suppressing activity of HEXIM1 is not prerequisite for glucocorticoid resistance of these genes (Fig. 8A). Furthermore, the fact that 159–167A binds GR and suppresses GR recruitment to the target gene (Figs. 6C and 8B) again emphasizes the importance of the suppression of GR recruitment through direct GR-HEXIM1 interaction in the mechanisms of glucocorticoid resistance by HEXIM1. These results highlighted the role of HEXIM1 in P-TEFb-independent and gene-selective suppression of mRNA expression. The bimodal roles of HEXIM1 may differentially contribute to suppressing mRNA expression in a gene context-dependent manner. In this line, it should be noted that other transcription factors, such as estrogen receptor (45) and CCAAT/enhancer binding protein α (44), which were shown to directly interact with HEXIM1, may also be controlled by HEXIM1 through a P-TEFb-independent mechanism. Moreover, the interaction of HEXIM1 with these transcription factors has been shown to be a molecular basis for various physiological or pathological actions of HEXIM1 (44, 45).

Our previous observation revealed that HEXIM1/GR complexes reside in a distinct subnuclear area (30). Given this, HEXIM1 might prevent intranuclear GR from accessing to the promoter and decrease the amount of available GR for transcription. Since we revealed that the central and C-terminal regions of HEXIM1 are indispensable for its proper nuclear localization and GR repression (Refs. 41 and 48, and data not shown), HEXIM1 might anchor at as yet unknown but saturable subnuclear structure via these regions. Increasing evidence indicates that the C-terminal region of HEXIM1 possesses various functions, *e.g.* P-TEFb-binding (57, 58), self-oligomerization (59–61), and interaction with transcription factors (44, 51). Recently, nucleophosmin was shown to bind HEXIM1 via BR and promote its degradation (62). Taken together, it may be indicated that subnuclear localization and function of HEXIM1 might be tightly controlled via multimodal interactions among distinct HEXIM1 domains and various nuclear machineries to elicit fine tuning of transcriptional control of gene expression. In any case, an important question to be solved is how multiple functions of HEXIM1 are rationally regulated in a gene- or tissue-dependent manner.

Expression levels of HEXIM1 vary in different tissues and are modulated during differentiation and development as well as in response to extracellular stimuli (see "Introduction"). Disturbances of tissue-specific glucocorticoid responses have been implicated in pathophysiology of rheumatoid arthritis, osteoarthritis, Crohn's disease, ulcerative colitis, asthma, AIDS, osteoporosis, and metabolic syndromes (63). Numbers of proteins have been shown to affect GR activity at different steps of GR signaling pathway and indicated to be potentially involved in the pathogenesis of such diseases that have relations to disturbed glucocorticoid responses in particular tissues (63). HEXIM1-mediated repression of GR might be one of such mechanisms and play pathological roles in certain diseases. On the other hand, glucocorticoids are still indispensable in treatment for a numerous diseases (4, 64). However, the desired therapeutic effects are often accompanied by severe side effects. Pharmacological alteration of the expression levels of HEXIM1, if possible, might indirectly modulate glucocorticoid effects in a tissue-specific manner and enable selective expression of pharmacological actions of glucocorticoids in given tissues. Along with development of selective GR modulators (26), HEXIM1 might also be considered as a drug target for tissue-specific modulation of GR actions.

MATERIALS AND METHODS

Reagents and Antibodies

DEX, troglitazone (TGZ), 3MC, and TSA were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-6 was from Peprotech (London, UK). Other reagents were from Nacalai Tesque (Kyoto, Japan) unless otherwise specified. Polyclonal antibodies against CDK9, STAT3, HSF1, CycT1, PPARγ, GR, and HA-peptide were from Santa Cruz Biotechnology, Inc. (sc-484, sc-7179, sc-9144, sc-8127, sc-7196, sc-8992, and sc-805, respectively; Santa Cruz, CA). Polyclonal anti-AhR antibodies were from Biomol (SA-210; Plymouth Meeting, PA). Polyclonal anti-FLAG-peptide antibodies and monoclonal anti-α-actinin antibody were from Sigma-Aldrich (F7425 and A5044, respectively). Monoclonal anti-GR antibody was from BD Biosciences (San Jose, CA). Monoclonal anti-RNAPII antibody was from Covance Laboratories, Inc. (MMS-126R; Princeton, NJ). Rabbit antihuman HEXIM1 antiserum and rabbit antimouse HEXIM1 antiserum were generated against a peptide corresponding from 39–53 amino acids of human HEXIM1 (RVPEEDSRWQSRAFP) and 55–69 amino acids of mouse HEXIM1 (SGSRPGQEGEGGLKH), respectively. Polyclonal anti-HEXIM1 affinity-purified antibodies were obtained from antihuman HEXIM1 antiserum with immunogen-immobilized affinity matrix (Kitayama Labes, Ina, Japan).

Cell Culture and Transfection

HepG2, 293, COS7, and HeLa cells were from RIKEN cell bank (Tsukuba, Japan) and maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and antibiotics in a humidified atmosphere at 37 C with 5% CO_2. Before transfection, cells were washed twice with PBS, and media were replaced with phenol red-free Opti-MEM I (Invitrogen). Transient transfection was performed using TransIt-LT1 transfection reagent (Panvera, Madison, WI) as described previously (65). Total amounts of plasmids to transfect were kept constant by adding empty vector.

Western Blot Analysis

Whole-cell extracts or nuclear extracts were prepared as described previously (30), resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gels, and blotted to polyvinylidene fluoride membranes. The membranes were incubated with Blocking One (Nacalai Tesque) at room temperature for 1 h, incubated with specific antibodies diluted in Blocking One (1:500 dilution for HA-peptide or 1:2000 for the others) at 4 C for 18 h, and then, washed three times with TBS-T (25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.1% Tween 20), incubated with secondary antibodies conjugated to horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) at room temperature for 30 min, washed three times with TBS-T, and detected with Chemi-Lumi One L (Nacalai Tesque) according to manufacturer's instruction.

Recombinant DNA and Adenoviruses

Expression plasmids for FLAG-tagged HEXIM1 (wild-type and dBR+SV) were described previously (30). pFLAG-CMV2-derived mammalian expression plasmids for mutant FLAG-HEXIM1 (159–167A and 168–177A) were generous gifts from Dr. Q. Zhou (University of California, Berkeley, CA). cDNA fragments for wild-type (SR), 159–167A (SR), 168–177A (SR), and dBR+SV (SR) HEXIM1 were generated by a standard PCR protocol using custom-designed primers and subcloned into pCMV-HA (TaKaRa, Otsu, Japan) or pFLAG-CMV2 (Sigma-Aldrich) expression plasmid using blunt-ended *Eco*RI and *Xho*I sites. The expression plasmid for human PPARγ, pCMX-6His-PPARγ, was generated by cloning appropriate PCR fragments into pCMX-6His vector (65). The PPAR response element (PPARRE)-driven reporter plasmid p3xPPARRE-LUC was a kind gift from Dr. E. A. Jansson (Karolinska Institutet, Stockholm, Sweden). All plasmids constructed above were verified by DNA sequencing. Recombinant adenoviruses encoding double-stranded hairpin RNAs for siRNA

against HEXIM1, AdsiHEXIM1, or control siRNA, Adsictrl, were constructed by subcloning expression cassettes from pSilencer3.1-H1 neo-derived expression plasmids (30) into adenoviral genome using Adenovirus Expression Vector Kit (TaKaRa) according to the manufacturer's instruction. Recombinant adenoviruses prepared from 293 cells were purified with Virakit AdenoMini-24 (Virapur, San Diego, CA) and titrated using Adeno-X Rapid Titer Kit (TaKaRa).

qRT-PCR

Total RNA was prepared with Sepasol-RNA I super (Nacalai Tesque), reverse-transcribed with oligo-dT primer using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). qRT-PCR was performed with the LightCycler TaqMan Master, Universal ProbeLibrary Set, Human, and LightCycler ST300 systems (Roche, Indianapolis, IN) according to manufacturer's instructions. Expression levels of mRNA were calculated on the basis of standard curves generated for each gene. mRNA for *gapdh* was used as an internal control. Sequences of primers used in this study are shown below:

atp1a1: 5′-ccctggctgctttccttt-3′ and 5′-ggcacagaaccacccaggta-3′

scnn1a: 5′-aaccaggtctcctgcaacc-3′ and 5′-gaaagtatagcagtttccatacatcg-3′

sgk1: 5′-cctgagcttatgaatgccaac-3′ and 5′-gccaaggttgatttgctgag-3′

adh1a: 5′-aaggcccatgaagttcgtatt-3′ and 5′-ccacgtggtcatctgtgc-3′

cyp1a1: 5′-cccagctcagctcagtacct-3′ and 5′-ggagattgggaaaagcatga-3′

junb: 5′-atacacagctacgggatacgg-3′ and 5′-gctcggtttcaggagtttgt-3′

hspa1a: 5′-ggagtcctacgccttcaaca-3′ and 5′-ccagcaccttcttcttgtcg-3′

fos: 5′-ctaccactcacccgcagact-3′ and 5′-aggtccgtgcagaagtcct-3′

gapdh: 5′-agccacatcgctcagaca-3′ and 5′-gcccaatacgaccaaatcc-3′

ChIP

ChIP assay was performed with ChIP Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer's instructions with minor modification. First, HepG2 cells were cultured in phenol red-free Opti-MEM I for 24 h for hormone depletion. Then, the cells were treated with 1 μM DEX or 0.1% ethanol (vehicle) for the indicated time periods. After treatment, the cells were cross-linked in 1% formaldehyde for 10 min at 37 C. Cross-linking was stopped with addition of glycine to medium to a final 125 mM for 5 min at 37 C, after which the cells were rinsed with ice-cold PBS twice and harvested. Cell pellets were collected and resuspended in SDS-lysis buffer (50 mM Tris, pH 8.0; 1% SDS; 10 mM EDTA; 1 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; 800 nM aprotinin; 15 μM E-64; 20 μM leupeptin-hemisulfate; 50 μM bestatin; and 10 μM pepstatin A) for 10 min at 4 C. Chromatin was sheared to an average size of 500 bp by sonication of the lysate using a Bioruptor Ultrasonicator (Cosmo-Bio, Tokyo, Japan). Lysates corresponding to 2×10^6 cells were diluted 10-fold in ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris, pH 8.1; and 167 mM NaCl) and precleared with Salmon Sperm DNA/Protein A Agarose beads (Upstate Biotechnology) at 4 C for 30 min. Supernatants were then collected and incubated with 5 μg of anti-GR polyclonal antibodies or anti-RNAPII monoclonal antibody at 4 C overnight. To collect immune complex, Salmon Sperm DNA/Protein A Agarose beads were added and further incubated at 4 C for 1 h. The beads were then washed twice each with Low-Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris, pH 8.1; and 150 mM NaCl), High-Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris, pH 8.1; and 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25 M LiCl; 1% Nonidet P-40; 1% deoxycholate; 1 mM EDTA; and 10 mM Tris, pH 8.1), and Tris-EDTA buffer. Protein-chromatin complex was eluted with elution buffer (10 mM dithiothreitol, 1% SDS, and 0.1 M $NaHCO_3$), and reversal of cross-link of eluates was performed in 200 mM NaCl at 65 C for 6 h, after which proteins were digested with proteinase K at 45 C for 1 h. Precipitated DNA fragments were recovered by QIAquick DNA purification kit (QIAGEN, Chatsworth, CA) and quantified with qRT-PCR using appropriate primer sets. Sequences of primers used in this study are shown below:

sgk1 −1238: 5′-acctcctcacgtgttcttgg-3′ and *sgk1* −982: 5′-caagcaaggctgaaaaatcc-3′ for GR

sgk1 −173: 5′-cctctcaatggggacagaac-3′ and *sgk1* +85: 5′-ccttagcagcctcagtttttca-3′ for RNAPII

atp1a1 −732: 5′-cgcccttcagattctcattt-3′ and *atp1a1* −447: 5′-ggactcagggatgctgga-3′ for GR

atp1a1+156: 5′-ccctagctccctccacttg-3′ and *atp1a1* +239: 5′-tcgctggagaatcagagagaa-3′ for RNAPII

FLAG-Affinity Purification

HeLa cells (2.5×10^6) were transfected with 4 μg of pFLAG-CMV2-derived expression plasmids. After 4 h, media were replaced with DMEM supplemented with 10% fetal calf serum. After 32 h, cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (wt/vol) Nonidet P-40, 1 μM dithiothreitol, 0.5 μM phenylmethylsulfonyl fluoride], centrifuged at 20,000 × *g* for 20 min. Supernatant was diluted in FAR buffer [16.7 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.33% (wt/vol) Nonidet P-40, 0.33 μM dithiothreitol, 0.17 μM phenylmethylsulfonyl fluoride], applied to anti-FLAG M2-agarose beads (Sigma-Aldrich), incubated for 2 h at room temperature. The beads were washed three times with FAR buffer. Bound proteins were eluted with SDS-sample loading buffer and subjected to Western blot analysis using anti-FLAG peptide, anti-CycT1, and anti-CDK9 antibodies.

Luciferase Assay

COS7 cells (1×10^6) were transfected with 2 μg of reporter plasmids (p2xGRE-LUC or p3xPPARRE-LUC), 2.5 ng of expression plasmids for the receptors (pCMX-6His-GR or pCMX-6His-PPARγ), and pCMV-HA-derived HEXIM1 expression plasmids. After 4 h, media were replaced with fresh phenol red-free Opti-MEM I, and infected with recombinant adenoviruses at multiplicity of infection (MOI) of 100. After 20 h, cells were treated with 100 nM DEX, 100 nM TGZ or vehicle (0.1% ethanol), and further cultured for 18 h. Cells were lysed in Cell Culture Lysis Reagent (Promega Corp., Madison, WI), and cellular luciferase activity was measured by using Luciferase Assay System (Promega). Relative light units were normalized to the protein amounts determined with BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

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Activation of Mitochondrial Biogenesis by Hormesis

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Mitochondria play a major role in oxidative energy production, reduction–oxidation reaction (redox) control and calcium homeostasis. Although mitochondria contain DNA with mitochondrial-specific genes, most mitochondrial proteins are encoded by the nDNA, synthesized in the cytosol, and imported into mitochondria. The expression of nuclear genes that encode mitochondrial proteins that function in metabolic pathways such as the trichloroacetic acid cycle (TCA), oxidative phosphorylation, heme synthesis, and in mitochondrial DNA replication and transcription (eg, mitochondrial transcription factor A [Tfam]), is coordinately regulated by the transcriptional coactivators PPARγ coactivator (PGC)-1α and PGC-1β through activation of nuclear respiratory factor (NRF)-1 and NRF-2.[1]

In their recent publications, Piantadosi et al provided insight into the mechanisms underlying the interaction between mitochondria-derived reactive oxygen species (ROS) signaling and mitochondrial biogenesis. First, lipid hydroperoxide regulates Tfam expression through phosphorylation of NRF-1 via Akt activation, which promotes nuclear translocation of NRF-1 and binding to the Tfam promoter.[2] Second, carbon monoxide (CO) induced mitochondrial biogenesis via activation of Akt1/PKB and guanylate cyclase, which augmented gene and protein expression of NRF-1 and NRF-2, PGC-1α, and TFAM.[3] CO-induced mitochondrial ROS result in the activation of AKT. Third, the anthracycline anticancer agent doxorubicin suppresses the nuclear program for mitochondrial biogenesis, and its associated intrinsic antiapoptosis proteins, leading to severe mitochondrial DNA (mtDNA) depletion and apoptosis. CO inhalation or heme oxygenase (Hmo)1 overexpression prevented doxorubicin-induced mtDNA depletion and apoptosis via activation of AKT and guanylate cyclase.[4] Lastly, new work in this issue of *Circulation Research*[5] sheds light on the role of NF-E2–related factor (Nrf)2 as a key transcriptional regulator in mitochondrial ROS-dependent induction of NRF-1 mRNA.

There is increasing evidence to suggest that ROS may be a double-edged sword: although they can be toxic to cells, they may also play an important role in cell signaling involved in the antioxidant defense network. ROS are generated from many sources including the Nox family of NADPH oxidases, xanthine oxidase, and mitochondria, where ROS are produced as a byproduct of oxidative energy production. ROS are very unstable and cannot penetrate lipid membranes; they are therefore retained within the compartment in which they are produced. However, ROS can attack neighboring polyunsaturated fatty acids of the membrane and trigger a chain reaction of lipid peroxidation, resulting in the generation of lipid hydroperoxides and α, β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (4-HNE) (Figure). They are highly electrophilic and react with biomolecules, such as proteins and nucleic acids, generating various adducts. By virtue of their increased chemical stability, these lipid peroxidation products can diffuse greater distances compared with their precursor ROS and can propagate and amplify oxidative injury. Thus, lipid peroxidation products have been implicated in the development and progression of a variety of pathological events such as oxidization of LDL, atherosclerosis, ischemia/reperfusion injury, Alzheimer's disease, cancers, and cell senescence.

However cells are able to sense macromolecular damage and counteract stress-induced damage to reestablish homeostasis. Electrophilic lipid peroxidation products can trigger a cascade of stress resistant pathways in both a tissue- and cell type–specific manner. The induction of stress-protective mechanisms by stress is referred to as "stress-response hormesis."[6] The principle of stress-response hormesis can be seen in many contexts. For example, the ninja, a group of spies and assassins in feudal Japan, were known to regularly take sublethal doses of poison to build their capacity to detoxify xenobiotics and thus protect themselves against assassination with poison. In cell culture, 4-HNE kills cells at a high dose, whereas pretreatment of cells with low-dose 4-HNE upregulates endogenous antioxidant and phase II enzymes, conferring greater tolerance against subsequent oxidative insult.[7] An effect of stress-response hormesis may also be seen in clinical studies that have tested antioxidant supplements for prevention of cardiovascular events[8] and cancers[9] based on the principle that they should prevent oxidative stress-induced macromolecular damage. In both clinical studies, antioxidant supplements may have failed to identify a beneficial effect because this inevitably attenuates the cell-signaling pathways necessary for protection against oxidative stress and reestablishment of redox homeostasis.[6]

Following the induction of oxidative stress, 2 basic leucine zipper transcription factors, Nrf2 and activating transcription factor (ATF)4, are activated at the posttranscriptional level and induce the expression of genes encoding proteins that function as antioxidants and enzymes involved in phase II detoxification and glutathione biosynthesis. Under nonstressed conditions, Nrf2 is tethered in the cytoplasm by Keap1. This complex directs Nrf2 polyubiquitination and degradation. On oxidative stress, Nrf2 is liberated from

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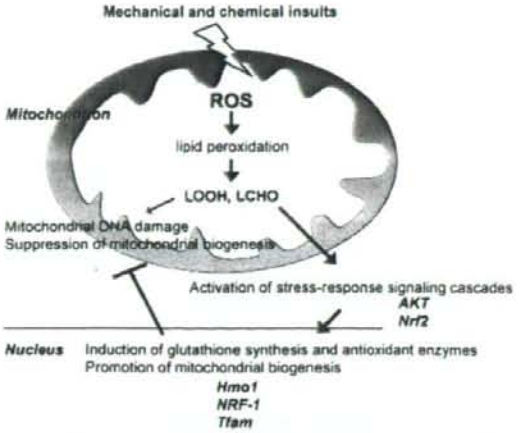

Figure. The generation of ROS and the subsequent oxidative modification of biomolecules are inevitable events in aerobic organisms. The polyunsaturated fatty acids in membrane lipids are particularly vulnerable to ROS attack and they undergo peroxidation. These lipid peroxidation products form protein and DNA adducts and have biphasic properties, in that high doses cause overt toxicity, whereas low doses interact with genetic signaling systems that upregulate gene expression to counteract stressor challenges and to re-establish homeostasis. LOOH indicates lipid hydroperoxides; LCHO, lipid aldehydes.

Keap1 and enters the nucleus, where it can form a heterodimer with the small Maf transcription factor Nrf2 to stimulate the expression of antioxidant response element–containing genes, including NAD(P)H:quinine oxidoreductase, heme-oxygenase 1, γ-glutamylcysteine synthetase, glutathione *S*-transferase, glutathione peroxidase, glutathione reductase, cysteine glutamate transporter, and multidrug resistance–associated protein 1.[11] Oxidative stress leads to the phosphorylation of the α subunit of translation initiation factor 2 (eIF2α). Phosphorylation of eIF2α inhibits general protein synthesis but specifically upregulates translation of ATF4. ATF4 forms homodimers and heterodimers with members of the AP-1 and C/EBP family of proteins to regulate the expression of genes involved in amino acid metabolism which provide the precursor amino acids necessary for glutathione biosynthesis, such as phosphoserine amino transferase, phosphoserine phosphatase, cystathione γ-lysate, and methylenetetrafolate dehydrogenase[12] Thus, Nrf2 and ATF4 coordinately regulate glutathione biosynthesis and the glutathione redox cycle.

Intense muscular contractile activity by exercise results in oxidative stress, as indicated by altered muscle and blood glutathione concentrations and increases in protein, DNA, and lipid peroxidation. Interestingly, it was recently reported that excess vitamin C supplements decrease training efficiency via the reduction of the exercise-induced expression of PGC-1, NRF-1, and Tfam.[13] This observation further suggests that ROS cannot only be considered to be toxic byproducts; they also play an important role in the cell signaling that regulates expression of genes involved in mitochondrial biogenesis. Piantadosi et al first demonstrated a role for Nrf2 in ROS-mediated induction of NRF-1. The NRF-1 promoter contains multiple antioxidant response element motifs and mitochondrial-derived ROS enhance Nrf2 binding to the NRF-1 promoter via AKT-mediated derepression of Nrf2 nuclear translocation. In the heart, however, the role of Nrf2 signaling in the basal expression, as well as the induction of antioxidants in pathological circumstances remains unclear.

Mitochondrial DNA copy number and mitochondrial gene expression are reduced in heart failure. Not surprisingly, concomitant downregulation of PGC-1α, NRF-1, and Tfam in the failing heart is observed.[14] The mechanism by which pathophysiological cues downregulate PGC-1/NRF-1/Tfam expression have only begun to be resolved,[15] but it is tempting to speculate that rescue of PGC-1/NRF-1/Tfam expression may have beneficial effects on cardiac function. Indeed, transgenic overexpression of Tfam in the heart ameliorates the decrease in mitochondrial DNA copy number and mitochondrial complex enzyme activities in the hearts and attenuates left ventricular remodeling and failure after myocardial infarction.[16]

The novel concept that mitochondrial biogenesis seems to be triggered by mitochondrial ROS generation is intriguing. To move present knowledge toward more general applicability, the physiological and pathological relevance of mitochondrial ROS-mediated transcriptional and posttranscriptional activation of NRF-1 via AKT, in the setting of postneonatal normal growth, exercise-challenged, pressure-challenged, ischemic, and failing heart need to be clarified.

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None.

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KEY WORDS: lipid metabolites ■ mitochondria ■ oxidative stress ■ redox ■ signaling pathways ■ hormesis ■ stress response

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REVIEW

Molecular mechanisms underlying the onset of degenerative aortic valve disease

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Abstract Morbidity from degenerative aortic valve disease is increasing worldwide, concomitant with the ageing of the general population and the habitual consumption of diets high in calories and cholesterol. Immunohistologic studies have suggested that the molecular mechanism occurring in the degenerate aortic valve resembles that of atherosclerosis, prompting the testing of HMG CoA reductase inhibitors (statins) for the prevention of progression of native and bioprosthetic aortic valve degeneration. However, the effects of these therapies remain controversial. Although the molecular mechanisms underlying the onset of aortic valve degeneration are largely unknown, research in this area is advancing rapidly. The signaling components involved in embryonic valvulogenesis, such as Wnt, TGF-β_1, BMP, and Notch, are also involved in the onset of aortic valve degeneration. Furthermore, investigations into extracellular matrix remodeling, angiogenesis, and osteogenesis in the aortic valve have been reported. Having noted avascularity of normal cardiac valves, we recently identified chondromodulin-I (chm-I) as a crucial anti-angiogenic factor. The expression of chm-I is restricted to cardiac valves from late embryogenesis to adulthood in the mouse, rat, and human. In human degenerate atherosclerotic valves, the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases and angiogenesis is observed in the area of chm-I downregulation. Gene targeting of chm-I resulted in VEGF expression, angiogenesis, and calcification in the aortic valves of aged mice, and aortic stenosis is detected by echocardiography, indicating that chm-I is a crucial factor for maintaining normal cardiac valvular function by preventing angiogenesis. The present review focuses on the animal models of aortic valve degeneration and recent studies on the molecular mechanisms underlying the onset of degenerative aortic valve disease.

Keywords Angiogenesis · Calcification · Cardiovascular · MMP · VEGF

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Introduction

Several lines of evidences suggest that the mechanism of aortic valve degeneration is similar to that of atherosclerosis, and accordingly, more people are suffering from degenerative aortic valve disease. Until recently, clinicians and researchers have focused mainly on surgical treatments for valvular heart disease, and the underlying mechanisms have remained largely unknown. Nevertheless, progress in basic research on atherosclerosis and the recent development of animal models of degenerative aortic valve disease have advanced basic research on aortic valve degeneration. We review the published studies on the molecular mechanisms underlying the onset of degenerative aortic valve disease and present our recent finding that the angiogenesis inhibitor chondromodulin-I (chm-I) is critical for maintaining normal cardiac valvular function.

Recent studies on the onset of aortic valve degeneration

Previous immunohistologic studies [1–8] have suggested that the mechanism of degeneration of native as well as bioprosthetic aortic valves is similar to that underlying atherosclerosis, i.e., involving the destruction of the endothelial layer overlying the valve, followed by invasion into the valve of inflammatory cells, such as monocytes and T lymphocytes, accumulation of low density lipoprotein within the valve, release of inflammatory cytokines, proliferation of valvular interstitial cells, extracellular matrix remodeling, and eventual calcification. More recently, it has been suggested by several groups [9–18] that apoptosis of endothelial cells and myofibroblasts in the valve, elastin degradation and collagen synthesis by matrix metalloproteinases and/or cathepsins, and enhanced osteogenesis contribute to aortic valve degeneration. Based on these findings, angiotensin-II-converting enzyme inhibitors or HMG CoA reductase inhibitors (statins) were tested for their effects on preventing the degeneration of native and bioprosthetic aortic valves. However, recent results from prospective, randomized clinical trials of these drugs are controversial [19–23]. Although statins appear to be effective in slowing the rate of progression of aortic stenosis when hyperlipidemia is present, further prospective studies are needed. Therefore, it is necessary to investigate new molecular mechanisms of disease onset to develop novel strategies for preventing valvular heart disease.

Investigations into degenerative aortic valve disease have traditionally focused on pathologic and observational studies of degenerating valves rather than on the molecular mechanisms behind the onset of degeneration. This is partly due to the lack of appropriate animal models of degenerative aortic valve disease. Rajamannan et al. [24] first observed that hypercholesterolemia caused by a high-cholesterol diet induced apoptosis of the rabbit aortic valve. Since 2002, several groups have reported on "the aortic stenosis model" in which wild-type rabbits fed a cholesterol-enriched diet with or without vitamin D_2 for 2 to 6 months exhibit proliferation of valvular interstitial cells, bone matrix formation, and calcification of the aortic valves [25–28]. Tanaka et al. [29] examined aged apolipoprotein E (ApoE)-deficient mice and found that more than half of the ApoE knockout mice older than 43 weeks showed aortic stenosis by echocardiography. Apoptosis and chemokine expression were increased and green fluorescent protein (GFP)-positive cells made up 16% of the total cell population in these stenotic valves after GFP-bone marrow transplantation, which shows that cells derived from the bone marrow contribute to aortic valve remodeling. Moreover, Drolet et al. [30] reported that a high-fat/high-carbohydrate diet for 4 months significantly reduced aortic valve area and increased valve leaflet thickness in wild-type C57BL/6J mice as well as in low-density lipoprotein receptor-deficient mice. There are certain difficulties associated with research into degenerative cardiac valve disease: the development of aortic valve degeneration takes a long time in these animal models, and the methods available to analyze the valves are restricted to immunohistochemistry, Western blotting, and echocardiography. However, the development of the new animal models mentioned above and the establishment of in vitro culturing techniques for valvular interstitial cells from large animals will advance research on the molecular mechanisms for the onset of degenerative aortic valve disease.

Although there are few basic reports regarding the molecular mechanisms of aortic valve degeneration, the recent reports are reviewed here (Fig. 1).

Extracellular matrix

Tenascin-C is an extracellular matrix component and a glycoprotein that is expressed in developmental bone tissue and atherosclerotic plaque. Jian et al. [31] reported on immunohistologic studies showing that the expression levels of tenascin-C, matrix metalloproteinase-2 (MMP-2), and alkaline phosphatase activity were increased in human calcified aortic valves. Furthermore, an in vitro study revealed that MMP-2 expression and its protease activity were significantly increased by the addition of tenascin-C to cultured human or ovine aortic valvular interstitial cells. From these data, Jian and colleagues raised the possibility that tenascin-C and MMP-2 are involved in the progression of aortic valve calcification. The secreted bone matrix protein osteopontin was detected in calcified human aortic valves by Western blotting and immunohistochemistry [32]. Steitz et al. [33] paradoxically observed accelerated calcification of aortic valve leaflets after subcutaneous implantation into osteopontin knockout mice in vivo and mineral dissolution of osteopontin following physically blocking of hydroxyapatite crystal growth in vitro, which led to the conclusion that osteopontin promotes regression of ectopic calcification. Interestingly, transcription factor Egr-1, which is known to induce tenascin-C and osteopontin, was also upregulated in calcified human aortic valves, as compared to normal valves [34]. Egr-1 upregulation appears to be protein kinase-C-dependent, although major upstream stimulation remains unclear.

MMPs and tissue inhibitor of matrix metalloproteinases (TIMPs) promote extracellular matrix remodeling. Fondard et al. [35] reported that the expression and activity levels of MMP-3, MMP-9, and TIMP-1 were increased in calcified human aortic valves, as measured by immunohistochemistry, enzyme-linked immunosorbent assay, and zymography. Another group [36] also observed increased expression of TNF-α and MMP-1 in human stenotic valves.

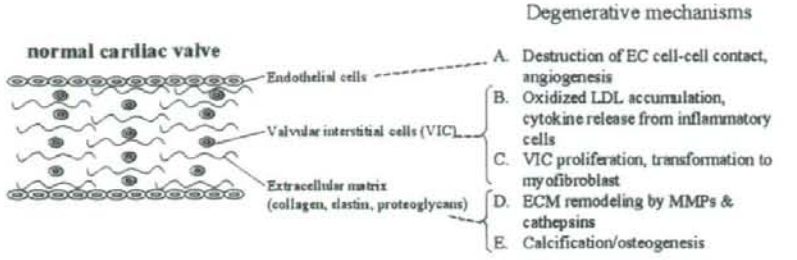

Fig. 1 Schematic of the degenerative mechanisms in the cardiac valve. The normal cardiac valve consists of the superficial endothelial cells, valvular interstitial cells, and extracellular matrices. The putative degenerative mechanisms for each component are shown (*A–E*). The table is a summary of the several factors cited in the present review, including the function, the mechanism (corresponding to *A–E* above), and the effect on degeneration of the cardiac valve

factor	function	mechanism	effect on degeneration
Tenascin-C	MMP-2 expression/activity ↑	D	degenerative
Fibulin-4	maintain TGF-β signal, ECM integrity	D	protective
Filamin-A	maintain cytoskeletal architecture	D	protective
MMPs (-1, -2, -3, -9)	elastin degradation, collagen synthesis ↑	D	degenerative
TIMPs	MMP activity ↓	D	protective
Wnt3/β-catenin		E	degenerative
$TGF\beta_1$		A, C, D, E	degenerative
BMP2		C, E	degenerative
Notch1	Runx2 activity ↓	E	protective
RANKL	MMP-1/2, cathepsin K, Runx2 activity ↑	D, E	degenerative
osteoprotegerin		E	protective
β_2 AR agonist	Runx2 expression in VIC ↓	E	protective
P21 WAF1/CIP1, 14-3-3 sigma	cell cycle ↓	C	protective
Chondromodulin-I		A	protective

Fibulin-4 protein is known to maintain the structure of the extracellular matrix. Fibulin-4 knockout mice show aortic valve stenosis due to thickening of the aortic valve leaflets as well as ascending aortic aneurysm or aortic dissection due to disarrangement of elastic fibers [37]. Transcriptome analysis of the aortas of fibulin-4 knockout mice revealed attenuated TGF-β signaling as the main cause of these knockout phenotypes, which indicates that the intensity of TGF-β signaling is related to the onset of aortic valve stenosis.

Myxomatous cardiac valvular dystrophy, which is one of the most common indications for valvular surgery, sometimes shows X-chromosome-linked inheritance. Kyndt et al. [38] used a positional cloning approach to identify the gene that encodes X-linked familial myxomatous valvular dystrophy and reported three missense mutations of the Filamin A gene in patients with this disease. Filamin A is a lining protein that bridges cytoskeletal actin and cellular membrane proteins, such as β-integrin, and it is known to amplify $TGF\text{-}\beta_1$ signaling.

Wnt, $TGF\text{-}\beta_1$, BMP, and Notch signaling

These signals are not only important for cardiac valvulogenesis at the embryonic stage but are also reported to be involved in valve degeneration.

Caira et al. [39] examined 40 subjects with normal human valves, 23 patients with myxomatous degeneration of the mitral valve, and 50 patients with calcified aortic valves (including the bicuspid valves) for changes in Wnt signaling and signaling related to osteoblast differentiation using reverse transcription polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry. In the degenerated valves, RT-PCR revealed that the messenger RNA (mRNA) expression levels of Sox9 and Cbfa1, which are crucial transcription factors for osteoblast differentiation, and the

mRNA expression levels of bone matrix protein osteopontin and osteocalcin were significantly increased. Moreover, in the calcified aortic valves, the expression levels of Wnt3, its receptor low-density lipoprotein receptor-related protein 5 (Lrp5), β-catenin, and osteocalcin in the calcified area were two to fourfold higher than those in normal valves, as assessed by Western blotting or immunohistochemistry. These findings strongly suggest that activation of the ossification process by Wnt signaling is involved in valve degeneration, especially aortic valve calcification [40].

Walker et al. [41] observed that TGF-β_1 stimulation in vitro accelerated the differentiation of cultured pig aortic valvular interstitial cells into α-smooth muscle, actin-positive myofibroblasts, and the remodeling of extracellular matrices. Another group [42] reported that stimulation of canine valvular interstitial cells with TGF-β_1 increased calcified nodule formation above the level seen in the control or in those cells that were treated with BMP-2. Quantitative PCR showed that the expression levels of TGF-β_1, alkaline phosphatase, and MMP-9 mRNA were significantly upregulated in human calcified aortic valves and that TGF-β_1 stimulation promoted apoptosis, MMP-9 and alkaline phosphatase activities, and osteocalcin expression in ovine and human aortic valvular interstitial cells in vitro [5, 43].

The expression levels of BMP-2 and bone sialoprotein are increased in calcified human aortic valves [44]. In contrast, the BMP antagonist Noggin is expressed in the outflow tract of the ventricle, the AV canal, and the future right ventricle of the murine embryonic heart. Choi et al. [45] analyzed the phenotypes of Noggin knockout mice and found thickening of the endocardial cushion and left ventricle outflow tract due to cell proliferation at the embryonic stage. Based on the results of an in vitro study, it has been speculated that augmentation of BMP signaling induces the endocardial–mesenchymal transition or promotes cell migration from the neural crest. As expected, gene targeting in mice of Smad6, which specifically inhibits BMP signaling, results in hyperplasia and thickening of the cardiac valves [46].

In the zebrafish embryo, transient overexpression of Notch1 causes cardiac valve thickening, whereas inhibition of Notch1 signaling interrupts valvulogenesis due to a decrease in the epithelial–mesenchymal transition needed for valve formation [47]. Garg et al. [48, 49] identified a Notch1 mutation by genetic linkage analysis of autosomal-dominant familial bicuspid calcified aortic valve disease. In vitro studies have revealed that Notch1 blocks the activation of Cbfa1 (Runx2), which is a crucial transcription factor for osteoblast differentiation, and that Hairy-related transcription factor (Hrt) activated by Notch1 signaling forms a complex with Cbfa1 and inhibits its transcriptional activation. These data demonstrate the molecular mechanism of enhanced aortic valve calcification through Notch1 mutation. Notch1 missense mutations have been found not only in cases of familial bicuspid aortic valve but also in 4% of sporadic disease cases [50].

Considering recent reports on the relationship between valve degeneration and Wnt, TGF-β_1, BMP, and Notch signaling, we speculate that signaling intensity may define valve degeneration and calcification.

Other components

The components of the receptor activator of NF-κB ligand (RANKL) signaling pathway, including its receptor RANK, and the downstream enzyme cathepsin K are expressed in developing cardiac valves, and NFATc1 is specifically required for the expression of RANKL and cathepsin K during valvulogenesis [51]. Kaden et al. [52, 53] reported that RANKL expression was increased, whereas osteoprotegerin was decreased in human calcified aortic valves, both of which are involved in bone turnover and vascular calcification. Moreover, RANKL stimulation promoted cell proliferation, MMP-1 and MMP-2 activities, the DNA binding activity of Cbfa-1, alkaline phosphatase activity, osteocalcin expression, and matrix calcification in human aortic valve myofibroblasts in vitro, which suggests that the RANKL/osteoprotegerin system regulates valvular calcification. The sympathetic nervous system is known to regulate bone metabolism via β_2 adrenergic receptors. Osman et al. [54] examined the expression of β_2 adrenergic receptors in human aortic valvular interstitial cells in vitro; immunohistochemistry revealed the expression of β_2 adrenergic receptors and RANK in these cells. Furthermore, stimulation of valvular interstitial cells with the selective β_2 adrenergic receptor agonist salmeterol decreased the levels of Cbfa1 mRNA and osteocalcin protein and alkaline phosphatase activity, which indicates that the sympathetic nervous system acting via the β_2 adrenergic receptor is protective against aortic valve calcification.

Golubnitschaja et al. [55, 56] investigated the relationship between cell cycle gene expression and valvular calcification and reported that the mRNA and protein levels of the cell cycle checkpoint genes *p21WAF1/CIP1* and *14-3-3 sigma* were downregulated several-fold in α-actin-positive cells of the human calcified aortic valve. They also observed a higher cell density in the calcified aortic valve than in the normal valve and speculated that increased cell density with consequent calcification might be due to the downregulation of these G1 and G2 checkpoint genes.

In 2007, two groups [57, 58] independently reported that the use of ergot dopamine agonists (Pergolide, Cabergoline) for the treatment of Parkinson's disease significantly increased the risk of valvular regurgitation in humans. In the study of Zanettini et al. [57], 5.6%, 0%, and 23.4–28.6% of the control group, the non-ergot dopamine agonists group, and the ergot dopamine agonists group,