RBE significantly decreased the transcriptional activity in serum containing (Fig. 2*C*) or deprived (Fig. 2*D*) mDCT cells.

*Runx3 is involved in the upregulation of endogenous ATRAP gene expression in mDCT cells.* To inhibit the binding of the runt-related transcription factors (Runx1, Runx2, and Runx3) to RBE, we performed transient transfection studies using a decoy ODN method that has been well described (7, 20). A double-stranded decoy ODN was transfected into mDCT cell cultures to interfere with Runx binding to RBE, and the effects on the endogenous expression of Runx and ATRAP were subsequently analyzed. The results of real-time quantitative RT-PCR analysis showed that the ATRAP mRNA level had a tendency of decrease as the result of RBE-decoy transfection despite there being no apparent change in the expression of Runx mRNA (Fig. 3*A*). Interestingly, serum starvation upregulated Runx3 mRNA levels, and this upregulation was not affected by RBE-decoy transfection (Fig. 3*A*). Furthermore, transfection with the RBE-decoy ODN decreased the serum starvation-induced upregulation of the ATRAP protein levels in mDCT cells (Fig. 3*B*). These results indicate that Runx binding sites are involved in the transcriptional activation of ATRAP gene by serum starvation in mDCT cells.

To further determine which Runx protein is responsible for the serum starvation-induced activation of ATRAP expression

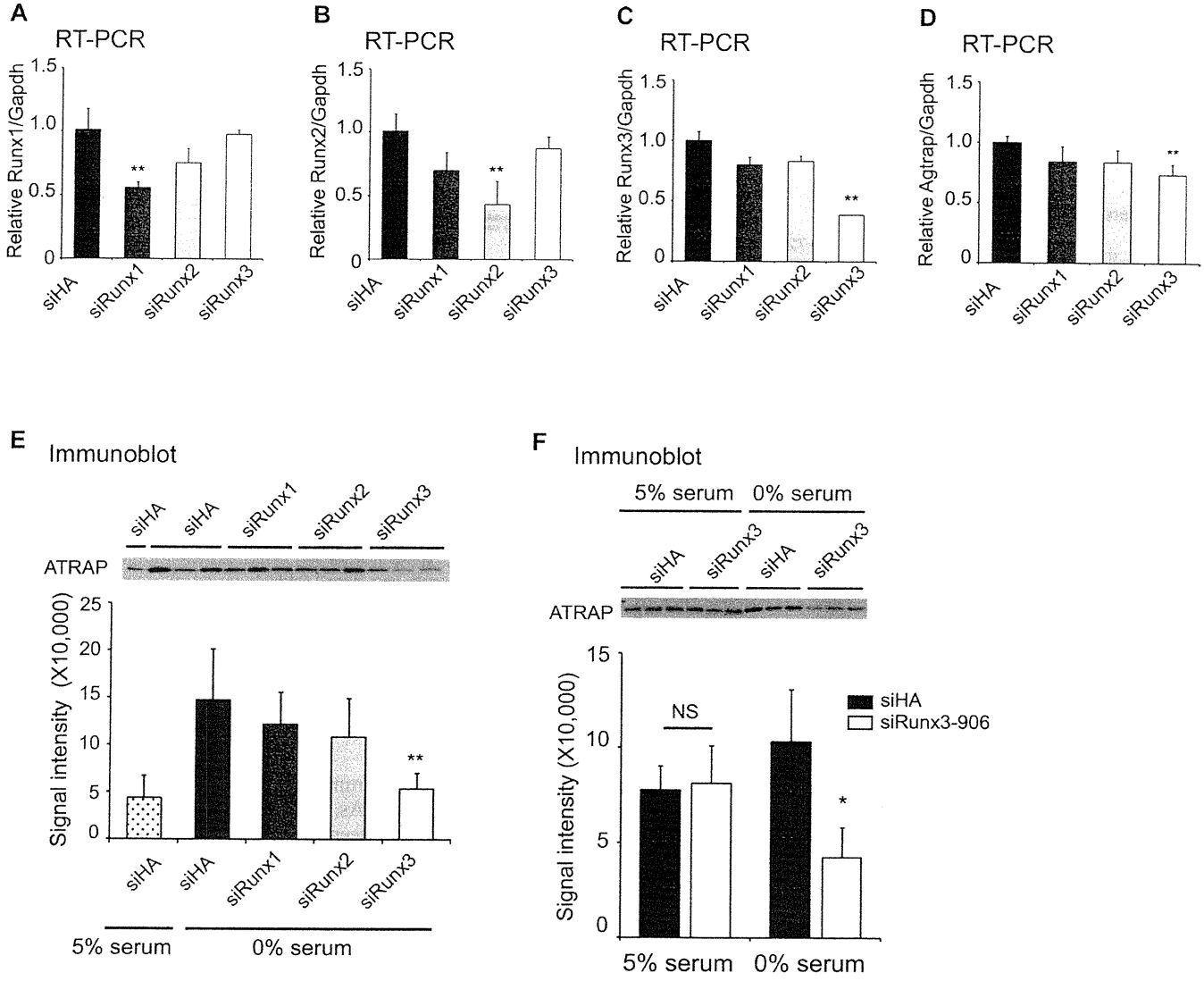



Fig. 4. Specific knockdown of Runx3 by small interference (si) RNA decreases endogenous ATRAP gene expression in mDCT cells. Quantitative real-time RT-PCR analysis showing the effects of respective siRNAs transfection on the relative Runx1 (*A*), Runx2 (*B*), and Runx3 (*C*) mRNA levels. RNA quantity was normalized to the signal generated by constitutively expressed GAPDH and is expressed relative to those achieved with extracts derived from mDCT cells transfected with control siHA ($n = 3$). $^{**}P < 0.01$, vs. siHA. *D*: quantitative real-time RT-PCR analysis showing the effects of respective siRNA transfection on the relative ATRAP mRNA level. RNA quantity was normalized to the signal generated by constitutively expressed GAPDH and expressed relative to those achieved with extracts derived from mDCT cells transfected with control siHA ($n = 3$). $^{**}P < 0.01$, vs. siHA. *E*: immunoblot showing the effects of respective siRNA transfection on ATRAP protein expression in the total membrane fraction of the deprived (0% serum) mDCT cells ($n = 3$). siHA was used as a control. $^{**}P < 0.01$, vs. siHA transfected into the deprived mDCT cells. *F*: immunoblot showing the effects of respective siRNA transfection on ATRAP protein expression in the total membrane fraction of the serum containing (5% serum) or deprived (0% serum) mDCT cells ($n = 3$). siHA was used as a control. $^{*}P < 0.05$, vs. siHA. Immunodetection of the membrane stained by Coomassie blue dye served as an internal control for the determination of equal protein loading.

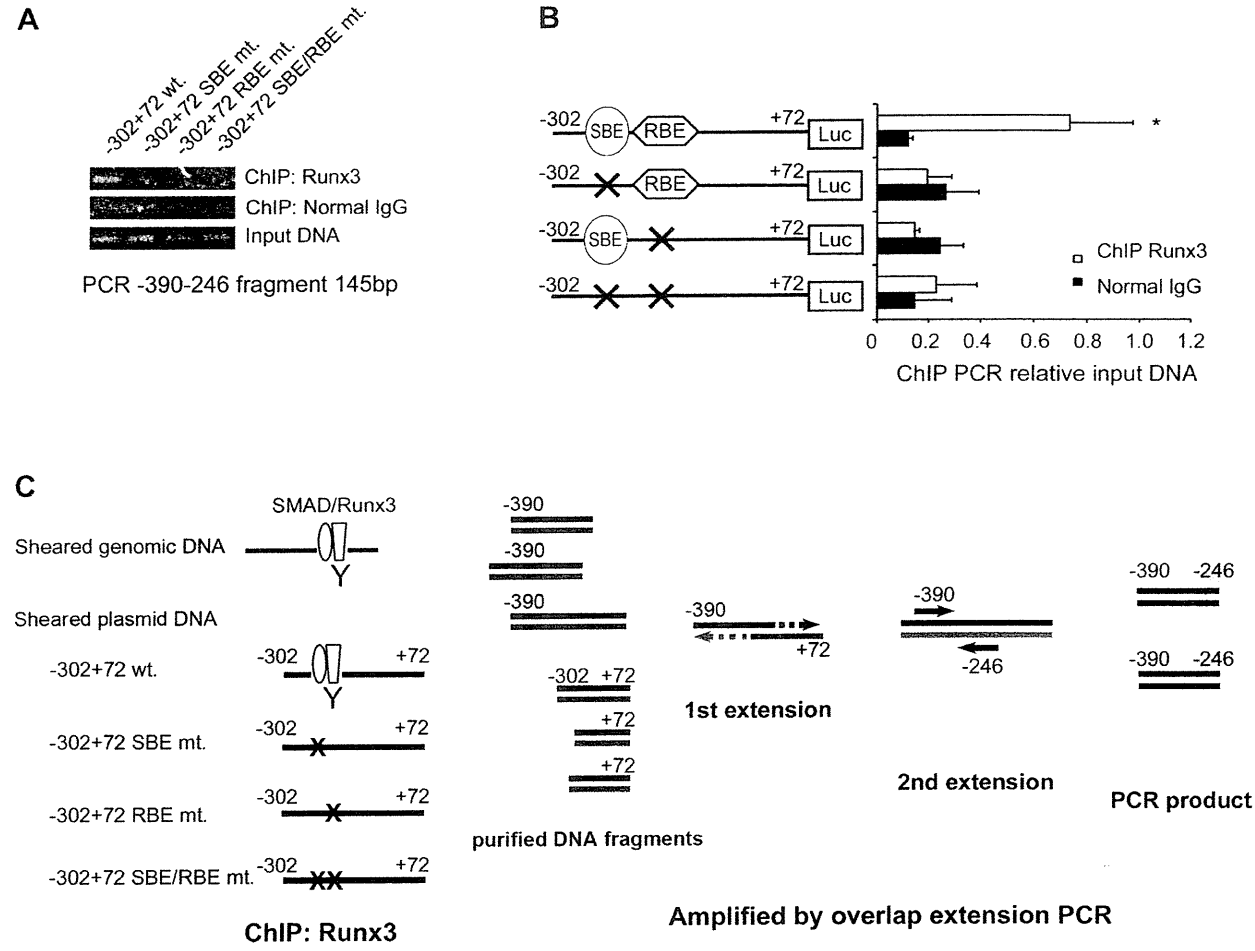


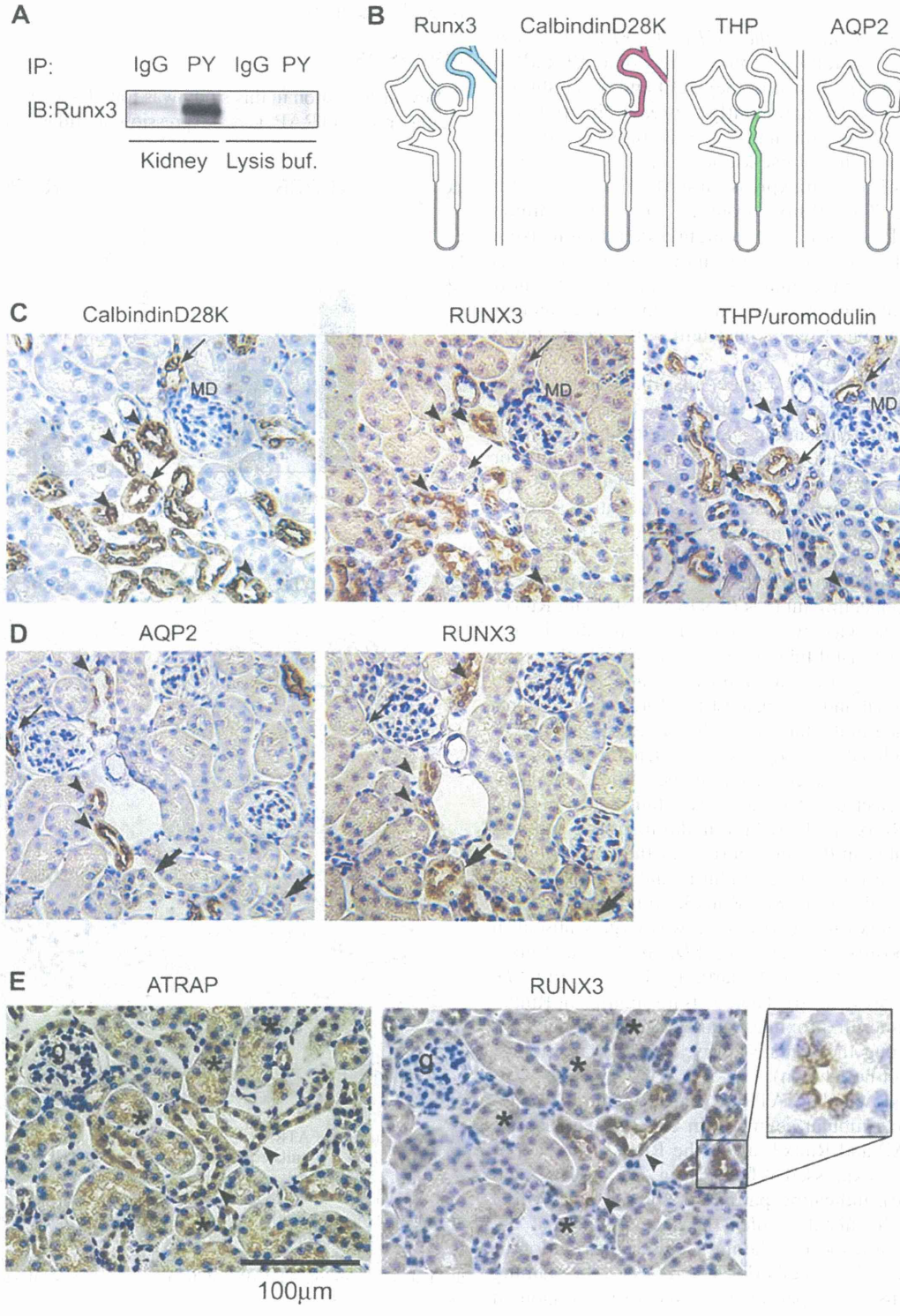

Fig. 5. Runx3 specifically binds to the ATRAP promoter in mDCT cells in ChIP assay. *A*: representative ethidium bromide staining showing the amplified ATRAP promoter fragment of 145 bp containing SBE and RBE in the anti-Runx3 antibody-bound immunoprecipitates in the mDCT cells, which were transiently transfected with the wild-type or mutated ATRAP promoter (−302 to +72)-luciferase hybrid genes, on 3% agarose gel. *B*: quantitative real-time PCR analysis showing a specific enrichment of the ATRAP promoter fragment containing wild-type SBE and RBE in the anti-Runx3 antibody-bound immunoprecipitates. The relative values of enrichment were calculated relative to the input DNA ($n = 3$). Normal IgG was used as a control. $^*P < 0.05$, vs. normal IgG. *C*: schematic explanation of the overlap extension PCR detection in the ChIP assay. Briefly, mDCT cells transiently transfected with the wild-type or mutated ATRAP promoter-containing plasmids were treated with paraformaldehyde to cross-link the protein-DNA complexes. Cell lysates were sonicated to reduce the DNA fragments to an average size of ~500 bp. Following immunoprecipitation with anti-Runx3 antibody or normal rabbit IgG, DNA was purified from the antibody-bound and unbound fractions, and the enrichment of the ATRAP promoter fragment in the bound fraction was assayed by the overlap extension PCR method. The overlap extension PCR analysis was performed using the forward primer (−390), which anneals to genomic DNA, and the reverse primer (−246), which anneals to both genomic and plasmid DNA.

in mDCT cells, we examined the effect of Runx siRNA transfection on endogenous ATRAP gene expression. The mRNA levels of Runx1 (Fig. 4*A*), Runx2 (Fig. 4*B*), and Runx3 (Fig. 4*C*) were significantly decreased after transfection with their respective siRNA in serum-starved mDCT cells. Importantly, among these Runx siRNAs, only the siRNA reduction of Runx3 resulted in a significant decrease in the levels of ATRAP mRNA (Fig. 4*D*). Furthermore, Runx3 knockdown caused a significant

Fig. 6. Runx3 immunostaining is observed mainly in the DCT and connecting tubules of the mouse kidney, and the Runx3-immunopositive (IP) distal tubules expressed the ATRAP protein. *A*: immunoblot (IB) showing that the anti-Runx3-specific antibody recognized the apparent molecular mass of the major band as ~44 kDa, which was consistent with the predicted molecular mass in the mouse kidney extracts immunoprecipitated using the anti-PY motif antibody (PY, kidney), compared with the control normal IgG (IgG, kidney). *B*: schematic localization of Runx3, CalbindinD28K, Tamm-Horsfall protein (THP) and aquaporin 2 (AQP2) along the nephron segments. *C*: consecutive sections showing the immunostaining of CalbindinD28K, Runx3, and THP, respectively. The distal tubules immunopositive for all of the CalbindinD28K, Runx3, and THP immunostaining are designated by arrowheads, and those positive for CalbindinD28K and THP, but not for Runx3, are designated by arrows. MD, macula densa. *D*: consecutive sections showing the immunostaining of aquaporin 2 (AQP2) and Runx3, respectively. The distal tubules immunopositive for both the AQP2 and Runx3 immunostaining are designated by arrowheads, while those positive for AQP2 but not for Runx3 are designated by thin arrows, and those positive for Runx3, but not for AQP2, are designated by thick arrows. *E*: consecutive sections showing the immunostaining of ATRAP and Runx3, respectively. The distal tubules immunopositive for both ATRAP and Runx3 are designated by arrowheads, with Runx3 immunostaining at a higher magnification shown on the *right*. Scale bars indicate 100 μm. g, Glomerulus; *proximal convoluted tubules. Original magnification: ×400.

suppression of the serum starvation-induced upregulation of ATRAP protein expression (Fig. 4*E*), while the inhibitory effect of Runx3 knockdown on ATRAP expression was not observed in the serum-containing mDCT cells (Fig. 4*F*).

ChIP assays were also performed to examine the association of Runx3 with the ATRAP promoter (Fig. 5). The mDCT cells were transiently transfected with the wild-type or mutated ATRAP promoter (−302 to +72)-luciferase hybrid genes, which were the same constructs as used in the promoter assays in (Fig. 2). The immunoprecipitates obtained by cross-linking ChIP assay were subjected to overlap extension PCR analysis using the forward primer (−390), which anneals to genomic



DNA, and the reverse primer (−246), which anneals to both genomic and plasmid DNAs. The ChIP assay results support a direct interaction of Runx3 with the ATRAP promoter in mDCT cells. Notably, since mutations of either SBE or RBE significantly decreased the occupancy of the ATRAP promoter by Runx3, neither Runx3 nor SMAD by itself is sufficient, and a combinatorial interaction of Runx3 and SMAD appears to be necessary for the efficient binding of Runx3 to the ATRAP promoter.

*Runx3 is expressed mainly in the DCT and CNT in the mouse kidney.* While we previously demonstrated that ATRAP is highly expressed in the mouse kidney (29) and other studies have shown that Runx3 is abundantly expressed in epithelial cells of the gastrointestinal tract (4, 14), there is no report of Runx3 expression in the mouse kidney. Thus, we examined endogenous Runx3 protein expression in the kidney of adult C57BL/6J mice. The Runx proteins share a common COOH-terminal PY-motif, and immunoprecipitation using an anti-PY motif antibody and the mouse kidney extracts followed by immunoblot analysis revealed that the anti-Runx3-specific antibody recognized an apparent molecular mass of ~44 kDa, which was consistent with the predicted molecular mass (Fig. 6*A*).

We then examined Runx3 protein distribution in normal adult mouse kidneys sections by immunohistochemistry. We found the Runx3 immunostaining sites to be localized to renal tubules in the cortex, with no involvement of the medulla or papilla (data not shown). To identify the specific tubular segments in the Runx3 immunostaining, consecutive sections were stained for Runx3 and markers specific to the tubular segments (Fig. 6*B*). We used a specific antibody against calbindin-D, a calcium-binding protein that is expressed primarily in the DCT and connecting tubules (CNT) (16, 30), and Runx3 protein immunoreactivity was observed specifically in the calbindin-D positive distal tubules (DCT and CNT) other than the macula densa (Fig. 6*C*, calbindinD28K and Runx3). We also used a polyclonal antibody against the Tamm-Horsfall protein that is expressed in the thick ascending limbs of the loop of Henle, and a polyclonal antibody against aquaporin 2, which is specifically expressed in the collecting tubules. Runx3 immunostaining was not detected in the Tamm-Horsfall protein-positive tubules (Fig. 6*C*, Runx3 and THP/uromodulin, arrow) and was detected only weakly in the early portion of the distal tubules, which were positive for both calbindin-D and Tamm-Horsfall protein (Fig. 6*C*, calbindinD28K, Runx3, and THP/uromodulin, arrowhead). Runx3 immunostaining was largely absent in the aquaporin 2-positive tubules (Fig. 6*D*, AQP2 and Runx3, arrow) and when evident was only partially observed (Fig. 6*D*, AQP2 and Runx3, arrowhead). There was no significant Runx3 staining in the glomeruli, proximal tubules, collecting ducts, or vasculature including the arcuate artery, interlobular arteries, and arterioles (data not shown).

We investigated whether ATRAP and Runx3 are coexpressed in the same tubular segment. In consecutive sections stained for ATRAP and Runx3, all of the Runx3-immunopositive distal tubules expressed ATRAP (Fig. 6*E*, ATRAP and Runx3, arrowhead), indicating partial coexpression of Runx3 with ATRAP in the distal tubules (DCT and CNT) of the mouse kidney. With respect to the intracellular distribution of Runx3 in the distal tubular cells (DCT and CNT), the staining of Runx3 was intense in the perinuclear and cytosolic region on the apical side at higher magnification (Fig. 6*E*, Runx3). Finally, we examined the effects of UUO on Runx3 and ATRAP expression. ATRAP mRNA expression was significantly downregulated in the affected kidney after 7 days of UUO, with a concomitant decrease in ATRAP immunostaining (Fig. 7, *A* and *B*). On the other hand, while the Runx3 mRNA expression was upregulated in the affected kidney after 7 days of UUO, the distal tubules were not immunopositive for Runx3 (Fig. 7, *A* and *B*).

## DISCUSSION

Serum starvation in this study was found to be a major positive regulator of ATRAP gene expression in mDCT cells. Serum

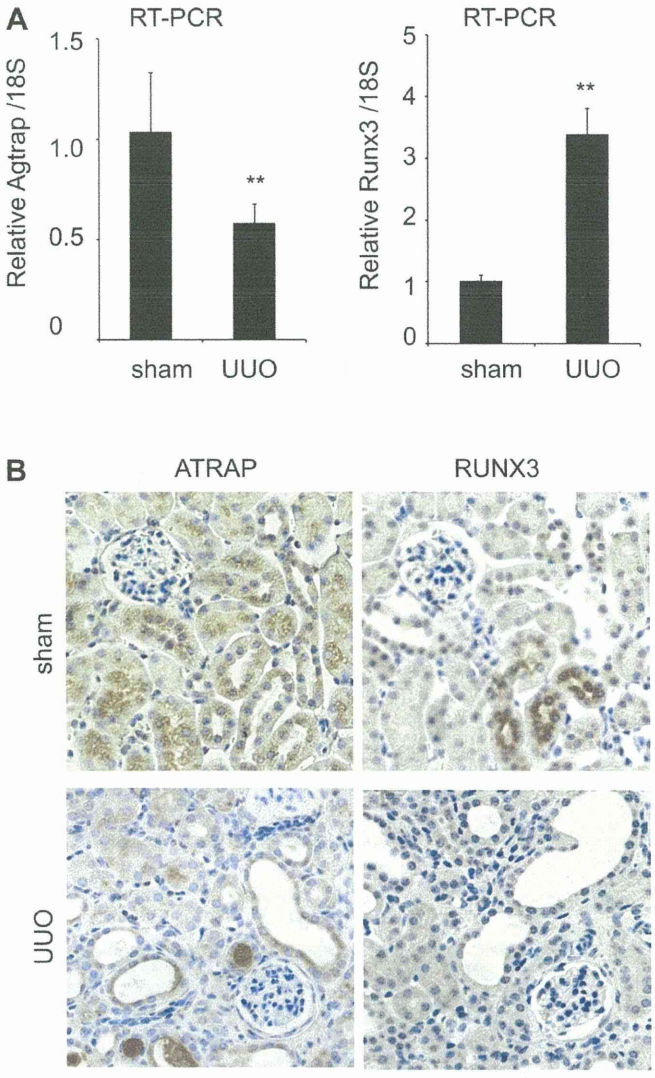



Fig. 7. Regulation of ATRAP and Runx3 mRNA and protein expression in the affected kidney by unilateral ureteral obstruction (UUO). *A*: quantitative real-time RT-PCR analysis showing the effects of UUO on the relative ATRAP and Runx3 mRNA levels. RNA quantity was normalized to the signal generated by the constitutively expressed 18S rRNA and is expressed relative to those achieved with extracts derived from sham-operated kidney ($n = 3$). $^{**}P < 0.01$, vs. sham. *B*: consecutive sections showing the effects of UUO on the immunostains of ATRAP and Runx3 in the affected kidney. Original magnification: ×400.

starvation is known to reduce cellular proliferation and induce differentiation in cultured cells, including renal tubule cells (3), and this may include increased ATRAP expression in mDCT cells. Since the starvation-induced increase in basal ATRAP expression was inhibited by treatment with either actinomycin D or cycloheximide (Fig. 1, *F* and *G*), it is likely that the serum starvation induced activation of basal ATRAP expression involves de novo mRNA and protein synthesis. Sequence analysis of the 5′-flanking region of the ATRAP gene using the TF Search program and Signal Scan algorithm revealed the absence of a TATA box and the presence of a GC-rich region extending >200 nt from the initiating methionine. The percentage of GC dinucleotides in this region ranged between 65 and 80%, with a CG:GC ratio of 0.94, thus fulfilling the length and base composition criteria for a canonical CpG island specifically located at the transcriptional start site (32).

The results of the transient transfection assay demonstrate that the proximal promoter region from −302 to +72 of the ATRAP gene is sufficient to drive luciferase reporter gene expression in deprived mDCT cells. Mutation of either SBE or RBE significantly decreased the promoter activity of the ATRAP gene, but a mutation of both SBE and RBE did not further decrease the promoter activity in the transfection assay (Fig. 2). Similarly, mutations of SBE or RBE alone or together significantly decreased the binding of Runx3 to the ATRAP promoter to a similar extent in the ChIP assay (Fig. 5). These results suggest that the combinatorial interaction of SBE and RBE is important for the binding of Runx3 to the ATRAP promoter and transcriptional activation of the proximal promoter of the ATRAP gene by Runx3. This is consistent with previous studies showing that the intranuclear targeting of the Runx-Smad complex to transcriptionally active SBE and RBE adjacent sites is necessary for transcriptional regulation by Runx and SMAD proteins (12, 37). A previous study also reported a similar coordinated action of SMAD and Runx3 drives the transcription of the GLγ2b gene in response to TGFβ1 stimulation (24).

With respect to the functional role of Runx3 in the expression of the ATRAP gene, the results of the RBE-decoy transfection experiment and Runx siRNA-mediated endogenous Runx knockdown assay indicate that it is Runx3 among the Runx family transcription factors that is critically involved in the activation of ATRAP gene expression. Interestingly, multiple RBEs are present throughout the mouse ATRAP locus, including both the coding and noncoding regions (data not shown). A previous study showed that Runx2 directly regulated ribosomal biogenesis through multiple RBEs in the ribosomal RNA locus by DNA binding and indirectly by affecting chromatin histone modification (36). The results of the present study indicate that the direct DNA binding of Runx3 to RBE is involved in the activation of ATRAP gene expression in mDCT cells, but further studies are needed to elucidate the molecular mechanism of Runx3-mediated transcriptional activation of the ATRAP gene promoter.

The Runx3 gene is reported to be expressed in the mesenchymal elements; it controls the proper development of gastric endothelial cells by apoptosis and also acts as a tumor suppressor gene (5). In this study, we showed that in vitro Runx3 as well as ATRAP is expressed in mDCT cells and, in vivo, in the distal tubules of the mouse kidney. To the best of our knowledge, this is the first report demonstrating that Runx3 is expressed in renal tubular cells. Furthermore, the intrarenal localization of Runx3 does not completely coincide with that of ATRAP in terms of the tubular segments. While the expression of the Runx3 protein was mainly localized in the DCT and CNT of the distal tubules, the ATRAP protein was widely distributed along the renal tubules (3, 29).

Our previous studies showed that the intracellular localization of ATRAP also does not completely coincide with that of the AT1 receptor, the binding partner of ATRAP, in the tubular segments (29). The results of in vitro experiments showed that ANG II treatment induces substantial colocalization of ATRAP and AT1 receptor in cardiovascular cells (1, 28), thereby suggesting that ATRAP and the AT1 receptor can have other effectors, as reported (10). Therefore, the results of the present study suggest that there are other factor(s), such as coactivator(s), which cooperate with both Runx3 and ATRAP. Further molecular screens for both Runx3 and ATRAP are needed to identify such putative additional partners for these molecules, which may act cooperatively or independently in terms of timing and specific cellular locations in the kidney.

Finally, UUO is a well-established experimental model of progressive tubulointerstitial fibrosis. UUO leads to changes in renal hemodynamics, inflammatory responses in the kidney, and tubular hypertrophy and interstitial fibrosis of the affected kidney (23). The renin-angiotensin system is also known to be activated in UUO, and the results of the present study showed a significant downregulation of Runx3 and ATRAP expression in the affected kidney, suggesting a possible role of Runx3 in the regulation of ATRAP in vivo.

The limitations of the present study include the lack of data on the effect of serum starvation on ATRAP binding to the AT1 receptor and its subsequent internalization. Further studies are needed to elucidate the function of ATRAP on AT1 receptor signaling under starvation conditions, and these will be taken up in due course. Caution should be used in interpreting the finding of this study in terms of the pathophysiology in humans, and further studies are warranted to investigate the role of Runx3 in ATRAP regulation under various physiological and/or pathological conditions.

In summary, the results of the present study demonstrate the Runx3-mediated basal transcriptional activation of ATRAP gene in renal distal tubular cells is dependent on its DNA binding. Furthermore, the results of immunohistochemical analysis show a partial colocalization of Runx3 and ATRAP proteins in the distal tubules of the mouse kidney. These findings suggest an interesting molecular link between Runx3, one of the important Runt-related transcription factors in the determination of cell lineage and differentiation (35), and ATRAP, a newly emerging component of the renin-angiotensin system, a link that is likely to play a role in the regulation of ATRAP gene expression in the renal distal tubules.

## ACKNOWLEDGMENTS

We are indebted to Dr. P. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA) for providing us with the mDCT cells. The authors also thank Emi Maeda and Hiroko Morinaga for technical assistance and helpful discussion. Pacific Edit reviewed the manuscript prior to submission.

## GRANTS

This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture; by a Health and Labor Sciences Research grant; and by Salt Science Research Foundation Grants 1033, 1134; the Kidney Foundation, Japan (JKFB11-25); and Strategic Research Project of Yokohama City University.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Hypertension Research (2011) 34, 1249–1250

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COMMENTARY

# Day-by-day home-measured blood pressure variability: another important factor in hypertension with diabetic nephropathy?

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*Hypertension Research* (2011) **34**, 1249–1250; doi:10.1038/hr.2011.149; published online 25 August 2011

The number of patients with diabetes is increasing, and cardiovascular complications are the most common cause of death in these patients. Thus, it would be of considerable value to identify the mechanisms involved in the cardiovascular events associated with diabetes. Ambulatory blood pressure (BP) monitoring has allowed an easier and more accurate determination of the circadian rhythm of BP under different pathophysiological conditions. The circadian pattern of BP in patients with diabetes has been found to exhibit a blunted nocturnal decrease in BP, which is associated with autonomic neuropathy and nephropathy.[1] The loss of nocturnal BP dipping has been considered a risk factor for the progression of nephropathy and of prognostic value with respect to target organ damage and cardiovascular morbidity in both diabetic and hypertensive patients.[2–4]

Ambulatory BP monitoring allows the acquisition of valuable information on not only the average 24-h BP but also the variations in the BP values that happen during the course of daily life. Previous studies on ambulatory BP monitoring have shown that BP variability is a complex phenomenon that involves both short- and long-lasting changes. Thus, the 24-h BP varies not only because of a reduction in BP during nighttime sleep and an increase in the morning, but also because of sudden, rapid and short-lasting changes that occur during the daytime and, to a lesser extent, at night. This phenomenon of short-term BP variability has been shown to depend on sympathetic vascular modulation and on atherosclerotic vascular changes.[5] Several previous animal studies have shown that exaggerated short-term BP variability without significant changes in mean BP induced chronic cardiovascular inflammation and remodeling (Figure 1).[6] Short-term BP variability is also thought to be clinically relevant because hypertensive patients with similar 24-h mean BP values exhibit more severe organ damage when the short-term BP variability is greater.[5,7–13]

Several clinical studies have provided epidemiological evidence of the greater accuracy of home BP monitoring compared with clinical BP monitoring for the prognosis of fatal and non-fatal cardiovascular disease in long-term follow-up surveys and in cross-sectional studies. There is a general consensus that home BP monitoring is more convenient, available and less costly than ambulatory BP monitoring, but the superiority of ambulatory BP monitoring for special clinical problems is also clearly recognized. These special clinical problems include (1) the detection of non-dippers or the need for sleep pressures in chronic renal disease, autonomic neuropathies and sleep apnea and (2) the estimation of short-term BP variability.[14] Surveys of both physicians and patients suggest that home BP monitoring is both appreciated and recognized as a valuable strategy. Several experts in the field of hypertension research and care have published appeals to expand the use of home BP monitoring for routine care and to have it supported by health care systems.

In the current issue of *Hypertension Research*, Ushigome *et al.*[15] evaluated the association of home-measured BP variability with overt nephropathy by a cross-sectional analysis in 858 Japanese patients with type 2 diabetes. The patients measured their BP three times every morning and three times every evening for 2 weeks. The home-measured BP variability was expressed as the day-by-day BP variability calculated using the within individual coefficient of variation of all home BP values. The day-by-day BP variability in the morning and that in the evening were calculated separately. The authors showed that the day-by-day BP variability correlated with macroalbuminuria (urine albumin-to-creatinine ratio $\geqslant 300\,mg\,g^{-1}$ creatinine) independent of the known risk factors in Japanese patients with type 2 diabetes. Concerning home-measured BP variability, a previous study showed that high day-by-day BP variability is associated with increases in total, cardiovascular and stroke mortality, independent of BP value and other cardiovascular risk factors in the general population of the Ohasama study.[16] For type 2 diabetes, while high short-term BP variability during ambulatory BP monitoring is reported to be associated with atherosclerosis and proteinuria in hypertensive patients with type 2 diabetes,[8,17,18] the study by Ushigome *et al.* adds further information about the clinical relevance of home-measured BP variability in the pathophysiology of diabetic nephropathy. Although the hypothesis that home-measured BP variability favors the development of nephropathy in type 2 diabetes is appealing, the cross-sectional nature of this study makes it impossible to evaluate the causal relationships between day-by-day BP variability and diabetic nephropathy, as acknowledged by the authors.[15] Further studies, such as outcome studies focusing on whether a therapeutic intervention

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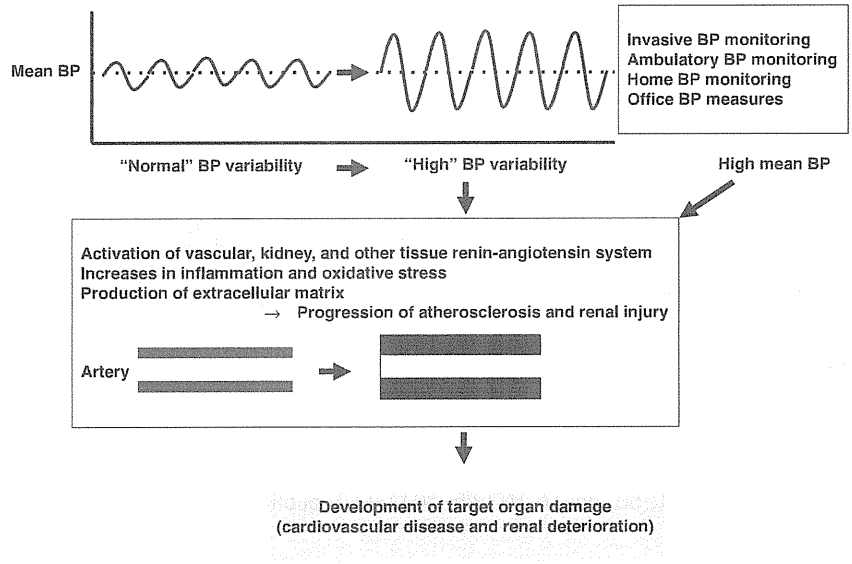


**Figure 1** Schema showing the proposed effects of BP variability by various BP measures on the progression of vascular atherosclerosis and the development of target organ damage.

that reduces day-by-day BP variability also carries additional prognostic benefits through the concomitant suppression of the development of diabetic nephropathy, are warranted to confirm the prognostic value of home-measured BP variability.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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