

# TonEBP stimulates multiple cellular pathways for adaptation to hypertonic stress: organic osmolyte-dependent and -independent pathways

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<sup>1</sup>Department of Medicine, University of Maryland, Baltimore, Maryland; <sup>2</sup>Department of Physiology, Chungnam National University, Daejeon, Republic of Korea; <sup>3</sup>Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania; and <sup>4</sup>Department of Pathology, University of California, La Jolla, California

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**Lee SD, Choi SY, Lim SW, Lamitina ST, Ho SN, Go WY, Kwon HM.** TonEBP stimulates multiple cellular pathways for adaptation to hypertonic stress: organic osmolyte-dependent and -independent pathways. *Am J Physiol Renal Physiol* 300: F707–F715, 2011. First published January 5, 2011; doi:10.1152/ajprenal.00227.2010.—TonEBP (tonicity-responsive enhancer binding protein) is a transcription factor that promotes cellular accumulation of organic osmolytes in the hypertonic renal medulla by stimulating expression of its target genes. Genetically modified animals with deficient TonEBP activity in the kidney suffer from severe medullary atrophy in association with cell death, demonstrating that TonEBP is essential for the survival of the renal medullary cells. Using both TonEBP knockout cells and RNA interference of TonEBP, we found that TonEBP promoted cellular adaptation to hypertonic stress. Microarray analyses revealed that the genetic response to hypertonicity was dominated by TonEBP in that expression of totally different sets of genes was increased by hypertonicity in those cells with TonEBP vs. those without TonEBP activity. Of over 100 potentially new TonEBP-regulated genes, we selected seven for further analyses and found that their expressions were all dependent on TonEBP. RNA interference experiments showed that some of these genes, asporin, insulin-like growth factor-binding protein-5 and -7, and an extracellular lysophospholipase D, plus heat shock protein 70, a known TonEBP target gene, contributed to the adaptation to hypertonicity without promoting organic osmolyte accumulation. We conclude that TonEBP stimulates multiple cellular pathways for adaptation to hypertonic stress in addition to organic osmolyte accumulation.

tonicity-responsive enhancer binding protein; heat shock protein 70; asporin; insulin-like growth factor binding proteins; lysophospholipase D

CELLS IN THE RENAL MEDULLA are bathed in hypertonic interstitium. The hypertonicity is due to hyperosmotic concentration of sodium chloride, which routinely reaches 1,000 mosmol/kgH<sub>2</sub>O in rat inner medulla during antidiuresis (4). While cultured cells die at such extreme hypertonicity, cells in the renal medulla in situ do not display any measurable sign of cell death (32, 36). It has been shown that cultured cells can be acclimated to the extreme hypertonicity when ambient tonicity is raised slowly (7, 16). The slow increase in ambient tonicity allows increased cellular accumulation of organic osmolytes (6). Major organic osmolytes in the mammalian kidneys are *myo*-inositol, betaine, taurine, sorbitol, and glycerophosphorylcholine (GPC) (11). The transcription factor TonEBP (tonicity-responsive enhancer binding protein) is a central regulator of the osmoprotective

organic osmolyte accumulation (5, 15). Activated by hypertonicity, TonEBP stimulates transcription of those genes encoding the sodium/*myo*-inositol cotransporter (SMIT), sodium chloride/betaine cotransporter (BGT1), sodium chloride/taurine cotransporter (TauT), aldose reductase (AR, for biosynthesis of sorbitol), and a phospholipase named neuropathy target esterase (NTE, for biosynthesis of GPC). In the renal medulla, cellular accumulation of *myo*-inositol, betaine, taurine, sorbitol, and GPC prevents harmful elevation of intracellular potassium concentration (4). Severe medullary atrophy is observed in those mice whose TonEBP gene is inactivated (20) or whose kidney cells express dominant negative form of TonEBP (17), demonstrating the critical role of organic osmolyte accumulation for the survival in hypertonic environments.

In this study, we uncovered new TonEBP-regulated genes whose expression was stimulated by hypertonicity. When many of these genes were individually knocked down, cells displayed reduced hypertonic adaptation without reducing cellular content of *myo*-inositol, the major organic osmolyte in the cells used, suggesting that TonEBP action involved organic osmolyte-independent pathways in addition to the organic osmolyte-dependent pathway.

## METHODS

**Cell culture.** Wild-type and *TonEBP*<sup>ΔΔ</sup> mouse embryonic fibroblast (MEF) cells (12) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate (Invitrogen), 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen). Where indicated, medium tonicity was raised by adding 75 to 300 mM NaCl as indicated.

**Lactate dehydrogenase release assay.** At the end of treatment, culture medium was collected, and the adhering cells were lysed in TBS containing 2% Triton X-100. Lactate dehydrogenase (LDH) in culture medium and cell lysates was quantified using a commercial kit (LDH Cytotoxic Dection Kit; Clontech, Mountain view, CA).

**Immunoblotting.** Cells were washed with ice-cold PBS and lysed for 30 min at 4°C in lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Lysates were cleared by centrifugation at 15,000 g for 5 min, separated on SDS-polyacrylamide gels, and transferred to PVDF membrane. Renal samples were prepared from four 10-wk-old male mice of C57BL6 strain (Harlan, Indianapolis, IN). Use of the animals was approved by the Institutional Animal Care and Use Committee of the University of Maryland. The kidneys were perfused for 15 s with PBS via retrograde perfusion of the aorta to rinse out the blood. Cortex, outer medulla, and inner medulla were excised and homogenized in 1% SDS, 1 mM orthovanadate, and 10 mM Tris (pH 7.5). The

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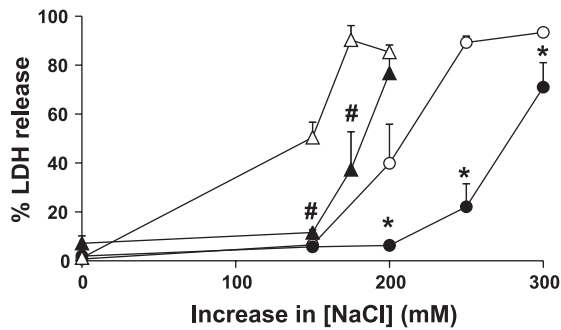


Fig. 1. Differential sensitivity to hypertonic stress in wild-type (circles) vs. *TonEBP*<sup>ΔΔ</sup> MEF cells (triangles). TonEBP, tonicity-responsive enhancer binding protein. Cells had been pretreated for 1 day in isotonic (open symbols) or hypertonic medium (75 mM NaCl added; filled symbols) before they were switched for 1 day to isotonic medium or hypertonic medium containing extra 150–300 mM NaCl. Cell death was assessed by %LDH (lactate dehydrogenase) release: %LDH in medium over total LDH (LDH in medium + LDH in adherent cells). Values are means ± SD; n = 4 or 5. \*P < 0.05 vs. corresponding isotonic pretreatment.

homogenates were cleared by centrifugation and processed as the cell lysates. Immunoblotting was performed as described (30), with the addition of the following antibodies (dilution used): anti-Enpp2 (1:500; Cayman, Ann Arbor, MI); anti-IGFBP5, anti-IGFBP7, anti-Npr1, and anti-CryAB (all 1:500; R&D Systems, Minneapolis, MN); and anti-CDO1 (1:1,000; Abcam, Cambridge, MA). Enhanced chemiluminescence assay was performed to visualize horseradish peroxidase by means of a commercial kit.

**RNA interference.** The following list of Dicer-substrate small interfering (si)RNAs was purchased from Integrated DNA Technologies (Coralville, IA). Target sequences of siRNAs were (last two nucleotides are DNA as shown in small letters): TonEBP (CCAGUCCUACAAU-GAUACACUga), SMIT (GCCUUGUACUUAAGGAGAAUUActa), BGT1 (AGAUAGAAAUGUCAUCAAGAGCUtg), asporin (CCCAAU-CAUUAGCAGAACUCAGaa), AR (GGCCGUGAAAGUUGCUAUU-GACUtg), Hsp70 (GGCACCGAUUACUGUCAAGGUUAtt), CryAB (CAGAGAGCUAGUGAAAACAAGACCat), IGFBP5 (CCACUAAA-GUGCAAUGUUUCCUGca), IGFBP7 (CCCACUAACACUUUAU-UACAGCCag), Enpp2 (GCCUUUAUAGACCAAUCUUAUUAAUAta), Npr1 (CCAAGACAGCAUACUUAUAGGGCaa), and CDO1 (GGAAGUUUAAUCUGAUGAUUCUGtg). Scrambled siRNA did not target any sequence in the human, mouse, or rat transcriptomes. MEF cells were transfected for 1 day using 10 nM siRNA and Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. Transfected cells were cultured for another day in fresh culture medium before further treatment or analysis.

**Microarray analysis.** Wild-type and *TonEBP*<sup>ΔΔ</sup> MEF cells were cultured for 1 day in isotonic or hypertonic medium. RNA samples from four independent experiments over four passages were equally pooled for analysis. The pooled samples were labeled and hybridized to Affymetrix Mouse Gene 1\_0 st v.1 gene chips using the service provided by University of Pennsylvania Microarray Facility. Robust Multi-Array normalized probe set intensities and Affymetrix annotations were loaded into the microarray tool Spotfire DecesionSte for further analysis. Duplicate probes were averaged, and the hypertonic-to-isotonic ratio for both cell types was calculated. Only one assay was performed for each pooled sample. All microarray data are MIAME compliant and have been deposited in the Gene Expression Omnibus database under accession no. GSE25816.

**Ribonuclease protection assays.** Ribonuclease protection assays (RPA) were performed using a commercial kit (RPA III kit; Ambion, Austin, TX). An RPA probe for SMIT was made to nucleotides 779–1162 of NM\_017391 (NCBI accession no.); BGT1, 643–1035 of NM\_133661; asporin, 287–558 of NM\_025711; AR 363–644 of

NM\_009658; CryAB, 623–915 of NM\_009964; IGFBP5, 1322–1632 of NM\_010518; IGFBP7, 538–770 of NM\_008048; Enpp2, 333–603 of NM\_015744; Npr1, 994–1255 of NM\_008727; and Sgk1, 1446–1714 of NM\_011361. A probe for mouse cyclophilin (Ambion) was used as the loading control to correct for RNA loading.

**Myo-inositol assay.** Cells grown on Cluster 6 plates were washed and extracted with 0.5 M perchloric acid. The extracts were neutralized using 1 N NaOH. Protein content was assayed using a commercial reagent (Bio-Rad Protein Assay) using bovine serum albumin as a standard. Myo-inositol was determined using an enzymatic colorimetric assay (3) and was expressed as nanomoles per milligram of protein.

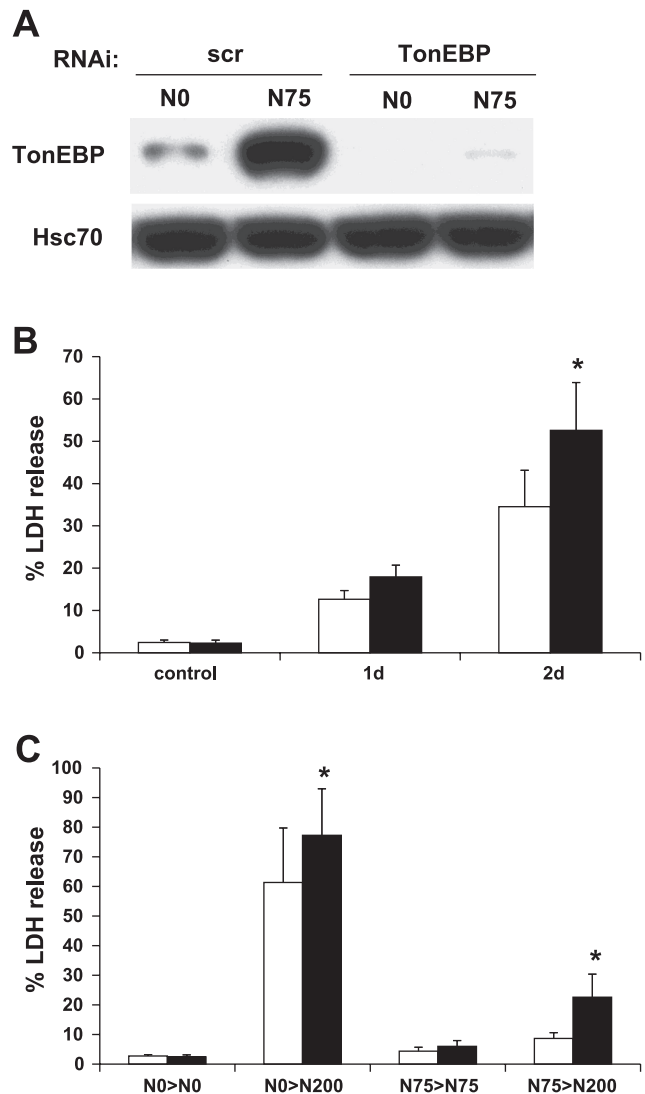


Fig. 2. Effects of siRNA-mediated TonEBP knockdown on sensitivity to hypertonic stress. **A:** wild-type MEF cells were transfected with scrambled (scr) or TonEBP-targeted siRNA (TonEBP). Transfected cells were cultured for 1 day in isotonic (N0) or hypertonic medium (N75, 75 mM NaCl added) and immunoblotted for TonEBP and Hsc70. **B:** wild-type MEF cells transfected with scrambled (open bars) or TonEBP-targeted siRNA (solid bars) were cultured for 0 (control), 1 (1d), or 2 days (2d) in hypertonic medium containing additional 150 mM NaCl. %LDH release was measured as in Fig. 1. **C:** MEF cells were transfected as in **B** and then cultured for 1 day in isotonic medium (N0) or N75, followed by another day in N0, N75, or N200 (200 mM NaCl added) as indicated. Values are means ± SD; n = 3–5. \*P < 0.05 vs. cells transfected with scrambled siRNA.

## RESULTS

*TonEBP promotes cellular adaptation to hypertonic stress.* TonEBP was originally identified on the basis of its ability to stimulate transcription of those genes that encoded proteins involved in cellular accumulation of organic osmolytes (15). As expected from the osmoprotective function of organic osmolytes, it was reported that MEF cells established from mice homozygous for the inactive mutant allele (*TonEBP $\Delta$* ) failed to survive in hypertonic conditions (12). We decided to characterize these cells to gain further insight into the cellular adaptation to hypertonic stress.

We subjected wild-type and *TonEBP $\Delta/\Delta$*  MEF cells to hypertonic conditions and measured the release of LDH to monitor cell death. When directly switched to varying degrees of hypertonicity (open symbols in Fig. 1), both cell types displayed dose-dependent cell death. *TonEBP $\Delta/\Delta$*  cells displayed greater sensitivity to hypertonic stress than wild-type cells: maximum cell death was reached at  $\sim$ 650 mosmol/kg (175 mM NaCl added) for *TonEBP $\Delta/\Delta$*  cells compared with  $\sim$ 800 mosmol/kg (250 mM NaCl added) for wild-type cells. These observations show that TonEBP increases the resistance to hypertonicity by shifting the cell death curve to higher tonicity.

A mild hypertonicity made by addition of 75 mM NaCl (N75) was well tolerated by both wild-type and *TonEBP $\Delta/\Delta$*  MEF cells in that less than 5% of LDH was released in a day (data not shown). Pretreatment for 1 day in N75 made both types of cells more resistant to hypertonic stress (filled symbols in Fig. 1): maximum cell death was reached at  $\sim$ 700 mosmol/kg (200 mM NaCl added) for *TonEBP $\Delta/\Delta$*  cells,

whereas maximum cell death was yet to be reached at  $\sim$ 900 mosmol/kg (300 mM NaCl added) for wild-type cells. The ability of *TonEBP $\Delta/\Delta$*  cells to adapt to hypertonicity might be due, at least in part, to residual expression of some the TonEBP-regulated genes (see next paragraph).

The establishment of *TonEBP $\Delta/\Delta$*  MEF cells involved continuous rounds of cell doubling for a prolonged period of time. This raised concerns that their phenotype might be due to indirect effects of long-term TonEBP deficiency. To examine direct effects of TonEBP, we acutely knocked down TonEBP with siRNA in wild-type MEF cells. Figure 2A shows that we were able to routinely knock down  $\sim$ 90% of TonEBP. In these cells, significantly increased sensitivity to hypertonicity and reduced adaptation to hypertonicity were observed (Fig. 2, B and C). The effects were much smaller than what was observed in *TonEBP $\Delta/\Delta$*  cells. This was likely due to residual TonEBP activity as indicated by modest upregulation of TonEBP-regulated genes at the level of mRNA (Fig. 3, B vs. A) and protein (Fig. 4, B vs. A). It should also be noted that, even in *TonEBP $\Delta/\Delta$*  cells, some of the TonEBP-regulated genes were clearly expressed and might have contributed to the adaptation to hypertonic stress, albeit with significantly less efficiency. Taken together, these data demonstrate that TonEBP promotes cellular adaptation to hypertonic stress.

*Identification of new TonEBP-regulated genes.* In order to screen TonEBP-regulated genes, we examined the wild-type and *TonEBP $\Delta/\Delta$*  cells. We analyzed changes in mRNA expression after the cells had been cultured for 1 day in N75 for two reasons. First, both wild-type and *TonEBP $\Delta/\Delta$*  cells tolerated

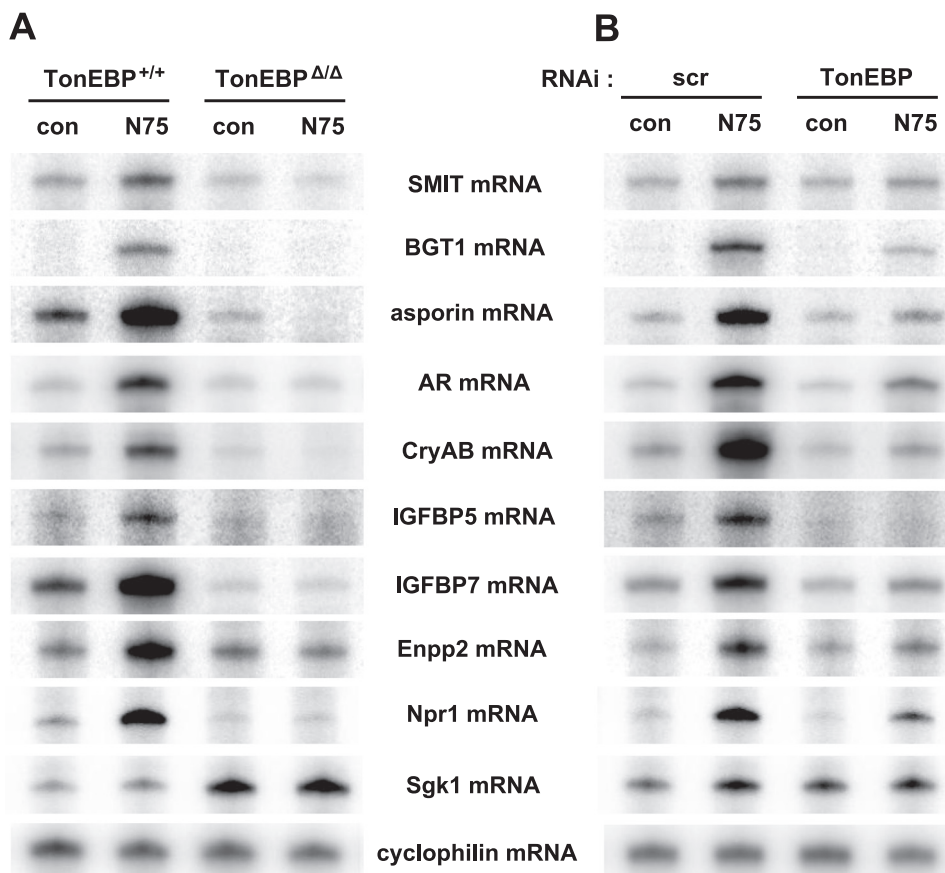


Fig. 3. Effects of TonEBP deficiency on mRNA expression of selected genes. A: wild-type (*TonEBP<sup>+/+</sup>*) and *TonEBP $\Delta/\Delta$*  MEF cells were cultured for 1 day in isotonic (N0) or hypertonic medium (N75). B: wild-type MEF cells were transfected with scrambled (scr) or TonEBP-targeted siRNA (TonEBP) followed by cultured for 1 day in N0 or N75. RPA was performed to detect mRNA for the genes shown. For definitions, see next page. Cyclophilin mRNA showed similar pattern in all the siRNA-transfected cells. Representative results from 3 independent experiments are shown.

this mild hypertonicity without significant cell death; therefore, we could eliminate the effect of cell death. Second, since these cells became adapted, i.e., more resistant, to hypertonicity, changes in gene expression profile should provide insight into cellular pathways involved.

We examined mRNA expression of over 18,000 genes in wild-type and *TonEBP $\Delta\Delta$*  cells cultured in isotonic medium or N75 using microarray analysis as described in METHODS. In response to the mild hypertonicity, 114 genes were upregulated more than twofold; i.e., mRNA abundance increased more than twofold in wild-type cells. None of those genes were upregulated in the *TonEBP $\Delta\Delta$*  cells, suggesting that they were direct transcriptional targets of TonEBP. We decided to investigate those genes because they were likely to be involved in the adaptation to hypertonicity. As expected, the list of 114 genes included those genes involved in organic osmolyte metabolism: AR, SMIT, and BGT1. Other genes involved in organic osmolyte accumulation, TauT and NTE, were not detected in MEF cells (data not shown). The list also included Hsp70, a TonEBP target gene (34), but not Sgk1 (serum- and glucocorticoid-inducible kinase-1) (9), as its induction was less than twofold (see below for more detail).

We first examined known TonEBP target genes in MEF cells. As expected, AR, SMIT, BGT1, and Hsp70 showed dramatic induction in response to hypertonicity in a TonEBP-

dependent manner: reduced expression in *TonEBP $\Delta\Delta$*  cells (Fig. 3A) and in response to TonEBP knockdown (Fig. 3B). On the other hand, Sgk1 mRNA showed only a moderate induction (<2-fold) in response to hypertonicity, which was not suppressed by TonEBP deficiency or knockdown (Fig. 3). This observation suggests that in MEF cells Sgk1 is not a direct target of TonEBP, which contrasts with recent findings in rat renal medullary cells that suggest that Sgk1 is a TonEBP target gene (9).

We could find commercially available antibodies to only 7 of the 114 genes: asporin,  $\alpha$ B-crystallin (CryAB), insulin-like growth factor-binding protein-5 and -7 (IGFBP5 and IGFBP7), ectonucleotide pyrophosphatase/phosphodiesterase-2 (Enpp2), natriuretic peptide receptor 1 (Npr1), and cysteine dioxygenase-1 (CDO1). All the RPA probes except for those targeted to CDO1 detected specific transcripts (Fig. 3). The six genes showed increased mRNA abundance in response to hypertonicity in wild-type cells, as expected. The hypertonicity-induced increase in mRNA abundance was completely suppressed in *TonEBP $\Delta\Delta$*  cells (Fig. 3A) or significantly blunted in response to TonEBP knockdown (Fig. 3B). These data suggest that all six genes are transcriptionally regulated by hypertonicity in a TonEBP-dependent manner.

Figure 4 shows immunoblot analysis of the cells used in Fig. 3. All of the commercial antibodies except for that against

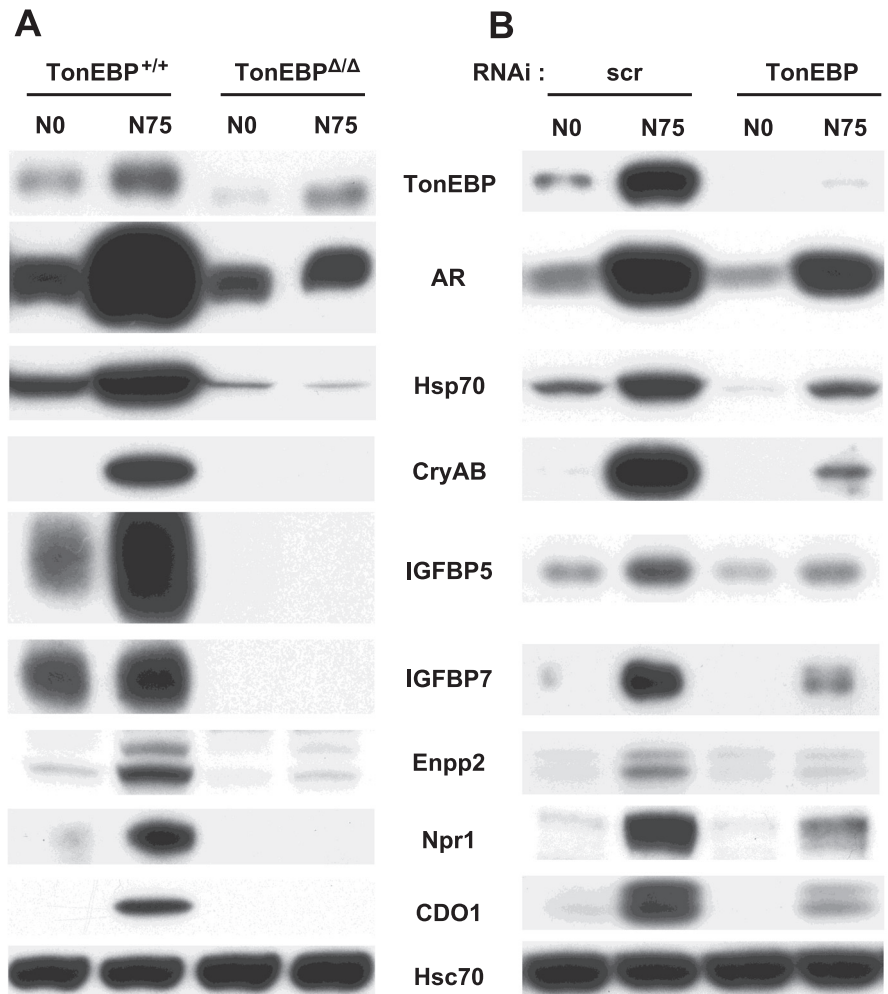


Fig. 4. Effects of TonEBP deficiency on protein expression of TonEBP-target genes. *A*: wild-type (*TonEBP<sup>+/+</sup>*) and *TonEBP $\Delta\Delta$*  MEF cells were cultured for 1 day in isotonic (N0) or hypertonic medium (N75). *B*: wild-type MEF cells were transfected with scrambled (scr) or TonEBP-targeted siRNA (TonEBP) followed by culture for 1 day in N0 or N75. Immunoblotting was performed, and representative results of 3 independent experiments are shown. Hsc70 showed a similar pattern in all the siRNA-transfected cells.

asporin detected specific bands. In *TonEBP*<sup>ΔΔ</sup> cells, the smaller TonEBP protein produced from the *TonEBP*<sup>Δ</sup> allele was observed. The smaller protein is inactive due to a deletion in the DNA binding domain (12). In *TonEBP*<sup>ΔΔ</sup> cells, protein expression of TonEBP target genes was generally reduced in isotonic conditions: moderate decrease in AR and Enpp2 and dramatic decreases in Hsp70, IGFBP5, and IGFBP7. In addition, hypertonicity-induction of protein expression was absent except for slight induction of AR and Enpp2. Less dramatic but essentially similar results were obtained from wild-type MEF cells whose TonEBP was knocked down. Thus, protein expression of the TonEBP-regulated genes generally paralleled the mRNA level, demonstrating that the transcriptional regulation by TonEBP was largely translated into changes in protein level.

We also examined expression of the newly identified TonEBP-regulated genes in mouse kidneys (Fig. 5). Expression of CryAB, IGFBP5, IGFBP7, and Npr1 followed the osmotic gradient along the corticomedullary axis: low in the isotonic cortex and high in the hypertonic inner medulla. On the other hand, expression of Enpp2 and CDO1 was not higher in the medulla, demonstrating that expression of TonEBP-regulated genes was not necessarily elevated in the hypertonic renal medulla under euhydrated conditions.

*Role of TonEBP-regulated genes in cellular adaptation to hypertonic stress: organic osmolyte-dependent and -independent pathways.* We tested the hypothesis that TonEBP promoted the adaptation to hypertonic stress via upregulation of its target genes. For this, we individually knocked down TonEBP-regulated genes with specific siRNA and examined its effects using the protocol described above (Fig. 2C): pretreatment in N75 followed by a challenge with N200. We were able to verify significant knockdown of all the genes studied at the level of protein (Fig. 6B) or, when antibody was not available, at the level of mRNA (Fig. 6A).

Among the genes involved in organic osmolyte accumulation, knockdown of SMIT resulted in a clear reduction in the

hypertonic adaptation (Fig. 7) in association with reduced *myo*-inositol accumulation (Fig. 8). On the other hand, knockdown of BGT1 was without significant effects on the hypertonic adaptation (Fig. 7), in association with increased *myo*-inositol accumulation (Fig. 8). Since betaine concentration in the medium was very low (~10 μM coming from 10% serum), we examined the effects of raising betaine concentration. Both wild-type and *TonEBP*<sup>ΔΔ</sup> cells, in which BGT1 mRNA expression was undetectable (Fig. 3A), displayed the same betaine concentration dependence: when betaine concentration exceeded 3 mM, both cells displayed dramatic but comparable increase in resistance and adaption to hypertonic stress (data not shown). Since the *K<sub>m</sub>* of betaine for BGT1 is ~0.5 mM (35), the protective effects of betaine observed over 3 mM might be unrelated to the transport function of BGT1. In the case of AR, we suspect that inefficient knockdown contributed to the lack of effect on the hypertonic adaptation (Fig. 7) in that ~30% AR remained (Fig. 6B). Taken together, the data suggest that *myo*-inositol is the major functional organic osmolyte in MEF cells because relatively inefficient knockdown of SMIT mRNA (Fig. 6A, ~50% reduction) led to a significant reduction in *myo*-inositol accumulation and hypertonic adaptation.

Next, we examined other genes regulated by TonEBP (Fig. 7). Knockdown of Hsp70 significantly reduced hypertonic adaptation. Among the newly identified TonEBP-regulated genes, knockdown of asporin, IGFBP5, IGFBP7, or Enpp2 resulted in a significant defect in the adaptation. Enpp2 is a major factor responsible for producing serum lysophosphatidic acid (LPA) (2). Addition of LPA to the medium did not affect the acclimation in *TonEBP*<sup>ΔΔ</sup> cells (data not shown), raising doubts that Enpp2 action was mediated by LPA production. On the other hand, knockdown of CryAB, Npr1, or CDO1 did not affect the acclimation. Since all these proteins were substantially knocked down (Fig. 6B), it was unlikely that the lack of effect was due to residual activity.

We investigated whether the proadaptive action of asporin, Hsp70, IGFBP5, IGFBP7, and Enpp2 was related to promotion of organic osmolyte accumulation. Individual knockdown of these genes did not affect expression of TonEBP (Fig. 6B and data not shown for asporin knockdown), or SMIT mRNA or AR (Fig. 9). On the other hand, BGT1 mRNA expression was reduced by 30–60%. The decrease was not more than that observed in those cells transfected with BGT1-targeted siRNA (Fig. 6A). For example, BGT1 mRNA abundance decreased by 55 ± 12 and 60 ± 7% (*n* = 3, *P* > 0.3) in response to BGT1 and Enpp2 knockdown, respectively. In addition, BGT1 did not contribute to the adaptation to hypertonicity in MEF cells (see above). Surprisingly cellular accumulation of *myo*-inositol increased when these genes were knocked down (Fig. 8), even though SMIT mRNA expression did not change (see above). Although it is possible that the total cellular content of organic osmolytes decreased due to depletion of some other organic osmolytes, we believe this is unlikely, because *myo*-inositol was functionally the most prominent organic osmolyte, as discussed above. Taken together, the data presented here suggest that the proadaptive action of asporin, Hsp70, IGFBP5, IGFBP7, and Enpp2 is independent of organic osmolyte accumulation.

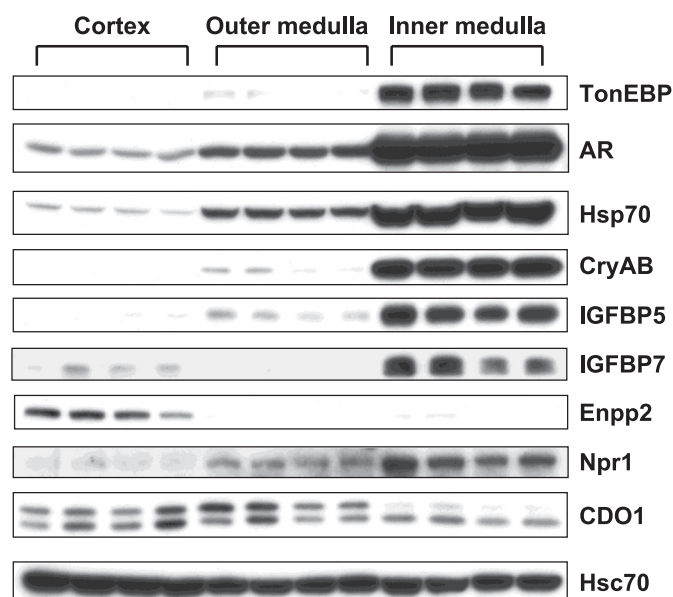


Fig. 5. Expression of TonEBP-regulated genes in mouse kidneys. Cortices, outer medullae, and inner medullae were dissected from 4 euhydrated mice and immunoblotted.

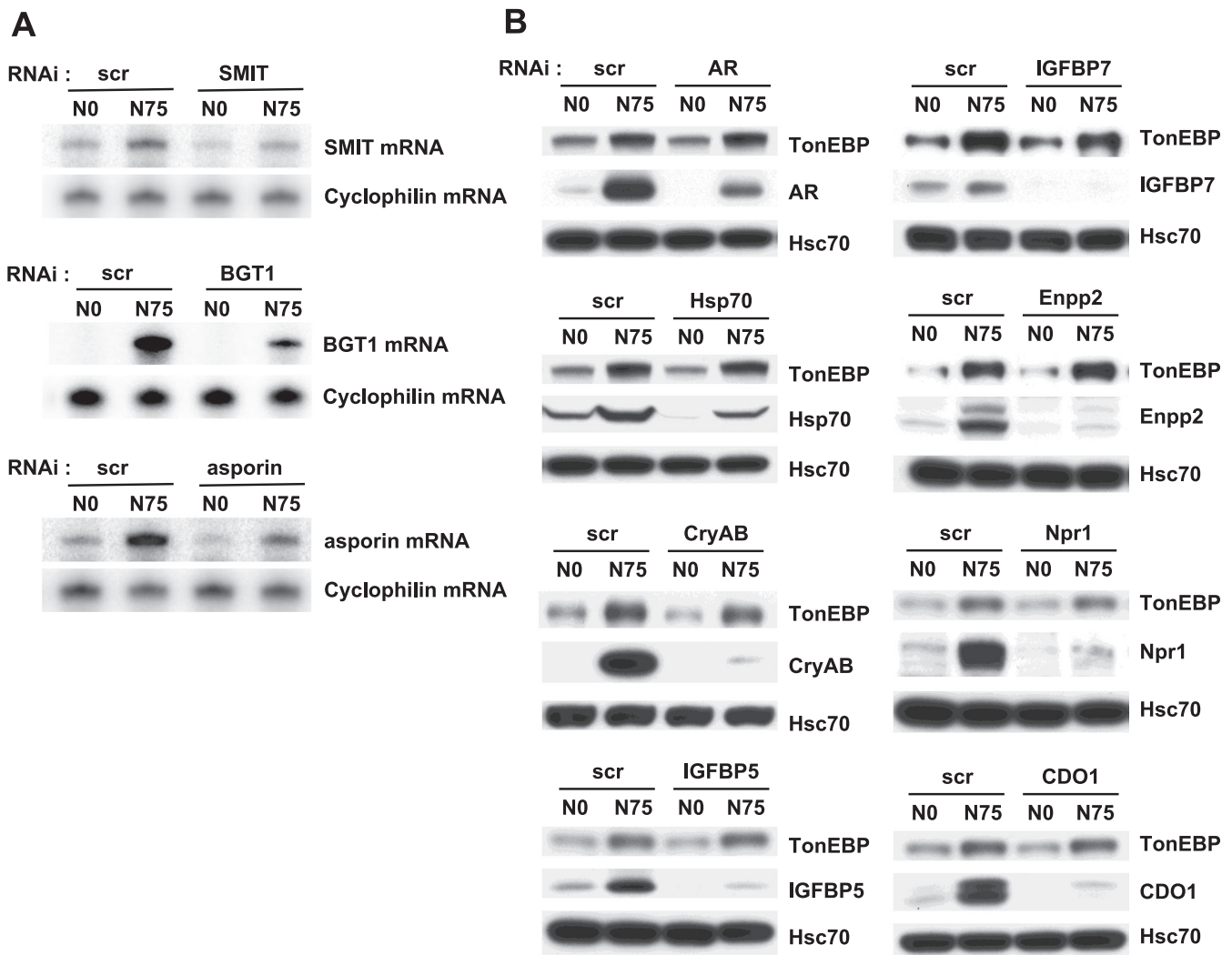


Fig. 6. Knockdown of TonEBP regulated genes. Wild-type MEF cells were transfected with scrambled (scr) or targeted siRNA for various genes indicated. Transfected cells were cultured for 1 day in N0 or N75 before analysis by RPA (A) or immunoblotting (B).

## DISCUSSION

Here, we have shown using gene deletion and RNA interference in MEF cells that TonEBP promotes both resistance and adaptation to hypertonic stress. We focused on the role of TonEBP in adaptation to hypertonic stress because the assay we used was better suited for this purpose. We have found that TonEBP dictates the genetic response to hypertonicity and stimulates expression of over 100 genes during the adaptation. As expected, the list of the upregulated genes includes known TonEBP target genes such as SMIT, BGT1, AR, and Hsp70. We selected and further analyzed seven genes that had not been previously shown to be regulated by TonEBP. We have found that all of these genes are TonEBP-dependent genes, either direct transcriptional targets of TonEBP or indirectly regulated by TonEBP, since their mRNA and protein expression is reduced when TonEBP is knocked down. The biggest surprise is the finding that some of the TonEBP-dependent genes promote cellular adaptation to hypertonic stress without increasing organic osmolyte accumulation. We conclude that multiple TonEBP target genes individually contribute to the cellular acclimation to hypertonic stress through organic os-

molyte accumulation and other yet-to-be-identified pathways. Since all the cells in the renal medulla express a high level of TonEBP (8) and many of the newly identified TonEBP-regulated genes are highly expressed in the renal medulla, the cellular pathways described here must be relevant to the renal medulla.

On the basis of reduced mRNA and protein expression in response to TonEBP knockdown, we identified seven new TonEBP-regulated genes: asporin, CryAB, IGFBP5, IGFBP7, Enpp2, Npr1, and CDO1. When some of these genes, asporin, IGFBP5, IGFBP7, and Enpp2 and Hsp70 were individually knocked down, the hypertonic adaptation was compromised without reducing *myo*-inositol accumulation. In fact, *myo*-inositol accumulation increased in response to the gene knockdown (Fig. 8) without a significant increase in SMIT mRNA expression (Fig. 9). While these observations demonstrate organic osmolyte-independent proadaptive action, the unexpected increase in *myo*-inositol accumulation might represent a compensatory reaction to maintain cell survival.

CryAB is a small molecular chaperone in the small heat shock protein family. CryAB is widely expressed in animal

tissues and is involved in many processes, including normal heart function (37) and protection from neurotoxins (27). Although overexpression of CryAB is known to protect cultured cells from hypertonic stress (22), we did not find protective effects of CryAB when it was knocked down. This might have been due to limitations of the assay we used. Given the prosurvival activity of CryAB (21), it is possible that CryAB in the renal medulla provides protection from other, nonosmotic stresses.

The protective effect of Hsp70 described here was unexpected, because it had previously been reported that heat induction of Hsp70 did not provide protection from subsequent hypertonic stress (1). In *Caenorhabditis elegans*, heat shock proteins are not induced by hypertonicity (28). On the other hand, cell lines that express little or extremely low levels of Hsp70 display increased sensitivity (cell death) to hypertonic stress (26, 31). This is associated with activation of protein kinases, capases, and lysosome-mediated apoptotic pathways (10, 19, 26). We are not sure whether any of these changes contributed to the reduced acclimation to hypertonicity in those cells whose Hsp70 was knocked down, because a substantial amount of Hsp70 (up to 50%) remained (Fig. 6B).

IGFBPs bind to and modulate the actions of insulin-like growth factors (29); they also have a variety of other functions. For example, IGFBP5 stimulates cell growth independently of IGF action (24). IGFBP7 inhibits cell proliferation acting through autocrine/paracrine pathways (33). Although we do not understand how IGFBP5 and IGFBP7 promote the hypertonic adaptation, high expression of these proteins in the renal medulla supports the idea that they provide osmoprotective function in vivo.

Enpp2, an ectoenzyme as well as a lysophospholipase D, is a major factor determining serum levels of LPA (2). Acting through its membrane receptors, LPA regulates a variety of cellular pathways including cell proliferation (23). However, our data do not support the notion that Enpp2 promotes the

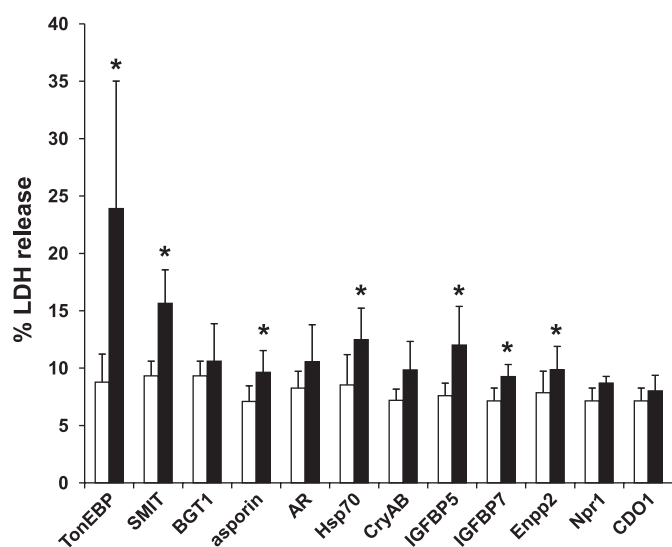


Fig. 7. Effects of TonEBP-regulated genes on cellular adaptation to hypertonicity. Wild-type MEF cells transfected with scrambled siRNA (open bars) or siRNA targeted to the genes indicated (solid bars) as described in Figs. 2 and 6. Transfected cells were cultured for 1 day in N75 followed by another day in N200. %LDH release was measured and shown as means  $\pm$  SD;  $n = 3-7$ . \* $P < 0.05$  vs. cells transfected with scrambled siRNA.

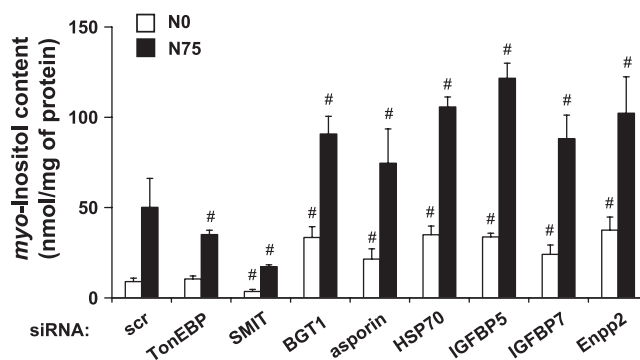


Fig. 8. Myo-inositol content in wild-type MEF cells transfected with scrambled siRNA (scr) or siRNA targeted to individual genes as indicated. Transfected cells were cultured for 1 day in N0 (open bars) or N75 (solid bars) before myo-inositol assay. Values are means  $\pm$  SD;  $n = 4$ . # $P < 0.01$  vs. corresponding scr.

acclimation to hypertonic stress via LPA. Likewise, we do not understand how asporin promotes the acclimation. Asporin belongs to a family of leucine-rich repeat proteins associated with the cartilage matrix. A variation in the aspartate repeat is associated with osteoarthritis (13). Asporin inhibits TGF- $\beta$  signaling by blocking its interaction with type II receptor (25).

Npr1 is a membrane-bound guanylate cyclase that serves as the receptor for both atrial and brain natriuretic peptides. In the inner medullary collecting duct, activation of Npr1 reduces water permeability by inhibiting the apical trafficking of aquaporin 2 (14). A recent report showed that TonEBP stimulated expression of Npr1 via activation of Sgk1 (9). In the MEF cells used here, we found that Sgk1 induction by hypertonicity was not reduced by TonEBP gene deletion or gene knockdown. Our data are consistent with the view that Npr1 is a direct target of TonEBP without involving Sgk1. CDO1 converts cysteine to cysteine sulfinic acid, which in turn can be converted to taurine, an organic osmolyte. The lack of contribution by CDO1 to the acclimation to hypertonic stress suggests that MEF cells do not synthesize enough taurine for osmoprotection.

The contribution of multiple TonEBP target genes in the adaptation to hypertonic stress is similar to recent findings from *C. elegans* (28). Although more than 300 genes were found to be regulated by hypertonicity in *C. elegans*, individual inhibition of these tonicity-regulated genes by RNAi, including the gene *gpdh-1*, which encodes the rate-limiting enzyme for biosynthesis of glycerol, the major organic osmolyte in *C. elegans* (18), was without effect on whole animal hypertonic stress resistance. However, inhibition of two GATA-type transcription factors, *elt-2* and *elt-3*, which are broadly required for tissue-specific osmosensitive gene expression, resulted in a dramatic reduction in survival under hypertonic conditions (note that *C. elegans* does not express TonEBP homologs). Thus, collective action of multiple genes involved in multiple pathways is an evolutionarily conserved feature of cellular adaptation to hypertonicity in both *C. elegans* and mammals.

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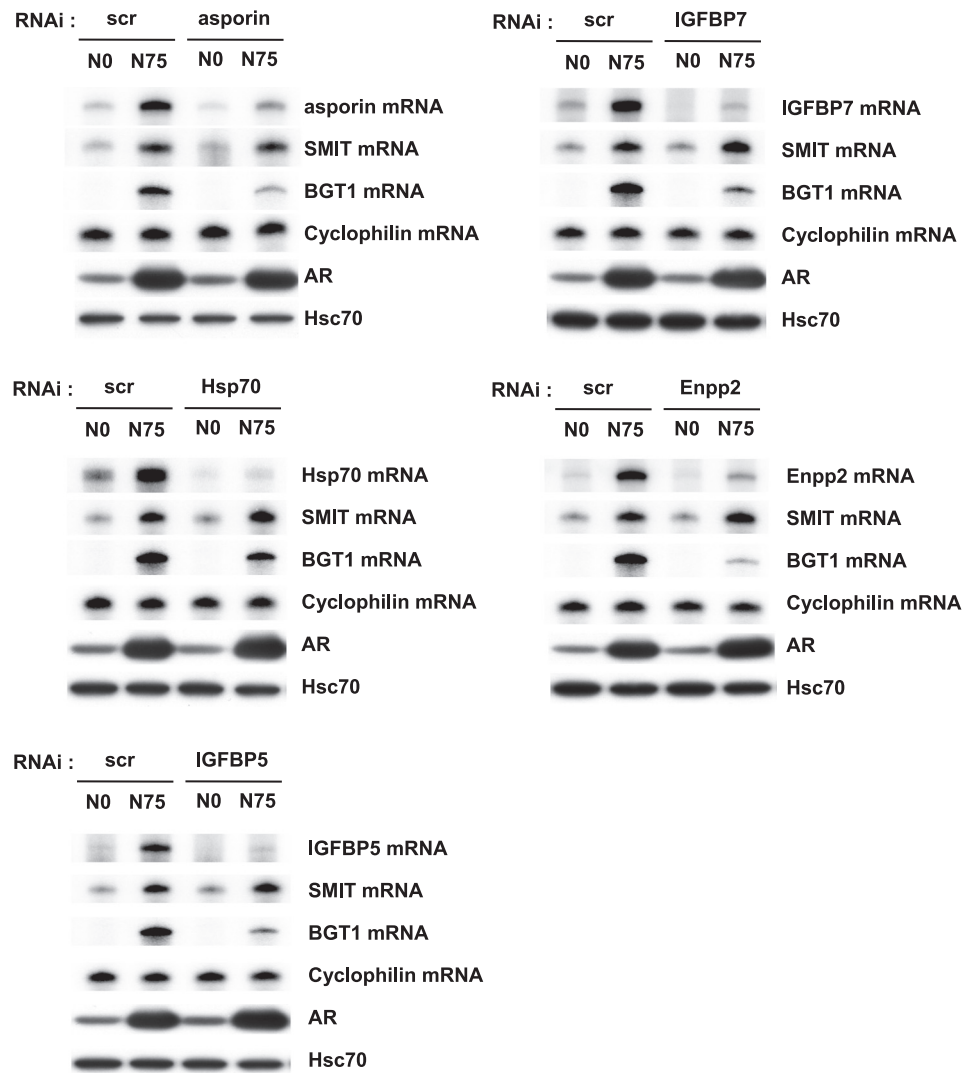


Fig. 9. Expression of SMIT mRNA, BGT1 mRNA, and AR in wild-type MEF cells transfected with scrambled (scr) or targeted siRNA for asporin, Hsp70, IGFBP5, IGFBP7, or Enpp2, as indicated. Transfected cells were cultured for 1 day in N0 or N75. RPA and immunoblotting were performed as described in Fig 6.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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