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Expression and function of the Delta-1/Notch-2/Hes-1 pathway during experimental acute kidney injury in rats

by

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ABSTRACT

The Notch signaling pathway is important for embryogenesis and cellular differentiation and proliferation. The Notch system consists of several Notch receptors and Notch ligands (Delta and Jagged). The activated Notch is cleaved to the cytoplastic domain of Notch (NICD). NICD translocates into the nucleus and induces basic-loop-helix proteins such as Hes or Hey. The functional roles of the Notch signaling pathway in acute kidney injury (AKI) are not well known. In this study we sought to clarify the significance of the Notch signaling pathway in AKI using a rat ARF model in vivo and cultured renal tubular cells (NRK-52E cells) in vitro. The left renal artery was clamped for 60 min and the left kidney was excised at 0.5, 1, 2, 3, 6, 12, 24, 48, and 72 h after reperfusion. RT-PCR analysis of ligands and target genes from total RNA and protein isolated from total kidney after AKI revealed increases in the mRNA expressions of Delta-1 (ligand) and Hes-1 (target gene). Western blot analyses after the induction of ischemia revealed elevated protein expressions of Delta-1, Hes-1, and cleaved Notch-2. Immunohistological examination identified co-localization of Delta-1 and cleaved Notch-2 and Hes-1 expressions in the same segments of the renal proximal tubules. In separate experiments, recombinant Delta-1 was synthesized and plated onto the wells of culture plates in vitro. The number of renal tubular cells in the presence of Delta-1 was higher than the number counted under the control condition. Taken together, these data suggest that the Delta-1/Notch-2/Hes-1 pathway is activated after ischemic AKI, and that Delta-1 plays a role in the proliferation of renal tubules. The Delta-1/Notch-2/Hes-1 signaling pathway may regulate the regeneration and proliferation of renal tubules in AKI.

Key words: acute kidney injury, proximal tubule, ischemia, regeneration

INTRODUCTION

Ischemic acute kidney injury (AKI) is the most common form of AKI in the adult population. Prominent morphologic features of ischemic AKI include effacement and loss of proximal tubule brush border, patchy loss of tubular cells, focal areas of proximal tubular dilation and distal tubular casts, and areas of cellular regeneration (1). The molecular basis of the events leading to tubular regeneration after AKI is only partially established. The mechanisms that lead to renal cell proliferation and regeneration must be better understood before novel therapeutic strategies for the treatment of ischemic AKI can be explored. Evidence has suggested that regeneration processes may recapitulate developmental processes in order to restore organ or tissue function (2, 3). Adult tubular epithelial cells have a potent ability to regenerate after cellular damage (4). In organisms which have suffered ischemic renal damage, normally quiescent cells de-differentiate and acquire the ability to proliferate through enhancements in DNA synthesis (5, 6).

Regeneration processes may be similar to developmental processes. Embryonic genes such as Wnt-4 and Ets-1 are markedly induced in the mature kidney after ischemic renal injury and apparently play crucial roles in the regeneration and repair of the organ (7-9). Though initially identified in *Drosophila* (10), the Notch signaling pathway is now known to function in all metazoa as a major pathway leading to the determination of cellular identity during developmental stages (11, 12). The expression of the pathway has been identified in the mesonephric duct during embryonic development. Notch is a transmembrane protein that interacts with ligands of the Jagged and Delta families (12, 13). There are four Notch members in mammals (Notch 1–4), two Jagged-like genes, and three Delta-like genes (14). The interaction

of these ligands with Notch activates the proteolytic cleavage of the Notch intracellular domain (NICD) (15). Transport of NICD to the nucleus allows the domain to bind to a transcription factor, RBP-Jk. The RBP-Jk NICD complex activates the transcription of a number of effectors such as Hes and Hey, two families of basic helix loop helix genes (16). Notch signaling is reported to play a key role in nephrogenesis (17, 18). Chen and Al-Awqati identified the expressions of Notch-1 and Notch-2 in the epithelial cells of the developing glomerulus and the expression of Notch-2 in comma- and S-shaped bodies (tubular components of the nephron) (19). The same group demonstrated the expression of Delta-1 in the tubules and in early pretubular aggregates, and the expression of Jagged-1 in the collecting ducts and endothelial cells of the developing glomerulus (19). Recent experiments by Cheng et al., using conditional knockout mice demonstrated that the differentiation of proximal nephron structures requires the presence of Notch-2 but not Notch-1 (20).

The Notch signaling pathway is also induced in mature organs after injury. Notch-1 and Jagged-1 proteins are upregulated in the rat liver following partial hepatectomy (21), and NICD levels are significantly elevated in the brain after cerebral ischemia reperfusion (22). With regard to the renal injury, one study reported an upregulation of Jagged-1 expression in the kidney of mice with ureteral obstruction (23). Little is known, however, about the expression and functional roles of the Notch signaling pathway in kidney after ischemic injury.

In this study we hypothesized that the Delta/Notch/Hes pathway is activated after ischemic AKI and plays a role in the proliferation of renal tubules. We tested this hypothesis by examining whether the Notch signaling pathway is activated in proximal tubules after ischemic AKI and whether the pathway regulates the proliferation of renal tubular cells.

RESULTS

The mRNA expression of ligands (Delta-1, -4, and Jagged-1, -2) and target proteins (Hes and Hey families) after ischemic renal failure

To screen the renal genes for ligands (Delta-1, -3, -4, and Jagged-1, -2) and target proteins (Hes and Hey families) in this system, we conducted an RT-PCR analysis of rat kidney mRNA after ischemia/reperfusion. The left renal artery was clamped for 60 min and the left kidney was excised at 0.5, 1, 2, 3, 6, 12, 24, 48, and 72 h after reperfusion. Sham-operated kidney (0 h) and neonatal whole body were used for control and positive control, respectively. Delta-1, -4, Jagged-1, and Jagged-2 mRNA expressions were screened by RT-PCR. Preliminary experiments produced no positive band for Delta-3 at any time point in the injured kidney, or even in the positive control (data not shown). The mRNA level of Delta-1 was dramatically elevated during from 1 to 24 h after the ischemia/reperfusion injury (Figure 1A, B). Quantitation of ligand mRNA transcripts by real-time quantitative PCR revealed 5.7-fold (1 h), 11.1-fold (3 h), 13.5-fold (6 h), 9.2-fold (12 h), and 3.5-fold (24 h) increases in Delta-1 mRNA levels compared with the 0 h value (control). The other ligands (Delta-4, Jagged-1, and Jagged-2), in contrast, exhibited no significant changes (Figure 1A).

RT-PCR screening for the expressions of Hes-1, -5, -7, Hey-1, and -2 mRNA revealed a significant upregulation of the mRNA expression of Hes-1 after ischemia/reperfusion injury in the target genes (Figure 1C). Quantitative real-time RT-PCR assay revealed 5.4-fold (3 h), 6.8-fold (6 h), 8.0-fold (12 h), and 4.7-fold (24h) increases in Hes-1 mRNA levels compared with the 0 h value (control) (Figure 1D). The mRNA expressions of the other target genes were very weak and showed no significant changes during ischemia/reperfusion (Figure 1C). Preliminary experiments

produced no positive band for Hes-2, -3, -4, -6, and Hey-L at any time point in the injured kidney, or even in the positive control (data not shown). On the basis of these data, we decided to focus on Delta-1 and Hes-1 in our ischemia/reperfusion system in the following experiments.

The protein expression of Delta-1, Notch-2, and Hes-1 after ischemic renal failure

Western blot analyses were performed after ischemia/reperfusion of the kidneys to detect the protein levels of Delta-1 and Hes-1 (Delta-1 and Hes-1 mRNA expression levels sharply increased) as well as those of Notch-1, Notch-2, Notch-3, and Notch-4. The Delta-1 protein expression level increased dramatically but transiently compared to the control level (sham-operated, at 0 h) 2–6 h after ischemia/reperfusion (Figure 2A). Later, 24–72 h after ischemia/reperfusion, the intensity of the Delta-1 band gradually decreased. Quantitative analysis using a densitometer revealed 3.5-fold (6 h), 6.2-fold (12 h), and 3.9-fold (24 h) increases in the Delta-1 protein levels compared to the control level (Figure 2B). The expression level of cleaved Notch-2 (activated forms of the receptors) was significantly elevated 2–48 h after ischemia/reperfusion; in contrast, the expression of full-length Notch-2 decreased 2–48 h after ischemia/reperfusion (Figure 2A). In the preliminary experiments, we performed western blot analysis by using the anti-full-length Notch-2-specific and anti-cleaved Notch-2 antibodies. However these antibodies are not specific for either the cleaved activated Notch-2 or full-length Notch-2 but recognize both forms. In the subsequent experiments, we decided to use the Notch-2 antibody (Code: 100-401-406, Rockland), which recognizes all forms of Notch-2. Based on the data obtained in the western blot analyses, we used the Notch-2 antibody (Code: 100-401-406, Rockland) in the following studies on Notch-2.

. Quantitative analysis using a densitometer revealed 2.5-fold (3 h), 4.7-fold (6 h), and 4.2-fold (12 h) increases in the cleaved Notch-2 protein level compared to the control level. On the other hand, 0.55-fold (3 h), 0.35-fold (6 h), and 0.47-fold (12 h) decreases were observed in the full-length Notch-2 protein level compared to the control level (Figure 2B). The expression levels of both the forms of Notch-2 returned to the basal level 72 h after ischemia/reperfusion. The expression of Hes-1 was weak in the control kidney and increased dramatically but transiently 6–24 h after ischemia/reperfusion. Later, 72 h after ischemia/reperfusion, this upregulation of Hes-1 was no longer observed (Figure 2A). Quantitative densitometric analysis revealed 3.7-fold (6 h), 3.8-fold (12 h), and 3.2-fold (24 h) increases in the Hes-1 protein level compared to the control level (Figure 2B). No increases were detected in the expression levels of cleaved Notch-1, cleaved Notch-3, full-length Notch-3 (data not shown), cleaved Notch-4, or full-length Notch-4 (data not shown) under our experimental conditions. Immunoblotting was performed, with actin as a loading marker (Figure 2A). The protein levels of Delta-1, Hes-1, and cleaved Notch-2 remained unchanged 6–12 h after the sham operation in the control animals (data not shown).

Immunohistochemical examination of Delta-1 in ischemic renal injury.

We next performed immunohistological studies on Delta-1 using confocal microscopy. A low-power view revealed Delta-1 expression in the outer medulla and cortical renal tubules at 12 h after ischemia/reperfusion, but not in the inner medulla. When the anti-aquaporin-1 antibody was used as a marker of the proximal tubules, the Delta-1 expression was found to be colocalized with aquaporin-1 in the outer medulla and cortex after ischemia/reperfusion (Figure 3A, B). In the control kidneys, aquaporin-1 expression was clearly observed in the renal cortex and medulla, but

Delta-1 expression was not observed (Figure 3A, B). Only minimal staining of aquaporin-1 was observed in the inner medulla of the ischemic/reperfusion and control kidneys. A high-power view of samples stained with antibodies for both Delta-1 and aquaporin-1 revealed positive staining for both these proteins in the same proximal tubular cells in the outer medulla. Furthermore, Delta-1 was expressed in the cytoplasm of tubular cells (Figure 4). These results demonstrated the prevalence of Delta-1 expression in the proximal tubules, mainly the S3 segment, of the renal cortex at 12 h after ischemia/reperfusion.

Immunohistochemical examination of Notch-2 in ischemic renal injury.

Immunohistochemical studies on Notch-2 performed by using confocal microscopy revealed a clear expression of Notch-2 in the outer medulla and cortical renal tubules at 12 h after ischemia/reperfusion, but no expression was observed in the inner medulla. Positive, albeit weak, Notch-2 expression was observed in the renal cortices of the control. The expression of Notch-2 was found to be colocalized with that of aquaporin-1 in the outer medulla and cortex after ischemia/reperfusion (Figure 5A, B). Based on the data obtained in the western blot analyses, we used the Notch-2 antibody (Code: 100-401-406, Rockland) in the immunohistochemical studies on Notch-2. A high-power view of samples stained with antibodies for both Notch-2 and aquaporin-1 revealed positive staining in the same proximal tubular cells in the outer medulla. Notch-2 was detectable in the cytoplasm and the nuclei in the ischemic/reperfused kidney and in the membrane of the control kidney (Figure 6A).

Next, a double-staining examination of Notch-2 and Delta-1 confirmed the colocalization of Notch-2 and Delta-1 in the same renal tubules (Figure 6B). These results demonstrate the prevalence of the expression of Notch-2 in the segments

(mainly the S3 segment) of the renal tubules positive for Delta-1 expression at 12 h after ischemia/reperfusion.

Immunohistochemical examination of Hes-1 in ischemic renal injury.

The immunohistological studies on Hes-1 were performed using confocal microscopy. Hes-1 expression was observed in the cortical and outer medullary renal tubules at 12 h after ischemia/reperfusion, but was not observed in the inner medullary renal tubules. It was also not observed in the renal cortices of the control rats. The expression of Hes-1 was found to be colocalized with that of aquaporin-1 in the outer medulla and cortex after ischemia/reperfusion (Figure 7A, B). A high-power view of the samples stained with antibodies for both Hes-1 and aquaporin-1 revealed positive staining in the nuclei of the same proximal tubular cells in the outer medulla (Figure 8A). Next, we performed a double-staining examination of Hes-1 and Delta-1. As shown in Figure 8B, the expression of Hes-1 was colocalized in the same renal tubules with that of Delta-1. These results demonstrate the prevalence of Hes-1 expression in the same segments (mainly the S3 segment) of the renal cortex positive for Delta-1 expression at 12 h after ischemia/reperfusion and the presence of Hes-1 staining in proximal tubular cells.

Antibody specificity for Delta-1, Notch-2, and Hes-1 in immunohistological and western blot analyses

We performed immunohistological analysis by using blocking peptides in order to verify the specificity of the Delta-1, Notch-2, and Hes-1 antibodies. As shown in Figure 9 A–C, the immunohistological signals for Delta-1, Notch-2, and Hes-1 diminished significantly in the presence of the blocking peptide for each antibody. Further, we performed western blot analyses by using blocking peptides in order to confirm the specificity of the Delta-1, Notch-2, and Hes-1 antibodies. As shown in

Figure 9 D–F, the bands obtained for Delta-1, Notch-2, and Hes-1 at 6 h after ischemia/reperfusion diminished significantly in the presence of the blocking peptide for each antibody. These results confirmed the antibody specificity.

Delta-1 stimulated cellular proliferation and Hes-1 expression in NRK-52E cells.

Next, we used a culture system with NRK-52E cells to examine the functional role of the Delta-1 system in renal tubular cells. To examine whether this culture system mimics the in vivo ischemia/reperfusion model, we investigated the expressions of Delta-1 and Hes-1 mRNA by RT-PCR and the expressions of Delta-1, cleaved Notch-2, and Hes-1 protein by Western blot analysis. Hypoxia (12 h) upregulated the mRNA expressions of Delta-1 and Hes-1 by 1.8-fold and 4.5-fold over the levels in control NRK-52E cells, respectively (Figure 9A, B). In the Western blot analysis, hypoxia (12 h) upregulated the protein expressions of Delta-1, cleaved Notch-2, and Hes-1 by 2.5-fold, 2.3-fold, and 4.1-fold over the control levels, respectively (Figure 9C, D).

Next, to clarify the function of Delta-1 pathway in renal tubule cells, recombinant Delta-1 was used to stimulate NRK-52E cells as described previously (24). Recombinant Delta-1 was plated onto the cell culture plates and NRK-52E cells were cultured in the wells. The cell morphologies were observed and cell counts were taken for 5 days. The NRK-52E cell count in the presence of Delta-1 was significantly increased over the control level (Figure 10A, B). Figure 10A shows a typical morphology of an NRK-52E cell in this experiment. The morphology changed only slightly, whereas the cell count increased significantly. We also examined the effects of Delta-1 on the cell proliferation of NRK-52E cells by [^{3}H]-thymidine uptake. Recombinant Delta-1 stimulated [^{3}H]-thymidine uptake to 205 % dose-dependently

(Figure 10C). Taken in sum, these results demonstrate a Delta-1-induced increase in the proliferation of NRK-52E cells. To explore further, we also examined the signal transduction of recombinant Delta-1 in NRK-52E cells. Delta-1 upregulated the mRNA expression of Hes-1 by 2.2-fold over the control level in NRK-52E cells (Figure 11A, B). In Western blot analysis, Delta-1 upregulated the protein expressions of cleaved Notch-2 and Hes-1 by 3.2-fold and 4.5-fold, respectively, compared to the controls (Figure 11C, D). Taken together, the results of these experiments confirmed that Delta-1 stimulation upregulated Hes-1 and cleaved Notch-2 in NRK-52E cells. Thus, we have established that Delta-1 stimulates Notch-2/Hes-1 pathway in renal tubular cell.

DISCUSSION

In this study, we demonstrate that the Delta-1/Notch-2/Hes-1 pathway is strongly upregulated in the proximal tubules of rat kidney after ischemic acute kidney injury (AKI). Our findings also demonstrate that the Notch system induces the proliferation of renal tubular cells.

The recovery from AKI requires the replacement of damaged cells with new ones to restore the integrity of the tubular epithelium. The regeneration processes are characterized by proliferation of dedifferentiated cells, followed by differentiation of daughter cells into the required cell phenotype (3–5). Noting that a similar phenomenon occurs during embryogenesis, our group and some others have postulated that these regeneration processes may redeploy certain parts of the genetic program executed during organogenesis in order to reestablish proper tissue function in the kidney (3, 5, 7, 8). A previous study by our group revealed the expression of the developmental gene *Wnt-4* in ischemic AKI and confirmed that this gene promotes the

proliferation of renal tubular cells (7). We also reported that Ets-1 is upregulated in the proximal tubules in the recovery phase of AKI and highly expressed in the embryonic kidney (8). These previous papers suggest that certain developmental genes are reexpressed during the recovery from AKI and play key roles in tubular regeneration. To confirm this hypothesis, we examined the expression patterns and functions of the Delta/Notch/Hes pathway in an AKI model and renal tubular cells.

This study is the first to demonstrate upregulation of Delta-1 expression in the early phase of ischemic AKI. The Delta-1 expression was localized exclusively in the proximal tubule. The Notch system includes many kinds of ligands (Delta-1, Delta-3, Delta-4, Jagged-1, and Jagged-2) and target genes (*Hes-1*, *Hes-2*, *Hes-3*, *Hes-4*, *Hes-5*, *Hes-6*, *Hes-7*, *Hey-1*, *Hey-2*, and *Hey-L*). For our study, we were required to perform a reverse transcription-polymerase chain reaction (RT-PCR) analysis of these ligands and target gene mRNAs for screening purposes (Figure 1). We detected strong bands for Delta-1 and Hes-1 after ischemia/reperfusion, but no bands were detected for Delta-3, Delta-4, Jagged-1, Jagged-2, Hes-2, Hes-3, Hes-4, Hes-5, Hes-6, Hes-7, Hey-1, Hey-2, or Hey-L under our experimental conditions. The protein expressions of Delta-1 and Hes-1 also increased dramatically during the ischemia/reperfusion. Next, western blot analyses for Notch-1, Notch-2, Notch-3, and Notch-4 proteins revealed a significant increase in the expression level of cleaved Notch-2 and decrease in that of full-length Notch-2 during ischemia/reperfusion; however, no significant change was observed in the cleaved Notch-1, cleaved Notch-3, or cleaved Notch-4. It was shown that the reduction in the level of full-length Notch-2 was associated with increase in that of cleaved Notch-2. Chen and Al-Awqati identified the expressions of Delta-1 and Notch-2 in the prospective proximal tubules of embryonic kidney (19). Further, Chang et al. found that the distal tubules developed in Notch-2-deficient kidneys lacked

proximal tubules and podocytes, whereas those developed in Notch-1-deficient kidneys were normal (20). Our results agree with those of other recent studies on the importance of Notch-2 in the regeneration of the proximal tubule. The detection of Delta-1 and cleaved Notch-2 upregulation in proximal tubules after AKI suggests that these proximal tubules are at least partially embryonic in character. Our present study is also the first to demonstrate the significant induction of the effecter protein Hes-1 in the proximal tubule after AKI. The expressions of Hes-1, Hes-5, Hey-1, and Hey-L have been identified in the developing nephron, but they have not been analyzed to screen for Hes-1 in AKI (17–20). The functional difference between the Hes and Hey families has not been clarified (26, 27). Our experiments detected no clear induction of Hes-5, Hes-7, Hey-1, or Hey-2, as shown in Figure 1. Further studies are necessary to gain a more precise understanding of the expressions of the Delta/Notch/Hes pathway after ischemia/reperfusion injury.

In the next round of experiments for our study, we examined the immunohistology of Delta-1, Notch-2, and Hes-1 under a confocal microscope. Delta-1 expression was mainly observed in the S3 segment of the proximal tubules at 12 h after ischemia/reperfusion, and the expressions of the Notch-2 and Hes-1 were apparently colocalized with that of Delta-1 in the proximal tubules of the renal cortex and outer medulla at the same time point (Figures 3–8). In addition, immunohistochemical investigations and western blot analyses performed using blocking peptides confirmed the antibody specificity for Delta-1, Notch-2, and Hes-1. The proximal tubular cells have been established to be highly sensitive to ischemic injury and regenerate in the nephron segments (27, 28). Our immunohistological studies clearly identified the expressions of Delta-1, cleaved Notch-2, and Hes-1 in the same segments after ischemia/reperfusion. Our in vivo results, meanwhile, strongly suggested that this

pathway is capable of regulating the proliferation and regeneration of proximal tubular cells. The Notch pathway reportedly plays a key role in the regeneration of the liver and brain after injury (21, 22). Nonetheless, our in vivo study did not clarify the functional role of the Delta-1/Notch-2/Hes-1 pathway in cellular proliferation in renal tubular cells. Our next step, therefore, was to prepare a cell culture system using recombinant Delta-1.

The purposes for preparing the cell culture were to examine the functional role of Delta-1 signaling in renal tubular cells (NRK-52E cells). In some instances, it may be inappropriate to extend the in vitro results to in vivo conditions. First, we performed an RT-PCR and western blot analysis of NRK-52E cells under hypoxic conditions to determine whether hypoxic stress activates Delta-1, Notch-2, and Hes-1. We found that the mRNA and protein expressions of Delta-1, cleaved Notch-2, and Hes-1 were upregulated in NRK-52E cells under hypoxic conditions (Figure 9). This is the first known evidence of hypoxia-induced upregulation of Delta-1 and Hes-1 in renal cells. These data suggest that the findings of the NRK-52E cells under hypoxic conditions were similar to those of the abovementioned in vivo studies on the induction of the Delta-1/Notch-2/Hes-1 pathway. Next, to clarify the function of the Delta-1 pathway, recombinant Delta-1 was used to stimulate NRK-52E cells. This is the first study to demonstrate Delta-1-induced increases in the cell counts and proliferation of renal tubular cells. In the final experiment, the interactions between Delta-1 and Notch-2 and that between Delta-1 and Hes-1 were clarified when we observed that Delta-1 stimulation increased the expressions of cleaved Notch-2 and Hes-1 proteins in NRK-52E cells. Our in vitro data suggest that the Delta-1/Notch-2/Hes-1 pathway plays a role in the regeneration of renal tubular cells in AKI. Other data, meanwhile, revealed that the Delta/Notch/Hes pathway also plays multiple roles in cell migration and cell

identification during development (11, 18, 29). The use of other experimental approaches in the future will help broaden the understanding of the functional roles of the Delta/Notch/Hes pathway in AKI.

In summary, our study has produced 2 novel findings: First, the Delta-1/Notch-2/Hes-1 pathway is activated after ischemic AKI; second, Delta-1 plays a role in the proliferation of renal tubules. The Delta-1/Notch-2/Hes-1 pathway may regulate the regeneration and proliferation of renal tubules in AKI. Further studies are necessary to gain a more precise understanding of the molecular mechanisms of renal recovery after ischemia/reperfusion injury.

MATERIAL AND METHODS

Induction of ARF. Male Sprague-Dawley (SD) rats (Saitama Experimental Animal Supply, Saitama, Japan) weighing 150g-200g were anaesthetized intraperitoneally with sodium pentobarbital (30 mg/kg) at surgery. The left renal artery was occluded with Sugita aneurysm clips (Mizuho Ikakogyo, Tokyo, Japan) for 60 min. The clamps were removed, the incisions were closed, and the rats were sacrificed at 0.5, 1, 2, 3, 6, 12, 24, 48, and 72 h (n=5). The left kidney was quickly removed and processed for histological evaluation, protein extraction, or RNA extraction (7, 8). Age- and weight-matched SD rats received sham operations without clamping of the renal arteries, at 0, 6, 12, and 24 h (n=3).

Cell and cell culture and exposure to hypoxia. NRK-52E cells (renal tubular cells of adult rat kidney) originally purchased from American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 50 IU/ml penicillin and 10% heat-inactivated fetal calf serum (FCS; Gibco). Once grown, the cells were cultured at 37˚C in either 20% O_2

and 5% CO_2 (the normoxic condition) or in a hypoxic chamber (Bellow Glass, Inc., Vineland, NJ) containing 0% O_2 and 5% CO_2 (the hypoxic condition) (8).

Isolation of kidney tissue and histological examination. The rats were anesthetized with pentobarbital at indicated times after the ischemic event. Their kidneys were perfused with sterile phosphate-buffered saline (PBS). The left kidney was quickly excised. For immunohistochemical studies, the kidney was removed after in vitro perfusion with 4% paraformaldehyde in a phosphate buffer and immersed overnight in the same fixative at 4˚C. The fixed tissue was cryoprotected by immersion in 20% sucrose in PBS at 4˚C and then shock-frozen in liquid nitrogen. Frozen 7-□m sections were cut with a cryostat, thaw-settled on APS-coated slides, mounted with an aqueous mounting medium (Mount-Quick Aqueous; Daido Sangyo, Tokyo, Japan), and examined under a confocal laser microscope (Carl Zeiss Japan, Tokyo, Japan). Frozen sections prepared in the manner described above were used for immunohistochemistry (7). Immunohistochemical staining was performed using an anti-Delta-1 specific antibody (Abcam Inc., Cambridge, MA, ab10554), anti-Notch-2 specific antibody (Rockland, Philadelphia, PA, #11172), anti-Hes-1 specific antibody (Chemicon International Inc., Dundee, UK, AB5702), and anti-aquaporin-1-specific antibody (Abcam Inc., Cambridge, MA, ab9566) as markers for proximal tubules, as described previously (30, 31). The secondary antibodies were an anti-goat IgG FITC-conjugated antibody, an anti-rabbit IgG Cy3-conjugated antibody, an anti-rabbit IgG FITC-conjugated antibody, an anti-sheep IgG Cy3-conjugated antibody, and an anti-goat IgG Cy3-conjugated antibody (Sigma, St. Louis, MO). The sequences of the blocking peptides used were as follows: Delta-1, CGEEWSQDLHSSGRTDLRYS; Notch-2, CRDASNHKRREPVGQD; and Hes-1,

TPDKPKTASEH. The blocking peptide (10 μg/ml) was preloaded onto the histology sections or immunoblot membranes prior to addition of the antibody.

Western blot analysis. Fifty μg of protein samples (total renal tissue or NRK-52E cells) prepared as described above were denaturated at 100 ^{o}C for 5 minutes in an SDS sample buffer and separated on 7.5% or 10/20% polyacrylamide electrophoresis gel (32). The proteins were transferred to a nitrocellulose membrane, blocked for 1 hour with 5% (wt/vol) fat-free milk powder, and probed with the primary antibody. Western blot analysis was performed using an anti-Delta-1 specific antibody, anti-Notch-2 specific antibody, anti-Hes-1 specific antibody, anti-cleaved Notch-1 specific antibody (Cell signaling technology inc., Val-1744), anti-Notch-3 specific antibody (Santa Cruz Biochemic inc., M-20, sc-7424), anti-Notch-4 specific antibody (Santa Cruz Biochemic inc., M-19, sc-8646), and anti-actin specific antibody (Santa Cruz Biochemic inc., H-300, sc-10731). The primary antibodies were detected using horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG and donkey anti-rabbit IgG, and visualized by the Amersham ECL system (Amersham Corp., Arlington Heights, IL). In the preliminary experiments, we performed western blot analysis by using the anti-full-length Notch-2-specific and anti-cleaved Notch-2 antibodies. However these antibodies are not specific for either the cleaved activated Notch-2 or full-length Notch-2 but recognize both forms. In the subsequent experiments, we decided to use the Notch-2 antibody (Code: 100-401-406, Rockland, Philadelphia, PA), which recognizes all forms of Notch-2. Based on the data obtained in the western blot analyses, we used the Notch-2 antibody (Code: 100-401-406, Rockland) in the immunohistochemical studies on Notch-2.

Real-Time Quantitative PCR. A reverse transcription-polymerase chain reaction (RT-PCR) analysis with RNA extracted from the ischemia/reperfusion

kidneys was carried out as previously described (33). The primers used are described in Table 1. Total RNA was harvested from renal tissue using TRI-REAGENT™ (Life Technologies, Gaithersburg, MD) (33). One μg of total RNA samples was used for the RT-PCR, as follows. The real-time quantitative PCR method was used to accurately detect the changes in Delta-1 and Hes-1 gene copies. The RT-PCR of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as a positive control. A three-step PCR was performed for 35 cycles. The samples were denatured at 94˚C for 30 sec, annealed at 58˚C for 30 sec, and extended at 72˚C for 30 sec. The PCR products were subcloned to the TA cloning vector ™ (Promega, Biotec, Madison, WI) as described previously (33). The plasmids containing ligand cDNA and GAPDH cDNA were used to make standard curves of quantitative PCR (34). The linear curves between the PCR products and cDNA quantities (10pg/□l-100ng/□l) with the use of Delta-1, Hes-1, and GAPDH cDNA plasmids were observed in the ranges used.

Effects of Delta-1 on the cell count and cell proliferation. The effect of Delta-1 on cell growth was examined as described previously (35). The recombinant Notch ligand protein, Delta-1, was synthesized as described previously (24). Briefly, partial cDNAs encoding the extracellular domain of Delta-1 was fused in-frame to a sequence of IgG-Fc. The gene fusion was inserted into an expression vector and electroporated into Chinese hamster ovary cells. The chimeric protein was purified from conditioned media by affinity chromatography. Delta-1 was immobilized in the wells of a 96-well culture plate (Becton-Dickinson Labware, Franklin Lakes, NJ. USA) and in the wells of a 10 cm culture plate by loading the wells with Delta-1 dissolved at a concentration of 5.0 μg/ml in phosphate-buffered saline (PBS) at 37˚C for 2 h. The amount used had been confirmed to exert the maximum effect in preliminary experiments. PBS alone was placed in the wells under similar conditions

as a control for Delta-1. The wells were then blocked with PBS containing 5% bovine serum albumin and washed with PBS. The cells (10^4 cells/well) were cultured in 0.1 ml of DMEM. The cells in the 96-well culture plate were counted by microscopy or protein, and the mRNA from the 10 cm culture plate was harvested for 1-5 days. The cells were plated in 24-well plates and incubated in a medium with recombinant Delta-1 at indicated concentrations for 3 days. For the last 12 h, the cells were pulsed with 1 μCi [^{3}H] thymidine (Amersham, Arlington Heights, IL). After the incubation, the cells were redissolved in 0.5 M NaOH, and counted in an Aquasol-2 scintillation cocktail (NEN Research Products, Boston, MA) (8).

Statistics. The results given as means ± SEM. The differences were tested using a two-way analysis of variance followed by the Scheffe's test for multiple comparisons. Two groups were compared by the unpaired *t* test. $P<0.05$ was considered significant.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. RT-PCR analysis of mRNA expression of ligands (Delta-1, -4, and Jagged-1, -2) and target proteins (Hes and Hey families) after ischemic renal failure.

Bilateral renal arteries were clamped for 60 min and kidneys were excised at 0.5, 1, 2, 3, 6, 12, 24, 48, and 72 h after reperfusion. Sham-operated rats at 0 h served as controls. Extracted total RNAs were subjected to quantitative PCR using the LightCycler real-time PCR for the estimation of relative Delta-1, -4, and Jagged-1, -2 mRNA levels and the ratio of to GAPDH mRNA, as described in the Methods section. (A) The representative agarose gels are shown. (B) The Delta-1/GAPDH mRNA ratio

is shown. Each column with a bar represents the mean ± SEM (n=5). *, P<0.05 vs. control rats. (C) Extracted total RNAs were subjected to quantitative PCR using the LightCycler real-time PCR for the estimation of relative Hes-1, -5, -7, Hey-1, and Hey-2 mRNA levels and the ratio of to GAPDH mRNA, as described in the Methods section. The representative agarose gels are shown. (D) The Hes-1/GAPDH mRNA ratio is shown. Each column with a bar represents the mean ± SEM (n=5). *, P<0.05 vs. control rats.

Figure 2. Western blot analyses of protein expression after ischemic renal failure.

(A) Extracted protein (50 □g) from renal tissue was separated by SDS-PAGE gels. Delta-1, Notch-2, Hes-1, cleaved Notch-1, cleaved Notch-3, and cleaved Notch-4 protein levels were detected by western blot analysis. In the preliminary experiments, we performed western blot analysis by using the anti-full-length Notch-2-specific and anti-cleaved Notch-2 antibodies. However these antibodies are not specific for either the cleaved activated Notch-2 or full-length Notch-2 but recognize both forms. In the subsequent experiments, we decided to use the Notch-2 antibody (Code: 100-401-406, Rockland), which recognizes all forms of Notch-2. Western blots of actin as loading controls are shown. (C: control, M: molecular weight marker) (B) Quantitative analyses using a densitometer were performed for Delta-1, full-length Notch-2, cleaved Notch-2, and Hes-1. Each bar represents the mean ± SEM. (n=6) *P<0.05 vs control (0 h) by the ANOVA test.

Figure 3. Immunohistochemical examination of Delta-1 after ischemic renal failure.

(A) Immunohistochemical analyses of the renal cortex were performed at a low-power view (X100) by using antibodies against Delta-1 and aquaporin-1 and in a

merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures). (B) Immunohistochemical analyses of the renal medulla were performed at a low-power view (X100) with antibodies against Delta-1 and aquaporin-1 and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures). (IM, inner medulla; OM, outer medulla) (Delta-1; AQP1, aquaporin-1; Merge, merged condition)

Figure 4. Immunohistochemical examination (high-power views) of Delta-1 in the renal outer medulla of ischemic/reperfused kidneys.

Immunohistochemical analyses of the renal outer medulla were performed at a high-power view (X600) by using antibodies against Delta-1 and aquaporin-1 and 4, 6-diamidino-2-phenylindole (DAPI) and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures).

Figure 5. Immunohistochemical examination of cleaved Notch-2 in the renal cortex of ischemic/reperfused kidneys.

(A) Immunohistochemical analyses of the renal cortex were performed at a low-power view (X100) by using antibodies against Notch-2 and aquaporin-1 and in a merged condition at 12 h after ischemic injury (upper figure) and in control kidneys (lower figure). (B) Immunohistochemical analyses of the renal cortex were performed at a low-power view (X100) using antibodies against Notch-2 and aquaporin-1 and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures). (IM, inner medulla; OM, outer medulla) (Notch-2, cleaved Notch-2; AQP1, aquaporin-1; Merge, merged condition)

Figure 6. Immunohistochemical examination (high-power views) of Notch-2 in the renal outer medulla of ischemic/reperfused kidneys.

(A) Immunohistochemical analyses of the renal outer medulla were performed at a high-power view (X600) by using antibodies against Notch-2 and aquaporin-1 and 4, 6-diamidino-2-phenylindole (DAPI) and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures). (B) Immunohistochemical analyses of the renal outer medulla were performed at a high-power view (X600) by using antibodies against Notch-2 and Delta-1 and DAPI and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures).

Figure 7. Immunohistochemical examination of Hes-1 in the renal cortex of ischemic/reperfused kidneys.

(A) Immunohistochemical analyses of the renal cortex were performed at a low-power view (X100) by using antibodies against Hes-1 and aquaporin-1 and in a merged condition at 12 h after ischemic injury (upper figure) and in control kidneys (lower figures). (B) Immunohistochemical analyses of the renal medulla were performed at a low-power view (X100) by using antibodies against Hes-1 and aquaporin-1 and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures). (IM, inner medulla; OM, outer medulla) (Hes-1; AQP1, aquaporin-1; Merge, merged condition)

Figure 8. Immunohistochemical examination (high-power views) of Hes-1 in the renal outer medulla of ischemic/reperfused kidneys.

(A) Immunohistochemical analyses of the renal outer medulla were performed at a high-power view (X600) by using antibodies against Hes-1 and aquaporin-1 and 4′,6-diamidino-2-phenylindole (DAPI) and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures). (B) Immunohistochemical analyses of the renal outer medulla were performed at a high-

power view (X600) by using antibodies against Hes-1 and Delta-1 and DAPI and in a merged condition at 12 h after ischemic injury (upper figures) and in control kidneys (lower figures).

Figure 9. Immunohistochemical investigations and western blot analyses of the specificity of antibodies for Delta-1, Notch-2, and Hes-1.

(A) Immunohistochemical analyses of the renal cortex were performed under a high-power view (600X) by using antibodies against Delta-1 at 12 h after ischemic injury (left figure) and in the presence of the blocking peptide for Delta-1 (right figure). (B) Immunohistochemical analyses of the renal cortex were performed under a high-power view (600X) by using antibodies against Notch-2 at 12 h after ischemic injury (left figure) and in the presence of the blocking peptide for Notch-2 (right figure). (C) Immunohistochemical analyses of the renal cortex were performed under a high-power view (600X) by using antibodies against Hes-1 at 12 h after ischemic injury (left figure) and in the presence of the blocking peptide for Hes-1 (right figure). (D–F) Protein (50 μg) extracted from the renal tissue at 6 h after ischemia/reperfusion was resolved over SDS-PAGE gels. Western blot analyses were performed in the absence or presence of the blocking peptide. The Delta-1 (D), Notch-2 (E), and Hes-1 (F) protein levels were detected by performing western blot analyses in the absence (left lanes) and presence of blocking peptides (right lanes). The western blots obtained for actin that was used as the loading control are also shown.

Figure 10. RT-PCR analysis of the expressions of Delta-1 and Hes-1 mRNA and the protein expressions of Delta-1, Cleaved Notch-2, and Hes-1 of NRK-52E cells under a hypoxic condition.

(A). Extracted RNA (1 μg) from NRK-52E cells was used for quantitative RT-PCR analyses for Delta-1, Hes-1, and GAPDH mRNA levels. Typical agarose gels are

shown. (B) Quantitative analyses were performed for Delta-1 and Hes-1 mRNAs. Each bar represents the mean ± SEM. (n=6) *P<0.05 vs control by the ANOVA test. (C). Extracted protein (50 μg) from NRK-52E cells was separated by SDS-PAGE gels. Delta-1, cleaved Notch-2, and Hes-1 protein levels were detected by Western blot analysis. Western blots of actin as loading controls are shown. (D) Quantitative analyses using a densitometer were performed for Delta-1, cleaved Notch-2, and Hes-1. Each bar represents the mean ± SEM. (n=6) *P<0.05 vs control by the ANOVA test.

Figure 11. Delta-1-stimulated cell numbers of renal tubular cells (NRK-52E).

Recombinant Delta-1 was plated onto each well of a 96-well culture plate. NRK-52E cells were cultured for five days. The morphologies and cell counts were observed for 5 days. (A) Typical morphology of NRK-52E cell in control (left panel) and in a Delta-1-stimulated condition (right panel). (B) The NRK-52E cell count in the presence of Delta-1 was significantly higher than the control level. (C) We examined the effects of recombinant Delta-1 on the cell proliferation of NRK-52E cells by [^{3}H]-thymidine uptake. Each bar represents the mean ± SEM. (n=6) *P<0.05 vs control by the ANOVA test.

Figure 12. RT-PCR analysis of Hes-1 mRNA and protein expressions of cleaved Notch-2 and Hes-1 in the presence of Delta-1 of NRK-52E cells.

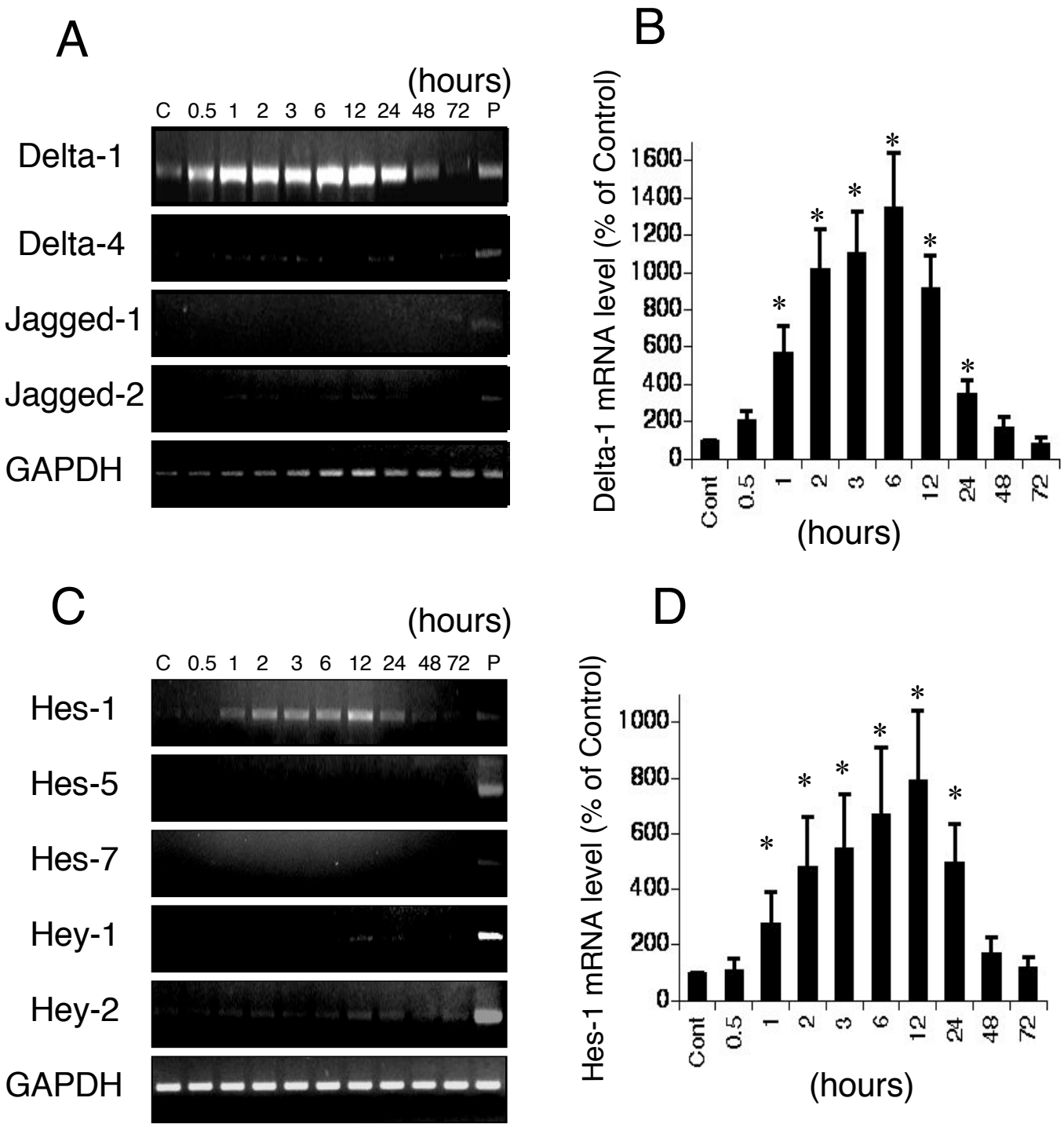
(A) Extracted RNA (1 μg) from NRK-52E cells was used for quantitative RT-PCR analyses for Hes-1 and GAPDH mRNA levels in the presence of Delta-1 and in a control condition. Typical agarose gels are shown. (B) Quantitative analyses using a densitometer were performed for Hes-1 mRNAs in the presence of Delta-1 and in a control condition. Each bar represents the mean ± SEM. (n=6) *P<0.05 vs control

by the ANOVA test. (C). Extracted protein (50 μg) from NRK-52E cells was separated by SDS-PAGE gels. Cleaved Notch-2 and Hes-1 protein levels were detected by Western blot analysis in the presence of Delta-1 and in a control condition. Western blots of actin as loading controls are shown. (D) Quantitative analyses using a densitometer were performed for cleaved Notch-2 and Hes-1. Each bar represents the mean ± SEM. (n=6) *P<0.05 vs control by the ANOVA test.

Table 1. Primers for RT-PCR

Delta-1	(upper) 5’-CAACCCCATCCGATTCCCCT-3’ (lower) 5’-GTCACAATATCCATGTTGGT-3’
Delta-4	(upper) 5’-GAAATTCACTTATCAGCCAA-3’ (lower) 5’-CAGGGGATGGTGCAGGT-3’
Jagged-1	(upper) 5’-GTCTGCAAAGAAGGCTGGGA-3’ (lower) 5’-GCCACACCAGACCTTGGAGC-3’
Jagged-2	(upper) 5’-TGCACACACAACACCAATGA-3’ (lower) 5’-CACTGGGCTGAGGGGACAGC-3’
Hes-1	(upper) 5’-GGTGGCTGCTACCCCAGCCA-3’ (lower) 5’-GGTAGGTCATGGCGTTGATC-3’
Hes-5	(upper) 5’-GGTGGAGATGCTCAGTCCCAAGGA-3’ (lower) 5’-TAACCCTCGCTGTAGTCCTGGTG-3’
Hes-7	(upper) 5’-GGTCCCAAGATGCTGAAGCCGTTGGTGGA-3’ (lower) 5’-CAGGCACTCGCGGAAGCCGGACAAGTA-3’
Hey-1	(upper) 5’-TCGAGAAGCGCCGACGAGACCGA-3’ (lower) 5’-CAGCAGCGGGTGTGCGATGTGTGGGT-3’
Hey-2	(upper) 5’-GTCTGCAAAGAAGGCTGGGA-3’ (lower) 5’-GCCACACCAGACCTTGGAGC-3’
GAPDH	(upper) 5’-AGATCCACAACGGATACATT-3 (lower) 5’-TCCCTCAAGATTGTCAGCAA-3’

Figure 1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(hours)
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Delta-1
Delta-4
Jagged-1
Jagged-2
GAPDH
B
Delta-1 mRNA level (% of Control)
1600
1400
1200
1000
800
600
400
200
0
Cont 0.5 1 2 3 6 12 24 48 72
(hours)
C
(hours)
C 0.5 1 2 3 6 12 24 48 72 P
Hes-1
Hes-5
Hes-7
Hey-1
Hey-2
GAPDH
D
Hes-1 mRNA level (% of Control)
1000
800
600
400
200
0
Cont 0.5 1 2 3 6 12 24 48 72
(hours)

Figure 2

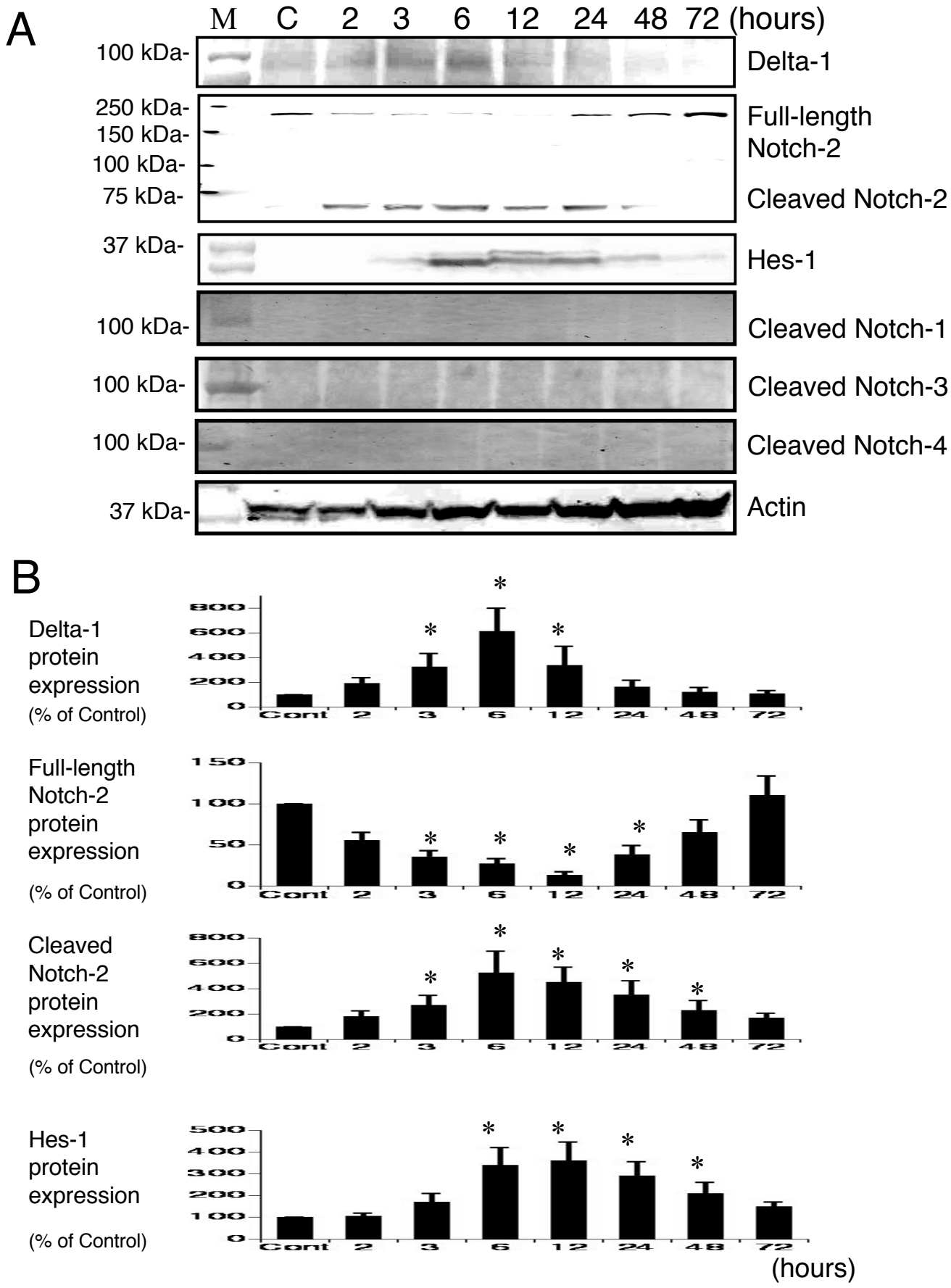
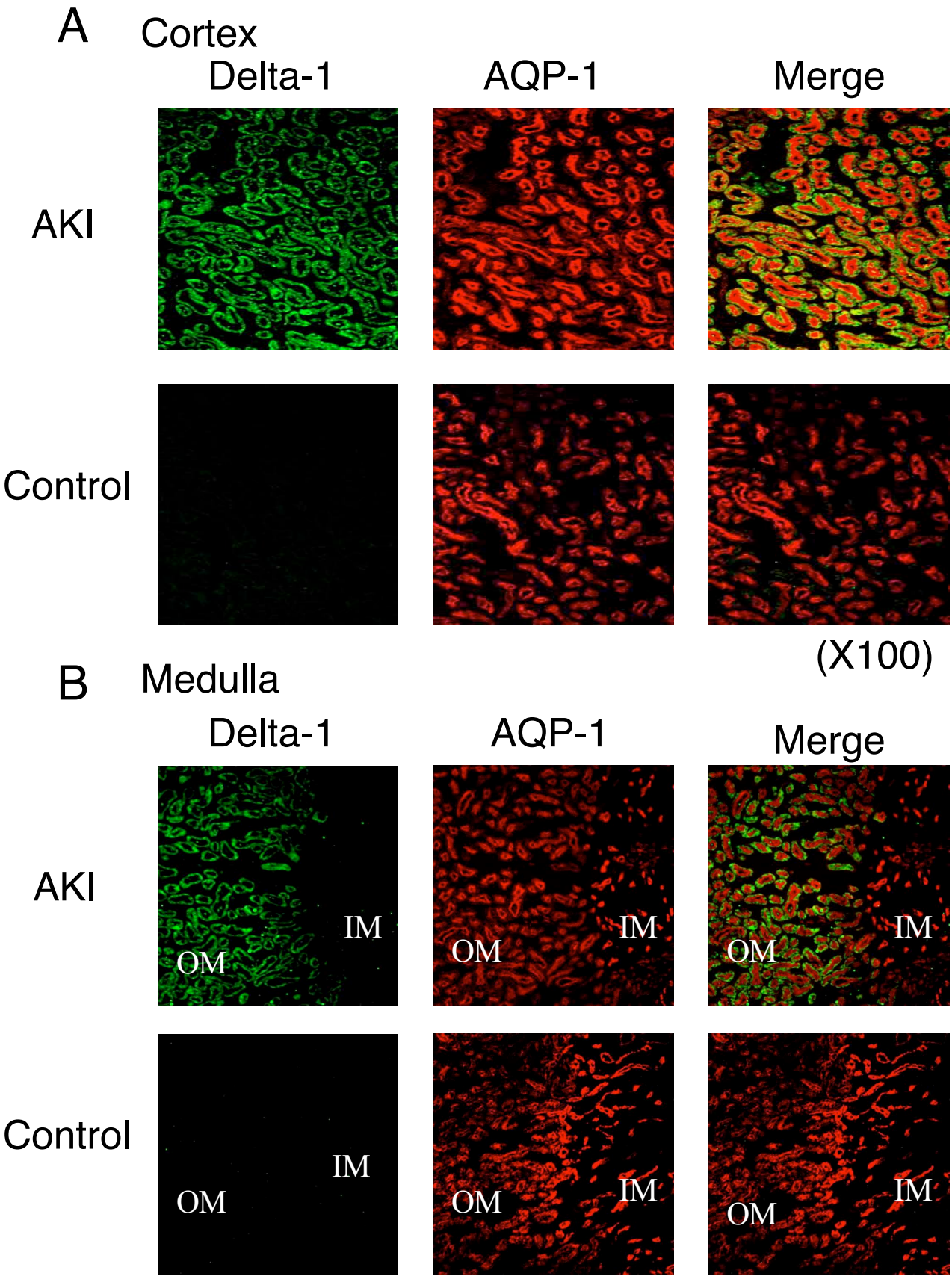

A
M C 2 3 6 12 24 48 72 (hours)
100 kDa-
Delta-1
250 kDa-
150 kDa-
100 kDa-
75 kDa-
Full-length Notch-2
Cleaved Notch-2
37 kDa-
Hes-1
100 kDa-
Cleaved Notch-1
100 kDa-
Cleaved Notch-3
100 kDa-
Cleaved Notch-4
37 kDa-
Actin
B
Delta-1 protein expression (% of Control)
Full-length Notch-2 protein expression (% of Control)
Cleaved Notch-2 protein expression (% of Control)
Hes-1 protein expression (% of Control)
Cont 2 3 6 12 24 48 72
(hours)

Figure 3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Cortex
Delta-1
AQP-1
Merge
AKI
Control
(X100)
B
Medulla
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AQP-1
Merge
AKI
OM
IM
Control
OM:Outer Medulla
IM:Inner Medulla
(X100)

Figure 4

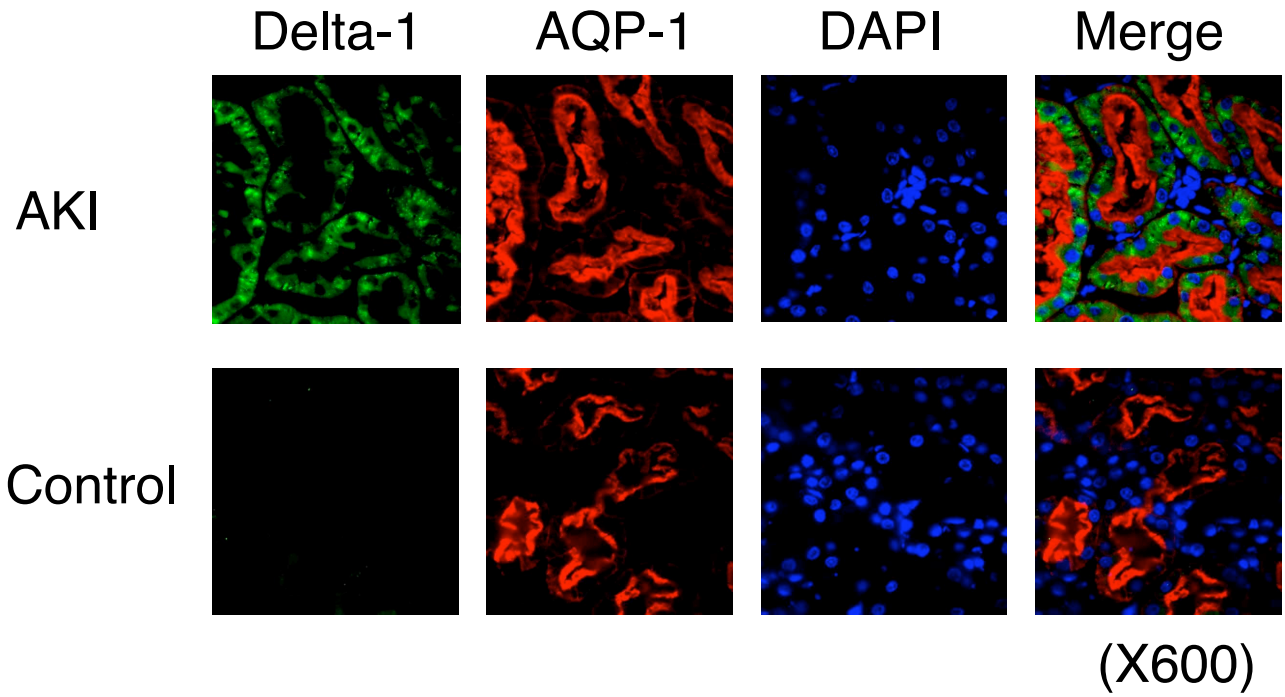
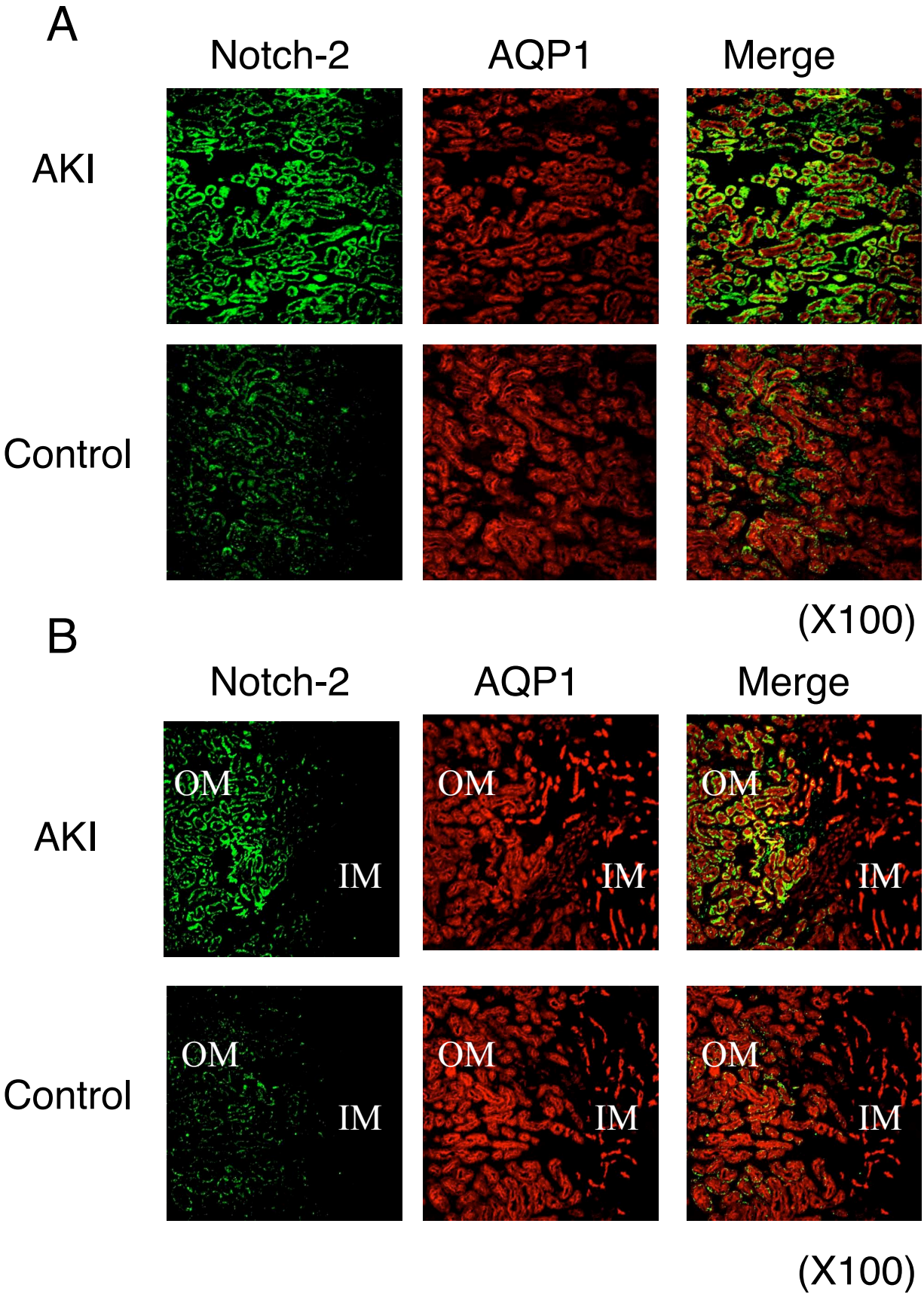


Figure 5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AQP1
Merge
AKI
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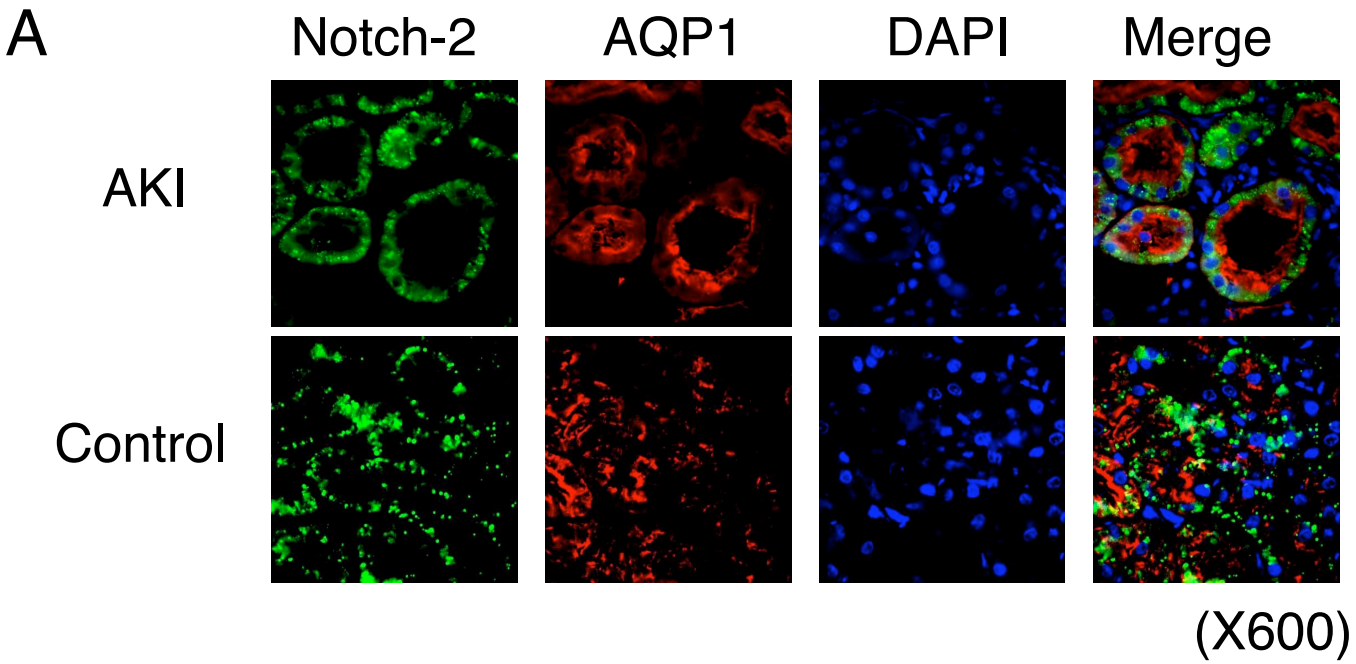
AKI
OM
IM
Control
OM
IM
(X100)

Figure 6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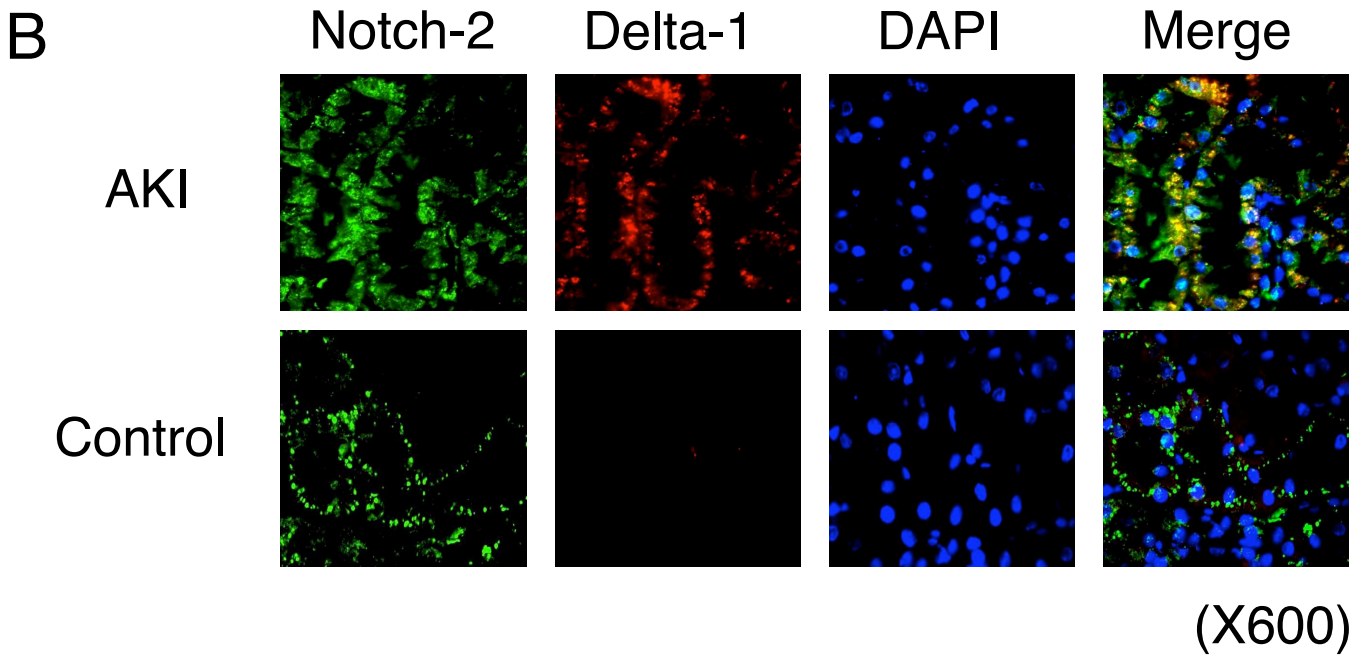
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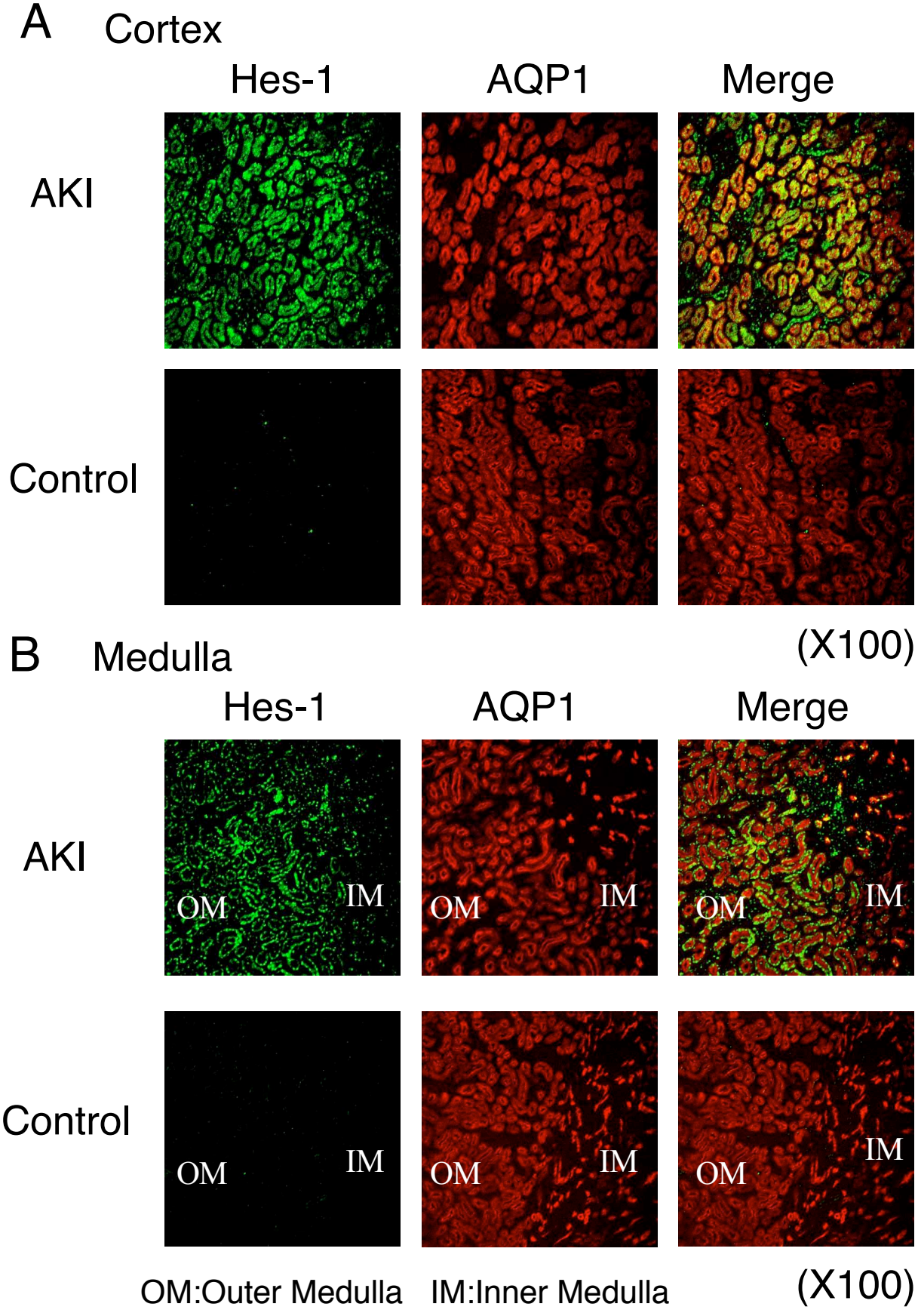
Notch-2
Delta-1
DAPI
Merge
AKI
Control
(X600)

Figure 7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Hes-1
AQP1
Merge
AKI
Control
(X100)
B Medulla
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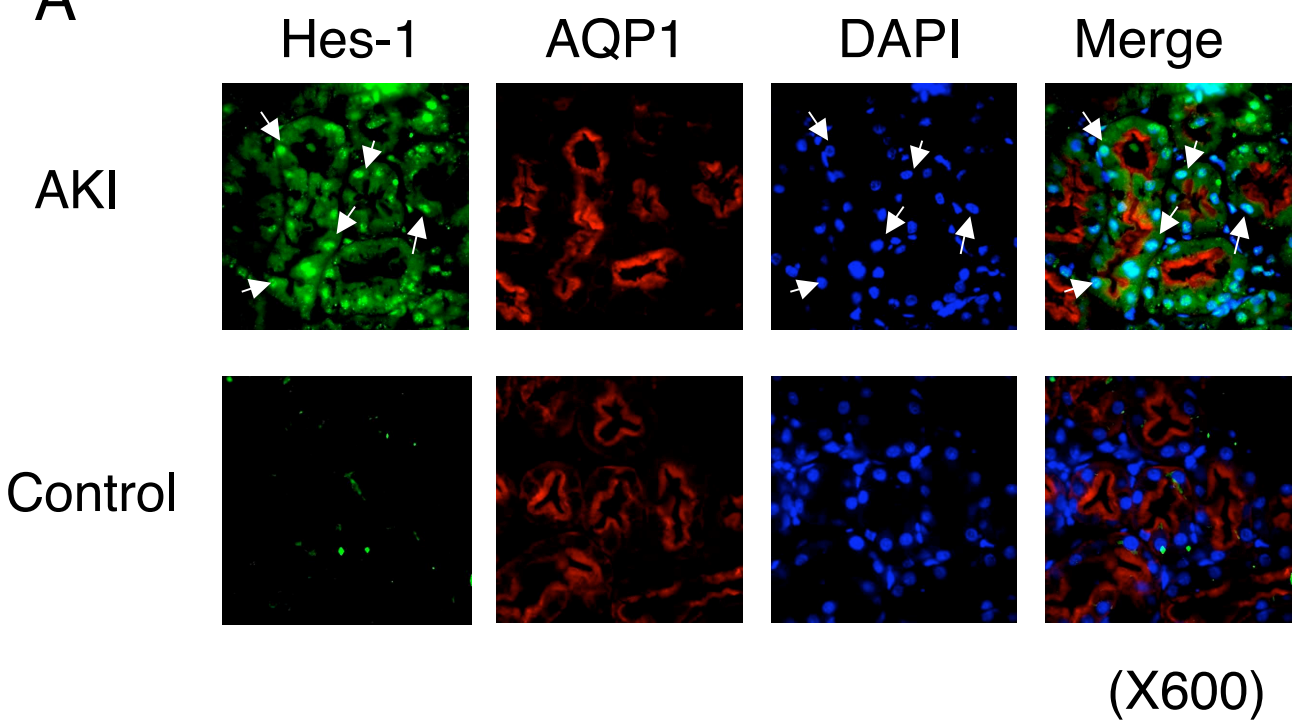
AQP1
Merge
AKI
OM
IM
Control
OM
IM
OM:Outer Medulla
IM:Inner Medulla
(X100)

Figure 8


A
Hes-1
AQP1
DAPI
Merge
AKI
Control
(X600)

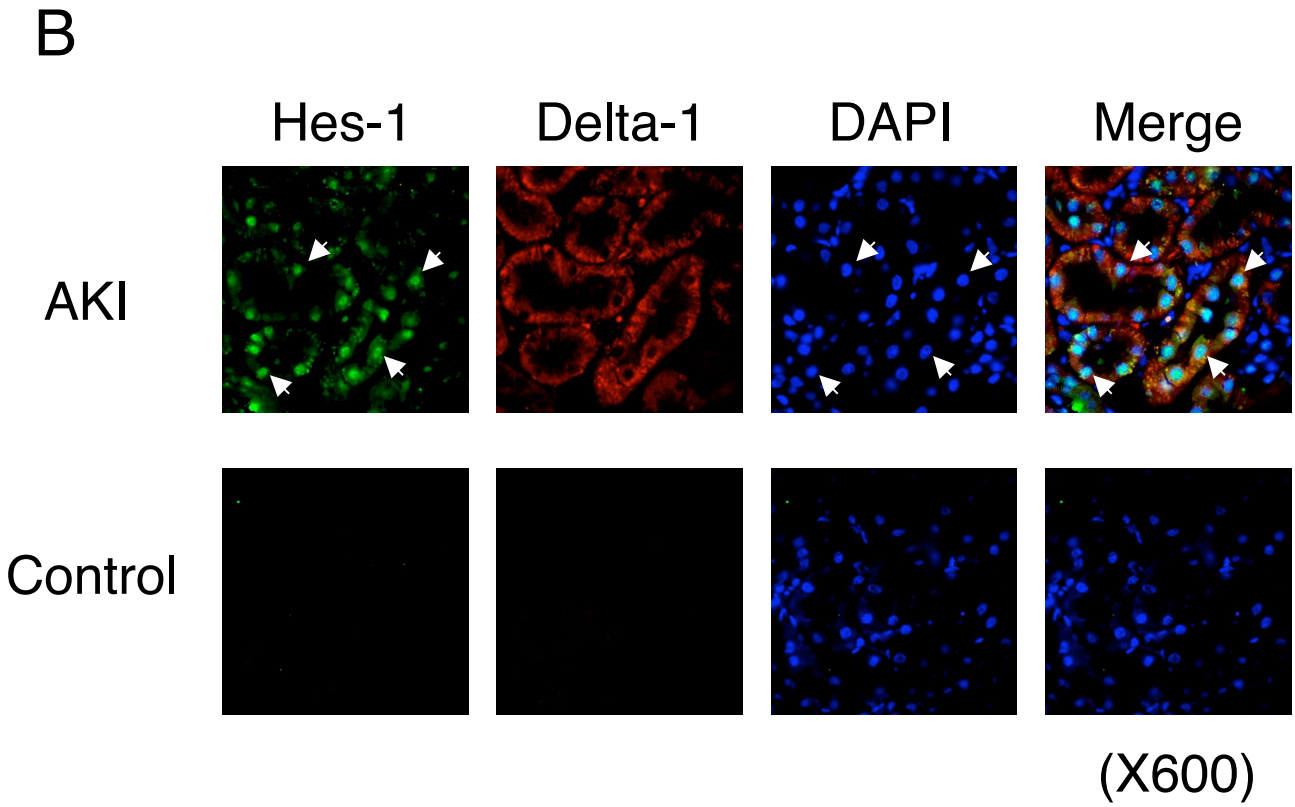

B
Hes-1
Delta-1
DAPI
Merge
AKI
Control
(X600)

Figure 9

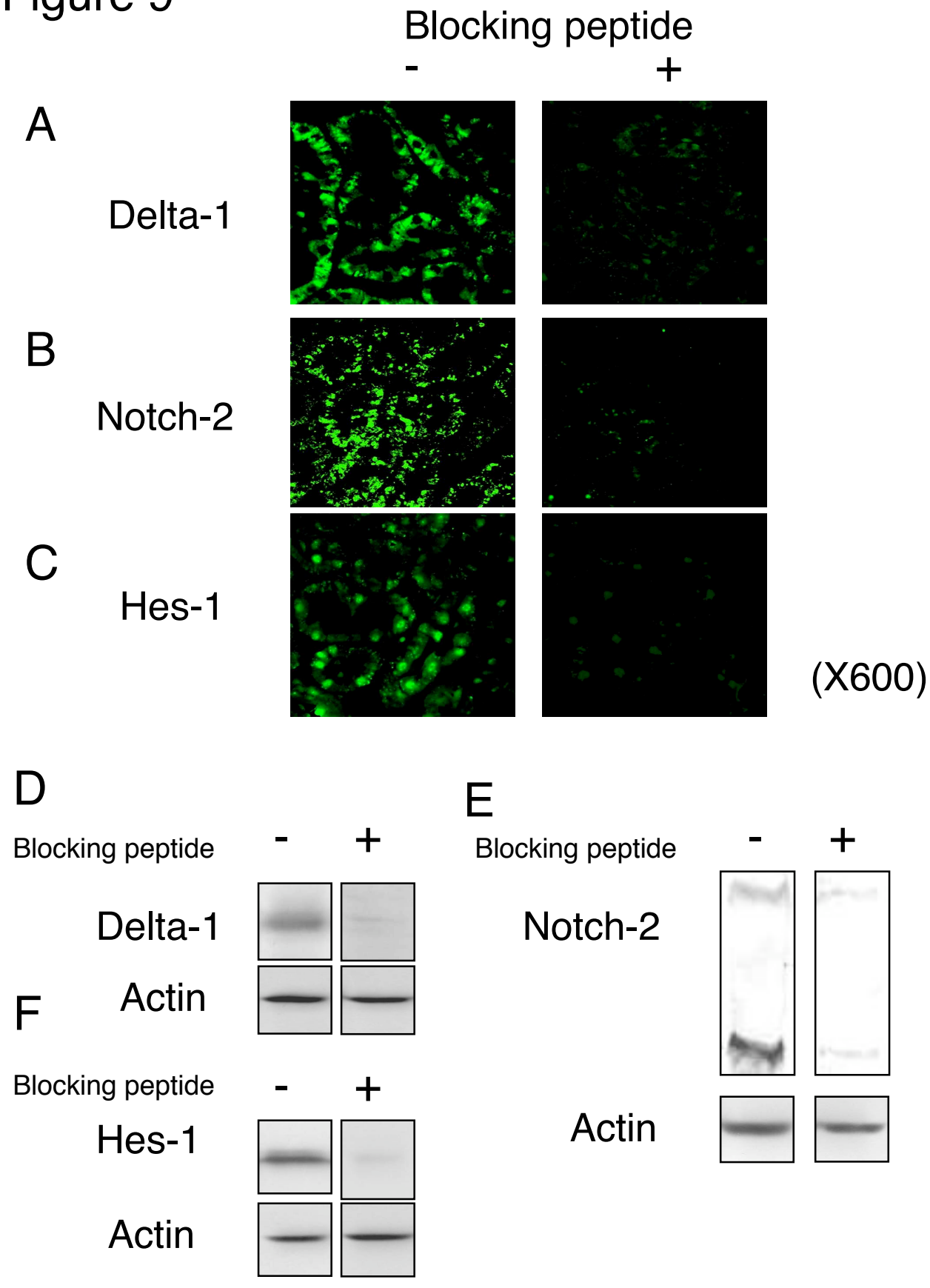

Blocking peptide
-
+
A
Delta-1
B
Notch-2
C
Hes-1
(X600)
D
Blocking peptide
-
+
Delta-1
Actin
E
Blocking peptide
-
+
Notch-2
Actin
F
Blocking peptide
-
+
Hes-1
Actin

Figure 10

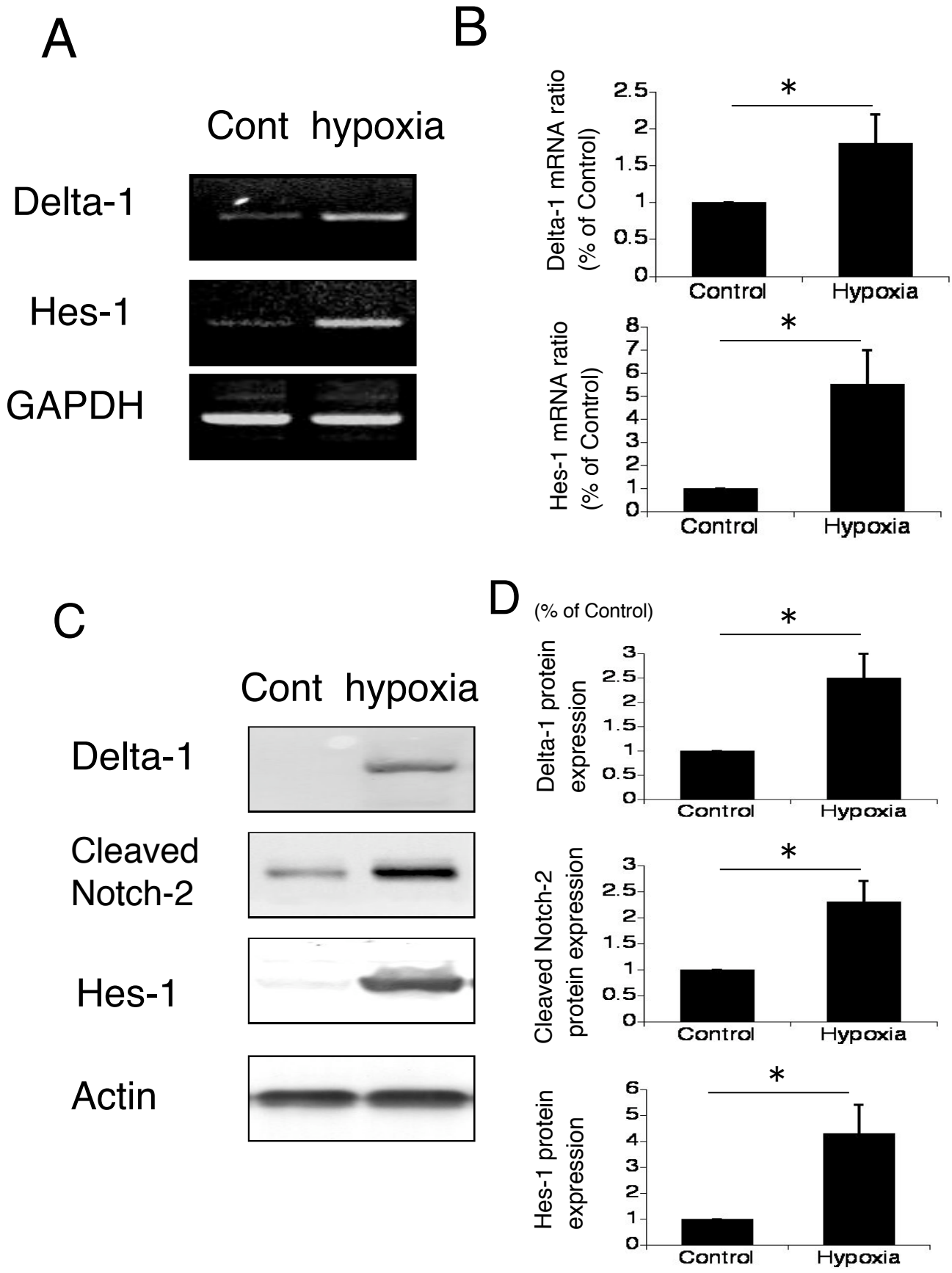

A
Cont hypoxia
Delta-1
Hes-1
GAPDH
B
Delta-1 mRNA ratio (% of Control)
2.5
2
1.5
1
0.5
0
*
Control
Hypoxia
Hes-1 mRNA ratio (% of Control)
8
7
6
5
4
3
2
1
0
*
Control
Hypoxia
C
Cont hypoxia
Delta-1
Cleaved Notch-2
Hes-1
Actin
D
(% of Control)
Delta-1 protein expression
3
2.5
2
1.5
1
0.5
0
*
Control
Hypoxia
Cleaved Notch-2 protein expression
3
2.5
2
1.5
1
0.5
0
*
Control
Hypoxia
Hes-1 protein expression
6
5
4
3
2
1
0
*
Control
Hypoxia

Figure 11

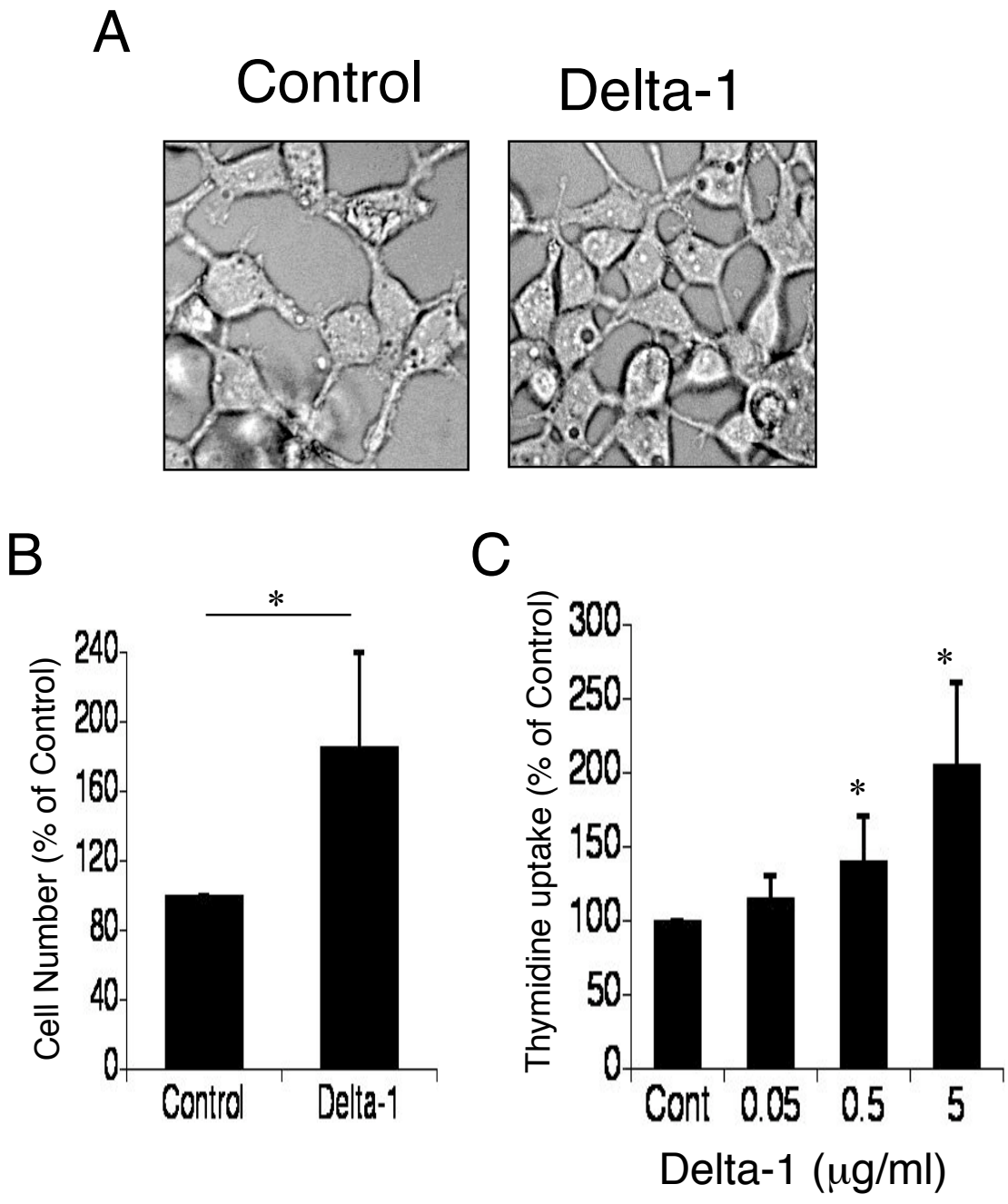
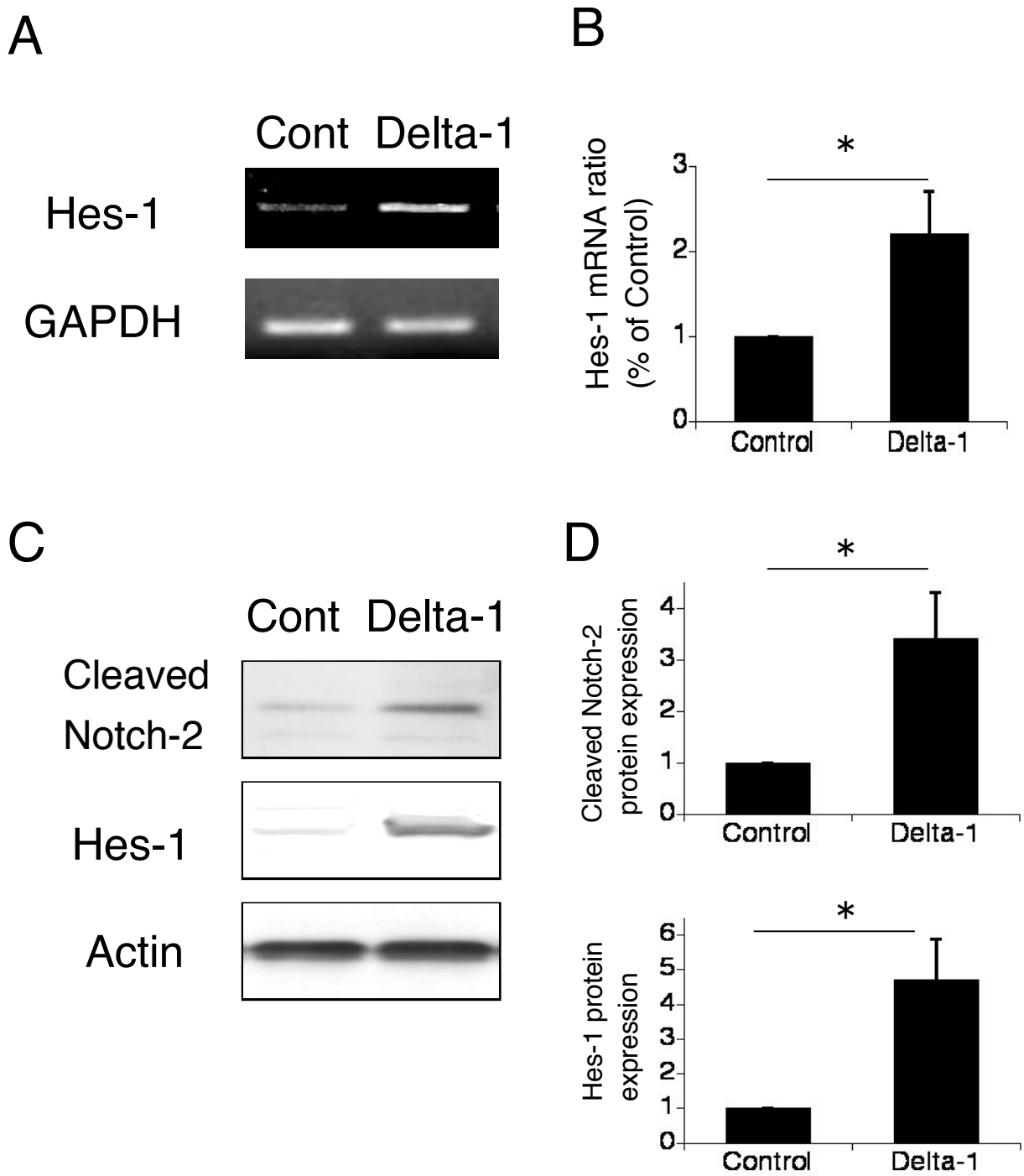

A
Control
Delta-1
B
Cell Number (% of Control)
240
200
160
120
80
40
0
*
Control
Delta-1
C
Thymidine uptake (% of Control)
300
250
200
150
100
50
0
*
*
Cont
0.05
0.5
5
Delta-1 (μg/ml)

Figure 12


A
Cont Delta-1
Hes-1
GAPDH
B
Hes-1 mRNA ratio (% of Control)
*
Control
Delta-1
C
Cont Delta-1
Cleaved Notch-2
Hes-1
Actin
D
Cleaved Notch-2 protein expression
*
Control
Delta-1
Hes-1 protein expression
*
Control
Delta-1