

# Spinal Cord Injury Markedly Altered Protein Expression Patterns in the Affected Rat Urinary Bladder during Healing Stages

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## INTRODUCTION

Acute traumatic spinal cord injury (SCI) is a destructive neurological disorder that causes defects in sensory and autonomic functions as well as motor paralysis distal to the lesion (1). Sensory and autonomic dysfunctions often lead to urinary bladder impairment, which can be a major cause of morbidity for those with SCI (2). The molecules involved in the impairment of affected spinal cord or neural tissue itself have been relatively well-studied (3-5), but little has been reported about the expression and changes in relevant proteins in sensory and autonomic dysfunctions induced by SCI such as neurogenic bladder.

Neurogenic bladder is any dysfunctional condition of the urinary bladder caused by SCI or other lesions of the central ner-

The influence of spinal cord injury (SCI) on protein expression in the rat urinary bladder was assessed by proteomic analysis at different time intervals post-injury. After contusion SCI between T9 and T10, bladder tissues were processed by 2-DE and MALDI-TOF/MS at 6 hr to 28 days after SCI to identify proteins involved in the healing process of SCI-induced neurogenic bladder. Approximately 1,000 spots from the bladder of SCI and sham groups were visualized and identified. At one day after SCI, the expression levels of three protein were increased, and seven spots were down-regulated, including heat shock protein 27 (Hsp27) and heat shock protein 20 (Hsp20). Fifteen spots such as S100-A11 were differentially expressed seven days post-injury, and seven proteins including transgelin had altered expression patterns 28 days after injury. Of the proteins with altered expression levels, transgelin, S100-A11, Hsp27 and Hsp20 were continuously and variably expressed throughout the entire post-SCI recovery of the bladder. The identified proteins at each time point belong to eight functional categories. The altered expression patterns identified by 2-DE of transgelin and S100-A11 were verified by Western blot. Transgelin and protein S100-A11 may be candidates for protein biomarkers in the bladder healing process after SCI.

**Key Words:** Spinal Cord Injuries; Urinary Bladder, Neurogenic; Proteomic Analysis; Biological Markers; S100-A11; Transgelin

vous system (CNS). Cortical control over the sacral reflex voiding center is impaired after suprasacral SCI, and subsequent neurogenic detrusor overactivity and/or detrusor-sphincter dys-synergia leads to functional urethral obstruction, urinary retention, and bladder hypertrophy (6). Microarray analysis of gene expression in the bladder after SCI revealed that mRNA levels of molecules involved in tissue remodeling were up-regulated in the post-SCI bladder (7).

In this study, we used a proteomic analysis to identify differentially expressed proteins in the bladder after SCI. The proteins from bladder tissues in spinal cord injured rat and sham control were prepared and processed for 2-DE and MALDI-TOF/MS. To find new biomarkers for the healing process in the post-SCI bladder, dynamic changes in protein profiles identified by 2-DE were

analyzed at different time points. Our results may provide insight into the novel candidate target proteins involved in the healing stages of the bladder after SCI and underlying pathophysiological mechanisms between SCI and neurogenic bladder.

## MATERIALS AND METHODS

### Animals

The female Sprague-Dawley rats (250-300 g, 12 weeks old,  $n = 57$ ) were purchased from Koatech Co. (Seoul, Korea). Animal experiments were performed according to the Gyeongsang National University Animal Care and Use Committee (GLA-090701-R0051).

### Spinal cord injury

Female rats have relatively short urethra compared to males and were preferentially used for SCI animals suffering from neurogenic voiding dysfunction. For contusion SCI ( $n = 40$ ), the ninth thoracic cord segment (T9) of rats was exposed under anesthesia and injury was produced by dropping a 2.0 mm diameter rod with 10 g weight from 25 mm height onto the exposed spinal cord using the New York University (NYU) Impactor (New York, NY, USA). Sham-operated rats ( $n = 17$ ) experienced the same operation as the spinal cord injured rats without contusion injury, and animal care after SCI was performed, as previously described (8). The bladder post-injury was manually voided twice daily until voluntary voiding occurred. The operated rats were randomized and divided into separate groups: 1) early phase of recovery, 6 hr ( $n = 5$ ), 1 day ( $n = 6$ ) and 2 days ( $n = 6$ ) after SCI; 2) intermediate recovery phase, 7 days ( $n = 6$ ) and 9 days ( $n = 6$ ) after SCI; 3) late phase of recovery, 21 days ( $n = 5$ ) and 28 days ( $n = 6$ ). The rats in the control group were sacrificed at the same time intervals as the SCI groups.

### Assessment of locomotor behavior

The Basso, Beattie, and Bresnahan (BBB) test (9), a behavioral test for the assessment of hindlimb locomotion after SCI, was performed to evaluate the recovery of locomotor function post-contusion injury with scores from zero (no observable movement) to a maximum of 21 (normal locomotion). The test was performed on immediately after SCI and then continued for four weeks at 6 hr, 1 day, 2, 7, 9, 21, and 28 days post-SCI by two blinded observers. The data were presented as the mean  $\pm$  SD (sham group [ $n = 17$ ] and contusion group [ $n = 40$ ]). Data were evaluated using a one-way ANOVA with a paired t-test using GraphPad Prism 4 for Windows, GraphPad Software, Inc. (La Jolla, CA; www.graphpad.com). Statistical significance was set at  $P < 0.05$ .

### Protein sample preparation for 2-DE

Bladder tissues of spinal cord injured rats were homogenized in suspension buffer (0.1 g tissue/mL buffer, 50 mM Tris-HCl, pH

7.2, 50 mM EDTA and protease inhibitor cocktail) and processed by centrifugation at  $100,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant was precipitated with trichloroacetic acid, and the resulting pellet was dried and dissolved in lysis buffer (8 M urea, 4% CHAPS, 100 mM DTT and 2% ampholytes pH 3-10) with sonication. After centrifugation at  $15,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , the final supernatant was used as a 2-DE sample. Protein concentration was measured with the Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

### Two-dimensional electrophoresis

Samples containing 50  $\mu\text{g}$  (for analytical gel) or up to 600  $\mu\text{g}$  (for micropreparative gel) proteins were diluted with rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.5% IPG buffer [pH 4-7] and bromophenol blue), and applied to IPG gel strips (Bio-Rad) at 50 V for 14 hr. Isoelectric focusing (IEF) in the first dimension was performed with 18 cm IPG strips (pH 4-7) using Protean IEF Cell (Bio-Rad). After completion of IEF, the strips were placed in an equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and bromophenol blue) containing 1% DTT for 15 min, and transferred to the equilibration solution containing 2.5% iodoacetamide before placing them on a SDS gel (7.5%-17.5%,  $185 \times 200 \times 1$  mm). Separation in the second dimension was performed in Tris-glycine buffer containing 0.1% SDS, and the separated gel was visualized with silver staining for analytical gel and/or Coomassie blue staining for micropreparative gel, as previously described (10).

### Image analysis and protein identification

Protein patterns in the gel were recorded as digitalized image using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, Bio-Rad) and matching of the gel image was performed with PDQuest software (version 6.2.1, Bio-Rad). For each sample, four gels were run. Differentially expressed proteins in separate gels ( $P < 0.05$  by Student's t-test) were identified for each bladder sample at different healing stages of the spinal cord injured rat compared to the sham control sample. For all spot intensity calculations, normalized values were used.

For protein identification, the desired gel pieces were excised after staining, and processed for tryptic digestion, as previously described (10). Target preparation was performed by solution-phase nitrocellulose method (11). We used a Voyager-DE<sup>TM</sup> (delayed extraction) STR biospectrometry workstation (Applied Biosystems, Foster City, CA, USA) for MALDI-TOF/MS. Proteins were identified by peptide mass fingerprinting with the search program MS-FIT (<http://prospector.ucsf.edu/prospector>).

### Western blot analysis

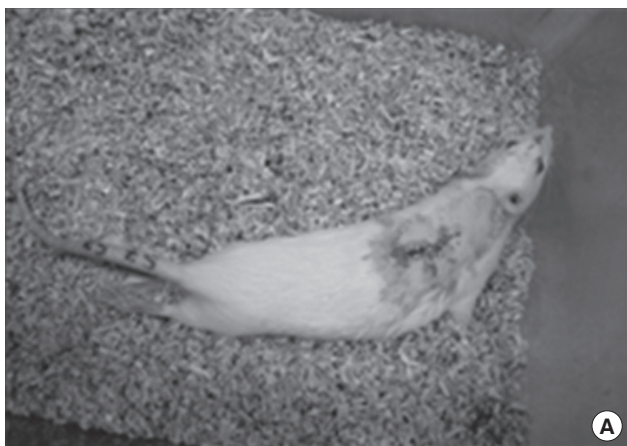
Total proteins (20  $\mu\text{g}$ ) from urinary bladder in spinal cord injured rat were separated on a 12% SDS-gel electrophoresis and subsequently transferred to nitrocellulose membrane by a Tank

transfer system (Bio-Rad) for 1 hr at 340 mA. After blocking in 5% skim milk/TBST (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl and 0.1% Tween 20) for overnight at 4°C, the membrane was incubated with rabbit polyclonal antibody against transgelin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or S100 alpha (Abcam, Cambridge, MA, USA) for 1 hr at room temperature and washed three times in TBST for 10 min each. Then the membrane was incubated with goat anti-rabbit IgG HRP (Santa Cruz Biotechnology) for 1 hr and the unbound antibodies were washed with TBST solution thrice for 10 min. Proteins were visualized with an ECL detection system (Pierce Chemical, Rockford, IL, USA).

## RESULTS

### Assessment of locomotor behavior of hindlimb in spinal cord injured rats

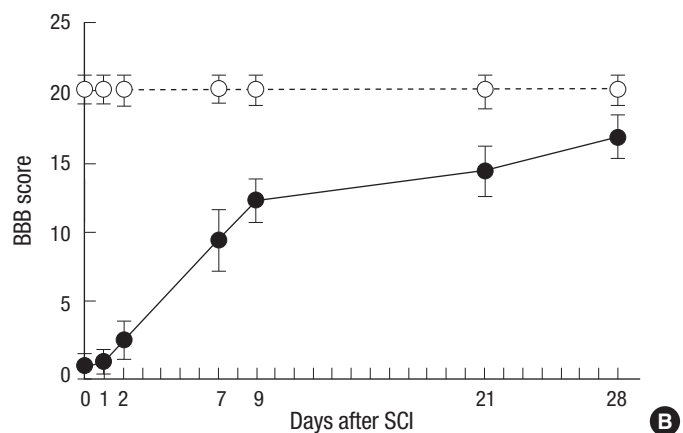
To assess behavioral outcome of rats with or without contusion SCI, 57 female rats were used to validate the BBB score (9). Fig. 1A shows a picture of spinal cord injured rat with sutured muscles and skin overlying in the back. The hindlimbs of rat post-injury were observed as drooped and paralyzed state. The BBB score of rats in SCI groups and sham control was estimated at different time points (Fig. 1B). The rats in sham group sustained normal hindlimb locomotion and the score maintained almost constant level around from 19 to 21 throughout the experimental period (open circle). However, the BBB score of rats at 1 day after contusion injury appeared near zero, and the score subsequently started to improve from 2 days after SCI and achieved 50% recovery between 7 and 9 days post-injury ( $P < 0.05$ ). The score of rats with the contusion injury continued to recover to the 80% values of sham control group until 28 days post-injury (closed circle).



### Identification and validation of differentially expressed proteins in urinary bladder after contusion

To investigate the differentially expressed proteins at healing stages of urinary bladder after SCI, proteomic analysis was performed in the bladder tissues of SCI-affected and sham control rats. All operated animals were evaluated hindlimb locomotor recovery by BBB test (9). The sacrificed rats owing to unsuitable urinary evacuation in the course of recovery process were excluded for the study.

Two-dimensional electrophoretic analysis of the bladder tissue proteins in rat after contusion SCI was carried out at the indicated time points post-injury (6 hr, 1, 2, 7, 9, 21, and 28 days) ranging from five to six animals in each experimental group, and the expression patterns of spots were compared using PDQuest image analysis software. The individual scanned images were matched to form a composite image of the sham and affected tissues. Approximately 1,000 protein spots (616-1,069 spots from each time point) were visualized in a gel (pH range of 4-7 and molecular mass ranges of 10-100 kDa) (Fig. 2). The overall protein expression patterns in the SCI-affected urinary bladder (neurogenic bladder) were quite dissimilar depending on the healing stages after injury, showing significant loss of protein spots in the early phase of recovery (1 and 2 days post-injury), considerable recovery of the spots in the intermediate of mid-phase (7 and 9 days post-injury), and almost complete rescue of the protein spots comparable to the unaffected sham control tissues in the late phase of recovery (21 and 28 days) (A-H in Fig. 2). Spots representing proteins that were up- or down-regulated with more than two-fold difference in 2-DE gels at indicated time points were selected, and further analyzed by comparing their expression patterns. Individual protein spots were picked out, digested with trypsin, and identified by MALDI-TOF/mass spectrometry (Table 1). The criteria used to accept identifications includ-

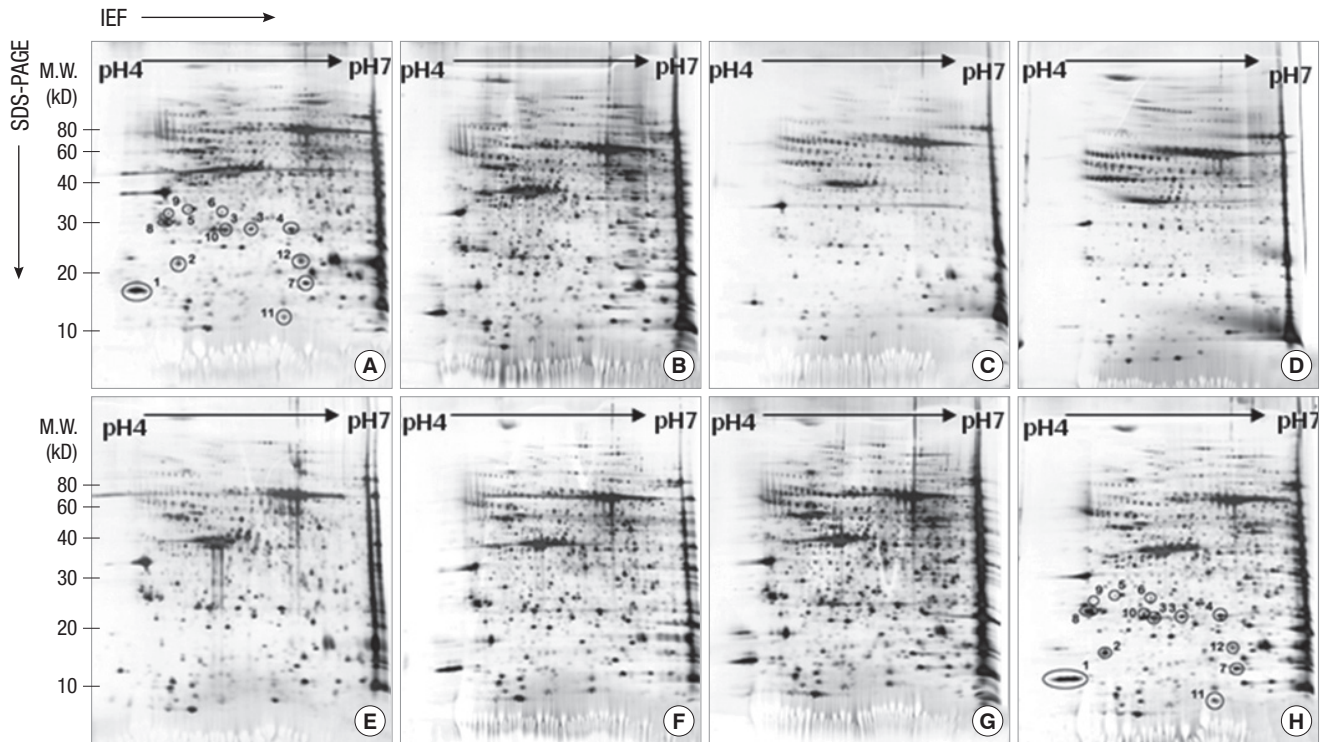


**Fig. 1.** Evaluation of spinal cord injury by locomotor behavior scoring. **(A)** A photograph of spinal cord injured rat. Sutured muscles and skin in the back after surgery are shown, and hindlimbs are drooped and paralyzed. **(B)** Basso Beattie Bresnahan (BBB) open field locomotor rating scale is scored in the course of healing process after contusion SCI. Adult rats ( $n = 5-6$  animals per group) were contused at T-9 exposed spinal cord with a 250 gram weight drop device from a 25 mm height using the New York University (NYU) Impactor. Locomotor behavior was evaluated before injury and then for up to 28 days with different time intervals. The graph exhibits functional hindlimb recovery of rats in contusion group compared with those of sham control group. Data were presented as mean  $\pm$  SD and  $P < 0.05$  in both groups.



ed the extent of sequence coverage, the number of peptides matched, the probabilistic score and also the corresponding

values of *pI* and *Mr*. The mean intensity of each spot was calculated by its silver stain intensity and the relative intensities be-



**Fig. 2.** Comparison of protein expression patterns of rat urinary bladder after contusion SCI at different time points. The bladder tissue proteins (50 µg) from each stage (sham control [A], 6 hr [B], 1 day [C], 2 days [D], 7 days [E], 9 days [F], 21 days [G], and 28 days [H] post-injury) were separated on pH 4–7 IPG strips (17 cm, Bio-Rad) in the first dimension and on 7.5%–17.5% linear-gradient SDS-PAGE in the second dimension, and visualized by silver nitrate staining. Protein names specified by Arabic numerals (A and H) are listed in Table 1.

**Table 1.** Identification of differentially expressed spots by MALDI-TOF/MS in rat bladder post-SCI at indicated time points

SSP	Accession #*	Protein name*	MW/pI	Masses matched	Sequence coverage	P value
<b>Up-regulated proteins</b>						
<b>1 day after SCI</b>						
236	P63102	14-3-3 protein zeta/delta (8)	27771/4.7	5/35 (14%)	20.8%	0.003
520	P05545	Serine protease inhibitor A3K	46562/5.3	11/22 (50%)	26.2%	0.072
5628	P02770	Serum albumin	68731/6.1	22/50 (42%)	37.0%	0.017
<b>2 days after SCI</b>						
207	P63102	14-3-3 protein zeta/delta (8)	27771/4.7	5/35 (14%)	20.8%	0.000
212	P63610	Tropomyosin alpha-3 chain (9)	29007/4.7	6/28 (21%)	22.1%	0.000
602	P05545	Serine protease inhibitor A3K	46562/5.3	10/28 (35%)	27.9%	0.002
1601	P05544	Serine protease inhibitor A3L	46278/5.2	8/21 (38%)	20.6%	0.001
2702	P01048	T-kininogen 1	47776/6.1	10/25 (40%)	25.8%	0.014
4708	P20059	Hemopexin	51351/7.6	13/33 (39%)	27.0%	0.390
5106	P04639	Apolipoprotein A-I	30062/5.5	12/38 (31%)	42.1%	0.006
5505	P02680	Fibrinogen gamma chain	50633/5.6	9/33 (27%)	21.2%	0.027
<b>7 days after SCI</b>						
205	P62261	14-3-3 epsilon	29174/4.6	10/23 (52%)	43.5%	0.001
211	Q63610	Trophomyosin alpha-3 chain (9)	29007/4.7	6/28 (21%)	22.1%	0.010
218	P63102	14-3-3 zeta/delta (8)	27771/4.7	9/27 (22%)	31.4%	0.054
220	Q5ZLQ6	14-3-3 beta/alpha	28055/4.8	7/24 (29%)	38.6%	0.000
1726	P10148	T-kininogen 2	47776/6.1	10/25 (40%)	25.8%	0.387
2118	53733577	Rho GDP dissociation inhibitor (10)	23408/5.1	9/27 (33%)	53.4%	0.004
4103	P42930	Heat shock protein 27 (Hsp27) (3)	22893/6.1	9/21 (43%)	43.2%	0.001
5006	Q6B345	Protein S100- A11 (11)	11065/5.6	4/23 (17%)	43.9%	0.000
5507	P11884	Aldehyde dehydrogenase, mitochondrial	56489/6.6	10/35 (29%)	21.8%	0.129
5510	P02770	Serum albumin	68731/6.1	8/28 (29%)	16.9%	0.157

(continued to the next page)

**Table 1.** (continued from the previous page) Identification of differentially expressed spots by MALDI-TOF/MS in rat bladder post-SCI at indicated time points

SSP	Accession #*	Protein name*	MW/pI	Masses matched	Sequence coverage	P value
<b>Up-regulated proteins</b>						
9 days after SCI						
235	P63102	14-3-3 zeta/delta (8)	27771/4.7	9/27 (22%)	31.4%	0.042
1117	Q64122	Myosin regulatory light chain 2, smooth muscle isoform (2)	19723/4.8	7/33 (21%)	31.6%	0.049
1213	Q5ZLQ6	14-3-3 beta/alpha	28055/4.8	7/24 (29%)	38.6%	0.000
21 days after SCI						
3209	Q5I0D1	Glyoxylase domain-containing protein 4	33268/5.1	7/40 (17%)	27.9%	0.119
28 days after SCI						
42	Q64122	Myosin regulatory light chain 2, smooth muscle isoform (2)	19723/4.8	7/33 (21%)	31.6%	0.107
228	P63102	14-3-3 zeta/delta (8)	27771/4.7	11/39 (28%)	31.0%	0.000
5117	P31232	Transgelin (12)	22603/8.9	13/38 (34%)	44.3%	0.062
<b>Down-regulated proteins</b>						
6 hr after SCI						
2624	P06761	78 kDa glucose-regulated protein (GRP)	72348/5.1	19/36 (53%)	35.9%	0.082
1 day after SCI						
36	Q64119	Myosin light polypeptide 6 (1)	16975/4.5	7/28 (25%)	37.1%	0.027
1120	Q64122	Myosin regulatory light chain 2, smooth muscle isoform (2)	19723/4.8	7/33 (21%)	31.6%	0.000
1333	P02651	Apolipoprotein A-IV	44457/5.2	9/21 (42%)	25.8%	0.021
1426	P10719	ATP synthase subunit beta, mitochondrial	56354/5.2	8/21 (38%)	21.9%	0.009
5217	P42930	Heat shock protein 27 (Hsp27)	22893/6.1	10/37 (27%)	40.8%	0.086
6137	P97541	Heat shock protein 20 (Hsp20)	17505/6.1	6/30 (20%)	35.2%	0.000
6214	P97532	3-mercaptopyruvate sulfurtransferase	32940/5.9	5/25 (20%)	19.5%	0.005
2 days after SCI						
1202	P14668	Annexin A5 (5)	35745/4.9	8/32 (25%)	24.8%	0.030
2210	P02650	Apolipoprotein E (6)	35754/5.2	6/31 (19%)	21.5%	0.001
5412	P70564	Serpin B5	42064/5.8	9/17 (47%)	31.2%	0.194
5920	157822133	Vinculin	116616/5.8	16/46 (56%)	31.9%	0.000
6009	P07632	Superoxide dismutase [Cu-Zn] (7)	15912/5.9	7/21 (33%)	43.5%	0.002
8014	Q7M0E3	Dextrin	18534/8.2	28/34 (76%)	44.8%	0.000
8318	P07943	Aldose reductase	35798/6.3	14/34 (41%)	40.8%	0.012
7 days after SCI						
1514	P10719	ATP synthase subunit beta, mitochondrial	56354/5.2	21/37 (57%)	44.8%	0.012
2204	P97697	Inositol monophosphatase-1	30512/5.2	7/26 (27%)	27.4%	0.065
5305	P42123	L-lactate dehydrogenase B chain	36613/5.7	7/25 (28%)	24.3%	0.007
6513	P11884	Aldehyde dehydrogenase, mitochondrial	56489/6.6	11/21 (52%)	26.2%	0.079
7509	Q8VIF7	Selenium binding protein	52533/6.1	16/31 (52%)	41.1%	0.011
21 days after SCI						
1202	P14668	Annexin A5 (5)	35745/4.9	8/32 (25%)	24.8%	0.009
1622	P69897	Tubulin beta-5 chain	46971/4.8	9/27 (33%)	20.5%	0.236
28 days after SCI						
1717	P06761	78 kDa glucose-regulated protein (GRP)	72348/5.1	19/36 (53%)	35.9%	0.082
2210	P02650	Apolipoprotein E (6)	35754/5.2	6/24 (25%)	25.6%	0.080
2217	P06866	Haptoglobin	38564/6.1	9/25 (36%)	21.0%	0.084
5501	Q9QX79	Fetuin B	41533/6.7	9/24 (38%)	21.7%	0.111

\*Protein names and accession numbers are listed according to the SWISS-PROT database. SSP, standard spot numbers; SCI, spinal cord injury; MW, molecular weight; pI, isoelectrical point. Arabic numerals in parentheses specified identified proteins in Fig. 2.

tween urinary bladder tissues after SCI and sham control were compared. All of these proteins were found to have a statistical significance.

At 6 hr post-injury, of the beginning stage in the healing process of urinary bladder after SCI, 78 kDa glucose regulated protein (GRP) was only identified as a decreasingly expressed protein spot (Table 1). Of 81 up-regulated and 87 down-regulated protein spots in the SCI bladder tissues at 1 day post-injury ten spots were identified including up-regulated 14-3-3 zeta/delta, and down-regulated heat shock protein 27 (Hsp27), heat shock protein 20 (Hsp20), myosin light polypeptide 6 and myosin regulatory light chain 2 (Table 1). At 2 days after injury, 15 spots

from 166 differentially expressed spots (108 increased and 58 decreased spots) in the SCI bladder were identified and revealed such as up-regulated apolipoprotein A-I and hemopexin, and down-regulated annexin A5, superoxide dismutase and aldose reductase (Table 1). At 7 days of the intermediate stage of recovery in the bladder after injury, ten proteins showed increased expressions, that is, three isoforms of 14-3-3 protein (epsilon, zeta/delta, beta/alpha) and rho GDP dissociation inhibitor (GDI), and five proteins such as aldehyde dehydrogenase and selenium binding protein were decreasingly expressed (Table 1). At 9 days after injury, three spots from 86 differentially expressed spots in the bladder post-SCI were identified as up-regulated

14-3-3 zeta/delta, myosin regulatory light chain 2 and 14-3-3 beta/alpha (Table 1). At 21 days of the late healing stage in the bladder post-SCI, two repressed proteins including annexin A5 and tubulin beta-5 chain were identified from 30 differentially expressed protein spots, whereas three increased proteins (14-3-3 protein zeta/delta, myosin regulatory light chain 2, and transgelin) and four repressed proteins (78 kDa GRP, apolipoprotein E, haptoglobin, and fetuin B) were revealed after 28 days of injury from 38 up-regulated and 15 down-regulated protein spots, respectively (Table 1).

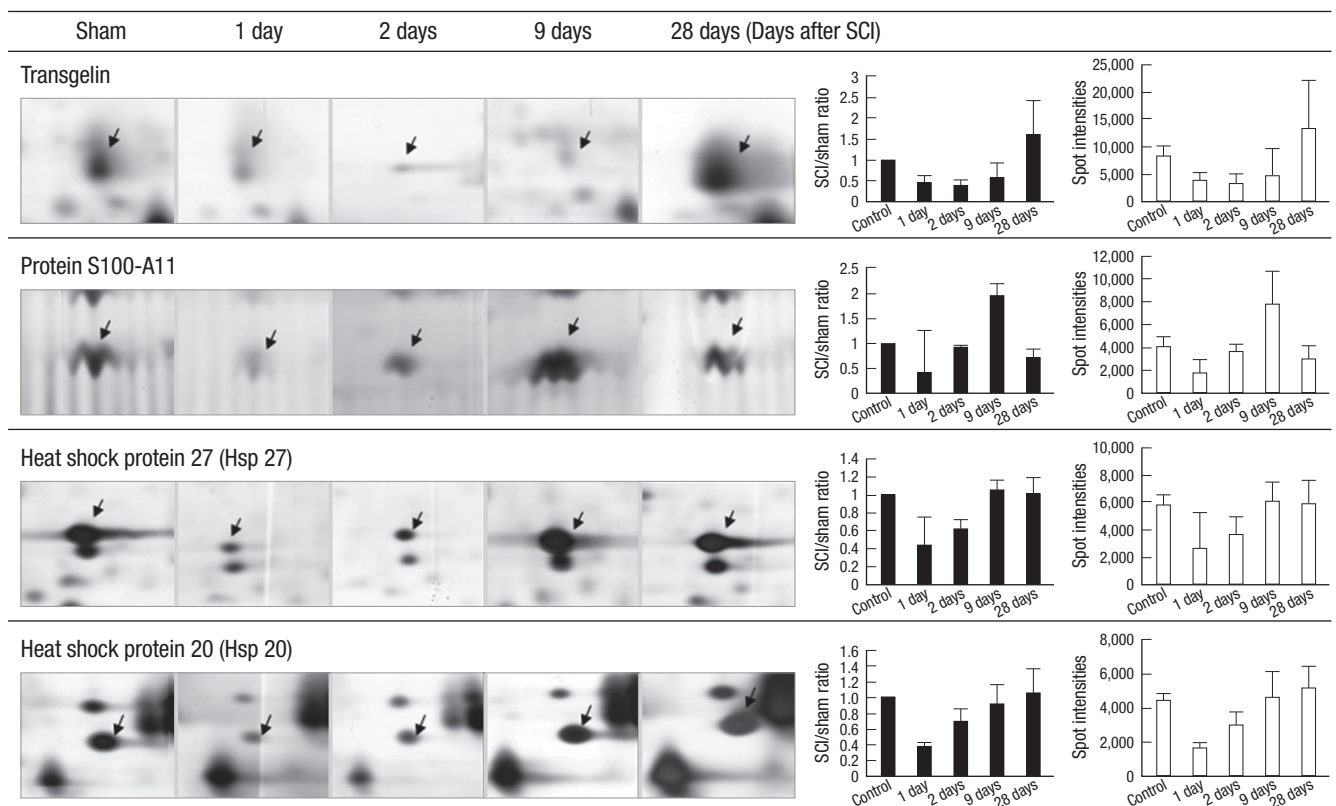
Of these identified proteins with altered expression level 14-3-3 zeta/delta, myosin regulatory light chain 2, Hsp27, Hsp20, transgelin, and protein S100-A11 were continuously expressed with some fluctuation in the course of healing process of urinary bladder after SCI. Especially, the expression of protein S100-A11 showed peak around 9 days post-injury, the mid-phase of recovery, while the level of transgelin, Hsp27, and Hsp20 increased continuously up to the late phase of recovery in the bladder post-injury (Fig. 3). These data suggest that the recovery status of urinary bladder after SCI is often associated with stress response proteins in signal transduction such as molecular chaperone, inducing metabolic and biochemical alterations (Table 1). The expression level of each protein in both injury-affected urinary bladder and sham control tissues was indicated by estimating

of the density values (Fig. 2). Their predicted values of isoelectric point (pI) and molecular weight (MW) are summarized in Table 1, and the values were compatible to those of acquired from the gels. The sequence coverage of proteins isolated from the peptide mass matching in a program was acceptable (20%-40% ranges).

Of four proteins exhibited in Fig. 3, transgelin, which is expressed below half amount of sham control at 1 day post-injury, showed continuously elevated protein expression in bladder up to 28 days after SCI. While protein S100-A11, which is also expressed below half amount of sham control at 1 day post-SCI, displayed peak expression at 9 days post-injury, which is the mid-point of recovery phase and the initial point of spontaneous voiding after SCI, but the expression was declined thereafter. Based on the 2-DE results, transgelin and S100-A11 were further confirmed their expressions in the bladder after SCI by Western blot analysis. The expression level of transgelin and S100-A11 was well agreement with the protein intensities shown in 2-DE gel (Figs. 3, 4). Beta-actin was used as a loading control for the Western blot.

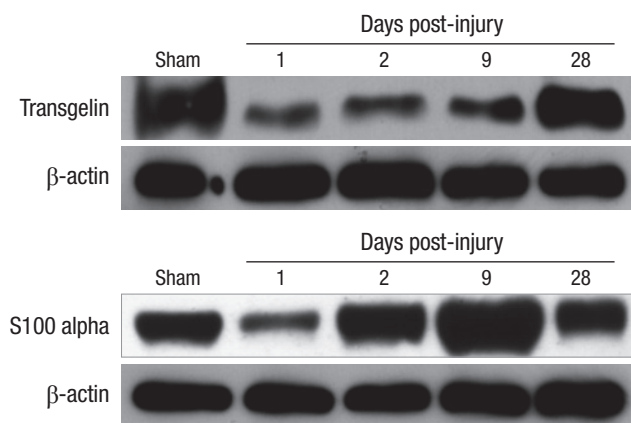
#### Functional annotation of the proteins expressed in urinary bladder post-injury

The 54 proteins identified from the bladder tissue after SCI were



**Fig. 3.** Expression profiles of selected protein spots in the bladder post-injury at different time points. Representative spots specified at each stage (spot intensity from PDQuest software), identified as transgelin, protein S100-A11, Hsp27, and Hsp20 are shown in the healing course of rat bladder after contusion spinal cord injury. The bar graphs exhibited the expression level of each protein at indicated time points compared with the sham control. Data are presented mean  $\pm$  SD ( $n = 5$  per group),  $P < 0.05$  (ANOVA).

annotated to achieve biological and functional information of the changed proteins. Fig. 5 shows the alteration of protein expressions in a certain category during recovery of the bladder tissue after SCI (A, 1 day post-SCI; B, 2 days post-SCI; C, 9 days

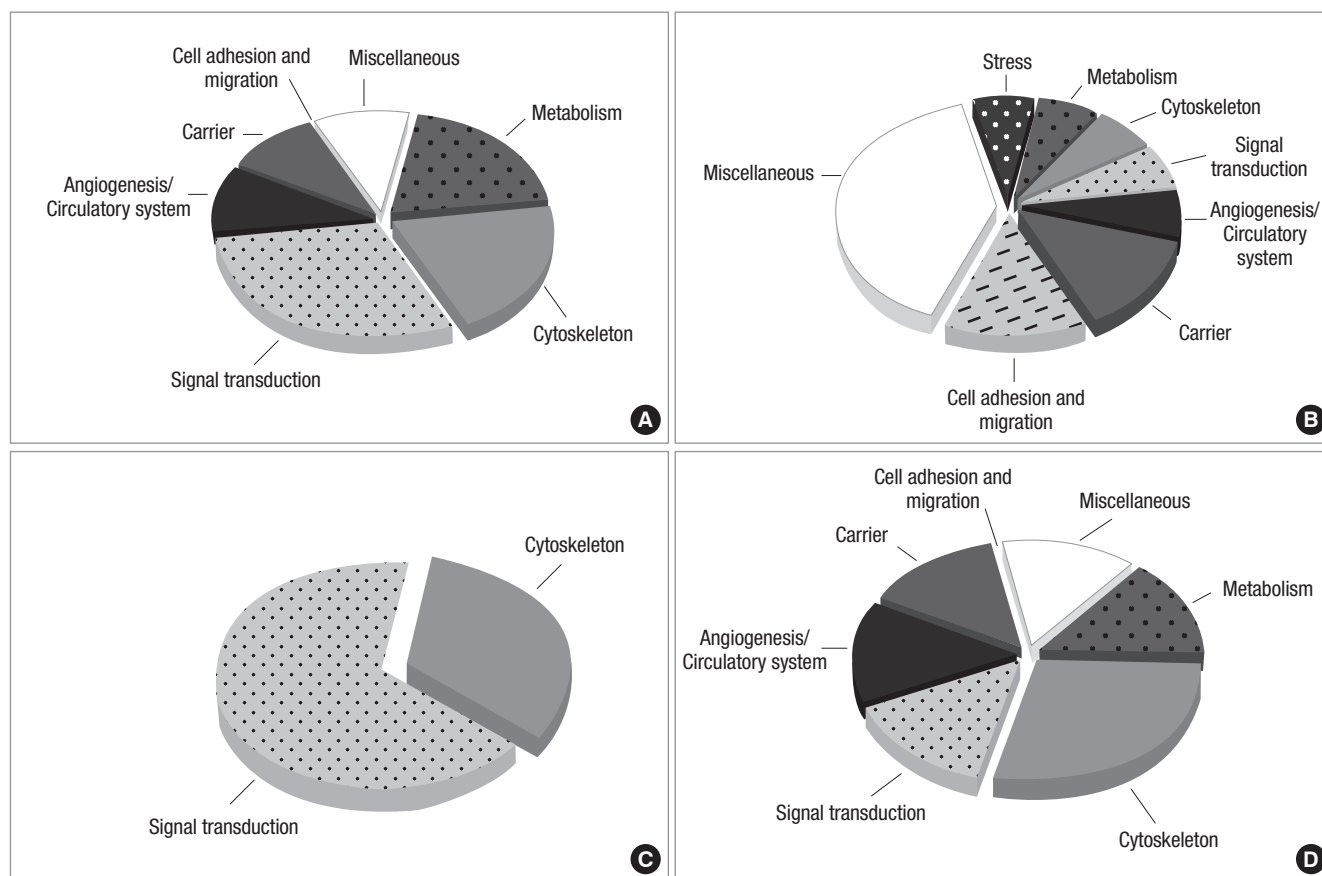


**Fig. 4.** Validation of differentially expressed proteins in the rat bladder after contusion injury by Western blot analysis. Selected proteins in rat bladder tissues after SCI and sham control were analyzed and confirmed by Western blot at each time points. The expression levels of transgelin and S100-A11 in the protein blot are well agreement with quantitative 2-DE results (Fig. 3), respectively.  $\beta$ -actin was used as a loading control.

post-SCI; D, 28 days post-SCI). At 2 days post-SCI, the proteins for signal transduction, cytoskeleton and metabolism were quite decreased with more than three-fold, but cell adhesion and migration related proteins were considerably increased compared to the bladder tissues at 1 day post-injury. However, the protein expression patterns at 9 days post-injury, the mid-point of recovery in the bladder post-SCI, turned to comprise most altered proteins involved in signal transduction and cytoskeleton as seen in Fig. 5C. The increased proteins for the active recovery at 9 days post-SCI seemed to significantly decreased thereafter, and proteins related with angiogenesis/circulatory system, carrier, and metabolism were fairly returned and positioned in the rest of protein category at 28 days post-injury (Fig. 5D).

## DISCUSSION

Spinal cord injury relevant to thoracic region from contusion to complete transection initially affects the lower urinary tract function (12). The loss of normal descending control inhibits normal micturition, requiring manual bladder evacuation. The bladder responds by enlarging, and in transection models this is known to initiate the afferent plasticity and subsequent inter-



**Fig. 5.** Functional manifestations of differentially expressed proteins in rat bladder tissues after SCI. The expression profiles and functions of the proteins were categorized into eight different subgroups. Values exhibited the number of identified proteins in each category. (A) to (D) shows total proteins expressed at indicated time points: (A) 1 day post-injury, (B) 2 days post-injury, (C) 9 days post-injury, and (D) 28 days post-injury.



neuronal alterations resulting in reflex bladder development (13, 14). The same mechanisms occurred after incomplete contusion injury in the first week after SCI (15). The bladder pathologies after SCI comprised not only changes in mechanical properties but also changes in tissue morphology such as hypertrophy and fibrosis (16). In addition, functional deficiencies including bladder areflexia, hyperreflexia, and detrusor-sphincter dys-synergia could be induced in the SCI bladder. However, the molecular mechanisms underlying the observed changes in the bladder following SCI have not yet been elucidated.

In the present study, differentially expressed proteins in the rat bladder after SCI compared with normal bladder were identified at different time points from 6 hr to 28 days post-injury. For this purpose, proteomic analysis using 2-DE, MALDI-TOF/mass spectrometry and peptide mass fingerprinting were utilized.

The protein expression in the SCI-affected urinary bladder showed different profile according to the recovery phase after injury. In the early acute phase (6 hr, 1, and 2 days post-injury), 2-DE gel images of injury-affected bladder tissue exhibited significant loss of protein spots compared to sham control except those of 6 hr (Fig. 2A to D). At this stage, cytoskeletal proteins such as myosin light polypeptide 6, myosin regulatory light chain 2, vinculin, and destrin were remarkably decreased, suggesting SCI as a significant stress to smooth muscle function of urinary bladder. The expression level of Hsp20 and Hsp27 involved in the signal transduction and molecular chaperone machinery was also decreased at 1 day post-injury but gradually recovered their own expression same as sham control up to 28 days after SCI (Fig. 3). On the contrary, the expression of two types of serine protease inhibitor (A3K and A3L) and apolipoprotein (A-I and E) was up-regulated at this stage.

The characteristics of the intermediate phase of recovery (7 and 9 days post-injury) were manifested as up-regulated cytoskeletal proteins and adaptor signaling molecules, and down-regulated metabolic enzymes. Protein S100-A11 (calcium binding protein), tropomyosin alpha-3 chain playing roles in actin filament binding and regulation of smooth muscle contraction, and myosin regulatory light chain 2 (smooth muscle isoform) involved in the cytoskeletal system are increasingly expressed, implicating of the initiation of spontaneous voiding at this mid-stage (Table 1). One of the adaptor molecules, 14-3-3 protein family, has seven isoforms in mammalian. The mammalian 14-3-3 proteins are involved in many intracellular processes including cell cycle regulation, metabolism control, apoptosis, and control of gene transcription (17). Pull-down assays of the 14-3-3 proteins demonstrated the association with phosphopeptide analogs of Hsp20. The binding of 14-3-3 protein to phosphopeptide analogs of Hsp20 prevented the association of cofilin with 14-3-3, suggesting Hsp20 as a modulator of actin cytoskeletal dynamics by competing with the actin depolymerizing protein cofilin for binding to the scaffolding protein 14-3-3. Especially,

this association could be modulated by phosphopeptide motifs only within Hsp20 molecule. These data also suggest the possibility that cyclic nucleotide dependent relaxation of smooth muscle might be mediated by a thin filament (actin) regulatory process (18). Three isoforms of 14-3-3 zeta/delta, epsilon, and beta/alpha was continuously increased in expression at mid-stage of the recovery (Table 1) and possible association of each 14-3-3 molecule with Hsp20 should be studied for the future project. Metabolic enzymes at this stage are generally down-regulated, suggesting the priority of the recovery of the smooth muscle contractility in the affected bladder due to SCI.

In the late phase of recovery (21 and 28 days) post-SCI, the protein spots found in unaffected sham control tissues were considerably recovered (A, G and H in Fig. 2). The iron saving molecules such as haptoglobin was no more urgently needed for the late stage of recovery. However, the expression of cytoskeletal proteins such as myosin regulatory light chain 2 and transgelin was considerably increased, and 14-3-3 zeta/delta protein was up-regulated (Table 1). Throughout recovery stages after SCI, four proteins such as transgelin, Hsp27, Hsp20, and protein S100-A11 were continuously expressed as shown in Fig. 3. Three proteins (transgelin, Hsp27 and Hsp20) exhibit similar expression patterns; marked decrease at day 1 after injury and gradual regaining of the protein amounts until late healing stages. On the other hands, protein S100-A11 decreased at 1 day post-injury and markedly increased protein expression at mid-point of recovery phase after injury. We discuss these four proteins for their biochemical and molecular properties and functional implications as below.

Transgelin, also called smooth muscle (SM)-specific SM22 protein, is 22 kDa cytoskeleton related protein. It binds to and co-localizes with F-actin, indicating that it may be involved in cell differentiation by stabilizing the cytoskeleton through actin binding (19, 20). SM22 $\alpha$  plays a functional role in inducing F-actin bundling and forming strong stress fibers, which are essential structures for cell contraction (21). As shown in Figs. 3, 4, the level of the transgelin expression was diminished 1 day post-SCI compared to sham control, suggesting the close relationship of transgelin with the bladder smooth muscle contraction. This finding suggests decreased contractility of detrusor muscle due to SCI. Proteomic investigation on chronic irritation of the rat bladder such as interstitial cystitis revealed remarkable up-regulation of four nuclear proteins containing inflammation-associated calgranulin A and marked down-regulation of one nuclear protein (contractility-associated transgelin) compared with normal rat bladders.

Western blot analysis exhibited that time-course expression of transgelin showed maximal decrease of expression at 4 days after brief (90 sec) intravesical HCl irritation and returned to normal expression at 28 days after the treatment (22). The decreased expression of transgelin and restoring to control level at



4 weeks post-injury are validated not only in chronic bladder irritation in rat by HCl but also in the rat bladder after contusion SCI. Down-regulated transgelin expression was also observed by proteomic investigation after partial bladder outlet obstruction (23). On the other hands, an interesting study about the effects of SCI on alterations in gene expression and respective protein products using human skeletal muscle was reported 2 days and 5 days post-SCI. These data suggest that the primary signaling networks activated in the days post-SCI are those involved in protein degradation (ubiquitin proteasome pathway), and those which may function to protect skeletal muscle from rapid degradation (metallothioneins) (16). Hence, it is suggested that there can be some different protein expression patterns in the recovery after SCI between skeletal muscle and smooth muscle.

S100 proteins belong to the EF-hand calcium-binding protein family, which are expressed in cell- and tissue-specific manners, regulate a variety of cellular processes via interaction with different target proteins (24). S100-A11 (previously named S100C or calgizzarin) is a novel and relatively unknown member of the large family of S100 proteins, and exhibits several unique properties. S100-A11 was found to regulate the cytoskeletal function via calcium-dependent interaction with annexin I (25). S100-A11 expression was significantly increased in leiomyoma, human uterine smooth muscle tumors (26). Suppression of S100-A11 by small interfering RNA (siRNA) led to apoptosis, and the overexpression of S100-A11 inhibited apoptosis in human uterine smooth muscle tumor cells, suggesting S100-A11 has an anti-apoptotic function. Investigation of the molecules participating in the metabolism of calcium/calmodulin may provide new insights into the pathophysiology of various smooth muscle diseases as well as cardiac and skeletal muscle diseases (26). S100-A11 protein expressed in the bladder tissue after SCI is shown with peak increase in the mid-point of recovery phase post-injury and decreases thereafter, suggesting that it can be associated with signal transduction of calcium/calmodulin relevant muscle contraction and inhibition of apoptosis in the initiation stage of spontaneous voiding in the smooth muscle of bladder tissue after SCI (Figs. 3, 4).

In the past few years, many proteins have been found to be involved in the injured spinal cord tissue (3, 5). Of these proteins, Hsp27 belong to the small heat shock protein family is differentially expressed both in spinal cord tissue itself and SCI-affected bladder tissue (Fig. 3). Exercise-induced Hsp27 mRNAs expression in the soleus muscle is dependent on time after SCI in rats (27). The expression level of Hsp27 in the spinal cord after SCI varied with the injury type and duration of recovery (5, 16).

Another small heat shock protein, Hsp20, is expressed in nearly all mammalian tissues. Hsp20 expression in smooth muscles is variable. Low levels of Hsp20 expression in the normal bladder are converted to substantial expression when the bladder is obstructed (28). Low or no expression of Hsp20 is associated with

diminished cyclic nucleotide-induced relaxation, suggesting phosphorylation of Hsp20 can be a dominant mechanism of smooth muscle relaxation (29). The expression of Hsp20 in smooth muscle of the affected bladder after SCI exhibited similar pattern shown in Hsp27 (Fig. 3).

In conclusion, this study displays a proteomic profiling of the affected bladder after contusion SCI at seven time points from 6 hr to 28 days. During entire recovery phase post-injury, 54 proteins related with SCI-affected bladder tissue are identified. Of four proteins which are continuously expressed with different patterns, two proteins including transgelin and S100-A11 are confirmed their expression level by Western blot analysis. The next goal for the future study will be focused on Hsp20 and 14-3-3, two intriguing proteins identified by this proteome research, elucidating biochemical and pathophysiological relationship between two molecules in the affected urinary bladder after SCI.

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## AUTHOR SUMMARY

### Spinal Cord Injury Markedly Altered Protein Expression Patterns in the Affected Rat Urinary Bladder during Healing Stages

Ji-Young Lee, Bong Jo Kim, Gyujin Sim, Gyu-Tae Kim, Dawon Kang, Jae Hun Jung, Jeong Seok Hwa, Yeon Ju Kwak, Yeon Jin Choi, Young Sook Park, Jaehee Han, Cheol Soon Lee and Kee Ryeon Kang

We investigated the influence of spinal cord injury (SCI) on the protein expressions in rat urinary bladder using proteomic analysis. The bladder tissues were processed for 2-DE and MALDI-TOF/MS from 6 hr to 28 days after the contusion SCI between T9 and T10 SCI. 54 proteins were found to show altered expression levels, and among them, transgelin, S100-A11, Hsp27, and Hsp20 were continuously expressed with variation in entire recovery period post-injury. The changes in transgelin and S100-A11 were confirmed by Western blot analysis. The results suggest that transgelin and protein S100-A11 might be involved in the healing process of bladder after SCI.