Regulation of connexin 43 by basic fibroblast growth factor in the bladder: transcriptional and behavioral implications

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Running Head: Bladder bFGF-Cx43 axis in transcription and behavior

Key words: connexin 43, bFGF, bladder, AP-1, micturition behavior

Abbreviations:

BOO: bladder outlet obstruction

Cx43: connexin 43

Cx45: connexin 45

bFGF: basic fibroblast growth factor

ZT: zeitgeber time

POD: post-operative day

DMEM: Dulbecco's modified Eagle's medium

FCS: fetal calf serum

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

ERK: extracellular signal regulated kinase

pERK: phospho-extracellular signal regulated kinase

Fwd: Forward

Rev: Reverse

RT-PCR: reverse transcription-polymerase chain reaction

BSMC: bladder smooth muscle cells

TSS: transcription start site

ABSTRACT

Purpose: Basic fibroblast growth factor (bFGF) is a candidate causative factor of detrusor overactivity in bladder outlet obstruction (BOO), through up-regulation of connexin 43 (Cx43), a gap-junction protein. This study addresses the transcriptional and behavioral implications of this axis.

Materials and Methods: *Cx43* and *Cx45* mRNA expression was assessed by real-time RT-PCR in the bladder of a rat BOO model and in cultured rat bladder smooth muscle cells (BSMC) with and without bFGF treatment. The involvement of the ERK1/2-AP-1 pathway was evaluated by immunofluorescent study and a promoter-reporter assay in BSMC. The effect of bFGF on micturition behavior was measured in unrestrained rats under a 12 h light/dark cycle, using a controlled release system from gelatin hydrogels fixed on the bladders. The expression of ERK1/2 and Cx43 protein was assessed by western blotting of rat bladder protein.

Results: *Cx43*, but not *Cx45*, mRNA expression was increased in the bladder of the BOO model and in BSMC treated with bFGF. PD98059, a MEK inhibitor, blocked the stimulatory effect of bFGF on Cx43 protein expression and promoter activity, which was also decreased by mutation or deletion of an AP-1 cis-element of the Cx43 promoter. *In vivo* application of bFGF on the bladder increased the urinary frequency during the latter half of the dark phase, i.e., the late active phase of rats ($F=5.1$; $P<0.05$, by two-way ANOVA). The expression of pERK1/2 and Cx43 protein was elevated in the bladder.

Conclusions: The ERK1/2-AP-1-Cx43 axis could be a potential therapeutic target for increased urinary frequency.

INTRODUCTION

Overactivity of the obstructed bladder is a highly prevalent and recognized clinical condition.[1] In an experimental rat model, Saito et al reported that unrestrained rats with BOO under conditions of a 12 h light/12 h dark cycle, showed increased urinary frequency in the dark and active phase.[2] However, the mechanism underlying this phenomenon has not yet been elucidated clinically or experimentally.

Cx43 and Cx45 are major gap junction proteins in the bladder. Up-regulation of Cx43 has been implicated in detrusor overactivity in rats with BOO, in association with increased intercellular transmission of electrical and chemical excitation through gap junctions.[3-5] Bladders retrieved from BOO rats show hypersensitivity on a cystometrogram, and this is blocked by a gap junction inhibitor.[6]

Basic FGF is a multifunctional growth factor, known to be elevated in urine under various clinical pathological conditions of the bladder, including voiding dysfunction.[7,8] We have previously shown that bFGF is produced by the urothelium of the obstructed rat bladder and that Cx43 protein expression is up-regulated via ERK1/2 activation in BSMC. The elevation of Cx43 causes bladder overactivity in muscle strip tests, corresponding to increased micturition frequency in a cystometrogram under anesthesia.[5] However, in this previous study, the precise mechanism of Cx43 protein up-regulation by bFGF was not fully investigated. In addition, the behavioral implications of these phenomena are unknown.

Transcriptional control is one of the most important steps in the regulation of Cx43 protein, although many steps are involved in the pathway from DNA to protein in its tissue dependent expression.[9] Several factors are reported to regulate Cx43

promoter activity, such as transcriptional factors Sp1 and AP-1, signaling molecules such as cyclic AMP and retinoids, and signal transduction pathways such as Wnt and the Ras-Raf-MAPK pathway.[9-13]

Since AP-1 is known to be a major target of stretch-induced gene expression in BSMC, and is activated by pERK1/2,[14,15] and the AP-1 cis-element is regulated by bFGF,[16] we hypothesized that the bFGF-ERK pathway regulates the expression of Cx43 in the obstructed bladder via AP-1. *In vitro*, the transcriptional regulation of Cx43 promoter activity by bFGF was studied in cultured BSMC. *In vivo*, we investigated the impact of BOO on *Cx43* and *Cx45* mRNA expression. We also investigated the effect of bFGF on micturition behavior using our original system, in which bFGF incorporated in gelatin hydrogels is control-released by gel degradation over 1 week. We applied this system, which was originally used to accelerate tissue regeneration in various organs,[17-20] to analyze the micturition behavior of unrestrained rats.[5]

MATERIALS AND METHODS

Cell culture

BSMC were isolated from 9-week-old female Sprague-Dawley rats (Japan SLC Inc, Shizuoka, Japan) using a procedure described previously.[17,21] Cells after two passages were seeded at 6×10^4 cells/2 ml in 6-well plates for RNA extraction and 1×10^4 cells/100 μl in 96-well plates for the promoter-reporter assay.

Animals

Seven-week-old female Sprague-Dawley rats weighing 170 to 190 g were purchased from Japan SLC Inc. Animals were treated in accordance with NIH animal care guidelines and all animal experiments were approved by the Kyoto University Animal Experiment Committee.

Rat bladder outlet obstruction model

Partial BOO was created in nine rats using methods previously described.[5,17] Briefly, a longitudinally incised 2 mm-long PE200 polyethylene catheter (BD Intramedic, Sparks, MD) was placed around the proximal urethra. Three sham-operated rats underwent similar procedures, but the catheter was removed before closing the abdominal incision. After 5, 7, and 14 days in the BOO group (n=3 for each day) and 14 days in the sham group, bladders were removed and weighed. All

samples were immediately placed in RNA*later* solution (Ambion, Austin, TX).

Behavioral effect of bFGF on rat bladders using gelatin hydrogels as release carriers

A gelatin hydrogel, containing human recombinant bFGF (Kaken Pharmaceutical Co. Tokyo, Japan) (0 or 10 μg/site), was fixed to the bladder of unrestrained rats (n=8 for each group) as previously described.[5,17] The rats were maintained under the conditions of a 12 h light/12 h dark cycle (8 am light on [ZT=0] and 8 pm light off [ZT=12]) throughout the experiment. This included a 7-day adaptation period before the operation with free access to water and food. In six animals in each group, urinary frequency was measured for 11 days under the same conditions upon an electronic balance system as described previously.[22] Two rats in each group were sacrificed on POD 7 for western blotting.

Immunoblotting

Whole cell lysates from bladder tissue were lysed with radio-immunoprecipitation assay buffer containing protease inhibitors. The protein content of the cell lysates was measured using the BioRad Protein Assay Kit. Cell lysates were resolved by sodium dodecylsulfate polyacrylamide electrophoresis and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were incubated with antibodies against pERK1/2 (1:1000, CST, Beverly MA), ERK1/2 (1:1000, CST), Cx43 (1:200, Zymed, San Francisco, CA) and GAPDH (1:2000, CST) as an

internal control. After incubation with HRP-conjugated anti-rabbit secondary antibodies (Pierce, Rockford, IL), immunoreactive proteins were visualized using a Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Each band was quantified by densitometry using Image J 1.42q software (NIH, Bethesda, MD).

RNA extraction and real-time RT-PCR

BSMC were treated with bFGF 10 μg/ml for 36 h following serum depletion in DMEM containing 0.5% FCS for 48 h. Total RNA was extracted from BSMC and the bladders of the BOO model, and mRNA expression of *Cx43* and *Cx45* was evaluated by real-time RT-PCR (n=4). Total RNA extraction and cDNA synthesis were carried out using the RNeasy Mini Kit and SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) as described previously.[17] The primers used were as follows: rat Cx43 Fwd: CCATCCAAAGACTGCGGAT and Rev: GTAATTGCGGCACGAGGAA; Cx45 Fwd: CAGAGCCAACCAAAACCCA and Rev: GAAAGCCCACCTCAAACACA; and β-actin Fwd: GAAGCTGTGCTATGTTGCCCT and Rev: TTCTGCATCCTGTCAGCAATG. These primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in 25-μl reactions containing SYBR Green PCR Master Mix using the 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixtures were denatured at 95°C for 10 min, followed by 40 PCR cycles. Each cycle consisted of the following three steps: 94°C for 15 s, 57°C for 15 s, and 72°C for 1 min. Each sample was

normalized against an internal 18s ribosomal RNA control[19] in BSMC and β-actin was used in the BOO model.

Immunofluorescent study

BSMC were treated with bFGF 10 μg/ml with and without PD98059 (Calbiochem, San Diego, CA), the MEK inhibitor, 5 μM for 48 h following serum depletion in DMEM containing 0.5% FCS for 48 h. Cells were washed twice and fixed with 4% paraformaldehyde, followed by heating in 0.01 M citrate buffer (pH 6.0) for 20 min for antigen retrieval. Samples were incubated with antibody against Cx43 (1:100) in Canget Signal solution A (Toyobo, Osaka, Japan) for 12 h at 4°C. The binding was detected using biotinylated anti-rabbit secondary antibody and Alexa Fluor 555-conjugated streptavidin, and samples were counterstained with 4, 6-diamidino-2-phenylidole.

Promoter-reporter assay

The mouse pGL-2-Cx43 promoter-reporter (pCx43 -1686/+165-Luc), constructed by Dr. Lye SJ,[23] was a kind gift from Dr. Jian Yao (University of Yamanashi, Yamanashi, Japan), and the appropriate negative and control constructs, pGL-2 basic and pRL-TK respectively, were purchased from Promega (Madison, WI). Site directed mutagenesis deletion and truncation of the AP-1 site of pCx43 were performed using a mutagenesis basal kit (Takara Bio, Shiga, Japan) according to the protocol. Promoter constructs were transfected into rat BSMC as follows. BSMC were transfected with 240 ng pCx43-Luc and 1 ng pTK-RL using Fugene6 (Roche,

Indianapolis, IN) in DMEM-10% FCS for 24 h. Cells were treated with bFGF (0, 10, and 50 ng/ml; n=3 for each concentration) in DMEM-0.5% FCS for 24 h. To further investigate the effect of factors mediating effects downstream of bFGF, cells were treated with 50 ng/ml bFGF with or without 5 μM PD98059 (n=6). Lysates were harvested 48 h post-transfection and luciferase activity was measured using the dual luciferase assay reagent (Promega).

Statistical analysis

Data were analyzed with one-way ANOVA in multiple comparisons and Mann-Whitney U-test for the relative mRNA expression and luciferase assay using SPSS ver.11.0.1J software (SPSS inc., Chicago, IL). For comparing the urinary frequency with and without bFGF, t-test and two-way repeated measures ANOVA were used. A P value < 0.05 was accepted as significant.

RESULTS

Cx43 mRNA expression is increased in the BOO model and in BSMC treated with bFGF

In the bladder of the BOO model, *Cx43* mRNA expression was increased after the operation compared with sham-operated controls, in parallel with an increase in bladder weight (Figs. 1A, B). There was no increase in *Cx45* mRNA expression. In cultured BSMC, *Cx43* mRNA expression was also significantly increased with stimulation of bFGF for 36 h (Fig. 1C), with no increase in expression of *Cx45*.

Basic FGF up-regulates Cx43 transcription via the ERK1/2-AP-1 pathway

Immunofluorescence showed increased Cx43 protein expression in BSMC with bFGF treatment, and this was blocked by the ERK1/2 inhibitor, PD98059 (Fig. 2A), replicating our previous results by immunoblots.[5] The influence of transcriptional regulation of Cx43 protein expression by the bFGF-ERK1/2 pathway was assessed by a promoter-reporter assay using BSMC. Cx43 promoter activity was significantly up-regulated upon stimulation of bFGF (50 ng/ml) (Fig. 2B), and this was blocked by PD98059 (Fig. 2C).

We then focused on two putative AP-1 sites ‘TGAGTCA’ and ‘TGACTCA’ located at 43 and 995 base pairs, respectively, upstream from TSS of the Cx43 promoter, which are evolutionally conserved among humans, rats and mice.[10,11] The distal site was considered unrelated to the effect of bFGF, as truncation mutants of

this site were still responsive to bFGF stimulation (data not shown). Mutation and deletion of the other proximal AP-1 site (Fig. 2D) blocked the effect of bFGF on Cx43 promoter activity (Fig. 2E).

Basic FGF induces urinary frequency in the late active phase of unrestrained rats

Unrestrained rats treated with 10 μg/site bFGF in the bladder showed significantly increased urinary frequency in the latter half of the dark phase, i.e., the late active phase of rats, compared with rats without bFGF stimulation on POD 8 (17.7 ± 2.5 vs.10.8 ± 1.5; $P<0.05$, t-test; Figs. 3A, B). In diachronic analysis, rats with bFGF stimulation urinated more frequently than those without bFGF only in this late active phase ($P<0.05$, by two-way repeated measures ANOVA; Figs. 3C, D). Western blotting showed that the expression of pERK1/2 and Cx43, especially the phosphorylated forms, was increased in bladder tissue with bFGF stimulation at ZT 12 and 24 (Fig. 4). There were no significant differences in micturition volume and total urination volume (data not shown).

DISCUSSION

The present study demonstrates that bFGF up-regulates *Cx43* transcription in BSMC via the ERK1/2-AP-1 pathway, and that this mechanism may underlie Cx43 overexpression in the obstructed bladder. Such phenomena might be associated with increased urinary frequency during the active phase in rats with BOO.

A major finding in this study is the differential transcriptional regulation of Cx43 and Cx45 by bFGF. Basic FGF is a candidate signaling molecule for pathological conditions of the bladder, as it has been reported to be elevated in the urine from patients with voiding dysfunction.[7,8] In the rat BOO model, although it can be difficult to detect bFGF in urine,[24] we have reported its up-regulation in the urothelium.[5,17] In cultured BSMC, bFGF up-regulated Cx43, but not Cx45, which is the other major gap junction protein in the bladder, replicating our findings in an *in vivo* release model.[5] This finding is particularly interesting because similar differential modulation of gap junction proteins was also observed in the bladder of the BOO model. If the hypersensitivity in the BOO model is related to increased gap junction function by bFGF, our results suggest that this is more likely to be associated with Cx43 than with Cx45.

Another major finding was the identification of the ERK-AP-1 pathway for transcriptional regulation of *Cx43* mRNA by bFGF. As reported in our previous study, an increase of Cx43 protein by bFGF was blocked by PD98059, the ERK inhibitor. Since this ERK-dependency was also observed in promoter activity, and AP-1 is a known downstream transcription factor of ERK, we investigated the involvement of two putative evolutionally conserved AP-1 sites in the Cx43

promoter. A promoter-reporter assay using deleted, mutated, or truncated constructs suggested that the proximal AP-1 site could be involved in the response to bFGF. Although the proximal AP-1 site in the Cx43 promoter has been implicated in transcriptional activity,[9-11] no upstream signaling has been reported thus far, and our findings illustrate the physiological role of AP-1 for Cx43 regulation.

Interestingly we found that bFGF, by itself, could induce altered micturition behavior typically observed in the BOO model. Basic FGF increased daytime urinary frequency in unrestrained rats, as reported previously in the BOO model.[2] Our bFGF control-release model has several common features with the short-term BOO model, representing the compensated phase of BOO, in which up-regulation of bFGF has been reported.[5,17,25] In these control-release and short-term BOO models, a proliferation of SMC, a decrease in the collagen I/III ratio, and an increase in Cx43 protein expression have been reported.[5,17,25] Our results could extend such biochemical similarity to behavioral aspect.

One unexpected finding in our study was the unaffected urinary frequency during the sleeping phase, because clinically, nocturia has been recognized as a sign and a major problem related to BOO, typically seen in the elderly with prostate hypertrophy, and it is included in the International Prostate Symptom Score. Despite elevated pERK and Cx43 levels at ZT 12 and 24, the urinary frequency of the rats remained undisturbed during the sleeping phase. Although urinary frequency is one of the major symptoms in BOO,[1] the distinction between daytime and nighttime frequency (i.e., nocturia) has been recently emphasized.[2,26,27] While there is an association of nocturia with benign prostatic hyperplasia, recent epidemiological studies suggest that the etiology of nocturia is not always related to BOO, and is far

more diverse and multi-factorial, including the sleep-awake cycle and urine production by the kidneys.[27] Therefore, unaffected micturition frequency during the sleeping phase observed in the bFGF control-release model and the BOO model,[2] may suggest that the early phase of BOO does not affect nighttime urinary frequency, and this may not be contradictory with clinical observations in patients with voiding problems.

We observed an increased expression of Cx43 protein and its phosphorylated form, which has been associated with stabilized gap junction function,[5] on POD 7. This corresponded to elevated pERK1/2 levels, which could be interpreted as the accumulative effect of bFGF that continues until around POD 7.[18] At approximately POD 7, urinary frequency of the rats reaches a peak, while the decrease in frequency by POD 11 could be associated with the loss of bFGF release from the gelatin hydrogel;[18] this suggests that the effect of bFGF on the bladder is reversible. This reversible alteration in urinary frequency is a major difference between our model and the BOO model, which is associated with complex mechanical over-distention and ischemic change.[28] In the long-term BOO model, defined as a decompensated state associated with chronic ischemic change, bFGF is not elevated anymore,[25] and Cx43 has an altered cellular localization.[28,29] On the other hand, relief from BOO does not return urinary frequency on a cystometrogram to normal levels after 28 days from relief,[29] although *Cx43* mRNA expression returns to normal levels by this time. Such complex features of the BOO model have made it difficult for researchers to analyze its pathogenesis mechanistically. Therefore, our control-release system is a valuable model that enables a more accurate determination of the *in vivo* effect of bFGF on Cx43 expression.

Finally, although our study demonstrates an effect of bFGF on Cx43 regulation, there may be other factors associated with the pathological state of bladder dysfunction regulating the expression of Cx43,[30] because bFGF could not be elevated in every patients with such diseases.[8] Our results indicate that various other AP-1 regulating factors could be possible candidates for Cx43 up-regulation, and such factors could be candidate targets for innovative therapies against bladder diseases in further studies in the future.

CONCLUSIONS

The present findings may explain the relationship between the elevation of bFGF in the obstructed bladder and the associated lower urinary tract symptoms. They also suggest that the ERK1/2-AP-1-Cx43 axis could be a potential therapeutic target for medical treatment of bladder overactivity.

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LEGENDS

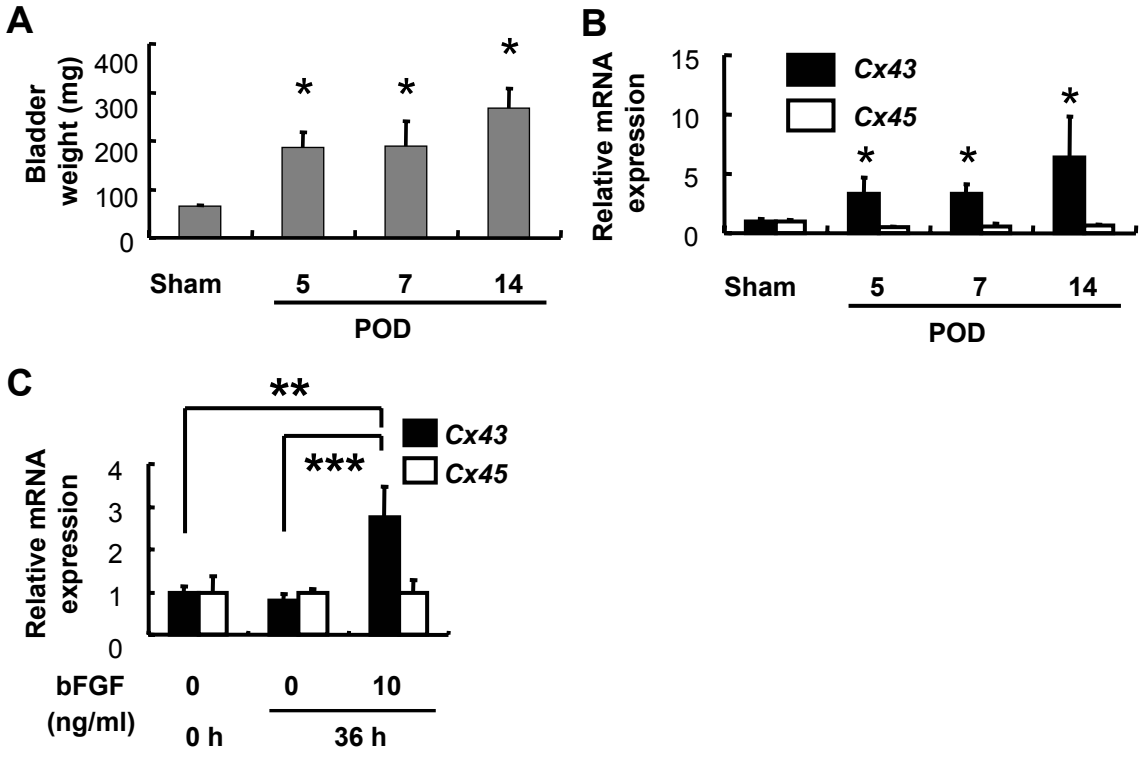
Fig. 1 *Cx43* mRNA expression is increased in the BOO model and in BSMC treated with bFGF, but the expression of *Cx45* is not. **A**: Bladder weights of sham-operated rats on POD 14 and BOO model rats retrieved at 5, 7, and 14 days postoperatively (n=3 for each point). **B**: Relative mRNA expressions of *Cx43* and *Cx45* in the same animals. For relative expression, each value for shams was set as 1. **P*<0.05 compared with shams. **C**: Relative mRNA expressions of *Cx43* and *Cx45* in rat BSMC treated with 10 ng/ml of bFGF for 36 h following 48 h of serum depletion (n=4 for each point, ****P*<0.001, ***P*<0.005 compared with controls by ANOVA). Each value at 0 h was set as 1. Error bars indicate s.d.

Fig. 2. Basic FGF stimulates Cx43 promoter activity through the ERK1/2-AP-1 pathway in rat BSMC. **A**: Immunofluorescence shows that inhibition of ERK1/2 by PD98059 blocks up-regulation of Cx43 protein by bFGF. **B**: Promoter-reporter assay shows dose dependent activation of the Cx43 promoter. **C**: PD98059 blocks Cx43 promoter activation by bFGF. **D**: The conserved putative AP-1 site of the Cx43 promoter. **E**: Alterations of the AP-1 site block Cx43 promoter activation with bFGF. The value of WT without bFGF was set as 1. **P*<0.05, ***P*<0.01 and ****P*<0.005 by ANOVA. Error bars indicate s.d.

Fig. 3. Basic FGF increases urinary frequency during the late active phase in unrestrained rats. **A**: Daily micturition pattern of unrestrained rats (n=12) before the operation. **B**: A significant difference in the urinary frequency can be noted between

rats with and those without control-released bFGF during the latter half of the dark phase, i.e., the active phase of rats, on POD 8. **C**: Urinary frequency in the latter half of the light phase, i.e., the sleeping phase of rats, is not affected by bFGF throughout the study period. **D**: Controlled release of bFGF significantly increased urinary frequency during the late active phase. Note that this difference diminishes by POD 11. †$P<0.05$ by two-way ANOVA. Error bars indicate s.e.m.

Fig. 4. Basic FGF up-regulates pERK1/2 and Cx43 protein expression. Expression of pERK and Cx43 in the bladder at ZT 12 and 24 on POD 7 was assessed by western blotting. P, phosphorylated; NP, non-phosphorylated.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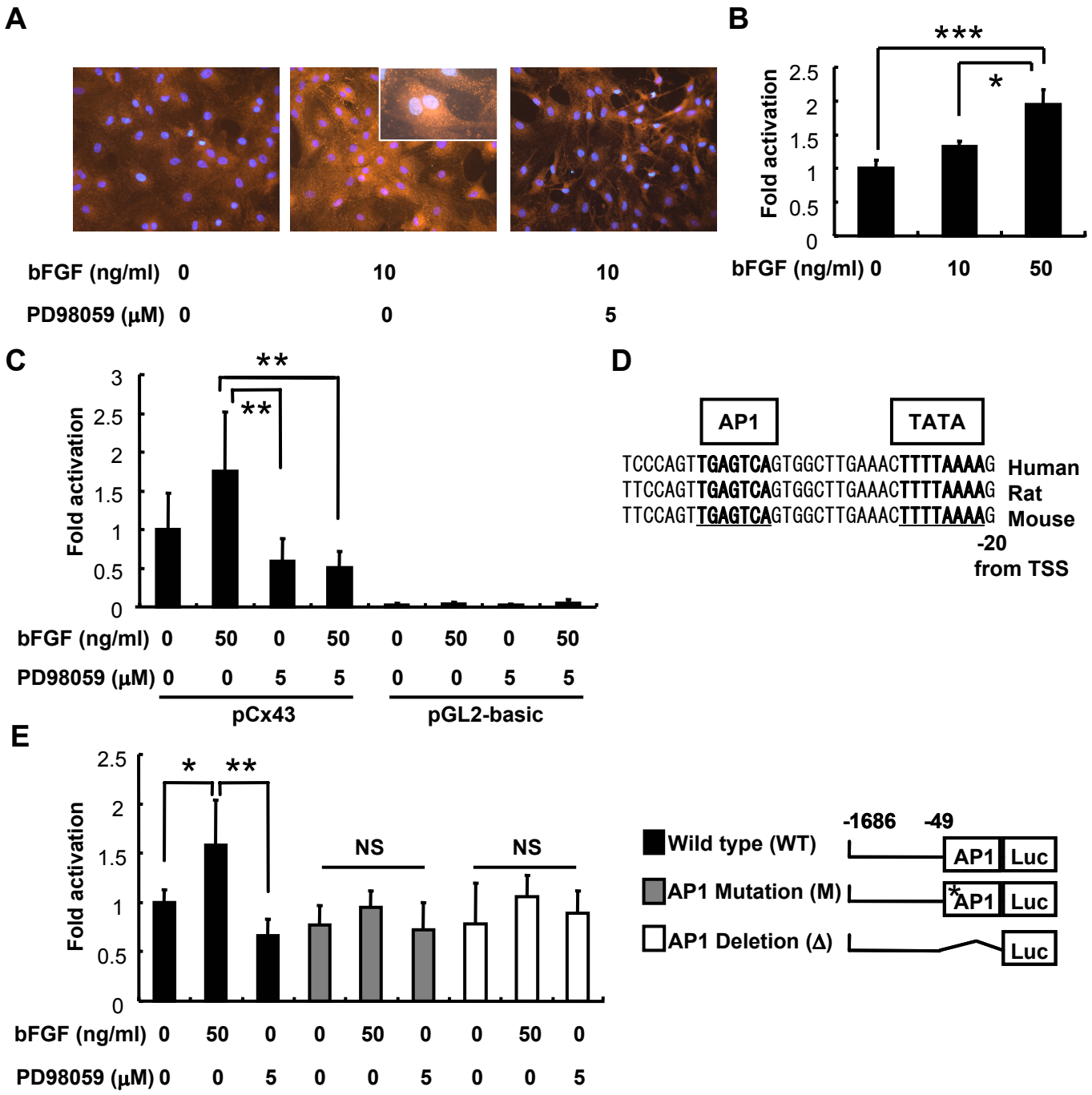
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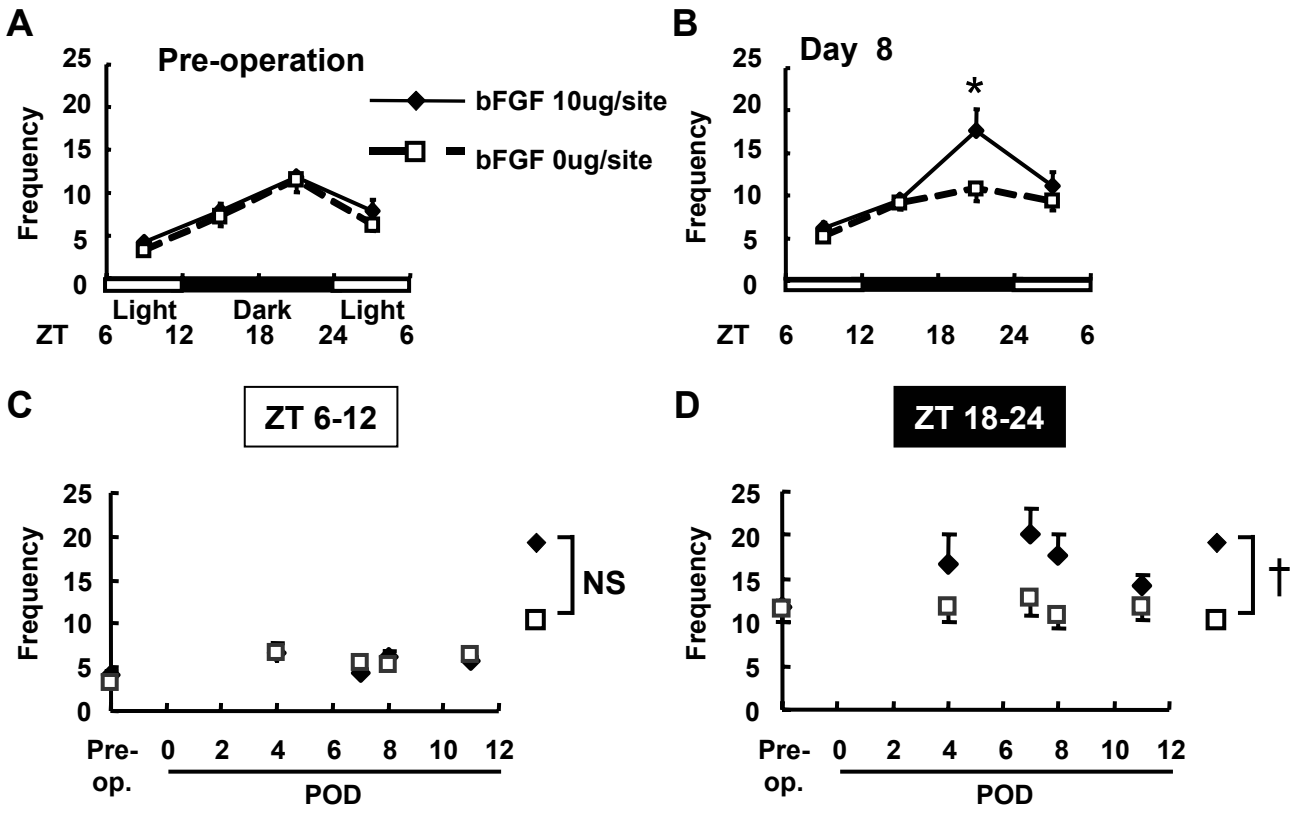
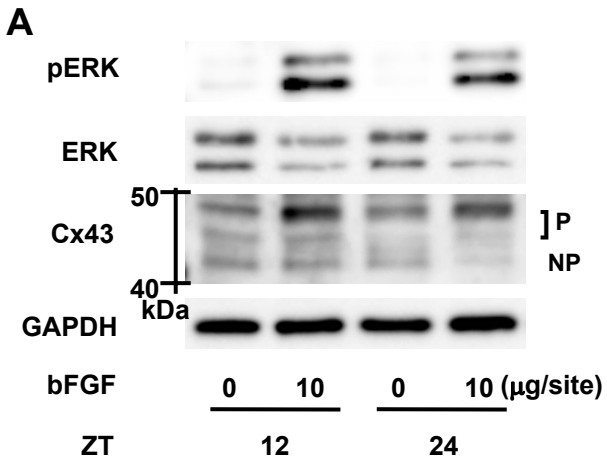

A
Pre-operation
bFGF 10ug/site
bFGF 0ug/site
Frequency
25
20
15
10
5
0
Light
Dark
Light
ZT 6 12 18 24 6
B
Day 8
*
Frequency
ZT 6 12 18 24 6
C
ZT 6-12
Frequency
NS
Pre-op.
0 2 4 6 8 10 12
POD
D
ZT 18-24
Frequency
†
Pre-op.
0 2 4 6 8 10 12
POD


A
pERK
ERK
50
Cx43
40
kDa
]P
NP
GAPDH
bFGF
0
10
0
10 (μg/site)
ZT
12
24