Bcl3: a regulator of NF-κB inducible by TWEAK in acute kidney injury with anti-inflammatory and anti-apoptotic properties in tubular cells.

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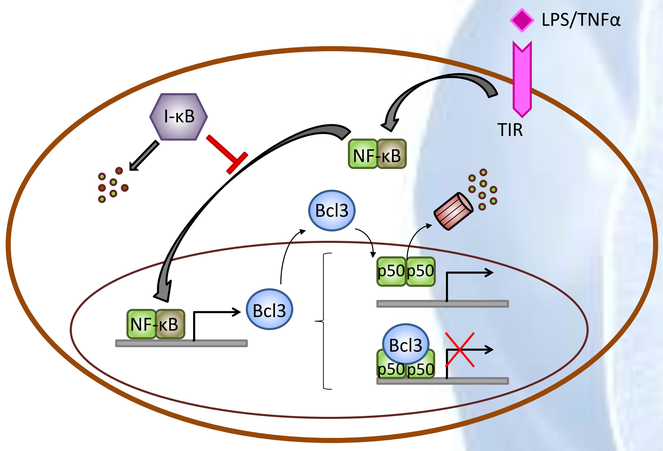
INTRODUCTION

Acute kidney injury (AKI) is characterized by an acute rise in serum creatinine levels or a decrease in urine output and is associated with the progression of chronic kidney disease (CKD) and to an increase in short-term and long-term mortality. The TNF super-family cytokine TWEAK is a key promoter of acute and chronic kidney injury. Tubular cell death or injury is the main morphological feature of AKI. TWEAK promotes both the early canonical NF-κB pathway activation and the prolonged activation of the non-canonical pathway. NF-κB activation promotes inflammation, regulates fibrosis and may protect from cytokine-induced cell death. Bcl3 is a member of the IκB family of NF-κB regulatory proteins that, unlike other IκBs, is a predominantly nuclear protein containing a trans-activation domain, and it can be recruited to NF-κB-responsive promoters, resulting in transcriptional activation or repression depending on the subunit composition of NF-κB complexes.

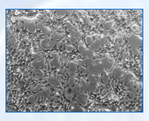


OBJECTIVE

The aim of this work is to characterize the regulation of Bcl3 expression and Bcl3 function in kidney cells.

CONCISE METHODS AND MATERIALS

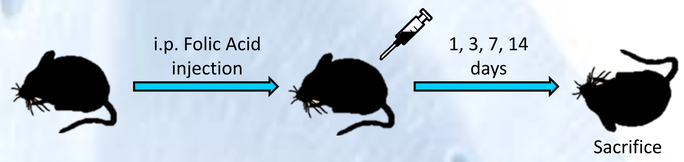
Cells and reagents



MCTs mouse tubular epithelial cells were grown on RPMI 1640 medium with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, in 5% carbon dioxide at 37°C. For experiments cells were rested in serum-free media 24 hours prior to the addition of stimuli. Cells were stimulated with TWEAK (100ng/ml), interferon-γ (INFγ) (30 UI/ml) and TNFα (30ng/ml).

Experimental models

· Experimental AKI: C57/BL6 mice (12- to 14-week-old) received a single i.p. injection of folic acid 250 mg/kg in sodium bicarbonate 0.3 mol/L (AKI) or vehicle alone (controls) and were sacrificed 1, 2, 3, 7 and 14 days later. Kidneys were cold saline perfused *in situ* before removal. One kidney from each mouse was fixed in buffered formalin, embedded in paraffin and stained with hematoxylin-eosin or used for immunohistochemistry. The other kidney was snap-frozen in liquid nitrogen for protein and RNA studies.

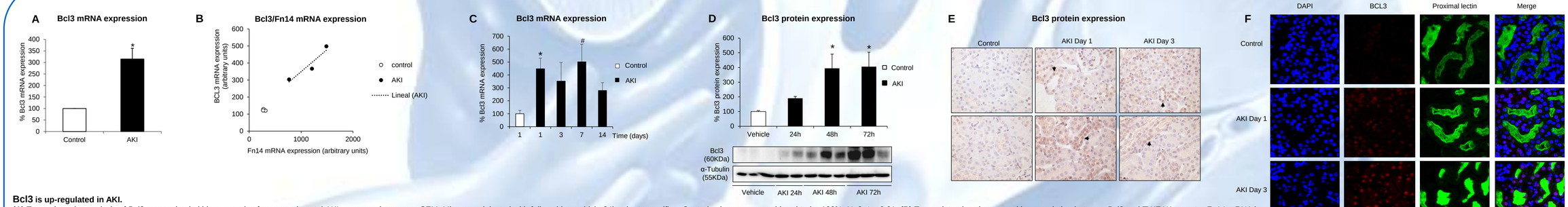


· TWEAK model: healthy 12- to 14-week-old mice were sacrificed 48 or 72 hours after a single i.p. injection of either 0.5 µg/mouse TWEAK or vehicle (200 µl 0.9% NaCl). Renal tissue was processed as in the previous model. The study was approved by the IIS-FJD animal ethics committee and followed Directive 2010/63/EU on the protection of animals used for scientific purposes.

Cell death assays

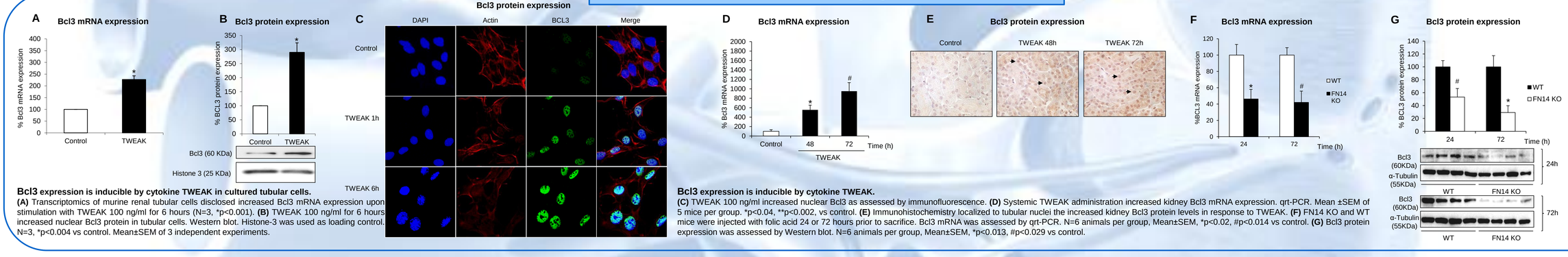
For assessment of apoptosis cells were rested in serum-free media for 24 hours and then stimulated for 24 hours. A lethal cytokine cocktail (TWEAK/TNFα/INFγ) was used as a positive control. For morphological characterization of apoptosis cells were fixed with formalin and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to observe the typical apoptosis morphological changes. For assessment of hypodiploid apoptotic cells, adherent cells were pooled with spontaneously detached cells and incubated in 100 µg/mL propidium iodide (PI), 0.05% NP-40, 10 µg/mL RNAse A in PBS at 4°C for >3 hours. This assay permeabilizes the cells, allowing PI to stain both alive and dead cells. The percentage of apoptotic cells with decreased DNA staining (hypodiploid cells) was counted by flow cytometry using BD CellQuest Software.

Bcl3 expression is increased in experimental AKI



Bcl3 is up-regulated in AKI.
(A) Transcriptomics analysis of Bcl3 expression in kidney samples from experimental AKI, expressed as mean±SEM. Mice were injected with folic acid or vehicle 24h prior to sacrifice. Control values were considered to be 100%. N=3, *p<0.01. **(B)** Transcriptomics show a positive correlation between Bcl3 and TWEAK receptor Fn14 mRNA in AKI. **(C)** Kidney Bcl3 mRNA levels. qrt-PCR. Mean ±SEM. *p<0.004, #p<0.02 vs control. **(D)** Bcl3 protein assessed by Western blot. Mean±SEM, *p<0.002 vs control. **(E)** Bcl3 protein immunohistochemistry. Note nuclear Bcl3 staining in AKI tubules (arrowheads). **(F)** Bcl3 protein immunofluorescence. Tetranogolobus lotus lectin stains proximal tubular cells. Note nuclear Bcl3 staining in AKI proximal and non-proximal tubules.

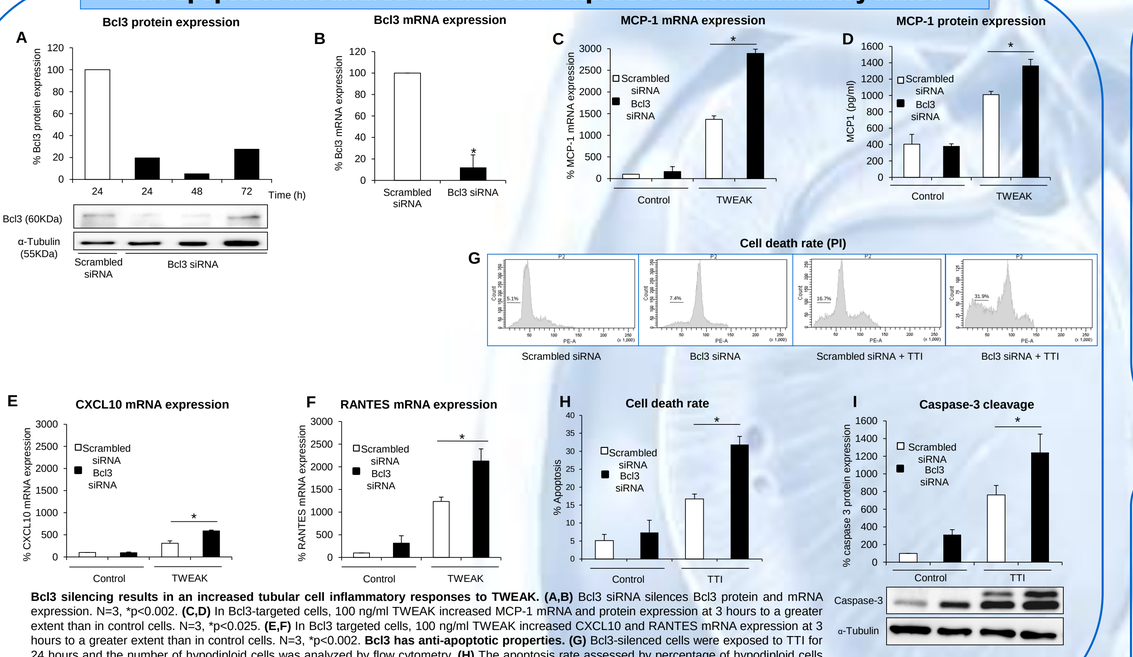
Bcl3 expression is inducible by cytokine TWEAK



Bcl3 expression is inducible by cytokine TWEAK in cultured tubular cells.
(A) Transcriptomics of murine renal tubular cells disclosed increased Bcl3 mRNA expression upon stimulation with TWEAK 100 ng/ml for 6 hours (N=3, *p<0.001). **(B)** TWEAK 100 ng/ml for 6 hours increased nuclear Bcl3 protein in tubular cells. Western blot. Histone-3 was used as loading control. N=3, *p<0.004 vs control. Mean±SEM of 3 independent experiments.

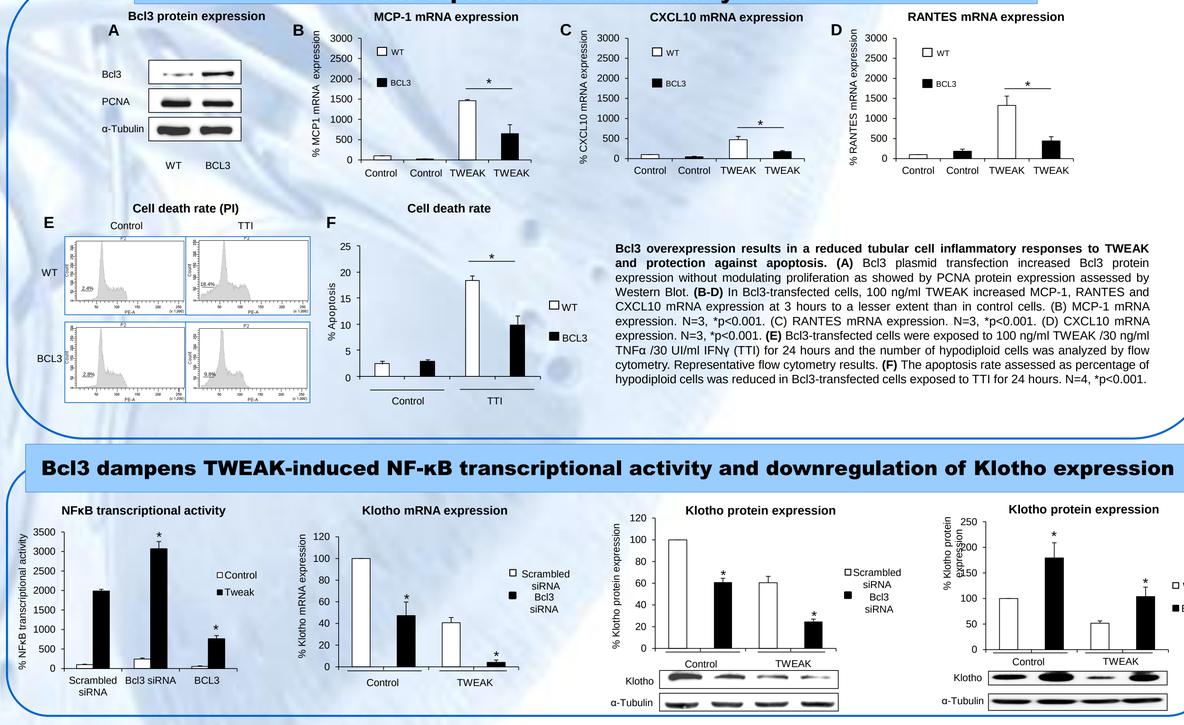
Bcl3 expression is inducible by cytokine TWEAK.
(C) TWEAK 100 ng/ml increased nuclear Bcl3 as assessed by immunofluorescence. **(D)** Systemic TWEAK administration increased kidney Bcl3 mRNA expression. qrt-PCR. Mean ±SEM of 5 mice per group. *p<0.04, **p<0.002, vs control. **(E)** Immunohistochemistry localized to tubular nuclei the increased kidney Bcl3 protein levels in response to TWEAK. **(F)** FN14 KO and WT mice were injected with folic acid 24 or 72 hours prior to sacrifice. Bcl3 mRNA was assessed by qrt-PCR. N=6 animals per group, Mean±SEM, *p<0.02, #p<0.014 vs control. **(G)** Bcl3 protein expression was assessed by Western blot. N=6 animals per group, Mean±SEM, *p<0.013, #p<0.029 vs control.

Bcl3 silencing increases inflammatory gene expression in response to TWEAK and apoptosis in cultured tubular cells exposed to an inflammatory milieu



Bcl3 silencing results in an increased tubular cell inflammatory responses to TWEAK. (A,B) Bcl3 siRNA silences Bcl3 protein and mRNA expression. N=3, *p<0.002. **(C,D)** In Bcl3-targeted cells, 100 ng/ml TWEAK increased MCP-1 mRNA and protein expression at 3 hours to a greater extent than in control cells. N=3, *p<0.025. **(E,F)** In Bcl3 targeted cells, 100 ng/ml TWEAK increased CXCL10 and RANTES mRNA expression at 3 hours to a greater extent than in control cells. N=3, *p<0.002. **Bcl3 has anti-apoptotic properties. (G)** Bcl3-silenced cells were exposed to TTI for 24 hours and the number of hypodiploid cells was analyzed by flow cytometry. **(H)** The apoptosis rate assessed by percentage of hypodiploid cells was increased in Bcl3-silenced cells exposed to TTI for 24 hours. N=3, *p<0.002. **(I)** Caspase-3 cleavage was assessed by Western blot. Bcl3-silencing increased caspase-3 cleavage in response to TTI. N=3, *p<0.025.

Bcl3 over-expression prevents inflammation and apoptosis in cultured tubular cells exposed to an inflammatory milieu



Bcl3 overexpression results in a reduced tubular cell inflammatory responses to TWEAK and protection against apoptosis. (A) Bcl3 plasmid transfection increased Bcl3 protein expression without modulating proliferation as showed by PCNA protein expression assessed by Western Blot. **(B-D)** In Bcl3-transfected cells, 100 ng/ml TWEAK increased MCP-1, RANTES and CXCL10 mRNA expression at 3 hours to a lesser extent than in control cells. (B) MCP-1 mRNA expression. N=3, *p<0.001. (C) RANTES mRNA expression. N=3, *p<0.001. (D) CXCL10 mRNA expression. N=3, *p<0.001. **(E)** Bcl3-transfected cells were exposed to 100 ng/ml TWEAK /30 ng/ml TNFα /30 UI/ml IFNγ (TTI) for 24 hours and the number of hypodiploid cells was analyzed by flow cytometry. Representative flow cytometry results. **(F)** The apoptosis rate assessed as percentage of hypodiploid cells was reduced in Bcl3-transfected cells exposed to TTI for 24 hours. N=4, *p<0.001.

Bcl3 dampens TWEAK-induced NF-κB transcriptional activity and downregulation of Klotho expression

CONCLUSSIONS

- ✓ **Bcl3 is constitutively expressed in tubular cells in the kidney.**
- ✓ **Bcl3 suppresses chemokine production in tubular cells in a pro-inflammatory environment.**
- ✓ **Bcl3 prevents cytokine-induced cell death.**
- ✓ **Bcl3 suppresses TWEAK-induced klotho downregulation in tubular cells.**
- ✓ **The observation that Bcl3 inhibits the pro-inflammatory action of NF-κB-activating cytokines while preventing cytokine-induced cell death postulates Bcl3 as a potential therapeutic target in kidney disease.**

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54 ERA
Experimental AKI I
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