THE LYSINE METHYLTRANSFERASE SET9 IS CRITICAL FOR TGF B-1 SIGNALLING AND WOUND HEALING IN FIBROSIS

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Introduction & Objectives

Fibrosis is the histological endpoint of all forms of chronic kidney disease (CKD) and results from aberrant wound healing. It's two variants, glomerulosclerosis and tubulointerstitial fibrosis, replace normal renal parenchyma, leading to end stage renal failure (ESRF), necessitating dialysis or renal transplantation.

It is estimated that the worldwide prevalence of ESRF will double by 2030, making it imperative to slow down or reverse fibrosis if were are to sustain patients with CKD, as the costs of dialysis and transplantation are beyond the reach of many.

Fibroblast cells are responsible for normal wound healing and fibrosis, which is characterised by :

- Alterations in fibroblast contractility, mobility and migration through regulation of the cellular cytoskeleton, such as actin reorganisation.
- Excessive deposition of extracellular matrix (ECM) proteins such as fibronectin and collagens.

TGFB-1 / SMAD3 are key factors in these processes.

Here we describe a novel interaction between the SMAD3 protein and the lysine methyltransferase SET9 (SETD7). We investigated this interaction further with respect to suitability as a therapeutic target in the treatment of CKD.

Methods

- Immunoprecipitation: Cells were treated with or without TGF B-1, lysed in RIPA buffer then immunoprecipitated with protein G dynabeads cross-linked to SET9 antibodies. Proteins resolved on SDS PAGE were then subject to western blotting.
- Nuclear and cytoplasmic fractions were separated using Igepal CA-630 and sequential centrifugation. Fractions were then subject to western blotting.
- Immunofluorescence: Cells were seeded on glass coverslips in 6 well plates then treated with TGFB-1. Cells were fixed, blocked and stained with the stated primary antibodies then Alexa Fluor 488 (green) or 568 (red) secondary antibodies.
- Wound Healing: Cells were seeded into chambers to create a reproducible wound. After 24h the chambers were removed and cells treated as shown. Cells were imaged at 4h intervals over 48h and wound closure interpreted as % surface coverage was charted.
- Global gene expression was performed with Illumina Mouse WG6V2.0 BeadChip technology on total RNA from either SET9 +/+ or SET9 -/- Mouse Embryonic Fibroblasts (Giuseppe Testa, Milan, Italy). Cut off 1.5-fold change in expression and p value stringency <0.05 was applied. DAVID was used for online functional annotation gene clustering with genelists meeting above criteria with respect to SET9 -/- cells.

SET9 interacts with SMAD3 modulating SMAD3 stability

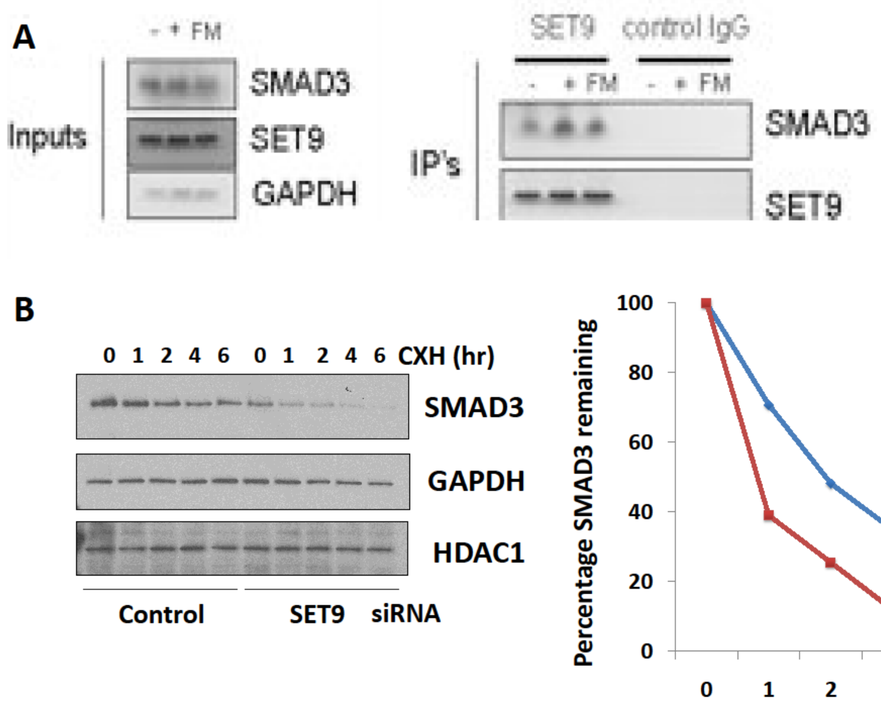


Figure 1: (A) HKC8 tubular cells were treated with 2ng/ml TGFB-1, where indicated, vehicle control, or full medium for 24 hr. Cells were then immunoprecipitated with SET9 antibodies or rabbit immunoglobulin G (IgG) control. Proteins then resolved on SDS PAGE were subject to western blotting with antibodies as indicated. **(B)** HKC8 cells were transfected with either non-silencing control or SET9 siRNA prior to treatment with cycloheximide (CXH) for 0-6hr prior to western blotting as shown. Graph demonstrates SMAD3 half-life.

SET9 is required for SMAD3 nuclear import

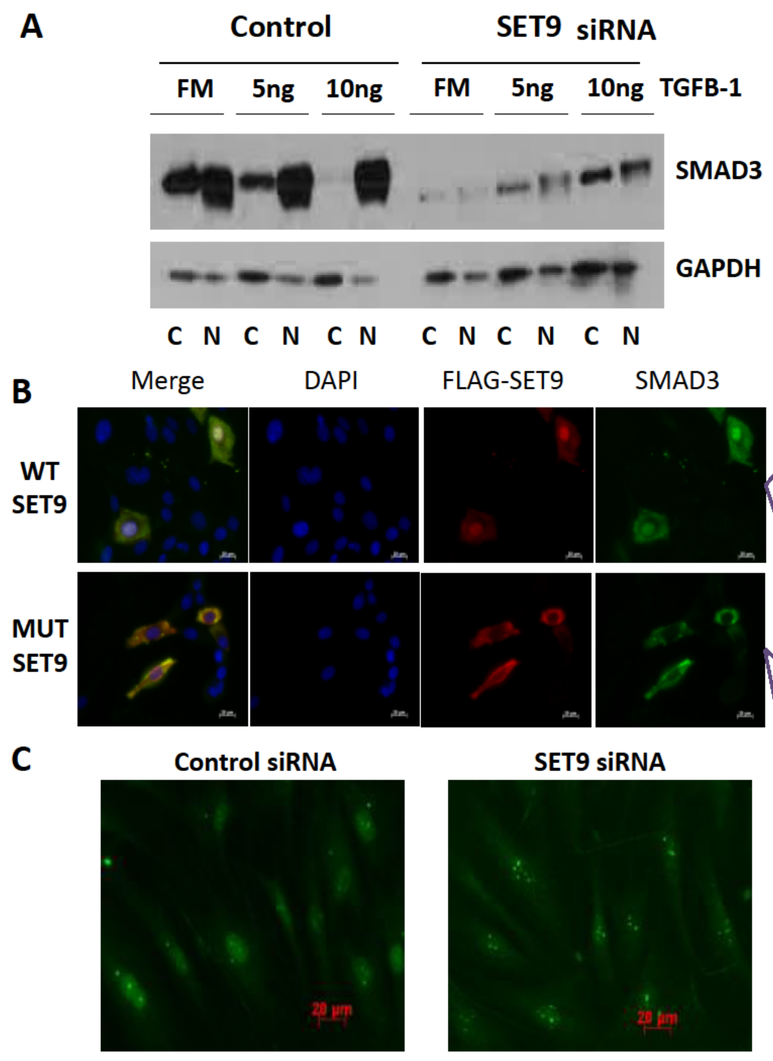
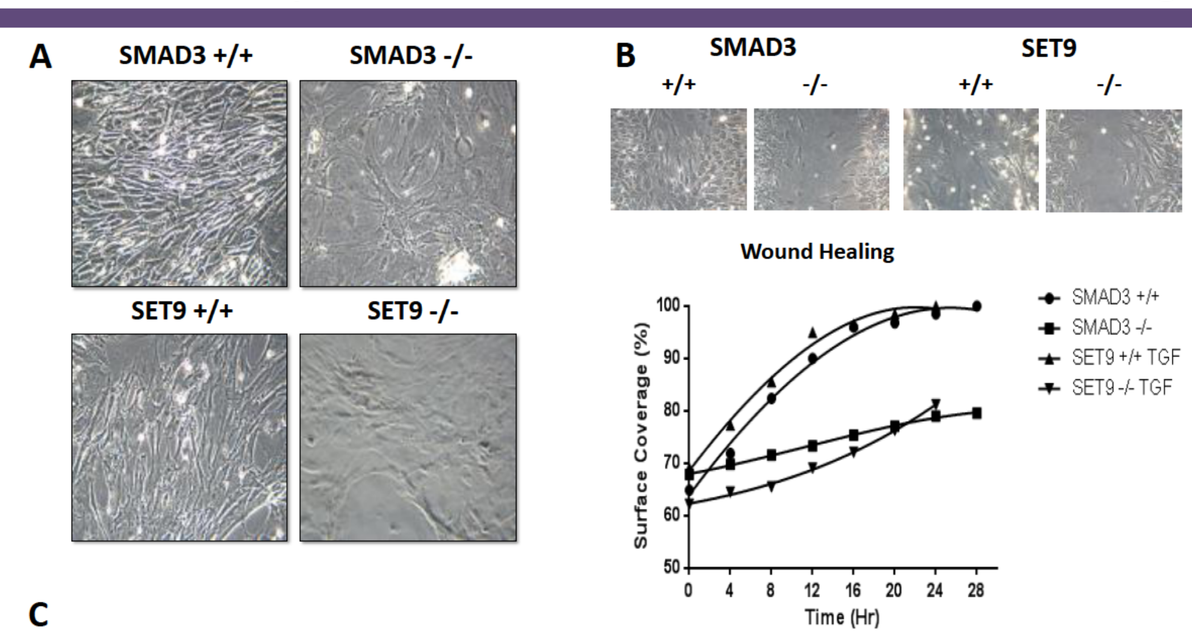


Figure 2: (A) Nuclear and cytoplasmic fractions generated from HKC8 cells transfected with either control or SET9 siRNA, treated with or without TGF B-1. Lysates were subject to western blotting for SMAD3 and GAPDH. **(B)** HKC8 cells were transfected with WT or methylase deficient MUT FLAG-SET9 then treated with 5ng/ml TGFB-1 for 24h. Immunofluorescence staining was acquired for SMAD3 (green), SET9 (red) and DAPI (blue). **(C)** Fibroblast cells were transfected with either Control or SET9 siRNA and immunofluorescence staining was performed for SMAD3 (green).

Loss of SET9 impairs fibroblast transformation and wound healing



Process	No. genes	Process rank
Cytoskeleton	50 genes	1 / 151
Ubl conjugation	34 genes	2 / 151
Angiogenesis	18 genes	3 / 151
ECM	15 genes	16 / 151
Protease inhibitors	5 genes	28 / 151
Extracellular	58 genes	67 / 151

Figure 3: (A) Phenotype Expt: Smad3 or SET9 +/+ and -/- MEFs were treated with 10ng/ml TGFB-1 for 72h. **(B) Wound healing Assay:** Smad3 or SET9 +/+ and -/- MEFs were seeded into wounding chambers, after 24h chambers were removed and cells treated with TGFB-1. Images are wounds at 24h. Images of the wounds taken at 4h intervals for 48h and wound closure plotted on the graph. **(C) Gene expression in SET9 in MEFs:** SET9 -/- and +/+ MEFs were treated with or without TGF B-1. Genes upregulated in +/+ cells compated to -/- cells were listed for analysis by gene ontology in DAVID, as shown in the table.

Conclusions

- SMAD3 interacts with the lysine methyltransfease SET9, regulating SMAD3 stability and nuclear import.
- SMAD3 and SET9 -/- MEFs demonstrate identical phenotypes in response to TGF B-1 and are both required for wound healing.
- SET9 regulates a broad gene expression programme in response to TGF B-1, particularly the cytoskeleton.
- SET9 represents a therapeutic target for fibrotic diseases, including CKD.

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