Abstract # 80

Transcriptional regulation of the human type III TGFβ receptor.

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Abstract

Renal cell carcinoma (RCC) accounts for 3% of all malignancies reported each year and is the sixth leading cause of cancer death. Early diagnosis of this disease is essential for intervention and cure due to the lack of effective treatments available for later stages and metastatic disease. Previous publications from this laboratory have identified through the utilization of genomic profiling, that loss of the type II and III TGFβ receptors in the progression of this disease lead to loss of TGFβ responsiveness and suggests a role for dysregulated TGFβ signaling in RCC progression and metastasis. Restoration of the type II and III receptors in cell lines derived from metastatic RCC restored TGFβ-mediated responses, inhibited anchorage independent growth, decreased cell proliferation and completely blocked tumor growth in athymic nude mice (Copland *et al.* 2003). Restoration of TGFβ receptor type III (TβRIII) alone demonstrated induction of apoptosis in RCC cell lines (Margulis *et al.* 2007). Therefore modulation of the expression level of TβRIII in RCC metastatic disease is an attractive method for intervention. Two promoters have been identified that regulate the expression of the TβRIII gene; the distal promoter and the proximal promoter which reside 45kB and 25kB upstream of the transcriptional start site respectively. Examination of these promoters through deletion and point mutation analysis utilizing normal renal and RCC cell lines has identified a number of putative transcription factor binding sites with the potential to regulate TβRIII expression. One such site in the distal promoter accounts for more than 90% of the promoters activity. Further evaluation of these putative transcriptional regulators of TβRIII are being investigated utilizing ChIP and EMSA assays. Functionality is being tested by silencing putative transcription factors using lentiviral shRNA in normal renal epithelial cells with the expectation that TβRIII mRNA levels will be attenuated. Over-expression of transcriptional factors in RCC cell lines are being examined for their potential to restore TβRIII mRNA and protein levels. Inhibition of methyltransferases and HDACs in RCC cells lead to a 3-fold induction in TβRIII RNA expression suggesting that gene silencing is another process involved. Taken together, these investigations will identify novel molecular targets able to regulate the expression of TβRIII and allow for a better understanding of the events leading to its loss in RCC.

Background

Renal cell carcinoma (RCC) accounts for 3% of all malignancies reported each year and is the sixth leading cause of cancer death in the US

Risk factors include smoking, obesity, high-fat diet and genetic and hereditary history

Early diagnosis of this disease is essential for intervention and cure due to the lack of effective treatments available for later stage and metastatic disease.

Loss of responsiveness to TGFβ signaling has been proposed to play a prominent role in RCC carcinogenesis and progression. Loss of the type III Transforming Growth Factor-β (TβRIII) receptor (Betaglycan) has been identified to cause a loss of TGFβ responsiveness

TβRIII is a putative tumor suppressor and its expression had been identified to be lost or reduced in a number of cancers; prostate cancer [1], renal cell carcinoma (RCC) [2], ovarian cancer [3], breast cancer [4], non small cell lung cancer [5]

Loss of TβRIII has been hypothesized to be an early event in renal cell cancer (RCC)

A clearer understanding of the molecular events that lead to loss of TβRIII expression in renal cell cancer would allow for identification of putative therapeutic pathways that through manipulation would allow for re-expression of the type III receptor and the return of TGFβ responsiveness

Representation of TβRIII promoter

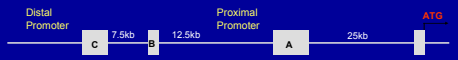


Figure 1. Graphical representation of the human type III TGFβ receptor.

Expression of TβRIII in experimental cell lines

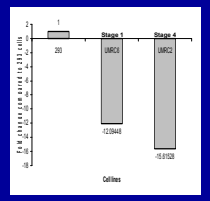


Figure 2. Real Time PCR analysis of TβRIII expression levels in experimental cell lines. Cells were grown to 90% confluence in 10% FBS, washed with PBS and RNA extracted using RNAqueous. 20ng of RNA were used in the Real Time reaction and samples measured in triplicate. TβRIII expression levels are compared back to those of 293 cells.

Identification of transcription factor response elements in the TBRIII 0.58kB distal promoter

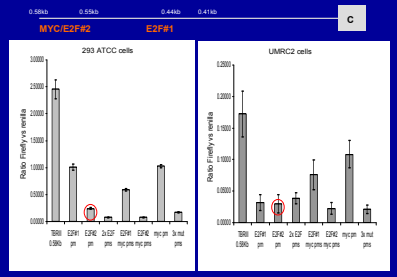


Figure 3. Point mutations of putative binding sites in the proximal promoter of TβRIII lead to decreased expression. 293 and UMRC2 cells were grown to 70% confluence before undergoing Fugene6 transient transfection with full length and point mutated luciferase reporter constructs. Point mutation constructs were created using the Quikchange II XL site-directed mutagenesis kit (Stratagene). After a 24 hour incubation period proteins were collected and analyzed. Results are expressed as a ratio of Firefly activity normalized to the Tk renilla transfection control vector.

Analysis of the TβRIII proximal promoter

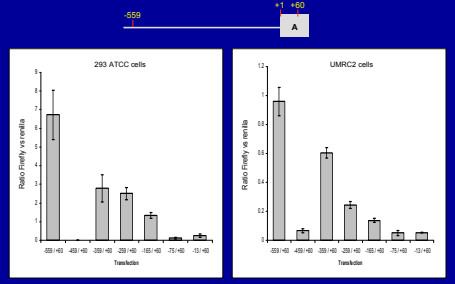
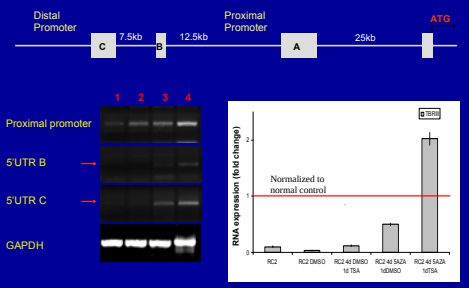


Figure 4. Identification of enhancer regions in the proximal TβRIII promoter. 293 and UMRC2 cells were grown to 70% confluence in complete media before undergoing Fugene6 transient transfection with proximal promoter full length and deletion constructs. Cells were harvested 24 hours post transfection and luciferase activity analyzed. Results are expressed as a ratio of Firefly activity normalized to the Tk renilla transfection control vector.

Promoter regulation of endogenous TβRIII mRNA



1. UMRC2 cells 4 day DMSO
2. UMRC2 cells 4 day DMSO 1 day TSA
3. UMRC2 cells 4 day 5'AZA 1 day DMSO
4. UMRC2 cells 4 day 5'AZA 1 day TSA

Figure 5. Demethylation and inhibition of HDACs return expression of TβRIII promoter and RNA expression in the UMRC2 RCC cell line. Cells were treated as described with TSA and 5'AZA at concentrations of 0.5μM and 10μM respectively. RNA was extracted using RNAaqueous (Ambion) and analyzed for proximal and distal promoter expression by RT-PCR. Analysis of expression levels of TβRIII compared to normal matched controls of UMRC2s were also evaluated by Real Time PCR techniques. Results were normalized to the NK2 normal renal cell controls.

Conclusions

- Mutations of the E2F binding sites of the TβRIII distal promoter interferes with its expression suggesting a regulatory role for E2Fs.
- Silencing of E2F1 in the UMRC2 RCC cell line leads to a two fold re-expression of TβRIII suggesting that E2F1 is working as a transcriptional repressor (Data not shown).
- However, expression of the TβRIII distal promoter is not observed in our normal renal cell model system.
- The proximal promoter sequence contains both activating and repressing elements. Proximal promoter expression is seen in our normal renal cell model system and in RCC and is also induced by TSA and 5'AZA treatment.
- DNA demethylation leads to re-expression of TβRIII message and in combination with histone deacetylation induces a 2-fold return of expression above normal patient matched TβRIII levels.

Future Directions

- Identification of transcription factor family members and other proteins that are binding to the -559/-459 region of the proximal promoter that are modulated between normal renal cells, tumor cells and 5'AZA/TSA treated tumor cells
- Creation of 50 and 25bp deletion mutants of the -559/-359 sequence of the proximal promoter to identify regions of transcriptional activation and repression
- shRNA silencing of TBRIII in normal renal tissue cultures to identify if the loss of TBRIII is sufficient to move cells towards a malignant phenotype and to identify downstream targets of TBRIII loss in normal renal cells.

Acknowledgements

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References

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