

RESEARCH LETTER

TRANSIENT INHIBITION OF SMAD1/SMAD4 TRANSLATION USING ANTISENSE OLIGODEOXYNUCLEOTIDES IN VITRO

Dear MJM,

During normal fetal development, the ureteric bud (UB) branches out of the mesonephros (primitive fetal kidney) and undergoes branching morphogenesis to give rise to the ureter, renal pelvis, calyces, and collecting tubules of the mature kidney [1]. Molecular pathways that regulate this branching are either inhibitory or stimulatory, and both are essential for a normal growth pattern [2]. In 40% of end stage paediatric renal disease, the UB fails to develop appropriately, most probably due to defects in these pathways [3].

The inhibitory pathway of the Bone Morphogenetic Protein-2 (BMP-2) belongs to the TGF- β superfamily. Gupta et al. have shown that BMP-2 is a very potent inhibitor of renal branching morphogenesis, both in cell culture models and in cultured murine embryonic kidney explants [5, 6]. BMP-2 exerts its effects via intracellular messengers known as SMAD proteins (or MAD-H proteins, the human homologues of the drosophila Mothers Against Decapentaplegic gene). Activation of the BMP-2 receptor phosphorylates SMAD1(R-SMAD), which binds SMAD4 (Co-SMAD), and translocates to the nucleus where it is postulated to regulate gene expression [5, 11 Fig.1]. It is true that BMP-2 inhibits tubule formation and branching, but it is still unknown whether SMAD proteins are essential for this action [5].

Previous work has shown that SMAD1 and SMAD4 are expressed in developing kidneys, but their function is still unknown [12]. Since they are downstream effectors of BMP-2, we hypothesized that SMADs mediate inhibitory signals for UB development, such that blockade of either one will promote branching morphogenesis. In simpler terms, in a kidney that is not branching very well, we hypothesize that the inhibition of the SMAD1 and/or SMAD4 proteins will promote branching morphogenesis and allow the kidney to develop normally.

In order to block SMAD1 and SMAD4 translation, we opted to use Antisense Oligodeoxynucleotides (oligos) purchased from Biognostik Canada, Montreal, Quebec. These oligos are DNA sequences complementary to the SMAD mRNA, and it is postulated that they can bind to it and inhibit protein translation. These phosphorothioated second generation molecules are considered DNAase resistant, minimally toxic, and independent of lipid agents that facilitate cell entry. Cell culture wise, the mouse inner medullary collecting duct-3 (mIMCD-3) cell line was used [5]. This is an excellent model for the study of UB development because it originates from the UB, expresses

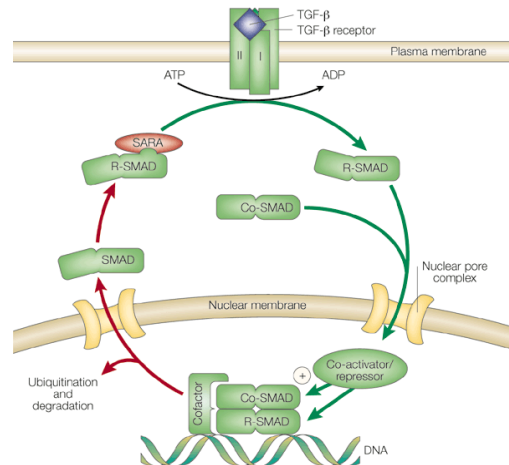


Figure 1. The signaling cascade of the TGF- β family via the R-SMAD (SMAD-1) and the Co-SMAD (SMAD-4) from Massagué J., Nature Reviews Molecular Cell Biology 1; 169-178 (2000)

SMAD1 and SMAD4, and develops 3D tubular structures when cultured on a type I collagen matrix. The cells were cultured in 6-well plates in 1ml of DMEM (5% FCS and 1% P/S). Seeding concentration was 87,000 cells/ml and cells were given 24 hrs to attach properly. The antisense and control oligos for SMAD1 and SMAD4 were added at $t=24$ hrs and refreshed with the media every 24 hrs. Cells were harvested at $t=120$ hrs for Western immunoblot analysis as per the technique used by Chen et al [5]. Preliminary dose response assays using fluorescent microscopy had shown that the optimal oligo concentration for uptake with minimum toxicity was in the vicinity of $3\mu\text{M}$ - $5\mu\text{M}$. Both these concentrations were used to conduct the antisense experiments described above.(Fig. 2).Two different oligo sequences were used against each of SMAD1 and SMAD4. Hence there were four sets of experiments taking place, each set comprising a unique anti-SMAD sequence with its scrambled and sense control sequences.

Western analysis was performed to measure SMAD concentrations after treatment with the oligos. Some of the gels showed a slight decrease in cytoplasmic SMAD protein, but none of the gels showed any remarkable attenuation of SMAD expression (Fig. 3). Each set was repeated five times and the results were perfectly reproducible. There could be multiple reasons for the failed downregulation of the SMAD proteins. Failed entry into the cell could be ruled out because all immunofluorescence microscopy clearly shows that the FITC-oligos were present in the cytoplasm (Fig. 2). Likewise, it is not very likely that the oligos were being degraded intracellularly because they are DNAase resistant sequences. Moreover, the observed toxicity at $18\mu\text{M}$ hints to the fact that the oligos are present and active within the cells. One probable reason is a problem

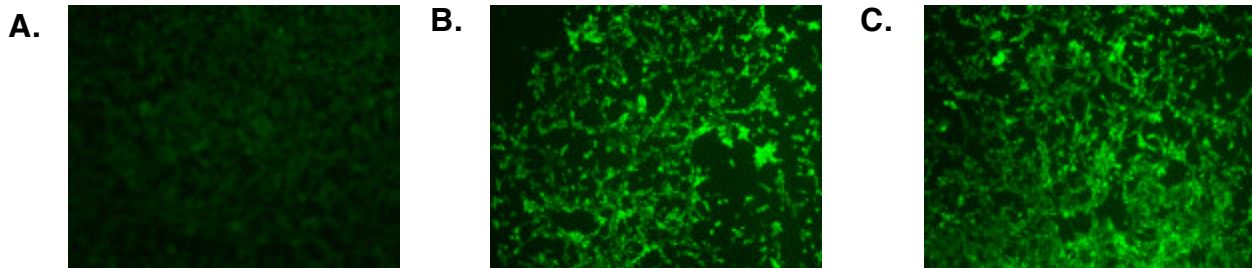


Figure 2. mIMCD-3 cells cultured on 8-well NUNC Labtek II Chamber microslides as visualized under fluorescence microscopy after the addition of FITC labeled oligos. A. Control well shows residual fluorescence. B. $1.0\mu\text{M}$ FITC-oligo. C. $3.0\mu\text{M}$ FITC-oligo shows maximal uptake with no notable toxicity. Uptake was shown without toxicity up to $18.0\mu\text{M}$ of oligo.

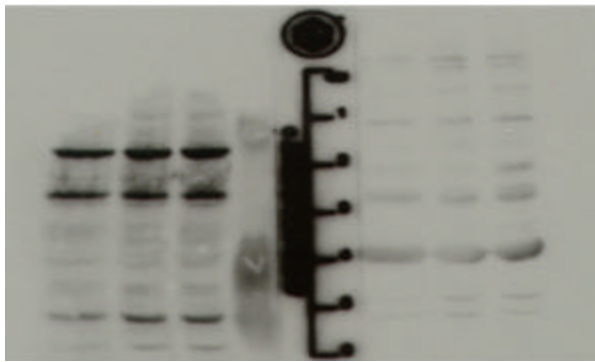


Fig. 3. Western blot analysis using Anti-SMAD1 antibodies after treatment with anti-SMAD1 oligos shows no difference in SMAD-1 levels between the Control (Ctrl), Antisense treated (AS), and Scrambled treated (SC) wells.

with antisense binding Oligonucleotide manufacturers report that there is a 1/10 to 1/8 chance for the anti-sense sequence being active in inhibiting mRNA translation. This low precision is attributed to tertiary folding and steric hindrance between the antisense DNA and the target mRNA. A possible solution for this problem would be to keep on trying different sequences until one eventually succeeds in blocking SMAD1/4 translation.

Although we could not knock-out the desired proteins, this study represents progress in antisense SMAD experiments. We were able to show that the antisense oligos used in this experiment are indeed accessible to kidney cells, and constitute a promising means of knocking out SMAD1/4, given the right DNA sequence. We have determined the optimal uptake concentrations for these oligos as well as the borderline toxic concentrations. We have also been capable of devising improved cell culture protocols that lead to optimal growth of the mIMCD-3 cell line.

In short, this project has set the practical basis for working with antisense oligos in an mIMCD-3 system. Future work may be aided by the findings of this project in terms of cell culture regimens, dosage requirements, and oligodeoxynucleotide sequences.

Eventually, once appropriate anti-sense sequence is

established, we will be able to determine the role of SMAD1 and SMAD4 in renal branching morphogenesis. From there, various studies may be devised to investigate possible *in utero* therapy for congenital renal defects.

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REFERENCES

1. De Vries, L., Zheng, B., Fischer, T., Elenko, E., and Farquhar, M. (2000) The Regulator of G-protein signalling family. *Annu. Rev. Pharmacol. Toxicol.* 40, 235-271
2. Panetta, R., Guo, Y., Magder, S. and Greenwood, M. T. (1999) Regulators of G-protein signalling (RGS) 1 and 16 are induced in response to bacterial lipopolysacchiride and stimulate c-fos promoter expression. *Biochem. Biophys. Res. Commun.* 259, 550-556
3. Grant, S. L., Lassegue, B., Griendling, K. K., Fukai, M. U., Lyons, P. R. and Alexander, R. W. (2000) Specific regulation of RGS2 messenger RNA by Angiotensin II in cultured vascular smooth muscle cells. *Molec. Pharmacology*, 57, 460-467.
4. Fong, C. W., Zhang, Y., Neo, S. Y., and Lin, S. C. (2000) Specific induction of RGS16 mRNA by protein kinase C in CEM leukaemia cells is mediated via tumour necrosis factor in a calcium-sensitive manner. *Biochem. J.*, 352, 747-753
5. Chen, C., Zheng, B., Han, J., and Lin, S.-C. (1997) Characterization of a novel mammalian RGS protein that binds to Ga proteins and inhibits pheromone signalling in yeast. *J. Biol. Chem.* 272, 8679-8885
6. Tamirisa, P., Blumer, K. J., Muslin, A. J. (1998) RGS4 Inhibits G-protein Signaling in Cardiomyocytes. *Circulation*, 99, 441-447
7. Limbird, L. E., and Vaughan, D. E. (1999) Augmenting b receptors in the heart: Short-term gains offset by long term pains? *Proc. Natl. Acad. Sci.* 96, 7125-7127
8. Zhang, S., Watson, N., Zahner, J., Rottman, J. N., Blumer, K. J., and Muslin, A. J. (1998) RGS3 and RGS4 are G-protein inhibitors in the heart. *J. Mol. Cell Cardiol.* 30, 269-276
9. Owen, V. J., Burton, B. J., Mullen, A. J., Birks, E. J., Barton, P., and Yacoub, M. H., (2001) Expression of RGS3, RGS4 and Gi

- alpha 2 in acutely failing donor hearts and end-stage heart failure. *European Heart Journal*, 22, 1025-1020
10. Kong, Janice (2001) Master of Science (MSc) Thesis, McGill University.
 11. Rogers, J. H., Tsirka, A., Kovacs, A., Blumer, J. K., Dorn, G. W., Muslin, A. J. (2001) RGS4 reduces contractile dysfunction and hypertrophic gene induction in Gαq overexpressing mice. *J. Mol. Cell Cardiol.* 33, 209-218
 12. <http://www.ncbi.nlm.nih.gov/Genbank/index.html>
 13. Greenwood M.T., Hukovic, N., Kumar, U., Panetta, R., Hjorth, S.A., Srikant, C.B., Patel, Y.C. (1997) Ligand binding pocket of the human somatostatin receptor 5: mutational analysis of the extracellular domains. *Mol. Pharmacol.* 52, 807-814
 14. Greenwood, M.T., Guo, Y., Kumar, U., Beausejourns, S., Hussain, S.N. (1997) Distribution of protein inhibitor of neuronal nitric oxide synthase in rat brain. *Biochem. Biophys. Res. Commun.* 238, 617-621
 15. Mumberg, D., Muller, R., and Funk, M. (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* 22, 5767-5768
 16. Dohlman, H. G., Song, J., Ma, D., Courchesne, W. E., and Thorner, J. (1996) Sst2, a Negative regulator of pheromone signalling in the yeast *Saccharomyces cerevisiae*: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein α subunit) *Mol. Cell. Biol.* 16, 5194-5209
 17. Gietz, D., St Jean, A., Woods, R. A., Schiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* 20, 1425
 18. Druey, K. M., Blumer, K. J., Kang, V. H., Kehrl, J. H. (1996) Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. *Nature.* 379, 742-746

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