Transfection of transformed human trabecular meshwork TM5, and primary human NTM210-05, NTM486-04, NTM174-04, and NTM153-00 cells with Metafectene Easy

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Glucocorticoids (GCs), a family of steroids, exert their effects through their interaction with glucocorticoid receptors (GR). Human GR exists in two forms, GRα and GRβ. GRα is known to bind to GCs and regulate expression of thousands of genes. However, GRß which does not bind GC, has been reported to suppress GRα activity, and implicated in several glucocorticoid resistance diseases including asthma, arthritis, and inflammatory bowel disease. Moreover, GRβ also appears to play a critical role in regulating glucocorticoid responsiveness in the trabecular meshwork (TM) cells by affecting the transcriptional activity of the GRα response. Therefore, knock-down of GRß may be of therapeutic uses. In the current report, metafectene was used to transfect primary and transformed human trabecular meshwork cells with siRNAs of GRβ receptor.

1 Materials and methods

1.1 Materials

Metafectene Easy was a gift and subsequently purchased from Biontex Laboratories GmbH (Munich, Germany), Lipofectamine™ 2000 was purchased from Invitrogen Inc. (Carlsbad, CA). Dexamethasone (DEX) was obtained from Sigma-Aldrich Corp (St. Louis, MO). Polyclonal anti-GRβ and polyclonal antibodies were purchased from Affinity Bioreagents (Golden, CO). psiRNA-hH1GFPzeo (empty vector) was purchased from InvivoGen (San Diego, CA). GR β-siRNA#1: 5' CATGAAAATGTTATGTGGTTT 3', GR β-siRNA#3: 5' TTTCCGAGTTCTTGTTTCAGG 3', GR β-siRNA#4: 5' AACCAGAAAGCACATCTCACA 3', and Non-targeting #1: Cat.#: D001206-13-05 were custom-made by Eurogenetec Inc. (San Diego, CA). pGRE-Luc plasmid was obtained from BD Clontech (Palo Alto, CA).

1.2 Cells

The human transformed NTM-5, and primary NTM210-05, NTM486-04, NTM174-04, and NTM153-00 trabecular meshwork cells were obtained from Alcon Ltd (Fort Worth, TX). The cells were cultured in 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin, and glutamate (Invitrogen, Grand Island, NY). Confluent cells were used in most studies as described (1-3).

2 Plasmid and Transfections

2.1 Transfection of GRβ siRNA

When cells were approximately 70-80% confluent, they were transfected with plasmids that once inside the cell produce; scrambled siRNA, GRb-siRNA#1, GRb-siRNA#3, and GRb-siRNA#4. Oligo DNA sequences that will produce; siRNAs #1, 3, 4, were inserted into the empty vector (psiRNA-hH1GFPzeo) and subsequently used for transfection experiments designed for luciferase assay. A stock of plasmid DNA and Metafectene Easy was prepared where 10 µg of plasmid DNA was mixed with 10 µl of Metafectene Easy (1:1).

2.2 Luciferase Assay

Luciferase assay was used to determine the glucocorticoid responsiveness after transient knockdown of GRβ using GRβ-siRNA#1,3, & 4 in TM5 and four primary TM cell lines; NTM210-05, NTM486-04, NTM174-04, and NTM153-00. Cells (1 x 10^5) were seeded in 12-well-plates for 24 hours in 1ml of DMEM media. TM cells 70-80% confluent were transfected with 800ng/well of plasmid DNA (empty vector or siRNA), 400ng/well of a mercury luciferase reporter pGRE-Luc (BD Clontech, Palo Alto, CA), and also co-transfected with 50ng/well of pSV40-β -Galactosidase as a control for 10 hours in serum-free and antibody-free DMEM. The cells were washed with serum-free DMEM two times and were treated for 24 hours with vehicle (Ethanol) or DEX (dexamethasone, 100nM/well, dissolved in ethanol) (Sigma Chemical, St Louis, MO) in serum-free DMEM. Whole cell lysates were prepared and luciferase activity performed with a luminometer. This activity was normalized with the reading of pSV40-β-Galactosidase (Promega, Madison, Wisconsin, USA) which served as a control for normalizing the transfection efficiency of the plasmid into the cells. Data were plotted as a fold change from each basal activity of control vehicle treatment and represent the "mean±SE" of results in two independent experiments repeated 2-4 times per experiment.

2.3 siRNA transfection for western blot

TM cells 70-80% confluent were transfected with 10 µg of scrambled siRNA, GRβ-siRNA#1, GRβ-siRNA#3, and GRβ-siRNA#4 for 10 hours, respectively in NTM5 cell line in serum-free and antibiotic-free DMEM in 100mm dish. The media was changed to complete medium for 72 hours and GRβ was immunoprecipiated and immunoblot analysis was performed.

3 Results and discussion

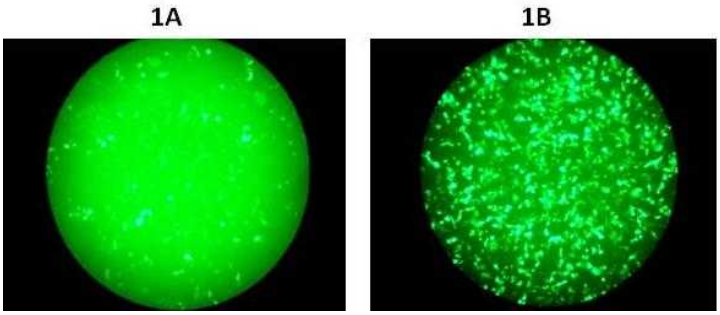
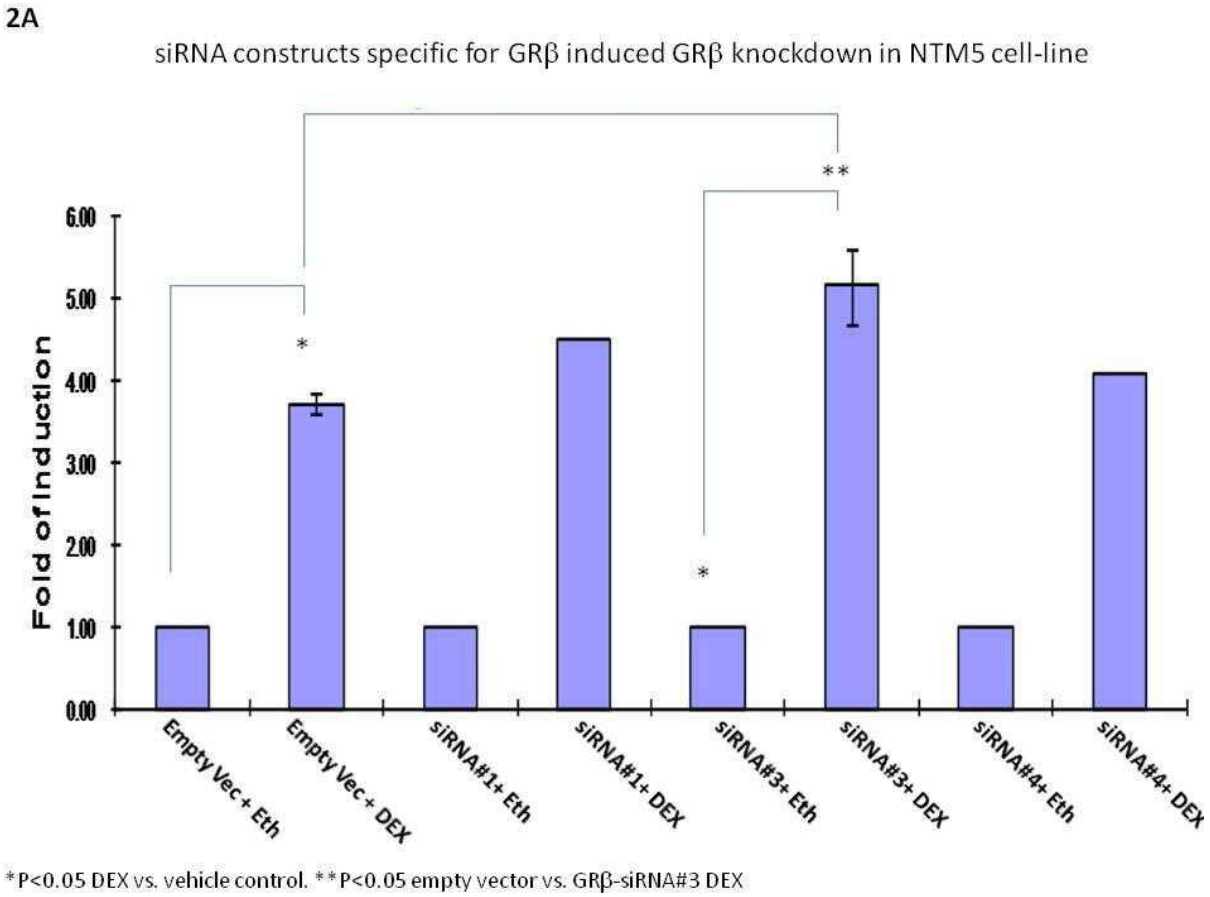
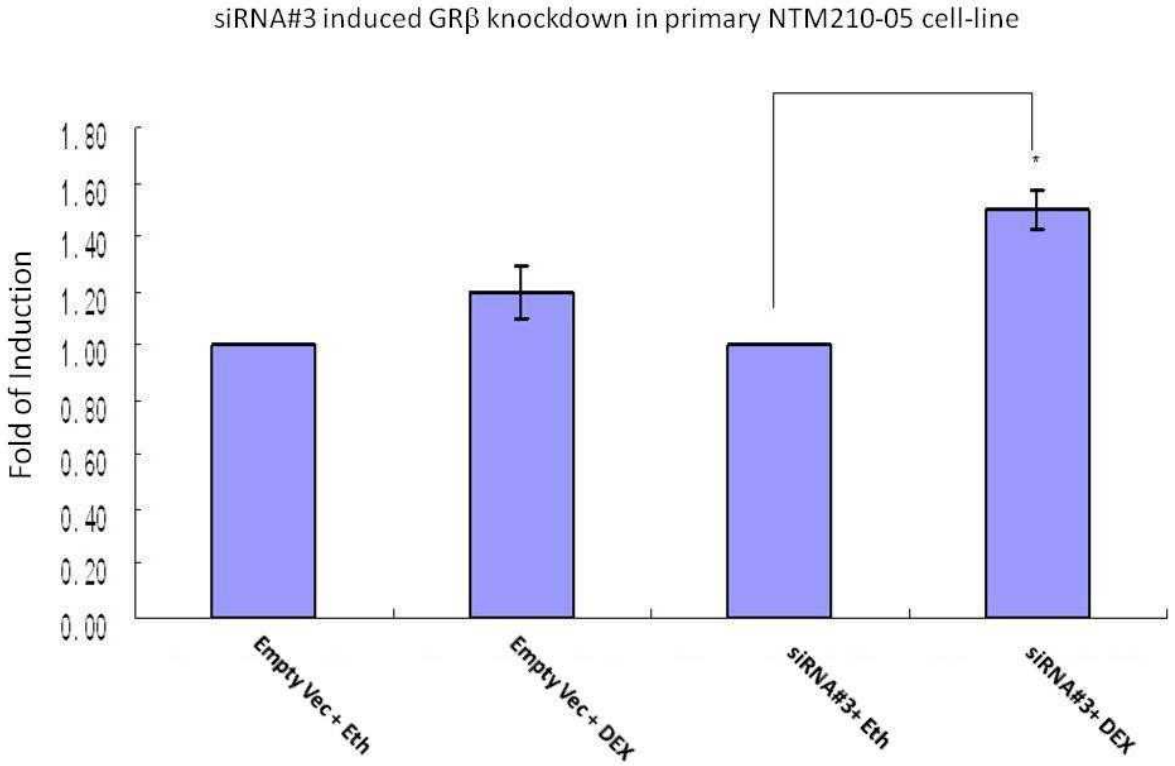


Fig. 1. Metafectene Easy which has at least twice the transfection efficiency as Lipofactamine 2000. Ten microgram of empty vector-GFP (psiRNA-hH1GFPzeo, InvivoGenCA) was mixed with equal volume of metafectene or Lipofactamine 2000 (1:1 ratio) in DMEM-serum free media. As shown, Metafectene Easy produced ~ 70% fluorescent cells (B) vs. ~ 30% fluorescent cells in Lipofactamine transfected cells (A). No toxicities were observed in using Metafectene Easy.



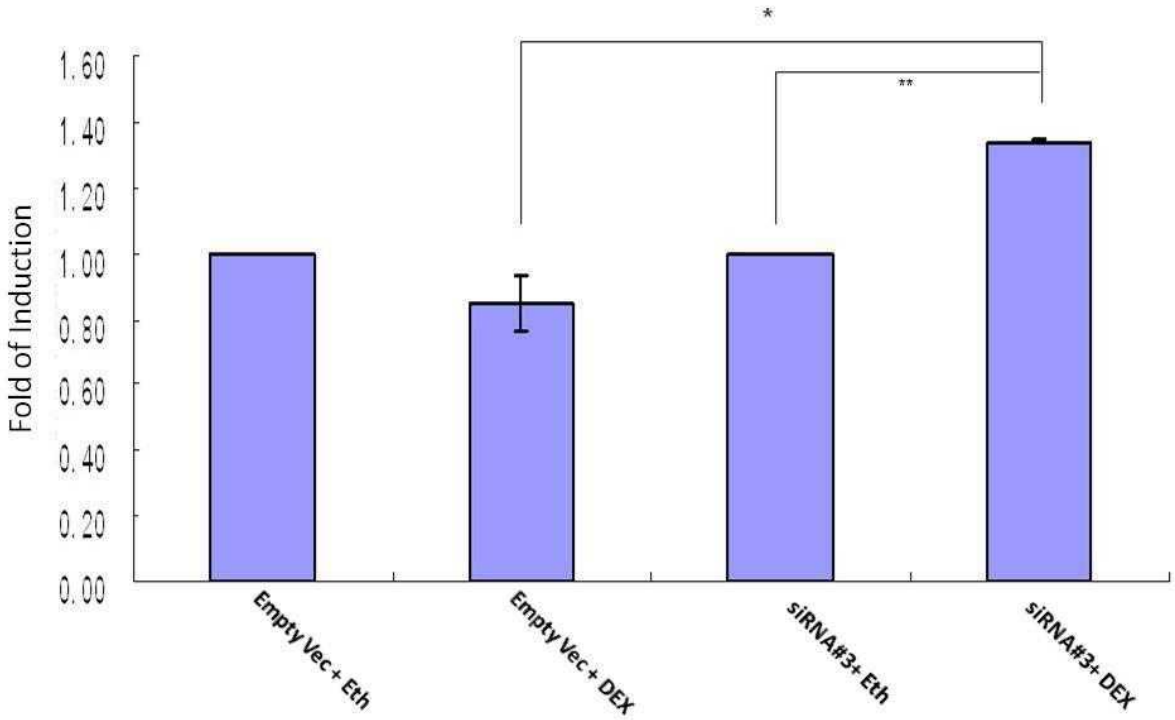
*P<0.05 DEX vs. vehicle control. **P<0.05 empty vector vs. GRβ-siRNA#3 DEX

2B



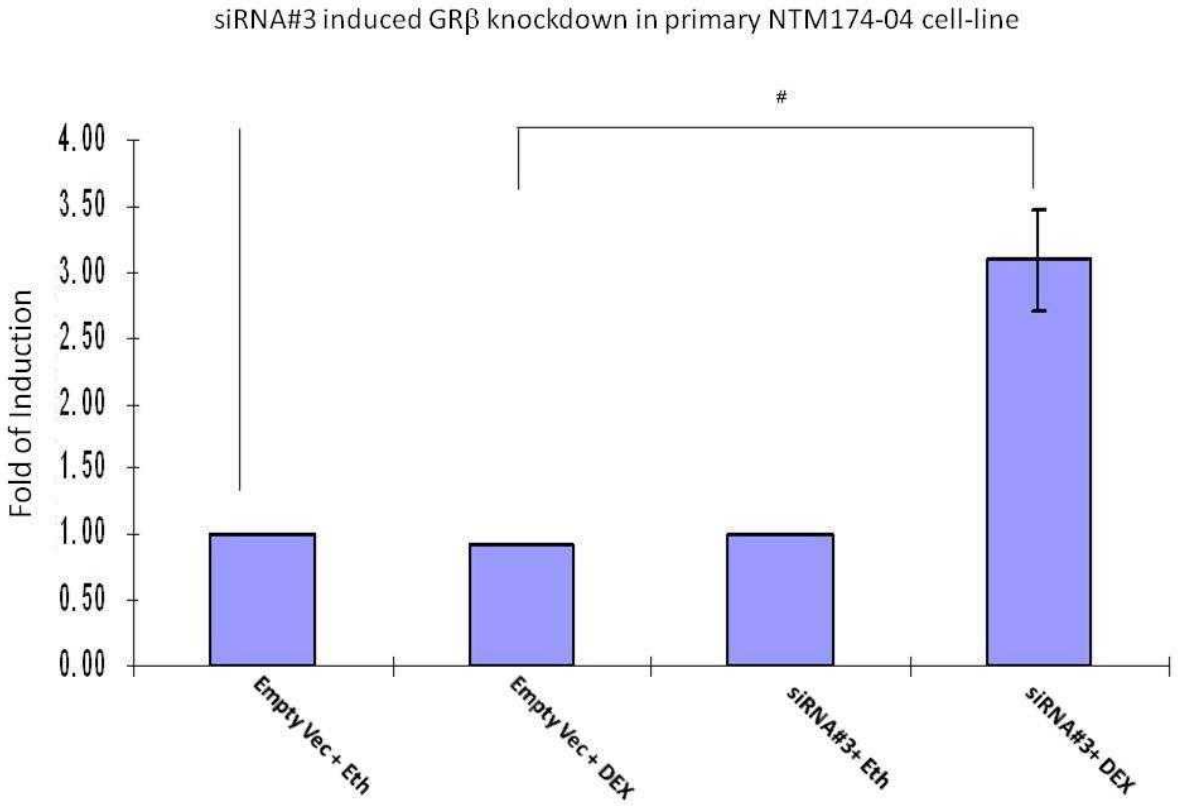
*P<0.05 vs. vehicle control

2C



*P<0.01 DEX vs. vehicle control. **P<0.05 empty vector vs. GRβ-siRNA#3 DEX

2D



*P<0.05 empty vector DEX vs. GRβ-siRNA#3 DEX

2E

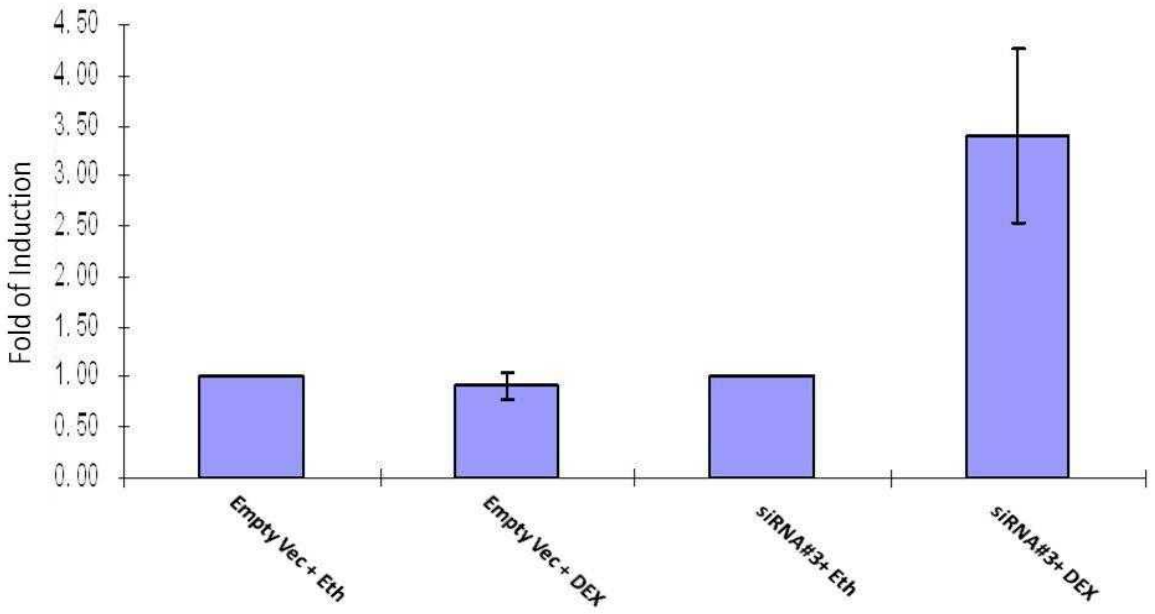


Figure 2. Luciferase assay was used to determine the glucocorticoid responsiveness after transient knockdown of GRβ in TM cell lines (NTM-5, primary NTM210-05, NTM486-04, NTM174-04, and NTM153-00). In 12-well-plates, 100,000 cells were seeded for 24 hours. NTM5 cells were transfected with 800ng/well of empty vector and three siRNA structures, 400ng/well of a mercury luciferase reporter pGRE-Luc (BD Clontech, Palo Alto, CA), and also co-transfected with 50ng/well of pSV40-β-Galactosidase as a control for 10 hours. The cells were washed with serum-free DMEM two times. The cells were treated for 24 hours with vehicle (Ethanol) or DEX (Dexamethasone, 100nM/well, dissolved in ethanol) (Sigma Chemical, St Louis, MO) in serum-free DMEM. Whole cell lysates were prepared and luciferase activity performed with a luminometer. This activity was normalized with the reading of pSV40-β-Galactosidase (Promega, Madison, Wisconsin, USA) which serves as a control for normalizing the transfection efficiency of the plasmid into cells. Data were plotted as fold change from each basal activity of control vehicle treatment and represent the "mean±SE" of results in two independent experiments repeated 2-6 times per experiment.

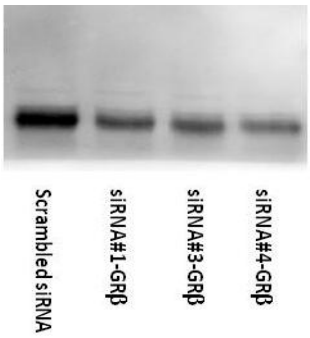


Figure 3. Western blot analysis after transient knockdown of GRβ in TM5 cell line. In a 100-mm dish, 200,000 cells were seeded for 24 hours. Twelve microliters of siRNA stock (10 μM) were mixed with 588 μl of serum-free DMEM media. In a separate tube, 12 μl of Metafectene Easy were mixed with 588 μl of serum-free DMEM media. After 5 minutes, Metafectene Easy mixture was added to siRNA mixture and mixture was allowed to sit for 20 minutes at room temperature. At the end of 20 minutes, 5 ml of serum-free media was added (6 ml final volume) and the entire mixture was added to dish (final concentration of siRNA is 20 nM). The cells were incubated for 24 hours then 6 ml of DMEM media containing 10% FBS and antibiotics were added. After 72-96 hours, GRβ was immunoprecipiated using rabbit anti-GRβ and samples were run on a 7.5% SDS-PAGE followed by western blot. As shown, all 3 siRNAs significantly decreased GRβ protein levels.

4 Conclusion / summary:

Metafectene Easy was highly effective in transfecting both transformed as well as primary human trabecular meshwork cells without noticeable toxicities.

5 References:

1. Pang IH. Shade DL, Clark AF, Steely HT, DeSantis L. Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. Curr. Eye Res. 13:51-63, 1994
2. Steely HT, Browder SH, Julian MB, Miggans ST, Wilson KL, Clark AF. The effect of dexamethasone on fibronectin expression in cultured trabecular meshwork cells. IVOS. 33:2242-2250, 1992.
3. Zhang X, Ognibene CM, Clark AF, Yorio T. Dexamethasone inhibition of trabecular meshwork cell phagocytosis and its modulation by glucocorticoid receptor beta. Exp Eye Res. 84(2):275-284, 2007.