Human non-functioning pituitary tumors invasiveness: inhibitory effects of dopamine receptor type 2 (DRD2) agonist and cofilin involvement

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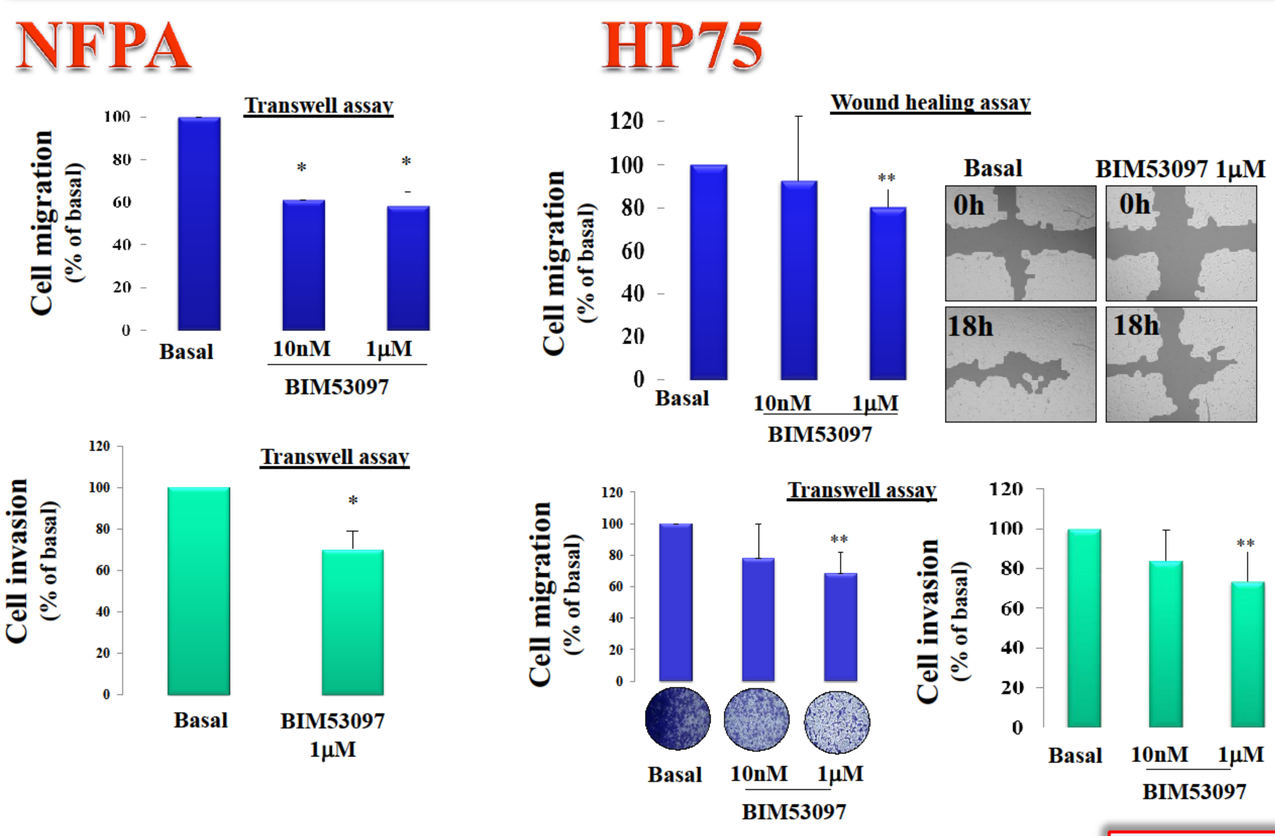
Non-functioning pituitary adenomas (NFPAs) are epithelial tumors that, although benign in nature, frequently present local invasiveness that strongly reduces neurosurgery success. Medical therapy is still under debate, although evidences indicate that dopamine (DA) receptor 2 (DRD2) agonists induce tumor shrinkage in some patients, and inhibit in vitro proliferation of NFPA cultured cells.

Aims of this study were: 1) to evaluate the effect of DR2D agonist BIM53097 on migration and invasion of NFPA cells, and 2) to investigate the molecular mechanisms regulating the motility of these cells, focusing on the role of cofilin, a protein controlled by small GTPases of the Rho family and involved in actin reorganization.

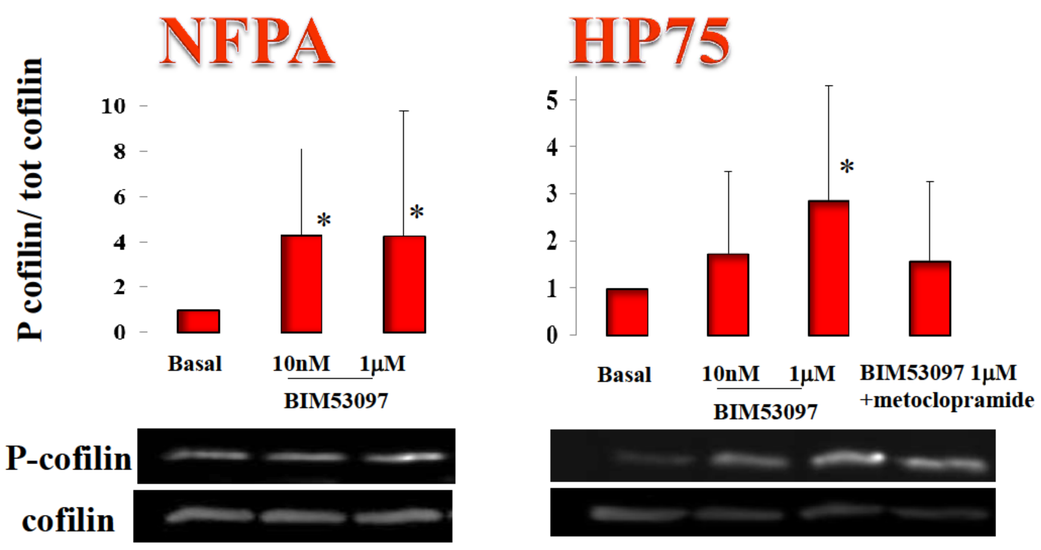
Materials and Methods

- **Pituitary cell culture:** NFPA tissues were enzymatically dissociated in DMEM + 2mg/mL collagenase at 37 C for 2h, and cultured in DMEM 10%FBS, glut, pen/strep. Human non functioning pituitary tumor cell line HP75 were grown in DMEM, 15% HS, 2,5% FBS, glut, pen/strep.
- **Transwell migration and invasion assays:** cells were plated in serum-free medium, with or w/o increasing concentrations of BIM53097, into the upper chamber of a transwell insert with porous polycarbonate membrane (pore diameter 8 µm), and allowed to migrate towards the lower compartment, filled with DMEM with 10% FBS. A negative control with serum-free medium in lower chamber was used in each experiment and substracted from each value. For primary NFPA cells, growing in suspension, the cells migrated into the lower chamber were stained with Calcein AM and fluorescence was measured. For HP75 cells non-migratory cells were mechanically removed from the upper side of the membrane with cotton swabs whereas migratory cells were stained with Crystal Violet solution, extracted with 10% acetic acid, and measured at 560 nm. To measure cell invasion, the upper chamber of a transwell insert was coated with 0.025 µM of Collagen IV.
- **Wound healing assay:** Two cross-shaped scratches were introduced to the confluent monolayer of HP75 cells with a sterile pipette tip. After PBS washing, cells were incubated in a complete medium BIM53097. Pictures of the four cross points per well were taken immediately after scratch ($t = 0$) and after 18h ($t = 18$) at 5X magnification and the percentage of the open area was analyzed with the TScratch software for automated image analysis.
- **Western Blot Analysis:** Total proteins were extracted, separated on SDS/polyacrylamide gels and transferred to a nitrocellulose filter. P-cofilin (Ser3) or cofilin antibody (1:1000) and an anti-rabbit HRP-linked antibody were used to detect levels of P-cofilin and total cofilin. Chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Upland, CA) and analyzed using the image analysis program NIH ImageJ.
- **Cell transfection:** To generate expression vector coding for wild-type or mutated cofilin, human wild type cofilin cDNA was subcloned into Sac1/Kpn1 cloning site of pGFP-N3 expression vector. Point mutations were introduced into cofilin cDNA by PCR-based mutagenesis replacing Ser3 with Ala (S3A) or Asp (S3D). cDNA were inserted into pGFP-N3 in frame with the GFP coding sequence, with no intervening in-frame stop codons. Transient transfections of mutated cofilins were performed in HP75 cultured cells using Lipofectamine 3000 reagent. For wound healing assay, cells were transfected for 24h in p6 well and then transferred in p24 well at a density of 50000 cells/well.

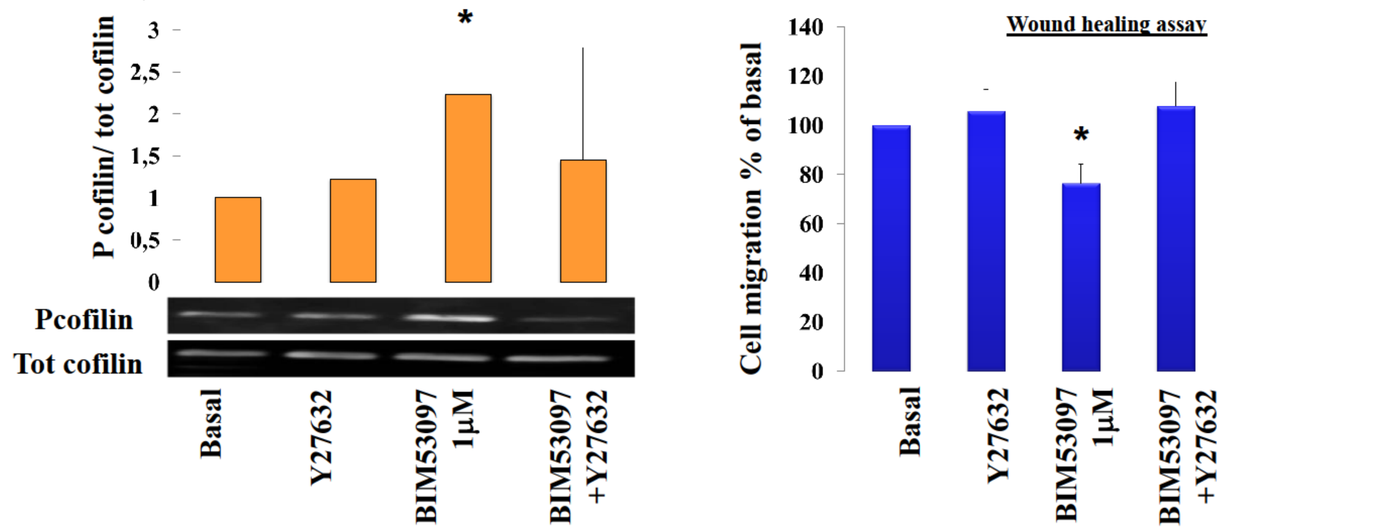
1. DRD2 agonist reduced NFPA cells migration and invasion



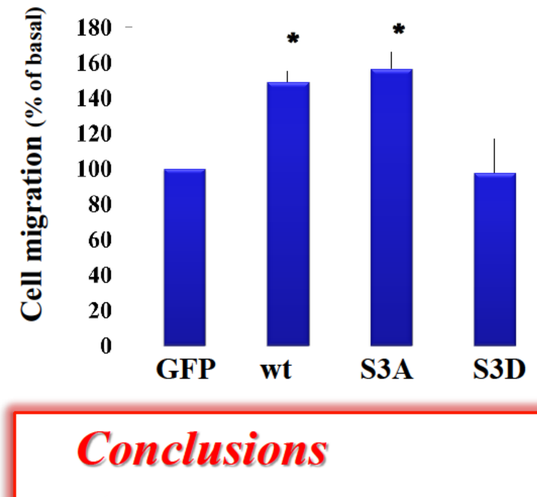
2. DRD2 agonist increased cofilin phosphorylation in NFPA cells



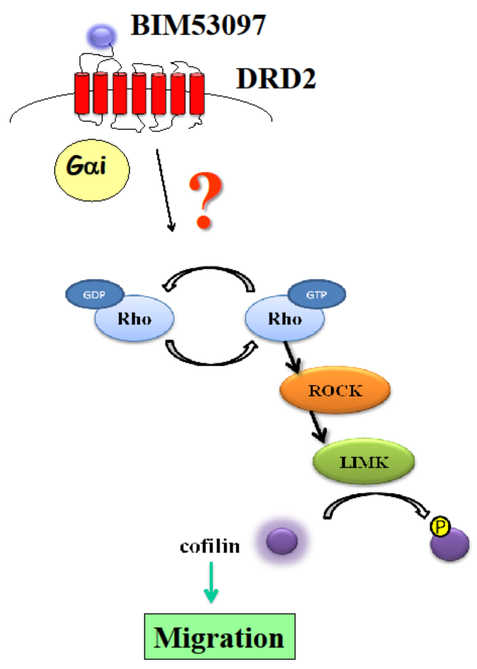
3. ROCK inhibitor Y27632 reversed the ability of BIM53097 to increase cofilin phosphorylation and to reduce cell migration

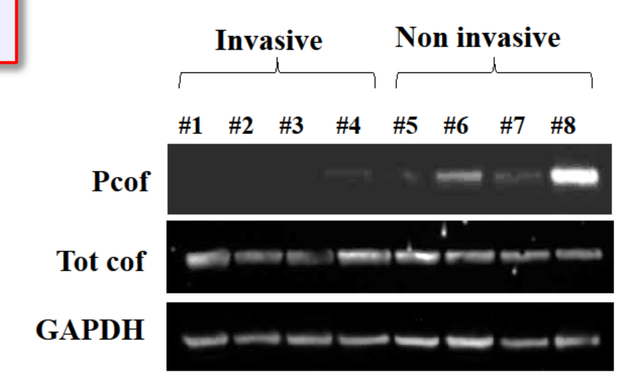


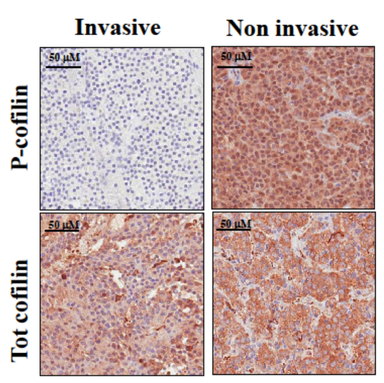
4. The overexpression of wt or phospho-deficient (S3A) cofilin increased HP75 cell migration

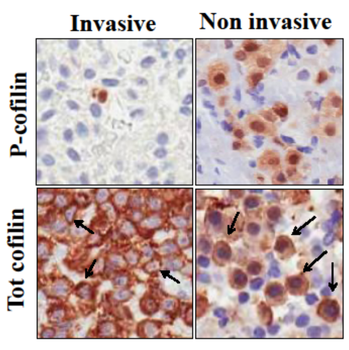


5. Cofilin phosphorylation is reduced in invasive NFPAs









Conclusions

- ✓ **DRD2 agonist reduces migration and invasion of NFPA cells through a molecular mechanism that involves ROCK-dependent phosphorylation of cofilin**
- ✓ **NFPA invasiveness is associated with low phosphorylation levels of cofilin, suggesting that cofilin phosphorylation status might be a molecular marker associated with the invasive behaviour of NFPAs**

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