

# THE GLOBAL mTOR INHIBITOR TORIN1 IS MORE EFFECTIVE THEN THE mTORC1 INHIBITOR, EVEROLIMUS, ALONE OR IN COMBINATION WITH HDACi, IN SUPPRESSING NEUROENDOCRINE TUMORS CELL PROLIFERATION

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**Background:** RAD001 (everolimus) blocks proliferation and interacts with the PI3K/Akt/mTOR pathway in different neuroendocrine tumour (NETs) cell lines; however, its effects are limited by non-targeting mTORC2, which is responsible for the compensatory activation of Akt and development of resistance to mTORC1 inhibitors. Torin1, a globally inhibitor of both mTORC1 and mTORC2, seems to impair cell proliferation to a greater degree than rapamycin; however, its effects in NET cells are largely unknown. Histone deacetylases inhibitors (HDACi) represent a new class of anti-cancer agents, based on potency and specific HDAC target of inhibition.

**Aims:** To examine the effects of Torin1 vs. everolimus, alone or in combination with HDACi, on cultured NET cells.

**Methods:** Two NETs cell lines (BON1 and RIN) as well as cells extracted from human NETs after surgical excision were treated with everolimus, Torin1 and HDACi. Proliferation assays were used to determine the effects of the drugs on cell proliferation. Western blotting was used to analyze the expression of p-Akt, cyclin D1, cyclin D3, p27 and cleaved PARP, HIF 1- $\alpha$ , VEGF.

**Results:** Treatment of NETs cells with HDACi (AN-7 and LBH589) inhibited cell viability with a greater effect observed with LBH589. Incubation of NETs cells with everolimus resulted in a significant dose-dependent decrease in viable cell number. Incubation of cells with everolimus 50nM in combination with AN-7 (80mM) or LBH589 (10nM) exerted a greater decrease in viable cell number (up to 50% decrease;  $P < 0.0001$ ) than either of the drugs individually. This effect was also observed in cultured cells derived from two patients with NETs. Torin1 significantly inhibited cell viability to a greater degree than everolimus in combination with HDACi (up to 65%;  $p < 0.0001$ ).

**Conclusions:** In NETs cells, Torin1 shows a greater inhibitory effect on tumor cell proliferation than did the combination of everolimus with an HDACi.

## ACTIVATION AND ROLE OF PI3K AND PI4K IN MAPK ACTIVATION DURING GnRH ACTIONS

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Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide that serves as a key regulator of the reproductive system. Interaction of GnRH with its receptor (GnRHR), which is a G-protein coupled receptor (GPCR), leads to intracellular mechanisms that include activation of Mitogen-activated protein kinase (MAPK) cascades to mediate the expression of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Here we describe the role of PI3K and PI4K in GnRH-induced ERK1/2 phosphorylation in the  $\alpha$ T3-1 and L $\beta$ T2 gonadotrope cell lines. Therefore, we incubated  $\alpha$ T3-1 and L $\beta$ T2 cells for 1h with Wortmannin (WT) at 10nM and 10 $\mu$ M or LY294002 (LY) at 10 $\mu$ M and 100 $\mu$ M, doses known to inhibit PI3K and PI4K respectively, before stimulation with GnRH (100nM) or PMA (100nM) for 10min. Wortmannin gave a significant inhibition of ERK1/2 activation by GnRH or PMA at the two doses examined, with a more pronounced inhibition observed in the more mature L $\beta$ T2 cells. LY294002 also gave a significant inhibition of ERK1/2 activation by GnRH at the two doses examined in L $\beta$ T2 cells. For further examination we co-transfected  $\alpha$ T3-1 cells with a dominant negative (DN) form or wild-type (wt) of PI4K110 (PI4KIII $\beta$ ) with ERK-GFP. Indeed, while the wt-PI4K had no significant effect, the DN-PI4K markedly reduced the effect of GnRH on ERK1/2 phosphorylation. Finally, we examined GnRH-induced Akt activity which is a downstream effector of PI3K target. Therefore,  $\alpha$ T3-1 cells were treated with GnRH (100nM) for increasing period until 240min. The basal phosphorylation of Akt in both sites was markedly high, reduced rapidly within 5min of stimulation and remained low for 60min (Ser473) and for 15min (Thr308). After 240min of stimulation the level of phosphorylation returned almost to its basal level at both sites. Hence, we conclude that PI3K and PI4K seem to play a role in GnRH-induction of ERK1/2 activation in pituitary gonadotrope cells.

## **mTOR INHIBITOR TORIN1 INDUCES ANTIPROLIFERATIVE EFFECTS IN MtT/E CELL LINE AND HUMAN PITUITARY TUMORS**

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As for many tumor types, it has been shown that the Akt pathway is overexpressed and activated in human pituitary tumors. Thus, pituitary tumors may be sensitive to the anti-proliferative effects of mTOR inhibitors. However, non-functioning pituitary tumors are rapamycin-resistant. Torin1, second-generation ATP-competitive mTOR kinase inhibitor (TKI), suppresses both mTORC1 and mTORC2 complexes. To evaluate the *in vitro* effects of mTOR inhibitor Torin1 on pituitary cells, a rat non-secreting pituitary tumor cell line, MtT/E, and human non-functioning pituitary adenoma (NFGPA) cells were used.

Treatment of MtT/E cells with Torin1 induced a significant dose- and time-dependent decrease of cell viability and cell number. Incubation of cells from four NFGPAs with Torin1 significantly reduced the number of viable cells by 25-45%. The anti-proliferative effects of Torin1 on pituitary tumor cells were found to be mediated by G0/G1 cell cycle arrest associated with cyclin D1 and cyclin D3 suppression, apoptosis reflected by increased fraction of cleaved caspase and subG1 events and autophagy tested with an autophagy marker, LC3. Expression of phosphorylated-p70S6K and phosphorylated-Akt was significantly reduced by Torin1. Interestingly, the protein expression of the negative regulator of PI3K, the PTEN phosphatase, was significantly decreased by Torin1 in MtT/E cells.

Our results show that Torin1 potently inhibits pituitary cell proliferation suggesting that TKIs may be a promising antiproliferative therapy for pituitary adenomas. This therapeutic manipulation may have beneficial effects particularly for patients harboring invasive pituitary tumors unresponsive to current treatments.

## CRF RECEPTOR TYPE 2 ACTIVATION IN THE VMH IS REQUIRED FOR ENERGY BALANCE REGULATION FOLLOWING METABOLIC CHALLENGES

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**Background:** Corticotropin-releasing factor (CRF) and its related Urocortins are key regulators of energy balance. Stressful stimuli, food deprivation and leptin administration remarkably alter hypothalamic CRF type 2 receptor (CRFR2) expression, suggesting an important role for this receptor in regulating energy homeostasis during challenge.

**Methods:** To examine the role of CRFR2, expressed by the ventromedial hypothalamus (VMH), in modulating energy balance, a lentiviral-based system for site-specific knockdown (KD) of CRFR2 was established and VMH-specific CRFR2 KD mice were generated. Mice were tested both on basal conditions and following exposure to physiological perturbations to homeostasis.

**Results:** Reduced expression of VMH-CRFR2 did not affect basal metabolic parameters suggesting that under basal state VMH-CRFR2 does not play a crucial role in maintaining energy homeostasis. In the 24h period following food deprivation challenge, in order to regain energy homeostasis, control mice increased their food intake and reduced their physical activity. In contrast, CRFR2 KD mice increased their food intake only up to 75% of the control mice and maintained similar activity levels. Meal structure analysis showed that CRFR2 KD mice failed to increase their meals number and to prolong their meal duration. Moreover, CRFR2 KD mice showed reduced respiratory exchange ratio during the light phase compared to control group. This maladaptive recovery suggests that hypothalamic CRFR2 signaling is essential for re-establishing homeostasis following metabolic challenge. In addition, insulin tolerance test revealed reduced insulin sensitivity in CRFR2-KD mice which could be due to reduced suppression of the counterregulatory responses.

**Conclusions:** Our results support an important role for VMH-CRFR2 neurons in the control of food intake and energy expenditure in response to homeostatic challenge and suggest a role for these neurons in glucose sensing.

## MIRNA ABLATION IN POMC NEURONS LEADS TO SEVERE OBESITY AND GLUCOCORTICOID DEFICIENCY

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MicroRNAs have important roles in neuronal differentiation and survival, as well as in neuronal plasticity in the mature nervous system. To investigate the role of miRNA in the central hub connecting stress response and appetite regulation, we generated a transgenic mouse model in which Dicer was specifically deleted from POMC expressing neurons, using a cre-lox system. On PND1, POMC-mRNA expression is similar in wild type and Dicer-KO mice but at six weeks of age no POMC neurons could be detected in the arcuate nucleus by ICH, indicating that miRNAs are essential for survival of these neurons. Similarly, POMC-mRNA expression in the hypothalamus of Dicer-KO mice was reduced by 96% from the levels in WT animals ( $p=0.0009$ ). AgRP, Leptin receptor and NPY mRNA levels were reduced by 87% ( $p=0.003$ ), 32% ( $p = 0.02$ ), and 26% ( $p=0.09$ ) respectively. POMC and CRFR1 mRNA levels were undetectable in the anterior pituitary gland of Dicer-KO mice. Consequently, basal and stress-induced corticosterone levels were undetectable in these mice. Weight gain and fat mass were significantly more prominent in Dicer-KO mice: 15-20 week- old Dicer  $-/-$  and  $+/-$  males weighted  $42\pm 2.9$  and  $32 \pm 0.5$ g respectively ( $p=0.003$ ) and had  $21.1\pm 3.4$  % and  $9.3 \pm 0.9$ % fat mass ( $p=0.0029$ ). Although free thyroxine levels were similar between the groups, total tri-iodothyronine levels were significantly higher in the Dicer-KO ( $45.4\pm 3.7$  ng/ml) in comparison to wild type mice ( $32.7\pm 4.4$  ng/ml;  $p= 0.017$ ). Dicer-KO were glucose intolerant, responding with higher glucose levels after a glucose load ( $357\pm 34$  vs.  $247\pm 13$  mg% at 30 min ( $p=0.007$ ). Nevertheless, total ( $p =0.009$ ), HDL ( $p=0.003$ ) and LDL ( $p=0.001$ ) cholesterol levels were significant lower in Dicer-KO, whereas there were no differences in triglyceride values. In conclusion, postnatal POMC neuronal death due to microRNA ablation leads to development of obesity and increased fat mass despite glucocorticoid deficiency.

## **GnRH-STIMULATION OF GONADOTROPIN A SUBUNIT (GSU) GENE EXPRESSION VIA CALCINEURIN INVOLVES ACTIVATION OF A NFAT TRANSCRIPTION FACTOR AND THE TORC COACTIVATOR**

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GnRH induces gonadotropin gene transcription through activating various protein kinases and increasing levels of intracellular calcium which activates calmodulin. We previously reported that GnRH also activates the calcium/calmodulin-dependent phosphatase, calcineurin, and in this study we investigated its role in transcription of the gonadotropin  $\alpha$  subunit (GSU) gene. Calcineurin over-expression is sufficient to induce promoter activity, and is required for both basal activity and the GnRH effect. We mapped the calcineurin responsive region to within 80 bp, which includes a region, comprising TTTCCTGTT, previously reported to be important for basal and GnRH-induced promoter activity, although the binding factor was never identified. To clarify which calcineurin target is responsible for transducing these effects, we examined Nuclear Factor of Activated T-cells (NFAT) transcription factors which bind a TTTC sequence, and saw that GnRH induces nuclear import of NFAT3 in a calcineurin-dependent manner. NFAT3 was detected at the GSU promoter only after GnRH treatment, and its knock-down substantially reduced GnRH-stimulated GSU promoter activity. In some cells, calcineurin also targets the CREB coactivator, Transducer of Regulated CREB activity (TORC), and we found that TORC1 over-expression induced GSU promoter activity, while its knock-down abolished the GnRH response. However, activation of the GSU by GnRH was not affected by over-expression of dominant negative CREB, suggesting that a different factor is responsible for TORC recruitment to this promoter. Although we expected GnRH to induce nuclear accumulation of TORC, this did not occur, and TORC appeared to shuttle between the cytoplasm and nucleus constantly. However GnRH did induce changes in the TORC protein, inducing an initial degradation of TORC, much of which is already N-terminal truncated, and facilitating a transcription-independent increase in levels of intact TORC. As the N-terminus of TORC was previously shown to interact with various transcription factors, this likely represents a means of TORC activation, through a mechanism that has yet to be elucidated but appears to involve calpain cleavage and the proteasome.

## ACTH SECRETING PITUITARY MACROADENOMAS: OUTCOME OF MULTIMODAL THERAPY

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**Introduction:** Cushing's disease is rare, with an incidence of 1/1,000,000. It is commonly associated with a pituitary microadenoma, however, 4-10% of patients have a macroadenoma. Patients with macroadenomas have higher plasma ACTH levels, reduced suppression after dexamethasone and lower surgical cure rates, necessitating radiation and medical therapies.

**Aim of the study:** To evaluate the biochemical and anatomical presentation of pts with ACTH secreting pituitary macroadenomas, the need of different therapeutic options and the long- term remission rates.

**Methods:** Multicenter retrospective study. Clinical, biochemical, radiological and therapeutic data were retrieved from charts.

**Results:** Twelve patients (5 males and 7 females) with ACTH secreting pituitary macroadenomas (mean size  $27.7 \pm 10.3$  mm) were included. Mean age at diagnosis was  $41.4 \pm 11.7$  y and mean follow up was  $6.5 \pm 4.2$  y (range 2-15 y). At diagnosis, mean plasma ACTH and mean urinary free cortisol were  $21.6$  pmol/L  $\pm 15.6$  (nl:2-10.1) and  $1185 \pm 1616$  nmol/day (nl:20-208 nmol/day), respectively. Three patients had evidence of visual field defects, and 5/12 patients had partial hypopituitarism. Transphenoidal surgery was the primary therapy in 11/12 pts and medical treatment with pasireotide in one. Postoperative remission rate was 36% (4/11 pts). Reoperation was performed in one patient. Six patients were referred to pituitary radiotherapy, two had conventional radiotherapy and 4 had stereotactic radiotherapy.

**Conclusions:** Transsphenoidal surgery alone frequently fails to cure Cushing's disease caused by ACTH secreting pituitary macroadenomas, therefore, postoperative pituitary irradiation and/or medical therapy is often necessary and effective.