

**Progress report for ESA Research Higher Degree Scholarship  
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Project title: PPAR $\gamma$  and XIAP as potential targets for combination treatment of ovarian granulosa cell tumours

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Hypothesis: XIAP antagonism sensitises GCT cells to PPAR $\gamma$ -mediated apoptosis

Aims:

1. Analyse PPAR $\gamma$  and XIAP protein expression and clinicopathological features in GCT, other ovarian cancers and post-menopausal ovaries using tissue microarray and immunohistochemistry
2. Identify novel PPAR $\gamma$ -regulated genes in GCT and their contribution to inhibiting apoptosis and/or differentiation
3. Identify proteomic changes upon PPAR $\gamma$  activation and XIAP inhibition

Background:

Granulosa cell tumours (GCT) of the ovary are the most common type of ovarian sex-cord stromal tumours which contribute to approximately 5% of all ovarian cancers<sup>1</sup>. This study focuses on the adult GCT which are more common among the two subtypes – adult GCT (95%) and the rare juvenile GCT (5%). Clinical manifestations of these tumours include endocrine symptoms such as precocious breast development (in prepubertal females), menstrual irregularities (in reproductive age group) and postmenopausal vaginal bleeding<sup>1</sup>. Patients who present with early-stage disease have a good prognosis and are managed by surgical removal of the tumour. However, the characteristic indolent growth and late relapse of GCT and the lack of efficacious treatment modalities for those with advanced or recurrent tumours contribute to a high mortality rate of greater than 80%<sup>1</sup>.

GCT exhibit a phenotype similar to that of proliferating granulosa cells (GC) of the preovulatory follicle. This includes the expression of functional FSH receptors with FSH binding, and synthesis of estrogen and inhibin<sup>2</sup>. Given that GCT are endocrine tumours, our laboratory has screened for the expression of 48 nuclear receptors (NR) in these tumours<sup>3</sup>. Surprisingly, overexpression was observed in a NR normally associated with terminal differentiation and apoptosis, the peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ )<sup>3</sup>. PPAR $\gamma$  binds to DNA at specific sites as obligate functional heterodimers with RXR $\alpha$  which is also expressed in GCT and cell lines. Overexpression of PPAR $\gamma$  has also been reported in other human malignancies<sup>4</sup>. PPAR $\gamma$  agonists, such as the thiazolidinediones, including rosiglitazone (RGZ) and pioglitazone, are clinically used as anti-diabetic drugs. In addition, the effects of TZD-induced growth arrest and apoptosis on various cancers are currently being

explored in clinical trials<sup>4</sup>. In two GCT-derived cell lines, KGN and COV434, we observed no changes in PPAR $\gamma$ -mediated transactivation using a PPAR $\gamma$  responsive reporter and treatment with RGZ and retinoic acid (RA; ligand for RXR $\alpha$ ). Our subsequent studies have revealed that this is due to constitutive NF- $\kappa$ B activity in GCT cells (Chu *et al*, unpublished observations).

The NF- $\kappa$ B signalling pathway plays an important role in apoptosis, inflammation, immunity and cancer. In GCT, not only does constitutive NF- $\kappa$ B signalling transrepress PPAR $\gamma$  activity, but it also upregulates one of its effector proteins, the X-linked inhibitor of apoptosis protein (XIAP)<sup>5</sup>. XIAP is the most potent caspase inhibitor among the eight members of the Inhibitor of Apoptosis Protein (IAP) family, of which the baculovirus IAP repeat (BIR) domain is well conserved. XIAP consists of three BIR domains and a RING (Really Interesting New Gene) domain. The BIR domains of XIAP directly bind the caspases; BIR2/caspase-3 and -7 binding<sup>6</sup>, BIR3/caspase-9 binding<sup>7</sup>. In addition, the RING domain exerts E3 ubiquitin ligase activity and mediates degradation of caspase-3 and -9 to inhibit apoptosis<sup>8</sup>. Thus XIAP can inhibit both the intrinsic and extrinsic apoptotic pathways. Regulation of XIAP expression is via an endogenous XIAP inhibitor, the second mitochondrial activator of caspases (Smac), which binds and blocks the BIR2 and BIR3 domains to inhibit their caspase-binding activity. Since the inhibition of apoptosis is a critical mechanism of tumorigenesis, it has been suggested that overexpression of XIAP leads to heightened anti-apoptotic activity, thus playing an important role in the oncogenic process. It has thus become an attractive therapeutic target<sup>5</sup>. There are XIAP inhibitors, embelin and smac mimetics (SM) (synthetic analogues of the endogenous Smac) currently in clinical trials for various cancers<sup>5</sup>.

Using two GCT-derived cell lines, KGN and COV434 as *in vitro* models of GCT, we have shown that inhibition of NF- $\kappa$ B (using BAY11-7082), removes NF- $\kappa$ B transrepression and restores PPAR $\gamma$ -mediated transactivation with combined RGZ and RA treatment. My current research has demonstrated the same effect when XIAP is inhibited using either embelin or SM and PPAR $\gamma$  is activated with RGZ/RA. Furthermore, we observed a significant reduction in cell viability and proliferation as well as a significant induction in cell apoptosis with combined PPAR $\gamma$  activation and XIAP or NF- $\kappa$ B inhibition. It is important to note that singular treatment with any of the agents does not have any effects on cell apoptosis or proliferation. Given that PPAR $\gamma$  is an anti-proliferative transcription factor in GC, it is of prime interest to explore the mechanism by which PPAR $\gamma$  can cause apoptosis in GCT.

#### Aim 1: Tissue microarray (TMA) and immunohistochemistry (IHC)

*Research Strategy:* To determine whether PPAR $\gamma$  and XIAP expression can provide prognostic and/or diagnostic information for GCT, an evaluation of the cellular localisation of PPAR $\gamma$  and XIAP will be performed using TMA and IHC. The TMA contain duplicate tissue cores from 79 GCT and epithelial ovarian carcinoma (EOC) (n = 34), as well as histologically normal ovaries (n = 5) and benign ovarian diseases (n = 7). I will perform IHC on the human TMA using two independent antibodies for both PPAR $\gamma$  and XIAP. Intensity of staining will be graded arbitrarily as 0 (no), 1+ (weak), 2+ (moderate) or 3+ (strong) using the Aperio ImageScope v11.2.0.780 software. The results will be analysed with other clinicopathological features, such as

stage, grade of tumour, histology (poorly differentiated vs well-differentiated), age at diagnosis and menopausal status, by multivariate analysis and univariate Cox proportional hazards regression analysis. Additionally, correlation with expression for cIAP1, cIAP2, survivin and XIAP target proteins, caspase-3, -8 and -9, will be determined.

*Progress to date:* Using IHC and TMA, I have generated the first systematic review of XIAP, cIAP1 and cIAP2 protein expression across a variety of ovarian pathologies including GCT, EOC and normal post-menopausal ovary (manuscript in preparation). In GCT and EOC, XIAP was predominantly expressed, as compared to cIAP1 and cIAP2. In contrast, I saw minimal immunostaining for XIAP in benign ovarian pathologies and normal post-menopausal ovary. This suggests a possible link between malignancy and XIAP expression levels for GCT and EOC. Further analysis of clinicopathological features will give a more informative illustration of the prognostic significance of XIAP overexpression. Similarly, I observed strong immunostaining for PPAR $\gamma$  in GCT and EOC but not in benign ovarian cancers or post-menopausal ovary.

#### Aim 2: Gene expression and signalling pathway analysis

*Research Strategy:* By removing the constraints of NF- $\kappa$ B transrepression, we have shown that treatment with combined PPAR $\gamma$ /RXR $\alpha$  ligands may be of therapeutic benefit. Given that PPAR $\gamma$  is an anti-proliferative transcription factor in GC, I will explore the gene expression signature upon activation of PPAR $\gamma$  in GCT cells to better understand the molecular mechanism by which PPAR $\gamma$  can cause apoptosis in GCT cells in the KGN and COV434 cells lines. Cells will be treated with BAY11-7082 (NF- $\kappa$ B inhibition) alone, or in combination with RGZ and RA (PPAR $\gamma$  and RXR $\alpha$  activation). RNA will be extracted from the cells for cDNA microarray analyses using Agilent Whole Genome Microarray. Regulated genes will be subjected to further analysis based on (i) the magnitude of response, (ii) expression abundance and (iii) functional information. Pathway analysis will be applied for determining known structural or functional groups. Changes in gene expression will be validated using qRT-PCR and Western blot analysis in the cell lines. The functional significance of prioritised PPAR $\gamma$ -regulated genes will be determined by examining (i) gene expression profiles, (ii) IHC, (iii) gene knock-down or re-expression and (iv) signalling pathway analysis using reporter activity assays.

*Progress to date:* We have treated KGN and COV434 cell lines with BAY11-7082 (NF- $\kappa$ B inhibition) alone, or in combination with RGZ and RA (PPAR $\gamma$  and RXR $\alpha$  activation). RNA has been extracted from the cells for subsequent cDNA microarray analyses using Agilent Whole Genome Microarray at the Monash Health Translation Precinct. It is anticipated this analysis will be performed in December 2015.

#### Aim 3: Stable isotope labelling with amino acids in cell culture (SILAC)

*Research Strategy:* Given the previous findings that combined PPAR $\gamma$  activation and XIAP inhibition induces apoptosis in GCT cells, we sought to identify PPAR $\gamma$  and XIAP regulated proteins which could be made potential therapeutic targets using the novel SILAC proteomic approach. SILAC involves culturing cells in arginine- and

lysine-free media supplemented with light or heavy arginine and lysine isotopic peptides. Isotopic tags are incorporated into proteins which allow relative quantification by mass spectrometry. KGN and COV434 will be treated with PPAR $\gamma$  agonists and XIAP inhibitors either alone or in combination. Additionally, KGN and COV434 cells expressing caspase binding mutants of XIAP will also be used in this analysis. The cell lysates will be mixed together, digested with trypsin and analysed in-house using Orbitrap mass spectrometer. Differentially expressed proteins will be identified using the MASCOT protein database. The functional significance of differentially expressed proteins will be further examined as mentioned previously.

*Progress to date:* After culturing KGN and COV434 in media containing either heavy or light arginine and lysine, there was greater than 99% incorporation of these heavy or light amino acids in the two cell lines. Cells labelled with heavy amino acids have been treated for 24 hours with SM or embelin (XIAP inhibition) in combination with RGZ and RA (PPAR $\gamma$  and RXR $\alpha$  activation), with cells labelled with light amino acids used as vehicle treated control. A reciprocal approach has also been performed. Using Orbitrap mass spectrometer analysis at the Adelaide Proteomics Centre, I have identified 32 upregulated and 21 downregulated proteins after the combined treatment of XIAP inhibition and PPAR $\gamma$  activation. The protein that showed the greatest fold increase in expression was acyl-CoA desaturase. I have subsequently validated this finding using both western blots for protein and RT-PCR for mRNA, and found that the combination treatment increase the expression at both protein and message level, consistent with the mass spectrometry findings. I am currently investigating the mRNA expression of other candidates using digital QPCR (Fluidigm Biomark HD System). Further, the analysis of the regulated proteins also identified several candidate proteins which potentially can be therapeutically targeted. Validation of these regulated proteins is currently being pursued. In order to determine if these are potential therapeutic targets, I will examine the effects of inhibitors or agonists to these candidates in KGN and COV434 cells.

Awards / presentations during scholarship period

**Novartis Junior Scientist Award**, Endocrine Society of Australia (ESA), Australia

- **Leung DTH**, Fuller PJ, Chu S. Combined PPAR $\gamma$  and XIAP treatment sensitises granulosa cell tumours to PPAR $\gamma$ -mediated apoptosis. 58<sup>th</sup> Annual Scientific Meeting of the Endocrine Society of Australia, Adelaide, Australia 2015. *id #27606*

**Poster Competition – 2<sup>nd</sup> Prize in Endocrinology (Basic Science)**, Research Week, Monash Health and Monash Health Translational Precinct, Australia

- **Leung DTH**, Chu S, Fuller PJ. PPAR $\gamma$  agonists augment anticancer effects of XIAP inhibition on human granulosa cell tumour-derived cells. Research Week 2015, Monash Health and Monash Health Translational Precinct, Melbourne, Australia. *PO15*

**Leung DTH**, Fuller PJ, Chu S. PPAR $\gamma$  and XIAP as potential targets for combination treatment of ovarian granulosa cell tumours. Australia Society for Medical Research (ASMR) Student Research Symposium, Melbourne, Australia 2015. *POS50*

## Publications

- Alexiadis M, Chu S, **DTH Leung**, Gould J, Jobling T, Fuller PJ. Transcriptomic analysis of stage I versus advanced adult granulosa cell tumours. *Oncotarget* – reviewed; acceptance considered subject to revision (Nov 2015)
- **DTH Leung**, Fuller PJ, Chu S. Impact of FOXL2 mutations on signalling in ovarian cancer. *International Journal of Biochemistry and Cell Biology* – currently under review (Nov 2015)

## Manuscript in preparation (first author):

- Immunohistochemical analysis of XIAP and PPAR $\gamma$  in ovarian tissues
- Gene expression and proteomic analysis of GCT-derived cells following XIAP inhibition and PPAR $\gamma$  activation

## References

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2. Fuller PJ, Chu S. Signalling pathways in the molecular pathogenesis of ovarian granulosa cell tumours. *Trends in endocrinology and metabolism: TEM.* 2004 Apr;15(3):122-8.
3. Alexiadis M, Eriksson N, Jamieson S, Davis M, Drummond AE, Chu S. Nuclear receptor profiling of ovarian granulosa cell tumors. *Horm Cancer.* 2011;2:13.
4. Smallridge RC, Copland JA, Brose MS, Wadsworth JT, Houvras Y, Menefee ME, et al. Efatutazone, an oral PPAR-gamma agonist, in combination with paclitaxel in anaplastic thyroid cancer: results of a multicenter phase 1 trial. *The Journal of clinical endocrinology and metabolism.* 2013 Jun;98(6):2392-400.
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6. Lukacs C, Belunis C, Crowther R, Danho W, Gao L, Goggin B, et al. The structure of XIAP BIR2: understanding the selectivity of the BIR domains. *Acta crystallographica Section D, Biological crystallography.* 2013 Sep;69(Pt 9):1717-25.
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8. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science.* 2000 May 5;288(5467):874-7.