12-Month Report for ESA Research Higher Degree Scholarship
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Name: Tan Hung (Dilys) Leung

Institution: Hudson Institute of Medical Research and Monash University, VIC, Australia

Supervisors: Dr Simon Chu, Professor Peter Fuller

Project title: PPARγ and XIAP as potential targets for combination treatment of ovarian granulosa cell tumours

Hypothesis: XIAP antagonism sensitises GCT cells to PPARγ-mediated apoptosis

Aims:

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

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 (2). Given that GCT are endocrine tumours, our laboratory has screened for the expression of 48 nuclear receptors (NR) in these tumours (3). Surprisingly, overexpression was observed in a NR normally associated with terminal differentiation and apoptosis, the peroxisome proliferator-activated receptor-gamma (PPARγ) (3). PPARγ binds to DNA at specific sites as obligate functional heterodimers with RXRα which is also expressed in GCT and cell lines. Overexpression of PPARγ has also been reported in other human malignancies

(4). PPARγ 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 (4). In two GCT-derived cell lines, KGN and COV434, we observed no changes in PPARγ-mediated transactivation using a PPARγ responsive reporter and treatment with RGZ and retinoic acid (RA; ligand for RXRα). Our subsequent studies have revealed that this is due to constitutive NF-κB activity in GCT cells (Chu *et al*, unpublished observations).

The NF-κB signalling pathway plays an important role in apoptosis, inflammation, immunity and cancer. In GCT, not only does constitutive NF-κB signalling transrepress PPARγ activity, but it also upregulates one of its effector proteins, the X-linked inhibitor of apoptosis protein (XIAP) (5). 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 (6), BIR3/caspase-9 binding (7). In addition, the RING domain exerts E3 ubiquitin ligase activity and mediates degradation of caspase-3 and -9 to inhibit apoptosis (8). 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 (5). There are XIAP inhibitors, embelin and smac mimetics (SM) (synthetic analogues of the endogenous Smac) currently in clinical trials for various cancers (5).

Using two GCT-derived cell lines, KGN and COV434 as *in vitro* models of GCT, we have shown that inhibition of NF-κB (using BAY11-7082), removes NF-κB transrepression and restores PPARγ-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 PPARγ 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 PPARγ activation and XIAP or NF-κB 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 PPARγ is an anti-proliferative transcription factor in GC, it is of prime interest to explore the mechanism by which PPARγ can cause apoptosis in GCT.

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

Research Strategy: To determine whether PPARγ and XIAP expression can provide prognostic and/or diagnostic information for GCT, an evaluation of the cellular localisation of PPARγ 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 PPARγ 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 PPARγ 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-κB transrepression, we have shown that treatment with combined PPARγ/RXRα ligands may be of therapeutic benefit. Given that PPARγ is an anti-proliferative transcription factor in GC, I will explore the gene expression signature upon activation of PPARγ in GCT cells to better understand the molecular mechanism by which PPARγ can cause apoptosis in GCT cells in the KGN and COV434 cells lines. Cells will be treated with BAY11-7082 (NF-κB inhibition) alone, or in combination with RGZ and RA (PPARγ and RXRα 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 PPARγ-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-κB inhibition) alone, or in combination with RGZ and RA (PPARγ and RXRα activation). RNA has been extracted from the cells for subsequent cDNA microarray analyses using Agilent Whole Genome Microarray at the Monash Health Translation Precinct. This analysis is currently being pursued.

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

Research Strategy: Given the previous findings that combined PPARγ activation and XIAP inhibition induces apoptosis in GCT cells, we sought to identify PPARγ 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 PPARγ 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 spectrometor. 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. I will also investigate the mRNA expression of other candidates using digital QPCR (Fluidigm Biomark HD System). This is a high-throughput digital PCR analysis which profiles the expression of 48 genes (including 3 housekeeping genes) in 48 samples (including 2 no RT control). The genes of interest comprise of those that are involved in metabolism, apoptosis, cell cycle and steroidogenesis.

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 (PPARγ and RXRα 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 PPARγ activation. The protein that showed the greatest fold increase in expression was acyl-CoA desaturase. I have subsequently validated this finding using western blots for protein detection, and found that the combination treatment increases the expression at the protein level, consistent with the mass spectrometry findings.

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. One of these proteins is fascin, an actin-bundling protein which is implicated in cell motility and tumour metastasis (9). In order to determine if fascin is a potential therapeutic target, I will examine the effects of a fascin inhibitor to this protein in KGN and COV434 cells.

Utilizing the Fluidigm Biomark HD System, I have observed a significant upregulation of metabolic genes such as *SCD* and *ACSL3* following the combined XIAP and PPARγ treatment. Similarly, apoptosis induced by the combined treatment was confirmed by the upregulation of pro-apoptotic genes such as *BNIP3L*.

Given PPARγ has an important role in metabolism and the proteomic changes I observed from the SILAC study, it has led us to investigate the metabolic consequences of the combined treatment. I have optimised the cell numbers and the

concentration of two metabolic stressors, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and oligomycin, for the Seahorse XF cell energy phenotype test. The cell energy phenotype test assesses the metabolic pathway (mitochondrial respiration or glycolysis) the cells use under basal and stressed conditions. I will also use the Seahorse XF cell mito stress test to measure changes in mitochondria function following the combined treatment.

Awards received during scholarship period (2015 – 2016)

1. **Charitable Grants Program (AUD $3,000),** Australian Chinese Medical Association of Victoria, Australia

2. **MCCC Research Travel Grant (AUD $2,000),** Monash Comprehensive Cancer Consortium, Australia
   - attend US ENDO 2017 in Orlando, Florida and visit to Prof. Ken Korach's laboratory at the National Institute of Environmental Health Sciences in North Carolina

3. **Novartis Junior Scientist Award**, Endocrine Society of Australia (ESA), Australia
   - **Leung DTH**, Fuller PJ, Chu S. Combined PPARγ and XIAP treatment sensitises granulosa cell tumours to PPARγ-mediated apoptosis. 58th Annual Scientific Meeting of the Endocrine Society of Australia, Adelaide, Australia 2015. *id #27606*

4. **Poster Competition – 2nd Prize in Endocrinology (Basic Science)**, Research Week, Monash Health and Monash Health Translational Precinct, Australia
   - **Leung DTH**, Chu S, Fuller PJ. PPARγ 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*

Presentations during scholarship period (2015 – 2016)

1. **Leung DTH**, Fuller PJ, Chu S. Combined PPARγ and XIAP treatment alters invasive properties of ovarian granulosa cell tumours. Research Week, Monash Health and Monash Health Translational Precinct, Melbourne, Australia 2016. *EN4*

2. **Leung DTH**, Fuller PJ, Chu S. Combined PPARγ and XIAP treatment sensitises granulosa cell tumours to PPARγ-mediated apoptosis. Australia Society for Medical Research (ASMR) Student Research Symposium, Melbourne, Australia 2016. *P2A03*

3. **Leung DTH**, Fuller PJ, Chu S. PPARγ 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 during scholarship period (2015 – 2016)

- **Leung DTH**, Fuller PJ, Chu S. Impact of FOXL2 mutations on signalling in ovarian granulosa cell tumours. Int J Biochem Cell B. 72: 51-54.
- Alexiadis M, Chu S, **Leung DTH,** Gould J, Jobling T, Fuller PJ. Transcriptomic analysis of stage I versus advanced adult granulosa cell tumours. Oncotarget. 7(12): 14207-19.
- Fuller, P.J., **Leung, D.T.H.** and Chu, S. Genetics and genomics of ovarian sex-cord stromal tumors. Clinical Genetics. (Epub)

Manuscript in preparation (first author):

- PPARγ activation augments anticancer effects of XIAP inhibition in ovarian granulosa cell tumours
- Regulation of the constitutive NF-κB and AP-1 signalling in in ovarian granulosa cell tumours

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9.   Tan VY, Lewis SJ, Adams JC, Martin RM. Association of fascin-1 with mortality, disease progression and metastasis in carcinomas: a systematic review and meta-analysis. BMC Med. 2013;11:52.