Annual Progress Report

Scholar name: Pui Shi PANG
Institution: The University of Melbourne, Department of Medicine, Austin Health & Northern Health
Award commenced on 25th February 2009
Award ends on 25th February 2011 (duration: 2 years)

Title of research project:
Investigating non-classical signalling pathways of the androgen receptor (AR)

Name of other researchers working on this project:
Helen MacLean, Rachel Davey, Michele Clarke, Jarrod Skinner

Research activities completed in the award period (February 2009 – January 2011)

A)   Laboratory work

1. *In vitro*:
    Ligand binding (Aim 1.1) – Completed 2009
    Second messenger activation (Aim 1.4.1) – Completed 2009
    AR gene and protein expression (Aim 1.5.1) – Completed 2009
    AR nuclear localisation (Aim 1.2) – Completed 2010
2. *In vivo*:
    AR expression (Aim 1.5.2) – Completed 2009
    Classical target gene expression (Aim 1.3.2) – Completed 2010
    Baseline second messenger phosphorylation characterisation (Aim 2.2.1)
    –   Completed 2009
    Baseline non-classical target gene characterisation (Aim 2.3.1) – Completed 2009
    Validation of orchidectomy surgery and androgen administration (Aim 2.1)
    – Completed 2010

B)   Conference presentation:

ESA 2009 oral presentation (Novartis Junior Scientist Award session)
Pang TPS, Davey RA, MacLean HE.
"Investigating non-classical signalling pathways of the androgen receptor."
Proc. Endocrine Society of Australia Annual Scientific Meeting, Volume 52, p.82, 2009.

ESA 2010 oral presentation (Novartis Junior Scientist Award session)
Pang TPS, Davey RA, Michelle MV, MacLean HE.
"Investigating non-genomic signalling pathways of the androgen receptor."
Proc. Endocrine Society of Australia Annual Scientific Meeting, Volume 53, p.80, 2010.

Research activities to be completed in the award period (January 2011 – February 2011)

A)   Laboratory work
1. *In vitro*:
    Classical target gene expression (Aim 1.3)
    Non-classical target gene expression (Aim 1.4.2)
2. *In vivo:*
    Orchidectomy and androgen replacement study (Aim 2.2.2 and 2.3.2)

RNA and protein isolation from tissues of orchidectomised mice
(Aim 2.2.2 and 2.3.2)
Mass of tissues in orchidectomised mice with or without androgen replacement
(Aim 3.1)

B)   Paper
        Pang et al. "A physiological role for non-genomic androgen actions in the absence of androgen receptor deoxyribonucleic acid binding activity" submitted to American Journal of Physiology – Endocrinology and Metabolism in Jan 2011

Research activities to be completed by August 2011

A)   Laboratory work
        1. *In vivo* (orchidectomised mice with or without androgen replacement):
            Non-classical target gene expression (Aim 2.3.2) – expected completion April 2011
            Second messenger phosphorylation (Aim 2.2.2) – expected completion July 2011
            Microarray analyses (Aim 3.3) – expected completion July 2011

B)   Papers
        1. Orchidectomy study (fat) – expected completion July 2011
        2. Orchidectomy study (bone) – expected completion Sep 2011
        3. Orchidectomy study (other tissues) – expected completion July 2011

C)   PhD thesis – August 2011

Progress made on the aims and hypothesis of research project

Aims and hypotheses:
The aim of this project is to investigate the hypothesis that androgen action is mediated through non-classical androgen receptor (AR) pathways in muscle, bone and fat. Androgens are well established to play important roles in both reproductive and non-reproductive tissues in males. Their anabolic actions include increasing muscle strength and bone mineral density [1], and reducing body fat mass [2]. Androgens produce their effects by binding to the AR. However, the mechanisms of AR action are not fully understood. The well-characterised classical AR signalling pathway involves the AR binding to DNA to regulate gene transcription [3]. However, some *in vitro* studies [4, 5] have provided evidence to suggest that the AR can act via alternative signalling pathways, that is non-classical signalling pathways. It has been proposed that the non-classical androgen actions involve phosphorylation of second messengers, such as ERK and CREB [5], and regulation of target gene transcription in the absence of AR directly binding to DNA [6]. Given that the physiological relevance of non-classical AR signalling remains unknown, my project aims to study the non-classical AR signalling *in vitro* and *in vivo* and to show the physiological importance of non-classical androgen actions *in vivo*.

I hypothesise that androgens act via both the classical and non-classical pathways *in vivo* and that the non-classical AR signalling pathways play an important physiological role in androgen-mediated actions in muscle, bone and fat. I will test this hypothesis using our unique AR knockout (ARKO) mouse model [7]. Our ARKO mice express a mutant AR, which has in-frame deletion of the 2nd zinc finger of the DNA-binding domain [7]. The mutant AR cannot bind to DNA and cannot activate the classical signalling, but theoretically retains non-classical signalling.

Hypotheses to be tested:
The mutant AR lacking the 2nd zinc finger of the DNA-binding domain:
1) cannot bind to DNA, but retains the ability to bind ligand and activate the non-classical AR signalling pathway *in vitro*,
2) is expressed normally *in vivo*,
3) can activate non-classical pathways *in vivo*.
4) mediates physiologically significant non-classical pathways *in vivo*.

Specific aims to test the hypotheses:
1) To validate the ARKO mouse model *in vitro* by determining the DNA binding and ligand binding ability of the mutant AR and characterising the non-classical AR signalling in ARKO male genital skin fibroblasts (GSFs).
2) To validate the ARKO mouse model *in vivo* by determining mutant AR protein expression.
3) To investigate the non-classical AR signalling *in vivo* by comparing the degree of phosphorylation of second messengers and level of non-classical target gene expression in muscle, bone and fat from orchidectomised ARKO males with or without androgen treatment.
4) To determine the physiological relevance of non-classical signalling in muscle, bone and fat.

Brief background:

Androgens, the androgen receptor and classical AR signalling pathway
Androgens, including testosterone and dihydrotestosterone (DHT), are steroid male sex hormones [8]. They are produced in the testes and the adrenal cortex in men [8]. They act via the AR, a ligand-dependent transcription factor [3], which regulates gene transcription. The AR protein is expressed in most tissues, but the level of expression may vary [9]. Compared with the reproductive tissues, lower levels of AR are expressed in non-reproductive tissues [9]. The AR contains three functional domains, the amino terminal domain, the ligand-binding domain and the DNA-binding domain [3].

The classical signalling pathway is the well-characterised mechanism of AR action. Briefly, androgens bind to the ligand-binding domain to activate the AR. The activated AR then translocates to nucleus and binds with the two zinc fingers within the DNA binding domain to short palindromic DNA consensus sequences, known as androgen response elements (ARE), to up-regulate target gene transcription [10] (e.g. *Ornithine decarboxylase 1*, *Odc1* [11]).

Non-classical AR signalling pathway
Recently, novel AR intracellular signalling pathways, which do not involve binding of the AR to DNA, have been proposed. Some biological effects, including phosphorylation of second messengers, such as ERK, CREB [5] and Akt [12], can be detected within minutes after androgen treatment *in vitro* [4], which are too rapid to involve gene transcription. In addition, *in vitro* studies have demonstrated that regulation of some androgen target genes, such as *matrix metalloproteinase 13* (*Mmp13*) [13] and *nerve growth factor receptor* (*Ngfr*) [6], do not require the AR to bind to DNA. In these cases, the AR binds and sequesters the transcription factors that normally up-regulate these genes, causing repression of gene transcription [6]. Although a number of non-classical pathway models have been proposed, the physiological relevance of these non-classical actions remains controversial, since most evidence of the non-classical signalling are limited to *in vitro* studies.

Our ARKO mouse model
Our laboratory has developed a unique mouse model, which was designed to inactivate the classical AR signalling pathway but to retain non-classical AR pathways. In this mouse model, exon 3 of the *AR* gene, which encodes the 2nd zinc finger of the DNA-binding domain, is deleted [7]. Since the deletion of exon 3 is in-frame, the remainder of the AR protein is theoretically translated normally

and functions properly. ARKO male mice are androgen insensitive and have female external genitalia [7]. This phenotype demonstrates that the mutant AR, lacking the 2nd zinc finger, cannot activate the classical signalling pathway.

Research progress:

Aim 1: To validate the ARKO mouse model *in vitro and in vivo*

1.1. Analysis of ligand binding activity

To demonstrate that the mutant AR retains the ability to bind ligand, androgen receptor binding assays [14] on GSF cell lines established from 9-12 week old WT and ARKO males (n=3/genotype) were performed. Cultured GSFs were treated with 0.01-2 nM ^{3}H-DHT in the presence or absence of excess unlabeled DHT to determine the total and non-specific AR binding respectively. The maximum ligand binding capacity and affinity of AR for ligand of WT and ARKO GSFs were calculated by Scatchard analysis [15] of binding data.

Prediction: The mutant AR will retain ligand binding activity.

Progress to date:
- This section is completed.
- I have completed AR binding assays for two WT GSF cell lines and one ARKO cell line, with 3 independent assays performed for each cell line.
- The maximum ligand binding capacity (B_{max}) of the two WT cell lines are 85 fmol/mg protein and 93 fmol/mg protein. The B_{max} of the ARKO cell line is 88 fmol/mg protein.
- The ligand binding affinity (K_d) of the two WT cell lines are 0.34 nM DHT and 1.39 nM DHT. The K_d of the ARKO cell line is 0.28 nM DHT.
- My AR binding assay results were combined with those previously completed by my supervisors to give three cell lines/genotype.
- Our data show that both the maximum number of binding sites (B_{max}) and binding affinity (K_d) of the mutant AR is normal.
- B_{max}: WT: 85.4 ± 4.3 fmol ^{3}H-DHT/mg protein, ARKO: 85.4 ± 4.3 fmol/mg protein
- K_d: WT: 0.67 ± 0.36 nM, ARKO: 0.90 ± 0.36 nM
- **These results show that the mutant AR retains ligand binding activity.**

1.2. Analysis of nuclear trans-localisation of the mutant AR

Immunocytochemistry was performed to localise AR in GSF cell lines established from 9-12 week old WT and ARKO males (n=2/genotype) after overnight treatment with vehicle or 100 nM DHT, using the anti-AR antibody (Ab) PG-21, FITC-conjugated secondary Ab and nuclei counter-stained with propidium iodide.

Prediction: The mutant AR will translocate to the nucleus after androgen treatment.

Progress to date:
- This section is completed, with experiments performed by a RA in my laboratory.
- WT cell lines demonstrated strong AR nuclear localisation in the presence of DHT, while ARKO cell lines showed reduced nuclear localisation of the AR in response to DHT treatment.
- **These results show that deletion of the DNA binding domain of the AR reduces but does not abolish nuclear localisation ability of the AR.**

1.3. Analysis of classical target gene expression in fibroblasts with androgen treatment

To demonstrate that the mutant AR cannot activate the classical signalling pathway, Q-PCR was performed to measure the expression of the classical AR target gene *Odc1* in GSF after overnight treatment with vehicle and 10 nM DHT.

Prediction: Up-regulation of *Odc1* expression after overnight 100 nM DHT treatment in WT but not in ARKO fibroblasts.

Progress to date:
- A number of experiments have been completed and *Odc1* expression is not up-regulated by overnight DHT treatment in WT fibroblasts.
- However, extensive studies have shown that *Odc1* is up-regulated by androgens.
- This suggests that *Odc1* levels are not reduced to the baseline in fibroblasts before vehicle or DHT treatment. It is possible that other substances, such as phenol red in media, also induce the expression of *Odc1*.
- Fibroblasts are now growing in phenol red-free media.

1.4. Analysis of non-classical AR signalling

1.4.1. To demonstrate that the mutant AR retains the ability to activate non-classical signalling, the degree of phosphorylation of ERK1/2 and Akt in WT and ARKO GSFs (n=3/group) was measured by Western analysis after androgen treatment. Cultured GSFs were treated with 100 nM DHT or vehicle, in the absence or presence of 100 μM bicalutamide (an AR antagonist) for 1, 5 or 30 minutes. Total protein was isolated from GSFs and immunoblotting was peformed to detect ERK1/2, p-ERK1/2 (^{202}Thr,^{204}Tyr), Akt and p-Akt (^{473}Ser). Signal intensity was quantitated by densitometric analysis using the Fuji-LAS chemiluminescent detection system.
Prediction: The degree of phosphorylation of second messengers induced by androgens in ARKO GSFs will be same as that in WT.

Progress to date:
- This section is completed.
- I have shown that the degree of phosphorylation of ERK was 1.3 to 2-fold higher in two WT cell line after one minute of DHT treatment compared to vehicle ($p<0.05$), and there was a trend of increasing of ERK phosphorylation after one minute treatment in the third WT cell line ($p=0.057$).
- In the ARKO cell line, the degree of ERK phosphorylation was variable among the three cell lines tested. ERK phosphorylation was 25% higher after one minute DHT treatment in one cell line, and 17% lower after 30 minutes treatment ($p<0.05$). Another cell line showed increased ERK phosphorylation after 30 minutes of DHT treatment, while, in contrast, the third ARKO cell line had a 25% reduction in ERK phosphorylation after five minutes of DHT treatment ($p<0.05$).
- The variability may be caused by an alteration in AR conformation that reduces its affinity for intermediate second messengers, such as Src, or stability of the AR-Src complex.
- DHT-induced ERK phosphorylation was abolished after one minute bicalutamide treatment in both WT (↓ 80%) and ARKO (↓ 60%) cell lines.
- In WT cell lines, Akt phosphorylation was not regulated by DHT, except for an increase (17%) in one of the three cell lines after one minute of DHT treatment. Since Akt response is variable in WT cell lines, Akt phosphorylation in ARKO cell lines were not investigated further.
- **These results show that ERK phosphorylation induced by androgens is AR-mediated and occurs in both WT and ARKO GSFs.**

1.4.2. To demonstrate that the mutant AR retains the ability to repress the expression of the non-classical target gene, *Mmp13*, in the absence of DNA-binding. Q-PCR was performed to measure the expression of *Mmp13* in cultured GSFs treated with 10 nM DHT or vehicle, in the absence or presence of 100 μM bicalutamide (an AR antagonist) for overnight.

Prediction: Down-regulation of *Mmp13* expression after overnight 10 nM DHT treatment in both WT and ARKO fibroblasts.

Progress to date:
- This section will be carried out after completion of section 1.3.

1.5. Analysis of AR expression in ARKO

1.5.1. To demonstrate that the mutant AR is expressed normally in fibroblasts, Western analyses using an anti-AR antibody were performed to detect the AR in protein isolated from cultured GSFs (n=5/genotype). Q-PCR was performed to measure the AR gene expression in cultured GSFs (n=6/genotype). **Prediction: AR gene and protein levels in ARKO GSFs will be the same as that in WT.**

Progress to date:
- This section is completed.
- Our data show that AR gene and protein levels are normal in ARKO GSF.
- **These results show that the mutant AR is expressed at normal level in GSF.**

1.5.2. To demonstrate that the mutant AR is translated and expressed normally in our ARKO mouse model, we performed Western analysis using an anti-AR antibody to detect the AR protein in protein isolated from kidney, muscle, bone and fat of 9-12 week old WT and ARKO (n=4/genotype). It is necessary to determine the AR protein level in the ARKO tissues since mRNA containing mutations can be degraded by the process of nonsense-mediated decay [16]. **Prediction: The AR protein level in ARKO tissues will be same as that in WT.**

Progress to date:
- This section is completed.
- My data show that AR protein levels in ARKO kidney, fat and bone are normal, but are significantly higher ($p<0.05$) in muscle of ARKO compared to WT.
- The results of the Western analyses match our previous findings that expression of the *AR* gene is higher in ARKO muscle compared to WT.
- **Our data demonstrate that the mutant AR protein is translated and expressed *in vivo* and suggests that the autologous down-regulation of the *AR* gene occurs in muscle.** A previous study [17] has demonstrated that the AR can repress *AR* gene expression, via binding of the AR to ARE in the regulatory region of the *AR* gene promoter, in a tissue-specific manner [18, 19]. Therefore, the mutant AR cannot bind to the *AR* gene promoter and repress *AR* gene expression in muscle.

Aim 2: To investigate the non-classical AR signalling pathway *in vivo*

ARKO and WT males will undergo orchidectomy. Since androgens in male rodents are exclusively produced in the testis [20], orchidectomy will stop androgen production. Therefore, both classical and non-classical AR signalling will be abolished in these orchidectomised males, due to the lack of ligand for the AR. One group of these orchidectomised mice will be treated for 10 weeks with DHT to activate the putative non-classical pathways, while the other group will receive vehicle, using intraperitoneal implants of silastic tubing containing DHT or empty implants, as we have previously described [21]. WT males will also be orchidectomised as controls.

2.1. Validation of orchidectomy surgery and androgen administration

To validate the orchidectomy surgery and androgen administration, Q-PCR was performed to measure the expression of the classical androgen responsive gene, *Odc1*, in kidneys of WT and ARKO mice that underwent orchidectomy or sham operation with DHT or empty implants.

Prediction: *Odc1* levels in kidneys of orchidectomised WT mice with DHT replacement are higher than that in orchidectomised WT mice with empty implants. *Odc1* is expressed at similar levels in kidneys in ARKO mice with or without DHT treatment.

Progress to date:
- This section is completed.
- We found increased *Odc1* levels in kidneys of orchidectomised WT mice with DHT implants compared to orchidectomised WT mice with empty implants.
- We found identical *Odc1* levels in kidneys between orchidectomised ARKO mice with DHT and empty implants.
- **Our data demonstrated that the orchidectomy surgery successfully removed endogenous androgens in mice and therefore, *Odc1* expression levels were reduced to baseline levels. Administration of androgens induces *Odc1* expression in WT but not in ARKO. This demonstrates that the mutant AR expressed in ARKO is unable to activate the classical signalling pathway.**

2.2. Phosphorylation of second messengers

2.2.1. To determine whether the non-classical second messenger pathways remain at baseline in ARKOs, Western analyses were performed to detect ERK1/2, p-ERK1/2, CREB, p-CREB, Akt and p-Akt in proteins isolated from kidney, muscle, bone and fat harvested from non-orchidectomised 9-12 week old WT and ARKO males (n=4/genotype).

Prediction: All ARKO tissues will show a normal degree of second messenger phosphorylation, as the non-classical AR signalling is retained in ARKOs.

Progress to date:
- This section is completed.
- I have demonstrated that the degree of phosphorylation of ERK, CREB and Akt in all ARKO tissues examined was the same as that in WT, except for Akt phosphorylation in muscle and CREB phosphorylation in fat.
- The degree of phosphorylation of Akt and CREB is higher in ARKO muscle and fat compared to that in WT respectively ($p < 0.05$; $p < 0.05$). This may be due to the knockout of the classical AR pathway, which could regulate phosphorylation of second messengers in these tissues, or secondary consequences of loss of classical signalling.
- **Our results indicate that, in the presence of circulating androgens, the ARKOs have normal activation of second messenger pathways in most tissues *in vivo*.** Therefore, this is consistent with my prediction, that the non-classical signalling pathways that phosphorylate second messengers are retained in ARKO mice.

2.2.2. To determine whether phosphorylation of second messengers in ARKOs is mediated by androgens via the non-classical pathway, Western analysis will be performed to detect the total and phosphorylated second messengers in protein isolated from tissues of 17 week old orchidectomised WT and ARKO males with or without DHT treatment plus sham controls. **If phosphorylation of second messengers is androgen-dependent and is activated via the non-classical AR pathway *in vivo*, we predict that the degree of phosphorylation of second messengers in orchidectomised**

mice with androgen treatment will be higher than those without androgen treatment in both WT and ARKO males.

Progress to date:
- We have collected kidney, muscle, bone, liver, brain, heart, spleen and fat from 50 orchidectomised WT and 56 orchidectomised ARKO males, with DHT or control implants. I am now isolating protein form these tissues and will perform Western analysis.

2.3. Expression of non-classical target genes
I have shown that the expression of *Ngfr* is 57-fold higher in ARKO testis compared to WT. This demonstrated that the mutant AR cannot repress *Ngfr* expression, so an intact DNA binding domain is required to bind and sequester the transcription factor AP-1 [6].

2.3.1. To determine whether genes regulated by the AR in the absence of DNA binding, require an intact AR DNA binding domain, Q-PCR was performed to measure the expression of a non-classical AR target, *Mmp13*, in bone of non-orchidectomised 9-12 week old WT and ARKOs (n=6/genotype).

Progress to date:
- This section is completed.
- I have demonstrated that expression of *Mmp13* in ARKO bone is normal.
- **These results show that the mutant AR can repress *Mmp13* expression. Therefore, an intact DNA binding domain is not required to bind and sequester the transcription factors of *Mmp13*. The fact that the mutant AR can still repress *Mmp13* but not *Ngfr*, demonstrates that indirect transrepression of different target genes by the AR varies in its dependence on the DNA-binding domain.**

2.3.2. To confirm that expression of these non-classical target genes is androgen-dependent *in vivo*, Q-PCR will be performed to measure *Mmp13* and *Ngfr* gene expression in bone and testis of 17 week old orchidectomised WT males with or without androgen treatment. **If the expression of these non-classical genes is repressed by androgens, we predict that the level of gene expression will be lower in orchidectomised mice with androgen treatment than those without androgen treatment.**

Progress to date:
- We have collected kidney, muscle, bone, liver, brain, heart, spleen and fat form 50 orchidectomised WT and 56 orchidectomised ARKO males, with DHT or control implant. I am now isolating RNA from tissues and will perform Q-PCR.

Aim 3: To determine the physiological relevance of non-classical AR signalling in muscle, bone and fat

In order to characterise the physiological importance of non-classical AR signalling, the muscle mass, fat mass, testis mass and bone histomorphometry of orchidectomised ARKO mice treated with DHT will be compared with untreated orchidectomised ARKOs. Since orichidectomised ARKOs treated with androgens will activate non-classical signalling, while orchidectomised ARKOs without treatment will not (Aim 3), any physiological difference between these two groups of mice must arise via activation of non-classical AR pathways. These experiments will provide the first evidence to support the hypothesis that non-classical AR signalling pathways are physiological relevant. **If the non-classical AR actions are physiologically relevant, we predict that one or more of the following will occur in DHT-treated ARKOs: increased muscle mass, decreased fat mass, increased bone mineral density.**

3.1. Muscle and fat mass in orchidectomised ARKOs with or without DHT treatment

Orchidectomised ARKO mice (after 12 weeks DHT or empty implant) will be sacrified and the mixed-fibre gastrocnemius muscle, fast-twitch extensor digitorum longus muscle, slow-twitch soleus muscle, subcutaneous fat and infrarenal fat will be excised and weighed. WT orchidectomised males (DHT or control) will also be examined to confirm the effectiveness of the orchidectomy and DHT implants.

Progress to date:
- We have collected and weighed these tissues form 50 orchidectomised WT and 56 orchidectomised ARKO males, with DHT or control implant.
- Preliminary data demonstrated that DHT treatment increases the mass of heart, kidney and muscle and decrease spleen mass in WT, and no effect of DHT treatment on organ mass have been observed in ARKO.

3.2. Bone histomorphometry

Quantitative histomorphometry will be carried out in the metaphysis of the distal femur using standard resin embedding techniques [22]. 5 µm thick longitudinal sections will be stain using a modified von Kossa silver technique [23] to identify mineralised matrix and subsequently counterstained with hematoxylin and eosin for osteoclast, osteoblast and osteoid surface calculations. Trabecular bone volume, trabecular thickness and trabecular number will be determined using a Leica Quantimet image analysis system. Bone resorption (osteoclast surface) and bone formation (osteoclast surface, osteoid surface (unmineralised bone matrix)) will de determined manually as a percentage of trabecular bone surface occupied by osteoclasts, osteoblasts or osteoid using an ocular mounted Weibel II graticule at x200 magnification [24]. Bone formation and mineral apposition rates will be assessed in unstained sections by calcein labelling. Calcein, an antibiotic that is incorporated with newly mineralised bone, will be injected at 10 and 3 days prior to sacrifice.

Progress to date:
- Calcein injection was given to 50 orchidectomised WT and 56 orchidectomised ARKO males prior to sacrifice. Analyses will be performed by Michele Clarke.

3.3. Microarray analyses

If I can demonstrate any physiological effects of the non-classical AR signalling pathway, the mechanisms underlying these effects will be investigated in more detail. One of these approaches is to identify genes regulated by non-classical AR pathways by microarray. Microarray analyses will be performed on RNA from bone and muscle of orchidectomised ARKO with or without androgen treatment.

Microarray analyses will be performed by the Australian Genome Research Facility. I will use the Affymetrix mouse genome 430 2.0 array, with independent triplicate RNA samples (15 µg total RNA/ sample) from each group. Normalised expression intensity data will be analysed using GeneSpring software, to identify transcripts for which a >2-fold differences between groups.

Progress to date:
- This study will be carried out in the final 6 months of this project.

Progress made in the areas of methods of research, study design, selection criteria, data collection, statistical analysis

- Data collection, analyses, manuscript written and submitted to American Journal of Physiology – Endcrinology and Metabolism.
- Experimental groups and size of the orchidectomy study are
  - A) ARKO male, sham-operated, n=18
  - B) ARKO male, orchidectomised, control implants, n=18
  - C) ARKO male, orchidectomised, DHT implants, n=18
  - D) WT male, sham-operated, abdominal incision made, n=12
  - E) WT male, orchidectomised, control implants, n=12
  - F) WT male, orchidectomised, DHT implants, n=12
- Statistical analysis: One way ANOVA will be used to compare data between sham ARKO males and orchidectomised ARKO males with control or DHT implants.

Report on Expenditure of the Scholarship funds for the first six months of the project

Scholarship funds are paid to Pui Shi Pang, which support her university tuition fees and daily expenses.

References:

1. Chen, J., J. Kim, and J.T. Dalton, Discovery and therapeutic promise of selective androgen receptor modulators. Mol Interv, 2005. **5**(3): p. 173-88.
2. Herbst, K.L. and S. Bhasin, Testosterone action on skeletal muscle. Curr Opin Clin Nutr Metab Care, 2004. **7**(3): p. 271-7.
3. MacLean, H.E., G.L. Warne, and J.D. Zajac, Localization of functional domains in the androgen receptor. J Steroid Biochem Mol Biol, 1997. **62**(4): p. 233-42.
4. Estrada, M., et al., Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Endocrinology, 2003. **144**(8): p. 3586-97.
5. Unni, E., et al., Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. Cancer Res, 2004. **64**(19): p. 7156-68.
6. Kallio, P.J., et al., Androgen receptor-mediated transcriptional regulation in the absence of direct interaction with a specific DNA element. Mol Endocrinol, 1995. **9**(8): p. 1017-28.
7. Notini, A.J., et al., Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model. J Mol Endocrinol, 2005. **35**(3): p. 547-55.
8. Boron, W., Boulpaep EL, The Male Reproductive System, in Medical Physiology. 2003, Elsevier Science: Philadephia. p. 1124-31.
9. Manolagas, S.C. and S. Kousteni, Perspective: nonreproductive sites of action of reproductive hormones. Endocrinology, 2001. **142**(6): p. 2200-4.
10. Beato, M. and J. Klug, Steroid hormone receptors: an update. Hum Reprod Update, 2000. **6**(3): p. 225-36.
11. Persson, L., J.E. Seely, and A.E. Pegg, Investigation of structure and rate of synthesis of ornithine decarboxylase protein in mouse kidney. Biochemistry, 1984. **23**(16): p. 3777-83.
12. Ghosh, P.M., et al., Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. Endocr Relat Cancer, 2005. **12**(1): p. 119-34.
13. Schneikert, J., et al., Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-modulation of matrix metalloproteinase expression. J Biol Chem, 1996. **271**(39): p. 23907-13.
14. MacLean, H.E., et al., Abnormal androgen receptor binding affinity in subjects with Kennedy's disease (spinal and bulbar muscular atrophy). J Clin Endocrinol Metab, 1995. **80**(2): p. 508-16.
15. Scatchard, G., The attraction of proteins for small molecules and ions. Ann NY Acad Sci, 1949. **51**: p. 660.
16. Frischmeyer, P.A. and H.C. Dietz, Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet, 1999. **8**(10): p. 1893-900.
17. Quarmby, V.E., et al., Autologous down-regulation of androgen receptor messenger ribonucleic acid. Mol Endocrinol, 1990. **4**(1): p. 22-8.
18. Lin, T.M. and C. Chang, Cloning and characterization of TDD5, an androgen target gene that is differentially repressed by testosterone and dihydrotestosterone. Proc Natl Acad Sci U S A, 1997. **94**(10): p. 4988-93.
19. Zhu, L.J., et al., Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. Biol Reprod, 2000. **63**(2): p. 368-76.
20. Wilson, J.D., et al., Androgen physiology: unsolved problems at the millennium. Mol Cell Endocrinol, 2002. **198**(1-2): p. 1-5.
21. Axell, A.M., et al., Continuous testosterone administration prevents skeletal muscle atrophy and enhances resistance to fatigue in orchidectomized male mice. Am J Physiol Endocrinol Metab, 2006. **291**(3): p. E506-16.
22. Baldock, P.A., et al., Comparison of three methods for estimation of bone resorption following ovariectomy in the distal femur and the proximal tibia of the rat. Bone, 1999. **24**(6): p. 597-602.
23. Page, K., Bone and preparation of bone sections, in J.D. Bancroft AS, Bone and preparation of bone sections. 1997: Churchill Livingstone, London. p. 223-248.
24. Davey, R.A., et al., Effects of amylin deficiency on trabecular bone in young mice are sex-dependent. Calcif Tissue Int, 2006. **78**(6): p. 398-403.