Anterior prostate epithelial AR inactivation modifies estrogen receptor expression and increases estrogen sensitivity

Ulla Simanainen,1 Keely McNamara,1 Yan Ru Gao,1 Stephen McPherson,2 Reena Desai,1 Mark Jimenez,1 and David J. Handelsman1
1ANZAC Research Institute, University of Sydney, Sydney, NSW; and 2Australian Prostate Cancer Research Centre - Queensland, Institute of Biomedical Health and Innovation, Queensland University of Technology, Brisbane, Queensland, Australia

Submitted 8 October 2010; accepted in final form 24 June 2011

ALTHOUGH THE PROSTATE is a classically androgen-dependent organ (8), it is also sensitive to exogenous estrogens and endogenous estradiol (E2) (20, 22). Effects of androgens and estrogens are mediated via nuclear receptors, androgen receptor (AR) for androgens and estrogen receptor (ER) for estrogens. Overexpression of AR, but also the lack of epithelial AR, leads to increased epithelial proliferation, including development of prominent hyperproliferative foci (39, 43, 46). Estrogens are also believed to play a role in the origins and progression of prostate hyperplasia and cancer but the mechanism(s) remains incompletely defined (9, 32). In rodents, developmental or pharmacological exposure to estrogens can induce prostatic pathology (13, 24, 31, 35). The metaplastic effects of estrogens are mediated via ERα (31, 36), whereas the ERβ-mediated signaling is believed to inhibit cell proliferation (23, 36).

In hormone-dependent tissues like prostate and breast, the expression of steroid receptors is typically responsive to changes in circulating steroids (17, 18). AR and ERs are expressed in both the neonatal and adult prostate (28, 29, 47) with the expression levels in epithelial and stromal compartments changing during prostate development (1, 11, 28, 31). During the first 4 wk of life in rodents, increasing testicular hormone secretion (25) produces an increase in prostatic expression of AR and a decrease in ERα to virtually undetectable levels (28). On the other hand, the epithelial ERβ expression is initially weak but increases with more prominent nuclear location with increasing age (28). This hormonal regulation is exemplified in luteinizing hormone receptor knockout (LuRKO) mice lacking postnatal androgen production (49), in which pubertal androgen treatment induces prostate growth and strong AR and ERβ expression (37). In addition, the ERα expression remains detectable in prostates of mice lacking postnatal androgen (4, 37) but is reduced upon androgen treatment. These observations suggest that the expression of ERs in prostatic epithelium is responsive to androgens that and changes in AR signaling may contribute to estrogen-induced pathology in rodents (37). However, the regulation of estrogenic action in the prostate and how these may be related to androgenic activity are not well understood.

We have contrasted models of castration-induced deprivation of AR activation in prostatic stromal and epithelial compartments with the selective inactivation of prostate epithelial AR knockout (PEARKO) mouse model (39). These models were used to characterize AR-mediated regulation of ERα and ERβ expression in adult mouse prostate and the role of AR in regulating prostatic epithelial sensitivity to estrogen.

MATERIALS AND METHODS

Mice

Prostate epithelium-specific exon 3 deletion of AR [Tg(Pbsn-cre × ARfEx3)], denoted PEARKO, were produced and genotyped as previously described (39), and the F1 generation of experimental PEARKO and littermate control (Pbsn-cre transgene with wild-type AR) males, denoted WT, were used for all experiments. All animal procedures were approved by the Animal Welfare Committee of the Sydney South West Area Health Service within relevant NHMRC guidelines for animal experimentation.
**Experimental Design**

We contrasted the intact WT (normal AR signaling), castrated (lack of stromal and epithelial AR signaling), and PEARKO males (lack of epithelial AR signaling) to determine the role of epithelial AR expression in regulating ER expression and estrogen sensitivity of the prostate. At 8 wk of age, male mice (5 mice per group) were castrated or sham operated through a scrotal incision. Two weeks after castration, mice were implanted subdermally with Silastic tubing containing E2 that generates sustained circulating levels of E2 (4). In the present study, the implants were filled with crystalline E2 diluted 1:1,000 with cholesterol by recrystallization. Castration-only groups received empty Silastic tubing. All males were euthanized at 3 wk after castration/sham surgery (7 days after E2 implantation).

**Sample Collection**

Mice were killed by cardiac exsanguinations under ketamine-xylazine anesthesia. Prostate lobes were dissected free of fat and connective tissue and weighed separately. Anterior prostate (AP) was weighed separately. Castration/sham surgery (7 days after E2 implantation).

**Histology and Stereology**

Serial 5-μm sections were cut from fixed, paraaffin-embedded prostate lobes. Every 10th section was stained with hematoxylin and eosin (H&E). CASTGRID v1.10 (Olympus Albertslund, Denmark) software generated counting frames and a point grid used for stereological, unbiased analysis of proliferation index as previously described (39, 41, 42). Briefly, sections were mapped manually for tissue boundaries and sampling was conducted at unbiased, uniform, random intervals along x- and y-axes. At least 200 counts/tissue compartments (stroma, lumen, and epithelia) were obtained using the point grid. Point counts were combined to get a reference volume for each lobe, and relative volumes for each tissue compartment were determined. Stroma-to-epithelium ratio was determined by dividing the relative stromal volume with relative epithelial volume to empirically identify gross histological changes in cell content within the prostate.

**Immunohistochemistry**

Immunohistochemistry for high-molecular-weight cytokeratins (HMWCK) as a marker for basal epithelial cells, cytokeratin 10 (CK10; marker for keratinized epithelia), smooth muscle α-actin (SMαActin; marker for smooth muscle), as well as ERα and ERβ was performed on 5-μm-thick dewaxed paraaffin sections. Tissues containing both distal and proximal ends from control and PEARKO for each treatment group (n = 4) were stained in parallel to allow comparative evaluation of positive immunostaining. Antibodies used were mouse anti-HMWCK (1:100; Dako, Glostrup Denmark), mouse anti-CK10 (1:300; Dako), mouse anti-SMαActin (1:1,000; Sigma, Sydney, Australia), rabbit anti-ERα (1:150; Santa Cruz Biotechnology, Santa Cruz, CA) for ERα and anti-ERβ (1:100; NCL, Leica) for ERβ. Microwave-induced antigen retrieval was done with pH 9 antigen retrieval solution (Dako) for ERβ and 0.01 M citrate buffer (pH 6) for all other antibodies, with addition of 0.01% trypsin incubation (5 min at room temperature) for HMWCK and CK10. Anti-ERα and -ERβ positivity was detected using an anti-rabbit Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), and HMWCK and CK10 using biotinylated anti-mouse (Dako) secondary antibody. The staining was visualized by color development with 3,3’-diaminobenzidine tetrahydrochloride chromogenic substrate (Leica). Negative controls received PBS instead of the primary antibody.

CASTGRID v1.10 (Olympus Albertslund, Denmark) software was used for unbiased stereological analysis of epithelial cells positive for ERα as previously described for epithelial proliferation index (39). At least 200 cells were counted from at least two separate sections. Adjacent sections were immunostained for basal cell marker HMWCK to identify the basal cells. In addition, intensity of positive ERα and ERβ immunostaining was graded separately in distal and proximal epithelium and stroma, using an arbitrary scale ranging from no apparent staining to strong staining.

No antigen retrieval was required for SMαActin, and the positive SMαActin immunoreactivity was visualized with an EnVision Rabbit/Mouse (Permanent Red) kit (DAKO, Carpentaria, CA). Sections were counterstained with Harris hematoxylin.

Cell proliferation was determined using proliferating cell nuclear antigen (PCNA) kits (Zymed, San Francisco, CA) according to the manufacturer’s instructions (antigen retrieval with 0.01 M citrate buffer, pH 6). Sections were counterstained with Harris hematoxylin.

**RNA Extraction and RT-PCR**

Total RNA was extracted using TRIzol reagent (Sigma) according to the manufacturer’s instructions. RNA was treated with DNase (Invitrogen Australia Pty, Mount Waverley, Australia) and cDNA synthesized with Omniscript reverse transcriptase (Qiagen) from 250 ng of total RNA, respectively, using oligo(dT) and random hexamer mix (Invitrogen). Final reverse transcription reactions were diluted 1:5 for storage at −20°C.

**Real-Time RT-PCR**

Quantitative real-time RT-PCR analyses for Erα, Erβ, and β-actin (housekeeping gene) were analyzed using primer sequences previously described (45). The β-actin housekeeping gene was chosen as it showed no significant differences between WT and PEARKO samples when cDNA was generated from 500 ng of mRNA and β-actin expression measured by real-time RT PCR (data not shown). β-Actin primers were provided in SABioscience’s RT2 Real-Time PCR kit (Jomar Bioscience Pty, SA, Australia). Real-time RT-PCR for all analytes was performed on cDNA using SABioscience’s RT2 Real-Time PCR kits. Real-time RT-PCR analysis was performed using theRotor-Gene 2000 System (Corbett Research, Mortlake, Australia) as previously described (39).

**Serum E2 Levels**

The serum E2 levels in intact and E2-treated PEARKO and WT males were measured by liquid chromatography-tandem mass spectrometry (LC-MS-MS) (10) as adapted for mouse serum and tissues (21). Serum (100 μl) was extracted with 3:2 (vol/vol) of hexane:ethyl acetate fortified with estradiol-2,4,16,16-d4 (d4-E2) as internal standards. The organic layer, separated by freezing the aqueous layer, was dried and reconstituted in 1.2 ml of 20% methanol in PBS prior to injection onto a C18 column for analysis (1 ml). The detection limit for E2 was 5 pg/ml. All samples were measured in the same assay.

**Statistics**

Statistical analysis was performed using one-way ANOVA and the least significant difference (LSD) method as a post hoc test. P values < 0.05 were considered statistically significant. Data are expressed as means ± SE unless otherwise specified. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL).

**RESULTS**

**Prostate Epithelial AR Inactivation Modifies ER Expression**

ERα-immunopositive luminal epithelial cells were only sporadically detectable in APs of intact WT males (Fig. 1A). Castration significantly increased the ERα expression, leading to consistent nuclear and some cytoplasmic ERα immunopositivity in the epithelium (Fig. 1B). In contrast to intact WT,
strong epithelial ERα immunopositivity was commonly observed in intact PEARKO males lacking the epithelial AR, particularly in cells abutting the basement membrane of acini (Fig. 1C), and ERα immunopositivity was strongly identifiable with HMWCK-positive basal epithelial cells (Fig. 1D). In some acini of PEARKO prostate, ERα was also detectable in the HMWCK-negative luminal epithelial cells (Fig. 1, C and D). While the analysis concentrated on the most estrogen responsive lobe AP, the ERα immunopositivity was also increased in dorsolateral prostate (DLP; Fig. 1, E and F) of...
PEARKO compared with WT. ERα expression in VP was not affected by epithelial AR inactivation (data not shown).

ERβ immunoreactivity was strongly expressed in luminal epithelial cells of APs from intact WT, castrated, and PEARKO males (Fig. 1, G–I). Clusters of intensely stained ERβ-positive epithelial cells were detectable in PEARKO tissues but not in intact WT (Fig. 1, G, I, and K). The strongly ERβ-positive foci in PEARKO males coincided with the lesions of prominent epithelial stratification observed in AP of PEARKO males (Fig. 1, I–L).

To further demonstrate the effect of prostate epithelial AR inactivation on ER expression, ERα and ERβ expressions were quantified at protein and mRNA levels in WT and PEARKO. In stereological quantification of ERα immunopositivity, the percentage of epithelial cells expressing ERα protein was significantly higher in the AP of PEARKO compared with intact WT ([stereology; n = 5), B, C (mRNA; n = 8 for AP and n = 5 for DLP]). ERβ expression was not significantly different between AP of intact WT and PEARKO. mRNA expression analyzed by real-time RT-PCR and presented relative to β-actin. Values are means ± SE. *WT and PEARKO values significantly different.

**Prostate Epithelial AR Inactivation Increases Estrogen Responsiveness**

**Organ weights and body weights.** To determine whether modified ER expression in PEARKO prostate influences its estrogen sensitivity in vivo, the prostates of PEARKO and WT mice were analyzed in untreated state following castration or after combined castration plus E2 treatment. PEARKO males had significantly smaller AP (Fig. 3A) than WT; however, 3 wk after castration the weights of both PEARKO and WT APs were significantly reduced compared with intact weights (Fig. 3A) but were not significantly different from each other (Fig. 3A). E2 treatment of PEARKO males after castration resulted in significantly increased AP weight compared with castration-only PEARKO males (67 ± 3% increase in weight from castrate level; P < 0.008), whereas E2 treatment did not significantly increase AP weight in castrated WT control males (17 ± 6% increase from castrate level) (Fig. 3A). Similarly, E2 treatment did not significantly increase DLP or ventral prostate

---

**Fig. 2.** Comparison of ERα and ERβ expression in AP (A and B) and dorsolateral prostate (DLP; C) of intact WT and PEARKO males. %Epithelial cells positive for ERα expression was significantly increased in AP (A and B) and DLP (C) of PEARKO males compared with WT [A (stereology; n = 5), B, C (mRNA; n = 8 for AP and n = 5 for DLP)]. ERβ expression was not significantly different between AP of intact WT and PEARKO. mRNA expression analyzed by real-time RT-PCR and presented relative to β-actin. Values are means ± SE. *WT and PEARKO values significantly different.

---

**Fig. 3.** AP weight (absolute/relative) and stroma to epithelial ratio (stereology) in PEARKO and littermate controls following castration (21 days; Cast) or castration + E2 (estradiol) treatment (14 days castration + 7 days E2; Cast + E2). A: AP weights (mg). B: relative AP weights [mg AP/g body wt (bw)] in intact WT (open bars) or PEARKO (filled bars) males and following Cast or Cast + E2. C: prostate stroma-to-epithelial ratio in intact, Cast, and Cast + E2 APs of WT and PEARKO males. Values are means ± SE; n ≥ 5 for both WT and PEARKO. *WT and PEARKO values significantly different within treatment (P < 0.05); aCast + E2 significantly different from intact; bCast + E2 significantly different from Cast.
Body weights of WT and PEARKO mice were significantly reduced by castration ($P < 0.05$) and returned to intact levels by subsequent E2 treatment; however, no significant differences were observed in body weight between the genotypes at any stage (data not shown). As both WT and PEARKO mice showed similar body weights at all stages of treatment, no significant effect on relative AP weight (mg tissue/g body wt) could be attributed to the genotypes of the animals (Fig. 3B). Stereological analysis of H&E-stained sections of APs from intact PEARKO mice revealed significantly higher relative epithelial volumes compared with intact WT controls ($53 \pm 3$ vs. $38 \pm 3\%$, $P = 0.001$, $n = 5$), illustrating the greater epithelial proliferation known to occur in PEARKO prostate (39). Increased epithelial volume resulted in significantly lower stromal-to-epithelial ratio in APs of intact PEARKO males (Fig. 3C).

CASTRATE. As expected, 21 days of castration reduced the volume of epithelium in both WT and PEARKO APs, illustrating the loss of epithelial cells upon castration-induced androgen deprivation (19) and resulting in significantly increased stroma-to-epithelium ratios in the control ($P = 0.001$) and PEARKO ($P < 0.001$) tissues compared with intact APs (Fig. 3C). The relative volumes of stroma and epithelium in the castrated mice were not significantly different between genotypes (Fig. 3C).

CASTRATE + E2. In AP of PEARKO males, E2 treatment for 7 days (following 14 days of castration) increased the relative volume of epithelium from castration-only levels, resulting in a significant ($P = 0.002$) decrease in stroma-to-epithelium ratio compared with castrate (Fig. 3C), indicating significant structural changes in PEARKO prostate already upon 7 days of E2 treatment. In contrast, no effect of E2 treatment following castration was observed in AP of WT males compared with castration only WT organs (Fig. 3C).

### Structural Differentiation of Epithelium and Stroma After E2 Treatment

Following castration and E2 treatment, the PEARKO AP epithelium demonstrated evidence of hyperproliferation with extensive multilayering and abnormal keratinization of epithelial cells compared with WT littermates (Fig. 4, A–C). In WT littermates, E2 treatment following castration induced patchy epithelial proliferation with PCNA-positive cells identified in discrete, discontinuous areas of the epithelium (Fig. 4B). In contrast, APs from similarly treated PEARKO males demonstrated a continuous layer of proliferating epithelial cells that was detected abutting the basement membrane (Fig. 4B).

Some evidence of increased basal cells (positive for HMWCK) and possible squamous metaplasia (SQM; positive for CK10) was apparent in WT prostates following castration and E2 treatment (Fig. 4C), yet the extent of epithelial cell multilayering was much less apparent than in PEARKO mice (Fig. 4C). In the most severely affected prostatic ducts of PEARKO mice, abnormal multilayering completely replaced the luminal epithelial layer with cells positive for the basal cell marker HMWCK (Fig. 4C). Ducts with less extensive SQM showed evidence of cellular debris within the lumen (Fig. 4C). In WT APs, no ducts showing complete replacement of normal secretory epithelium with SQM were detected. Expression of HMWCK and CK10 overlapped in squamous cells; however, CK10 immunoreactivity was absent from basal cells abutting the basement membrane (Fig. 4C).

Despite the apparent changes occurring within the epithelium, no obvious changes were observed in the prostatic stroma following E2 treatment, as SMα-Actin staining demonstrated continuous smooth-muscle layers surrounding the prostatic ducts of both PEARKO and WT (data not shown). No staining was detected in the negative controls for any of the immunostaining (data not shown).

### Similar serum E2 Levels in WT and PEARKO

Serum E2 levels were below the detection limit of 5 pg/ml serum in intact WT and PEARKO males. E2 treatment for 7 days following 2 wk of orchidectomy resulted in similar serum E2 levels between E2-treated WT control ($31 \pm 14$ pg/ml, $n = 5$) and PEARKO ($28 \pm 9$ ng/ml, $n = 5$) males.

### DISCUSSION

Interaction between AR and ER signaling is an essential determinant for the growth of the prostate gland (34) and may be involved in the origins of human prostate disease (7, 16). In the present study, we utilized the PEARKO mouse model to determine if postnatal epithelial-specific inactivation of AR influenced prostate expression of ERs and/or in vivo prostate estrogen sensitivity. We focused on AP as the mouse prostate lobe most sensitive to exogenous estrogens (36). Our results...
suggest that ERα expression in the prostate epithelial cells is regulated by local, epithelia-specific, androgen-dependent mechanisms, and this imbalance in the AR- and ER-mediated signaling sensitizes the mature PEARKO prostate to exogenous estrogens.

Our current results demonstrate the novel finding that ERα expression in prostatic epithelium is modified by the specific inactivation of epithelial AR activity. This was shown by the increased numbers of ERα-positive epithelial cells in the AP and DLP of the intact PEARKO compared with very low ERα...
expression in WT epithelium of mature prostate [present study; (26)]. Similarly to PEARKO, the removal of the cognate AR ligands by castration results in increased epithelial ER\(\alpha\) expression [present study; (26)]. The ER\(\alpha\)-positive cells in PEARKO can be localized to luminal and basal epithelial cells, whereas in WT the ER\(\alpha\) expression was very low with only rare ER\(\alpha\)-positive luminal epithelial cells. We (39) previously reported that most, if not all, epithelial cells of the adult PEARKO model express a mutated, inactive AR, whereas the stroma expresses solely wild-type AR. Therefore, the results strongly suggest that ER\(\alpha\) expression in the prostate epithelial cells is regulated by local, epithelium-specific, AR-dependent mechanisms. Interestingly, multiple ER\(\alpha\)-positive luminal epithelial cells were located within some, but not all, acini, further implicating local mechanisms in regulating ER\(\alpha\) expression. The luminal ER\(\alpha\) positivity was detected in smaller acini, and it is hypothesized that these acini may contain higher proportions of immature ER\(\alpha\)-positive epithelial cells, supported by dominant epithelial ER\(\alpha\) expression in the neonatal, immature rodent prostate when the AR expression is low (28). However, further studies are required to confirm our hypothesis. In addition, we cannot exclude the possibility that the lack of AR signaling in the epithelium is also regulating the stromal microenvironment and the paracrine communication circuit between prostate epithelium and stroma (15), thereby indirectly influencing the ER\(\alpha\) expression in the epithelial cells. This may be relevant for prostate cancer progression where the relative level of epithelial-stomal AR expression is suggested as a prognostic factor (12).

In contrast to ER\(\alpha\), ER\(\beta\) is strongly expressed in adult prostate epithelium (27, 32) of both intact WT and PEARKO males. However, the PEARKO males had clusters of strongly ER\(\beta\)-positive epithelial cells that were not detected in the intact WT prostates. These clusters of highly ER\(\beta\)-positive epithelial cells coincided with prominent epithelial stratification and elongation of nuclei. Epithelial stratification has been suggested as a precursor of mouse prostate intraepithelial neoplasia (mPIN) and in other prostate cancer models shown to progress to invasive carcinoma (38). These clusters may reflect a loss of AR-regulated balance in epithelial proliferation and survival (39). However, it is unclear whether the expression of ER\(\beta\) is acting to promote or retard this aberrant growth. Regardless, the presence of strong ER\(\beta\) in PEARKO was not capable of suppressing the epithelial proliferation, at least not to the WT level (39). Therefore, our results and others’ (2, 5, 37, 48) support the concept that the epithelial turnover is controlled via a finely tuned balance between AR-, ER\(\alpha\)-, and ER\(\beta\)-mediated signaling.

The imbalance between the steroid hormone activation during prostate maturation may also sensitize the prostate to hormonal pathogenesis (14, 33, 37). This is supported by the present finding of enhanced in vivo sensitivity of PEARKO prostate to estrogens. Estrogen sensitivity was assessed in castrated PEARKO and WT control males treated with the same dose of E2 that resulted in similar serum E2 levels. The PEARKO prostate showed an increased sensitivity to E2, as demonstrated by the significantly greater increase in AP weight above the castrate level, prominent epithelial proliferation, and severe SQM observed in PEARKO but not in littermate WT controls. Interestingly, despite the increased ER\(\alpha\) expression in the DLP of PEARKO compared with WT, the DLP weight was not increased by E2 treatment above castrate levels in either WT or PEARKO males. This is consistent with previous studies reporting AP as the most estrogen responsive of the rodent prostate lobes (36).

The induction of SQM in prostatic epithelium is a characteristic response to high doses of exogenous estrogens acting via ER\(\alpha\) in mouse prostate (31, 36). For example, 7 days of DES treatment induces one to two layers of keratinized epithelium in the basal cell layer of AP (36), an outcome also observed in our similarly treated WT control males. However, continuation of high-dose DES treatment for 21 days was reported to result in significantly increased epithelial multilayering and keratinization similar to the pathology observed in AP of PEARKO mice after only 7 days of treatment with E2. We have previously shown that the PEARKO prostate epithelium remains immature (39) and lead to altered structural response of PEARKO prostate to castration (40). Therefore, it is suggested that the cells highly susceptible to E2-induced, ER\(\alpha\)-mediated epithelial metaplasia may persist in PEARKO prostate following castration. This enhanced in vivo responsiveness to E2 may explain the recent finding of increased PEARKO prostate susceptibility to acute testosterone-, but not dihydrotestosterone-induced epithelial disorganization and aberrant epithelial hyperplasia (40).

The present results demonstrate that local, epithelium-specific AR inactivation significantly increases prostatic responsiveness to estrogens. This may have implications for issues such as environmental endocrine disruptors that could modify the AR activity within the prostate and thereby lead or predispose to aberrant and tumor growth of the prostate later in life (30, 33). Furthermore, ER\(\alpha\) expression in the epithelial cells is regulated by AR-specific mechanisms within the prostate epithelium and is enhanced by the removal of either the cognate AR ligands (by castration) or by epithelial AR inactivation. Understanding this role of AR may be particularly relevant to androgen refractory human prostate cancers, as increased response to estrogens has been shown to play a role in acquired androgen independence (3), and these cases of prostate cancer can manifest high levels of ER\(\alpha\) expression (6). The lobe-specific effects observed in the present study are in accord with previous studies on estrogen actions in rodent prostate (35); however, the implications of the lobe specificity to men with prostate cancer are not clear. Yet, in men, the emergence of ER\(\alpha\) expression in prostate cancer and possibly the estrogen sensitivity appear to be zone dependent, with greater ER\(\alpha\) in peripheral zone compared with transitional zone (44). This suggests that, depending on the research questions under consideration, different mouse prostate lobes may provide the most appropriate model and demonstrate the necessity for further evaluation of selective, tissue- and cell-specific regulation of intraprostatic hormone actions.

ACKNOWLEDGMENTS

We thank Dr. Fen Wang for the generous supply of the Probasin Cre [Tg(Pbsn-cre)] mice, Dr. Jeffrey Zajac for collaboration with ARflox mice, and Lucy Yang for technical help.

GRANTS

This study was supported by Cancer Institute NSW Early Career Fellowship, NHMRC and a Reginald Maney Lake and Amy Laura Bonamy Scholarship.
REFERENCES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


