J Clin Endocrin Metab. First published ahead of print September 13, 2013 as doi:10.1210/jc.2013-2291

Overexpression of aromatase associated with loss of heterozygosity of the *STK11* gene accounts for prepubertal gynecomastia in boys with Peutz Jeghers Syndrome

Seungmin Ham[1,2], Sarah J. Meachem[1,3], Catherine S. Choong[6,8], Adrian K. Charles[7,8], Gareth S. Baynam[8,9,10,11], Timothy W. Jones[6,8], Nirukshi U. Samarajeewa[1,4], Evan R. Simpson[1,5], Kristy A. Brown[1,4]

[1]Metabolism & Cancer Laboratory, Prince Henry's Institute, Departments of [2]Obstetrics and Gynecology, [3]Anatomy and Developmental Biology, [4]Physiology, [5]Biochemistry and Molecular Biology, Monash University, Clayton, Victoria. Departments of [6]Paediatric Endocrinology and [7]Paediatric Pathology, [8]School of Paediatrics and Child Health, [9]Princess Margaret Hospital for Children, University of Western Australia, [10]Institute for Immunology and Infectious Diseases, Murdoch University, [11]Genetic Services of Western Australia, King Edward Memorial Hospital, Perth, Western Australia.

Context: Peutz Jeghers Syndrome (PJS) is an autosomal dominant disorder which arises as a consequence of mutations in the *STK11* gene that encodes LKB1. PJS males often have estrogen excess manifesting as gynecomastia and advanced bone age. We and others have previously described an increase in testicular aromatase expression in PJS patients. However, the underlying mechanism has not yet been explored.

Objective: The aim of this study was to characterize the role of LKB1 in regulating the expression of aromatase in boys with PJS via signaling pathways involving AMPK and CRTCs.

Patients: We studied testicular biopsies from two boys with *STK11* mutations; a 13-year-old boy and an unrelated 4-year-old boy with prepubertal gynecomastia and advanced bone age, as well as breast tissue from the 13-year-old boy.

Results: Loss of heterozygosity (LOH) of *STK11*, measured by the absence of LKB1 immunofluorescence, was observed in Sertoli cells of abnormal cords of testis samples from affected individuals. This was associated with loss of p21 expression and decreased phosphorylation of AMPK, known downstream targets of LKB1, as well as the increased expression of aromatase. Similar results of low LKB1 expression in cells expressing aromatase were observed in the mammary epithelium from one of these individuals. Nuclear expression of the CRTC proteins, potent stimulators of aromatase and known to be inhibited by AMPK, was significantly correlated with aromatase.

Conclusions: LOH of the *STK11* gene leads to an increase in aromatase expression associated with an increase in CRTC nuclear localization, thereby providing a mechanism whereby PJS results in increased endogenous estrogens in affected males.

Peutz Jeghers Syndrome (PJS) is an autosomal dominant condition characterized by gastrointestinal (GI) harmartomatous polyps and mucocutaneous pigmentation (1). Prepubertal males may have estrogen excess manifesting as gynecomastia and an advanced bone age that is typically associated with Sertoli cell lesions of the testes of affected individuals (2–4). Gynecomastia in prepubertal boys is rare and most often arises as a consequence of endocrine abnormalities involving the increased endogenous production of estrogens within the testis or at ex-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received May 19, 2013. Accepted September 9, 2013.

Abbreviations:

tragonadal sites (5). Aromatase is the key enzyme responsible for the conversion of androgens to estrogens and breast growth in boys is a direct result of the dysregulation of the balance between androgens and estrogens (6).

Most PJS cases occur as a consequence of mutations in the Serine Threonine Kinase 11 (*STK11*) gene (1, 7). These range from point mutations, located in the kinase domain and C-terminal domain, to more substantial alterations including complete truncations of the catalytic domain. *STK11* encodes the Liver kinase B1 (LKB1) protein. LKB1 is a tumor suppressor through its interaction with p53 to increase the expression of cell cycle arrest proteins including p21, and is a key regulator of energy homeostasis by directly phosphorylating and activating AMP-activated protein kinase (AMPK) (7). We have established a link between LKB1 and aromatase in the female breast involving cyclic AMP-responsive element binding protein-regulated transcription coactivator 2 (CRTC2) (8). By directly phosphorylating AMPK, LKB1 inhibits the nuclear translocation of CRTC2 within breast adipose tissue and decreases the cAMP-dependent activation of aromatase promoter PII. In breast cancer, tumor-derived inflammatory factors, which include prostaglandin E2, decrease the expression and activity of LKB1 causing the increased nuclear localization of CRTC2 and increased expression of aromatase. Three CRTCs exist, but the role of CRTC1, CRTC2, and CRTC3 in the regulation of aromatase expression in the testis has not yet been established. We postulated that similar events account for prepubertal gynecomastia observed in PJS.

The aim of this study was to characterize the role of LKB1 in regulating the expression of aromatase in the testis of two boys with PJS via signaling involving pAMPK and CRTC1, CRTC2, and CRTC3, as well as examine this relationship in breast tissue.

Case Reports

Patient one

Patient one presented initially at 1 year of age with bleeding per rectum associated with GI hamartomatous polyps. During polypectomy he was noted to have hyperpigmented macules of the lips and oral mucosa, supporting a provisional diagnosis of PJS. There was no family history suggesting PJS. The patient was lost to follow up for some years but represented with per rectal bleeding at nine years of age. He was referred for endocrine assessment at 12 years five months of age for investigation of prepubertal gynecomastia characterized by firm nontender breast tissue corresponding to Tanner stage three, which had developed over the previous two and a half years. Breast tissue was palpable beneath prominent areola bilaterally and testes measured 4 ml bilaterally. At the chronological age of 12 years and five months bone age was advanced corresponding closest to the male standard for 14 years in Greulich and Pyle (standard deviation ~10.5 months). Serum estradiol levels were elevated at 30 pmol/l (9). A testicular ultrasound identified microlithiasis with no evidence of testicular tumors, consistent with previous PJS reports (10). The patient was offered treatment with an aromatase inhibitor but declined this medical therapy. Mastectomy and a biopsy of the testis were performed at 13 years 3 months of age. A heterozygous mutation in *STK11* (910delC) was identified on analysis of genomic DNA at 12.5 years of age confirming the clinical diagnosis of PJS. Height at 13 years 5 months of age was 171 cm (height standard deviation score (SDS) +1.3). Final adult height at 18.1 year of age was 183 cm (SDS +1.0).

Patient two

Patient two was referred for assessment of prepubertal gynaecomastia at 4 years of age. The available interval of follow up is approximately 6 years from first presentation. There was a family history of PJS associated with a known *STK11* mutation, c.180C>A p.Tyr60X (11), affecting his mother. The same mutation was identified in gDNA from Patient two as part of endocrine investigations confirming PJS at 4 years of age. Height at a chronological age of 4 years and one month of age was 112.5 cm (SDS +2.3). The associated bone age corresponded nearest to the male standard for five years in Greulich and Pyle (standard deviation ~ 6.6 months). Growth velocity prior to initial presentation is not known. Letrozole (a third generation oral aromatase inhibitor) was commenced at 4 years of age to treat gynaecomastia and reduce the rate of skeletal maturation. Aromatase inhibition therapy continued without interruption until 10 years of age, the dose throughout this interval was 2.5 mg/d. Growth velocity averaged 4.45 cm per year during the 6 year period of letrozole therapy. At ten years two months of age, his height was 139.2 cm (SDS +0.1). At 10 years chronological age, bone age corresponded closest to the male standard for 10 years in Greulich and Pyle. Serum estradiol levels were assayed at four years of age prior to initiation of letrozole treatment and again at nine years of age while on letrozole therapy. On both occasions serum estradiol levels were below the limits of detection. Estrone levels were not determined on either occasion. On ultrasound examination at four years six months of age, testes were enlarged, 3.5 ml on the right and 2.8 ml on the left. Microlithiasis was identified. Bilateral mastectomy and biopsy of the testis were performed at five years one month of age. Annual testicular ultrasound find-

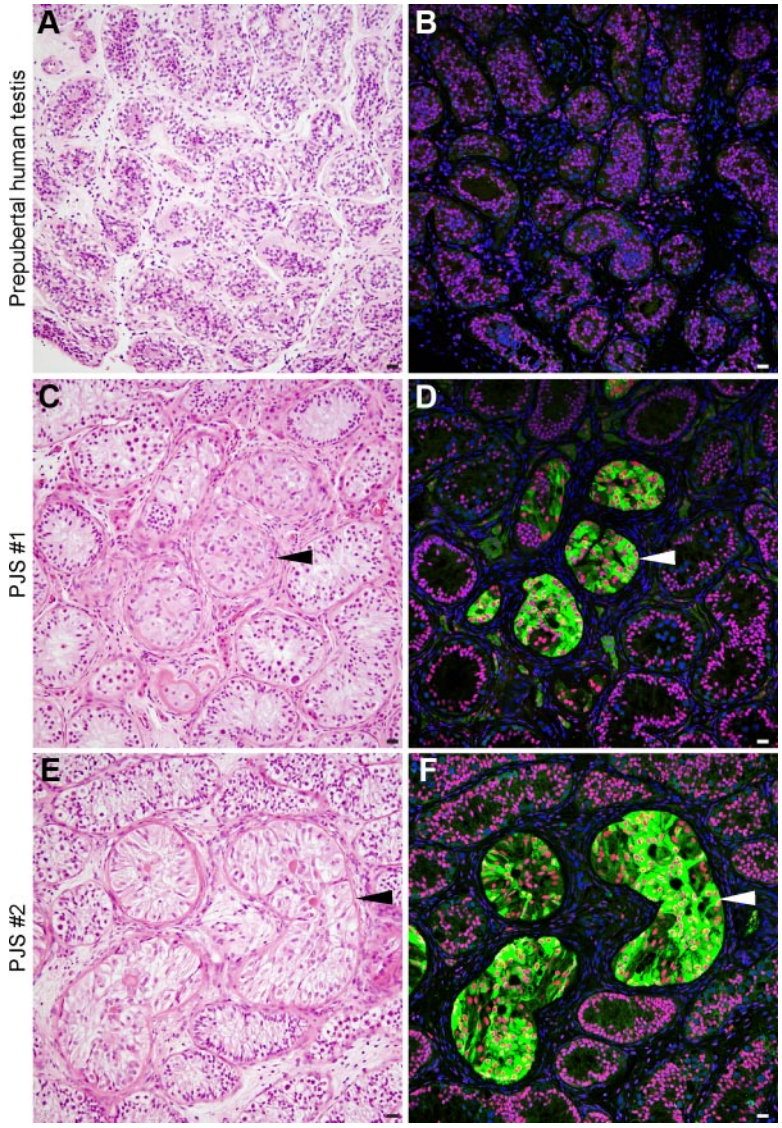

Figure 1. Testis cord structure and aromatase immunofluorescence of healthy prepubertal, and PJS testis samples. Panels A, C & E: H&E staining was performed to assess hitopathology and identify abnormal cords in PJS patient samples (C and E, black arrows). Panel B, D & F: Aromatase immunofluorescence (green) was strongest in abnormal cords from PJS patient samples (D & F, white arrows). Somatic cell marker, GATA4, appears pink; nuclear stain, TOPRO3, appears blue. Scale bar represents 20 μm.

ings did not change significantly over the follow-up period of six years.

Informed consent for publication of these case studies was obtained according to the institutional guidelines of Princess Margaret Hospital for Children's Human Research Ethics Committee and the Southern Health Human Research Ethics Committee.

Materials and Methods

Genetic and Biochemical studies

In the setting of a mutation search of *STK11*, denaturing high pressure liquid chromatography (DHPLC) analysis was performed to screen for *STK11* mutations. Genomic sequencing was performed to confirm any mutation. Testing for a known familial mutation was performed by restriction enzyme digest. Serum estradiol was assayed by Siemens Coat-A-Count Estradiol, Siemens Health Care Diagnostics Inc, Los Angeles, USA. This is a sensitive estradiol assay that uses a Double Antibody Estradiol sequential radioimmunoassay (RIA). The lower limit of detection is 20 pmol/l. For Patient two, an additional measurement was performed at age 9 years using Liquid Chromatography–Tandem Mass Spectrometry (Mayo Clinic Laboratories) where the lower limit of detection is 10 pg/ml (or 36.7 pmol/l).

Detection of LKB1 expression and activity

The LKB1 antibody (Cat #3050, Cell Signaling) used in Western blotting and immunofluorescence binds to a region between amino acids 340 and 450 of human LKB1, indicating that it will not detect the proteins associated with the germline mutations identified for both patients. To determine the effect of mutation on protein expression and stability, mutations were introduced into a construct containing the open reading frame with in-frame flag tag at the 5′ end using site-directed mutagenesis. Proteins were overexpressed in COS-7 cells and detected by Western blotting using anti-flag (Cat #2368, Cell Signaling) or anti-LKB1 antibodies. Proteins were visualized with the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE) after conjugation with a secondary antibody (Alexa Fluor 680 goat antirabbit antibody, Invitrogen, Melbourne, Australia). Protein stability was also assessed by Western blotting of protein extracts from cells treated for zero, four, and eight hours with 200 μM cycloheximide (CHX) to inhibit protein translation. Aromatase promoter PII reporter assays were performed as previously described (8) to determine the effect of CRTC1, CRTC2, and CRTC3 on aromatase expression. Briefly, COS-7 cells were seeded at 2×10^5 per well in six-well plates and incubated overnight prior to transfection. Cells were cotransfected with a promoter II-reporter construct, and expression vectors for CRTCs and LKB1 using Fugene6 (Roche applied science, NSW, Australia) followed by serum-starvation for 24 hours with serum-free media containing 0.1% BSA. After starvation, luciferase reporter assays were performed using the Dual-Glo luciferase assay system (Promega, WI, USA) as described by the manufacturer. The effect of LKB1 on the CRTC-mediated activation of PII was also assessed.

Histology

Testicular biopsies were obtained from Patient one and Patient two when they were aged 13 and four, respectively, and compared to normal archival testicular tissue from a four year-old boy. Breast tissue from Patient one was obtained at five years of age and compared to breast tissue derived from adult male and healthy and tumor-bearing female. Formalin-fixed paraffin-embedded tissue, sectioned (5 μm), was stained using hematoxylin and eosin (H&E) or immunofluorescence was performed using a protocol adapted from Tarulli et al (12). Immunohistochemistry for aromatase on breast tissue was performed using the Vectastain ABC-HP kit and 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, USA) as a substrate, adapted from Samarajeewa et al (13). Antibodies used were as follows: LKB1 (Cat #3050), CRTC1 (Cat #2501), CRTC2 (Cat #3826), CRTC3 (Cat #2768), pAMPK (Cat #2535s), and p21 (Cat #2947s), and were purchased from Cell Signaling. Somatic cell nuclear marker GATA4 antibody (Cat #14–9980) was purchased from eBioscience. Aromatase mouse monoclonal primary antibody 677 was obtained from Prof. Dean P. Edwards (Baylor College of Medicine). Secondary antibodies and TOPRO3 nuclear stain (Cat #T3605) were purchased from Invitrogen.

Statistical analyses

Reporter assays and Western blotting were performed at least three times and results are displayed as mean ± SEM. Analysis was performed by a two-tailed Student's t test using GraphPad Prism version five. Statistical significance was identified with $P < .05$. For Figure 2F, statistically significant differences were identified using different letters.

Results

Abnormal testis cords and heterogeneous aromatase staining

Both patients displayed similar morphology upon histopathological assessment of testicular biopsies. Notably, abnormal cords with thickening of the basement membrane and the peritubular stroma, and enlarged cells containing ovoid nuclei, wispy cytoplasm and prominent central nucleoli were observed (Figure 1C & E). Invaginations of stroma into the tubular epithelium were also recognized in some areas. The surrounding cords showed evidence of increased Sertoli cells with occasional germ cells. These histopathological findings are consistent with the Sertoli cell proliferations found in previously published cases of PJS (3). VASA staining confirmed the presence of germ cells within the normal cords (data not shown). No evidence of malignancy was discovered. Abnormal cords contained cells positive for GATA4, a somatic cell marker that is specific for Sertoli cells within cords, which also displayed intense staining for aromatase (Figure 1D & F) compared to healthy prepubertal testicular tissue (Figure 1B) and normal cords within PJS patient samples. Upon closer examination, aromatase immunoreactivity was found to be heterogeneous within abnormal cords.

Effect of mutations on LKB1 protein stability and expression within cords

A heterozygous mutation in the *STK11* gene was identified for each patient. For Patient one, a frameshift mutation p.Arg304Glyfs*32 was identified. As a result, the LKB1 protein has complete loss of function of the C-terminal domain and is truncated (Figure 2A). The truncation of this protein with predicted molecular weight of 38 kDa was confirmed when the protein was overexpressed and visualized by Western blot using an antiflag antibody (Supplementary Figure 1A). Stability assays demonstrate that this mutation significantly impairs protein stability when compared to the full-length protein (Figure 2C). For Patient two, a nonsense mutation was identified at c.180C>A p.Tyr60X. This mutation leads to complete loss of the kinase and C-terminal domains (Figure 2A) and the protein, with predicted molecular weight of 8 kDa, is undetectable in cells transfected with a flag-tagged construct (Supplementary Figure 1A). Full length LKB1 protein expression, as demonstrated by the presence of LKB1 immunofluorescence, was present in cells within normal cords from sections from Patient one (Figure 2B). Abnormal cords displayed heterogeneous expression of LKB1 with some cells displaying intense LKB1 staining (Figure 2B, white arrow) and others with apparent loss of heterozygosity (LOH), where LKB1 staining was absent (Figure 2B, black arrow). Cells within abnormal cords which displayed LKB1 staining had low to undetectable expression of aromatase. This was evident from the lack of yellow staining. *STK11* LOH was also examined in patient sections by staining for LKB1 downstream targets, pAMPK and p21 (Figure 2D & E, respectively). Staining for pAMPK revealed strong punctate expression localized to a single region within the cell cytoplasm, whereas p21 staining was predominantly nuclear. In cells where pAMPK and p21 were present (Figure 2D & E, respectively, white arrows), aromatase staining was low to undetectable. Conversely, cells which displayed intense aromatase staining had low to undetectable staining for LKB1, p21, and pAMPK (Figure 2B, D, E, respectively, black arrows). Results for Patient two were similar (Supplementary Figure 1B, C &D).

LKB1 inhibits the CRTC-mediated activity of aromatase PII and increased aromatase expression is associated with an increase in CRTC nuclear staining

Results from reporter assays demonstrated that CRTC1, CRTC2, and CRTC3 significantly increase the activity of aromatase promoter PII in vitro (Figure 2F; blue bars) with CRTC3 causing the most dramatic effect. Full length LKB1 significantly inhibited the CRTC-mediated

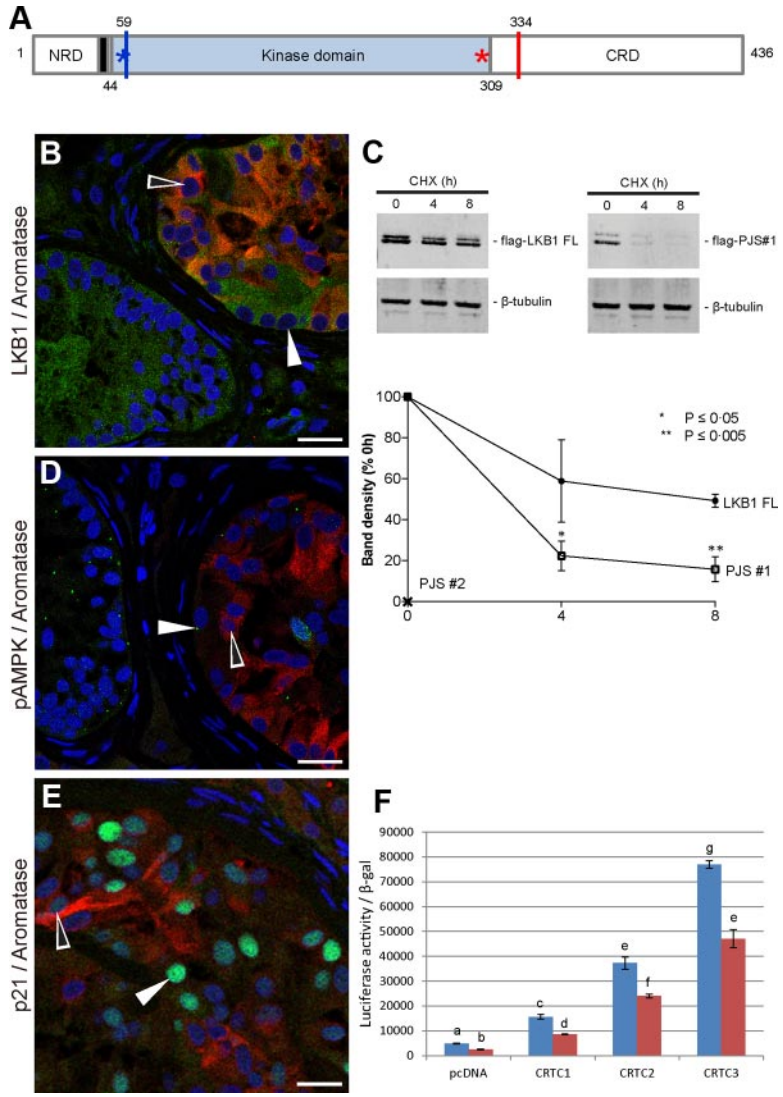

Figure 2. In vitro and in vivo assessment of LKB1 expression and activity (A) Diagram of the location of STK11 mutations on the primary structure of the LKB1 protein. Asterisks represent location of identified mutation and lines represent location of stop codon for PJS Patient one (red) and Patient two (blue). (B) Immunofluorescence for LKB1 (green) and aromatase (red). (C) Stability assay for PJS1 and PJS2 LKB1 proteins. Western blot (top) and densitometric analysis (bottom) of LKB1 protein abundance of full length (FL), Patient one (PJS#1) and Patient two (PJS#2) proteins after cycloheximide (CHX) treatment to inhibit protein translation. (D) Immunofluorescence for pAMPK (green) and aromatase (red). (E) Immunofluorescence for p21 (green) and aromatase (red). (F) Reporter assay demonstrating that CRTC1, CRTC2, and CRTC3 increase aromatase promoter II (PII) activity (blue bars) and that full length LKB1 significantly inhibits the CRTC-mediated activation of PII (red bars). Different letters denote statistical differences ($P \leq .05$) between groups. Three comparisons were made i) between transfected and nontransfected LKB1 (red vs blue bars) ii) between CRTCs tranfected without LKB1 (blue bars) and iii) between CRTCs transfected with LKB1 (red bars). Immunofluorescence images are presented for Patient one. Patient two images available in Supplementary 1. Blue fluorescence represents nuclear stain TOPRO3. Scale bar represent 20 μm. NRD: N-terminal regulatory domain, CRD: C-terminal regulatory domain.

activation of PII (Figure 2F; red bars). Staining for CRTC1 and CRTC3 was detectable by immunofluorescence in Patient one testes sections within normal and abnormal cords (Figure 3A & E, respectively). CRTC2 staining was undetectable (Figure 3C). CRTC1 and CRTC3 subcellular localization was associated with intensity of aromatase staining (Figure 3A & E), where cells with high nuclear CRTC immunoreactivity had high aromatase staining (filled arrows) and cells with weak nuclear CRTC immunoreactivity had low aromatase staining (empty arrows). Quantification of fluorescence intensity revealed a significant positive correlation between average intensity of aromatase and nuclear CRTC1 and CRTC3 (Supplementary Figure 3). A positive correlation between nuclear CRTC3 and aromatase was also found for Patient two (Supplementary Figure 2).

Aromatase is increased in epithelial cells with LKB1 LOH from PJS breast tissue

Immunohistochemistry demonstrated that aromatase is expressed in PJS breast tissue from Patient one (Figure 4A). Aromatase immunoreactivity was higher than that of tissue obtained from non-PJS gynecomastia and healthy female breast (Figure 4C and D, respectively) and was comparable to levels seen in female postmenopausal breast cancer (Figure 4B). Multichannel confocal images also demonstrated that similar to testicular tissue, aromatase immunoreactivity was undetectable in cells where LKB1 was high, and elevated in cells were apparent LKB1 LOH had occurred (Figure 4E). All three CRTCs were detectable in PJS breast tissue (Figure 4F-H) and nuclear CRTC fluorescence intensity was significantly positively correlated with aromatase average intensity (Supplementary Figure 3).

Discussion

This report describes a mechanism which explains the clinical findings of prepubertal gynecomastia in two unrelated male patients. Namely, increased expression of aromatase resulting from nuclear localization of CRTC proteins due to LOH of the *STK11* gene and decreased phosphorylation of AMPK.

The age of onset and severity of bilateral breast en-

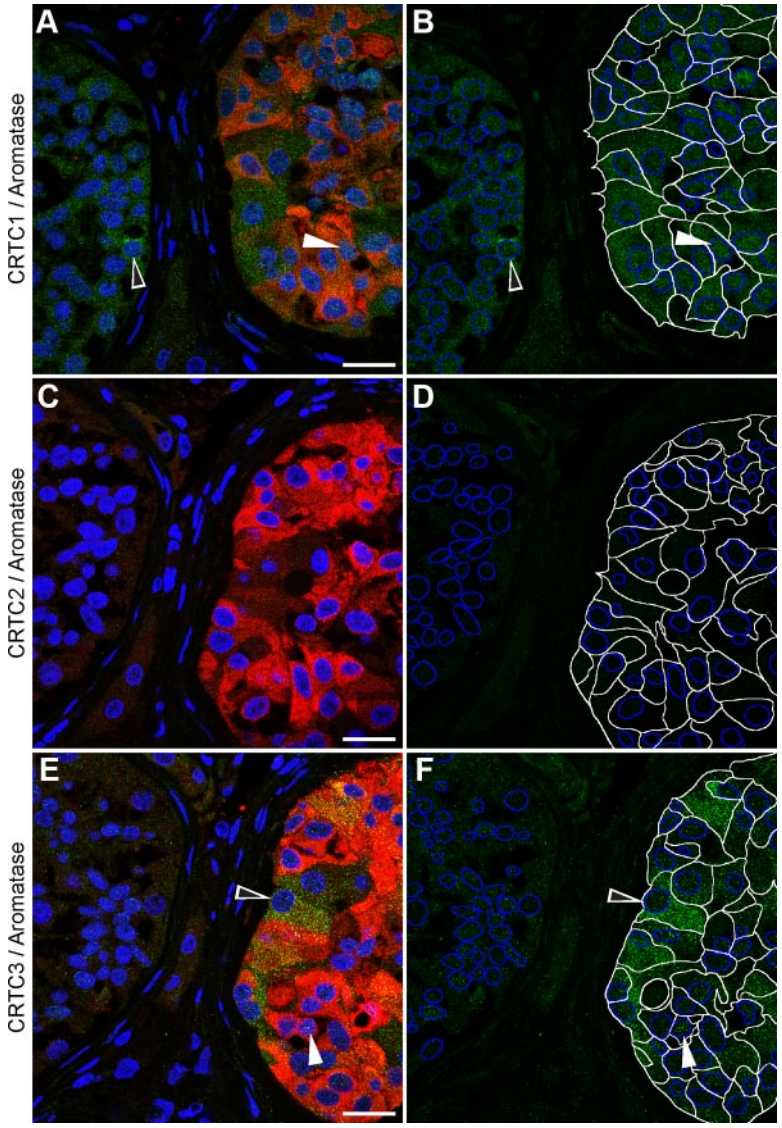

Figure 3. Localization of CRTC1, CRTC2 and CRTC3 in relation to aromatase in PJS testis Immunofluorescence for CRTC1 (green, A, B), CRTC2 (green, C, D), CRTC3 (green, E, F) and aromatase (red, A, C, E) was examined in Patient one testis sections. Blue fluorescence represents nuclear stain TOPRO3. Scale bar represent 20 μm. Right column (B, D, F) contains green staining alone to permit visualization of subcellular localization. Contours of cells were drawn in white and contours of nuclei were drawn in blue.

largement were different in each patient. Although they both developed harmatomatous polyps at an early age, prepubertal gynecomastia was evident in Patient two at four years of age and developed from approximately eight years of age in Patient one. The mutation identified for Patient two (c.180C>A) causes the complete loss of the kinase and C-terminal regulatory domains, and the protein is undetectable when overexpressed, suggesting that it is rapidly degraded. This boy had a much earlier onset of breast enlargement compared with Patient one, who has a mutation which causes loss of the C-terminal domain with significantly impaired protein stability, raising the possibility of a correlation between the genotype and phenotype. Function of the C-terminal regulatory domain of LKB1 is not yet fully understood. Truncation of this domain does not appear to affect the ability of LKB1 to phosphorylate AMPK in vitro or cause cell cycle arrest (14). Nonetheless, several mutations which lead to early stop codons or frameshifts within the C-terminal regulatory domain have been identified in patients with PJS or sporadic cancers (7), suggesting that the impaired stability of the Patient one protein may account, at least in part, for the observed PJS phenotype. A study by Nony et al suggests that proteosomal, rather than lysosomal, degradation accounts for LKB1 protein degradation (15). On the other hand, the impact of C-terminal mutations on LKB1 mRNA stability has also been proposed. It has been suggested that truncated forms of LKB1 may be expressed at very low levels in vivo due to nonsense-mediated decay, a mechanism which causes the degradation of mRNA containing premature stop codons (16, 17). LKB1 and aromatase expression was heterogeneous in abnormal testis cords from both PJS patients. Cells expressing full length LKB1 and its downstream targets pAMPK and p21 did not express aromatase. Reciprocally, Sertoli cells displaying intense aromatase staining had low to undetectable levels of LKB1, pAMPK, and p21. This suggests that impaired LKB1 protein expression is necessary for the abnormal induction of aromatase within the testis. Bardeesy et al (18) have suggested that LOH of *STK11* is necessary for the development of PJS-like features in LKB1+/- mice. These animals which develop GI polyps, similar to that in PJS, had either lost the wild-type LKB1 allele or had completely lost LKB1 expression in cells isolated from the polyps. This was also demonstrated to be the case for LKB1 +/- mice which develop hepatocellular carcinomas, where LKB1 mRNA and protein expression were absent (19). Loss of the homologous normal allele of *LKB1/STK11* has also been described in humans, including one report demonstrating LOH in aggressive breast cancer (20). Based on our immunofluorescence data, we speculate that decreased ex-

pression of LKB1 is due to *STK11* LOH within human testicular Sertoli cells of PJS patients, and that this leads to an increase in aromatase expression and the development of abnormal cords.

Although serum estradiol levels were not elevated in Patient two (both prior to and during letrozole treatment) and only mildly elevated in Patient one, both displayed signs of estrogen excess with advanced bone age and bilateral prepubertal breast enlargement. Neither were obese individuals. Increased gonadal expression of aromatase has been demonstrated in PJS patients and testicular expression of aromatase has been suggested to be the cause of gynecomastia in prepubertal boys with PJS (21–23). Lefevre et al noted normal plasma estrogen levels in one of their patients with prepubertal gynaecomastia, and varying levels of plasma estradiol in their review of the literature identifying 22 patients with PJS and testicular tumors (24).

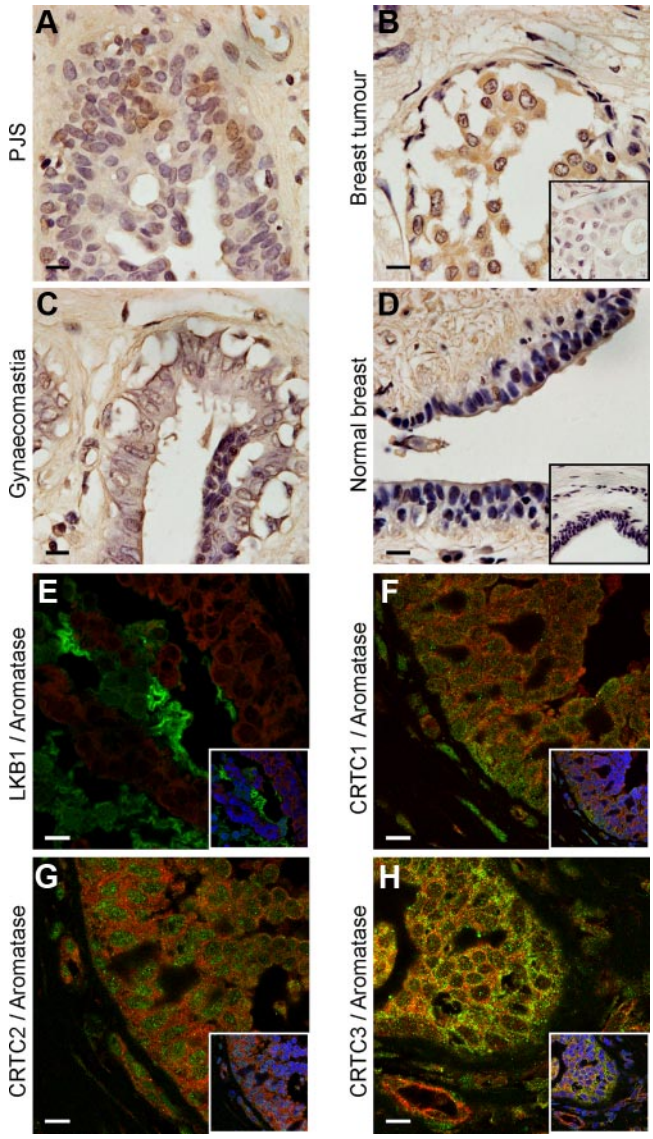

Figure 4. Immunostaining for aromatase, LKB1 and CRTC proteins in PJS breast tissue. (A-D) Immunohistochemistry demonstrating aromatase staining (brown) in breast tissue from PJS Patient one (A) compared to female postmenopausal breast tumor (B), non-PJS gynecomastia (C) and normal female breast (D). Insets represent negative controls were primary antibody was ommitted, hematoxilin nuclear stain (blue). (E-H) Multichannel confocal images examining colocalization of aromatase (red) and LKB1 (E; green), CRTC1 (F; green), CRTC2 (G; green) and CRTC3 (H; green) in breast tissue of PJS Patient one. Hoescht nuclear stain (blue).

Although estrone levels were not assessed in either of our patients, it is possible that increased testicular aromatase activity may have resulted in elevated levels of other estrogens, eg, estrone, as has been observed in kindred with gain of function aromatase mutations (25, 26). Neither of our patients had a bone biopsy, it is conceivable that the bone age advancement observed in these two patients may have resulted in part from a local autocrine action of increased aromatase activity (27, 28). Normalization of bone age and growth in Patient two who was treated with an aromatase inhibitor despite low serum estradiol levels raises the possibility of local inhibition of aromatase activity in bone to account for these clinical observations (29, 30).

We have shown that LKB1 is a negative regulator of aromatase in the female breast (8). It is conceivable that altered expression of LKB1 in breast tissue also contributes to breast enlargement observed in these two patients and findings presented in the present manuscript support that hypothesis. In particular, our results suggest that LKB1 LOH also occurs in the mammary epithelium and this is associated with the increased nuclear expression of CRTC proteins and aromatase. A mechanism whereby this occurs involves LKB1 activating AMPK by directly phosphorylating the alpha subunit at Thr172. As a result, AMPK phosphorylates CRTCs, coactivators of CREB and potent stimulators of aromatase expression, and causes their cytoplasmic sequestration via interactions with 14–3–3 proteins. Our previous research in the breast has demonstrated that decreased expression of LKB1 is sufficient for CRTC2 to enter the nucleus where it can activate the promoter PII-dependent transcription of aromatase (8). Interestingly, it is also aromatase promoter PII which is used to drive gonadal expression of aromatase, and we and others have demonstrated that PII-specific transcripts are increased in the PJS testis (31, 32).

The luciferase assay demonstrates that all three CRTCs induce PII activity in vitro and that LKB1 significantly suppresses the CRTC-dependent activation of aromatase PII. CRTC2 staining was absent from testicular tissue biopsied from both patients and control prepubertal testis tissue (data not shown) consistent with data from Conkright et al (33) demonstrating that CRTC2 mRNA expression in the testis is low when compared to CRTC1 and CRTC3. We did not observe a relationship between cy-

toplasmic CRTC1 staining and aromatase in Patient two. In vitro reporter assays demonstrated that CRTC1 caused the lowest fold induction to promoter PII compared to other CRTCs. CRTC3, on the other hand, potently stimulated PII activity in vitro and, consistent with these findings there was increased cytoplasmic staining for CRTC3 colocalized with intense aromatase staining within abnormal cords of both patients. Conversely, cells which had low to undetectable levels of aromatase had low nuclear staining for CRTC3. In the breast, all three CRTCs were detectable and their nuclear localization was positively correlated with aromatase immunoreactivity. Our previous work in the breast also demonstrated that CRTC overexpression was associated with an increase in aromatase transcript expression, whereas knockdown of CRTCs is sufficient to decrease aromatase activity in breast stromal cells (34).

Taken together, our results suggest that the normal allele of *STK11* in PJS patients encodes a protein capable of causing CRTC cytoplasmic sequestration and as a result inhibits aromatase expression (Figure 5). Loss of LKB1 expression is then associated with an increase in CRTC nuclear localization and aromatase expression and hence estrogen biosynthesis in the testis and breast. This is the first report to describe a mechanism whereby loss of LKB1 is associated with development of breast tissue in the prepubertal male.

Acknowledgments

This research was supported by NHMRC (Australia) Program Grant GNT494802 and Project Grant GNT1005735 and by the Victorian Government Operational Infrastructure Support Program. K.A.B. is supported by NHMRC (Australia) Career Development Award GNT1007714. E.R.S. is supported by an NHMRC Senior Principal Research Fellowship GNT0550900. C.S.C is a recipient of a School of Pediatrics and Child Health, University of Western Australia Telethon Foundation Investigator Grant.

Address all correspondence and requests for reprints to: Dr. Kristy A. Brown, Email: kristy.brown@princehenrys.org, Address: Prince Henry's Institute, P.O. Box 5152, Clayton, Victoria Australia, 3168, Tel: + 61 3 9594 4333, Fax: + 61 3 9594 6125.

Disclosure Summary: The authors have nothing to disclose.

This work was supported by .

References

1. **Beggs AD, Latchford AR, Vasen HF, Moslein G, Alonso A, Aretz S, Bertario L, Blanco I, Bulow S, Burn J, Capella G, Colas C, Friedl W, Moller P, Hes FJ, Jarvinen H, Mecklin JP, Nagengast FM, Parc Y, Phillips RK, Hyer W, Ponz de Leon M, Renkonen-Sinisalo L, Sampson JR, Stormorken A, Tejpar S, Thomas HJ, Wijnen JT, Clark SK, Hodgson SV.** Peutz-Jeghers syndrome: a systematic review and recommendations for management. *Gut*. 2010;59:975–986.
2. **Alikasifoglu A, Gonc EN, Akcoren Z, Kale G, Ciftci AO, Senocak ME, Yordam N.** Feminizing Sertoli cell tumor associated with Peutz-Jeghers syndrome. *J Pediatr Endocrinol Metab*. 2002;15:449–452.
3. **Ulbright TM, Amin MB, Young RH.** Intratubular large cell hyalinizing sertoli cell neoplasia of the testis: A report of 8 cases of a distinctive lesion of the Peutz-Jeghers syndrome. *American Journal of Surgical Pathology*. 2007;31:827–835.
4. **Hertl MC, Wiebel J, Schafer H, Willig HP, Lambrecht W.** Feminizing Sertoli cell tumors associated with Peutz-Jeghers syndrome: an increasingly recognized cause of prepubertal gynecomastia. *Plast Reconstr Surg*. 1998;102:1151–1157.
5. **Ma NS, Geffner ME.** Gynecomastia in prepubertal and pubertal men. *Curr Opin Pediatr*. 2008;20:465–470.
6. **Carlson HE.** Approach to the patient with gynecomastia. *J Clin Endocrinol Metab*. 2011;96:15–21.
7. **Alessi DR, Sakamoto K, Bayascas JR.** LKB1-dependent signaling pathways. *Annu Rev Biochem*. 2006;75:137–163.
8. **Brown KA, McInnes KJ, Hunger NI, Oakhiil JS, Steinberg GR, Simpson ER.** Subcellular Localization of Cyclic AMP-Responsive Element Binding Protein-Regulated Transcription Coactivator 2 Provides a Link between Obesity and Breast Cancer in Postmenopausal Women. *Cancer Research*. 2009;69:5392–5399.
9. **Ankarberg-Lindgren C, Norjavaara E.** Twenty-four hours secretion pattern of serum estradiol in healthy prepubertal and pubertal boys as determined by a validated ultra-sensitive extraction RIA. *BMC Endocrine Disorders*. 2008;8:10.
10. **Ulbright TM, Amin MB, Young RH.** Intratubular large cell hyalinizing sertoli cell neoplasia of the testis: a report of 8 cases of a distinctive lesion of the Peutz-Jeghers syndrome. *Am J Surg Pathol*. 2007;31:827–835.
11. **Baynam G, Schofield L, Goldblatt J.** 2011 A child with an STK11 mutation and Sotos syndrome-like features: can STK11 mutations produce a Sotos syndrome phenocopy? BMJ Case Reports
12. **Tarulli GA, Meachem SJ, Schlatt S, Stanton PG.** Regulation of testicular tight junctions by gonadotrophins in the adult Djungarian hamster in vivo. *Reproduction*. 2008;135:867–877.
13. **Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, Fox SB, Simpson ER, Brown KA.** HIF-1alpha stimulates aromatase expression driven by prostaglandin E2 in breast adipose stroma. *Breast Cancer Res*. 2013;15:R30.
14. **Fogarty S, Hardie DG.** C-terminal Phosphorylation of LKB1 Is Not Required for Regulation of AMP-activated Protein Kinase, *BRSK1, BRSK2, or Cell Cycle Arrest. Journal of Biological Chemistry*. 2009; 284:77–84.
15. **Nony P, Gaude H, Rossel M, Fournier L, Rouault JP, Billaud M.** Stability of the Peutz-Jeghers syndrome kinase LKB1 requires its binding to the molecular chaperones Hsp90/Cdc37. *Oncogene*. 2003;22:9165–9175.
16. **van der Velden YU, Wang L, Zevenhoven J, van Rooijen E, van Lohuizen M, Giles RH, Clevers H, Haramis AP.** The serine-threonine kinase LKB1 is essential for survival under energetic stress in zebrafish. *Proc Natl Acad Sci U S A*. 2011;108:4358–4363.
17. **Forcet C, Etienne-Manneville S, Gaude H, Fournier L, Debilly S, Salmi M, Baas A, Olschwang S, Clevers H, Billaud M.** Functional analysis of Peutz-Jeghers mutations reveals that the LKB1 C-terminal region exerts a crucial role in regulating both the AMPK pathway and the cell polarity. *Hum Mol Genet*. 2005;14:1283–1292.
18. **Bardeesy N, Sinha M, Hezel AF, Signoretti S, Hathaway NA, Sharpless NE, Loda M, Carrasco DR, DePinho RA.** Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. *Nature*. 2002;419:162–167.
19. **Nakau M, Miyoshi H, Seldin MF, Imamura M, Oshima M, Taketo MM.** Hepatocellular carcinoma caused by loss of heterozygosity in Lkb1 gene knockout mice. *Cancer Research*. 2002;62:4549–4553.
20. **Nakanishi C, Yamaguchi T, Iijima T, Saji S, Toi M, Mori T, Miyaki**

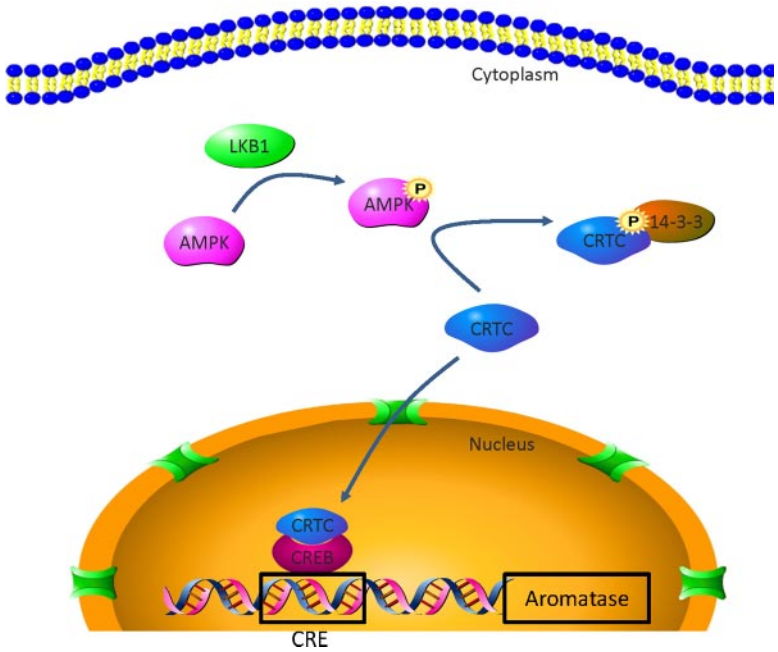

Figure 5. Model of aromatase regulation by LKB1 in the testis LKB1 is a negative regulator of aromatase via the activation of AMPK and the cytoplasmic sequestration of the CREB coactivator CRTC. In PJS patients, LKB1 function is lost and CRTC enters the nucleus where it can upregulate aromatase expression.

M. Germline mutation of the LKB1/STK11 gene with loss of the normal allele in an aggressive breast cancer of Peutz-Jeghers syndrome. *Oncology*. 2004;67:476–479.
21. **Hasan MA, Al Wahadneh AM, Izzat M, Khanan M.** Sertoli Cell Tumor of Testes in a Child with Peutz-Jeghers syndrome. *Saudi J Kidney Dis Transpl*. 1997;8:36–39.
22. **Coen P, Kulin H, Ballantine T, Zaino R, Frauenhoffer E, Boal D, Inkster S, Brodie A, Santen R.** An Aromatase-Producing Sex-Cord Tumor Resulting in Prepubertal Gynecomastia. *N Engl J Med*. 1991; 324:317–322.
23. **Brodie A, Inkster S, Yue W.** Aromatase expression in the human male. *Mol Cell Endocrinol*. 2001;178:23–28.
24. **Lefevre H, Bouvattier C, Lahlou N, Adamsbaum C, Bougneres P, Carel JC.** Prepubertal gynecomastia in Peutz-Jeghers syndrome: incomplete penetrance in a familial case and management with an aromatase inhibitor. *Eur J Endocrinol*. 2006;154:221–227.
25. **Shozu M, Sebastian S, Takayama K, Hsu WT, Schultz RA, Neely K, Bryant M, Bulun SE.** Estrogen excess associated with novel gain-of-function mutations affecting the aromatase gene. *N Engl J Med*. 2003;348:1855–1865.
26. **Stratakis CA, Vottero A, Brodie A, Kirschner LS, DeAtkine D, Lu Q, Yue W, Mitsiades CS, Flor AW, Chrousos GP.** The aromatase excess syndrome is associated with feminization of both sexes and autosomal dominant transmission of aberrant P450 aromatase gene transcription. *J Clin Endocrinol Metab*. 1998;83: 1348–1357.
27. **Simpson ER, Davis SR.** Minireview: aromatase and the regulation of estrogen biosynthesis–some new perspectives. *Endocrinology*. 2001;142:4589–4594.
28. **Sjogren K, Lagerquist M, Moverare-Skrtic S, Andersson N, Windahl SH, Swanson C, Mohan S, Poutanen M, Ohlsson C.** Elevated aromatase expression in osteoblasts leads to increased bone mass without systemic adverse effects. *J Bone Miner Res*. 2009;24:1263–1270.
29. **Shulman DI, Francis GL, Palmert MR, Eugster EA.** Use of aromatase inhibitors in children and adolescents with disorders of growth and adolescent development. *Pediatrics*. 2008;121:e975–983.
30. **Stocco C.** Tissue physiology and pathology of aromatase. *Steroids*. 2012;77:27–35.
31. **Bulun SE, Rosenthal IM, Brodie AM, Inkster SE, Zeller WP, DiGeorge AM, Frasier SD, Kilgore MW, Simpson ER.** Use of tissue-specific promoters in the regulation of aromatase cytochrome P450 gene expression in human testicular and ovarian sex cord tumors, as well as in normal fetal and adult gonads. *J Clin Endocrinol Metab*. 1994;78:1616–1621.
32. **Young S, Gooneratne S, Straus FH, 2nd, Zeller WP, Bulun SE, Rosenthal IM.** Feminizing Sertoli cell tumors in boys with Peutz-Jeghers syndrome. *Am J Surg Pathol*. 1995;19:50–58.
33. **Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M.** TORCs: Transducers of Regulated CREB Activity. *Molecular cell*. 2003;12:413–423.
34. **Samarajeewa NU, Docanto MM, Simpson ER, Brown KA.** CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes. *Horm Cancer*. 2013; 4:233–241.