An IGF1/insulin receptor substrate-1 pathway stimulates a mitotic kinase (cdk1) in the uterine epithelium during the proliferative response to estradiol

Michael P Walker, Richard P DiAugustine, Ernest Zeringue, Maureen K Bunger, Martina Schmitt, Trevor K Archer and R Gregg Richards

Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, National Institutes of Health, Mail Drop D4-01, PO Box 12233, Research Triangle Park, North Carolina 27709, USA

(Correspondence should be addressed to T K Archer; Email: archer1@niehs.nih.gov)

Abstract

Estrogens are potent mitogens for some target organs, such as the uterus, and cancers that develop in this organ might be linked to the proliferative action of these hormones. However, the mechanism by which estrogens influence the cell cycle machinery is not known. We found that a null mutation for the insulin receptor substrate (IRS)-1, a docking protein that is important for IGF1 signaling, compromised hormone-induced mitosis in the uterine epithelium; BrdU incorporation was not affected. This selective effect on mitosis was associated with a reduction in uterine cyclin B-associated kinase activity; cyclin A-associated kinase activity was not changed. The null mutation also reduced the extent of hormone-induced phosphorylation of endogenous uterine histone H1, as determined with phospho-specific antiserum. Uterine epithelial cyclin dependent kinase (cdk)1 was induced

in response to hormone, but the level of the kinase protein, as determined by immunoblotting, was noticeably less in the *irs 1* null mutant than that in the wild-type (WT) mouse, especially around the time of peak mitosis (24 h). Since IRS-1 binds/activates phosphatidylinositol 3-kinase (PI3K), the absence of this docking protein could impair signaling of a known pathway downstream of AKT that stimulates translocation of cell cycle components. Indeed, we found that phosphorylation of uterine AKT (Ser473) in *irs 1* null mutants was less than that in WTs following treatment. Based on earlier studies, it is also possible that an IGF1/IRS-1/PI3K/AKT pathway regulates posttranslational changes in cdk1. This model may provide insights as to how a growth factor pathway can mediate hormone action on cell proliferation.

Journal of Endocrinology (2010) **207**, 225–235

Introduction

It is well known from laboratory studies of rodents that estradiol (E₂) is a potent mitogen for uterine luminal and glandular epithelial cells (Tong & Pollard 2002). This mitotic action extends to other congeners of E₂ and may contribute to the increased risk of endometrial carcinoma observed in postmenopausal females provided with estrogen replacement therapy (Gambrell *et al.* 1983). The proliferative response to E₂ occurs naturally during the ovarian cycle and during early pregnancy. Hormone interaction with the uterine estrogen receptor- α (ER α) is apparently essential for this action since ovariectomized ER α knockout (α ERKO) mice exhibit negligible uterine cell DNA synthesis or proliferation in response to E₂ (Couse *et al.* 1995). Injection of E₂ into the ovariectomized rodent increases both the expression of cell cyclins and the activation of cyclin-dependent kinases (cdk). Transcripts for uterine cyclins D (D1–D3), E, A, and B increase, with each cyclin having a specific temporal pattern following estrogen exposure (Altucci *et al.* 1997,

Tong & Pollard 1999). In mammalian cells, cyclin A is reported to function during S phase, as well as during the G₂/M transition, whereas cyclin B controls entry into mitosis (Minshull *et al.* 1990, Nurse 1990). There is limited knowledge of the local pathways that link activation of the estrogen receptor with the uterine epithelial cell cycle machinery. For over two decades, investigators have considered the notion that hormone-induced growth factors can serve as mediators of cell proliferation. In this regard, experimental evidence is compiling that supports a functional role for the insulin-like growth factor 1 (IGF1) in estrogen-induced proliferation of uterine epithelial cells. E₂ is known to increase the level of IGF1 transcripts in the uterus of some mammals. For instance, *IGF1* mRNA increases by 14-fold in the uterus of the ovariectomized rat as early as 6 h after treatment with E₂ (Murphy *et al.* 1987). This elevation of the uterine IGF1 ligand leads to activation of the IGF1 receptor and downstream signaling pathways in the uterus (Richards *et al.* 1996, 1998, Klotz *et al.* 2000). Other studies show that the insulin receptor substrate-1 (IRS-1), which functions as

a primary substrate for the insulin and IGF1 receptors, is critical for the mitogenic response of these factors (Waters *et al.* 1993, Rose *et al.* 1994, Bruning *et al.* 1997). The omission of IRS-1 in embryonic fibroblasts by null mutation results in a marked reduction in IGF1-stimulated cell proliferation and phosphatidylinositol 3-kinase (PI3K) activity (Bruning *et al.* 1997). *In situ* hybridization studies suggest that the interaction of IGF1 with the uterine epithelial IGF1 receptor occurs through a paracrine-type mechanism, with IGF1 originating in proximal stromal cells (Baker *et al.* 1996, Cooke *et al.* 1997). A complex containing the IGF1R, IRS-1, and PI3K occurs in uterine extracts of mice treated with E₂ or IGF1 (Richards *et al.* 1998). Formation of this hormone-induced complex is severely compromised in α ERKO mice (Klotz *et al.* 2000) and also in IGF1^{m/m} mice (Richards *et al.* 1998), which have a deficiency in IGF1 (Lembo *et al.* 1996).

It is well known that IGF1 can stimulate proliferation of a variety of cells *in vitro* (Lowe 1991). This growth factor was originally referred to as a 'G₁ progression factor' as an outcome of these earlier studies (Leof *et al.* 1982, Campisi & Pardee 1984). However, a more recent investigation of cell cycle kinetics in fibroblasts derived from *igf1*^{-/-} null mice reveals a large increase in the duration of the G₂/M phase when compared with that of wild-type (WT) cells (Sell *et al.* 1994). Moreover, when intact *igf1*^{-/-} null female mice are treated with E₂, the proportion of uterine epithelial cells in the G₁ and S phases by 20 h is not different between WT and mutant mice; however, cell number and mitotic figures are markedly reduced in the *igf1*^{-/-} null mice (Adesanya *et al.* 1999). This is the direct evidence that IGF1 provides a mitogenic stimulus in the uterus in response to E₂ and does so by influencing transit time through G₂/M, rather than G₁.

The major goal of this study is to identify components of the cell cycle in the uterus that are regulated by the hormone-induced IGF1 signaling pathway. To achieve this, we use the uterine epithelium of hormone-treated ovariectomized mice as an experimental model (Fagg *et al.* 1979). This model permits analysis of signaling and cell cycle proteins in the uterine epithelium, while providing the permissive micro-environment for E₂ to stimulate cell proliferation (Cooke *et al.* 2002). Since adult *igf1*^{-/-} null mutant mice are extremely small and can exhibit a high frequency of neonatal lethality (Liu *et al.* 1993), *irs1*^{-/-} null mutants (Araki *et al.* 1994) were selected for most of the experiments. As described above, the uterus of the *irs1*^{-/-} mouse should have a compromised IGF1 signaling pathway. Our initial experiments used *igf1*^{-/-} mice to establish that this ligand is critical for E₂-stimulated association of PI3K (p85) with IRS-1. Following this, we sought to determine whether *irs1*^{-/-} null mutants exhibit compromised mitosis in the uterine epithelium in response to E₂, as was previously observed with *igf1*^{-/-} null mutants (Adesanya *et al.* 1999). Finally, we attempted to identify what component(s) of the cell cycle might be regulated by the IGF1/IRS-1 pathway. Our findings indicate that the IGF1/IRS-1 pathway contributes to the mitogenic action of E₂ by stimulation of cyclin B-associated

cdk1 kinase. This finding may have broad implications with regard to the mechanism by which IGF1 provides a stimulus for cell proliferation in various tissues or neoplasias.

Materials and Methods

Mice, treatments, and tissue collection

IRS-1 WT (*irs1*^{+/+}) and IRS-1 null mutant (*irs1*^{-/-}) mice on a C57Bl/6×129Sv background were generated in our laboratory from heterozygous IRS-1 breeding pairs obtained from Taconic (Germantown, NY, USA) with the permission of Dr Ronald Kahn (Joslin Diabetes Center, Boston, MA, USA). Null mutants were also generated by breeding *irs1*^{-/-} males with *irs1*^{+/+} females. The weight of female *irs1*^{-/-} mice was ~70% that of the corresponding WTs. IGF1 WT (*igf1*^{+/+}) and IGF1 null mutant (*igf1*^{-/-}) mice were generated from heterozygous IGF1 breeding pairs (MF1×129/Sv hybrids) provided by Dr Argiris Efstratiadis (Columbia University, New York, NY, USA). Tail genomic DNA was screened by PCR to determine the genotype. All of the WT, *irs1*^{-/-}, and *igf1*^{-/-} mice were ovariectomized at 8–13 weeks of age. Procedures for surgery, treatments, and euthanasia were conducted in compliance with the guidelines of the NIEHS, National Institutes of Health Animal Care and Use Committee.

To assess 5-bromo-2-deoxyuridine (BrdU) incorporation, ovariectomized WT and *irs1*^{-/-} mice (*n*=8 per group) were treated with E₂ (0.08 µg/g) or vehicle (4% v/v ethanol PBS) s.c. and killed after 18 h; BrdU (Sigma, 0.1 mg/g i.p.) was injected 2 h before euthanasia. For mitosis experiments, mice (*n*=4 per group) were killed 22 h after hormone treatment; the anti-mitotic compound demecolcine (Colcemid, Sigma, 5 µg/g i.p.) was injected 2 h before euthanasia. Uteri were excised, and a 5 mm long transverse section in the middle of each horn was taken and immersed in 10% neutral-buffered formalin. Paraffin sections were prepared by standard procedures. BrdU was detected in sections with a rat anti-BrdU monoclonal antibody that was part of a Vector kit (no. PK4004). All mice were treated with hormone no earlier than 14 days after ovariectomy.

Apoptotic cells were identified in uterine sections with a TACS 2 TdT (TBL) *In Situ* Apoptosis Detection kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions; negative and positive control slides were included in the kit.

Epithelial cell extracts

Mice were treated with hormone as described above and euthanized at various time points after treatment. The specific intervals (up to 48 h) following treatment for the different experiments are shown in the figures. For the 0 time point, mice were treated with 4% ethanol PBS and immediately killed. Uterine epithelial cells were removed as described

previously (Fagg *et al.* 1979, Tong & Pollard 1999). Briefly, uteri were slit lengthwise and placed in 15 ml tissue culture tubes with chilled extraction buffer (1% Triton X-100, 150 mM NaCl, 1 mM Na₃VO₄, 1 mM NaF, 50 mM Na₂MoO₄, 20 µg/ml aprotinin, 20 µg/ml leupeptin, and 4 µg/ml 4-amido-phenylmethylsulfonyl fluoride in 50 mM Tris-HCl, pH 7.4). A volume of 580 and 400 µl buffer was used for each WT and *irs1*^{-/-} uterus respectively. The uteri were agitated in the presence of seven glass beads (3.0 mm diameter) on a Vortex-Genie mixer (30 s on/30 s off) for a total of 5 min. The resulting cell suspension was drawn off and then disrupted ultrasonically (3 × 5 s bursts) followed by brief centrifugation. The supernatant was transferred to a microfuge tube and stored at -80 °C. Protein concentration was determined with a Pierce BCA assay kit (Pierce Chemical, Rockford, IL, USA) with bovine albumin (2 mg/ml) as a standard.

Western immunoblotting and immunoprecipitation

To remove endogenous IgGs prior to immunoprecipitation, aliquots of supernatants were cleared by incubating with 100 µl washed protein A-Sepharose (CL-4B, Amersham Pharmacia Biotech) for 30 min at 4 °C. For immunoprecipitation, the cleared aliquots (300–450 µg protein) were incubated with 5 µg antibody for 2 h at 4 °C. After incubation with the antibody, the supernatants were incubated with the protein A-Sepharose for an additional 2 h at 4 °C. Antibody/protein A-Sepharose pellets were washed three times with the extraction buffer and subjected to kinase assays or boiled for 5 min in Laemmli sample buffer. After boiling, the precipitates were stored at -20 °C. Aliquots of mouse uterine epithelial cell extracts or immunoprecipitates were boiled for an additional 2 min, subjected to SDS-PAGE, and then transferred to polyvinylidene fluoride (Immobilon-P) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with either 5% bovine albumin/Tris-buffered saline (TBS) with 0.1% Tween 20 or 5% nonfat dry milk/PBS and incubated with a dilution of specific antibody. For cdk1, where cytokeratin 18 was used as a loading control, the membrane was cut just above the 37 kDa protein marker. The lower portion (cdk1) was blocked with 1% BSA/TBS-Tween, and the upper portion (cytokeratin 18) was blocked with 5% nonfat dry milk/PBS.

Antibodies (catalog number) to IRS-1 (06-248), p85 (06-195), cdk1 (06-141), and cdk2 (05-596) were purchased from Upstate (Temecula, CA, USA); antibodies to cyclin A (sc-596), cyclin B1 (sc-595), and cytokeratin 18 (sc-28264) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies to AKT (9272) and phospho-AKT (Ser473) (9271) were from Cell Signaling Technology (Beverly, MA, USA).

Each membrane was then incubated with a HRP-conjugated donkey anti-rabbit (NA9340) (Amersham Biosciences) or goat anti-mouse IgG secondary antibody (A3682) from Sigma.

Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Six trials were used to obtain immunoblots of uterine cdk1 and cdk2 for up to 30 h after E₂ treatment of WT versus *irs1*^{-/-} mice. Four trials were used to examine uterine cdk1 levels in both the groups 24 h after E₂ treatment with cytokeratin 18 as a loading control. Four trials were also used to analyze p-AKT/AKT in both the groups in a time-course study. In these experiments, different extracts were analyzed for each trial. Densitometric scans of immunoblot bands were made with an Alpha Innotech FluorChem FC2 Imaging System.

Microarray analysis

Fold changes in mouse uterine cdk1 and cdk2 transcripts following treatment with E₂ were retrieved from the microarray data offered by Sylvia C Hewitt and Dr Bonnie Deroo (NIEHS, NIH). Details of the experimental design have been published (Hewitt *et al.* 2003), and the repository for the microarray data is accessible at NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>); accession numbers GSE4664 and GSE4615.

Quantitative real-time PCR

Uteri were obtained from control (0 h; *n* = 4 for each group) and estrogen-treated mice (24 h; *n* = 6 for each group). Each uterus removed was immediately placed on ice in a tube containing 1.5 ml RNAlater Stabilization Reagent (Ambion, Austin, TX, USA). RNA was extracted with the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Uteri were transferred to the RLT buffer (0.6 ml per uterus) containing β-mercaptoethanol (15 µl/ml), and homogenized with an Omni Model TH-115 hand-held homogenizer (7 mm sawtooth probe with three bursts at full power, each at ten seconds). RNA isolation was followed by a DNA removal step with DNase I. One microgram total RNA was then reverse transcribed using a Gene Amp PCR 29 System 9700 (Applied Biosystems, Foster City, CA, USA) and the SuperScript First-Strand Synthesis System 30 (Invitrogen).

Quantitative real-time PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) on a Bio-Rad iCycler (Bio-Rad). The primers for mouse cdk1 were 5'-GGA-CCT-CAA-GAA-GTA-CCT-GGA-C-3' (forward) and 5'-CCC-TGG-AGG-ATT-TGG-TGT-AAG-3' (reverse). The primers for mouse actin were 5'-TGA-CAG-GAT-GCA-GAA-GGA-GA-3' (forward) and 5'-CGC-TCA-GGA-GGA-GCA-ATG-3' (reverse). The cDNAs were amplified for 40 cycles (95 °C/10 min → 95 °C/15 s → 60 °C/30 s). Controls included the omission of template or primers. Melt curves of both the amplicons indicated the presence of a single product and the absence of primer-dimer formation. Relative quantitation of cdk1 expression was determined by the comparative C_t method (Pfaffl 2001) with *actin* as the endogenous reference gene.

Real-time PCR efficiencies for the cdk1 ($E=1.97$) and actin ($E=1.98$) were determined from the Q5 Optical System Software (Bio-Rad).

Cyclin-associated kinase assay

Uterine epithelial extracts were prepared as described above and stored at -80°C . Equal amounts of WT and KO extracts ($\sim 400\ \mu\text{g}$ protein) were diluted to 0.5 ml with the extraction buffer. After preclearing the sample with protein A-Sepharose, 5 μg anti-cyclin B (Upstate) or anti-cyclin A (Santa Cruz) were reacted with the sample for 2.5 h. Protein A-Sepharose was added, and the immunoprecipitates collected were washed three times with the extraction buffer and then three times with the cdk1/cdk2 kinase assay buffer (20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, 25 mM glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol). The kinase reaction was carried out according to the instructions of the cdk1/cdk2 kinase assay kit (Upstate). Kinase activity of the immunoprecipitate was carried out in the assay buffer containing 10 μCi [$\gamma\text{-}^{32}\text{P}$]ATP (3000 Ci/mmol), 90 μM ATP, and 14 mM MgCl_2 . Histone H1 (0.4 $\mu\text{g}/\mu\text{l}$) was the exogenous substrate. The mixture was incubated for 10 min at 30°C . The assay was stopped by placing the reaction tubes on ice and adding gel sample loading buffer. Sample proteins were separated on 4–20% Tris Ready Gels (Bio-Rad) and transblotted to Immobilon-P membrane. Five separate trials, each with different uterine extracts, were used for the kinase assays. Detection was made by autoradiography and phosphoimaging (Storm 860 Molecular Imager).

In situ histone (H1) phosphorylation

Nuclei were isolated from whole uteri following homogenization in a detergent-containing buffer, as described previously (Archer *et al.* 1991). Nuclei were resuspended in 0.2 M H_2SO_4 and proteins in the soluble fraction precipitated with 5 volumes of ethanol. Ten micrograms isolated histones were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Immunoblotting procedures were similar to those described above. The membrane was probed with antibodies (1–2 $\mu\text{g}/\text{ml}$) developed in rabbits against mouse H1.3 amino acids 15–27 containing a phosphate at Thr18 (Deterding *et al.* 2008). A test of the antibody against chromatographically purified H1 isoforms revealed recognition of only H1.3 and H1.4 isoforms in mouse cells; the antibody did not detect an H1.3 peptide phosphorylated at Ser173. Three separate trials, each with different uterine extracts from WT and *irs1*^{−/−} uteri, were used for the *in situ* histone phosphorylation experiments.

Statistical analysis

Statistical significance was determined by the Student's *t*-test; two-sided $P \leq 0.05$ was considered statistically significant.

Results

IGF1 is required for E₂-induced IRS-1 activation

Since we intended to use the *irs1*^{−/−} mice to compromise the IGF1R signaling pathway, it was important to ascertain at the outset that the IRS-1 docking protein requires IGF1 for activation of PI3K in response to E₂. To demonstrate this, ovariectomized female WT and *igf1*^{−/−} mice were treated with E₂ (6 h), and the immunoprecipitates of IRS-1 from uterine epithelial extracts were evaluated for the PI3K regulatory subunit (p85). As shown in Fig. 1, the immunoprecipitates of uterine IRS-1 from WT mice (lanes 1–4) revealed the expected increased association of p85 as a result of hormone treatment. In contrast, uterine IRS-1 from hormone-treated *igf1*^{−/−} mice (lanes 5–9) was not associated with p85. These findings indicate that the IGF1 ligand is a required intermediate for estrogens to activate IRS-1 and stimulate binding of p85 to IRS-1 in the uterus. In addition, these data rule out the participation of another IGF1R ligand, e.g. IGF2, that might function in the absence of IGF1 to stimulate the activation of IGF1R and IRS-1 (Osborne *et al.* 1989).

*E₂-stimulated uterine epithelial proliferation is compromised in *irs1*^{−/−} mice*

Since IGF1 was critical for E₂-induced activation of uterine IRS-1, mice with an IRS-1 null mutation could provide insights as to the importance of an IGF1/IGF1R/IRS-1 pathway in the proliferative response of uterine epithelial cells to E₂. In order to determine this, ovariectomized adult *irs1*^{−/−} and WT (*irs1*^{+/+}) female mice were treated with E₂, and the uterine epithelium was examined for the proportion of cells in the S phase and in the mitosis. The proliferative response of the uterine epithelial cells to E₂ in the adult mouse is considered to begin with a drastic shortening of the G₁–S

Figure 1 Binding of the PI3K regulatory subunit (p85) to uterine IRS-1 in wild-type (WT) and *igf1*^{−/−} mice treated with estradiol. Ovariectomized mice were treated with estradiol (E₂) or vehicle (C, control), and uterine epithelial extracts were obtained 6 h later. Aliquots of the extracts were incubated with anti-IRS-1 antiserum. Immunoprecipitates were separated by SDS-7.5% PAGE and then immunoblotted with anti-p85 antiserum (upper panel) or anti-IRS-1 antiserum (lower panel). Other details of the treatment, extraction, and immunoblotting procedures are described in Materials and Methods. Each lane represents analyses of a different animal.

Figure 2 BrdU uptake and mitosis in uterine epithelial cells of estradiol-treated wild-type and *irs1*^{-/-} mice. (A) Mice were treated with estradiol and killed 18 h later. BrdU was injected 2 h prior to euthanasia. Standard methods were used to prepare uterine sections for examination by light microscopy. BrdU was detected in epithelial cells with a rat anti-BrdU monoclonal antibody. The estrogen-stimulated incorporation of BrdU into uterine epithelial cells of *irs1*^{-/-} (KO) mice was not significantly different ($P>0.05$) from that of wild-type (WT) mice. (B) Mitosis in uterine epithelial cells was determined by injecting demecolcine into mice 20 h after hormone treatment, followed by euthanasia at 22 h. Further details of the procedures used are described in Materials and Methods. In (A and B), each bar represents the mean $\pm$ S.D.; n is the number of mice used in each group. For both BrdU uptake (A) and mitosis (B), ~ 500 epithelial cells were counted in each uterine section. The mitotic index of the KO mice was significantly different from that of WT mice at $P<0.005$.

transition rate. The epithelial cells exhibit a near-synchronous wave of DNA synthesis beginning about 10 h after hormone treatment; this is followed by mitosis that peaks around 20–24 h. To identify cells in the S phase, BrdU was injected into animals that were previously treated with E₂ 16 h earlier. Negligible incorporation of BrdU into uterine epithelial cells occurred when WT or mutant mice were injected with vehicle. Incorporation of BrdU was apparent in both the groups of mice, and the proportion of cells that incorporated BrdU (labeling index) in *irs1*^{-/-} mice (70 ± 13 , mean $\pm$ S.D.) was not significantly different ($P>0.05$) from that of WT mice (79 ± 8 , mean $\pm$ S.D.; Fig. 2A). The fraction of epithelial cells undergoing mitosis was determined at 22 h after hormone treatment. At this time, epithelial cells of the *irs1*^{-/-} mice had approximately one-sixth the frequency of mitotic figures (1.8 ± 1.2 , mean $\pm$ S.D.) as that of WT mice (10.5 ± 0.3 , mean $\pm$ S.D.; $P<0.005$; Fig. 2B). These data suggest that the IRS-1 null mutation affects the rate of transit of uterine epithelial cells through G₂/M without impairing recruitment of cells into G₁/S in response to E₂. The effects obtained with the *irs1*^{-/-} mice with regard to hormone stimulation of the uterine epithelial cell cycle are similar to that reported previously for *igf1*^{-/-} mice (Adesanya *et al.* 1999). In both cases, the null mutation affected the number of cells in mitosis but not the cells in the S phase. This functional overlap indicates that IGF1 and IRS-1 are part of the same signaling pathway that mediates hormonal stimulation of mitosis of cells in the uterine epithelium. The decrease in the number

of mitotic figures observed at 22 h after E₂ treatment in *irs1*^{-/-} mice could not be explained by a difference in apoptosis. Evaluation of uterine sections at this time point for detection of fragmented DNA *in situ* end labeling showed no detectable apoptosis in the epithelium of WT or *irs1*^{-/-} mutant mice (data not shown).

The IRS-1 null mutation reduces the E₂-induced increase in cdk1 (p34) protein but not in mRNA

The significant decrease in hormone-induced mitosis in the uterine epithelium of *irs1*^{-/-} mice prompted us to consider an effect of the mutation on the major mitotic kinase cdk1. In the mammal, cdk1 kinase is poorly expressed in normal quiescent cells but accumulates during the S phase and peaks during the G₂ and M phases of the cell cycle (Kim *et al.* 1992, Loyer *et al.* 1994). As shown in Fig. 3A, microarray analysis of WT mouse uterine RNA reveals that the level of cdk1 transcripts increases markedly by 24 h after treatment with E₂, whereas the level of cdk2 transcripts was essentially invariable during this period. The influence of E₂ on uterine cdk1

Figure 3 Changes in uterine cdk1 transcripts in wild-type and *irs1*^{-/-} mice after treatment with estradiol. (A) Total RNA was isolated from whole uteri of C57BL/6 (ovariectomized) mice at the indicated time points after treatment with estradiol. The plots compare the estradiol-induced fold changes in uterine gene expression of cdk1 against that of cdk2, as determined by microarray analyses. Details of the microarray are described in Materials and Methods. The plots were constructed from values given at the website in Hewitt *et al.* (2003). (B) Total RNA was isolated from whole uteri of ovariectomized wild-type and *irs1*^{-/-} mice before and after treatment with estradiol. cDNA was prepared from 1 μ g RNA and used for quantitative real-time PCR. Quantitation was made by the comparative C_t method with *actin* as the endogenous reference gene. After calculating the C_t (cdk1 minus *actin*), the values (C_t) were determined relative to that for untreated wild types. Each bar represents the mean $\pm$ S.D.; n is the number of mice used in each group. Note that the mean value for the untreated IRS-1 (KO) was <1.0 . Cdk1 transcript level was increased in wild-type (WT) and KO animals 24 h after hormone treatment; the value for KO (24 h) was significantly greater than that of WT (24 h) at $P<0.05$. Further details of the procedures and primer sequences are described in Materials and Methods.

mRNA in WT mice was compared with that of *irs1*^{-/-} by quantitative PCR analyses using the comparative (C_t) method with *actin* as the reference gene. As shown in Fig. 3B, *cdk1* transcripts increased in both the groups by 24 h after estrogen treatment. Interestingly, the level of uterine *cdk1* mRNA in the IRS-1 null mutants was significantly greater ($P<0.05$) than that of the WT mice at this time point.

To compare the levels of *cdk1* protein, the extracts of WT and mutant uterine epithelium were prepared at various time points after hormone treatment and evaluated by immunoblotting. In accordance with the microarray and quantitative PCR data, *cdk1* (p34) protein in the quiescent uterine epithelium of ovariectomized mice (time 0), WT or *irs1*^{-/-}, was below the level of detection (Fig. 4A). When mice were treated with E₂, uterine *cdk1* increased and could be readily detected by 18 h in WT mice. In contrast, the uterine *cdk1* abundance in *irs1*^{-/-} (KO) mice was generally much less than that observed in WT mice at the corresponding

intervals after hormone treatment (Fig. 4A). In the four trials that analyzed the extracts from uteri obtained 24 h after estrogen treatment, the levels of *cdk1* protein in mutants were significantly less ($P<0.005$) than that of WT: KO (0.41 ± 0.09 , mean $\pm$ s.d.) versus WT (1.16 ± 0.14 , mean $\pm$ s.d.); KO/WT = 0.35 (Fig. 4B).

For both WT and mutants, the level of hormone-induced *cdk1* was highest at the 30-h time point, which is after peak mitosis in this model. It is not known whether treatment with E₂ alone ('unopposed') favors this pattern, which might differ considerably from that of the intact female exposed to sequential cycles of estrogens and progestins. Levels of *cdk2* (p33) kinase, which forms a heterodimer with cyclin A or cyclin E, were also examined in the uterine extracts of WT and *irs1*^{-/-} mice treated with hormone. As shown in Fig. 4 (upper panel), *cdk2*, in contrast to *cdk1*, was detected in the extracts before and after hormone treatment. Hormone treatment did not elevate uterine *cdk2* protein levels, which is in accordance with the microarray findings (Fig. 3A). The discrepancy observed for *cdk1* in WT and *irs1*^{-/-} mice was not apparent for *cdk2*.

*The stimulation of AKT phosphorylation and cyclin B-associated kinase by E₂ is reduced in the *irs1*^{-/-} mutant*

Since hormone-induced mitosis was inhibited by the *irs1*^{-/-} null mutants, we wanted to resolve whether the mutation compromised mitotic kinase activity. *Cdk1* acquires the potential to become a mitotic kinase in mammalian cells when bound to cyclin B (Meijer *et al.* 1989). It is possible that the observed reduction in *cdk1* (protein) in mutants prevented accumulation of enough mitotic kinase for mitosis. Alternatively, the mutation might affect posttranslational changes that regulate *cdk1*. Activation and inhibition of *cdk1* kinase are known to occur by phosphorylation and dephosphorylation of specific sites, and it is likely that some of these modifications are integrated with growth factor pathways. The activation of AKT (Ser473 phosphorylation) can be demonstrated in our experimental model, as shown by the time-course study in Fig. 5. AKT phosphorylation is clearly evident in the extracts from WT uterine epithelium obtained at 12 and 18 h following E₂ treatment. Based on densitometric scans obtained at the 12-h time point, the p-AKT/AKT in *irs1*^{-/-} mice (0.55 ± 0.04 , mean $\pm$ s.d.) was significantly less ($P<0.005$) than that of WT mice (1.31 ± 0.16 , mean $\pm$ s.d.); KO/WT = 0.42. For the time-course experiment shown in Fig. 5, the p-AKT/AKT KO/WT ratios at 18 and 24 h after hormone treatment were similar to that reported at 12 h (data not shown). After 24 h, the level of AKT phosphorylation was very weak in both the groups (Fig. 5). These findings support that an IRS-1-dependent pathway is utilized for the hormonal activation of uterine epithelial AKT. Other (non-IRS-1) signaling pathways probably account for the phosphorylation of AKT observed in mutant animals.

To determine whether the IRS-1 null mutation can affect mitotic kinase activity, uterine cyclin B-associated kinase

Figure 4 Time-course analyses of *cdk1* and *cdk2* (immunoreactivity) in uterine epithelial extracts of wild-type and *irs1*^{-/-} mice. Ovariectomized wild-type (WT) and *irs1*^{-/-} (KO) mutant mice were treated with estradiol, and uterine epithelial extracts were obtained at the time points indicated. Mice at the 0 time point did not receive hormone. Proteins (~30 µg) in aliquots from the extracts were separated by SDS-PAGE and immunoblotted for *cdk1* or *cdk2*. Further details of the extraction and immunoblotting are described in Materials and Methods. In (A), immunoblots of uterine *cdk1* and *cdk2* are compared for up to 30 h after estradiol treatment. The results are representative of six different trials. Extracts for each trial (time course) were prepared on separate days; each lane represents an extract from one mouse uterus. In (B), immunoblots of *cdk1* from wild-type (WT) and *irs1*^{-/-} (KO) mouse uterine extracts are compared. All the extracts were obtained from uteri 24 h after hormone treatment. Extracts from each of the four pairs of blots (WT versus KO) were prepared on different days; each lane represents an extract from one mouse uterus. Cytokeratin 18 (ck18), an epithelial marker, was a loading control. As determined by densitometry, KO *cdk1* levels (0.41 ± 0.09 , mean $\pm$ s.d.) were significantly less ($P<0.005$) than that of WT (1.16 ± 0.14 , mean $\pm$ s.d.); KO/WT = 0.35.

Figure 5 AKT phosphorylation in the uterine epithelium of estradiol-treated wild-type mice and *irs1*^{-/-} (KO) mice. Ovariectomized wild-type (WT) and *irs1*^{-/-} (KO) mice were treated with estradiol, and uterine epithelial extracts were obtained at the time points indicated. Mice at the 0 time point did not receive hormone. Each lane represents analyses of one uterus. Equal amounts of extracted protein (300–400 µg) were used for WT and KO mice in each experiment. AKT was immunoprecipitated from extracts with 5 µg anti-AKT antibody and immunoblots made with anti-phospho-AKT (Ser 473) and anti-AKT antibodies. Further details are described in Materials and Methods. The results shown are representative of four separate trials with different animals. Densitometric scans of the 12-h time point revealed that the uterine p-AKT/AKT in KO mice (0.55 ± 0.04 , mean $\pm$ s.d.) was significantly less ($P < 0.005$) than that of WT mice (1.31 ± 0.16 , mean $\pm$ s.d.); KO/WT = 0.42.

activity was examined for up to 48 h following treatment of the mice with E₂. The phosphorylation of the histone H1 (exogenous) substrate was clearly evident at 20 h after hormone treatment in WT samples; however, much less kinase activity was observed at 48 h (Fig. 6A). This temporal pattern is in keeping with the importance of cdk1/cyclin B kinase activity for entry into the mitotic phase of the cell cycle (Lew & Kornbluth 1996). The corresponding time-course study with samples from *irs1*^{-/-} uteri revealed much less phosphorylation of histone H1 than that obtained with WT samples. As determined by phosphoimaging, the cyclin B-associated kinase activity at 20 h after E₂ treatment in *irs1*^{-/-} samples ($1.25 \pm 0.17 \times 10^4$, mean $\pm$ s.d.) was significantly less ($P < 0.005$) than that of WT samples ($3.16 \pm 0.22 \times 10^4$, mean $\pm$ s.d.); KO/WT = 0.38. This effect of the IRS-1 null mutation on hormonal activation of cyclin B-associated kinase activity was not observed with cyclin A-associated kinase activity. As shown in Fig. 6B, the ³²P-labeling of the histone H1 substrate by kinase associated with cyclin A precipitates was comparable in the extracts from WT and *irs1*^{-/-} mice. Immunoblots were made of cyclin B and cyclin A in the extracts obtained after 20 h. The amount of each cyclin appeared comparable in WT and *irs1*^{-/-} samples (Fig. 6B). Therefore, it is unlikely that the disparity between hormone-induced cyclin B-associated kinase activity in *irs1*^{-/-} and WT uteri could be explained by lower cyclin B levels.

Phosphorylation of H1 histone during mitosis entry is considered a downstream event of cdk1/cyclin B (Langan *et al.* 1989). Therefore, we examined the effect of the IRS-1 null mutation on hormone-induced phosphorylation of endogenous H1 histone with a phospho-specific (pThr18)

antiserum to a cognate sequence of phospho (Thr18) mouse histone H1.3. As shown in Fig. 6C, three major bands corresponding to H1 isoforms were detected in the acid extracts of isolated uterine nuclei from WT and *irs1*^{-/-} mice. Twenty hours following hormone treatment, an immunoreactive band was observed in WT extracts that comigrated with the H1.3/H1.4 isoforms. In contrast, hormone-induced phosphorylation of these isoforms isolated from *irs1*^{-/-} mice was much less than that observed with WT mice. This finding suggests that the activation of the IGF1/IRS-1 pathway by E₂ can result in phosphorylation of endogenous histone.

Figure 6 Cyclin A- and cyclin B-associated kinase activities in the wild-type and *irs1*^{-/-} uterine epithelium. (A) Uterine epithelial extracts were obtained from ovariectomized wild-type (WT) and *irs1*^{-/-} (KO) mice after the indicated interval following treatment with estradiol. Mice at the 0 time point did not receive estradiol. Cyclin B was immunoprecipitated from equal amounts (~400 µg protein) of extracts from WT and KO uteri and analyzed for histone H1 kinase activity. More details of the immunoprecipitation and the kinase assay are described in Materials and Methods. The kinase samples were run on 4–20% Tris Ready Gels and then transblotted to Immobilon-P membrane. Specific quantitation of the ³²P-labeled histone in the 20 h WT and KO samples was made by phosphoimaging; $n = 5$ for each group. Cyclin B-associated kinase activity associated with KO uterine samples ($1.25 \pm 0.17 \times 10^4$, mean $\pm$ s.d.) was significantly less ($P < 0.005$) than that of WT samples ($3.16 \pm 0.22 \times 10^4$, mean $\pm$ s.d.); KO/WT = 0.38. (B, left) Cyclin A- and cyclin B1-associated kinase activities were measured in uterine epithelial extracts obtained 20 h after hormone treatment. (B, right) Immunoblots were made of cyclin A and cyclin B1 in uterine epithelial extracts obtained from wild-type and mutant mice 20 h after hormone treatment. (C) Histones were enriched from whole uterine nuclear fractions, separated by 16% SDS-PAGE, and transblotted. The upper panel represents staining with Coomassie Blue; in the lower panel, the blot was probed with antiserum to mouse phospho (Thr18) histone H1.3/1.4 as described in Materials and Methods. Veh, represents extracts from vehicle-treated, or control, mice. The data in A and B are representative of five separate trials; the data in C are representative of three separate trials. Each trial used extracts from different mice, and each lane represents an extract from a single mouse uterus.

Discussion

The use of the whole animal as an experimental model is important in accurately resolving the qualitative and quantitative responses of hormones. Cell cultures are generally inappropriate for studying estrogen-stimulated proliferation because of the requirement for normal epithelial/stromal interactions (Cooke *et al.* 1997, 2002). Cell cultures may not only be refractory to hormone action but also impose artifacts as a consequence of immortalization, high rate of cell turnover, or inappropriate activation of signaling pathways. This later aspect is particularly important in the present investigation where we are trying to link an E₂-induced growth factor pathway with components of the cell cycle.

Since E₂ treatment of ovariectomized *igf1*^{-/-} female mice failed to stimulate binding of p85 (PI3K) to IRS-1 in uterine epithelial cells, mice with the IRS-1 null mutation should reflect the importance of this adaptor protein as well as of IGF1 receptor signaling in response to E₂. Earlier studies in this laboratory showed that PI3K was the only SH2 protein that bound to uterine IRS-1 following E₂ treatment (Richards *et al.* 1998). Thus, the absence of IRS-1 should – barring any adaptive changes in the null mutant – selectively deprive the uterine epithelial cell of that pool of PI3K that becomes catalytically active by binding to IRS-1 as a consequence of E₂ stimulation of the IGF1 receptor pathway. It was previously reported that stimulation of PI3K activity by IGF1 in *irs1*^{-/-} mouse embryonic fibroblasts was reduced by 70% when compared with that of *irs1*^{+/+} cells (Bruning *et al.* 1997). Mouse embryonic cells, along with uterine cells, contain IRS-2, which can also bind and activate PI3K (Sun *et al.* 1995). This IRS isoform does not functionally supplant IRS-1 in the embryonic fibroblasts. In addition, after E₂ treatment, uterine IRS-2 is degraded through an IGF-stimulated, proteasome-dependent pathway, while levels of IRS-1 remain essentially unchanged (Richards *et al.* 2001).

The IRS-1 null mutation did not significantly alter the fraction of uterine epithelial cells undergoing DNA synthesis in response to E₂; however, this mutation markedly reduced the fraction of cells undergoing mitosis. These data are very similar to the cytokinetic response to E₂ in uterine cells of *igf1*^{-/-} mice (Adesanya *et al.* 1999). In a broad sense, these findings indicate that a growth factor pathway can regulate a specific stage, e.g. mitosis, of the cell cycle. Thus, we can propose that an IGF1R/IRS-1/PI3K pathway stimulates the rate of mitosis in the uterine epithelium in response to estrogens. There are several reports where cell lines were used to demonstrate that a signaling pathway downstream from PI3K/AKT functions in the G₂→M transition of the cell cycle. In synchronized HeLa or MDCK cells, AKT activity was maximal during the G₂/early M phase and then dropped precipitously as cells completed mitosis (Shtivelman *et al.* 2002). Inhibition of PI3K in these cells prevents the activation of cyclin B-associated kinase and elicits G₂ arrest that can be abolished by activated AKT. In HEK293 cells, inhibition of

PI3K also induces a G₂ cell cycle arrest that could be alleviated by activated AKT. Mouse embryonic fibroblasts with an AKT1 null mutation revealed a delayed transition from G₂/M to G₁ (Kandel *et al.* 2002). In this study, the reduction in PI3K/AKT activation caused by the IRS-1 null mutation apparently led to a loss of specific stimulation of the G₂→M transition. The component(s) of the cell cycle regulated by the hormonally stimulated IRS-1/PI3K/AKT that would specifically influence the mitotic phase is unknown.

In accordance with the reduced rate of E₂-induced mitosis in uterine cells of *irs1*^{-/-} mice, there was a marked reduction in cyclin B-associated kinase activity. Other cdks cannot supplant the function of cdk1 as a mitotic kinase in mammalian cells (Nurse 1990, Nigg 1993). On the other hand, cdk1 can associate with A- and B-type cyclins in mammalian cells to phosphorylate histone H1 (Loyer *et al.* 1994, Swank *et al.* 1997). Since levels of cyclin A, cdk2, and cyclin A-associated H1 kinase activity were not affected by the IRS-1 null mutation, it is likely that cyclin A predominantly functions in uterine cells as a complex with cdk2. This would favor the notion that IGF1R signaling in these cells targets cdk1, and not other cdks, such as cdk2. The level of cdk1 in the IRS-1 null mutants prior to mitosis was less than that in WTs, which could delay the formation of the threshold concentration of the mitotic kinase (heterodimer). Since the lower levels of cdk1 protein could not be explained by a corresponding decrease in transcript, altered signaling in the mutant might have reduced translation or half-life of cdk1. Earlier studies showed that IRS-1 and PI3K were required for insulin to stimulate eIF-4E/4E-BP-1 phosphorylation and translation in cultured cells (Mendez *et al.* 1996). Using phospho-specific antisera, we demonstrated that the translation effectors mechanistic target of rapamycin, tuberlin, 4E-BP1, eIF-4E, p70S6 kinase, and glycogen synthase kinase 3 are activated in response to E₂; however, we could not demonstrate an effect of the IRS-1 null mutation on specific phosphorylation of these translational components (data not shown). To our knowledge, this specific effect of the IGF1R signaling on cdk1 protein levels has not been previously reported. One study did suggest that translational control of cdk1 (p34^{cdc2}) was associated with meiotic competence of mouse oocytes; incompetent oocytes have abundant *cdk1* mRNA but low levels of cdk1 protein (deVantery *et al.* 1997). Our experimental model may have allowed us to identify changes in cell cycle components that do not occur or are obscured by most cells in culture. With many immortalized cell lines, for instance, the cdk1 protein is expressed at all stages of the cell cycle (McGowan *et al.* 1990, Welch & Wang 1992, Draetta & Beach 1998). However, there are aspects of cdk1 transcripts in mammalian cell cultures that overlap with those observed in animal studies. For instance, cdk1 transcription in HeLa cells is elevated in the S and G₂ phases and low in the G₁ phase of the cell cycle, based on nuclear run-on assays (Dalton 1992). Likewise, other studies with HeLa cells revealed that the abundance of *cdk1* mRNA and rate of synthesis of cdk1 protein (p34) increase dramatically as the cells pass through S/G₂ (Welch & Wang 1992).

Since the KO/WT for p-AKT/AKT was similar at different times (12, 18, and 24 h) after hormone treatment, it is likely that the IRS-1 null mutation does not affect the rate of p-AKT dephosphorylation at Ser473. During the period when uterine AKT phosphorylation was elevated in WT and *irs1*^{-/-} mice, AKT was essentially unchanged from that of untreated mice; the increased level of AKT observed after 24 h following treatment with E₂ is in keeping with the known increase in AKT transcripts in response to this hormone (Hewitt *et al.* 2003). It was reasonable to expect in our model that the IRS-1 null mutation would cause a pronounced reduction in AKT activation. Previous reports suggest that enzymes that regulate phosphorylation and activity of the mitotic kinase are proximal targets of AKT. For instance, phosphorylation at Thr14 and Tyr15 of cdk1 by Myt1, a member of the Wee family of kinases, inhibits the kinase activity of cdk1 (Mueller *et al.* 1995). In a study with starfish oocytes, it was shown that AKT, acting downstream from PI3K, phosphorylates Myt1 and downregulates its activity, which favors the meiotic G₂/M phase transition (Okumura *et al.* 2002). A related study showed that AKT can phosphorylate Wee 1 in 293T or HeLa cells, which promotes cytoplasmic localization at the kinase (by binding to 14-3-3q), and G₂/M cell progression (Katayama *et al.* 2005). Thus, it is possible that a deficient PI3K/AKT signaling pathway in the IRS-1 null mutants increases phosphorylation at Thr14/Tyr15 of cdk1 and thereby compromises its kinase activity. Alternatively, AKT might also be activating cdc25C, which dephosphorylates Thr14/Tyr15 of cdk1. Inhibition of PI3K by LY294002 in HeLa cells delays hyperphosphorylation of cdc25C and cells entering mitosis (Dangi *et al.* 2003). However, it is still not known whether a PI3K/AKT pathway is activating for cdc25C, directly or indirectly. In another study, wortmannin, a potent inhibitor of PI3K, partially inhibited cyclin B translation and inhibited dephosphorylation of the tyrosine (inhibitory) site of cdk1 of sea urchin oocytes (Salaün *et al.* 2002). In the later study, the effects of wortmannin on cyclin B synthesis (triggered by fertilization) were also correlated with the effects of this compound on cap-dependent translation machinery, which included 4E-BP/eIF-4E dissociation. AKT facilitates the G₂/M phase transition of mouse PC12 neuronal cells (Lee *et al.* 2005). Stable expression of a dominant negative form of AKT suppressed expression of both cdk1 and cyclin B1 at the mRNA and protein levels without affecting the cyclin A or cdk2 protein levels. A constitutively active form of AKT (myristoylated AKT) elevated cyclin B and cdk1 mRNA and protein levels and could override cell growth arrest at G₂/M (induced by etoposide).

The present finding that the hormone-induced phosphorylation of an endogenous histone H1 isoform in WTs clearly exceeded that of *irs1*^{-/-} mice is in accordance with the observed decrease of cyclin B-associated kinase activity in the mutant animals. The purified cdk1/cyclin B heterodimer phosphorylates specific sites on histone H1 *in vitro* (Swank *et al.* 1997). Earlier studies had also provided evidence to show that the cdks are the major *in vivo* kinases for H1 at cell cycle

transitions with cdk1 implicated as the G₂ kinase for H1 (Langan *et al.* 1989). The phosphorylation of histone H1 *in vivo* by a cdk is considered to destabilize H1–chromatin interactions and, thereby, facilitate a more open chromatin structure (Contrera *et al.* 2003). Thus, by regulating cyclin B-associated kinase activity an IGF1R/IRS-1/PI3K-stimulated signaling cascade mediates hormonal effects on the state of chromatin condensation. A query to consider for postmenopausal females receiving prolonged exposure to estrogens is whether the perturbation of chromatin structure by this signaling pathway facilitates genomic instability and increased risk of endometrial cancer (Enders & Maude 2006).

Our cumulative findings support that the stimulation of uterine epithelial cell proliferation by E₂ is mediated by an IGF1/IRS-1/PI3K/AKT pathway, and that this pathway targets the mitotic kinase cdk1/cyclin B. A specific goal of future work should determine whether impairment of AKT activity by the IRS-1 null mutation affects the activity of enzymes or other proteins that regulate posttranslational changes or catalytic activity of cdk1. Other cell populations might have mechanisms to regulate mitotic kinase activity that are similar to or overlap with those observed in this study. In a broad sense, our experimental findings are probably part of a more general phenomenon, where individual growth factors exert stage-specific regulation of the cell cycle by activating or suppressing specific cyclin-associated kinases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by the Intramural Research Program of the National Institute of Environmental Health Sciences, NIH; project number Z01 ES071006-09.

Acknowledgements

We thank Drs C Ronald Kahn and Argiris Efstratiadis for providing mice with an IRS-1 null allele and IGF1 null allele respectively. We thank Jesse DeGraff for supervising the breeding of the mutant mice. The assistance given by Janice Hicks, Maureen Trogon, Ervin Smallwood, Colleen Anna, and Wes Gladwell is also greatly appreciated. We thank James Clark and Page Meyers for reliably preparing ovariectomized mice throughout the study. Sylvia C Hewitt and Dr Bonnie Deroo are gratefully acknowledged for providing the mouse uterine microarray data. The critique of the manuscript by Tom Gray, Sylvia C Hewitt, and Dr Grace E Kissling is also greatly appreciated.

References

- Adesanya OO, Zhou J, Samathanam C, Powell-Braxton L & Bondy CA 1999 Insulin-like growth factor 1 is required for G₂ progression in the estradiol-induced mitotic cycle. *PNAS* **96** 3287–3291. (doi:10.1073/pnas.96.6.3287)

- Altucci L, Addeo L, Cicatiello L, Germano D, Pacilio C, Battista T, Cancemi M, Petrizzi VB, Bresciani F & Weisz A 1997 Estrogen induces early and timed activation of cyclin-dependent kinases 4, 5, and 6 and increases cyclin messenger ribonucleic acid expression in rat uterus. *Endocrinology* **138** 978–984. (doi:10.1210/en.138.3.978)
- Araki E, Lipes MA, Patti ME, Bruning JC, Haag BL III, Johnson RS & Kahn CR 1994 Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. *Nature* **372** 186–190. (doi:10.1038/372186a0)
- Archer TK, Cordinley MG, Wolford RG & Hager GL 1991 Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumor virus promoter. *Molecular and Cellular Biology* **11** 688–698.
- Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellvire AR & Efstratiadis A 1996 Effects of an Igf1 gene null mutation on mouse reproduction. *Molecular Endocrinology* **10** 903–918. (doi:10.1210/me.10.7.903)
- Bruning JC, Winnay J, Cheatham B & Kahn CR 1997 Differential signaling by insulin receptor substrate 1 (IRS-1) and IRS-2 in IRS-1-deficient cells. *Molecular and Cellular Biology* **17** 1513–1521.
- Campisi J & Pardee AB 1984 Post-translational control of the onset of DNA synthesis by an insulin-like growth factor. *Molecular and Cellular Biology* **4** 1807–1814.
- Contrera AA, Hale TK, Stenoien DL, Rosen JM, Mancini MA & Herrera RE 2003 The dynamic mobility of histone H1 is regulated by cyclin/cdk phosphorylation. *Molecular and Cellular Biology* **23** 8626–8636. (doi:10.1128/MCB.23.23.8626-8636.2003)
- Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB & Cunha GR 1997 Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *PNAS* **94** 6535–6540. (doi:10.1073/pnas.94.12.6535)
- Cooke PS, Buchanan DL, Kurita T, Lubahn DB & Cunha GR 2002 Role of stromal-epithelial interactions in hormonal responses in the uterus. In *The Endometrium*, pp 151–166. Eds SR, Glasser, JD Alpin, LC Giudice & S Tabibzadeh. London: Taylor & Francis.
- Couse JF, Curtis SW, Washburn TF, Lindzey J, Golding TS, Lubahn DB, Smithies O & Korach KS 1995 Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. *Molecular Endocrinology* **9** 1441–1454. (doi:10.1210/me.9.11.1441)
- Dalton S 1992 Cell cycle regulation of the human cdc2 gene. *EMBO Journal* **11** 1797–1804.
- Dangi S, Cha H & Shapiro P 2003 Requirement for phosphatidylinositol-3 kinase activity during progression through S-phase and entry into mitosis. *Cellular Signalling* **15** 667–675. (doi:10.1016/S0898-6568(03)00002-0)
- Deterding LJ, Bunger MK, Banks GC, Tomer KB & Archer TK 2008 Global changes in and characterization of specific sites of phosphorylation in mouse and human histone H1 isoforms upon CDK inhibitor treatment using mass spectrometry. *Journal of Proteome Research* **7** 2368–2379. (doi:10.1021/pr700790a)
- Draetta G & Beach D 1998 Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* **54** 17–26. (doi:10.1016/0092-8674(88)90175-4)
- Enders GH & Maude SL 2006 Traffic safety for the cell: influence of cyclin-dependent kinase activity on genomic stability. *Gene* **371** 1–6. (doi:10.1016/j.gene.2005.11.017)
- Fagg B, Martin L, Rogers LA, Clark BF & Quarmby VE 1979 A simple method for preparing pure samples of uterine cells. *Journal of Reproduction and Fertility* **57** 335–345. (doi:10.1530/jrf.0.0570335)
- Gambrell RD, Bagnell CA & Greenblatt RB 1983 Role of estrogens and progesterone in the etiology and prevention of endometrial cancer: review. *American Journal of Obstetrics and Gynecology* **146** 696–707.
- Hewitt SC, Deroo BJ, Hansen K, Collins J, Grissom S, Afshari CA & Korach KS 2003 Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. *Molecular Endocrinology* **17** 2070–2083. (doi:10.1210/me.2003-0146)
- Kandel ES, Keen JS, Majewski N, DiCristofano A, Pandolfi PP, Feliciano CS, Gartel A & Hay N 2002 Activation of Akt/protein kinase B overcomes a G₂/M cell cycle checkpoint induced by DNA damage. *Molecular and Cellular Biology* **22** 7831–7841. (doi:10.1128/MCB.22.22.7831-7841.2002)
- Katayama K, Fujita N & Tsururo T 2005 Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle progression at G₂/M transition. *Molecular and Cellular Biology* **25** 5725–5737. (doi:10.1128/MCB.25.13.5725-5737.2005)
- Kim YH, Proust JJ, Buchholz MJ, Chrest FJ & Nordin AA 1992 Expression of the murine homologue of the cell cycle control protein p34^{cdc2} in T lymphocytes. *Journal of Immunology* **149** 17–23.
- Klotz DM, Curtis Hewitt S, Korach KS & DiAugustine RP 2000 Activation of a uterine insulin-like growth factor I signaling pathway by clinical and environmental estrogens: requirement of estrogen receptor- α . *Endocrinology* **141** 3430–3439. (doi:10.1210/en.141.9.3430)
- Langan TA, Gautier J, Lohka M, Hollingsworth R, Moreno S, Nurse P, Maller J & Sclafani RA 1989 Mammalian growth-associated H1 histone kinase: a homolog of cdc2 + /cdc28 protein kinases controlling mitotic entry in yeast and frog cells. *Molecular and Cellular Biology* **9** 3860–3868.
- Lee S-R, Park JH, Park EK, Chung CH, Kang S-S & Bang OS 2005 Akt-induced promotion of cell-cycle progression at G₂/M phase involves upregulation of NF- κ B binding activity in PC12 cells. *Journal of Cellular Physiology* **205** 270–277. (doi:10.1002/jcp.20395)
- Leombo G, Rockman HA, Hunter JJ, Steinmetz H, Koch WJ, Ma L, Prinz MP, Ross K Jr, Chien KR & Powell-Braxton L 1996 Elevated blood pressure and enhanced myocardial contractility in mice with severe IGF-1 deficiency. *Journal of Clinical Investigation* **98** 2648–2655. (doi:10.1172/JCI119086)
- Leof EB, Wharton W, van Wyk JJ & Pledger WJ 1982 Epidermal growth factor (EGF) and somatomedin C regulate G₁ progression in competent BALB/c-3T3 cells. *Experimental Cell Research* **141** 107–115. (doi:10.1016/0014-4827(82)90073-8)
- Lew DJ & Kornbluth S 1996 Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. *Current Opinion in Cell Biology* **8** 795–804. (doi:10.1016/S0955-0674(96)80080-9)
- Liu JP, Baker J, Perkins AS, Robertson EJ & Efstratiadis A 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (IGF-1) and type 1 IGF receptor (IGF1r). *Cell* **75** 59–72. (doi:10.1016/S0092-8674(05)80084-4)
- Lowe WL 1991 Biological actions of the insulin-like growth factor. In *Insulin-Like Growth Factors: Molecular and Cellular Aspects*, pp 49–85. Ed. D LeRoith. Boca Raton, FL: CRC Press.
- Loyer P, Glaire D, Cariou S, Baffet G, Meijer L & Guguen-Guillouzo C 1994 Expression and activation of cdk1 (1 and 2) and cyclins in the cell cycle progression during liver regeneration. *Journal of Biological Chemistry* **269** 2491–2500.
- McGowan CH, Russell P & Reed SI 1990 Periodic biosynthesis of the human M-phase promoting factor catalytic subunit p34 during the cell cycle. *Molecular and Cellular Biology* **10** 3847–3851.
- Meijer L, Arion D, Golsteyn R, Pines J, Brizuela L, Hunt T & Beach D 1989 Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. *EMBO Journal* **8** 2275–2282.
- Mendez R, Myers MG Jr, White MF & Rhoads RE 1996 Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Molecular and Cellular Biology* **16** 2857–2864.
- Minshull J, Goldsteyn R, Hill CS & Hunt T 1990 The A- and B- type cyclin associated cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. *EMBO Journal* **9** 2865–2875.
- Mueller PR, Coleman TR, Kumagai A & Dunphy WG 1995 Myt1: a membrane-associated inhibitory kinase that phosphorylates cdc2 on both threonine-14 and tyrosine-15. *Science* **270** 86–90. (doi:10.1126/science.270.5233.86)
- Murphy LJ, Murphy LC & Friesen HG 1987 Estrogen induces insulin-like growth factor-1 expression in the rat uterus. *Molecular Endocrinology* **1** 445–450. (doi:10.1210/mend-1-7-445)

- Nigg EA 1993 Cellular substrates of p34^{cdc2} and its companion cyclin-dependent kinases. *Trends in Cell Biology* **3** 296–301. (doi:10.1016/0962-8924(93)90011-O)
- Nurse P 1990 Universal control mechanism regulating onset of M-phase. *Nature* **344** 503–508. (doi:10.1038/344503a0)
- Okumura E, Fukuhara T, Yoshida H, Hanada S, Kozutsumi R, Mori M, Tachibana K & Kishimoto T 2002 Akt inhibits Myt1 in the signaling pathway that leads to meiotic G₂/M-phase transition. *Nature Cell Biology* **4** 111–116. (doi:10.1038/ncb741)
- Osborne CK, Coronado EB, Kitten LJ, Arteaga CI, Fuqua SA, Ramasharma K, Marshall M & Li CH 1989 Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-1 receptor. *Molecular Endocrinology* **3** 1701–1709. (doi:10.1210/mend-3-11-1701)
- Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45 (online). (doi:10.1093/nar/29.9.e45)
- Richards RG, DiAugustine RP, Petrusz P, Clark GC & Sebastian J 1996 Estradiol stimulates tyrosine phosphorylation of the insulin-like growth factor-1 receptor and insulin receptor substrate-1 in the uterus. *PNAS* **93** 12002–12007. (doi:10.1073/pnas.93.21.12002)
- Richards RG, Walker MP, Sebastian J & DiAugustine RP 1998 Insulin-like growth factor-1 (IGF-1) receptor–insulin receptor substrate complexes in the uterus. Altered signaling response to estradiol in the IGF-1^{mv/m} mouse. *Journal of Biological Chemistry* **273** 11962–11969. (doi:10.1074/jbc.273.19.11962)
- Richards RG, Klotz DM, Bush MR, Walmer DK & DiAugustine RP 2001 E₂-induced degradation of uterine insulin receptor substrate-2: requirement for an IGF-1-stimulated, proteasome-dependent pathway. *Endocrinology* **142** 3842–3849. (doi:10.1210/en.142.9.3842)
- Rose DW, Saltile AR, Majumdar M, Decker SJ & Olefsky JM 1994 Insulin receptor substrate 1 is required for insulin-mediated mitogenic signal transduction. *PNAS* **91** 797–801. (doi:10.1073/pnas.91.2.797)
- Salaün P, LeBreton M, Morales J, Belle R, Boulben S, Mulner-Lorillon O & Cormier P 2002 Signal transduction pathways that contribute to CDK1/cyclin B activation during the first mitotic division in sea urchin embryos. *Experimental Cell Research* **296** 347–357. (doi:10.1016/j.yexcr.2004.02.013)
- Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, Rubin R, Efstratiadis A & Baserga R 1994 Effect of a null mutation of the type 1 IGF receptor gene on growth and transformation of mouse embryo fibroblasts. *Molecular and Cellular Biology* **14** 3604–3612.
- Shtivelman E, Sussman JS & Stokoe D 2002 A role for PI 3-kinase and PKB activity in the G₂/M phase of the cell cycle. *Current Biology* **12** 919–924. (doi:10.1016/S0960-9822(02)00843-6)
- Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG Jr, Glasheen EM, Lane WS, Pierce JH & White MF 1995 A role for IRS-2 in insulin and cytokine signaling. *Nature* **377** 173–177. (doi:10.1038/377173a0)
- Swank RA, Th'ng JPH, Guo X-W, Valdez J, Bradbury EM & Gurley LR 1997 Four distinct cyclin-dependent kinases phosphorylate histone H1 at all of its growth-related phosphorylation sites. *Biochemistry* **36** 13761–13768. (doi:10.1021/bi9714363)
- Tong W & Pollard JW 1999 Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E-and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice. *Molecular and Cellular Biology* **19** 2251–2264.
- Tong W & Pollard JW 2002 Female sex steroid hormone regulation of cell proliferation in the endometrium. In *The Endometrium*, pp 94–109. Eds SR Glasser, JD Aplin, LC Giudice & S Tabibzadeh. London: Taylor & Francis.
- deVantery C, Stutz A, Vassalli JD & Schorderet-Slatkine S 1997 Acquisition of meiotic competence in growing mouse oocytes is controlled at both translational and posttranslational levels. *Developmental Biology* **187** 43–54. (doi:10.1006/dbio.1997.8599)
- Waters SB, Yamauchi K & Pessin JE 1993 Functional expression of insulin receptor substrate-1 is required for insulin-stimulated mitogenic signaling. *Journal of Biological Chemistry* **268** 22231–22234.
- Welch PJ & Wang JYJ 1992 Coordinated synthesis and degradation of cdc2 in the mammalian cell cycle. *PNAS* **89** 3093–3097. (doi:10.1073/pnas.89.7.3093)

Received in final form 18 August 2010

Accepted 26 August 2010

Made available online as an Accepted Preprint
26 August 2010