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Loss of Estrogen Receptor Alpha and its Contribution to Tumorigenesis in Breast Cancer

By Christine Couldrey, PhD, and Jeffrey E. Green, PhD

ESTROGENS PLAY A PIVOTAL ROLE IN SEXUAL DEVELOPMENT, REPRODUCTION, AND PHYSIOLOGical processes in a variety of tissues, including mammary, pituitary, bone, and liver tissue, as well as the cardiovascular system.[1] However, estrogens also are involved in various pathological processes such as breast and endometrial cancer.[2] At the present time, the primary treatment regimen for breast cancer involves endocrine therapies that inhibit estrogen signaling. One of the major challenges to improving the treatment of breast cancer is understanding and overcoming the resistance to endocrine therapy that often develops during the course of treatment.

Estrogen elicits its effect through the activation of estrogen receptors (ER). These receptors belong to a super family of nuclear hormone receptors that includes retinoic acid, progesterone, glucocorticoid, androgen, and thyroid hormone receptors. To date, two genes that encode ERs have been identified (*ERα* and *ERβ*). These genes are expressed in a tissue-specific and temporal manner, and are influenced by estrogen and other hormones.[3-5] ERα is the major form of ER in the mammary gland and in breast cancers; therefore, the following discussion will focus on the loss of ERα expression in breast cancer.

Molecular Mechanisms of Estrogen Receptor Expression

The *ERα* gene is made up of eight exons, encoding a protein of 595 amino acids that can be divided into six conserved functional domains. *(See Figure 1.)* Estradiol (the most potent form of estrogen) elicits its effect by binding to ER, causing a conformational change in the protein, displacement of heat shock proteins, and phosphorylation of the receptor. These changes allow receptor homodimerization and subsequent binding to cis-acting elements within enhancers of target genes *(see Figure 2)*, such as *c-myc*, progesterone receptor, and *TGF-α*, ultimately leading to the proliferation of ER-positive cells.

INSIDE

Approximately two-thirds of human breast cancers express ER, and their growth is stimulated by estrogen. For these tumors, therapeutic strategies include estrogen ablation or the administration of anti-estrogens (e.g., tamoxifen). The remaining one-third of breast cancers lack ER. These tumors can grow in the absence of estrogen, rarely respond to hormonal therapy, and are associated with less differentiated, more aggressive tumors and shorter disease-free survival.[6] Given the importance of ER and the fact that all current therapies act through ER, defining how and why tumors become ER-negative is a critical step in improving therapeutic outcomes. Unfortunately, the molecular mechanisms underlying the lack of ER expression are poorly understood.

Loss of ERα During Tumorigenesis

A number of factors influencing ERα expression have been identified.[7] Loss of functional ERα may occur because of aberrations in the *ER* gene (i.e., mutations or rearrangements), or because of changes at the transcriptional, post-transcriptional, or translational levels.

Genomic Alterations

The human *ERα* gene is located on chromosome 6q25. Although homozygous deletion containing this region of chromosome 6 has not been reported, loss of heterozygosity (LOH) has been noted in 80-90% of breast cancers. However, mutation of one allele and loss or replacement of a chromosomal segment on the other were not found to be accompanied by changes in ER expression.[8]

Point mutations that cause functional alterations of the ER protein also are rare. One study of 188 breast cancer patients that used single-stranded polymorphism analysis, denaturing gradient gel electrophoresis, and DNA sequencing reported that loss of ER was not the result of mutations in the coding region of the *ERα* gene.[9] Together, these results suggest that genetic alterations in the *ERα* gene at the DNA level might account only for a small portion of hormone independence. However, it is important to note that breast tumors are a heterogenous population that includes normal and malignant cells; current technologies may not be sufficiently sensitive to identify *ER* mutations that occur infrequently.

Splicing Variants of ER mRNA

In contrast to the minimal number of mutations that have been identified, a significant number of *ERα* splicing variants have been found.[10] Moreover, *ERα* splicing variants have been found to occur at a high frequency in breast carcinomas. Alternative splicing, resulting in deletion of one or more exons (2, 3, 4, 5, or 7), has been detected and the resulting biological properties have been examined. An in-depth description of all splicing variants is beyond the scope of this review; however, they can be categorized into three groups: constitutively active variants, dominant-negative variants, and inactive variants.[8,10,11] However, the true in vivo, functional significance of these variants is complex, because the variants always are found in conjunction with wild type ERα mRNA and they frequently are detected in normal as well as malignant tissues.

As a further level of complexity, the human *ERα* gene has two major promoters. Each of these promoters yields unique transcripts that encode the same size gene product; both promoters are potentially active, although usage varies across cell and tissue types.[8,10] To date, no firm correlations have been made between promoter usage and ERα loss in the progression of breast cancer.

Methylation of the *ERα* Gene

Given the small proportion of cancers with ER-negative phenotypes that can be explained by disruptions at the genomic level, other mechanisms must exist for the loss of ERα expression. Analysis of human breast cancers indicates that the loss of ERα protein is due to a lack of ERα mRNA.[10] It is possible that reduced levels

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of ERα mRNA are the result of reduced levels of active transcription cofactors required for the transcription of this gene. However, to date, this possibility has not been widely demonstrated. Another, more commonly studied, epigenetic mechanism for transcriptional failure is DNA hypermethylation of the *ERα* gene.

The methylation of CpG islands of DNA induces a dilation of a major groove and a kink in a minor groove at the opposite side of the double helix loop. This conformational change in the chromosome alters the interaction between DNA and histone particles. DNA methylation in the promoter region of many genes is associated with transcriptional silencing of the gene either through a direct effect or via a change in the chromatin conformation that inhibits transcription.

The *ERα* gene has been studied as a target for silencing via methylation. Initial studies that focused on the methylation status of the body of the gene failed to show any correlation between methylation and ERα expression.[8] However, more recent studies directed at the CpG island in the 5' transcriptional regulatory region and first exon of the gene have established a clear correlation between *ERα* CpG island methylation and lack of *ERα* gene expression in breast cancer cell lines and primary breast tumors.[12] A functional role for methylation is supported by studies in which two human ER-negative breast cancer cell lines were treated with demethylating agents (5-Aza-2'deoxycytidine or 2-Azacytidine), resulting in the demethylation of the *ERα* gene CpG islands, re-expression of the *ERα* gene, and production of ER.[13] Together, these data suggest that *ERα* gene CpG island methylation may play a role in gene silencing in at least a subset of ER-negative human breast cancers. Although, given the complex nature of transcription, it is likely that other transcriptional regulators of ER also are involved in breast tumor progression.

ERα Degradation—mRNA and Protein

It is possible that reduction of ERα mRNA seen in ER-negative breast cancer is due to decreased mRNA stability and therefore, is a possible cause of the ER-negative phenotype. However, few studies have addressed this issue and data are not sufficient to draw any conclusions.

Determination of ERα status in normal and malignant tissue is most commonly performed by immunostaining for the protein. The absence of ER also may be due to enhanced degradation of the protein. However, given the previously described evidence showing that steady-state mRNA levels are severely reduced, control of ERα expression appears to be at the RNA levels. Thus, few studies have examined ERα protein degradation.

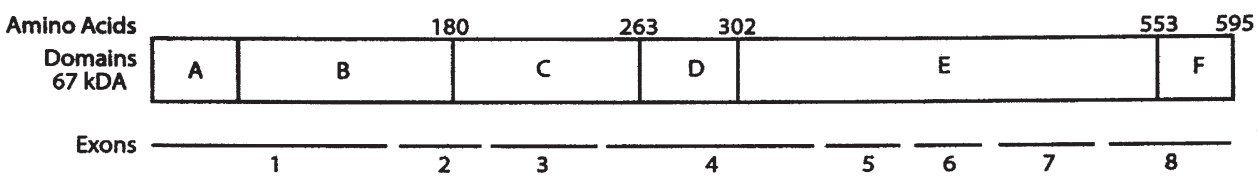
Use of Animal Models—Why Use Animal Models?

Although a significant amount of breast cancer research has been performed on human tissue, research using human tissues from biopsies and tumor removal has several severe limitations. First, the amount of excised tissue is limited, and must be used primarily for assessing tumor characteristics (prognosis) by a pathologist. Second, after biopsy/tumor removal from the patient, a significant length of time often elapses before the tumor can be stored in a manner such that DNA, protein, and particularly, RNA are not degraded. Furthermore, tissue treatment required by the pathologist often is not compatible with subsequent molecular analysis. Third, it is extremely difficult to obtain multiple samples from the same patient, making time course studies virtually impossible. Finally, the ethical issues involved and the difficulty of obtaining consent are complex when dealing with human tissue.

During the past 10 years, numerous mouse models of mammary cancer have been generated through chemical carcinogen, knockout, or transgenic technologies. These models have overcome the problems associated with the use of human tissue (i.e., significant quantities of tumors may be collected and tumor progression and histopathology are reproducible). However, other problems have arisen. The main question of current concern is how similar these tumors are to those seen in human breast

Figure 1

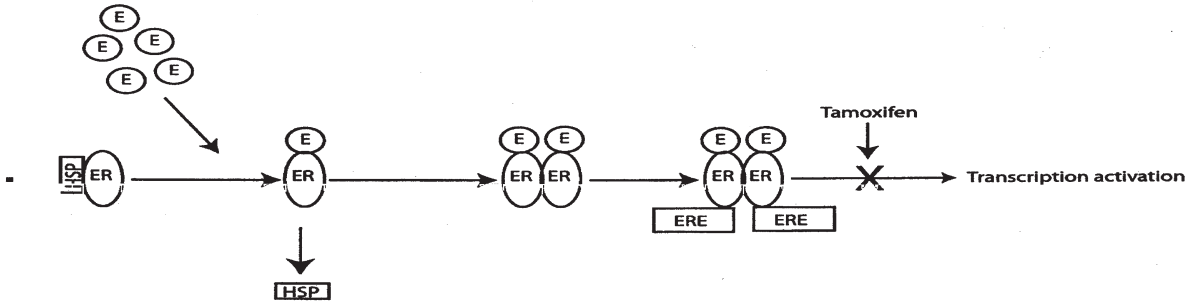
Structure of the *ER* gene and protein



Exons 1-8 and functional domains A-F, encompassing the 595 amino acids of the 67-kDA protein. A/B: constitutive transcriptional activation function; C: DNA binding domain containing two zinc finger motifs and a nuclear localization signal; D: hinge region; E: estrogen-inducible transactivation function; F: unknown function.

Figure 2

Estrogen-induced expression



Estrogen (E) binding to estrogen receptor (ER) causes release of heat shock proteins (HSP) and phosphorylation, receptor homodimerization, binding to estrogen response elements (ERE) within the enhancers of estrogen-responsive genes, and finally, transcriptional activation of target genes. Tamoxifen blocks interaction of the active receptor with its cofactors.

cancers. While no single model can answer every question given the variety of human breast cancers seen today, certain models are well-suited to studying particular aspects of mammary tumorigenesis. The C3(1)/Tag model of mammary cancer has proven to be useful.

C3(1)/Tag Transgenic Mouse Model

All female mice, heterozygous or homozygous for the C3(1)/Tag transgene, develop mammary carcinomas.[14] The transgene is expressed in mammary epithelial ductal cells without stimulations of pregnancy or artificial hormones. Expression that is not dependent on pregnancy is unlike many other transgenic mouse models of breast cancer that use hormone-responsive promoters to drive oncogenes and require one or more pregnancies to express the transgene. Pregnancy-induced tumorigenesis does not closely mimic breast cancer in humans, in whom pregnancy is a well-accepted protective factor. Furthermore, mammary lesions develop during a predictable time course, with many histological similarities to human ductal carcinoma in situ and infiltrating ductal adenocarcinomas.[15]

It has been shown recently that the C3(1)/Tag transgene is not estrogen-responsive. These studies also led to the discovery that ERα expression appears to be lost during mammary tumor progression, a detrimental prognostic factor in human breast cancer as described above. Loss of ERα in the C3(1)/Tag model appears to be the first demonstration of ERα loss in a transgenic mouse model, although it remains to be determined whether other models also display this phenotype. ERα was detected by immunohistochemistry and western blotting in normal mammary epithelial cells and low-grade mammary intraepithelial neoplasia (MIN).[16] Tumors that were classified as high-grade MIN showed a reduction in ERα protein. ERα expression in invasive carcinomas was low to undetectable. Northern blot analysis of mammary gland and invasive carcinoma mRNA from these mice correlated with protein levels. Thus, it appears that ERα loss in mammary tumors from C3(1)/Tag mice is not the result of increased ERα protein degradation, but rather a result of a decrease in steady-state ERα mRNA, either through a reduction in stability or reduced transcription. Given the literature linking *ERα* gene hypermethylation with decreased *ERα* transcription in breast cancer and prostate cells lines,[12,17] it is possible that hypermethylation also is the cause of ERα loss in C3(1)/Tag tumors. Thus, given the clinical implications of ERα loss outlined above, this may be an extremely useful model for studying the poorly understood molecular mechanisms of ERα loss.

Although the mouse is used extensively as a model organism to study ERα regulation in vivo, the complete structure of the *ERα* gene has not been elucidated. Given the complex structure of the human gene with two alternative promoters and numerous mRNA variants, it is possible that the mouse *ERα* gene may exhibit similar features. Studies recently published and studies in progress indicate that the mouse *ERα* gene is a complex transcription unit with tissue-specific promoter usage and alternate splicing.[18] This complexity, along with gaps in our knowledge of *ERα* structure, has made it difficult to study methylation in the mouse *ERα* gene.

ERα Expression—A Potential Target for Breast Cancer Treatment?

The discussion presented above illustrates the desperate need for a greater understanding of why a significant proportion of human breast cancers stop expressing *ERα* and subsequently become refractory to treatment. The use of mouse models of breast cancer enables studies of tumor development and progression, and the

development of new therapies for breast cancer treatment that prevent the loss of ERα, or cause re-expression of the gene so that endocrine therapies remain/become effective in halting tumor progression. *(Dr. Couldrey is Postdoctoral Fellow, and Dr. Green is Head, Transgenic Oncogenesis Group, Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD.)* ❖

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Phenol Sulfotransferases Join List of Low-Penetrance Breast Cancer Susceptibility Genes

By Kornelia Polyak, MD, PhD, and Pankaj Seth, PhD

BREAST CANCER IS ONE OF THE MOST PREVALENT CANcers in women and comprises 18% of all female cancers worldwide. The search for hereditary breast cancer genes has identified two high-penetrance cancer susceptibility genes, *BRCA1* and *BRCA2*.[1] Disappointingly, mutations of *BRCA1* and *BRCA2* are found very rarely in sporadic cases, which make up 90% of all breast cancers. The search to find additional high-penetrance genes is ongoing, but it appears that the majority of breast cancer cases may be due to multiple low-penetrance genes and the influence of environmental or endogenous

carcinogen exposure *(see Table)*. The identification of these genes represents a major challenge, and most of the low-penetrance cancer susceptibility genes have been identified based on a candidate approach. This requires prior knowledge of the gene sequence and function. This approach, therefore, precludes the identification of novel genes.

Table

Low-penetrance breast cancer susceptibility genes

Gene	Putative Mechanism	Reference
GSTM1	Altered detoxification of carcinogens	12,13
CYP1A1	Metabolism of estrogen and polycyclic aromatic hydrocarbons	14
SOD2	Metabolism of superoxide anions	15
CYP17	Metabolism of steroid hormones	16
ERa	Altered estrogen signaling	16,17
NAT2	Metabolism of aromatic and heterocyclic amines	18
AR	Altered androgen signaling	19
SULT1A	Metabolism of carcinogens and endogenous hormones	6
COMT	Metabolism of catechol estrogens	20,21
XRCC1, XRCC3	Base excision repair	22
H-ras-VNTR	Proto-oncogene/Altered transcription/Linkage disequilibrium	23

Recently developed genomics technologies, including DNA arrays and serial analysis of gene expression (SAGE), coupled with the completion of the human genome sequence, will enable large, unbiased, population-based studies that may identify novel genes associated with modest increases in breast cancer risk.[2] However, these studies require the analysis of large cohorts and are likely to take years to complete. However, a combination of these two different approaches can analyze downstream targets of known breast cancer risk factors or cancer preventive agents using genomics technologies.

Phenol sulfotransferases recently were identified as a new group of low-penetrance breast cancer susceptibility genes by analyzing the response of breast cancer cells to tamoxifen using SAGE, and they represent one of the first examples of the feasibility of this type of approach.

Hormonal Factors and Breast Cancer

Animal models and human epidemiological studies support the role of hormonal factors, particularly estrogen, in the development of breast cancer.[3] In addition, one of the most important phenotypic and prognostic features of breast carcinomas is the presence or absence of hormone receptors. Clinical trials have shown that anti-estrogen (tamoxifen) therapy decreases the risk of second primary breast cancers in women with invasive breast cancer and decreases breast cancer incidence in high-risk patients, proving the importance of estrogens in tumor development and identifying tamoxifen as a breast cancer preventive agent.[4,5]

However, little is known about the mechanisms that account for the tumorogenic effects of estrogen and the cancer preventive effects of tamoxifen. The best documented biological property of estrogen is its ability to activate the transcription of genes. Therefore, determining the gene expression profiles following estrogen and tamoxifen treatment will enable a better understanding of the tumor-promoting effects of estrogen and the chemopreventive effects of tamoxifen.

Sulfotransferases and Breast Cancer

To determine the global cellular response of breast cancer cells to estrogen and tamoxifen in a comprehensive and unbiased way, Seth and associates generated SAGE libraries from an estrogen-dependent human breast cancer cell line (ZR75-1) prior to and following estrogen or tamoxifen treatment.[6] Interestingly, the gene encoding SULT1A phenol sulfotransferase, a metabolic enzyme involved in the metabolism of environmental carcinogens and steroid hormones (e.g., estrogen), was identified as one of the tamoxifen-induced genes.

Sulfotransferases are enzymes involved in the metabolism of xenobiotics and endogenous chemicals (steroids, catecholamines, and iodothyronines).[7,8] Sulfation is a common step in phase II metabolism and generally leads to detoxification, but certain compounds can become mutagenic once sulfonated. In humans, the phenol sulfotransferase family consists of three highly related genes (*SULT1A1*, *SULT1A2*, and *SULT1A3*) localized on chromosome 16p.[9]

Inherited differences in the enzymatic activity of drug-metabolizing enzymes (e.g., glutathione transferases and N-acetyltransferases) have been shown to influence cancer risk, and differences in sulfotransferase activity similarly may influence breast cancer risk. In fact, several animal and in vitro studies have found an association between high sulfotransferase activity and the risk of developing chemically induced cancers.[10] Interestingly, exon 7 of the *SULT1A1* gene contains a

functionally relevant polymorphism that significantly influences its enzymatic activity.[11] There are two major alleles (high-activity SULT1A1*1 and low-activity SULT1A1*2) that encode proteins with an approximately 10-fold difference in enzymatic activity. Similarly, there are several functionally relevant polymorphisms in the highly related, but much less active *SULT1A2* gene. The induction of SULT1A by tamoxifen, a known breast cancer preventive agent, together with the known inherited variability in SULT1A enzymatic activity, led the authors to formulate a hypothesis that polymorphism in phenol sulfotransferase genes might influence the risk of breast cancer.

To test this hypothesis, the authors analyzed the distribution of the low- and high-activity SULT1A1 alleles in 444 breast cancer patients from three different cohorts and 227 controls (healthy blood donors, male and female) free of malignancy using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach. Two of the cohorts comprised 378 early-onset breast cancer patients (cohort 1: 280 cases, < 40 years of age at diagnosis; and cohort 2: 98 cases, < 57 years of age at diagnosis); the third cohort included 66 sporadic breast cancer patients (ages 24-89 years).

Most of these patients had no family history of breast cancer and some had been shown not to be carriers of mutant *BRCA1* or *BRCA2* genes. The authors found no difference in SULT1A1 allele or genotype frequency between breast cancer patients and healthy controls, indicating that polymorphism in *SULT1A1* does not influence breast cancer risk in this cohort. However, these early-onset breast cancer patients already may have a strong predisposition to breast cancer due to some unidentified moderate or high cancer susceptibility genes, the effect of which may mask the milder effect of a *SULT1A1* polymorphism. Thus, further studies are needed to determine whether *SULT1A1* polymorphism influences breast cancer risk in unselected patient populations.

Next, the authors analyzed the age of onset of breast cancer in relation to *SULT1A1* genotype. Interestingly, in cohort 1, homozygotes for the low-activity allele had an earlier onset, whereas in cohort 2, homozygotes for the high-activity allele had an earlier onset. The differing effect of *SULT1A1* on the age of onset in the two patient populations could be due to differences in exogenous (exposure to various chemicals) or endogenous (estrogen levels) factors. Alternatively, *SULT1A1* polymorphism may influence the penetrance of other breast cancer susceptibility genes. This later hypothesis is strongly supported by the finding that 27 patients with other cancer (in addition to breast cancer) were all homozygous or heterozygous carriers of the high-activity SULT1A1 allele. These additional cancers were of diverse origin and included ovarian, colorectal, thyroid, and basal cell carcinomas; osteosarcomas; melanomas; and Hodgkin lymphomas. None of these multiple cancer patients had Li-Fraumeni, Cowden, or Gorlin syndrome, and only one of the breast-ovarian cancer patients had a germline *BRCA1* mutation. The high frequency of the high-activity SULT1A1 allele in patients with multiple cancers may indicate that high phenol sulfotransferase activity increases overall cancer risk or that *SULT1A1* modifies the penetrance of certain high-penetrance cancer susceptibility genes. This latter hypothesis can be tested by analyzing the *SULT1A* genotype of known hereditary cancer syndrome patients (e.g., *BRCA1*, *BRCA2*, PTEN, APC, *p53*, and PTCH mutation carriers).

Another important point made in the paper is the suggestion that the patient's *SULT1A1* genotype may influence the effectiveness of certain breast cancer preventive agents like tamoxifen. First, tamoxifen may increase SULT1A protein levels and, therefore, enzymatic activity. Depending on the *SULT1A* genotype, this effect may be beneficial or harmful. Second, tamoxifen itself may be metabolized and presumably inactivated by *SULT1A*. Thus, in patients with high-activity SULT1A alleles, the biological dose of tamoxifen may be lower than in patients who are homozygous for low-activity SULT1A alleles. Testing the *SULT1A* genotype of patients participating in tamoxifen prevention trials and correlating this with clinical outcome is the best way to answer this question. This issue is particularly important because most of these patients are at high risk of breast cancer due to genetic or environmental factors, and the effects of both could be influenced by *SULT1A*.

Conclusion

Phenol sulfotransferases have joined the growing list of low-penetrance breast cancer susceptibility genes *(see Table)*. These genes include glutathione transferases (*GSTM1*), cytochrome P450 family members (*CYP1A1* and *CYP17*), N-acetyl-transferase 2 (*NAT2*), catechol-O-methyl transferase (*COMT*), manganese superoxide dismutase (*SOD2*), and components of the estrogen (*ER*) and androgen (*AR*) signaling pathways.

As this list indicates, most of these low-penetrance breast cancer susceptibility genes are involved in the metabolism of steroid hormones, environmental carcinogens, or both. This finding appears to confirm the hypothesis that the most important risk factor for sporadic cancers is lifestyle and its interaction with the individual's genotype. Although patients cannot change

their genetic composition, they clearly can change their behavior and significantly decrease their cancer risk. Future goals of molecular epidemiology include determining the individual's susceptibility to cancer based on detailed genotyping, which will be administered like a routine blood test, and designing preventive therapies accordingly. It remains to be determined whether this is an achievable goal, but due to the recently developed genomics technologies and the complete sequence of the human genome, we now have the tools to start addressing these questions. *(Dr. Polyak is Assistant Professor of Medicine, and Dr. Seth is Postdoctoral Fellow, Department of Adult Oncology, Dana-Farber Cancer Institute, Boston, MA.)* ❖

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Functional Significance of the Polo-like Kinase Family

By El Mustapha Bahassi, PhD and Robert F. Hennigan, PhD

POLO-LIKE KINASES (PLKS) ARE A FAMILY OF CONSERVED serine-threonine kinases that are emerging as important regulators of cell cycle progression.[1,2] The founding member of this family is the *polo* gene of *Drosophila melanogaster*.[3] Mutation of the *polo* gene confers a pleiotropic phenotype, initially observed to include defects in spindle formation and chromosome segregation,[3,4] and more recently cytokinesis.[5] Homologues of *Drosophila polo* have been found in a wide range of eukaryotes. These proteins, termed polo-like kinases, are characterized by a conserved N-terminal catalytic domain and a C-terminal region, the polo-box, that is involved in subcellular localization.[6] Homologues of *Drosophila polo* include Plo1+ in *Schizosaccharamyces pombe* and cdc5p in *Saccharomyces cerevisiae*. An amphibian homologue (Plx1) has been described in *Xenopus*, and three *Plk* genes (designated *Plk1*, *Plk2*, and *Plk3*) have been found in mammalian cells.[1,7] Of these, *Plk1* is most similar in structure and function to the yeast and *Drosophila* polo kinases. Plks have been found to have critical roles in three distinct mitotic functions: M-phase entry, M-phase exit, and cytokinesis.[1,2]

M-phase Entry

At the onset of mitosis, the eukaryotic cells undergo profound structural rearrangements that are regulated by protein phosphorylation. Prominent among the kinases responsible for regulating entry into mitosis is the p34cdc2 kinase, the first member of the evolutionarily conserved cyclin-dependent kinase (cdk) family. p34cdc2 activation at the G2/M transition requires dephosphorylation of threonine 14 and thyrosine 15 residues,[8,9] which is accomplished by a dual specificity phosphatase cdc25C.[10,11] While the key role of cdc2 kinase in triggering entry into M phase is well-established, evidence also points to the involvement of the Plks in regulating the activation of this system. The *Xenopus* polo-like kinase (Plx1) associates with and phosphorylates cdc25C,[12] and its immunodepletion or immunoinhibition blocks the conversion of p34cdc2 into its mitotic form. Conversely, microinjection of Plx1 into oocytes accelerates activation of both cdc25C and mitosis-promoting factor (MPF). However, overexpression of *Plk1* in HeLa cells does not cause premature mitosis, arguing against a role for *Plk1* in triggering p34cdc2 activation.[13] Instead, because Plks also must be activated by upstream kinases, it is thought that *Plk1* participates in a regulatory feedback loop that amplifies the activity of p34cdc2 and does not function as the initiating event in p34cdc2 activation.[1]

Anaphase Entry

In addition to participating in the G2/M transition described above, the Plks appear to play an essential role during M phase. Recent studies identify Plks as important upstream regulators of the ubiquitin-dependent proteolytic degradation machinery that controls passage through mitosis.[14-16] This machinery controls both anaphase onset and M-phase exit by catalyzing degradation of inhibitors of sister chromatid separation (termed Pds1p in *S. cerevisiae*[17] and cut2p in *S. pombe*[18]) and mitotic cyclins, respectively. How the proteolytic degradation of these and other proteins is regulated in time and space is not fully understood. Current studies focus primarily on the multiprotein anaphase-promoting complex/cyclosome (APC/C), and on proteins related to *Drosophila* fizzy and its budding yeast homologue cdc20p.[19] APC is an E3 ubiquitin ligase which catalyzes the polyubiquitination of specific substrates, thereby targeting them for destruction by the proteasome. Fizzy/cdc20p and related proteins are thought to serve as substrate and/or cell cycle stage-specific, positive regulators of APC/C.

In *S. cerevisiae*, the polo-kinase cdc5p is both a regulator and a target of APC/C.[19] Cdc5 mutants fail to complete mitosis because they fail to ubiquitinate mitotic cyclins. This indicates that cdc5p acts as a positive regulator of cyclin-specific APC/C activity. Conversely, cdc5p levels drop as wild-type yeast cells exit M phase, and this clearly results from APC/C-dependent degradation of cdc5p.[19] Substrate recognition by APC/C depends on specific protein motifs, termed destruction boxes.[20] Two such motifs are present in the extreme amino terminus of cdc5p, suggesting that it is, itself, a target for APC. However, no obvious destruction boxes can be discerned in Plks from other species, suggesting that organisms undergoing cytokinesis (or fission) may utilize alternative mechanisms to reduce Plk levels upon exit from mitosis. The exit from M-phase arrest in *Xenopus* egg extracts requires active Plx, which in turn is required for activation of APC.[15] Kotani and colleagues showed that PKA and MPF-activated *Plk1* plays a critical role in late mitosis progression by controlling APC activity.[16] MPF-activated *Plk1* phosphorylates at least three subunits of APC (cdc16, cdc27, and tsg24), which activates APC to ubiquitinate cyclin B. Conversely, PKA phosphorylates cdc27 and tsg24 and suppresses

APC activity. In fission yeast, APC activation appears to be inhibited by the PKA pathway,[21] whereas protein phosphatase 1 (PP1) appears to regulate APC and is essential for initiating anaphase.[22] Therefore, at least two events are required for APC activation at metaphase-anaphase transition: APC phosphorylation by MPF-activated Plks and dephosphorylation of PKA phosphorylation sites in APC subunits by a specific serine/threonine phosphatase.

Cytokinesis

In addition to the described roles for Plks during entry into and exit from mitosis, Plks also may be important regulators of cytokinesis. In *Drosophila*, *polo* mutants cause cytokinesis defects at different stages of spermatogenesis.[5] In addition, *polo* co-localizes with a kinesin-related motor protein, called Pavarotti, that is required for the organization of the central spindle, formation of a contractile ring, and cytokinesis.[23] *Plk1* has been localized to the spindle poles and centromeres in early mitosis and to the central spindle and mid-body during telophase and cytokinesis in animal cells.[24,25] In *S. cerevisiae*, overexpression of the non-catalytic C-terminal domain of cdc5 results in a severe cytokinesis defect that is dependent upon an intact polo box.[6] Overexpression of either wild type or catalytically-inactive *Plk1* in mammalian cells increases the frequency of multinucleated cells, suggesting that *Plk1* interacts with proteins that mediate cytokinesis.[13] The molecular mechanism by which Plks regulate cytokinesis is unclear because critical targets for the polo kinases in cytokinesis are unknown. However, *Drosophila* polo is able to phosphorylate purified tubulin in vitro and the mammalian *Plk1* can make a stable complex with α, β, and γ tubulin in both interphase and mitotic cells.[26,27]

Other Polos, Other Functions

Much of the data regarding the function of polo kinases in the cell cycle was derived from lower eukaryotes and is most applicable to mammalian *Plk1*. Indeed, expression of human *Plk1* compensates for the cdc5p mutation in *S. cerevisiae*, suggesting that *Plk1* and cdc5p are functionally homologous. The function of the other mammalian Plk family members (i.e., *Plk2* and *Plk3*) is less certain. In contrast to *Plk1*, both *Plk2* and *Plk3* are immediate early genes, implying a function in interphase cells. Overexpression of murine *Plk1* results in oncogenic transformation,[28] whereas *Plk3* overexpression inhibits cell growth by inducing apoptosis.[7] However, some functional overlap between *Plk1* and *Plk3* must exist because *Plk3* also can compensate for the *S. cerevisiae* cdc5p mutation.[29,30] *Plk2* and *Plk3* also have been shown to function in the dendrites and somata of postmitotic neurons.[31] Deregulated expression of *Plk3* induces a change in cell morphology due to the disruption of the cellular F-actin network, and *Plk3* co-immunoprecipitates and co-localizes with Ca2+/integrin-binding protein Cib.[32] These data suggest a role for Plks in the signaling network that controls cellular adhesion and a function for polo kinases outside the cell cycle.

Conclusion

It is clear that the polo kinases are key regulators of the cell cycle that are conserved from lower eukaryotes through mammalian species. However, the existence of multiple homologues of the Plks in mammalian species suggests a diversification of function that is poorly understood. The finding that *Plk1* levels generally are increased in rapidly proliferating cells has suggested that *Plk1* is a proliferation marker with prognostic value in human lung and head and neck cancers.[33-35] Overexpression of a dominant-negative *Plk1* mutant containing a functional polo-box caused apoptosis specific to tumor cells.[36] These data raise the intriguing possibility that conditional expression of the polo-box domain may selectively inhibit endogenous polo kinases. Since the polo-box is a unique and essential domain for polo kinase function, these inhibitors may provide tools to selectively target proliferating cells and are good candidates for antitumor agents. The possible involvement of the polo kinase family with the regulation of several different aspects of mitosis makes understanding the function of all polo kinases important in gaining insight into the process of oncogenesis and evaluating their potential as future targets for anticancer therapies. *(Dr. Bahassi is Postdoctoral Assistant and Dr. Hennigan is Assistant Professor, Department of Cell Biology, University of Cincinnati College of Medicine, The Vontz Center for Molecular Studies, Cincinnati, OH.)* ❖

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Funding News

Zebrafish Mutagenesis and Screening Tools

Over the past couple of decades, molecular genetic and mutational analysis research in nonvertebrate systems such as the worm and fruit fly have yielded important information on genetic signals, enzymes, and transitional regulators. Although this information is important for understanding the molecular events in invertebrate systems, many features of patterning and morphogenesis in vertebrates are distinct and require study in vertebrate models. The zebrafish model is one vertebrate model currently available that has several advantages, including early embryonic patterning, nervous system development, and aspects of cell fate and lineage determination.

The National Institutes of Health has issued a program announcement inviting researchers interested in advancing mutagenesis screening in the zebrafish model to detect and characterize genes, pathways, and phenotypes of interest in development, aging, organ formation, behavior, and disease processes, including cancer. This program announcement uses the modular grant and just-in-time concepts. Applications and funds are distributed through the RO1 process with grants issued in amounts of $250,000 per year in modules of $25,000. All foreign, domestic, public, private for profit, and non-profit organizations, including universities, hospitals, and research institutions, are eligible. There are three deadlines per year: June 1, Oct. 1, and Feb. 1. More information on this program announcement is available at: http://www.grants.nih.gov/grants/guide/pa-files/PA-01-070.html.

Metals in Medicine

The National Institutes of Health has issued a program announcement to encourage research that bridges inorganic chemistry and medicine. The focus of this program is to elucidate the mechanism by which organisms transition medal ions and the roles of these metals in cellular regulation and cell-cell signaling. A secondary area of interest is the interaction of synthetic organic complexes with living systems and their components. This announcement encourages collaboration between chemists, biochemists, and cell and molecular biologists. All foreign, domestic, public, private for profit, and non-profit organizations, including universities, hospitals, and research institutions, are eligible. This program announcement will use the existing RO1 research project grant mechanism with awards made in the amount of up to $250,000 per year in $25,000 modules. There are three deadlines per year: June 1, Oct. 1, and Feb. 1. More information on this grant is available at: http://www.grants.nih.gov/grants/guide/pa-files/PA-01-071.html. ❖

Correction

The pages of the January 2001 issue of Cancer Research Alert *were numbered incorrectly. They were numbered pages 81-92, creating a four-page overlap with the December issue (pages 73-84). We are sorry for any inconvenience this may have caused.* ■

CME Questions

18. Estrogen receptors are:
a. cell membrane hormone receptors.
b. cytosolic hormone receptors.
c. nuclear hormone receptors.
d. mitochondrial receptors.

19. Sulfotransferases are enzymes involved in the metabolism of:
a. xenobiotics and endogenous chemicals.
b. steroids.
c. catecholamines.
d. All of the above

20. Polo-like kinases:
a. are a family of serine proteases.
b. play a role in the regulation of the cell cycle.
c. were first identified in the polio virus.
d. are not evolutionarily conserved.

In Future Issues: XAGE-1

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A monthly update of developments in preclinical oncology research for the clinician and researcher

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