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Estrogen and progesterone are critically involved in both the development of the mammary gland and the pathogenesis of breast cancer, functions thought to rely on their regulation of cell proliferation. This project aims to complement studies of cell cycle control in breast cancer cells in tissue culture by addressing cell cycle control mechanisms in the normal mammary gland. Of particular interest are the roles of cyclin D1 and p27, which have been implicated in steroid regulation of proliferation, and are aberrantly expressed in a subset of breast cancers. We have obtained preliminary data which suggest that the impaired mammary development in cyclin D1^{-/-} mice does not arise simply due to a slower proliferation rate in the cyclin D1^{-/-} epithelial cells and are currently investigating the response of these cells to steroid treatment. Ongoing experiments are also investigating the effects of loss of p27 on cyclin-CDK complex formation and activity in mammary epithelium. To date our results argue that loss of p27 has no substantial effects on mammary development or steroid-mediated cell proliferation. If more detailed analyses confirm this conclusion, lack of p27 expression in clinical breast cancer may be a surrogate marker for another molecular lesion conferring adverse outcome.

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Introduction

Steroid hormones, in particular estrogen and progesterone, have diverse physiological functions and are critically involved in both the development of the mammary gland and the pathogenesis of breast cancer. A central component of the physiological effects, and very probably the oncogenic actions, of steroid hormones is their regulation of cell proliferation. Substantial progress has been made in recent years towards defining their effects on cell cycle control mechanisms, using established breast cancer cell lines in tissue culture. However, the role *in vivo* of targets identified from studies *in vitro*, and the degree to which the mechanisms defined in cancer cells are applicable to normal mammary epithelium are unanswered questions which are addressed by this project. The overall aim of this project is to characterise the molecular mechanisms controlling epithelial cell proliferation and differentiation within the mouse mammary gland. Of particular interest are steroid hormone regulation of these mechanisms and the contribution of key cell cycle regulatory genes identified in other models i.e. cyclin D1 and p27. These genes have been implicated in steroid control of cell cycle progression *in vitro* and their aberrant expression in a subset of breast cancers has prognostic consequences. The specific aims are to:

- (i) Identify the cell cycle control pathways activated in mammary epithelial cells during pregnancy and following treatment with the steroid hormones, estrogen and progesterone.
- (ii) Investigate the role of the CDK inhibitor p27 in mammary gland development and differentiation.

Body of Report

Progress made in the first 12 months of the granting period is described below, with reference to the tasks in the Statement of Work

Task 1: To define the effects of steroid hormone treatment on CDK activity and complex formation in wild-type and cyclin D1^{-/-} mice.

Initial experiments were aimed at establishing the optimal time and dose of steroid treatment, since although this is a commonly used experimental approach, little information on the effects of such steroid treatment on cell cycle kinetics in mammary epithelium was available. Preliminary experiments using daily subcutaneous injections to deliver either estrogen, progesterone or both showed no significant increase in DNA synthesis within 48 h treatment. Since it was thus necessary to deliver the steroids over a longer time-frame, all subsequent experiments have been performed using commercially available slow-release pellets implanted under the skin of the upper back. The doses chosen were: 25 mg progesterone/20-day release pellet and 0.025 mg 17 β -estradiol/20-day release pellet. Higher doses of estradiol were no more effective at inducing a proliferative response.

Long-term experiments revealed that after 18-20 days treatment of wild-type female mice with a combination of estradiol and progesterone, the mammary gland had developed to a stage comparable with early-to-mid pregnancy, displaying increased ductal branching and development of alveolar buds compared with placebo-implanted controls. This was accompanied by increased DNA synthesis as determined by quantitation of bromodeoxyuridine (BrdU)-positive cells in sections of paraffin-embedded mammary glands.

Increased DNA synthesis was also apparent after 7 or 12 days of treatment. Subsequent shorter term experiments revealed that DNA synthesis began to increase after 3 days of steroid treatment, and was maximal after 4-6 days. These experiments thus established the timecourse of DNA synthesis after steroid treatment and provided material for initial examination of cyclin, CDK and CDK inhibitor expression and regulation. In the course of other experiments we discovered that DNA synthetic rates in mice during estrus were very low, <0.1%, as was cyclin protein expression. Although we had previously ovariectomised mice before steroid treatment, this argued that it was unnecessary and we have omitted it from subsequent experiments.

Western blots of lysates from steroid-treated mammary glands revealed increases in cyclin D1 and cyclin A expression and little change in the expression of cyclin E, Cdk2, Cdk4 or p27. The p27-related CDK inhibitor p21 appears to be expressed at very low levels in the mammary gland. Measurement of cyclin D1 by immunohistochemistry using a biotinylated anti-cyclin D1 mouse monoclonal antibody confirmed that increased numbers of cyclin D1-positive epithelial cells were present within 2 days steroid treatment, suggesting that the increased expression observed in mammary gland lysates was not simply due to an increased proportion of epithelial cells. It will be necessary to establish this more rigorously by normalising expression to the levels of an epithelial cell-specific marker, e.g. the epithelial-specific keratins 8 and 18. Some difficulties were encountered in identifying anti-keratin antibodies suitable for Western blotting. However recent correspondence with an investigator whose research focusses on keratin function has yielded some suggestions of suitable antibodies which should resolve this problem. Overall, the changes in cyclin expression were not of large magnitude despite an increase in DNA synthetic rate from <0.1% to 10-20%, nor were significant changes in cyclin E-Cdk2 activity demonstrated at early timepoints. It thus appears that analysis of whole mammary gland lysates lacks sensitivity, limiting its utility in this context.

Since we have previously found that gel filtration chromatography offers increased resolution in examining cyclin-CDK complex formation in breast cancer cells in culture (5, 7, 9), we also used this technique with mammary gland lysates. In preliminary experiments this yielded much cleaner Western blots than unfractionated lysates. Ongoing experiments are examining whether increased sensitivity is also apparent in kinase assays. We interpret the improved sensitivity of Western blotting following separation as being in part due to separation of cellular proteins from lipid and other contaminants in the mammary gland lysates and so also plan to trial more simple fractionation of the lysates.

While these experiments were ongoing, we began an examination of a key physiological model of steroid-dependent cell proliferation: mammary gland development during pregnancy. While initial development of the mammary gland in cyclin D1^{-/-} mice appears normal, these mice fail to develop alveoli in mid-to-late pregnancy and consequently do not produce milk (2, 8). The lack of development may indicate a specific requirement for cyclin D1 function for lobuloalveolar development, perhaps downstream of the progesterone or prolactin receptors since mice lacking these receptors display a similar phenotype (3, 6). However an alternative explanation is that proliferation simply proceeds at a much reduced rate and there is thus insufficient time for full mammary development before parturition (1).

As a first step towards distinguishing between these possibilities we have compared the degree of development and cellular proliferation within the mammary epithelium of cyclin D1^{-/-} and wildtype mice during pregnancy. Whole-mount analysis of the glands has confirmed that the cyclin D1^{-/-} mice display restricted alveolar bud development relative to wild type mice. The degree of epithelial DNA synthesis as assessed by BrdU immunohistochemistry follows a bimodal distribution during pregnancy, with an initial peak of up to 30-40% BrdU-positive cells at day 3 of pregnancy, consistent with published data (10). The initial peak in DNA synthesis is of similar magnitude in cyclin D1^{-/-} mice but thereafter the proportion of BrdU-positive cells appears to be significantly reduced relative to wild type. These novel data are more consistent with a specific requirement for cyclin D1 than with an overall decrease in the rate of proliferation. Further analysis of material from these experiments will include examination of expression of cyclin D1 and other key genes during pregnancy by immunohistochemistry, and Western analysis and measurement of CDK activity in lysates. This will both characterise cyclin-CDK expression and activity during pregnancy where there is to date very little data available, and provide some insights into alterations in cell cycle control mechanisms which might account for the defect in mammary gland development in cyclin D1^{-/-} mice.

While mammary gland development during pregnancy is steroid hormone-dependent and the experiments described above are thus expected to yield important insights into steroid regulation of cell proliferation in the mammary gland, they do not allow distinction between the effects of individual steroids. Therefore to complement these studies, in ongoing experiments we have also begun to collect mammary glands from animals treated with estradiol, progesterone or both for 6 days, compared with placebo-implanted littermate animals. This timepoint is when maximum DNA synthesis occurs following pellet implantation and thus we will be able to identify whether absence of cyclin D1 alters the proliferative response to the individual steroids, and if so, what alterations in cell cycle control mechanisms accompany this response. Depending on the results of these analyses, we may also use the material for DNA microarray analysis using our in-house Affymetrix GeneChip facility to identify genes involved in steroid-mediated epithelial proliferation.

Task 2: To define the effects of lack of p27 on mammary development.

The sterility of female p27^{-/-} mice precludes ready examination of mammary gland development during pregnancy. Therefore, a series of transplant experiments was performed in collaboration with Mr Matthew Naylor in Dr Chris Ormandy's laboratory, to determine whether this apparent difference was a property of the mammary epithelium or its hormonal environment. Fragments of mammary gland tissue from donor wild type and p27^{-/-} mice were transplanted into contralateral mammary fat pads "cleared" of endogenous epithelium by surgical removal of the rudimentary ductal structure present in the 3 week old recipient animals. The recipient Rag1^{-/-} mice are deficient in both T and B lymphocytes and thus accept allografts, but have apparently normal endocrinology. Twelve weeks after transplant, when the recipient mice were mature, they were mated and transplanted glands were collected in parallel with an endogenous #3 gland at various stages through pregnancy and at parturition. Of a total of >30 transplanted glands, only 2 failed to take. Some problems with *Helicobacter* infection present in the recipient mice at supply were encountered, necessitating

premature culling of some animals. This has now been controlled, by acidification of the drinking water and antibiotic treatment.

Mammary gland development and the degree of cellular proliferation have been assessed by whole mount analysis and BrdU immunoreactivity, respectively, in multiple recipient animals at the following stages of pregnancy: virgin, 6-7 days pregnant, 14 days pregnant. In addition, we have examined single recipient animals at term, 1 day post-partum and during involution. At no stage of pregnancy was a difference in mammary gland morphology between p27^{-/-} glands and transplanted or endogenous wild-type glands observed. This was confirmed by counting the number of branchpoints and alveolar buds within three 1 mm² areas selected randomly from the periphery of the gland.

BrdU immunohistochemistry was then performed. Quantitation by two independent observers, counting >2000 cells/gland showed that the proportion of BrdU-positive epithelial cells was consistently higher in p27^{-/-} mammary glands compared with wild type transplants or endogenous glands, indicating an increased rate of DNA synthesis. However, microscopic examination of haematoxylin and eosin-stained sections failed to reveal any differences in tissue architecture between wild type and p27^{-/-} glands, nor were there any apparent increases in the number of cells within the epithelium. This suggests either that the increased DNA synthesis does not result in increased cell division, perhaps due to compensatory alterations in the length of other cell cycle phases, or that increased cell death accompanies an increased rate of cell division. In future analysis of these tissues the TUNEL assay will be utilized to assess the extent of cellular apoptosis. Since these experiments did not provide evidence for alterations in differentiated function i.e. milk production, in p27^{-/-} epithelium, our original plan to examine the expression of markers of differentiation has not been pursued. Instead, we have begun to address Task 3.

Task 3: To define the effects of lack of p27 on CDK activity and complex formation following steroid hormone treatment

To examine more closely the composition and regulation of cyclin-CDK complexes in proliferating mammary epithelium lacking p27, we used steroid treatment to stimulate cell cycle progression. This aspect of the research plan was initiated earlier than originally planned and in consequence will be completed earlier than anticipated. Ovariectomised wildtype and p27^{-/-} animals were implanted with both estradiol and progesterone pellets, compared with placebo pellets. Mammary glands were collected after either 4 days, when the initial round of DNA synthesis was maximal, or 18 days, when more extensive development of the gland could be observed. Whole mount analysis revealed a simple ductal system within the mammary glands of placebo-treated animals and more extensive ductal and lobulo-alveolar development in the mammary glands of steroid-treated animals. There was no significant difference in gross morphology, number of ductal branches or number of alveolar buds between wild type and p27^{-/-} mice at either timepoint, consistent with our observations in transplanted mammary glands.

Very low levels of BrdU immunoreactivity (<0.1% positive cells) were observed within the mammary glands of placebo-implanted wild type and p27^{-/-} control animals following 4 and 18 day treatment, whereas following steroid treatment 10-20% of epithelial cells were BrdU-

positive. However, in contrast with the results obtained in transplanted glands during pregnancy, no significant difference in the degree of DNA synthesis in wildtype and p27^{-/-} glands was found after comparison of 4-7 animals/group. Similarly, initial Western analysis of mammary gland lysates suggests that although various cell cycle proteins are upregulated following steroid hormone treatment, there is no difference between wild type and p27^{-/-} animals. This will, however, be confirmed following fractionation of the mammary gland lysates by gel filtration chromatography, since this was found to be more sensitive than Western blotting and in addition should reveal what differences, if any, in cyclin-CDK complex formation occur in the absence of p27. The reasons why increased proliferation is apparent in transplanted mammary glands but not in steroid-treated glands are unclear at this stage, nor is it clear why our results diverge from those in a recently published study addressing similar issues (4). We are currently evaluating strategies to resolve these questions. In preliminary experiments we are also attempting primary culture of mammary epithelial cells since this may offer a more sensitive model in which to examine changes in cyclin-CDK complex formation.

Key Research Accomplishments

- Development and validation of methods for immunohistochemical analysis of cyclin D1 expression and DNA synthesis (via measurement of BrdU incorporation).
- Characterisation of timecourse of DNA synthesis after implant of steroid pellets, a necessary basis for the design of future experiments.
- Analysis of cyclin, CDK and CDK inhibitor expression in wildtype, cyclin D1^{-/-} and p27^{-/-} mammary gland lysates following steroid treatment and preliminary evaluation of gel filtration chromatography of these lysates.
- Measurement of DNA synthesis in the mammary gland during pregnancy in wildtype and cyclin D1^{-/-} mice and collection of material for future analysis of cyclin, CDK and CDK inhibitor expression and function
- Demonstration that loss of the CDK inhibitor p27 appears to have little effect on mammary development and proliferation, with the exception of increased DNA synthesis in transplanted p27^{-/-} epithelium during pregnancy.

Reportable Outcomes

Significant developmental effort has been required this year to establish and validate model systems to be used in the remainder of the grant and in consequence no manuscripts, abstracts or presentations have yet arisen from the work. We are confident, however, that these will be forthcoming within the near future.

Conclusions

During the first 12 months of this project we have focussed on two main aspects of the research plan, aimed at understanding the roles of cyclin D1 and p27 in mammary epithelial cell proliferation and differentiation, since these proteins are implicated in steroid control of cell proliferation and are commonly aberrantly expressed in breast cancer. First, we have characterised the model system we will use to investigate steroid effects on mammary

epithelial cell proliferation and begun studies to identify the causes of the specific defect in mammary epithelial cell proliferation and differentiation which leads to impaired mammary development in cyclin D1^{-/-} mice. Results to date suggest that the impaired development does not arise simply due to a slower proliferation rate in the cyclin D1^{-/-} epithelial cells. Further addressing the question of the role of cyclin D1 in control of mammary epithelial cell proliferation will be the principal component of the project over the remainder of the grant. In the light of developments since the proposal was drafted we are currently considering strategies which will extend and complement the studies planned at the time of grant submission. These include the use of transcript profiling to identify steroid-responsive pathways in mammary epithelium which may be defective in cyclin D1^{-/-} animals. These studies are expected to provide insight into how aberrant expression of cyclin D1 might affect the biology of breast cancer cells.

Our second major theme of research has been investigation of the role of p27 in mammary proliferation and differentiation. Ongoing experiments are aimed at identifying whether loss of p27 alters cyclin-CDK complex formation and activity in mammary epithelium. To date our results argue that loss of p27 has no substantial effects on mammary development or steroid-mediated cell proliferation. If more detailed analyses confirm this conclusion, lack of p27 expression in clinical breast cancer may be a surrogate marker for another molecular lesion conferring adverse outcome.

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