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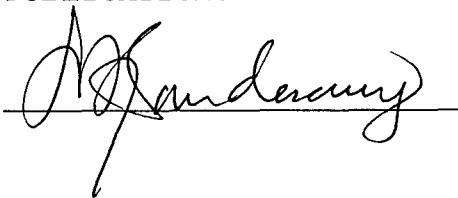
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13. ABSTRACT (Maximum 200 Words) Several protein kinases involved in mammary gland development have been associated with human breast cancer and murine animal models of mammary gland carcinogenesis. To identify other protein kinases expressed in the mammary gland which may be involved in these processes, an RT-PCR degenerate oligonucleotide screen was performed on several transformed mammary epithelial cell lines and tissue derived from the mammary gland during different developmental stages. Forty-one kinases were identified, of which three were novel. One of these novel kinases, <i>Krct</i> , does not belong to any previously described subfamily of kinases. Analysis of the expression of murine <i>Krct</i> , demonstrates <i>Krct</i> expression in all tissues analyzed during all stages of development. Despite this wide expression pattern, <i>Krct</i> is preferentially expressed in the epithelium of several tissues including the mammary gland. Since many kinases regulate cellular proliferation and differentiation, it will be important to determine <i>Krct</i> 's role in these processes. An analysis of the expression pattern of <i>Krct</i> during proliferation and differentiation has been initiated. Preliminary results demonstrated <i>Krct</i> mRNA levels may be cell cycle regulated. However, <i>Krct</i> protein levels in a serum starve/refeed experiment remained constant. Therefore it is unclear if <i>Krct</i> is regulated in a cell cycle dependent fashion. Further analysis of <i>Krct</i> with proliferation and differentiation assays <i>in vitro</i> and <i>in vivo</i> will determine if <i>Krct</i> is involved in these cellular processes.				
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FOREWORD

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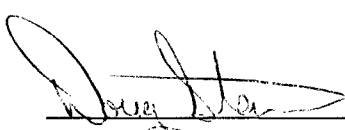
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Introduction:

Epidemiological evidence suggests breast cancer risk is closely related to development of the mammary gland. Additionally, development of the mammary gland through fetal life, puberty, pregnancy and post-lactational regression involves cellular processes such as differentiation, proliferation and apoptosis(1-3). Protein kinases are key regulatory molecules of these cellular processes(4). Several protein kinases have been shown to contribute to mammary gland development and breast cancer, including c-erb-B2/Neu, the epidermal growth factor (EGF) receptor, c-Src and Met(4-11).

To identify other kinases involved in both of these processes, we performed RT-PCR using degenerate oligonucleotides to catalytic subdomains VII and IX conserved in all protein kinases. Forty-one kinases were identified after screening several transformed mammary epithelial cell lines and developmental time points in the mammary gland by this method. Three of these kinases were novel, one of which, H51, does not fit into any known kinase subfamily and has closest homology to a serine/threonine kinase in yeast with unknown function.

The H51 PCR product obtained from this screen was used to isolate a full-length cDNA. The kinase encoded by this cDNA was renamed from H51 to Krct and will henceforth be referred to as such. After isolation of full length *Krct*, analysis of its expression pattern, chromosomal localization and verification of its phosphotransferase activity were performed and published(12). Since many kinases are involved in cellular proliferation and differentiation, we hypothesize that Krct may also be involved in these processes. To investigate this hypothesis, *in vitro* and *in vivo* proliferation and differentiation assays will be performed.

Specific Aims:

Aim 1: Determine the role played by Krct in proliferation.

- A. Generate antisera specific for Krct.**
- B. Characterize the expression of Krct *in vitro* as a function of cell proliferation.**
- C. Overexpress Krct to assess its effects on proliferation.**
 - i. Assessing the effects of Krct overexpression on proliferation *in vitro*.**
 - ii. Assessing the effects of Krct overexpression on proliferation *in vivo*.**

Aim 2: Determine the role played by Krct in differentiation.

- A. Characterize the expression of Krct in an *in vitro* differentiation system.**
- B. Overexpress the novel kinase, Krct, to assess its effects on differentiation *in vitro*.**
- C. Overexpress the novel kinase, Krct, to assess its effects on differentiation *in vivo*.**

Aim 3: Determine the potential role played by Krct in carcinogenesis in the mammary gland.

- A. Assessing the effects of Krct on transformation *in vitro*.**
- B. Assessing the tumorigenicity of Krct overexpressing cells.**
- C. Determine the effects of Krct overexpression on carcinogenesis *in vivo*.**

Task1. Determine the role played by Krct in proliferation: months 1-36.

- Generate antisera to Krct: months 1-14.
- Generate pTetO.Krct and pTetO.LacZ constructs: months 1-7.
- Characterize Krct expression during proliferation in HC11 cells: months 8-16.
- Generate and characterize stable transfectants in HC11 cells: months 17-24.
- Perform Northern and Western analysis to assess proliferation rates: months 25-36.
- Perform FACS and 3H-Thymidine studies to assess proliferation rates: months 25-36.
- Create MMTV.Krct transgenic construct: months 1-6.
- Create founder mice for transgenic construct: months 7-10.
- Characterize changes in proliferation rates in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: months 10-36.
- Characterize changes in proliferation rates in transgenic mice by Northern and Western analysis: months 12-36.

Generate antisera to Krct (months 1-14). Since Krct regulation is likely to occur at the translational level due to upstream AUG codons in the 5'-untranslated region (UTR) in its mRNA, it will therefore be important to determine Krct protein levels. Therefore, antisera were generated to two different regions of the protein. GST-Krct fusion constructs were generated to the middle and C-terminal regions of Krct. These constructs were expressed in *E. coli* and purified using glutathione sepharose beads and cleaved with a peptidase supplied by the manufacturer. The liberated and purified Krct peptides were injected into two rabbits each. The resulting antisera were then affinity purified and tested for its ability to recognize Krct protein by Western analysis and its ability to immunoprecipitate (IP) Krct. All four antisera generated were able to detect Krct by Western analysis and IP Krct (Fig. 1). With these antisera, we will be able to determine if protein levels are cell cycle regulated similar to *Krct* mRNA.

Generate pTetO.Krct construct (months 1-7). Since most genes regulated by the cell cycle are themselves regulators of the cell cycle, over-expression studies will be performed to determine if Krct over-expression perturbs the cell cycle and therefore alters proliferation rates. To inducibly over-express Krct, the tetracycline-inducible plasmid-based expression system will be used. A modified Krct cDNA was generated for over-expression. PCR-based truncation of the 5'UTR was performed to remove all upstream AUG codons and place an in-frame stop codon upstream of the initiation codon. This modified cDNA was subcloned into a plasmid between the TetO regulatory sequence and an SV40 3'UTR containing an intron for more efficient mRNA expression in mammalian cells. This expression construct will be used to generate inducible, stable clones of Krct for proliferation and differentiation assays.

Characterize Krct expression during proliferation in HC11 cells (months 8-16). To determine Krct's role in proliferation, *Krct* mRNA expression levels in actively growing, quiescent, and synchronized cells have been determined. *Krct* is expressed at similar levels in actively growing cells and confluent cells in two different mammary epithelial cell lines, HBI2 and 16MB9A cells. However, upon serum starvation in these cell lines, *Krct* mRNA levels significantly decrease. After refeeding, *Krct* levels remain low until just after *Cyclin A* induction where *Krct* expression dramatically increases (Fig. 2). Despite the observation that *Krct* mRNA levels don't change when cells become quiescent, its change in expression upon serum starvation/release demonstrates *Krct* is likely a cell cycle regulated gene. Additional Northern analysis needs to be performed to follow up this observation. Since *Krct* expression levels do not

peak during this time course, serum starve/refeed experiments need to be performed again and cells harvested at later times.

Protein expression levels of Krct during a serum starve/refeed experiment of HC-11 cells were determined. Krct levels did not change during the time course. This is not consistent with mRNA levels seen in HBI2 and 16MB9A cells. This brings into question whether Krct is regulated during the cell cycle. Further synchronization experiments analyzing Krct protein and mRNA from matched samples in these three cell lines will need to be performed to address these disparate results.

Generate and characterize stable transfectants in HC11 cells: (months 17-24).

Generation of inducible Krct clones has proven to be difficult. The pTetO.Krct construct was transfected into HC-11 cells previously transfected with pTetO.rtTA which has previously been demonstrated to inducibly express rtTA in a doxycycline-dependent manner. After puromycin selection for pTetO.Krct transfectants, clones were isolated, expanded and characterized for the ability to overexpress Krct. Cells were induced with 1 ug/ml of doxycycline for 48 hours. After screening over 30 Krct clones by Western analysis, we have identified one clone that overexpresses Krct by 5 to 10 fold (Fig. 3). One clone is not sufficient to prove any potential phenotype observed is due to transgene expression and not a result of disrupting the expression of a gene at the integration site. Therefore at least one more clone will need to be generated to verify observations made with the first clone.

Perform Northern and Western analysis to assess proliferation rates: (months 25-36). Perform FACS and 3H-Thymidine studies to assess proliferation rates: (months 25-36). Proliferation rates were analyzed by doubling time analysis. The HC-11-TetO.Krct overexpressing clone, as well as 5 control cell lines containing the empty vector, were grown in the presence and absence of 1ug/ml of Dox. Cell doubling times were identical between all cell lines over 5 passages. Therefore the proliferation rates for this one inducible clone is normal.

Create MMTV.Krct transgenic construct (months 1-6) and create founder mice for transgenic construct (months 7-10). To further study Krct's potential role in proliferation, transgenic animals are to be generated with full length Krct cDNA placed under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. The MMTV-Krct transgenic construct has been created and injected into oocytes from FVB mice. Injections were performed by the University of Pennsylvania Transgenic Mouse Core Facility. Southern analysis of mice generated confirmed the generation of four transgene positive animals, two females and two males. One female animal died in the process of breeding these transgene positive animals with wild type FVB mice to verify germline transmission of the transgene in these four potential founders. Autopsy analysis of this female shows her to have had only one kidney and to have been pregnant at the time of death. Presumably the stress from the pregnancy on her only kidney resulted in her death and not an effect from the transgene. The other three transgene positive animals are able to pass the transgene.

Analysis of transgene expression by Northern Hybridization showed no transgene expression. Therefore an RNase A protection assay was performed. This too showed no expression for all three founder lines. The lack of any transgenic founder lines which express Krct and the difficulty in generating over-expressing Krct cell lines led us to speculate that Krct may be toxic to cells when overexpressed and therefore an inducible transgenic animal would be preferred.

We have therefore generated a TetO-Krct transgene suitable for the generation of transgenic mice. This construct is composed of a Tetracycline operon driving transcription of a bicistronic mRNA containing Krct upstream of an IRES element and a luciferase reporter gene

and an SV40 3' UTR with intron and polyadenylation signal. The advantage of this construct is that transgene expression can be rapidly determined after Doxycycline administration by a simple luciferase assay. To date we have screened 38 potential founders for transgene incorporation by PCR and have not identified any transgene positive animals. Further injections are in progress and screening will continue.

Characterize changes in proliferation rates in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: (months 10-36). As a result of our inability to generate any Krct transgenic mice which express, this task cannot be completed.

Characterize changes in proliferation rates in transgenic mice by Northern and Western analysis: (months 12-36). As a result of our inability to generate any Krct transgenic mice which express, this task cannot be completed

Task2: Determine the role played by Krct in differentiation: months 1-36.

- Characterize Krct during HC11 cell differentiation: months 8-17.
- Perform Northern and Western analysis to detect changes in H51 and B-casein levels: months 18-26.
- Assess Krct overexpression on differentiation *in vivo*: months 10-36.
- Characterize changes in differentiation in transgenic mice by Northern and Western analysis: months 10-36.
- Characterize changes in differentiation in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: months 10-36.

Characterize Krct during HC11 cell differentiation. Perform Northern and Western analysis to detect changes in H51 and B-casein levels: (months 8-26). Over-expression of protein kinases has been shown to affect differentiation of the mammary gland. To investigate the role of Krct in differentiation, we will analyze Krct expression during HC-11 differentiation, this mammary epithelial cell line will express β -casein when stimulated with prolactin, dexamethasone, and insulin. Protein extracts and RNA samples during HC-11 differentiation have been generated. Cells were stimulated with 1ug/ml of both prolactin and dexamethasone and 10% fetal calf serum. Cells were harvested for RNA and protein at 0 hours while actively growing, and 6hrs, 1,2,3,4,5,7, and 10 days post induction. Cells were all harvested during the same passage. Krct expression has been determined by Western analysis. Krct protein levels appear to increase slightly through day 4 of induction and decrease thereafter to levels significantly lower than that in uninduced states. Northern analysis of *Krct* and β -casein expression during differentiation will be performed to determine the differentiation states of the cells at each time point.

Once the expression profile of *Krct* relative to β -casein induction is determined during the normal differentiation of HC-11 cells, the effects of Krct over-expression on HC-11 differentiation will be examined using the same inducible system described in Task 1.

Characterize Krct during mammary gland development by Western analysis. Northern analysis reveals that *Krct* mRNA varies two fold during mammary gland development. *Krct* mRNA levels appear to rise steadily during pregnancy and peaks during lactation. Since *Krct* has a large 5'UTR with upstream AUG's, we hypothesize it might be translationally

regulated and therefore mRNA levels may not accurately reflect protein levels. We therefore analyzed Krct protein expression levels during mammary gland development using our AB1003 antisera which was raised against the C-terminus of the protein. Western analysis reveals that the 35 kDa Krct protein appears to have increased expression during mid and late pregnancy with its peak expression seen during late pregnancy (Fig. 4). This is in contrast to RNA levels where *Krct* expression continues to increase through lactation. Interestingly, a very intense 45 kDa band is detected during pregnancy and peaks during lactation. Is this upper band actually a modified form of Krct? Preliminary evidence suggests it might be. A band at approx 45 kDa in an IVT of Krct exactly co-migrates with the upper band seen in the mammary gland during lactation by Western analysis. Experiments are currently underway to identify what the modification is that would cause a shift in the apparent molecular weight of Krct by approximately 10 kDa.

Assess Krct overexpression on differentiation *in vivo*: (months 10-36). As a result of our inability to generate any Krct transgenic mice which express, this task cannot be completed

Characterize changes in differentiation in transgenic mice by Northern and Western analysis: (months 10-36). As a result of our inability to generate any Krct transgenic mice which express, this task cannot be completed

Characterize changes in differentiation in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: (months 10-36). As a result of our inability to generate any Krct transgenic mice which express, this task cannot be completed

Task3: Determine the potential role played by a novel serine/threonine kinase in carcinogenesis in the mammary gland: months 1-36.

- Generate and characterize 3T3 fibroblast cell lines containing pTetO.Krct and pTetO.LacZ constructs: months 7-14.
- Perform focus formation study: months 15-22.
- Perform soft agar experiment: months 15-22.
- Determine the effects of Krct overexpression on carcinogenesis by injecting nude mice: month 12
- Characterize changes in tumor status of nude mice by gross dissection, and hematoxylin and eosin stained histologic sections: months 10-36.
- Characterize changes in tumor status of transgenic mice by gross dissection, and hematoxylin and eosin stained histologic sections: months 7-36.

Generate and characterize 3T3 fibroblast cell lines containing pTetO.Krct and pTetO.LacZ constructs: (months 7-14). Several kinases have been demonstrated to function as oncogenes in human cancers and murine animal models of mammary gland carcinogenesis(11, 13-16). To investigate if Krct has a role in mammary gland carcinogenesis, we will use 3T3 cells and transfect them with a CMV-Krct construct. This approach will be used instead of generating stable cell lines in 3T3 cells that inducibly overexpress Krct due to the extreme difficulty in generating stable over-expressing Krct cell lines.

Perform focus formation study: (months 15-22). During the time it has taken to generate one inducible Krct clone, a focus formation assay was performed by transiently transfecting a CMV-Krct construct into 3T3-L1 cells. Cells were cultured until confluent and fed

once every two days for two weeks and stained. No foci were observed on plates transfected with CMV-Krct or with the empty vector as a control. This experiment will need to be repeated since no foci formed. It is possible that 3T3-L1 cells may not transform as easily as NIH3T3 cells.

We will therefore use rat embryo fibroblasts (REF) which are known to be transformed when activated H-Ras and myc are co-transfected. We will co-transfect activated H-Ras and myc with and without Krct into REFs and monitor for focus formation. This will test if Krct is able to modify the co-transforming capabilities of Ras and myc. This is a more sensitive assay to test the transformation capabilities of a gene with unknown transforming potential. It will test the ability of Krct to synergize with known oncogenes or to form foci directly. Additionally, this will test the ability of Krct to inhibit the co-transforming capabilities of Ras and myc.

Perform soft agar experiment: (months 15-22). The usefulness of this test is predicated on the ability of Krct to modify the ability of REF cells to form foci upon transfection with Ras and myc. Only if Krct modifies focus formation will this test need to be performed.

Determine the effects of Krct overexpression on carcinogenesis by injecting nude mice: (month 12). Characterize changes in tumor status of nude mice by gross dissection, and hematoxylin and eosin stained histologic sections: (months 10-36). This task has not been performed since *in vitro* experiments have not been completed. As a result, the use of animals for the determination of Krct tumorigenesis is not warranted at this time.

Characterize changes in tumor status of transgenic mice by gross dissection, and hematoxylin and eosin stained histologic sections: (months 7-36). As a result of our inability to generate any Krct transgenic mice which express, this task cannot be completed.

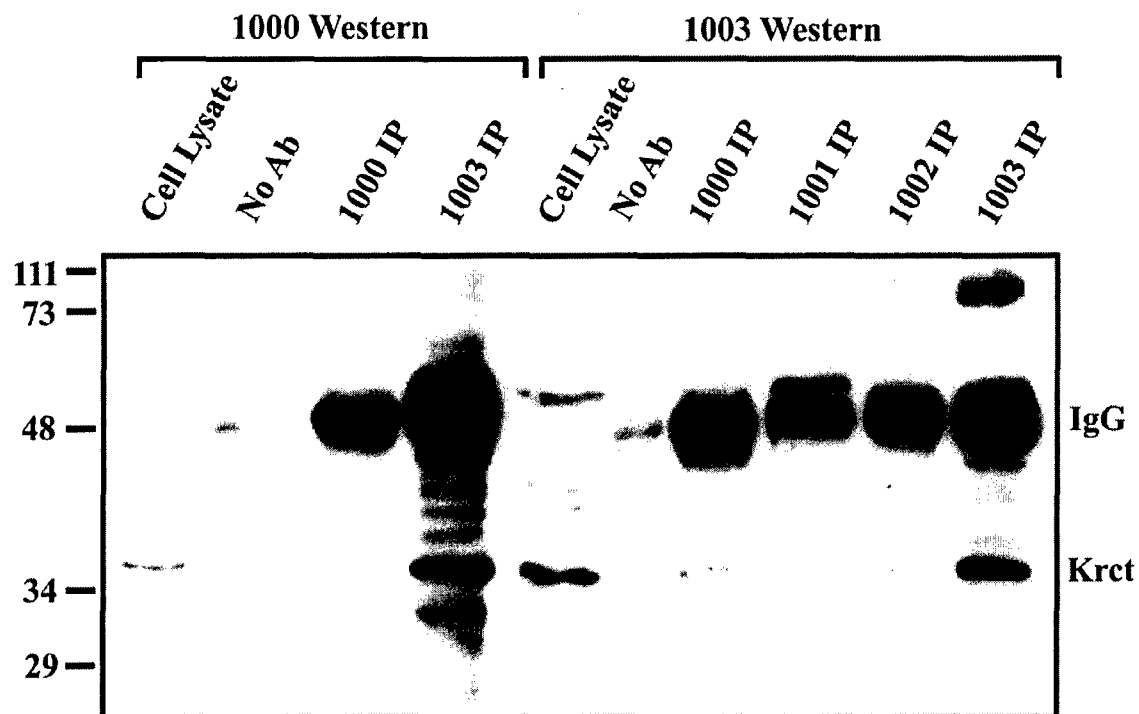


Figure 1. Krcr Immunoprecipitation (IP) and Western Analysis. IP's were performed with each of the four antisera raised against Krcr peptides (1000, 1001, 1002, 1003). All IP's performed used 2ug of affinity purified antisera and 500ug of cell lysate. Western analysis was performed with antisera 1000 and 1003 on the indicated samples. Both antisera recognize Krcr in cell lysates. 1000, 1002, and 1003 antisera can IP Krcr protein while the 1003 antisera appears to IP more Krcr protein than does 1000 or 1002.

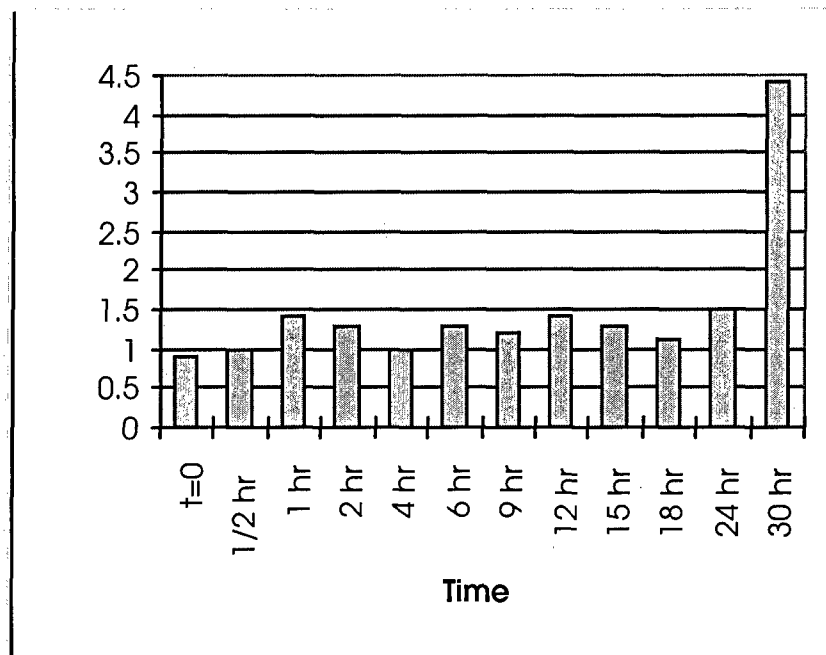
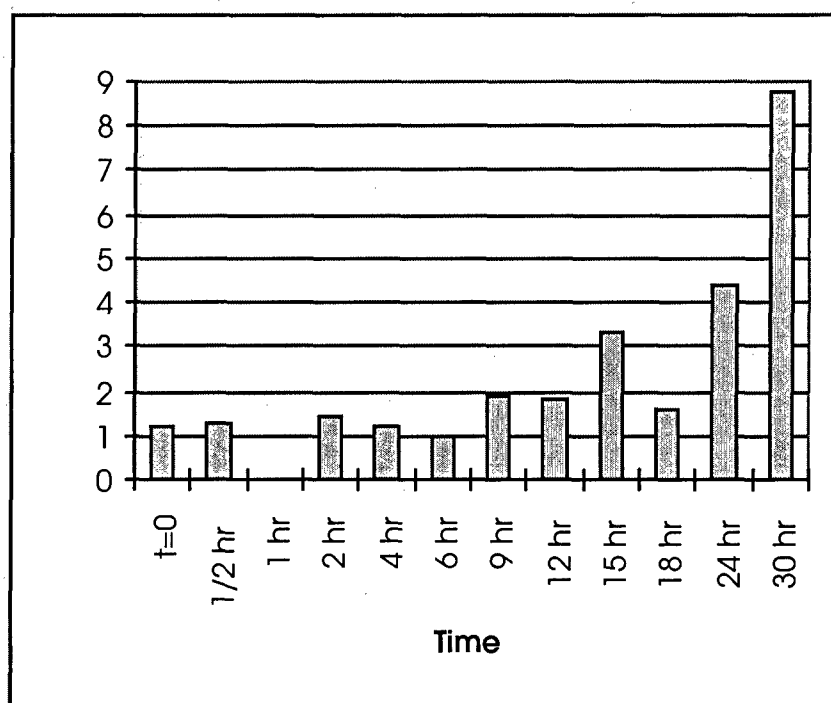
A.**B.**

Figure 2. Krct Cell Cycle Analysis. Cells in both experiments were starved for 48 hours and then refed in media containing 10% serum. Cells were harvested at the various times indicated. Krct expression was normalized using ARPP-PO (A) Krct expression in HBI2 cells. Krct expression increases at 30 hours. (B) Krct expression in 16MB9A cells. Krct expression increases at 24 hours.

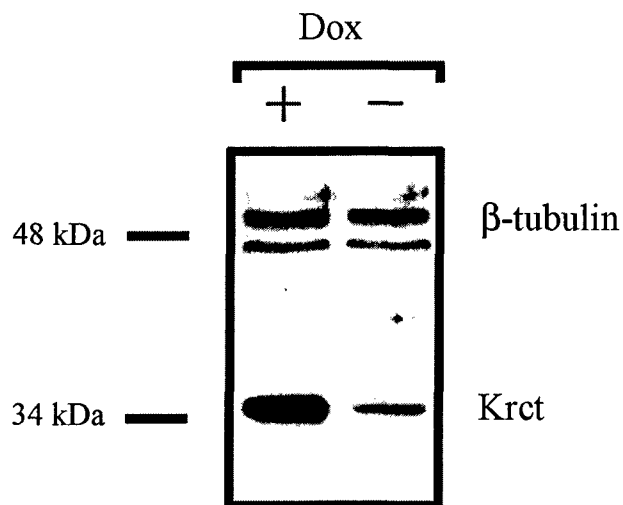


Figure 3. Doxycycline induction of a Krt inducible clone. Western analysis was performed on a dox-inducible Krt clone in the presence and absence of 1ug/ml of Doxycycline for 48 hours. 20 ug of cell extracts from induced and uninduced samples were analyzed with AB1003, a polyclonal antisera recognizing Krt. 5 to 10 fold more protein is seen for Krt in induced cell extracts when compared to uninduced extracts. Monoclonal antibodies recognizing β -tubulin were used to demonstrate equal loading.

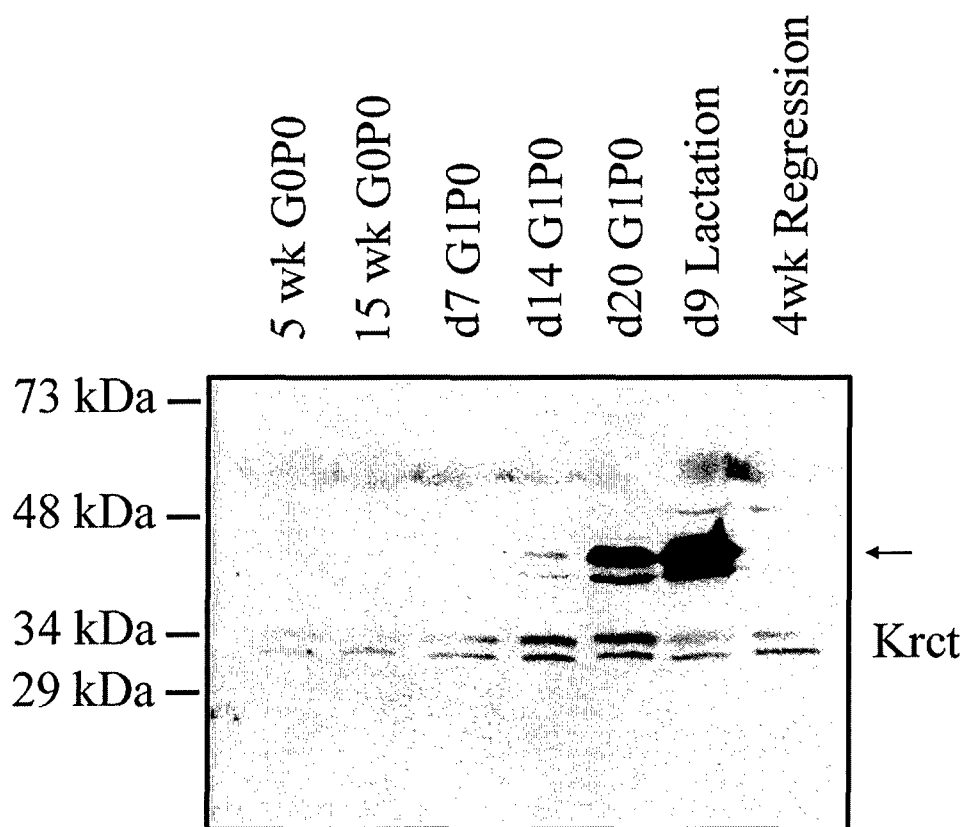


Figure 4. Western analysis of Krct during mammary gland development. 20ug of protein extracts from the indicated stages of murine mammary gland development were screened for Krct expression using polyclonal antisera raised against the C-terminus of Krct (Ab1003). Expression of the 35 kDa Krct protein parallels the expression pattern of *Krct* mRNA during pregnancy, but during lactation, mRNA still increases while protein levels decrease. Interestingly, a 45 kDa protein is detected during pregnancy with its expression peaking during lactation. This protein co-migrates with a 45 kDa protein seen in IVT products and may be a modified form of Krct. If so, this may indicate that Krct has a role in regulating events during late pregnancy and lactation.

Key Research Accomplishments:

- Generated antisera to two independent regions of Krct which recognize Krct by Western analysis and Immunoprecipitation.
- Generated the TetO-Krct expression construct responsive to rtTA and tetracycline.
- Generated the MMTV-Krct expression construct for over-expression of Krct in the mammary gland of transgenic mice.
- Determined that Krct mRNA levels are regulated during the cell cycle in response to serum starvation/refeeding in two mammary epithelial cell lines.
- Generated three lines of mice carrying an MMTV-Krct transgene.
- Generated one HC-11 cell line clone in which Krct can be inducibly overexpressed in response to doxycycline.
- Identified a potentially modified form of Krct which is differentially regulated during mammary gland development.

Reportable Outcomes:

- (1) Stairs DB, Perry Gardner H, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Chodosh LA. Cloning and characterization of Krct, a member of a novel subfamily of serine/threonine kinases. *Hum Mol Genet* (1998) 7(13):2157-2166.
- (2) Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, Wang M. Mammary gland development, reproductive history, and breast cancer risk. *Cancer Res* (1999) 59(7 Suppl):1765-1771s; discussion 1771s-1772s.

Conclusions:

Analysis of Krct expression using proliferation and differentiation assays as well as determining what affects over-expression of Krct has on these processes will yield valuable insight into Krct's potential role in mammary gland development and carcinogenesis. Preliminary results from analysis of Krct expression during the cell cycle demonstrate that Krct mRNA levels are regulated. Krct expression in the serum-starved state is low compared to actively dividing cells. Upon refeeding serum-starved cells, Krct expression rises dramatically just after Cyclin A induction in the cell cycle occurs. This finding, while suggestive of a possible role for Krct in proliferation, is not conclusive. Follow-up Western analysis experiments showed that Krct protein levels do not change during the cell cycle in HC-11 cells. Differences seen between Northern and Western analysis may be explained in several ways. Krct's 5'UTR contains elements known to regulate translational efficiency, and therefore protein levels need not reflect mRNA levels. Alternatively, different cell lines were used for protein and Northern

analysis. While unlikely, Krct expression may differ during the cell cycle in these cell lines. Further experiments will address the disparate results seen. Use of the inducible Krct cell line will be valuable in determining if Krct might play a role in cell cycle regulation.

Western analysis of Krct during mammary gland development has identified a 45 kDa protein that may be Krct. Krct is normally a 35 kDa protein, but a 45 kDa band is detected by antisera raised against the C-terminus of Krct. This 45 kDa protein is temporally regulated during mammary gland development. Expression of this protein is first detected during early pregnancy and peaks during lactation. Evidence that suggests this protein might be Krct is based on the migration of this potentially novel form of Krct co-migrating with an upper form of Krct seen during *in vitro* transcription/translation (IVT) reactions. Experiments are currently underway to determine what modification is responsible for the shift in apparent size of Krct in IVT reactions and during mammary gland development. If Krct is indeed modified during pregnancy and into lactation, this spatial regulation would suggest a very large increase in protein accumulation during stages of mammary gland development when the gland is differentiating. This would therefore suggest a potential role for Krct in differentiation of mammary epithelial cells.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

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