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Award Number: DAMD17-99-1-9284

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TITLE: The Role of the Integrin-Linked Kinase (ILK) in Mammary Gland Tumorigenesis and Metastasis

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REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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20010810 086

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 074-0188		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Derations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Rudet. Paperwork Reduction Project (0704-0188), Washington, DC 20503						
1. AGENCY USE ONLY (Leave blan	k) 2. REPORT DATE	3. REPORT TYPE AND	DATES COVERE	D		
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6. AUTHOR(S)						
Donald E. White						
Doctor William Mul.						
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)			8. PERFORMING ORGANIZATION REPORT NUMBER			
McMaster University						
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9. SPONSORING / MONITORING A	GENCY NAME(S) AND ADDRESS(ES	6)	10. SPONSORING / MONITORING			
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U.S. Army Medical Research and Fort Detrick Maryland 21702-5	012					
Fort Detrick, Maryland 21702-5	012					
11. SUPPLEMENTARY NOTES		1999 99 9	L			
12a. DISTRIBUTION / AVAILABILITY STATEMENT				12b. DISTRIBUTION CODE		
Approved for Public Release; Distribution Unlimited						
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The results of the experiment were submitted as a manuscript to the journal Oncogene. In addition to this main						
research objective, we have also shown that mammary-specific expression of a kinase-dead allele of ILK does						
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14. SUBJECT TERMS	and the state of t			15. NUMBER OF PAGES		
Breast Cancer, Tumorigenesis, Integrin, Murine Mammary Gland,				37		
Transgenic, Metastasis						
17. SECURITY CLASSIFICATION	18. SECURITY CLASSIFICATION	19. SECURITY CLASSIF	ICATION	20. LIMITATION OF ABSTRACT		
Unclassified	Unclassified	Unclassif	ied	Unlimited		
NSN 7540-01-280-5500			Stan	dard Form 298 (Rev. 2-89)		
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INTRODUCTION

The research objective outlined in the original proposal was to determine the role of the integrin-linked kinase (ILK) in the induction and progression of metastatic mammary tumors. This work was initially based on the observation that overexpression of ILK in cultured epithelial cells results in changes characteristic of oncogenic transformation, including anchorageindependent growth, suppression of cell death in suspension, invasion of extracellular matrices, and tumorigenicity in nude mice (Hannigan et al., 1996; Novak et al., 1998; Radeva et al., 1997; Wu et al., 1998). In addition, ILK protein levels have been found to be elevated in a variety of human tumors and tumor cell lines, suggesting an important physiological role for this protein in the initiation and progression of human cancers. As a result of these observations, we decided to directly evaluate the role of ILK in mammary tumorigenesis, by deriving transgenic mice that express ILK under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). Expression of the MMTV/ILK transgene resulted in both a hyperplastic mammary gland phenotype and focal mammary tumors in these mice, demonstrating that the mammary-specific overexpression of ILK can indeed facilitate malignant transformation of the mammary epithelium. Given that ILK protein levels are elevated in human tumor samples, this result may have important implications for understanding the molecular mechanisms of breast cancer.

RESEARCH ACCOMPLISHMENTS

1) The overexpression of ILK in the mammary epithelia of transgenic mice results in the induction of mammary hyperplasias and mammary tumors

The major goal outlined in the original research proposal was to determine whether the overexpression of ILK in the mammary epithelium could result in the induction of mammary tumors. This was accomplished by the establishment of transgenic mice expressing ILK under the transcriptional control of the MMTV-LTR, which has known promoter/enhancer activity in the murine mammary epithelium. The appearance of mammary tumors in these MMTV/ILK mice confirmed that the overexpression of ILK could indeed result in the induction of mammary tumors *in vivo*, which may have clinical significance given that elevated ILK protein levels are found in human tumors and tumor cell lines. The results of this experiment are presented in a submission to the journal Oncogene, entitled "Mammary epithelial-specific expression of the integrin linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice" (see letter and manuscript, Appendix 1).

2) Overexpression of a kinase-dead (KD) allele of ILK does not inhibit tumor formation by activated erbB-2

A second major goal during the funding period was to determine if the kinase activity of ILK was necessary for the induction of tumorigenesis in other transgenic mouse models. This was accomplished by first generating transgenic mice expressing a kinase-dead (KD) allele of ILK in the mammary epithelium, under the transcriptional control of the MMTV-LTR (Figure 1, Appendix 2). These MMTV/ILK-KD mice were then crossed with our MMTV/erbB-2 strains,

to determine if expression of the ILK-KD allele could inhibit tumor formation by the MMTV/erbB-2 transgene. The rationale behind this cross was based on reports showing that ILK may play a direct role in growth factor receptor signaling (Tu et al., 1998; 1999), and that there is a direct correlation between ILK and erbB-2 expression in mice (Xie et al., 1998). Since erbB-2 is upregulated in a large proportion of human breast cancers, the MMTV/erbB-2 transgenic mice provided a relevant model for testing the upstream role of ILK kinase activity in oncogenesis. However, by measuring the kinetics of tumor formation in these MMTV/erbB-2/ILK-KD bitransgenic mice, we determined that expression of the ILK-KD allele does not have a detectable impact on the kinetics of tumor induction by activated erbB-2 (Figure 2, Appendix 2).

3) Co-expression of ILK does not compensate for a lack of PI-3' kinase signaling in PyV mT-induced tumorigenesis

The original proposal also described an experiment to determine whether elevated expression of ILK could complement a mutant PyV mT oncogene that is decoupled from the PI-3' kinase signaling molecule (Webster *et al.*, 1998), given that ILK is thought to be a direct downstream target of PI-3' kinase (Delcommenne *et al.*, 1998). Complementation of the mT mutant by ILK would be revealed by an increase in mutant mT-induced tumor kinetics and metastasis, to levels seen in mice expressing the wild-type PyV mT antigen, which signals through PI-3' kinase (Webster *et al.*, 1998). This experiment was performed by breeding the MMTV/ILK mice with those expressing the mutant mT antigen, also under control of the MMTV-LTR. The experiment was designed ultimately to confirm the importance of ILK in the PI-3' kinase/Akt pathway *in vivo*. The results of this experiment, however, revealed that elevated expression of ILK was not sufficient to complement the defect observed in the PyV mT strains, in terms of tumor kinetics and metastasis (Figure 3, Appendix 2). These results would suggest that perhaps there is a requirement for other PI-3' kinase targets, such as PDK1 and the Rac/Rho small GTPases (Currie *et al.*, 1999; Rodriguez-Viciana *et al.*, 1997), for the induction of metastatic tumors resembling those induced by the wild-type mT antigen.

TRAINING ACCOMPLISHMENTS

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Over the past 2 years, I have acquired skills pertaining to the construction and analysis of transgenic mice, specifically those involving models of mammary tumorigenesis. These skills include the underlying molecular biological manipulations, as well as biochemical analysis and immunohistochemistry. In addition to these basic laboratory skills, I have gained a great deal of knowledge regarding the histology and histopathology of normal and cancerous mammary gland tissue. By working with Dr. Bill Muller and our collaborator, Dr. Robert Cardiff (UC Davis), I now have a basic understanding of the progression of metastatic disease, and have acquired a knowledge of the variety of tumor types arising in the mammary glands of both humans and transgenic mouse models. My understanding of cancer biology now encompasses the molecular and cellular changes occuring during the tumorigenesis process, including pre-neoplastic events, metaplasia and epithelial-to-mesenchymal transition, as well as the mechanisms of tumor invasion and metastasis. In addition, since ILK overexpression seems to have an impact on the differentiative state of the murine mammary gland, I now have a much more thorough understanding of mammary gland development and the molecular mechanisms behind the

specialized functions of this tissue. Dr. Cardiff is only one of several collaborators whom we can access as a resource in Dr. Muller's lab.

KEY RESEARCH ACCOMPLISHMENTS

1

- Generation and characterization of mice expressing ILK in the mammary epithelium, under transcriptional control of the MMTV-LTR, and demonstration of tumorigenesis in those mice
- Generation and characterization of mice expressing a kinase-dead (KD) allele of ILK, also in the mammary epithelium, under control of the MMTV-LTR
- Interbreeding of the MMTV/ILK-KD mice with MMTV/erbB-2 mice, to assess role of ILK in erbB-2-induced mammary tumorigenesis
- Interbreeding of the MMTV/ILK mice with mice expressing MMTV/mT transgene decoupled from the PI-3' kinase pathway, to assess role of ILK in PI-3' kinase/Akt pathway *in vivo*

REPORTABLE OUTCOMES

- Manuscript (submitted to the journal Oncogene)—"Mammary epithelial-specific expression of the integrin linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice." Authors: D White, R D Cardiff, S Dedhar and WJ Muller (see letter and manuscript, Appendix 1).
- Oral presentation—Canadian Breast Cancer Research Initiative, Reasons for Hope Meeting, Loews LeConcorde Hotel, Quebec City, Quebec, May 3-5, 2001 (see abstract, page 36, Appendix 3)
- Abstract—Oncogene Meeting, Salk Institute, San Diego, California, June 22-25, 2000 (see abstract, page 37, Appendix 3)

CONCLUSIONS

Funding for this project was provided in order to assess the role of ILK in mammary tumorigenesis. The most important result after 2 years of funding is the demonstration that the overexpression of ILK in the mammary epithelia of transgenic mice results in the induction of mammary gland hyperplasias and mammary tumors. This result is directly relevant to the understanding of the molecular mechanisms behind invasive breast cancer, since elevated levels of ILK protein have been found in human tumors and tumor cell lines (Chung et al., 1998; Janji et al., 1999).

The induction of a hyperplastic and tumor phenotype in the mammary glands of the MMTV/ILK mice confirms the results of cell culture experiments, which implicated ILK as a potential oncogene. Consistent with the results of those experiments, the hyperplastic phenotype of the MMTV/ILK mice was accompanied by the phosphorylation of key signaling proteins, known to be downstream targets of ILK (see manuscript, Appendix 1). The targets include PKB/Akt, MAP kinase and GSK-3 β , an inhibitor of the Wnt signaling pathway. Given the roles of these proteins in known survival and proliferative pathways (see manuscript, and references

therein, Appendix 1), it is likely that they contributed to the hyperplastic phenotype of the MMTV/ILK mice. Given the focal nature of the tumors, however (see manuscript, Appendix 1), additional genetic events are likely required for progression from a hyperplastic to a tumor phenotype. The MMTV/ILK mice may therefore provide a model for multi-step oncogenesis.

In addressing another major goal of this project, we found that the mammary-specific expression of a kinase-dead (KD) allele of ILK did not inhibit tumorigenesis induced by activated erbB-2. This result is not unexpected, given that the erbB-2 receptor binds to multiple signaling molecules, and may therefore induce tumorigenesis through multiple or redundant signaling pathways. In addition, the ILK-KD allele expressed in the bitransgenic mice retains protein interaction domains recently shown to be important for the cellular functions of ILK (Nikolopoulos et al., 2001; Tu et al., 2001; Zervas et al., 2001). A more informative approach in addressing the role of ILK in oncogene-mediated tumorigenesis would therefore involve the mammary-specific excision of the ILK coding sequence, using the cre/lox recombination system.

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Appendix 1

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Manuscript submitted to Oncogene

THE INSTITUTE FOR MOLECULAR BIOLOGY & BIOTECHNOLOGY



May 7, 2001 Dr. E. Premkumar Reddy Ph.D. The Fels Institute for Cancer Research and Molecular Biology 3307 North Broad St. Philadelphia, PA 19140 USA

Dear Dr. Reddy:

Enclosed you will find our manuscript entitled "Mammary epithelial-specific expression of the integrin linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice" which we would like to be considered for publication in Oncogene.

In this manuscript we describe the phenotypic consequences of mammary epithelial -specific expression of ILK in transgenic mice. Elevated expression of ILK in the mammary glands of these transgenic strains was initially correlated with the induction of mammary epithelial hyperplasias that was further correlated with the constitutive activation of the PKB, GSK-3B and MAP kinase signaling pathways. However, a proportion of these female animals eventually developed focal mammary tumours after a long latency period. Interestingly, these tumors exhibited features of epithelialmesenchymal transition (EMT). Given the potential importance of ILK expression in sporadic human breast cancer, these observations provide the first direct in vivo evidence that mammary epithelial expression of ILK can result in the induction of mammary tumors. Thank you in advance for considering this manuscript for publication in Oncogene.

Yours Sincerely

11

William J. Muller Professor

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Mammary epithelial-specific expression of the integrin linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice

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Running title: Mammary tumorigenesis in MMTV/ILK transgenic mice

Keywords: integrin linked kinase; ILK; transgenic mice; mammary epithelium; breast cancer; tumorigenesis

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Abstract

The integrin-linked kinase (ILK) is a cytoplasmic effector of integrin receptors, involved in the regulation of integrin binding properties as well as the activation of cell survival and proliferative pathways, including those involving MAP kinase, PKB/Akt and GSK-3β. Overexpression of ILK in cultured intestinal and mammary epithelial cells has been previously shown to induce changes characteristic of oncogenic transformation, including anchorage-independent growth, invasiveness, suppression of anoikis and tumorigenicity in nude mice. In order to determine if ILK overexpression can result in the formation of mammary tumors in vivo, we generated transgenic mice expressing ILK in the mammary epithelium, under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). By the age of 6 months, female MMTV/ILK mice developed a hyperplastic mammary phenotype, which was correlated with the constitutive phosphorylation of PKB/Akt, GSK-3β and MAP kinase. Focal mammary tumors subsequently appeared in 34% of the animals at an average age of 18 months. Given the focal nature and long latency of the tumors, however, additional genetic events are likely required for tumor induction in the MMTV/ILK mice. These results provide the first direct demonstration of a potential oncogenic role for ILK, which is upregulated in human tumors and tumor cell lines.

Introduction

The normal growth and development of the mammay epithelium depends on interactions between the epithelial cells with the adjacent extracellular matrix (ECM). This interaction is mediated primarily through the integrin family of receptors (Streuli and Edwards, 1998; Schmeichel et al., 1998), which play critical roles in modulating the mechanical aspects of cell adhesion, such as in the assembly and remodeling of the ECM, as well as promoting the proliferation, differentiation and survival of the epithelial cells. The regulation of cell proliferation, differentiation and survival by integrin receptors is achieved through the activation of various signaling pathways, such as those involving MAP kinase (MAPK) and PI3K-PKB/Akt (Reviewed in Giancotti and Ruoslahti, 1999). Mutations which affect the properties of integrin receptors and cytoplasmic effector molecules may result in the deregulation of these integrin-mediated signaling pathways and the subsequent loss of anchorage dependence for epithelial cell proliferation and survival (Zutter et al., 1998; Shaw, 1999). Such mutations may have dramatic pathological consequences, and are indeed an important contributing factors in the growth and spread of mammary tumors (Zutter et al., 1998; Shaw, 1999).

As a result, understanding the role of cytoplasmic effectors in the regulation of integrin binding properties and signaling pathways is important for understanding the initiation and progression of mammary gland tumors. One such proximal effector of integrin signaling is the integrin linked kinase (ILK), a 59 kilodalton ankyrin repeatcontaining serine/threonin protein kinase, which interacts with the cytoplasmic domains of $\beta 1$ and $\beta 3$ integrin subunits (Hannigan et al., 1996). ILK has been shown to be an important effector of both integrin and growth factor receptor signaling, in a manner dependent on PI3K activity (Delcommenne et al., 1998; Troussard et al., 1999; Tu et al., 1999; Persad et al., 2000; 2001a). When overexpressed in cultured epithelial cells, ILK induces the phosphorylation and inactivation of GSK-3 β , a negative regulator of the Wnt signaling pathway. The phosphorylation of GSK-3 β results in the activation of the AP-1 and β -catenin/LEF-1 transcription factors (Delcommenne et al., 1998; Novak et al., 1998; Troussard et al., 1999), and the subsequent expression of mesenchymally-related genes. ILK overexpression also results in the suppression of apoptosis in both intestinal epithelial and mammary epithelial cells, through the phosphorylation and activation of the anti-apoptotic PKB/Akt kinase (Delcommenne et al., 1998; Atwell et al., 2000).

In addition to playing a role in these intracellular signaling pathways, ILK has also been shown to regulate the adhesive properties of cells. In this regard, epithelial cells overexpressing ILK exhibit reduced adhesion when plated on fibronectin, collagen or laminin (Hannigan et al., 1996), show elevated levels of fibronectin matrix assembly in culture (Wu et al., 1998), and have disrupted epithelial cell-cell contacts (Hannigan et al., 1996). More importantly, the overexpression of ILK in cultured epithelial cells results in several changes characteristic of oncogenic transformation, including anchorage-independent growth and survival (Hannigan et al., 1996; Radeva et al., 1997; Atwell et al., 2000), invasiveness in 3-dimensional culture, and tumorigenicity in nude mice (Wu et al., 1998).

Whereas these studies suggest that the elevated expression of ILK may be involved in promoting the oncogenic conversion of mammary epithelial cells in vitro, the in vivo role of ILK in mammary tumorigenesis remains to be elucidated. As a result, we have derived transgenic mice expressing ILK in the mammary epithelium, under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer, in order to directly evaluate the role ILK in mammary tumor progression. Mammary epithelial-specific expression of ILK was initially associated with induction of mammary epithelial hyperplasias in female transgenic mice by the age of 6 months. However, a subset of older female transgenic mice subsequently developed focal mammary tumors. Interestingly, histological analyses of these mammary tumors revealed that a proportion of the tumors exhibited evidence of epithelial-to-mesemchymal transition, consistent with the ability of ILK to mesenchymally transform mammary epithelial cells in culture (Somasiri et al., 2001).

The results of this experiment therefore provide the first direct evidence that elevated expression of ILK may result in the induction of mammary tumors <u>in vivo</u>. These results may have clinical significance in the treatment of human breast cancer, considering the observation that ILK is upregulated in a variety of human tumors and tumor cell lines (Chung et al., 1998; Janji et al., 1999; 2000).

Results

Mammary hyperplasia and alveolar development in transgenic mice expressing elevated levels of ILK in the mammary epithelium

To assess the oncogenic effects of elevated ILK expression in mammary epithelial cells in vivo, one cell mouse zygotes were microinjected with an expression cassette in which the full length cDNA for human ILK was placed under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Figure 1a). A total of 6 founder animals were generated. To determine which of these transgenic strains were expressing the MMTV/ILK transgene, RNA isolated from the mammary epithelia of nulliparous mice was subjected to RNase protection analyses with an antisense riboprobe directed to SV40 component of the transgene (Figure 1a). Using this approach, we identified 3 independent lines of mice expressing the MMTV/ILK

transgene in the virgin mammary gland (Figure 1b). In addition to the mammary epithelium, lower levels of transgene-specific transcript were detected in the salivary gland, seminal vesicle and epididymus (data not shown), consistent with the tissue-specific pattern of transgene expression in other MMTV-based transgenic strains (Dankort et al., 1999).

To explore whether mammary epithelial-specific expression could perturb normal mammary gland development, we performed wholemount analyses on the mammary glands of virgin female transgenic mice at various stages of mammary gland development. Although early ductal development (8 weeks of age) in these strains was comparable to control female FVB/N mice (data not shown), the mammary glands of a proportion of virgin transgene carriers displayed aberrant development by the age of 6 months. This phenotype consisted of mild ductal and acinar hyperplasia, with an unusual number of secondary and tertiary branches and small, spiculated side buds (Figure 2a). Interestingly, the appearance of these glands resembled those of wild-type mice during early pregnancy (data not shown), inconsistent with the nulliparous state of the transgenic animals. Microscopic examination of a section of these glands revealed a multi-layered epithelium (Figure 2b) containing an unusually high number of abnormal ring mitotic figures (Figure 2b, inset), and which was disorganized with respect to the normal columnar arrangement (Figure 2b). After 12 months of age, the phenotype of the glands was more severe, consisting of an unusual number of well developed alveolar units, with tight clusters resembling hyperplastic alveolar nodules (HAN) (Figure 2c). In addition, secretory vacuolization was apparent in the histological sections of these older virgin mice, again resembling a partially lactating phenotype (Figure 2d).

To assess whether the observed mammary epithelial abnormalities reflected activation of known targets of ILK, we performed biochemical analyses on mammary tissue extracts from 6 month-old nulliparous female control (FVB/N) and MMTV/ILK transgenic mice. One important downstream target of ILK is the PKB/Akt serine kinase, which is phosphorylated on serine 473 in response to elevated levels of ILK expression (Delcommenne et al., 1998; Persad et al., 2001). To determine whether PKB/Akt was constitutively phosphorylated in response to MMTV/ILK expression, mammary tissue extracts from 6 month-old nulliparous female MMTV/ILK and FVB/N mice were subjected to immunoblot analysis with phosphospecific antisera directed to serine 473 of PKB/Akt. The results revealed that the mammary glands derived from the MMTV/ILK mice contained elevated levels of phosphorylated PKB/Akt protein, in comparison to glands from FVB/N control mice (compare lanes 4-9 with 1-3, Figure 3a). The differences in the extent of phosphorylation were not due to differences in the levels of total PKB/Akt protein, which were comparable between transgenic and control mice (Figure 3a, lower panel).

Another important target of ILK kinsase activity is the serine/threonine kinase GSK-3 β (Delcommenne et al., 1998; Persad et al., 2001*b*), a negative regulator of the Wnt signaling pathway which is inhibited by phosphorylation (Cross et al., 1995). To determine whether GSK-3 β was constitutively phosphorylated in the MMTV/ILK mice, the identical tissue lysates were subjected to immunoblot analysis with phosphospecific antibodies directed against serine 9 of GSK-3 β . As with PKB/Akt, the results showed that the phosphorylation of GSK-3 β was elevated in the MMTV/ILK-derived mammary extracts, relative to those of control FVB/N mice (compare lanes 4-9 with 1-3, Figure 3b). Again, the increase in GSK-3 β phosphorylation could not be ascribed to differences in the levels of total GSK-3 β protein (Figure 3b, lower panel).

Finally, given the recent observation that ILK can activate the MAPK signaling pathway in cultured epithelial cells (Troussard et al., 1999; Huang et al., 2000), we also examined the state of activation of the MAPK signaling pathway in these ILK-induced epithelial hyperplasias, as indicated by the phosphorylation status of MAPK (p44/42 Erk1/2). Consistent with the phosphorylation of both PKB/Akt and GSK-3 β , increased phosphorylation of MAPK was detected in the ILK-expressing mammary extracts, relative to the control FVB/N mammary glands (compare lanes 4-9 with 1-3, Figure 3c). The difference in the state of phosphorylation of MAPK could not be attributed to differences in levels of MAPK protein, since the extracts contained comparable levels of total MAPK protein (Figure 3c, lower panel). Taken together, these observations suggest that the induction of mammary epithelial hyperplasias in the MMTV/ILK mice is correlated with the concerted activation of pathways involved in epithelial cell proliferation and survival, and which are known to be targets of ILK activity in culture.

Elevated expression of ILK predisposes the mammary epitheliun to tumorigenesis

In spite of the hyperplastic phenotype, no gross abnormalities nor reproductive problems were observed in the female MMTV/ILK mice during the first year of their life. After 1 year of age, however, we began to notice the appearance of focal mammary tumors in female mice from all 3 founder lines that expressed the MMTV/ILK transgene (Figure 4a). In our best characterized strain (line 363), 34% of female animals developed focal mammary tumors with an average latency of 560 days (Figure 4b, Table 1). By contrast, no mammary tumors were observed in age-matched female FVB/N control mice (Table 1). As shown in Figure 4c, the induction of mammary tumors in the MMTV/ILK mice was accompanied by an increase in the overall levels of total ILK protein, in comparison to adjacent mammary gland (Figure 4c, compare lanes 2,4,6,8,10,12 and 14 to lanes 1,3,5,7,9,11 and 13, upper panel). These differences in the levels of ILK protein

were not due to variation in protein loading, as both tumor and adjacent mammary gland expressed comparable levels of β -actin loading control (Figure 4c, lower panel).

The tumors from the MMTV/ILK mice revealed a somewhat polymorphic pathology, ranging from well differentiated papillary adenocarcinomas (Figure 5a-d), to undifferentiated spindle cell tumors (Figure 5i-l). The tumors were invasive, with nests and cords of cells infiltrating a dense connective tissue stroma and adjacent skeletal muscle (not shown). In addition, we observed distal pulmonary metastases in 21% of the tumor-bearing animals (Table 1). Well differentiated adenocarcinomas were characterized by large cells with hyperchromatic, pleomorphic nuclei, and contained regions of squamous metaplasia (Figure 5a). Interestingly, in several of the tumors differentiated epithelial cells were interspersed within regions of mesencymal-like cell populations (Figure 5e-h), as indicated by the expression pattern of epithelial markers (Figure 5f-h) and the mesenchymal marker vimentin (not shown). Similarly, the spindle cell tumors consisted of cells exhibiting a mesenchymal morphology, but which expressed markers suggesting a combination of both epithelial and mesenchymal characteristics (Figure 5i-l). The presence of mesenchymal-like cell populations, particularly within tumors containing well defined glandular elements, therefore argues that tumorigenesis in these MMTV/ILK mice may involve an epithelial-to-mesenchymal transition.

Discussion

The interaction between the extracellular matrix and a tumor cell has been implicated as an important event in promoting both the growth of a tumor and invasion of surrounding tissue. In this regard, the integrin receptors and their coupled signaling pathways are thought to play a critical role in tumorigenesis (Zutter et al., 1998; Shaw, 1999). One cytoplasmic effector of integrin signaling which has been implicated in tumor progression is the integrin-linked kinase (ILK) (Dedhar, 2000; Yoganathan et al., 2000). By deriving transgenic mice expressing ILK under the transcriptional control of the MMTV promoter/enhancer, we have provided direct evidence that the overexpression of ILK can result in the induction of mammary carcinomas <u>in vivo</u>.

Expression of the MMTV/ILK transgene in the mammary epithelia of the transgenic strains resulted in both mammary tumor formation, as well as a hyperplastic mammary gland phenotype in a proportion of the mice. In addition, by performing immunoblot analysis on protein lysates from 6 month old virgin mammary glands, we detected elevated phosphorylation levels of PKB/Akt, GSK-3 β and MAPK, which were correlated with the hyperplastic state of the glands. Given their role in cell proliferation and cell survival pathways, the phosphorylation of these proteins, and the concomitant activation of the respective signaling pathways, by ILK overexpression likely contributed

directly to the increase in mammary epithelial content in the transgenic mice. Tumor induction, however, likely involved additional events, given the long latency and focal nature of the tumors. Consistent with this hypothesis, Hutchinson et al. have shown recently that the mammary epithelial expression of activated PKB/Akt is indeed insufficient to induce mammary tumors in transgenic mice (Hutchinson et al., 2001).

The phenotypes of the MMTV/ILK-induced tumors were variable, ranging from papillary adenocarcinomas to undifferentiated spindle cell tumors. Most of the tumors, however, exhibited evidence of an epithelial-to-mesenchymal transition, which may provide a clue as to the molecular mechanisms involved in tumor induction following expression of the MMTV/ILK transgene. In this regard, it should be noted that the MMTV-dependent expression of the transgene was dramatically reduced in a majority of late stage MMTV/ILK-induced tumors analysed, relative to adjacent mammary gland (data not shown). Silencing of the transgene in these tumors possibly reflects the activation of a genetic program that is incompatible with the epithelial-specific MMTV promoter/enhancer. Furthermore, the reduction in transgene expression occurs despite an increase in total ILK protein levels in the tumors. Tumor cells undergoing an epithelialto-mesenchymal transition must therefore initiate a series of genetic events that lead to the upregulation of the endogenous ILK promoter, perhaps contributing to the long latency and incomplete penetrance of tumor formation in the MMTV/ILK mice. The transition from an epithelial to a mesenchymal phenotype in the tumors of the MMTV/ILK mice is consistent with the induction of a mesenchymal phenotype in cultured mammary epithelial cells expressing elevated levels of ILK (Somasiri et al., 2001).

Interestingly, a similar tumor phenotype has recently been described in mice expressing a casein kinase 2 alpha (CK2 α) transgene, also under the transcriptional control of the MMTV promoter/enhancer (Landesman-Bollag et al., 2001). The tumors from these MMTV/CK2 α mice exhibited comparable kinetics, penetrance and histologically diverse phenotype as those seen in the MMTV/ILK strains, with both spindle cell tumors and adenocarcinomas appearing in 30% of the mice, at an average age of 23 months (Landesman-Bollag et al., 2001). Moreover, as in the MMTV/ILK strains, transgene expression in the MMTV/CK2 α mice has been replaced by elevated levels of endogenous CK2 α protein in a majority of the tumors examined. It is intrguing to note that like ILK, CK2 α has been implicated in modulating the Wnt signaling pathway, as well as cellular adhesion and cell spreading (Song et al., 2000; Seger et al., 2001). The similarity between the MMTV/CK2 α and MMTV/ILK mice may therefore reflect overlapping mechanisms of tumorigenesis, involving either the regulation of intracellular signaling pathways, such as the Wnt pathway, or the regulation of integrin binding properties and cellular adhesion.

A similar phenotype has also been described in transgenic mice expressing the matrix metalloproteinase MMP-3 in the mammary epithelium (Sternlicht et al., 2000). In this case, expression of an MMP-3 transgene, driven by the whey acidic protein (WAP) gene promoter, induced focal mammary tumors at an average age of 18 months. The histological and cytological appearance of these WAP/MMP-3-induced tumors was again comparable to those of the MMTV/ILK strains, consisting of both moderately to well differentiated adenocarcinomas, with a proportion of the tumors revealing some degree of epithelial-to-mesenchymal transition (Sternlicht et al., 2000; Figure 5, this paper). Indeed, a tumor cell line generated from 1 undifferentiated cytokeratin-positive tumor gave rise to a spindle cell tumor in nude mice (Sternlicht et al., 2000).

In addition to similar tumor kinetics and phenotype, the WAP/MMP-3 and MMTV/ILK strains of mice were comparable with regards to the presence of hyperplastic mammary glands resembling those of parous mice. In this regard, a proportion of virgin glands from both transgenic strains exhibited increased ductal branching and lobuloalveolar development, with regions of secretory vacuolization apparent in histological sections (Sternlicht et al., 2000; Figure 2, this paper). As with the MMTV/CK2 α mice, the similarity between the WAP/MMP-3 and MMTV/ILK models may again reflect overlapping mechanisms of epithelial transformation. Indeed, a recent report by Troussard *et al.* describes the upregulation of MMP-9 expression following the overexpression of ILK in cultured mammary epithelial cells (Troussard et al., 2000).

The induction of mammary tumors by expression of an ILK transgene, possibly involving the activation of a mesenchymal pathway, may reflect the normal physiological role of ILK in development. For example, a survey of human tissues revealed that ILK mRNA and protein is primarily expressed in cells of mesenchymal origin, most notably cardiac and skeletal muscle (Hannigan et al., 1996; Chung et al., 1998). Consistent with its expression in muscle tissue, experiments by Huang et al. and Deng et al. recently revealed a role for ILK in the regulation of myogenic differentiation (Huang et al., 2000), and in the phosphorylation of myosin light chain during smooth muscle contraction in chickens (Deng et al., 2001). Similarly, a drosophila orthologue of ILK has been found to be expressed primarily in the mesoderm of the developing Drosophila embryo (Zervas et al., 2001). Taken together, these observations suggest that the transformation of epithelial cells by elevated expression of ILK may result from the overexpression of a protein primarily involved in the establishment and maintenance of a mesenchymal phenotype. Further studies are required to elucidate the biological role of ILK and the molecular basis for ILK-dependent transformation, particularly since this protein has been found to be elevated in human cancers and cancer cell lines (Chung et al., 1998; Janji et al., 1999; 2000).

Materials and methods

Generation and identification of transgenic animals

The 1.8 kb full length cDNA for human ILK (Hannigan et al., 1996) was subcloned into the EcoRI site of plasmid p206, harbouring the MMTV-LTR and the polyadenylation sequence of the SV40 early region (Sinn et al., 1987). The expression cassette was then prepared and injected into one cell zygotes of FVB/N mice, as described previously (Webster et al., 1998). To identify transgenic animals, genomic DNA was isolated from 0.5cm clippings of mouse tails (Muller et al., 1988), and PCR amplified using an ILKspecific forward primer (CATGTATGCACCTGCCTG) and an SV40-specific reverse primer (TATGTCACACCACAGAAG), to generate a transgene-specific amplification product. PCR conditions included a 30 second annealing step at 52 °C, and a 1 minute extension at 72°C, for 30 cycles.

RNA expression analysis

To identify mice expressing the MMTV/ILK transgene, total mammary gland RNA was prepared by homoginization in 4M GIT, followed by gradient sedimentation through CsCl (Chirgwin et al., 1979). Transgene expression was determined by RNase protection analysis, using a riboprobe specific for the SV40 polyadenylation signal, as described previously (Webster et al., 1998). A riboprobe specific for PGK-1 (Webster et al., 1998) was used as an internal control to standardize for total RNA content.

Protein extraction and western blot analysis

Mammary gland and tumor samples were flash frozen in liquid nitrogen, and lysed in buffer containing 50mM HEPES, pH 7.5, 150mM NaCl, 10% glycerol, 1% triton X-100, 1mM EGTA, 2mM EDTA, 10mM NaF, 10mM Napyrophosphate, 1ug/ul leupeptin, 1 ug/ul aprotinin and 1mM Na orthovanadate. Protein concentrations were determined using the Bio-Rad protein assay kit. Samples (20-40 μ g) were then electrophoresed through a 12% PAG, and transferred to an immobilin-P nylon membrane. Membranes were blocked in 3% nonfat dried milk in 1xTBS, 0.05% Tween-20, incubated in primary antibody overnight at 4°C, washed in TBS/0.05% Tween-20, and incubated with HRPconjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1 hour at room temperature. Secondary antibody was visualized using ECL reagent, according to the manufacturer's instructions. In the case of phospho-protein analysis, blots were stripped in 2%SDS/β-mercaptoethanol/Tris (pH 6.8) at 70°C for 30 minutes, blocked, and reprobed with antibodies recognizing total (phosphorylated and unphosphorylated) protein. Primary antibodies used for immunoblotting included rabbit anti-ILK (Upstate Biotechnology) and mouse anti- β -actin (Sigma), used as an internal control for protein loading. Phospho-PKB/Akt, phospho-GSK-3 β and phospho-MAPK blots were performed with rabbit polyclonal antibody kits from New England Biolabs. Primary antibodies were used at a dilution of 1:1000, and secondary antibodies were used at a dilution of 1:2500.

Histological and whole mount analysis

For histological analysis, mammary and tumor tissue samples were fixed overnight in Bouin's fixative (Accustain, Sigma Diagnostics), blocked in paraffin, and sectioned at 5μ m thickness. Sections were then stained with hematoxylin and eosin to facilitate examination. Mammary gland whole mounts were prepared by mounting the left abdominal mammary fat pads on glass slides, and processing them according to a protocol described previously (Webster et al., 1998). Immunohistochemistry for cytokeratin 8, E-cadherin and smooth muscle actin was performed at the Center for Comparative Medicine, University of California at Davis, Davis, California.

Acknowledgements

The authors would like to thank Judy E Walls (UC Davis School of Medicine) for excellent technical support. This work was supported by the US Department of Defense Breast Cancer Research Initiative DAMD 17-99-1-9284 (D White) and DAMD 17-99-1-9286 (WJ Muller), by grants from the National Cancer Institute of Canada (S Dedhar), and by grant 5JB-0014 from the State of California Breast Cancer Research Program (RD Cardiff).

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Figure 1 Targeted expression of ILK in the mammary gland of transgenic mice. (a) Expression cassette used in the microinjection of one cell zygotes, for the generation of MMTV/ILK mice. The 1.8kb EcoRI fragment of human ILK cDNA was placed downstream of the MMTV-LTR promoter/enhancer, in order to drive expression in the mammary epithelium. The polyadenylation signal of SV40 (SV40 poly A) was included to ensure efficient processing of the RNA transcript. (b) Confirmation of MMTV/ILK transgene expression in 3 independent founder lines of transgenic mice, by ribonuclease protection analysis of total mammary gland RNA, using a riboprobe generated against the transgene-specific SV40 sequence, as shown in (a). A ribonuclease protection riboprobe specific for the phosphoglycerate kinase (PGK) RNA message was used as an internal control for total RNA levels.



Figure 2 Mammary-specific expression of the MMTV/ILK transgene induces mammary hyperplasia and alveolar development in nulliparous female transgenic mice. (a) Whole-mount of mammary gland from 6 month old nulliparous female MMTV/ILK mouse, showing mild ductal and acinar hyperplasia. (b) Section of mammary gland from 6 month old nulliparous female mouse, showing multi-layered and disorganized ductal epithelium, with abnormal ring mitotic figure (inset). (c) Whole-mount of mammary gland from 18 month old nulliparous female MMTV/ILK mouse, showing extensive lobulo-alveolar development. (d) Section of mammary gland taken from 18 month old nulliparous female MMTV/ILK mouse, showing epithelial hyperplasia and secretory vacuolization. (e) Whole-mount of mammary gland from 6 month old nulliparous female FVB/N mouse, showing normal pattern of branching and alveolar development. (f) Section of mammary gland from 12 month old nulliparous female FVB/N mouse, showing normal glandular epithelial content.



Figure 3 Expression of MMTV/ILK transgene induces phosphorylation of downstream signaling proteins in mammary glands of transgenic mice. Protein lysates from 6 month old virgin female FVB/N (lanes 1-3) and MMTV/ILK (lanes 4-9) mice were subjected to SDS-PAGE and blotted with polyclonal antibodies recognizing (a) the phosphorylated form of serine residue 473 of PKB/Akt (upper panel), (b) the phosphorylated form of serine residue 9 of GSK-3 β (upper panel), and (c) the phosphorylated form of MAPK (p44/42 Erk1/2) (upper panel). Levels of total protein were determined by stripping and reprobing the same membranes with (a) anti-PKB/Akt (lower panel), (b) anti-GSK-3 β (lower panel), and (c) anti-MAPK (lower panel) polyclonal antibodies.



Figure 4 Expression of an MMTV/ILK transgene in the mammary epithelium of FVB/N mice induces mammary tumour formation. (a) Photograph showing focal tumour (indicated by arrow) arising from the right abdominal mammary gland of a female MMTV/ILK transgenic mouse, aged 18 months. (b) Kinetics of tumour formation in female MMTV/ILK mice. Tumours appeared in 14/41 female mice from line 363, at an average age of 560 days (18.6 months). (c) Elevated levels of total ILK protein in mammary tumours from MMTV/ILK mice. Mammary tumour lysates from 7 mice (lanes 2,4,6,8,10,12,14) were analysed for total ILK levels by immunoblotting, using an anti-ILK polyclonal antibody recognizing both human and mouse isoforms. ILK levels in adjacent glands from the same mice (lanes 1,3,5,7,9,11,13) are shown for comparison. The membrane was probed with anti- β -actin antibody to control for protein loading (lower panel).

Table 1 Mammary tumor kinetics in MMV/ILK transgenic mice

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MMTV/ILK founder line	tumor incidence	tumor onset (average)	lung metastases	tumor phenotype
ILK 363	14/41 (34%)	560 days (18.6 months)	3/14	adenocarcinoma (12*); spindle cell tumor (2)
ILK 2189	3/9 (33%)	440 days (14.7 months)	1/3	adenocarcinoma; spindle cell tumor (2)
ILK 1934	1/10 (10%)	364 days (12 months)	0/1	adenocarcinoma
FVB/N	0/21			none**

* adenocarcinomas show a degree of differentiation, from high to low, including mixed tumors containing both epithelial- and mesenchymal-like cell populations. The adenocarcinoma from ILK line 1934 was poorly differentiated. **An ovarian tumor appeared in 1 virgin FVB/N mouse at 20 months of age.



Figure 5 MMTV/ILK expression induces a diverse range of mammary tumour phenotypes in transgenic mice. (a-d) Well differentiated papillary adenocarcinoma. (e-h) Mixed tumour consisting of populations of epithelial cells interspersed within regions of cells of more mesenchymal-like phenotype. (i-l) Spindle cell tumour. Cell type-specific populations of tumours shown in panels a, e and i were identified morphologically, and by immunohistochemical analysis using antibodies to cytokeratin 8 (b,f,j), E-cadherin (c,g,k), smooth muscle actin (d,h,l), cytokeratin 14 (not shown) and vimentin (not shown). Tumour sections in panels a, e and i were stained with hematoxylin and eosin for visualization.

Appendix 2

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Figure 1-3



Figure 1. Expression of a kinase-dead (KD) allele of ILK in the mammary epithelium of FVB/N mice. Expression of an MMTV-ILK-KD transgene in the mammary epithelium of line #1414 was confirmed by RNase protection (lane 3). An FVB control and a non-expressing founder (lanes 1,4), as well as a mouse expressing the wild-type ILK transgene(lane 2), are shown as negative and positive controls for transgene expression, respectively. The KD allele of ILK was provided by Dr. Shoukat Dedhar of the University of British Columbia. This allele contains a glutamic acid to lysine substitution at position 359, in the conserved kinase domain of ILK (Novak et al., 1998). The mutant cDNA was cloned into an expression vector, downstream of the MMTV-LTR, and injected into the pronuclei of fertilized egges. Protection of phosphoglycerate kinase (PGK) transcripts are shown as internal controls for RNA integrity.



Figure 2. Kinetics of tumor formation in MMTV/erbB-2 mice (open circles, n=3), versus MMTV/erbB-2 x MMTV/ILK-KD bitransgenic mice (closed circles, n=3).



Figure 3. Kinetics of tumor formation in MMTV/PyV mT Y315/322F mice (open circles, n=5), versus MMTV/PyV mT Y315/322F x MMTV/ILK bitransgenic mice (closed circles, n=6).

Appendix 3

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Meeting abstracts

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Induction of mammary tumours in transgenic mice expressing the integrin-linked kinase (ILK) in the mammary epithelium

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The integrin-linked kinase (ILK) was first identified as a 59 kilodalton molecule associated with the cytoplasmic domains of \$1- and \$3-integrins. Subsequent analysis revealed an important role for ILK in both cell survival and oncogenic pathways, including the activation of Akt through phosphorylation of serine residue 473, and stabilization of β-catenin. Overexpression of ILK in cultured epithelial cells resulted in changes characteristic of oncogenic transformation, including anchorage-independent growth, invasiveness, and tumourigenesis in nude mice. In order to test the impact of ILK overexpression in vivo, we generated transgenic mice expressing ILK in the mammary epithelium, under the transcriptional control of the MMTV promoter/enhancer. By the age of 6 months, over 50% of these MMTV/ILK mice developed mild mammary hyperplasia, which progressed to extensive lobulo-alveolar development after 12 to 18 months, with focal mammary tumours appearing in 34% of mice between the ages of 12 to 24 months. Although the mechanism of ILK-induced tumourigenesis has not been established, analysis of mammary epithelium from young virgin animals revealed elevated levels of Akt-ser 473 phosphorylation, MAPK phosphorylation, and alterations in β-catenin phosphorylation. Considering the long latency of tumour formation, however, additional genetic events are likely required. These mice provide the first direct demonstration of the oncogenic potential of ILK overexpression in vivo, which may have physiological relevance to human cancers given that elevated levels of ILK are often observed in tumours and tumour cell lines.

Tumorigenesis in Transgenic Mice Expressing the Integrin-Linked Kinase (ILK) in the Mammary Epithelium

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The integrin-linked kinase (ILK) is a 59K serine-threonine kinase, identified by virtue of its association with the cytoplasmic domains of β 1- and β 3-integrins. Transformation of cultured epithelial cells by overexpression of ILK suggested that ILK might contribute to tumorigenesis, invasiveness and metastasis *in vivo*. In order to test this hypothesis in a physiological context we generated mice expressing the full-length ILK cDNA in the mammary epithelium, under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat. Focal mammary tumors appeared in 36% of female animals between the ages of 18 and 24 months, and pulmonary metastases were observed in 50% of these mice. In addition, increased phosphorylation of PKB/Akt on serine 473 was confirmed by immunoblot analysis of whole mammary gland, recapitulating the PKB/Akt-specific phosphorylation observed following ILK overexpression in culture. These experiments possibly provide the first direct demonstration of ILK's potential to induce tumorigenesis when overexpressed *in vivo*.