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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The major objective of our DOD sponsored research program is to assess the role of the integrin linked kinase in mammary tumorigenesis and metastasis. Based on previous observation that ILK is overexpressed in human breast cancers, we have derived several independent transgenic strains that express ILK in the mammary epithelium. Genetic and biochemical analysis of these strains revealed that female transgenic strains developed focal mammary tumors after a long latency period. Interestingly mammary tumors derived from these strains have undergone epithelial-mesenchymal transition (EMT). The results of these studies provide direct evidence that overexpression of ILK may be an important step in mammary tumor progression.				
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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 anchorage-independent 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 invasive 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.

BODY

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 have been published in the journal *Oncogene*, in an article 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 manuscript, Appendix 1).

As discussed in the manuscript, a majority of the MMTV-ILK-induced tumors show evidence of epithelial-to-mesenchymal transition (EMT), including the loss of epithelial markers and downregulation of the epithelial-specific MMTV promoter (Appendix 1). These changes are accompanied by upregulation of endogenous ILK (manuscript, Appendix 1, and Figure 1, Appendix 2), which is expressed throughout the tumors (Figure 1, Appendix 2). These changes are consistent with the induction of a mesenchymal phenotype in cultured mammary epithelial cells overexpressing ILK, and suggest that ILK may be involved in promoting the more aggressive phenotype of tumors undergoing EMT.

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 2, 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 3, 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 4, 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.

KEY RESEARCH ACCOMPLISHMENTS

- 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 the PI-3' kinase/Akt pathway in vivo

REPORTABLE OUTCOMES

- Publication—White DE, Cardiff RD, Dedhar S and WJ Muller. (2001). Mammary epithelial-specific expression of the integrin linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. *Oncogene*, 20: 7064-7072 (see manuscript, Appendix 1).
- Oral presentation—Gordon Research Conference on Mammary Gland Biology, Il Ciocco, Italy, April 28-May 3, 2002 (see abstract, page 26, Appendix 3)
- Oral presentation—Canadian Breast Cancer Research Initiative, Reasons for Hope Meeting, Loews LeConcorde Hotel, Quebec City, Quebec, May 3-5, 2001 (see abstract, page 27, Appendix 3)
- Abstract—Oncogene Meeting, Salk Institute, San Diego, California, June 22-25, 2000 (see abstract, page 28, Appendix 3)

CONCLUSIONS

Funding for this project was provided in order to assess the role of ILK in mammary tumorigenesis. The most important and published result after 3 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 suggests that the elevated levels of ILK observed in invasive human tumors and tumor cell lines (Chung et al., 1998; Janji et al., 1999) may be physiologically relevant to the formation and/or growth of human cancers, and is therefore directly relevant to the understanding of the molecular mechanisms behind invasive breast cancer. In addition, we have found that ILK overexpression is associated with the loss of epithelial markers in a majority of the MMTV-ILK-induced tumors (manuscript, appendix 1). This result is consistent with a report demonstrating that ILK can induce an epithelial-to-mesenchymal transition in cultured mammary epithelial cells (Somasiri et al., 2001), and suggests that ILK overexpression may be involved in the behaviour of tumors exhibiting an invasive mesenchymal phenotype.

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

Manuscript



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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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-3 β . 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 accompanied by the constitutive phosphorylation of PKB/Akt, GSK-3 β 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. *Oncogene* (2001) 20, 7064–7072.

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

Introduction

The normal growth and development of the mammary 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 their 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 repeat-containing serine/threonine 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 (Delcomenne *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 inhibition of GSK-3 β , a

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negative regulator of the Wnt signaling pathway (Delcomenne *et al.*, 1998). The inhibition of GSK-3 β by ILK results in the activation of the AP-1 and β -catenin/LEF-1 transcription factors, and the subsequent expression of mesenchymally-related genes (Delcomenne *et al.*, 1998; Novak *et al.*, 1998; Troussard *et al.*, 1999). 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 (Delcomenne *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 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; Attwell *et al.*, 2000), invasiveness in 3-dimensional culture, and tumorigenicity in nude mice (Wu *et al.*, 1998).

Whereas these studies demonstrate that the elevated expression of wild-type ILK can transform mammary epithelial cells *in vitro*, the potential for ILK overexpression to induce mammary tumorigenesis *in vivo* 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. By using this approach, we were able to directly assess the oncogenic potential of ILK overexpression in a physiological context. Analysis of these MMTV/ILK mice revealed that the mammary epithelial-specific overexpression of ILK was initially associated with the 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-mesenchymal 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 *in vivo*. These results may have significance for the understanding and perhaps management of human breast cancer, considering that ILK is upregulated in a variety of human tumors and tumor cell lines (Chung *et al.*, 1998; Janji *et al.*, 1999, 2000; Graff *et al.*, 2001).

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 of the FVB/N strain 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). To determine which lines of the resulting transgenic founders were expressing the MMTV/ILK transgene, RNA isolated from the mammary epithelia of 10-week-old virgin female mice was subjected to ribonuclease protection analysis using an antisense riboprobe directed to the SV40 component of the transgene (Figure 1a). Using this approach, we identified three independent lines of mice expressing the MMTV/ILK transgene in the 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 (Webster *et al.*, 1998). A comparison of ILK protein levels between mammary glands of female non-transgenic FVB/N mice (lane 1) and those of MMTV/ILK line 363 (lane 2) is shown in Figure 1c.

To explore whether mammary epithelial-specific overexpression of ILK 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 approximately 55% of virgin transgene carriers, from all three founder lines, 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 which was disorganized with respect to the columnar arrangement of a normal mammary gland epithelium (Figure 2b). In addition, a proportion of the luminal epithelial cells exhibited abnormal mitotic structures, including ring-like figures (Figure 2b, inset. For a full size image, go to <http://ccm.ucdavis.edu/tgmouse/preprint/Muller/ILK1/mitoses/mitosisBW.htm>). 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

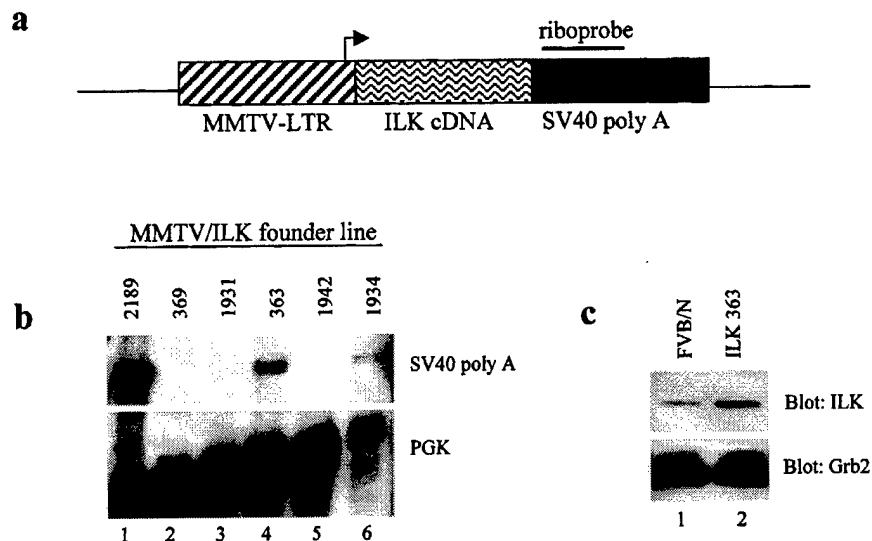


Figure 1 Targeted expression of ILK in the mammary gland of transgenic mice. (a) Expression cassette used for the generation of MMTV/ILK mice. The 1.8 kb *EcoRI* fragment of wild-type human ILK cDNA was placed downstream of the MMTV-LTR promoter/enhancer, in order to drive expression in the mammary epithelium. 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 three 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. (c) ILK protein levels in mammary glands of non-transgenic FVB/N mice (upper panel, lane 1) and MMTV/ILK line 363 (upper panel, lane 2) were compared by immunoblotting total mammary gland lysates with anti-ILK polyclonal antibody. Levels of Grb2 were used as an internal control for protein loading (lower panel)

vacuolization was apparent in the histological sections of these older virgin mice, again resembling a partially lactating phenotype (Figure 2d).

To determine whether the observed mammary epithelial abnormalities reflected the 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 (Delcomenne *et al.*, 1998; Persad *et al.*, 2001a). 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 kinase activity is the serine/threonine kinase GSK-3 β (Delcomenne *et al.*, 1998; Persad *et al.*, 2001b), a negative regulator of

the Wnt signaling pathway, and which is inhibited by phosphorylation on serine residue 9 (Cross *et al.*, 1995). To determine whether GSK-3 β was constitutively phosphorylated in the MMTV/ILK mice, the same tissue lysates were subjected to immunoblot analysis with phospho-specific 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 the mammary glands of the 6-month-old transgenic mice, 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

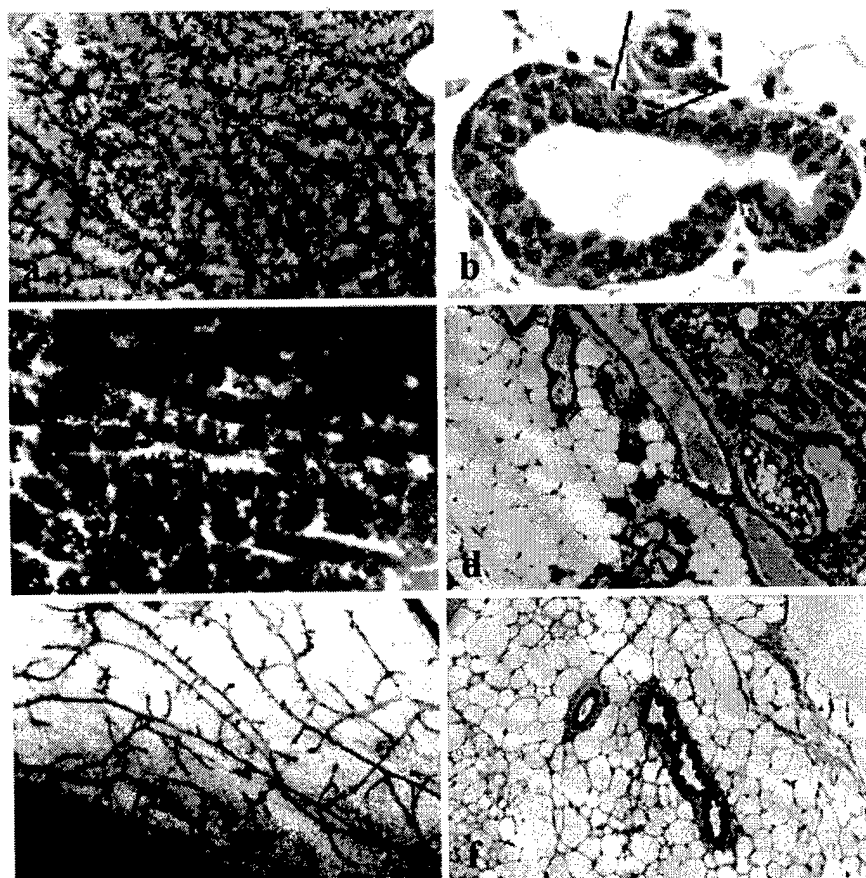


Figure 2 Mammary-specific expression of the MMTV/ILK transgene induces mammary hyperplasia and alveolar development in nulliparous female transgenic mice. (a) Whole-mount of mammary gland from 6-month-old nulliparous female MMTV/ILK mouse, showing mild ductal and acinar hyperplasia. (b) Section of mammary gland from 6-month-old nulliparous female mouse, showing multi-layered and disorganized ductal epithelium, with abnormal ring mitotic figure (Inset. See text for link to higher resolution black and white and color images). (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

panel). Taken together, these observations suggest that the concerted activation of pathways involved in epithelial cell proliferation and survival, which have been shown to be targets of ILK activity in culture, may be contributing to the induction of mammary epithelial hyperplasias in the MMTV/ILK mice.

Elevated expression of ILK predisposes the mammary epithelium 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 three founder lines that expressed the MMTV/ILK transgene. In our best characterized strain (line 363), 34% of female animals

developed focal mammary tumors with an average latency of 560 days (Figure 4a, Table 1). By contrast, no mammary tumors were observed in age-matched female FVB/N control mice (Table 1). As shown in Figure 4b, 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 4b, 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 4b, lower panel).

The tumors from the MMTV/ILK mice revealed a somewhat diverse phenotype, ranging from well differentiated papillary adenocarcinomas (Figure 5a-d), to undifferentiated spindle cell tumors (Figure 5i-

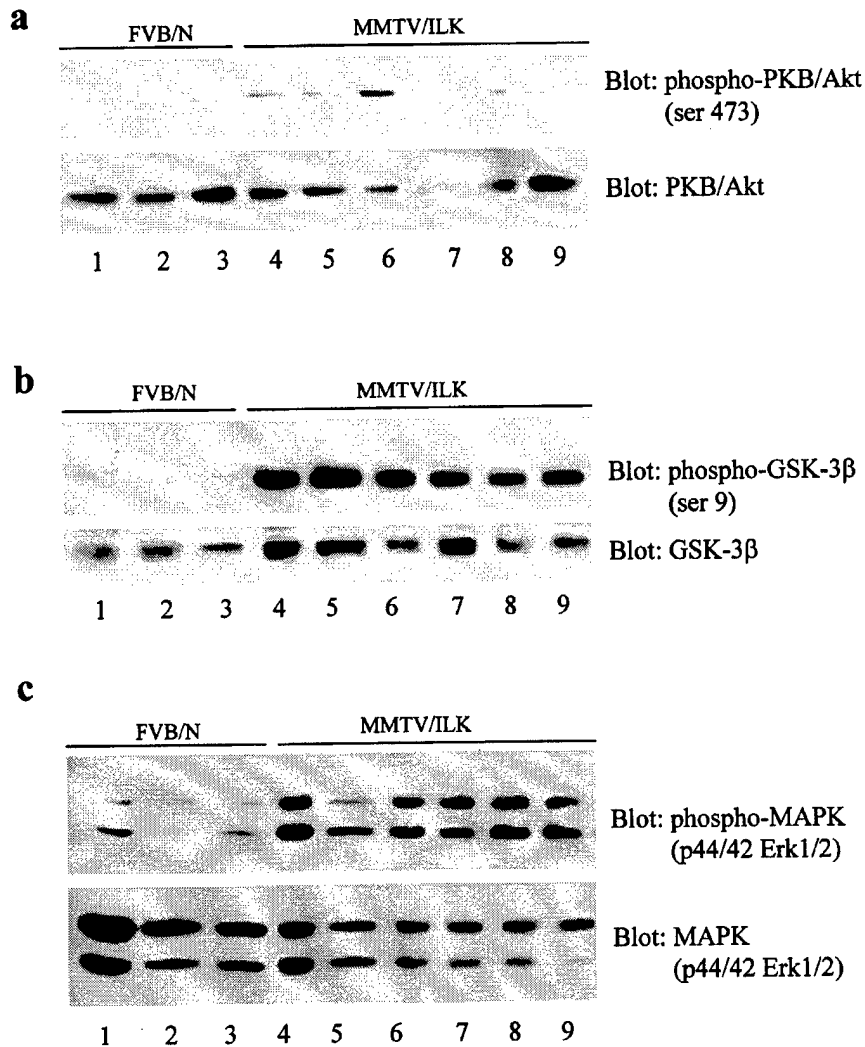


Figure 3 Expression of MMTV/ILK transgene induces phosphorylation of downstream signaling proteins in mammary glands of transgenic mice. Mammary gland lysates from 6-month-old virgin female FVB/N (lanes 1–3) and MMTV/ILK (lanes 4–9) mice were subjected to SDS–PAGE and blotted with polyclonal antibodies recognizing (a) the phosphorylated form of serine residue 473 of PKB/Akt (upper panel), (b) the phosphorylated form of serine residue 9 of GSK-3β (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-3β (lower panel), and (c) anti-MAPK (lower panel) polyclonal antibodies

l). The tumors were invasive, as determined by microscopic examination of tumor sections, which revealed nests and cords of tumor cells infiltrating the dense connective tissue stroma and adjacent skeletal muscle (data not shown). In addition, we observed distal pulmonary metastases in 21% of the tumor-bearing animals (Table 1). Several of the tumors consisted of differentiated epithelial cells interspersed within regions of mesenchymal-like cell populations (Figure 5e–h), as indicated by the expression pattern of the epithelial markers cytokeratin 8 (CK8) and E-cadherin (Figure 5f,g), and the mesenchymal markers vimentin and CK14 (data not shown). Interestingly, the

expression of smooth muscle actin (SMA) in these tumors (Figure 5h) suggests the presence of myoepithelium, which is often retained in tumors undergoing epithelial-to-mesenchymal transition. Similarly, SMA was detected in the spindle cell tumors (Figure 5l), whereas CK8 and E-cadherin were not (Figure 5j,k), again suggesting that the establishment of these tumors involved an epithelial-to-mesenchymal conversion. 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.

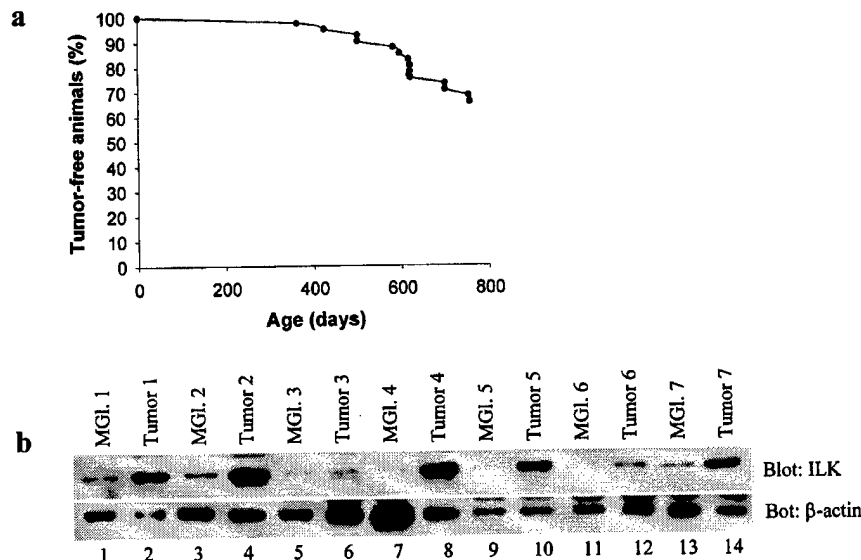


Figure 4 Expression of an MMTV/ILK transgene in the mammary epithelium of FVB/N mice induces mammary tumor formation. (a) Kinetics of tumor formation in female MMTV/ILK mice. Tumors appeared in 14/41 female mice from line 363, at an average age of 560 days (18.6 months). (b) Elevated levels of total ILK protein in mammary tumors from MMTV/ILK mice. Mammary tumor lysates from seven mice (lanes 2,4,6,8,10,12 and 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 and 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 MMTV/ILK transgenic mice

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 one virgin FVB/N mouse at 20 months of age

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 wild-type ILK can result in the induction of mammary tumors *in vivo*.

Expression of the MMTV/ILK transgene in the mammary epithelia of the transgenic mice initially

resulted in mammary gland hyperplasias in a proportion of the animals examined. This hyperplastic phenotype appeared by the age of 6 months, at which time we detected elevated levels of PKB/Akt, GSK-3 β and MAPK phosphorylation. Given their role in cell proliferation and cell survival pathways, the phosphorylation of these proteins, and the concomitant activation of the associated signaling pathways, may have contributed directly to the increase in mammary epithelial content in these MMTV/ILK transgenic mice. Tumor induction, however, likely involved additional events, given the relatively lower penetrance, as well as the long latency and focal nature of the tumors. Consistent with this hypothesis, Hutchinson *et al.* (2001) have shown recently that the mammary epithelial expression of activated PKB/Akt is indeed insufficient to induce mammary tumors in transgenic mice.

The phenotypes of the MMTV/ILK-induced tumors were variable, ranging from papillary adenocarcinomas

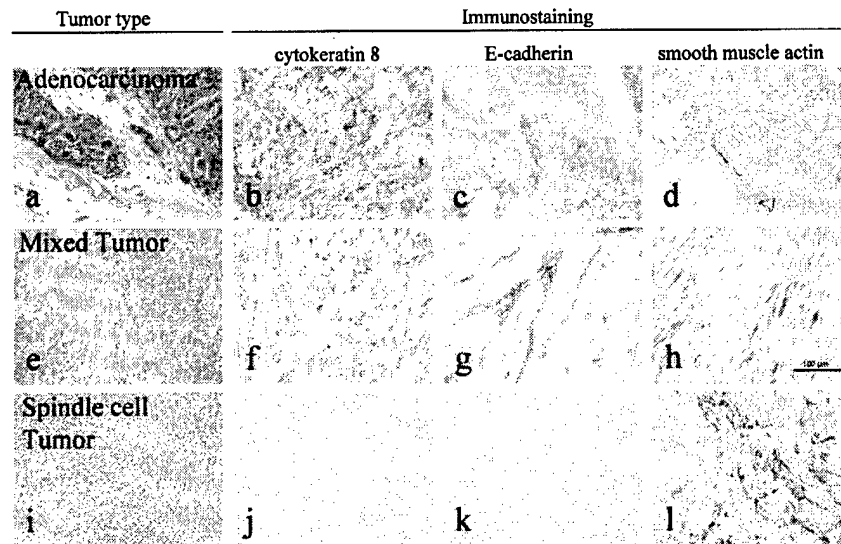


Figure 5 MMTV/ILK expression induces a diverse range of mammary tumor phenotypes in transgenic mice. (a–d) Well differentiated papillary adenocarcinoma, characterized by large cells with hyperchromatic, pleomorphic nuclei, and containing regions of squamous metaplasia. The cells of this tumor express the epithelial markers cytokeratin 8 (CK8) and E-cadherin (b,c), revealing the well differentiated phenotype. (e–h) Mixed tumor expressing both epithelial markers CK8 and E-cadherin (f,g) and the mesenchymal markers vimentin and CK14 (data not shown). Note that this tumor has retained the myoepithelial marker smooth muscle actin (SMA) (h), which is characteristic of tumors undergoing an epithelial-to-mesenchymal transition. (i–l) Spindle cell tumor, again retaining the myoepithelial marker SMA (l), but negative for the epithelial markers CK8 and E-cadherin (j,k). Tumor sections in panels a, e and i were stained with hematoxylin and eosin for visualization.

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 epithelial-to-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 intriguing 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 and Figure 5). Indeed, a tumor cell line generated from one 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 glands from virgin female mice representing both transgenic strains exhibited increased ductal branching and lobulo-alveolar development, with regions of secretory vacuolization apparent in histological sections (Sternlicht *et al.*, 2000 and Figure 2). 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.* (2000) describes the upregulation of another matrix metalloproteinase, MMP-9, following the overexpression of ILK in cultured mammary epithelial cells.

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 expressed at highest levels 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.* (2000) and Deng *et al.* (2001) recently revealed a role for ILK in the regulation of myogenic differentiation, and in the phosphorylation of myosin light chain during smooth muscle contraction in chickens. 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. It is clear that further studies are required regarding both the biological and oncogenic roles of ILK, 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; Graff *et al.*, 2001).

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, harboring 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 0.5 cm clippings of mouse tails (Muller *et al.*, 1988), and PCR amplified using an ILK-specific forward primer (CATGTATGCACCTGCCTG) and an SV40-specific reverse primer (TATGTACACACCACAGAAG), to generate a transgene-specific amplification product. PCR conditions included a 30 s annealing step at 52°C, and a 1 min 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 homogenization 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 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 2 mM EDTA, 10 mM NaF, 10 mM Na pyrophosphate, 1 μ g/ μ l leupeptin, 1 μ g/ μ l aprotinin and 1 mM 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 1 \times TBS, 0.05% Tween-20, incubated in primary antibody overnight at 4°C, washed in TBS/0.05% Tween-20, and incubated with HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1 h 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 min, 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 5 μ 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, USA.

Acknowledgments

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Appendix 2

Figures 1-4

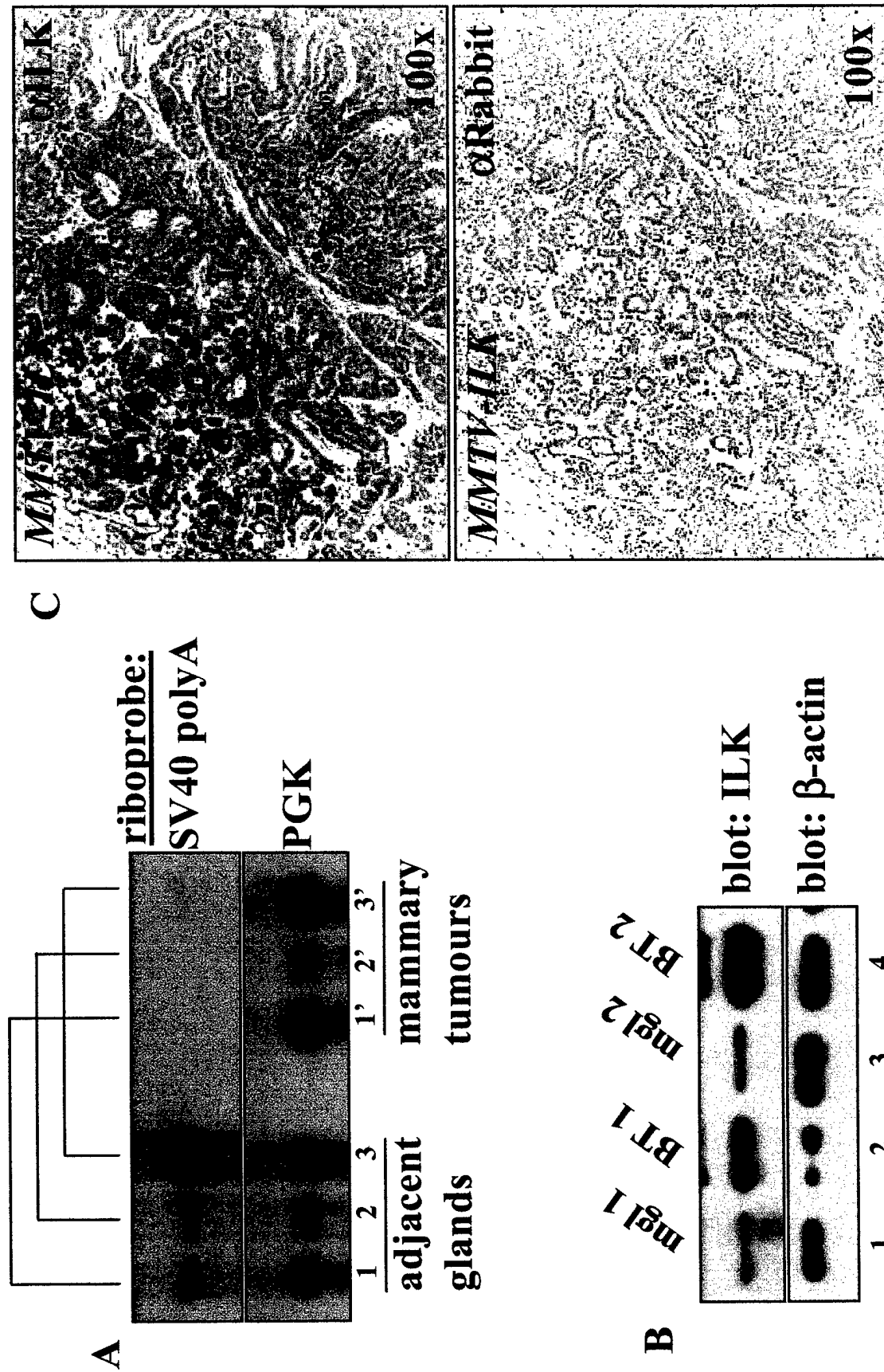


Figure 1 Endogenous ILK is overexpressed in MMTV-ILK-induced tumors, which show evidence of EMT. (A) RNase protection analysis showing downregulation of the epithelial-specific MMTV promoter during the transition from mammary gland hyperplasia (lanes 1, 2 and 3) to invasive mammary tumour (lanes 1', 2' and 3'). (B) Western blot showing upregulation of endogenous ILK in mammary tumors (lanes 2 and 4) versus adjacent gland (lanes 1 and 3) from MMTV-ILK mice. (C) Immunohistochemistry showing expression of ILK throughout MMTV-ILK-induced tumor.

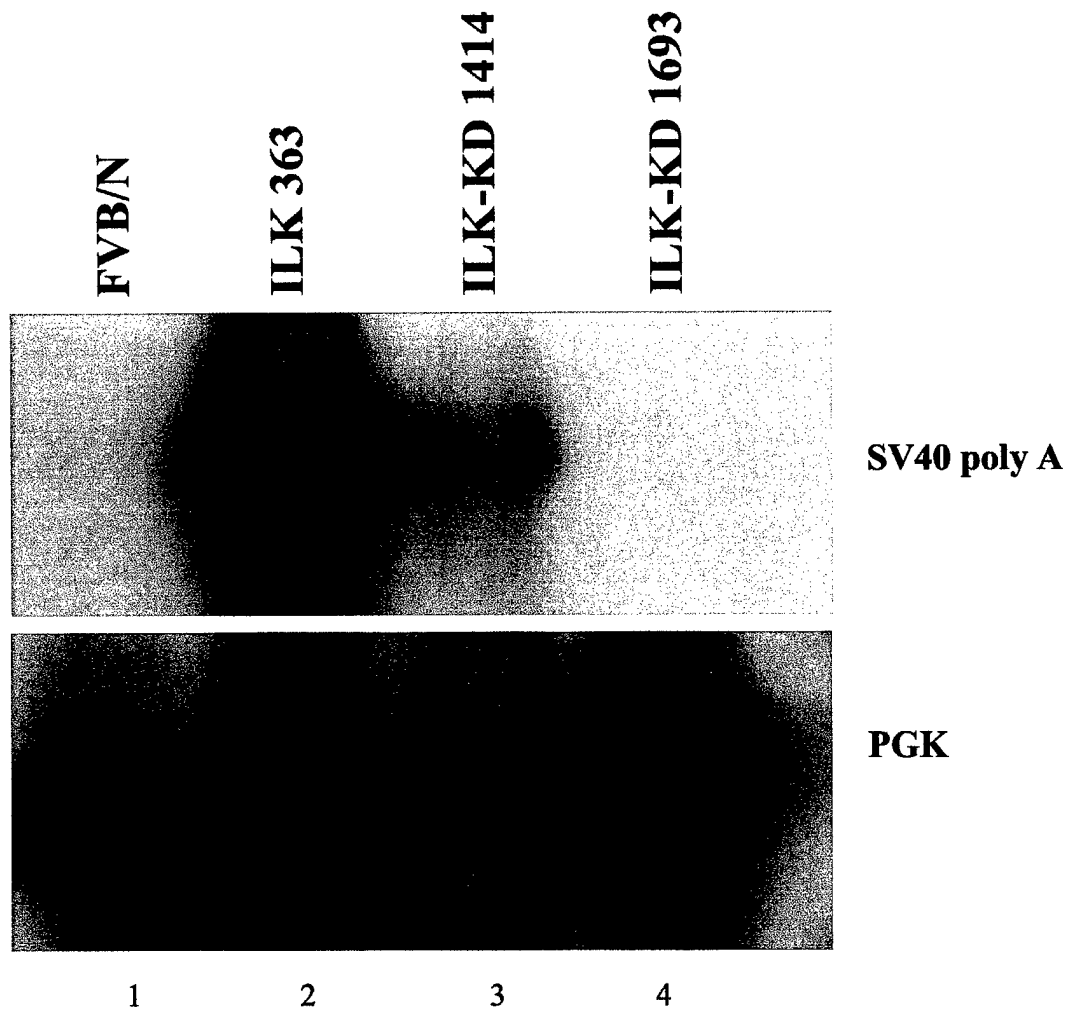


Figure 2. Expression of a kinase-dead (KD) allele of ILK in the mammary epithelium of FVB/N mice. Expression of an MMTV-ILK-KD transgene in the mammary epithelium of line #1414 was confirmed by RNase protection (lane 3). An FVB control and a non-expressing founder (lanes 1,4), as well as a mouse expressing 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 eggs. Protection of phosphoglycerate kinase (PGK) transcripts are shown as internal controls for RNA integrity.

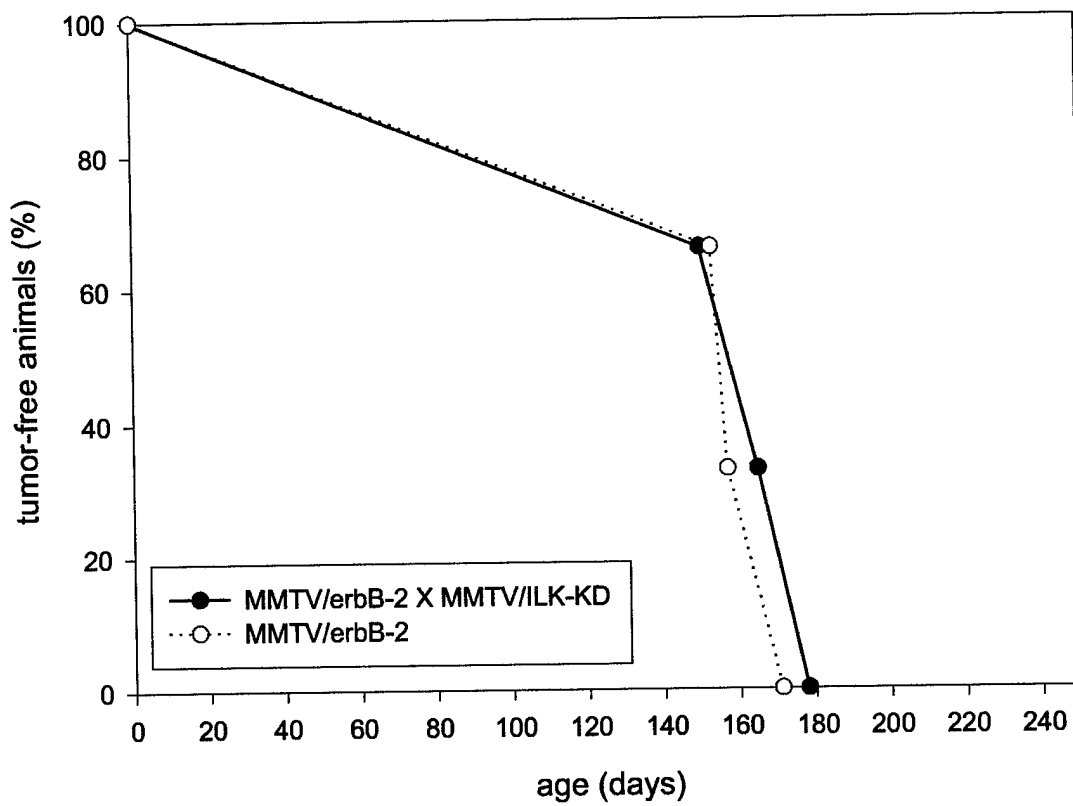


Figure 3. Kinetics of tumor formation in MMTV/erbB-2 mice (open circles, n=3), versus MMTV/erbB-2 x MMTV/ILK-KD bitransgenic mice (closed circles, n=3).

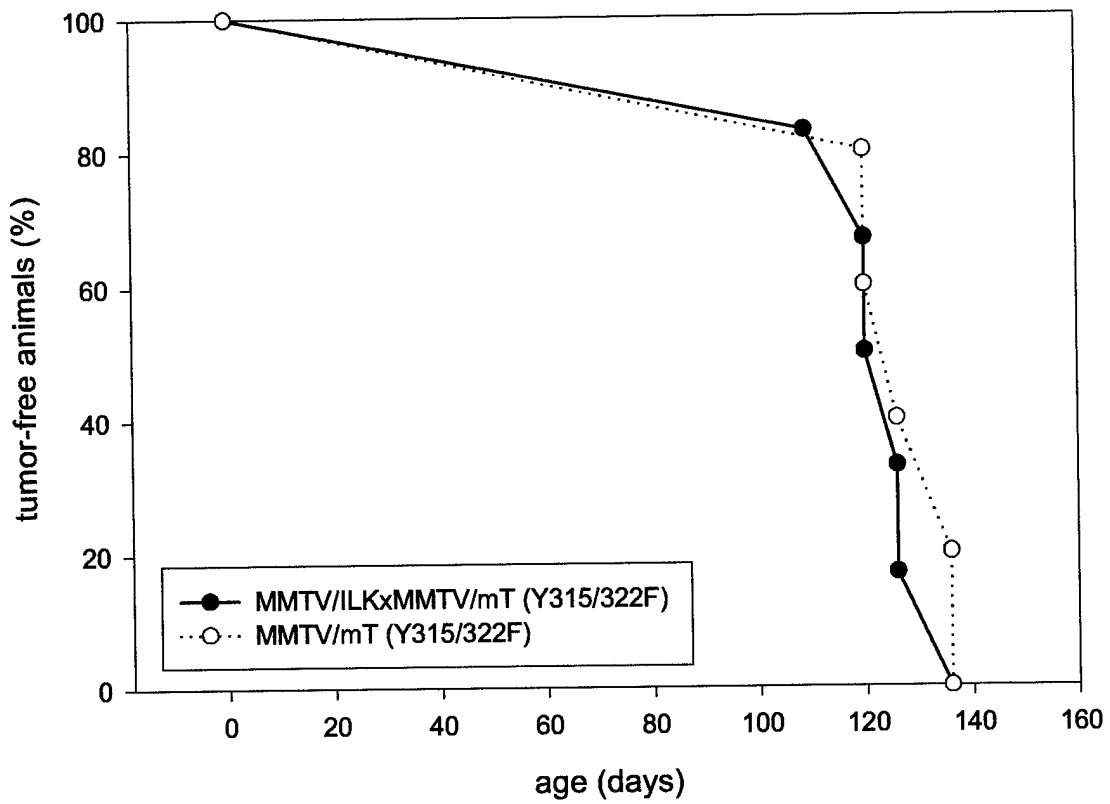


Figure 4. Kinetics of tumor formation in MMTV/PyV mT Y315/322F mice (open circles, n=5), versus MMTV/PyV mT Y315/322F x MMTV/ILK bitransgenic mice (closed circles, n=6).

Appendix 3

Meeting Abstracts

Integrin-Linked Kinase in Mammary Gland Development and Tumorigenesis

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The architecture and function of the mammary gland depends critically upon interactions between the mammary epithelial cells and the extracellular matrix (ECM). Genetic events which disrupt the regulation of these interactions can result in developmental irregularities and have been associated with tumorigenesis. These epithelial cell-ECM interactions are mediated by the integrin family of transmembrane proteins, such as the β 1-integrin, and their cytoplasmic effectors, such as the integrin-linked kinase (ILK). β 1-integrin is required for normal mouse mammary gland development, and both β 1-integrin and ILK have been implicated as playing important roles in the transformation and metastatic behaviour of mammary epithelial cells. Our goal is to further elucidate the roles of β 1-integrin and ILK in mammary tumorigenesis and development *in vivo*, using transgenic mouse models and the cre/lox recombination system. The results of 2 experiments regarding ILK are included here. In one of these experiments, we wanted to determine if ILK played a role in the development of the murine mammary gland, given its role in modulating integrin-mediated cell adhesion and survival. Mice were generated containing loxP recombination sites flanking ILK coding sequences, and these "floxed" ILK mice were then crossed with an MMTV/cre-expressing line. Excision of the ILK coding sequences by the cre recombinase did not prevent mammary gland formation or lactation, but resulted in a reduction in the rate of ductal migration during mammary tree outgrowth during puberty. The results of this experiment provide the first demonstration of a physiological function for ILK in mammary gland development. Another major objective of our research was to determine if the overexpression of ILK could transform the mammary epithelium *in vivo*, since ILK has been previously shown to induce changes characteristic of oncogenic transformation in cultured mammary epithelial cells, and has been found to be elevated in various human cancers exhibiting an invasive phenotype. To test the effects of ILK overexpression *in vivo*, we generated transgenic mice expressing an MMTV/ILKcDNA transgene. Female mice from this line indeed developed a hyperplastic mammary gland phenotype by the age of 6 months, and focal mammary tumors, showing evidence of EMT, appeared in 34% of the animals after 1 year. These results demonstrate that ILK can transform the mammary epithelium *in vivo*, and suggest that the elevated levels of ILK in human tumors is physiologically relevant to tumor formation and progression. In a related set of experiments, we are currently using floxed ILK and floxed β 1-integrin alleles to determine whether these molecules are necessary for oncogene-driven tumorigenesis *in vivo*. In this regard, we will present data concerning a requirement for β 1-integrin during oncogenic transformation in the MMTV/PyV mT mouse model of mammary tumorigenesis.

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 $\beta 1$ - and $\beta 3$ -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 tumorigenesis 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 tumorigenesis 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.