



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

Donald E White¹, Robert D Cardiff², Shoukat Dedhar^{3,4} and William J Muller^{*.5}

¹MOBIX and Department of Medical Sciences, McMaster University, Hamilton, Ontario, Canada L8S 4K1; ²Center for Comparative Medicine, University of California at Davis, Davis, California, CA 95616, USA; ³British Columbia Cancer Agency, Jack Bell Research Centre, Vancouver, British Columbia, Canada V6H 3Z6; ⁴Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada V6H 3Z6; ⁵MOBIX and Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada L8S 4K1

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 (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 inhibition of GSK-3 β , a

*Correspondence: WJ Muller, MOBIX, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1; E-mail: mullerw@mcmaster.ca
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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; Atwell *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

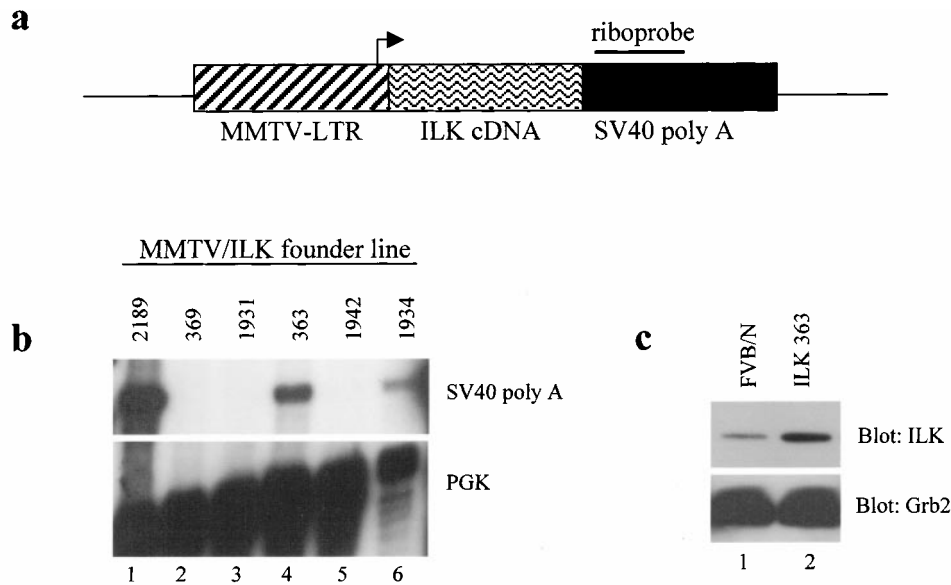


Figure 1 Targeted expression of ILK in the mammary gland of transgenic mice. **(a)** Expression cassette used for the generation of MMTV/ILK mice. The 1.8 kb *EcoRI* fragment of wild-type human ILK cDNA was placed downstream of the MMTV-LTR promoter/enhancer, in order to drive expression in the mammary 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 (Delcommenne *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 (Delcommenne *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

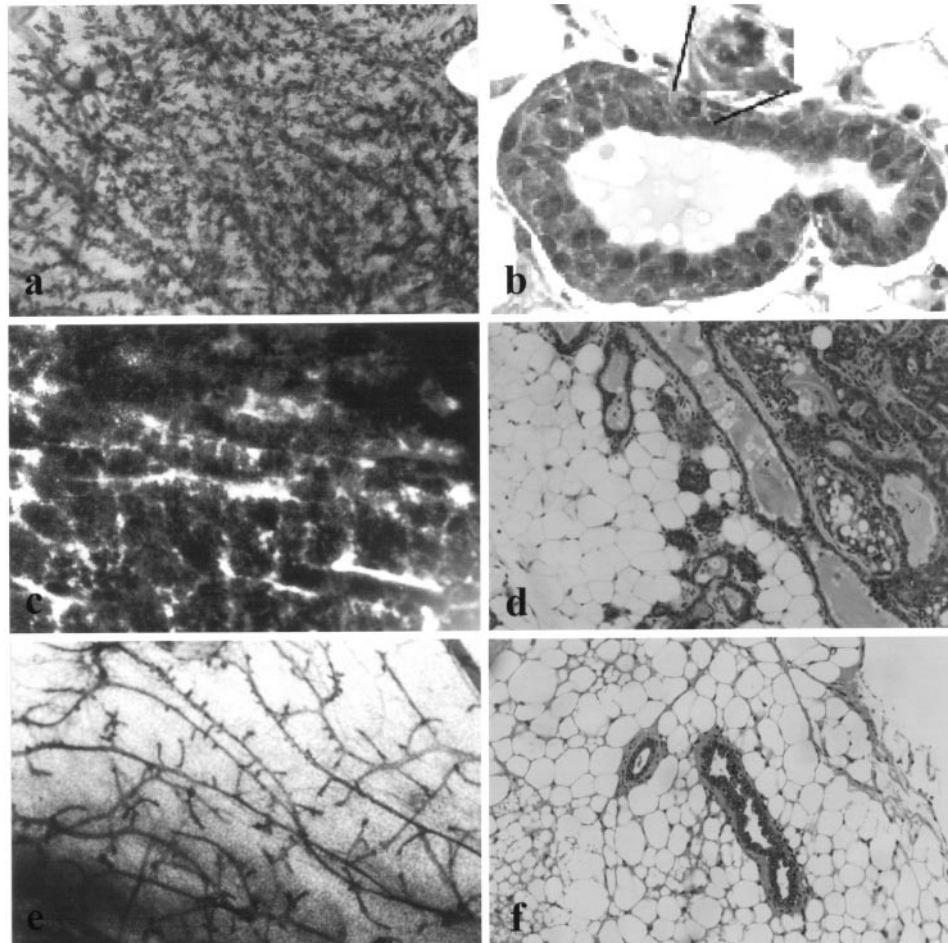


Figure 2 Mammary-specific expression of the MMTV/ILK transgene induces mammary hyperplasia and alveolar development in nulliparous female transgenic mice. (a) Whole-mount of mammary gland from 6-month-old nulliparous female MMTV/ILK mouse, showing mild ductal and acinar hyperplasia. (b) Section of mammary 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–

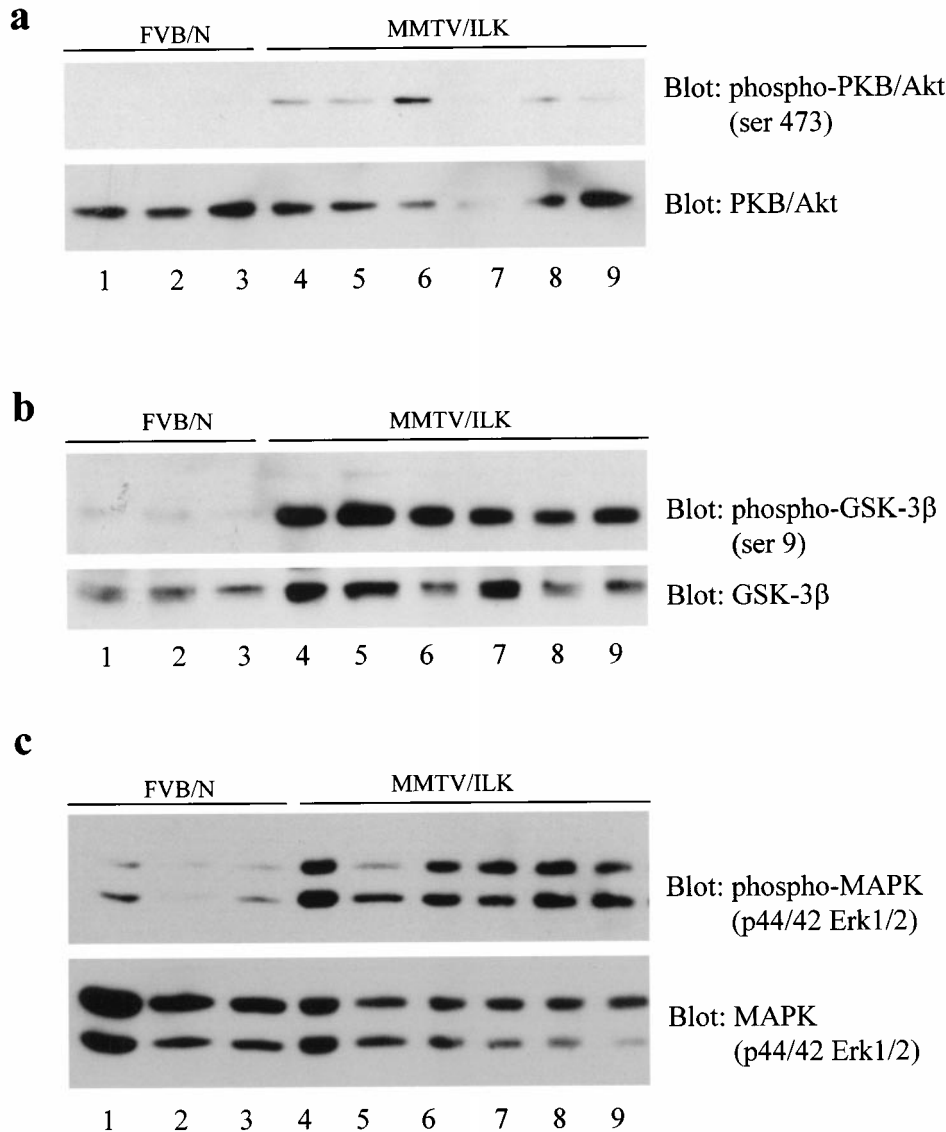


Figure 3 Expression of MMTV/ILK transgene induces phosphorylation of downstream signaling proteins in mammary glands of transgenic mice. Mammary gland lysates from 6-month-old virgin female FVB/N (lanes 1–3) and MMTV/ILK (lanes 4–9) mice were subjected to SDS–PAGE and blotted with polyclonal antibodies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

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.

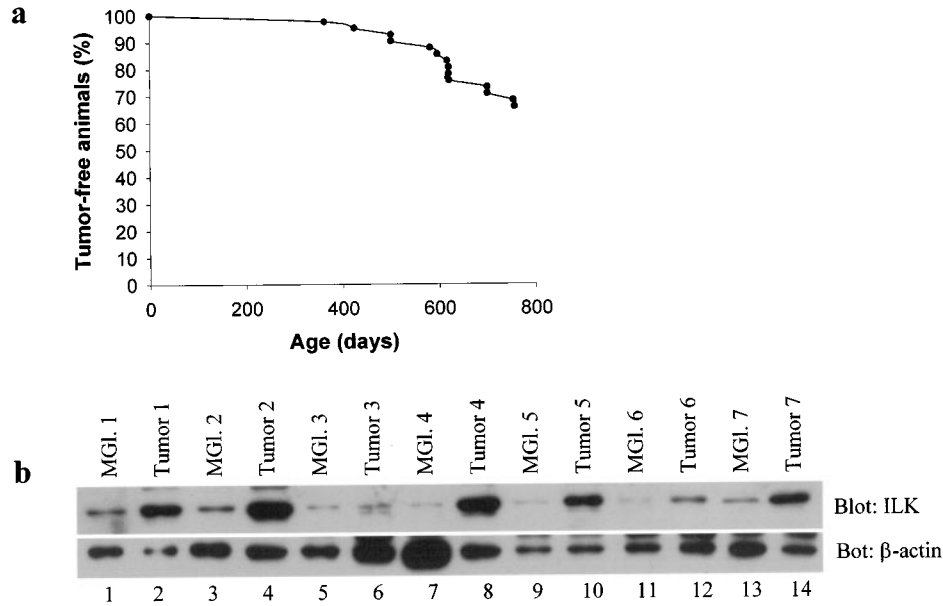


Figure 4 Expression of an MMTV/ILK transgene in the mammary epithelium of FVB/N mice induces mammary tumor formation. **(a)** Kinetics of tumor formation in female MMTV/ILK mice. Tumors appeared in 14/41 female mice from line 363, at an average age of 560 days (18.6 months). **(b)** Elevated levels of 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

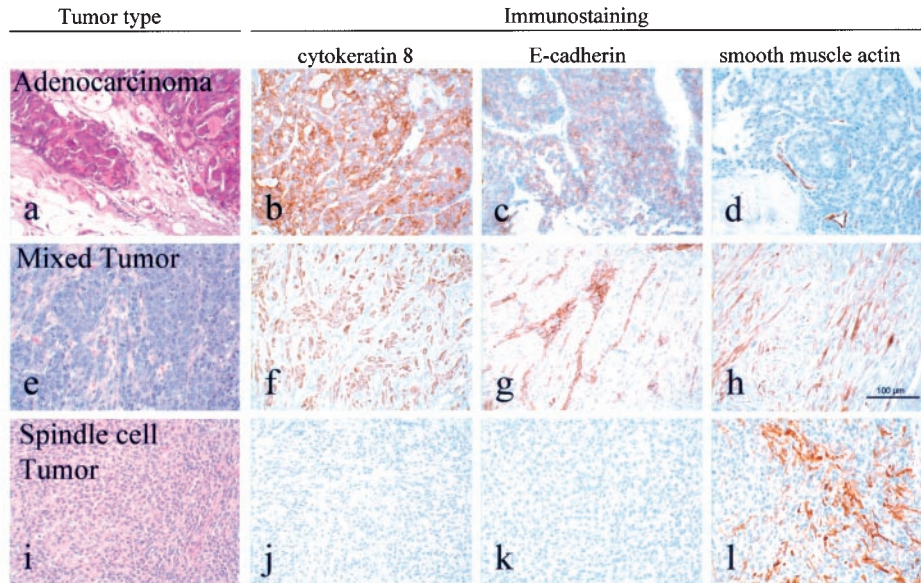


Figure 5 MMTV/ILK expression induces a diverse range of mammary tumor phenotypes in transgenic mice. (a–d) Well differentiated papillary adenocarcinoma, characterized by large cells with hyperchromatic, pleomorphic nuclei, and containing regions of squamous metaplasia. The cells of this tumor express 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 *Eco*RI 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 (TATGTCACACCACAGAAG), 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/ul leupeptin, 1 μ g/ul 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.

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