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Tumorigenesis and Metastasis

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FOREWORD

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Introduction

The primary objective of this proposal is to elucidate the role of the integrin-linked kinase (ILK) in the induction of metastatic mammary tumors. Recent observations have suggested that overexpression of ILK in established mammary epithelial cells can induce anchorage independent growth (Novak *et al.*, 1998). Consistent with these observations, we have observed that mammary tumors derived from activated erbB-2 strains express elevated levels of ILK. To directly assess the oncogenic potential of ILK in the mammary gland, we have derived transgenic mice that express ILK under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. Analyses of several of these MMTV/ILK strains has revealed that mammary specific expression of ILK initially results in the development of mammary epithelial hyperplasias that eventually progress toward full malignancy. These observations suggest that mammary specific expression of ILK can predispose the primary epithelial cell to undergo malignant transformation. Another important objective of the proposal was to assess the effect of ablating ILK function on tumor progression. To accomplish this objective we have derived transgenic mice that express a kinase defective ILK under the transcriptional control of the MMTV promoter. We are currently interbreeding these MMTV/dominant negative ILK strains to our MMTV/erbB-2 and MMTV/polyomavirus (PyV) middle T (mT) strains to assess the importance of ILK in mammary tumorigenesis and metastasis.

Body

Phenotypic and biochemical characterization of the MMTV/ILK and MMTV/kinase dead ILK strains.

During the course of the last year we have completed derivation and analyses of transgenic mice expressing ILK under the transcriptional control of the MMTV promoter. Of the six founder animals that we initially derived, analyses of RNA from the mammary glands of female progeny from these founders with a RNase protection probe directed to SV40 component of the transgene revealed that three of these strains expressed appreciable levels of ILK (Figure 1B, Appendix 1). Because line 363 expressed elevated levels of ILK transcript, we chose to assess the tissue specific expression of ILK in the various organs from both male and females from this strain. To this end 20 ug of total RNA from brain, epididymus, heart, Kidney, liver, lung, mammary gland, ovary, salivary gland, seminal vesicles, spleen and testis. The results revealed that transgene transcripts could be detected in the male reproductive tract (seminal vesicles, epididymus), salivary and mammary glands (Fig. 1C). The observed pattern of transgene expression was not due to differences in integrity or levels of RNA since many tissues expressed comparable levels of PGK transcript (Fig. 1C) The tissue specific expression of the 363 ILK strain conforms to what has previously been observed in other MMTV/ oncogene strains (Dankort and Muller, 1996; Muller *et al.*, 1990; Muller *et al.*, 1988). Together these observations indicate that we derived several independent transgenic strains that express elevated levels of the ILK transgene in mammary epithelium.

To assess whether mammary epithelial expression of ILK was associated with mammary specific abnormalities, we monitored a cohort of 41 female animals From the ILK 363 strain for the appearance of palpable mammary tumors. Although mammary epithelial expression of the ILK transgene was not initially associated with tumor induction, wholemount analyses of virgin

mammary glands revealed extensive mammary epithelial hyperplasias. However focal mammary tumors began to appear in 39% of the older female transgenic mice. The age of onset varied between a 365 to 617 days of age (Fig. 2). Histological analyses of these tumors revealed that they resembled invasive mammary adenocarcinomas that occasionally metastasized to the lung tissue (Fig. 3). Consistent with these observations, we have just begun to observe the appearance of mammary tumors in the ILK 2189 strain suggesting that tumor induction is not dependent on integration site of the transgene. Together these observations argue that elevated expression of ILK can predispose the primary mammary epithelial cell to malignant transformation. However because the mammary tumors were focal in origin and arose after a long latency period, mammary epithelial expression of ILK is not sufficient to induce mammary tumors. Rather these observations suggest that tumor progression in these MMTV/ILK strains require additional genetic events.

To further characterize the molecular basis for ability of ILK to induce transformation, we measured the levels of ILK protein in both normal adjacent mammary and tumor epithelia. To accomplish this, protein extracts from both normal and tumor epithelia from the MMTV/ILK strains to immunoblot analyses with ILK specific antisera. The results of these analyses revealed that tumor epithelia from the MMTV/ILK strains expressed elevated levels of ILK by comparison to the adjacent normal epithelium (Fig. 4, top panel). Because elevated levels of ILK have been associated with increased levels of cyclin D1 (Radeva *et al.*, 1997), we performed immunoblot analyses with cyclin D1 specific antisera. These analyses revealed that the tumor samples expressed elevated levels of cyclin D1 (Fig. 4, bottom panel). Given the elevated expression of both of ILK and cyclin D1 in these tumors, it is conceivable that co-expression of ILK and cyclin D1 is required for efficient tumor induction in these MMTV/ILK strains.

Another important signaling molecule that is thought to play a role in ILK tumorigenesis is the Akt/PKB serine kinase. Previous biochemical analyses have revealed that Akt is phosphorylated on serine 473 by ILK in established epithelial cells (Delcommenne *et al.*, 1998). To explore whether Akt is constitutively phosphorylated in the mammary epithelium of the MMTV/ILK strains, mammary tissue extracts from either ILK 363 or parental FVB strain were subjected to immunoblot analyses with phosphospecific antibodies directed to serine 473 of Akt. The results revealed that mammary epithelia derived from the ILK strains possessed constitutively phosphorylated Akt whereas Akt was not phosphorylated in the parental FVB strain (Fig. 5). The differences in phosphorylation status of Akt in these tissues was not due to differences in the levels of Akt since both sets of tissue samples possessed comparable levels of Akt protein. These observations confirm that one the important downstream *in vivo* targets for ILK is the Akt serine kinase.

Another important goal of the last year was to generate transgenic mice that express a dominant negative form of ILK in the mammary epithelium. In collaboration with Dr. S. Dedhar, we placed a kinase dead version of ILK bearing mutation in the conserved kinase domain (Novak *et al.*, 1998) in MMTV expression cassette (MMTV-KD-ILK) (Figure 1A) and microinjected the construct into one cell mouse embryos. The results of the first series of microinjection experiments resulted in the generation of independent founder strains. Although the phenotypic analyses of these strains has not been completed, preliminary RNase protection analyses of the mammary epithelium from female of the ILK-KD 1414 strain revealed the presence of transgene specific transcripts albeit at lower levels than the ILK 363 strain (Fig. 6). We are currently performing detailed wholemount analyses to assess whether mammary epithelial expression of dominant negative ILK can affect normal mammary gland development

in these strains. In addition we plan to derive further MMYV /KD-ILK strains to confirm that any observed mammary gland defect is not restricted to a single expressing line.

Co-expression of ILK and a mutant PyV mT de-coupled from the PI-3' kinase signaling molecule does not accelerate PyV mT induced tumor progression.

Another important experimental goal during the last funding period was to determine whether elevated expression of ILK could complement tumor progression in transgenic mice expressing a mutant PyV mT oncogene that is decoupled from the PI-3' kinase signaling molecule (Webster *et al.*, 1998). Because ILK is thought to be a direct downstream target of the PI-3' kinase, we hypothesized that elevated expression of ILK could conceivably complement the tumor defect observe in transgenic mice expressing a mutant PyV mT oncogene de-coupled from the PI-3'kinase signaling pathway. To this end, the MMTV/ILK mice were interbred with the mutant PyV mT strains to derive cohorts of females that co-express both transgenes. The results of these analyses revealed that elevated expression of ILK was not sufficient to complement the defect observed in the PyV mT strains (Fig. 7). One possible explanation for the observed lack of complementation is that the concerted activation of other PI-3' kinase targets is required for efficient tumor induction. Indeed it has previously been demonstrated that PI-3' kinase products can activate other enzymes such as PDK1 and the Rac/Rho small GTPases (Currie *et al.*, 1999; Rodriguez-Viciana *et al.*, 1997). Alternatively because activation of ILK is dependent on the products of the PI-3' kinase, the basal levels of PI-3' kinase activity generated by the mutant PyV mT oncogene may not be sufficient to activate the ILK transgene. Future experiments with more suitable mammary tumor models may allow us to address the role of ILK in mammary tumor progression.

In this regard we have performed immunoblot analyses on mammary tumors derived from the MMTV/activated erbB-2 strains (Siegel *et al.*, 1999) with antibodies directed against ILK. These results of these analyses revealed that tumors derived from activated erbB-2 strains expressed elevated levels of ILK (Fig. 8). Given the potential importance of ILK in erbB-2-induced mammary tumor progression (Xie *et al.*, 1998), we are currently interbreeding the MMTV/ILK with the MMTV/activated erbB-2 strains to assess whether elevated expression of ILK can accelerate mammary tumor progression in these strains. Conversely we are planning to interbreed the MMTV/activated erbB-2 strain to transgenic mice expressing the dominant negative ILK construct (MMTV/KD-ILK) in the mammary gland to assess whether a functional ILK is required for efficient tumor progression. The results of these crosses should provide important insight into the role of ILK in erbB-2 induced tumorigenesis.

Key Research Accomplishments

- **Abstract-Oncogene Meeting**
- **Generation and characterization of MMTV/ILK strains**
- **Generation and characterization of MMTV/dominant negative ILK strains**
- **Interbreeding of MMTV/ILK with mutant PyV mT oncogene mice de-coupled from the PI-3' kinase signaling molecule**

Reportable Outcomes - Abstract-Oncogene Meeting (See appended abstract).

Conclusions

The first year of funding has been extremely productive. Firstly we have demonstrated that mammary epithelial expression of ILK is capable of inducing metastatic mammary tumors. However because these tumors arose in a stochastic fashion and were focal in origin, mammary specific expression of ILK is not sufficient for mammary tumor progression. We have further demonstrated that tumor progression in these MMTV/ILK strains is associated with elevated expression of ILK protein. Given that activation of ILK is associated with stimulation of Akt serine kinase and induction of elevated cyclin D1 (Delcommenne *et al.*, 1998; Radeva *et al.*, 1997), we also examined whether the levels and activity of these key signaling molecules were affected in these strains. Consistent with the previous *in vitro* analyses, we have demonstrated that ILK mammary tissues exhibit constitutive phosphorylation of Akt on serine residue 473. Although these analyses do not discriminate whether Akt is a direct or indirect target of ILK, they suggest that ILK is involved in activating Akt. Because Akt has been implicated as important cell survival signal (Alessi *et al.*, 1996; Brunet *et al.*, 1999; Delcommenne *et al.*, 1998; Franke *et al.*, 1997; Webster *et al.*, 1998), it is conceivable that activation of Akt by ILK may be involved in the observed mammary tumor phenotype in the MMTV/ILK strains.

Another possible means by which ILK influences tumor progression is through regulation of cell cycle progression (Radeva *et al.*, 1997). Indeed consistent with conclusion, we have observed that tumors induced by ILK express elevated levels of cyclin D1. In this regard, it of interest to note that another target of ILK is β -catenin transcription factor signaling pathway (Delcommenne *et al.*, 1998) which has been implicated as a key transcriptional regulator of cyclin D1 promoter (Lin *et al.*, 2000; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999). Thus ILK may also contribute to tumorigenesis by activating the expression of cyclin D1. Future studies designed to assess the relative contribution of these signaling pathways may provide important insight into the molecular basis for ILK induced tumorigenesis.

In addition to assessing the effects of overexpression of ILK on tumor progression, we have also been interested in determining the effects of ablating ILK activity on tumor induction. Indeed we have demonstrated that erbB-2 induced mammary tumors express elevated levels of ILK. To accomplish this we have generated transgenic mice that express a kinase inactive (dominant negative inhibitor) ILK molecule in the mammary epithelium. Although we are in the preliminary stages of analyses of strains, we ultimately plan to interbreed the MMTV/KD-ILK to MMTV/activated erbB-2 to assess whether ILK function is required for erbB-2 induced mammary tumors. The results of these studies should have important implications in both the diagnosis and treatment of human breast cancer.

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Appendices

Abstract – Oncogene Meeting

Figures

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

Donald E. White¹, Shoukat Dedhar³, Robert D. Cardiff⁴ and William J. Muller^{1,2}

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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*.

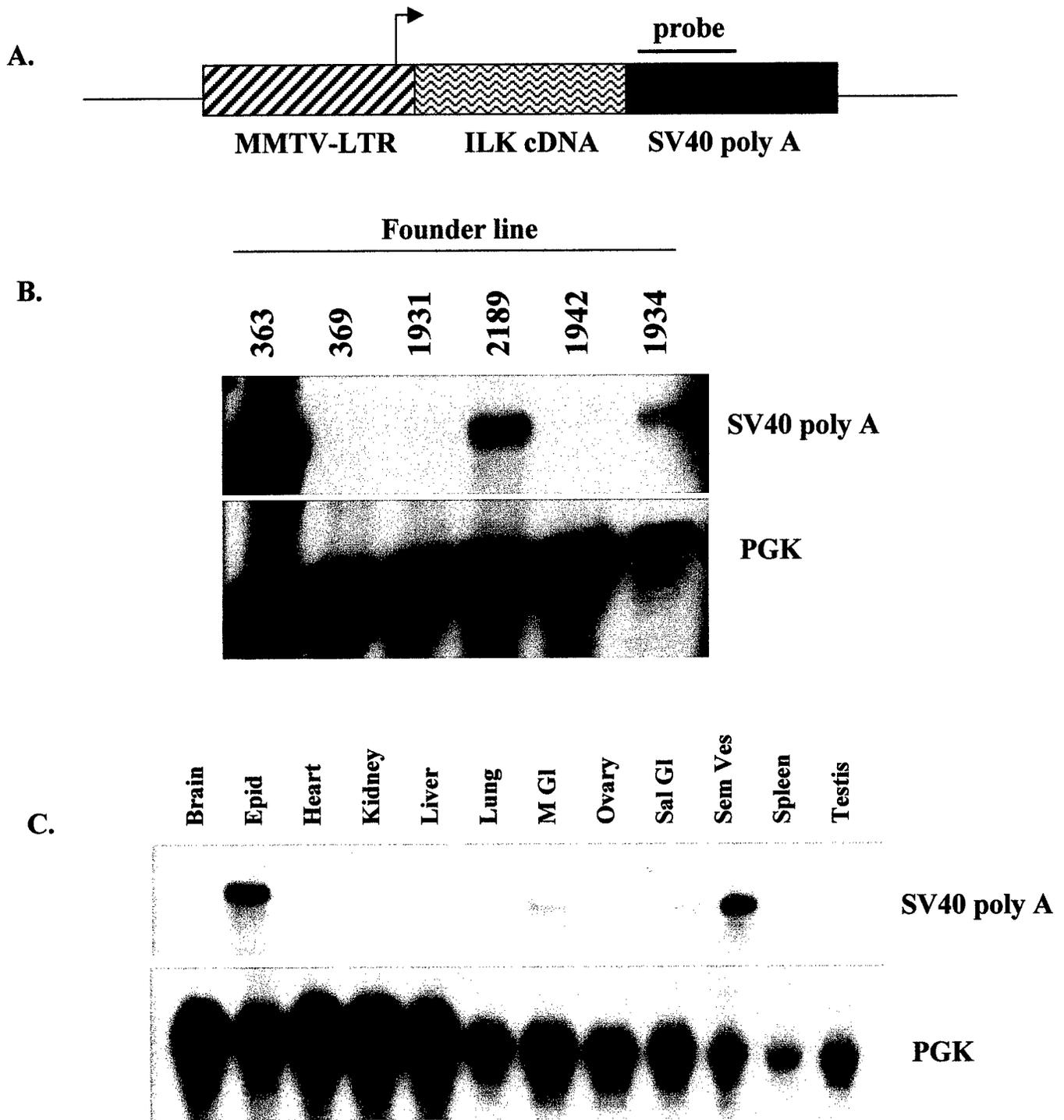


Figure 1. Tissue-specific expression of ILK in the mammary gland of transgenic mice. (A) Structure of the ILK transgene. (B) Expression of the ILK transgene in the mammary epithelium of 3 founder lines, as assessed by RNase protection. (C) Tissue distribution of transgene expression in both male and female mice. RNA antisense probes produce protected fragments of the poly adenylation region from SV40 (SV40 poly A), as well as that of a PGK internal control. M. GI: mammary gland; Sal. GI: salivary gland; Sem. Ves: seminal vesicle.

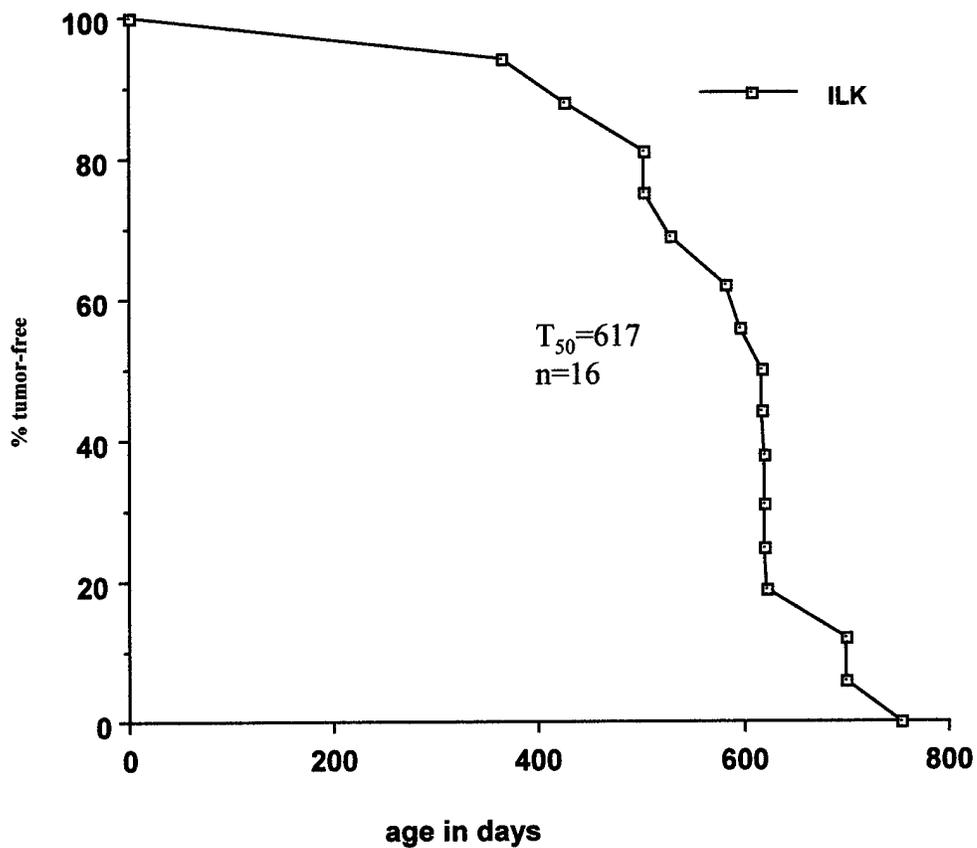
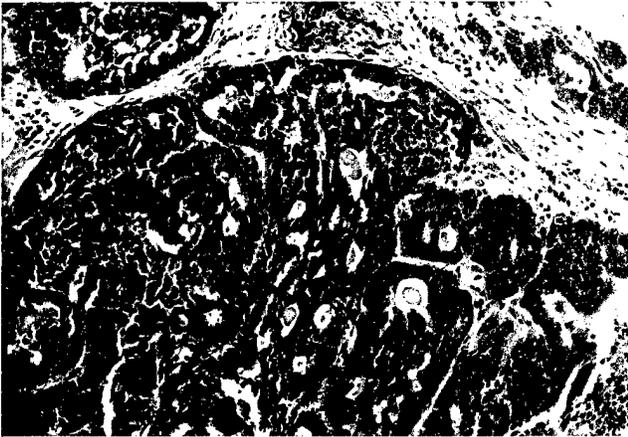


Figure 2. Kinetics of tumor formation in MMTV/ILK mice, line 363. Median age of onset (T_{50}) is 617 days, as determined from 16 tumor-bearing mice ($n=16$). Tumor incidence was calculated as 39% (16/41).

A.



B.

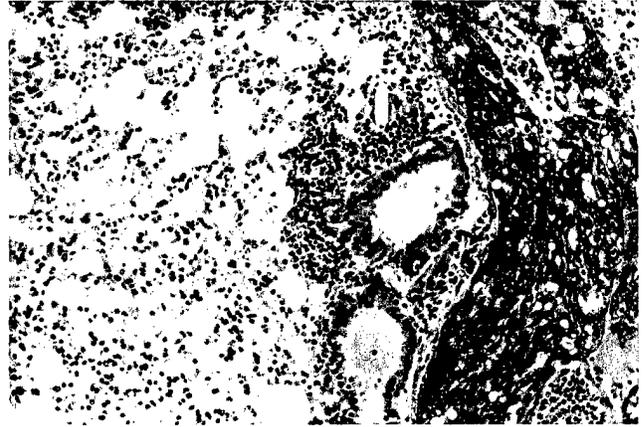


Figure 3. Hematoxylin and eosin stained tissue sections of (A) mammary adenocarcinoma and (B) lung taken from female mouse expressing the MMTV/ILK transgene (200x).

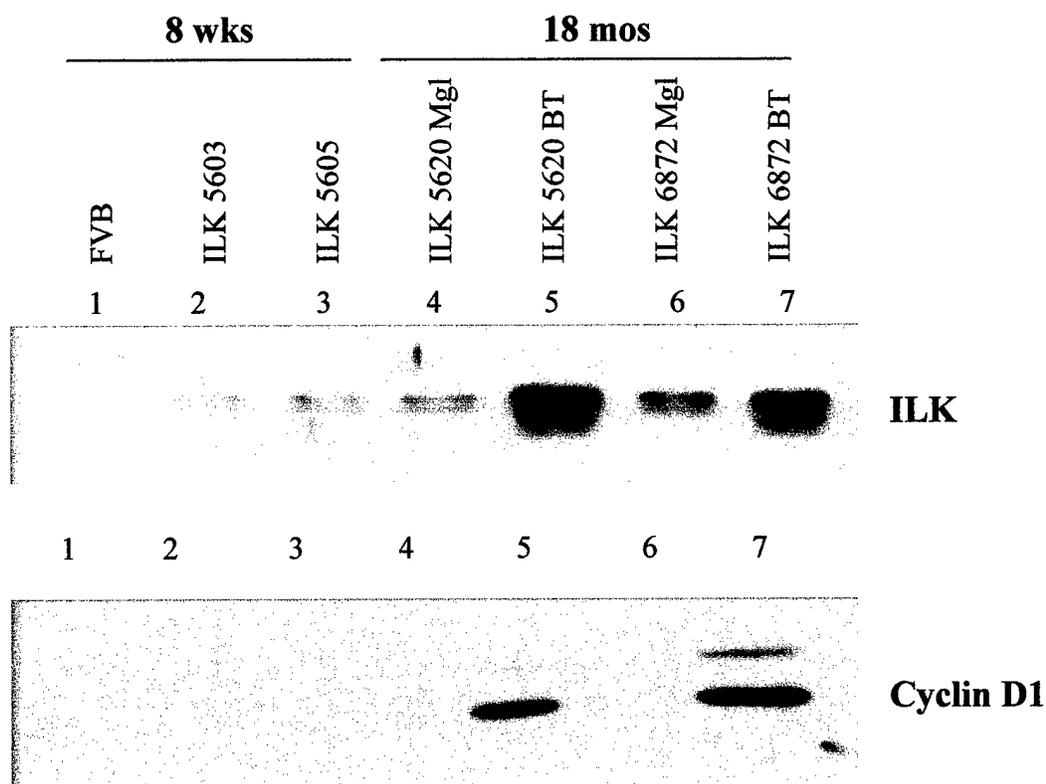


Figure 4. Upregulation of ILK and cyclin D1 in mammary tumours induced by MMTV-ILK transgene expression. Representative immunoblot showing protein levels in 5ug of normal virgin FVB (lane 1) and transgenic glands, aged 10 weeks (lanes 2,3), and in tumour (lanes 5,7) and adjacent mammary gland (lanes 4,6) tissue taken from 2 mice, aged 18 months. All transgenic mice are of the line #363, and are indicated by the label ILK, plus the ear-tag number.

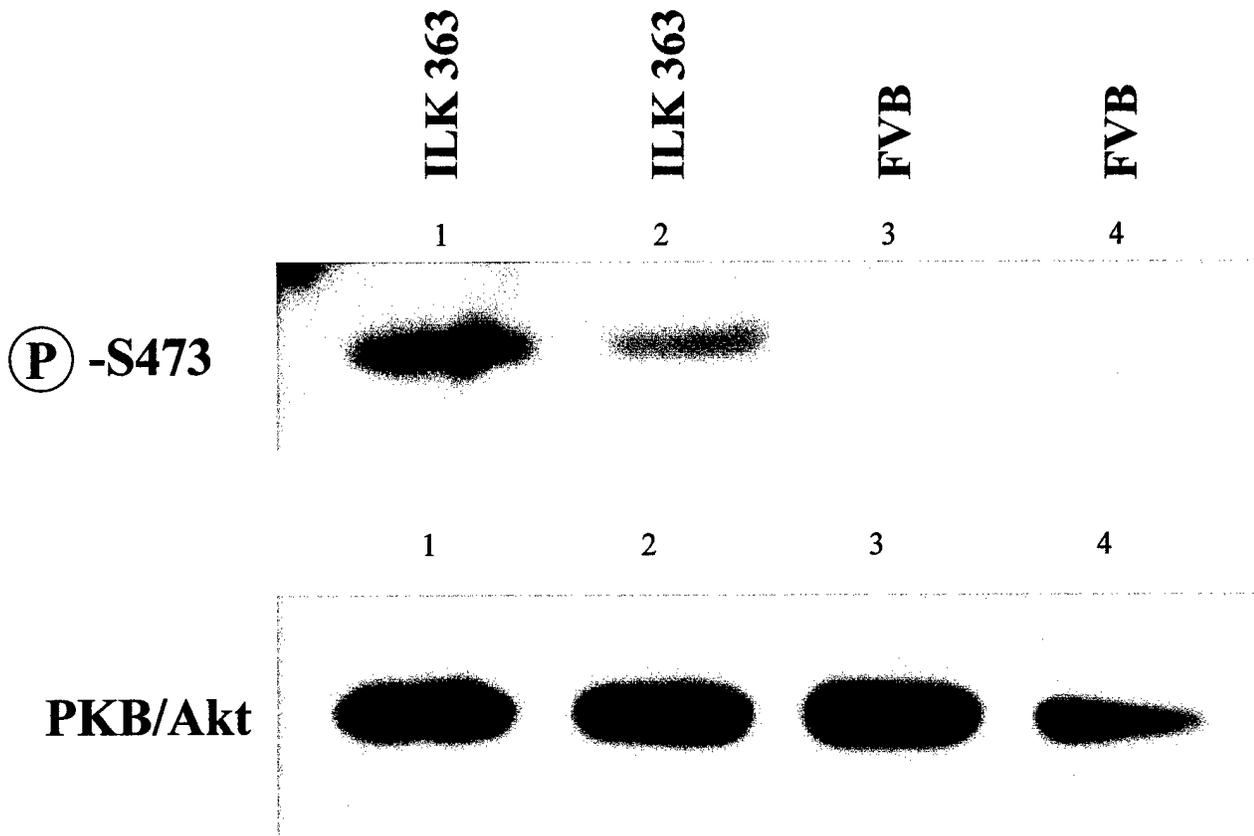


Figure 5. Comparison of phospho-Akt (S473) levels between mammary glands of 2 female ILK transgenic mice (line #363) (lanes 1,2) and 2 FVB control mice (lanes 3,4), 8 weeks of age. The lower panel shows total Akt levels in the same samples.

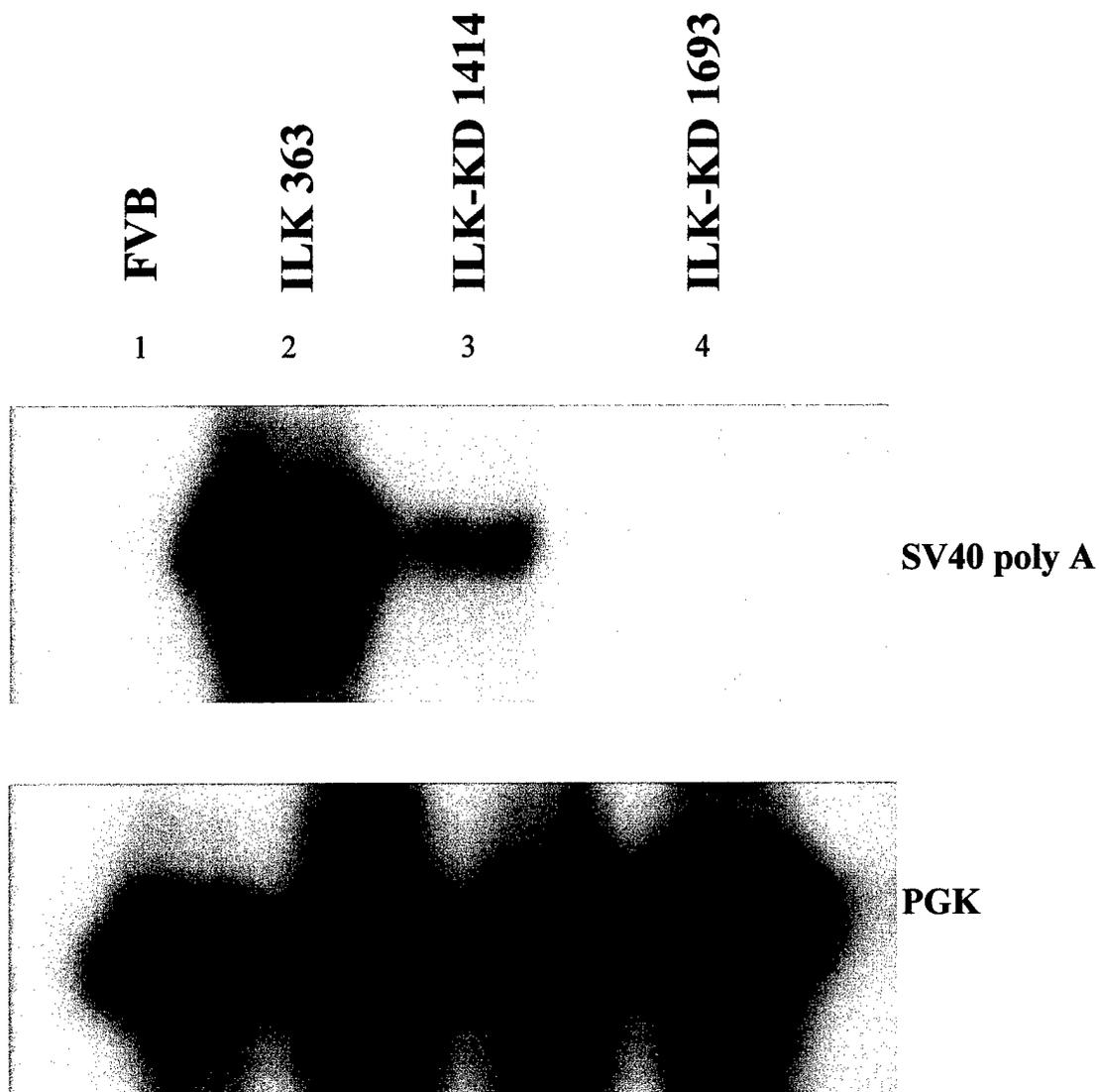


Figure 6. Expression of a kinase-dead (KD) allele of ILK in the mammary epithelium. Expression of an MMTV-ILK-KD transgene in the mammary epithelium of line #1414 was confirmed by RNase protection (lane 3). An FVB control and a non-expressing founder (lanes 1,4), as well as a mouse expressing the wild-type 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.

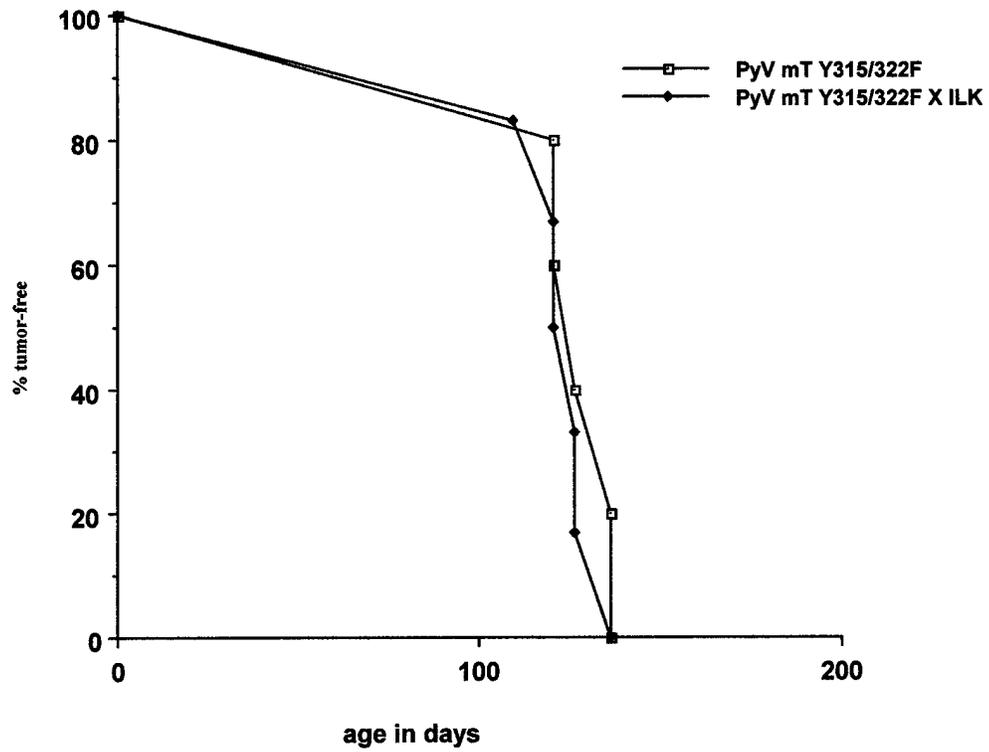


Figure 7. Kinetics of tumor formation in MMTV/PyV mT Y315/322F mice (n=5), versus MMTV/PyV mT Y315/322F x MMTV/ILK bitransgenic mice (n=6).

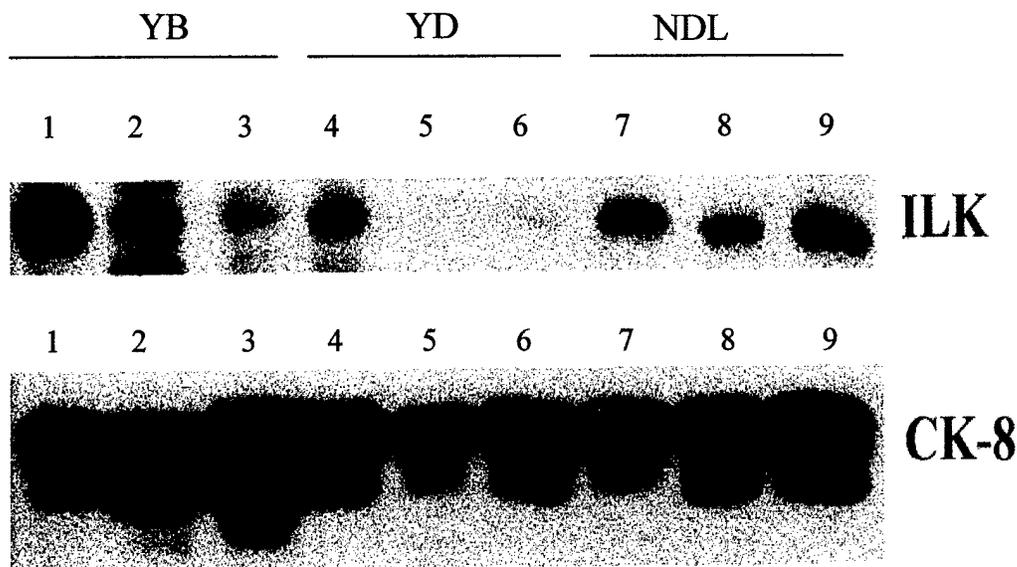


Figure 8. ILK levels are elevated in tumors expressing an activated erbB2/neu allele (NDL) containing all five tyrosine residues known to be phosphorylated in response to erbB2/neu kinase activity (lanes 7,8,9). By comparison, tumors expressing an allele deficient in all tyrosine residues except for site D (YD) have relatively lower levels of ILK (lanes 4,5,6). These YD-induced tumors have a longer latency than those induced by the activated NDL allele, and are less metastatic. An allele containing only tyrosine residue B (YB) induces tumors which, by comparison, are more metastatic to those induced by the YD allele, and appear earlier. ILK levels in the YB-induced tumors are shown in lanes 1,2 and 3. A cytokeratin-8 (CK-8) blot (lower panel) provides a control for protein loading and epithelial cell content.