

**Activated type I TGF $\beta$  receptor (Alk5) kinase confers enhanced survival to mammary epithelial cells and accelerates mammary tumor progression**

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## **Abstract**

PENDING.

## Introduction

The transforming growth factor-betas (TGF $\beta$ s) are members of a large superfamily of pleiotropic cytokines that also includes the activins and the bone morphogenetic proteins (BMPs). Members of the TGF $\beta$  family regulate complex physiological processes such cell proliferation, differentiation, adhesion, cell-cell and cell-matrix interactions, motility, and cell death, among others (Massague, 1998). Dysregulation of TGF $\beta$  signaling contributes to several pathological processes including cancer, fibrosis, and auto-immune disorders (Massague et al., 2000). The TGF $\beta$ s elicit their biological effects by binding to type II and type I transmembrane receptor serine-threonine kinases (T $\beta$ RII and T $\beta$ RI) which, in turn, phosphorylate Smad 2 and Smad3. Phosphorylated Smad2/3 associate with Smad4 and, as a heteromeric complex, translocate to the nucleus where they regulate gene transcription. The inhibitory Smad7 downregulates TGF $\beta$  signaling by binding to activated T $\beta$ RI and interfering with its ability to phosphorylate Smad2/3 (Derynck and Zhang, 2003; Shi and Massague, 2003). Signaling is also regulated by Smad proteolysis. TGF $\beta$  receptor-mediated activation results in multi-ubiquitination of Smad2 in the nucleus and subsequent degradation of Smad2 by the proteasome (Lo and Massague, 1999). Activation of TGF $\beta$  receptors also induces mobilization of a Smad7-Smurf complex from the nucleus to the cytoplasm; this complex recognizes the activated receptors and mediates their ubiquitination and internalization via caveolin-rich vesicles, leading to termination of TGF $\beta$  signaling (Di Guglielmo et al., 2003). Other signal transducers/pathways have been implicated in TGF $\beta$  actions. These include the extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (Jnk), p38 mitogen-activated protein kinase (MAPK), protein phosphatase PP2A, phosphatidylinositol-3 kinase (PI3K), and the family of Rho GTPases [reviewed in (Derynck and Zhang, 2003)]. Although signaling by Smads has been shown to be causally associated with the anti-proliferative effect of TGF $\beta$  (Datto et

al., 1999; Liu et al., 1997), the role of non-Smad effectors on mediating the cellular effects of TGF $\beta$  is less well characterized.

TGF $\beta$  can behave as both a tumor suppressor and a tumor promoter. Its tumor suppressor role can be explained by its ability to inhibit mitogenesis (Alexandrow and Moses, 1995), induce apoptosis (Siegel and Massague, 2003), maintain tissue architecture (Engle et al., 1999), inhibit genomic instability (Glick et al., 1996), and induce replicative senescence (Boulangier et al., 2004; Kordon et al., 1995). Consistent with its anti-proliferative effect, overexpression of active TGF $\beta$ 1 or T $\beta$ RI in the mammary gland of transgenic mice delays gland ductal extension and alveolar development (Jhappan et al., 1993; Pierce et al., 1993; Siegel et al., 2003) and protects from oncogene- or carcinogen-induced transformation (Pierce et al., 1995). In addition, attenuation of autocrine growth control by expression of dominant-negative T $\beta$ RII in the mouse mammary gland results in enhanced propensity for carcinogen-induced and spontaneous mammary cancers (Bottinger et al., 1997; Gorska et al., 2003). Overexpression of TGF $\beta$ 3 in alveolar epithelium of lactating mice causes alveolar cell apoptosis whereas Smad3-null mammary epithelium exhibits impaired post-lactational involution and cell death (Nguyen and Pollard, 2000; Yang et al., 2002), further consistent with the tumor suppressive role of TGF $\beta$ . However, other data suggest that TGF $\beta$  can also act as a survival factor in normal mammary epithelium. During pregnancy, there is a 3-fold increase in apoptosis in ductal and alveolar cells from *Tgf $\beta$ 1* heterozygote compared to wild-type mouse mammary glands (Ewan et al., 2002). Post-pubertal mice in which *Tgf $\beta$ 2* was conditionally deleted in the mammary gland also exhibit a much higher rate of apoptosis compared to controls (Forrester, 2005).

Other reports using cancer cells and/or transgenic mammary tumors support a role for TGF $\beta$  in the suppression of apoptosis (Huang et al., 2000; Muraoka et al., 2003). Conditional overexpression of active TGF $\beta$ 1 in a triple transgenic model expressing Polyomavirus middle T antigen in the mammary

gland (MMTV/PyVmT) inhibits apoptosis and accelerates metastases (Muraoka-Cook et al., 2004). In MMTV/PyVmT transgenic mice, systemic administration of a soluble T $\beta$ RII increases tumor cell apoptosis and reduces metastases (Muraoka et al., 2002a). Also in transformed cells, treatment with TGF $\beta$  enhances survival via Akt-induced phosphorylation of FKHRL1 (Shin et al., 2001). TGF $\beta$  has been shown to activate PI3K and its target Akt (Bakin et al., 2000; Higaki and Shimokado, 1999; Yi, 2005). Although the signaling programs regulating TGF $\beta$ -mediated protection from cell death are not yet clear, in both mesenchymal and epithelial cells, this protection is blocked by the PI3K inhibitor LY294002 and/or expression of dominant-negative Akt (Horowitz et al., 2004; Muraoka-Cook et al., 2004; Shin et al., 2001). These data are consistent with the ability of TGF $\beta$  to behave as a tumor promoter in late phases of transformation [reviewed in (Wakefield and Roberts, 2002)].

Therefore, to further examine the role of active TGF $\beta$  signaling in mammary epithelial cell survival *in vivo*, we generated transgenic mice expressing a mutant type I receptor (Alk5) containing a substitution of threonine 204 with aspartic acid (Alk5<sup>T204D</sup>), which results in constitutive activation of the receptor serine-threonine kinase in the absence of added ligand (Wieser et al., 1995). Expression of mutant Alk5<sup>TD</sup> was directed to mammary epithelium using the mouse mammary tumor virus (MMTV) promoter. Although ductal extension was delayed during mammary gland morphogenesis, apoptosis was markedly reduced in pubertal terminal endbuds (TEBs) and during post-lactational involution. Primary mammary epithelial cells from transgenic glands contained reduced levels of Smad2/3/4 and higher levels of c-myc compared to wild-type cells, and were insensitive to TGF $\beta$ -induced anti-mitogenesis. They also exhibited high levels of ligand-independent PI3K, Akt, and Rac1 activities, enhanced survival and adhesion to collagen I and fibronectin, and increased integrin expression. Treatment with an Alk5 kinase inhibitor upregulated Smad2/3 levels, reduced PI3K activity and P-Akt, and inhibited cell adhesion and survival, suggesting a causal relation between Alk5<sup>TD</sup> and the observed phenotypic changes. Finally,

although  $Alk5^{TD}$ -expressing mice did not develop mammary tumors, bigenic MMTV- $Alk^{TD}$  x Neu mice developed tumors that were more metastatic than those occurring in MMTV-Neu mice. These data suggest that dysregulated TGF $\beta$  signaling can confer PI3K-dependent enhanced survival to non-transformed mammary epithelial cells and accelerate metastatic progression of oncogene-induced cancers.

## Results

*Activated Alk5 reduces apoptosis and increases periductal fibrosis in the mouse mammary gland.* We generated two transgenic mouse lines expressing a cDNA construct encoding mutant Alk5<sup>T204D</sup> (Fig. 1A). The Alk5 construct was tagged with a haemagglutinin (HA) epitope at its carboxy-terminus. Expression of Alk5<sup>TD</sup> was directed to mammary epithelium using MMTV promoter. Of the 11 founder animals tested, 3 animals were identified as having genomic transgene insertion, 2 of which were able to transmit the transgene to their offspring (Fig. 1B). Expression of the HA-tagged Alk5<sup>TD</sup> in the mammary glands of 12-week old virgin females from both transgenic lines was confirmed by Alk5 immunoblot of HA precipitates from mammary gland extracts (Fig. 1C). Multiple tissues were screened for transgene expression by HA and Alk5 western analysis. Expression of HA-tagged Alk5 was limited to the mammary tissues from both transgenic lines. Although Alk5 expression was detected in kidney, thymus, heart, and liver extracts, this expression was from the endogenous but not the HA-tagged Alk5 protein (Fig. 1D). Using immunofluorescence with HA and Alk5 antibodies, both HA and Alk5 expression were detected in the mammary epithelium of MMTV-Alk5<sup>TD</sup> mice but not in WT mice (Fig 1E). HA and Alk5 expression was diffuse in mammary epithelium, suggesting homogenous expression of the transgene.

Overexpression of active TGFβ1 in the mammary gland of transgenic mice has been shown to delay ductal extension and alveolar development. Similar to MMTV-TGFβ1<sup>S223/225</sup> mice, mammary whole mounts from pubertal MMTV-Alk5<sup>TD</sup> mice showed that ductal extension into the mammary fat pad was initially delayed compared to age-matched WT siblings (Fig. 2A). However, by 20 weeks of age the ductal tree had completely permeated the mammary fat pad in MMTV-Alk5<sup>TD</sup> mice. This delay in ductal outgrowth may have been due to a decrease in cellular proliferation within the highly mitotic TEBs at the distal ends of the developing ductal tree. However, proliferation measured by the proportion of cells

in the TEBs from 7-week old mice that stained positive for proliferating cell nuclear antigen (PCNA) was similar in WT and transgenic mice (Fig. 2B; **Rebecca: Any quantitation? They look the same to me**). The TEBs also exhibit high levels of apoptosis, which contributes to the canalization of the ductal network. At mid-puberty (7 weeks), TEBs from MMTV-Alk5<sup>TD</sup> glands displayed a >3-fold reduction in TUNEL+ cells compared to TEBs from age-matched WT siblings. Despite the reduced rate of cell death, canalization of the ductal tree was not impaired in MMTV-Alk5<sup>TD</sup> mice (Fig. 2C).

Histological analysis of 20-week old transgenic glands at 20 weeks of age showed an abundant periductal eosinophilic infiltrate which was not present in age-matched WT siblings or in MMTV-TGFβ1<sup>S223/225</sup> mice (Fig. 3A). Since TGFβ signaling results in secretion of ECM proteins, we speculated that active Alk5 signaling would enhance secretion of ECM components. Masson's trichrome staining revealed marked collagen deposition surrounding the transgenic ducts but this accumulation was much less around WT ducts (Fig. 3B). We next determined if enhanced periductal stroma was associated with an increase in myoepithelial cells. Using immunohistochemistry for smooth muscle actin (SMA), a marker of these cells, a single-cell layer of myoepithelium was detected surrounding ducts, in direct apposition to the ductal epithelium. It is unclear from these data whether the epithelial or myoepithelial cells are synthesizing the excess in collagen. Staining with antibodies against active TGFβ1 showed clear excess of active ligand throughout the periductal space (Fig. 3D), suggesting the possibility that TGFβ-stimulated resident fibroblasts could **secrete?** the excess in periductal collagen.

To examine the effect of Alk5<sup>TD</sup> on mammary development during pregnancy, we examined pregnant mammary glands at 16.5 days post-coitum (d.p.c). Both WT and transgenic mammary glands exhibited abundant lobuloalveolar structures filled with proteinaceous secretions. However, epithelial content was less and adipose tissue more abundant in glands from MMTV-Alk5<sup>TD</sup> compared to WT mice (Fig. 4A-C). By day 10 of lactation, the secretory epithelium in MMTV-Alk5<sup>TD</sup> mammary glands

appeared indistinguishable from that in WT glands (Fig. 4D-F). Interestingly, an excess in stroma was apparent in transgenic glands examined at 16.5 d.p.c. and at day 10 of lactation (Fig. 4C,F). **Rebecca:** Where the pups OK? Should we make a comment re the small weight of the pups in our study? I remember that caught your eye initially. It is possible that the reason Siegel et al. (PNAS 2003 – 2<sup>nd</sup> page) did not see delayed involution is because they had such a potent Alk5 (double mutant ... remember?) that they may have seen both an anti-proliferative and a **pro-apoptotic** effect. Indeed some of their pups died. I don't think ours did but I remember they were small.

The reduced apoptosis in MMTV-Alk5<sup>TD</sup> TEBs suggested the possibility of reduced programmed cell death during post-lactational involution and architectural remodeling of the mammary gland, the other physiological state of the gland during which epithelial apoptosis is high. Mammary gland involution was induced after 10 days of lactation by withdrawing pups from nursing mothers. We then examined mammary gland whole mounts and histology at 1, 3, 5, 7, and 21 days post-forced wean (d.p.f.w.; Fig. 5A,B). These revealed a delay in the elimination of mammary epithelium in MMTV-Alk5<sup>TD</sup> glands compared to involuting WT glands. Again, an excess of ECM and collagen surrounding ducts and persistent lobuloalveolar structures was evident in transgenic but not WT mammary glands at 21 d.p.f.w. (Fig. 5C). TUNEL assays were employed to determine if the delayed involution was due to a reduced rate of apoptosis. Apoptosis in post-lactational mammary epithelium can be detected as early as day 1, peaks at day 3, and completes between days 7 and 10 (ref here). The fraction of total nuclei undergoing apoptosis in MMTV-Alk5<sup>TD</sup> glands was decreased 3-fold compared to WT glands at 3 d.p.f.w. but both were approximately equal at 7 d.p.f.w. (Fig. 5D). These results suggest that active Alk5 was inducing survival signals that counteracted epithelial apoptosis during post-lactational involution.

***Activated Alk5 downregulates expression of Smads and induces PI3K/Akt signaling.*** To determine possible mechanisms by which Alk5<sup>TD</sup> expression modulates cell survival and the ECM, we examined

protein levels of molecules that mediate TGF $\beta$  signaling in whole mammary gland lysates from 12-week old virgin mice. By immunoblot analysis, Smad2, Smad3, and Smad4 levels were decreased in MMTV-Alk5<sup>TD</sup> compared to WT glands, while levels of the inhibitory Smad7 were elevated (Fig. 6A). The reduced Smad2 content in transgenic glands was confirmed by immunohistochemistry (Fig. 6B). However, the steady-state Smad2 RNA levels were roughly similar between both types of glands (Fig. 6C). Consistent with the different levels of Smad2, treatment with TGF $\beta$  of WT PMECs markedly induced Smad2 phosphorylation within 30 min and inhibited their proliferation. These effects were not observed in PMECs from transgenic glands (Fig. 6D). In addition, c-myc was elevated in MMTV-Alk5<sup>TD</sup> mammary glands as well as Ser473 phosphorylated active Akt without a change in total Akt protein levels. Treatment with TGF $\beta$  eliminated detectable c-myc levels in WT but not in the transgenic cells (data not shown). Levels of  $\beta$ -tubulin and p21<sup>Cip1</sup> were similar in glands of both genotypes (Fig. 6A). Since TGF $\beta$  can activate phosphatidylinositol-3 kinase (PI3K) and its target kinase Akt (above), we examined PI3K catalytic activity in mammary gland lysates. The regulatory subunit of PI3K, p85, was precipitated from cells; p85-containing immune complexes were tested for their ability to stimulate formation of 3' phosphorylated inositol lipids *in vitro*. MMTV-Alk5<sup>TD</sup> exhibited markedly higher levels of ligand-independent, p85-associated PI3K activity compared to WT PMECs (Fig. 6E). **Interestingly, added ligand did not induce PI3K in WT PMECs (?).**

We next attempted to increase Smad2 expression in PMECs expressing the Alk5<sup>TD</sup> transgene by transducing them adenoviruses encoding FLAG-tagged Smad2 **and Smad4**. Western analysis of extracts from Ad.Smad2-transduced cells suggested that the ectopic Smad2 was not expressed in MMTV-Alk5<sup>TD</sup> cells (Fig. 7A), even though expression of  $\beta$ -galactosidase was widely detected in cells transduced with an Ad.lacZ control (Fig. 7B). This result plus the maintained levels of Smad2 RNA (Fig. 6C) suggested a post-transcriptional mechanism for Alk5-mediated downregulation of Smad2 protein levels. Receptor-

mediated activation of Smad2 has been shown to result in ubiquitination and subsequent degradation of Smad2 in the proteasome (Lo and Massague, 1999). Thus, we examined if treatment of MMTV-Alk5<sup>TD</sup> PMECs with the proteasome inhibitor PS-341 (Adams, 2004) would restore Smad2 protein levels and function. Treatment with PS-341 for 24 h restored Smad2 expression and detectable P-Smad2 levels in cells transduced or not with Ad.Smad2 (Fig. 7C). In addition, treatment with PS-341 downregulated the enhanced basal levels of c-myc observed in the Alk5<sup>TD</sup>-expressing cells. Furthermore, ligand-independent transcriptional activity in MMTV-Alk5<sup>TD</sup> PMECs transiently transfected with a reporter construct containing twelve Smad binding elements in tandem, p(CAGA)<sub>12</sub>-Lux, was enhanced 17-fold by PS-341 alone. PS-341 did not increase p(CAGA)<sub>12</sub>-Lux expression in WT cells although it modestly enhanced ligand-induced reporter activity (Fig. 7D). Consistent with these data, proteasome inhibition restored the ability of exogenous TGFβ to inhibit cell cycle progression of Alk5<sup>TD</sup>-expressing cells. In the absence of PS-341, TGFβ did not increase the G1 phase or reduce the S phase fraction in Alk5<sup>TD</sup> cells. However, addition of PS-341 alone to Alk5<sup>TD</sup> cells increased the G1 fraction from 72 to 91% and reduced S phase from 15 to 6% after 24 h (Fig. 7E).

Additional changes were displayed by Alk5<sup>TD</sup>-expressing PMECs. These consisted in membrane ruffling and lamellopodia, loss of an epithelial morphology (Fig. 8A), and increased motility through transwells (data not shown) in the absence of added growth factors, all features consistent with an epithelial-to-mesenchymal transition (EMT) and activation of Rho GTPases (Hall, 1998). RhoA, Rac1, and Cdc42 activity was measured by precipitating the GTP-bound forms of these proteins using GST fusions containing rhotekin (for RhoA) or the p21-activated kinase (Pak) binding domain (PBD; for Rac1 and Cdc42). Active RhoA and Cdc42 were equal in both Alk5<sup>TD</sup>-expressing and WT PMECs but active Rac1 was clearly higher in transgene-expressing cells (Fig. 8B). Treatment with the PI3K

inhibitor LY294002 inhibited both basal Rac1 activity and cell motility through transwells (data not shown).

MMTV-Alk5<sup>TD</sup> cells also exhibited increased adhesion and low evidence of cell death upon increasing passages in culture. Therefore, we next examined cell adhesion to different ECM proteins. Binding to fibronectin- or collagen I-coated dishes was markedly increased in MMTV-Alk5<sup>TD</sup> compared to WT cells, while adhesion to laminin was approximately equal in both cell types (Fig. 8E). Since fibronectin and collagen I bind integrins and TGF $\beta$  regulates integrin expression, we measured integrin expression in both cell types. By flow cytometry, we found elevated expression of the integrins  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 5, and  $\beta$ 1 in MMTV-Alk5<sup>TD</sup> compared to WT cells (Fig. 8D).

***Alk5 kinase inhibitor downregulates PI3K in MMTV-Alk5<sup>TD</sup> mammary cells.*** To determine if active Alk5 was causally associated with the enhanced PI3K activity in transgene-expressing cells, we utilized the small molecule Alk5 serine/threonine kinase inhibitor LY580276. LY580276 is a para-fluorophenyl substituted dihydropyridopyrazole that is highly selective for Alk5 (IC<sub>50</sub> 0.18  $\mu$ M, Ki 37 nM) relative to T $\beta$ RII (IC<sub>50</sub> >20  $\mu$ M), p38 $\alpha$  (IC<sub>50</sub> >20  $\mu$ M) and a panel of 39 additional kinases with an IC<sub>50</sub> >10  $\mu$ M. It inhibits TGF $\beta$ -stimulated p3TP-Lux reporter activity in Mink lung cells (IC<sub>50</sub> 96 nM) and NIH3T3 proliferation (IC<sub>50</sub> 39 nM)(Peng, 2005). The crystal structure of LY580276 in Alk5<sup>T204D</sup> has been solved which confirms binding at the receptor's ATP site (Sawyer et al., 2004). In dose-dependent fashion, treatment with the Alk5 inhibitor for 24 h restored Smad2 levels while reducing basal P-Smad2, P-Akt, and c-myc in MMTV-Alk5<sup>TD</sup> cells. In addition, preincubation for 1 h with 2-10  $\mu$ M LY580276 blocked both basal and ligand-induced P-Smad2 and P-Akt in a dose-dependent manner (Fig. 9A). Treatment with LY580276 also inhibited p85-associated PI3K activity in MMTV-Alk5<sup>TD</sup> cells. A similar level of inhibition was seen with LY294002, a small molecule that directly inhibits p110, the catalytic subunit of PI3K (Fig. 9B). Finally, treatment with the Alk5 inhibitor reversed the increased adhesion to collagen I

and fibronectin and markedly enhanced cell death with no effect on binding to laminin (Fig. 9C) or a detectable change in the expression of integrins  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 5$ , and  $\beta 1$  (Fig. 9D), supporting in part a causal association between Alk5-induced PI3K and the enhanced cell adhesion and survival. Similar results were obtained with LY294002.

***Activated Alk5 accelerates oncogene-induced mammary tumor progression.*** Despite the pro-survival effect of the Alk5 transgene product, we have not observed mammary hyperplasias or cancers in MMTV/Alk5<sup>TD</sup> as old as 2 years of age. Recently, overexpression of active TGF $\beta$ 1 or an active mutant of T $\beta$ RI containing T204D and L193A/P194A (to prevent binding of the FKBP-12 inhibitor) have been shown to increase extravascular metastases in bigenic mice also expressing c-Neu or mutant Neu, respectively (Muraoka et al., 2003; Siegel et al., 2003). Therefore, we cross-bred MMTV-Alk<sup>TD</sup> with MMTV/Neu mice. Whole mounts of mammary glands from 7-week old MMTV/Neu mice showed the previously described precocious alveolar budding and branching of primary ducts. In glands from age-matched bigenic mice, Alk5<sup>TD</sup> failed to delay ductal extension but appeared to accelerate ductal branching (Fig. 10A).

Neu and Alk5<sup>TD</sup> x Neu mice were examined weekly to detect the presence of mammary tumors. Tumor latency was similar in both genotypes (251 vs. 269 days, respectively) with 100% of mice of either genotype developing mammary tumors by day \_\_\_\_ **Incheol???**. Overall, the bigenic tumors were of a higher histological grade with more necrosis and evidence of local invasion (data not shown). Approximately 60 days after the initial palpation of a mammary tumor, some bigenic mice exhibited signs of respiratory distress. Examination of the lungs 68 days after initial tumor palpation, revealed presence of lung surface metastases in 4/5 (80%) bigenic vs. 1/9 (11%) MMTV-Neu mice ( $p=0.0048$ ). However, there were no differences in the number of tumors per mouse or their calculated cumulative tumor volume (Figs. 10B,C). Finally, we examined TGF $\beta$  receptor levels in PMECs harvested from

established tumors of both genotypes by subjecting them to affinity cross-linking with  $^{125}\text{I}$ -TGF $\beta$ 1. All three TGF $\beta$  receptor proteins were detectable in MMTV-Alk5<sup>TD</sup> x Neu PMECs (Fig. 10D),

## Discussion

We have examined the biochemical and cellular effects of aberrant TGF $\beta$  signaling in mammary epithelium by overexpressing a constitutively active mutant of Alk5 in the mouse mammary gland. Expression of Alk5<sup>T204D</sup> in mammary epithelial cells resulted in a delay in ductal extension during gland morphogenesis as well as a reduction in apoptosis during TEB formation at puberty and during post-lactational involution. The last two are physiological states of the gland during which the rate of cell death is high. PMECs isolated from transgenic glands exhibited a markedly lower content of Smad2/3 protein compared to controls, high constitutive PI3K and Akt activities, and were insensitive to TGF $\beta$ -mediated growth arrest (Fig. 6) and transcriptional reporter activity (Fig. 7). Treatment of the transgenic mammary cells with a proteasome inhibitor increased steady-state Smad2/3 protein levels and ligand-independent Smad transcriptional reporter activity as well as inhibited basal cell proliferation in the absence of exogenous TGF $\beta$  (Fig. 7). These results suggest that mutant Alk5 was increasing Smad proteolysis and are consistent with the observation that TGF $\beta$  receptor activation results in ubiquitination and proteasome-mediated degradation of Smad2 (Lo and Massague, 1999).

In addition, c-myc protein levels were higher in Alk5<sup>TD</sup>-expressing cells compared to controls and they were unresponsive to added TGF $\beta$ . The downregulation of c-myc is required for the anti-mitogenic action of TGF $\beta$  in order to deprive the cell of growth-promoting functions and facilitate the induction of the Cdk inhibitors p15<sup>INK4B</sup> and p21<sup>CIP1</sup> (Alexandrow and Moses, 1995; Chen et al., 2001). A complex of Smad3, Smad4, E2F4/5, and the transcriptional repressor p107 has been shown to mediate TGF $\beta$ -induced downregulation of c-myc (Chen et al., 2002). Treatment with the proteasome inhibitor PS-341 simultaneously upregulated Smad2/3, downregulated c-myc, and induced cell cycle arrest in the Alk5<sup>TD</sup>-expressing cells. Therefore, we speculate that the low Smad3 levels contributed to derepression (and a higher level) of c-myc in the transgenic cells, further antagonizing the anti-proliferative effect to TGF $\beta$ .

In this study, overexpression of active Alk5 resulted in high levels of constitutive PI3K activity in mammary epithelium. PI3K increases the formation of intracellular 3' phosphoinositol lipids, signal transducers involved in the regulation of cell cycle progression, glucose metabolism, cell motility, EMT, and inhibition of apoptosis (Vivanco and Sawyers, 2002). TGF $\beta$  stimulates PI3K activity as well as phosphorylation and activation of the PI3K-dependent Akt serine-threonine kinase (Bakin et al., 2000; Higaki and Shimokado, 1999). Both T $\beta$ RII and Alk5 have been shown to associate indirectly with p85. Although the association of p85 with T $\beta$ RII is constitutive, the Alk5-p85 interaction is ligand-dependent (Yi, 2005). TGF $\beta$ -mediated cell motility and protection from apoptosis are blocked by LY294002 and/or expression of dominant-negative Akt (Bakin et al., 2000; Horowitz et al., 2004; Muraoka-Cook et al., 2004; Shin et al., 2001). Inhibition of PI3K has also been shown to reverse the fibroblastoid phenotype of TGF $\beta$ -treated Ras transformed hepatocytes to an epithelial phenotype (Gotzmann et al., 2002), further suggesting that PI3K is an effector of the transforming effects of TGF $\beta$ . By repressing the induction of p21<sup>CIP1</sup>, activation of the PI3K/Akt pathway can inhibit the cytostatic effect of TGF $\beta$  (Seoane et al., 2004). In addition, phosphorylated Akt can sequester Smad3 and prevent its phosphorylation, association with Smad4, and nuclear translocation, hence blocking TGF $\beta$ -induced apoptosis (Conery et al., 2004; Remy et al., 2004).

Consistent with the pro-survival effect of the PI3K pathway in mammary epithelium, mice deficient on the phosphatase PTEN, the negative regulator of PI3K, develop breast hyperplasias and carcinomas (Stambolic et al., 2000). In addition, overexpression of active mutants of Akt1 in the mammary gland of transgenic mice results in delayed post-lactational involution and gland remodeling (Ackler et al., 2002; Hutchinson et al., 2001; Schwertfeger et al., 2001), similar to that observed in the MMTV-Alk5<sup>TD</sup> mice. Supporting a causal association between active Alk5 and aberrant PI3K activity and function, treatment with a small molecule inhibitor of the Alk5 kinase inhibited PI3K and P-Akt and adhesion/survival in

transgene expressing PMECs (Fig. 9). Therefore, the data herein show for the first time *in vivo* and in nontumor cells that TGF $\beta$  can signal to PI3K and inhibit apoptosis.

In MMTV/Alk5<sup>TD</sup> mice, the low levels of Smad2/3 and the enhanced PI3K activity may have cooperated to negate the anti-proliferative effect of TGF $\beta$ . However, ductal extension during gland formation was still dampened (Fig. 1A) and the mice exhibited a transient reduction in mammary secretory epithelium during pregnancy (Fig. 4A-C). Interestingly, the weight of the pups from transgenic mothers was lower than pups from control mice (Rebecca??), suggesting the presence of a subtle lactational defect previously reported in WAP-TGF $\beta$ 1 transgenics (Boulanger et al., 2004; Jhappan et al., 1993). Siegel *et al.* reported a marked lactational deficiency in transgenic mice expressing another ‘gain-of-function’ mutant of Alk5 under MMTV promoter regulation (Siegel et al., 2003). In addition to T204D, this mutant contained two missense mutations, L193A and P194A, which prevent binding of the Alk5 inhibitor FKBP12 (Charng et al., 1996). It is possible that this mutant kinase was more potent than the Alk5<sup>T204D</sup> mutant used in our studies resulting in a more severe anti-mitogenic and pro-apoptotic effect, thus leading to lactational deficiency.

Finally, to determine if aberrant TGF $\beta$  signaling modulated oncogene-induced tumor progression, we cross-bred MMTV-Alk5<sup>TD</sup> with MMTV-Neu mice. Mice expressing c-Neu in mammary epithelium develop mammary hyperplasias and carcinomas with a median latency of 6 months; approximately 60% of tumor-bearing transgenic mice develop lung metastases (Guy et al., 1992). Median tumor latency, number of tumors per mouse, and the calculated cumulative tumor volume were not different in MMTV-Alk5<sup>TD</sup> x Neu bigenics compared to MMTV-Neu mice. However, the number of lung metastases was greater and the timing at which they became clinically apparent was much shorter in bigenic compared to mice bearing Neu-expressing mammary tumors. These results are not completely original as it has been reported already that overexpression of active TGF $\beta$ 1<sup>S223/225</sup> or a different mutant of Alk5

(T204D/L193A/P194A) under the control of the MMTV promoter in transgenic mice also expressing c-Neu or mutant Neu in mammary epithelium, respectively, accelerates lung metastases from oncogene-induced breast carcinomas (Muraoka et al., 2003; Siegel et al., 2003). Nonetheless, these results suggest that, although the MMTV-Alk5<sup>TD</sup> mice did not develop mammary tumors after long follow-up, the mutant Alk5 kinase may provide a ‘gain-of-function’ effect that synergizes with oncogene-induced transformation.

Why is Alk5-TD more potent than active TGFb-S223/225 or added ligand?? Is Alk5-TD sensitive to degradation as per DiGuglielmo’s paper in Nat. Cell Biol.?

Roy: What should I say about the integrins?? I am at a loss here. Does PI3K induce integrins?

What are we going to say about the fibrosis???? Is there a connect with the increased secretion of collagen?? Plus, excess the excess stroma may increase survivability. It’s been shown experimentally.

Although it is generally accepted that TGFβ is both a tumor suppressor and a tumor promoter, the general dogma has been that, in normal cells, the cytostatic effects of TGFβ are dominant over mitogenic signals whereas in established cancer cells, the anti-apoptotic and mitogenic action of oncogenes can subvert the growth inhibitory action of TGFβ. The data presented in this report would imply an alternative to this dogma. The phenotypic changes exhibited by the Alk5<sup>TD</sup>-expressing PMECs, i.e., increased survival, adhesion, motility, constitutive Rac1 and PI3K/Akt signaling, and lack of response to TGFβ-induced growth inhibition, taken together, suggest a partial transformed phenotype. These occurred in cells harvested from histologically normal mammary glands containing dysregulated TGFβ receptor signaling. They suggest that, in certain contexts, i.e., when low Smad2/3 and/or high PI3K/Akt signaling are present, the tumor ‘promoting’ effects of TGFβ may occur in ‘normal’ epithelial cells before evidence of histological or cytological transformation is present. **Still need a better punch line!!**

## Materials and Methods

**Generation and analysis of transgenic mice.** MMTV-TGF $\beta$ 1<sup>S223/225</sup> founders in an FVB strain background were provided by Harold Moses (Vanderbilt University). The 1.02 kb constitutively active human Alk5<sup>T204D</sup> cDNA fragment including the carboxy-terminal HA-tag was subcloned into the pBS-MMTVpA-GSK vector (D'Cruz et al., 2001)(provided by Lewis Chodosh, Univ. of Pennsylvania). The linearized transgene was injected into one-cell stage FVB mouse embryos which were transplanted into pseudo-pregnant females. All resulting pups were screened for presence of the transgene using primer pairs P1 and P2 (from the MMTV sequence), or P3 and P4 (from human Alk5). P1: 5'-CCCAAGGCTTAAGTAAGTTTTTGG-3'; P2: 5'-GGGCATAAGCACAGATAAAACACT-3'; P3: 5'-TTAAGATATTCTCCTCTAGAGAAGA-3'; P4: 5'-AATCTCTATGAGCAATGGCTGGCTT-3'. Three independently-derived transgenic founders were identified, two of which transmitted the transgene to their offspring. All subsequent studies were performed in age-matched females of the F1 or F2 generation. For studies of timed pregnancies, mating pairs were established and mice were monitored daily for vaginal plugs, the presence of which would indicate 0.5 days post coitus (d.p.c.). Mammary glands were harvested at 16.5 d.p.c. For studies with lactating mammary glands, these were harvested 10 days post-partum (lactation day 10). For mammary involution studies, pups were withdrawn from nursing mothers on day 10 and gland involution assessed on days 1, 3, 5, 7, and 21 post forced wean (d.p.f.w.). In some cases, FVB MMTV-Neu mice (Jackson Laboratories, Bar Harbor, ME) were crossed with MMTV-Alk5<sup>TD</sup> mice to generate MMTV-Neu x Alk5<sup>TD</sup> bigenic mice, from which primary mammary epithelial cells were harvested as described below. Mice were genotyped by polymerase chain reaction analysis of genomic DNA as previously described (Muraoka et al., 2003). Only age-matched virgin female mice were analyzed. Mice were monitored weekly by palpation to determine the presence of mammary tumors.

***Histological analysis, TUNEL, and immunofluorescence.*** Mammary glands were harvested and immediately fixed in 10% formalin (VWR Scientific). Hematoxylin-stained whole mounts of #4 (right inguinal) mammary glands were prepared as previously described (Muraoka et al., 2001). Paraffin-embedded mammary glands were sectioned (5  $\mu$ m), rehydrated, and stained with Mayer's hematoxylin and eosin B-phloxine or with Masson's trichrome stain (all from Sigma, St. Louis, MO). Proliferation was determined by immunohistochemistry (IHC) using an antibody against proliferating cell nuclear antigen (PCNA; Neomarkers, Fremont, CA) as described (Muraoka et al., 2003). Detection of apoptosis by TUNEL analysis was performed using the Apoptag Detection Kit (Serologicals Corp., Norcross, GA.) according to manufacturer's instructions. Immunofluorescence for Smad2 and active TGF $\beta$ 1 was performed using fresh frozen tissue cryosections as described previously (Ehrhart et al., 1997) using the following antibodies: active TGF $\beta$ 1 (cat # AF-101-NA, lot FS08, R&D Systems, Minneapolis, MN), Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), and secondary antibodies labeled with Alexa 488 or 594 fluorochromes (Molecular Probes, Eugene, OR). Images were captured using a scientific-grade 12-bit charged coupled device (KAF-1400, 1317x1035 6.8-mm<sup>2</sup> pixels) digital camera (Xillix, Vancouver, Canada). Internal standardization was achieved by comparing only images stained with the same antibodies in the same experiment, captured with identical parameters, and scaled and displayed identically.

***Western and Northern analyses.*** Mammary glands, tumors, or cell cultures were harvested and homogenized as previously described (Muraoka et al., 2003). Total protein (20  $\mu$ g) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Western analyses were performed using the following antibodies: Alk5 (V-22), E-cadherin (H-108), integrin  $\beta$ 1 (M-106), pan-cytokeratin (H-240),  $\alpha$ -actinin (H-300), Smad4 (B-8), Smad7 (H79), c-myc (N-262), and HA (Santa Cruz Biotechnology, Santa Cruz, CA); total and P-MAPK (Promega, Madison,

WI); total and S473 P-Akt and P-Smad2 (Upstate Biotechnology, Lake Placid, NY); Smad2/3 (BD Biosciences Pharmingen, San Diego, CA); smooth muscle actin and Smad3 polyclonal (Zymed, Inc., San Francisco, CA); p21 (ab-2, Oncogene Sciences). Immunoprecipitations were performed as previously described (Muraoka et al., 2001). Total RNA was harvested from mammary glands using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's directions. Northern analysis using total RNA (20 $\mu$ g) was performed as described (Muraoka et al., 2002b).

***Isolation and culture of primary mammary epithelial cells (PMECs).*** Mammary glands were digested at 37°C for 4 h in 3 mg/ml collagenase A (Sigma) in PBS (pH 7.4). The cell suspension was plated on dishes coated with Growth Factor-Reduced Matrigel (BD Biosciences Pharmingen) in PMEC media [Dulbecco's Modified Eagle's Medium:F12 (50:50; GibcoBRL), 10% FCS and 50 ng/ml insulin (Clonetics)] and cultured at 37°C in 5% CO<sub>2</sub>. Human recombinant TGF $\beta$ 1 was from R&D Systems. PS-341 was provided by Julian Adams (MilleniumPharmaceuticals, Cambridge, MA).

***Affinity labeling of TGF $\beta$  receptors.*** To determine presence of TGF $\beta$  receptors, PMECs were affinity-labeled with 100 pM <sup>125</sup>I-TGF $\beta$ 1 (specific activity, 3925 Ci/mmol; NEN Life Science Products, Inc., Boston, MA) as described (Dumont et al., 2003). All samples were fractionated using 3-12% gradient SDS-PAGE followed by autoradiography.

***TGF $\beta$  transcriptional reporter assays.*** PMECs (0.5x10<sup>6</sup> per well in 6 well plates) were transfected with 2  $\mu$ g pCAGA-Lux. After 24 h, cells were treated for 24 h with 2 ng/ml TGF $\beta$ 1 with or 1  $\mu$ M PS-341 or both. Firefly and *Renilla reniformis* luciferase activities were determined by using the Promega dual-luciferase assay system. The data were normalized utilizing the ratio of firefly to *R. reniformis* luciferase as previously described (Dumont et al., 2003).

***Transwell Invasion Assays.*** Cells were labeled with the lipophilic dyes Sp-DiOC18(3) (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Labeled cells were seeded in the

upper chamber of transwells fitted with Matrigel-coated, 8- $\mu$ M pore polycarbonate filters (Corning Inc. Life Sciences, Acton, MA). Lower chambers contained 2.5% FCS with or without 2 ng/ml TGF $\beta$ 1. After 24 h, cells were scraped from upper filter surfaces and cells on the lower surfaces were photographed using fluorescence microscopy. Fluorescence was quantified using Scion Image software.

***Rho, Rac1, Cdc42, and PI3K activity assays.*** For Rac1 assays, extracts were prepared in 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP-40, 5% glycerol, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2  $\mu$ g/ml aprotinin, and 2  $\mu$ g/ml leupeptin. Lysates (500  $\mu$ g) were incubated at 4°C with 2  $\mu$ g GST-bound Pak-binding domain (PBD) or rhotekin, (both from Upstate Biotechnology). As a positive control, 100 mM GTP $\gamma$ S (Sigma) was added to the lysis buffer in which extracts were prepared. Affinity precipitation was performed using 10  $\mu$ l of glutathione-Sepharose beads (Pharmacia) for 1 hour at 4°C. Samples were washed five times with Mg<sup>2+</sup> buffer (Upstate Biotechnology), suspended in 20  $\mu$ l of Laemmli sample buffer, and analyzed by western analysis using antibodies against RhoA, Cdc42, GST (all from Santa Cruz Biotechnology) and Rac1 (Transduction Laboratories).

PI3K activity was measured as described previously (Carpenter et al., 1997). In brief, cells were lysed with 1% NP40 in buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, and 0.1 mM Na orthovanadate) and a protease inhibitor cocktail (Roche). Lysates were precipitated with a p85 antibody (Upstate Biotechnology) and protein A sepharose 4B (Sigma). After washes, the immunoprecipitates were resuspended in 80  $\mu$ l of assay buffer (20 mM Hepes, 150 mM NaCl, 0.1 mM EDTA) containing 10  $\mu$ l of crude brain phosphoinositides (PI, 2mg/ml; Sigma) and 10  $\mu$ l of substrate [500  $\mu$ M cold ATP with 10  $\mu$ Ci/1  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP (sp. act. 6000 Ci/mmol; Amersham, Piscataway, NJ) in 200 mM Hepes, pH 7.5, and 50 mM MgCl<sub>2</sub>]. Following gentle

agitation for 10 min at room temperature, the reaction was terminated by the addition of 100  $\mu$ l of 1 N HCl and 200  $\mu$ l of chloroform:methanol (1:1). The radiolabeled lipids were extracted, concentrated, and separated by TLC using silica gel plates pretreated with 1% w/v K oxalate in a solvent system of n-propanol:2M acetic acid (65:35 v/v). Incorporation of  $^{32}$ P into PI was detected by autoradiography.

**Adhesion assays.** Adherent PMECs treated with 10  $\mu$ M LY580276 or DMSO vehicle for 24 h were lifted using EDTA and resuspended up to a density of  $10^5$  cells/ml in serum-free DMEM with or without LY580276. A 1-ml cell suspension was added to each of a 35-mm well coated with poly-D lysine, collagen I, laminin I, or fibronectin (Biocoat, Franklin Lakes, NJ). After 2 h, non-adherent cells were removed with 3 washes with PBS, and cells were photographed with \_\_\_\_\_ Incheol???

**Integrin expression.** PMECs were harvested by trypsinization when 70% confluent, washed with PBS, and resuspended in DMEM/10% FCS at a density of  $2 \times 10^7$  cells/ml. Primary antibody (2  $\mu$ l) directed against specific integrins was added to  $2 \times 10^6$  cells for 30 min on ice. Cells were washed with PBS x3, resuspended in 0.1 ml PBS, and then incubated with either phycoerythrin (PE)-conjugated (2  $\mu$ l; Caltag Laboratories, Burlingame, CA) or fluorescein-conjugated (1:250; Jackson ImmunoResearch Labs, Inc.) secondary antibodies for 1 h on ice. Cells were washed and resuspended in serum-free DMEM; PE+ cells were sorted using a FACS Calibur flow cytometer (Becton Dickinson) as described (REF). Integrin-specific antibodies were as follows:  $\alpha 1$  (555001),  $\alpha 2$  (553819),  $\alpha 5$  (555615), and  $\beta 1$  (550530)(all from BD Biosciences Pharmingen).

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## Figure Legends

**Figure 1.** Generation of MMTV-Alk5<sup>TD</sup> transgenic mice. **A.** Diagram of the linearized MMTV-Alk5<sup>TD</sup> transgene, including the MMTV-LTR promoter/enhancer, Ras leader sequence, HA-tagged human Alk5<sup>TD</sup> cDNA, and SV40 polyadenylation sequence cassette. Restriction sites are indicated. [P1, P2] and [P3, P4] are forward and reverse primer pairs, respectively, used for PCR-based genotyping. **B.** Founder animals were screened for transgene (Tg) sequences using primer pair [P1, P2]. **C.** Alk5 western analysis of IgG or HA immunoprecipitates (IP) of mammary gland lysates from 12-week old virgin F1 offspring. MWs are shown in kDa at right of each panel. **D.** Western analysis of whole tissue extracts from 12-week old virgin mice using an Alk5, HA, or  $\beta$ -tubulin antibodies. **E.** Immunofluorescence using HA or Alk5 antibodies. Nuclei are counterstained in DAPI as shown to the right of each antibody panel.

**Figure 2.** Delayed ductal extension and reduced apoptosis in MMTV-Alk5<sup>TD</sup> and MMTV-TGF $\beta$ 1<sup>S223/225</sup> mammary glands. **A.** Hematoxylin-stained whole mounts of (#4) mammary glands harvested at 4, 7, 12, and 20 weeks of age. Arrowheads indicate the centrally-located lymph node. Direction of ductal outgrowth is indicated below. **B.** PCNA IHC as a measure of proliferation within TEBs of 7-week old mice. Scale bars=25  $\mu$ m. **C.** Immunohistochemical detection of TUNEL+ nuclei in TEBs of 7-week old virgin mice. Mean percent of TUNEL+ nuclei ( $\pm$  S.D.) is indicated ( $n=8$  per group). Scale bar=50  $\mu$ m.

**Figure 3.** Periductal fibrosis in MMTV-Alk5<sup>TD</sup> mammary glands. **A.** H&E stained sections of mammary glands harvested from 20-week old virgin females. Arrows point to ductal epithelium which in MMTV-Alk5<sup>TD</sup> mammary is surrounded by a thick eosinophilic layer. **B.** Trichrome staining of glands from 20-week old virgin mice. Arrows, periductal collagen deposition. Scale bars=50  $\mu$ m. **C.** Immunofluorescent analysis of smooth muscle actin expression to detect myoepithelial cells in 20-week old virgin female mice. Arrows, myoepithelial (SMA+) cells. Scale bars=50  $\mu$ m. **D.** Immunofluorescent detection of the

active TGF $\beta$ 1 in mammary glands of 20-week old virgin mice as described in Methods. DAPI counterstaining for total nuclei is shown to the right of each panel.

**Figure 4.** Altered morphogenesis in MMTV-Alk5<sup>TD</sup> pregnant mammary glands. **A-C.** Whole mounts of glands harvested at 16.5 d.p.c. stained with hematoxylin (**A**), H&E stained sections at low power (**B**) and high power (**C**). Arrowhead, central lymph node. Asterisk indicates milk-filled lumen. Arrow indicates fibrotic region. Scale bars=50  $\mu$ m. **D-F.** Hematoxylin-stained whole mounts of glands harvested at day 10 of lactation (**D**), H&E staining of sections at low power (**E**) and high power (**F**). Arrows indicate periductal stroma.

**Figure 5.** Delayed post-lactational involution in MMTV-Alk5<sup>TD</sup> mice. **A.** Hematoxylin-stained whole mounts of glands harvested 7 and 21 d.p.f.w. Arrows, lymph node. Insets represent a high power magnification of an Alk5<sup>TD</sup> gland with lobuloalveolar structures. **B.** H&E-stained sections at 21 d.p.f.w. **C.** Trichrome staining of glands at 21 d.p.f.w. **D.** Detection of apoptotic cells in mammary glands at 3 and 7 d.p.f.w. Mean percent of TUNEL+ nuclei ( $\pm$  S.D.) is shown ( $n=8$  per group).

**Figure 6.** Smad protein downregulation and PI3K hyperactivity in MMTV-Alk5<sup>TD</sup> mammary glands. **A.** Western analysis of mammary gland lysates from 12-week old virgin mice. Antibodies used are indicated at left. **B.** Immunofluorescent localization of Smad2 in mammary ducts from 20-week old virgin females. Total nuclei were counterstained with DAPI. **C.** Northern analysis of total RNA harvested from mammary glands of 20-week old virgin mice. RNA blots were probed with a mouse Smad2 cDNA. The ethidium-stained RNA gel is shown in bottom panel. **D.** Left: Smad2 phosphorylation in PMECs cultured in serum-free medium  $\pm$  TGF $\beta$ 1 (2 ng/ml) for the indicated times. Antibodies used for immunoblot analysis are shown at left. Right:  $5 \times 10^4$  PMECs per well were seeded onto 6-well dishes in full medium and treated with the indicated concentrations of TGF $\beta$ 1 for 7 days. Medium and ligand were replenished every other day. Each bar represents the mean cell number  $\pm$  S.D. of three wells. **E.**

PI3K activity *in vitro*. PMECs from wild-type and MMTV-Alk5<sup>TD</sup> mice were treated or not with 2 ng/ml TGFβ1 for 1 h and lysed. Cell lysates were precipitated with p85 antibodies and immune complexes tested for their ability to generate phosphorylated inositides *in vitro* as indicated in Methods. PIP, PIP2, and PIP3 are identified by the arrows. Where indicated, the PI3K inhibitor Wortmannin (Wn) was added to the kinase reaction *in vitro* as a positive control.

**Figure 7.** Smad2/3 protein levels and function are restored by inhibition of the proteasome. **A.** Western analysis of lysates from PMECs infected with Ad.Smad2 or with Ad.Smad2/Ad.Smad4. Antibodies used for western analysis are shown at left. **B.** β-galactosidase staining of PMECs infected with Ad.LacZ. **C.** Western analysis of uninfected PMECs or infected with Ad.Smad2. Where indicated, PMECs were cultured in the presence of 1 μM PS-341 for 24 h. Antibodies used for western analysis are shown at right. **D.** PMECs were transfected with 2 μg p(CAGA)<sub>12</sub>-luciferase and treated with 2 ng/ml TGFβ1 with or without 1 μM PS-341 for 24 h. Dual luciferase activity in cell extracts was determined as described in Methods. Each bar represents the mean ± S.D. relative luciferase activity (RLU) of 4 wells. **E.** PMECs were cultured for 24 h in 0.5% serum with 2 ng/ml TGFβ1 or 1 μM PS-341, where indicated. Propidium iodide-stained nuclei were sorted for DNA content by flow cytometry as described in Methods.

**Figure 8.** Increased cell adhesion and integrin expression in MMTV-Alk5<sup>TD</sup> cells. **A.** Photomicrograph of PMECs cultured in 0.5% serum. **B.** Affinity precipitation of PMEC lysates using GST-rhotekin (for RhoA) and GST-PBD (for Rac1 and Cdc42) followed by western analysis of precipitates using the antibodies indicated at right. WT PMEC lysates were incubated in the presence of GTPγS prior to affinity precipitation as a positive control. **C.** Adherence of PMECs after 2 h to dishes coated with fibronectin, collagen I, or laminin (*n*=3). **D.** Integrin expression in PMECs was determined by flow cytometry using integrin-specific antibodies as indicated in Methods. The mean level of expression in

WT PMECs was given a value of 1 such that the expression level in MMTV-Alk5<sup>TD</sup> PMECs is shown relative to the control.

**Figure 9.** Reversal of Alk5<sup>TD</sup> effects with Alk5 kinase inhibitor. **A.** PMECs from MMTV-Alk5<sup>TD</sup> mice were incubated for the indicated times with increasing concentrations of LY580276 in the presence or absence of TGF $\beta$ . Cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. **B.** PI3K catalytic activity. MMTV-Alk5<sup>TD</sup> cells were treated with LY580276 or the PI3K inhibitor LY294002 for 24 h and lysed; cell lysates were precipitated with p85 antibodies and tested in a PIP-forming assay as indicated in Methods. Wortmannin was added to the *in vitro* reaction as a positive control. **C.** PMECs from Alk5<sup>TD</sup> mice were preincubated with LY580276 for 24 h (Incheol?) and then tested for their adherence after 2 h to collagen I-, fibronectin-, and laminin-coated dishes. **D.** Cells in simultaneously treated plates were subjected to flow cytometry with integrin-specific antibodies and FITC-conjugated anti-hamster IgG as indicated in Methods. The red overlays in the control panels represent histograms from cells treated with secondary antibody only. The green overlays in the LY580276-treated panels represent histograms from untreated cells for comparison.

**Figure 10.** Active Alk5 accelerates oncogene-induced mammary tumor metastases. **A.** Whole mounts of (#4) mammary glands from 7-week old WT, Neu, Alk5<sup>TD</sup> and Alk5<sup>TD</sup> x Neu mice. Inset illustrates better the increased lateral branching in mice expressing the Neu transgene. **B.** H&E-stained lung section from a representative Alk5<sup>TD</sup> and Alk5<sup>TD</sup> x Neu mouse 68 days after initial detection of a mammary tumor. **C.** Upper left: Mammary tumor latency of MMTV-Neu mice ( $n=14$ ) and MMTV-Alk5<sup>TD</sup> x Neu bigenics ( $n=13$ ). Upper right: Lung surface metastases per mouse. Lower left: Number of tumors per mouse of each genotype. Lower right: Tumor volumes in mm<sup>3</sup>. Each data point represents an individual tumor volume calculated by the formula [volume = width<sup>2</sup> x length/2]. The horizontal bars represent the mean values. For all these panels, statistical significance was calculated using the Student's *t* test. **D.** TGF $\beta$

receptor levels. PMEC from MMTV-Neu, MMTV-Alk5<sup>TD</sup>, and from bigenic mice were crosslinked with <sup>125</sup>I-TGFβ1 and then resolved by gradient SDS-PAGE as indicated in Methods. The locations of the type I (Alk5), II, and III TGFβ receptors are indicated. The double Alk5 band likely represents endogenous type I receptor as well as the HA-tagged Alk5 transgene product. MWs are shown at the right.

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