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We report that MMTV-DNIIR female mice have accelerated mammary gland differentiation during early pregnancy with impaired development during late pregnancy and lactation followed by delayed post-lactational involution. Mammary tumors, mostly carcinoma *in situ*, developed spontaneously in the MMTV-DNIIR mice with a long median latency (27.5 months). Invading carcinoma cells in MMTV-DNIIR animals showed loss of DNIIR transgene expression as determined by *in situ* hybridization. The data indicate that signaling from endogenous TGF-ßs not only plays an important role in normal mammary gland physiology but also can also suppress the early stage of tumor formation and contribute to tumor invasion once carcinomas have developed.

The ability of the TGF-ß signaling pathways to inhibit proliferation of many epithelial cells while stimulating proliferation fibroblasts remains a conundrum. We found that the absence of RhoA and $p160^{ROCK}$ activity in fibroblastic NIH3T3 cells and its presence in mammary epithelial NMuMG cells can at least partially explain the difference in the TGF-ß growth response. TGF-ß stimulation of $p160^{ROCK}$-mediated inactivation of the cdk-activating phosphatase, cdc25A, blocks G1-S progression in NMuMG cells. These results provide novel evidence that TGF-ß signaling through RhoA and $p160^{ROCK}$ links signaling components for epithelial transdifferentiation with regulation of cell cycle progression.

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Introduction

A number of studies have implicated the TGF-ß family, TGF-ß1, TGF-ß2 and TGF-ß3, in the regulation of mammary gland morphogenesis during puberty, lactation and involution. All three TGF-ß ligands are expressed in various parts of the mammary gland during different physiologic states in both the epithelium and the stroma (Robinson et al., 1991). Silberstein and Daniel (Silberstein and Daniel, 1987) provided the first direct evidence for TGF-ß involvement in mammary gland development by demonstrating that experimentally implanted TGF-ß1 causes rapid and reversible inhibition of terminal end bud growth.

Transforming growth factor-ß1 (TGF-ß1) causes growth inhibition in many non-transformed epithelial cell types (Akhurst and Derynck, 2001). The mechanism by which this occurs involve the binding and activation of TGF-ß type I and type II receptors, for the subsequent induction of parallel downstream signaling pathways (Blain and Massague, 2000). The established paradigm of TGF-ß type I receptor phosphorylation of Smad2 and Smad3 in conjunction with Smad4 recruitment is important in TGF-ß-mediated G1 cell cycle arrest (Kretzschmar and Massague, 1998). The SMAD signaling pathway up-regulates the expression of cyclin-dependent kinase (cdk) inhibitors, $p16^{INK4}$, $p15^{INK4}$, $p27^{Kip1}$, and $p21^{Cip1}$, thereby suppressing cyclin D and/or cyclin E associated hyper-phosphorylation of Rb and S phase progression (reviewed in (Massague et al., 2000)). Additionally, SMAD signaling is associated with the transcriptional down-regulation of the growth stimulatory factor, c-myc (Alexandrow and Moses, 1995; Feng et al., 2002; Yagi et al., 2002). This in turn down-regulates the cdk activating phosphatase, cdc25A (Jinno et al., 1994), a transcriptional target of c-myc (Bernardi et al., 2000; Galaktionov et al., 1996). However, fibroblastic NIH3T3 cells exhibit both TGF-ß-mediated SMAD activation and *c-myc* down-regulation, yet proliferation of these cells is stimulated by TGF-ß (Koskinen et al., 1991). Earlier findings suggest that growth stimulation of NIH3T3 cells by TGF-ß is due to increased cyclin E associated kinase activity (Sgambato et al., 1997).

We previously described TGF-ß activation of RhoA in various cell types with the exception of NIH3T3 cells (Bhowmick et al., 2001). Thus, we hypothesized that RhoA signaling may be involved in TGF-ß-mediated cell cycle arrest. The role of RhoA in cytoskeletal organization is well established, and it has also emerged as a mediator of cell cycle progression (Kimura et al., 2000; Olson et al., 1998; Song et al., 2000). The activation-state of RhoA is positively regulated by guanine exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (Hall, 1998). Downstream effector proteins of RhoA, such as $p160^{ROCK}$, PKN, mDia, rotekin, and citron, have been reported to mediate many specific processes (Aspenstrom, 1999). The role of $p160^{ROCK}$ and mDia are better understood as mediators of the formation and maintenance of stress fibers. Recently, PKN activation has been implicated in the delay of G2-M progression (Misaki et al., 2001). However, since physiological activation of RhoA is primarily a component of cytokine signaling, where other cytokine-specific signals are present, RhoA activity occurs in both a stimulus- and context-dependent manner.

The effect of endogenous TGF-ß signaling on mammary gland development has been examined in transgenic mice by expression of a truncated, kinase defective, dominant-negative type II TGF-ß receptor (DNIIR) driven by MMTV. Notably, we showed previously that DNIIR expression may not block all signaling downstream of the receptor at low expression levels

(Bhowmick et al., 2001). However, the resultant phenotype includes increased lobuloalveolar development in virgin female mice (Bottinger et al., 1997b; Gorska et al., 1998). MMTV-DNIIR mice developed by one group did not develop mammary tumors spontaneously, but did demonstrated enhanced mammary tumorigenesis in response to administration of the chemical carcinogen, 7,12-dimethylbenz(Abdollah et al.)anthracene.

In the present study, we have extended studies with the previously reported MMTV-DNIIR mice ((Gorska et al., 1998) to show for the first time that inhibition of TGF-ß signaling in mammary epithelial cells impairs full lactational differentiation and retards post-lactational involution. It is further demonstrated that multiparous MMTV-DNIIR female mice spontaneously develop mammary carcinomas with a long median latency (>2 yrs). Tumor invasion was not observed in carcinoma cells expressing the DNIIR transgene in the MMTV-DNIIR mice and bigenic mice also expressing the MMTV-TGF-α transgene. The data support the hypotheses that signaling from endogenous TGF-ßs not only plays an important role in normal mammary gland physiology but also can suppress the early stage of tumor formation and contribute to tumor invasion once carcinomas have developed.

Further, we define the role of RhoA in the context of TGF-ß regulation of the G1 cell cycle arrest. Our results suggest that TGF-ß-mediated $p160^{ROCK}$ activation is involved not only in epithelial to mesenchymal transdifferentiation (EMT) as we demonstrated previously (Bhowmick et al., 2001), but also the phosphorylation of cdc25A and inhibition of cdk2 activity. This suggests a common pathway by which cells regulate growth inhibition and the actin cytoskeleton organization.

Body

An important result of the current study was the spontaneous development of mammary tumors in mice expressing the DNIIR transgene indicating that endogenous TGF-ß signaling suppresses mammary tumor formation initiated by endogenous events. This is the first demonstration that abrogation of signaling from endogenous TGF-ßs can result in accelerated tumor formation. Most of the mammary tumors arising in the MMTV-DNIIR mice were non-invasive and were diagnosed as ductal carcinoma in situ (DCIS) based on the consensus criteria developed by the Mouse Models of Human Cancer Consortium (Cardiff et al., 2000). Of the 18 mammary tumors that developed in 15 mice (four mice had two separate mammary tumors), 13 were DCIS and five were invasive; two were invasive adenocarcinomas, and three were invasive adenosquamous carcinomas. It is of interest that adenocarcinomas tended to develop in older mice; three of the four adenocarcinomas developed in mice that were over two years of age at the time of first detection of the tumor. The typical histological appearance of DCIS while illustrates the appearance of a differentiated adenocarcinoma with areas of stromal invasion.

We (Bakin et al., 2000; Bhowmick et al., 2001) and others (Oft et al., 1998) have shown that TGF-ß can induce loss of adherens junctions in epithelial, and this is thought to be an important component of the complex set of changes necessary for epithelial cells, including carcinoma cells, to become motile and invasive. For this reason, we examined non-invasive and invasive carcinomas for presence of adherens junctions by immunohistochemical staining for ß-catenin, an important member of the adherens junction complex. DCIS tumors and well differentiated areas within invasive adenocarcinomas demonstrated junctional localization of ß-catenin, whereas areas of invasion consistently showed loss of junctional staining for ß-catenin, indicating that adherens junctions were absent in the invading cells. Strikingly, the areas of invasion were absent in DNIIR expression, as determined by *in situ* hybridization.

Previous studies have indicated that TGF-ß signaling in carcinoma plays an important role in the invasive capability of the cells (Dumont and Arteaga, 2000; McEarchern et al., 2001; Muraoka et al., 2002). Thus, we were interested in determining whether expression of the DNIIR transgene was lost in areas of carcinoma invasion. This was determined by northern blot analysis of RNA extracted from tumors and by in situ hybridization (ISH) using a transgene-specific probe as previously described (Gorska et al., 1998). We were able to extract sufficient tumor RNA for northern blotting from 10 of the 15 animals. All of the seven DCIS samples examined showed transgene expression, whereas one of three RNA samples from invasive carcinomas was negative by northern analysis. By ISH, tumors exhibiting the DCIS pattern consistently showed transgene expression similar to that observed in non-tumor ductal epithelial cells. However, poorly differentiated adenocarcinomas frequently showed no ISH evidence of transgene expression, and all areas of tumor invasion showed no transgene expression even when other areas of the same tumor had evidence of transgene expression. The data are consistent with TGF-ß signaling in carcinoma cells being involved in the processes of invasion.

The TGF-ßs inhibit proliferation of a variety of normal cell types, including most epithelial cells and hematopoietic cells (Akhurst and Derynck, 2001). However, non-transformed dermal fibroblasts and fibroblastic NIH3T3 cells are growth stimulated by TGF-ß (Clark et al., 1997; Sgambato et al., 1997). We reported that restoration of RhoA signaling through $p160^{ROCK}$ in

NIH3T3 cells converts the growth response to TGF-ß treatment from growth stimulation to growth inhibition. It was further demonstrated that inhibition of $p160^{ROCK}$ in NMuMG cells blocks the G1 arrest induced by TGF-ß. Investigations of the down stream effectors of RhoA/ $p160^{ROCK}$-mediated growth arrest demonstrated that TGF-ß rapidly stimulated $p160^{ROCK}$ translocation to the nucleus, phosphorylation, and inhibition of the cdk-activating phosphatase, cdc25A (Hoffmann et al., 1994; Jinno et al., 1994). This was associated with inhibition of cyclin E/cdk2 kinase activity, which likely plays a causal role in the inhibition of G1-S progression. These results provide evidence that signaling through RhoA and $p160^{ROCK}$ is important in TGF-ß inhibition of cell proliferation. (see appendix for details)

Key Research Accomplishments

- Defined a novel signaling pathways downstream of the TGF-ß receptor that involves RhoA and p160ROCK contributing to growth inhibition. P160ROCK inhibits cdc25A by phosphorylation. This pathway complements the known SMAD signaling pathway.
- We have demonstrated that partial blockage of TGF-ß signaling in mammary epithelial cells by DNIIR in female mice causes precocious mammary gland during puberty and early pregnancy with impaired development during late pregnancy and lactation and retarded post-lactational involution.
- We showed that abrogation of signaling from endogenous TGF-ßs can result in accelerated spontaneous mammary tumor development.
- We provide additional *in vivo* data supporting the hypothesis that, after tumors have developed, TGF-ß signaling can enhance carcinoma cell invasion and metastasis.

Reportable Outcomes

Research

Manuscripts

- Gorska A.E., Jensen R.A., Shyr Y., Aakre M.E., **Bhowmick N.A.,** Moses H.L.* (2003) Transgenic mice expressing a dominant-negative mutant type II TGF-ß receptor show impaired mammary development during pregnancy and lactation and enhanced mammary tumor formation. *Submitted.*
- **Bhowmick N.A.***, Ghiassi M., Brown K., Singh V., Aakre M., and Moses H.L. (2003) Cell cycle arrest by transforming growth factor-ß requires RhoA-mediated p160ROCK activation. *In revision, PNAS.*

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Abstracts

- **Bhowmick N.,** Chitil A., Gorska, A.G., Plieth D., Neilson E.G., Davidson J., Moses H.L. (2002) Limiting TGF-ß signaling in fibroblasts results in prostate intra-epithelial neoplasia. (oral) AACR special conference, TGF-ß Superfamily: Roles in the Pathogenesis of Cancer and Other Diseases. SanDiego, CA.

- **Bhowmick N.,** Chitil A., Dumont, N., Plieth D., Neilson E.G., Davidson J., Moses H.L. (2002) Limiting TGF-ß signaling in fibroblasts results in mammary and prostate gland displasia. American Cancer Society -Schilling Research Conference. Aptos, CA.

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- Aventis Pharmaceuticals, Leadership Development in Cancer Progam Award, Renewal
- AACR-AFLAC Scholar-in-Training Award

Products

CDNA construct, cell lines, and animal models developed

- Generated retroviral constructs for P160ROCK, cdc25A, and Ost

Conclusion

In summary, we have demonstrated that partial blockage of TGF-ß signaling in mammary epithelial cells in female mice causes precocious mammary gland during puberty and early pregnancy with impaired development during late pregnancy and lactation and retarded post-lactational involution. This is a common feature of transgenic expressing an oncogene in mammary epithelial (Guy et al., 1992; Kwan et al., 1992; Liu et al., 2001; Matsui et al., 1990; Sinn et al., 1987) and may contribute to the accelerated development of mammary tumors. Indeed, we show that abrogation of signaling from endogenous TGF-ßs can result in accelerated spontaneous mammary tumor development. While the effect of TGF-ß signaling in the mammary gland will likely be contextual depending on initiating events, these results indicate that signaling from endogenous TGF-ßs suppresses formation of mammary tumors initiated by endogenous events. Further, we provide additional *in vivo* data supporting the hypothesis that, after tumors have developed, TGF-ß signaling can enhance carcinoma cell invasion and metastasis. This provides a potentially important target for therapy (Muraoka et al., 2002).

The results of the present study are somewhat different from those reported by another group with a line of MMTV-DNIIR mice developed in their laboratory (Bottinger et al., 1997b). Mammary tumors did not develop spontaneously. There are several potential reasons for the lack of spontaneous mammary carcinomas in the previous study in comparison to the present report, including strain differences (FVB/N strain in the previous study vs. C57BL/6.DBA/2 in the present study) and level of transgene expression. However, enhanced mammary tumorigenesis was observed in response to administration of the chemical carcinogen, 7,12-dimethylbenz[a]anthracene by Bottinger et al (Bottinger et al., 1997a).

We previously reported that RhoA and $p160^{ROCK}$ are essential in TGF-ß-mediated EMT of NMuMG cells (Bhowmick et al., 2001) in accord with its well-established role in actin cytoskeletal organization (Hall, 1998). The context dependence of RhoA-$p160^{ROCK}$ activation in TGF-ß-mediated EMT and growth arrest is particularly intriguing. Here we also provide evidence for RhoA and $p160^{ROCK}$ involvement in TGF-ß-induced growth inhibition. However, Rho-family GTPases have been implicated previously in the positive regulation of cell cycle progression through the G1 phase, specifically, in Ras-mediated transformation as well as LPA stimulation of RhoA (Kranenburg and Moolenaar, 2001). The apparent discrepancy illustrates the complexity of the biological effects of RhoA and $p160^{ROCK}$ activation.

References

Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997). TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem *272*, 27678-27685.

Akhurst, R. J., and Derynck, R. (2001). TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol *11*, S44-51.

Alexandrow, M. G., and Moses, H. L. (1995). Transforming growth factor beta and cell cycle regulation. Cancer Res *55*, 1452-1457.

Aspenstrom, P. (1999). Effectors for the Rho GTPases. Curr Opin Cell Biol *11*, 95-102.

Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L., and Arteaga, C. L. (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem *275*, 36803-36810.

Bernardi, R., Liebermann, D. A., and Hoffman, B. (2000). Cdc25A stability is controlled by the ubiquitin-proteasome pathway during cell cycle progression and terminal differentiation. Oncogene *19*, 2447-2454.

Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L., and Moses, H. L. (2001). Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell *12*, 27-36.

Blain, S. W., and Massague, J. (2000). Different sensitivity of the transforming growth factor-beta cell cycle arrest pathway to c-Myc and MDM-2. J Biol Chem *275*, 32066-32070.

Bottinger, E. P., Jakubczak, J. L., Haines, D. C., Bagnall, K., and Wakefield, L. M. (1997a). Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12- dimethylbenz-[a]-anthracene. Cancer Res *57*, 5564-5570.

Bottinger, E. P., Jakubczak, J. L., Roberts, I. S., Mumy, M., Hemmati, P., Bagnall, K., Merlino, G., and Wakefield, L. M. (1997b). Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. Embo J *16*, 2621-2633.

Cardiff, R. D., Anver, M. R., Gusterson, B. A., Hennighausen, L., Jensen, R. A., Merino, M. J., Rehm, S., Russo, J., Tavassoli, F. A., Wakefield, L. M., *et al.* (2000). The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene *19*, 968-988.

Clark, R. A., McCoy, G. A., Folkvord, J. M., and McPherson, J. M. (1997). TGF-beta 1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event. J Cell Physiol *170*, 69-80.

Dumont, N., and Arteaga, C. L. (2000). Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. Breast Cancer Res 2, 125-132.

Feng, X. H., Liang, Y. Y., Liang, M., Zhai, W., and Lin, X. (2002). Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta- mediated induction of the CDK inhibitor p15(Ink4B). Mol Cell *9*, 133-143.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc. Nature *382*, 511-517.

Gorska, A. E., Joseph, H., Derynck, R., Moses, H. L., and Serra, R. (1998). Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. Cell Growth Differ *9*, 229-238.

Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. (1992). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A *89*, 10578-10582.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509-514.

Hoffmann, I., Draetta, G., and Karsenti, E. (1994). Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G1/S transition. Embo J *13*, 4302-4310.

Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. (1994). Cdc25A is a novel phosphatase functioning early in the cell cycle. Embo J *13*, 1549-1556.

Kimura, K., Tsuji, T., Takada, Y., Miki, T., and Narumiya, S. (2000). Accumulation of GTP-bound RhoA during cytokinesis and a critical role of ECT2 in this accumulation. J Biol Chem *275*, 17233-17236.

Koskinen, P. J., Sistonen, L., Bravo, R., and Alitalo, K. (1991). Immediate early gene responses of NIH 3T3 fibroblasts and NMuMG epithelial cells to TGF beta-1. Growth Factors *5*, 283-293.

Kranenburg, O., and Moolenaar, W. H. (2001). Ras-MAP kinase signaling by lysophosphatidic acid and other G protein- coupled receptor agonists. Oncogene *20*, 1540-1546.

Kretzschmar, M., and Massague, J. (1998). SMADs: mediators and regulators of TGF-beta signaling. Curr Opin Genet Dev *8*, 103-111.

Kwan, H., Pecenka, V., Tsukamoto, A., Parslow, T. G., Guzman, R., Lin, T. P., Muller, W. J., Lee, F. S., Leder, P., and Varmus, H. E. (1992). Transgenes expressing the Wnt-1 and int-2 proto-oncogenes cooperate during mammary carcinogenesis in doubly transgenic mice. Mol Cell Biol *12*, 147-154.

Liu, C. H., Chang, S. H., Narko, K., Trifan, O. C., Wu, M. T., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. (2001). Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J Biol Chem *276*, 18563-18569.

Massague, J., Blain, S. W., and Lo, R. S. (2000). TGFbeta signaling in growth control, cancer, and heritable disorders. Cell *103*, 295-309.

Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L., and Coffey, R. J. (1990). Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell *61*, 1147-1155

McEarchern, J. A., Kobie, J. J., Mack, V., Wu, R. S., Meade-Tollin, L., Arteaga, C. L., Dumont, N., Besselsen, D., Seftor, E., Hendrix, M. J., *et al.* (2001). Invasion and metastasis of a mammary tumor involves TGF-beta signaling. Int J Cancer *91*, 76-82.

Misaki, K., Mukai, H., Yoshinaga, C., Oishi, K., Isagawa, T., Takahashi, M., Ohsumi, K., Kishimoto, T., and Ono, Y. (2001). PKN delays mitotic timing by inhibition of Cdc25C: possible involvement of PKN in the regulation of cell division. Proc Natl Acad Sci U S A *98*, 125-129.

Muraoka, R. S., Dumont, N., Ritter, C. A., Dugger, T. C., Brantley, D. M., Chen, J., Easterly, E., Roebuck, L. R., Ryan, S., Gotwals, P. J., *et al.* (2002). Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest *109*, 1551-1559.

Oft, M., Heider, K. H., and Beug, H. (1998). TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol *8*, 1243-1252.

Olson, M. F., Paterson, H. F., and Marshall, C. J. (1998). Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. Nature *394*, 295-299.

Robinson, S. D., Silberstein, G. B., Roberts, A. B., Flanders, K. C., and Daniel, C. W. (1991). Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. Development *113*, 867-878.

Sgambato, A., Doki, Y., Schieren, I., and Weinstein, I. B. (1997). Effects of cyclin E overexpression on cell growth and response to transforming growth factor beta depend on cell context and p27Kip1 expression. Cell Growth Differ *8*, 393-405.

Silberstein, G. B., and Daniel, C. W. (1987). Reversible inhibition of mammary gland growth by transforming growth factor-beta. Science *237*, 291-293.

Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. (1987). Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell *49*, 465-475.

Song, Y., Wong, C., and Chang, D. D. (2000). Overexpression of wild-type RhoA produces growth arrest by disrupting actin cytoskeleton and microtubules. J Cell Biochem *80*, 229-240.

Yagi, K., Furuhashi, M., Aoki, H., Goto, D., Kuwano, H., Sugamura, K., Miyazono, K., and Kato, M. (2002). c-myc is a downstream target of the Smad pathway. J Biol Chem *277*, 854-861.

TGF-ß-induced RhoA and p160^{ROCK} activation is involved in the inhibition of cdc25A with resultant cell cycle arrest

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Running Title: **TGF-ß-mediated p160^{ROCK} inhibition of cdc25A**

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The ability of the TGF-ß signaling pathways to inhibit proliferation of many epithelial and hematopoietic cells while stimulating proliferation of fibroblasts remains a conundrum. Here we report that absence of RhoA and p160^{ROCK} activity in fibroblastic NIH3T3 cells and its presence in epithelial NMuMG cells can at least partially explain the difference in the TGF-ß growth response. Further, evidence is presented for TGF-ß stimulated p160^{ROCK} translocation to the nucleus and phosphorylation and inhibition of the cdk-activating phosphatase, cdc25A, with resultant inhibition of cdk2 activity and G1-S progression in NMuMG cells. These results provide novel evidence that signaling through RhoA and p160^{ROCK} is important in TGF-ß inhibition of cell proliferation and links signaling components for epithelial transdifferentiation with regulation of cell cycle progression.

Transforming growth factor-ß1 (TGF-ß1) causes growth inhibition in many non-transformed epithelial cell types (Akhurst and Derynck, 2001). The mechanism by which this occurs involve the binding and activation of TGF-ß type I and type II receptors, for the subsequent induction of parallel downstream signaling pathways (Massague et al., 2000). The established paradigm of TGF-ß type I receptor phosphorylation of Smad2 and Smad3 in conjunction with Smad4 recruitment is important in TGF-ß-mediated G1 cell cycle arrest (Kretzschmar and Massague, 1998). The SMAD signaling pathway up-regulates the expression of cyclin-dependent kinase (cdk) inhibitors, $p16^{INK4}$, $p15^{INK4}$, $p27^{Kip1}$, and $p21^{Cip1}$, thereby suppressing cyclin D and/or cyclin E associated hyper-phosphorylation of Rb and S phase progression (reviewed in (Massague et al., 2000)). Additionally, SMAD signaling is associated with the transcriptional down-regulation of the growth stimulatory factor, c-myc (Alexandrow and Moses, 1995; Feng et al., 2002; Yagi et al., 2002). This in turn down-regulates the cdk activating phosphatase, cdc25A (Jinno et al., 1994), a transcriptional target of c-myc (Bernardi et al., 2000; Galaktionov et al., 1996). However, fibroblastic NIH3T3 cells exhibit both TGF-ß-mediated SMAD activation and *c-myc* down-regulation, yet proliferation of these cells is stimulated by TGF-ß (Koskinen et al., 1991). Earlier findings suggest that growth stimulation of NIH3T3 cells by TGF-ß is due to increased cyclin E associated kinase activity (Sgambato et al., 1997).

We previously described TGF-ß activation of RhoA in various cell types with the exception of NIH3T3 cells (Bhowmick et al., 2001a). Thus, we hypothesized that RhoA signaling may be involved in TGF-ß-mediated cell cycle arrest. The role of RhoA in cytoskeletal organization is well established, and it has also emerged as a mediator of cell cycle progression (Kimura et al., 2000; Olson et al., 1998; Song et al., 2000). The activation-state of RhoA is positively regulated by guanine exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (Hall, 1998). Downstream effector proteins of RhoA, such as $p160^{ROCK}$, PKN, mDia, rotekin, and citron, have been reported to mediate many specific processes (Aspenstrom, 1999). The role of $p160^{ROCK}$ and mDia are better understood as mediators of the formation and maintenance of stress fibers. Recently, PKN activation has been implicated in the delay of G2-M progression (Misaki et al., 2001). However, since physiological activation of RhoA is primarily a component of cytokine signaling, where other cytokine-specific signals are present, RhoA activity occurs in both a stimulus- and context-dependent manner.

Here we define the role of RhoA in the context of TGF-ß regulation of the G1 cell cycle arrest. Our results suggest that TGF-ß-mediated $p160^{ROCK}$ activation is involved not only in epithelial to mesenchymal transdifferentiation (EMT) as we demonstrated previously (Bhowmick et al., 2001a), but also the phosphorylation of cdc25A and inhibition of cdk2 activity. This suggests a common pathway by which cells regulate growth inhibition and the actin cytoskeleton organization.

Results

TGF-ß-mediated growth arrest involves RhoA activation. Treatment of NMuMG cells, with TGF-ß results in growth inhibition, however NIH3T3 cells are growth simulated when treated with TGF-ß (Sgambato et al., 1997). To identify TGF-ß signaling pathways involved in growth stimulation of NIH3T3 cells, we initially analyzed SMAD signaling in both cell types. We found the SMAD pathway to be active, as illustrated by 3TP-Lux (containing PAI-1 and collagenase promoter elements) and CAGA (SMAD binding consensus site) reporter activation (Figure 1A). Both cell lines showed an 10-fold 3TP-Lux and an 3-fold induction of CAGA reporter activity. As a transcriptional reporter for RhoA activity we also examined induction in serum response element (SRE) by TGF-ß and found that NMuMG cells showed a 3-fold induction while NIH3T3 cells had little TGF-ß-mediated activation. The transcriptional reporter experiments were supported by western blotting for the phosphorylation of Smad2, indicating Smad2 activation by 1 h of TGF-ß treatment of both NMuMG and NIH3T3 cells (Figure 1B). The expression levels of Smad2 did not change in either cell line for the 12h of TGF-ß treatment. However, TGF-ß stimulated GTP-RhoA accumulation in NMuMG cells by 5 min, but did

not induce RhoA activation in NIH3T3 cells, as has been previously shown (Figure 1C) (Bhowmick et al., 2001a). The total RhoA expression levels did not change, and lysophosphatidic acid (LPA) stimulated RhoA activation in both NMuMG and NIH3T3 cells.

We next examined the disparity in TGF-ß signaling and growth inhibition in NIH3T3 cells by artificially stimulating the RhoA signaling pathway. Initially, ^{3}H-thymidine incorporation of NIH3T3 cells retrovirally transduced with wt-RhoA was compared to that of NIH3T3 cells infected with empty virus. In the presence of increasing concentrations of TGF-ß, there was a 25% increase in ^{3}H-thymidine incorporation of NIH3T3 cells, while wt-RhoA over-expressing NIH3T3 cells were no longer growth stimulated (Figure 2A). We hypothesized that a more physiological method of RhoA activation might be required to mimic the transient RhoA activation by TGF-ß in NMuMG cells. Thus a RhoA-GEF, Ost, was retrovirally transduced into NIH3T3 cells. Sixty percent growth inhibition was achieved in Ost expressing NIH3T3 cells in the presence of 10 ng/ml TGF-ß. To further examine the role of RhoA activation in the presence and absence of TGF-ß, we performed flow cytometry on rapidly growing NIH3T3 and Ost expressing NIH3T3 cell. Treatment with TGF-ß or expression of Ost alone did not significantly alter the cell cycle profile compared to control cells (Figure 2B). However, in Ost-expressing NIH3T3 cells TGF-ß elevated the G1 fraction of cells by 2 fold. In contrast, TGF-ß increased the fraction of Ost-expressing NIH3T3 cells in the G1 phase of the cell cycle two-fold indicating a G1 arrest. The retroviral infection of GFP cDNA showed greater than 95% infection efficiency in the NMuMG and NIH3T3 cells ((Bhowmick et al., 2001a) and data not shown),

Conversely, when RhoA signaling was specifically inhibited by the stable introduction of the RhoA-binding domain of rhotekin (RBD) (Ren et al.. 1999; Welsh et al., 2001) in NMuMG cells, TGF-ß-mediated growth inhibition was abrogated (Fig 3A). The results of these studies suggest a role for RhoA activation in TGF-ß-mediated cell cycle arrest.

P160^{ROCK} contributes to TGF-ß-mediated growth inhibition. We examined two downstream effectors of RhoA signaling, PKN and p160^{ROCK}, for their possible involvement in TGF-ß-mediated growth inhibition. Dominant-negative PKN and p160^{ROCK} constructs were retrovirally transduced into NMuMG cells. Dominant-negative PKN-transduced NMuMG cells displayed a similar dose response of TGF-ß-mediated growth inhibition as control vector infected cells (Figure 3B). In addition, treatment with a PKN inhibitor, 6-thioguanine, gave similar results (data not shown). However, TGF-ß treatment of dominant-negative p160^{ROCK} (Ishizaki et al., 1997) expressing cells displayed no growth inhibition. There was little effect on ^{3}H-thymidine incorporation by the expression of dominant-negative p160^{ROCK} alone, as reported by others similar results were found by the treatment of epithelial cell lines, MK, Mv1Lu, and NMuMG cells with a p160^{ROCK} specific inhibitor, Y27632 (Sahai et al., 1999; Uehata et al., 1997) (Figure 3C). Further, treatment with increasing concentrations of Y27632 in the presence of 1 ng/ml TGF-ß (a concentration sufficient to mediate ≥50% growth inhibition alone) showed antagonism of growth inhibition. Similarly, when TGF-ß concentrations were varied in the presence of 5 μM Y27632, there was reduced growth inhibitory response (data not shown).

To address whether Y27632-associated blocking of TGF-ß-mediated inhibition of DNA synthesis, as measured by ^{3}H-thymidine incorporation, correlated with effects on cell proliferation cell counting experiments were performed for a period of 3 days of treatment of NMuMG cells. While TGF-ß treatment resulted in reduced cell numbers, the combination treatment of Y27632 and TGF-ß displayed similar rates of proliferation with that of untreated cells grown in serum containing medium through the times course examined (Figure 3D). Together these results suggest a role for p160^{ROCK} activation in TGF-ß-mediated growth arrest.

TGF-ß-mediated phosphorylation of cdc25A involves p160^{ROCK} activity. Next we examined the impact of p160^{ROCK} signaling on TGF-ß-mediated regulation of select proteins involved in cell cycle

regulation at the G1/S boundary. This was achieved by examining rapidly growing NMuMG cells treated with 5 ng/ml TGF-ß for 0, 24, or 48 h either in the presence or absence of 5 μM Y27632. Cell lysates were examined for changes in Rb, $p160^{ROCK}$, cdc25A, and α-tubulin expression and phosphorylation by Western blotting. In the absence of TGF-ß, hyperphosphorylated Rb was present and there was high basal cdk2 activity, and cdc25A expression (Figure 4). TGF-ß treatment resulted in the appearance of predominantly hypo-phosphorylated Rb by 24 h while the co-treatment with Y27632 delayed this process by 24 h. TGF-ß did not alter the expression of $p160^{ROCK}$ but 48h treatment resulted in significant down regulation of cdc25A expression. Y27632 had little effect on the expression of $p160^{ROCK}$ or cdc25A. The expression level of α-tubulin was constant and used as a control for protein loading. To determine whether the delay in Rb hypophosphorylation resulting from the co-treatment of TGF-ß and Y27632 was due to altered cdk2 activity, *in vitro* kinases for immuno-precipitated cdk2 were performed. Y27632 antagonized TGF-ß-inhibition of cdk2 kinase activity. Thus, these studies suggest an important role for $p160^{ROCK}$ in TGF-ß-mediated regulation the state of Rb phosphorylation and cdk2 activity.

Since cdc25A is an important regulator of cdk2 activity, we chose to test whether TGF-ß can regulate cdc25A activity in a $p160^{ROCK}$-dependent manner by analyzing TGF-ß effects on the phosphorylation of cdc25A utilizing both *in vivo* and *in vitro* kinase assays. *In vivo* kinase assays were performed by metabolic labeling of NMuMG cells with [^{32}P]-orthophosphate followed by immuno-precipitation of cdc25A. This procedure showed low basal level of cdc25A phosphorylation in rapidly growing cells absent of exogenous TGF-ß (Figure 5A, lane 1). A successive increase in cdc25A phosphorylation was detected in cells 30 min, 1h, and 3 h following TGF-ß addition (Figure 5A, lane 2-4). The concomitant addition of Y23637 inhibited TGF-ß-stimulated cdc25A phosphorylation (Figure 5A, lane 5-6). However, since we were not able to co-precipitate cdc25A and $p160^{ROCK}$ (data not shown), it is possible that $p160^{ROCK}$ regulates cdc25A phosphorylation, but is not the kinase that directly acts on cdc25A. Thus, the regulation of cdc25A phosphorylation was directly determined by immuno-precipitating $p160^{ROCK}$ and cdc25A from NMuMG and NIH3T3 cells for use in *in vitro* kinase assays. $P160^{ROCK}$-associated cdc25A phosphorylation activity reached maximal levels by 1h of TGF-ß treatment and maintained an elevated level by 3h of treatment in NMuMG cells (Figure 5B). In contrast, TGF-ß did not stimulate $p160^{ROCK}$ activity in NIH3T3 cells, consistent with the lack of TGF-ß-mediated RhoA activity in these cells.

Further evidence for a role of RhoA signaling in cdc25A phosphorylation was determined by expressing Ost in NIH3T3 cells and examining TGF-ß-stimulation of cdc25A phosphorylation *in vivo*. [^{32}P]-orthophosphate labeled Ost-expressing NIH3T3 cells immuno-precipitated for cdc25A after 0, 1, and 3h of TGF-ß treatment showed increased phosphate labeling, whereas no labeling was observed in the absence of Ost expression (Figure 5C). This is in agreement with results from the *in vitro* kinase assay (Figure 5B). Together, these results indicate that TGF-ß activates cdc25A phosphorylation *in vivo* through a RhoA- and $p160^{ROCK}$-dependent manner.

TGF-ß inhibits cdc25A phosphatase activity. To better understand the mechanism of cdc25A phosphorylation, cell fractionation experiments were performed examining TGF-ß-mediated $p160^{ROCK}$ sub-cellular localization. Nuclear and cytoplasmic fractions were separated from NMuMG cells treated with TGF-ß over a time course of 12h. As a control, TGF-ß stimulation of Smad2 was examined, showing elevated detection in the nuclear fraction by 1h of treatment and persisting through 12h of treatment (Figure 6A). Nuclear translocation of $p160^{ROCK}$ was apparent at the 1h and 3h time points, while changes in the cytoplasmic expression of $p160^{ROCK}$ were not detectable within the 12 hours of TGF-ß incubation. Cdc25A was only detected in the nuclear fraction. The cell fractions were also examined for PCNA and RhoGDI expression as a control for nuclear and cytoplasmic

fractionations, respectively. There was no perceptible cytoplasmic expression of PCNA, and there was minimal RhoGDI detected in the nuclear fraction.

The impact of TGF-ß-mediated cdc25A phosphorylation on its enzymatic activity was determined by *in vitro* phosphatase assays. NMuMG cells were harvested after treatment with TGF-ß (5 ng/ml) through a 6 h time course in the presence and absence of Y23637 (5 μM). We found that the incubation of the phosphorylated-histone H1 substrate with immuno-precipitated cdc25A from rapidly growing cells showed efficient phosphatase activity. In contrast, cdc25A from TGF-ß-treated cells had diminished phosphatase activity as early as 30 min with progressively greater inhibition up to 6 h (Figure 6B). Treatment with Y23637 antagonized the TGF-ß inhibition of cdc25A de-phosphorylation of histone-H1. Together these results describe a mechanism by which TGF-ß may rapidly downregulate cdc25A activity to allow cell cycle arrest.

Discussion

The TGF-ßs inhibit proliferation of a variety of normal cell types, including most epithelial cells and hematopoietic cells (Akhurst and Derynck, 2001). However, non-transformed dermal fibroblasts and fibroblastic NIH3T3 cells are growth stimulated by TGF-ß (Clark et al., 1997; Sgambato et al., 1997). Here we report that restoration of RhoA signaling through $p160^{ROCK}$ in NIH3T3 cells converts the growth response to TGF-ß treatment from growth stimulation to growth inhibition. It is further demonstrated that inhibition of $p160^{ROCK}$ in NMuMG cells blocks the G1 arrest induced by TGF-ß. Investigations of the down stream effectors of RhoA/ $p160^{ROCK}$-mediated growth arrest demonstrated that TGF-ß rapidly stimulated $p160^{ROCK}$ translocation to the nucleus, phosphorylation, and inhibition of the cdk-activating phosphatase, cdc25A (Hoffmann et al., 1994; Jinno et al., 1994). This was associated with inhibition of cyclin E/cdk2 kinase activity, which likely plays a causal role in the inhibition of G1-S progression. These results provide evidence that signaling through RhoA and $p160^{ROCK}$ is important in TGF-ß inhibition of cell proliferation.

The NIH3T3 cells make an interesting model system to examine TGF-ß signaling since they are responsive to Smad signaling, yet refractory to TGF-ß-mediated growth inhibition and RhoA activation (Figure 1). Because we were specifically interested in TGF-ß-mediated RhoA signaling which is associated with a transient increase in RhoA activation, a constitutively active RhoA (V14RhoA) was purposely not expressed. We also wanted to avoid epithelial differentiation of NIH3T3 cells, as has been reported to occur under V14RhoA expression (Sander et al., 1999). The introduction of wild type RhoA attenuated TGF-ß-mediated growth stimulation. But, the expression of the Rho GEF, Ost, in NIH3T3 cells resulted in TGF-ß-stimulated RhoA activity and the rescue of growth inhibitory properties of TGF-ß (Fig 2). Although Ost is clearly not the endogenous GEF in these cells, the data suggest Ost is able to couple to the TGF-ß signaling pathway. It further suggested that TGF-ß regulation of cyclin E associated kinase activity is a result of the lack of TGF-ß-mediated RhoA activation. We also found the coincident inhibition of TGF-ß-stimulated RhoA activity and growth inhibition achieved by the expression of the rhotekin RhoA-binding domain (RBD) (Ren et al., 1999; Welsh et al., 2001) in NMuMG cells (Fig 2). These findings complement recent studies showing that blocking RhoA and $p160^{ROCK}$ activity resulted in early cyclin D1 expression and accelerated G1-S progression (Welsh et al., 2001) and further support our hypothesis for the requirement of RhoA activity in TGF-ß-mediated growth arrest. Further, RhoA signaling is an immediate mechanism of cdc25A enzymatic inhibition through post-translational modification that precedes the previously described TGF-ß-mediated transcriptional down regulation of cdc25A (Iavarone and Massague, 1997).

Thus a two step model for TGF-ß-mediated G1 checkpoint inhibition can be proposed where there is an initial mechanism for growth inhibition by way enzymatic inhibition of cdc25A followed by a secondary response associated with the various transcriptional up-regulation of cdk-inhibitory

proteins (Datto et al., 1995; Reynisdottir et al., 1995; Toyoshima and Hunter, 1994) and down-regulation of *c-myc* (Coffey et al., 1988) and *cdc25A* genes (Figure 7). The potential for TGF-ß-mediated phosphorylation and inhibition of cdc25A phosphatase activity is illustrated by the *in vivo* and *in vitro* kinase assays (Figures 5) and cdc25A phosphatase assay (Figure 6B), respectively. As suggested by the cell fractionation results in Figure 6A, $p160^{ROCK}$ translocates to the nucleus in order to inactivate cdc25A. $P160^{ROCK}$ translocation is presumably mediated through its activation by RhoA and the presence of a bipartite nuclear localization signal sequence at position 1020aa-1037aa. An analogous "two-wave" concept of G1-S inhibition during genotoxic stress is also found to target cdc25A (Bartek and Lukas, 2001). Chk1 and Chk2 proteins are reported to inactivate cdc25A by serine phosphorylation as a result of DNA damage (Falck et al., 2001; O'Neill et al., 2002; Sanchez et al., 1997). This may suggest that the preservation of genomic integrity by growth arrest is a fundamental response to both TGF-ß-mediated transdifferentiation and DNA damage.

The context dependence of RhoA-$p160^{ROCK}$ activation in TGF-ß-mediated EMT and growth arrest is particularly intriguing. We previously reported that RhoA and $p160^{ROCK}$ are essential in TGF-ß-mediated EMT of NMuMG cells (Bhowmick et al., 2001a) in accord with its well-established role in actin cytoskeletal organization (Hall, 1998). Here we also provide evidence for RhoA and $p160^{ROCK}$ involvement in TGF-ß-induced growth inhibition. However, Rho-family GTPases have been implicated previously in the positive regulation of cell cycle progression through the G1 phase, specifically, in Ras-mediated transformation as well as LPA stimulation of RhoA (Kranenburg and Moolenaar, 2001). The apparent discrepancy illustrates the complexity of the biological effects of RhoA and $p160^{ROCK}$ activation.

Experimental Procedures

Cell Culture. The NMuMG and NIH3T3 cells were purchased from the American Type Culture Collection (Rockville, MD) and propagated in DMEM with 10% fetal bovine serum. Insulin (10 μg/ml) was supplemented to the NMuMG media. The dominant-negative $p160^{ROCK}$ cDNA (from Dr. Shuh Narumiya, Kyoto University) subcloned into pBabe, retroviral vector, was transfected into amphotrophic into retrovirus producing Phoenix cells (from Dr. Gary Nolan, Stanford U.). Conditioned media was allowed to incubate with target cells for 24 h after which point the media was replaced for subsequent experiments (Bhowmick et al., 2001a; Kinsella and Nolan, 1996). Experiments were performed 48 h after transfection or infection.

RhoA-GTP assay. Activation of RhoA was detected in NMuMG and NIH3T3 cells by adsorbing cell lysates to Rho binding domain of rhotekin (RBD) to enrich for GTP-bound RhoA as described previously (Bhowmick et al., 2001a; Ren et al., 1999). Adsorbed and non-adsorbed lysates were then western blotted for RhoA (26C4, Santa Cruz Biotechnology, Santa Cruz, CA).

Thymidine Incorporation, Flow Cytometry, and Sequential Cell Counting. As described previously, cells treated for 48 h with TGF-ß were pulsed with ^{3}H-thymidine two hours prior to harvest. [^{3}H] incorporation was measured by scinilation counting to assess DNA synthesis (Bhowmick et al., 2001a). Cells treated for 24h with or without TGF-ß were stained with propidium iodide in sodium citrate buffer (containing 0.1% Triton X-100 and 5 μg/ml RNAse A) for 30 min prior to flow cytometric. A total of 50,000 cells per sample were analyzed by flow cytometry on a FACS-Calibur (Becton Dickinson Biosciences, San Jose, CA) and cell cycle profiles determined using Cell Quest software.

Sequential cell counts were performed through a 5 day time course using a Coulter counter.

Luciferase Reporter Assay. 3TP-Lux, CAGA, and SRE transcriptional activation was analyzed in cells transfected with the luciferase (firefly) reporter construct cDNAs indicated in conjunction with a CMV-driven renal luciferase plasmid (Promega Inc.). The firefly luciferase activity determined by luminometer measurements were normalized to renela activity and data are reported in relative luminescent units.

Immuno-precipitation and Western blot Analysis. Cells subjected to indicated treatments were lysed by sonication in HEPES lysis buffer [pH 7.5], as described previously (Bhowmick et al., 2001b). Cell lysates of equivalent protein amounts (400 μg) were immuno-precipitated with the indicated antibodies and protein G-sepharose beads (Sigma). The immuno-precipitants or cell lysates directly (40 μg) were separated by SDS-PAGE for subsequent immuno-detection by blotting on to PVDF membranes. Western blots were visualized by chemiluminescence (Pierce Inc., Rockford, IL) or alkaline phosphatase development (Sigma).

***In vivo* Phosphorylation of Cdc25A.** Rapidly growing cells were pre-incubated in phosphate-free DMEM supplemented with 0.2% FBS for 1h prior to [^{32}P]-orthophosphate metabolic labeling (0.9 mCi/ml) for an additional 1h. Then the cells were further incubated in the presence or absence of TGF-ß (25 ng/ml) or Y27632 (5 μM). The cells were subsequently, washed, harvested, and cdc25A immuno-precipitated (F-6, Santa Cruz Biotechnology, Santa Cruz, CA) for SDS-PAGE separation and visualized by auto-radiography.

***In vitro* Phosphorylation.** Cells incubated in the absence or presence of TGF-ß (5 ng/ml) for the indicated times were lysed and immuno-precipitated with anti-p160^{ROCK} antibody (Santa Cruz) for determining kinase activity *in vitro*. Cdc25A was immuno-precipitated from lysate of untreated cells for the p160^{ROCK} kinase assay. The protein G-sepharose beads from the two immuno-precipitations were mixed in reaction buffer containing 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 40 μM ATP, and [γ^{32}P]-ATP and incubated at 30°C for 15 min. Similarly cdk2 activity was determined from immuno-precipitated lysate using histone H1 (Sigma) as substrate. The products were terminated in Lameli buffer, resolved by SDS-PAGE, and visualized by auto-radiography.

Cell Fractionation. Rapidly growing NMuMG and NIH3T3 cells incubated with TGF-ß (5 ng/ml) were harvested through a 12h time course by the Nuclear and Cytoplasmic Extraction Reagent (NE-PER) from Pierce Inc., per manufacturer instructions. The respective nuclear and cytoplasmic fractions were analyzed by western blot analysis. Immuno-detection of proliferating cell nuclear antigen (PCNA, Santa Cruz) and RhoGDI (Santa Cruz) were used as a controls to identify nuclear and cytoplasmic cell fraction contamination, respectively.

Cdc25A Phosphatase Assay. As a substrate for the *in vitro* phosphatase reaction, histone H1 (10 μg) was phosphorylated as above with [γ^{32}P]-ATP and immuno-precipitated cdk2 from rapidly growing NIH3T3 cells. The reactions were carried out for 15 min at 30°C in a total volume of 20 μl containing tyrosine phosphorylated-histone H1 and immuno-precipitated cdc25A from TGF-ß and/or Y23637 NMuMG cells in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM DTT (Saha et al., 1997). The reactions were stopped by adding Lameli buffer, and analyzed by auto-radiography of SDS-PAGE.

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Akhurst, R. J., and Derynck, R. (2001). TGF-beta signaling in cancer--a double-edged sword, Trends Cell Biol *11*, S44-51.

Alexandrow, M. G., and Moses, H. L. (1995). Transforming growth factor beta and cell cycle regulation, Cancer Res *55*, 1452-7.

Aspenstrom, P. (1999). Effectors for the Rho GTPases. Curr Opin Cell Biol *11*, 95-102.

Bartek, J., and Lukas, J. (2001). Mammalian G1- and S-phase checkpoints in response to DNA damage, Curr Opin Cell Biol *13*, 738-47.

Bernardi, R., Liebermann, D. A., and Hoffman, B. (2000). Cdc25A stability is controlled by the ubiquitin-proteasome pathway during cell cycle progression and terminal differentiation, Oncogene *19*, 2447-54.

Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L., and Moses, H. L. (2001a). Transforming Growth Factor-beta1 Mediates Epithelial to Mesenchymal Transdifferentiation through a RhoA-dependent Mechanism, Mol Biol Cell *12*, 27-36.

Bhowmick, N. A., Zent, R., Ghiassi, M., McDonnell. M., and Moses, H. L. (2001b). Integrin ß1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity, J Biol Chem.

Clark, R. A., McCoy, G. A., Folkvord, J. M., and McPherson, J. M. (1997). TGF-beta 1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event, J Cell Physiol *170*, 69-80.

Coffey, R. J., Jr., Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B. E., and Moses, H. L. (1988). Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta, Mol Cell Biol *8*, 3088-93.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism, Proc Natl Acad Sci U S A *92*, 5545-9.

Falck, J., Mailand, N., Syljuasen, R. G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis, Nature *410*, 842-7.

Feng, X. H., Liang, Y. Y., Liang, M., Zhai, W., and Lin, X. (2002). Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta- mediated induction of the CDK inhibitor p15(Ink4B), Mol Cell *9*, 133-43.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc, Nature *382*, 511-7.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton, Science *279*, 509-14.

Hoffmann, I., Draetta, G., and Karsenti, E. (1994). Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G1/S transition, Embo J *13*, 4302-10.

Iavarone, A., and Massague, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15, Nature *387*, 417-22.

Ishizaki, T., Naito, M., Fujisawa, K., Maekawa, M., Watanabe, N., Saito, Y., and Narumiya, S. (1997). p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions, FEBS Lett *404*, 118-24.

Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. (1994). Cdc25A is a novel phosphatase functioning early in the cell cycle, Embo J *13*, 1549-56.

Kimura, K., Tsuji, T., Takada, Y., Miki, T., and Narumiya, S. (2000). Accumulation of GTP-bound RhoA during cytokinesis and a critical role of ECT2 in this accumulation, J Biol Chem *275*, 17233-6.
Kinsella, T. M., and Nolan, G. P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus, Hum Gene Ther *7*, 1405-13.
Koskinen, P. J., Sistonen, L., Bravo, R., and Alitalo, K. (1991). Immediate early gene responses of NIH 3T3 fibroblasts and NMuMG epithelial cells to TGF beta-1, Growth Factors *5*, 283-93.
Kranenburg, O., and Moolenaar, W. H. (2001). Ras-MAP kinase signaling by lysophosphatidic acid and other G protein- coupled receptor agonists, Oncogene *20*, 1540-6.
Kretzschmar, M., and Massague, J. (1998). SMADs: mediators and regulators of TGF-beta signaling, Curr Opin Genet Dev *8*, 103-11.
Massague, J., Blain, S. W., and Lo, R. S. (2000). TGFbeta signaling in growth control, cancer, and heritable disorders, Cell *103*, 295-309.
Misaki, K., Mukai, H., Yoshinaga, C., Oishi, K., Isagawa, T., Takahashi, M., Ohsumi, K., Kishimoto, T., and Ono, Y. (2001). PKN delays mitotic timing by inhibition of Cdc25C: possible involvement of PKN in the regulation of cell division, Proc Natl Acad Sci U S A *98*, 125-9.
O'Neill, T., Giarratani, L., Chen, P., Iyer, L., Lee, C. H., Bobiak, M., Kanai, F., Zhou, B. B., Chung, J. H., and Rathbun, G. A. (2002). Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach, J Biol Chem *30*, 30.
Olson, M. F., Paterson, H. F., and Marshall, C. J. (1998). Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1, Nature *394*, 295-9.
Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton, Embo J *18*, 578-85.
Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta, Genes Dev *9*, 1831-45.
Saha, P., Eichbaum, Q., Silberman, E. D., Mayer, B. J., and Dutta, A. (1997). p21CIP1 and Cdc25A: competition between an inhibitor and an activator of cyclin-dependent kinases, Mol Cell Biol *17*, 4338-45.
Sahai, E., Ishizaki, T., Narumiya, S., and Treisman, R. (1999). Transformation mediated by RhoA requires activity of ROCK kinases, Curr Biol *9*, 136-45.
Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25, Science *277*, 1497-501.
Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999). Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior, J Cell Biol *147*, 1009-22.
Sgambato, A., Doki, Y., Schieren, I., and Weinstein, I. B. (1997). Effects of cyclin E overexpression on cell growth and response to transforming growth factor beta depend on cell context and p27Kip1 expression, Cell Growth Differ *8*, 393-405.
Song, Y., Wong, C., and Chang, D. D. (2000). Overexpression of wild-type RhoA produces growth arrest by disrupting actin cytoskeleton and microtubules, J Cell Biochem *80*, 229-40.
Toyoshima, H., and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21, Cell *78*, 67-74.
Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension [see comments], Nature *389*, 990-4.
Welsh, C. F., Roovers, K., Villanueva, J., Liu, Y., Schwartz, M. A., and Assoian, R. K. (2001). Timing of cyclin D1 expression within G1 phase is controlled by Rho, Nat Cell Biol *3*, 950-7.

Yagi, K., Furuhashi, M., Aoki, H., Goto, D., Kuwano, H., Sugamura, K., Miyazono, K., and Kato, M. (2002). c-myc is a downstream target of the Smad pathway, J Biol Chem *277*, 854-61.

Figure Legend

Figure 1. TGF-ß-mediated RhoA activation in NMuMG cells, but not NIH3T3 cells. (*A*) NMuMG and NIH3T3 cells were transfected with 3TP-Lux, CAGA, and SRE luciferase-conjugated reporter constructs in combination with CMV-renela expression vector. Thereafter, the cells were incubated with or without TGF-ß before measuring luciferase activity. Luciferase activity was normalized for renela activity and mean ± S.D. of relative luciferase units (RLU) in triplicate measurements repeated two times. (*B*) NMuMG and NIH3T3 cells were incubated with TGF-ß for the indicated times, lysed, and equal protein amounts of total cell lysates were analyzed by western blotting with an antibody against the phosphorylated form and total Smad2 protein. (*C*) GTP loaded RhoA was measured by adsorbing cell lysates to GST-RBD beads and western blotted for RhoA. As a positive control, NMuMG cells treated for 5 min with LPA were assayed.

Figure 2. RhoA activation is involved in TGF-ß-mediated growth arrest. (*A*) NIH3T3 cells expressing RhoA, Ost, or control cDNA constructs were incubated with TGF-ß for 48h and pulsed with ^{3}H-thymidine for 2h prior to harvesting for measurement of thymidine incorporation. TGF-ß-mediated RhoA-GTP loading of Ost expressing NIH3T3 cells was examined (inset). The results are representative of at least 2 experiments done in triplicate ± S.D. (B) RhoA activation was examined through a time course of 15 min TGF-ß treatment in NIH3T3 cells expressing empty vector or Ost. Flow cytometry was performed on similar NIH3T3 cells in the presence or absence of TGF-ß.

Figure 3. Growth inhibition of NMuMG cells by TGF-ß requires RhoA-p160^{ROCK} activity. (*A*) NMuMG cells expressing RBD or control cDNA constructs were assayed for thymidine incorporation in the presence of increasing concentrations of TGF-ß. (*B*) Similarly NMuMG cells expressing a dominant-negative PKN (DNPKN), dominant-negative p160^{ROCK} (DNROCK), or control vector were subjected to thymidine incorporation assays. (*C*) Thymidine incorporation studies were done in MK, CCL64, Mv1Lu, and NMuMG cells in the presence of varying concentrations of TGF-ß or Y23637. (D) Cells treated as indicated were subjected to sequential counts during a 3 day period in triplicate ± S.D.

Figure 4. TGF-ß-mediated Rb hypo-phosphorylation involves p160^{ROCK} activity. (*A*) NMuMG cells treated with TGF-ß (5 ng/ml) for 0 or 24h in the presence or absence of Y23637 (5 μM) were harvested and analyzed by western blotting for the expression of Rb, p160^{ROCK}, cdc25A, and ß-tubulin. (*B*) *In vitro* kinase activity of immuno-precipitated cdk2 was determined by examining [^{32}P] labeling of histone H1 substrate. Cdk2 kinase activity was also examined in the presence of 5 μM, 2.5 μM, and 1 μM Y23637 in the presence of 5 ng/ml TGF-ß. Western blots for cdk2 were also done from the same cell lysate as a control.

Figure 5. p160^{ROCK} phosphorylates cdc25A. (*A*) NMuMG cells treated with TGF-ß and/or Y23637 were metabolically labeled with [^{32}P]-orthophosphate and cdc25A was immuno-precipitated from equal amounts of extracts. (*B*) *In vitro* kinase activity of immuno-precipitated p160^{ROCK} from TGF-ß treated NMuMG and NIH3T3 cells using cdc25A as substrate. Western blot for cdc25A was also done from the same cell lysate as a control. (*C*) *In vivo* p160^{ROCK} kinase activity was determined from NIH3T3 cells expressing Ost or control cDNA. The result is representative of at least 3 independent experiments.

Figure 6. P160^{ROCK} mediates cdc25A inhibition. (*A*) Nuclear and cytoplasmic fractions were isolated from TGF-ß treated NMuMG cells. Equivalent amounts of protein were western blotted for Smad2, p160^{ROCK}, cdc25A, PCNA, and RhoGDI. (*B*) Histone H1 phosphorylated by cdk2 using [γ^{32}P]-ATP

was incubated with immuno-precipitated cdc25A from NMuMG cells. The subsequent phosphorylation-state of histone H1 was analyzed by electrophoresis and auto-radiography.

Figure 7. Model for the TGF-ß two step down-regulation of cdc25A. Step 1, RhoA and SMAD proteins are activated. Subsequently $p160^{ROCK}$ is activated and translocates to the nucleus and inactivates cdc25A activity. Step 2, later RhoA and SMAD signaling results in the transcriptional (dotted line) regulation of *cyclinD*, cdk inhibitors and *c-myc* (Kretzschmar and Massague, 1998; Welsh et al., 2001; Yagi et al., 2002). Which in turn either physically (solid line) regulate cyclin D/cdk4/cdk6 activity or transcriptionally affect *cdc25A* expression (Iavarone and Massague, 1997; Kretzschmar and Massague, 1998).