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PRINCIPAL INVESTIGATOR: Xiao-Fan Wang, Ph.D.

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Xiao-Fan Wang, Ph.D.

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Durham, North Carolina 27710

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13. ABSTRACT *(Maximum 200 words)*

The overall goal of this research project is to explore the roles of TGF-ß and its signaling receptors in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. We have investigated the importance of the type II TGF-ß receptor (RII) in mediating the growth-inhibitory effect of TGF-ß in MCF-7 breast carcinoma cells through reconstituting an autocrine system of TGF-ß by the re-expression of RII in those cells. The results indicated that the loss of RII consists of a key step in breast carcinogenesis and restoration of the pathway could potentially reverse the tumorigenic phenotype of breast cancer cells. In addition, work is in progress to determine if two inhibitors of the cyclin-dependent kinases, p15 and p21, serve as effectors of the TGF-ß growth-inhibitory pathway in breast epithelial cells and whether estrogen could have an effect on this pathway. Results from further analysis in this direction will not only significantly contribute to an understanding of the molecular events leading to breast carcinogenesis, but also aid in the development of new therapeutics for breast cancer.

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A. INTRODUCTION

Breast cancer is the most common cancer in women in the United States. Endocrine therapy has proven to be beneficial in approximately one-third of breast cancer patients. However, the tumors inevitably progress to a state of hormone insensitivity and no longer respond to conventional endocrine therapies. Therefore, it is necessary to identify other molecular markers to monitor the pathological process of the disease in order to better evaluate patient prognosis and to elucidate molecular mechanism of breast cancer initiation, progression and metastasis in order to develop new reagents for subsequent treatments. The proposed study of TGF-ß growth inhibitory signaling pathways in breast carcinogenesis will significantly benefit both of these purposes.

The overall goal of this research project is to explore the roles of TGF-ß and its signaling receptors in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. TGF-ß's are a group of multifunctional polypeptide hormones which play important roles in many normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration. The effects of TGF-ß are highly diverse and cell specific. In normal breast epithelium, TGF-ß acts primarily as an inhibitor of proliferation. Loss of the TGF-ß growth inhibitory response in these normal cells may lead to a selective growth advantage which in turn could lead to uncontrolled proliferation in a subsequently transformed cell population. Since the TGF-ß signaling receptors are crucial components mediating TGF-ß growth regulatory effects, alterations in the expression profile of these receptors may be a cause of changes in TGF-ß responsiveness in breast epithelial cells. Furthermore, available evidence suggests that the expression of TGF-ß receptors may be a target subject to regulation by steroid hormones, such as estrogen. We intend to determine if this type of interactive regulation between two growth signaling pathways in breast epithelial cells, one positive and one negative, plays a significant role in the control of cell proliferation and subsequent tumorigenesis.

The other important aspect of TGF-ß function, besides its growth regulatory activity, is its potent effect on the activation of genes involved in extracellular matrix formation, cell adhesion and migration. The loss of growth inhibitory response to TGF-ß, while retaining gene activation responses in breast cancer cells, could give tumors a great growth advantage and metastasis potential. It is possible that different arms of the TGF-ß responsiveness (growth regulation vs. induction of specific genes) are controlled by different signaling pathways. A hypothesis will be tested whether the type II receptor is primarily responsible for initiating a cascade toward the growth regulatory pathway while the type I receptor for the gene induction pathway, even though both receptors are simultaneously required for mediating any TGF-ß signals across the membrane. Alternatively, the two arms of TGF-ß signaling pathways may diverge downstream of the two receptors at the level of substrates/effectors that interact specifically with the individual receptors. We intend to put our efforts on identifying and isolating these cellular components by using several strategies that have been effective in other studies.

In the last twelve months, our work has been focused on two main areas: evaluation of the functional role of the type II TGF-ß receptor in breast carcinogenesis and examination of the specific TGF-ß growth responses and their modulation by estrogen in breast cell lines. These studies were proposed as Specific Aims 1 & 2 in the original proposal and the progress in these areas are reported below. We have encountered certain technical difficulties in addressing the remaining questions outlined in the proposal for Year 1-2. Our effort in trying to overcome those difficulties is also reported below.

B. PROGRESS REPORT

Many breast cancer cell lines have lost their responsiveness to the TGF-ß growth-inhibitory signal to potentially gain a growth advantage. In certain cases, this loss of responsiveness was the result of loss of signaling receptors for TGF-ß, particularly the type II receptor (RII). To evaluate whether the loss of the RII consists a key event in the tumorigenic phenotype of those cancerous cells, we examined the role of TGF-ß RII in reversing the malignant phenotype of human breast cancer MCF-7 cells in collaboration with Dr. M. Brattain's lab at Medical College of Ohio. Parental MCF-7 cells were insensitive to TGF-ß and expressed undetectable levels type II receptor expression on cell surface. Stable transfection of a RII expression vector yielded three clones with various levels of exogenous RII mRNA and protein levels. Expression of RII also increased TGF-ß binding to RI in all three clones. Proliferation of RII-positive clones was inhibited by exogenous TGF-ß in a dose-dependent manner, whereas the control clones remained TGF-ß-insensitive. The RII transfectants were growth arrested in monolayer culture at saturation densities which were 41-66% of that of the Neo controls. They also showed reduced clonogenicity in soft-agarose. Tumorigenecity in ovariectomized estrogen-supplemented nude mice was delayed in transfectants with low RII levels. Transfectants expressing high levels of RII showed a large reduction in tumorigenicity as well as a longer delay in tumor formation. Tumor growth was associated with loss of exogenous RII expression in transfectants. These results indicate that when systems for TGF-ß signal transduction are intact, reconstitution of the TGF-ß receptor system can lead to reversion of malignancy in breast cancer cells lacking RII (appendix 1).

The conclusion that TGF-ß and its signaling receptor RII may play an important role in tumorigenesis has been reinforced by the recent finding that the RER colorectal cancer genotype, which is characteristic of hereditary nonpolyposis colorectal cancers (HNPCC), is closely associated with the loss of RII and consequently an escape from the negative growth regulation by TGF-ß in those cells. Again, in collaboration with Dr. Brattain's lab, an attempt was made to directly test the importance of RII mutations in RER colon carcinoma cells in determining the malignant phenotype by the restoration of RII expression in those cells. Similar to the studies in MCF-7 cells, stable RII transfectants of the RER+ HCT116 cells showed reduced clonogenicity in both monolayer culture and soft agarose; they were growth arrested at a lower saturation density than control cells, indicating the alterations in growth parameters of the transfected cells were due to the acquisition of autocrine-negative activity; tumorigenicity in athymic mice was reduced and delayed. These results indicate that reconstitution of TGF-ß autocrine activity by re-expression of RII can reverse malignancy in RER colon cancers, thus further verifying the notion that the malignancy of both breast cancer and HNPCC can be directly associated with the loss of RII expression (ref. 1, appendix 2).

The tasks outlined in the original proposal or Statement of Work also include histological studies of preisolated breast carcinoma specimens and the examination of TGF-ß receptor content in breast primary cultures and cell lines. Progress in undertaking those tasks has been limited primarily due to the lack of certain specific reagents, namely high titer specific antibodies against the type II and type I TGF-ß receptors which are absolutely necessary if the stated goals are to be achieved. We have tested and failed to detect the expression of the two receptors in breast cell lines through either Western blot analysis or histological staining by using all six commercially available antibodies. Those antibodies apparently could only be used to detect the overexpressed type II or type I receptors in receptor cDNA-transfected COS cells. This has been a common problem for all the researchers who have attempted to study the expression of endogenous TGF-ß receptors due to their low abundance in almost all cell types and tissues. In an attempt to eventually overcome this difficulty, we have in the past year developed four polyclonal antibodies: three against different domains of the type II TGF-ß receptor and one against the type I receptor. We have also established a collaboration with Dr. Carlos L. Arteaga

of the Dept. of Medical Oncology at Vanderbilt University, who is an expert in the special field of the roles of TGF-ß in breast carcinogenesis, to address this question. Our preliminary results indicate that at least one of the type II receptor antibodies, raised against the extracellular domain of the receptor, appears to recognize a single band about the right size of the type II receptor in MCF-7 cells by both immunoprecipitation and Western blot analyses (data not shown). We are in the process of confirming those results by examining several more human cell lines which are known to response to TGF-ß and thus presumably express the type II receptor. If this antibody is proven to be capable of detecting the type II receptor in those cell lines, we will extend the study to include the histological examination of the receptor content in both primary breast cells in culture and the preisolated breast carcinoma specimens. We are still in the process of assessing the capability of the anti-type I receptor antibody in recognizing endogenously expressed type I receptor in breast cell lines.

We have initiated studies on the examination of specific TGF-ß gene responses and growth responses, and modulation of these effects by estrogen and antiestrogens in breast cell lines, as outlined in the Statement of Work for Year 1-2. The main purpose of this examination is to determine the interactions between the growth-inhibitory pathway of TGF-ß and the growth-stimulatory pathway of estrogen in both normal and transformed breast epithelial cells. To achieve this goal, we needed to establish a system in which the biological effects of both TGF-ß and estrogen are well defined so that the biochemical nature of their pathways could be analyzed at the molecular level. For practical reasons, we initiated our studies on breast cell lines rather than on the primary breast epithelial cell cultures. We have screened a large number of available breast cell lines, including multiple sublines of the MCF-7 cell line, because many of them may have accumulated different genetic alterations while they are in culture for a long peroid of time. We treated those cell lines with TGF-ß, estrogen, estrogen plus progestron, or TGF-ß plus estrogen, respectively, and measured DNA synthesis using the ^{3}H-thymidine incorporation assay. Based on those studies, we have found three MCF-7 sublines to be responsive to both the TGF-ß growth-inhibitory signal as well as the estrogen growth-stimulatory signal (shown as Fig. 1 and 2). In at least two of these MCF-7 sublines, the TGF-ß growth-inhibitory effect was significantly reduced by the presence of estrogen, suggesting the estrogen signaling pathway may override the TGF-ß pathway. A separate breast cancer cell line, SKBR3, was found to be non-responsive to either TGF-ß or estrogen and has been used as a negative control in our studies. These results established a foundation upon which we could begin the task of dissecting the molecular mechanism of interactions between the two antagonizing pathways in breast epithelial cells. However, before we could study the question of how the interactions between the two pathways occur, we need to elucidate the molecular nature of the TGF-ß signaling pathway leading to cell cycle arrest in late G1 in those cells.

Beyond the level of TGF-ß signaling receptors, we still do not have adequete information on how the TGF-ß signal is transmitted to cause growth arrest in mammary epithelial cells. Previous examination of TGF-ß effects on the cyclin-cdk complexes by us and other groups has shown that at least part of the growth inhibitory effects of TGF-ß can be explained by a down regulation of the G1 cyclin dependent kinases (specifically cdk2 and cdk4) as well as a similar effect on their cyclin partners (specifically cyclin E). These G1 cdk's and cyclins appear to be essential for cells to progress through the G1 phase as shown by their temporal expression during the mid to late G1 as well as by dominant negative mutations of certain cdk's (specifically cdk2) which cause growth arrest of cells in G1. These experiments taken together suggest that TGF-ß may work through an inhibition of the members of the cell cycle machinery that are essential for G1 progression. There has been data of TGF-ß inhibition of cell cycle progression that can not be explained simply by cyclin/cdk down regulation. In addition to down regulation of cyclins and their kinase partners, for example, treatment of cells with TGF-ß appear to cause accumulation of factors which inhibit cyclin dependent kinase activity. A recently identified molecule, p27, has been shown to inhibit the function of cyclin E/cdk2 complexes upon

treatment of cells with TGF-ß. In addition to p27, there are several other small proteins, including p15 and p21, which have been recently cloned and shown to inhibit the activities of cyclin/cdk complexes (ref. 2, 3).

p21 is the best characterized of these small inhibitory molecules. It was originally cloned by three independent approaches: 1) by the two hybrid system using cdk2 as a bait molecule; 2) using a subtractive RNA cloning technique isolating molecules induced by p53; and 3) from microsequencing of a 21kd molecule which associates with the cyclin/cdk complexes upon immunoprecipitation. After the cloning, p21 was found to be induced by p53 and to act as a strong inhibitor of all cyclin/cdk complexes. p21 is present at high levels in senescent cells and overexpression of p21 causes growth arrest of cells in G1. All these lines of evidence make p21 an excellent candidate for an effector of the TGF-ß pathway by acting to inhibit the cyclin kinase activity and cause growth arrest in G1. If this is the case, then p53, a molecule which is known to be a important regulator of G1 progression, would also be implicated in the TGF-ß growth regulatory pathway.

In collaboration with Dr. Y. Xiong's lab, we have investigated this possibility by determining the RNA and protein levels of p21 in TGF-ß treated and untreated human kerotinacyte line, HaCaT, which is highly sensitive to TGF-ß growth inhibition. Results from Northern blot analysis, immunoprecipitation with specific antibodies against p21 as well as cdk4 and cyclin D, and Western blot analysis indicated that p21 level is increased significantly by TGF-ß treatment. We have further demonstrated that TGF-ß causes a rapid and significant transcriptional induction of the p21 promoter. These results suggest that p21 as a vital regulator of the cell cycle progression, can respond to both intracellular signals, such as DNA damage, and extracellular signals, such as TGF-ß. Previous findings have implicated $p27^{Kip1}$ as an effector mediating the TGF-ß growth inhibitory effect. More recently, p15, or MTSII (multiple tumor suppressor II), is induced in the same HaCaT cell line upon TGF-ß treatment. Taken together these results demonstrate that a single extracellular antiproliferative signal, TGF-ß, can act through multiple signaling pathways to elicit a growth arrest response (ref. 4). Our recent studies have further demonstrated that TGF-ß may act through a single pathway involving the transcription factor Sp1 and its family members to activate both the p21 and p15 promoters (ref. 5, 6).

We have since extended the study of TGF-ß regulation on cell cycle control to mammary epithelial cells to determine whether p21, p15, or p27 serve as downstream effectors for the TGF-ß growth inhibitory signal. We are currently using two mammary cell lines as model systems in the study: the human MCF-7 cells and the TGF-ß type II receptor transfectants derived from them as described above; and the mouse NMuMG cells derived from normal mammary gland (CRL 1636) which have been shown to be responsive to TGF-ß growth inhibitory signal. Any potential similarities and differences between the two types of cells in TGF-ß signal transduction may provide us with a unique opportunity to dissect and elucidate the complex functional interactions among those molecules and their potential roles in breast tumorigenesis. Our preliminary results indicate that the p21 protein was induced by TGF-ß in the RII transfectant of MCF-7 cells (data not shown), suggesting the TGF-ß mediated growth arrest in MCF-7 breast cancer cells is potentially mediated by p21. In the immediate future, we will focus our effort on three specific questions: 1. whether the levels and/activities of p21, p15, or p27 are induced upon TGF-ß treatment; 2. if p21 and/or p15 is induced, whether the TGF-ß signal acts to transcriptionally activate their promoters; 3. whether a common TGF-ß responsive element is contained in the p21 and the p15 promoters. Based on the results generated in those studies, we will determine whether the action of estrogen affects the induction or activation of these Cdk inhibitors. We will also use antiestrogens in those studies to determine the effects observed are specific for the action of estrogen. The breast cancer cell line SKBR3 will be used as a negative control in those analyses.

C. CONCLUSIONS

The findings that the loss of type II TGF-ß receptor is closely associated with the tumorigenic phenotype in both breast and colon carcinomas strongly support the notion that the TGF-ß growth-inhibitory signal may play an important role in maintaining a normal control in cell proliferation. Alterations in the expression of this key component, RII, consists a key step in the multistep process of carcinogenesis. Moreover, a restoration of the RII expression in these tumor cells may reconstitute the negative-growth loop and consequently reverse the tumorigenic phenotype of the cancerous cells. Further studies in this direction, particularly in combining with the original proposed analysis of the estrogen effect on the expression of RII in breast epithelial cells, will yield valuable information regarding the molecular events controlling both the normal and malignant growth of those cells.

The lack of specific reagents, the high titer antibodies against the two types of TGF-ß receptors, has limited the progress in undertaking the tasks of histological studies of preisolated breast carcinoma specimens and the examination of TGF-ß receptor content in both breast cell lines and primary cell cultures. However, our effort in overcoming this difficulty has yielded promising preliminary results and significant progress could be made in the coming years in addressing those questions. In the meantime, we have made significant progress in establishing a biological system in which the important question of how the two antagonizing growth-regulatory pathways of TGF-ß and estrogen interact in breast epithelial cells.

The findings that the inhibitors to the cyclin-dependent kinases, p15 and p21, may serve as effectors for the TGF-ß growth inhibitory pathway opens a great opportunity in studying cell cycle control in breast epithelial cells. Current studies focus on the dissection and elucidation of the signaling pathway linking the TGF-ß receptors at the plasma membrane and the cell cycle control machinery in the nucleus , as well as the point where estrogen may interfere with the signaling. Results from further analysis in this direction will not only significantly contribute to an understanding of the molecular events leading to breast carcinogenesis, but also aid in the development of new therapeutics for breast cancer.

D. APPENDIX

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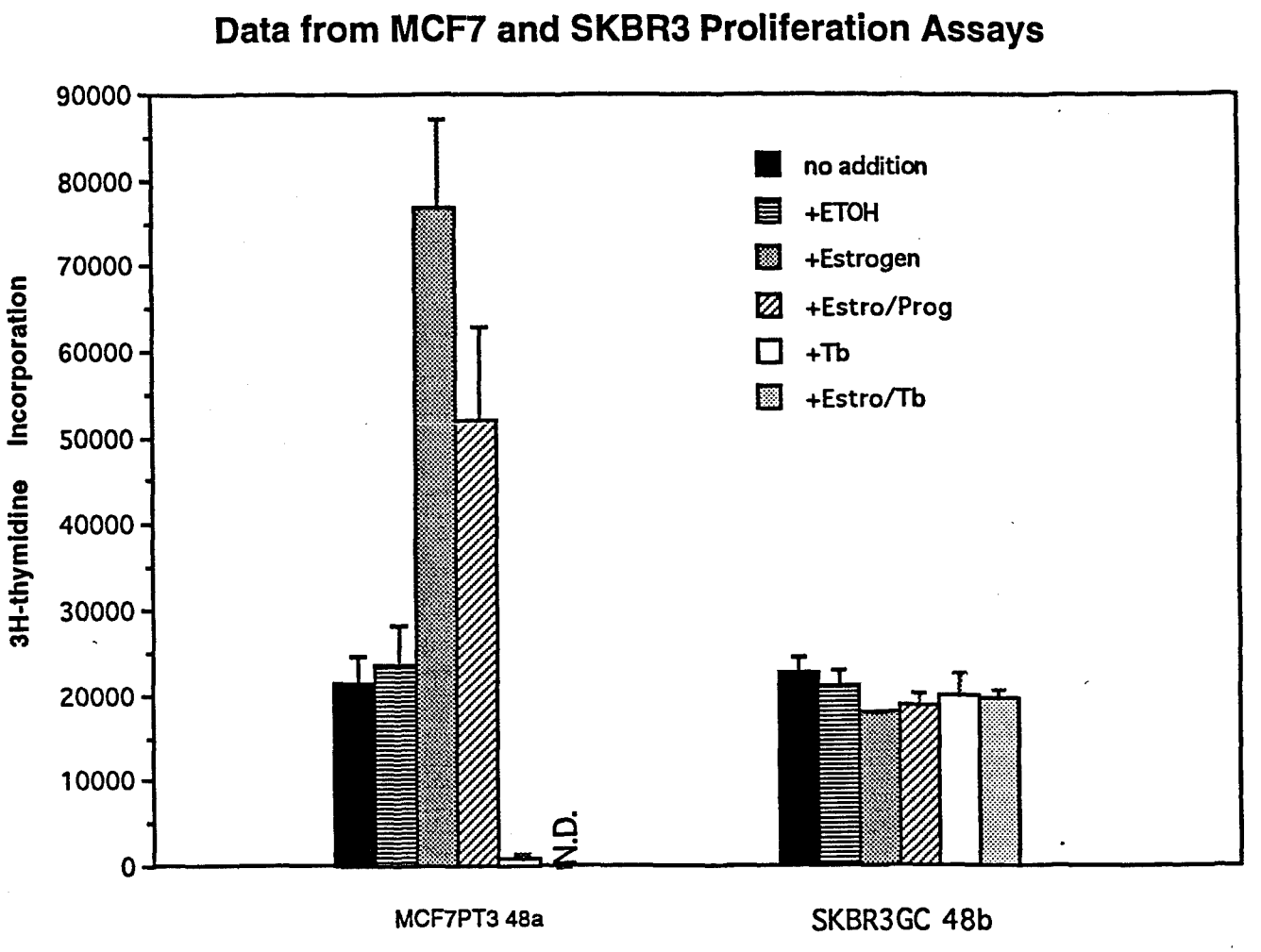

Fig. 1. MCF7 or SKBR3 cells were plated into 12-well plates at a density of 20,000 cells per well and treated for 20 hours with 100pM TGF-ß1, 10nM estrogen, 10nM progestron, or as indicated. The cells were labeled for the last 2 hours with 4 µCi of [^{3}H]thymidine, fixed in 10% TCA and lysed in 0.2 M NaOH. [^{3}H]thymidine incorporation into the DNA was measured with a scintilation counter.

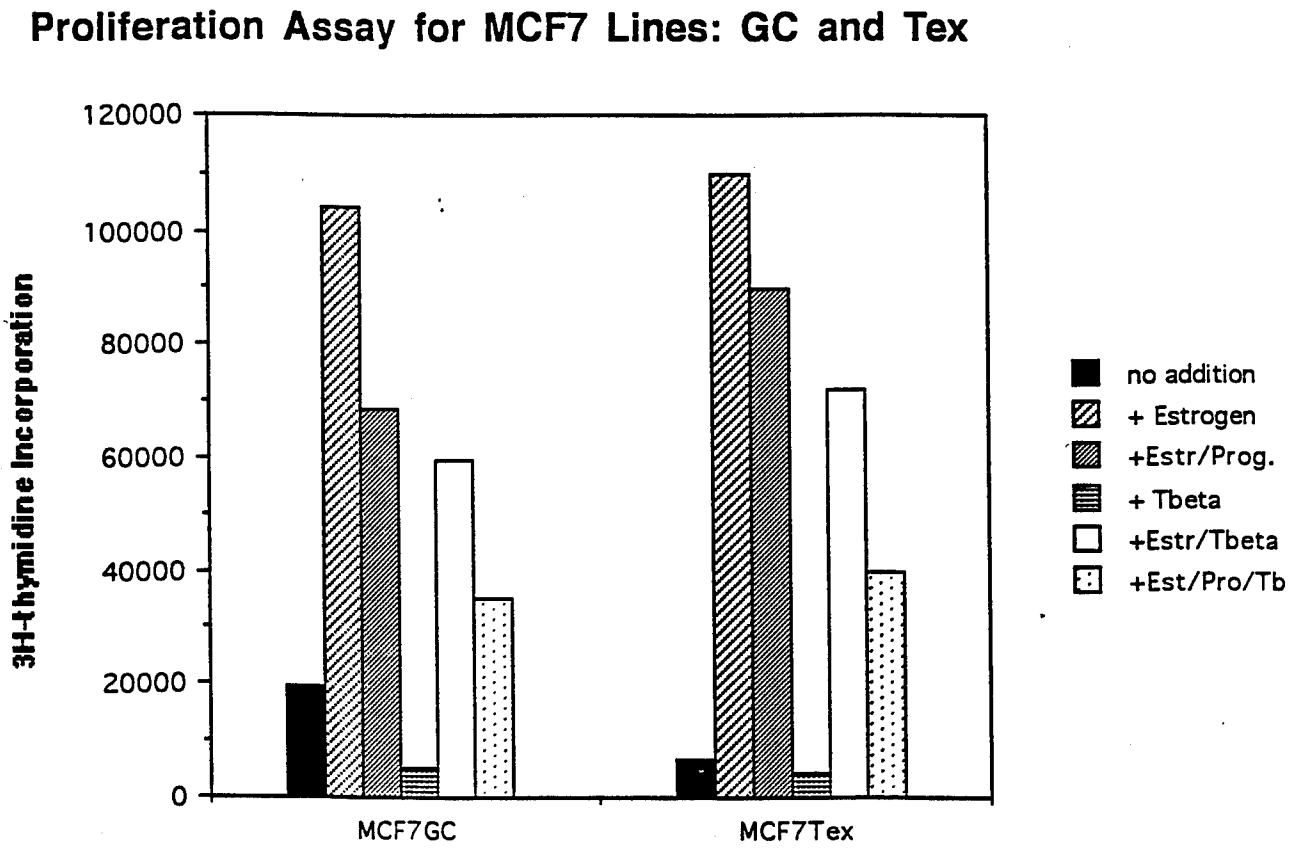

Fig. 2. Two MCF7 subline cells were plated into 12-well plates at a density of 20,000 cells per well and treated for 20 hours with 100pM TGF-ß1, 10nM estrogen, 10nM progestron, or as indicated. The cells were labeled for the last 2 hours with 4 µCi of [^{3}H]thymidine, fixed in 10% TCA and lysed in 0.2 M NaOH. [^{3}H]thymidine incorporation into the DNA was measured with a scintilation counter.

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Expression of Transforming Growth Factor β Type II Receptor Leads to Reduced Malignancy in Human Breast Cancer MCF-7 Cells*

(Received for publication, June 28, 1994, and in revised form, August 5, 1994)

LuZhe Sun‡, Gengfei Wu‡§, James K. V. Willson¶, Elizabeth Zborowska¶, Junhua Yang‡, Indunilka Rajkarunanayake‡, Jing Wang‡§, Larry E. Gentry‡, Xiao-Fan Wang‖, and Michael G. Brattain‡

From the ‡Medical College of Ohio, Department of Biochemistry and Molecular Biology, Toledo, Ohio 43614, the ¶Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, and the ‖Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

The role of transforming growth factor (TGF) β type II receptor in reversing the malignant phenotype of human breast cancer MCF-7 cells was examined. MCF-7 cells were insensitive to TGFβ_1 and expressed undetectable levels of cell surface TGFβ type I receptor (RI) and type II receptor (RII) by cross-linking with ^{125}I-TGFβ_1. Stable transfection of a RII expression vector yielded 3 transfectants with varying levels of exogenous RII mRNA and protein levels. Expression of RII also increased TGFβ_1 binding to RI in all 3 clones. Proliferation of RII-positive clones was inhibited by exogenous TGFβ_1 in a dose-dependent manner, whereas the control clones remained TGFβ-insensitive. The RII transfectants were growth arrested in monolayer culture at saturation densities which were 41–66% of that of the Neo controls. They also showed reduced clonogenicity in soft-agarose. Tumorigenicity in ovariectomized, estrogen-supplemented nude mice was delayed in transfectants with low RII levels. Transfectants expressing high levels of RII showed a large reduction in tumorigenicity as well as a longer delay in tumor formation. Tumor growth was associated with loss of exogenous RII expression in transfectants. The results indicate that when systems for TGFβ signal transduction are intact, reconstitution of the TGFβ receptor system can lead to reversion of malignancy in cells lacking RII.

Transforming growth factor β (TGFβ)[1] has been shown to regulate cell differentiation, proliferation, and extracellular matrix formation (Massagué, 1990; Moses *et al.*, 1990; Roberts and Sporn, 1991). Although in most systems active TGFβ represents a small percentage of total secreted TGFβ, it has been shown to act as an autocrine-negative growth regulator as evidenced by TGFβ neutralizing antibody stimulation of several cell lines (Arteaga *et al.*, 1990; Hafez *et al.*, 1990; Singh *et al.*, 1990). We have employed a TGFβ antisense expression technique to suppress autocrine TGFβ activity in two colon carcinoma cell lines (Wu *et al.*, 1992, 1993). Repression of endogenous TGFβ expression by antisense TGFβ_1 RNA increased tumorigenicity in nude mice by the transfected colon carcinoma cell lines. These results suggested that loss of autocrine TGFβ activity contributes to malignant progression and implied that restoration of TGFβ responsiveness in cells which had lost the capacity for inhibition could lead to reversal of malignant progression.

TGFβs elicit their effects by binding to cell surface receptors. Three major types of TGFβ-binding proteins are known to be widely distributed in most TGFβ-responsive cells. They are referred to as type I (RI), type II (RII), and type III or β-glycan (RIII). RI and RII are glycoproteins of 53- and 75-kDa, respectively, whereas RIII is a proteoglycan of 280–330 kDa (Massagué, 1990, 1992). Molecular cloning and functional analyses have shown that both RI and RII are serine/threonine kinase receptors necessary for TGFβ signal transduction (Lin *et al.*, 1992; Franzén *et al.*, 1993; Bassing *et al.*, 1994; Wrana *et al.*, 1992). Sequence analysis of RIII revealed that it has a large extracellular domain with a relatively small cytoplasmic domain that contains no obvious signaling motif (Lopez-Casillas *et al.*, 1991; Wang *et al.*, 1991). Recent studies showed that RIII is involved in ligand presentation to TGFβ signaling receptors (Lopez-Casillas *et al.*, 1993; Moustakas *et al.*, 1993). Thus, direct involvement of RI and RII in TGFβ signal transduction would suggest that loss of functional RI and/or RII expression could also contribute to the loss of autocrine TGFβ activity resulting in tumor progression.

Several TGFβ-resistant cell lines (Kimchi *et al.*, 1988; Arteaga *et al.*, 1988) have been shown to have low or undetectable RI and/or RII. MCF-7 cells are of particular interest in this regard as the TGFβ produced by these cells which lack the type II receptor has been implicated as contributing to progression (Arteaga *et al.*, 1993a).

Although a number of studies have demonstrated autocrine-negative growth regulatory activity of TGFβ isoforms both *in vitro* as well as *in vivo* (Brattain *et al.*, 1993), other studies also showed that TGFβ_1 overexpression may have a tumor-promoting effect. For example, overexpression of active TGFβ_1 in the E1A-transformed human embryonic kidney cell line 293 which is insensitive to TGFβ growth inhibition resulted in increased tumor incidence and enhanced tumor growth in nude mice (Arrick *et al.*, 1992). In this case and that of MCF-7 cells mentioned above, the insensitivity of the malignant cells to TGFβ growth inhibitory effect indicated that the overproduced TGFβ_1 presumably acted in a paracrine fashion with host cells to promote tumor growth.

We hypothesized that if the TGFβ insensitivity of MCF-7

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§ Performed as part of the requirements for the Ph.D. degree at Medical College of Ohio.

** To whom correspondence should be addressed. Tel.: 419-381-4324; Fax: 419-382-7395.

[1] The abbreviations used are: TGFβ, transforming growth factor-β; FBS, fetal bovine serum; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

cells was due to low TGFβ receptor expression, restoration of expression should restore the sensitivity of the cells to TGFβ growth inhibitory activity and reduce the malignancy. Since TGFβ binding to RI requires RII, loss of TGFβ binding to both receptors could in fact be due to the loss of RII expression alone as in the case of chemically-mutagenized mink lung epithelial cells (Laiho *et al.*, 1990; Wrana *et al.*, 1992). Recently, Inagaki and co-workers (1993) showed that loss of TGFβ sensitivity of a human hepatoma line after continued exposure to low $TGF\beta_1$ concentrations was due to loss of the RII gene and transfection of RII expression vector into the cells restored TGFβ sensitivity. We now show that re-expression of RII in MCF-7 cells also restored TGFβ sensitivity. More importantly, the RII-transfected cells showed reduced cloning efficiency in soft-agarose and reduced tumorigenicity in nude mice, indicating that restoration of TGFβ response can lead to reversion of malignancy in cells which have lost the capacity to respond to TGFβ-negative regulatory effects.

MATERIALS AND METHODS

Cell Culture—MCF-7 cells were originally obtained from the Michigan Cancer Foundation and stored frozen. The cell line was adapted to McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), pyruvate, vitamins, amino acids, and antibiotics (Mulder and Brattain, 1989). Working cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and routinely checked for mycoplasma contamination. MCF-7 limiting dilution clones were obtained by diluting the parental cells into 96-well tissue culture plates at 0.5 cell/well.

RII Expression Vector Construction and Transfection—The TGFβ RII cDNA (~3 kilobases) (Lin *et al.*, 1992) was subcloned by blunt-ended ligation into a tetracycline-controllable expression system kindly provided by Dr. H. Bujard at University of Heidelberg, Germany (Gossen and Bujard, 1992). This system consists of two vectors. One expresses a tetracycline-controlled transactivator (tTA) which is a fusion protein of the tetracycline repressor and the C-terminal domain of protein 16 of herpes simplex virus, and is constitutively expressed under control of human cytomegalovirus promoter. The other vector contains a multiple cloning site to accept any cDNA to be expressed followed by an SV40 polyadenylation sequence. The promoter region upstream of the multiple cloning site contains a minimal human cytomegalovirus promoter with heptamerized tet-operators. This promoter has no activity without the tTA. However, when the tetracycline repressor of the tTA binds to the tet-operators, the virion protein 16 domain of the tTA can activate the minimal human cytomegalovirus promoter activity to a very high level, whereas binding of tetracycline to the repressor domain of the tTA can almost completely inhibit tTA binding to the promoter. The orientation of the subcloned RII cDNA was checked by restriction enzyme digestion. In order to select stable transfectants, a neomycin-resistant gene under control of the mouse β-globin promoter was subcloned into the tTA-containing plasmid. This plasmid (1 μg) and the RII-containing plasmid (10 μg) were linearized and transfected into one of the MCF-7 limiting dilution clones designated MCF20 with a Bio-Rad electroporator at 250 V and 960 microfarads. The control cells were transfected with the Neo-containing plasmid (1 μg) and the cloning plasmid (10 μg) without RII cDNA. The transfected cells were plated in 10-cm tissue culture plates in McCoy's 5A medium containing 10% FBS for 2 days. Selection of stable transfectants were carried out by adding Geneticin (G418 sulfate; Life Technologies, Inc.) to the medium at 600 μg/ml. After 2 weeks, antibiotic-resistant clones were ring-cloned and expanded for screening of RII expression. Some of the control clones were ring-cloned and others were pooled. They were designated as MCF 20-Neo clone or MCF 20-Neo pool, respectively.

RNA Analysis—Total RNA from MCF-7 cells in culture and xenografts from tumor-bearing nude mice was extracted by guanidine thiocyanate homogenization and ultracentrifugation through a cesium gradient essentially as described by Chirgwin *et al.* (1979). In order to measure RI and RII mRNA levels, we constructed RI and RII riboprobe plasmids for RNase protection assays.

A fragment of human RI cDNA (588 bp) was obtained by polymerase chain reaction using the sense primer 5′-GACCAGTGTGCTTCGTCTGC-3′ and the antisense primer 5′-GCTGGCTTTCCTTGGGTACC-3′ according to the sequence published by Franzén *et al.* (1993). The polymerase chain reaction fragment was confirmed to be RI cDNA by restriction enzyme analysis and DNA sequencing, and was cloned into pBSK(−) plasmid (Stratagene Cloning Systems). After cutting the plasmid with *Hin*fI, an antisense riboprobe was synthesized *in vitro* using T_3 RNA polymerase which protects a 164-bp fragment of RI mRNA during RNase digestion. The RII riboprobe plasmid was constructed by subcloning a piece of RII cDNA from the 5′ end of the expression vector into pBSK(−) plasmids. This piece of DNA contained, in addition to 264-bp RII cDNA (*Nar*I-*Pst*I), some of the vector DNA sequence (18 bp) which is transcribed into recombinant RII mRNA when the RII expression vector is transfected into MCF-7 cells. As a result, the transfected RII mRNA could be distinguished from endogenous RII mRNA in RNase protection assays because the antisense riboprobe synthesized by T_3 RNA polymerase *in vitro* protected a larger fragment (282 bp) when hybridized with the transfected RII mRNA than when hybridized with endogenous RII mRNA.

RNase protection assays were performed as described previously (Wu *et al.*, 1993). Briefly, radioactive riboprobes were allowed to hybridize with the RI and RII mRNA in 40 μg of total RNA. The hybridization mixture was then treated with RNase A and RNase T_1 followed by proteinase K treatment. The protected fragments of the probes were analyzed by urea-PAGE and visualized by autoradiography. Actin mRNA protected by an actin riboprobe was used to verify equal sample loading as described previously (Wu *et al.*, 1993).

Receptor Cross-linking—Human $TGF\beta_1$ was purchased from R & D Systems (Minneapolis, MN) and iodinated by the chloramine T method as described by Ruff and Rizzino (1986). Binding and cross-linking of 200 pM ^{125}I-$TGF\beta_1$ to a cell monolayer on 35-mm tissue culture wells were performed as described by Segarini *et al.* (1987). Labeled cell monolayers were solubilized in 200 μl of 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride. Equal amounts of cell lysate protein were electrophoresed in 4–10% gradient SDS-PAGE under reducing conditions and exposed for autoradiography.

Mitogenesis Assay—[^{3}H]Thymidine incorporation was used to determine TGFβ sensitivity of control and RII-transfected cells to exogenous TGFβ treatment. The cells were plated in 24-well plates at a density of 1.5×10^4 cells per well in the presence of various concentrations of $TGF\beta_1$ (0.2–25 ng/ml). After 4 days of incubation, cells received a 2-h pulse with [^{3}H]thymidine (7 μCi; 46 Ci/mmol; Amersham Corp.). DNA was then precipitated with 10% trichloroacetic acid and the amount of [^{3}H]thymidine incorporated was analyzed by liquid scintillation counting in a Beckman LS7500 Scintillation Counter.

Soft-agarose Assay—Soft-agarose assays were performed as described previously (Wu *et al.*, 1992) to compare the clonogenic potential of control and RII-transfected cells. Briefly, cells (2×10^3 or 6×10^3) were suspended in 1 ml of 0.4% sea plaque-agarose in McCoy's medium containing 10% FBS and plated on top of a 1-ml underlayer of 0.8% agarose in the same medium in 35-mm tissue culture plates. The plates were incubated for 2 weeks in a humidified atmosphere of 5% CO_2 at 37 °C. Cell colonies were visualized by staining with 1 ml of *p*-iodonitrotetrazolium violet (Sigma).

Tumorigenicity Study—Cells from exponential cultures of MCF-7 transfectants were inoculated subcutaneously behind the anterior fore limb of 4-week-old athymic mice (athymic *nu*/*nu*-CWRU Cancer Center athymic mouse colony). Each mouse was inoculated on both sides. Mice had been ovariectomized and 1.7 mg of 17β-estradiol 60-day extended release pellets (Innovative Research of America, Toledo, OH) were placed subcutaneously as described by Gottardis *et al.* (1988) 1 week prior to cell inoculation. Mice were maintained in a pathogen-free facility. Growth curves for xenografts were determined by externally measuring tumors in two dimensions using a caliper. Volume (V) was determined by the following equation, where L is length and W is the width of the xenograft: $V = (L \times W^2) \times 0.5$. Xenografts were resected from anesthetized mice, flash frozen in liquid nitrogen, and pulverized for total RNA extraction.

RESULTS

MCF-7 Resistance to Inhibition by TGFβ—Arteaga *et al.* (1988) reported that estrogen receptor-positive breast cancer cells, including MCF-7 cells, were insensitive to TGFβ growth inhibitory activity and had reduced TGFβ binding. Since different phenotypes of MCF-7 cells have been observed from different laboratories (Osborne *et al.*, 1987), we initially examined the TGFβ sensitivity of the MCF-7 cells available in this laboratory. As shown in Fig. 1, our MCF-7 cells were resistant to $TGF\beta_1$ whereas mink lung CCL-64 cells showed a dose-dependent growth inhibition by $TGF\beta_1$. Receptor cross-linking to ^{125}I-$TGF\beta_1$ revealed that MCF-7 cells did not express detectable

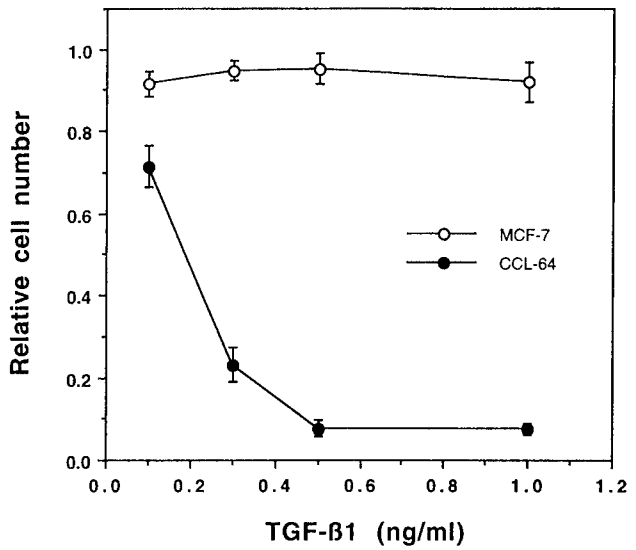

FIG. 1. **TGFβ insensitivity of MCF-7 cells.** MCF-7 cells (5×10^3 cells/well) and mink lung epithelial CCL-64 cells (2×10^3 cells/well) were plated in 96-well culture plates in the presence of TGF-β_1 at various concentrations in McCoy's 5A medium containing 10% FBS. After 4 days of incubation, the colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) assay (Carmichael *et al.*, 1987)) was used to determine relative cell numbers. Each point is the mean ± S.E. of four replicate wells.

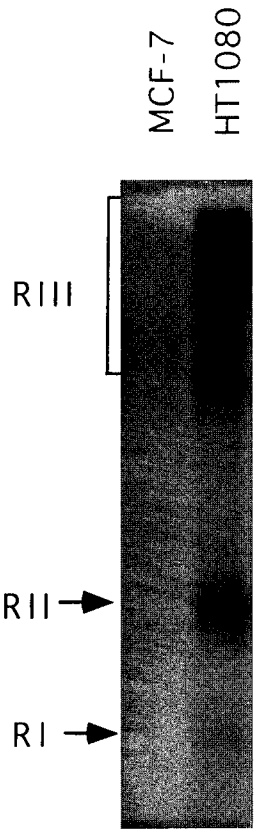

FIG. 2. **Measurement of cell surface TGFβ receptor expression.** Monolayer confluent cultures of MCF-7 cells and human fibrosarcoma HT1080 cells (used as a positive control) in 35-mm wells were incubated in the presence of 200 pM ^{125}I-TGFβ_1 for 3 h at 4 °C. The receptor-bound ^{125}I-TGFβ_1 was cross-linked with disuccinimidyl suberate. Cell lysates were electrophoresed in 4–10% gradient SDS-PAGE under reducing conditions. The ^{125}I-TGFβ_1-linked TGFβ receptor type I (RI), type II (RII), and β-glycan (RIII) were visualized after autoradiography.

levels of RI and RII on the cell surface while HT1080 human fibrosarcoma cells used as a positive control expressed substantial amounts of both receptor subtypes under the same conditions (Fig. 2). Therefore, the lack of cell surface RI and RII expression was apparently responsible for TGFβ insensitivity. This MCF-7 phenotype is essentially the same as that described by Arteaga *et al.* (1988, 1993a).

Expression of RII—Since it was shown that TGFβ binding to RI requires RII (Wrana *et al.*, 1992) and overexpression of RII in a TGFβ-resistant hepatoma cell line restored TGFβ sensitivity (Inagaki *et al.*, 1993), it was possible that the low level of TGFβ binding to RI in MCF-7 cells was merely due to lack of RII expression and restoration of RII alone might restore TGFβ sensitivity. Therefore, we transfected tetracycline-regulatable RII expression plasmids into a MCF-7 limiting dilution clone designated clone 20. Initial screening for RII positive transfectants with RNase protection assays yielded 3 clones (designated MCF 20-RII 1, 8, and 21, respectively) expressing transfected RII mRNA (Fig. 3). MCF 20-RII 8 expressed the lowest level of transfected RII whereas MCF 20-RII 1 expressed the highest level of RII. The untransfected parental clone (MCF 20) and the control transfectants (MCF 20-Neo pool) expressed a low level of endogenous RII. It appears that the expression of the transfected RII increased endogenous RII mRNA levels in MCF 20-RII 1 and 21. Since it is not possible to distinguish endogenous protein from transfected RII protein, the significance of this RNA band is not clear. However, it does raise the possibility that the type II receptor can be autoregulated through the restoration of the TGFβ signaling pathway in MCF-7 cells. All the clones expressed similar levels of RI mRNA. Consistent with mRNA levels, MCF 20-RII 1 also expressed a higher level of RII protein than MCF 20-RII 21 as shown by receptor cross-linking assays (Fig. 4, *lanes 3* and *7*). MCF 20-RII 8 expressed the lowest level of RII protein (data not shown). Similar to MCF-7 parental cells, the MCF 20 clone and a MCF 20-Neo clone showed very low levels of both RI and RII expression (Fig. 4, *lanes 1* and *2*). However, overexpression of RII also increased TGFβ_1 binding to RI as shown in MCF 20-RII 1 and 21. The increased ^{125}I-TGFβ binding to RI and RII could be competed by 100-fold cold TGFβ_1 as shown in *lanes 4* and *8* of Fig. 4. As expected, addition of tetracycline in the tissue culture medium for 4 days before the receptor cross-linking assay showed a dose-dependent suppression of RII expression as well as RI binding (Fig. 4, *lanes 5, 6, 9,* and *10*). Thus, our results also suggest that TGFβ binding to RI requires RII in MCF-7 cells.

Restoration of TGFβ Sensitivity—We next examined whether overexpression of RII as well as increased TGFβ binding to RI and RII could restore the sensitivity of the transfectants to TGFβ growth inhibitory activity. When the three RII transfectants were plated in 24-well culture plates in the presence of TGFβ_1 at concentrations from 0.2 to 25 ng/ml for 4 days, DNA synthesis was significantly inhibited in a dose-dependent manner (Fig. 5). The IC_{50} was approximately 0.4 ng/ml for MCF 20-RII 1, 0.8 ng/ml for MCF 20-RII 21, and 0.9 ng/ml for MCF 20-RII 8. The 40% inhibition of clone 1 at 0.2 ng/ml is similar to the 50% inhibition of CCL64 cells at this level of TGFβ exposure. Although MCF 20-RII 8 had a lower RII expression level than MCF 20-RII 1 or MCF 20-RII 21, it appears that this level of RII expression is adequate to confer a growth inhibitory response to exogenous TGFβ_1 in this type of assay. Both MCF 20 and MCF 20-Neo controls were insensitive to the treatment with TGFβ_1 over the same range of concentrations as the parental MCF-7 cells shown in Fig. 1.

Growth Arrest at Low Cell Density—Growth curves of the Neo pool and the three RII transfectants were generated to determine whether RII expression led to alteration of growth parameters in tissue culture (Fig. 6). Growth rates (doubling

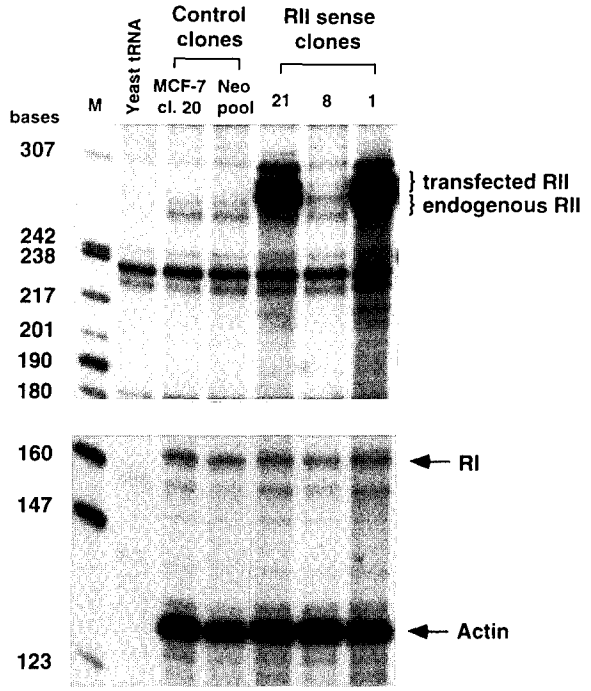

FIG. 3. **Expression of TGFβ RII mRNA in MCF 20 transfectants.** MCF-7 limiting dilution clone 20 (MCF 20) was stably transfected with RII expression plasmids and selected with Geneticin. The stable clones were compared to untransfected MCF 20 and transfection-control MCF 20-Neo pool for RII mRNA levels with RNase protection assays. Three RII sense clones designated MCF 20-RII 8, 21, and 1, respectively, were obtained which expressed increasing amounts of transfected RII mRNA with relatively similar amounts of endogenous RII and RI mRNA levels. Actin mRNA levels were used to verify equal sample loading.

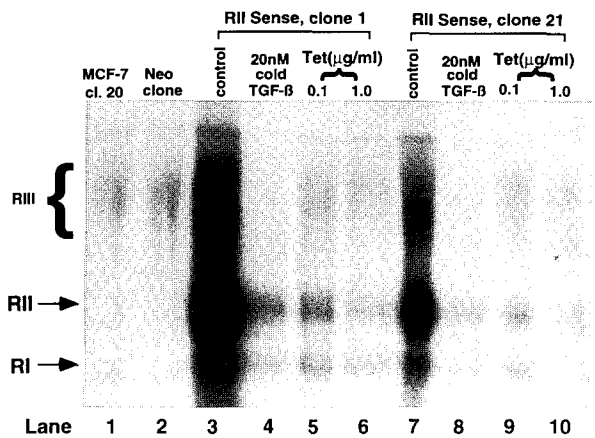

FIG. 4. **Expression of TGFβ RII protein in MCF 20 transfectants.** Receptor cross-linking assays were used to verify that RII clones expressing transfected RII mRNA also expressed RII protein. Confluent monolayer cultures of the MCF 20, a MCF 20-Neo clone, and MCF 20-RII 1 and 21 were incubated with 200 pM ^{125}I-TGFβ$_1$ alone (*lanes 1–3* and *7*) or in the presence of 20 nM cold TGFβ$_1$ (*lanes 4* and *8*) for 3 h at 4 °C. For *lanes 5, 6, 9,* and *10,* the cells were cultured in the presence of tetracycline at 0.1 or 1 μg/ml for 4 days before the receptor cross-linking assay. The media were replenished once during the 4-day period. The receptor-bound ^{125}I-TGFβ$_1$ was cross-linked with disuccinimidyl suberate. Cell lysates containing equal amounts of protein were electrophoresed in 4–10% gradient SDS-PAGE under reducing conditions. The ^{125}I-TGFβ$_1$-linked TGFβ receptors were visualized after autoradiography.

time) of the MCF 20-Neo pool and RII transfectants were similar at the exponential growth phase. The growth of the three RII transfectants reached plateau phase on day 9, while the growth of the MCF 20-Neo pool reached plateau phase on day 11. As a result, the saturation densities of the RII transfectants were only 41–66% that of the MCF 20-Neo pool on day 11. MCF 20-RII 1 (with the highest RII expression) had the lowest cell density and MCF 20-RII 8 (with the lowest RII expression) had the highest cell density among the RII transfected clones.

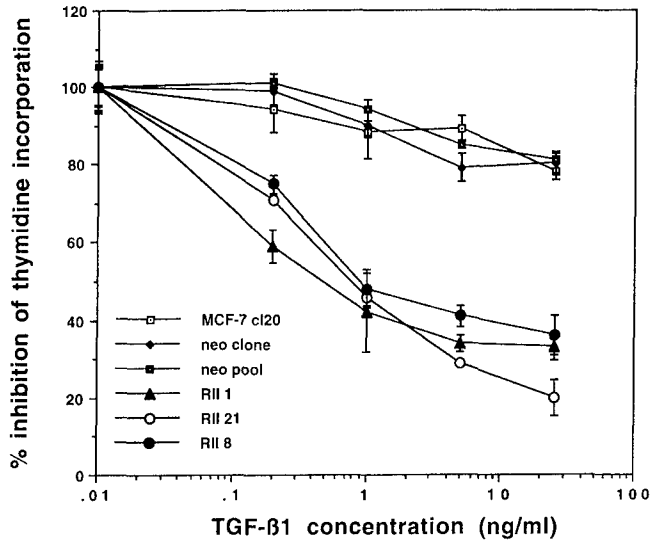

FIG. 5. **Growth inhibition of RII transfectants by TGFβ$_1$.** Untransfected MCF 20, MCF 20-Neo clone, MCF 20-Neo pool, and the three RII transfectants were plated in 24-well plates at 1.5 × 10^4 cells per well in the presence of various concentrations of TGFβ$_1$. DNA synthesis was assayed 4 days later by measuring [^{3}H]thymidine incorporation during a 2-h pulse. The thymidine incorporation in the presence of TGFβ$_1$ was calculated as percent of the thymidine incorporation in the absence of TGFβ$_1$ for each clone. Each point is the mean ± S.E. of 3 replicates.

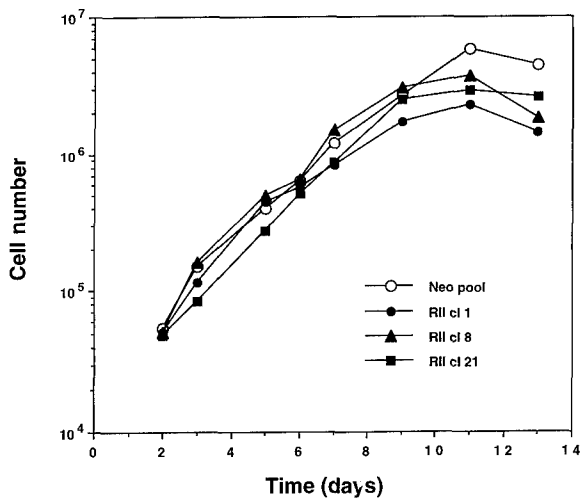

FIG. 6. **Growth curves of MCF 20-Neo pool and RII transfectants.** MCF 20-Neo pool and the three RII transfectants were plated in 6-well tissue culture plates at 8 × 10^4 cells per well. Cell numbers of duplicate wells were determined using a hemeocytometer at each time point and the average of the two numbers are presented. The mean variation between the duplicate measurements was 9.3% with a standard deviation of 3.6%.

Anchorage Independent Growth—To assess the effect of RII expression on the malignant properties of MCF-7 cells, we compared the ability of the control and RII-transfected cells to form colonies in soft-agarose as this property is considered to be reflective of malignancy. Control cells and each of the RII transfectants were tested for anchorage independent growth in 6-well culture plates at 2 × 10^3 and 6 × 10^3 cells/well. After 2 weeks of incubation, culture growth was terminated and colonies were visualized by staining with *p*-iodonitrotetrazolium violet staining. As shown in Fig. 7, the cloning efficiencies of the MCF 20-Neo pool and the MCF 20-Neo clone were similar at both cell numbers. In contrast, all 3 RII transfectants showed a

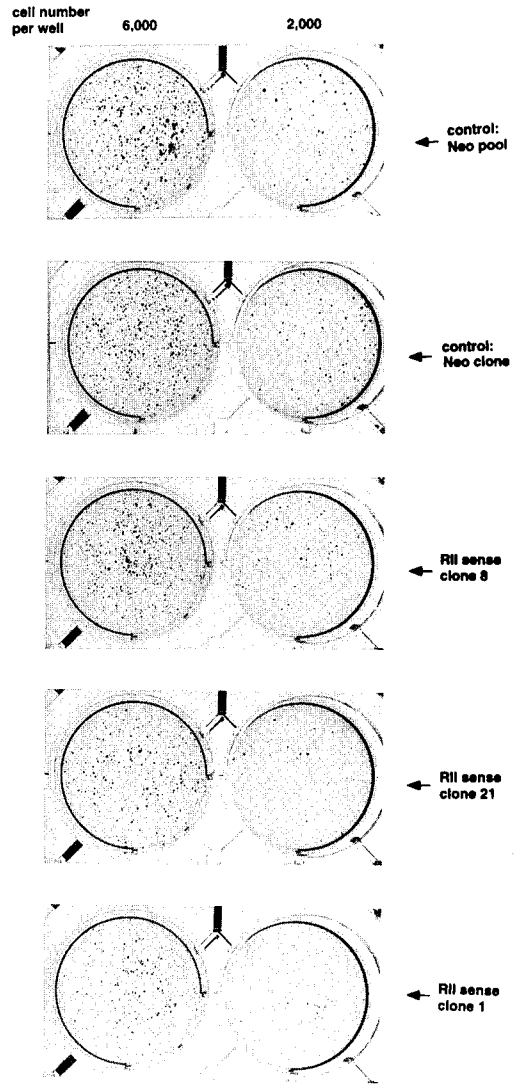

FIG. 7. **Anchorage independent colony formation in soft-agarose of MCF 20 controls and RII transfectants.** Exponentially growing cells (2×10^3 or 6×10^3) were suspended in 1 ml of 0.4% sea plaque-agarose in McCoy's 5A medium containing 10% FBS and plated on top of a 1-ml underlayer of 0.8% agarose in the same medium in 6-well tissue culture plates. After 2 weeks of incubation, cell colonies were visualized by staining with 1 ml of *p*-iodonitrotetrazolium violet.

significant reduction of cloning efficiency. Similar to the pattern of reduced saturation density in monolayer growth MCF20-RII 1 showed the greatest reduction of anchorage independent growth while MCF 20-RII 8 showed the least effect of transfection on cloning efficiency among the 3 clones.

Tumorigenicity—Reduction of cloning efficiency in soft-agarose suggested to us that restoration of TGFβ sensitivity might also render MCF-7 cells less tumorigenic. To test this hypothesis, we inoculated exponentially growing cells of MCF 20-Neo pool, MCF 20-RII 8, and MCF 20-RII 21 into ovariectomized estrogen-supplemented nude mice at 5×10^6 cells per site and followed progression of xenograft formation. MCF 20-Neo pool formed xenografts in 10 of 10 inoculation sites and all grew rapidly in estrogen-supplemented nude mice. Xenograft formation of the two RII transfectants was delayed compared to the MCF 20-Neo pool (Fig. 8). Consistent with *in vitro* data, MCF 20-RII 8 produced progressively growing xenografts at all sites inoculated (10/10) while MCF 20-RII 21 resulted in xenografts in 7 of 10 sites. Mean delay in time to xenograft formation >200 mm^3 was 11 days for MCF 20-RII 8 and 30 days for MCF 20-RII 21 compared to the time to xenograft formation by the MCF

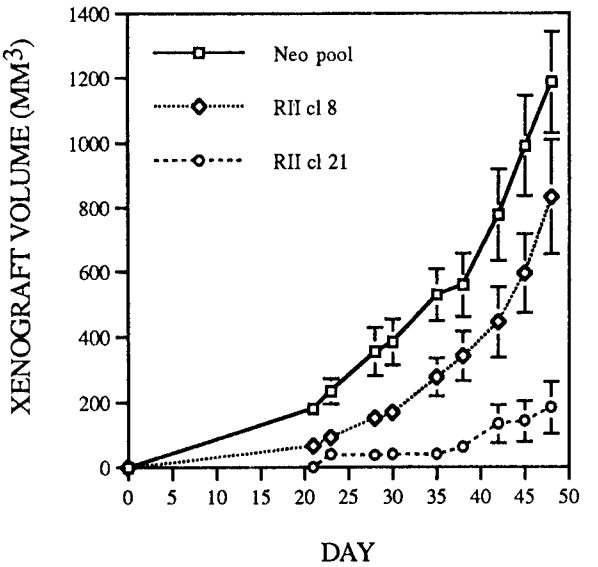

FIG. 8. **Xenograft growth curves of MCF 20-Neo pool and MCF 20-RII clones.** Exponentially growing cells (5×10^6) of MCF 20-Neo pool, MCF 20-RII 8, and MCF 20-RII 21 were subcutaneously inoculated behind the anterior forelimb of ovariectomized athymic nude mice supplemented with 17β-estradiol. Tumors were measured externally on the indicated days in two dimensions using a caliper. Volume was determined from the equation: $V = (L \times W^2) \times 0.5$, where L is length and W is width of the tumor. Each point represents a mean volume ± S.E. of 10 xenografts.

20-Neo pool. In fact, during the first 3 weeks of the experiment, MCF 20-RII 21 was almost nontumorigenic at all sites. MCF 20-RII 8 formed tumors at a much slower pace than the MCF 20-Neo pool. It was not until 3 to 4 weeks after inoculation that the xenografts of MCF 20-RII 8 started to show a similar growth rate to those of the MCF 20-Neo pool.

Since the xenografted transfectants were no longer under Neo selection, it was hypothesized that resumption of tumor growth might have been due to the loss of expression of RII. Therefore, total RNA samples were prepared from xenografts at the termination of the experiment and analyzed for RII mRNA with a human-specific RII riboprobe. Among the xenografts examined, all 3 from MCF 20-RII 8 and two out of four from MCF 20-RII 21 had lost transfected RII expression (Fig. 9). In addition, the transfected RII mRNA levels were significantly reduced in the other two xenografts from MCF 20-RII 21. Thus, it appears the increased tumorigenicity of MCF 20-RII 8 and 21 at the later stages of the xenograft experiment was due to reduction or loss of the transfected RII expression.

DISCUSSION

A number of studies have indicated that at least in some situations TGFβ can contribute to tumor growth and malignancy (Arrick *et al.*, 1992; Steiner and Barrack, 1992; Arteaga *et al.*, 1993a; Chang *et al.*, 1993) while others have indicated that TGFβ autocrine activity acts in a suppressive manner with respect to malignant progression (Wu *et al.*, 1992, 1993). Most of the studies have employed manipulation of autocrine/paracrine TGFβ activity by manipulating TGFβ expression. Such studies have thus been focused on the balance between TGFβ inhibitory actions on tumor cells *versus* TGFβ effects on the microenvironment which might promote tumor growth by a variety of effects on stromal cells and/or the immune system. In this study we examined the role of RII in modulating tumor cell growth in the presence of autocrine/paracrine TGFβs. The MCF-7 cell line provided a good model system for this approach in that it was insensitive to growth inhibitory effects of TGFβ apparently due to low RII expression (Arteaga *et al.*, 1993a).

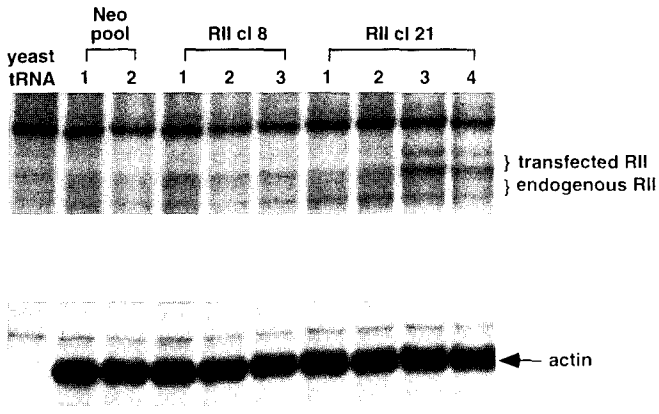

FIG. 9. **RII mRNA levels in xenografts.** At the termination of the tumorigenicity experiments, total RNA was extracted from randomly selected xenografts formed by MCF 20-Neo pool, MCF 20-RII 8, and MCF 20-RII 21. RNase protection assays were performed using radioactive [^{32}P]UTP-labeled human RII and actin antisense riboprobes with 40 μg of total RNA to determine transfected and endogenous RII mRNA levels in the indicated xenografts. Actin mRNA levels were used to verify equal sample loading.

Our study showed that overexpression of RII restored TGFβ sensitivity and reduced the malignancy of MCF-7 cells. Furthermore, although the three RII transfectants were similarly sensitive to exogenous TGFβ$_1$ proliferative inhibitory effects (Fig. 5), their growth patterns *in vitro* and *in vivo* were inversely related to their transfected RII expression levels. These results show that at least in MCF-7 cells, TGFβ receptor levels play an important role in determining malignant properties. However, an intriguing question raised by this study is why the cells expressing different TGFβ receptor levels showed similar responsiveness to the growth inhibitory effects of exogenous TGFβ$_1$. Since sensitivity to growth factors is dependent on the capacity of the effectors downstream from the receptor, it is possible that the levels of TGFβ receptors in MCF 20-RII 8 were sufficient to maximally mobilize the intracellular signal transduction components when activated by high TGFβ concentrations. Therefore, the higher levels of cell surface TGFβ receptor in MCF 20-RII 1 and MCF 20-RII 21 appear to confer sensitivity at lower TGFβ concentrations *in vitro*. The result of tumorigenicity of transfected cells appears to be reflective of this difference in sensitivity at low TGFβ concentrations and may, therefore, reflect low *in vivo* concentrations of active TGFβ in the tumor microenvironment.

TGFβ isoforms have been shown to be potent growth inhibitory agents in several cancer cell lines *in vitro* (Roberts and Sporn, 1991; Moses, 1992; Brattain *et al.*, 1993). Studies on the role of TGFβ in controlling tumor cell growth *in vivo* have been contradictory. Overexpression of TGFβ$_1$ in TGFβ-insensitive cells has indicated increased tumorigenicity (Arrick *et al.*, 1992; Arteaga *et al.*, 1993a). Twardzik and co-workers (1989) reported that subcutaneous, peritumoral injection of TGFβ$_1$ and β$_2$ inhibited the growth and promoted the differentiation of A549 lung cancer cells which are sensitive to TGFβ *in vitro*. We showed that suppression of autocrine TGFβ-negative activity by antisense TGFβ$_1$ RNA significantly increased tumorigenicity of TGFβ-sensitive colon cancer cell lines, indicating autocrine-negative TGFβ activity has a role in counteracting tumor progression (Wu *et al.*, 1992, 1993). In contrast to the above observations, intraperitoneal injection of TGFβ$_1$ was shown to have no significant effect on the growth of tumors produced by human breast cancer MDA-MB-231 cells which are sensitive to growth inhibitory activity of TGFβ *in vitro* (Zugmaier *et al.*, 1991). More recently, several studies showed that the growth of different tumor cells was inhibited *in vitro*, but promoted *in vivo* by TGFβs. Overexpression of latent TGFβ$_1$ in the R3327-MATLyLu prostate cancer epithelial cells was shown to significantly stimulate tumor growth and metastasis *in vivo* although the cells were growth inhibited *in vitro* (Steiner and Barrack, 1992). Another study by Chang *et al.* (1993) showed that Meth A sarcoma cells were growth inhibited by overexpression of active TGFβ$_1$ *in vitro*. However, exogenous TGFβ promoted Meth A cell tumor growth and the TGFβ$_1$ overexpressing cells were also more tumorigenic *in vivo*. Arteaga and co-workers (1993b) also reported that an anti-TGFβ antibody which neutralized all three TGFβ isoforms inhibited TGFβ-sensitive MDA-231 human breast cancer cell tumorigenicity and increased mouse spleen natural killer cell activity. These results led to the suggestion that the net effect of TGFβs on tumor growth *in vivo* is promoting rather than suppressing perhaps due to *in vivo* effect on angiogenesis, the expression of extracellular matrix proteins and their cell surface receptors, or the local suppression of immune surveillance.

Our unpublished data[2] showed that RII expressing clones and Neo controls produced similar levels of TGFβ$_1$. Yet, the RII expressing clones were less tumorigenic. It may be that the increased TGFβ receptor expression in MCF-7 cells enhanced autocrine/paracrine TGFβ-negative activity on the tumor cells, as well as reducing the TGFβ effect on the microenvironment of the tumor host by sequestering TGFβs. Along these lines RII expression also led to large increases in cell surface expression of type I and type III receptors in transfected cells (Fig. 4). As such, it may be that the model systems which were shown to be stimulated *in vivo* by TGFβ overexpression lack sufficient TGFβ receptor expression to sequester the high levels of the ligand.

Loss of TGFβ response has been shown to be associated with tumor development and/or tumor progression in a number of cancer cell lines including squamous carcinoma cells (Masui *et al.*, 1986; Shipley *et al.*, 1986), retinoblastoma cells (Kimchi *et al.*, 1988), estrogen receptor-positive breast cancer cells (Arteaga *et al.*, 1988), colon carcinoma cells (Hoosein *et al.*, 1989), and prostate adenocarcinoma cells (Murphy *et al.*, 1991). While some TGFβ-insensitive squamous carcinoma cells and prostate adenocarcinoma cells were shown to retain TGFβ receptors (Masui *et al.*, 1986; Murphy *et al.*, 1991), the majority of the studies showed that loss of TGFβ sensitivity was associated with reduced TGFβ receptor expression including highly-progressed colon carcinoma cells. It remains to be determined whether reduced expression of TGFβ receptors is the only event contributing to the loss of TGFβ sensitivity in these cancers. However, our study indicates that reduced expression of TGFβ type II receptor alone was sufficient to confer TGFβ insensitivity and a more malignant phenotype. Therefore, altered TGFβ receptor expression is one of the mechanisms by which neoplastic cells may undergo malignant progression via the escape of negative growth regulation by TGFβ.

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Demonstration That Mutation of the Type II Transforming Growth Factor β Receptor Inactivates Its Tumor Suppressor Activity in Replication Error-positive Colon Carcinoma Cells*

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Jing Wang‡§¶, LuZhe Sun‡§‖, Lois Myeroff, Xiaofan Wang‡‡, Larry E. Gentry‡, Junhua Yang‡, Jiurong Liang‡, Elizabeth Zborowska**, Sanford Markowitz**, James K. V. Willson**, and Michael G. Brattain‡ §§**

*From the ‡Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43699, the ‡‡Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710, and the **Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106*

Escape from negative growth regulation by transforming growth factor β (TGF-β) as a result of the loss of TGF-β type II receptor (RII) expression has been found to be associated with the replication error (RER) colorectal cancer genotype, which is characteristic of hereditary nonpolyposis colorectal cancers. The RER-positive HCT 116 colon carcinoma cell line was examined for RII mutations. A 1-base deletion was found within a sequence of 10 repeating adenines (nucleotides 709–718), which resulted in a frameshift mutation. Although it is reasonable to predict that the loss of RII function would be an important determinant of malignancy, the large number of potential mutations in cells of this phenotype raises the possibility that an RII mutation may not be a key event in the tumorigenic phenotype of these cells. One way to test directly the importance of RII mutations in determining the malignant phenotype would be to restore its expression. If restoration of expression leads to diminished tumorigenicity, it would indicate that RII mutation is an important determinant of malignancy in the RER phenotype. To determine whether restoration of RII would lead to reversal of malignancy in RER colon cancers, an RII expression vector was transfected into the HCT 116 cell line. RII stable clones showed mRNA and protein expression of transfected RII. The fibronectin mRNA level was increased by exogenous TGF-β_1 treatment in a dose-dependent manner in RII-positive clones, whereas the control cells remained insensitive. The RII transfectants showed reduced clonogenicity in both monolayer culture and soft agarose. They were growth arrested at a lower saturation density than control cells. TGF-β_1-neutralizing antibody stimulated the proliferation of RII-transfected but not control cells, indicating that the alterations in the growth parameters of the transfected cells were due to the acquisition of autocrine-negative activity. Tumorigenicity in athymic mice was reduced and delayed in RII transfectants. These results indicate that reconstitution of TGF-β autocrine activity by reexpression of RII can reverse malignancy in RER colon cancers, thus verifying that the malignancy of hereditary nonpolyposis colorectal cancer can be directly associated with the loss of RII expression.

Transforming growth factor β (TGF-β)[1] is a multifunctional polypeptide that regulates a number of cellular processes including growth, differentiation, deposition of the extracellular matrix, and immunosuppression (Roberts and Sporn, 1991; Massagué, 1990; Moses *et al.*, 1990). TGF-β exerts its effects through binding to specific cell surface proteins. Three major types of TGF-β receptors have been identified in most cells by receptor affinity labeling assays (Roberts and Sporn, 1991; Massagué, 1990). These receptors have been termed type I (RI), type II (RII), and type III (RIII). RI and RII are glycoproteins of 53 and 75 kDa, respectively, whereas RIII is a proteoglycan of 280–330 kDa. Both RI and RII are transmembrane serine/threonine kinase receptors indispensable for TGF-β signaling (Lin *et al.*, 1992; Wrana *et al.*, 1992; Franzén *et al.*, 1993; Bassing *et al.*, 1994). RIII is a membrane protein lacking a cytoplasmic protein kinase domain (Wang *et al.*, 1991; Morén *et al.*, 1992). The direct involvement of RI and RII in TGF-β signal transduction would suggest that loss of functional RI and/or RII expression could contribute to the loss of TGF-β responsiveness.

An important feature of normal growth regulation is the balance of autocrine-negative and -positive signals regulating the cell cycle. TGF-β has been shown to act as an autocrine-negative growth regulator as evidenced by TGF-β-neutralizing antibody stimulation of several cell lines (Arteaga *et al.*, 1990; Hafez *et al.*, 1990; Singh *et al.*, 1990). Accordingly, cells that lose the ability to express or respond to TGF-β are more likely to exhibit uncontrolled growth and to become tumorigenic. Previous work in our laboratory showed that repression of endogenous TGF-β expression by antisense TGF-β_1 RNA led to malignant progression of colon cancer cells (Wu *et al.*, 1992, 1993). TGF-β antisense transfected cells retained sensitivity to exogenous TGF-β, thus suggesting that the loss of autocrine TGF-β function was a key feature in the development of these

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§ These authors contributed equally to the work.

¶ Performed as part of the requirements for the Ph.D. degree at the Medical College of Ohio.

‖ Present address: Dept. of Pharmacology, University of Kentucky College of Medicine, Lexington, KY 40536.

§§ To whom correspondence should be addressed. Tel.: 419-381-4324; Fax: 419-382-7395.

[1] The abbreviations used are: TGF-β, transforming growth factor β; RI, RII, and RIII, receptor types I, II, and III, respectively; RER, replication error(s); bp, base pair(s); MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); ECM, extracellular matrix.

transfectants to a more progressed phenotype. The loss of TGF-β receptors in association with the inability to respond to TGF-β has been reported for some tumor cell lines (Arteaga *et al.*, 1988; Kimchi *et al.*, 1988). In particular, some strains of MCF-7 cells appear to be resistant to TGF-β because of the loss of RII expression (Arteaga *et al.*, 1993; Sun *et al.*, 1994). Reconstitution of the autocrine TGF-β loop by reexpression of TGF-β receptors in this breast cancer cell line restored TGF-β sensitivity and reversed malignancy (Sun *et al.*, 1994). Reexpression of RII by complementation of a bladder cancer and a colon carcinoma cell line also led to reversal of tumorigenicity (Geiser *et al.*, 1992). Transfection of an RII expression vector into a human hepatoma cell line with a receptor defect restored TGF-β sensitivity, but *in vivo* tumorigenicity was not addressed in this study (Inagaki *et al.*, 1993). Taken together, these studies indicated that TGF-β has a significant suppressive role in malignancy.

Hereditary nonpolyposis colorectal cancer has been found to have a high incidence of microsatellite instability (termed RER), which is characterized by genetic alteration of simple repeated sequences (Aaltonen *et al.*, 1993, 1994; Thibodeau *et al.*, 1993; Lindbolm *et al.*, 1993). Recently, RER was found to be associated with mismatch repair defects, which are responsible for markedly elevated gene mutation rates (Aaltonen *et al.*, 1994; Fishel *et al.*, 1993; Leach *et al.*, 1993). We have shown that TGF-β RII is a downstream mutation target resulting in the disruption of growth regulation of this hereditary form of colon cancer in both cell lines and primary tissues as 9 of 10 RER tumors showed loss of RII transcript, whereas 48 of 53 non-RER tumors expressed the receptor mRNA (Markowitz *et al.*, 1995). Disruption of the RII gene has been noted in gastric cancer, which also has a high incidence of RER (Park *et al.*, 1994; Eshleman and Markowitz, 1995). These studies indicate that RII is a tumor repressor gene in gastrointestinal cancers.

RII is of particular interest as a suppressor gene because of the negative growth regulatory activity of TGF-β. In view of the association of the RER colorectal cancer phenotype with the loss of RII, it is important to determine whether reconstitution of RII would lead to reversal of tumorigenicity. This would provide direct evidence that RII mutation and/or loss of transcript contributes to the malignancy of this hereditary form of cancer. In this report we describe mutation of RII in the RER-positive HCT 116 colon cancer cell line. Stable transfection of an RII expression vector into HCT 116 cells reversed both *in vitro* and *in vivo* malignant properties, thus indicating that the malignancy of hereditary nonpolyposis colorectal cancer is directly associated with loss of RII expression.

MATERIALS AND METHODS

Cell Culture—The HCT 116 human colon carcinoma cell line was established *in vitro* from a primary tumor and maintained as described previously (Brattain *et al.*, 1984; Boyd *et al.*, 1988). HCT 116 clones were obtained by limiting dilution in 96-well tissue culture plates at a density of 0.5 cells/well.

RII cDNA Sequencing—RII from cell line HCT 116 and normal tissue control was isolated as follows. Five μg of total RNA was reverse transcribed into cDNA with murine Moloney leukemia virus reverse transcriptase primed with random hexamers. One-fourth of the cDNA reaction was used in a polymerase chain reaction with the sense primer 297 (5′-CGCTGGGGGCTCGGTCTATG-3′) and the antisense primer 55 (5′-CCACTGTCTCAAACTGCTCT-3′) using the cycle 95 °C for 30 s, 61 °C for 1 min, 70 °C for 3 min for 35 cycles, followed by a 70 °C 10-min final extension. The 861-bp product covering the 5′ half of the RII coding region was purified by electrophoresis on a SeaPlaque low melting agarose gel and Elutip (Schleicher & Schüll). The purified product was then sequenced directly with Sequenase using the (antisense) primer 847 (5′-CTGATGCCTGTCACTTGAAA-3′) and electrophoresed on a 6% polyacrylamide, 7 M urea gel.

RII Transfection—The TGF-β RII cDNA (~3 kilobases) was cloned into the pRC/CMV expression vector (Invitrogen) as described previously (Lin *et al.*, 1992). The plasmid (10 μg) was linearized and transfected into a typical limiting dilution clone from HCT 116. Electroporation was carried out at 250 V, 960 microfarads with a Gene Pulser (Bio-Rad). The control cells were similarly transfected with 10 μg pRC/CMV plasmid lacking the RII cDNA.

RNA Analysis—To determine TGF-β_1, RI, and RII mRNA levels, RNase protection assays were performed as described previously (Wu *et al.*, 1993; Sun *et al.*, 1994). The RII riboprobe plasmid was constructed by inserting a piece of human RII cDNA from the 5′ end of the expression vector into the pBSK(−) plasmid. This piece of DNA contained 264 bp of human RII cDNA and 18 bp of vector DNA. The vector DNA is transcribed into recombinant RNA when the RII expression vector is transfected into HCT 116 cells. As a result, the transfected mRNA could be distinguished from the endogenous RII mRNA because the antisense riboprobe synthesized by T3 RNA polymerase *in vitro* protected a larger fragment (282 bp) when hybridized with transfected RII mRNA than when hybridized with the endogenous 264-bp RII mRNA (Sun *et al.*, 1994). The ^{32}P-labeled probes were hybridized with 40 μg of total RNA. The hybridization mixture was then digested with RNase A and RNase T1 followed by proteinase K treatment. The protected fragments of the probes were analyzed by urea-polyacrylamide gel electrophoresis and visualized by autoradiography. Actin mRNA protected by an actin riboprobe was used to normalize sample loading as described previously (Wu *et al.*, 1993).

To study the effect of exogenous TGF-β_1 on fibronectin expression, exponentially growing RII clones and the NEO pool were treated with various concentrations of TGF-β_1 for 24 h. Total RNA was then extracted for detection of fibronectin mRNA using an RNase protection assay. The fibronectin antisense riboprobe for RNase protection assay was synthesized *in vitro* from a 232-bp *Bam*HI-*Pvu*II fragment of human fibronectin cDNA inserted into pGEM3Z(−) plasmid using T7 RNA polymerase.

Receptor Cross-linking—Simian recombinant TGF-β_1 was purified from conditioned medium of transfected Chinese hamster ovary cells as described by Gentry *et al.* (1988). Purified TGF-β_1 was iodinated by the chloramine-T method (Ruff and Rizzino, 1986). The cell monolayers were incubated with 400 pM ^{125}I-TGF-β_1 on 35-mm tissue culture wells at 4 °C for 3.5 h with shaking followed by cross-linking with disuccinimidyl suberate (Segarini *et al.*, 1987). Labeled cell monolayers were solubilized in 200 μl of 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride. Equal amounts of cell lysate protein were electrophoresed on a 4–10% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and exposed for autoradiography.

Plating Efficiency Assay—To study the effects of RII expression on clonogenic potential at low seeding density, HCT 116 RII transfectants and control cells were plated in 24-well plates at a density of 300 cells/well with McCoy's serum-free medium. TGF-β_1-neutralizing antibody (R&D Systems, Minneapolis) was added to the medium at a final concentration of 10 μg/ml to determine the autocrine TGF-β activity. Cell colonies were visualized by staining with MTT (Sigma).

Soft Agarose Assays—Soft agarose assays were performed as described previously (Wu *et al.*, 1992) to compare the clonogenic potential of control and RII-transfected cells in semisolid medium. Briefly, cells were suspended at 3×10^3 cells/ml in 1 ml of 0.4% SeaPlaque agarose in McCoy's 5A serum-free medium and plated on top of 1 ml of 0.8% agarose in the same medium in triplicate in six-well tissue culture plates. Plates were incubated for 2–3 weeks at 37 °C with 5% CO_2 in a humidified incubator. Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotetrazolium violet staining (Sigma).

Tumorigenicity—Tumorigenicity studies were performed as described previously (Wu *et al.*, 1992, 1993; Sun *et al.*, 1994). Briefly, cells from exponential cultures of HCT 116 transfectants and control cells were inoculated subcutaneously behind the anterior forelimb of 4-week-old athymic mice (athymic nu/nu-CWRU, Cancer Center athymic mouse colony). Mice were maintained in a pathogen-free environment. Growth curves for xenografts were determined by externally measuring tumors in two dimensions. Volume was determined by the equation $V = (L \times W^2) \times 0.5$, where V = volume, L = length, and W = width.

RESULTS

Mutation of RII—The wild type RII cDNA has a repeat sequence of 10 adenines at nucleotides 709–718 (Fig. 1 and Lin *et al.*, 1992). Sequencing of the 5′ half of RII cDNA from the RER-positive HCT 116 cells showed a homozygous loss of one A (no wild type sequences detected) in the polyadenine tracts (Fig. 1). This mutation predicts an inactive truncated receptor

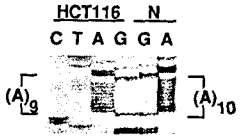

FIG. 1. **Mutant RII sequence in HCT 116 cells.** Five μg of total RNA from HCT 116 cells was transcribed into cDNA by reverse transcriptase-polymerase chain reaction as described under "Materials and Methods." The purified product was then sequenced directly with Sequenase using the antisense primer and electrophoresed on a 6% polyacrylamide, 7 M urea gel. The sequence from this primer covers the polyadenine tract (nucleotides 709–718).

of 161 amino acids, with the terminal 34 amino acids different from the wild type sequence. Similar RII mutations resulting from 1- and 2-bp deletions within this poly(A) tract have been detected previously in both RER-positive colon cancer cells and primary RER-positive colon cancer tissues (Markowitz *et al.*, 1995). It has been shown that frameshift mutations located in the 5′ half of mRNA transcripts can accelerate the decay of mRNAs (Hagan *et al.*, 1995). This likely accounts for the reduced RII transcript levels in HCT116 (Fig. 2) and in previously characterized RER-positive colon cancer cell lines with similar RII mutations.

Expression of RII—Several positive clones (designated as RII clones 17, 21, 26 and 37) with varying levels of transfected RII mRNA expression were obtained (Fig. 2). RII clones 17 and 37 expressed the highest levels of transfected RII. A pool of control plasmid-transfected clones (the NEO pool) expressed little or no endogenous RII mRNA. Consistent with mRNA levels, RII clones 17 and 37 also expressed the highest levels of RII protein as shown by receptor cross-linking assays (Fig. 3, from *left*, *second*, *fourth*, *fifth*, and *sixth lanes*), whereas the cell surface receptor expression of RII was undetectable in the NEO pool (Fig. 3, *first lane*). It appears that restoration of RII expression increased cell surface expression of RI, which is consistent with previous reports that the presence of RII is required for TGF-β binding to RI (Wrana *et al.*, 1992). The specificity of RI and RII was shown by competing ^{125}I-TGF-β_1 binding to RI and RII with 50-fold unlabeled TGF-β_1 (Fig. 3, *third lane*). This indicated that transfected RII can be expressed and bind ligand in HCT 116.

Increased Fibronectin mRNA Expression—We determined whether expression of RII, as well as increased TGF-β binding to RI, could restore the TGF-β sensitivity of the RII transfectants. One of the cellular responses to TGF-β is induction of fibronectin expression (Ignotz and Massagué, 1986). Consequently, exponential phase RII clone 17 and the NEO pool were treated with 1.0 and 5.0 ng/ml TGF-β_1 for 24 h. Fibronectin mRNA levels increased in RII-transfected cells in a dose-dependent manner, whereas fibronectin mRNA levels remained the same in the NEO pool cells (Fig. 4). Similar results were also observed in RII clone 37. These observations indicated that RII expression in HCT 116 cells restored TGF-β_1 sensitivity of extracellular matrix (ECM)-associated molecules (ECM pathway).

Growth Arrest at Low Cell Density—Growth curves for RII clones 17 and 37 and the NEO pool were generated to determine whether RII expression led to alteration of growth parameters in tissue culture (Fig. 5). Growth rates in the exponential growth phase were essentially similar for the NEO pool and RII clones, but some delay in reaching log phase was observed in RII transfectants. The NEO pool had a slightly higher saturation density than the RII-transfected clones.

Plating Efficiency Assay—To assess further the effects of RII expression on cell growth properties, we compared the ability of the control and RII-transfected cells to expand and form colonies at low seeding density. As shown in Fig. 6. RII clones 17

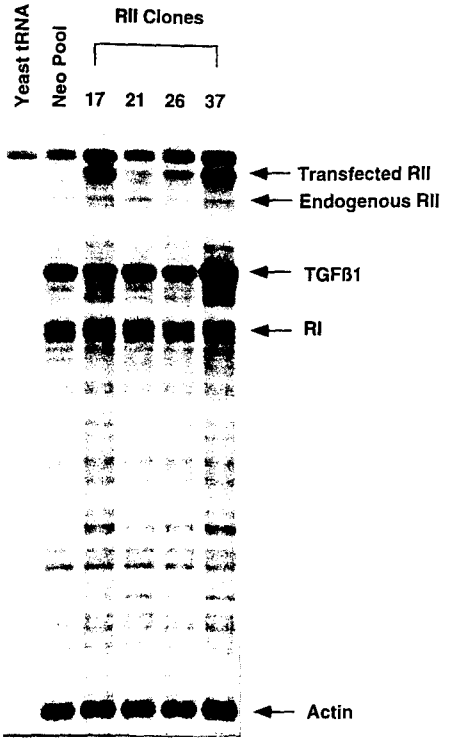

FIG. 2. **Expression of TGF-β RII mRNA in HCT 116 transfectants.** A typical HCT 116 limiting dilution clone was stably transfected with an RII expression vector and selected in geneticin. The stable clones (designated RII clone 17, 21, 26, and 37) were compared with the NEO transfection control pool for RII mRNA levels by RNase protection assay. Endogenous RI, RII, TGF-β_1, and transfected RII mRNA levels are shown. Actin mRNA levels were used for normalization.

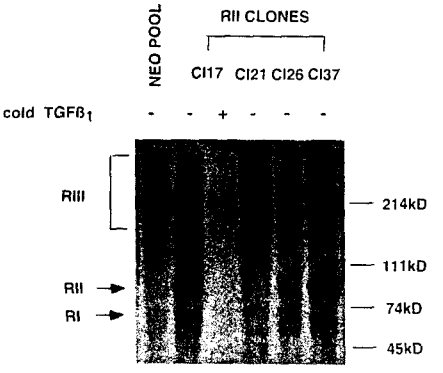

FIG. 3. **Cell surface expression of TGF-β receptors in HCT 116 transfectants.** Receptor cross-linking assays were used to verify cell surface expression of RII. Confluent monolayer cultures of HCT 116 NEO pool and RII clones 17, 21, 26, and 37 were incubated with 400 pM ^{125}I-TGF-β_1 alone (from *left*, *first*, *second*, *fourth*, *fifth*, and *sixth lanes*) or in the presence of 20 nM cold TGF-β_1 (*third lane*) as described under "Materials and Methods." Electrophoresis of 150 μg of protein was performed (4–10% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions).

and 37 showed a significant reduction of cloning efficiency (38 and 22% of that of the NEO pool, respectively). HCT 116 cells expressed high levels of TGF-β_1 mRNA (Fig. 2) and secreted TGF-β_1 protein (0.4 ng/ml/10^6 cells). Taken together with the growth curves, it suggested that endogenous TGF-β was acting in an autocrine-negative manner to inhibit cell proliferation. If this were true, TGF-β-neutralizing antibody should be able to uncouple the TGF-β autocrine loop and increase cell proliferation as described previously (Wu *et al.*, 1992, 1993). TGF-β_1-neutralizing antibody increased RII clone 17 cloning efficiency as reflected by increases in the number and size of colonies relative to the control antibody-treated RII clone 17 cells (Fig.

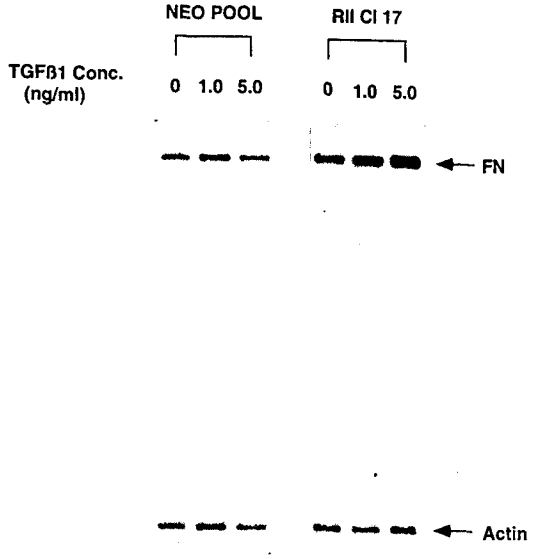

FIG. 4. **Fibronectin mRNA induction by TGF-β_1 in HCT 116 RII transfectants.** HCT 116 NEO pool and RII clone 17 were treated in log phase with 0, 1.0, and 5.0 ng/ml TGF-β_1 for 24 h. Total RNA was isolated, and RNase protection assay for fibronectin was performed. Actin mRNA levels were used for normalization.

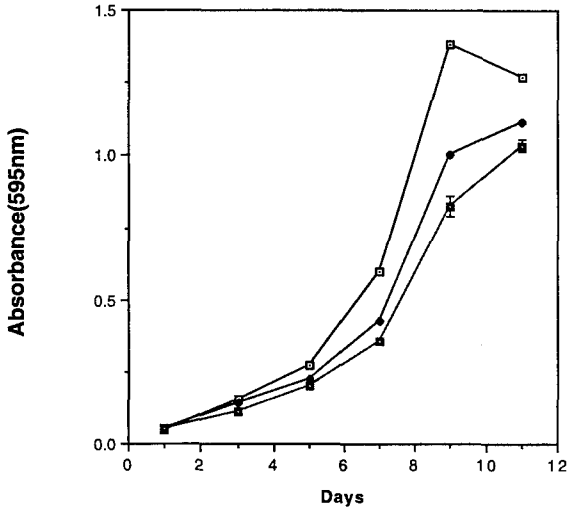

FIG. 5. **Growth curves of HCT 116 NEO pool (□) and RII clones 17 (♦) and 37 (■).** Cells were plated at 1,500 cells/well in 96-well plates in 0.1 ml of serum-free medium. The relative cell number was determined using the MTT assay (Carmichael *et al.*, 1987). Values are means ± S.E. of 12 replicates.

7*A*). The proliferation of RII clone 17 was stimulated by 60% after neutralizing antibody treatment, whereas the NEO pool showed no significant response (Fig. 7*B*). Similar results were seen with RII clone 37 (data not shown).

Anchorage-independent Growth—The ability to form colonies in soft agarose is reflective of malignant transformation. Therefore, to assess the effect of the restoration of autocrine TGF-β activity on the malignant properties of HCT 116 RII transfectants, we compared the ability of the NEO pool and RII-transfected clones to form colonies in soft agarose. As shown in Fig. 8, RII clones 17 and 37 had a significantly lower cloning efficiency in semisolid medium than the NEO pool.

Tumorigenicity—Reduction of the ability for anchorage-independent growth suggested that restoration of autocrine TGF-β activity might also render HCT 116 cells less tumorigenic. To test this hypothesis, we inoculated exponentially growing cells of the NEO pool and RII clones 17 and 37 into nude mice at 5 × 10^6 cells/site and followed progression of xenograft formation. The NEO pool cells formed xenografts in 10 of 10 inoculation sites, and all grew rapidly. Xenograft formation of RII clones 17 and 37 was delayed compared with the NEO pool (Fig. 9). The time needed to form xenografts of >100 mm^3 was 4 days for the NEO pool and 14 days for RII clones 17 and 37; once formed, RII clones 17 and 37 tumors grew at a slower rate than the NEO pool tumors. By day 20, the xenograft size of RII clones 17 and 37 was less than 20% of that of the NEO pool. The experiments assessing the tumorigenicity of RII clones 17 and 37 were performed at different times. Consequently, curves of the NEO controls in these two separate experiments are shown in Fig. 9. This provides a demonstration of the reproducibility of this model system.

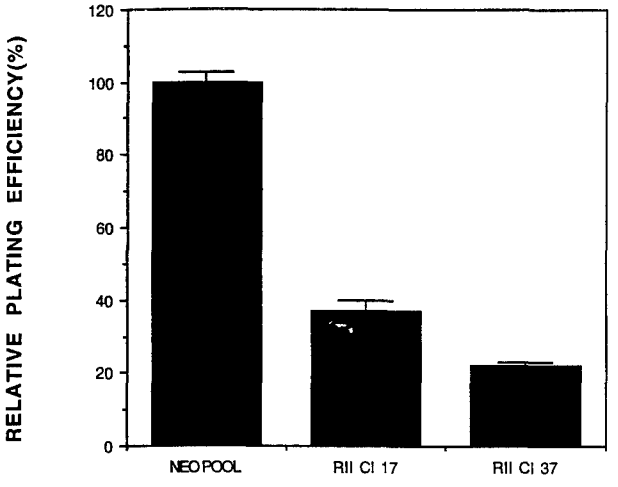

FIG. 6. **Plating efficiency of HCT 116 NEO control and RII clones 17 and 37.** Cells were plated in 24-well plates at 300 cells/well in 1 ml of McCoy's 5A serum-free medium. Cell colonies were observed by MTT staining after 2 weeks of incubation. This was followed by suspension in dimethyl sulfoxide. Relative cell numbers were then determined by the resultant absorbance at 595 nm. Values are the means ± S.E. of four replicates.

DISCUSSION

Many cancers are believed to develop through a series of sequential pathologic steps (Filmus and Kerbel, 1993) that reflect the progressive accumulation of mutations (Fearon and Vogelstein, 1990). Early malignant models of colorectal carcinoma, which are unaggressive and well differentiated, retain some responsiveness to TGF-β growth inhibition, whereas their highly aggressive counterparts do not (Hoosein *et al.*, 1989). Several studies have shown that the progression from adenomas to carcinomas is accompanied by a reduced responsiveness to the growth inhibitory effects to TGF-β (Hoosein *et al.*, 1989; Manning *et al.*, 1991; Markowitz *et al.*, 1994). Since malignant progression in cancer is thought to be related to an accumulation of genetic defects, it was of interest to correlate TGF-β resistance to specific gene mutations or alterations of gene expression. An obvious mechanism that can be proposed when cells develop resistance to TGF-β effects is the loss or significant reduction of TGF-β receptor expression. Our recent studies showed that TGF-β RII, but not RI, can be a downstream mutation target in hereditary nonpolyposis colon cancers which are characterized by high incidence of RER. Although inactivation of TGF-β RII correlated with DNA repair-defective RER colon tumors (Markowitz *et al.*, 1995), it had not been shown that the loss of the receptor had a direct impact on the malignant phenotype of the cells. To show that the loss of RII expression is directly associated with malignancy of hereditary nonpolyposis colorectal cancer, we restored RII expression in HCT 116 cells. Our results showed that RII expression led to reconstitution of a TGF-β autocrine-negative loop in HCT

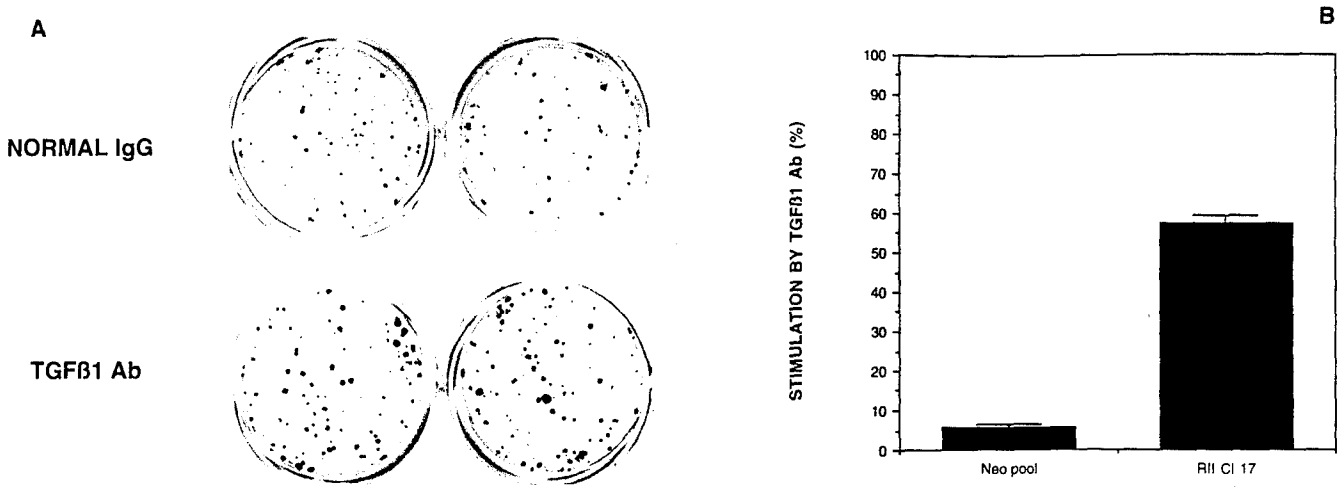

FIG. 7. **Autocrine TGF-β activity in HCT 116 RII transfectants.** HCT 116 NEO pool and RII clone 17 cells were plated in 24-well plates at 300 cells/well in the presence of 10 μg/ml normal IgG or 10 μg/ml TGF-β_1-neutralizing antibody. Cell colonies were stained and photographed, and the relative cell number was determined as described in Fig. 6 after a 2-week incubation. *Panel A* depicts the effectiveness of the TGF-β_1-neutralizing antibody in the clonogenicity of RII clone 17. *Panel B* shows the quantitation of the colony formation of the NEO pool and RII clone 17 cells. Stimulation by TGF-β_1-neutralizing antibody is expressed as the percent increase of absorbance relative to normal IgG-treated cells. The values are the means ± S.E. of four replicates.

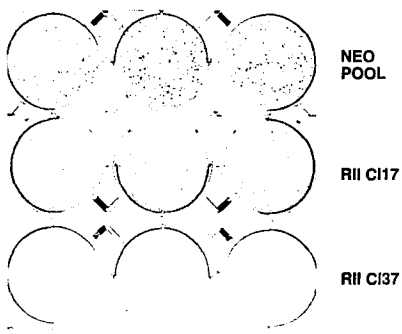

FIG. 8. **Anchorage-independent colony formation in soft agarose of HCT 116 NEO control and RII transfectants.** Exponentially growing cells (3×10^3) were suspended in 1 ml of 0.4% SeaPlaque agarose in McCoy's 5A serum-free medium and plated on top of a 1-ml underlayer of 0.8% agarose in the same medium in a six-well plate. Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotetrazolium violet after 2 weeks of incubation.

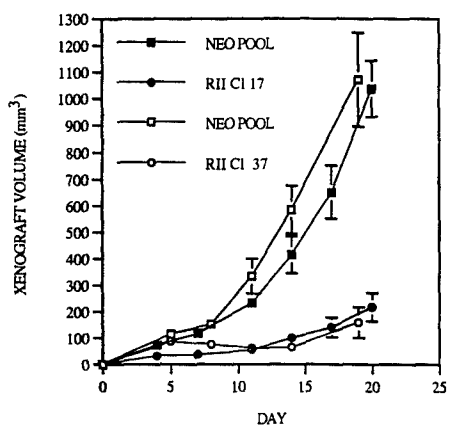

FIG. 9. **Xenograft formation by HCT 116 NEO pool and RII clones 17 and 37.** Exponentially growing cells (5×10^6) were inoculated subcutaneously in athymic nude mice. Tumors were measured externally on the indicated days in two dimensions using calipers. Tumor volume was determined by the equation $V = (L \times W^2) \times 0.5$, where L = the length and W = the width of the tumor. Values are the means ± S.E. of 10 xenografts. Experiments for RII clones 17 and 37 were performed at different times; consequently, the NEO control for each experiment is shown.

116 cells and reversed both its *in vitro* and *in vivo* malignant properties.

In addition to restoration of autocrine-negative activity, the RII-transfected cells displayed sensitivity to exogenous TGF-β for induction of the ECM pathway. However, RII-transfected cells were insensitive to growth inhibition by exogenous TGF-β (data not shown). Segregation of growth inhibition and ECM protein induction in response to exogenous TGF-β has been observed in other colon carcinoma cells (Geiser *et al.*, 1992) as well as other model systems (Ebner *et al.*, 1993). As such, the TGF-β signal transduction pathways for these two types of TGF-β responses may diverge downstream of receptor binding, and the effectors of ECM induction may be more sensitive than growth inhibition effectors. The ECM response is completely absent from untransfected HCT 116 cells, whereas RII clone 26 with a low level of exogenous RII expression showed only 20% as much induction of fibronectin as RII clones 17 and 37 following TGF-β treatment. Thus, the ECM response appears to be a function of available receptors in this model system. Exogenous TGF-β may not be inhibitory because the pathways capable of transducing the inhibitory pathway are saturated by the endogenous TGF-β produced by HCT 116 RII-transfected cells, whereas pathways capable of transducing the ECM pathway are not saturated by these levels of TGF-β and hence are capable of responding to exogenous TGF-β_1. For example, HCT 116 cells may have a low potential for induction of *cdk* inhibitors such as p15 or p27 (Ewen *et al.*, 1993; Hannon and Beach, 1994; Polyak *et al.*, 1994a; Slingerland *et al.*, 1994) which allows for saturation of the pathway by autocrine-negative TGF-β activity. Presumably, the ECM pathway would not be dependent upon the induction of these inhibitors but rather upon specific transactivation factors associated with ECM molecule transcription (Polyak *et al.*, 1994b).

Our finding that the tumorigenicity of HCT 116 is reversible upon restoration of wild type RII expression establishes that RII is a tumor suppressor gene in RER colon cancer. Thus the inactivation of RII in HCT 116 by a 1-base truncation of a polyadenylate sequence is an event that directly promotes tumor progression. Deletions and insertions in repetitive DNA sequences are characteristic of RER tumors (Ionov *et al.*, 1993; Aaltonen *et al.*, 1993; Kim *et al.*, 1994), and the shortening of polyadenylate sequences in RER tumors is particularly common (Chen *et al.*, 1995). The occurrence of a polyadenylate tract within the RII coding region thus renders it particularly vulnerable to mutation in a cell with the RER mutator phenotype.

RII inactivation is thus both a consequence of the RER mutator defect and a mechanism by which the RER defect is able to drive tumor progression forward.

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