

# Overexpression of activin A in stage IV colorectal cancer

S Wildi, J Kleeff, H Maruyama, C A Maurer, M W Büchler, M Korc

## Abstract

**Background and aims**—Activins and inhibins are dimeric polypeptides that belong to the transforming growth factor beta (TGF- $\beta$ ) superfamily and that bind to transmembrane receptors with serine/threonine kinase activity. The aim of this study was to characterise, in colon cancer cell lines and in normal and malignant human colon tissues, levels of expression of inhibin subunits that are involved in activin/inhibin dimer formation, and of the type I and II activin receptors (actRI and actRII).

**Methods**—Expression of inhibin subunits and activin receptors was analysed by northern blot analysis. Inhibin  $\beta$ A and activin receptor expression were also assessed by use of polymerase chain reaction (PCR). In addition, activin A/inhibin  $\beta$ A localisation in human colon samples was assessed by immunohistochemistry and in situ hybridisation.

**Results**—Inhibin  $\beta$ A mRNA was expressed in CaCo2 cells but not in SW 837 or SW 1463 cells whereas inhibin  $\beta$ B and inhibin  $\alpha$  were below the level of detection. In contrast, all four activin receptors were present in the three cell lines. Colon cancers overexpressed inhibin  $\beta$ A mRNA in comparison with normal colon, and this overexpression was greatest in stage IV tumours. ActRI $\beta$  mRNA levels were slightly higher in the normal colon than in cancer tissues. By immunohistochemistry and in situ hybridisation, activin A and inhibin  $\beta$ A mRNA were present in the mucosal epithelial cells in normal tissues from patients with stage I disease but were either absent or weakly present in normal tissues from patients with stage IV disease. Conversely, they were present at weak to moderate levels in stage I cancers but at high levels in stage IV cancers.

**Conclusions**—Our findings indicate that activin A is overexpressed in human colorectal tumours, especially in stage IV disease, raising the possibility that activin A may have a role in advanced colorectal cancer.

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Keywords: colorectal cancer; activin  $\beta$ A; northern blot analysis

Colorectal cancer is the second most common cause of cancer death and the fourth most prevalent carcinoma in the Western world. In the USA there were approximately 131 000 new cases of colorectal cancer and 55 000

deaths from this disease in 1997.<sup>1</sup> Patient complacency in reporting symptoms often leads to a delayed diagnosis and contributes to disappointing survival results. Despite the general improvement in surgical therapy, almost 50% of colorectal cancer patients still die of metastatic tumour, and the five year survival rate for patients with metastasis is only 9%.<sup>2,3</sup> While the best predictor for survival in this disease remains the initial histopathological staging of the tumour,<sup>4,5</sup> the basis for this disease aggressiveness may be due in part to alterations in several regulatory pathways that lead to enhanced tumorigenesis. Thus as many as 70% of these patients harbour mutations in the APC and p53 tumour suppressor genes, approximately 50% harbour mutations in the K-ras oncogene, whereas approximately 15% exhibit mutations in the Smad4 gene and in mismatch repair genes.<sup>6</sup>

Loss of tumour suppressor gene function in colon cancer and other malignancies is compounded by ineffective negative growth regulation that is normally exerted by growth suppressing factors. One component of this negative growth regulation is represented by the transforming growth factor beta (TGF- $\beta$ ) superfamily of growth factors and receptors.<sup>7,8</sup> It is known that TGF- $\beta$ s play an important regulatory role in the development of normal colonic epithelium,<sup>9</sup> and that high serum levels of TGF- $\beta$ 1 in patients with colorectal cancer is associated with disease progression.<sup>10,11</sup> However, colon cancer cells are resistant to TGF- $\beta$  mediated growth inhibition as a consequence of the presence of Smad4 mutations, and less commonly Smad2 mutations.<sup>3</sup> In addition, colon cancers often exhibit microsatellite instability, resulting in mutations in the type II TGF- $\beta$  receptor, which also leads to loss of responsiveness to the growth inhibitory actions of TGF- $\beta$ s.<sup>12,13</sup>

The importance of the TGF- $\beta$  pathway in colon carcinogenesis is underscored by two observations. Firstly, there is a marked increase in colon cancer frequency in a family with a germline mutation in the type II TGF- $\beta$  receptor gene.<sup>14</sup> However, family members do not exhibit an increased incidence of extracolonic malignancies.<sup>14</sup> Secondly, Smad3 mutant mice uniformly develop metastatic colorectal cancer.<sup>15</sup> In spite of these observations, little is

**Abbreviations used in this paper:** TGF- $\beta$ , transforming growth factor beta; actRI, activin receptor type I; actRII, activin receptor type II; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; BSA, bovine serum albumin; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; RT, reverse transcription; PBS, phosphate buffered saline.

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known about the potential role in colorectal cancer of other members of the TGF- $\beta$  family that also signal through Smad dependent pathways. Therefore, in the present study we sought to characterise expression of the activin/inhibin members of the TGF- $\beta$  family and the activin/inhibin receptors in colon cancer cell lines and in sporadic colon cancers in comparison with normal colon tissue.

The activin/inhibin family consists of various dimers of three subunits of inhibin  $\beta$ A, inhibin  $\beta$ B, and inhibin  $\alpha$ . Inhibin  $\beta$ A and inhibin  $\beta$ B are closely related with 64% amino acid sequence homology whereas inhibin  $\alpha$  shares 27% and 23% amino acid homology with inhibin  $\beta$ A and inhibin  $\beta$ B, respectively.<sup>16-18</sup> Inhibin  $\beta$ A and  $\beta$ B form homo- and heterodimers via disulphide bond linkage yielding the biologically active activin A ( $\beta$ A- $\beta$ A), activin B ( $\beta$ B- $\beta$ B), and activin AB ( $\beta$ A- $\beta$ B). Furthermore, inhibin  $\beta$  subunits can form dimers with the inhibin  $\alpha$  subunit, generating inhibin A ( $\beta$ A- $\alpha$ ) and inhibin B ( $\beta$ B- $\alpha$ ).

Activins and inhibins contribute to normal growth and development, and participate in the regulation of gonadal and extragonadal functions.<sup>16-18</sup> They signal through transmembrane serine-threonine kinase receptors which can be divided into two distinct subgroups, type I and type II receptors.<sup>18-21</sup> Two type I receptors for activin have been described.<sup>18-21</sup> ActRIb appears to be specific for activin whereas actRI may be shared by other members of the TGF- $\beta$  superfamily. Furthermore, two type II receptors have been identified in humans (actRII and actRIIb) and activin appears to have a higher affinity for actRIIb than for actRII. Activation of appropriate intracellular signalling pathways is dependent on the presence of both type I and II receptors and of Smad2, -3, and -4 proteins. Thus activation of TGF- $\beta$  and activin receptors leads to phosphorylation of Smad2 and/or Smad3, complex formation of phosphorylated Smad2 or Smad3 with Smad4, and subsequent translocation of these hetero-oligomeric complexes into the nucleus where they modulate gene transcription.<sup>7,8</sup>

We now report that two of three tested colon cancer cell lines express activin receptors but are generally resistant to the growth inhibitory actions of activin A. We also demonstrate that colon carcinomas overexpress activin A *in vivo*, and that this overexpression is most pronounced in stage IV disease.

## Materials and methods

### MATERIALS

The following materials were purchased: fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Leibovitz L-15 medium, trypsin solution, and penicillin solution from Irvine Scientific (Santa Ana, California, USA); Genescreen membranes from New England Nuclear (Boston, Massachusetts, USA); restriction enzymes, Genius 3 non-radioactive nucleic acid detection kit, and Genius 4 RNA and random primed labelling kits from Ambion (Austin, Texas, USA); [ $\alpha$ -<sup>32</sup>P] dCTP from Amersham (Arlington Heights,

Illinois, USA); polymerase chain reaction (PCR) primers from Bio Synthesis, Inc. (Lewisville, Texas, USA); RT-Kit and DNA molecular weight markers from Gibco BRL (Gaithersburg, Maryland, USA); and a highly specific affinity purified mouse monoclonal antibody to the human  $\beta$ A subunit of inhibin/activin from Serotec (Raleigh, North Carolina, USA). All other reagents were from Sigma (St Louis, Missouri, USA). SW 837, SW 1463, and CaCo2 human colon cancer cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA). Activin A was a gift from Dr AF Parlow (NIDDK's National Hormone and Pituitary Program).

### CELL CULTURE AND PROLIFERATION ASSAY

SW 837 and SW 1463 human colon cancer cell lines were routinely grown in Leibovitz L-15 medium, supplemented with 10% FBS and 100 U/ml penicillin (complete medium), whereas CaCo2 was grown in DMEM supplemented with 15% FBS and 100 U/ml penicillin (complete medium). To perform growth assays, cells were plated overnight at a density of 10 000 cells/well in 96 well plates and subsequently incubated in serum free medium (Leibovitz/DMEM containing 0.1% bovine serum albumin (BSA), 5  $\mu$ g/ml transferrin, 5 ng/ml sodium selenite, and antibiotics) or complete medium. Incubations were continued for the indicated times in the absence or presence of activin A, prior to the addition of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 62.5  $\mu$ g/well) for four hours.<sup>22</sup> Cellular MTT was solubilised with acidic isopropanol. Optical density was measured at 570 nm with an ELISA plate reader (Molecular Devices, Menlo Park, California, USA), as previously reported.<sup>23</sup>

### TISSUE SAMPLES

Normal colon tissue samples as well as cancer samples (n=41) were obtained from patients with colorectal cancer undergoing surgery for their disease (see table 1). Normal tissue was taken from the same patients at a distance of at least of 10 cm from the tumour site. According to the TNM classification of the Union Internationale Contre le Cancer, there were 13 stage I cancers, seven stage II cancers, seven stage III cancers, and 14 stage IV cancers. Freshly removed tissue samples were fixed in Bouin or 10% formaldehyde solution and paraffin embedded for histological analysis. Tissue samples were also frozen in liquid nitrogen immediately on surgical removal and stored at -80°C until use for RNA extraction. All studies were approved by the ethics committee of the University of Bern and by the human subjects committee of the University of California, Irvine.

### CONSTRUCTION OF VECTORS

cDNAs encoding sequences corresponding to activin receptor I (actRI) and the inhibin  $\alpha$  subunit were produced by PCR amplification of single stranded cDNA that was reversed transcribed from human placenta RNA. The PCR products were generated using standard

Table 1 Summary of clinical characteristics of 41 patients undergoing surgery for colorectal cancer

	Stage* I (n=13)	Stage II (n=7)	Stage III (n=7)	Stage IV (n=14)	Overall (n=41)
Sex (M:F)	5:8	5:2	4:3	9:5	23:18
Age (mean (range))	72.4 (54–87)	66.6 (54–76)	64 (49–82)	70.1 (54–87)	68.3 (49–87)
Localisation of tumour	Colon: 2 Rectum: 10 Caecum: 1	Colon: 5 Rectum: 2	Colon: 3 Rectum: 2 Sigmoid: 2	Colon: 6 Rectum: 6 Sigmoid: 2	Colon: 16 Rectum: 20 Sigmoid: 4 Caecum: 1
Grading	G1: 1 G2: 11 Unknown: 1	G2: 5 G3: 1 Unknown: 1	G2: 6 G3: 1	G2: 10 G3: 3 Unknown: 1	G1: 1 G2: 32 G3: 5 Unknown: 3

\*According to the TNM classification of the Union Internationale Contre le Cancer.

conditions, subcloned into pGEM3Zf (actRI) and Bluescript-IISK+ (inhibin  $\alpha$ ) vectors, and authenticity was confirmed by sequencing. The actRI cDNA consisted of a 401 bp fragment (nucleotides 172–572) and the inhibin  $\alpha$  cDNA consisted of a 405 bp fragment (nucleotides 478–882), based on the published human sequences.<sup>19–21</sup> The primers consisted of a three nucleotide stretch followed by a restriction site (underlined) and the respective cDNA sequence. The actRI primer pair was sense 5'-GACGGATCCTAAGCGTCACACTGCCAAG, and antisense 5'-CAGAAGCTTTT CCTGCTCATAAACCTGG. The inhibin  $\alpha$  primer pair was sense 5'-GACGGATCCA GATACATGTTCCGGCCATC, and antisense 5'-CAGGAATTCAGAGCAGAGG GA-GACCAAG. A 1.7 kb ClaI-XbaI fragment of human actRIb cDNA, a 2.6 kb XbaI-BamHI fragment of mouse actRII cDNA (93% homology to human), and a 1.7 kb EcoRI-BamHI fragment of mouse actRIIb cDNA (91% homology to human) were gifts from Dr J Massagué (Memorial Sloan-Kettering Cancer Center New York, New York, USA). A 380 bp activin  $\beta$ A (91% homology to human) (nucleotides 1025–1404; Genbank accession X69619) and 270 bp activin  $\beta$ B (89% homology to human) (nucleotides 180–449; Genbank accession X69620) fragments of mouse origin were gifts from Dr M O'Connor (University of Wisconsin, Wisconsin, USA). Authenticity was confirmed by sequencing.

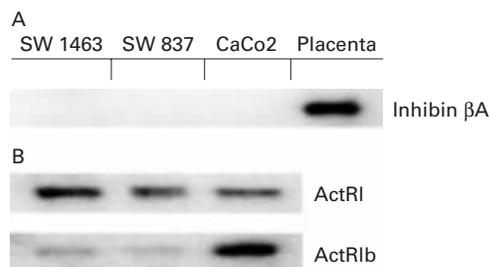
**RNA EXTRACTION AND NORTHERN BLOT ANALYSIS**  
Total RNA was extracted by a guanidinium thiocyanate phenol chloroform method.<sup>24</sup> Where indicated, polyA<sup>+</sup>-RNA was prepared by affinity chromatography on oligo-dT cellulose. RNA was size fractionated on 1.2% agarose/1.8 M formaldehyde gels, electrotransferred onto nylon membranes, and cross linked by UV irradiation. Blots were prehybridised and hybridised with cDNA probes and washed under high stringency conditions as previously reported.<sup>25–26</sup> Blots were then exposed at  $-80^{\circ}\text{C}$  to Kodak BiomaxMS films and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands. A BamHI 190 kb fragment of mouse 7S cDNA that hybridises with human cytoplasmic RNA was used to confirm equal RNA loading and transfer.<sup>26</sup>

#### PCR ANALYSIS

PCR amplified products were fractionated on a 1% agarose gel containing ethidium bromide

(0.1  $\mu\text{g}/\text{ml}$ ) and visualised by UV transillumination. The gels were incubated in 0.25 M HCl for 30 minutes at  $23^{\circ}\text{C}$ . DNA was transferred by capillary action in 0.4 M NaOH to Nylon membranes (Hybond N+). Hybridisation was performed with [ $\alpha$ -<sup>32</sup>P]-dCTP labelled cDNA in 10 ml hybridisation solution (0.75 M NaCl, 5 mM EDTA, pH 8.0, 50 mM sodium phosphate, pH 7.4, 25% formamide, 5 $\times$  Denhart's solution, 10% dextran sulphate, 1% SDS, and 1% salmon sperm DNA). The membranes were washed twice at  $23^{\circ}\text{C}$  in 2 $\times$  SSC and twice at  $42^{\circ}\text{C}$  in 0.1 $\times$  SSPE, 2% SDS, and subsequently exposed at  $-80^{\circ}\text{C}$  to Kodak BiomaxMS films for the appropriate time. Primers for the PCR analysis consisted of a three nucleotide stretch followed by a restriction site (italised) and the respective cDNA sequence. The primer pairs were: sense, 5'-AGTGGATCCAAAAGATGGCCACAAA CCTG and antisense, 5'-GTAGAATTCTTT CCTCCTCAAATGGCAAC (actRII); sense, 5'-AGTGGATCCACATGGTGGAGAAGAG GGTG and antisense, 5'-GTAGAATTCCGGG TGCTATGATCCA-GTCGT (activin  $\beta$ B); sense, 5'-AGTGGATCCGCACCTTGAAGAA GAGACCCG and antisense, 5'-GTAGAATT CCTGCTGGAGACAGGGAAGAC (activin  $\beta$ A).

To perform quantitative reverse transcription (RT)-PCR, total RNA (2  $\mu\text{g}/\text{sample}$ ) pooled from each tumour stage and the corresponding normal tissue samples (n=41) were reverse transcribed using the SuperScript Preamplification System for First Strand cDNA Synthesis, and subjected to two rounds of PCR amplification.<sup>27</sup> In the first PCR reaction, two composite primers were used. Each composite primer had the target gene primer sequence attached to a short stretch of sequence designed to hybridise to opposite strands of a heterologous DNA fragment, thus generating a PCR product of a different size than the target DNA. This PCR product that has the same primer templates as the target DNA was used as the internal standard. Concentrations of the PCR were measured and diluted to 100 attomole/ $\mu\text{l}$ . In the second step, dilution of the control PCR product was then amplified together with the target cDNA using the gene specific primers. Competitive PCR was carried out under standard conditions for 27 cycles. RT-PCR products were size fractionated on a 2% agarose gel and stained with ethidium bromide. The bands were scanned

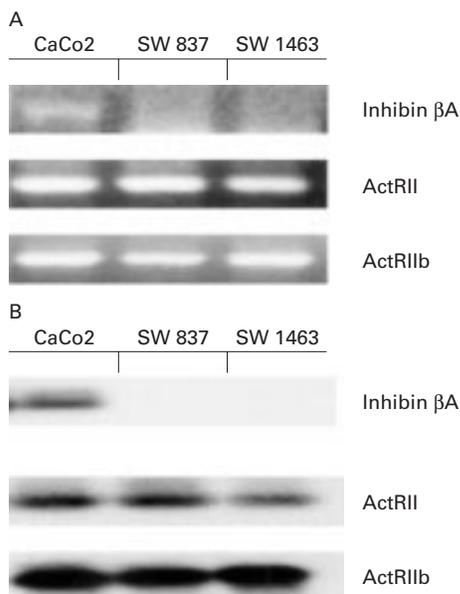


**Figure 1** Northern blot analysis of PolyA<sup>+</sup>-RNA. PolyA<sup>+</sup>-RNA (2 µg/lane) was prepared from the cell lines SW 1463, SW 837, and CaCo2, and hybridised with <sup>32</sup>P labelled cDNA probes (500 000 cpm/ml) specific for inhibin βA (A), or with <sup>32</sup>P labelled cDNA probes (500 000 cpm/ml) specific for actRI and actRIb (B). Human placenta RNA served as a positive control for the inhibin βA transcript. Exposure times were 12 hours.

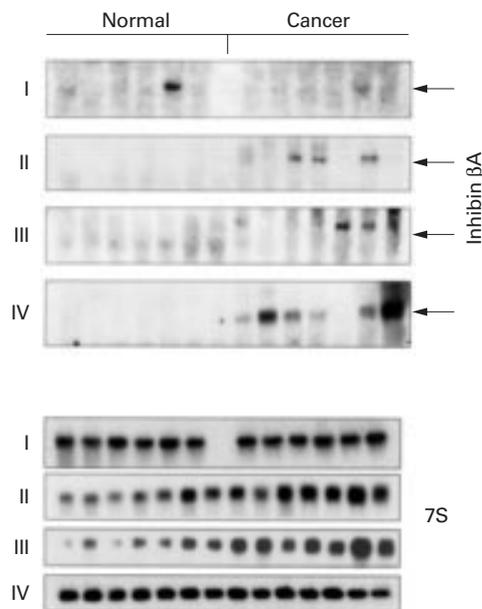
and quantitated by a gel video system (Eagle-Eye II; Stratagene). The relative concentration of the target cDNA was calculated by comparison with the standard values. We used the following primer pair: sense 5'-GCACTTG AAGAAGAGACCCG and antisense 5'-CTG CTGGAGACAGGGAAGAC.

#### IMMUNOBLOTTING

After washing with phosphate buffered saline (PBS, 4°C), cells were solubilised in lysis buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium vanadate, 50 mM sodium fluoride, 100 µg/ml benzamidine, 10 µg/ml leupeptin, and 1 mM phenylmethylsulphonyl fluoride. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membranes. Membranes were incubated for



**Figure 2** Polymerase chain reaction (PCR) analysis. RNA from the cell lines (SW 1463, SW 837, and CaCo2) was reverse transcribed and subjected to PCR using primers for inhibin βA, actRII, and actRIIb, as described in the materials and methods section. (A) Ethidium bromide staining. (B) Southern blot analysis. Filters were hybridised with α-<sup>32</sup>P labelled cDNA fragments (500 000 cpm/ml) which covered the corresponding amplified regions. Exposure time was five hours.



**Figure 3** Expression of inhibin βA in human colon. Total RNA (20 µg/lane) from 28 (seven/stage) colon cancers and the corresponding normal colon mucosa samples were subjected to northern blot analysis. (A) A <sup>32</sup>P labelled inhibin βA cDNA probe (500 000 cpm/ml) was used to perform hybridisations. Exposure time was 12 hours. (B) A <sup>32</sup>P labelled 7S cDNA probe (50 000 cpm/ml) was used to confirm equivalent loading of lanes. Exposure time was six hours. Roman numerals on the left indicate disease stage.

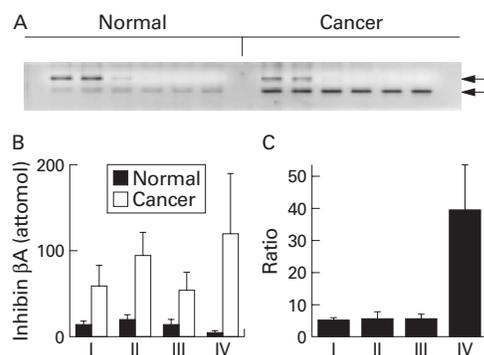
90 minutes with a highly specific affinity purified mouse monoclonal antibody to the human βA subunit of inhibin/activin, corresponding to the 82–114 residues of the peptide. Membranes were then washed and incubated with a secondary antibody against mouse for 60 minutes. Following additional washing, visualisation was performed by enhanced chemiluminescence.

#### IMMUNOHISTOCHEMISTRY

The same highly specific monoclonal antibody to the human βA subunit of inhibin/activin that was used for immunoblotting was also utilised for immunohistochemistry. Paraffin embedded sections (4 µm) from colon cancer and the corresponding normal colon tissues were subjected to immunostaining using the streptavidin-peroxidase technique.<sup>25 26</sup> Endogenous peroxidase activity was blocked by incubation for 30 minutes with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 15 minutes (23°C) with 10% normal goat serum and incubated for 16 hours at 4°C with activin antibody (10 ng/ml) in PBS containing 1% BSA. Bound antibodies were detected with biotinylated goat antimouse IgG secondary antibodies and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer's haematoxylin. Sections incubated without primary antibody did not yield positive immunoreactivity.

#### IN SITU HYBRIDISATION

To perform in situ hybridisation, tissue sections (4 µm thick) were placed on 3-aminopropyl-methoxysilane coated slides, deparaffinised,



**Figure 4** Quantitative reverse transcription-polymerase chain reaction (RT-PCR) of inhibin  $\beta A$  mRNA. First strand cDNA synthesis was performed using pooled total mRNA from each stage (I-IV) and analysed by quantitative RT-PCR as described in the materials and methods section. (A) Representative ethidium bromide gel (stage III). The upper arrow indicates dilution series whereas the lower arrow denotes amplified inhibin  $\beta A$  mRNA from pooled stage III RNA samples. (B) Concentrations of inhibin  $\beta A$  mRNA in normal and cancerous colon tissues. (C) Ratio cancer/normal in activin  $\beta A$  mRNA expression. Vertical bars denote SEM values.

and incubated at 23°C for 20 minutes with 0.2 N HCl and at 37°C for 15 minutes with 10  $\mu\text{g/ml}$  of proteinase K. The sections were then postfixed for five minutes in PBS containing 4% paraformaldehyde, and incubated twice with PBS containing 2 mg/ml glycine and once in 50% (V/V) formamide/2 $\times$  SSC for one hour. Hybridisation was performed in a moist chamber for 16 hours at 50°C, following addition of 100  $\mu\text{l}$  of hybridisation buffer that contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris HCl (pH 7.6), 0.25% SDS, 200  $\mu\text{g/ml}$  yeast tRNA, 1 $\times$  Denhart's solution, 10% dextran sulphate, 40% formamide, and the digoxigenin labelled riboprobe (100 ng/ml), as previously reported.<sup>28-31</sup> The probes were labelled with digoxigenin-UTP by T3 or T7 RNA polymerase using the Genius 4 RNA labelling kit. Sections were then washed at 42°C with 50% formamide/2 $\times$  SSC for 20 minutes, 2 $\times$  SSC for 20 minutes, and 0.2 $\times$  SSC for 20 minutes. The Genius 3 non-radioactive nucleic acid detection kit was used for immunological detection.<sup>28-31</sup> Sections were washed with buffer 1 solution (100 mM Tris HCl and 150 mM NaCl, pH 7.5) and incubated with 1% (W/V) blocking reagent in buffer 1 solution for 60 minutes at 23°C. After incubation for 30 minutes at 23°C with a 1:2000 dilution of an alkaline phosphatase conjugated polyclonal sheep antidigoxigenin Fab fragment antibody, sections were washed twice for 15 minutes at 23°C with buffer 1 solution containing 0.2% Tween 20, and equilibrated for two minutes with 100 mM Tris HCl, 100 mM NaCl, and 50 mM MgCl<sub>2</sub> at pH 9.5. Sections were then incubated with colour solution containing nitroblue tetrazolium and X-phosphate in a dark box for 2-3 hours. After the reaction was stopped with TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), the sections were mounted in aqueous mounting medium.

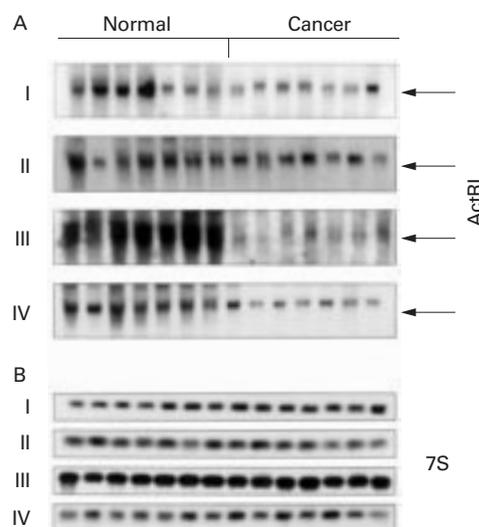
## Results

### EFFECTS OF ACTIVIN A ON COLON CANCER CELL GROWTH

After a 48 hour incubation in serum free medium, activin A inhibited the growth of SW 1463 colon cancer cells in a dose dependent manner. In three experiments, maximal inhibitory effects of 19 (1)% (mean (SEM)) ( $p < 0.05$ ) occurred at a concentration of 100 ng/ml of activin A. A comparable growth inhibitory effect ( $p < 0.05$ ) also occurred in medium supplemented with 10% FBS. However, activin A had no effect in CaCo2 or SW 837 cells, irrespective of the absence or presence of serum. Furthermore, all three cell lines were resistant to the growth inhibitory effects of TGF- $\beta 1$ .

### EXPRESSION OF THE ACTIVIN-INHIBIN SUBUNITS AND ACTIVIN RECEPTORS IN HUMAN COLON CANCER CELL LINES

Northern blot analysis of polyA<sup>+</sup>-RNA isolated from SW 837, SW 1463, and CaCo2 colon cancer cell lines failed to reveal the presence of activin/inhibin  $\beta A$ ,  $\beta B$ , or  $\alpha$  mRNA transcripts. In contrast, the inhibin  $\beta A$  transcript was readily evident in placenta RNA which was used as a positive control (fig 1A). Furthermore, activin  $\beta A$  was not detectable in all three cell lines using western blotting. As a positive control we used activin  $\beta A$  that was readily detected by the antibody exhibiting a single band of the expected size (not shown). Northern blot analysis of polyA<sup>+</sup>-RNA revealed that the 4.0 kb actRI mRNA moiety was present at relatively equal levels in all three cell lines (fig 1B). Furthermore, all three cell lines also expressed the 5.2 kb actRIb mRNA transcript (fig 1B). CaCo2 cells expressed the highest levels of actRIb whereas SW 837 and SW 1463 cells expressed relatively low levels of this



**Figure 5** Expression of actRI in human colon. Total RNA (20  $\mu\text{g/lane}$ ) from 28 (seven/stage) colon cancers and the corresponding normal colon mucosa samples were subjected to northern blot analysis. (A) A <sup>32</sup>P labelled actRI cDNA probe (500 000 cpm/ml) was used to perform hybridisations. Exposure time was 12 hours. (B) A <sup>32</sup>P labeled 7S cDNA probe (50 000 cpm/ml) was used to confirm equivalent loading of lanes. Exposure time was six hours. Roman numerals on the left indicate disease stage.

mRNA moiety (fig 1B). ActRII and actRIIb mRNA transcripts were below the level of detection by northern blotting of polyA<sup>+</sup>-RNA (not shown).

To further assess expression of activin  $\beta$ A and type II activin receptors in colon cancer cell lines, a highly sensitive RT-PCR analysis was performed. This analysis revealed the presence of an inhibin  $\beta$ A mRNA transcript in CaCo2 cells but not in SW 837 or SW 1463 cells (fig 2A). In contrast, actRII and actRIIb transcripts were detected by RT-PCR in all three cell lines. Authenticity of the PCR products was confirmed by Southern blotting using

$\alpha$ -<sup>32</sup>P labelled actRII, actRIIb, and activin  $\beta$ A cDNA fragments which covered the amplified regions (fig 2B).

#### NORTHERN BLOT ANALYSIS IN HUMAN COLON TISSUES

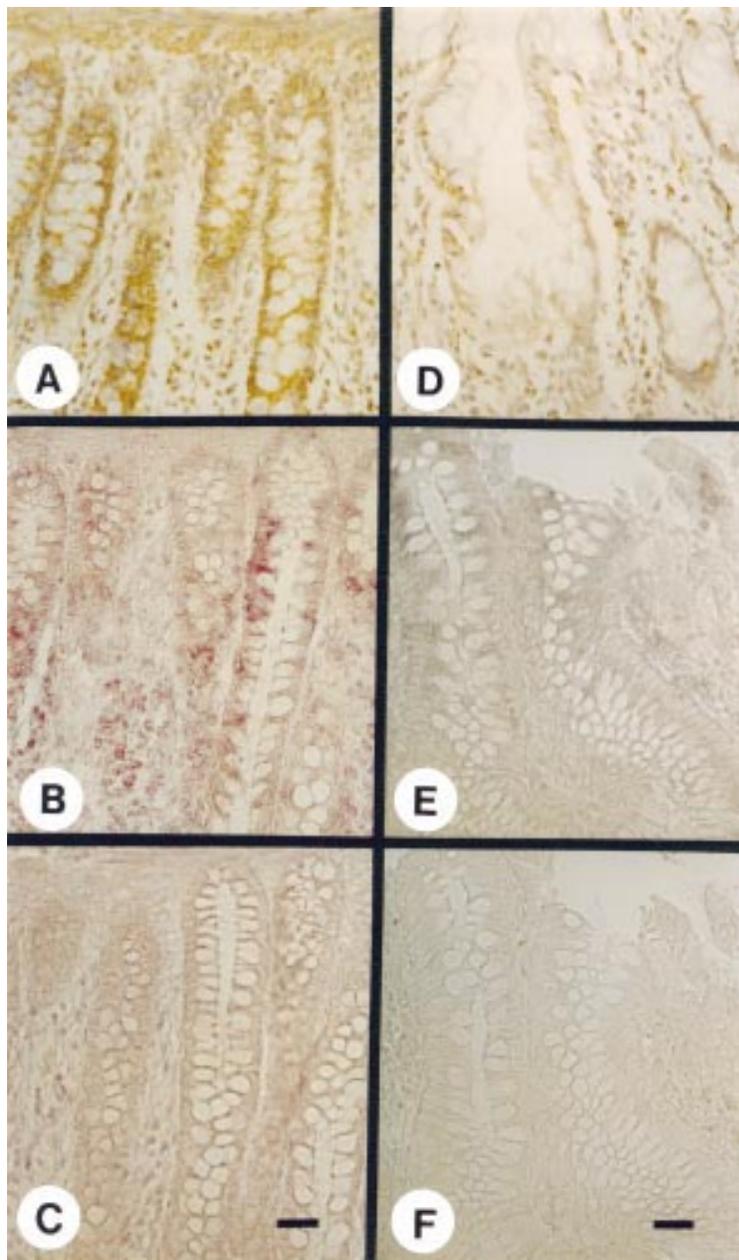
Next, we examined tissue samples of 41 patients undergoing surgery for colorectal cancer. The clinical characteristics of the patients are summarised in table 1. None of the patients received preoperative chemotherapy or radiation therapy. Northern blot analysis of total RNA isolated from the normal portion of the colon of these patients revealed that the 6.5 kb inhibin  $\beta$ A mRNA moiety was visible in only two samples. In contrast, inhibin  $\beta$ A was strongly expressed in 26 of 41 cancer tissues. Moreover, the frequency of overexpression of inhibin  $\beta$ A mRNA tended to correlate with disease stage. Thus, six of 13 stage I samples (46%) and 13 of 14 stage IV samples (93%) expressed high levels of the inhibin  $\beta$ A mRNA. A representative set of northern blots is shown in fig 3.

To quantify the differences in inhibin  $\beta$ A expression in normal and cancer samples, quantitative RT-PCR was carried out. For all four stages of disease, inhibin  $\beta$ A mRNA levels were elevated in the cancer samples compared with levels in the respective control tissues (fig 4). When the data were expressed as a ratio of optical density for cancer versus normal samples, stages I–III exhibited five to sixfold increases in levels of inhibin  $\beta$ A compared with the respective normal tissues (fig 4). There was a dramatic 40-fold increase in this ratio in stage IV disease (fig 4). In contrast, inhibin  $\beta$ B and  $\alpha$  mRNA transcripts were below the level of detection by northern blotting in all 41 normal and cancer samples.

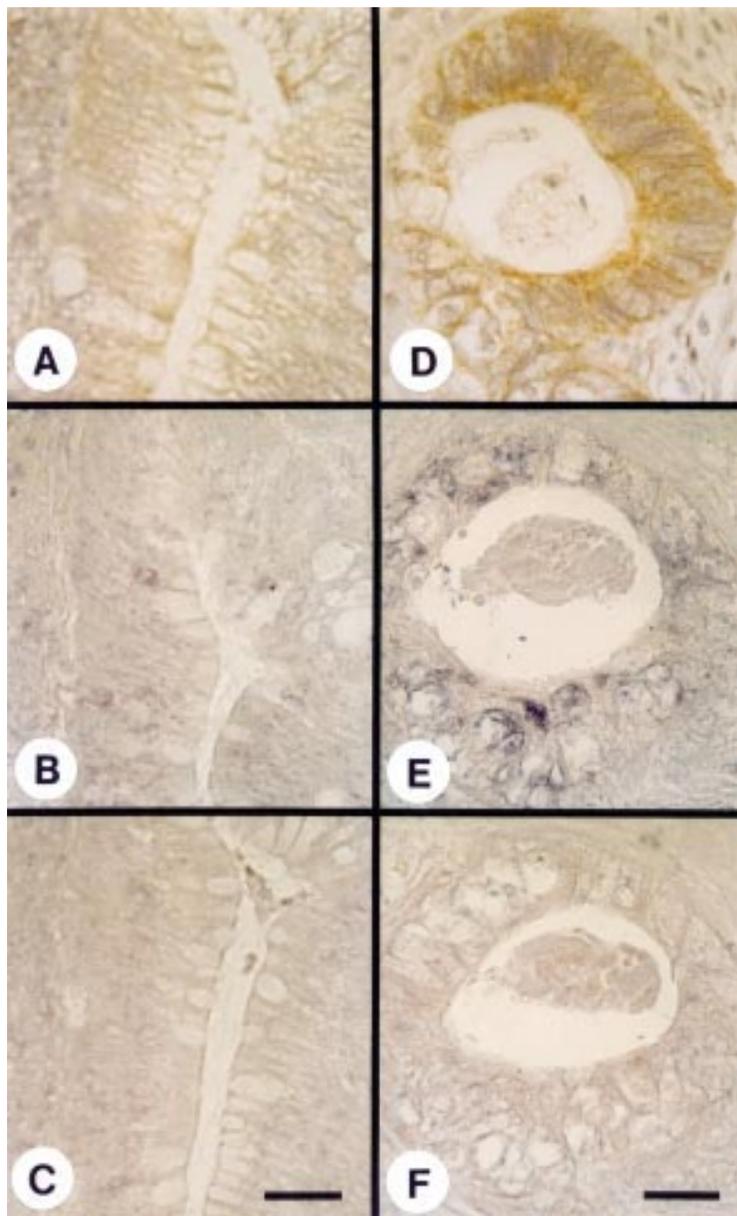
We next determined expression levels of the different activin receptors in the same tissue. The 4.0 kb actRI mRNA transcript was present in all 41 cancer and normal tissue samples (fig 5). Densitometric analysis indicated that by comparison with the normal colon there was a 1.7-fold decrease in actRI mRNA levels in stage I and II disease, a 3.1-fold decrease in stage III disease, and a 1.8-fold decrease in stage IV disease. The actRIb and actRIIb mRNA transcripts were below the level of detection by northern blotting of total RNA in both the normal and cancer tissues (data not shown). In contrast, the 3.0 and 6.0 kb act RII mRNA transcripts were visible in eight of 13 normal and in eight of 13 cancer samples in stage I disease, and in 10 of 14 normal, and in 11 of 14 cancer samples in stage IV disease, respectively. Overall, actRII mRNA levels were similar in normal and cancer samples in all four stages.

#### IMMUNOHISTOCHEMISTRY AND IN SITU HYBRIDISATION

The site of expression in the colon samples of inhibin/activin  $\beta$ A was assessed by immunohistochemistry and in situ hybridisation. Moderately strong inhibin/activin  $\beta$ A immunoreactivity was present in the mucosa from the normal colon of patients with stage I disease where it was most evident in the epithelial cells lining the crypts. A sample with relatively strong



**Figure 6** Immunohistochemical and in situ hybridisation analysis in the normal colon. Moderate inhibin/activin  $\beta$ A immunoreactivity was readily evident in the normal mucosa from patients with stage I disease (A). This sample also expressed relatively high levels of inhibin  $\beta$ A mRNA by northern blot analysis. In contrast, inhibin/activin  $\beta$ A immunoreactivity was relatively weak in normal mucosa from patients with stage IV disease (D). In situ hybridisation revealed a moderate inhibin  $\beta$ A mRNA signal (B) in stage I disease and a very weak inhibin  $\beta$ A mRNA signal in stage IV disease (E). The sense probe (C, F) did not yield any specific signal. Scale bars, 25  $\mu$ m.



**Figure 7** Immunohistochemical and in situ hybridisation analysis in colon cancer. Only faint immunoreactivity for inhibin/activin  $\beta$ A was visible in stage I disease (A) whereas stage IV disease exhibited strong inhibin/activin  $\beta$ A immunoreactivity (D). In situ hybridisation revealed a weak inhibin  $\beta$ A mRNA signal in stage I disease (B) and a strong signal in stage IV disease (E). The sense probe (C, F) did not yield any specific signal. Scale bars, 25  $\mu$ m.

immunoreactivity that also expressed relatively high levels of inhibin  $\beta$ A by northern blot analysis is shown in fig 6A. In contrast, only faint inhibin/activin  $\beta$ A immunoreactivity was evident in this cell type in the normal mucosa

from patients with stage IV disease (fig 6D). Conversely, faint inhibin/activin  $\beta$ A immunoreactivity was only occasionally evident in the cancer cells from stage I disease (fig 7A) whereas strong inhibin/activin  $\beta$ A immunoreactivity was present in the cancer cells from stage IV disease (fig 7D, table 2).

In situ hybridisation was performed in 21 colon tissues to determine the exact site of expression of inhibin  $\beta$ A mRNA. In normal tissues, the in situ hybridisation signal corresponding to inhibin  $\beta$ A mRNA was relatively strong in three of three stage I samples (fig 6B) and either absent (n=3) or very weak (n=2) in stage IV samples (fig 6E). In contrast, inhibin  $\beta$ A mRNA was expressed at low levels in two of seven samples from patients with stage I disease, and at high levels in three of four samples from patients with stage IV disease. In situ hybridisation with sense probes did not produce any specific signal in either the normal (fig 6C, F) or cancer samples (fig 7C, F). The results for the cancer samples are summarised in table 3.

### Discussion

Activins were initially characterised as stimulators of follicle stimulating hormone production from the anterior pituitary.<sup>32-33</sup> Subsequently, they were detected in reproductive and other tissues where they were found to exert important endocrine, paracrine, and autocrine actions that contribute to the regulation of cell proliferation, development, and differentiated functions.<sup>16-17</sup> The multifunctional nature of activins is underscored by the finding that activin A inhibits growth in a number of cell lines, including prostate cancer cell line, vascular endothelial cells, mammary epithelial cells, and hepatocytes<sup>34-38</sup> but stimulates the growth of BALB/c 3T3 fibroblasts, granulosa cells, erythropoietic progenitor cells, and ovarian cancer cell lines.<sup>39-42</sup> Interestingly, inhibin  $\alpha$  deficient mice exhibit high levels of circulating activin and develop gonadal stromal tumours,<sup>43-44</sup> raising the possibility that increased activin expression may be tumorigenic under certain circumstances. This concept is supported by the findings that pancreatic, prostate, and ovarian cancers overexpress activin A,<sup>28-45-46</sup> and that patients with endometrial and cervical cancers have high serum levels of activin A.<sup>47</sup>

In the present study we determined that CaCo2 colorectal cancer cells expressed inhibin  $\beta$ A mRNA whereas SW 837 and SW 1463 did not. All four activin receptors were present in these three cell lines. However, only

**Table 2** Summary of expression of activin/inhibin ligands and receptors by northern blotting of normal and cancer tissues from 41 patients undergoing surgery for colorectal cancer disease

	Stage I		Stage II		Stage III		Stage IV	
	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer
Inhibin $\beta$ A	2/13	6/13	0/7	3/7	0/7	4/7	0/14	13/14
Inhibin $\beta$ B	—	—	—	—	—	—	—	—
Inhibin $\alpha$	—	—	—	—	—	—	—	—
ActRI	13/13	13/13	7/7	7/7	7/7	7/7	14/14	14/14
ActRIb	—	—	—	—	—	—	—	—
ActRII	8/13	8/13	0/7	4/7	3/7	1/7	10/14	11/14
ActRIIb	—	—	—	—	—	—	—	—

—, below the level of detection.

Table 3 Expression of inhibin  $\beta$ A in colon cancer tissues, analysed by different methods

	Northern blot analysis <sup>1</sup>	Immunohistochemistry <sup>2</sup>	In situ hybridisation <sup>3</sup>
Stage I	6 of 13 samples (46%)	1 of 5 samples (20%)	2 of 7 samples (29%)
Stage IV	13 of 14 samples (93%)	4 of 5 samples (80%)	5 of 6 samples (83%)

<sup>1</sup>Positive inhibin  $\beta$ A mRNA expression.

<sup>2</sup>Positive inhibin/activin  $\beta$ A immunoreactivity within the cancer cells.

<sup>3</sup>Positive inhibin  $\beta$ A in situ hybridisation signals within the cancer cells.

CaCo2 cells readily exhibited both actRI and actRIIb mRNA transcripts by northern blotting whereas ActRII and actRIIb were only readily detectable by PCR analysis. It is not surprising therefore that exogenous activin A did not alter the growth of either SW 837 or CaCo2 cells. None the less, activin A exerted a slight but significant growth inhibitory effect in SW 1463 cells, and this inhibitory effect was not altered in the presence of serum. These results differ from our findings in pancreatic cancer cell lines, some of which are growth stimulated in the presence of serum and growth inhibited in its absence.<sup>28</sup> These observations suggest that colon cancer cells are generally resistant to the actions of activin A, or are slightly growth inhibited by this member of the TGF- $\beta$  family. This resistance could be due to perturbations in the Smad signalling cascade or to the fact that our cell lines expressed low levels of actRII and actRIIb that could only be detected by PCR. Indeed, low levels of activin receptors have been implicated in the resistance of breast cancer cells to activin.<sup>48</sup>

Analysis of human colon cancer samples revealed that inhibin  $\beta$ A, which forms homodimers to yield activin A, is overexpressed in colon cancer, most notably in stage IV disease. In stage I colorectal disease, only 46% of all patients expressed the inhibin  $\beta$ A subunit whereas almost all patients with stage IV disease (93%) showed enhanced expression of this subunit in the cancer cells. Furthermore, in normal tissue samples from the same patients we detected inhibin  $\beta$ A only in stage I cases whereas in stages II–IV this transcript was below the level of detection by northern blot analysis.

To further characterise this difference in expression, we performed quantitative RT-PCR of pooled total RNA for every stage. There was a stage related increase in the cancer to normal ratio of inhibin  $\beta$ A mRNA levels. Thus in stage IV disease there was a decrease in inhibin  $\beta$ A mRNA levels in normal samples and an increase in cancer samples. Consequently, there was a 40-fold increase in the ratio of cancer to normal inhibin  $\beta$ A transcript. These observations were consistent with the immunohistochemical data which revealed faint activin  $\beta$ A immunoreactivity in one of five cancer samples from stage I disease, and strong activin  $\beta$ A immunoreactivity in four of five cancer samples from stage IV disease. This was a complete reversal of the findings in the normal tissues inasmuch as activin  $\beta$ A immunoreactivity was of moderate intensity in the normal mucosa from stage I disease but either absent or only weakly present in the normal mucosa from stage IV disease. The in situ hybridisation data confirmed that inhibin  $\beta$ A mRNA was expressed at higher levels in the normal mucosa of stage I disease by comparison

with stage IV disease. It also demonstrated that the strongest and most abundant inhibin  $\beta$ A mRNA signal was seen in the cancer cells of patients with stage IV disease. Taken together, these observations indicate that colon cancer cells acquire an increased capacity to express inhibin  $\beta$ A with disease progression while the normal colonic mucosa progressively loses this capacity.

ActRI expression was decreased in colorectal cancer by comparison with normal controls, especially in stage III disease. In contrast, actRII expression was similar in both groups whereas actRIb and actRIIb were below the level of detection. The decrease in actRI levels and the apparent absence of actRIb and actRIIb raises the possibility that cancer cells in colon carcinomas are resistant to activin mediated growth inhibition. For example, levels of the type I TGF- $\beta$  receptor are decreased in approximately 30% of pancreatic cancer and this decrease has been shown to be associated with decreased responsiveness to TGF- $\beta$ 1.<sup>49</sup> Taken together with the fact that about 20% of sporadic colorectal cancers exhibit Smad4 mutations and 7% exhibit Smad2 mutations,<sup>6 50 51</sup> these findings suggest that a variety of perturbations in the activin  $\beta$ A signalling pathway in colon cancer cells may render them resistant to activin mediated growth inhibition.

Current molecular models for colorectal cancer implicate serial genetic alterations as the basis of cancer formation. These alterations include perturbations in the APC/ $\beta$ -catenin pathway, overexpression of COX2, p53, and DNA mismatch repair gene mutations, K-ras mutations, and microsatellite instability that is associated with mutations in the type II TGF- $\beta$  receptor.<sup>6</sup> Approximately 90% of colorectal cancers with microsatellite instability and 15% of sporadic colorectal cancers harbour mutations in this receptor.<sup>6</sup> Mutations in either the type II TGF- $\beta$  receptor or in the Smad4 or Smad2 genes<sup>50 51</sup> render colon cancer cells resistant to the growth inhibitory actions of TGF- $\beta$ .<sup>6</sup> Ostensibly, the cancer cell derived TGF- $\beta$  dimers can then exert paracrine effects to promote angiogenesis, suppress cancer directed immune mechanisms, and alter the extracellular matrix in a manner that enhances cancer spread and metastasis.<sup>52</sup> In this context, it is noteworthy that deletion of the *Smad4* genes through homologous recombination in HCT116 human colorectal cancer cells that originally contained two normal *Smad4* alleles results in loss of both TGF- $\beta$ 1 and activin responsiveness.<sup>53</sup> Furthermore, among all TGF- $\beta$  family members, activin most resembles TGF- $\beta$  in its mode of receptor activation and receptor structure.<sup>54</sup> Taken together with the finding that inhibin  $\beta$ A/activin A is markedly overexpressed in stage

IV colon cancer, these observations raise the possibility that, like TGF- $\beta$ , activin A may act via paracrine mechanisms to promote colon cancer spread. In addition, our findings suggest that activin A may serve as a marker of advanced colorectal cancer.

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