Synergistic Induction of Tumor Antigens by Wnt-1 Signaling and Retinoic Acid Revealed by Gene Expression Profiling*

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Novel drug targets can be identified by differential analysis of RNA transcripts isolated from cancer cell lines and tissues. We have extended this approach by analyzing differences in gene expression resulting from the drug treatment of transformed and nontransformed cells. A mouse mammary epithelial cell line (C57MG), which conditionally expresses the Wnt-1 proto-oncogene, was left untreated or treated with retinoic acid in the presence or absence of Wnt-1 expression. The experiment was performed in triplicate, and RNA extracted from the four samples was analyzed by hybridization to over 12,000 unique oligonucleotide probe sets. Reproducible alterations in gene expression that occurred in response to retinoic acid, Wnt-1, or retinoic acid plus Wnt-1 relative to untreated cells were identified. Greater attention was given to genes encoding cell surface antigens that were selectively up-regulated by the combination of Wnt-1 and retinoic acid. These genes included the tumor necrosis factor family 4-1BB ligand, ephrin B1, stra6, autotaxin, and ISLR. Administration of retinoic acid to mice bearing tumors driven by activation of the Wnt-1/β-catenin pathway resulted in increased expression of stra6 in the tumors but not in normal tissue. In principal, the therapeutic index of antibodies directed against these antigens should be enhanced by co-administration of retinoic acid.

The aberrant growth and survival of cancer cells is attributed to underlying genetic defects that alter normal cellular homeostasis. In the case of colorectal cancer, inactivation of the adenomatous polyposis coli tumor suppressor occurs early in tumor progression and provides a growth advantage resulting from the inappropriate activation of genes such as cyclin D, matrilysin, and c-myc (1–3). These genes are targets of T cell factor/lymphoid enhancer factor (TCF/LEF)1 transcription factors that are activated in their interaction with β-catenin, a protein that is normally down-regulated by adenomatous polyposis coli (4, 5). The up-regulation of this signaling pathway in cancer can also result from missense mutations in the β-catenin gene that render the β-catenin protein refractory to down-regulation by adenomatous polyposis coli (6, 7). Mutations in β-catenin have been identified in a wide variety of human tumors and are particularly prevalent in human hepatocellular cancer (5). Activation of β-catenin signaling also occurs when the cell surface frizzled receptors are stimulated by the secreted Wnt ligands (8). Although it is not known whether the Wnt ligands themselves contribute to human cancers, early experiments have demonstrated that their overexpression in mouse mammary tissue was tumorigenic (9). Thus, Wnt signaling represents a mechanism that contributes to the progression of a high percentage of human cancers for which appropriate animal and cell culture models are available.

Signals emanating from the Wnt receptors are thought to proceed via the activation of disheveled, which in turn, negatively regulates glycogen synthase kinase 3β (10). This kinase normally phosphorylates the regulatory subunit of β-catenin that targets the protein for ubiquitin-dependent degradation (11). Negative regulation of glycogen synthase kinase 3β thus increases the stability of β-catenin and prolongs its activation of the TCF/LEF transcription factors (12, 13). Although the activation of the TCF/LEF transcription factors by β-catenin is well established, there remain additional mechanisms independent of these transcription factors by which β-catenin might engage gene activation. One of these alternative mechanisms has been proposed by Byers and colleagues (14) in a study investigating the potential for cross-talk between signaling by retinoic acid receptors (RARs) and β-catenin. In accord with this proposal, we have recently demonstrated that the retinoic acid-responsive gene stra6 was activated upon Wnt-1 expression (15). Moreover, stra6 was synergistically induced by a combination of retinoic acid and Wnt-1. The synergistic activation of stra6 does not require the ectopic expression of intracellular signaling components and is therefore mediated entirely by endogenous signaling molecules responsive to their corresponding receptors. This suggests that genuine cross-talk might occur between the RAR and Wnt signaling pathways under normal physiological or pathological states.

That the induction of stra6 expression by retinoic acid was more robust on an oncogenic background prompted us to consider potential therapeutic applications where this effect could be exploited. As the stra6 gene codes for a cell surface protein, an appropriate application would be immunotherapy in cancer. The proven utility of therapeutic antibodies in treatment of human cancer in the clinic has stimulated intense activity aimed at the development and refinement of immunotherapeutics. Ideally these therapies require the presence of cell surface...
Genes Synergistically Induced by Wnt-1 and RAR Signaling

antigens expressed on the cancer cells at significantly higher levels than that present on normal tissues throughout the body. Such criteria for differential expression on tumors relative to normal tissue will obviously limit the number of antigens considered desirable as targets for immunotherapy. Therefore, reagents that selectively enhance the level of antigen expression on cancer cells relative to normal cells could conceivably improve the therapeutic index for immunotherapeutics directed against these antigens. To this end we performed a screen to identify antigens that are preferentially up-regulated by the treatment of Wnt-1-transformed cells with retinoic acid.

EXPERIMENTAL PROCEDURES

Cell Culture—C57MG, C57MG-Wnt-1, and C57MG cells with tetracycline-repressible Wnt-1 expression were grown as described previously (15). For the array analysis the C57MG cells with tetracycline-repressible Wnt-1 expression were grown in 10-cm dishes until ~60% confluent. Cells were washed with phosphate-buffered saline, cultured in tetracycline-free medium for 48 h either in the presence of 1 μM all-trans-retinoic acid (ATRA, Spectrum Laboratory Products) or an equal volume of Me2SO and then harvested. Control cells were maintained in a medium containing tetracycline either the absence or presence of 1 μM ATRA or Me2SO. All dishes were simultaneously harvested, and total RNA was extracted. The growth and treatment of cells and the purification of RNA from cells was performed three independent times. Human colon adenocarcinoma cell line WiDr was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum.

Total RNA Extraction—Cells were lysed in 3.5 ml of lysis buffer (4 mM guanidine thiocyanate, 25 mM sodium citrate, 0.5% N-laurylsarcosine, 0.7% 2-mercaptoethanol) and layered on 1.5 ml of a 5.7 M cesium chloride, 50 mM EDTA solution. Following centrifugation at 150,000 × g overnight, the RNA pellet was dried, resuspended in water, phenol-chloroform-extracted, and ethanol-precipitated. The RNA was resuspended in water to a final concentration of 1 mg/ml and stored at -70 °C.

Oligonucleotide Array—Approximately 10 μg of each RNA sample served as starting material for the preparation of biotinylated cRNA required for oligonucleotide array analysis on the Affymetrix system. cRNA targets were prepared according to previously described protocols (16). Following hybridization, the arrays were washed and stained with streptavidin-phycocerythrin and then scanned with the Gene Array Scanner (Agilent Technologies). Default parameters provided in the Affymetrix data analysis software package were applied in determining the signal intensities, referred to as average differences, and the fold differences for the approximately 12,000 probe sets represented on the Affymetrix Mu74 A chip. Each array image was scaled to an average difference of 1500. The average differences obtained with probes derived from cells expressing Wnt-1 in the absence or presence of ATRA, or treated with ATRA in the absence of Wnt-1 expression, were baseline-corrected against average differences obtained from untreated control cells to generate the -fold difference value for each gene call.

Reverse Transcription-PCR (RT-PCR) Analysis—Confirmation of gene expression was performed using quantitative RT-PCR as described previously (15). Fold induction was obtained by using the ΔΔCt method in which all samples are first normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample. Relative normalized units were then compared between the experimental sample and its control. Mouse and human GAPDH and str6 primer and probe sequences have been described previously (15). Mouse ephrin A-3 forward primer, 5'-AATACAACTC-3' and probe, 5'-CTTGGCTGTGCCAGTTCA-3'; probe, 5'-GGACAGACCCTCCTGGAA-3'; probe, 5'-CCCTGCCGTCAGTCACCA-3'; Mouse retSDR1: forward primer, 5'-TTGGTCATAGTGTTCTCCTAGCTA-3'; reverse primer, 5'-GGACAGACCCCTCCTGGAA-3'; probe, 5'-CAGCCT-CACGTGTTGCATAGCATTCA-3'; human retSDR1: forward primer, 5'-TTGTCATAATTGCTTCTAAGTCAATGACTAAATAC-3'; reverse primer, 5'-ATGTTTCTAATGGTCGCTGACTGACTGAC-3'; probe, 5'-CAGCCTCATATGATTGCTGAC-3'; probe, 5'-TCAGCTTCAGCTGACAATCG-3'; Human Tumor Growth in Vivo—Mammary tumor tissue from a Wnt-1 transgenic mouse was excised and minced into 2 × 2-mm sections in Hanks’ balanced salt solution. Individual tumors were surgically transplanted into the no. 23 mammary fat pad of wild-type syngeneic hosts (C57Bl6). Animals with tumors of 100–200 mm3 were randomly assigned to one of three treatment groups. Two groups received peritumoral injections of either 100 (n = 3) or 400 (n = 2) mg/kg ATRA suspended in olive oil, while a control group (n = 4) received vehicle alone. Eight hours after injection, animals were sacrificed, and tumors and adjacent normal mammary tissue were harvested for RT-PCR analysis.

For human colon xenografts, 5 × 106 WiDr cells in Hanks’ balanced salt solution were injected subcutaneously in the right dorsal flank of female athymic nude mice in a final volume of 0.2 ml. After tumors of 200–500 mm3 were established, animals were randomly assigned to one of four treatment groups. ATRA was administered per os to three groups (eight mice per group) at either 45, 135, or 400 mg/kg, while a control group (n = 4) received vehicle alone. Twelve hours after RA administration, animals were sacrificed, and tumors and normal murine colon tissues were harvested for RT-PCR analysis.

Northern Blottings—Northern blottings were performed as described previously using the Northern Max kit from Ambion (15). RNA was hybridized with 5′-labeled probes corresponding to nucleotides 880–1360 for ephrin B1, 100–370 for 4-IBB ligand, 260–730 for ISLR, and 260–940 for autotaxin.

Western Blotting—Following indicated treatment, cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM sodium fluoride, and Complete protease inhibitor mixture (Roche Molecular Biochemicals) and protein equivalents were subjected to SDS-PAGE and immunoblotting. Blots were incubated with 0.5 μg/ml anti-ephrin B1 goat polyclonal antibody (R&D Systems, Inc.) or 1 μg/ml anti-Myc monoclonal antibody (17). Blots were developed using the ECL system (Amersham Biosciences). Luciferase Assays—Cos-7 cells were transfected with 0.5 μg of the indicated expression plasmid 0.1 μg of RLuc, and either 0.05 or 1 μg of AMMTV-TREgal luciferase reporter construct (provided by S. Beyers) or 0.5 μg of pTopflash (provided by H. Clevers) using Effectene (Qiagen) transfection reagent per the manufacturer’s instructions. Expression plasmids for β-catenin and TCF/LEF have been described elsewhere (18). Cells were treated with 1 μM ATRA or Me2SO as control on the day of transfection and harvested 72 h later. Luciferase activity was the 10 μl of lysate was analyzed in duplicate using the Promega Dual-Luciferase Reporter Assay System and a Tropix TR717 microplate luminometer.

RESULTS

The mouse C57MG mammary epithelial cell line undergoes morphological transformation in response to expression of various Wnt genes (19). A version of this cell line that was engineered to conditionally express Wnt-1 in response to the removal of tetracycline from the culture medium was used for the gene expression profiling experiments. To identify genes that were preferentially activated by the combination of retinoic acid and Wnt-1 signaling, four different conditions were established for the treatment of cells. As a control, cells were left in medium containing tetracycline plus Me2SO, the vehicle for retinoic acid. A second dish of cells was treated with 1 μM ATRA in the presence of tetracycline, while a third dish of cells received Me2SO, and the tetracycline was removed to activate expression of Wnt-1. Finally a fourth dish of cells was treated with retinoic acid, and the tetracycline was removed. Following a 48-h incubation period, cells were harvested, and RNA was extracted and purified. Biotinylated cRNA synthesized from the RNA was hybridized to the Affymetrix Mouse Gene Chip Me74 A containing over 12,000 probe sets and six different conditions were surgically transplanted into one experimental, as initiated from the growth and treatment of cells, was performed three independent times. Data are presented for mRNA transcripts that underwent at least a 2-fold increase relative to those from untreated cells in all three experiments.

Treatment of the C57MG cells with retinoic acid alone re-
resulted in the robust activation of numerous genes (Table I). Some of these genes, such as decorin (20), \(11\beta\)-hydroxysteroid dehydrogenase 1A (\(11\beta\)-HSD1A) (21), autotaxin (22), COUP-TF1 (23), 3-O-sulfotransferase (24), Abca1 (25), cathepsin B (26), and ceruloplasmin (27) have been reported previously to be up-regulated by retinoic acid in other cell types. The identification of these genes in our screen demonstrates that the C57MG cells respond appropriately to retinoic acid and suggests that common end points for retinoic acid signaling exist in diverse cell types. Additional genes induced by retinoic acid, such as \(retSDR1\) and aldehyde dehydrogenase 3, code for enzymes that are involved in the metabolism of retinoic acid and might, therefore, represent feedback or feed-forward responses to retinoic acid itself (28). Increased expression of receptor-interacting protein 140, which has been shown to interact with retinoic acid receptors and suppress their activity, might represent another feedback mechanism (29). Several other genes that were induced by retinoic acid, such as angiotensinogen \(\gamma\) and 3-\(O\)-sulfatransferase, bear no obvious connection to retinoic acid receptor signaling or metabolism and have not been previously reported to be activated by receptors responsive to retinoic acid.

To identify genes induced by Wnt-1, tetracycline was removed from the cells in the absence of retinoic acid. Under these conditions, 12 transcripts were identified that were expressed at 2-fold or higher levels relative to control cells in all three experiments (Table II). This is likely an underestimate of the number of genes that are actually induced by Wnt-1 signaling in these cells due to some amount of leaky Wnt-1 expression. Other genes, including the Wnt-1 target cyclin D1 (2), were identified in only two of the three experiments or fell short of the 2-fold cutoff. Two of the genes identified in our screen, the TCF1 and BTEB2 transcription factors, were previously reported to be targets of Wnt signaling (30, 31). Splice variants of TCF1 that bind DNA but lack \(\beta\)-catenin binding sites provide negative feedback in Wnt signaling and thus function as tumor suppressors. In addition, one of the Wnt receptors, frizzled-2, was also up-regulated by Wnt signaling in the C57MG cell line.

Some of the genes up-regulated by Wnt-1, such as \(GADD45\) \(\gamma\) and 4-1BB ligand, might represent a response to inappropriate growth signaling due to the overexpression of Wnt-1. The 4-1BB ligand (CD137) is a tumor necrosis factor superfamily member that is expressed on various human carcinoma cell lines and stimulates T cell responses in tumor rejection (32). \(GADD45\) \(\gamma\) can be induced by genotoxic stress and promotes growth suppression and apoptotic cell death (33). Two additional genes up-regulated by Wnt-1, semaphorin E and autotaxin, have been implicated in human tumor progression as factors contributing to the motility and metastatic spread of cancer cells (34–36).

### Table I

**Genes induced by retinoic acid**

| Gene                                      | GenBank™ acc. no. | Avg. -fold change | S.D. |
|-------------------------------------------|------------------|------------------|-----|
| Angiotensinogen                           | AF045887         | 27.63            | 17.34 |
| retdSR1                                   | X95281           | 25.67            | 28.78 |
| Ribonuclease 1                            | X60103           | 18.60            | 10.87 |
| LPS-binding protein                       | X99347           | 15.10            | 6.74  |
| Decorin                                   | X53929           | 8.07             | 4.46  |
| 11\(\beta\)-HSD1A                         | X85292           | 7.97             | 4.02  |
| Autotaxin                                 | AW129933         | 7.90             | 5.72  |
| Type 2 deiodinase                         | AF096875         | 7.63             | 5.71  |
| COUP-TF                                   | X74134           | 7.00             | 2.65  |
| Aldehyde dehydrogenase 3                 | AF033063         | 6.43             | 3.07  |
| 3-O-Sulfotransferase                      | AF019385         | 6.13             | 3.97  |
| Ficolin-A                                 | AB007813         | 5.87             | 3.69  |
| Lyssosomal acid lipase                    | Z31689           | 5.60             | 2.46  |
| Osteoglycin                               | D31951           | 4.43             | 2.15  |
| Aco 1                                     | AJ845514         | 4.40             | 2.12  |
| Kallikrein                                | V00829           | 4.33             | 1.22  |
| Cathepsin B                               | A851255          | 3.90             | 1.21  |
| Complement component C3                   | K02782           | 3.83             | 2.67  |
| Precollogen, type IV, o 5                 | Z35168           | 3.53             | 1.01  |
| Dicyglycerol acyltransferase              | AF076752         | 3.40             | 1.45  |
| Chaperonin containing TCP-1 e subunit     | AW048733         | 3.33             | 1.88  |
| VEGFC                                     | U73620           | 3.10             | 0.95  |
| Glutathione S-transferase, o 1            | L06047           | 3.10             | 0.80  |
| Ceruloplasmin                             | U49430           | 3.03             | 1.30  |
| Sorting nexin 10                         | A174884          | 2.90             | 1.10  |
| Receptor-interacting protein 140          | AF053062         | 2.83             | 1.62  |
| Megakaryocyte potentiating factor         | D86370           | 2.73             | 1.35  |
| Annexin VI                                | X13460           | 2.67             | 1.50  |
| MGS7                                      | AW124268         | 2.63             | 0.75  |
| Prefoldin                                 | AB023957         | 2.60             | 0.44  |
| Myristoylated alanine-rich PKC substrate   | M69474           | 2.60             | 0.78  |
| Rho guanine nucleotide exchange factor    | AJ010045         | 2.43             | 0.55  |
| Solute carrier family 12, member 2        | U13174           | 2.40             | 0.61  |
| Guanyluate nucleotide-binding protein 3 (Gbp3) | AW047476       | 2.37             | 0.58  |
| Lysosomal membrane glycoprotein 2         | AW047743         | 2.37             | 1.16  |
| Reticoblastoma-1                          | D28941           | 2.37             | 0.35  |
| Caspase 3                                 | M26391           | 2.37             | 0.40  |
| Acyl-CoA synthase                         | U54803           | 2.37             | 1.07  |
| Lysozyme                                  | AA619027         | 2.30             | 0.36  |
| TM6P1                                     | M23017           | 2.30             | 0.56  |
| Mad4                                      | AA881018         | 2.23             | 0.47  |
| Interleukin 1-\(\beta\)-converting enzyme | L28095           | 2.00             | 0.26  |
In a previous study we identified *stra6* as a gene activated by Wnt-1 signaling (15). *stra6* encodes a cell surface protein that was originally identified in a screen designed to detect mRNA transcripts induced by retinoic acid receptor signaling (37). Although we found that retinoic acid induced the expression of *stra6*, we also demonstrated its synergistic activation by a combination of Wnt-1 and retinoic acid. Therefore, we were interested in whether other cell surface proteins in addition to Stra6 could be activated in a similar manner. Consistent with our previous findings, the present screen identified *stra6* as a gene modestly activated by either retinoic acid or Wnt-1, while the combination of these agents resulted in expression levels greatly exceeding that observed with either agent alone (data not shown). In addition to *stra6*, 10 transcripts were increased by the combination of agents compared with addition of either single agent including four genes encoding cell surface proteins (Table III). Ephrin B1, which encodes a transmembrane ligand for the Eph family of tyrosine kinase receptors (38), was synergistically induced by RA and Wnt-1. The results for ephrin B1 were confirmed by RT-PCR, Northern blot, and Western blot analysis (Fig. 1A). Induction of ephrin B1 differed somewhat from Stra6 in that it was induced by co-treatment of Wnt-1 and RA but not significantly by either treatment alone. By contrast, the gene coding for the transmembrane 4-1BB ligand was moderately activated by Wnt-1 and not by RA but synergistically activated by both agents (Fig. 1B).

The gene coding for *ISLR*, a transmembrane protein with immunoglobulin and leucine-rich repeats (39), was also highly activated by the combination of Wnt-1 and ATRA (Fig. 1C). *ISLR* was moderately activated by either ATRA or Wnt alone, but this did not occur in all three of the gene profiling experiments, thus precluding *ISLR* as an entry in either Table I or II. This variability may be due to low base-line values for *ISLR* gene expression, thus inflating the -fold change variation with only small changes in the base-line value. The variability in the induction of *ISLR* by single agents was also apparent upon confirmation by RT-PCR using the same RNA samples applied in the gene profiling experiments. Induction of *ISLR* by either ATRA or Wnt-1 alone was seen in only two of the three experiments (not shown), and Northern blotting with RNA obtained from a single experiment revealed modest up-regulation by Wnt-1 but not ATRA alone (Fig. 1C). The reason for the variation in the activation of *ISLR* by ATRA or Wnt alone is not known but might relate to the time course of gene induction or to the density of cultured cells during stimulation. In any case, *ISLR* was consistently activated by the combination of Wnt-1 and RA. We also identified autotaxin as a gene synergistically induced by Wnt-1 and ATRA (Fig. 1D). Autotaxin is a secreted molecule that also exits as a membrane-bound nucleotide phosphodiesterase with the catalytic site oriented outside of the cell (40). Expression of autotaxin has been shown to contribute to the invasive phenotype of transformed cells (36).

The canonical Wnt-1 pathway involves the interaction of β-catenin with TCF/LEF transcription factors. However, it is not known whether these transcription factors participate in the synergy observed with Wnt-1 and retinoic acid. To delineate potential differences between the signaling components required for the canonical Wnt-1 pathway and those that mediate the synergy with ATRA, we compared the activation of a retinoic acid-responsive reporter element (RARE) to that of the β-catenin/TCF-responsive reporter TopFlash. Expression of wild type or the S45Y oncogenic mutant of β-catenin activated the RARE and greatly potentiated the effects of retinoic acid (Fig. 2). Expression of the S45Y oncogenic mutant of β-catenin resulted in a strong activation of RARE when combined with retinoic acid. However, co-transfection of LEF with β-catenin resulted in the complete inhibition of the β-catenin-mediated activation of RARE (Fig. 2A). This effect was likely due to sequestration of β-catenin by LEF. By contrast, co-transfection of LEF with β-catenin greatly enhanced the activation of a TCF/LEF-responsive reporter in the same cell line (Fig. 2A). We also tested deletion mutants of β-catenin lacking amino-terminal (ΔN) or carboxyl-terminal (ΔC) structure. The ΔC mutant, which exhibits only weak TCF-dependent signaling activity (41), was also ineffective in the RARE assay. The ΔN-β-catenin also had no effect on the retinoic acid-responsive reporter (Fig. 2B), but this mutant activates the TCF-responsive reporter in vitro and is oncogenically active when expressed in vivo (42). Thus significant differences likely exist between the mechanism by which β-catenin activates canonical Wnt-1 signaling targets and the mechanism by which it potentiates the activation of retinoic acid-responsive targets.

In a previous report, we demonstrated that the ability of Wnt-1 alone to activate *stra6* was inhibited by a pan-antagonist of RAR signaling (15). This suggests that, even in the absence of exogenously added retinoic acid, Wnt-1 was dependent upon basal RAR activity for the induction of *stra6*. To determine whether this was also the case for additional genes that were synergistically induced by Wnt-1 and retinoic acid, we examined the effects of the RAR and retinoid X receptor pan-antagonist on their activation. As we had observed previously with *stra6*, increasing amounts of the RAR and retinoid X receptor pan-antagonist (Fig. 3) inhibited the induction of *ISLR* by Wnt alone. This was not observed with autotaxin, another gene that was moderately activated by Wnt alone and synergistically activated by Wnt plus retinoic acid. The results suggest that a subset of genes induced by Wnt-1 are strictly dependent upon retinoic acid receptor activity, while another subset of genes synergistically induced by ATRA plus Wnt can be activated independently by Wnt signaling. Interestingly an examination of human genomic sequence revealed that two of the genes that fall into the first category, *stra6* and *ISLR*, reside adjacent to each other on human chromosome 15 and mouse chromosome 9. That these two genes reside proximal to each other and behave in a similar manner with respect to their activation by Wnt and RA suggests that they are coordinately regulated.

Our results with the C57MG cell line suggest that tumors driven by Wnt signaling might respond to retinoic acid by expressing high levels of mRNA transcripts that are synergistically induced by Wnt and retinoic acid. To examine this, mammary tumors derived from Wnt-1 transgenic mice were transplanted to the mammary fat pad of naïve mice that were subsequently administered retinoic acid. Our initial analysis was focused on the expression of the *stra6* gene as it exhibited

### Table II

| Gene                | GenBank™ acc. no. | Avg. -fold change | S.D. |
|---------------------|-------------------|-------------------|------|
| Autotaxin-t         | AW122933          | 4.27              | 1.52 |
| 4-1BB ligand        | L15435            | 3.53              | 1.21 |
| EIG180              | AB022857          | 3.00              | 0.26 |
| Semaphorin E        | XX5994            | 2.90              | 0.90 |
| BTEB2 (IKLF)        | AA611766          | 2.80              | 0.26 |
| G28K GTP-binding protein | AW121294      | 2.73              | 0.61 |
| SSG-1 steroid-sensitive gene-1 protein | AW122012     | 2.57              | 1.10 |
| TCF-1               | A1019193          | 2.57              | 0.81 |
| GADD45 γ            | AF055638          | 2.43              | 0.25 |
| frizzled-2          | AW123618          | 2.23              | 0.23 |
| eot-2               | J04103            | 2.20              | 0.40 |
| TSA-1               | U47737            | 2.13              | 1.01 |

*Acc., accession; Avg., average; IKLF, intestinal-enriched Kruppel-like factor.*
the greatest degree of synergistic activation in the in vitro settings. Administration of retinoic acid but not vehicle control resulted in the up-regulation of stra6 mRNA in the mammary tumors but did not have a significant effect on stra6 expression in normal mammary tissue (Fig. 4A). To ensure that the administration of retinoic acid was effective at promoting gene induction in normal mammary tissue we measured the expression of retSDR1, which was induced by retinoic acid alone in the untransformed C57MG mammary cell line (Table I). Consistent with our cell culture experiments, retSDR1 was strongly induced in normal mammary tissue by retinoic acid alone, and no further induction was achieved in the presence of Wnt-1 expression (Fig. 4B). To determine whether Wnt-1 and retinoic acid would synergistically activate stra6 in human tumors, tumors derived from the colorectal cancer cell line WiDr were grown in nude mice that received retinoic acid or vehicle. Human stra6 mRNA expression was induced in the tumors resected from mice treated with retinoic acid relative to those from mice treated with vehicle as assessed using human-specific stra6 primers (Fig. 5A). Moreover, no significant increase in mouse stra6 was observed in normal colon tissue obtained from retinoic acid-treated mice, while expression of the control gene mouse retSDR1 was elevated (Fig. 5B). These cancer models indicate that the expression of stra6 can be preferentially induced in tumors relative to normal tissues by the administration of retinoic acid.

**DISCUSSION**

Gene expression profiling is a powerful method for identifying potential drug targets that are overexpressed in diseased tissue relative to healthy tissue. The approach is ideally suited for the identification of gene products whose overexpression is selected for during tumor progression. In particular, cell surface antigens differentially expressed on cancer cells can serve as specific targets for therapeutic antibodies. To some extent, the expression level of the target antigen on cancer cells relative to other vital tissues in the body will determine the therapeutic index of these therapies. Here we have further exploited the genetic differences between cancer and normal cells by screening for target antigens that are preferentially induced

### TABLE III

| Gene                      | GenBank™ acc. no. | Wnt S.D. | RA S.D. | S.D. |
|---------------------------|------------------|----------|---------|------|
| Autotaxin                 | AW129933         | 6.57     | 4.45    | 3.57 |
| BTEB2                     | AA611766         | 1.83     | 0.29    | 4.10 |
| Groucho-related gene 1 protein (Grg1) | U61362 | 2.67     | 0.45    | 1.80 |
| M-Ras                     | AB004879         | 2.75     | 0.92    | 5.83 |
| 4-1BB ligand              | L15435           | 3.55     | 0.92    | 12.43|
| Spasmolytic polypeptide   | U78770           | 12.85    | 3.32    | 5.47 |
| Ribonuclease 1             | X69103           | 19.57    | 4.56    | 1.90 |
| Ephrin B1                 | U07602           | 2.80     | 0.14    | 8.87 |
| ISLR                      | AB024538         | 11.80    | 7.59    | 5.20 |
| ZO-1                      | DI4340           | 1.73     | 0.06    | 1.95 |

**FIG. 1. Synergistic induction of genes coding for cell surface proteins.** Candidate genes identified by oligonucleotide array analysis were confirmed by RT-PCR (histogram), by Northern blotting (inset), and in the case of ephrin B1, by immunoblotting. RT-PCR was performed on RNA obtained from the three separate experiments in which C57MG cells were stimulated under the indicated conditions. The mean fold-change is presented relative to the −RA−Wnt control. The results of Northern blotting, performed with RNA from a single experiment, are presented using the same sample order as indicated for RT-PCR. Protein extracted from mouse lung tissue (M.L.) was used as positive control for the ephrin B1 immunoblot.

**FIG. 2. Activation of a RARE by β-catenin and retinoic acid.** A and B, plasmids expressing green fluorescent protein (GFP), LEF, wild-type β-catenin (wt), or β-catenin containing a missense mutation (S45Y) or a deletion of the amino-terminal (ΔN) or carboxyl-terminal (ΔC) sequence were co-transfected with a RARE or TopFlash into COS cells that were subsequently treated with retinoic acid (+) or vehicle control (−). Transactivation of the reporter is displayed as average fold-change of normalized luciferase units compared with untreated green fluorescent protein-transfected cells for three independent experiments. An immunoblot showing the relative expression of each of the Myc-tagged β-catenin proteins and LEF is presented below each RARE histogram.
We have chosen Wnt-1 as the oncogenic driver because this signaling pathway is hyperactivated in a high percentage of human cancer (5). Recent evidence demonstrating that Wnt signaling cooperates with retinoic acid receptor signaling prompted us to screen for potential drug targets that were preferentially activated by the combination of these two signals (14, 15). Accordingly several genes, including five that code for cell surface proteins, exhibited synergistic activation by Wnt and retinoic acid.

We chose the mouse C57MG mammary epithelial cell line to perform the gene expression profiling experiments as this cell is known to undergo transformation in vitro in response to Wnt signaling (19). The identification of TCF1, a known target of Wnt signaling in epithelial cells (31), as a gene induced by Wnt-1 in our screen indicates that the C57MG is a valid cell line for studying the activation of genes by Wnt-1. The C57MG cells also respond well to retinoic acid, which was apparent from the induction of several genes by ATRA that were previously shown to be induced by retinoids in other types of cells. Thus the genes induced by the combination of retinoic acid and Wnt likely represent the outcome of genuine signal transduction mediated by the intersection of these two pathways. Moreover, the ectopic expression of intracellular signaling components was not required to observe the synergy between Wnt and ATRA but occurred in response to the activation of endogenous receptors by their cognate ligands present in the cell culture medium. Thus there might exist genuine developmental or pathological states under which specific genes are regulated by so-called cross-talk between Wnt and retinoic acid receptor signaling.

Our results and previous work by others (14) suggest that the intersection between retinoic acid receptor signaling and Wnt signaling occurs at the level of β-catenin, which potentiated the activation of an RAR-responsive promoter element by retinoic acid. Our results are consistent with this proposal and also demonstrate that the TCF/LEF transcription factors that bind β-catenin in the canonical Wnt pathway are not involved in the potentiation of RARE activity by β-catenin. No consensus binding sites for TCF/LEF are contained in the RAR-responsive element, and LEF did not facilitate β-catenin activity...
but instead inhibited it. Therefore β-catenin might enhance RAR-dependent gene transcription by recruiting coactivators to or displacing corepressors from the RAR transcriptional complex. It is also conceivable that the contribution of β-catenin to gene activation by retinoids occurs by more than one mechanism. We found that two of the genes, stra6 and ISLR, that were synergistically induced by ATRA and Wnt could not be induced by Wnt-1 alone in the presence of RAR antagonists. On the other hand, some genes behaved like autotaxin in that Wnt plus ATRA resulted in gene activation exceeding that observed by either agent alone, but RAR antagonists did not inhibit their activation by Wnt. The finding that the stra6 and ISLR genes are adjacent to each other in the genome and behave in a similar fashion with respect to their activation by Wnt plus RA suggests that they might be co-regulated as part of a gene cluster.

An objective of our study was to identify genes coding for cell surface antigens that are induced more robustly by retinoic acid in the presence of an active Wnt-1 signal than in its absence. Ultimately these particular gene products might serve as antibody drug targets that exhibit enhanced differential expression in vivo, relative to normal cells, when tissues are exposed to retinoic acid. As a first test of this concept we administered retinoic acid to animals harboring either transplanted mouse mammary tumors derived from Wnt-1 transgenic animals or tumor xenografts derived from human colorectal cancer cells that lack a functional adenomatous polyposis coli tumor suppressor gene. In both cases up-regulation of stra6 was observed in the tumors upon treatment of the animals with retinoic acid. The mouse tumor model allowed us to analyze syngeneic normal mammary tissue for increases in stra6, and none was observed. The human colorectal cancer model demonstrated that human cancer cells respond to retinoic acid in vivo by expressing higher levels of stra6 mRNA transcript. Although the control tissue in this case was normal mouse colon, increased expression of stra6 was not observed following administration of retinoic acid. Importantly both models demonstrated that the influence of retinoic acid on stra6 expression was confined to the cells that exhibited hyperactive Wnt signaling.

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Synergistic Induction of Tumor Antigens by Wnt-1 Signaling and Retinoic Acid Revealed by Gene Expression Profiling

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