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Type-1 Insulin-Like Growth Factor Receptor Reexpression in the Malignant Phenotype of SV40-T-Immortalized Human Prostate Epithelial Cells Enhances Apoptosis

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The authors have previously shown that the type 1 insulin-like growth factor receptor (IGF-1R) is decreased in the transformation from benign to malignant human prostate epithelial cells in vivo. Further, in a well-described human SV40-T immortalized human epithelial cell system beginning with the immortalized, but rarely tumorigenic P69SV40-T cell line, to the highly tumorigenic and metastatic M12 subline, there is a similar decrease in IGF-1R number from 2.0×10^4 receptors per cell to 1.1×10^3 receptors per cell. When the IGF-1R was reexpressed in the M12 subline using a retroviral expression vector, M12-LISN, to a receptor number similar to that of the P69SV40-T parental cell line, the authors demonstrated a marked decrease in colony formation in soft agar in the M12-LISN cells vs the M12 control cells ($p \leq 0.01$), and a decrease in vivo tumor growth and metastases when injected either subcutaneously or an intraprostatic location ($p \leq 0.01$). This decrease in tumor volume was not because of a decrease in proliferative capacity, but was associated with an increase in apoptosis in baseline cultures and in response to the apoptotic-inducing agents 6-hydroxyurea, retinoic acid, and transforming growth factor β_1.

Key Words: IGF-1R; SV 40-T; HEPES; MTT.

Introduction

The insulin-like growth factor (IGF) system plays a critical role in the transformation process of cells from their benign to malignant state *(1,2)*. Most of the mechanisms by which IGFs influence the transformation process are mediated by signaling through the type 1 IGF receptor (IGF-1R) *(3–5)*. The IGF-1R is a homodimer consisting of two (and two ß subunits *(5)*. The α subunits are the ligand binding portions of the receptor, and the β subunits span the cell membrane and activate tyrosine kinases to begin intracellular signaling processes. The IGF-1R appears to have multiple functions in the transformation process. These functions include proliferation, tumorigenesis, anti-apoptotic, and pro-apoptotic activities *(6,7)*. Recently, O'Connor et al. have mapped the various functional domains of the IGF-1R using a series of IGF-1Rs altered by site-directed mutagenesis *(8)*. Their studies indicate that mutations in the β-subunit of the IGF-1R that occur at, before, or within the tyrosine kinase domain result in a receptor that has neither mitogenic nor transforming functions and, depending on the site of the mutation, may or may not retain its antiapoptotic function. Mutations that occur beyond amino acid 1250 do not affect the mitogenic function of the receptor, and have variable effects on the transforming and antiapoptotic activities. Point mutations in the C-terminal portion of the receptor at residues 1250/1251, 1251 and 1293/1294R result in a receptor that has mitogenic potential, but has lost transforming and anti-apoptotic functions. However, complete deletion of the β-subunit beyond amino acid 1229 results in a receptor that continues to support antiapoptotic, transforming, and mitogenic functions. This suggests that residues 1250/1251, 1251, and 1293/1294 describe a portion of the receptor that can act directly to promote cell death, or may alter other portions of the receptor by modulation of phosphorylation to suppress survival signals. The mechanisms by which these various domains of the IGF-1R may be activated are not known.

The authors have previously shown, using *in situ* hybridization and immunohistochemistry, that insulin-like growth factor-II (IGF-II) message and protein are increa-

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sed in the luminal prostate epithelium during the process of malignant transformation, whereas the IGF-1R mRNA and protein are significantly decreased in these same tissues *(9)*. These findings are of interest because in many malignancies an increase in both IGF-II and IGF-1R have been described *(9)*. The authors have also reported that re-expression of the IGF-1R in a malignant prostate epithelial cell results in decreased tumor size *(6)*. The purpose of the present study was to determine which properties of the IGF-1R were pre-sent in the transformed state when the IGF-1R was decreased, and what functions were lost or gained when the IGF-1R was re-expressed. In order to perform this study, we examined the effects of re-expressing high levels of the IGF-1R in the highly malignant M12 subclone of the P69SV40-T immortalized prostate tumor cell line *(6,10–13)*. An advantage of this system is the availability of the parental, poorly-malignant cell type as well as the malignant sublines for comparative examination of changes in cell components.

Materials and Methods

Materials

Tissue culture media, RPMI-1640, epidermal growth factor, dexamethasone, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Insulin-like growth factors-I and -II were gifts from Eli Lilly and Co. (Indianapolis, IN). The αIR3 monoclonal antibody to the human IGF-1R was obtained from Oncogene Science (Uniondale, NY). Gentamycin, fungizone, geneticin (G418), and deoxyribonuclease were obtained from GIBCO-BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Insulin, transferrin, and selenium were purchased as the additive ITS from Collaborative Research (Waltham, MA). The Vectastain ABC illumination kits were purchased from Vector Laboratories (Burlingame, CA). Nitrocellulose and electrophoresis reagents were purchased from BioRad Laboratories (Richmond, CA). Horseradish peroxidase-linked donkey antirabbit IgG, Enhanced Chemiluminescence detection reagents, and ^{125}I-IGF-I were from Amersham (Arlington Heights, IL). The BCA protein assay kit was from Pierce Biological (Rockford, IL). Human IGF-1R cDNA was obtained from ATCC (Rockville, MD). Each experiment was performed at least three separate times.

Cell Culture

The derivation of the P69SV40-T cell line and its subline M12 have been previously described by Bae et al. *(10, 11,13)*. Cells were cultured in RPMI-1640 supplemented with 23 mM HEPES, 10 ng/mL epidermal growth factor, 0.1 μM dexamethasone, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium. All cells used in these experiments were mycoplasma free as determined by the Gen-Probe Mycoplasma T.C. Rapid Detection System (Gen-Probe, San Diego, CA).

IGF Binding Studies

For determination of IGF receptor number and affinity, cells were cultured in 24-well plates until 90–100% confluent *(9)*. The wells were washed twice at 37°C for 15 min with the binding medium (RPMI with 1% BSA), and preincubated with binding medium for 1 h at 4°C. Cells were then incubated in duplicate with varying concentrations (0.05–2 n*M*) of ^{125}I - IGF-I for 3 h at 26°C in a total volume of 0.15 mL binding medium. Nonspecific binding was determined by adding a 500-fold molar excess of unlabeled IGF-I (0.025–1.0 μ*M*). Following the incubation period, the cells were incubated at 4°C for 15 min, and then rinsed quickly two times with ice-cold PBS and 0.1% BSA. Cells were solubilized with 0.2 mL 0.2 *M* NaOH, and cell-associated radioactivity was counted in a gamma counter using the entire 0.2 mL aliquot. The same 0.2 mL aliquot was then used for protein measurements using the BCA protein assay reagent kit. In each experiment, replicate wells were used to determine cell number.

Specific binding was calculated as the difference between total and non-specific binding and was corrected for the number of cells/well. Nonspecific binding represented about 5% of the total ^{125}I-IGF-I binding. The maximum binding capacity(B_{max}) and the apparent dissociation constant (K_d) of binding was determined by Scatchard plots subjected to linear regression analysis *(16)*. The number of receptors per cell was calculated using Avagadro's constant. Representative IGF-1R binding studies for each cell line were also performed with des(1-3)IGF-I to rule out measurement of cell surface associated IGFBPs.

Proliferation Assays

Cell proliferation was measured by conversion of a tetrazolium salt into a blue formazan product by viable cells using the Cell Titer 96 AQ_{ueous} kit, and quantitated by absorbence at 570 nm. In this assay, 2500 cells were added to each well of a 96-well plate. IGF-II(100 ng/mL) was added at the time of plating. After 72 h in culture, the tetrazolium salt and the dye solution were added and the plates read 2–3 h later. Each cell type was tested at least three times. The correlation between cell number and the tetrazolium assay in the authors' laboratory is $r = 0.97$.

Cell Lines

As the authors have previously described, LISN and LNL6 replication-deficient retroviruses were constructed as described by Kaleko et al. *(6,14)*. The LISN virus contained the 7 Kb human IGF-1R cDNA sequence driven by the retroviral long terminal repeat, and also contained the G418 resistance gene (neomycin phosphotransferase) driven by an internal SV40 promotor. The control LNL6 virus contained the Neo resistance gene without the IGF-

Table 1
Receptor Number and Receptor Affinity (Kd)
of the M12-LISN and M12-LNL6 Clones
Used in this Study and the Parental P69SV40-T Line

Clone	Receptor number (±SD)	Kd (±SD) nmolar
M12-LISN 1	$3.4 \times 10^4 \pm 0.22$	0.33 ± 0.04
M12-LISN 2	$1.8 \times 10^4 \pm 0.24$	0.25 ± 0.02
M12-LNL6 1	$1.8 \times 10^3 \pm 0.27$	0.28 ± 0.05
M12-LNL6 2	$0.8 \times 10^3 \pm 0.12$	0.31 ± 0.07

Each of the clones listed was derived from a separate infection of M12 cells with either LISN or LNL6 vectors.

1R sequence. Amphotropic retroviruses were prepared in PA317 packaging cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Virus-containing medium was harvested from these cells and the titers of infectious particles per mL were determined by assay of antibiotic-resistant colonies of NIH-3T3 cells.

To produce M12 cells expressing increased levels of the IGF-1R (M12-LISN) or control cells infected with the vector LNL6 (M12-LNL6), M12 cells were cultured in RPMI-1640 complete medium with 5% FCS. Cells were infected with either the LISN or LNL6 retroviral particles at a ratio of one viral particle to three M12 cells. Twenty-four hours after infection, the cells were transferred to 100-mm plastic culture dishes containing RPMI complete medium with 0.8 mg/mL G418. In addition to the infected M12 cells, control plates of M12 cells were treated with the same medium. After 7 d, no viable cells were present in the control plates. The infected cells were maintained in 0.8 mg/ml G418 for an additional week and subsequent cultures were maintained in RPMI complete medium containing 0.5 mg/mL G418. Receptor numbers per cell were determined by Scatchard analysis as described above. After initial transfection, clones expressing high levels of IGF-1R were picked using the method of D'Ambrosio *(15)*. After G418 selection, individual clones of the M12-LNL6 cells were selected at random and receptor numbers determined. For the experiments described in this paper, clones from two separate infections of M12 cells with either the LISN or LNL6 vectors were selected, M12-LNL6 1, M12-LNL6 2, M12-LISN 1, and M12-LISN 2. The IGF-1R number and affinity for these clones and the parental P69SV40-T cells are presented in Table 1.

Anchorage-Independent Growth

For studies of anchorage-independent growth of M12-LISN and M12-LNL6 lines, each well of a 24-well plate was first layered with 0.6% agarose-2X RPMI 1640. A top layer containing 10^6 cells suspended in 2X RPMI-supplemented 0.3% agar was then added. Plates were maintained at 37°C , 5% CO_2 for 21 d. Colonies greater than 50 μm in diameter were counted.

Assays of Tumor Growth in Vivo

Male athymic nude mice 6–8 wk old at the time of injection were used. To assess orthotopic growth capacity, the authors injected groups of mice (10/group) in an intraprostatc location with 2×10^6 cells as previously reported *(10,11)*. Briefly, mice were anesthetized with sodium pentobarbital. The lower abdomen was incised and the bladder and male accessory glands retracted anteriorly. Tumor cells were injected in 50 μL PBS into the dorsal prostate with a 30 gage needle. The wound was closed with sterile clips, and the mice were allowed to recover. Following injections, the mice were observed for 2 mo unless the animal was distressed or cachexic, necessitating immediate euthanasia. After euthanasia, the urogenital tract (consisting of the seminal vesicles, dorsal and ventral prostate glands, ampullary glands, bladder and ureter), lungs and diaphragm were collected, fixed in 10% formalin, paraffin embedded, serially sectioned, and stained with hematoxylin and eosin. Large tumors were defined as ≥ 2.0 cm^3 in volume, whereas small tumors were ≤ 0.5 cm^3 in volume. Lymph nodes were collected from the mesenteric and para-aortic areas and examined histologically only if they appeared enlarged at autopsy. Intra-abdominal tumors, growing on the peritoneal wall or attached to the surface were also collected and examined histologically. Large metastases were defined as ≥ 10 mm^3 while small metastases were ≤ 2 mm^3. All mice were maintained in a specific pathogen-free barrier facility. The in vivo experiments were conducted under a protocol approved by the Virginia Commonwealth Institutional Animal Care and Use Committee.

DNA Fragmentation Assay

All cells from culture dishes are collected and pelleted. A representative sample (1×10^6 cells per sample) are suspended in a 1:1 loading buffer:RNase and loaded into wells on a gel. The gel is poured in two part with 1,5% agarose in TBE buffer below the comb and 1% agarose, 2% SDS, and 64 μg/mL proteinase K above the comb. Samples are then electrophoresed and the DNA is visualized by staining with ethidium bromide.

In situ Detection of Apoptosis

This assay was performed on cell preparations grown directly on glass slides. The area to be studied was demarcated with a wax pencil. Endogenous peroxidases were inactivated with hydrogen peroxide. The specimens were end labeled with Klenow Labeling Reaction Mix (Oncogene Research Products, Cambridge, MA), and incubated at 37°C under a Parafilm® coverslip for 1.5 h in a humidified chamber. The labeling reaction was terminated, blocked with the Blocking Buffer supplied in FragEL™-Klenow DNA Fragmentation Detection Kit™ (Oncogene Research Products, Cambridge, MA). The endlabeled fragments were detected with 3'3 diaminobenzidene sup-

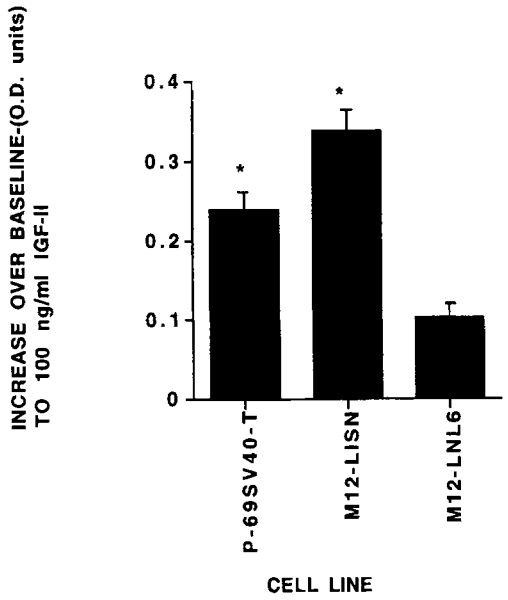

Fig. 1. Increase proliferation in response to 100 ng/mL IGF-II 72 h after the addition of the growth factor. Proliferation was measured by the MTT assay. *$p \leq 0.01$ compared to M12-LNL6 cells.

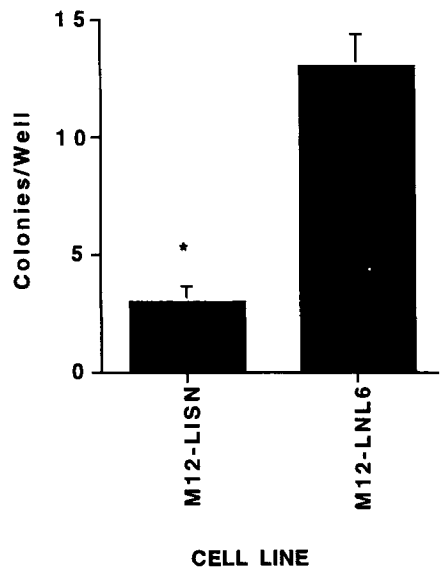

Fig. 2. Growth of the M12-LISN and M12-LNL6 clones in soft agar. Note the significant decrease in colonies formed by the M12-LISN cells compared to the M12-LNL6 cells. *p ≤ 0.01.

plied by the manufacturer and counterstained with 0.3% methyl green. Apoptotic cells were identified and quantitated per 100 total cells under light microscopy.

Statistical Analyses

Statistical analyses were performed using analysis of variance (ANOVA) or Student's *t*-test, as appropriate, with significance accepted at $p \leq 0.05$.

Results

Proliferation

As the authors have previously reported, the parental P69SV40-T cells demonstrated a brisk proliferative response to added IGF-I or -II, while the M12 cells had a much flatter slope in response to IGF *(16)*. Following the reexpression of the IGF-1R, the proliferative response of the M12-LISN cells was again similar in slope to that of the parental P69SV40-T cells, although the basal proliferation rate remained elevated at the level of the M12-LNL6 cells (Fig. 1).

Soft Agar Growth

Since preliminary data had demonstrated a decrease in growth of the M12-LISN clones when injected subcutaneously into athymic mice, we examined attachment-free growth of the cells in soft agar as an in vitro index of tumorgenicity. As seen in Fig. 2 there was a significant decrease in the number and size of colonies that formed in the soft agar in from M12-LISN cell compared to the M12-LNL6 controls, $p \leq 0.001$.

Table 2
Mice Injected with M12-LNL6 and M12-LISN Cells

Cells injected	Days to death ± sem	Weight at death (gms) ± sem
M12-LNL6	49.3 ± 5.2*	27.3 ± 1.1**
M12-LISN	68.0 ± 1.3	34.0 ± 1.2

The mice with the M12-LNL6 cells also weighed significantly less at time of euthanasia than did the M12-LISN animals, Table 2, $p \leq 0.01$.

Orthotopic (Intraprostatic) Tumor Growth

Following intraprostatic injection of the M12-LNL6 cells, all of the animals developed large prostatic tumors within 2 mo of injection, and all of the animals had large diaphragmatic and pulmonary metastases. In contrast, only 50% of the mice injected with M12-LISN cells developed large intraprostatic tumors, whereas the rest had tumors that were small. Fifty-percent of the M12-LISN animals developed visible diaphragmatic or pulmonary metastases. Furthermore, the mice that received the M12-LNL6 cells had to be sacrificed significantly earlier than those receiving the M12-LISN cells because of cachexia and distress, Table 2.

Apoptosis

When exposed to the apoptosis inducing agents 6-hydroxyurea, TGF-β, or retinoic acid, the parental P69SV40-T cells readily undergo apoptosis as determined by DNA fragmentation in agarose gels and a decrease in cell number, Fig. 3. The M12-LNL6 cells have a much decreased apoptotic response to these agents; however,

Table 3
Percentage of Cells Grown on Glass Slides that Were Demonstrated to have Apoptotic Nuclei by Labeling 3' Ends of DNA Fragments with Biotin Labeled UTP

Cell Line	% Apoptotic cells baseline	% Apoptotic cells 4 h after 6-OH urea	*P*
P69SV40-T	5.2 ± 1.4 (sem)	30.7 ± 4.3	$p \leq 0.01$
M12-LISN	4.2 ± 1.7	25.6 ± 2.4	$p \leq 0.01$
M12-LNL6	1.0 ± 0.3	1.7 ± 0.6	N.S.

P = baseline vs. 4 hr post addition of 6-OH urea.

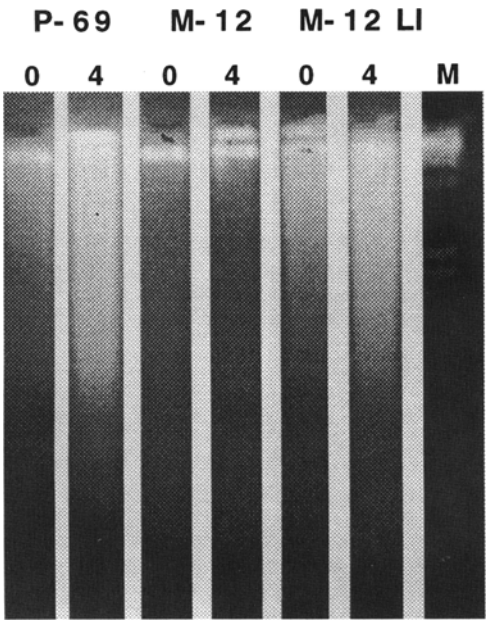

Fig. 3. Ethidium bromide stained gel of DNA from P-69, M12-LNL6, and M12-LISN cell lines collected at 0 and 6 h time periods following the addition of 6-OH urea. M = DNA marker. Note the absence of DNA fragmentation in the M12-LNL6(M-12) cells at the 6 h time period compared to the P69SV40-T(P69) and M12 LISN(M12-LI) cells.

when the IGF-1R is reexpressed in the M12-LISN cells appear to have again acquired the ability to undergo the apoptosis in response to 6-OH urea, Fig. 3. Retinoic acid and TGFβ1 produced similar results to 6-OH Since all of these studies were begun with cells that had been deprived of serum or growth factors for 16 to 24 hr, some degree of apoptosis may be seen in the P69SV40-T and M12-LISN in response to the loss of the anti-apoptotic effect of the growth factors.

Apoptosis was also determined in situ as described in Materials and Methods. The results of this study are seen in Table 3. These findings are consistent with those seen in the DNA fragmentation gel (Fig. 3) in that they show increased apoptosis in the basal state, and post 6-OH urea in P69SV40-T and M12-LISN cells, but significantly less in the M12-LNL6 cells.

Discussion

The IGF system has been shown to be a crucial element to the development of malignancy *(1,2)*. Evidence for the importance of the IGF system in transformation is seen when the IGF system is interrupted by antisense IGF-1R oligonucleotides or over-expression of inhibitory IGF binding proteins (IGFBP) *(1,17–19)*. When the IGF system is interrupted, proliferation of tumor cells in monolayers is decreased, colony formation in soft agar is inhibited, and tumor formation in immunocompromised mice is decreased. These effects of the IGF system are seen in transformed cells; however, over-expression of the IGF system alone is not enough to cause transformation in most cell lines, but is a component of the molecular events that are necessary for the transformation process.

In addition, to the positive effects on the transformation process, recent studies in breast and prostate epithelial cancers and myeloid leukemia have suggested that over-expression of the IGF-1R in these tumor cells may have a negative effect on tumor growth *(6,20,21)*. The present study provides further information as to how the IGF-1R may inhibit tumor growth.

The IGF system is composed of three major components including the ligands IGF-I and -II, two cell surface receptors IGF-1R, and the mannose 6-phosphate/type 2 IGFr, and six high-affinity IGFBPs. Although IGF independent actions have been proposed for several of the IGFBPs and the type 2 IGFr has been suggested, but not conclusively proven to have intracellular signaling mechanisms, most of the cellular activities of IGFs are mediated through the IGF-1R. The IGF-1R has been demonstrated to effect antiapoptotic, mitogenic, transforming, proapoptotic, metabolic, and differentiating functions *(22–26)*. Therefore, a single receptor must be able to mediate multiple actions. The mechanisms by which the receptor may mediate these effects may reside in two levels of signal tranduction in a cell. The first is alteration of the intracellular signaling pathways. The activation of these pathways to affect different functions of the IGF system has been recently reviewed *(27–29)*. With respect to the effect on apoptosis that was shown in the present study, the antiapoptotic effects of IGFs have been demonstrated to be mediated through both the phosphatidyl inositol-3(PI3) kinase and mitogen activated protein (MAP) kinase pathways *(27–30)*. A possible proapoptotic effect has been suggested to occur through the Crk II, Sos, Ras intracellular pathway *(31)*. However, the mechanism for the activation of this pathway independently of the PI-3' kinase and other portions of the MAP kinase pathway that are involved with antiapoptotic activity has not been identified.

A second level of signal transduction that may account for the multiple functions of the IGF-1R has been proposed to exist in the β subunit of the IGF-1R. Using site-directed mutagenesis, specific domains of the β-subunit have been

shown to cause either mitogenesis, transformation, protect against apoptosis, and induce apoptosis *(8,32)*. Selective signaling by specific regions of the IGF-1R β-subunit could account for the changes found in the present study. How certain regions of a receptor would be activated whereas other regions were quiescent is not known. Possibly phosphorylation of specific amino acids in the receptor would activate that region or lead to inhibition of another region. Regardless, the mechanisms of selective activation of regions of the IGF-1R remains an exciting possibility for regulation of multiple IGF functions.

In the study presented in this paper, as well as data from other laboratories, the concept of a multifunctional IGF-1R with both pro- and anti-apoptotic activities begins to evolve. This suggests the possibility that a tumor cell may select a level of IGF-1R expression that maximizes the mitogenic, antiapoptotic, and transformation properties of the receptor while obviating the differentiation and proapoptotic activities of the IGF-1R (Fig. 2). These data suggest a dual function of the IGF-1R in malignant cells. Strategies designed to decrease the properties that favor tumor formation and maintenance or enhance the differentiating and proapoptotic activities of the IGF-1R in a tumor may provide new treatment regimens for epithelial malignancies.

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