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13. ABSTRACT *(Maximum 200 Words)*

Studies with gene knockout mice have demonstrated M6P/IGF2R is an inhibitor of IGF2-dependent embryonic growth. Tumor-associated M6P/IGF2R mutations that disrupt IGF2 binding have been described suggesting that the IGF2 antagonist action of M6P/IGF2R may also play a role suppressing tumorigenesis. To investigate the role of M6P/IGF2R as an IGF2 antagonist in breast cancer, M6P/IGF2R was overexpressed in IGF2-sensitive MCF7 cells. Recombinant IGF1- and IGF2-dependent proliferation was compared between control transfected cells, cells constitutively overexpressing wildtype M6P/IGF2R and cells overexpressing an IGF2-binding or an M6P-binding defective mutant. Despite 5- to 10-fold overexpression of wildtype M6P/IGF2R, IGF2-dependent growth in either monolayer or in suspension culture was unaffected. To confirm this unanticipated negative result, MCF7 cell clones engineered to overexpress wildtype M6P/IGF2R under the control of a doxycycline inducible promoter were utilized. Ten- to 15-fold induction of M6P/IGF2R expression with doxycycline resulted in no change of growth rate for cells in the presence of IGF2 when compared with control cells. It has previously been demonstrated that affinity of IGF2 for M6P/IGF2R suppresses autocrine IGF2 activity (Ellis, M. J. C. *et al.,* Mol Endo 10:286-297, 1996). These data therefore suggests that M6P/IGF2R may operate to inhibit autocrine IGF2 within the secretory pathway but not efficiently suppress the action of IGF2 after it has been released from the cell.

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The aim of this investigation is to provide evidence to determine whether *mannose 6-phosphate insulin-like growth factor 2 receptor* (*M6P/IGF2R*) is a tumor suppressor gene. Genetic studies have identified M6P/IGF2R as a putative tumor suppressor. The gene locus at 6q25-26 is reportedly subject to loss of heterozygosity (LOH) accompanied by loss-of-function point mutations in the remaining allele of both liver and breast carcinomas (1,2). Furthermore, somatic mutations in coding region microsatellites of *M6P/IGF2R* have been documented in a variety of replication error positive (RER+) tumors of the gastrointestinal tract, brain and endometrium (3,4,5,6).

Physiologically, well-defined functions of M6P/IGF2R include: 1) to facilitate endocytosis of extracellular mannose 6-phosphorylated (M6P) proteins (7); to escort newly synthesized M6P proteins from the Golgi to endosomes (8); 3) to play a role in the proteolytic activation of transforming growth factor β (TGFβ), a negative growth regulator (9); and 4) to target insulin-like growth factor 2, a potent mitogenic peptide, for degradation thereby preventing insulin-like growth factor 1 receptor (IGF1R) activation (10). The cellular consequences of loss of M6P/IGF2R function are therefore potentially wide-ranging. It has been hypothesized that loss of M6P/IGF2R function may influence tumor growth by promoting tumor invasion through lysosomal enzyme misrouting, decreasing the growth inhibitory activity of TGFβ, and increasing the mitogenic and cell survival activities of IGF2 (11). Although M6P/IGF2R as a transport protein has been extensively studied, the role of M6P/IGF2R in growth regulation in the adult remains unclear. There is some evidence that the role of M6P/IGF2R as an IGF2 antagonist is critical in regulating cellular growth (10,11,12,13) but to date, there is no direct evidence to support the hypothesis that M6P/IGF2R function is important for suppression of tumor cell growth. This study addresses the hypothesis that M6P/IGF2R is a tumor suppressor in IGF2-sensitive malignancies.

Current Progress

This study employs IGF2-sensitive MCF7 breast cancer cells to investigate the consequence of overexpression of wildtype M6P/IGF2R or specific ligand binding deficient mutants that specifically bind either IGF2 or M6P proteins. Bovine M6P/IGF2R cDNA was employed since transfected bovine M6P/IGF2R can be distinguished from endogenous human M6P/IGF2R by a bovine-specific monoclonal M6P/IGF2R antibody. Furthermore, the human

and bovine M6P/IGF2R cDNA share 80% sequence identity (14) and studies investigating the M6P-sorting properties of M6P/IGF2R, the functional properties of the human and bovine M6P/IGF2R were identical (15,16). Wildtype M6P/IGF2R cDNA subcloned into the expression vector pcDNA3 was transfected into MCF7 cells. To identify the potential role of M6P/IGF2R in growth regulation, mutations were introduced in the receptor, which selectively disrupt either IGF2- or M6P-binding. IGF2*def*R deficient in IGF2 binding, and M6P*def*R deficient in M6P binding, in addition to an inactive frameshift negative control D95Stop, were also subcloned into pcDNA3 and transfected into MCF7 cells. cDNA constructs for bovine M6P-binding deficient and wt M6P/IGF2R were kindly provided by Dr N. Dahms (15). We hypothesized that increasing the M6P/IGF2R expression by transfection would be sufficient to observe M6P/IGF2R-mediated growth suppression. Preliminary data from previous reports indicate that the function of M6P/IGF2R as an IGF2 antagonist is important in suppression of IGF-stimulated MCF7 tumor cell growth. The SOW for months 25 to 36 proposed the generation of human M6P/IGF2R cDNA that incorporated mutations identified in human breast tumors. These mutants would then be subcloned into the inducible expression system and transfected in MCF7 cells in order that change in phenotype could be assessed. However, in the past year, several repeats of experiments reveal that overexpression of M6P/IGF2R does not significantly regulate exogenous IGF2-dependent growth and in the remaining months of this grant, this negative result will be further assessed.

Overexpression of M6P/IGF2R in MCF7 cells does not alter IGF2-dependent growth

MCF7 cells transfected to constitutively express wt and mutant M6P/IGF2Rdemonstrated a 5-fold increase (determined by densitometry not shown) of M6P/IGF2R over endogenous levels (Figure 1A). A Western blot with a bovine receptor specific monoclonal antibody and with a polyclonal antibody to M6P/IGF2R confirmed that transfected wt MCF7/IGF2R, IGF2defR and M6PdefR were expressed at similar levels in each pool. The effect of M6P/IGF2R overexpression on proliferation in low serum (.05% FBS) medium supplemented with either 10nM IGF1 or IGF2 was studied. Figure 1, panels B and C, present the compilation of four separate WST-1 proliferation assays. As expected IGF1-dependent growth was similar for all the transfectants (Figure 1B) since IGF1 has no affinity for M6P/IGF2R. However, overexpression of M6P/IGF2R did affect the IGF2-dependent growth rate of cells

overexpressing wildtype or either of the two mutant receptors when compared to cells transfected with the negative control (Figure 1C). The consequence of M6P/IGF2R overexpression on the anchorage-independent growth capability of cells was also investigated (Figure 1D). To control for any differences in cell plating among the four pooled cell lines in soft agar assays, the results are presented as the ratio of the number of colonies (greater than 60μm) formed from stimulation with 10 nM IGF1 to the number of colonies formed from stimulation with 10 nM IGF2. If overexpression of M6P/IGF2R negatively affects IGF2- but not IGF1-dependent growth of MCF7 cells as hypothesized, cells overexpressing wt M6P/IGF2R and M6P*def*R would form less colonies in response to IGF2 then cells expressing IGF2*def*R or the negative control. Thus, the ratio of IGF1-dependent growth:IGF2-dependent growth would be greater for those cells expressing wt or M6P*def*R compared to that of control cells. However, no difference was detected in the number of colonies from cells overexpressing the various receptor constructs; the IGF1:IGF2 ratio of colonies formed in soft agar after a 2 week period from cells expressing either wt M6P/IGF2R or M6P*def*R was not significantly different from cells expressing IGF2*def*R or the negative control.

To determine that exogenous M6P/IGF2R in MCF7 cells was appropriately localized in the Golgi and endosomal compartments, the transfected cells were grown on microscope slides,

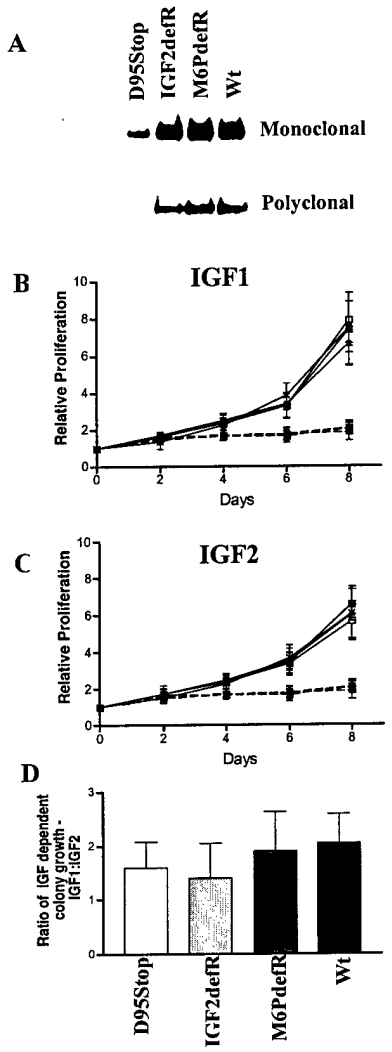

Figure 1. Constitutive M6P/IGF2R overexpression does not affect cell growth. **A**, Transfected MCF7 lysates were screened by Western blotting. **B**, **C**, Relative proliferation of cells expression the various M6P/IGF2R constructs □ D95Stop; ■ IGF2*def*R; X M6P*def*R; * Wt) was determined by WST-1 readings at the indicated time points for cells untreated (broken lines) or treated with 10nM IGF1 or IGF2 (solid lines). Values are an average of 4 independent experiments and the error bars indicate the SEM. **D**, Cells plated in soft agar were treated with 10nM IGF1 or 10nM IGF2 and the number of colonies counted after 14 days. The bar chart represents the average of ratios from 6 independent experiments and the error bars indicate the SEM.

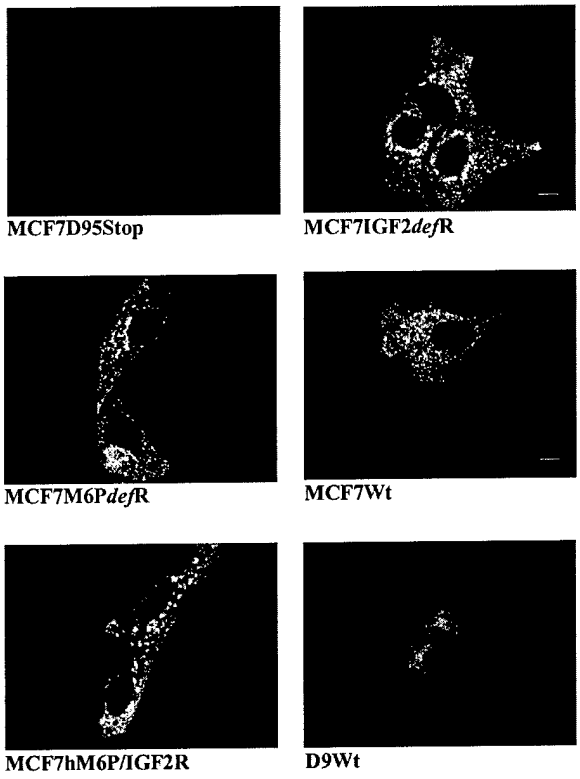

Figure 2. Subcellular localization of M6P/IGF2R expressed in MCF7 and D9 cells. MCF7 cells expressing bovine M6P/IGF2R receptor constructs, D95Stop, IGF2defR, M6PdefR or wt M6P/IGF2R, were cultured on glass coverslips, fixed and immunostained with an M6P/IGF2R polyclonal antibody and fluorescent secondary antibody. MCF7 cells expressing human wildtype M6P/IGF2R (hM6P/IGF2R) and D9 cells expressing bovine wildtype M6P/IGF2R were also stained. 600x magnification.

fixed, permeablized and probed with a polyclonal antibody. The stained cells were viewed by confocal microscopy (Figure 2). MCF7 cells transfected with the negative control D95Stop exhibited extremely low levels of M6P/IGF2R, indicating the low background of endogenous human M6P/IGF2R of MCF7 cells. The pattern of staining for wt M6P/IGF2R, M6P*def*R and IGF2*def*R constructs were identical and consistent with M6P/IGF2R localization at the Golgi and endosomal compartments. This distribution of M6P/IGF2R is indistinguishable from that of human wildtype M6P/IGF2R (hM6P/IGF2R) overexpressed in MCF7 cells or bovine wildtype M6P/IGF2R overexpressed in the murine fibroblast-like D9 cell line. Furthermore, the pattern of exogenous M6P/IGF2R localization shown here is similar to that observed in PD3881 transfected to overexpress human M6P/IGF2R and in normal rat fetal cardiac myocytes [Kang, 1999 #51]. We conclude then that the distribution of transfected M6P/IGF2R in MCF7 cells is normal.

The findings with pooled transfected MCF7 cells constitutively expressing M6P/IGF2R disproved our original hypothesis that the IGF2 antagonist action of M6P/IGF2R has a role in tumor suppression of IGF2 sensitive malignancies. To confirm this negative result, MCF7 clones transfected to overexpress wt and mutant M6P/IGF2R under the control of a doxycycline inducible promoter were utilized. A clonal population iWt1, was identified and expanded for further study. Western blot analysis of iWt1 lysates of cells treated without or with 1μg/ml doxycycline for 24 hours demonstrates overexpression of bovine wildtype M6P/IGF2R above

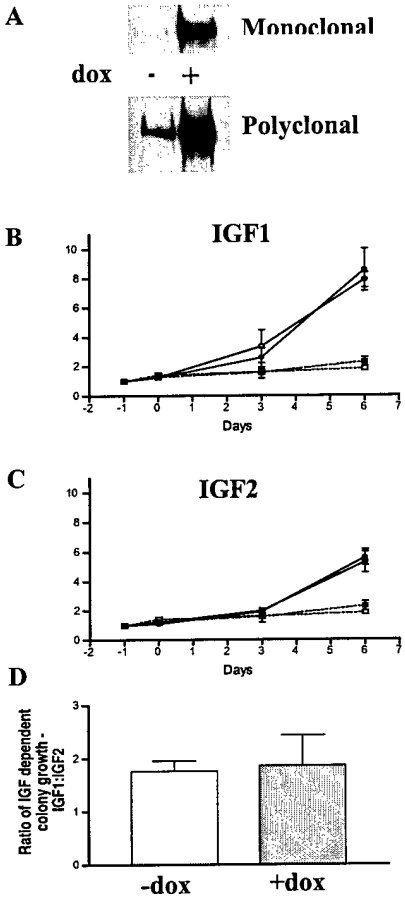

Figure 3 Inducible M6P/IGF2R overexpression does not affect cell growth. **A,** Western blot of whole cell lysates of MCF7 cells treated with or without 1 μg/ml doxycycline for 24 hours demonstrating inducible expression of functional wt M6P/IGF2R. **B, C,** Cells treated with or without doxycycline to induce wt M6P/IGF2R expression were given no treatment or treated with 40 nM IGF1 or IGF2 (●, +dox, + IGF; ○, -dox, -IGF; ■, +dox, SFM; □, -dox, SFM; broken line, SFM; solid line, IGF treatment) and proliferation determined by WST-1 at the times indicated. The graphs are representative of 3 experiments and the error bars represent the SEM of results in triplicate. **D,** Cells plated in soft agar were treated without or with doxycycline for 24 hours before treatment with 40 nM IGF1 or IGF2 and the colonies were counted after 14 days.

endogenous levels of human M6P/IGF2R. Confocal microscopy confirmed that the subcellular distribution of doxycycline induced wt M6P/IGF2R in iWt1 was identical to that shown in MCF7 pools constitutively overexpressing the receptor (data not shown). With this tightly regulated inducible expression model, WST-1 proliferation assays were repeated to study the effect of induced overexpressed M6P/IGF2R on the growth rate of cells compared to untreated controls in response to IGF1- and IGF2-dependent growth. iWt1 cells were plated in a 96-well dish and treated with or without doxycycline to induce M6P/IGF2R expression for 24 hours before IGFs were added. Figure 3B and 3C demonstrates that overexpression of wt M6P/IGF2R did not affect IGF dependent growth.

Anchorage independent growth was also unaffected by overexpression of M6P/IGF2R. iWt1 treated with doxycycline to induce M6P/IGF2R overexpression showed no retarded colony growth compared to untreated cells. The ratio of IGF-dependent colonies (greater than 60μm) was similar whether or not cells were treated with doxycycline to induce receptor overexpression (Figure 4D).

Key Research Accomplishments

1. MCF7 clones that inducibly express wt M6P/IGF2R and mutant M6P/IGF2R constructs.

Reportable Outcomes

Publications:

1) **Stacey A DaCosta,** Lisa M Schumaker and Matthew J Ellis. Mannose 6-Phosphate/ Insulin-like Growth Factor 2 Receptor, a bona fide Tumor Suppressor Gene or Just a Promising Candidate? *Journal of Mammary Gland Biology and Neoplasia 5(1):85-94, 2000*

2) Adam J Oates , Lisa M Schumaker, Sara B Jenkins, Amelia A Pearce, **Stacey A DaCosta,** Banu Arun and Matthew J C Ellis. The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R), a putative tumor suppressor gene. *Breast Cancer Research and Treatment 47:269-281, 1998.*

Abstracts:

1) Stacey A DaCosta, Lisa M Schumaker, Adam J Oates and Matthew J C Ellis. Mannose 6-Phosphate /Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) is an inefficient antagonist of extracellular IGF2. DOD Breast Cancer Research Era of Hope Meeting. Atlanta, GA. Abstract I-9 6/2000

2) Stacey A DaCosta, Lisa M Schumaker, Adam J Oates and Matthew J C Ellis. Mannose 6-Phosphate /Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) is an inefficient antagonist of extracellular IGF2. American Association of Cancer Research 91st Annual Meeting. San Francisco, CA Abstract #5002 4/2000.

3) Stacey A DaCosta, Lisa M Schumaker, Adam J Oates and Matthew J C Ellis. Mannose 6-Phosphate /Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) as a tumor suppressor gene. American Association of Cancer Research 91st Annual Meeting. New Orleans, LA Abstract #4230 4/1998

Conclusions

It has been determined that affinity for M6P/IGF2R suppresses autocrine IGF2 activation of IGF1R [ref]. This data together with the presented results suggest that M6P/IGF2R may inhibit autocrine IGF2 within the secretory pathway, but not efficiently suppress IGF2 after release from the cell. IWt1 cells were therefore transfected with a hygromycin –based IGF2 retrovirus. The resulting hygromycin resistant colonies were expanded and screened for IGF2 expression by dot blot and RNase protection assays. However, no colonies were obtained that expressed high levels of IGF2. The IGF2 cDNA was then subcloned into a zeomycin-based expression vector and iWt1 cells transfected with this plasmid. This will create a model in which to study the impact of M6P/IGF2R overexpression in an autocrine IGF2 model. iWt1 clones expressing IGF2 will be characterized and proliferation assays, soft agar assays and apoptosis assays will be done to determine any effect of M6P/IGF2R overexpression on the phenotype of these cells.

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