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13. ABSTRACT *(Maximum 200 words)*

A large number of recent studies have suggested that the insulin-like growth factor (IGF) system plays an important role in breast cancer progression. Mutational analyses of breast cancers have provided strong evidence to suggest that one component of the IGF system, the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) is a tumor suppressor. At least three functions of M6P/IGF2R exist. These include the targeting for degradation of the mitogenic factor, insulin-like growth factor 2 (IGF2), the trafficking of mannose 6-phosphate proteins from the Golgi to lysosomes, and the activation of the negative growth regulator, transforming growth factor β (TGFβ). It has been hypothesized that loss or aberrant expression of M6P/IGF2R may result in increased tumor growth rates due to the disruption of negative growth regulatory mechanisms mediated by M6P/IGF2R. This proposal seeks to provide evidence that M6P/IGF2R is a negative growth regulator and further support the hypothesis that M6P/IGF2R is a tumor suppressor.

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Steven D. Cook 8/13/98

PI - Signature / Date

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Introduction

The comments on the proposed investigation excited considerable debate between Dr Byers, Dr Ellis and myself. We were particularly concerned with the comment "the candidate is deserving of a more challenging project". We therefore considered several alternatives to the study originally presented. In this first year report I will summarize a new project direction that reflects my desire to investigate the role of insulin-like growth factors (IGF) in human breast cancer but rather than investigating the influence of insulin-like growth factors on adhesion and catenin function the project focuses on the mannose 6 phosphate/insulin-like growth factor 2 receptor, a potential breast cancer tumor suppressor gene. My thesis supervisors and I felt that the techniques and experimental designs outlined in this project provided a more focused approach and would provide a stronger basis for my future as a breast cancer researcher.

Insulin-like growth factor system and cancer

A biological role of the insulin-like growth factor system in the development of breast cancer is supported by a wide range of evidence. The insulin-like growth factors 1 and 2 (IGF1 and 2) are potent stimulators of cell growth in breast cancer cells both *in vivo* and *in vitro* (1, 2, 3). IGF2 is the predominant IGF present in breast tumors and is mainly the product of stromal cells such as fibroblasts (4, 5). Importantly, in transgenic studies, IGF2 overexpression targeted to the mouse mammary gland has been shown to induce breast tumor formation (6). This experiment provides persuasive evidence that aberrant IGF signaling can lead to breast malignancies. Most recently, it has been demonstrated that IGFs function as survival factors protecting cells from apoptosis-inducing agents (7, 8). This has led to the proposition that suppression of cell death is a key aspect of the contribution of IGF signaling to multistep carcinogenesis.

The growth and anti-apoptotic effects of IGF2 and IGF1 are mediated by the insulin-like growth factor 1 receptor (IGF1R), a receptor tyrosine kinase which has been

found to be overexpressed in breast cancer tumors and cell lines (9). IGF2 also interacts with a second receptor, the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R), which has been shown to target IGF2 for lysosomal degradation (10). M6P/IGF2R is therefore considered to be an IGF2 antagonist protein (11). Intriguingly, loss of heterozygosity at the *M6P/IGF2R* locus has been identified in breast and other malignancies, and loss-of-function point mutations have been detected in the remaining allele (12, 13, 14). These observations suggest *M6P/IGF2R* may be a tumor suppressor gene.

The M6P/IGF2R is a multifunctional transport protein which has both intracellular and cell surface functions. Intracellularly, it facilitates the transport of newly synthesized mannose 6-phosphorylated lysosomal proteins from the Golgi network to lysosomal compartments. At the cell surface, M6P/IGF2R binds and internalizes IGF2 and targets it for lysosomal degradation. M6P/IGF2R also plays a role in the proteolytic activation of TGFβ, a potent negative growth regulator (10). Furthermore, Kang *et al* (15) has recently reported that M6P/IGF2R binds to retinoic acid, a molecule which is essential for a variety of biological processes including development, cellular metabolism and the regulation of cell proliferation (16). The interaction between M6P/IGF2R and retinoic acid has been shown to modulate the trafficking and internalization functions of the receptor (15).

These data indicate that the cellular consequences of loss of M6P/IGF2R function are potentially wide-ranging. For example, it has been hypothesized that loss or aberrant cellular M6P/IGF2R expression may promote tumor cell growth by decreasing TGFβ activity, or by reducing IGF2-dependent IGF1R activation (10). It is therefore the focus of this investigation to provide evidence that M6P/IGF2R is a negative growth regulator of breast cancer cell proliferation.

This investigation therefore addresses the following hypothesis:

M6P/IGF2R is a negative regulator of the growth and survival of breast cancer cells.

To investigate this hypothesis, the following specific aims are proposed:

1) To establish an MCF7 based cell system to investigate the negative growth of M6P/IGF2R.

2) To investigate the growth regulatory properties of wildtype and ligand-binding deficient M6P/IGF2R mutant receptors in response to IGF2 and a number of growth factors including IGF1, insulin and heregulin.

3) To develop a human M6P/IGF2R-based system to investigate the phenotype of somatic mutations identified in human breast tumors.

Results and Discussion

The purpose of this study is to investigate the potential negative growth regulatory properties of the M6P/IGF2R in breast cancer cells. To achieve this goal the breast cancer cell line MCF7 has been transfected with expression constructs for wildtype M6P/IGF2R and M6P/IGF2R mutants, selectively defective in either mannose 6-phosphate, or IGF2 ligand-binding activities. Bovine M6P/IGF2R expression constructs have been employed in transfection studies with human cell lines to allow the distinction between the transfected M6P/IGF2R and the endogenous human M6P/IGF2R. The M6P-binding deficient bovine M6P/IGF2R (kindly provided by Dr N. Dahms) was engineered with two point mutations resulting in substitutions of arginine to alanine at positions 435 and 1334 (17). The IGF2-binding deficient bovine M6P/IGF2R construct (18) was generated in the laboratory by a

site-directed mutagenesis polymerase chain reaction approach which allowed a substitution of isoleucine with threonine at amino acid position 1581. As a negative control, a frame shift mutation encoding a nonfunctional truncated M6P/IGF2R was also synthesized. The binding selectivity of these receptors have been investigated in the Ellis laboratory and the details will soon be submitted for publication (19). This project will utilize these receptor constructs to evaluate the potential role of M6P/IGF2R as a negative growth regulator. We anticipate that information from these mutants will allow us to identify a cellular system in which to evaluate the significance of M6P/IGF2R mutations identified in human breast cancers.

Work Completed in Months 1 to 12.

1) Transfected the MCF7 cell line with four bovine M6P/IGF2R expression vectors containing wildtype and mutant cDNAs.

2) Investigated the IGF2 mediated growth response in MCF7 cells overexpressing wildtype and mutant M6P/IGF2R receptors.

To investigate the phenotype associated with overexpression of wildtype M6P/IGF2R, MCF7 cells were stably transfected with constitutive expression constructs for the wildtype M6P/IGF2R, the IGF2 ligand-binding mutant, the M6P ligand-binding mutant and the truncated negative control. Transfected cells were serum deprived and plated in triplicate in 96 well dishes at day zero. The cells were then treated with 5 nM or 10 nM IGF2 for a period of 6 days. XTT readings were performed at days 2, 4 and 6 (Figure 1). These studies demonstrated that IGF2-stimulated growth of MCF7 cells is reduced in the presence of elevated expression of wildtype M6P/IGF2R. Growth suppressor activity was also demonstrated when the M6P ligand-binding mutant was over expressed. However the IGF2 ligand-binding mutant receptor was inactive as a growth

suppressor molecule. These results suggest that M6P/IGF2R acting as an IGF2 antagonist has negative growth regulatory effects when expressed at high levels in MCF7 cells.

3) Developed an inducible expression system for bovine M6P/IGF2R in MCF7 cells.

The constitutive expression system achieved a relatively a low level of overexpression when estimated by Western blotting and immunofluorescence techniques. Of equal concern, receptor expression was lost within 2-3 passages following stable transfection of selected cell lines. We believe this may be due to the deleterious cellular effects of overexpressing M6P/IGF2R. Cells which express high levels of M6P/IGF2R may be being selected against, leading to a preponderance of cells which have lost M6P/IGF2R expression. In an attempt to overcome this problem the "Tet-On" expression system was employed. This system permits high levels of expression of a gene of interest following the addition of the inducer molecule, doxycycline. The Tet-On expression system has two components, the "regulator" plasmid pUHD 17-1 and the "response" plasmid pUHD 10-3. The response plasmid contains a promoter with a tetracycline-responsive element (TRE) upstream of a multiple cloning site where the gene of interest is cloned. The regulator plasmid, pUHD 17-1, constitutively expresses a reverse tetracycline-controlled transactivator (rtTA) fused to an activation domain, VP16 AD. In the presence of doxycycline (a tetracycline derivative), the rtTA fused to the VP16 AD activates transcription of the gene of interest cloned in the response plasmid pUHD 10-3. The wildtype receptor cDNA and the various mutated receptor cDNAs were cloned in the pUHD 10-3 plasmid.

The pUHD 10-3 plasmids containing the respective cDNAs were co-transfected with pBABEpuro, a plasmid which confers resistance to puromycin, into MCF7-RTA16 cells (containing the regulator plasmid). Following stable selection, multiple clones were obtained from the transfections of each of the four different receptor constructs. Following a doxycycline induction period of 24 hours, immunofluorescence staining was employed to

screen for clones expressing the bovine M6P/IGF2R. This screening technique was especially useful as it allowed the efficient and rapid screening of a large number of clones. Two clones which expressed high levels of the wildtype receptor upon induction with doxycycline were obtained and one clone expressing the IGF2 ligand-binding mutant and one clone expressing the M6P ligand-binding mutant were also identified.

Doxycycline-induced M6P/IGF2R expression was further investigated and quantified by Western blotting analysis. A monoclonal antibody specific for bovine M6P/IGF2R was used to determine the levels of the expression-construct derived protein. A rabbit polyclonal antibody which recognized both human and bovine M6P/IGF2R allowed for an assessment of the relative increase in total M6P/IGF2R levels when compared to endogenously expressed M6P/IGF2R (Figure 2). Bovine M6P/IGF2R can be detected 6 hours after doxycycline expression and peaks at approximately 48 hours, although the expression remains elevated until 96 hours after doxycycline treatment (Figure 3).

4) Cloned the human M6P/IGF2R cDNA into a constitutive expression vector and expressed M6P/IGF2R in receptor null mouse D9 cells.

D9 cells are murine fibroblast-like cells which lack endogenous M6P/IGF2R expression. The human M6P/IGF2R was expressed in these cells (Figure 4) to verify the expression construct was functional and to allow future determination of the IGF2 and mannose 6-phosphate ligand binding activities using assays optimized in the laboratory with the bovine receptor. This preliminary work is essential for studies of M6P/IGF2R mutations that occur in human malignancies.

Work to be completed in months 13 to 24.

1) Establish the growth effects of wildtype and mutated M6P/IGF2R using the inducible expression system.

The inducible clones are currently being used in growth assays (XTT colorimetric assays) to determine the effects of wildtype and mutated M6P/IGF2R on growth mediated by IGF2. We will also analyze growth in the presence of other growth factors, including heregulin, insulin and IGF1, to determine if the growth suppression mediated by M6P/IGF2R is restricted to IGF2 signaling, or if overexpression somehow alters other mitogenic pathways.

2) Develop and implement an animal protocol for the investigation of the phenotypic consequences of M6P/IGF2R in tumor xenografts.

MCF7 cells which inducibly express the M6P/IGF2R when treated with doxycycline will be introduced in mammary fat pads of nude mice. Palpable tumors will be allowed to develop and 50% of the mice will be administered doxycycline via drinking water. The effect of inducing M6P/IGF2R expression on the tumor will be assessed. **No animal experiments will be conducted until approval has been received.** We believe that *in vivo* experiments are essential to fully investigate the potential biological consequences of M6P/IGF2R overexpression, since some of the pathways that may be influenced by M6P/IGF2R activity may not be required during in vitro growth.

3) Determine the biochemical properties of the human M6P/IGF2R, i.e., its ability to bind IGF2 ligand and mannose 6-phosphorylated proteins.

IGF2 crosslinking studies and mannose 6-phosphate affinity chromatography will be used to investigate the IGF2 binding and the mannose 6-phosphate binding of the human receptor. These techniques already optimized in the Ellis laboratory.

4) Subclone the human M6P/IGF2R into the tetracycline inducible expression vector and transfect into breast cancer cell line.

The human cDNA will be cloned into the inducible system plasmids. *In vitro* assays previously optimized for the bovine receptor studies will be used to determine the growth effects on human M6P/IGF2R overexpression in MCF7 cells.

Work to be completed months 25 to 36.

1) Prepare human M6P/IGF2R cDNAs incorporating mutations that have been identified in human breast tumors.

Naturally occurring mutations which have been identified in human breast tumors will be introduced in the human M6P/IGF2R cDNA by a PCR site-directed mutagenesis approach.

2) Subclone mutants into inducible expression system vectors and transfect into MCF7 cells.

The mutant human M6P/IGF2R will be cloned into the Tet response plasmid to utilize the developed inducible system.

3) Determine phenotype of human M6P/IGF2R mutants in vitro and in vivo.

Assays previously described will be performed in order to determine the effect of the human M6P/IGF2R mutations in breast cancers.

Potential problems with the experimental design.

The principle objection to the experimental design described above is the choice of breast cancer cell line. As far as we have been able to determine (by Western blot and IGF2 crosslinking), MCF7 cells express wildtype M6P/IGF2R. Ideally the proposed series of transfection experiments would employ a breast cancer cell line that has lost M6P/IGF2R expression and/or function through somatic mutation. We are continuing to screen for such a cell line, however so far without success. We will continue to screen

breast cancer cell lines as they become available. Our current choice of MCF7 cells rests on the observation that these cells respond to IGF2 in serum-free media and therefore provide an opportunity to investigate the IGF2 antagonist properties of the receptor. We accept that any phenotype obtained may be masked by the presence of endogenous wildtype receptor. If we are unsuccessful in suppressing MCF7 cell growth with endogenous bovine M6P/IGF2R, we will consider approaches to reduce endogenous expression. For example, a ribozyme approach could be taken. There is considerable experience with this technique in this institution (20).

Conclusions

Data from transfection studies employing the M6P/IGF2R constitutive expression system have demonstrated that overexpression of wild type receptor and mutated M6P/IGF2R which has reduced affinity for M6P proteins, suppresses the IGF2-stimulated growth of the breast cancer cell line, MCF7. In contrast, mutated receptor which does not bind to the IGF2 ligand has no effect on growth rates. This findings would suggest that the role of M6P/IGF2R as an IGF2 antagonist is potentially an important mechanism in the negative regulation of growth in breast cancer cells.

Although preliminary experiments with the constitutive promoters have provided promising growth suppression results, the rapid loss of receptor expression have limited our ability to fully evaluate the growth regulatory role of the receptor in response to different mitogenic factors, and to do *in vivo* experiments. An inducible expression system has therefore been developed in which receptor expression is induced by doxycycline. Growth experiments are currently underway with this inducible expression system.

Experimental Methods

Construction of Inducible Plasmids:

MCF7 cells stably transfected with the Tet regulator plasmid pUHD 17-1 (MCF7-RTA16) were obtained. Full length cDNAs representing wildtype bovine M6P/IGF2R, the negative control, the IGF2 ligand binding mutant and the M6P ligand-binding mutant were each ligated into the multiple cloning site of the Tet responsive plasmid, pUHD 10-3 for eukaryotic expression. The pUHD10-3 plasmid was cleaved at a unique XbaI site and the end was blunted with dNTPs in a Klenow reaction. The blunted plasmid was then cleaved at a unique EcoRI site. The receptor constructs were excised from pcDNA3 plasmids with EcoRI and EcoRV restriction enzymes and the fragment (approximately 7 Kb) was ligated with the prepared pUHD 10-3. The nucleotide sequence of all receptors was confirmed by automated sequencing.

Cell culture and transfection.

For constitutive expression of the receptor constructs, MCF7 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) and transfected by lipofectamine according to the manufacturers instructions (Gibco BRL) and selected in 500μg/ml G418 for two weeks. Resistant colonies were then pooled and screened for M6P/IGF2R expression by western blotting. Experiments with the pooled cell lines were conducted within 3 passages and receptor expression repeatedly assessed by western blotting. For inducible expression of the receptor constructs, MCF7-RTA16 cells maintained in DMEM with 500μg/ml G418 were co-transfected by lipofectamine with the reporter plasmid containing the various receptor constructs and pBABEpuro and selected in 500 μg/ml G418 and 1.2μg/ml puromyocin for six weeks. Resistant clones were isolated and screened for M6P/IGF2R expression.

Immunofluorescence

The clones obtained from the transfection were treated with doxycycline for 24 hours before staining for the expression of the various receptor constructs. Immunofluorescence staining was performed by initially plating transfected cells on glass cover slips. After 24 to 48 hours, cells in culture were fixed by adding an equal volume of PBS containing 10% formalin and 0.1% Triton X 100. After a 5 minute incubation at room temperature, the fixative was removed and cells were incubated for an additional 5 minutes with a further volume of fixative. Fixative was then removed and cells were washed 3 times in PBS before being permeabilized with 0-5% Triton X 100 for 5 minutes at room temperature. Cells were again washed 3 times in PBS and then incubated for 20 minutes with 50 µl rabbit polyclonal antibody raised against bovine M6P/IGF2R. The cells were washed 3 times with PBS before being incubated with 50 µl of 1:200 dilution of Texas Red-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, Oregon). After 20 minutes cells were washed 3 times with PBS and the drained coverslip was inverted onto a glass slide containing Prolong Antifade mount (Molecular Probes). Stained cells were viewed using a Zeiss Fluorescence microscope and photographed.

M6P/IGF2R Western Blotting

An affinity-purified rabbit polyclonal antibody directed against M6P/IGF2R was generated by repeated immunization of rabbits with soluble M6P/IGF2R from bovine serum. Immune rabbit serum was then purified in a M6P/IGF2R affinity column. The bovine M6P/IGF2R monoclonal antibody DM86F was provided by Dr Peter Lobel. For Western blotting, cytoplasmic cell lysates were prepared with NP40 lysis buffer (1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.01 M Na_2PO_4 (pH 7.4), 150 nM NaCl) containing the following protease inhibitors: 0.5 mM PMSF, 10 µg/ml leupeptin, 1 mM benzamidine, 5 µg/ml pepstatin, 5 µg/ml aprotinin. Samples were mixed with NuPAGE sample buffer

Novex, San Diego, CA) in the absence of DTT and subjected to SDS-PAGE on 8 to 16% Tris-Glycine gradient cells (Novex). Proteins were electroblotted onto Hybond ECL nylon membranes (Amersham, Arlington Heights, IL), blocked with 3% BSA in TBST (10 mM Tris {pH 7.5}, 150 mM NaCl, 0.2% Polyoxyethylene-sorbitan monolaurate (Tween 20)) and probed with either concentrated DM86F hybridoma supernatant or a 1:5000 dilution of the affinity-purified rabbit polyclonal antibody followed by incubation with a horseradish peroxidase (HRP)-linked anti-murine or anti-rabbit antibody (Amersham, Arlington Heights, IL). HRP activity was visualized by chemiluminescence (ECL, Amersham).

XTT Proliferation assays

Growth factor-stimulated proliferation was measured using the Tetrazolium XTT assay (Polyscience, Warrenton, PA) essentially as described previously (11). Approximately 4,000 cells were plated in phenol red and serum free media. After a further 48 hours (time zero) IGF2, (Upstate Biotechnology, Lake Placid, NY) was added at the indicated concentrations.

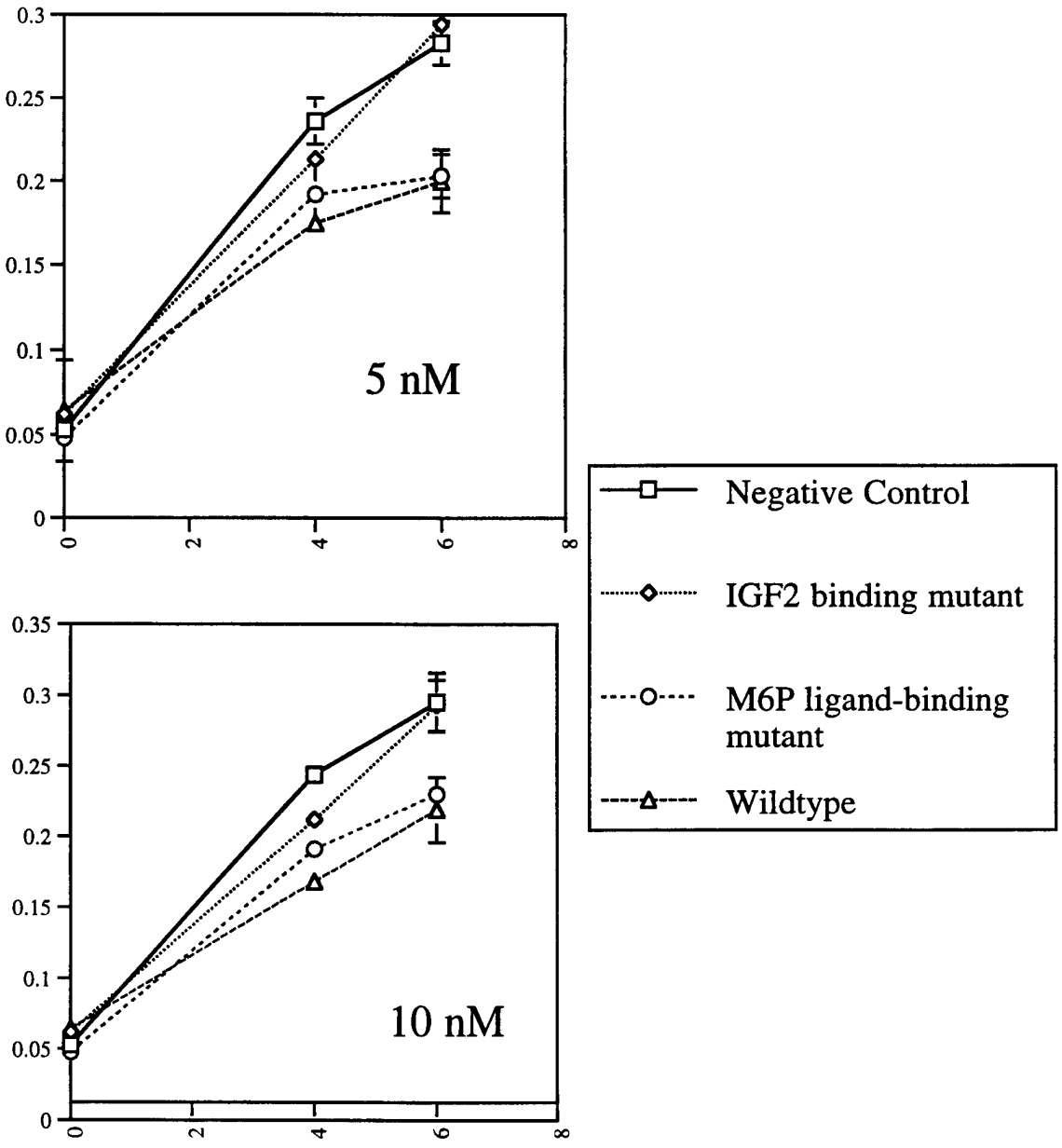

Figure 1. MCF7 cells transfected with each of the receptor constructs were plated in triplicate in serum-free medium. Cells were given a single dose of 5 nM or 10 nM IGF2. XTT proliferation assays were performed at the time points indicated.

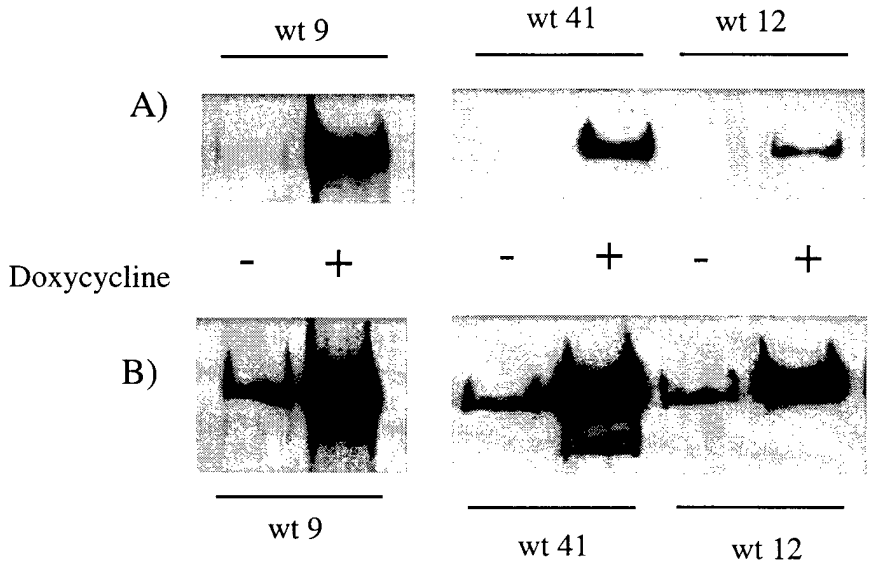

Figure 2. Inducible expression of wildtype M6P/IGF2R. 3 positive clones which inducibly express the wildtype M6P/IGF2R were expanded for further study (wt 9, wt 41 and wt 12). 25μg of whole cell extract was prepared from cells treated with 1μg/ml doxycycline in 10% FBS DMEM media, subjected to SDS-PAGE and transferred to nitrocellulose membrane. The levels of M6P/IGF2R were determined by first probing with (A) a monoclonal antibody and detected by autoradiography and the same blot was stripped and exposed to (B) a polyclonal anitbody followed by autoradiography.

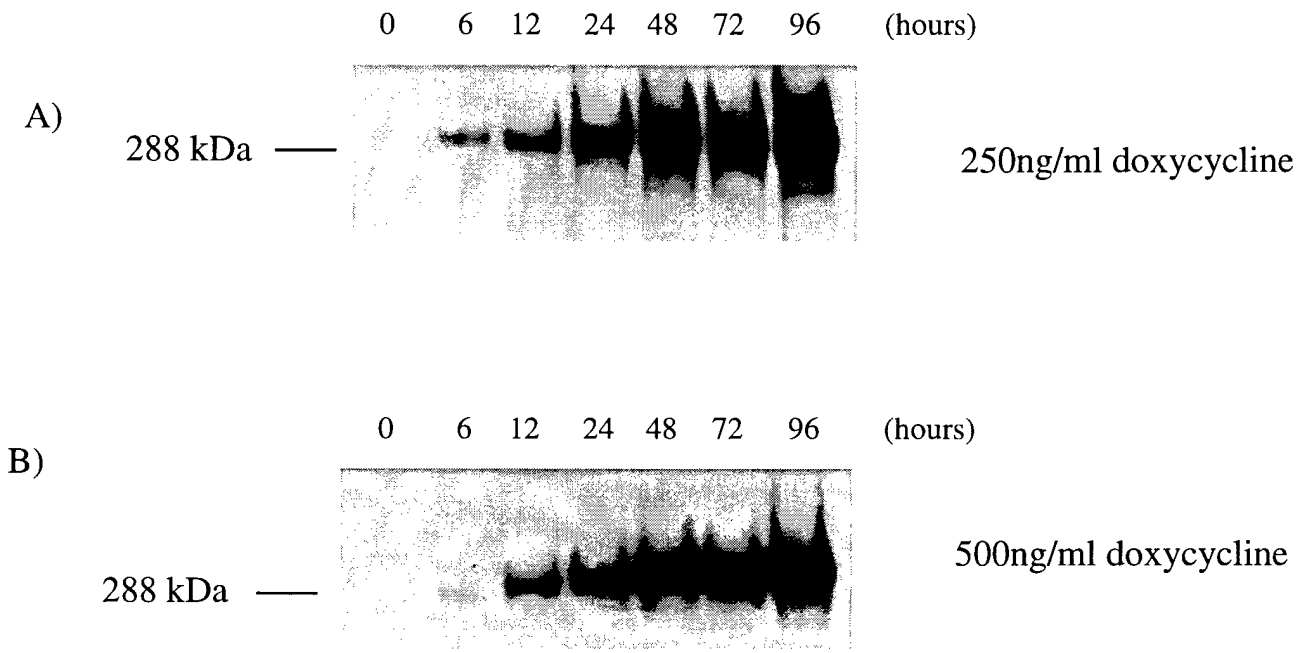

Figure 3. Inducible expression of wildtype M6P/IGF2R (wt 9) in MCF-7 cells. (A) Protein expression induced with 250 ng/ml of doxycycline. (B) Protein expression induced with 500ng/ml of doxycycline. 25µg of whole cell extract was prepared from wt 9 cells induced with doxycycline in 10% FBS DMEM media for 0 - 96 hours, subjected to SDS-PAGE and transferred to nitrocellulose membrane. The protein was detected by a monoclonal antibody for the induced bovine receptor.

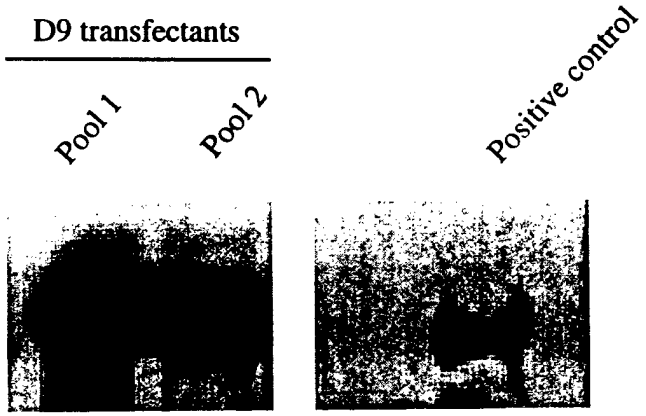

Figure 4. D9 cells were transfected with a constitutive vector containing the human M6P/IGF2R cDNA. 25μg of whole cell extract was prepared from pooled transfectant D9 cells, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The levels of expressed human M6P/IGF2R were determined by probing with a polyclonal antibody followed by autoradiography.

STATEMENT OF WORK

Work Completed in Months 1 to 12.

1) Transfected MCF7 cell line with four bovine M6P/IGF2R expression vectors containing wildtype and mutant cDNAs.

2) Investigated the response to IGF2 in MCF7 cells overexpressing wildtype and mutant M6P/IGF2R receptors.

3) Developed an inducible expression system for bovine M6P/IGF2R in MCF7 cells.

4) Cloned the human M6P/IGF2R cDNA into a constitutive expression vector and expressed M6P/IGF2R in receptor null mouse D9 cells.

Work to be completed in months 13 to 24.

1) Establish the growth effects of inducible expression of bovine M6P/IGF2R in MCF7 cells in vitro and investigate these growth effects of ligand binding mutants already established by the laboratory.

2) Develop an animal protocol for the investigation of the phenotypic consequences of M6P/IGF2R in vivo in tumor xenografts. Conduct pilot animal experiments.

3) Subclone the human M6P/IGF2R into a tetracycline inducible expression vector and compare growth effects of human and bovine M6P/IGF2R *in vitro*.

Work to be completed months 25 to 36.

1) Prepare human M6P/IGF2R cDNAs that incorporate mutants that have been identified in human breast tumors.

2) Subclone mutants into inducible expression vector and express in MCF7 cells expressing TET on transcription factor

3) Determine phenotype of human M6P/IGF2R mutants *in vitro* and *in vivo*.

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