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13. ABSTRACT *(Maximum 200 Words)*

Hepatocyte growth factor (HCF), also known as Scatter Factor, induces cell growth and cell movement, and is known to promote invasiveness of malignant cells. It also promotes angiogenesis. HGF is known to be produced by fibroblasts within breast tumors, while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves. HGF thus appears to act predominantly as a paracrine factor in breast cancer. High levels of HGF expression within breast tumors correlates with an aggressive tumor phenotype, and HGF has been found to be a powerful negative prognostic indicator for breast cancer. Expression of the c-Met protein by breast tumors in culture also correlates with an estrogen receptor negative phenotype and with loss of estrogen-dependent cell growth. Thus the HGF-c-Met ligand-receptor system may be important in controlling cell growth in breast tumors that have escaped estrogen regulation, a common occurrence in breast cancer patients who have lost responsiveness to anti-estrogen therapy. The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit the growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who have failed endocrine therapy. We will use two approaches for these studies: (1) an anti-sense strategy that uses vectors constructed in the U6 RNA expression plasmid and delivered by cationic liposomes and (2) a recombinant HGF antagonist molecule (truncated HGF/tHGF) produced in baculovirus and delivered through injection to the peritumoral area.

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Introduction

Hepatocyte growth factor (HGF), also known as Scatter Factor, induces cell growth (1) and cell movement (2), and is known to promote invasiveness of malignant cells (3). It also promotes angiogenesis (4). HGF is known to be produced by fibroblasts within breast tumors (5), while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves (6). HGF thus appears to act predominantly as a paracrine factor in breast cancer (7). High levels of HGF expression within breast tumors correlates with an aggressive tumor phenotype (8), and HGF has been found to be a powerful negative prognostic indicator for breast cancer (9). Expression of the c-Met protein by breast tumors in culture also correlates with an estrogen receptor negative phenotype (10,11) and with loss of estrogen-dependent cell growth. Thus the HGF-c-Met ligand-receptor system may be important in controlling cell growth in breast tumors that have escaped estrogen regulation, a common occurrence in breast cancer patients who have lost responsiveness to anti-estrogen therapy. The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit the growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who have failed endocrine therapy. We will use two approaches for these studies: (1) an antisense strategy that uses vectors constructed in the U6 RNA expression plasmid and delivered by cationic liposomes and (2) a recombinant HGF antagonist molecule (truncated HGF, tHGF) produced in baculovirus and delivered through injection to the peritumoral area. In vitro experiments examining effects of these agents on breast tumor growth will be followed by in vivo experiments using an s.c. model of tumor growth as well as growth in the mammary fat pad.

Body of Report

This research project was scheduled to start September 1, 1998. The month prior to this, the post-doctoral fellow whom we had recruited to work on this project, left the laboratory unexpectedly for personal reasons. Thus the first task to be undertaken was to identify another fellow. Unfortunately this took quite some time and a fellow did not join the laboratory until July of 1999. Some work on the project was performed in the meantime by undergraduate students and a technician in the laboratory. However, due to the lack of a dedicated fellow to perform the work, there has been a considerable delay in the progress. Thus, after the third year of the project, we are now at the point we hoped to be by the end of the second year. However, as discussed with the Program Officer, we are carrying over the salary monies that were not spent in the first year and now have a no-cost extension which should allow us to complete the aims by September 1, 2002.

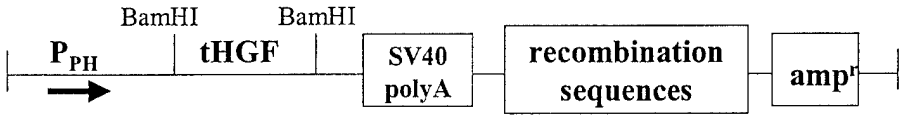
Progress to Date on Statement of Work shown in the next section.

1. Produce purified tHGF from baculovirus-infected insect cells

The viral stocks we had previously obtained from our collaborator, Dr. Zarnegar (Ref. 12), were very low titer and we were unable to reconstitute a high-titer viral stock We therefore cloned a new truncated (tHGF) plasmid vector for growth in baculovirus, as described last year, and have now succeeded in obtaining high titer stocks. These have been used to produce a first batch of purified tHGF.

pBlueBac-tHGF

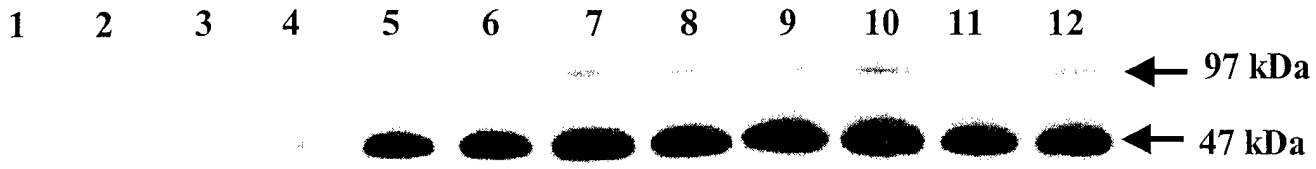
6.6kb



Important features of the pBlueBac-tHGF construct. P_{PH} represents the polyhedrin promoter which allow for efficient, high level expression of the recombinant protein. The BamHI site is part of the multiple cloning site which allowed for insertion of the tHGF gene. The SV40 polyadenylation site allows for increased mRNA stability. ORF1629 and *lac*Z Recombination sequences permits recombination of tHGF with Bac-N-Blue™ linear AcMNPV DNA, restores the essential ORF1629 for production of a viable, recombinant virus, and allows production of blue recombinant plaques for visual selection. The ampicillin resistance gene allows selection in *E. coli*.

The first step was to generate a high-titre stock for expression. We have produced the high-titre stock from 2 x 250ml cultures. A plaque assay was used to calculate the titre, which was 9×10^7 PFU. High 5 insect cells were infected with the high-titre virus, the supernatant was collected, and the truncated protein was purified. Both a time course of up to 6 days and a titration of multiplicity of infection were done. The optimal time is a 4 day exposure to baculovirus and the optimal MOI is 10. A western blot (shown below) was performed on a batch purification of tHGF using heparin-sepharose beads, showing the presence of the truncated HGF protein. A large scale purification is in progress, which will be done by column chromatography, and we aim to have 25 mg of tHGF for use in *in vitro* and *in vivo* studies over the next grant period.

Figure: Time Course of Expression of Recombinant tHGF



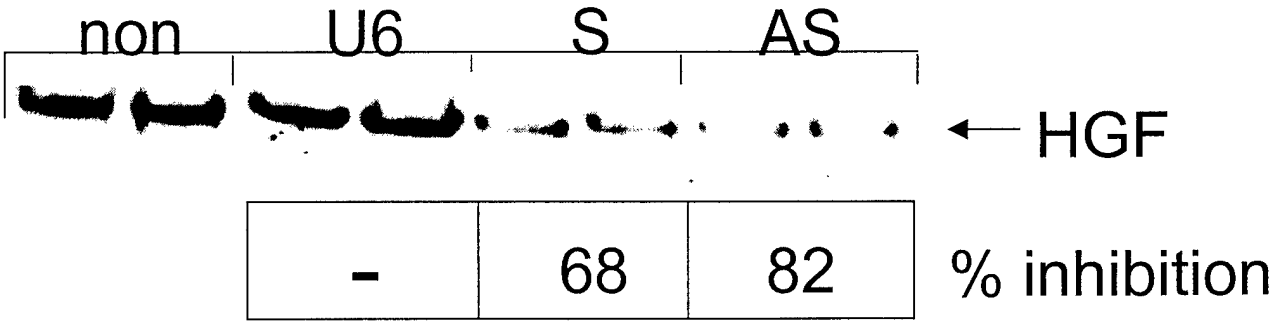
1. Day 0 MOI 5
2. Day 0 MOI 10
3. Day 1 MOI 5
4. Day 1 MOI 10
5. Day 2 MOI 5
6. Day 2 MOI 10
7. Day 3 MOI 5
8. Day 3 MOI 10
9. Day 4 MOI 5
10. Day 4 MOI 10
11. Day 6 MOI 5
12. Day 6 MOI 10

2. Produce sense and antisense constructs for c-Met and HGF and verify sequences

The constructs for the human genes were completed previously. During the past year, we also produced a murine antisense HGF vector that can be used in *in vivo* studies in the mouse. This goal is now completed.

We have previously described our results using the human HGF sense and antisense constructs in human normal lung fibroblast cell lines. In summary, we have shown a decrease in HGF protein levels in conditioned media from human cells transfected with the human HGF antisense construct. Since the *in vivo* experiments proposed involve mice, and the stromal cells that will grow in the xenotransplanted tumors will be murine in origin, we examined the *in vitro* effects of the human constructs in murine fibroblast cells. In four independent experiments, we did not observe a decrease in HGF protein levels from conditioned media isolated from murine fibroblasts transiently transfected with the human HGF antisense construct. This prompted us to examine the sequence for species-specific differences. We found four nucleotide differences out of forty between the mouse and human HGF sequences which might be a factor for this lack of response. We reconstructed these constructs using the mouse sequences following the same procedure as originally described. These new mouse constructs were then transiently transfected into murine NIH-3T6 cells and we observed an 82% decrease in HGF protein levels when the cells were transfected with the mouse HGF antisense construct versus the U6 control as shown in the Western blot. We also observed a decrease using the sense construct, as we have seen previously. This is a common occurrence with sense constructs. Our results suggest we should use the murine HGF AS and S constructs for *in vivo* experiments when the stromal cells will be of murine origin.

Transient Transfection of Mouse HGF Sense and Antisense Constructs in 3T6 Cells



3. **Optimize transfection conditions using breast cancer cells and human fibroblasts using a reporter gene and liposome preparations**

This part of the project was completed as described last year.

4. Transfer sense and antisense constructs into breast cancer cells and human fibroblasts and monitor expression of constructs.

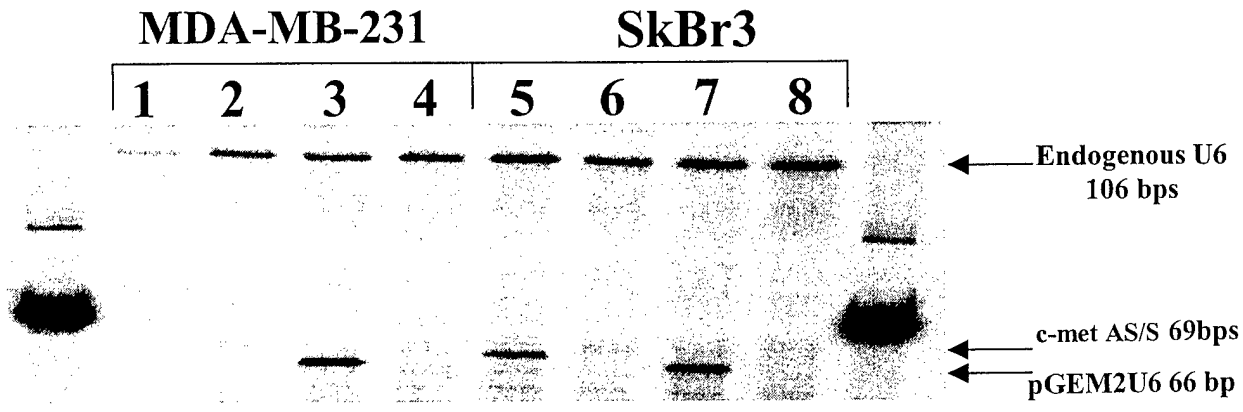
c-Met AS in Breast Cancer Cells:

We completed this part of the project during the past year. Primer extension was used to evaluate expression of the antisense c-Met vector in breast cancer cells, as shown in the figure below.

Figure: Expression of c-met constructs in breast cancer cell lines

Lanes Below	
1.MDA-MB-231	c-met antisense
2.	c-met sense
3.	pGEM2U6
4.	Untransfected
5.SkBr3	c-met antisense
6.	c-met sense
7.	pGEM2U6
8.	Untransfected

Outer lanes are mw markers; the large band is 72 bp.



As shown in the above figure, the MDA-MB-231 cells do not express the AS vector in vitro (lane 1), while the SKBR3 cell do (lane 5). This occurs even though we optimized the liposomes used, and we see uptake of green fluorescent protein when we test the liposome preparations. The empty vector is also expressed in both cell types (lanes 3 and 7). We do not have an explanation for this finding at this time. As described below, we could not see down-regulation of c-Met in the MDA-MB-231 cells, but we did observe it in SKBr3. Therefore, we are continuing in vivo experiments only with SkBr3 cells.

Expression of HGF AS in Human Fibroblasts: Completed as described previously.

5. Determine whether down-regulation of protein occurs after transfer of HGF and c-Met antisense vectors

Down-Regulation of HGF protein in Human Fibroblasts

Completed as described in last year's report. Up to 50% downregulation was observed at the protein level.

Down-Regulation of c-Met in Breast Cancer Cells

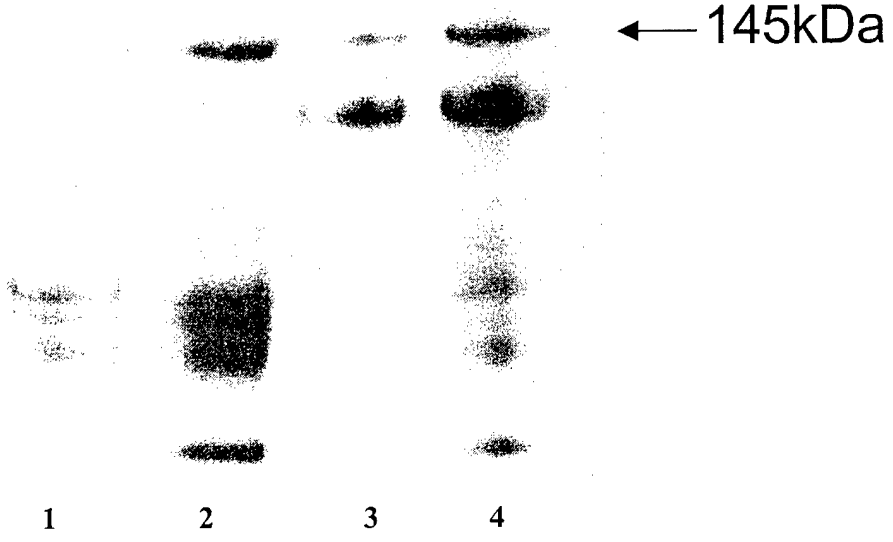
This was described last year. The effect was much less in SkBr3 breast cancer cells than lung cancer cells (about a 30% decrease in c-Met expression in breast cancer cells compred to 50-60% in lung ccanaer cells), even though the efficiency of transfer was the same (60%). As discussed above we determined the extent of c-Met AS vector expression in MDA-MB-231 breast cancer cells was not detectable. We also saw little or no effect on c-Met expression in MDA-MB-231 cells. We plan to proceed with *in vivo* studies only in SkBr3 cells.

Monitor *in vitro* Biological Effect of Antisense Plasmid Transfer

We had previously determined that human fibroblasts transfected with the HGF AS plasmid showed a decrease of 50% in amount of HGF protein secreted compared to empty vector control. We have now preliminary results that this decrease probably also results in a decreased biological activity for secreted HGF. To monitor the biological effects of the antisense construct in vitro, we examined phosphorylation levels of the c-met protein using an immunoprecipitation assay. Briefly, A549 lung cancer cells (derived from a bronchioloalveolar carcinoma) were plated at a density of 6×10^5 cells per 100 mm plate and allowed to attach overnight using fetal

bovine serum. The following day, the cells were serum-deprived to remove any growth factors derived from the serum. Recombinant HGF (50 ng), conditioned media that was isolated from normal fibroblast cells transiently transfected with the HGF antisense construct, or both treatments was added to the cells for 10 minutes. The amount of conditioned medium added from fibroblasts was the same amount as had previously given approximately 50 ng HG, in cells not treated with AS vector. The cells were then lysed and immunoprecipitated using an anti-phosphotyrosine antibody and protein A sepharose beads. The samples were separated on a 10% SDS-Tricine gel and transferred to nitrocellulose membrane. The membrane was then immunoblotted using an anti-c-met antibody followed by chemiluminescent substrate detection. The results show a 20-fold stimulation of the phosphorylated 145kDa c-met protein in the presence of rHGF. In the presence of conditioned media isolated from cells that had been transfected with the antisense construct, the levels of phosphorylated c-met decreased almost back to control levels (4.7-fold higher than control, 77% inhibition of rHGF stimulation). Both treatments combined resulted in a 20-fold stimulation over control. The smaller bands may represent degradation products or antibody heavy and light chains from the immunoprecipitation procedure. This experiment will be repeated using a breast cancer cell line as the target cell, once fully optimized, and we will compare conditioned medium from empty vector, and AS and S treatments, to verify the effect is reproducible and specific.

Detection of Phosphorylated c-Met Protein using Immunoprecipitation and Immunoblotting



Lane 1: control medium
Lane 2: 50 ng recombinant HGF
Lane 3: CM from AS treated fibroblasts
Lane 4: CM from AS treated fibrlbasts plus 50 ng recombinant HGF

6. Perform *in vivo* experiments using antisense vectors injected into peritumor area of s.c. tumors

Liposome optimization for *in vivo* experiments

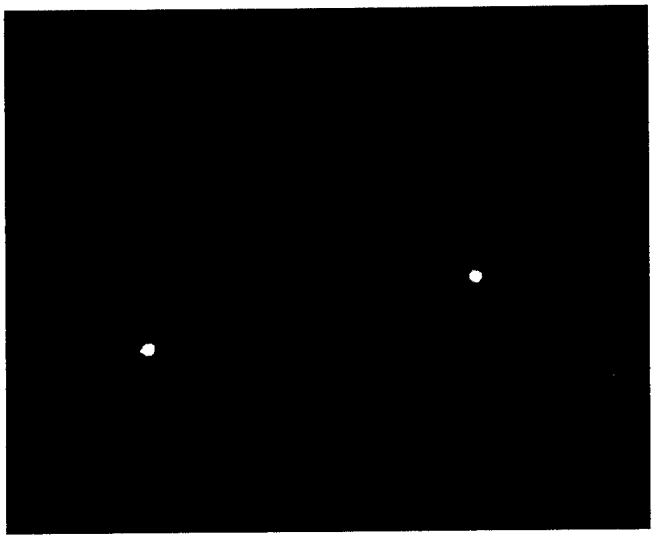
We were aware from the literature and from our collaborator, Dr. Huang, that conditions that are optimized for *in vitro* gene transfer are often not optimal for in vivo gene transfer. We first attempted to simulate *in vivo* conditions by growing breast cancer cells on monolayers of human fibroblasts, to make the cell-cell contacts more representative of a solid tumor. SkBr3 cells appear as round cells on top of the layer of elongated fibroblasts in the white field picture. Lipofectace showed the greatest uptake into both SkBr3 cells and fibroblasts as evidenced by the green fluorescence.

See accompanying color photographs.

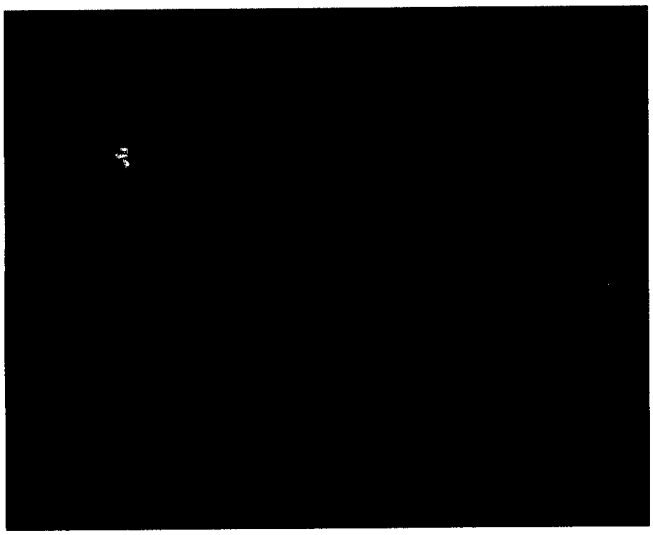
SkBr3

1. DOTAP

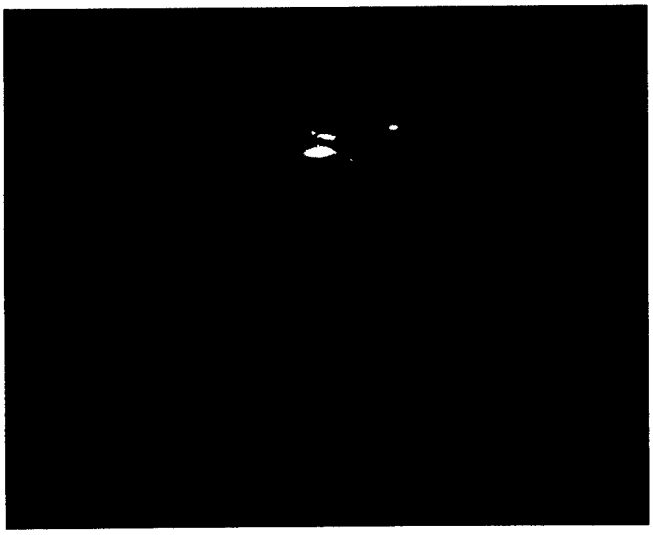
A.



B.

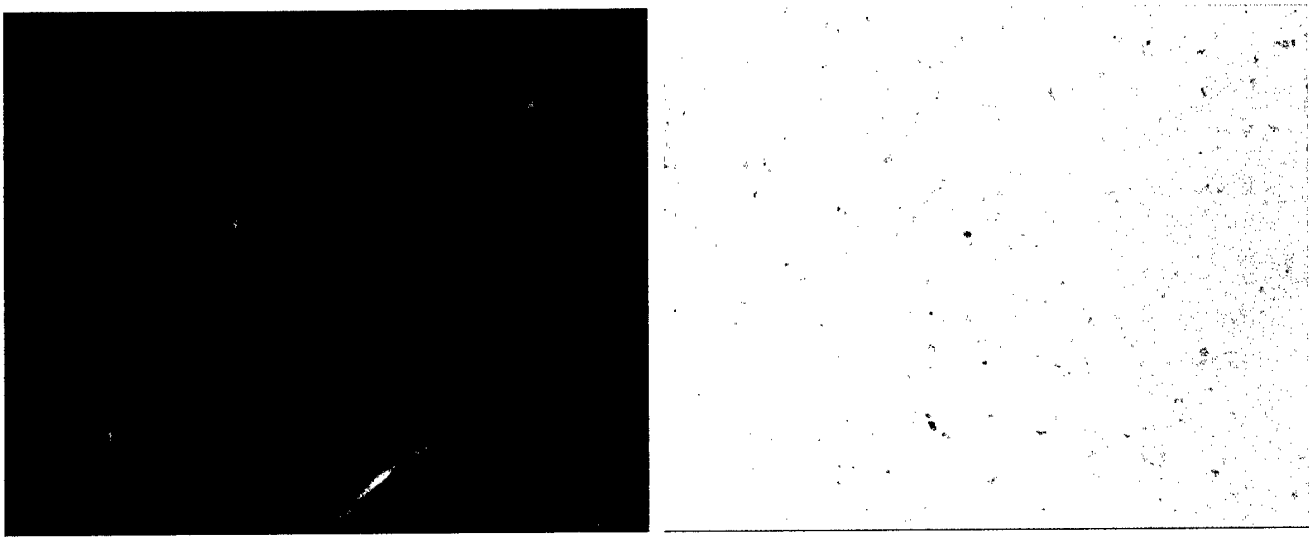


C.

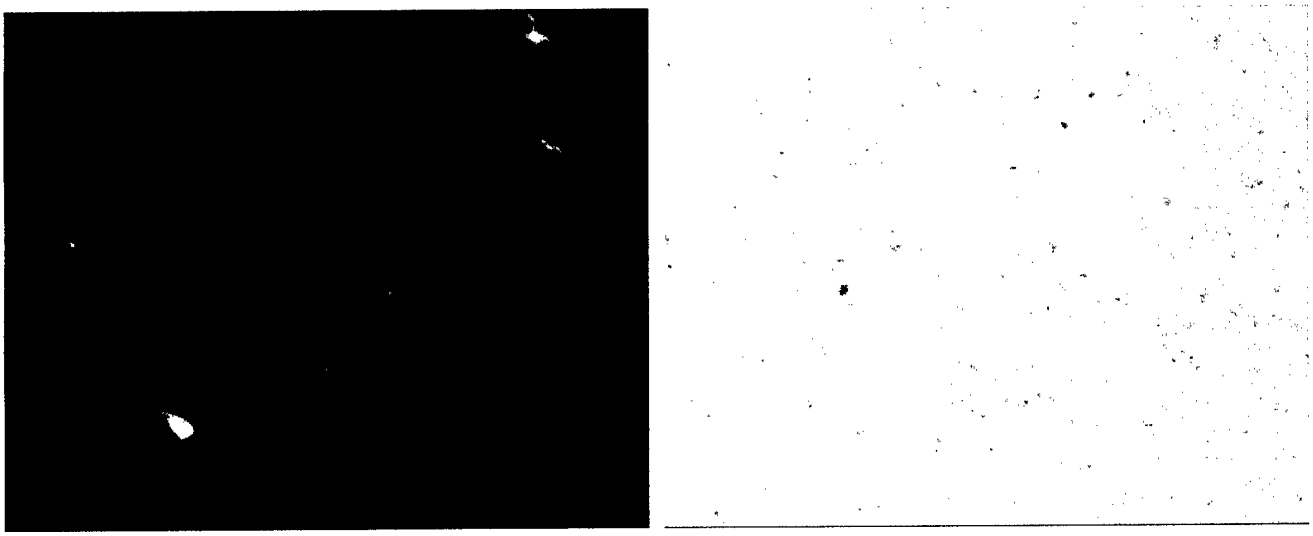


2. Lipofectace

A.



B.



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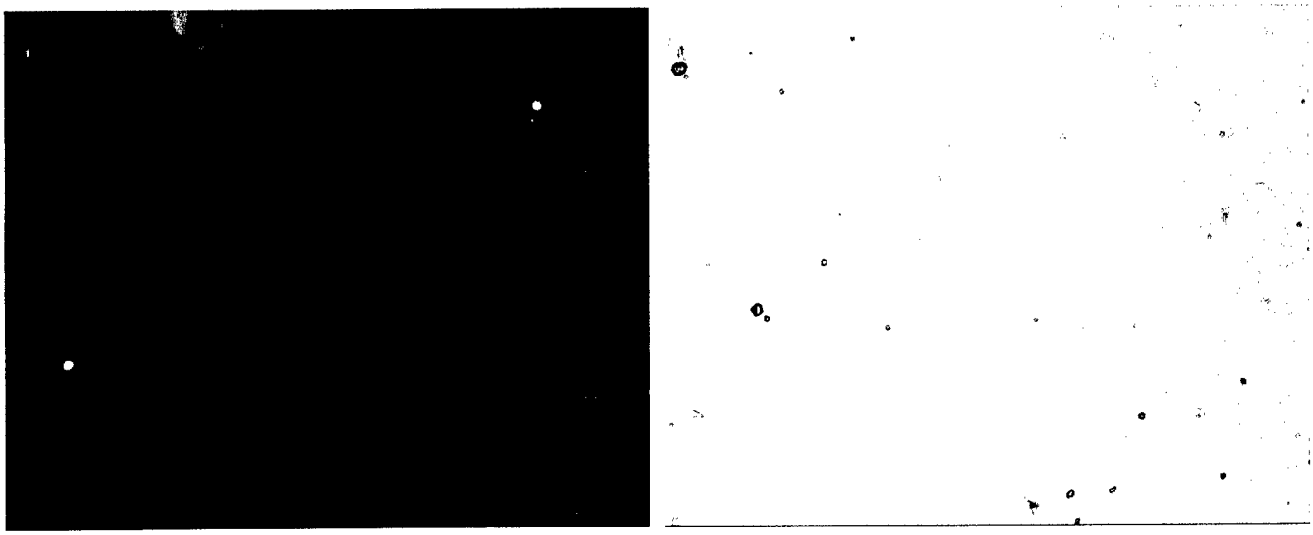
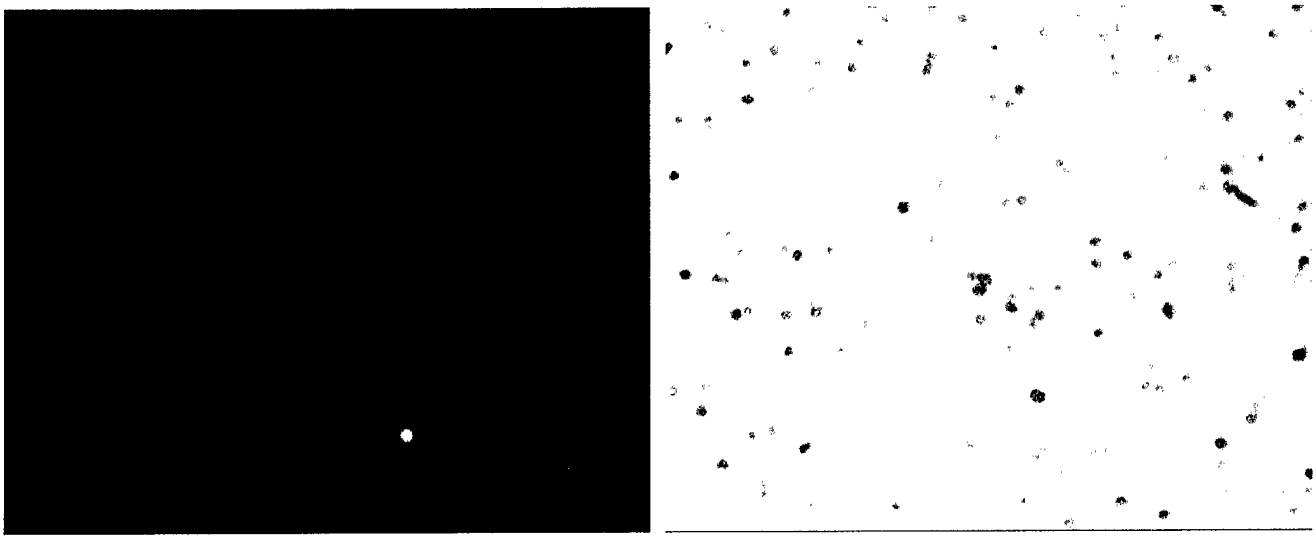


3. Lipofectamine

A.



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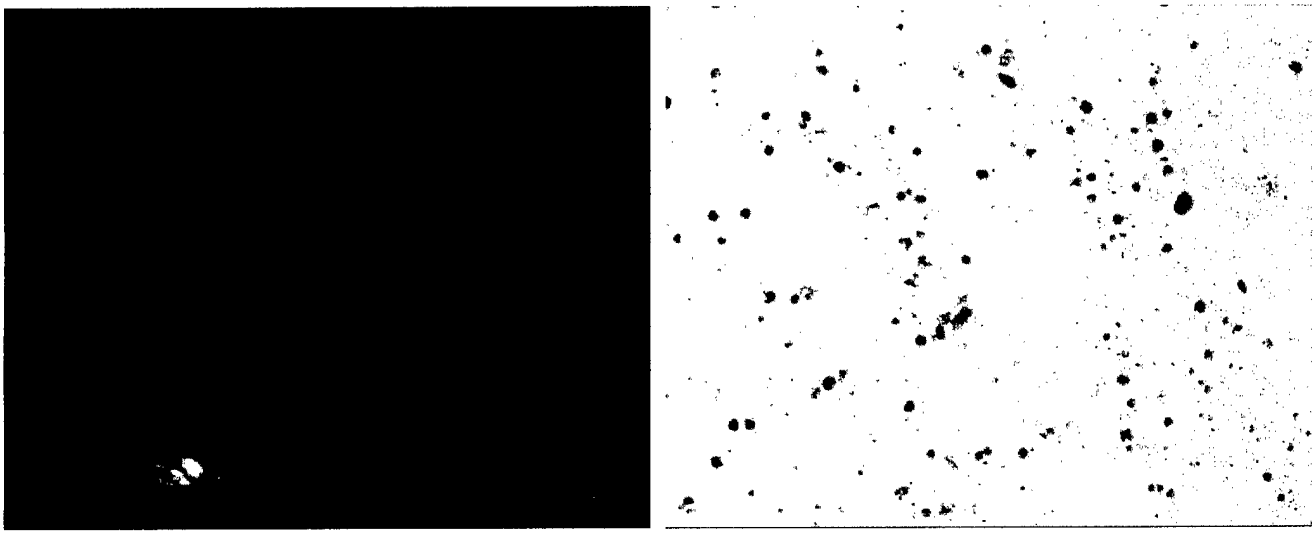

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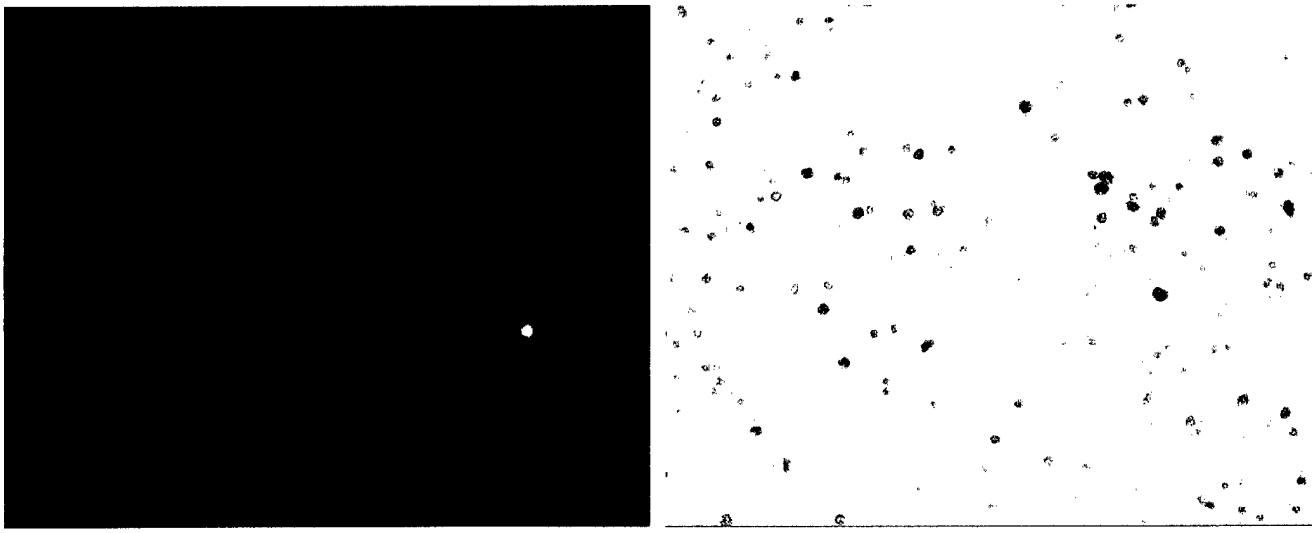
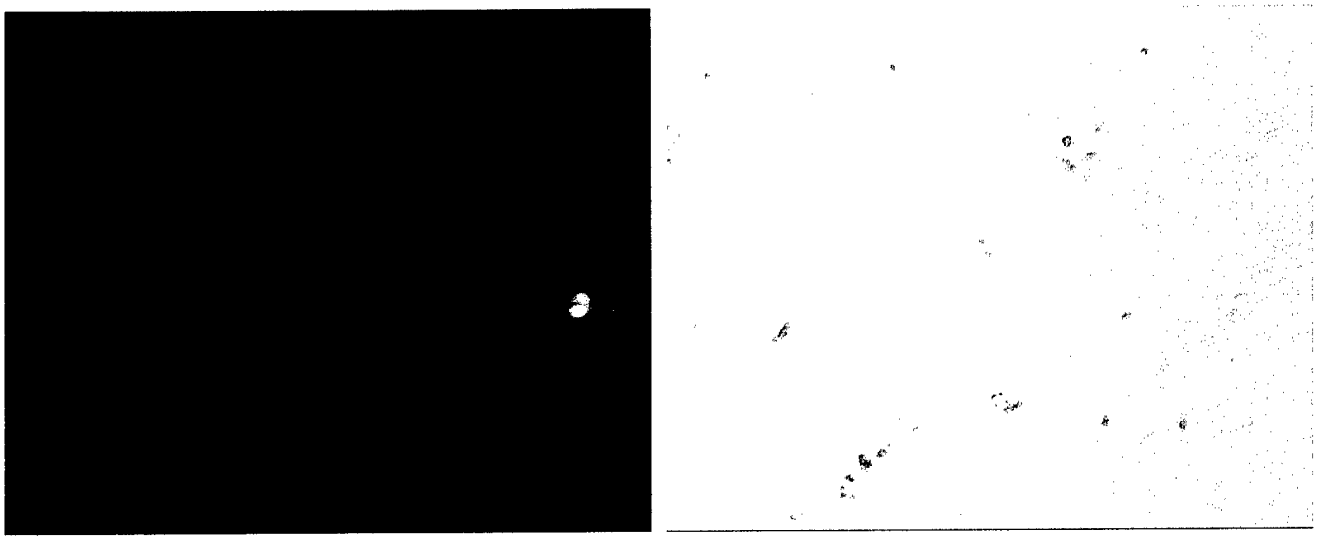


4.Superfect A

A.



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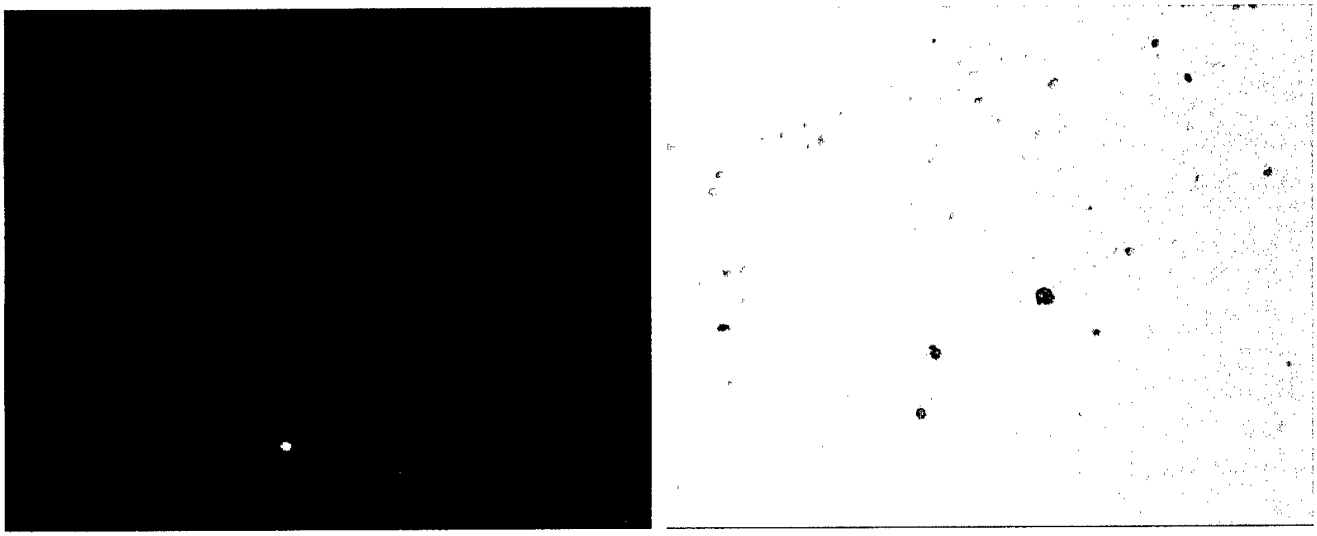
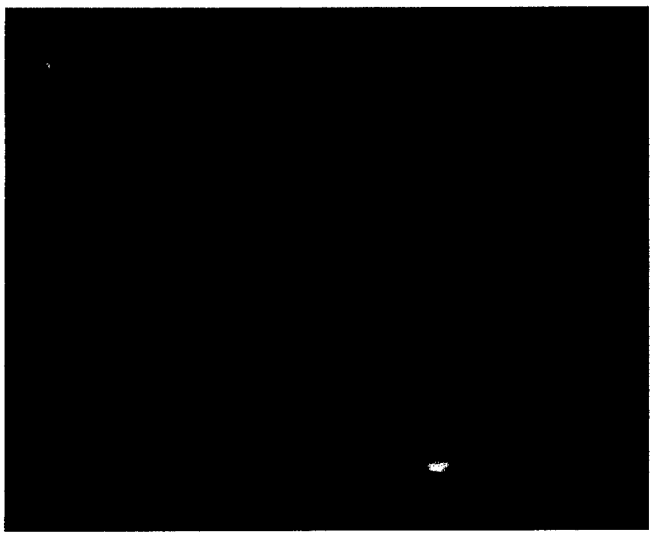


C.

4. Superfect B

A.



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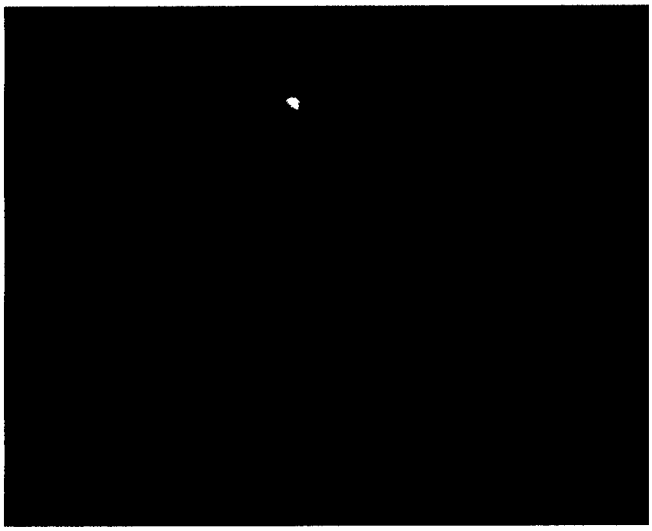

C.

Testing of Liposomes *in vivo*

However, injection of a luciferase vector encapsulated in liposomes into solid tumors of SkBr3 in immunocompromised mice yielded completely different results. The liposomes which we tested included: DC-Chol, LipofectACE (Gibco BRL), LipofectAMINE (Gibco BRL), Superfect (Qiagen), and DOTAP (Boehringer Mannheim). Once tumors grew to approximately 2 x 2 mm in diameter (14-21 days post-injection), we performed intratumoral injections of luciferase vector plasmid DNA complexed with different liposomes. We observed a 10-fold higher extent of luciferase activity in tumor extracts after injection of DC Chol liposomes compared to DOTAP, Lipofatamine, Lipofectase and Superfect. This is consistent with other results reported by Dr. Huang's laboratory. The DC-Chol liposomes, which were described in our grant application, are produced by Dr. Huang's laboratory as part of this project. 200 ml at 2 nmol/microliter have been produced for us to use in *in vivo* experiments.

Animal experiments: To extend our *in vitro* observations to an *in vivo* model, we used the antisense expression plasmids targeting c-met and HGF for direct intratumoral injection with DC-Chol liposomes to try to down-regulate tumor growth. We optimized the experiment using lung tumor cells, since they have given us the best response *in vitro*, and then performed the same experiments using breast cancer cells. Lung tumor cells (201-T, 4 X 10^6) were implanted into female scid mice (4-6 weeks old, Harlen Sprague Dawley). They were treated with 5 times weekly vector-liposome injections of empty vector or AS vector for three weeks (both HGF and c-Met). Tumors grew at approximately one-half the rate with AS treatments, and contained approximately 10 times as many apoptotic cells with AS treatment compared to empty vector. Expression analysis showed that vectors were expressed. C-Met protein in AS-treated tumors was down-regulated 50%, while HGF protein was not. Despite the fact that we could not demonstrate reduced HGF protein, this is an encouraging result, and we plan to proceed with a large *in vivo* experiments with SkBr3 cells using empty vector, AS, S, and DC-Chol liposomes alone. We will test the c-Met vectors alone in SkBr3 cells first, then, if an effect is observed in the breast cancer cells, we will carry out a combined experiment with c-Met and HGF antisense. We should also have the tHGF ready for *in vivo* testing in 6 months as well. If we see an effect with intratumoral injection in SkBr3 cells grown s.c., we will proceed with mammary fat pad experiments.

Key Research Accomplishments

1. The cationic liposome LipofectACE gave optimal gene transfer to human fibroblasts *in vitro*, as monitored by green fluorescent protein expression. For breast cancer cells, Lipofectamine gave the best gene transfer in standard in vitro cell culture to SkBr3 cells and Superfect to MDA-MB-231 cells. This was in contrast to our positive control lung cancer cells, in which the LipofectACE had given the best in vitro transfer efficiency. In co-culture with human fibroblast monolayers, LipofecACE gave the best transfer to SkBr3 cells. In the animals studies, however, we found that DC Chol liposomes gave the best transfer to s.c. SkBr3 tumors. Thus depending upon the system to be used for testing, the liposome must be optimized.

2. HGF sense and antisense vectors, as well as c-Met sense and antisense vectors, have been cloned into the U6 RNA expression system. The sequences have been verified, and the vectors target the first 40 bp of the HGF and cMet mRNA, starting at the ATG transcription start site. Plasmid DNA has been purified for optimal gene transfer. A murine HGF antisense vector was also produced and sequenced for in vivo studies, since the stromal cells in the tumors will be murine in origin. It is effective in reducing HGF expression in mouse fibroblasts.

3. An RNA protection assay and a primer extension strategy have been developed and reagents synthesized to carry out the assay for endogenous and transgene expression in cells transfected with vector DNA encalsulated into cationic liposomes. Expression of the U6 constructs as monitored by a ribonuclease protection assay and the primer extension method show that the transgenes are being transcribed to RNA in human fibroblasts and control lung cancer cells. Breast cancer cell line SkBr3 also shows expression of the antisense vector, but MDA-MB-231 cells do not.

4. Western analysis shows that several estrogen receptor negative human breast cancer cell lines express high levels of the c-met protein, whereas estrogen receptor positive human breast cancer cell lines are c-met negative. Therefore, the HGF-c-met system may be important in controlling cell growth in estrogen-independent breast cancer cells.

5. The HGF and c-met sense and antisense sequences have been transferred to target cells. A 50% down-regulation of HGF protein levels in normal fibroblasts using the antisense construct was observed by Western analysis. c-Met was down-regulated up to 30% in one breast cancer cell line and not at all in another, but the result was not always different from transfer of empty vector.

6. Methods have been developed to test these constructs in vitro for ability to prevent phosphorylation of c-Met. We have shown that conditioned medium from fibroblasts transfected with HGF AS vector show a lessened ability than expected to cause c-Met phosphrylation. This method will be used in the next grant period to determine how combined HGF and c-Met AS can reduce c-Met signaling.

7. tHGF, a truncated HGF that acts as a competitive inhibitor for c-Met, has been produced from a baculovirus vector. This protein will be used for *in vitro* and *in vivo* experiments to inhibit action of HGF.

8. Preliminary *in vivo* results show that the AS vectors can decrease tumor growth. The vectors are taken up and expressed by solid lung tumors grown s.c. The c-Met protein is down-regulated about 50%. The in vivo experiments will be expanded to the breast tumor cell line SkBr3.

Reportable Outcomes

We presented an abstract of our work on use of these vectors in gene transfer at the DOD Breast Cancer Research Program Era of Hope Meeting held in Atlanta, Georgia in June of 2000. A copy of the abstract, which appeared in the Proceedings from the meeting, is enclosed as an Appendix. We plan to publish our results after completion of in vivo experiments.

Conclusions

Progress on this project was initially delayed due to loss of personnel from the laboratory. Transfer of DNA vectors into human fibroblasts and breast cancer cells is feasible using cationic liposomes. Sense and antisense constructs in the U6 expression vector have been produced to target the HGF gene in human and murine fibroblasts and the c-Met gene in human breast cancer cells. These vectors are taken up by cells in standard cell culture at about 40-50% efficiency and are expressed, with variation between breast tumor cell lines noted. Down-regulation of the target protein occurs with varying degrees of efficiency. Methods have been developed to test these reagents for growth effects in vitro and in vivo. This novel approach may provide a new treatment option for patients with estrogen-independent breast tumors.

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LIPOSOME – MEDIATED DELIVERY OF AN ANTISENSE HGF VECTOR TO HUMAN FIBROBLASTS

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Hepatocyte growth factor (HGF) induces cell growth and movement and is known to promote invasiveness of malignant cells and angiogensis. HGF is produced by stromal cells within breast tumors while its receptor, c-met, is found on the breast tumor cells themselves. Increased HGF expression in tumor stroma is correlated with increased tumor aggression. We have shown by Western analysis that several estrogen receptor negative human breast cancer cell lines express high levels of the c-met protein, whereas estrogen receptor positive human breast cancer cell lines are c-met negative. Therefore, the HGF-c-met system may be important in controlling cell growth in estrogen-independent breast cancer cells. Our hypothesis is that disrupting the HGF/c-met pathway in estrogen independent breast cancer cells will inhibit their ability to grow *in vitro* and *in vivo*. The approach which we are using to study this hypothesis is to inhibit the action of HGF through the use of antisense (AS) constructs to both HGF and c-met. The HGF and c-met sense (S) and AS sequences have been cloned into a U6 expression cassette which allows for high expression of these sequences in target cells. Transfection conditions have been optimized in human fibroblasts and several breast cancer cell lines using green fluorescent protein and different liposome reagents. Use of lipofectACE and lipofectAMINE gave 60-70% transfection efficiencies in normal fibroblasts and breast cancer cells, respectively. Since HGF is a paracrine factor, we targeted human fibroblasts in culture with the HGF AS construct and c-met positive human breast cancer cells with the c-met AS construct. Expression of the U6 constructs as monitored by a rionuclease protection assay shows that the transgenes are being transcribed to RNA. A 50% down-regulation of HGF protein levels in normal fibroblasts using the AS versus the S construct was observed by Western analysis. This novel approach may provide a new treatment option for patients with estrogen-independent breast tumors.

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