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PRINCIPAL INVESTIGATOR: Judith Ilan, Ph.D.

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, Ohio 44106-7015

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Judith Ilan, Ph.D.

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Case Western Reserve University
Cleveland, Ohio 44106-7015

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We have focused our efforts on investigating the effectiveness of antisense to type I insulin-like growth factor receptor (IGF-IR) in reducing tumorigenicity in highly metastatic human breast cancer cells, MDA-MB-435S and murine breast cancer cells, EMT6 *in vitro* and *in vivo*. A new construct for antisense IGF-IR has been prepared and transfections were carried out in these cell lines. Clones expressing high levels of antisense IGF-IR and reduced IGF-IR RNA were selected for analysis. We have shown significant inhibition of *in vitro* growth in MDA-MB-435S cell clones carrying antisense to IGF-IR. An absence of colony formation in IGF-IR antisense cell clones indicates that IGF-IR plays a role in the transformed phenotype. An important finding was the dramatic inhibition in tumor growth that IGF-IR antisense clones exhibited in nude mice. Studies with the syngeneic animal model suggest that the immune system can be evoked by treating the EMT6 cells with an antisense strategy as reported for glioblastoma and teratocarcinoma. Downregulation in tissue type plasminogen (tPA) in IGF-IR antisense transfected EMT6 cells indicated a relationship between IGF-IR and tPA at the mRNA level. Our results demonstrate the importance of IGF-IR in breast cancer tumorigenesis and provide a basis for designing an antisense gene therapy for treatment of breast cancer.

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FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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Judith Hon
Principal Investigator's Signature

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Introduction:

In the United States approximately 183,000 new cases of breast cancer were diagnosed in 1995, with the number of deaths estimated at around 46,000 (Wingo et al., 1995). Ninety percent of patients dying with breast cancer have osteolytic bone metastases (Yoneda et al., 1994). Therefore, it is urgent to find treatments that will result in a cure. Current standard treatments include chemotherapy, irradiation and bone marrow transplantation. However, immunological approaches such as adoptive immunotherapy and rationally designed biological agents that attack specific proteins unique to the malignant cancer cells remain to be developed (Lippman, 1993). A number of proteins such as the insulin-like growth factors (IGFs), insulin-like growth factor receptors (IGF-IRs), transforming growth factors (TGFs) and plasminogen activators (PAs) have been shown to participate in the growth of breast cancer cells (Cullen et al., 1990; Dickson & Lippman, 1995; Mizoguchi et al., 1990). We intend to apply our experience in treating and preventing rat glioblastoma (Trojan et al., 1992; Trojan et al., 1993) and murine teratocarcinoma (Trojan et al., 1994) to develop a model for breast cancer.

During the past five years, my laboratory has been engaged with Dr. Joseph Ilan's laboratory in this school to develop gene therapy for treatment of rat glioblastoma and murine teratocarcinoma. This effort resulted in unanimous approval by the NIH Recombinant DNA Advisory (RAC) Committee and the FDA for a phase one gene therapy trial for human glioblastoma which is ongoing in the Gene Therapy Facility at CWRU. Our Breast Center is a comprehensive diagnostic and treatment center.

To assign a role of a gene product, one relies on naturally occurring mutants that fail to express the gene in normal fashion. Generation of cellular mutants has limited applicability due to the diploid nature of most genes and the lack of adequate mutant selection. Our experimental approach centers around the use of antisense RNA expression to produce phenocopies of a null mutation of IGFs, IGF receptors, TGFs and PAs. The antisense strategy bypasses inherent limitations of functional studies dependent upon natural mutant cells or artificially mutagenized cells. Inhibition of IGFs, IGF receptors, TGFs or PAs by antisense provides a direct approach for assessing their contribution to the tumorigenic phenotype of cancer cells both in culture and *in vivo*. The antisense RNA will either hybridize to the endogenous mRNA or disrupt its transcription or processing, thereby preventing the synthesis of protein product (Izant and Weintraub, 1985). Our vector includes the Epstein-Barr virus (EBV) replicative signals to permit self-replication and to maximize antisense transcript levels (Yates et al., 1985). We (Johnson et al., 1991) and others (Kiess et al., 1989) have reported that rat glioma C6 cells express high levels of IGF-I which is enhanced when the cells are grown in serum-free medium. We have observed that rats injected with transfected glioma cells which express antisense IGF-I RNA and which lack IGF-I protein continue to remain tumor-free for more than two years. In contrast, rats injected with parental (non-transfected) glioma cells consistently develop large tumors within a few weeks post-injection. These data demonstrate a transformation role for IGF-I, highlighting the fact that transformation phenotypes may arise through unexpected molecular mechanisms. It

represents a clear example of a dominant and essential role for a growth factor in malignant transformation.

Antisense strategy has been applied successfully to a growing set of genes in both cultured cells and transgenic animals (Katsuri et al., 1988; Munir et al., 1990). However, the antisense approach has frequently been complicated by incomplete inhibition of gene expression (Munir et al., 1990; Khokha et al., 1989). We (Johnson et al., 1991) and others (Kiess et al., 1989) have reported that rat glioma C6 cells express high levels of IGF-I which is enhanced when the cells are grown in serum-free medium. We have demonstrated effective antisense inhibition of endogenous IGF-I transcripts by Northern analysis, and inhibition of protein by immunocytochemistry. Inhibition of protein expression was linked to antisense expression in the same cells. Antisense IGF-I transcripts were prominently localized to the nuclei by *in situ* hybridization supporting an intranuclear mechanism for antisense RNA-mediated inhibition, in agreement with observations made by others (Kim and Wold, 1985: Stout and Caskey, 1990; Corenlissen, 1989; Munroe, 1988). A finding in our study was accumulation of mononuclear infiltrates, with a predominance of lymphoid cells, at sites of injection of antisense IGF-I transfected glioma cells before lesions disappeared (Trojan et al., 1992). The majority of cells were CD8 positive, suggesting antisense IGF-I inhibition rendered glioma cells highly immunogenic, and hence, loss of tumorigenicity had an immune basis. This is further supported by recent studies which show that glioblastoma cells transfected with antisense to IGF-I demonstrate a significant increase in major histocompatibility complex-I (MHC-I) when compared to untransfected cells by FACS techniques (Trojan et al., 1996; Shevelev et al., 1997. Appendix III). We have shown that prior injection of the antisense IGF-I transfected glioma cells can prevent development of glioblastoma upon subsequent challenge with parental glioma cells. Injection of the genetically engineered glioma cells into rats with established glioblastomas cures the rats. The findings are consistent with a role for the host immune response in the anti-tumor therapeutic effects (Trojan et al., 1993; Johnson et al., 1993). We have also utilized the antisense gene therapy approach to successfully treat mice with teratocarcinoma (Trojan et al., 1994). Due to the pluripotent nature of teratocarcinoma cells which includes breast adenocarcinoma-like cells, the availability of a syngeneic mouse strain and because they express IGFs and IGF receptors (Jing et., 1991), mouse teratocarcinoma cells provided a uniquely informative system with which to explore the effects on tumorigenicity of inhibiting insulin-like growth factor expression in an animal model.

Prior research has focused on the potential role of the IGFs as growth factors driving the proliferation of tumor cells that produce them. This has led to atempts to inhibit growth of such tumor cells with anti-IGF-I or anti-IGF-II antibodies. While some growth inhibition has been claimed using these antibodies *in vitro* (Minuto et al., 1987; Huff et al., 1986; Blatt et al., 1984) and *in vivo* (Gansler et al., 1989), tumor development could not be completely blocked using the anti-IGF antibodies. Moreover, since intracrine mechanisms (Heldin and Westermark, 1989) may well be involved here, there is no certainty that the effects of anti-IGF-I antibodies will parallel those of antisense IGF-I RNA in altering tumor immunogenicity in an immunologically intact animal.

Tumors that arise *de novo* are poorly immunogeinc, thereby escaping host anti-tumor responses (Hewitt et al., 1979). Our studies provide a potential therapeutic

approach toward enhancing tumor immunogenicity based upon antisense gene transfer. Alternative approaches based upon sense gene transfer have been reported for enhancing tumor cell immunogenicity (Fearon et al., 1988). However, loss of tumorigenicity of transfected tumor cells and tumor prevention using these cells were incomplete. The second approach involves enhancement of tumor immunogenicity by transferring into cells, genes expressing soluble cytokines, such as interleukin-2 (Fearon and Vogelstein, 1990) and interleukin-4 (Tepper et al., 1989; Golumbek et al., 1991). Results with this approach have been more promising than those with foreign antigen transfer. Indeed, it has been demonstrated that production of IL-2 by the mouse mammary sarcoma EMT6 transfected with a murine IL-2 cDNA is able to elicit rejection of the tumor, and that the rejection is associated with development of cytotoxic T-lymphocytes that can lyse the parental tumor (McAdam et al., 1994). In addition, human breast cancer cells MDA-MB-435 transduced with human IL-2 did not form tumors when injected into the mammary fat pad of nude mice (Su et al., 1994). Furthermore, it was shown that transfecting cultured murine melanoma cells with the co-stimulator B7 evoked an effective immune response which results in regression of the existing tumor in syngeneic animals (Townsend and Allison, 1993; Chen et al., 1992).

Many primary tumors and cell lines from tumors produce large amounts of IGFs and IGF receptors (Antoinades et al., 1992; Roholl et al., 1990; Williams et al., 1989; Gansler et al., 1988; Culouscou et al., 1987; Macaulay et al., 1990; Jing et al., 1991). Most important for this proposal are breast carcinomas which have also been shown to express IGFs and IGF receptors (Huff et al., 1986; Yee et al., 1988; Foekins et al., 1989; Brunner et al., 1990). In order to postulate an autocrine or paracrine role for IGFs in breast cancer, breast cancer cells must have appropriate receptors for these ligands. Cullen et al. (1991) examined breast cancer cell lines and tumor samples for mRNA expression of the insulin receptor as well as the type I and type II IGF receptors. All cell lines examined by this group expressed mRNA for these receptors. In addition, 6 of 7 breast tumor biopsy specimens expressed type-I IGF receptor mRNA and 11 of 11 tumor specimens expressed type-II IGF receptors. The monoclonal antibody (αIR3) which blocks binding to the type-I IGF receptor also blocked the mitogenic effects of both IGF-I and IGF-II, but not insulin. The αIR3 antibody was also able to block greater than 80% of radiolabeled IGF-II binding. Furthermore, αIR3 administered at the time of tumor cell inoculation could inhibit MDA-231 tumor formation in athymic mice which suggests that blockade of the IGF-IR can inhibit the growth of some breast cancer cells *in vivo* (Arteaga et al., 1989; Arteaga, 1992). This indicates that although both type-I and type-II receptors are expressed in breast cancer cells, the mitogenic response to both IGF-I and IGF-II is mediated by the type-I receptor (Cullen et al., 1990). It is likely that IGFs and the type-I IGF receptor play a pivotal role in the tumorigenicity of breast carcinoma. Since most breast tumors express IGF receptors, it is important to ascertain whether such tumors, upon inhibition of this gene expression, can be correlated with an analogous induction of an immunogenic phenotype. For this reason, we have initiated experiments to analyze the effect of blocking the expression of IGF receptors using antisense strategy in the human breast cancer cell line, MDA-MB-435S which was isolated from a patient with metastatic disease.

Breast cancer cells appear to possess certain intrinsic properties that facilitate the development of bone metastases. Almost all patients dying of breast cancer or with advanced breast cancer have bone metastases (Yoneda et al., 1994). Breast cancer cells may prefer bone to other organs because of the growth factors in the microenvironment. Bone has been shown to be a major source of IGFs, with humans having the highest concentration of total skeletal somatomedins of all species studied (Bautista et al., 1990). Indeed, IGF-II is the most abundant growth factor stored in bone matrix. Breast cancer cells have been shown to express type-I and type-II IGF receptors (Cullen et al., 1990). A recent study has demonstrated that highly metastatic murine carcinoma H59 cells expressing IGF-IR antisense RNA lost their ability to metastasize spontaneously to the liver or lung from primary subcutaneous tumors and could not colonize these organs, even when inoculated directly into their microvasculature (Long et al., 1995). These results implicate the IGF-IR in the control of tumor growth and show that IGF-IR can play a crucial role in the regulation of tumor cell potential to disseminate and form metastases in secondary organs. Therefore, targeting breast tumor cells with an antisense IGF-IR strategy could provide an effective anti-metastatic therapy for this disease.

Transforming growth factors (TGFs) are polypeptides which have important regulatory roles in angiogenesis, embryogenesis, inflammation and immunosuppression within both normal and transformed cells and tissues (MacCallum et al., 1994). The presence of transcripts for TGF-βs has been reported for rodent and human breast cancer cell lines (McAdam et al., 1994; Arrick et al., 1994). Furthermore, the expression of TGF-β in fresh human tumor tissue indicated that the majority of tumors expressing all three isoforms of TGF-β were derived from patients who had lymph node metastases, thereby suggesting a role of TGF-β in mammary cancer metastasis (MacCallum et al., 1994). Studies with an inbred rat model of mammary adenocarcinoma show that brief exposure of the tumor cells to TGF-β results in enhanced ability of these cells to form lung colonies when injected *in vivo* (Welch et al., 1990).

Degradative enzymes involved in tumor invasion play important roles in invasive and tissue remodeling processes, including angiogenesis and trophoblast implantation (Danø et al.,1985). Plasminogen activator is a serine protease which exists in two forms: tissue type (tPA) and urokinase-type (uPA) which have functional differences. uPA has been shown to be primarily involved in mediating tissue-remodeling processes (Moscatelli and Rifkin, 1988) whereas tPA is considered to play a key role in intravascular fibrinolysis (Rijken et al., 1982). It has been shown that expression of human recombinant PAs enhances invasion and experimental metastasis of H-*ras*-transformed NIH 3T3 mouse fibroblast cells (Axelrod et al.,1989). This study demonstrated that introduction into cells of a gene expressing high levels of either recombinant uPA or tPA yields a phenotype with a higher capacity to invade and furthermore indicates a causal role for PA-mediated proteolysis in this activity. A more recent report suggested that tPA may play a crucial role in the early stages of tumor growth and metastasis (Columbi et al., 1995). These authors showed that G361 human melanoma cells which produce only tPA and not uPA formed tumors and a number of metastases in several organs when injected into nude mice whereas human fibrosarcoma HT-1080 cells producing only uPA, formed relatively larger tumors but did not metastasize to distal sites. These results suggest that there is a relationship between the

metastatic potential of G361 cells and tPA. We have observed a significant reduction in the expression of t-PA and uPA in highly metastatic rat prostate adenocarcinoma cells (PA-III) after transfection with antisense to the type-I IFG-IR (Burfeind et al.,1996). Our data indicates the involvement of IGF-IR in the regulation of uPA and tPA expression in rat PA-III cells. We have initiated studies to examine the relationship of the IGF-IR to the expression of PAs in human and mouse breast cancer cells after transfection with the antisense IGF-IR construct.

Our experimental design for breast tumors and metastases provides for an efficient coupled *in vitro/in vivo* assay system to determine the role of certain growth factors in breast cancer tumor types that may be responsible for down-modulating tumor immunogenicity or other mechanisms thereby bringing about tumor regression. Since IGF receptors, TGF-βs and PAs are expressed by a number of breast cancers, it is important to ascertain whether such tumors, upon inhibition of one or more of these genes, can be correlated with an analogous induction of an immunogenic phenotype or other mechanisms that may inhibit metastasis. For this reason, we have focused our studies on blocking the expression of IGF-II, IGF-IR,TGF-β2 and PAs in human breast cancer cells, MDA-MB-435S and murine breast cancer cells, EMT6 using antisense expression plasmids.

Unpublished Data:

Body: In the second year of this project, we have continued to make progress in several of the proposed goals as well as in the additional areas that we presented in our progress report for 1995. In parallel with the studies we proposed in the application, we are utilizing syngeneic animal models as well as a metastatic human breast cancer model (Price and Zhang, 1989/1990, Price et al., 1990) to further elucidate the effects of our antisense strategy on tumorigenesis and metastatic processes.

A number of growth factors and their receptors are involved in tumorigenesis and influence the metastatic potential of cancer cells. Breast cancer cell lines and tumor samples have been shown to express IGF receptor (Cullen et al., 1991). Studies have shown that blockade of the type-I IGF receptors with the monoclonal antibody αIR3 can reduce the rate of cell growth of MCF-7 breast cancer cells (Rohlik et al., 1987) as well as inhibit formation of MDA-MB-231 breast tumors grown in nude mice (Arteaga et al., 1989).

TGF-β is secreted by a variety of cell types often associated with metastasizing tumor cells (Welch et al., 1990). Indeed, production of TGF-β seems to increase with breast cancer where this overproduction could contribute to aberrant tumor-host interactions (Dickson and Lippman, 1995). We have shown that EMT6 cells transfected with antisense TGF-β2 resulted in an reduction in tumor weight in sygeneic Balb/c mice but not in nude mice, underlining the important role of immune system in the inhibition of tumor by antisense TGF-β2.

Plasminogen activator is a protease implicated in both tumorigenesis and metastasis (Duffy et al.,1984; 1987). Treatment of MCF-7 cells with estradiol induces PAs (Butler et al.,1983; Katzenellenbogen et al.,1984). Estrogens and anti-estrogens modulate the production of PAs in human breast cancer cells (Katzenellenbogen et al.,1984; Busso et al.,1987). A variety of growth factors induce enhanced production of t-PA nad u-PA in various human tumors (Laiho and Keski-Oja, 1989). Modulation of PA expression in MCF-7 cells could be exerted through overexpression of growth factors by estradiol. IGFs and IGF-IR are potential candidates since estrogen could alter the expression of IGF ligands, receptors and binding proteins (Lee and Yee, 1995). The study of the relationship between IGF-IR and PAs may provide important information on the involvement of PA pathways in tumorigenesis and metastasis of breast cancer.

We have utilized several constructs which we prepared in the first year of the project to inhibit expression of IGF-II, IGF-IR and TGF-β2 in the human breast cancer cell line, MDA-MB-435S and the murine breast cancer cell line, EMT6. All constructs were assembled with the constitutive cytomegalovirus (CMV) or inducible mouse metallothionein-1 (MT-1) promoter with the appropriate insert and either a hygromycin (Hyg R) or neomycin (Neo R) resistant gene (Fig.1, Appendix I). The detailed preparation of the individual antisense constructs was described in the 1995 progress report.

We have previously reported the successful transfection of the human breast cancer cell line Hs578T with antisense to IGF-I and antisense to IGF-IR and the T47D cell line with antisense to IGF-II and antisense to IGF-IR. These cells displayed high levels of antisense expression with the appropriate construct. T47D cells form colonies in soft agar (Engel and Young,1978) and tumors in nude mice (Dickson et al.,1986).

Hs578T cells were reported to be invasive in Matrigel *in vitro*, as well as form tumors in nude mice (Thompson et al.,1992). However, we were unable to establish tumors in nude mice with either of these breast cancer cell lines which made it impossible to assess the effects of antisense inhibition in an animal model. Indeed, a report by Thompson et al. (1992) demonstrated that T47D cells did not form tumors in nude mice under their conditions. This may be due to an altered phenotype of the cells. Another possible explanation for the lack of tumor growth in nude mice of the T47D and Hs578T breast cancer cells, may be due to the culture conditions since we maintained the cells in serum free media for 24 hours prior to injection. We have also observed that MDA-MD-231 breast cancer cells would not form tumors when cultured under these conditions. We therefore, have directed our efforts to assess the effects of antisense inhibition in a highly metastatic human breast cancer cell line, MDA-MB-435S as well as a murine breast cancer cell line, EMT6 which form tumors under our culture conditions. The MDA-MB-435S cell line provides a model in which we can study the effects of antisense IGF-IR on tumorigenesis and metastasis. The EMT6 cell line has a syngeneic animal model in which we can test for immune responses evoked by injection of transfected cells. Here, we report our findings over the past year from experiments we carried out utilizing these two cell lines. Results from these studies will be presented at the American Association for Cancer Research in San Diego, CA in April 1997(Gan et al.,1997. Appendix II).

We have preliminary data from a study done in collaboration with Dr. Joseph Ilan's laboratory that inhibition of IGF-IR by antisense strategy suppresses insulin-like growth factor I production in C6 glioblastoma cells. It is therefore possible that the targeting of IGF-IR may bring about inhibition of insulin-like growth factors in breast cancer cells. Therefore, in this past year we have focused our efforts on investigating the effectiveness of antisense-IGF-IR in reducing tumorigenicity in human and murine breast cancer cells *in vitro* and *in vivo*. In addition we have carried out experiments with EMT6 cells in nude mice and syngeneic Balb/c mice to elucidate the possible role of the immune system in reducing the tumorigencity of the antisense IGF-IR transfected cells.

To test whether human MDA-MB-435S and mouse EMT6 breast cancer cells express IGF-IR, poly (A+) RNA derived from the parental cells was analyzed by Northern blot hybridization. Strong hybridization signals at 11-kb and 7-kb were observed in MDA-MB-435S and EMT6 parental cell lines (Fig. 1, Appendix I).

We have successfully transfected the MDA-MB-435S and EMT6 cells with the antisense construct for IGF-IR. We have also developed an alternative approach using a vector that could stably integrate into the host genome, thereby producing antisense IGF-IR mRNA capable of inhibiting the endogenous IGF-IR. When we utilized the episome based vectors containing the antisense-IGF-IR construct driven either by the inducible MT promoter or the constitutive CMV promoter for transfection of the MDA-MD-435S and EMT6 cells, the cells consistently failed to show significant inhibition in the mRNA levels of IGF-IR. While the episome based vector has been shown to work in C6 rat glioblastoma cells (Trojan et al.,1992), mouse PCC3 teratocarcinoma (Trojan et al.,1993), rat PAIII prostate cancer cells (Burfeind et al.,1996), and in mouse breast cancer EMT6 cells with an antisense-TGFβ construct (unpublished data), the results obtained following transfection of MDA-MB-435S cells and EMT6 cells with this vector

indicated that this vector was not efficacous as a delivery system for the antisense-IGF-IR into the breast cancer cells.

To circumvent these problems, we assembled a eukaryotic antisense IGF-IR expression vector pRcII/CMV. The pRc/CMV (Fig. 2A, Appendix I) was genetically modified to generate the construct pRcII/CMV (Fig. 2B, Appendix I). The new vector, pRcII/CMV which contains a constitutive cytomegalovirus (CMV) promoter was used for expression of the antisense cRNA of human IGF-IR in both human MDA-MB-435S cells and mouse EMT6 cells. The 0.7-kb cDNA fragment for IGF-IR was amplified by reverse transcription-PCR on total RNA prepared from the human breast cancer cell line T47D and subcloned into the expression vector in the antisense orientation (Fig. 2C, Appendix I). The presence of the IGF-IR cDNA insert in the antisense direction was confirmed by restriction mapping of the vector DNA (data not shown).

The MDA-MB-435S cells or EMT6 cells were transfected with the vector containing the IGF-IR cDNA in the antisense orientation (pRcII/IGF-IRAS) or control vector without an insert (pRcII/CMV). Several clones were isolated from the MDA-MB-435S and EMT6 cells transfected with antisense IGF-IR for further investigation. The expression of the 2-kb-long IGF-IR antisense-neomycin sense fusion transcript was determined by Northern blot analysis of total RNA isolated from MDA-MB-435S or EMT6 cells using either IGF-IR cDNA or neomycin cDNA as a hybridization probe (data not shown).

The expresssion of endogenous IGF-IR mRNA was determined in the MDA-MB-435S cell clones carrying the antisense IGF-IR (Fig. 3 Appendix I). The level of inhibition of IGF-IR in these clones was determined by comparing poly (A+) RNA from IGF-IR antisense-transfected cell clones to that obtained from MDA-MB-435S cells transfected with the control vector (without insert). Two different MDA-MB-435S cell clones (C8 and C9) displayed a strong expression of the 2-kB IGF-IR antisense-neomycin sense fusion transcript by Northern blot analysis (Figure 3A, Appendix I). The expression of IGF-IR mRNA in the antisense-transfected clones was reduced by 43 % (clone C8) and 56 % (clone C9) relative to the control-transfected clone (Fig. 3C, Appendix I). The same filter was rehybridized with a cDNA probe for chicken ß-actin to confirm the integrity and amount of poly (A+) RNA in the samples (Fig. 3B, Appendix I).

We carried out a MTT assay to determine the *in vitro* growth characteristics of the transfected MDA-MB-435S C8 and C9 cell clones. The *in vitro* growth rates of the antisense clones were compared to control-transfected cells (Fig. 4, Appendix I). The standard curves (MTT_{570} against cell numbers) of the different transfected cells were almost identical to one another (data not shown), therefore we could directly compare the MTT_{570} values of the different cell clones. A reduction in growth rates for the IGF-IR antisense clones, C8 and C9, was observed at serum concentrations of 1 % (Fig. 4A, Appendix I) and 10 % FBS (Fig. 4B, Appendix I). The differences between control-transfected cells and IGF-IR antisense-transfected cell clones were most pronounced on the day 5 assay, with 50% inhibition in growth of the C8 cell clone and 90% inhibition of growth in the C9 cell clone.

Anchorage-independent growth in the semisolid medium of soft agar is a strong indicator of the transformed phenotype. To assess the relative anchorage independence,

parallel cultures of MDA-MB-435S C8 and C9 cell clones were carried out in soft agar. The number of colonies that grew in soft agar was determined for each clone (Table 1, Appendix I). The C8 and C9 cell clones transfected with the antisense IGF-IR construct showed no colony formation (Figs 5B and 5C, Appendix I) whereas the control MDA-MB-435S cells transfected with the vector minus the IGF-IR insert displayed extensive colony formation (Fig 5A, Appendix I). The experiment was repeated with identical results. The reduction in IGF-IR expression suppresses anchorage independence of the cells suggesting roles for IGF-IR in the transformed phenotype.

To determine the effect of antisense IGF-IR suppression on tumorgenicity of MDA-MB-435S cells, female nude mice were injected *s.c.* over the left scapular region with 2 X 10^6 viable control-transfected cells (vector without insert, pRcII/CMV) or with the antisense IGF-IR expressing cell clones, C8 and C9 (pRcII/IGF-IRAS). Mice injected with the C8 and C9 antisense IGF-IR cell clones exhibited a delay in onset of tumor growth (Table 2, Appendix I) and a dramatic reduction in tumor size (Fig.6A, Appendix I). All of the mice (n=5) injected with cells from the C9 clone developed tumors by 11 weeks, however the tumors were over 90% smaller than those of the control group (Fig. 6B, Appendix I). Moreover, 3 out of 4 mice injected with cells from the C8 clone remained tumor free at the end of 11 weeks (Fig. 6B, Appendix I). A dramatic inhibition of tumor growth is apparent in mice injected with the MDA-MB-435S cell clones C8 (Fig.7A, Appendix I) and C9 (Fig. 7B, Appendix I) when compared to vector control transfected cells (Fig. 7C, Appendix I)

To further investigate the effectiveness of antisense IGF-IR, mouse EMT6 breast cancer cells were transfected with a vector in which IGF-IR cDNA is constitutively expressed in the antisense orientation and neomycin in the sense orientation (2-kb-long fusion transcript). The neomycin resistant mixed population of control-transfected and IGF-IR antisense-transfected EMT6 cells was obtained and further characterized. We found that the expression of IGF-IR mRNA in the antisense-transfected EMT6 cells was reduced by 30 % relative to control-transfected cells by Northern blot analysis (Figs. 8A and C, Appendix I). The filter was rehybridized with a chicken ß-actin cDNA probe to verify the amount and integrity of the RNA samples (Fig. 8B, Appendix I).

We have examined ability of antisense IGF-IR to inhibit tumor growth by injecting of 1 X 10^5 control-transfected EMT6 cells (vector without insert, pRcII/CMV) or IGF-IR antisense-transfected EMT6 cells (pRcII/IGF-IRAS) into the mammary fat pad region of female nude mice. Animals injected with antisense IGF-IR transfected EMT6 cells had significantly smaller tumor weights (60 % reduction) compared to the control group (Fig. 9, bar I. Appendix I). In a parallel experiment, 1 X 10^5 EMT6 cells treated as above were injected into the mammary fat pad region of syngeneic female Balb/c mice. The control-transfected cells formed tumors rapidly, and within 14 days the mice developed massive, solid, tumors at the injection site. This pattern of growth of control-transfected cells in syngeneic animals was similar to nude mice injected with the control-transfected cells. Balb/c mice injected with IGF-IR antisense-transfected EMT6 cells showed a dramatic inhibition of 80 % in tumor weight (Fig. 9, Bar 4. Appendix I). An important finding Is the significant inhibition in tumor growth observed in the syngeneic animals when compared to the nude mice receiving the antisense IGF-IR transfected cells. This results suggests that an immune response may be involved in the

enhanced inhibition of tumor growth in the syngeneic animal. As depicted in Fig. 10A a representative nude mouse injected with control cells (vector without insert, pRcII/CMV) developed a large tumor (arrow) whereas the representative nude mouse injected with antisense IGF-IR transfected tumor cells developed a significantly smaller tumor (Fig. 10B, Appendix I). Tumors from syngeneic animals injected with antisense IGF-IR transfected EMT6 cells (Fig. 10D, Appendix I) were significantly smaller than tumors from mice injected with the control transfected EMT6 cells (Fig. 10C, Appendix I).

We have isolated 9 clones of EMT6 transfected with antisense IGF-IR in order to select cells with low IGF-IR expression. We analyzed total RNA from antisense IGF-IR transfected EMT6 cell clones and EMT6 cells transfected with the control vector by Northern blot analysis. Fig. 11A (Appendix I) demonstrates the level of inhibition of IGF-IR in the 9 different clones. We have also examined the expression of tPA in these clones (Fig. 11C and 11E, Appendix I) and have observed that tPA expression is inhibited in the antisense IGF-IR transfected cell clones. We have selected 2 clones (M5 and M7) for further analyses *in vivo*.

We have tested the tumorigenicity of the cell clones M5 and M7 by injecting 1 X 10^5 cells from the control transfected EMT6 and the antisense transfected IGF-IR cell clones into nude mice (n=5). There is a significant inhibition of tumorigenesis in the mice injected with antisense IGF-IR transfected cell clones (Fig 12, Appendix I).

Although we have achieved considerable success with our antisense constructs, we hope to achieve a tight and regulatory control of the expression of the antisense IGF-IR gene. Therefore, we have recently cloned the antisense fragment into the tetracycline inducible vector construct pBI-G (Clontech). This newly developed system will allow us to tightly regulate the antisense gene. Heat shock, metallothionein and hormone induced promoters have been previously used to regulate the expression of the desired epigene. Although these approaches have the advantage of being able to regulate the expression of the gene of interest, they harbor inherent problems: the promoters can be "leaky" in the uninduced state or the stimuli themselves exert pleotropic effects on compatible endogenous promoters and proteins. The use of prokaryotic-inducible promoters in eukaryotic systems effectively eliminates these problems by the use of non-physiological inducers. Two such systems , the Tet-off and the Tet-on systems, have recently been developed by Bujard and coworkers (Gossen and Bujard, 1992; Gossen et al.,1995). The Tet-off system uses a chimeric protein that fuses portions of the transposon-5 tet repressor and the strong viral transactivator VP16. The tet-repressor moiety binds with high affinity and specificity to its prokaryotic DNA binding site (TRE), while the VP16-derived fragment transactivates the downstream target gene. In the presence of tetracycline, the chimeric protein binds the antibiotic and thereby loses the affinity for its DNA response element (Fig. 13A, Appendix I). The Tet-On system (Fig. 13B, Appendix I) is similar to the Tet-off system, but is based on a "reverse" Tet repressor (rTetR) result from a four amino acid changes in TetR (Gossen *et al.*, 1995).The rTetR binds to the TRE in the presence of tetracycline (Tc) or doxycyclinehydrochloride (Dox). rTet binds the TRE in the presence of Tc or Dox. Thus when fused to the VP16 activator domain, rTetR creates a "reverse" tTA (rtTA) that activates transcription in the presence of Tc or Dox. The maximal expression levels in the Tet systems are high and compare favorably with the maximal levels obtainable from the wild-type CMV promoter/ enhancer (Yin et

al.,1996). We have combined this binary tet-system with antisense technology in an attempt to specifically silence a target gene, namely, type-I insulin-like growth factor receptor. The steps for establishing cell lines with tetracycline-inducible genes are depicted diagramatically in Fig 14 (Appendix I). The 0.7kb antisense IGF-IR cDNA fragment amplified from T47D cells was subcloned into the pBIG tetracycline inducible vector in the antisense orientation. The presence of the IGF-IR cDNA was confirmed by restriction digests by a number of restriction enzymes. We are concurrently optimizing the conditions for stable and efficient expression of the antisense IGF-IR in human MDA-MB-435S cells as well as in murine EMT6 and MCak cell lines.

CONCLUSIONS

The long term goal of this study is to develop a gene therapy treatment to cure human breast cancer. In the first year we initiated studies on a highly metastatic human breast cancer cell line MDA-MD-435S. We were able to obtain tumors in nude mice when 2 X 10^6 cells were injected *s.c.* into 6 week-old female nude mice. In the second year of this study we have transfected the MDA-MB-435S cells with an antisense IGF-IR construct and have established stable clonal transfectants. These clones show reduced IGF-IR mRNA transcripts compared to the control transfected cells. The *in vitro* studies indicated that a 40% inhibiton of IGF-IR expression is sufficient to reduce growth rate and prevent the cells from forming anchorage independent colonies in soft agar. The mechanisms on how the inhibiton of IGF-IR could bring about such a phenotype is currently under investigation in our laboratory. Recent reports have suggested that overexpression of IGF-IR can inhibit apoptosis in C6 rat glioblastoma cells (Resnicoff et al.,1995), Balb/c 3T3 cells (Sell et al.,1995) and neuroblastoma cells (Singleton et al.,1996). Furthermore, inhibition of IGF-IR in Ewing's Sarcoma/ Peripheral Neuoectodermal tumor cell line (Scotlandi et al.,1996) induces apoptosis *in vitro*. A reduction in IGF-IR by the antisense approach may induce apoptosis in the MDA-MB-435S breast cancer cells. *In vitro* assays eg. DNA fragmentation assay (Eldadah et al.,1996) and TUNEL assay (Gavrieli et al.,1992) are being carried out in the human MDA-MB-435S and mouse EMT6 breast cancer cell lines to test whether these cells have a similar apoptotic response following IGF-IR inhibition.

When the antisense IGF-IR transfected MDA-MB-435S clones, C8 and C9 were injected into nude mice a significant delay in tumor onset and a reduction in tumorgenicity occurred. The experiment suggests that IGF-IR plays an important role in tumorigenesis in a metastatic human breast cancer cell line. Although all mice injected with C9 cells eventually developed tumors, the delay in the onset of tumor formation (Table 2, Appendix I) could be explained by a few cells subsequently losing their antisense IGF-IR expression. In addition, we could not rule out the involvement of the rudimentary immune system which exists in nude mice. We plan to further study the immune components involved in tumor inhibition with antisense IGF-IR by carrying a series of experiments on nude mice which have natural killer (NK) cells, and B cells, SCID mice which have only NK cells and SCID/ beige mice which lack both NK and B cells. This will provide a model system in which we could assess the contribution of immune factors involved in the delay and inhibition of tumor growth of antisense IGF-IR transfected human breast cancer cells.

Reduction in tumor growth was observed in nude and immunocompetent Balb/c mice injected with EMT6 cells transfected with antisense-IGFIR. In the immunocompetent Balb/c mice, the tumors were significantly smaller than in nude mice which could be due to the activation of the immune system by the antisense IGF-IR transfected cells in the syngeneic animals This is supported by previous reports that showed injection of C6 cells transfected with antisense IGF-I (Trojan et al.,1993) or C6 cells transfected with antisense IGF-IR (Reniscoff et al.,1994) into rats prevents subsequent wild-type tumorigenesis and induces regression of established tumors. More recently, Reniscoff et al. (1996) have demonstrated the stimulation of a cellular immune response in syngeneic rats following the injection of C6 IGF-IR antisense containing

cells. Further experiments need to be performed in order to address whether breast cancer cells can respond to antisense manipulation in a similar manner. We have isolated and are presently characterizing the IGF-IR transfected EMT6 clones which show significant inhibition of the IGF-IR expression for proliferation, anchorage-independent colony formation, invasiveness *in vitro* and tumorigenicity and immunogenicity *in vivo*. We intend to study the MHC profile in both the control transfected and antisense IGF-IR transfected cells. We also intend to carry out T cell proliferation and cytotoxicity assays to assess whether these cells can stimulate the immune the system.

We have shown that a correlation exists between the expression of IGF-IR and tPA at the RNA level in EMT6 cells. We plan to investigate whether this relationship exists in the MDA-MB-435S cells. The downregulation of PAs observed in the antisense IGF-IR transfected cells could play an important function in the inhibition of metastasis, therefore we plan to utilize a metastatic model of human breast cancer to study the effect of antisense IGF-IR inhibition. In the event of incomplete inhibition of tumor formation and metastasis with antisense to IGF-IR, we will assemble constructs for antisense tPA and cotransfect the breast cancer cells.

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Table 1. Supression of anchorage independent colony formation in antisense transfected MDA-MB-435S cells

Cells	No. of colonies
MDA-MB-435 S -pRCII/CMV	32 ± 4
MDA-MD-435 S pRCII/IGF-IRAS C8	0
MDA-MD-435 S pRCII/IGF-IRAS C9	0

Triplicate plates were seeded with 2500 cells each. Colonies were counted 4 weeks later. Results expressed were mean ± S.E.

Table 2. Tumorigenicity of the control transfected MDA-MB-435S cells and pRcII/IGF-IRAS transfectants.

Cells	Tumorigenicitiy[a]	Latency (weeks)
MDA-MD-435S pRcII/CMV	5/5	2
MDA-MD-435S pRcII/IGF-IRAS, C8	1/4	7
MDA-MD-435S pRcII/IGF-IRAS, C9	4/5	9

[a] Number of tumors versus number of sites injected.

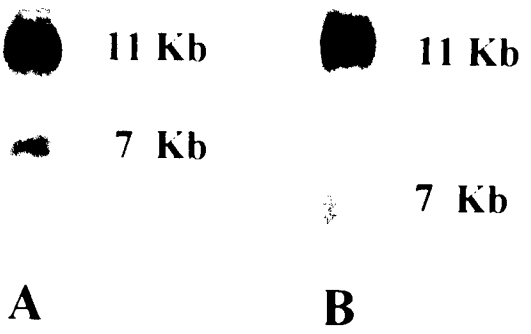

Figure 1. Expression of IGF-I receptor in MDA-MB-435S human breast cancer cells and EMT6 mouse breast cancer cells. Northern blot hybridization on poly A^+ RNA (10μg) isolated from (A) the human breast cancer cell line MDA-MB-435S and (B) the mouse breast cancer cell line EMT6. A radioactive labeled IGF-IR cDNA wasused for a probe.

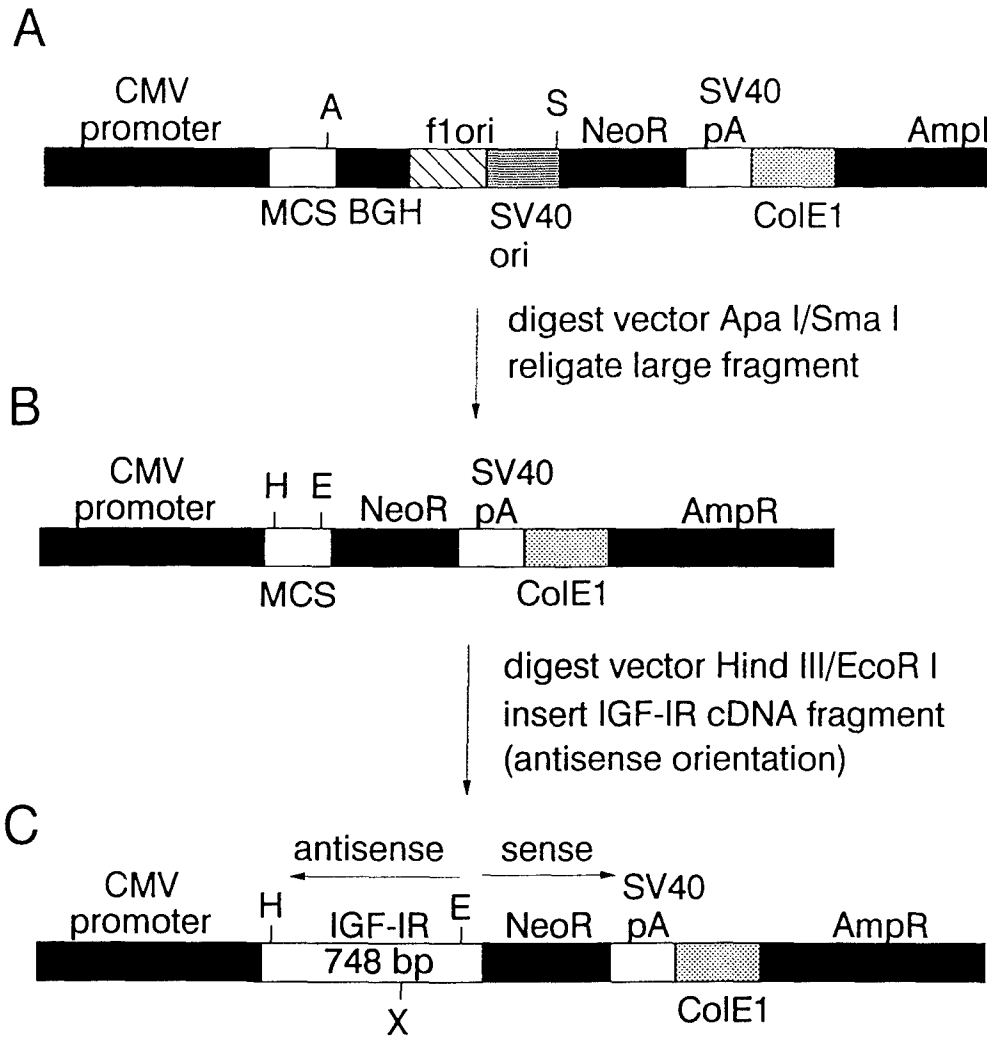

Figure 2. Schematic representation of the steps employed to construct the IGF-IR antisense vector pRcII/IGF-IRAS. The 748-bp-long human IGF-IR fragment was cloned into the vector pRcII/CMV in the antisense orientation upstream of the neomycin resistance gene. The transcription of both the IGF-IR antisense RNA and the neomycin sense RNA are under the control of the same constitutive human CMV promoter, thereby generating a 2-kb-long antisense-sense fusion transcript. CMV, cytomegalovirus; MCS, multiple cloning site; BGH, bovine growth hormone polyadenylation signal; f1 ori, f1 origin; SV 40 ori, Simian virus 40 origin of replication; NeoR, neomycin resistance gene; SV 40 pA, simian virus 40 polyadenylation signal; ColE1, ColE1 origin of replication; Amp^R, ampicillin resistance gene; A, *Apa*I; H, *Hind*III; E, *Eco*RI, S, *Sma*I.

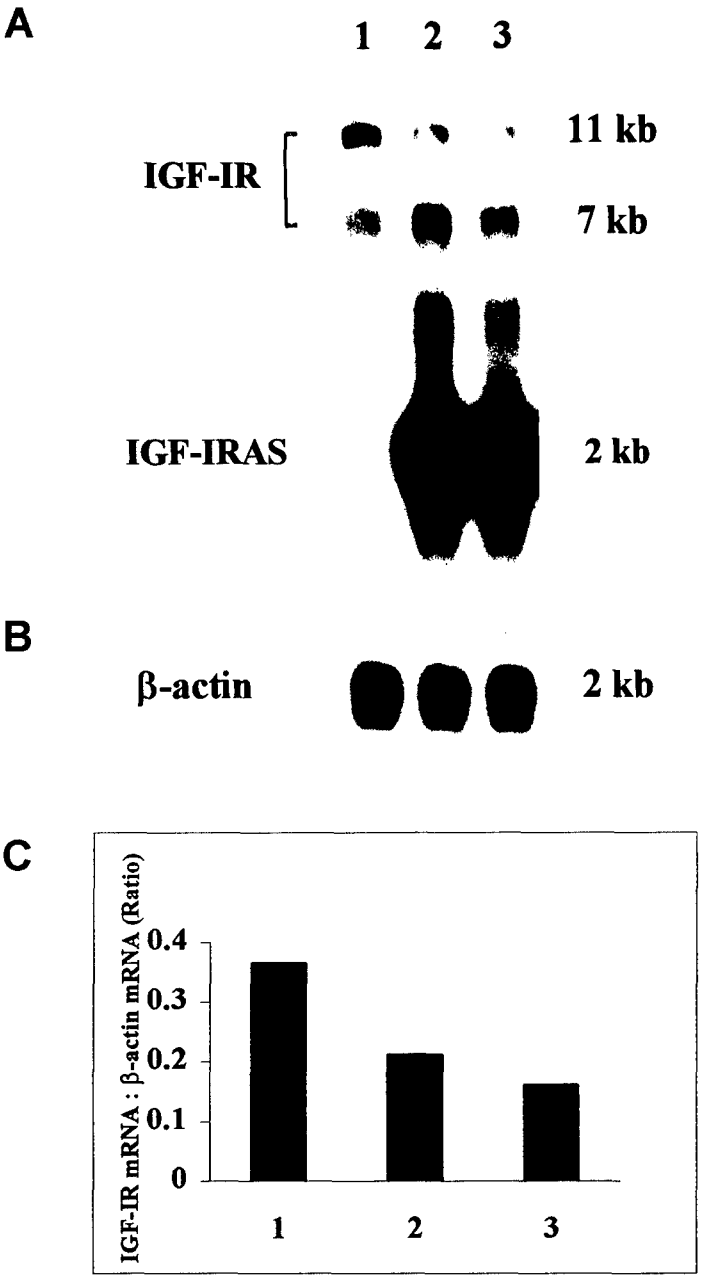

Figure 3. Northern blot analysis for IGF-IR and IGF-IR antisense expression in transfected MDA-MB-435S cells. (A) Poly (A)+ RNA (5μg per lane) derived from control-transfected (lane 1) and two IGF-IR antisense-transfected MDA-MB-435S cell clones C8 (lane 2) and C9 (lane 3) was analyzed using a ^{32}P-labeled IGF-IR cDNA as a hybridization probe. The molecular size of the endogenous IGF-IR transcripts and of the IGF-IR antisense-neomycin sense fusion cRNA (IGF-IRAS) is indicated on the right. The fusion transcript contains 0.75 kb IGF-IR sequence plus approximately 1.25 kb neomycin sequence. (B) Differences in loading and RNA integrity was assessed by reprobing the same filter with chicken β-actin. The autoradiographs were scanned using a laser densitometer SciScan 5000, and the difference in the expression of the mRNA transcripts was calculated relative to the β-actin standards. (C)The analysis for IGF-IR is shown in the bar graph. The numbers under the bar graph correspond to the numbers used in A and B.

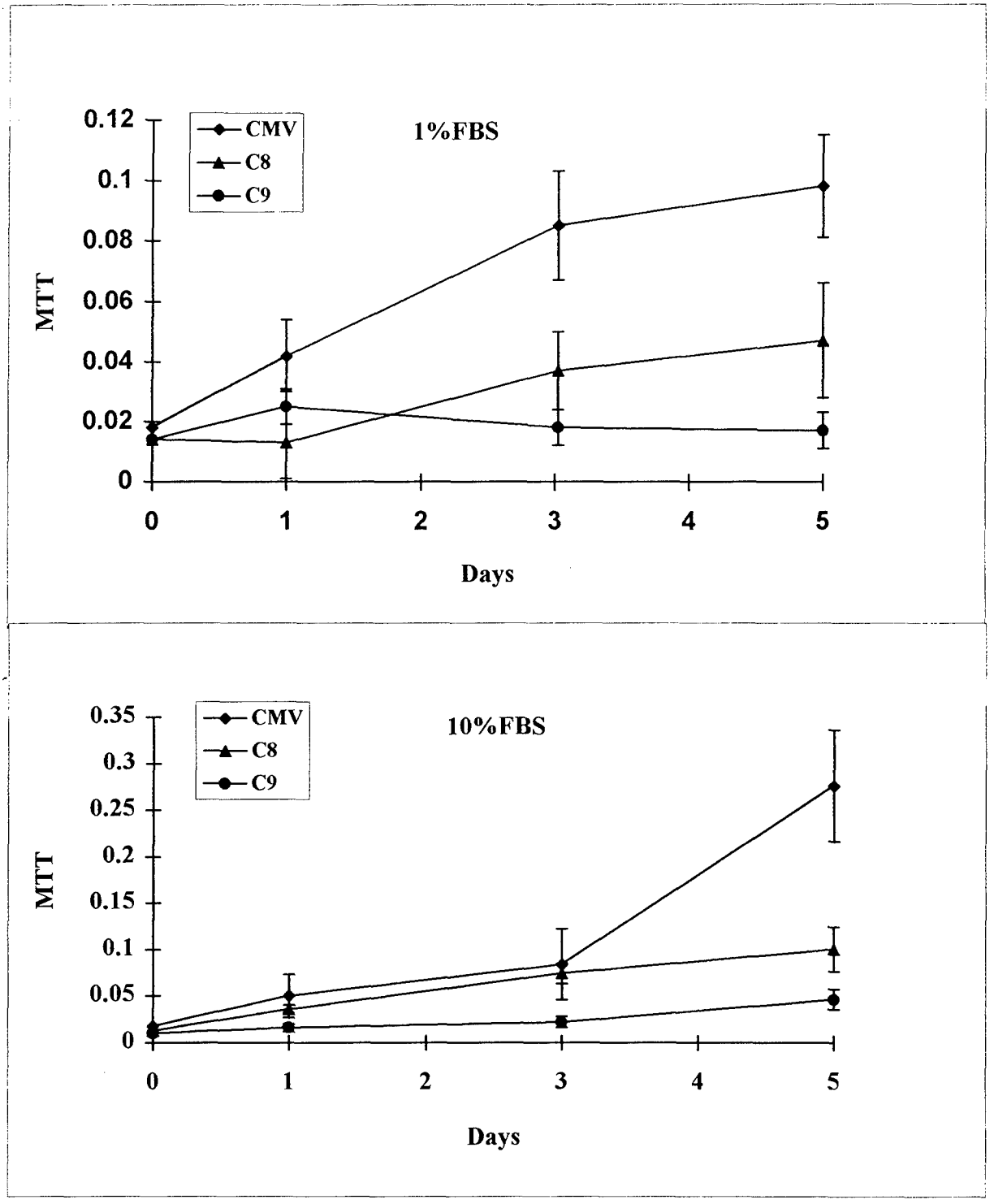

Figure 4. Growth rates of MDA-MB-435S cell clones transfected with pRcII/IGF-IRAS vector *in vitro*. Cells were grown in either (A) 1% FBS or (B) 10% FBS. Cell proliferation was measured using the MTT assay. Each point represents the mean of 6 wells. Standard errors are indicated.

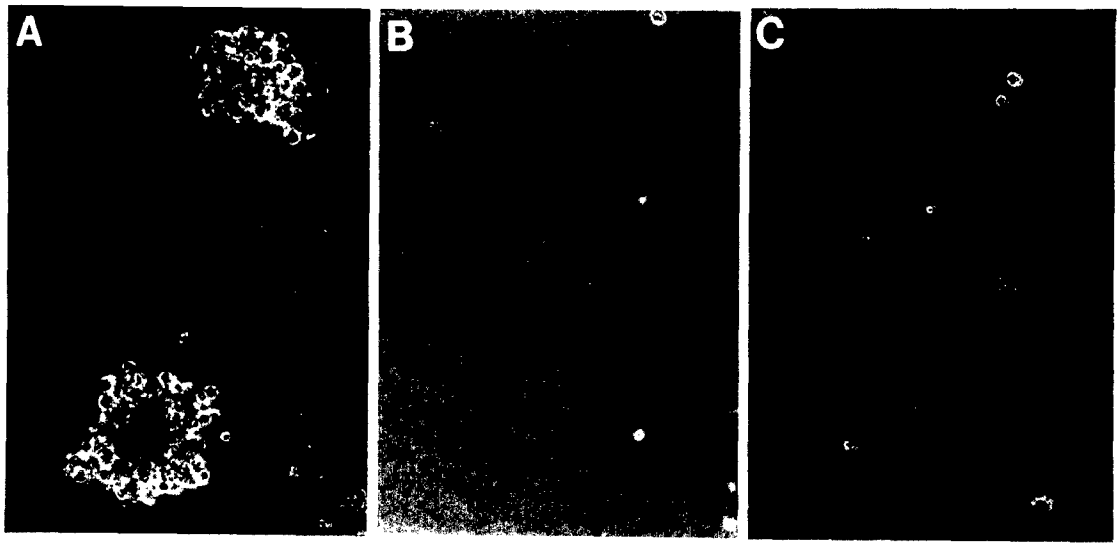

Figure 5. Anchorage-independent growth of MDA-MB-435S cell clones transfected with pRcII/IGF-IRAS. (A) control transfected cells (B) Clone C8 transfected with pRcII/IGF-IRAS and (C) Clone C9 transfected with pRcII/IGF-IRAS. All photographs were taken on day 23 of culture.

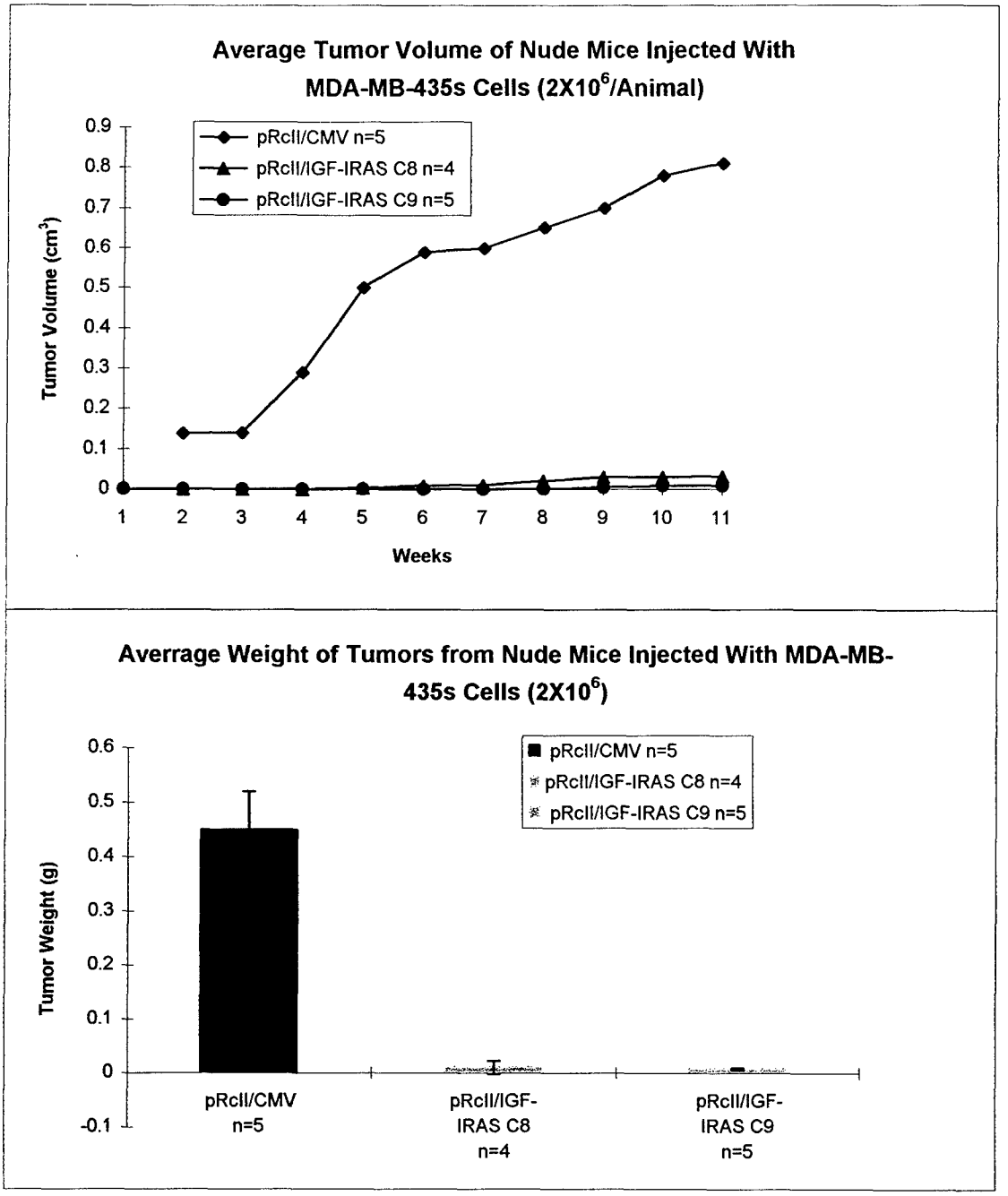

Figure 6. Effect of antisense IGF-IR treatment on tumor growth. (A) Delayed onset and inhibition of tumor growth by IGF-IR antisense-transfected MDA-MB-435S cells *in vivo*. Mice were assessed for tumor formation for 11 weeks following s.c. injection of 2 X 10^6 control transfected MDA-MB-435S cells and two antisense IGF-IR transfected MDA-MB-435S cell clones, C8 and C9. (B) Tumors were excised 11 weeks post injection and tumor weights were determined.

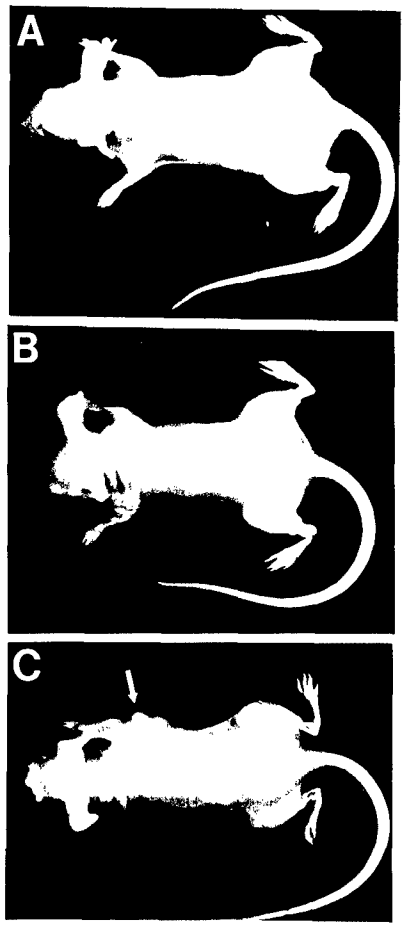

Figure 7. Tumor growth in nude mice. Tumors derived from the control transfected MDA-MB-435S cells (A), MDA-MB-435S cell clones C8 (B) and C9 (C) transfected with the antisense IGF-IR construct.

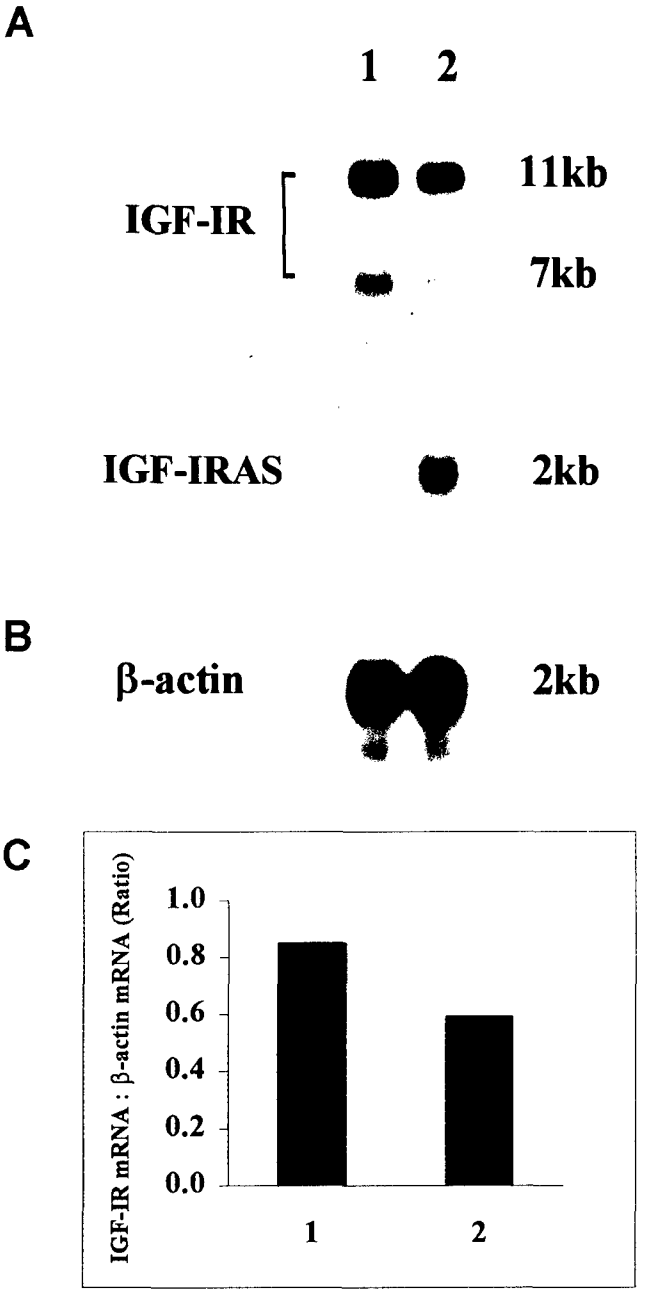

Figure 8. Northern blot analysis of IGF-IR mRNA in EMT6 cells transfected with the antisense IGF-IR construct. (A) Poly (A+) RNA from control-transfected (lane 1) and an antisense-transfected EMT6 cell population (lane 2) was analyzed using a radioactive labeled IGF-IR cDNA hybridization probe. The transcript size for IGF-IR mRNA and for IGF-IR antisense cRNA is indicated on the right. (B) The filter was stripped and rehybidized with a β-actin cDNA probe. A SciScan 5000 laser densitometer was used to scan the autoradiograph, and the difference in the expression of mRNA transcripts was calculated relative to the β-actin standards. Results of this analysis are shown in the bar graph. The numbers under the bar graph correspond to the numbers used in A-B.

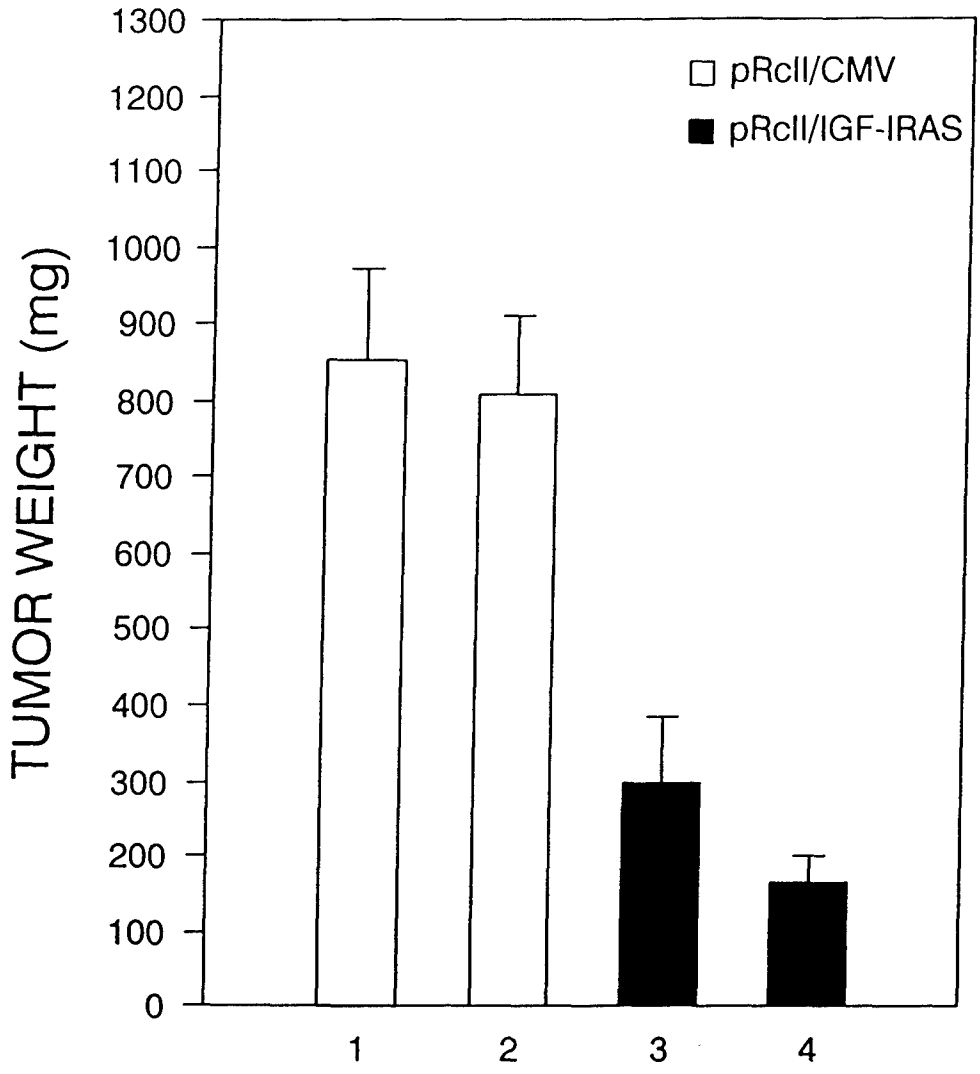

Figure 9. Comparison of tumor growth of IGF-IR antisense-transfected EMT6 cells in nude mice and syngeneic Balb/c mice. In the first experiment EMT6 cells transfected with the control vector (pRcII/CMV, Bar 1) or transfected with the antisense IGF-IR vector (pRcII/IGF-IRAS, Bar 3) were injected s.c. into the mammary fat pad region of 6-week-old female nude mice. In the second experiment control-transfected (Bar 2) or IGF-IR antisense-transfected (Bar 4) EMT6 cells were inject s.c. in the mammary fat pad region of 6-week-old female syngeneic Balb/c mice. The animals were sacrificed after 14 days. The tumors were excised, and tumor weights were recorded..

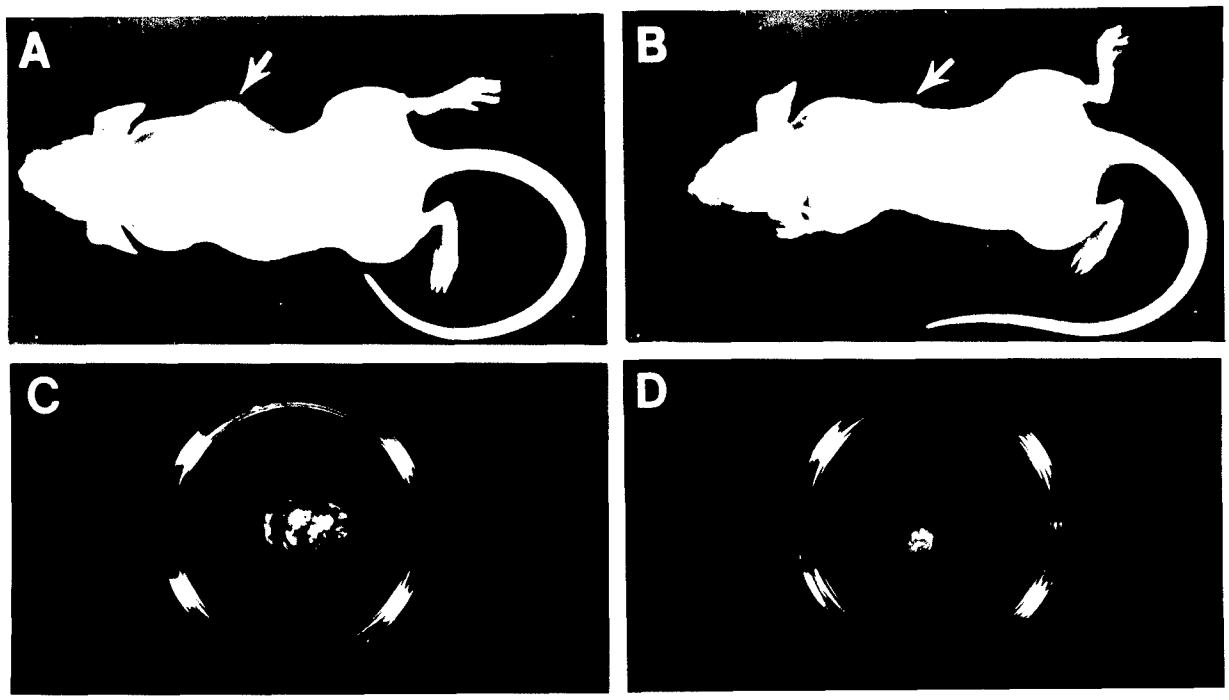

Figure 10. Tumor growth of EMT6 cells in nude mice and syngeneic Balb/c mice. Tumors derived from control-transfected (pRcII/CMV) cells (A), and EMT6 cells transfected with the construct expressing IGF-IR antisense RNA (B) in nude mice. Arrows indicate the positions of the tumors. Tumors excised from Balb/c mice that received control-transfected (C) or IGF-IR antisense-transfected EMT6 cells (D).

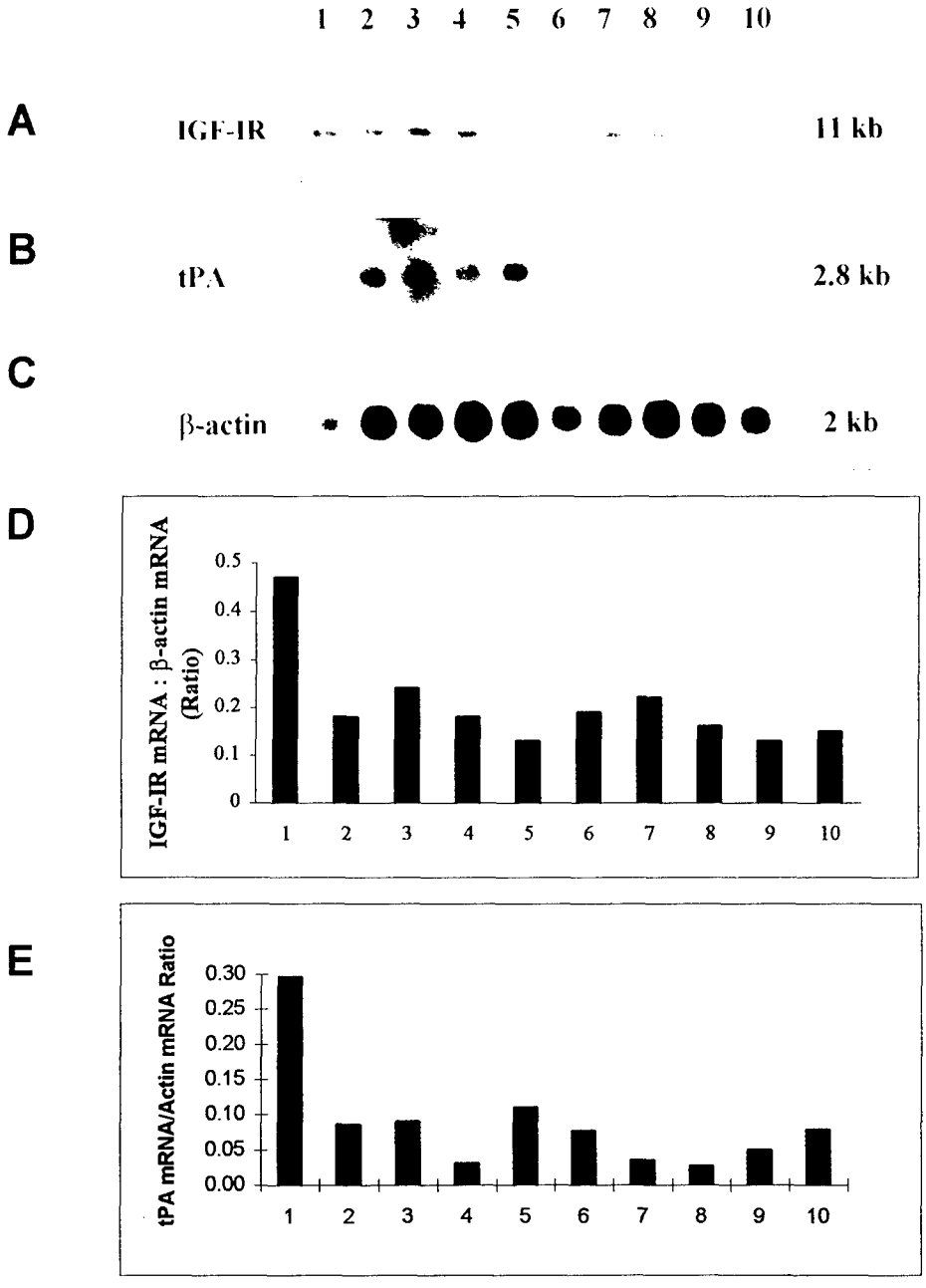

Figure 11. Northern blot analysis of total RNA from antisense IGF-IR transfected EMT6 cells. (A) Total RNA (20μg per lane) derived from control-transfected (lane 1) and antisense IGF-IR transfected EMT6 cell clones (lanes 2-10) was analyzed using a ^{32}P-labeled IGF-IR cDNA as a hybridization probe. The molecular size of the endogenous IGF-IR transcripts is indicated on the right. The fusion transcript contains 0.75 kb IGF-IR sequence plus approximately 1.25 kb neomycin sequence. (B) Lanes were normalized for differences in loading and RNA integrity by reprobing the same filter with chicken β-actin. The total RNA signals were scanned using the laser densitometer SciScan 5000, and the difference in the expression of the mRNA transcripts was calculated relative to the β-actin standards. The result of the analysis for IGF-IR (D) is shown in the bar graph. (C) The same blot probed with tPA cDNA fragment. (E) Graph depicting the normalised tPA expression. The numbers under the bar graph correspond to the numbers in A-B. (1- control transfected cells, 2- clone M2, 3- clone M3, 4- clone M4, 5- clone M5, 6- clone M6, 7- clone M7, 8- clone M8, 9- clone M9, 10- clone M10).

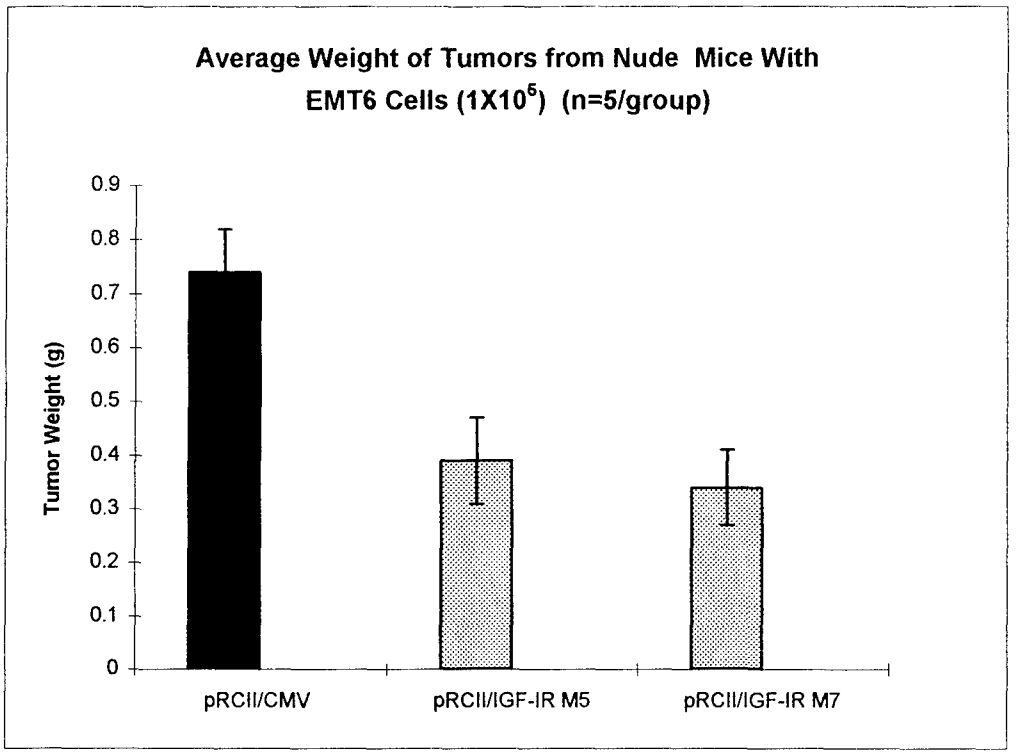

Figure 12. Suppression of tumorigenesis by antisense IGF-IR transfected EMT6 cells *in vivo.* Control transfected (pRcII/CMV) or antisense IGF-IR transfected EMT6 cell clones M5 and M7, respectively were injected s.c.into female nude mice. The mice were sacrificed 14 days post injection, the tumors were excised and tumor weights determined.

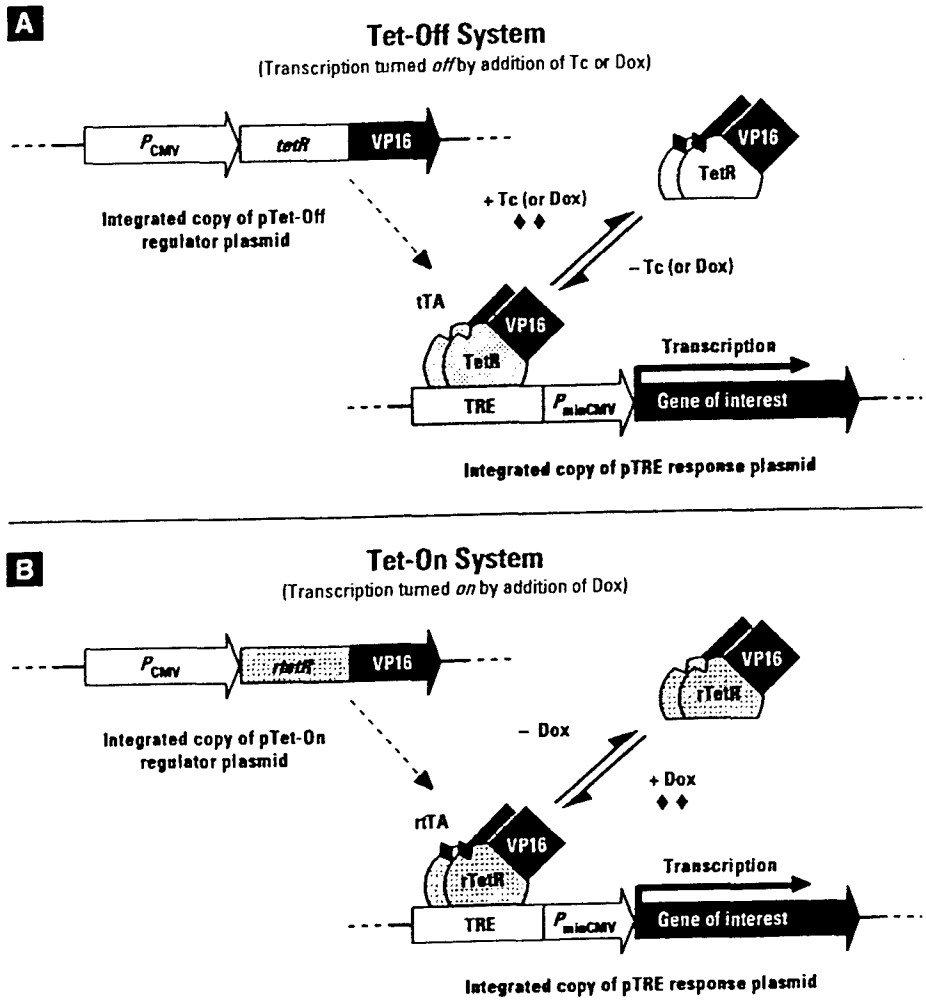

Figure 13. Schematic of gene regulation in the Tet-Off and Tet-On Systems. (A) Tet-Off. The tet-responsive transcriptional activator (tTA) is a fusion of the wild-type Tet repressor (TetR) to the VP16 activation domain (AS) of herpes simplex virus. The tet-responsive element (TRE) consists of seven copies of the 42bp tet operator (TetO). The TRE is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{mincmv}), which is silent in the absence of activation from the TRE. tTA binds the TRE- and thereby activates transcription of gene X- in the absence of tetracycline (Tc) or Tc derivatives such as doxycycline (Dox). (B) Tet-On. The "reverse" Tet repressor (rTetR) was created by 4 amino acid changes that reverse the protein's response to Tc and Tc derivatives. Thus, the reverse tet-responsive transcriptional activator (rtTA) binds TRE and activates transcription of Gene X (antisense IGF-IR in this project) in the presense of Dox.

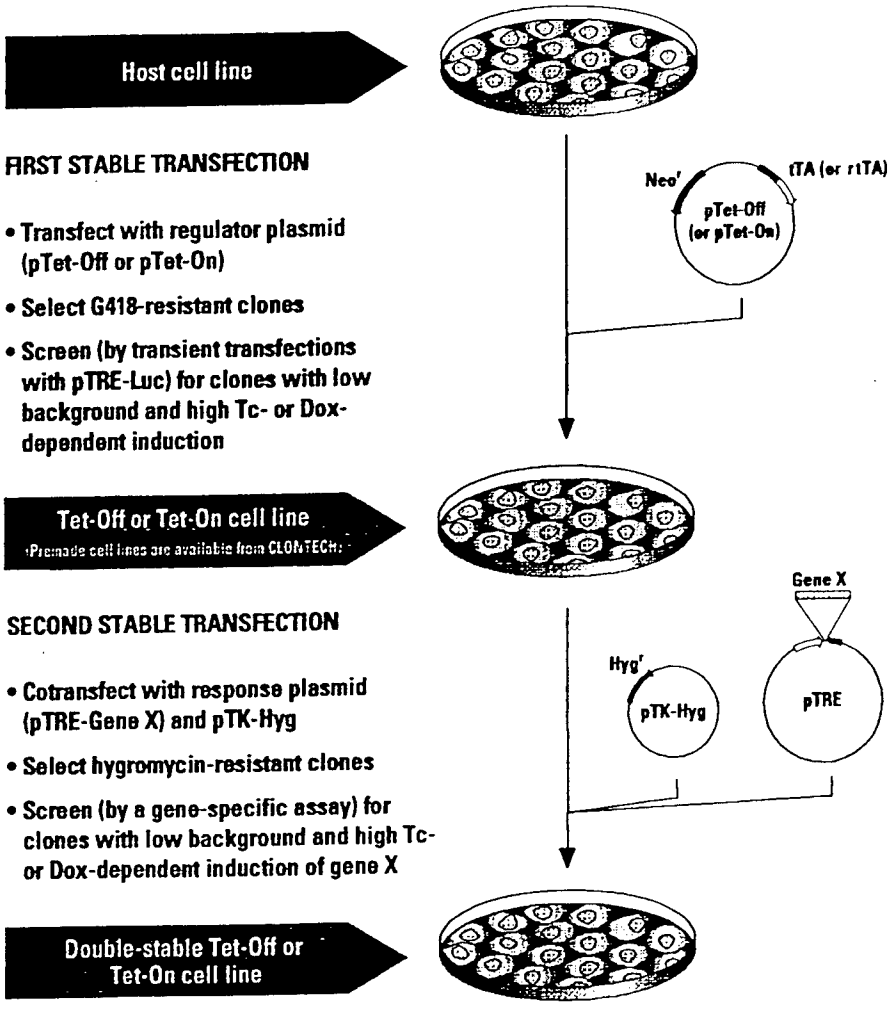

Figure 14. Overview of developing Tet-Off and Tet-On and double-stable Tet-Off and Tet-On cell lines. We will perform stable transfections into murine EMT6 and MCak breast cancer cell lines.

Abstract to be presented in April 1997 at the AACR Annual Meeting, San Diego, California

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<TITLE>Antisense to type I insulin-like growth factor receptor (IGF-IR) suppresses tumor growth of human breast cancer cells.
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<AFFIL>Department of Reproductive Biology. Case Western Reserve University. Cleveland, OH 44106.
<ABSTRACT>IGF-IR are expressed at relatively high levels in most cancer cells. We and others have previously shown the effectiveness of antisense RNA to IGF-IR in the inhibition of brain and prostate tumor growth *in vivo*. We now apply this antisense strategy to a human breast cancer cell line MDA-MB-435S and study the effects of inhibition of IGF-IR in human breast cancer cells. IGF-IR antisense RNA inhibits the expression of endogenous IGF-IR in stably transfected MDA-MB-435S clones. Cell proliferation assay and soft agar assay of the antisense IGF-IR transfected clones showed a decrease in growth rate and colony formation respectively. The level of inhibition of IGF-IR in the different transfected clones directly correlates with their growth rates and colony formation potential. Nude mice injected with the antisense transfected cells showed either a dramatic reduction or total absence of tumor growth after 11 weeks. These data provide support for the role of IGF-IR in human breast tumorigenesis and may be useful in developing an antisense gene therapy strategy for the treatment of breast cancer.
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Potential triple helix–mediated inhibition of IGF-I gene expression significantly reduces tumorigenicity of glioblastoma in an animal model

Alexander Shevelev,[1,2] Peter Burfeind,[3] Ekkehard Schulze,[1] Frauke Rininsland,[1] Thomas R. Johnson,[1] Jerzy Trojan,[1,4] Cheryl L. Chernicky,[3] Claude Hélène,[5] Judith Ilan,[3] and Joseph Ilan[1]

[1]Department of Pathology, [3]Department of Reproductive Biology, Case Western Reserve University School of Medicine, Cleveland, OH 44106-4943, and [5]Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, Institute National de la Santé et de la Recherche Médicale Unité 201, Unité Associée 481, 43 Rue Cue Cuvier, 75005 Paris, France.

Oligonucleotide-directed triple helix formation is a powerful approach to block transcription of specific genes. Although the oligonucleotide triplex approach is efficient for inhibiting gene expression in cultured cells, suppression is transient. We developed an approach which inhibits insulin-like growth factor-I (IGF-I) expression following stable transfection of C6 rat glioblastoma cells with a plasmid from which an RNA is transcribed that codes for the third strand of a potential triple helix. We tested the ability of this expression vector to inhibit IGF-I gene expression *in vitro* as well as its tumorigenesis in an animal. A dramatic reduction of IGF-I RNA and protein levels in cultured cells occurred following transfection of rat C6 cells with a eukaryotic expression plasmid encoding the oligopurine variant of the triple helix but not the oligopyrimidine or a control sequence. The cells transfected with the oligopurine variant displayed morphological changes, upregulation of major histocompatibility complex I, and increased expression of protease nexin I. Dramatic inhibition of tumor growth occurred in nude mice following injection of transfected C6 cells. To our knowledge, this is the first example of tumor growth inhibition in an animal model employing a triple helix approach.

* ***Key words: MHC-I; nexin-I; cell transfection; C6 cells.***

Insulin-like growth factor I (IGF-I), a wide spectrum growth and differentiation factor, plays an important role in mammalian development.[1] Many primary tumors and cell lines derived from tumors are known to produce large amounts of IGF-I.[2] We and others have previously reported that rat C6 glioblastoma cells express high levels of IGF-I, which is enhanced when the cells are grown in serum-free medium.[3,4] Furthermore, when C6 cells were transfected with a vector containing an antisense IGF-I cDNA transcriptional cassette driven by the mouse metallothionein-I promoter, they lost their tumorigenicity. Moreover, when antisense-transfected cells were injected into syngeneic animals with existing glioblastoma tumors, the established tumors regressed.[5] Studies in this animal model indicate that inhibition of IGF-I in cancer cells can have a profound effect on tumorigenesis.

Oligonucleotide-directed triple helix formation has recently emerged as a powerful approach to block transcription of specific genes (antigene strategy) and hence has great therapeutic potential.[6] The triplex strategy has certain advantages over the antisense RNA strategy, which include fewer and less regenerative targets (two alleles versus multiple copy mRNAs). When triplex-forming oligonucleotides are targeted to a functional promoter region of a test gene, they have been shown to selectively repress transcription in a dose-dependent manner and to interfere with the function of sequence-specific DNA binding proteins such as restriction enzymes and transcription factors, both *in vitro* and in cultured cells.[7–10]

Oligonucleotide binding to target DNA sequences with subsequent inhibition of the corresponding gene expression has been shown for several genes.[11–15] Although efficient, the suppression of the gene is transient with the oligonucleotide strategy.[16] We, therefore, de-

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Address correspondence and reprint requests to Joseph Ilan, PhD, Department of Pathology, Case Western Reserve University, School of Medicine, Biomedical Research Building, 10900 Euclid Avenue, Cleveland, OH 44106-4943.

[2]Present address: Laboratory of Genetic Engineering Institute of Experimental Cardiology, Cardiology Research Center, 121552 Moscow, Russia.

[4]Present address: Hepato-Biliary and Liver Transplant Center, Hospital Paul Brousse, Université Paris-Sud, 94804 Villejuif Cedex, France.

* Au/Ed: Key words ok?

veloped a new approach which involves stable transfection of the C6 cells with a plasmid, from which an RNA is transcribed that codes for the third strand of a potential triple helix. Here, we present the results obtained with a plasmid containing an oligopurine stretch of 23 bases, which is directed against a target sequence located between transcription start points and the start codon of the human IGF-I gene in its first exon.[17] In this study we tested the ability of an expression vector that continuously synthesizes a third RNA strand forming a potential triple helix structure in the 5′ untranslated region (5′-UTR) of the IGF-I gene to inhibit gene expression in cultured cells and tumorigenesis of C6 glioblastoma in an animal model. We observed inhibition of IGF-I expression in cultured cells, a reduction in cellular protein, and dramatic growth retardation of tumors in nude mice. To our knowledge, this is the first example of tumor growth inhibition in an animal model employing the triple helix approach to block transcription of a specific gene.

MATERIALS AND METHODS

Plasmids, oligonucleotides, and hybridization probes

The antisense expression construct, pAnti-IGF-I, used in this study was previously described.[2] For experiments concerning triplex formation, a plasmid capable of forming a triple helix *in vitro*, was assembled utilizing an oligopurine block of 23 bases located between the transcription and translation start points on the coding strand of the human IGF-I gene in its first exon (Fig 1A). This entire target sequence is completely conserved between human and rat. A *Kpn*I–*Eco*RI fragment (1,017 bp) of the human IGF-I gene containing this triple helix target sequence was subcloned into pBluescript II SK+ (Stratagene, La Jolla, Calif) multiple cloning site from the plasmid pIGF 1630/LUC kindly provided by Dr. Peter Rotwein. The resultant plasmid was designated pB5′hIGF-I.

The oligonucleotides: 5′-CTGGAAGCTTTCTTCTCCCTC TCTCTCTCTTCCGGATCCCCTC-3′ and 3′-GACCTTCGA AAGAAGAGGGAGAGAGAGAGAAGGCCTAGGG GAG-5′, were synthesized, annealed, and cloned into pBluescript II SK as shown in Figure 1B, yielding the plasmid pB triple helix (pB-TH). To prepare the expression plasmids designated as pMT-CT–triple helix (pMT-CT-TH) and pMT-AG–triple helix (pMT-AG-TH) coding for homopyrimidine and homopurine variants of the triple helix third strand, respectively, the cloned fragment in pB-TH was transferred in either direct or reverse orientation into the eukaryotic expression vector pMT/EP.[18] Briefly, pMT/EP contains the mouse metallothionein I promoter to drive the transcription of an inserted sequence, an SV40 polyadenylation signal and a gene conferring resistance to hygromycin.

Oligonucleotides 5′-TCTTCTCCCTCTCTCTCTCTTCC-3′ (O-CT and 5′-GGAAGAGAGAGAGAGGGAGAAGA-3′ (O-AG) were used to determine triple helix formation *in vitro* after ^{32}P-end labeling by T4 polynucleotide kinase.[19] A 500-bp rat IGF-I cDNA fragment,[20] kindly provided by Dr Liam Murphy, and chicken β-actin cDNA[21] were used for Northern blot hybridization.

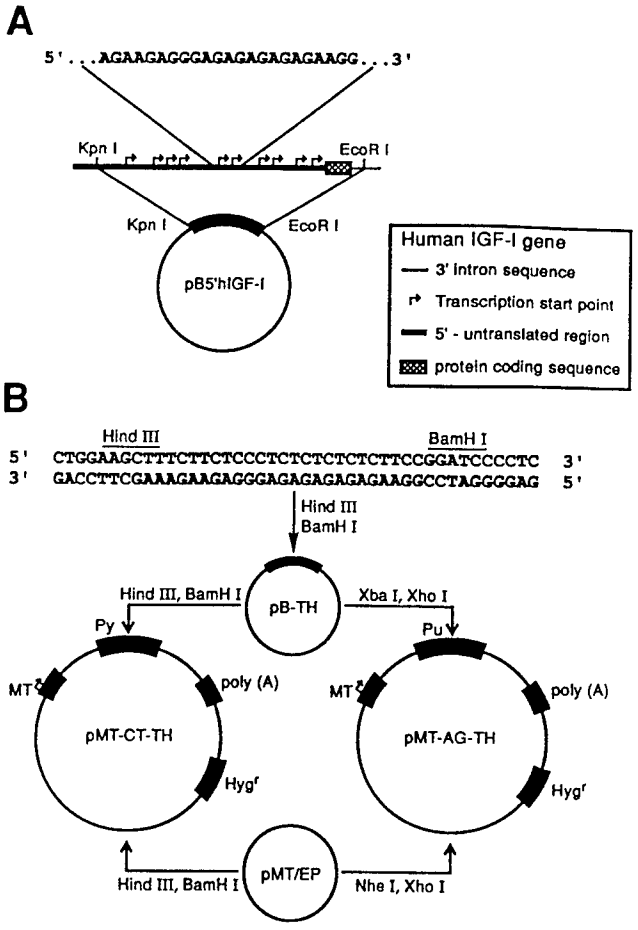

Figure 1. Schematic representation of plasmid construction. **A:** Triple helix target sequence: location on the map of the human IGF-I gene and scheme for cloning into the plasmid pB5′IGF-I. **B:** Construction of expression plasmids pMT-CT-TH and pMT-AG-TH coding for oligopyrimidine and oligopurine variants of the triple helix third strand, respectively. Py, oligopyrimidine; Pu, oligopurine; MT, mouse metallothionein-I promoter; Hyg^r, gene for hygromycin resistance; poly (A), SV40 polyadenylation sequence.

Cell culture, transfection, and animal injection

The C6 rat glioblastoma cell line[22] was obtained from the American Type Culture Collection (Rockville, Md) and passaged in Dulbecco's modified Eagle's medium in the presence of 10% fetal calf serum. In order to reduce intrinsic heterogeneity, the clone C6 (t1) was derived from the C6 cells. These cloned cells were transfected with plasmids pMT-CT-TH and pMT-AG-TH using Lipofectin (GIBCO/BRL, Gaithersburg, Md) according to the manufacturer's instructions. Transfected clones were selected individually in 96-well plates in the presence of 0.5 mg/mL Hygromycin B (Calbiochem, San Diego, Calif). The selection pressure was removed after 6 weeks.

HSD (nu/nu) mice (Case Western Reserve University Animal Resource Center) were used to assay tumor growth *in vivo*. Cells were trypsinized, washed in serum-free medium, and injected subcutaneously above the animals' hind leg. Nude mice were injected with 7.5×10^6 cells, and after 14 days the animals were sacrificed and the tumors were excised and weighed. Data are presented as mean ± SE.

Triple helix formation in vitro

Plasmid (pB5′hIGF-I) DNA was digested with appropriate enzymes and separated in a 1% agarose gel. The gel was dried, soaked in the hybridization mixture containing 5 × SSC (1 × SSC: 0.1 mM NaCl, 0.015 M sodium citrate), pH 5.0, 5 × Denhardt's solution (50 × Denhardt's: 1% Ficoll 400, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 1% sodium dodecyl sulfate (SDS), and 1 mM EDTA, incubated 90 minutes with 1 nM ^{32}P-labeled oligonucleotides (specific activity, 10^6 dpm/pmol) in the same mixture at 37°C and washed twice for 5 minutes in 2 × SSC, pH 5.0, 0.2% SDS at room temperature.

RNA isolation and analysis

Prior to RNA isolation cells were exposed to 50 μM $ZnSO_4$ in serum-free medium for 24 hours. Total cellular RNA was isolated from cultured C6 cells by the acid guanidine thiocyanate technique.[23] Agarose-formaldehyde electrophoresis, transfer to nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and hybridization were performed in the presence of 50% formamide. Final washing was done at 65°C in 0.5 × SSPE (1 × SSPE: 0.15 M NaCl, 2 mM NaH_2PO_4, 0.2 mM EDTA) and 0.5% SDS for 1 hour. DNA fragments used as hybridization probes were ^{32}P-labeled by random priming (Dupont NEN, Boston, MA) to the specific activity of 5 × 10^8 dpm/mg.

Differential display

This procedure was performed essentially as described.[24] Total RNA (after DNase treatment) was isolated from untransfected C6 cells, C6 cells transfected with pMT-CT-TH, C6 cells transfected with pMT-AG-TH and from C6 cells transfected with pAnti-IGF-I. RNA was reverse-transcribed using Moloney murine leukemia reverse transcriptase (GIBCO/BRL). One tenth of the cDNA was then amplified by polymerase chain reaction (PCR) using [^{35}S]dATP (Dupont NEN) in the reaction mixture. The amplification products were separated on a 6% polyacrylamide DNA sequencing gel. The PCR products representing differentially expressed mRNAs were excised from the gel and recovered by rehydration and subsequent precipitation. These were reamplified by PCR under conditions used in the initial RT-PCR minus the radioactive dNTP. After PCR one-third of the samples was run on a 1.5% agarose gel and stained with ethidium bromide. PCR bands of the expected size were cut from the gel, purified, and used either as probes for Northern blot analysis, or were cloned into the *Hin*cII site of vector pGEM3zf+ (Promega, Madison, Wis). Sequencing was carried out using the dideoxy chain termination method on double-stranded templates. The entire DNA sequence for both strands of the cloned DNA fragments was determined using the Sequenase system (U.S. Biochemical, Arlington Heights, Ill).

Flow cytometry and immunostaining

The C6 cells (1 × 10^5) were plated into 60-mm Petri dishes, grown for 48 hours, and incubated in serum-free medium for an additional 48 hours in the presence of 35 μM $ZnSO_4$. For flow cytometry, cells were removed from the plates with Ca^{+2}-free phosphate-buffered saline containing 1 mM EDTA. Mouse anti–rat major histocompatibility complex I (MHC-I) clone F.16.4.4 (Serotec, Oxford, UK) was used at a 1:1,000 dilution as primary antibody, followed by fluorescein-labeled goat anti–mouse IgG Fab (60529; Boehringer Mannheim, Indianapolis, Ind). A FACScan (Becton Dickinson, Mountain View, Calif) with LYSIS II software was used for data collection and analysis. The cell size of triple helix–transfected cells was compared to that of unmodified vector transfectants and C6(tl) parental cells by plotting forward versus side scattering and was found to be identical. Fluorescence data were visualized as a histogram. The mean fluorescent signal of the main peak (representing >90% of all measured cells) was divided by the mean fluorescent intensity obtained from C6(t1). For each clone, statistics from nine independent measurements obtained from three experiments are represented.

Immunostaining was performed using 10 μg/mL monoclonal antibody to IGF-I as primary antibody (Sm 1.2; Upstate Biotechnology, Lake Placid, NY). Cells were fixed with 4% paraformaldehyde for 5 minutes. Indirect immunoperoxidase staining was performed with a Vectastain Elite kit (PK 6102; Vector Laboratories, Burlingame, Calif) according to the manufacturer's instructions. Isotype-specific irrelevant IgG (X0931; Dako, Carpinteria, Calif) or antibody preincubated with recombinant human IGF-I (Upstate Biotechnology, Lake Placid, NY) at a 25:1 IGF-I:antibody molar ratio were employed as controls.

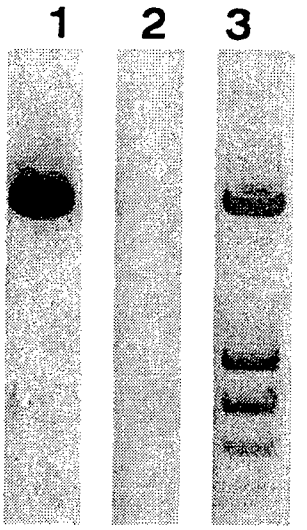

Figure 2. Oligonucleotide binding to the triple helix target sequence-bearing plasmid pB5′hIGF-I. Plasmid DNA (12 μg) was digested with *Bam*HI and *Dde*I, divided into the three portions, separated in a 1% agarose gel, and probed with ^{32}P-end-labeled oligonucleotides O-CT and O-AG under nondenaturing conditions followed by autoradiography. Lane 1, hybridization with the oligonucleotide O-AG. Lane 2, hybridization with the oligonucleotide O-CT. Lane 3, ethidium bromide staining of separated plasmid fragments.

RESULTS AND DISCUSSION

Triple helix formation in vitro *following incorporation of a target sequence into a plasmid*

To establish triple helix formation *in vitro*, plasmid pB5′h IGF-I containing the triple helix target sequence from the 5′-UTR of the human IGF-I gene was digested with *Bam*HI and *Dde*I and electrophoretically separated on an agarose gel (Fig 2, Lane 3). Following gel separation and hybridization with ^{32}P-labeled oligonucleotides O-AG or O-CT in nondenaturing conditions at 37°C,

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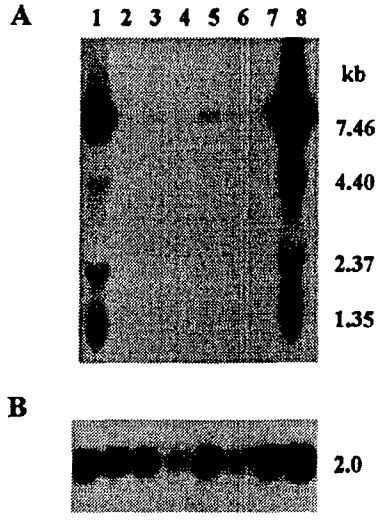

Figure 3. Northern blot analysis of total RNA from transfected C6 cells. **A:** Total RNA (30 μg per lane) derived from a pMT-CT-TH–transfected cell clone (Lane 1), six pMT-AG-TH–transfected cell clones (Lanes 2–7), and vector minus insert-transfected cells (Lane 8) was analyzed using rat IGF-I cDNA as a hybridization probe. **B:** Rehybridization of the same filter with a cDNA probe for β-actin to verify the quantity of RNA.

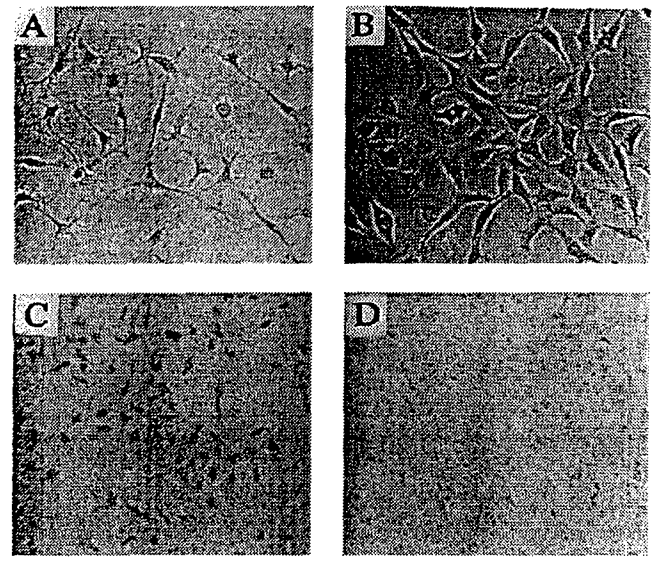

Figure 4. Photomicrographs of C6 cells. **A** depicts the normal morphology of a clone of parental C6 cells. **B** demonstrates the altered morphology of C6 cells that were transfected with the pMT-AG-TH plasmid. Phase contrast, ×1,000. **C** depicts parental C6 cells which display positive immunostaining for IGF-I protein. **D** depicts cells from a pMT-AG-TH–transfected clone treated with the same IGF-I–specific antibody as used in **C**. (Magnification, ×450).

only the fragment containing the target sequence gave a strong signal with the purine oligonucleotide O-AG probe (Fig 2, Lane 1). In contrast, none of the fragments gave a signal with the pyrimidine oligonucleotide O-CT probe (Fig 2, Lane 2). These data indicate that at least one variant of the synthetic third strand can bind to the double-stranded DNA at the 5′-UTR of the human IGF-I gene *in vitro* after incorporation of the target sequence into the plasmid. Our results are similar to previous studies that demonstrated specific binding of purine but not pyrimidine oligonucleotides at pH 7 and 37°C using gel retardation and footprinting techniques.[25]

Inhibition of IGF-I expression in cultured cells

Rat C6 (t1) glioblastoma cells, which were transfected with the plasmid pMT-AG-TH encoding the purine variant of the third strand of the potential triple helix, displayed significant inhibition of IGF-I gene expression. Six pMT-AG-TH–transfected clones (Fig 3A, Lanes 2–7) demonstrated dramatic suppression of IGF-I RNA levels after cultivating the cells for 2 months without hygromycin selection pressure indicating that stable integration of the vector DNA had occurred and that sequence delivery was continuous. On the other hand, the C6 cell clones containing the pMT-CT-TH plasmid encoding the pyrimidine variant (Fig 3, Lane 1) expressed IGF-I RNA at a level similar to that observed for cells that were transfected with the vector only in which the polylinker sequence was present instead of the oligopurine or oligopyrimidine sequence (Fig 3, Lane 8). Five additional pMT-CT-TH–transfected clones displayed approximately the same level of IGF-I RNA as parental and vector-transfected cells (data not shown).

There are only a few reports describing inhibition of endogenous gene expression by triplex forming oligonucleotides.[26,27] This inhibition is expected to be transient due to nuclease degradation of the oligonucleotide. Only when the oligonucleotide is cross-linked to its target sequence can an irreversible effect be expected as shown, eg, with oligonucleotide-psoralen conjugates.[28] Even in this case, the cross-links can be removed by repair enzymes and gene inhibition can be relieved. However, the inhibition of gene transcription was transient due to nuclease degradation of the oligonucleotide. The episomal-based pMT/EP vector that we utilized for assembling the plasmids can replicate in the cell and provide stability for continuously transcribing the third RNA strand which forms the potential triple helix. This vector has been employed in studies *in vitro* that showed inhibition of IGF II gene expression with an antisense approach in permissive mouse myogenic C2 cells.[29]

The morphology of the C6 cells was altered in all of the clones following transfection with the pMT-AG-TH plasmid. The pMT-AG-TH transfected C6 cells exhibited a fibroblastic spindle shape with reduced length and a decreased number of neurite-like extensions (Fig 4B) as compared to parental cells when the cells were incubated at a low serum concentration. However, when cells were incubated in 10% fetal calf serum the effect was barely apparent indicating that serum-derived IGF-I could restore the parental phenotype of the cells (Fig 4A). A study that utilized an antisense approach to inhibit IGF-I expression in C6 glioblastoma cells demonstrated morphological changes in the cells similar to the cellular alterations that we observed in the C6 cells

transfected with the pMT-AG-TH plasmid.[30] There was no difference in growth rate of pMT-AG-TH–transfected and vector-transfected or parental cells in culture at normal (10%) or reduced (1%) concentrations of serum. However, the pMT-AG-TH–transfected cells displayed significantly reduced levels of intracellular IGF-I protein as detected by immunoperoxidase staining (Fig 4C,D). Cellular staining was undetectable with an irrelevant IgG or with the primary antibody that was preabsorbed with recombinant IGF-I (data not shown). The decreased cellular staining for IGF-I correlates with the reduced levels of IGF-I RNA that were observed in C6 cells transfected with the pMT-AG-TH plasmid.

It has previously been demonstrated that a correlation exists between the inhibition of aromatase transcription and reduced protein levels in human MCF-7 breast cancer cells following treatment of the cells with a psoralen-linked triplet-forming oligonucleotide.[31] Moreover, a recent study showed inhibition of expression of the human oncogene *HER 2 (NEU)* which coincided with a significantly lower *HER 2* protein level by a triplehelix–forming oligonucleotide in MCF-7 cells.[32] However, in this study the inhibitory effect was overcome by a replenishment reaction by the second day followed by a slow return to the original condition of the cells.

Expression of MHC-I is enhanced in C6 cells transfected with the pMT-AG-TH plasmid

MHC-I which is known to play a key role in the immune system surveillance of neoplastic cells[33] was elevated in the pMT-AG-TH–transfected cell clones. Tumorigenic cells that exhibit a reduced expression of MHC-I may escape the host immune system and therefore have a metastatic advantage.[34,35] It has been demonstrated that MHC-I is down-regulated by IGF-I on FRTL-5 rat thyroid cells.[36] Conversely, artificial overexpression of MHC-I can lead to reduced tumorigenicity and metastatic properties in syngeneic animals.[37] We have previously observed an induction of MHC-I after IGF-I suppression in C6 cells with an antisense construct.[38] In the absence of fetal calf serum, cell clones transfected with pMT-AG-TH (Fig 5, Samples 2–7) as well as pAnti-IGF-I (Fig 5, Sample 8) showed MHC-I levels approximately twice those observed for control cells (Fig 5, Samples 1 and 9). The observation, that two independent methods of IGF-I gene suppression, triple helix and antisense strategies, both result in upregulation of MHC-I, suggests specific inhibition of IGF-I occurs by triplex formation in cultured cells.

Upregulation of protease nexin I in pMT-AG-TH–transfected cells

The differential display technique was utilized to identify whether gene expression is altered by suppression of IGF-I in RNA from pMT-AG-TH–transfected C6 cells and pAnti-IGF-I-transfected C6 cells. Differential display revealed prominent bands of the same size appearing in lanes corresponding to a pMT-AG-TH–transfected cell clone as well as pAnti-IGF-I–transfected cells (Fig 6A). When DNA from both bands were reamplified and sequenced the sequences were identical. Comparison of the probe sequence with the sequences in the GenBank database indicated that the DNA fragments represented a 160-bp portion of a cDNA sequence coding for rat protease nexin I (glia-derived nexin), a serine protease inhibitor. The nexin cDNA band was used as a probe for Northern hybridization to RNA prepared from pMT-AG-TH–transfected (Fig 6B, Lanes 2–7), pMT-CT-TH–transfected (Fig 6B, Lane 1), C6 vector-transfected (Fig 6B, Lane 8), and pAnti-IGF-I–transfected C6 cells. A transcript was identified which displayed markedly increased levels of nexin-I in pMT-AG-TH transfectants (Fig 6B) and pAnti-IGF-I transfectants (data not shown).

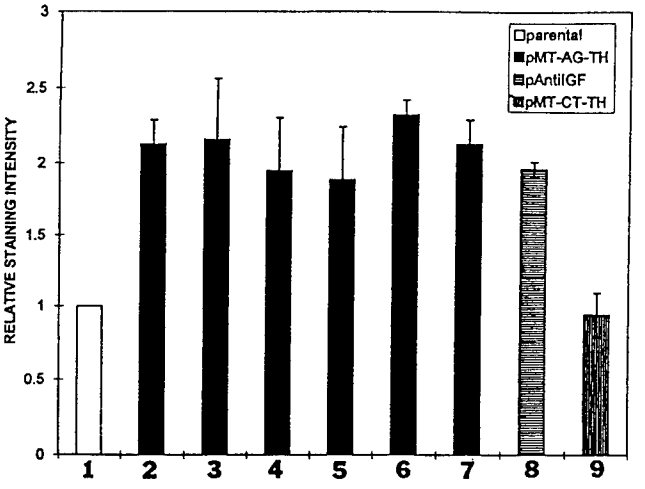

Figure 5. Flow cytometric analysis of MHC-I expression in C6 cells. Parental C6 cells (Lane 1), C6 cells from different clones transfected with the pMT-AG-TH plasmid (Lanes 2–7), C6 cells transfected with the antisense construct, pAnti-IGF-I (Lane 8), and C6 cells transfected with the pMT-CT-TH plasmid (Lane 9) were stained with a mouse anti-rat MHC-I monoclonal antibody.

Protease nexin I, which has been shown to be involved in the development and maturation of the central nervous system, is also expressed in rat and human cancer cell lines.[39,40] In solution, protease nexin I binds to and inhibits serine proteases such as thrombin, trypsin, urokinase-type plasminogen activator (uPA), and plasmin.[41] The plasminogen-plasmin system has been shown to play an important role in tumor growth, invasion, and metastasis, where uPA acts as an initiator of pericellular proteolysis of the extracellular matrix.[42] It has been demonstrated that protease nexin I can suppress tumor cell–mediated extracellular matrix degradation by inhibiting uPA activity in human fibrosarcoma (HT-1080) cells.[43] Hormones and growth factors such as interleukin-1 and vasoactive intestinal peptide have been shown to be positive regulators of protease nexin I expression, whereas angiotensin II, calcitonin gene–related peptide, and dexamethasone were found to be negative regulators.[44,45] In this study we have demonstrated that IGF-I

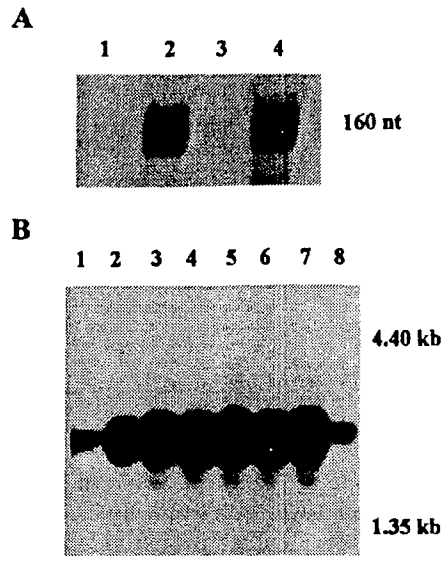

Figure 6. Expression of protease nexin I in C6 cells. **A** depicts an autoradiogram of a differential mRNA display using total RNA isolated from C6 cells transfected with vector minus the insert (Lane 1), C6 cells transfected with the antisense construct pAnti-IGF-I (Lane 2), C6 cells transfected with the homopyrimidine triplex expression construct, pMT-CT-TH (Lane 3), and C6 cells transfected with the homopurine triplex expression construct, pMT-AG-TH (Lane 4). **B** shows a Northern blot analysis that demonstrates expression of protease nexin I. The membrane used for Figure 3 was stripped and rehybridized with a cDNA fragment of rat protease nexin I. C6 cells from the clones transfected with the pMT-AG-TH plasmid are represented in Lanes 2–7. Lane 1 contains RNA from pMT-CT-TH–transfected C6 cells and Lane 8 contains RNA from vector-transfected C6 cells.

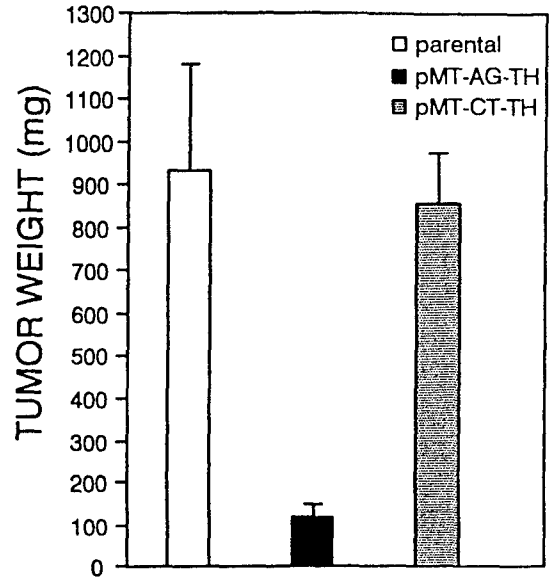

Figure 7. Suppression of tumorigenesis by pMT-AG-TH–transfected C6cells in nude mice. The animals were injected subcutaneously with 7.5×10^6 cells; parental (n = 4), pMT-AG-TH–transfected (n = 12) or pMT-CT-TH–transfected (n = 4). The tumor weights from the animals that received the six pMT-AG-TH–transfected clones (n = 2 mice/clone) were averaged because there was no significant variability in tumor size between mice.

acts as a negative modulator of protease nexin I expression in C6 glioblastoma cells. Elevated production of protease nexin I by inhibition of IGF-I expression results in suppression of serine protease activity, which is important for tumor invasion and dissemination, and may further decrease the tumorigenic potential of transfected C6 cells. This has been shown for maspin, another member of the serine protease inhibitor family, on a model of mammary carcinoma cells.[46]

Tumor growth in nude mice

The pMT-AG-TH–transfected cellular clones exhibited a suppressed growth rate when injected subcutaneously into nude mice (Fig 7). Fourteen days after injection into the nude mice, the weight of tumors resulting from pMT-AG-TH–transfected cells averaged 7-fold less than tumors from animals that were injected with control transfected cells. The mechanism for the inhibition of tumor growth that occurred with our triple helix approach may be similar to that observed with antisense strategies. It has been shown that a quantitative relationship exists between the level of type I IGF receptor and tumorigenesis in nude mice which is correlated to the extent of apoptosis in the animals.[47] Another possibility may be that an immune response occurred in the animals which inhibited tumorigenesis, because nude mice have a residual immune system containing both natural killer cells and B lymphocytes.[48] The suppression of IGF-I by the pMT-AG-TH plasmid appears to remain stable in the animals over an extended period of time, indicating the continuous nuclear delivery of pMT-AG-TH third strand sequences.

In conclusion, we have shown that plasmid pB5′hIGF-I containing the triple helix target sequence from the 5′-UTR of the human IGF-I gene can form a triple helix *in vitro* with the purine oligonucleotide variant, O-AG. Our results are supported by several other groups[28,49–51] who showed that antiparallel triplexes could be constructed *in vitro* provided that all strands were made of DNA. Therefore, incorporation of the potential triple-helix–forming target sequence into the pB5′hIGF-I plasmid does not affect the binding properties of the oligonucleotide O-AG.

Previous *in vitro* studies have shown that, under conditions where a DNA third strand containing G and A binds to an oligopurine · oligopyrimidine sequence, the isosequential RNA does not bind.[52] However, the conditions within cells might favor such a binding. In addition, proteins that recognize triple-helical structures[53] may stabilize RNA-directed triple helices *in vivo*. The results presented in this study show that an RNA strand containing a 23-nucleotide (nt) oligopurine sequence might be capable of forming a triple helix in cultured C6 cells with an oligopurine · oligopyrimidine sequence of the IGF-I gene. Two control RNA transcripts containing either the polylinker sequence or the

23-nt (C,T) sequence instead of the 23-nt (G,A) sequence did not effect IGF-I gene expression. Although we cannot exclude other mechanisms (eg, binding and sequestering of a regulatory protein factor by the (G,A) sequence of the RNA transcript or by the oligopurine · oligopyrimidine 23-bp sequence of the DNA vector), triple helix formation remains the most plausible mechanism for the inhibition of IGF-I gene expression. The use of the episome-based plasmid pMT-AG-TH imparts a stable phenotype consistent with continuous suppression of the IGF-I gene product. The suppression in IGF-I RNA levels and the coinciding reduction of cellular IGF-I protein indicates that the RNA strand, which forms the potential triple helix, can inhibit gene transcription in C6 cells for up to 2 months in culture.

The results presented in this study show that inhibition of IGF-I upregulates MHC-I expression in transfected C6 cells. In addition, the increased expression of protease nexin I may reduce the tumorigenic potential of the C6 cells which we observed when the pMT-AG-TH cells were injected into nude mice. Similar changes in gene expression were observed when the antisense approach was used to inhibit IGF-I transcription in cultured cells. These results are encouraging because they provide a basis for an alternative and possibly more efficient approach for inhibition of gene expression, which utilizes a triple helix strategy in an animal model. This could lead to the development of new gene therapy approaches for treatment of certain types of cancer.

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