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13. ABSTRACT (Maximum 200 Words)
The goal of the proposed work is to evaluate the cell growth inhibitory affects and apoptotic potential of HSPG gene therapy in vitro and in vivo. The HSPG syndecan-1 has proven to be a powerful inhibitor of tumor cell growth and loss of expression has been correlated with a poor prognosis in some cancers. Therefore, two c-myc tagged syndecan-1 gene cassettes have been constructed for the expression of syndecan-1 on the cell surface or for secretion and these constructs have been transfected into MDA-MB-231 cells and subcloned cell lines established. The MDA-MB-231 cells that overexpress syndecan-1 on the cell surface or secrete syndecan-1 grow at the same rate as cells transfected with empty vector however, in the absence of serum, the cells secreting syndecan-1 grow much slower suggesting a less transformed phenotype. However, both the cells that overexpress syndecan-1 on the cell surface and the cells that secrete syndecan-1 produce more colonies in soft agar than the vector only transfected control cells. Therefore, at the present time, the in vitro data are conflicting however, we have initiated in vivo studies to definitively determine if overexpression or secretion of syndecan-1 decreases tumorigenicity. This project is the first to use HSPG genes for anticancer therapy.

14. SUBJECT TERMS
Heparan sulfate proteoglycans, gene therapy, tumorigenesis

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INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) regulate normal and cancer cell behaviors by binding growth factors and by mediating cell adhesion and invasion. Current data strongly support the idea that HSPGs are a new class of tumor suppressors. Studies demonstrate the anti-tumor growth properties of three HSPGs, specifically syndecan-1, glypican-1, and betaglycan. Treatment with purified syndecan-1 produces strong growth suppression in multiple myeloma cell lines, a poorly differentiated squamous cell carcinoma cell line, human and murine mammary tumor cell lines, but not normal cell lines. In addition, treatment of multiple myeloma cells with purified syndecan-1 induces apoptosis. Many tumors display an alteration in cell surface HSPG expression. When syndecan-1 is lost from the surface of mammary epithelia, the cells lose epithelial morphology, invade collagen gels and show characteristics of neoplastic growth. When transfected with the syndecan-1 gene, transformed mammary epithelial cells regain morphology and lose neoplastic growth characteristics. *In vivo* experiments demonstrate reduced tumorigenicity of syndecan-1 or glypican-1 expressing multiple myeloma cells and betaglycan expressing breast cancer cells. We propose that HSPGs are excellent candidates for gene therapy applications for the treatment and possible eradication of breast cancer and therefore the purpose of the proposed work is to evaluate the affect of HSPG expression on breast cancer growth and progression. This work represents a novel use of HSPGs genes as anti-cancer therapy.

BODY

Task 1. Develop breast cancer gene therapy and evaluate *in vitro*.

- Engineer epitope tag containing human HSPG gene constructs for cell surface expression or secretion.
- Transfer epitope tagged HSPG gene construct into breast cancer cells and establish subcloned cell lines.
- Determine the affects of HSPG production on breast cancer growth.

As was shown in last year's annual report, syndecan-1 is expressed in roughly similar amounts on the cell surface of several breast cancer cell lines including MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-436, and Hs578t. Therefore, for the purpose of distinguishing native syndecan-1 from syndecan-1 expressed from transferred gene cassettes, a c-myc tag was incorporated into the human syndecan-1 gene construct. Using site directed mutagenesis the c-myc tag was inserted 3' to the DNA sequence

encoding the amino-terminal signal sequence and 5' to the glycosaminoglycan attachment sites. Two syndecan-1 gene cassettes were produced by PCR amplification of the full length syndecan-1 open reading frame or amplification of a truncated gene lacking the syndecan-1 cytoplasmic and transmembrane domains (Figure 1) and each cassette was inserted into the pcDNA 3.1 plasmid vector. The full length syndecan-1 construct allows for the production of the complete cell surface syndecan-1 proteoglycan. The truncated construct encodes a proteoglycan that will be secreted from cells due to the absence of the cytoplasmic and transmembrane domains.

The integrity of both modified gene cassettes was confirmed by DNA sequence analysis. Plasmid vector without an inserted gene, or plasmid vector containing either the full length, c-myc tagged human syndecan-1 gene or the truncated, c-myc tagged human syndecan-1 gene was attached to liposomes and transferred into MDA-MB-231 breast cancer cells. Following G418 selection, the batch transfectants were evaluated for trans-gene expression using antibody specific for human syndecan-1 (BB4 antibody) or antibody specific for the c-myc tag. Flow cytometric analysis revealed a population of the full length syndecan-1 transfected cells that overexpressed syndecan-1 and a population of cells that have the c-myc tag on the cell surface indicating that some cells do expressed the trans-gene. Vector only transfected cells do not overexpress syndecan-1 and do not stain with antibody specific for the c-myc tag. Dot blot analysis of media conditioned by the vector only, full length syndecan-1, and truncated syndecan-1 gene transfected cells indicates that the cells transfected with the truncated gene secrete syndecan-1 into the media at high levels whereas the other cell lines do not. Therefore each of the three batch transfected cells were subcloned by limiting dilution to isolate cells lines expressing the trans-genes. Subclones from each of the three transfectants were evaluated by flow cytometry and their conditioned media analyzed by dot blot. MDA-MB-231 cells transfected with vector only (neo) stain positively for human syndecan-1 on the cell surface and do not stain with the c-myc antibody (Figure 2). B4 and C4 subcloned cells transfected with the human syndecan-1, c-myc tagged trans-gene overexpress syndecan-1 and express c-myc on their cell surface. As expected, subclones of the cells transfected with the truncated syndecan-1 do not overexpress cell surface syndecan-1 and do not stain with the c-myc antibody (subclone C7). Dot blot analysis of the conditioned medias from cells transfected with the truncated syndecan-1 gene cassette were used to identify subclones that secrete syndecan-1 into the media (Figure 3).

No change in morphology was observed in the trans-gene expressing subclones when the cells were grown in a monolayer on tissue culture flasks. However, a change in morphology does occur when the cells were grown in suspension (Figure 4.) Normally adherent cells do not attach and form a monolayer on bacterial plates but instead grow

in suspension as spheroids. Comparison of the subclones grown in suspension reveals that the vector only transfected cells grow as a smooth spheroid and that the cells overexpressing syndecan-1 or producing truncated syndecan-1 produce bumpy spheroids. The significance of this morphologic change is at present unknown however, this result shows a change in phenotype in response to trans-gene expression.

To determine the affect of overexpression of syndecan-1 or the secretion of syndecan-1 on breast cancer cell growth, subclones of each cell line were evaluated in several assays. The cells were plated in triplicate on 96-well plates and incubated at 37°C 5% CO₂ and at the specified time points, cell growth was determined by MTT assay. Figure 5 shows the results for representative subclones. All the subclones tested including neo, syndecan-1 overexpressing subclones and soluble syndecan-1 expressing subclones grew at similar rates. However, some differences occur when the same cell lines are grown for 96 hours in the absence of serum or in the absence of estradiol (Figure 6). The cells that express soluble syndecan-1 (C7) grow significantly less in the absence of serum than the vector only cells (neo) or the cells that overexpress syndecan-1 on the cell surface (B4). Serum-independent growth is an important characteristic of transformed cells and therefore, this result suggest that cells producing soluble syndecan-1 may be less transformed. A slight but significant decrease in growth occurs in the absence of estradiol in the vector only transfected cells (neo) as compared to the trans-gene expressing subclones (B4 and C7). This is an interesting finding because MDA-MB-231 cells are thought to be estrogen receptor negative and therefore their growth would not be expected to respond to the removal of estradiol supplementation.

Another characteristic of transformed cells is anchorage-independent growth. Therefore, several subclones of each cell line were evaluated for growth in soft agar. MDA-MB-231 subcloned cells transfected with vector only produced colonies in soft agar however, subclones overexpressing syndecan-1 on the cell surface or secreting truncated syndecan-1 produced significantly more colonies (Figure 7). Therefore, the results of different assays to evaluate the growth characteristics of the MDA-MB-231 transfected cells are somewhat conflicting. The growth of the cell lines that produce soluble syndecan-1 is reduced in the absence of serum suggesting a less transformed phenotype however, these same cell lines display greater anchorage-independent growth than vector transfected cells suggesting increased tumorigenic potential. In addition, the cells that overexpress syndecan-1 on their cell surface grow the same as vector only transfected cells as measured in MTT assays but produce significantly more colonies in soft agar than the vector only transfected cells. Therefore the second task of this project, evaluation *in vivo*, will determine specifically the tumorigenic potential of these cells lines and indicate if indeed syndecan-1 overexpression or secretion affects

tumorigenicity.

Task 2. *In vivo* analysis of breast cancer gene therapy.

- Establish tumors in NUDE mice and evaluate HSPG expression and tumor burden.

Experiments are currently underway to evaluate the growth properties of the MDA-MB-231 transfected cells *in vivo*. Subcloned cells transfected with the vector only, overexpressing syndecan-1 or secreting syndecan-1 will be injected into nude mice and the tumor burden, syndecan-1 expression and metastasis will be determined.

KEY RESEARCH ACCOMPLISHMENTS

Task 1.

- Full length and truncated syndecan-1 gene constructs containing a c-myc tag have been produced by PCR amplification and cloned in plasmid vectors.
- The two c-myc tagged syndecan-1 constructs or an empty vector have been transfected into MDA-MB-231 cells to produce three cell lines.
- Subclones of each cell line including vector only, full length syndecan-1 and soluble syndecan-1 expressing cells, have been produced by limiting dilution.
- Overexpression of syndecan-1 has been confirmed by FAC analysis.
- Secretion of truncated syndecan-1 has been confirmed by dot blot of conditioned medias.
- Differences in spheroid morphology have been recognized.
- Growth characteristics of each subclone has been evaluated by MTT assay in complete media, serum free media and estradiol free media.
- Anchorage-independent growth of each subclone has been evaluated.

Task 2.

- Evaluation of the tumorigenicity of subcloned MDA-MB-231 cell lines that are transfected with vector only or either the full length human syndecan-1 gene or the truncated human syndecan-1 gene is underway.

CONCLUSIONS

The objective of the proposal is to transfer HSPG gene constructs into breast cancer cells lines and tumors growing in mice to test the ability of these tumor suppressor genes to slow growth and possibly eradicate tumors. During this year c-myc tagged human syndecan-1 gene constructs were transfected into MDA-MB-231 cells and trans-gene expression has been confirmed. Evaluation of the affects of trans-gene expression on growth of several subcloned cell lines has been performed and suggests conflicting results. Basic MTT analysis of the growth of these cell lines indicates similar rates of growth although in the absence of serum, expression of soluble syndecan-1 slows growth. When a hallmark of the transformed phenotype, anchorage-independent growth, was evaluated, cells that overexpress syndecan-1 on the cell surface or secrete truncated syndecan-1 produce more colonies suggesting improved tumorigenicity. To determine the true efficacy of syndecan-1 as a gene therapy for breast cancer the subclones are being evaluated *in vivo* to access the affect of syndecan-1 expression on tumorigenicity. This work represents the first attempt to use HSPG genes for the purpose of anti-cancer therapy.

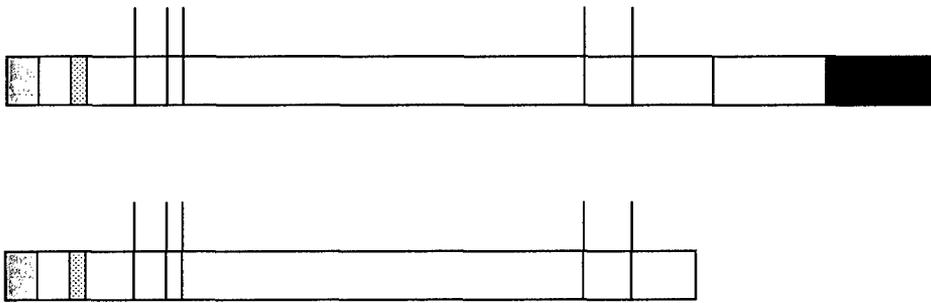
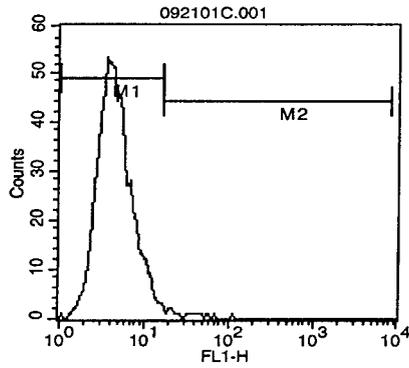
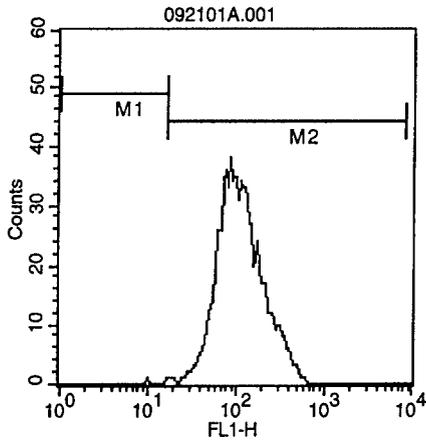


Figure 1. Full length and truncated syndecan-1 protein structure following expression of c-myc tag containing gene cassettes. The top illustration shows the full length syndecan-1 composed of an N-terminal signal sequence (gray box), an ectodomain (open box) containing glycosaminoglycan attachment sites (lines), a transmembrane domain (hatched box), and a cytoplasmic domain (black box). The c-myc tag (dotted box) was inserted in the ectodomain between the signal sequence and glycosaminoglycan attachment sites. The truncated form of syndecan-1 (bottom illustration) lacks the transmembrane and cytoplasmic domains.

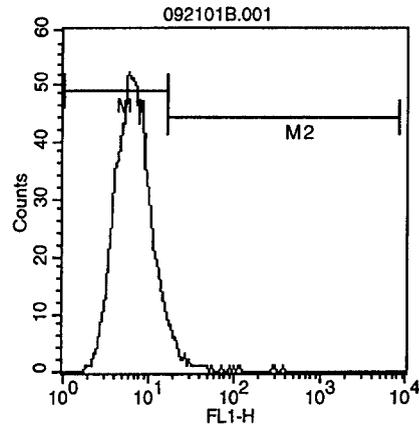
**231 neo
Negative control**



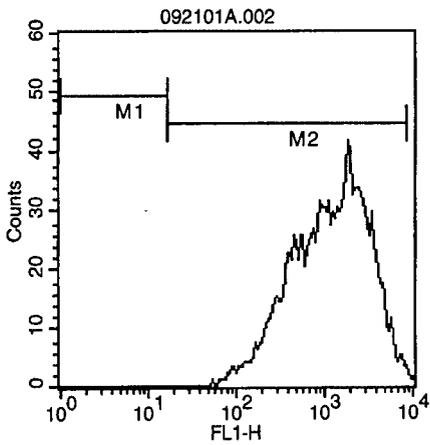
231 neo/BB4



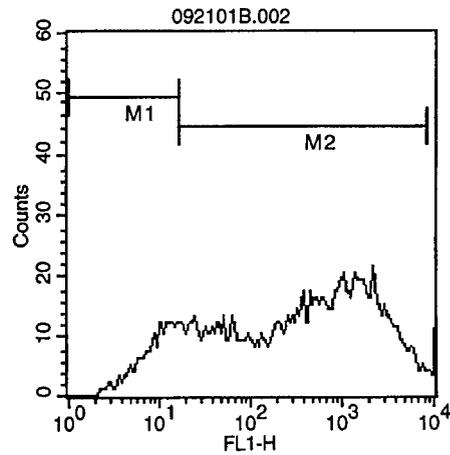
231 neo/c-myc



231 B4/BB4



231 B4/c-myc



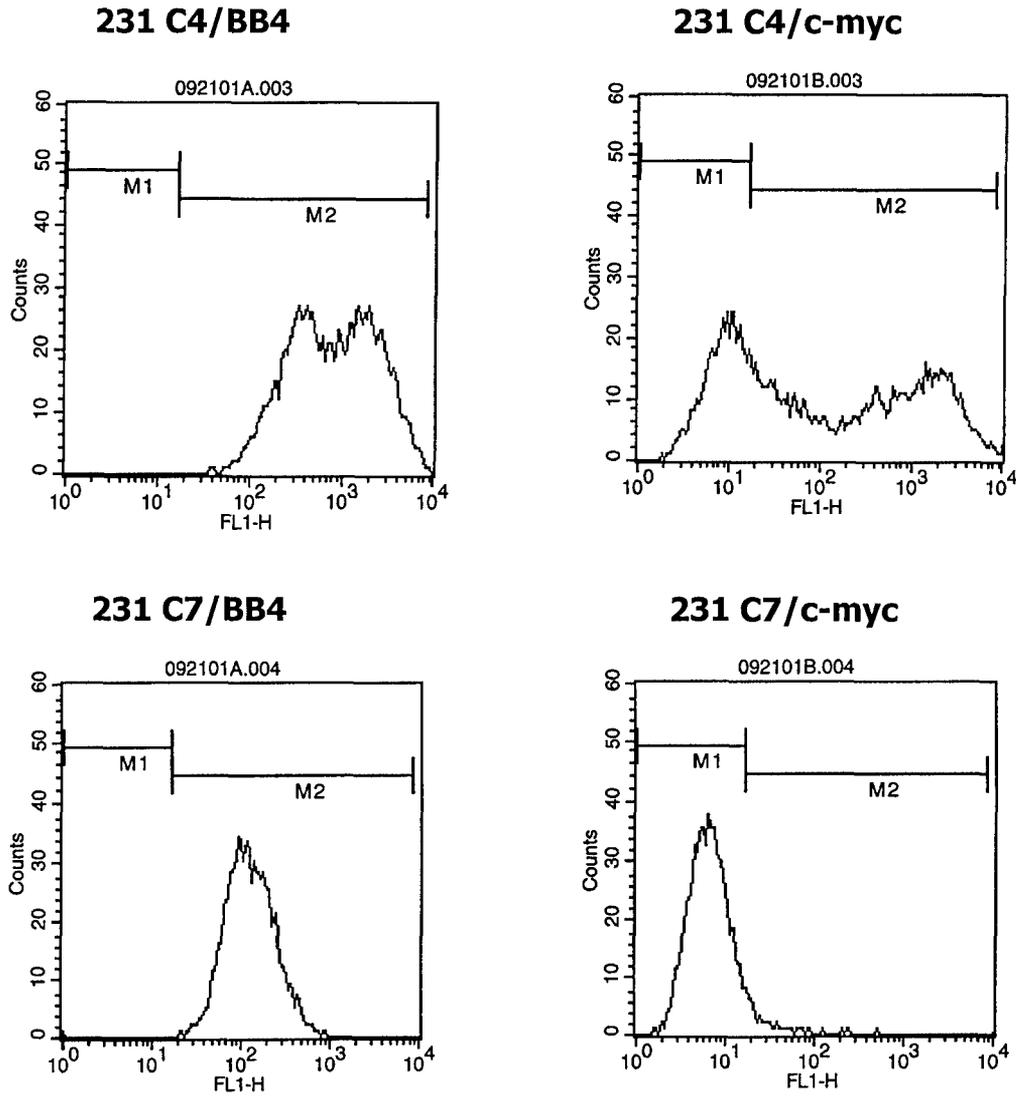


Figure 2. Cell surface expression of c-myc tagged human syndecan-1 on MDA-MB-231 subclones. MDA-MB-231 cells were transfected with vector only (231 neo), vector containing either a c-myc tagged human syndecan-1 gene cassette (231 B4 and 231 C4 subclones) or vector containing a truncated c-myc tagged human syndecan-1 gene (231 C7 subclone). Cell surfaces were stained with a secondary antibody (neo negative control), BB4 anti-human syndecan-1 antibody or a c-myc specific antibody and analyzed by flow cytometry. The X-axis of each graph indicates fluorescent intensity and the Y-axis reports cell counts.

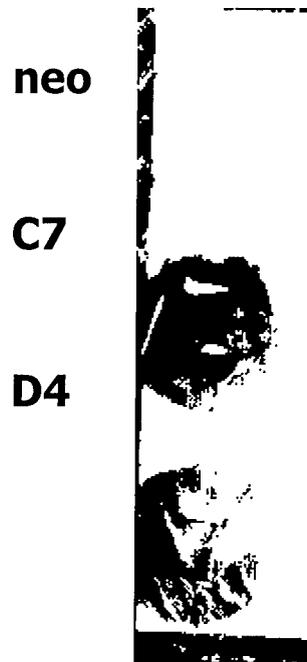
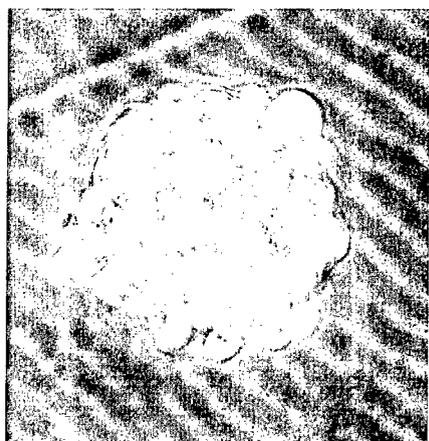
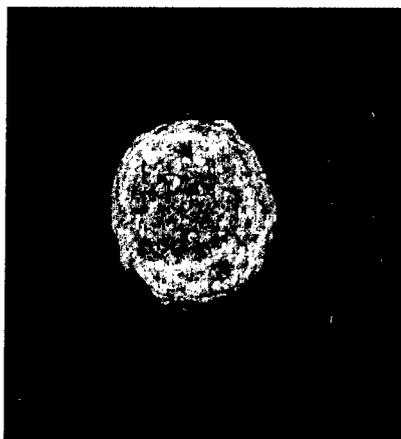
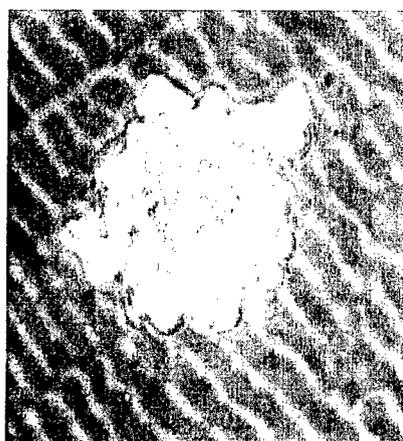


Figure 3. MDA-MB-231 cells transfected with a truncated human syndecan-1 gene cassette secrete syndecan-1 into the culture media. The media conditioned by two subclones (C7 and D4) contains syndecan-1 as determined by dot blot analysis using BB4 antibody. Cells transfected with the vector only (neo) do not.

231 neo



231 B4



231 C7

Figure 4. MDA-MB-231 cells transfected with full length syndecan-1 (B4) or truncated syndecan-1 (C7) produce spheroids with a bumpy morphology as compared to the smooth spheroids produced by cells transfected with vector only (neo). The cells were grown for several days on bacterial plates which prohibit attachment and the formation of monolayers but promote spheroid formation.

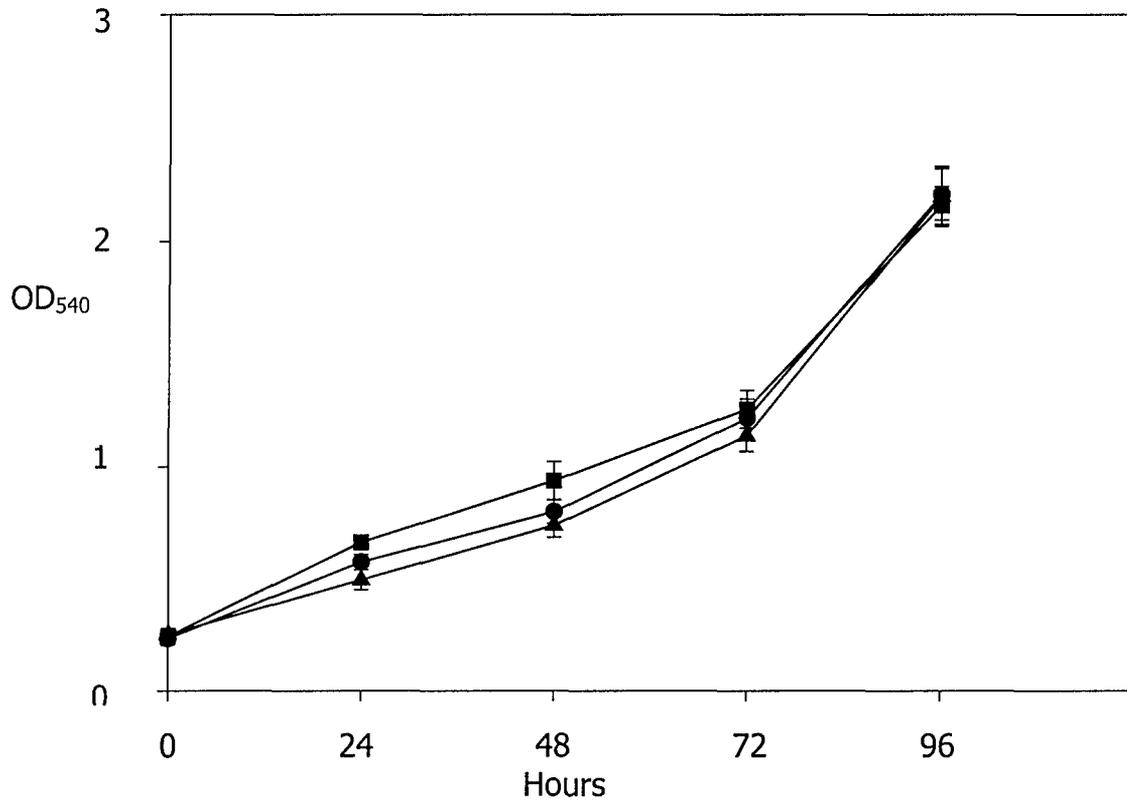
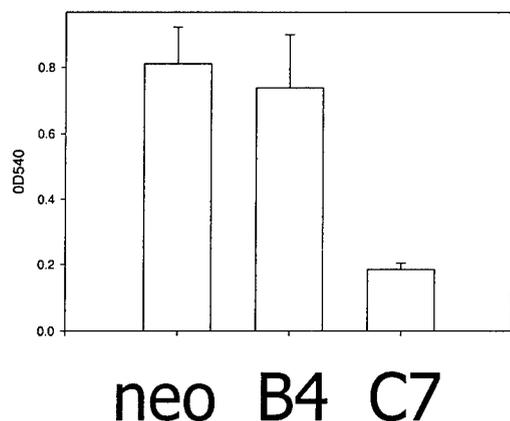


Figure 5. Overexpression of syndecan-1 or expression of soluble syndecan-1 does not affect cell growth. Transfected MDA-MB-231 cells including neo (circles), syndecan-1 overexpressing (squares) and soluble syndecan-1 expressing (triangle) cells were grown for the indicated periods of time and growth was assessed by MTT analysis. Increase in OD₅₄₀ value is indicative of cell growth. The cells were plated in triplicate and the experiment performed in duplicate. Bars indicate standard deviation.

A



B

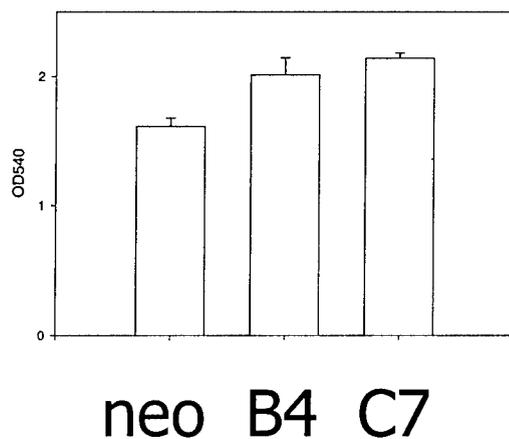


Figure 6. Transfected cell growth in serum or estradiol depleted media. MDA-MB-231 transfected cells were grown in the absence of sera (**A**) or estradiol (**B**) for 96 hours and growth was determined by MTT assay. Subcloned cells transfected with vector only (neo), full length syndecan-1 (B4) or truncated syndecan-1 (C7) were tested. Error bars indicate standard deviation of results of three wells.

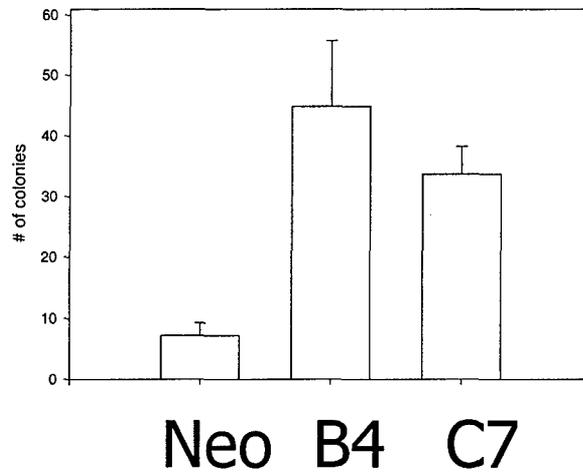


Figure 7. Overexpression of syndecan-1 (B4) or production of soluble syndecan-1 (C7) increases anchorage-independent growth. Colonies formed in three soft agar gels were counted in five separate fields. Error bars indicate standard deviation.



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FOR THE COMMANDER:

A handwritten signature in black ink, appearing to read "Phyllis M. Rinehart", is written over the typed name.

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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