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Identification of a novel insulin-like growth factor binding protein gene homologue with tumor suppressor like properties

Z. Cai, H.T. Chen, B. Boyle, F. Rupp, W.D. Funk *, D.A. Dedera

Nuvelo, Inc., 675 Almanor Ave., Sunnyvale, CA 94085, USA

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Abstract

Here we report the identification of a new insulin-like growth factor binding protein homologue, provisionally designated insulin-like growth factor binding related protein-4 (IGFBP-rP4). IGFBP-rP4 was found to be most closely related to IGFBP-7 with 52% amino acid homology and 43% amino acid identity, and shares a similar domain structure. Semi-quantitative RT-PCR expression analysis demonstrated a pattern of downregulation of this gene in multiple tumor samples including lung and colon cancer, compared to matched adjacent normal tissue. Western blotting revealed a protein of approximately 38 kDa expressed in both the cell pellet and secreted into the supernatant of transiently transfected Cos-7 cells. Cos-7 supernatants containing IGFBP-RP4 protein were observed to suppress the growth of HeLa cells in culture compared to vector controls. IGFBP-RP4 directly transiently transfected into HeLa cells also further confirmed the growth suppressive properties of this protein. Together these data suggest that IGFBP-RP4 may be a novel putative tumor suppressor protein.

Keywords: Tumor suppressor; Insulin-like growth factor binding related protein-4; Cancer

The insulin-like growth factor binding protein family of proteins binds to and in some cases modulates the growth effects of insulin-like growth factors (IGFs). IGFs have been demonstrated to have mitogenic properties and promote the survival of tumor cells in vitro and are found to be upregulated in prostate cancer patient sera (reviewed in [1]). Some members of the IGFBP family show tumor suppressor-like activities. IGFBP-3 introduced into lung cancer cells by stable transfection resulted in suppression of their growth in vitro [2]. IGFBP-3 introduced into non-small cell lung cancer cells was observed to suppress IGF-1 stimulated growth in vitro and to reduce tumor size in xenograft tumor models [3]. It has also been demonstrated that IGFBP-3 mutants that lack the ability to bind IGFs can independently induce apoptosis in prostate cancer cells [4].

The expression of IGFBP-7 (angiomodulin, IGFBP-rP1, mac 25) is down-regulated in breast cancer tissues [5] and expression is increased in senescent human mammary epithelial cells [6]. The expression of IGFBP-7 was also found to be reduced in uterine leiomyomata compared with adjacent myometrium [7]. IGFBP-7 has been demonstrated to bind to IGF-I and IGF-II albeit with lower affinity compared to IGFBP-3 [8]. M12 prostate cancer cells overexpressing IGFBP-7 have been demonstrated to have reduced tumorigenicity in nude mice and an increased propensity to undergo apoptosis [9]. IGFBP-7 suppresses cell growth in M12 cells by delaying cycle progression in the G1 phase, and unscheduled expression of cyclin A in G0/G1 may be contributing to increased apoptosis [10]. IGFBP-7 has also been demonstrated to

* Corresponding author.
E-mail addresses: wfunk@nuvelo.com (W.D. Funk), dedera1@aol.com (D.A. Dedera).

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bind to activin which may play a role in tumor growth suppression observed in Saos-2 (osteosarcoma), HeLa (cervical carcinoma), and P19 (murine embryonic carcinoma) cell lines [11].

Here we report the identification and characterization of a novel 278 amino acid IGFBP related protein provisionally designated IGFBP-RP4. Expression of this gene yields a protein of approximately 38 kDa in a Western blot assay. IGFBP-RP4 is observed to be a secreted protein found in the supernatants and cell pellet of transfected cells. Conditioned media from IGFBP-RP4 transfected Cos-7 cells were observed to suppress the growth of human cervical carcinoma cells (HeLa) in vitro. Finally, further confirmation of the growth suppressive properties of IGFBP-RP4 on tumor cells was demonstrated upon direct transfection of this gene into HeLa cells. These observations suggest that IGFBP-RP4 is a putative novel tumor suppressor protein.

Materials and methods

Cell lines and tissues. All cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and included the Cos-7 (an African Green Monkey kidney cell line) and HeLa cells (human cervical carcinoma cells). Culturing of cell lines was performed using standard protocols. All healthy tissue total RNAs were obtained from either Ambion or Clontech. Cancer patient tissues were obtained from the Cooperative Human Tissue Network (CHTN) or from Clontech and Ambion where noted. Patient tissue samples from the CHTN were snap-frozen shortly after surgical removal and used for total RNA isolation. Pathology information related to the tissues was obtained from the CHTN.

IGFBP-RP4 gene cloning. First strand cDNA was prepared using total RNA extracted from commercial healthy tissue total RNA from human testes. Oligonucleotide primers were designed that flank the predicted open reading frame and were used to amplify the corresponding cDNAs, which were subsequently cloned into the expression vector pcDNA3.1A (Invitrogen, Carlsbad, CA).

Real-time PCR (RT-PCR) expression analysis. All cell line RNA was isolated from cultured cells using the RNeasy Protect Total RNA isolation kit (Qiagen). Total RNA from frozen cancer tissues was obtained by first dissolving small pieces of frozen tissue in RNA lysis buffer obtained from the RNeasy Protect Total RNA isolation kit, followed by total RNA isolation.

Total RNA was reverse transcribed into cDNA using the Superscript First-strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA) in a 20 μl total volume and incubated at 42 °C for 1 h. After incubation, the reaction was diluted with 80 μl DEPC–H_2O. For real-time PCR, the SYBR Green protocol and reagents were used (Applied Biosystems) and PCR was performed in an ABI-5700 analyzer (Applied Biosystems). Five microliters of the diluted cDNA was used in each RT-PCR using the SYBR Green PCR master mix in a total reaction volume of 25 μl. Primers used to amplify the IGFBP-RP4 RNA were 5′-CGTGGAGAAAGGTCACGAAGT-3′ and 5′-ACTGGTACACACCCTCATCCT-3′ producing a 168 bp fragment. Amplification of eukaryotic translation elongation factor 1 α-1 (*Elf*) was used as an endogenous control in all samples. Primers used to amplify Elf RNA were 5′-ACTGTGCTGTCCTGAT TGTT-3′ and 5′-GCACTTGGCTCCAGCATGTT-3′. Primer specificity was determined by electrophoresing the amplified 184 and 289 bp products, respectively, on agarose gels and verifying the size. PCR amplification conditions used in these assays were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Relative quantities of RT-PCR product were determined using the protocol as described in the SYBR Green RT-PCR protocol (Applied Biosystems). Briefly, duplicate samples of each RNA were analyzed and the threshold cycle (C_t) was determined for IGFBP-RP4 RNA. The mean C_t values were normalized against the *Elf* endogenous control mean C_t value. To calculate the relative gene expression of each sample, the following formula was used $17(\text{mean } C_{t\ \text{SAMPLE}})/\text{mean } C_{t\ \text{Elf}}$. This formula normalized all mean sample C_t values to the equivalent of 17 cycles C_t value for *Elf*. Relative gene expression was then charted relative to expression of IGFBP-RP4 in the breast RNA sample, where the sample value was set at 1.

Western blotting of IGFBP-RP4. An antiserum was raised to a peptide LRKEDEGVYQCHAAN corresponding to the amino acid 232–247 region of the IGFBP-RP4 protein (Fig. 1). Purified peptides were injected into rabbits, with subsequent boosting using standard protocols. Purification of the peptide specific antibodies was accomplished using affinity purification to the peptides on CNBr-activate Sepharose 4B column.

Cellular lysates and supernatants containing the IGFBP-RP4 protein were produced by transient transfection of Cos-7 cells harvested after 48 h post-transfection using the Fugene 6 transfection system (Roche Biosystems). Supernatants were harvested, spun down, and aliquoted to microcentrifuge tubes with 3× Blue Loading Buffer (New England Biolabs) added to denature protein. Cell pellet lysates and supernatants were electrophoresed on a 16% Novex Tris–glycine polyacrylamide gel (Invitrogen) and then transferred onto a nitrocellulose membrane (Invitrogen). Western blotting was performed using the standard protocols. An anti-rabbit IgG-HRP was used as a secondary antibody. Chemiluminescent detection (Pierce) was used to visualize the bands. Similarly, IGFBP-V5 tagged protein lysates were produced and Western blotted using a conjugated anti-V5-HRP antibody (Invitrogen).

Tumor cell growth assays. Conditioned DMEM (Cellgro) from Cos-7 cells transfected with IGFBP-RP4V5 pCDNA3 was harvested 48 h post-transfection, filtered through a 0.22 μm PES (Polyethersulfone) 50-ml Tube Top Filter (Corning Incorporated), and concentrated to half volume by centrifuging in Centriprep YM10 (Millipore). Cells were incubated with a 50% concentration of conditioned media/fresh media on 80,000 HeLa cells seeded into 24-well cell culture plates. The assay was allowed to proceed for 72 h with the 50% conditioned media being replaced daily. Viable cell counts were determined daily using a hemocytometer and trypan blue dye exclusion. Each average cell count was determined by sampling independent wells at each time point. Five assays were performed: three assays were performed with six wells sampled per time point, one assay was performed with three wells per time point, and one assay with two wells per time point. The presence of IGFBP-RP4V5 in the media was demonstrated by Western blotting using protocols described above.

Cell growth of HeLa cells was also determined using cells transfected with the IGFBP-RP4 Trexi plasmid. The Trexi plasmid is a bicistronic retroviral expression plasmid that expressed both the IGFBP-RP4 and the yellow fluorescent protein (YFP) under the control of the CMV promoter. HeLa cells were directly transfected with the IGFBP-RP4 Trexi plasmid, incubated for 24 h, and then analyzed with a BD FACSVantage SE System (BD Biosciences), sorting out 1000 YFP fluorescing cells per well directly into a 96-well plate. The growth of the transfected HeLa cells was determined as before with trypan blue dye exclusion. The assay was allowed to proceed for 96 h with cell counts assayed daily from six independent wells per time point. Cells transiently transfected with Trexi vector alone were used as a negative control.

Results

IGFBP-RP4 gene characterization

The IGFBP-RP4 cDNA and protein sequences are shown in Fig. 1. IGFBP-RP4 is a protein of 278 amino acids with a signal peptide and conserved motifs found in the IGFBP family of proteins. The signal peptide runs from the N-terminus through G27, which is where the signal peptide cleavage site is predicted. Additionally, from amino acid D61 through S76, there is a conserved IGFBP family signature as predicted by the eMatrix software program. The Pfam software program predicts a Kazal serine-protease signature from C91 through C151, and an immunoglobulin (Ig) domain from G169 through A245. Fig. 2 shows the phylogenetic relationships as determined using the MegAlign software with IGFBP-RP4 and other IGFBP family members. IGFBP-RP4 shared highest homology and the closest phylogenetic relationship to the IGFBP-7 protein.

Expression of the IGFBP-RP4 RNA in healthy and tumor tissues

Assessment of IGFBP-RP4 RNA expression was examined using semi-quantitative real-time PCR. Human tissue RNAs derived from healthy tissues were assayed to determine the in vivo expression of the IGFBP-RP4 gene. IGFBP-RP4 was found to be expressed at the highest level in both the brain and testes, with lower levels of expression observed in the prostate, bladder, and lung (Fig. 3). Expression of IGFBP-RP4 in tumor tissue was examined using RT-PCR. Total RNA derived from lung, colon, prostate, stomach, and ovary tumors was compared to RNA derived from adjacent normal tissue from the same patient. Downregulation of IGFBP-RP4 expression was observed in all five colon tumor samples observed and four of six lung tumor samples (Table 1). Downregulation was also observed in stomach and ovary tumor tissue as well. The magnitude of mRNA

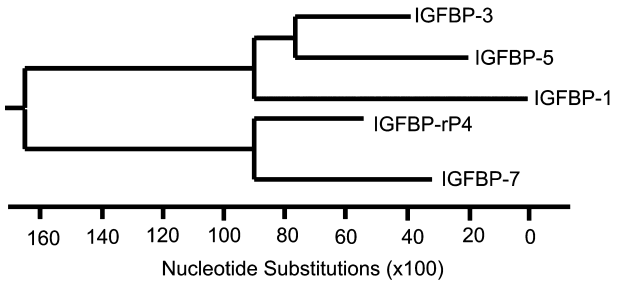

Fig. 2. Phylogenetic tree of IGFBP-RP4. A phylogenetic tree drawn from a multiple sequence alignment between IGFBP-RP4, IGFBP-1, IGFBP-3, IGFBP-5, and IGFBP-7 reveals that IGFBP-RP4 is most evolutionarily related to IGFBP-7 as compared to the other previously described IGFBP family members. IGFBP-RP4 has 54% homology to IGFBP-7 while the homology to IGFBP-1, IGFBP-3, and IGFBP-5 is 43%, 44%, and 40%, respectively. The tree was drawn using the ClustalW program provided in the MegAlign software from DNAStar.

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-78 ggg gcg gga gcg gtg act gct cgg cgg gcg cgg agc gga gcg cga agc aga ggc gcc gcc  -19
-18 gct gtc ccg gag caa gcc atg ccg cgc ttg tct ctg ctc ttg ccg ctg ctg ctt ctg ctg   42
                            M   P   R   L   S   L   L   L   P   L   L   L   L   L    (14)
 43 ctg ctg ccg ctg ctg ccg ccg ctg tcc ccg agc ctc ggg atc cgc gac gtg ggc ggc cgg   102
    L   L   P   L   L   P   P   L   S   P   S   L   G   I   R   D   V   G   G   R    (34)
103 cgc ccc aag tgt ggt ccg tgc cgg cca gag ggc tgc ccg gcg cct gcg ccc tgc ccg gcg   162
    R   P   K   C   G   P   C   R   P   E   G   C   P   A   P   A   P   C   P   A    (54)
163 ccc ggg atc tcg gcg ctc gac gag tgc ggc tgc tgc gcc cgc tgc ctg gga gcc gag ggc   222
    P   G   I   S   A   L   D   E   C   G   C   C   A   R   C   L   G   A   E   G    (74)
223 gcg agc tgc ggg ggc cgc gcc ggc ggg cgc tgt ggc ccc ggc ctg gta tgc gcg agc cag   282
    A   S   C   G   G   R   A   G   G   R   C   G   P   G   L   V   C   A   S   Q    (94)
283 gcc gct ggg gca gcg ccc gag ggc acc ggg ctc tgc gtg tgc gcg cag cgc ggc acc gtc   342
    A   A   G   A   A   P   E   G   T   G   L   C   V   C   A   Q   R   G   T   V   (114)
343 tgc ggc tcc gac ggt cgc tcg tac ccc agc gtc tgc gcg ctg cgc ctg cgc gct cgg cac   402
    C   G   S   D   G   R   S   Y   P   S   V   C   A   L   R   L   R   A   R   H   (134)
403 acg ccc cgc gcg cac ccc ggt cac ctg cac aag gcg cgc gac ggc cct tgc gag ttc gct   462
    T   P   R   A   H   P   G   H   L   H   K   A   R   D   G   P   C   E   F   A   (154)
463 cct gtg gtc gtc gtt cct ccc cga agt gtt cac aac gtc acc ggg gcg cag gtg ggc ctg   522
    P   V   V   V   V   P   P   R   S   V   H   N   V   T   G   A   Q   V   G   L   (174)
523 tcc tgt gaa gtg agg gct gtg cct acc cca gtc atc acg tgg aga aag gtc acg aag tcc   582
    S   C   E   V   R   A   V   P   T   P   V   I   T   W   R   K   V   T   K   S   (194)
583 cct gag ggc acc caa gca ctg gag gag ctg cct ggg gac cat gtc aat ata gct gtc caa   642
    P   E   G   T   Q   A   L   E   E   L   P   G   D   H   V   N   I   A   V   Q   (214)
643 gtg cga ggg ggc cct tct gac cat gag gcc acg gcc tgg att ttg atc aac ccc ctg cga   702
    V   R   G   G   P   S   D   H   E   A   T   A   W   I   L   I   N   P   L   R   (234)
703 aag gag gat gag ggt gtg tac cag tgc cat gca gcc aac atg gtg gga gag gct gag tcc   762
    K   E   D   E   G   V   Y   Q   C   H   A   A   N   M   V   G   E   A   E   S   (254)
763 cac agc aca gtg acg gtt cta gat ctg agt aaa tac agg agc ttc cac ttc cca gct ccc   822
    H   S   T   V   T   V   L   D   L   S   K   Y   R   S   F   H   F   P   A   P   (274)
823 gat gac cgc atg tga tgg aga aat gta cat gtt cta agt cat ttt cag tat ttt aca ccc   882
    D   D   R   M   *                                                               (278)
883 atg tta tga gat att tga ggt ggc tta taa gac ctg taa aaa aaa aaa a
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Fig. 1. cDNA and predicted protein sequences for IGFBP-RP4. The cDNA sequence and 278 aa protein sequence are shown. The nucleotides are numbered relative to the translation initiation codon, and the corresponding amino acid numbering is shown in parentheses. The signal peptide as predicted by the SignalP software (single underline). Conserved protein family regions for the IGFBP family (double underline), Kazal-type serine protease inhibitor family (bold text), and the immunoglobulin domain (dotted underline) are shown.

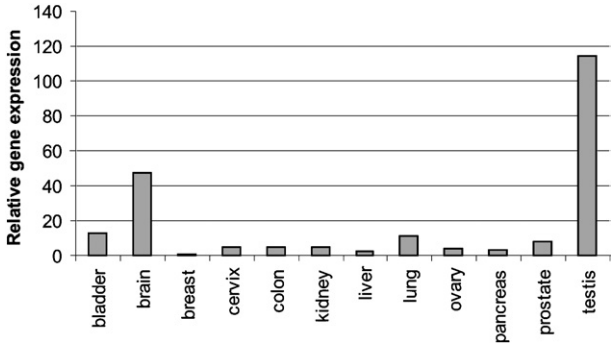

Fig. 3. Expression of IGFBP-RP4 mRNA in healthy tissues. Semi-quantitative RT-PCR was used to determine the relative expression of IGFBP-RP4 mRNA in healthy tissues where breast tissue is set at a value of 1.

Table 1
RT-PCR results for mRNA expression of IGFBP-RP4 in tumor and healthy tissues

Tissue	C_t normal[a,b]	C_t tumor	ΔC_t[c]
Lung 8036	28.02	27.35	−0.67
Lung 7981	29.33	31.00	1.66
Lung 7987	28.96	30.51	1.55
Lung 8040	26.09	29.91	3.81
Lung 8038	27.31	20.84	−6.47
Lung 8044	26.96	27.70	0.74
Colon 7413	31.15	33.09	1.95
Colon 7554	31.08	34.60	3.52
Colon 7932	29.73	30.30	0.57
Colon 8067	30.08	31.24	1.15
Colon (A)	26.74	28.99	2.25
Prostate 242	28.39	29.23	0.84
Prostate 238	30.60	28.61	−1.99
Stomach (A)	26.32	28.60	2.28
Ovary (A)	30.25	31.66	1.41

[a] All samples are the average of duplicate experiments.
[b] All C_t values are normalized to a C_t value of 17 for the elongation factor 1 α-1 (*Elf*) housekeeping gene.
[c] ΔC_t value = C_t tumor − C_t adjacent normal tissue.

downregulation varied from less than 2-fold difference to as much as 8-fold in colon 7554 and lung 8040 tumors. Although IGFBP-RP4 is not downregulated in every tumor tissue, an overall pattern of downregulation of gene expression is observed. This observed downregulated mRNA expression led us to test whether the IGFBP-rP4 protein displayed tumor-suppressor activities.

IGFBP-RP4 protein expression

The IGFBP-RP4 gene was modified to express the V5 epitope tag at its carboxy terminus in a pcDNA3 plasmid (IGFBP-RP4-V5). Cos-7 cells were transiently transfected with IGFBP-rP4-V5 or V5 vector controls. Conditioned medium supernatants were assessed by Western blotting using a polyclonal rabbit antisera raised to a carboxy-terminal 15mer peptide (6975A) or

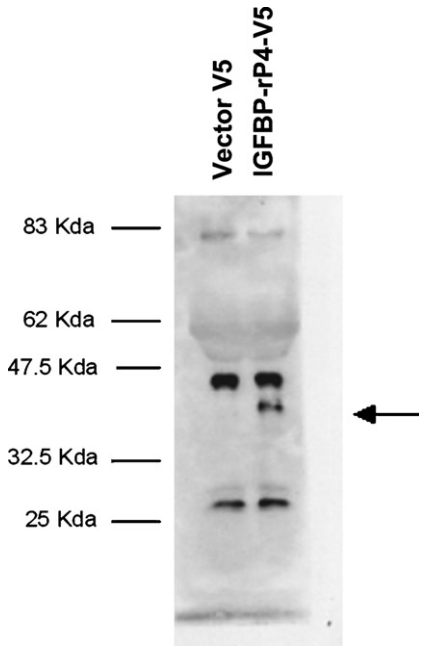

Fig. 4. Western blotting of Cos-7 cell supernatants collected 48 h after transient transfection. Western blot was probed with the 6975A peptide polyclonal antisera. Arrow indicates the IGFBP-RP4-V5 protein in the IGFBP-RP4-V5 transfected Cos-7 supernatants.

an anti-V5 monoclonal antibody (Figs. 4 and 5A, respectively). Fig. 4 demonstrates an approximately 38 kDa protein found in the IGFBP-rP4-V5 transfected cell supernatants and not found in V5 vector control transfected cells. Expression of IGFBP-rP4-V5 was also found to be present in the cell pellet (data not shown).

Suppression of tumor cell growth

Previous studies with IGFBP-7 protein demonstrated the ability to suppress the growth of HeLa cells in culture [11]. Since IGFBP-rP4 shares the greatest sequence similarity with IGFBP-7, HeLa cells were chosen as human tumor cells to assay tumor suppressor properties. An assay was set up in which the growth of HeLa cells could be examined while being incubated with 50% IGFBP-rP4-V5 conditioned media. Fig. 5A demonstrates the presence of IGFBP-rP4-V5 in the conditioned media used in this assay, as probed by the anti-V5 antibody. HeLa cells seeded at 80,000 cells per well were incubated with IGFBP-rP4-V5 containing media for 72 h. Viable cell counts were determined every 24 h for each time point and were determined averaging together six independent wells for each. Fig. 5B shows the results of the viable cell counts of a representative assay. HeLa cells incubated with IGFBP-rP4-V5 demonstrated a statistically significant reduction in cell growth at 72 h, with a greater than 36% reduction in HeLa cell growth, at a significance level greater than

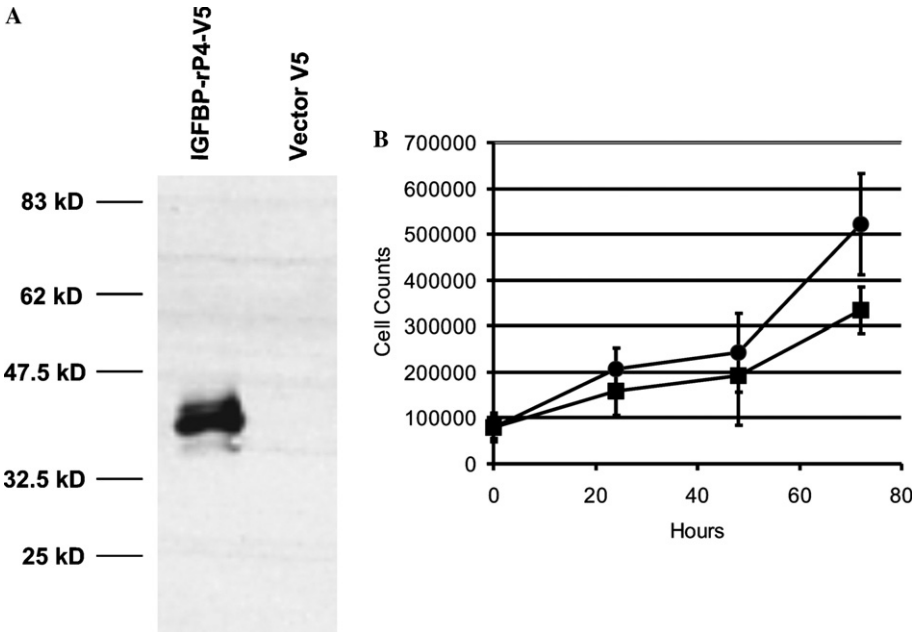

Fig. 5. (A) Conditioned media supernatants were Western blotted and probed with an HRP-anti-V5 tag antibody. The presence of IGFBP-RP4-V5 protein was demonstrated by a band of approximately 38 kDa in the IGFBP-RP4-V5 pCDNA3 transfected Cos-7 conditioned media and not in vector transfected controls. (B) Conditioned media (50%) from Cos-7 cells transiently transfected with pCDNA3 vector (circles) or with IGFBP-RP4-V5 pCDNA3 (squares) were incubated with 8×10^4 HeLa cells for 72 h. Viable cell numbers were determined at 24, 48, and 72 h time points using trypan blue dye exclusion. Each time point is the average of six independently sampled wells. *P* values were determined to be $\leqslant 0.0081$, 0.0562, and 0.0038 for the 24, 48, and 72 h time points, respectively.

99% as determined by a one-sided, paired student's *t* test. The 24 and 48 h time points in this assay also demonstrated an earlier reduction in HeLa cell growth but did not consistently achieve statistical significance (confidence interval <95%) between experiments. In all assays, cell growth for each time point was sampled from 6 independent culture wells.

A second assay was performed to determine if the HeLa cell growth suppression observed was a consequence of the IGFBP-RP4 protein. The IGFBP-RP4 gene was cloned into the Trexi vector which is a bicistronic expression vector also expressing the yellow fluorescent protein. HeLa cells were transiently transfected with IGFBP-RP4 Trexi or Trexi vector alone and analyzed by flow cytometry to identify transiently transfected cells expressing the YFP protein after 24 h. Fluorescing HeLa cells were then sorted into wells containing media in a 96-well plate, at 1000 cells per well. Viable cell counts were then conducted over the next 96 h. Fig. 6 shows the growth of cells transfected with IGFBP-RP4 Trexi or with the Trexi vector alone. A statistically significant reduction in cell growth was observed with IGFBP-RP4 Trexi transfected cells at 24, 48, 72, and 96 h post-transfection, with a greater than 99%, 99%, 99%, and 98% significance level, respectively (as determined by *t* test). Each time point was an average value determined from an independent sampling of six wells. Visual inspection of cells prior to counting revealed the presence of detached floating cells at apparent

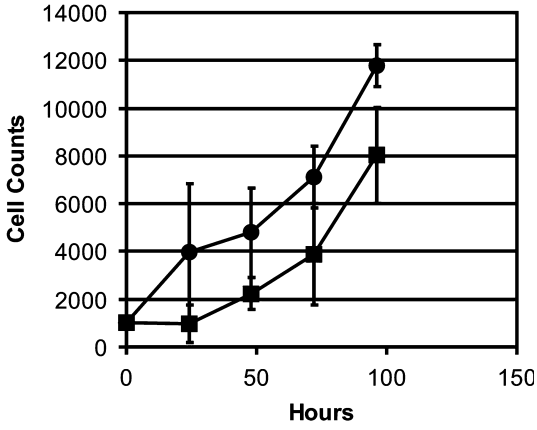

Fig. 6. Trexi vector (circles) or Trexi-IGFBP-RP4 (squares) transiently transfected into HeLa cells and 1000 cells sorted into individual wells. Each time point displayed is the average of six independent wells per time point. *P* values $\leqslant 0.0084$, 0.0053, 0.0083, and 0.0117 for 24, 48, 72, and 96 h time points, respectively.

greater numbers in the IGFBP-RP4 Trexi transfected cells compared to Trexi controls (data not shown), suggesting increased cell death.

Discussion

A number of IGFBP family members have been suggested to have tumor suppressor like properties.

IGFBP-3 has been shown to suppress the growth of breast cancer cells and the prostate cancer cell line PC-3 [1]. IGFBP-7, the family member that shares the greatest sequence similarity to IGFBP-rP4, had been demonstrated to suppress the growth of HeLa, P19 (murine embryonic carcinoma cells), and Saos-2 (osteosarcoma cells) when recombinant IGFBP-7 protein was added to the culture medium [11]. Furthermore, it was found that IGFBP-7 bound to activin A, suggesting a possible mechanism for the observed growth suppression. In a separate study, IGFBP-7 expressed at high levels in M12 prostate cancer cells resulted in growth suppression, decreased colony formation in soft agar, and decreased tumor formation ability in nude mice [10]. Similar to IGFBP-7, IGFBP-RP4 has been shown here to also have tumor suppressor like effects on HeLa cells. Although the mechanism of action was not determined here, the observation of many floating dead cells in the IGFBP-RP4 Trexi transfected cells might imply an apoptotic mechanism at work. Indeed, overexpression of IGFBP-7 in the M12 prostate cell line resulted in a delay in G1 and cyclin A associated apoptosis [10].

The expression profile observed with IGFBP-RP4 was suggestive of a tumor suppressor like functionality. Most tumor tissues examined demonstrated a downregulation of the IGFBP-RP4 gene when compared to adjacent, healthy tissues. Most notable was expression in colon and lung cancer tissues where IGFBP-RP4 was downregulated in five of five tissues and four or six tissues, respectively (Table 1). Similar patterns of expression can be found with IGFBP-7 in breast cancer [5], in uterine leiomyomata [7], and in prostate cancer [12]. The expression pattern demonstrated here along with the growth suppression observed in HeLa cells suggests that IGFBP-RP4 may be a secreted tumor suppressor protein. Further studies with recombinant protein and the expression of IGFBP-RP4 in in vivo tumor models will be needed to further verify this hypothesis.

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