A novel proteomic coculture model of prostate cancer cell growth

Dmitri Dvorzhinski[1, 2], Anu Thalasila[1, 2], Paul E. Thomas[1], Deirdre Nelson[1], Hong Li[3], Eileen White[1] and Robert S. DiPaola[1, 2, 4]

[1]The Cancer Institute of New Jersey, New Brunswick, New Jersey, USA
[2]The Dean and Betty Gallo Prostate Cancer Center
[3]Center for Advanced Proteomics Research
[4]Department of Medicine, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA

Chemotherapy and androgen ablation therapy are only temporarily effective against prostate cancer, and current studies are ongoing to test agents that target proteins responsible for autocrine and paracrine stimulated growth. Given limitations of current laboratory models to test the effect of these agents on cell growth and protein targets, we developed a coculture model that can distinguish paracrine stimulated growth and effects on proteins. We found that LNCaP prostate cancer cells and an immortalized rat prostate cell line transfected to over-express the antiapoptotic resistance protein Bcl-2 were stimulated to grow ($>$2-fold increase, $p < 0.01$) through autocrine effects from additional cells in an upper chamber of our system. Using a proteomic approach with a two-dimensional differential in gel electrophoresis method to increase fidelity, four proteins were found to increase after autocrine induced growth stimulation. These proteins were all identified by mass spectrometry as enzymes in the glycolytic pathway, validating the ability of this system to detect both clonogenic growth and the effect on proteins. These data, therefore, demonstrate a novel coculture model for further study of agents that target proteins in pathways of paracrine or autocrine stimulated cell growth.

1 Introduction

Androgen ablation and chemotherapy are only temporarily effective in advanced prostate cancer, secondary to the development of molecular mechanisms of tumor survival and drug resistance [1]. Our understanding of these molecular pathways has increased and is responsible for multiple therapeutic options currently under study, including agents that target paracrine or autocrine growth factor pathways [2, 3]. One difficulty, however, is the need to determine the most effective agents, or combination of agents, to maximize our chance of cure. The microenvironment of tumor cells, including autocrine and paracrine factors produced by tumor cells and stroma, is capable

of supporting events required for early growth and invasion. Initial clinical studies of agents that inhibit these growth factor pathways have demonstrated some beneficial clinical effects in solid tumors [4]. Studies of these agents, such as epidermal growth factor tyrosine kinase inhibitors, are now under clinical development in patients with prostate cancer [5]. Given only minimal clinical benefit to date, however, further studies are warranted to test the inhibition of multiple pathways, and therefore multiple agents in combination. Given the fact that most of these agents target proteins, laboratory models capable of detecting paracrine or autocrine growth effects and assessment of multiple proteins will be important to guide future clinical studies. Using a coculture proteomic approach with a two-dimensional differential in gel electrophoresis method (DIGE) to increase fidelity, we established a novel model capable of detecting paracrine and autocrine effects on growth and cellular proteins. This model can now be used for further study of agents that target proteins in pathways of paracrine or autocrine stimulated cell growth.

Correspondence: Robert S. DiPaola M.D., The Cancer Institute of New Jersey, 195 Little Albany St., New Brunswick, NJ 08901, USA
E-mail: Dipaolrs@umdnj.edu
Fax: $+$1-732-235-7493

Abbreviation: RP, rat prostate epithelial cells

2 Materials and methods

2.1 Cell growth

PC-3 (human androgen insensitive prostate cancer cell line), LNCaP (human androgen sensitive prostate cancer cell line) and human osteoblast (HOB) cells were obtained from the American type culture collection (Manassas, VA, USA). Experiments using a coculture model were performed with the clonogenic assay utilizing 6 well Transwell coculture plates (Corning, NY, USA). For the clonogenic assay, after 14 days clones were stained (0.1% methylene blue, 50% ethanol, 50% H_2O) and clone formation units counted on a Molecular Imager System (Bio-Rad, Hercules, CA, USA) using Quantity One software (Bio-Rad).

2.2 Transformed rat prostate cells

Primary Fisher baby rat prostate epithelial cells (RP) were surgically removed from 15 day old rats supplied from Taconic (Germantown, NY, USA). The prostates were treated with collagenase and cells were obtained by centrifugation. Cells were resuspended in DMEM with 10% fetal bovine serum, penicillin and streptomycin. The cells were cotransfected with an E1A expression plasmid, pCMV E1A, along with an expression plasmid for a temperature sensitive mutant p53 (val135). The epithelial nature of these cells was confirmed by Western blotting utilizing E-cadherin as an epithelial marker. RP E1A/p53 cells were transfected by electroporation with a linearized plasmid (pCDNA 3.1 Neo hBcl-2) which expressed the human Bcl-2 protein (RP/B cells). Confirmation of Bcl-2 expression was performed by immunoblotting with a Bcl-2 monoclonal antibody as previously described [6].

2.3 Protein sample preparation and labeling

To assess protein expression cultured cells were harvested and resuspended in lysis buffer (8 M urea, 40 mM TRIS, 4% w/v CHAPS (pH 8–8.5)). Cells were lysed at 4°C for 1 h with intermittent vortexing followed by centrifugation for 10 min at 4°C at $10\,000 \times g$. The supernatant was obtained and desalted by dialysis against 8 M urea, 40 mM Tris (pH 8–8.5) in mini-dialysis units (Slide-a-lyzer; Pierce Chemical, Rockford, IL, USA). Protein quantitation was performed with the BCA protein assay method (Pierce Chemical). Protein was labeled using DIGE dyes (Amersham Biosciences, Piscataway, NJ, USA), as previously described [7]. Cyanine dyes were reconstituted in 99.8% anhydrous DMF and added at 4°C in a ratio of 400 pmol CyDye to 75 ug protein. Typically, Cy2 was

used to label the internal standard (which comprised equal amounts of control and treated samples), Cy3 the control, and Cy5 the stimulated coculture (treatment).

2.4 2-D-DIGE

Protein samples were treated with equal volumes of $2 \times$ sample buffer (8 M urea, 4% w/v CHAPS, 130 mM DTT, 1% v/v Pharmalytes 3–10). The volume of the sample containing the standard, control, and stimulated coculture (treatment) labeled with Cy2, Cy3 and Cy5 was brought up to 200 μL with rehydration buffer (containing 8 M urea, 4% w/v CHAPS, 2 mg/mL DTT, 1% Pharmalyte 3–10) to rehydrate 11 cm Ready strips (Bio-Rad). The first dimension IEF was performed using an IPGphor IEF unit (Amersham Biosciences). The strips were actively rehydrated for 12 h at 20°C and 30 V and focused by the following 4 steps: 500 V for 500 Vh, 3000 V for 6000 Vh, 5000 V for 10 000 Vh, and maintained at 8000 V for 42 000 Vh. Prior to the second dimension, strips were equilibrated in 5 mL of equilibration buffer A (containing 8 M urea, 1% w/v SDS, 30% v/v glycerol, 100 mM Tris, pH 6.8 and 5 mg/mL of DTT) for 15 min followed by 5 mL of equilibration buffer B (containing 8 M urea, 1% w/v SDS, 30% v/v glycerol, 100 mM Tris, pH 6.8 and 4% iodoacetamide) for 15 min. The strips were then loaded and run on 10% pre-cast gels (Bio-Rad).

2.5 Image analysis and acquisition

The gel images were scanned on the Typhoon 9400 Imager (Amersham Biosciences). The Cy2 images were scanned with a 488 nm laser and emission filter of 520 nm BP30. Cy3 images were scanned using a 532 nm laser and an emission filter of 580 nm BP30. Cy5 images were scanned using a 633 nm laser and a 670 nm BP30 emission filter. The gel images were saved with Image Quant V.4.0 software (Molecular Dynamics, Sunnyvale, CA, USA). Images were analyzed using DeCyder V. 4.0 software specifically designed for use with DIGE technology, as previously described [7]. The sample/pooled internal standard gel image pairs were processed by the DeCyder differential-in-gel analysis software to codetect the spots and to quantitate the differences in the images. The codetection was done automatically by assigning the image pairs in the DeCyder batch processor. The estimated number of spots *per* gel was set to 1500 and the exclusion filter was set to exclude the periphery containing artifacts. The gel to gel matching of the standard spot maps from each gel followed by statistical analysis of protein change between samples were then performed using the DeCyder biological variation analysis software. Statistical analysis was done by comparing the control to treat-

ment groups. Student *t* test and 1 way ANOVA was performed for every matched spot set comparing average ratio and SD of protein abundance for a given spot. The Student *t* test was applied to the matched spots and data was filtered by the pick filter to obtain spots with an Av ratio of greater or less than 1.3 with a Student *t* test *p* value of 0.01 or less.

2.6 Protein identification by MS

Protein identification by MS analysis was on preparative gels loaded with 500 ug of proteins and post stained with Coomasie blue stain and destained with ethanol and acetic acid. The gel was scanned on an ImageMaster system (Amersham Biosciences). The preparative gel image was matched to the Cy dye image and spots in the picking list were identified and picked off the preparative gel by using a one touch manual spot picker (1.5 mm; The Gel Company, Sanfran, CA, USA). Protein in-gel digestion was performed based on a modified UCSF protocol (http://donatello.ucsf.edu/ingel.html). Gel plugs were washed with 500 uL of 25 mM ammonium bicarbonate, and 50% ACN in ammonium bicarbonate followed by shrinking the gel with 100% ACN. The gel samples were then dried in a Speed Vac for 10 min. The gel plugs were incubated with 200 ng of trypsin in 50 uL of 25 mM ammonium bicarbonate at 37°C overnight (16–18 h). The peptides were then extracted and vacuum dried at 45°C to reduce the volume to 10 uL. The peptides were desalted with C18 Zip Tips (Millipore, Bedford, MA, USA) and then recovered in 1–2 uL of 60% ACN/0.1% TFA. The sample was loaded onto an MALDI plate, following addition of matrix solution and allowed to air-dry. Samples were analyzed using a MALDI-TOF mass spectrometer (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA, USA). Protein identification was made using the protein database at the National Center for Biotechnology Information (NCBI) from the NIH (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?CMD=search&DB=protein).

3 Results

3.1 Coculture Model with Cancer Cell Lines

To determine if growth stimulation or inhibition could be detected in an *in vitro* model, tumor cells were assessed in a coculture system using a clonogenic assay (Fig. 1A). In this model, growth of cells in a lower chamber was assessed to determine the effect of various cells cocultured in the upper chamber. As shown in Fig. 1B, LNCaP cells cultured in a bottom chamber of the coculture system (LNCaP) were stimulated with LNCaP cells in the top chamber over 2-fold compared to a control
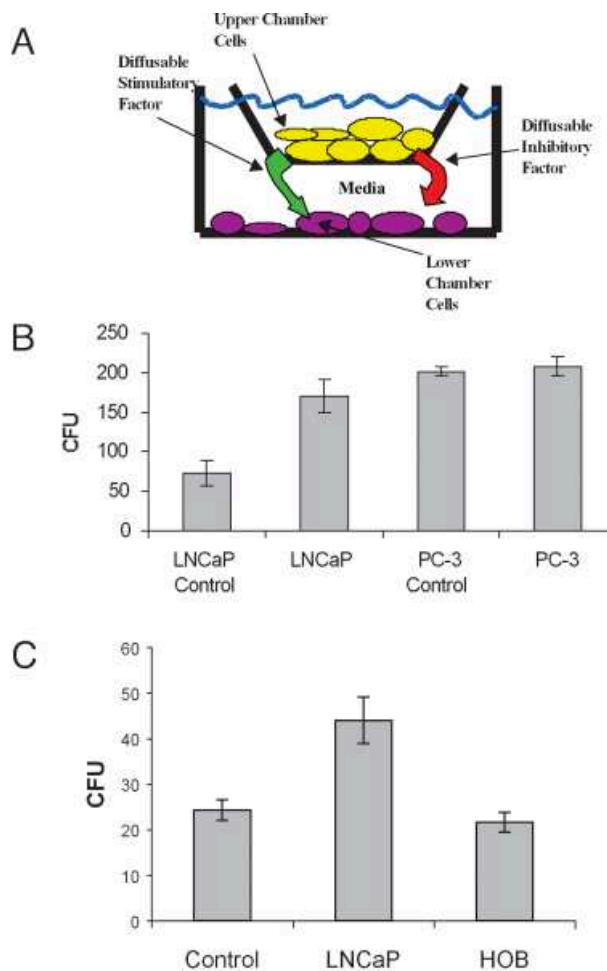

Figure 1. (A) Diagram of experimental coculture model, in which cells in the bottom chamber are assessed with various cells in an upper chamber. (B) Effect of coculture stimulation of LNCaP on LNCaP cells and PC-3 on PC-3 cells. LNCaP cells in the upper chamber stimulated LNCaP cells in the lower chamber compared to no cells in the upper well (control to left of LNCaP; *p* < 0.001). In contrast, PC-3 cells in the upper well did not stimulate PC-3 cells in the bottom well compared to PC-3 control (left of PC-3) with no PC-3 cells in upper well. (C) Effect of LNCaP cells or HOB cells in upper chamber on LNCaP cells in lower chamber. Experiments were performed in triplicate ± SEM.

(LNCaP control), without LNCaP cells in the upper chamber (*p* < 0.001). To determine if a similar effect occurred in a hormone refractory cell line, PC-3 cells were also cocultured, as shown in Fig. 1B. The hormone refractory cell line PC-3, both cocultured (PC-3) and control (PC-3 control), had increased overall growth compared to the hormone sensitive LNCaP cell line. In contrast to the growth stimulation noted with LNCaP cells in coculture, PC-3 cells cultured in a bottom chamber were not stimu-

lated with PC-3 cells cultured in the upper chamber (PC-3) compared to a control without PC-3 cells in the upper chamber (PC-3 control). As an additional control, immortalized human osteoblasts (HOB) were tested, since prior studies have demonstrated the lack of growth stimulation, or even growth inhibition, of LNCaP cells in coculture by osteoblasts [8]. As shown in Fig. 1C, LNCaP cells cultured in the bottom chamber with LNCaP cells in an upper chamber were again stimulated compared to LNCaP cells without additional cells in the upper chamber (control). In contrast, LNCaP cells in the bottom chamber were not stimulated by HOB cells cultured in the upper chamber (HOB).

3.2 Effect of coculture on protein expression in LNCaP cells

To determine effects of coculture growth stimulation on protein expression, we used 2-D-DIGE and DeCyder software statistical analysis (Amersham Biosciences). Gels contained protein from three separate conditions each labeled with a different fluorescent dye (Cy2, Cy3, and Cy5): LNCaP control, LNCaP cells stimulated in coculture, and an internal standard (derived from an equal amount of protein from each experiment) for statistical analysis across multiple gels. As shown in Fig. 2, LNCaP cells without (Fig. 2A) or with additional LNCaP cells in coculture (Fig. 2B) in the upper chamber were compared. The maximum number of spots detected was 983 and was automatically assigned as the master; 346 spots were confirmed as true spots with significant volume for detection, and after excluding artifacts. Protein selection using paired *t*-tests with a p value ≤ 0.01 demonstrated the six most optimal spots (p values 0.01 to 0.00015) that increased in LNCaP cells cocultured with LNCaP cells in the upper chamber (Fig. 2D) compared to the controls of

unstimulated LNCaP cells (Fig. 2C). All other changes were less significant, giving confidence that these six spots were most altered by coculture stimulation. The actual data with matched spot density changes between control and stimulated cells are presented in Fig. 3 for the six spots. Also shown in Fig. 3, are the computer generated 3D images of protein volume changes between control and stimulated cells. These images demonstrate that the six spots contained sufficient protein.

3.3 Protein identification

To identify the six spots selected with 2-D-DIGE and DeCyder analysis, protein was loaded on Commassie gels, which were matched to the original 2-D-DIGE for spot picking. After spot picking, following in-gel digestion, peptides were identified using MALDI-TOF and ESI MS/MS, as shown in Table 1. The six proteins were identified using a protein database with significant sequence coverage of 45%–73%. All proteins were found to be in only one critical pathway. They were identified as enolase, phosphoglycerate kinase, aldolase, and trios-phosphate isomerase. Two additional proteins were also identified as enolase and phosphoglycerate kinase (spots numbers 2 and 4), further confirming the consistency of these data as changes in one pathway; these two additional proteins likely represent post-translational modifications that could be a focus of study in future efforts.

3.4 Coculture model with epithelial cell line and effect of Bcl-2

Given the possibility that nonmalignant cells may be more sensitive to autocrine effects in coculture, transformed rat prostate epithelial cells were studied. Cells were also
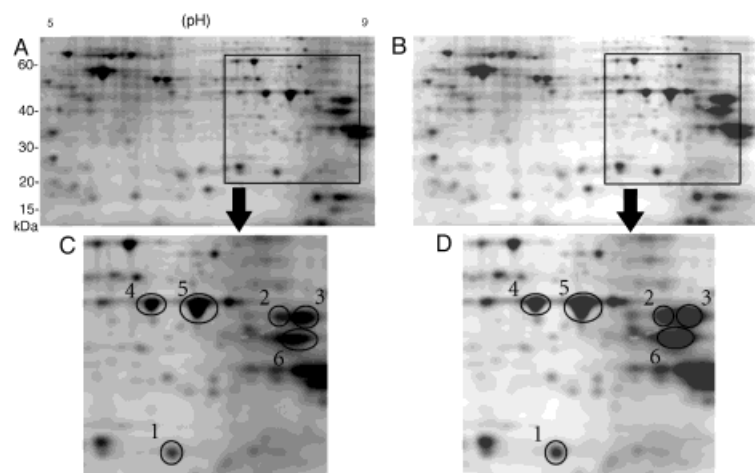

Figure 2. Effect of coculture on protein expression in LNCaP cells. LNCaP cells were isolated after culture for 72 h either without additional LNCaP cells (A and C), or after stimulation in coculture with additional LNCaP cells (B and D). To increase accuracy in determining the most significant protein changes, we used data from three individual experiments and duplicate gels that all had a common internal standard (six gels in total). As shown, the most significant protein changes (p values 0.01 to 0.00015) from the analysis (actual analysis is shown in Fig. 3) were represented by the six protein spots outlined.
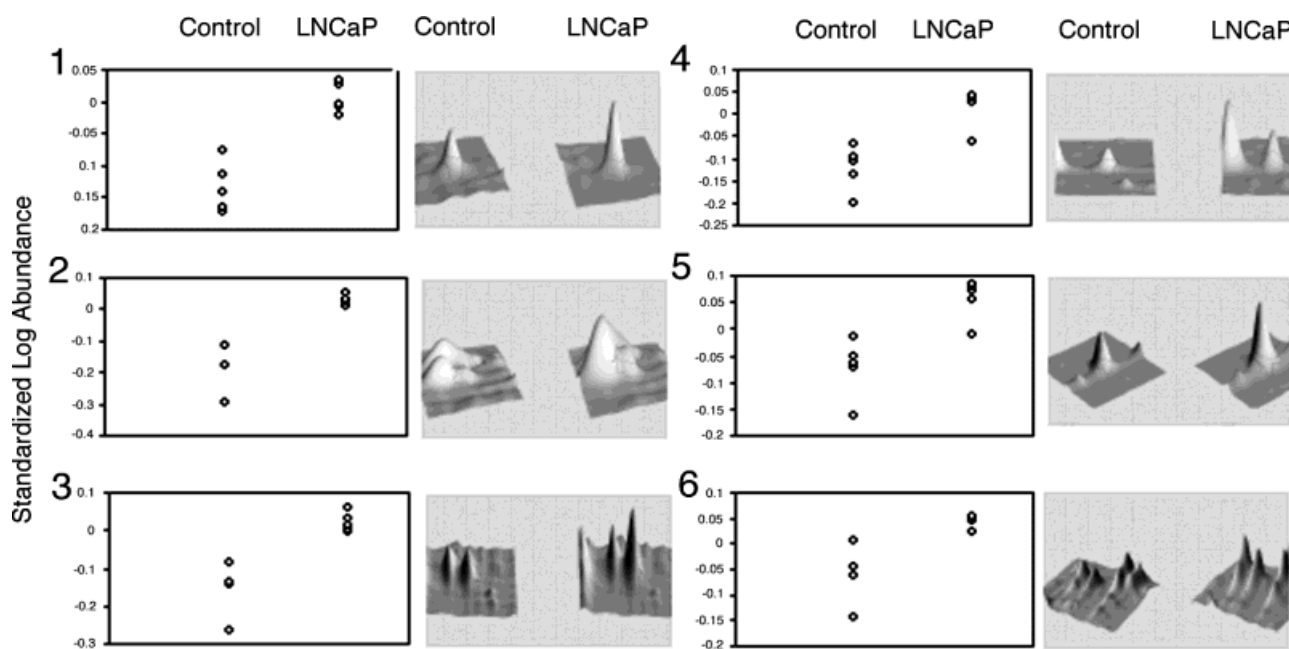
Figure 3. Decyder analysis of multiple experiments comparing LNCaP treated cells to untreated LNCaP (control) cells showing proteins with the most significant changes and 3D protein volume conformation. The protein numbers shown in Fig. 3 correspond to the numbered spots in Fig. 2. The actual *p* values of increase in each protein spot are listed in Table 1 with the NCBI accession numbers, theoretical p*I* and M_r.

Table 1. MS identification of proteins selected by 2-D-DIGE

Spot no.	*P* value for increase over control	Identification	Accession number (NCBI)[a]	p*I*	M_r	% Sequence coverage
1	0.00015	Triosephosphate isomerase 1	999892	6.5	26.5	73%
2	0.0016	Phosphoglycerate Kinase	4505763	8.6	44.6	45%
3	0.0017	Phosphoglycerate Kinase	129902	8.6	44.7	72%
4	0.0017	Enolase	4503571	7.0	47	48%
5	0.0025	Enolase	4503571	7.0	47	61%
6	0.010	Aldolase	229674	8.8	39.3	55%

a) NCBI accession numbers

transfected with a Bcl-2 expression vector (RP/B) or vector only control (RP/V), to begin to assess effects of resistance proteins commonly overexpressed in prostate cancer. Using immunoblotting, we assessed the difference in Bcl-2 expression in the Bcl-2 *vs.* vector transfected cells. As shown in Fig. 4, PC-3, LNCaP, and transfected RP/B cells overexpressed Bcl-2 protein abundantly compared to the vector only control (RP/V). To determine if growth stimulation could be detected in the *in vitro* model, as shown with LNCaP cells in Figs. 1B and C, we cocultured both RP/B and RP/V cells in a lower chamber with and without additional cells in the upper chamber. As shown in Fig. 5, the growth of RP/B cells or RP/V cells were
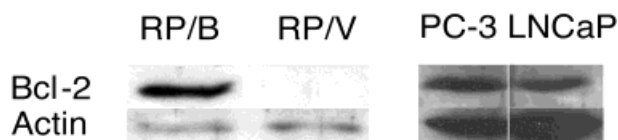

Figure 4. Transformed RP cells were derived from transfection of primary cells with vector encoding E1A (pCMVE1A) and the temperature sensitive mutant p53. Stable transfectants were made with the transformed cells with pcDNA3.1bcl-2 (RP/B) or vector control (RP/V). Bcl-2 was assessed by immunoblotting with a monoclonal antibody and actin control as previously described [6]. Bcl-2 was also assessed in PC-3 and LNCaP cells compared relative to actin.
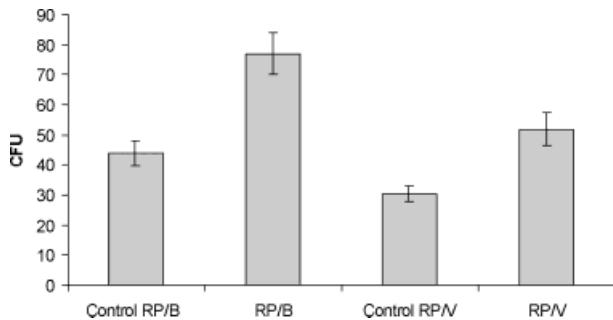
Figure 5. Effect of coculture on RP cells transfected with Bcl-2 (RP/B) or vector control (RP/V) compared with no cells in the top well (Control RP/B and Control RP/V respectively). Both RP/B and RP/V cells were stimulated to increase growth compared with controls ($p < 0.01$). All experiments were performed in triplicate $\pm$ SEM.

stimulated by additional cells in the upper chamber compared to control RP/B or control RP/V cells in the lower chamber without additional cells in the upper chamber ($p < 0.01$), similar to what was observed with LNCaP cells. Also shown in Fig. 5, Bcl-2 overexpression in RP/B cells was associated with increased growth in RP/B cells compared to RP/V cells overall, but growth stimulation occurred independently of overexpression of Bcl-2 in these cells. To begin to examine differences between the human LNCaP tumor cell line and the rat transformed epithelial cells, DIGE was also performed on RP/V and RP/B cell lines each with and without cocultured condi-

tions. As shown in Fig. 6, proteins found in the same region as spots 1 to 6 in LNCaP cells (Fig. 2) were not changed in RP/B cells in coculture (Fig. 6B) compared to RP/B control without coculture (Fig. 6A) or RP/V cells in coculture (Fig. 6D) compared to RP/V control without coculture (Fig. 6C).

4 Discussion

These data demonstrate a novel model capable of detecting paracrine and autocrine effects on cell growth and proteins. We found that LNCaP prostate cancer cells and an immortalized rat prostate cell line transfected to overexpress the antiapoptotic resistance protein Bcl-2 were stimulated to grow possibly through autocrine effects in our system. Using a proteomic approach with DIGE, four proteins were found to increase after autocrine induced growth stimulation in LNCaP cells. These proteins were all identified by MS as enzymes in the glycolytic pathway, validating the ability of this system to detect both clonogenic growth and the effect on proteins. Unique models capable of detecting growth factor stimulating effects on cells will be important to assess agents capable of modulating growth through inhibition of paracrine and autocrine pathway proteins. The novelty of our model compared to prior systems is the use of the same cells in coculture to detect autocrine effects, in addition to paracrine effects detected in systems with stroma in coculture; the use of cells in an upper chamber to get a
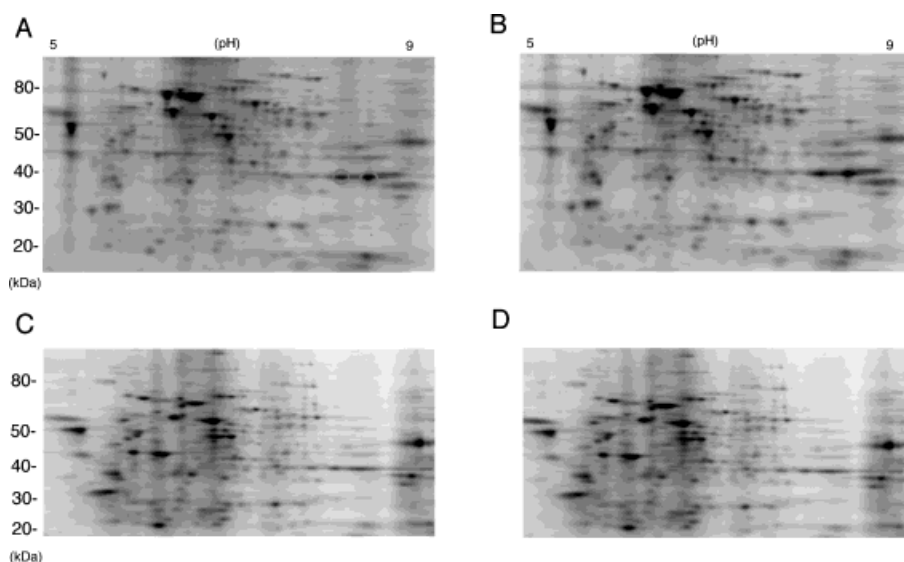

Figure 6. Effect of coculture on protein expression in RP/B and RP/V cells. RP/B or RP/V cells were isolated after culture for 72 h either without additional RP/B (A) or RP/V (C) cells, or after stimulation in coculture with additional RP/B (B) or RP/V cells (D). To increase accuracy in determining the most significant protein changes, we used data from three experiments with RP/B cells and a common internal standard. Proteins in a similar region to LNCaP changes in spots 1–6 were unchanged. Areas circled in A are in the same region as spots 4 and 5 of Fig. 2.

prolonged effect of autocrine secretion in media compared to prior studies using conditioned media; and the use of DIGE to improve our ability to detect effects at the protein level. The assessment of protein in such a model is especially important, since recent studies have demonstrated that the use of DNA microarray is not sufficient to detect many changes demonstrated by protein assessment [9]. Many prior coculture models have investigated the interaction between the stroma and tumor cells [8, 10, 11]. For example, Pinski *et al.* [8] demonstrated that immortalized human osteoblasts cocultured with LNCaP cells decreased cell growth, supporting the conclusion that paracrine inhibitory factors were produced by stroma to alter growth of tumor cells. The current study had a different focus, to study the interaction of tumor cells on tumor cells through release of autocrine factors or other effects on the microenvironment. As shown in Fig. 1, growth stimulation was detectable in this model, in contrast to prior studies demonstrating inhibition by stroma in coculture [8].

The detection of growth stimulation through media effects was further supported by the findings in our proteomic studies, as shown in Fig. 2, with the induction of multiple glycolytic enzymes. As shown in Fig. 3, the use of De-Cyder analysis with in gel comparisons revealed that the most significant protein changes detected were all enzymes in the glycolytic pathway. The use of DIGE allowed us to compare control and treatment groups within the same gel. Additionally, the use of a third Dye allowed us to label an additional pooled control that was loaded on all gels, allowing comparison with multiple repeated experiments [7]. This analysis is a powerful approach to limit nonspecific findings related to comparisons between gels, a major limitation of 2-D gel analysis comparing separate gels with only silver-staining, Coommassie staining, and Sypro Ruby staining [12, 13]. In fact, Tonge *et al.* [13] studied the variability in protein spot volume analyzed by DIGE in comparison to separate gel comparisons and found decreased variability in control protein with the use of DIGE. In addition to supporting the validity of LNCaP coculture as a model capable of detecting growth stimulation, this finding of glycolytic enzyme induction may have additional implications. The potential importance of glycolytic enzyme induction is highlighted in recent studies of early tumor progression and invasion. For example, Lu *et al.* [14] demonstrated that lactate and pyruvate stimulated accumulation of hypoxia-inducible factor 1alpha (HIF) in human glioma cell lines. They found that the accumulation of HIF under aerobic conditions required metabolism of glucose to pyruvate to prevent the degradation of HIF protein and to induce activation of several HIF activated genes, including vascular endothelial growth factor (VEGF), glucose

transporter 3, and aldolase A. Palayoor *et al.* [15] recently studied HIF as a potential target for therapy. They found that nonsteroidal anti-inflammatory agents reduced HIF in normoxic and hypoxic conditions associated with down-regulation of VEGF and glucose transporter-1. The preference of tumor cells for glycolysis will also result in increased acid production. Despite increased acid production, studies have demonstrated that tumor cells are capable of tolerating such conditions by maintaining a normal or alkaline pH [16]. Acidification of the extracellular environment may then lead to the destruction of normal tissue. Studies have demonstrated acid induced release of VEGF and degradation of extracellular matrix by proteolytic enzymes [16]. Despite the potential importance of the glycolytic phenotype in transformation and early invasion, the specific mechanisms that increase glycolysis early in cancer progression are unclear, and models to study these mechanisms as targets for intervention are needed.

We also found that this model of *in vitro* growth detected growth stimulation independent of Bcl-2 overexpression (Fig. 5). The overexpression of the antiapoptotic protein Bcl-2 is common in hormone refractory prostate cancer [1]. Additionally, agents that target the apoptotic pathway of Bcl-2 are commonly used and studied in the clinic for the treatment of prostate cancer [17, 18]. The finding of growth stimulation independent of Bcl-2 makes this model potentially more useful to test and discover agents that could add or synergize with agents that modulate Bcl-2, or other apoptotic pathway proteins. Additionally, the finding that protein expression changes between rat prostate epithelial cells (Fig. 6) and human cancer cells (Fig. 2) in coculture are different is not unexpected, supporting future opportunities to study such differences in these models.

5 Concluding remarks

In summary, these data established a simple proteomic coculture model of early growth simulation to study agents capable of modulating paracrine or autocrine stimulated growth. The effects of coculture stimulation in this model, were also independent of antiapoptotic pathways such as the overexpression of Bcl-2, providing a more specific test to discover agents capable of inhibiting autocrine mediated growth stimulation that is either dependent or independent of Bcl-2 expression. The finding of induction of multiple glycolytic enzymes in LNCaP cells demonstrates that this model can detect protein changes with DIGE associated with cell growth stimulation. These data, therefore, demonstrate a novel

coculture model for further study of novel agents and mechanisms responsible for the observed growth in this model.

6 References

[1] DiPaola, R. S., Patel, J., Rafi, M. M., *Hematol. Oncol. Clin. North Am.* 2001, *15*, 509–524.

[2] van der Poel, H. G., *Eur. Urol.* 2004, *45*, 1–17.

[3] Dancey, J. E., *Cancer Biol. Ther.* 2003, *2*, 601–609.

[4] Hainsworth, J. D., Mainwaring, M. G., Thomas, M., Porter, L. L., 3rd *et al.*, *Clin. Lung Cancer* 2003, *4*, 347–355.

[5] Blackledge, G., *J. Urol.* 2003, *170*, S77–83.

[6] DiPaola, R. S., Rafi, M. M., Vyas, V., Toppmeyer, D. *et al.*, *J. Clin. Oncol.* 1999, *17*, 2213–2218.

[7] Alban, A., David, S. O., Bjorkesten, L., Andersson, C. *et al.*, *Proteomics* 2003, *3*, 36–44.

[8] Pinski, J., Parikh, A., Bova, G. S., Isaacs, J. T., *Cancer Res.* 2001, *61*, 6372–6376.

[9] Waghray, A., Feroze, F., Schober, M. S., Yao, F. *et al.*, *Proteomics* 2001, *1*, 1327–1338.

[10] Greiff, A. H., Fischer, W. M., Sehgal, I., *Clin. Exp. Metastasis* 2002, *19*, 727–733.

[11] Brankin, V., Mitchell, M. R., Webb, B., Hunter, M. G., *Reprod. Biol. Endocrinol.* 2003, *1*, 55.

[12] Adam, B. L., Vlahou, A., Semmes, O. J., Wright, G. L., Jr., *Proteomics* 2001, *1*, 1264–1270.

[13] Tonge, R., Shaw, J., Middleton, B., Rowlinson, R. *et al.*, *Proteomics* 2001, *1*, 377–396.

[14] Lu, H., Forbes, R. A., Verma, A., *J. Biol. Chem.* 2002, *277*, 23111–23115.

[15] Palayoor, S. T., Tofilon, P. J., Coleman, C. N., *Clin. Cancer Res.* 2003, *9*, 3150–3157.

[16] Gatenby, R. A., Gawlinski, E. T., *Cancer Res.* 2003, *63*, 3847–3854.

[17] Thalasila, A., Poplin, E., Shih, J., Dvorzhinski, D. *et al.*, *Cancer Chemother. Pharmacol* 2003, *52*, 119–124.

[18] DiPaola, R. S., Aisner, J., *Semin. Oncol.* 1999, *26*, 112–116.