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14. ABSTRACT The cytokine interleukin-6 (IL-6) is strongly implicated in primary prostate cancer (PrCa) growth and the progression to bone metastasis. While expression and localization of IL-6 and its receptors gp130 and IL-6R have been studied in organ-confined PrCa, these key mediators of the IL-6/gp130 signaling pathway have not been previously assessed in prostatic bone metastases. Thus far, our investigations with archival patient biopsies revealed that all PrCa bone metastases examined (n=14) expressed IL-6 on an overwhelming majority of cells (78±5%). The IL-6 receptor (IL-6R) was expressed in 11/14 cases in 77±7% of PrCa cells. Activated (phosphorylated) gp130 was expressed in all but one case (13/14), and was expressed in the majority of cells (79±5%) in 9/14 cases. Importantly, when IL-6R was localized to cell membranes (4/11 cases), phospho-gp130 and IL-6 were also detected at the cell membrane. Thus, members of the IL-6/GP130 axis are present in a high proportion of bone metastatic PrCa cells in most cases. The importance of this pathway will be further elucidated by characterizing anti-IL-6-treated BM18 transplants. Understanding the role of IL-6/gp130 signaling in this disease may lead to identification of novel targets and therapeutic strategies to improve and extend the quality of life for PrCa patients.					
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Introduction

Prostate cancer (PrCa) is the leading cause of male cancer death in Western civilization. Interleukin-6 (IL-6) is a cytokine implicated in primary PrCa growth and survival post-androgen withdrawal. Moreover, IL-6 is strongly associated with bone metastasis, a major cause of morbidity in PrCa patients. This project aims to investigate the IL-6 axis in a novel *in vivo* PrCa xenograft model (BM18) of androgen-dependent growth (McCulloch et al., 2005) and osteosclerotic bone metastasis. The IL-6/gp130 signaling pathway will also be investigated using PrCa bone metastases from human patient biopsies. Pursuit of the aims outlined herein will greatly improve our understanding of the role of IL-6/gp130 signaling in prostate cancer growth, regression, survival post-androgen withdrawal and bone metastasis. This study provides a unique opportunity to examine the role of IL-6/gp130 signaling in a model that closely mimics human PrCa growth, progression and regression as well as osteosclerotic bone remodeling. Disease recurrence and osteosclerotic metastases are poorly understood processes that cause immense suffering, culminating in a painful demise. Understanding the role of IL-6/gp130 signaling molecules in this disease may lead to identification of novel targets and therapeutic strategies to improve and extend the quality of life for PrCa patients.

Body

Aim 1. Characterize the role of IL-6 in prostate cancer cell survival, post-androgen withdrawal

Task 1. To obtain BM18 tumor tissue that has regressed post-androgen withdrawal +/- IL-6 neutralization, or IL-6 neutralization alone, at time-points <1 week before the tumor regresses to non-detectable.

All animal experiments received ethical approval to be carried out at the St. Vincent's campus BioResources Centre when our laboratory was located at the Bernard O'Brien Institute of Microsurgery. Our laboratory has since been relocated to the Monash Institute of Medical Research (refer to my Request for Transfer letter dated 26 September, 2006). We immediately sought ethical approval for our animal experiments specifically to address Task 1 upon our arrival at the new facility. Unfortunately, despite vigorous pursuit of approval and consultations with key committee members over many months, approval has yet to be granted. However, we have been assured that approval is imminent. Therefore, we have been unable to pursue Task 1 thus far but are ready to initiate the required work as soon as approval has been granted and are confident that it can be completed within the timeframe of this fellowship.

Task 2. To characterize the expression of IL-6 related signaling molecules and apoptosis in archival BM18 tumor tissue:

2a) Carry out Western immunoblotting (15 antibodies) from protein (whole cell lysate or membrane/nuclear preparations) extracted from fresh and/or frozen BM18 tumour tissue.

Positive controls will include protein extracted from a cohort of readily available prostate cancer cell lines.

Thus far, optimization of detection by Western immunoblotting has been initiated for members of the IL-6/gp130 signaling pathway including IL-6, IL-6R, (activated) phospho-gp130, STAT1, STAT3, STAT6 and (activated) phospho-Jak2. All cell lysates were prepared in RIPA buffer with potent proteolytic and phosphatase inhibitors (Pierce Biotechnology) to preserve the structural integrity and phosphorylation status of all proteins. Proteins were separated by SDS-PAGE (Laemmli, 1970; Shapiro et al., 1967; Weber and Osborn, 1969) with a 4% stacking and 10% resolving gel and transferred to nitrocellulose membranes using standard protocols (Towbin et al., 1979). Membranes were then probed with antibodies and the targets visualized with the Li-

Cor Odyssey system (700 and 800nm color) or with the Pierce Biotechnology SuperSignal West Femto Chemiluminescence kit (grayscale).

A band of appropriate molecular mass (**x kDa**) corresponding to IL-6 was detected in BM18 tissue (Fig. 1a).

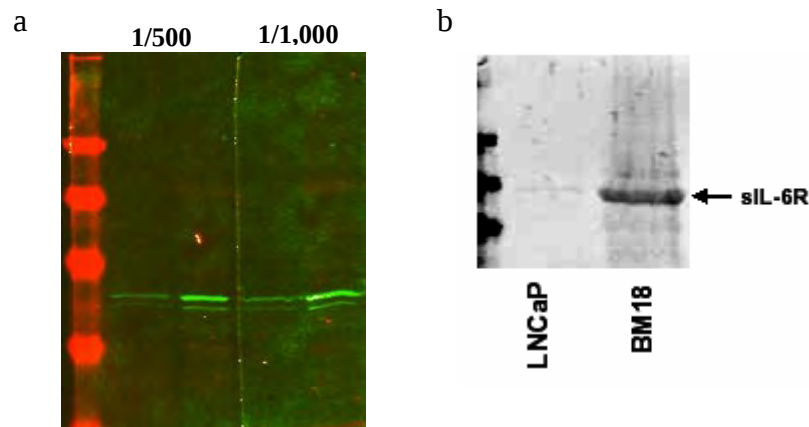


Figure 1 (a) Interleukin-6 was detected (Santa Cruz antibody) in BM18 xenograft tissue (lanes 2, 4) and LNCaP cells (lanes 3, 5), exhibiting doublet bands. The interleukin-6 receptor was detected (1/200, R&D Systems antibody) in BM18 xenograft tissue (b), with a molecular mass corresponding to the soluble form (sIL-6R).

Interestingly, the samples exhibited doublet IL-6 bands. Possible reasons for this unexpected finding may include post-translational modifications (e.g. phosphorylation, glycosylation) on some of the protein, partial digestion of the target due to inadequate proteolysis inhibition, non-specific bands and novel alternatively-spliced variants. IL-6 is not known to be post-translationally modified and the lysate was maintained at a maximum of 4°C with potent proteolysis inhibitors, rendering these explanations unlikely. To resolve the question of non-specificity, membranes could be blocked with IL-6 binding partners IL-6R and/or gp130 prior to IL-6 detection. The band that disappears would correspond to IL-6 while that which persisted would be non-specific. A more intriguing explanation would be provided if both bands disappeared, suggesting that the latter possibility (splice variants) was in evidence. Indeed, investigators have recently discovered novel IL-6 splice variants in peripheral blood mononuclear cells (Kestler et al., 1995) and renal cell carcinoma (Alberti et al., 2005). Confirmation of this explanation could be achieved by using the RT-PCR primers for human IL-6 designed by Kestler et al. to look for two different products.

Soluble receptors exist for several cytokines, allowing regulation of cytokine effects by sequestering their ligand to reduce its bioavailability (Muller-Newen et al., 1998). However, the

IL-6 signaling pathway is more complex. While membrane-bound IL-6R is restricted to the few cell types targeted by IL-6 in the ‘classical’ signaling pathway, the soluble form is an agonist that facilitates more promiscuous activation of IL-6 responses via the ubiquitously expressed gp130 co-receptor in a mechanism termed ‘transsignaling’ (Kallen, 2002). In BM18 xenograft tissue, the soluble form of IL-6R was detected by immunoblotting (Fig. 1b). Interestingly, osteoblasts appear to control their own responses to IL-6 by shedding membrane-bound IL-6R to its soluble form (Vermes et al., 2002) which could lead to enhanced activation of STAT3 resulting in bone formation (Itoh et al., 2006). It is perhaps no coincidence that BM18 exhibits induces an osteoblastic response and bone remodeling when co-transplanted with bone *and* expresses sIL-6R exclusively rather than membrane-bound IL-6R. Indeed, soluble IL-6R is important in many disease states, including cancer (Heinrich et al., 1998).

BM18 tissue was examined for the presence of the activated IL-6 co-receptor phospho-gp130 (Fig. 2).

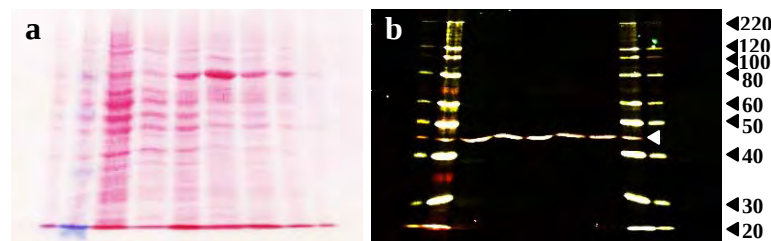


Figure 2 Nitrocellulose membranes were stained with Ponceau S to confirm equal protein loading and successful transfer (a) and probed for pan-actin (b, *white arrow head*) and phospho-gp130. Molecular mass standards (kDa, *black arrow heads*) were loaded in outer lanes surrounding 15µg protein samples from LNCaP, PC-3, BM18 05-21L, BM18 06-7 (androgen-independent subline) and BM18 05-08. Antibodies for pan-actin (Neomarkers) and phospho-gp130 (Santa Cruz) were utilized at 1/5,000 and 1/250, respectively.

Robust signals were detected for all samples at ~45kDa, corresponding to the housekeeping protein actin (~42kDa), thereby demonstrating successful technique. However, phospho-gp130 was not detected in any sample despite the expected expression of this protein in all samples (Palmer et al., 2004). This may have been due to relative lack of gp130 activation, although the detection of IL-6 and sIL-6R suggested that gp130 activation would be present and should therefore yield a detectable phospho-gp130 signal at least in some samples. Alternatively, other explanations include inadequate protein loading, poor transfer efficiency of high molecular mass

proteins as evidenced by the fainter Ponceau S staining at the top end of the membrane, and issues of antibody concentration or affinity. Possible solutions include loading more protein (30µg), using more primary antibody, a lower percentage gel and addition of 0.1% (w/v) SDS to the transfer buffer to facilitate greater transfer efficiency of high molecular mass proteins. All strategies are currently being vigorously pursued.

IL-6/gp130 signaling ultimately leads to activation of signal transducers and activators of transcription (STATs) -1 and -3 in the cytoplasm, whereupon homo- or hetero-dimerization of STATs occurs, followed by translocation to the nucleus and DNA binding of the dimer to modulate transcription of target genes (Heinrich et al., 1998). Western immunoblotting was utilized to determine the expression levels of STATs 1 and 3 in various PrCa cell lines and BM18 xenograft samples (Fig. 3).

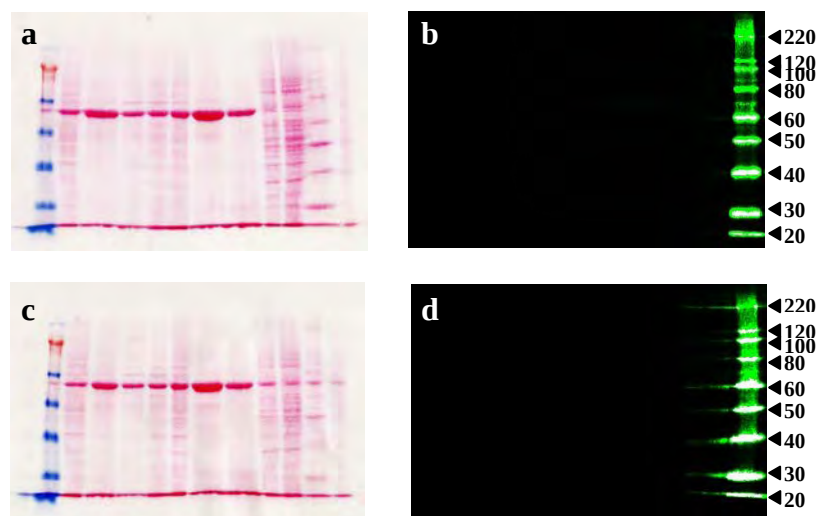


Figure 3 Protein samples (10µg) from LNCaP, PC-3, BM18 06-2L (regressed), BM18 05-3L (+androgen after regression), BM18 05-21L, BM18 06-7 (androgen-independent subline), BM18 05-1L (regressed), BM18 05-9 (+androgen after regression) and BM18 08-05 were separated by SDS-PAGE, transferred to nitrocellulose membranes, stained with Ponceau S (a, c) and probed for STAT1 (b) and STAT3 (c) (1/1,000, Cell Signaling Technology). Molecular mass standards were loaded in the final lane (kDa, *black arrow heads*).

Despite exposure beyond saturation levels, no signal for either STAT protein was visualized, possibly due to inadequate protein loading and/or primary antibody concentration. Therefore, higher protein loading and antibody concentration was utilized in the following attempt (Fig. 4).

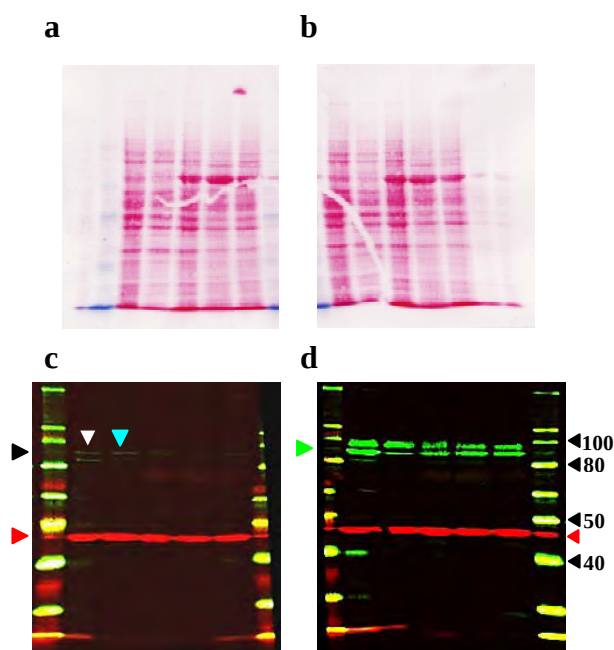


Figure 4 A set of protein samples (20 μ g) from LNCaP and PC-3 PrCa cell lines and BM18 05-21L, BM18 06-7 (androgen-independent subline) and BM18 05-08 xenograft tissues was loaded twice in tandem, separated by SDS-PAGE and transferred to nitrocellulose membranes as confirmed by Ponceau S staining (a, b). Using the Ponceau S as a guide, the membrane was cut in half vertically to allow probing with rabbit polyclonal antibodies (1/250, Cell Signaling Technology) to STAT1 (c) and STAT3 (d). Both membrane halves were probed for pan-actin (1/5,000 Neomarkers) as a positive control (*red arrow heads*).

A faint doublet of bands was detected in LNCaP cells (4c, *white arrow head*) corresponding to phosphorylated and unmodified STAT1 (~91kDa); PC-3 exhibited a single faint band of a size corresponding to phospho-STAT1 (4c, *turquoise arrow head*). There was no apparent STAT1 signal detected in the BM18 xenograft samples. Given the low signal intensity of the ‘positive controls’, presumably only very high expression would be detectable in the conditions tested. Therefore, neither the presence nor the absence of STAT1 in BM18 can be confirmed. Possible strategies to improve signal intensity in BM18 samples include the use of the Pierce Biotechnology SuperSignal West Femto Chemiluminescence system and/or amplification of the signal with an additional step utilizing biotinylated secondary antibodies followed by streptavidin-HRP. We are also attempting to acquire the SK-MEL-28 cell line used by the antibody manufacturer as a positive control.

Doublet bands of a size corresponding to unmodified and phosphorylated STAT3 (~86kDa) were readily detected in all samples (d, *green arrow head*), demonstrating that prostate cancer cell lines and xenograft tissues exhibit high levels of STAT3 protein and activity.

An analysis integrating three microarray datasets was recently reported, concluding that the gene encoding STAT6 was one of only two identified as a “robust marker” of prostate cancer (Xu et al., 2005). However, ‘classical’ STAT6 activation is initiated by IL-4 (Boothby et al., 1988) and/or IL-13 (Izuhara et al., 1996) but has not been reported to stem from IL-6 signaling. Therefore, STAT6 expression was examined as a potential prostate cancer marker responsive to non-IL-6-type cytokines to determine whether signaling via non-IL-6-type cytokine pathways was feasible in BM18 PrCa cells (Fig. 5).

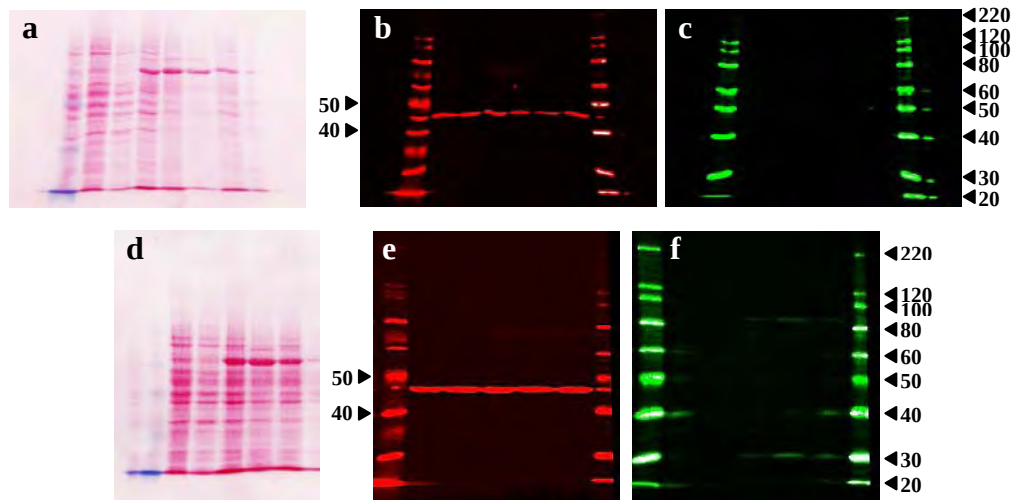


Figure 5 Transfer of 10 μ g protein from LNCaP, PC-3, BM18 05-21L, BM18 06-7 (androgen-independent subline), BM18 05-1L (regressed) and BM18 05-08 to nitrocellulose was confirmed by Ponceau S staining (a). The membrane was probed for pan-actin (b; 1/5,000, Neomarkers) and STAT6 (c; 1/250, Cell Signaling Technology). Ponceau S staining demonstrated equal loading and efficient transfer of 20 μ g protein samples from LNCaP and PC-3 PrCa cell lines and BM18 05-21L, BM18 06-7 (androgen-independent subline) and BM18 05-08 xenograft tissues, with molecular mass standards loaded on either end. Pan-actin (e) and STAT6 (f) were detected as described above.

Once again, pan-actin was a reliable positive control, demonstrating robust signals in all 10 μ g and 20 μ g samples (Fig. 5b, e). As there was no detectable STAT6 present in the 10 μ g samples (Fig. 5c), protein loading was doubled but still did not yield any specific bands corresponding to STAT6 (Fig. 5f). Without confirmation of antibody efficacy using a bona fide positive control,

the STAT6 status of these samples remains unresolved. We are attempting to acquire the Jurkat and HeLa cell lines used by the antibody manufacturer as positive controls. Lysates from these cell lines will enable confirmation of the STAT6-negative status of BM18 tissue or point to other technical obstacles such as low antigen abundance. If the positive control lysates also fail to yield STAT6 bands, attempts will be made to further amplify the signal as described for STAT1 detection (above).

Additional evidence consistent with an activated IL-6/gp130 signaling pathway includes the presence of the activated signal transducer phospho-Jak2, though its activation can also be stimulated by other IL-6-type cytokines (Stahl et al., 1994). BM18 samples were therefore examined for phospho-Jak2 expression (Fig. 6).

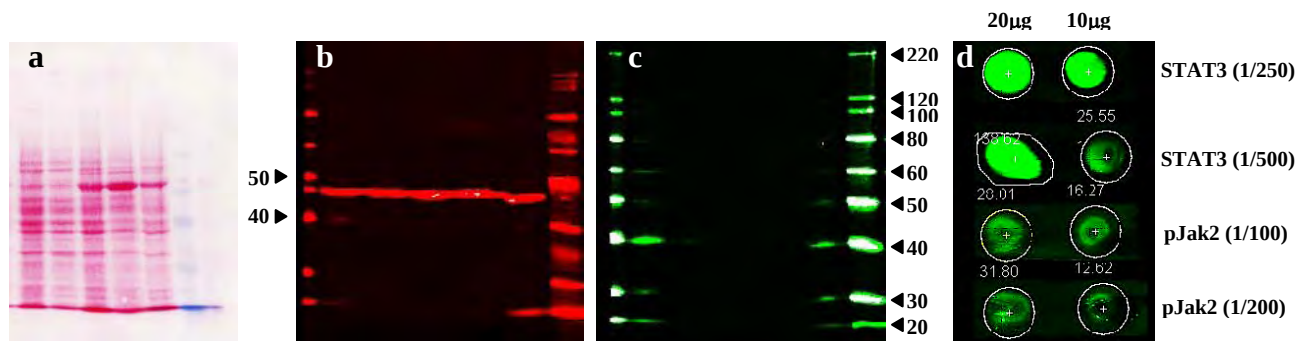


Figure 6 Protein samples (20µg) from LNCaP and PC-3 PrCa cell lines and BM18 05-21L, BM18 06-7 (androgen-independent subline) and BM18 05-08 xenograft tissues were transferred to nitrocellulose and visualized with Ponceau S (a). The membrane was probed for pan-actin (b; 1/5,000, Neomarkers) and phospho-Jak2 (c; 1/250, Cell Signaling Technology). Protein from LNCaP cells was examined for STAT3 and phosphor-Jak2 expression by dot blot (d).

While pan-actin was detected in all samples as usual (Fig. 6b), bands corresponding to the 125kDa phosphor-Jak2 were not in evidence at the top of the membrane (Fig. 6c). Though the 220 kDa molecular mass standard protein was faintly visible in the 800nm (green) channel (Fig. 6c), the Ponceau S staining of the membrane demonstrated that the transfer efficiency of the higher molecular mass proteins was completely inadequate (Fig. 6a). In order to circumvent the inadequate transfer efficiency and evaluate the affinity of the phospho-Jak2 antibody, dot blots were generate by spotting LNCaP lysate directly onto strips of nitrocellulose membrane and these were probed for STAT3 (positive control) and phosphor-Jak2 at various primary antibody dilutions (Fig. 6d). The STAT3 antibody yielded a robust signal at all protein amounts and antibody concentrations. In contrast to the previous attempt, the phospho-Jak antibody also gave

rise to a detectable signal, though the specificity cannot be ascertained without sizing the target. If the phosphor-Jak2 signal is specific, the results suggest that improving transfer efficiency could allow detection of this target in samples with as little as 10µg protein. The total amount of signal for each spot was determined as the integrated intensity using the Odyssey analysis software (version 2.1). Given the amount of protein spotted in the left column was double that of the right column, a 2:1 ratio of signal intensity would be expected. In both cases, the ratio was closest to 2:1 with the higher antibody concentration. Future efforts to detect phospho-Jak2 will focus on improving the transfer efficiency of high molecular mass proteins by lowering gel strength and adding 0.1% (w/v) SDS to the transfer buffer. If not successful, further optimization of transfer conditions may include reducing methanol in the transfer buffer to reduce shrinkage and tightening of gel pores and increasing voltage and/or time of transfer.

2b) Extract protein from fresh BM18 xenograft tissue collected from each of the experimental groups in Task 1b and examine key components of the IL-6 signaling cascade by Western blot analysis, optimized in Task 1b.

Please refer to Task 1 (above).

2c) Measure apoptosis and cellular proliferation in formalin fixed paraffin embedded BM18 xenograft tissue sections also collected from each of the experimental groups in Task 1b. (Months 4-12)

Please refer to Task 1 (above).

Aim 2. Describe the IL-6 signaling cytokines and their respective receptors in human prostate cancer bone metastases.

Task 1. To characterize key components of the IL-6 axis in PrCa bone metastases from patient biopsies, i.e. examining which cells express particular proteins of the axis:

1a) To perform immunohistochemistry on formalin-fixed, paraffin wax embedded PrCa bone metastasis tumours obtained from PrCa patient biopsies (currently n=40) to examine the expression and localization of the IL-6 family of cytokines and their respective receptors.

While expression and localization of IL-6 and gp130 has been studied in organ-confined PrCa, the key mediators of the IL-6/gp130 signaling pathway have not been previously assessed in

prostatic bone metastases. We therefore examined a cohort of 14 bone metastases from human patients for expression and localization of IL-6, IL-6R and phospho-gp130 using immunohistochemistry.

Table I Average frequency of cells positive for IL-6R, phospho-gp130 and IL-6 in human bone metastasis samples.

Sample	IL-6R	Phospho-gp130	IL-6
BM19	100	97	88
BM21	85	98	77
03R56	93	72	87
BM11	71	97	60
155938	100	27	98
BM5	61	75	75
244210	92	20	93
958580	97	25	80
BM18b	67	67	63
BM7	59	63	37
BM8	0	64	82
193970b	0	77	59
180606B	21	0	88
169463	0	1	98

All cells in all fields of at least three sections from each patient biopsy were scored for the frequency of IL-6R, phospho-130 and IL-6 positive cells.

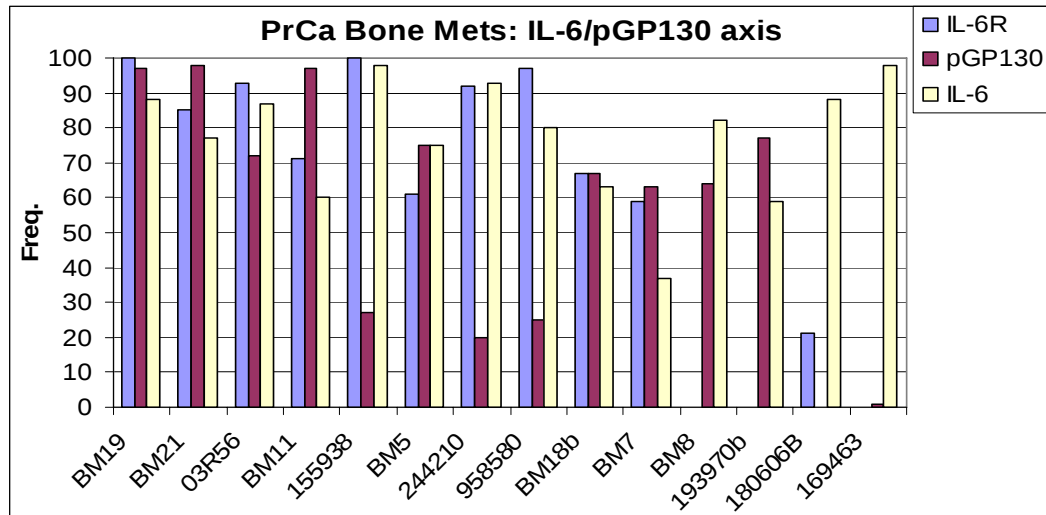


Figure 7 Frequency of cells positive for IL-6R, phospho-gp130 (phospho-gp130) and IL-6 in human patient bone metastasis biopsies.

Thus far, our investigations with archival patient biopsies have revealed that all PrCa bone metastases examined ($n=14$) expressed IL-6 on an overwhelming majority of cells ($78\pm5\%$). The IL-6 receptor was expressed in 11/14 cases by $77\pm7\%$ of PrCa cells. Activated phospho-gp130 was expressed in all but one case (13/14), and was expressed in the majority of cells ($79\pm5\%$) in

9/14 cases. Thus, members of the IL-6/gp130 axis are present in a high proportion of bone metastatic PrCa cells in most cases.

To measure the overall expression levels of these markers within the bone metastasis samples, all cells within all fields of at least three sections of each biopsy were scored from 0-3 with respect to staining intensity and the results averaged (Table II, Fig. 8).

Table II Average intensity of IL-6R, phospho-gp130 and IL-6 staining in human bone metastasis samples.

Sample	IL-6R	phospho-gp130	IL-6
03R56	59	36	57
958580	57	8	66
155938	37	10	81
244210	58	7	60
BM19	38	35	52
BM5	26	48	51
BM21	29	33	42
BM11	26	34	43
BM18b	27	22	45
BM8	0	22	53
193970b	0	46	23
180606B	13	0	54
BM7	20	24	21
169463	0	0	61

Cells with no signal were scored with intensity = 0. All positive cells were scored from 1 (low) to 3 (high). To calculate a measure of average overall intensity for each marker, the frequency of positive cells with a given intensity was multiplied by that intensity and the frequency-intensity products for the degrees of intensity 1-3 were averaged. For example, in scoring the intensity of IL-6R in sample 03R56, it was found that 40% of positive cells were intensity = 3 (40 x 3), 5% of positive cells were 2 (5 x 2) and 48% of positive cells were scored 1 (48 x 1). Therefore, the average overall intensity is

calculated as $((40 \times 3) + (5 \times 2) + (48 \times 1)) / 3 = 59$ of a possible 100 (Table II, row 2, column 2).

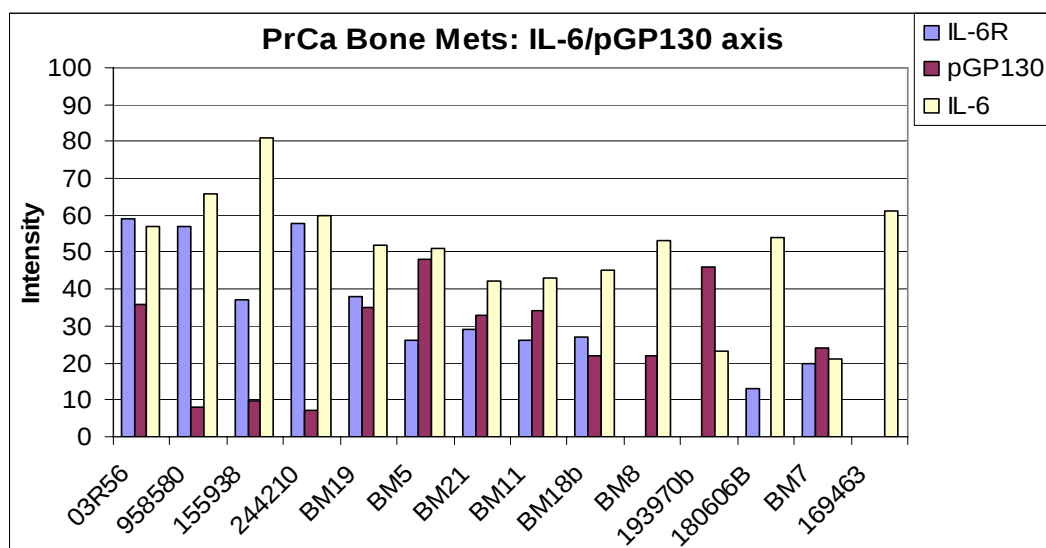


Figure 8 Intensity of staining in cells positive for IL-6R, phospho-gp130 (phospho-gp130) and IL-6 in human bone metastasis samples.

For IL-6R, the overall average intensity of positive cells in all samples was 35 ± 16 of a possible 100. For phospho-gp130, the overall average intensity of positive cells in all samples was 27 ± 14 of a possible 100, and for IL-6 the overall average intensity of positive cells was 51 ± 16 of a possible 100. Clearly, significant levels of IL-6R, phospho-gp130 and IL-6 were present in this cohort of bone metastases. Together with the high frequency of expression of these markers, these data provide strong presumptive evidence that the IL-6/gp130 signaling axis is an important pathway in prostate cancer bone metastases.

Key Research Accomplishments

- Successfully performed harvest and serial transplantation of BM18 xenografts into SCID mouse recipients
- Developed high-throughput method of assaying protein concentration in 96-well plate format using BMG Labtech's FLUOStar Optima fluorescent plate reader
- Achieved competency in SDS-PAGE and Western Immunoblotting techniques
- Achieved competency in near-infrared fluorescent detection of Western Immunoblot targets with visualization on the Li-Cor Odyssey imaging system
- Achieved competency in visualization of Western Immunoblot targets using the Pierce Biotechnology SuperSignal West Femto Chemiluminescence system
- Optimized detection of pan-actin, IL-6, sIL-6R and STAT3 in PrCa cell lines and BM18 xenograft tissue by Western immunoblotting
- Characterized frequency and intensity of IL-6R, phospho-gp130 and IL-6 expression in a cohort of human prostate cancer bone metastases that to our knowledge has only ever been reported in organ-confined prostate cancer

Reportable Outcomes

None to report thus far.

Conclusion

Training initiatives have been undertaken to achieve competency in some of the fundamental techniques required to undertake this research project, including BM18 xenograft harvest and transplantation, SDS-PAGE and Western Immunoblotting. BM18 xenograft were successfully harvested and serially transplanted to SCID mouse recipients with a 100% take rate.

Thus far, optimization of conditions for the detection of various IL-6/gp130 signaling molecules has progressed and revealed that BM18 xenografts express IL-6, the soluble form of the IL-6 receptor (sIL-6R and STAT3 in both unmodified and activated phosphorylated form; further optimization is required to determine the STAT1, phospho-gp130 and phospho-Jak2 status of BM18 PrCa cells.

Immunohistochemical studies of a cohort of human patient bone metastases has demonstrated conclusively for the first time in non-organ-confined prostate cancer that the members of IL-6/gp130 signaling complex are highly expressed in most cases.

Future work will endeavour to block IL-6 signaling with a neutralizing antibody in BM18 transplant recipients to determine the functional importance of the IL-6/gp130 signaling axis in PrCa growth, survival post-androgen withdrawal and osteosclerotic bone remodelling.

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