MODULATION OF PARACRINE INTERACTIONS BETWEEN PROSTATE CANCER CELLS AND BONE MARROW STROMAL CELLS BY TRANSFORMING GROWTH FACTOR-BETA SIGNALING DURING COLONIZATION OF BONE

by

Senem Kurtoglu

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

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Dedicated to my loving family.

This journey would not be possible without the unconditional support and encouragement of my family.

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LIST OF ABBREVIATIONS

AI	Androgen insensitive
ALK	Activin receptor-like kinase
ANOVA	Analysis of variance
AR	Androgen receptor
AS	Androgen-sensitive
ATF2	Activating transcription factor 2
BCa	Breast cancer
BMEC	Bone marrow endothelial cell
BMP	Bone morphogenetic protein
BMPRII	Bone morphogenetic protein receptor type II
BMSC	Bone marrow stromal cell
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
BSP	Bone sialoprotein
C4-2	LNCaP derivative, castrate resistant cell line derived from subcutaneous tumor grown in castrated athymic male mouse host
C4-2B	Castrate resistant cell line derived from spontaneous bone metastasis of orthotopically injected C4-2 in castrated athymic male mouse host

CAF	Cancer-associated fibroblast
cAMP	Cyclic adenosine 3',5'-monophosphate
CDK	Cyclin-dependent kinase
CgA	Chromogranin A
CM	Conditioned medium
c-Met	Receptor tyrosine kinase for hepatocyte growth factor
CR	Castrate-resistant
CRPC	Castrate-resistant prostate cancer
CXCR4	C-X-C chemokine receptor type 4
DAP	Death-associated protein
DKK-1	Dickkopf-related protein-1
DMEM	Dulbecco's Modified Eagle Medium
DN-TβRII	Dominant negative transforming growth factor beta receptor type II
DU145	Androgen receptor null prostate cancer cell line isolated from brain metastasis
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
Erk	Extracellular signal-regulated kinase
ET-1	Endothelin-1
EtOH	Ethanol
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
Fsk	Forskolin
HBME	Human bone marrow endothelial cell
HEK 293T	Human embryonic kidney cell line constitutively expressing the simian virus 40 large T antigen
HGF	Hepatocyte growth factor
HHT	Hereditary hemorrhagic telangiectasia
hK2	Human kallikrein 2
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL-6	Interleukin-6
JNK	c-Jun N-terminal kinase
LAP	Latency-associated protein
LLC	Large latent complex
LNCaP	Androgen sensitive human prostate cancer cell line derived from subclavicular lymph node metastasis
LTBP	Latent transforming growth factor beta-binding protein
MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCF7	Human breast adenocarcinoma cell line
MCF10A	Immortalized, non-transformed human mammary epithelial cell line

M-CSF	Macrophage colony-stimulating factor
MDA-MB-231	Highly metastatic human breast adenocarcinoma cell line
MMP	Matrix metalloprotease
NE	Neuroendocrine
NED	Neuroendocrine differentiation
NSE	Neuron specific enolase
NIH 3T3	Mouse embryonic fibroblast cell line
OC	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PC-3	Androgen receptor null prostate cancer cell line
PC-3M	Metastatic variant of PC-3 cell line
PCa	Prostate cancer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3-K	Phosphoinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PKA	Protein kinase A
PNGase F	Peptide-N-Glycosidase F
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTH	Parathyroid hormone

PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
Rb	Retinoblastoma
RGD	Arginine-glycine-aspartate motif
RIPA	Radioimmunoassay precipitation buffer
RPMI	Cell culture medium developed at Roswell Park Memorial Institute
Runx2	Runt-related transcription factor-2
SB-431542	Inhibitor of transforming growth factor beta receptor type I kinase (ALK-4/5/7) activity
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell-derived factor-1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	small-hairpin RNA
siRNA	small-interfering RNA
SLC	Small latent complex
Smad	Mothers against decapentaplegic homolog
TAK	Transforming growth factor beta-activated kinase
TβRI	Transforming growth factor beta receptor type I
TβRII	Transforming growth factor beta receptor type II
TβRIII	Transforming growth factor beta receptor type III

TGF-β	Transforming growth factor beta
TIEG	Transforming growth factor beta-inducible early gene
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TSP-1	Thrombospondin-1
uPA	Urokinase-type plasminogen activator
ZR-75-1	Osteoblastic human breast carcinoma cell line

ABSTRACT

Prostate cancer (PCa) is the most prevalent cancer and the second leading cause of cancer related death for men in the United States. Death is primarily due to bone metastasis with more than 80% of men who die of PCa having bone involvement at autopsy. The complex bone microenvironment may initially resist the newly resident PCa cells but PCa cells acquire adaptive changes that allow them to survive and grow in the "hostile" new microenvironment as they co-evolve in their genotypic and phenotypic characters with bone cells. We have previously shown that soluble factors released from immortalized human bone marrow stromal cells (BMSC) induce apoptosis of PCa cells, and the surviving cells undergo neuroendocrine differentiation (NED), characterized by morphological changes consistent with a neuroendocrine phenotype. The presence of neuroendocrine tumor cells in PCa is associated with aggressiveness, resistance to hormonal therapy, and poor prognosis. Using the LNCaP progression model of increasingly metastatic and castrate-resistant prostate cancer (CRPC) cell lines, I examined the influence of BMSC factors on PCa survival, using the HS-5 and HS-27a BMSC lines, which were characterized previously for their ability to support different stages of hematopoiesis. Neuroendocrine markers were elevated in PCa cells surviving HS-5 BMSC conditioned medium (CM) treatment, while differentiation markers such as androgen receptor (AR) and prostate-specific antigen (PSA) were decreased. PCa

cells that undergo NED using HS-5 BMSC CM or serum withdrawal had elevated phosphorylated-Smad2 levels. Furthermore, NED of PCa cells prevented HS-5 BMSC-induced apoptosis.

Cell death induced by BMSC CM was analyzed using live/dead analysis while the effect on cell growth was examined in soft agarose colony formation assays in the presence and absence of intact TGF-β signaling. Using immunoblotting and luciferase reporter assays, I measured levels and activity of phosphorylated-Smad2 in PCa cells surviving treatment with HS-5 BMSC CM. Treatment of PCa cells with the ALK-4, 5, and 7 kinase inhibitor, SB-431542, resulted in a significant reduction in HS-5-mediated cell death. Correspondingly, pre-treatment of HS-5 BMSC with TGF-β1 yielded a CM that elicited a marked reduction in PCa cell death. The ancillary TGF-β receptor endoglin levels were also decreased upon TGF-β1 stimulation of HS-5 cells suggesting the importance of endoglin in mediating BMSC-induced PCa cell death. Small interfering RNA-mediated knockdown of endoglin in HS-5 cells verified that the effect on PCa cell death was a direct result of the attenuation of endoglin. Futhermore, the loss of the cytoplasmic domain of endoglin in HS-5 cells attenuated BMSC-induced PCa cell death indicating the importance of the cytoplasmic domain in maintaining endoglin function and expression of the factor(s) responsible for PCa cell death.

Collectively, my findings indicate that 1) TGF-β signaling in PCa cells is induced during stimulation of NED, 2) TGF-β family cytokines secreted from HS-5 BMSC mediate PCa cell death, 3) TGF-β1 signaling in HS-5 BMSC alters paracrine signaling to promote PCa survival, 4) Endoglin is required for HS-5 BMSC-induced PCa cell death, 5) The cytoplasmic domain of endoglin is required for the expression of the factor(s) responsible for PCa cell death.

Chapter 1

INTRODUCTION

1.1 Prostate Cancer: Statistics and Current Therapies

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer related death for men in the United States, primarily because of metastatic disease. It is estimated that 1 in 7 men will be diagnosed with PCa in their lifetime. In 2014, approximately 233,000 men will be diagnosed with PCa, accounting for 27% of all cancer cases and 29,480 deaths (Siegel, Ma et al. 2014). An estimated 12-15% of patients have advanced PCa at diagnosis. While the 5-year survival rate for patients with localized PCa is nearly 100%, it is only 33% for patients with metastatic PCa (Mishra, Shiozawa et al.). Bone is the major target organ for PCa metastasis. Autopsies reveal the presence of bone metastases in >80% of the PCa patients with clinically evident metastases (Roudier, Vesselle et al. 2003, Shah, Mehra et al. 2004). Of all PCa metastases in bone, >90% are osteosclerotic lesions that are predominantly osteoblastic, causing the patients to experience bone pain, fractures and spinal cord compression (Cheville, Tindall et al. 2002).

If diagnosed early, PCa can be treated effectively through surgery or radiation therapy. If surgery or radiation is not a treatment option, then androgen ablation therapy may be used. Drugs that suppress the production or action of androgen impede cancer progression by starving the androgen sensitive (AS) PCa cells (Miyamoto, Messing et al. 2004). Standard therapies for metastatic PCa, such as androgen ablation through surgical or chemical castration have a disease-free interval of only 12-18 months, which is followed commonly by a more deadly, androgen insensitive (AI) or

castrate resistant (CR) PCa (Koutsilieris and Tolis 1985). The aggressive, CR PCa is responsible for the lethal phenotype of PCa, for which there is no curative therapy, but only palliation.

1.2 Prostate Cancer Colonization to Bone

Paget postulated the important role of the tumor microenvironment in the formation of distal metastases nearly a century ago and this remains the basic principle of metastasis (Ribatti, Mangialardi et al. 2006). He described the bone microenvironment as a specialized "*soil"* that favors the metastasis of certain cancer cells (or "*seeds"*), including PCa cells. Although the precise mechanisms by which PCa cells preferentially colonize bone remain unknown, it is well established that the bone microenvironment provides a fertile "*soil"* of cytokines and growth factors, which PCa utilizes to form metastatic lesions (Chung 2003). Within the skeleton, PCa often spreads to the sites where active bone remodeling is occurring, such as the axial skeleton and metaphyses of long bones (Brown, Cook et al. 2005, Schneider, Kalikin et al. 2005, Gomes, Buttke et al. 2009). Chemokines and growth factors produced by bone marrow stromal cells (BMSC) and resident bone cells, highly permeable and "leaky" bone marrow endothelium, and preferential adhesion to bone marrow endothelium may be among the factors that facilitate PCa colonization of bone. In fact, PCa cells preferentially adhere to the human bone marrow endothelial cells, HBME and BMEC, but not to other types of vascular endothelial cells (Lehr and Pienta 1998, Cooper, Graves et al. 2008). Phosphatase and tensin homolog (PTEN) function, which is frequently lost in advanced PCa, may contribute to bone tropism of PCa cells (Wu, McRoberts et al. 2007). Furthermore, the stromal cell-derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) pathway has been suggested to facilitate PCa

colonization of bone. SDF-1 expressed by bone marrow endothelial cells and osteoblasts might act as a chemoattractant for PCa cells expressing CXCR4 (Taichman, Cooper et al. 2002). αVβ3 integrin is highly expressed on metastatic PCa cells (Edlund, Miyamoto et al. 2001, Sikes, Nicholson et al. 2004, Stewart, Cooper et al. 2004) and αVβ3 integrin activated by the SDF-1/CXCR4 pathway might facilitate the adhesion of PCa cells to HBME cells (Sun, Fang et al. 2007). To this end, antibodies against CXCR4 have been shown to reduce the degree of intraosseous metastasis *in vivo* (Sun, Schneider et al. 2005).

It has been postulated that bone colonization requires PCa cells to exhibit osteomimetic properties, whereby they participate in bone remodeling by expressing proteins, such as osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OC), runt-related transcription factor-2 (Runx2), receptor activator of NF-κB ligand (RANKL), prostate-specific antigen (PSA), tissue-type and urokinase-type plasminogen activator (uPA), human kallikrein 2 (hK2), and cathepsins (Koeneman, Yeung et al. 1999, Morrissey and Vessella 2007). PCa cells may participate in bone remodeling by the attachment to osteoblasts and osteoclasts through non-collagenous bone matrix proteins, such as OPN, BSP, and OC (Young, Kerr et al. 1992, Koeneman, Yeung et al. 1999). PCa cells also may regulate the expression of these bone matrix proteins by expressing Runx2, which is an important transcription factor in osteoblast differentiation (Ducy, Zhang et al. 1997, Koeneman, Yeung et al. 1999). Growth factors, such as transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGF)-1 and 2, fibroblast growth factors (FGF)-1 and 2, and platelet-derived growth factor (PDGF) have abundant stores in the

bone matrix, and may directly induce osteoblast activity. Proteases produced by PCa cells, including PSA, uPA, hK2, and cathepsins have been shown to activate PDGF, latent TGF-β, and cleave IGFs from inhibitory binding proteins, such as IGF binding proteins (IGFBPs), thereby indirectly inducing osteoblast activity (Hauschka, Mavrakos et al. 1986, Koeneman, Yeung et al. 1999, Ustach and Kim 2005). There also is evidence suggesting that PCa cells produce dickkopf-related protein-1 (DKK-1), which inhibits Wnt-mediated osteoblastic response early in bone colonization. As metastasis progresses, DKK-1 expression is lost allowing for a Wnt-mediated osteoblastic response giving rise to osteosclerotic bone lesions, which are the predominant type of lesions seen in PCa (Hall, Kang et al. 2006, Hall and Keller 2006). Despite intensive research efforts, the mechanisms underlying preferential metastasis of PCa cells to the bone are not yet fully understood. Understanding the cellular interactions between PCa cells and the bone microenvironment will yield insight into how bone metastatic lesions originate and maintain themselves. This knowledge will provide novel targets for therapeutic intervention to prevent PCa metastasis.

A variety of studies suggest that prostatic epithelial cells undergo neuroendocrine (NE) differentiation during PCa progression. The presence of NE tumor cells in PCa is associated with aggressiveness, resistance to hormonal therapy, and poor prognosis (Di Sant'Agnese and Cockett 1994, di Sant'Agnese and Cockett 1996, Bonkhoff 2001). Neuroendocrine differentiation (NED) is determined by immunoreactivity for certain biomarkers such as neuron specific enolase (NSE), chromogranin A (CgA) (Abrahamsson 1999), bombesin, parathyroid hormone-related

protein (PTHrP), serotonin, and neurotensin (Palmer, Venkateswaran et al. 2008). The aggressiveness of PCa with NED may suggest paracrine stimulation of tumor cells by neurosecretory products of the NE tumor cells (di Sant'Agnese and Cockett 1996). In fact, NE tumor cells secrete a number of neurosecretory products with growth promoting properties, such as serotonin, bombesin, calcitonin, and PTHrP (Hansson and Abrahamsson 2001). Furthermore, published data from the Sikes lab suggest that conditioned medium (CM) from bone marrow stromal cells (BMSC) induces NED of androgen-sensitive and castrate-resistant PCa cells (Zhang, Soori et al. 2011).

NE cells are present in normal and malignant prostate tissue and have a pivotal role in growth, differentiation, and homeostasis (Hansson and Abrahamsson 2001). NE cells are considered non-proliferative and androgen-independent because they lack the expression of proliferation-associated markers and androgen receptor (AR), respectively (Bonkhoff, Stein et al. 1995). Because of this reason, it is very difficult to kill NE tumor cells by current cytotoxic or hormonal treatments.

1.3 Transforming Growth Factor-β Superfamily Signaling in Prostate Cancer Colonization to Bone

There is a tight balance between bone resorption and bone formation in normal bone remodeling; however, this balance is upset when PCa cells colonize the bone. It has been suggested that excess of active transforming growth factor (TGF)-β plays an important role in the “vicious cycle” of bone remodeling that takes place during PCa-bone interactions in the bone microenvironment (Guise 2000). In the “vicious cycle”, PCa cells in the bone microenvironment produce factors, such as parathyroid hormone-related protein (PTHrP), which stimulates RANKL expression by

osteoblasts. PCa cells also may produce RANKL, which subsequently binds to the RANK receptor on osteoclasts to induce osteoclast activity via the NF-κB pathway (Bonfil, Chinni et al. 2007). Activated osteoclasts release active TGF-β from the latent TGF-β complex through the activity of the matrix metalloproteases, MMP-2 and MMP-9, and by creating an acidic microenvironment that activates TGF-β via latent TGF-β-binding protein (LTBP)-1 cleavage (Oreffo, Mundy et al. 1989, Dallas, Rosser et al. 2002). This binding protein facilitates the deposition and storage of TGF-β in the bone matrix (Kwok, Qin et al. 2005). TGF-β expression can be further induced in osteoblasts by parathyroid hormone (PTH) (Oursler, Riggs et al. 1993). PTH also may regulate the expression of LTBP-1 in osteoblasts, suggesting its importance in regulating TGF-β bioavailability (Kwok, Qin et al. 2005). Active TGF-β stimulates production of PTHrP by PCa cells (Liao and McCauley 2006) and induces the expression of RANK that promotes monocyte differentiation into osteoclasts/osteoclastogenesis (Yan, Riggs et al. 2001). Interleukin-6 (IL-6) secreted by PCa cells further enhances PTHrP-induced osteoclastogenesis (de la Mata, Uy et al. 1995, Keller 2002). TGF-β also has been shown to repress Runx2 function in differentiating osteoblasts through Smad3 recruitment of histone deacetylases to Runx2-responsive elements, thereby inhibiting osteoblast differentiation (Alliston, Choy et al. 2001, Kang, Alliston et al. 2005). Furthermore, TGF-β enhances differentiation of hematopoietic cells and bone marrow-derived macrophages into osteoclasts induced by RANKL and macrophage colony-stimulating factor (M-CSF) (Sells Galvin, Gatlin et al. 1999, Kaneda, Nojima et al. 2000, Itoh, Udagawa et al. 2001). The effect of TGF-β on bone remodeling seems to be concentration dependent, as high levels of TGF-β repress RANKL expression by osteoblasts while increasing

osteoprotegerin (OPG) expression by osteoblasts and bone marrow stromal cells. OPG is a soluble decoy receptor for RANK-L that competitively occupies RANK binding sites, thereby reducing osteoclastogenesis (Murakami, Yamamoto et al. 1998, Takai, Kanematsu et al. 1998, Thirunavukkarasu, Miles et al. 2001, Sato, Futakuchi et al. 2008). TGF-β also increases endothelin-1 (ET-1) production by PCa cells, which stimulates osteoblast activity, and inhibits osteoclastic bone resorption (Chiao, Moonga et al. 2000, Guise, Yin et al. 2003, Mohammad and Guise 2003). These interactions promote the formation of woven bone; however, the osteoid produced is highly disorganized leading to decreased strength of bone that gives rise to bone fractures (Morrissey and Vessella 2007). Breaking the "vicious cycle" in bone metastasis by using anti-PTHrP antibodies has been shown to dramatically decrease breast cancer (BCa) colonization of bone (Guise 1997). This "vicious cycle" between PCa and resident bone and bone marrow cells disrupts normal bone homeostasis, giving rise to tumor growth therein.

Published data from the Sikes lab suggest that conditioned medium (CM) from bone marrow stromal cells (BMSC) induces apoptosis or neuroendocrine differentiation (NED) of PCa cells (Zhang, Soori et al. 2011). An important question to address is whether TGF-β signaling has a role in mediating BMSC-induced apoptosis and NED of PCa cells. TGF-β1, TGF-β2, TGF-β3 and activin, all have been shown previously to induce apoptosis in different model systems (Nguyen and Pollard 2000, Dunker and Krieglstein 2003, Edlund, Bu et al. 2003, Kim, Kim et al. 2009). Bone morphogenetic protein (BMP)-9 has been shown to induce apoptosis in PC-3 PCa cells through the up-regulation of prostate apoptosis response gene (Par)-4 (Ye,

Kynaston et al. 2008). The inhibitory effect of BMPs on tumor growth was illustrated in an *in vivo* study whereby the BMP receptor type II (BMPRII) knockout PC-3M cells were inoculated into nude mice (Kim, Lee et al. 2004). It has been demonstrated that exposure to activin-A converts pancreatic cells into neuron-like cells (Ohnishi, Ohgushi et al. 1995). Activin-A also resulted in morphological changes consistent with NED in LNCaP cells (Zhang, Zhao et al. 1997). Macrophages have been shown to induce NED of LNCaP cells through a tumor-derived BMP-6 and macrophage-derived interleukin-6 (IL-6) loop (Lee, Kwon et al. 2011). Previous studies have indicated that the loss of TGF-β1 receptor type II (TβRII) in the prostate stroma promotes PCa initiation, progression, and PCa bone metastatic growth (Bhowmick, Chytil et al. 2004, Li, Placencio et al. 2008, Li, Sterling et al. 2012). These studies provide evidence that the TGF-β superfamily signaling in the prostate stroma modulates the oncogenic potential of the adjacent epithelium. Therefore, it is reasonable to investigate the potential role of the TGF-β superfamily signaling in mediating BMSC-induced apoptosis and/or NED of PCa cells.

1.4 Mechanisms of Transforming Growth Factor-β Signaling

Transforming growth factor (TGF)-β is typically released from cells as a latent molecule and processed from a larger propeptide by cleavage in the trans-Golgi (Dubois, Laprise et al. 1995). TGF-β latency results from the continual association of TGF-β with its propeptide, following proteolytic processing of the precursor. This complex of dimeric, mature TGF-β non-covalently bound to its dimeric propeptide is called the small latent complex (SLC). The propeptide is also referred to as the latency-associated protein (LAP). The SLC associates with latent TGF-β-binding protein (LTBP) to form the large latent complex (LLC). LTBP regulates the

extracellular presentation of TGF-β by stabilizing latent TGF-β in the extracellular matrix where it is stored until needed. The dissociation of TGF-β from the LLC is required for binding of active TGF-β to its receptors. Latent TGF-β is activated by a number of proteases, including urokinase-type plasminogen activator (uPA) (Nunes, Gleizes et al. 1997), elastase (Taipale, Lohi et al. 1995), chymase (Taipale, Lohi et al. 1995), cathepsin (Lyons, Keski-Oja et al. 1988), calpain (Abe, Oda et al. 1998), MMP-9 (Yu and Stamenkovic 2000), kallikrein (Akita, Okuno et al. 2002), and PSA (Dallas, Zhao et al. 2005) as well as thrombospondin-1 (TSP-1) (Murphy-Ullrich and Poczatek 2000), and αV integrins. Specifically, LAPs of TGF-β1 and TGF-β3 contain an integrin binding motif called arginine-glycine-aspartate (RGD), with which many αV integrins, including αVβ1, αVβ3, αVβ5, αVβ6 (Annes, Chen et al. 2004), and αVβ8 interact, resulting in activation of TGF-β1 and TGF-β3 (Mamuya and Duncan 2012).

The TGF-β signaling cascade is initiated when active TGF-β family ligands (TGF-β1, TGF-β2, and TGF-β3) bind to a family of transmembrane serine-threonine kinases known as type I (TβRI) and type II (TβRII) receptors. The type I and type II receptors likely exist as homodimers on the cell membrane and assemble into heterotetramers in the presence of ligand (Yamashita, ten Dijke et al. 1994). Signaling downstream from the activated heterotetrameric receptor complex may lead to the activation of both Smad-dependent and Smad-independent pathways (Bierie and Moses 2006) (Figure 1).

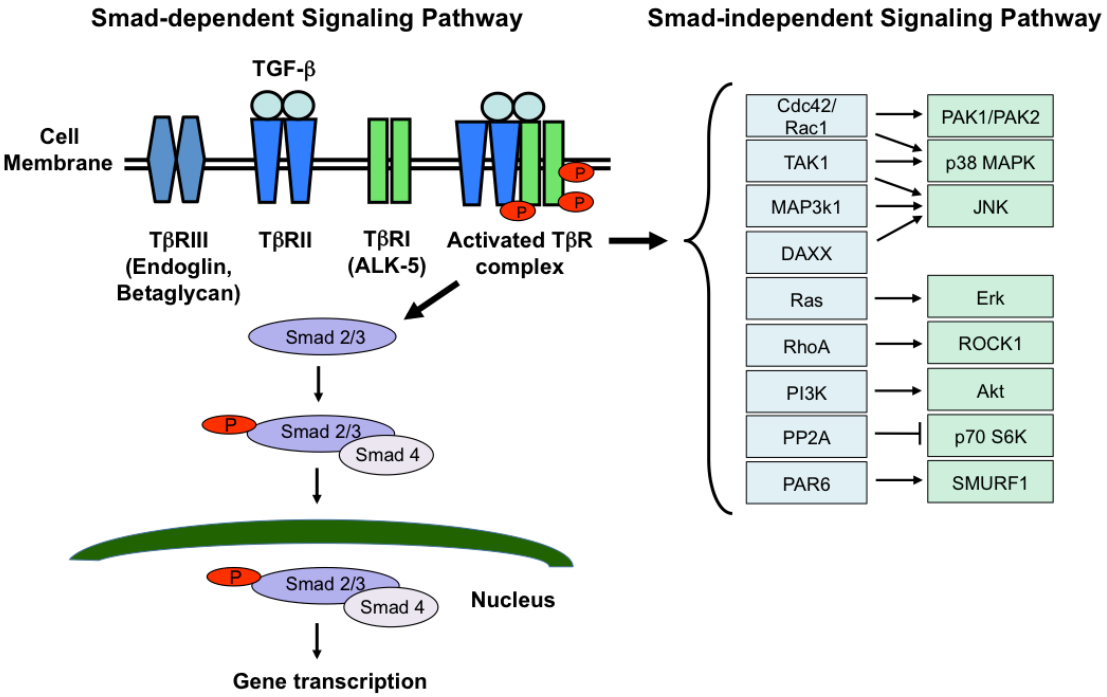


Figure 1: Smad-dependent and Smad-independent signaling pathways regulated by TGF-β.
In the Smad-dependent TGF-β signaling pathway, a heterotetrameric receptor complex is assembled when active TGF-β dimer first binds the type II receptor, TβRII. This subsequently allows the recruitment of the type I receptor, TβRI (ALK-5) to the TβRII receptor complex. The type II receptor phosphorylates and activates the type I receptor. The activated receptor complex phosphorylates and activates the receptor-activated (R-) Smads, Smad2 and Smad3. The activated R-Smads then form hetero-oligomeric complexes with the common mediator co-Smad (Smad4), which are translocated to the nucleus where they regulate gene transcription (left). In the Smad-independent TGF-β signaling pathway, the activated receptor complex activates a range of signaling pathways through interaction with effector proteins. Cdc42, cell division cycle 42; DAXX, death-associated protein 6; MAP3k1, mitogen-activated protein kinase, kinase, kinase 1; PAK, p21-activated kinase; PAR6, partitioning-defective protein 6; PI3K, phosphatidylinositol 3-kinase; PP2A, protein phosphatase 2A; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; SMURF1, Smad ubiquitination regulatory factor 1; TAK1, TGF-β-activated kinase 1 (right) (Adapted from F. Miles dissertation and (Bierie and Moses 2006)).

1.4.1 Transforming Growth Factor-β Signaling Through Smad-dependent Signaling Pathways

In the Smad-dependent transforming growth factor (TGF)-β signaling pathway, heterotetrameric receptor complexes are assembled when active TGF-β dimer first binds the TGF-β type II receptor. This subsequently allows the recruitment of the TGF-β type I receptor to the TGF-β type II receptor complex. The type II receptor phosphorylates the serines and threonines in the highly conserved 30–amino acid glycine-serine repeat (GS) domain of the type I receptors, activin receptor-like kinase-1 (ALK-1), ALK-2, or ALK-5, and thereby activates them. In most cells, signaling occurs through the TβRII-ALK-5 receptor complex, but in endothelial and cardiovascular cells, signaling also may occur through the TβRII-ALK-1 or TβRII-ALK-2 receptor complex. Ancillary receptors (TβRIII) such as endoglin and betaglycan, which share 70% homology, regulate TGF-β signaling by facilitating the binding of ligands to the type II receptor (Lopez-Casillas, Cheifetz et al. 1991). The receptor-activated (R-) Smads that are phosphorylated and activated by ALK-5 are Smads2 and 3 (Miyazawa, Shinozaki et al. 2002). Activated ALK-5 phosphorylates the carboxy-terminal Ser-Ser-X-Ser motif of the R-Smads (Abdollah, Macias-Silva et al. 1997). The activated R-Smads then form hetero-oligomeric complexes with the common mediator co-Smad (Smad4), which are translocated to the nucleus where they regulate gene transcription. On the other hand, ALK-1 and ALK-2 activate Smads1, 5, and 8, which also may complex with Smad4 before nuclear translocation. In the nucleus, the interaction of Smads with other transcription factors, co-activators, and co-repressors to generate transcriptional complexes is critical for the multifunctional nature of TGF-β signaling. Inhibitory (I-) Smads, specifically Smad7, interfere with Smad-receptor, or Smad-Smad interactions, and inhibit the intracellular signaling

(Reviewed in (Massague 1998, Massague, Seoane et al. 2005)). Phosphorylation of R-Smads can be inhibited pharmacologically with the use of inhibitors of type I receptor kinase activity, such as SB-431542. The structural similarity between ALK-4, ALK-5, and ALK-7 kinase domains contributes to the inhibition of ALK-4, and ALK-5 as well as ALK-7 when small-molecule inhibitors that have been designed to specifically attenuate ALK-5 kinase are used (Peng, Yan et al. 2005). SB-431542 is such an inhibitor and it has been shown to inhibit activation of ALK-4/5/7 kinases but fails to inhibit ALK-1/2/3/6 (Inman, Nicolas et al. 2002).

1.4.2 Transforming Growth Factor-β Signaling Through Smad-independent Signaling Pathways

In addition to Smad-dependent signaling, transforming growth factor (TGF)-β family ligands also may signal through Smad independent-pathways, such as those mediated by the mitogen-activated protein (MAP) kinases, Rho-like GTPases, phosphatidylinositol-3 (PI3) kinase, protein kinase A (PKA), and c-Src kinase. Mounting evidence suggests that Smad-independent pathways may either regulate cellular responses to TGF-β alone or converge onto Smads to regulate Smad activity. TGF-β activates three different MAP kinase pathways: the extracellular signal-regulated kinase (Erk) (Hartsough and Mulder 1995), the c-Jun amino-terminal kinase (JNK) (Engel, McDonnell et al. 1999), and the p38 MAP kinase (Hanafusa, Ninomiya-Tsuji et al. 1999). Activation of MAP kinase pathways is essential for the TGF-β-induced apoptosis, growth inhibition, and epithelial-mesenchymal transition (EMT) (Hartsough and Mulder 1995, Mazars, Lallemand et al. 2001, Yu, Hebert et al. 2002, Edlund, Bu et al. 2003). On the other hand, c-Src kinase was shown to contribute to resistance against the TGF-β1-induced apoptosis by suppressing MAP

kinases in the development of hepatocellular carcinoma (Park, Eom et al. 2004). Studies with a mutant TGF-β type I receptor that is incapable of activating Smads but still has intact kinase activity suggested that p38 MAP kinase is activated by TGF-β independently of Smads (Yu, Hebert et al. 2002). But Smads can interact and cooperate with transcription factors that are activated by the MAP kinase pathway, such as the JNK effector c-Jun and the p38 MAP kinase effector activating transcription factor 2 (ATF2), thereby generating an integrated transcriptional response (Zhang, Feng et al. 1998, Sano, Harada et al. 1999). Furthermore, TGF-β-activated kinase 1 (TAK1), a MAPKKK, has been shown to function upstream of the TGF-β-mediated activation of JNK and p38 MAP kinases (Yamaguchi, Shirakabe et al. 1995, Shim, Xiao et al. 2005). In studies done with TAK1-deficient mouse embryos, TAK1-deficient embryos exhibited defects in the developing vasculature. Such phenotype is strikingly similar to that exhibited by loss-of-function mutations in the TGF-β type I receptor ALK-1 and the type III receptor endoglin, suggesting that TAK1 may be an effector of TGF-β during vascular development (Jadrich, O'Connor et al. 2006). TGF-β induces activation of Rho-like GTPases such as Ras, RhoA, Rac1, and Cdc42, which are essential regulators of cytoskeletal organization, cell motility, and EMT (Bhowmick, Ghiassi et al. 2001, Edlund, Landstrom et al. 2002). Cross-talk with Rho and MAP kinase pathways may modulate TGF-β-induced Smad activation (Kamaraju and Roberts 2005, Chen, Crawford et al. 2006). TGF-β may induce phosphorylation and activation of Akt in a PI3 kinase dependent manner, and this is implicated in the TGF-β-mediated EMT and cell migration (Bakin, Tomlinson et al. 2000). Furthermore, the type II receptor was shown to be constitutively associated with the regulatory subunit of PI3 kinase, p85, and the type I receptor becomes

associated with p85 upon TGF-β stimulation in epithelial cells (Yi, Shin et al. 2005). Finally, TGF-β stimulated PKA activation was shown to contribute to the expression of fibronectin in mesangial cells (Wang, Zhu et al. 1998). Altogether, depending on the cellular context, the activated TGF-β receptor complex may relay signals through multiple intracellular pathways with a wide array of cellular responses.

1.5 Transforming Growth Factor-β1 as a Tumor Suppressor and Tumor Promoter in Cancer Progression

Transforming growth factor (TGF)-β1 is a multifunctional cytokine that can have varying effects on cancer. This appears to be dependent on cancer progression. It has been demonstrated to have suppressive effects on normal epithelial cells and early-stage cancer cells while having tumor-promoting effects on advanced cancers, although its effects are largely cell and context dependent (Bierie and Moses 2006). With loss of growth-inhibitory and pro-apoptotic effects in cancer cells, increased production of TGF-β1 may promote cancer progression in many different ways.

The first evidence for the role of TGF-β1 as a tumor-suppressor came from the studies that demonstrated inhibition of epithelial cell growth by TGF-β1 (Moses, Coffey et al. 1987). Despite the inhibition seen in normal epithelial cells, cancer cells exhibit resistance to the growth inhibitory effects of TGF-β1 as was first demonstrated by Hoosein *et al*. in colon cancer cells (Hoosein, McKnight et al. 1989). The opposing effects of TGF-β1 at early and late stages of tumorigenesis was further shown by Cui *et al.* in an *in vivo* skin tumor model, in which ectopic expression of TGF-β1 in keratinocytes increased resistance to TPA-induced benign skin tumor formation, whereas it enhanced the malignant phenotype by inducing the transformation of

benign skin tumors to highly invasive spindle cell carcinomas (Cui, Fowlis et al. 1996).

TGF-β1 inhibits proliferation of cells by blocking the progression of cells from the G_1 into the S phase of the cell cycle by: (1) inhibition of expression of proteins necessary for G_1/S progression, including c-Myc (Pietenpol, Holt et al. 1990, Pietenpol, Stein et al. 1990), cyclins A and D1 (Alexandrow and Moses 1995), cyclin dependent kinase 4 (CDK4) (Grady, Willis et al. 2006), and Cdc25A (Iavarone and Massague 1997), (2) induction of CDK inhibitors, including $p15^{Ink4b}$ (Hannon and Beach 1994), $p21^{Waf1/Cip1}$ (Moustakas and Kardassis 1998), and $p27^{Kip1}$ (Polyak, Kato et al. 1994), (3) inhibition of phosphorylation of retinoblastoma (Rb) (Brown, Roberts et al. 2004). Both Smad-dependent pathways and Smad-independent pathways, such as JNK and p38 MAP kinase have been implicated in the activation of TGF-β1 induced apoptosis (Sanchez-Capelo 2005). Potential mechanisms downstream from these pathways include induction of the pro-apoptotic genes, such as Bax, p53, and TGF-β-inducible early gene (TIEG) (Tachibana, Imoto et al. 1997), suppression of Bcl-xL, activation of caspase 3, release of cytochrome c (Freathy, Brown et al. 2000), and induction of death-associated protein (DAP) kinase (Jang, Chen et al. 2002) (Reviewed in Sanchez-Capelo (Sanchez-Capelo 2005)). Paradoxically, TGF-β1 may protect some cell types against apoptosis, such as NMuMG mouse mammary epithelial cells, 4T1 breast carcinoma cells, and HaCaT keratinocytes via Akt dependent regulation of the forkhead transcription factor (FKHRL1) (Shin, Bakin et al. 2001). Additionally, the variable apoptotic response of cells to TGF-β1 may depend on direct Akt interaction with Smad3. Akt sequesters Smad3 outside the

nucleus. This results in the inhibition of Smad3 phosphorylation, nuclear translocation, and the subsequent apoptotic response (Conery, Cao et al. 2004).

On the other hand, tumor-promoting effects of TGF-β1 may occur via its autocrine effects on cancer cells with deregulated pathways or through paracrine effects on host cells. Deregulation of TGF-β signaling can occur at multiple levels in the signaling pathway. Genetic and epigenetic alterations that inhibit TGF-β signaling have been identified in the genes of the pathway components including: *TGFBR2* (the gene encoding the TGF-β type II receptor), *TGFBR1* (the gene encoding the TGF-β type I receptor), *SMAD4*, and *SMAD2*. In addition, inhibitors such as Smad7 and Smurf2, and transcription factors that repress Smad signaling, including c-Ski, SnoN, and SIP1/ZEB-2 are overexpressed in many cancers (Markowitz and Roberts 1996, Wotton and Massague 2001, Elloul, Elstrand et al. 2005, Levy and Hill 2006). Mutations in *TGFBR2* are the most common mechanism identified for inactivating TGF-β signaling in many cancers (Garrigue-Antar, Munoz-Antonia et al. 1995, Markowitz, Wang et al. 1995, Knaus, Lindemann et al. 1996, Lucke, Philpott et al. 2001). Unlike *TGFBR2*, *TGFBR1* is a less common target for mutational inactivation in cancer. Although uncommon, *TGFBR1* mutations occur in prostate, head and neck, biliary, endometrial, ovarian, breast, cervical, and pancreatic cancer, and T-cell lymphomas (Nakashima, Song et al. 1999, Schiemann, Pfeifer et al. 1999, Knobloch, Lynch et al. 2001, Levy and Hill 2006). Furthermore, inactivating somatic mutations have been identified in *SMAD2* and *SMAD4* in many cancers (Levy and Hill 2006). *SMAD2* is mutated in cervical, colon, and non-small-cell lung cancer, and hepatocellular carcinoma. *SMAD4* mutations and deletions have been identified in a

wider array of cancers, including colorectal, pancreatic, non-small-cell lung, biliary, ovarian, cervical, breast, bladder, and esophageal cancer, and hepatocellular carcinoma, and mutations usually occur at later stages in carcinomas (Levy and Hill 2006). Unlike *TGFBR2, TGFBR1, SMAD4,* and *SMAD2,* mutations in *SMAD3* are not common in cancer, although its expression may be lost (Han, Kim et al. 2004). To date, only one group reported a missense mutation in *SMAD3* in colorectal cancer cell lines, which resulted in the inhibition of Smad3 protein translocation to the nucleus, and thereby a reduction in the TGF-β-induced transcriptional activation (Ku, Park et al. 2007).

Increased production of TGF-β1 by tumor cells can have paracrine effects on host cells that would promote tumor growth. TGF-β1 has many roles in the regulation of local immune mediators, angiogenesis, and stromal-epithelial interactions in the tumor microenvironment (Wojtowicz-Praga 2003). TGF-β1 was identified as a potent inhibitor of the immune response mediated by cytotoxic T-cells (Thomas and Massague 2005) and macrophages (Tsunawaki, Sporn et al. 1988). Abrogation of TGF-β signaling in the immune compartment by expression of a dominant-negative type II receptor in transplanted bone marrow progenitor cells elicits anti-tumor activity via a possible T-cell response in the hosts when challenged with injections of highly tumorigenic melanoma and PCa cells (Shah, Tabayoyong et al. 2002). Furthermore, TGF-β1 helps Fas ligand expressing tumor cells evade the immune system by inhibiting the activation of neutrophils, which typically eliminate these tumor cells (Chen, Sun et al. 1998).

TGF-β regulates the expression of vascular endothelial growth factor (VEGF), TGF-α, platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF-2) to induce angiogenesis in the tumor microenvironment, although its effects may be biphasic (Pepper 1997). One of the early examples for biphasic regulation of angiogenesis by TGF-β1 suggests that high levels of TGF-β1 inhibit capillary tube formation by endothelial cells *in vitro,* whereas low levels promote it (Pepper, Vassalli et al. 1993). Additionally, TGF-β1 or type II receptor knockout mice show defective vasculogenesis and embryonic lethality (Kulkarni, Huh et al. 1993, Dickson, Martin et al. 1995, Oshima, Oshima et al. 1996).

The importance of stromal-epithelial interactions in cancer progression is well established and TGF-β1 is a key player in the interplay between stromal and epithelial cells. Non-tumorigenic prostatic epithelial cell line, BPH-1 was recombined with carcinoma-associated fibroblasts (CAFs), which resulted in the formation of aggressive carcinomas in athymic mouse hosts (Hayward, Wang et al. 2001). Mice with humanized mammary fat fibroblasts overexpressing either TGF-β1 alone or in combination with hepatocyte growth factor (HGF) promote lesions derived from the human mammary epithelial cells (Kuperwasser, Chavarria et al. 2004). Paradoxically, stromal TGF-β signaling may facilitate tumor suppression of the adjacent epithelial cells. This was shown by conditional inactivation of *TGFBR2* in stromal fibroblasts that resulted in prostatic intraepithelial neoplasia (PIN) and gastric carcinoma in mice. The gastric carcinoma was accompanied by increased stromal cell expression of HGF and an up-regulation of phosphorylated (activated) c-Met receptor in the epithelium. This further suggests that HGF may have a role in TGF-β-mediated regulation of the

epithelium *in vivo* (Bhowmick, Chytil et al. 2004). Recent research done by the same group further suggests that conditioned medium from primary prostatic fibroblasts with a conditional *TGFBR2* knockout promotes PCa cell growth in bone and osteosclerotic bone lesions (Li, Sterling et al. 2012). Reports published by the Sikes lab further confirm the involvement of HGF in cancer progression by a novel c-Met-independent mechanism (Tate, Isotani et al. 2006). Altogether, these reports highlight the stage of the cancer and the cellular context as significant determining factors in the complexity of responses to the TGF-β signaling.

1.6 Endoglin: Implications in Cancer Progression

Endoglin, also known as CD105, is an ancillary TGF-β receptor that binds TGF-β1 and TGF-β3 but not TGF-β2, with high affinity through its association with TGF-β type I and type II receptors and thereby modulates the responses to these ligands. In addition to TGF-β1 and TGF-β3, endoglin also binds activin-A, and bone morphogenetic proteins (BMP)-2, 7 and 9 by interacting with their respective receptors, suggesting that it is a part of multiple receptor complexes of the TGF-β superfamily (Cheifetz, Bellon et al. 1992, Yamashita, Ichijo et al. 1994, Barbara, Wrana et al. 1999) (Figure 2). Endoglin is a 633 amino acid, 180kDa disulfide-linked, hypoxia-inducible, homodimeric trans-membrane glycoprotein expressed at high levels on vascular endothelial cells (Cheifetz, Bellon et al. 1992, Nassiri, Cusimano et al. 2011). Aside from its expression in endothelial cells, endoglin also is expressed on monocytes (Lastres, Letamendia et al. 1996), vascular smooth muscle cells (Adam, Clesham et al. 1998), macrophages (Lastres, Bellon et al. 1992), fibroblasts (St-Jacques, Cymerman et al. 1994), hematopoietic stem cells (Chen, Li et al. 2002), syncytiotrophoblasts of full-term placenta (Gougos, St Jacques et al. 1992), and BMS

cells (St-Jacques, Cymerman et al. 1994, Robledo, Hidalgo et al. 1996). Two isoforms termed long (L) and short (S) endoglin, differing in the composition of their cytoplasmic tails, have been characterized in humans and mice (Bellon, Corbi et al. 1993, Perez-Gomez, Eleno et al. 2005). The L-isoform, which is the predominant form in most tissues, has a large extracellular domain with 561 amino acid residues and a small constitutively phosphorylated cytoplasmic tail with 47 amino acid residues, whereas the S-isoform only has a 14 amino acid cytoplasmic tail (Lastres, Martin-Perez et al. 1994). Endoglin also can be shed from the cell surface, although the function of the soluble endoglin still remains unclear. In humans, the core protein is approximately 95kDa and is encoded by 14 exons spanning 40kbp of genomic DNA on chromosome 9q34 (Duff, Li et al. 2003, Fonsatti and Maio 2004).

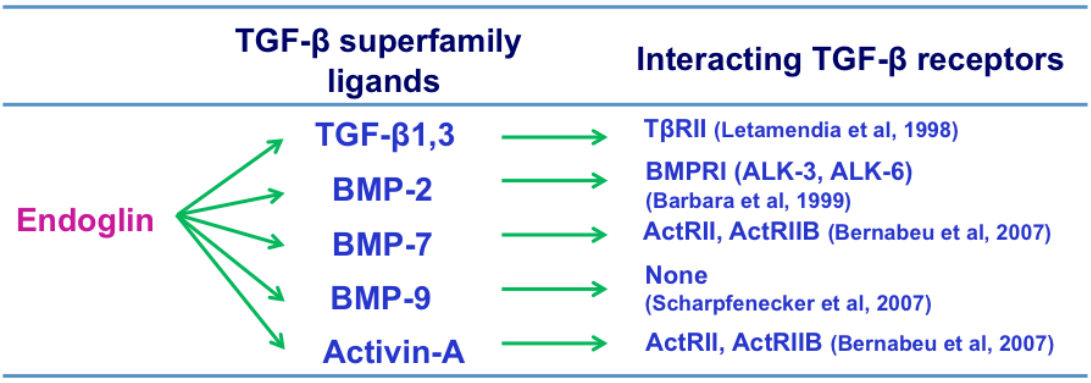


Figure 2: Endoglin is an ancillary TGF-β receptor.
Endoglin binds TGF-β1 and TGF-β3 through its association with TβRII and TβRI and thereby modulates the responses to these ligands. Endoglin also binds bone morphogenetic proteins (BMP)-2, 7 and 9, and activin-A by interacting with their respective receptors. BMPRI, bone morphogenetic protein receptor type I; ActRII, activin receptor type II; ActRIIB, activin receptor type IIB.

Endoglin is required for extraembryonic angiogenesis and heart development in mice. This was illustrated by the death *in utero* of endoglin knockout mice from defects in vascular development (Arthur, Ure et al. 2000). Interestingly, endoglin, TGF-β1 and TGF-β receptor type II knockouts all showed similar defects in vascular development, illustrating the complexity and interdependence of TGF-β signaling (Bourdeau, Dumont et al. 1999, Arthur, Ure et al. 2000, Li, Guo et al. 2001). The importance of endoglin in vascular homeostasis is further indicated by the association of mutations in the endoglin gene with Hereditary Hemorrhagic Telangiectasia (HHT) type I, also known as Osler-Weber-Rendu syndrome. HHT type I is an autosomal dominant vascular disorder characterized by bleeding from small vascular malformations called telangiectases, gastro-intestinal hemorrhages, as well as pulmonary, cerebral, and hepatic arteriovenous malformations (McAllister, Grogg et al. 1994). HHT type II, which causes similar vascular malformations, results from mutations in the ALK-1 gene (Johnson, Berg et al. 1996). The fact that both endoglin and ALK-1 have been linked to HHT suggests that they likely act in a common signaling pathway.

TGF-β has been shown to activate two distinct type I receptor pathways in endothelial cells: ALK-5 inducing Smad2/3 phosphorylation, and ALK-1 inducing Smad1/5/8 phosphorylation. Activation of the ALK-5 pathway leads to inhibition of cell proliferation and migration, whereas activation of ALK-1 pathway stimulates these responses (Goumans, Valdimarsdottir et al. 2002). Endoglin has an essential role in the balance of ALK-5 and ALK-1 signaling to regulate endothelial cell proliferation. Increased expression of endoglin regulates the switch between ALK-5

and ALK-1 signaling by promoting the TGF-β-ALK-1 and inhibiting the TGF-β-ALK-5 signaling, thereby promoting endothelial cell proliferation (Lebrin, Goumans et al. 2004). In these studies, ectopic expression of endoglin promoted endothelial cell proliferation via the TGF-β-ALK-1 signaling, whereas knocking down endoglin expression with a small interfering RNA (siRNA) resulted in impaired TGF-β-ALK-1 signaling and cell proliferation (Lebrin, Goumans et al. 2004). In accordance with this, endoglin expression is strongly up-regulated on proliferating endothelial cells *in vitro* and vascular endothelium in angiogenetic tissues *in vivo* (Duff, Li et al. 2003, Fonsatti and Maio 2004). Several reports suggest that the up-regulation of endoglin expression on tumor endothelium is correlated with poor prognosis in different cancers such as breast, cervical, colorectal, non-small cell lung, prostate, renal cell, endometrial and gastric carcinomas, and melanoma (Fonsatti and Maio 2004). In line with these findings, elevated plasma levels of soluble endoglin in patients with breast and colorectal cancers are correlated with metastasis (Li, Guo et al. 2001, Takahashi, Kawanishi-Tabata et al. 2001). The fact that endoglin is overexpressed on vascular endothelium in tissues undergoing angiogenesis suggests that endoglin could be a significant target in anti-angiogenetic therapy of cancer. Several preclinical studies and an ongoing phase I clinical trial support the potential of anti-endoglin monoclonal antibodies to be used as a therapeutic anti-angiogenetic strategy in cancer (Fonsatti, Nicolay et al. 2010). Furthermore, the elevated expression of endoglin in actively proliferating endothelial cells and its weak expression in quiescent endothelium suggest endoglin targeting as a potential strategy in cancer imaging (Fonsatti, Nicolay et al. 2010, Perez-Gomez, Del Castillo et al. 2010).

In contrast to its elevated expression on tumor endothelium and role in tumor angiogenesis, endoglin also may act as a suppressor of invasion and metastasis although the precise mechanism by which endoglin regulates these processes remains unclear. In line with these findings, endoglin expression has been shown to be lost on carcinoma cells, including that of breast (Henry, Johnson et al. 2011), esophageal squamous cell (Wong, Chan et al. 2008), and prostate (Liu, Jovanovic et al. 2002, Lakshman, Huang et al. 2011) associated with their malignant progression.

In PCa, elevated endoglin levels in tumor endothelium are correlated with a high Gleason score and biochemical recurrence (Kassouf, Ismail et al. 2004). In contrast, endoglin expression is lost in human metastatic PCa cells and this is correlated with increased cell migration and invasion (Liu, Jovanovic et al. 2002). Inhibition of PCa cell migration by endoglin is through both Smad-dependent (Craft, Romero et al. 2007) and Smad-independent pathways (Romero, Terzic et al. 2010). Furthermore, in a murine orthotopic model of human PCa, endoglin was shown to suppress PCa metastasis (Lakshman, Huang et al. 2011). The importance of endoglin in PCa-stromal cell interactions was demonstrated using a TRAMP (transgenic adenocarcinoma mouse prostate) mouse model where endoglin was deleted. In this study, endoglin was shown to be required for the infiltration of the tumor microenvironment by cancer-associated fibroblasts (CAFs) and subsequent neovascularization (Romero, O'Neill et al. 2011). The mechanisms underlying the diverse actions of endoglin in cancer progression are not yet fully understood. Elucidation of these mechanisms would yield insight into the events facilitating metastasis.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture and Reagents

LNCaP, C4-2, and C4-2B isogenic PCa cell lines were maintained in T-medium (Life Technologies, Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO).

DU145, MDA-MB-231, MCF7, ZR-75-1, and NIH 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech Inc., Manassas, VA) supplemented with 10% (v/v) FBS.

BPH1 cells were maintained in RPMI1640 medium (Mediatech Inc., Manassas, VA) supplemented with 5% (v/v) FBS. MCF10A cells were maintained in DMEM/F12 medium (Mediatech Inc., Manassas, VA) supplemented with 5% (v/v) horse serum, 1 ng/mL cholera toxin, 10 μg/mL insulin, 10 ng/mL epidermal growth factor and hydrocortisone 0.5 μg/mL. BMEC cells were maintained in RPMI1640 medium supplemented with 10% (v/v) FBS.

The immortalized human bone marrow stromal cell (BMSC) lines, HS-5 and HS-27a (Roecklein and Torok-Storb 1995) were purchased from ATCC (Manassas, VA), and maintained in DMEM supplemented with 10% (v/v) FBS. The dominant negative TGF-β type II receptor (DN-TβRII) stable transfectants and the pBabe vector-alone stable transfectants were maintained in the same culture medium as HS-5

supplemented with 2 μg/mL Puromycin (Gemini Bio Products, West Sacramento, CA).

TGF-β1 (R & D Systems, Minneapolis, MN) was used at a concentration of 5 ng/mL.

For SB-431542 treatments, PCa cells were pre-treated with SB-431542 at a concentration of 10 μM to neutralize ALK-4, 5, and 7 signaling or with vehicle (0.01% v/v EtOH) for 1 hour prior to treatment with TGF-β1 or HS-5 conditioned medium (CM).

2.2 Conditioned Medium Preparation

HS-5 and HS-27a cells were grown in DMEM with 10% FBS until confluent. Confluent cultures of HS-5 and HS-27a cells were placed in serum-free medium, and medium was collected every 48 hours for four days, and subsequently pooled and filtered through a 0.2 μm filter to remove cells and debris. HS-5 conditioned medium (CM) treatment of PCa cells was done in a 1:2 (v/v) ratio of HS-5 CM and T-medium with a final serum concentration of 2.5% for 24 hours as described previously (Zhang, Soori et al. 2011). The controls were cultured in a 1:2 ratio of T-medium and DMEM with a final serum concentration of 2.5%. For TGF-β1 pre-treatment experiments, subconfluent HS-5 cells were pre-treated with TGF-β1 (5 ng/mL) (R&D Systems, Minneapolis, MN), TGF-β1 after addition of SB-431542 (10 μM) (Tocris Bioscience, Bristol, UK), vehicle (4 mM HCL containing 1 mg/mL BSA), or not pre-treated at all in DMEM containing 10% FBS for four days. Subsequently CM was collected under serum-free conditions in the absence of TGF-β1 for one 48-hour cycle. For

preadaptation by serum starvation, subconfluent C4-2B cells were cultured in serum-free T-medium for 72 hours to induce neuroendocrine differentiation (NED) prior to treatment with HS-5 CM for 48 hours. Corresponding controls were cultured in T-medium supplemented with 5% FBS, followed by culture in DMEM and T-medium at a 1:2 ratio.

2.3 Live/Dead Assay

Subconfluent cultures were treated simultaneously with Calcein AM (125 nM) (Life Technologies, Grand Island, NY) and propidium iodide (4 μg/ml) (Sigma-Aldrich, St. Louis, MO) for 30 minutes to stain live and dead cells, respectively. Images were captured at four different fields per experiment using a Nikon 2000E inverted fluorescent microscope (Nikon, Inc., Melville, NY), and quantified using Adobe Photoshop CS6 version 13.0 (Adobe Systems, San Jose, CA) by measuring pixel intensity of red and green objects to obtain an average ratio of dead to live cells per field.

2.4 Reverse Transcription (RT)-PCR Analysis

Total cellular RNA from cell lysates was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA reactions were performed using 0.5 μg of RNA (SuperScript III First Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA). 50 ng of cDNA was used per 10 μl PCR reaction. RT-PCR was performed for 30 cycles with the following human endoglin primer set:

5′ oligo; 5′-CAACATGCAGATCTGGACCAC,

3′ oligo; 5′-CTTTAGTACCAGGGTCATGGC.

RNA was used in place of cDNA as a negative control with no other changes to reaction conditions. GAPDH was used as an expression level control with the following primer set:

5′ oligo; 5’-AAGGTCGGAGTCAACGGATTTGGT,

3′ oligo; 5’-ATGGCATGGACTGTGGTCATGAGT.

PCR products were visualized on a 1% (w/v) agarose tris-acetate buffered EDTA gel.

2.5 Western Blot Analysis

Subconfluent cell cultures were lysed in nuclear RIPA (1% v/v Nonidet-P40, 1% w/v sodium deoxycholate, 0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride) containing protease (Roche, Indianapolis, IN) and phosphatase (Thermo Scientific, Rockford, IL) inhibitor mixtures, and total cellular protein concentrations were determined via standard BCA protocol (Pierce, Rockford, IL). For TβRII analysis, lysates were treated subsequently with PNGase F purchased from New England Biolabs (Ipswich, MA) before subjecting to SDS-PAGE. Anti-TβRII (Cat. No. 06-227) antibody was purchased from Upstate Cell Signaling Solutions, and used at a dilution of 1:1000. Anti-phospho Smad2 (Ser465/467) (Cat. No. 3108) and anti-Smad2 (Cat. No. 3122) antibodies were purchased from Cell Signaling, and used at a dilution of 1:1000. Anti-NSE (Cat. No. M0873) antibody was purchased from Dako Cytomation (Denmark), and used at a dilution of 1:1000. Anti-endoglin (Cat. No. AB49228) antibody was purchased from Abcam (Cambridge, MA), and used at a dilution of 1:1000. Anti-GAPDH (Cat. No.

G9545) antibody was purchased from Sigma-Aldrich (St. Louis, MO), and used at a dilution of 1:10,000. Anti-actin (Cat. No. A2668) antibody was purchased from Sigma, and used at a dilution of 1:4000. Lysates were fractionated on SDS-PAGE for 50 minutes at 170 V and transferred onto nitrocellulose membranes overnight at 4^{o}C at 30 V, followed by staining with 0.5% (w/v) Ponceau-S in 0.1% (v/v) acetic acid in order to verify equal protein loading and transfer. Membranes were blocked in TBS-T with 4% (w/v) bovine serum albumin (BSA) for analysis of phospho-Smad2, Smad2, TβRII, NSE, GAPDH, and actin, and PBS-T with 5% (w/v) nonfat dry milk for analysis of endoglin. Primary antibodies were incubated overnight at 4^{o}C and membranes were incubated subsequently with species-specific horseradish-peroxidase (HRP) conjugated secondary antibodies. Membranes were stripped when necessary using Restore Plus Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL). Densitometric analysis of Western blot bands was performed using NIH ImageJ 1.47v software.

For immunoblotting for phospho-Smad2, cells were treated with forskolin at a concentration of 10 μM for 5 min, 15 min, 30 min, 60 min or 12 hours, or its vehicle (0.02% v/v DMSO) in T-medium containing 5% FBS or cultured in serum-free T-medium for 24 hours.

2.6 Soft Agarose Colony Formation Assay

Cells were seeded into 12-well culture plates (Greiner Bio-One, Monroe, NC) at a density of 2.5x10^{4} cells/well in 0.3% (w/v) agarose (1:1 ratio of UltraPure Agarose, Invitrogen, Carlsbad, CA and SeaPlaque Agarose, Cambrex Bio Science, Rockland, ME) in T-medium over a previously gelled layer of 0.6% (w/v) agarose in T-medium

and cultured for 28 days. Cells were treated with or without TGF-β1 and in the presence of SB-431542 or EtOH vehicle added 1 hour prior to TGF-β1 in a 1:2 combination of DMEM and T-medium supplemented with 2.5% (v/v) FBS. Alternatively, cells were treated with CM from HS-5 or HS-27a cells in T-medium supplemented with 2.5% (v/v) FBS. After 28 days, colonies were stained with 0.8 mM p-iodonitrotetrazolium violet (Sigma-Aldrich, St. Louis, MO) overnight at 37^{o}C under 5% CO_2 in a 95% humidified chamber. Colonies larger than 100 μm in diameter were counted using Volocity 3D Imaging Software (v5.4, PerkinElmer, Waltham, MA). Each experiment was performed at least three times in duplicate.

2.7 Luciferase Reporter Assay

The TGF-β1-responsive plasminogen activator inhibitor promoter-based luciferase reporter plasmid p3TP-luc was used to ascertain p-Smad2/3 promoter activity. PCa cells were transfected transiently with p3TP-luc (16 μg) and renilla (4 μg) luciferase using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were treated with HS5 CM for 24 hours, 24 hours post-transfection and luciferase activity was measured using a dual-luciferase kit as per manufacturer's recommendations (Promega, Madison, WI). Relative values of firefly luciferase were normalized to renilla luciferase after obtaining luminescence measurements.

2.8 Transfection by Electroporation

The truncated form of TGF-β type II receptor (TβRII) lacking the cytoplasmic domain (DN-TβRII) in pcDNA3.1 (provided by Dr. Takeshi Imamura (Katsuno, Hanyu et al. 2008)) was transfected into PCa cells by electroporation using ECM 830 Square Wave Electroporation System (BTX, Harvard Apparatus, Holliston, MA).

2 x 10^6 cells were resuspended in 400 µl T-medium supplemented with 5% (v/v) FBS (without antibiotics) with 10 µg plasmid DNA. The resuspended cells were electroporated in BTX disposable cuvettes (Model 640, 4 mm electrode gap) at the following settings: 1 pulse, 170 V, 70 msec in LV mode. Cells were then plated into 6-well culture plates (Greiner Bio-One, Monroe, NC) at a density of 1 x 10^6 cells/well in T-medium supplemented with 5% (v/v) FBS. At 24h post-transfection, cells were treated with HS5 CM and incubated for 24 hours.

2.9 DNA Fragmentation ELISA

PCa cells for apoptosis assays were seeded into 24-well plates (Greiner Bio-One, Monroe, NC) at a density of 1x10^5 cells/well. Cells were harvested using trypsin–EDTA. Cell pellets were collected and apoptosis assays were performed using Cell Death Detection ELISAplus (Roche, Indianapolis, IN) as per manufacturer's protocols.

2.10 Vector Construction

The truncated form of TGF-β type II receptor (TβRII) lacking the cytoplasmic domain (DN-TβRII) in pcDNA3.1 was subcloned into the retroviral vector pBabe. The empty vector pBabe was used as a negative control. The ΔC (C-terminal deleted) mutant of endoglin was provided in the retroviral pWZL vector by Dr. Calvin P. H. Vary (Conley, Koleva et al. 2004). The empty pWZL vector was used as a negative control. The constructs were confirmed by DNA sequencing analysis (GENEWIZ, Inc., South Plainfield, NJ).

2.11 Generation of Retroviral Transduced Cells

HEK 293T cells (ATCC, Manassas, VA) were grown to approximately 50% confluence and were transfected with pBabe, pBabe bearing DN-TβRII, pWZL or pWZL bearing ΔC endoglin construct along with a helper plasmid pPAM (Miller, Palmer et al. 1986) in a 1:1 ratio (10 μg each) using the $CaPO_4$ protocol according to Chen and Okayama (Chen and Okayama 1987). Cells were allowed to recover for 48 hours before collecting the media containing the viral particles. The viral particles were added to HS-5 cells to be stably transduced along with polybrene (at a final concentration of 10 μg/mL) and the cells were incubated overnight before the medium was removed and replaced with DMEM containing 10% FBS and 2 μg/mL Puromycin (for pBabe and pBabe bearing DN-TβRII) or 100 μg/mL Hygromycin B (for pWZL and pWZL bearing ΔC endoglin). Selection and expansion over the course of 3 weeks yielded a pooled cell population of resistant cells.

2.12 Statistical Analysis

Data presented are mean ± standard error of the mean. Group comparisons were performed by 1-way ANOVA, 2-way ANOVA, or unpaired two-tailed Student's *t* test as indicated for each experiment.

Chapter 3

TGF-β SIGNALING IS INDUCED DURING APOPTOSIS AND NEUROENDOCRINE DIFFERENTIATION OF PROSTATE CANCER CELLS MEDIATED BY BONE MARROW STROMAL CELLS

Partly as in the manuscript: Miles, Kurtoglu *et al*.

3.1 Introduction

Prostate cancer (PCa) metastasizes to bone in over 80% of patients with advanced disease (Roudier, Vesselle et al. 2003, Shah, Mehra et al. 2004). The high mortality associated with metastatic disease is a consequence of the re-programming of an otherwise hostile bone microenvironment allowing for colonization by the cancer, as the initially toxic microenvironment becomes altered as a result of circulating cytokines and reciprocal paracrine interactions between prostate and stromal cells. PCa cells, once in the bone, engage in the vicious cycle of bone turnover, which occurs at the interface of the endosteal bone surface and bone marrow stroma (Koeneman, Yeung et al. 1999). Growth of PCa cells in bone is supported by cytokines and growth factors released by bone remodeling, and simultaneous paracrine signaling by PCa cells prevents the normal limitations on remodeling activity (Koeneman, Yeung et al. 1999, Kopp, Avecilla et al. 2005, Loberg, Bradley et al. 2007). While it is clear that established bone metastases contribute to the lethal phenotype of PCa, the mechanisms allowing for PCa colonization of bone leading to osteosclerotic metastases are unclear. Particularly, it is unclear how paracrine signaling between tumor and stromal cells promotes tumor cell survival in the toxic

environment of the hematopoietic bone marrow stromal niche. One characteristic feature of aggressive, hormone-refractory and bone-metastatic disease is neuroendocrine differentiation (NED), which is associated with increased serum levels of neuronal specific enolase (NSE) and chromogranin A (CGA), and a decrease in levels of androgen receptor (AR) (Berruti, Dogliotti et al. 2001, Bonkhoff 2001, Kamiya, Akakura et al. 2003). Although neuroendocrine cells are withdrawn from the cell cycle, products of neuroendocrine PCa cells have the ability to act as mitogens for PCa (Jongsma, Oomen et al. 2000, Xiao, Qu et al. 2003), but the exact role of neuroendocrine cells in the bone microenvironment is unclear.

We have shown previously that conditioned medium (CM) from immortalized human bone marrow stromal cells (BMSC) is toxic to metastatic PCa cells. Stimulation with CM from HS-5 BMSC or HS-5 in combination with HS-27a BMSC induces NED, characterized by morphological changes consistent with a neuroendocrine phenotype. PCa cells that are not induced to undergo NED undergo apoptosis, although the mechanism is not clear (Zhang, Soori et al. 2011). It is well known that members of the TGF-β superfamily regulate growth and cytostasis in epithelial cells. Recently, we demonstrated that castrate-resistant prostate cancer (CRPC) cell lines show significant suppression of growth and motility upon exogenous treatment with TGF-β1. This likely involves signaling through Smad2 and Smad3, which are translocated to the nucleus after phosphorylation by the heterotetrameric TGF-β type I (ALK-5) and type II receptor complex (Miles, Tung et

al. 2012). Activin signaling has also been shown to suppress growth in LNCaP PCa cells (Carey, Sasur et al. 2004). Paradoxically, TGF-β1 is correlated with PCa progression (Wikstrom, Stattin et al. 1998, Shariat, Shalev et al. 2001) by unknown mechanism(s). Thus, it is clear that the role of TGF-β1 in cancer is quite complex. Because of the increased levels of TGF-β1 in PCa patients with bone metastasis and relevance of TGF-β superfamily signaling to regulation of PCa growth, I sought to analyze the contributions of TGF-β signaling to BMSC-mediated apoptosis and NED.

3.2 Results

3.2.1 Conditioned medium from HS-5 bone marrow stromal cells induces prostate cancer cell death

Our previous studies concluded that conditioned medium (CM) from bone marrow stromal cells (BMSC) induces apoptosis in PCa cells (Zhang, Soori et al. 2011). We examined further the influence of BMSC factors on PCa survival, using the HS-5 and HS-27a BMSC lines, which were characterized previously for their ability to support different stages of hematopoiesis, and consequently, are believed to represent separate anatomical regions of the bone marrow niche (Roecklein and Torok-Storb 1995, Calvi, Adams et al. 2003, Kopp, Avecilla et al. 2005). The BMSC-induced death effect was compared among the cell lines of the LNCaP progression model. This is an isogenic cell line model, which is composed of a series of cell lines with increasing metastatic and castrate-resistant potential. This cell line model begins with the poorly-metastatic and androgen sensitive LNCaP cells and progresses to the highly metastatic and castrate-resistant C4-2B cells. C4-2B cells were purified from

spontaneous bone metastases of C4-2 cells to the spine. C4-2 cells are capable of producing spontaneous osteoblastic/osteosclerotic bone lesions when inoculated orthotopically into castrated hosts, thereby resembling the predominant type of lesions observed in human metastatic PCa in bone (Thalmann, Anezinis et al. 1994, Wu, Hsieh et al. 1994, Thalmann, Sikes et al. 2000). Results from live/dead assays demonstrated an approximately 16-fold increase in dead cells in LNCaP, and progressively lower increases in C4-2 and C4-2B cell death (12 and 6-fold, respectively) after treatment with HS-5 CM for 24 hours (Figure 3A). Cell death effects were even more pronounced after 48 hours of treatment. Even more profound effects on cell growth in three-dimensional conditions were observed in soft agarose colony forming (SACF) assays, where there was between an 8 and 30-fold reduction in the percentage of colonies formed after four weeks of treatment with HS-5 CM, compared to HS-27a CM and control medium (Figure 3B).

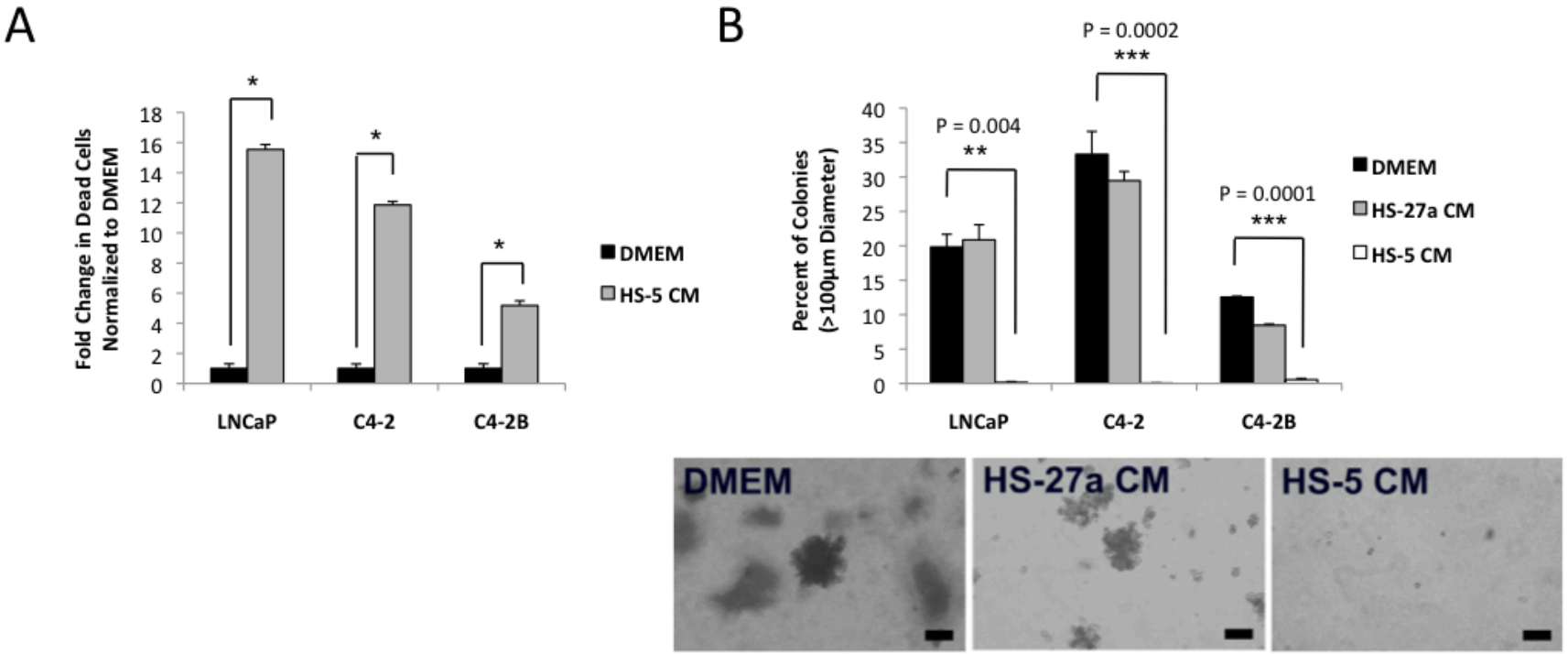
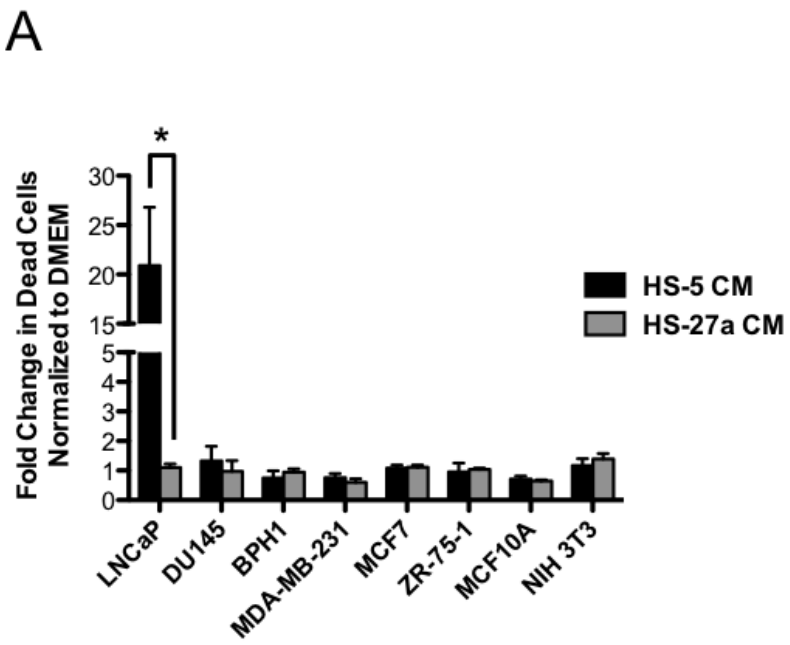

A
Fold Change in Dead Cells Normalized to DMEM
18
16
14
12
10
8
6
4
2
0
*
*
*
LNCaP
C4-2
C4-2B
DMEM
HS-5 CM
B
Percent of Colonies (>100µm Diameter)
40
35
30
25
20
15
10
5
0
P = 0.004
**
P = 0.0002
***
P = 0.0001
***
LNCaP
C4-2
C4-2B
DMEM
HS-27a CM
HS-5 CM
DMEM
HS-27a CM
HS-5 CM

Figure 3: Conditioned medium from HS-5 bone marrow stromal cells induces PCa cell death and growth inhibition.
A) Prostate cancer cells were treated for 24 hours in the presence of HS-5 conditioned medium and subsequently stained with Calcein AM/ethidium homodimer. Using fluorescence microscopy, four representative fields were captured, counted, and averaged to obtain a ratio of dead to live cells. Graph shows the average fold change of dead cells. B) Prostate cancer cells were cultured in a soft-agarose colony formation assay in the presence of HS-5, HS-27a, or control medium for four weeks. Colonies were subsequently stained using p-iodonitrotetrazolium (INT) violet and counted using Volocity 3D Imaging Software. Graph shows the average number of colonies with a diameter of at least 100μm. Representative images are shown of LNCaP colonies after treatment. Scale bar = 100μm. Data are representative of at least three independent experiments performed in duplicate. Statistical analysis was done using Student's *t* test. *P-value < 0.05, **P-value < 0.005, ***P-value < 0.0005.

3.2.2 Conditioned medium from HS-5, but not HS-27a, cells induces death of only osteoblastic/osteosclerotic prostate cancer cells

To study the specificity of the death effect, we tested LNCaP as well as various other cell lines. The cells were grown in HS-5 CM, HS-27a CM, or control medium for 24 hours before the assessment of live and dead cells by live/dead assay. CM harvested from HS-5 cells, but not HS-27a cells, induced death in LNCaP cells. This finding was consistent with the C4-2 and C4-2B sublines. No significant death was detected in DU145, BPH1, MDA-MB-231, MCF7, ZR-75-1, MCF10A, or NIH 3T3 that was used as a non-epithelial cell line control, upon treatment with either HS-5 or HS-27a CM (Figure 4A and B). This suggests the specificity of the death effect of HS-5 CM for osteoblastic/osteosclerotic PCa cells. A list of the cell lines used and their basic properties is given in Table 1.


A
Fold Change in Dead Cells Normalized to DMEM
30
25
20
15
5
4
3
2
1
0
*
HS-5 CM
HS-27a CM
LNCaP
DU145
BPH1
MDA-MB-231
MCF7
ZR-75-1
MCF10A
NIH 3T3

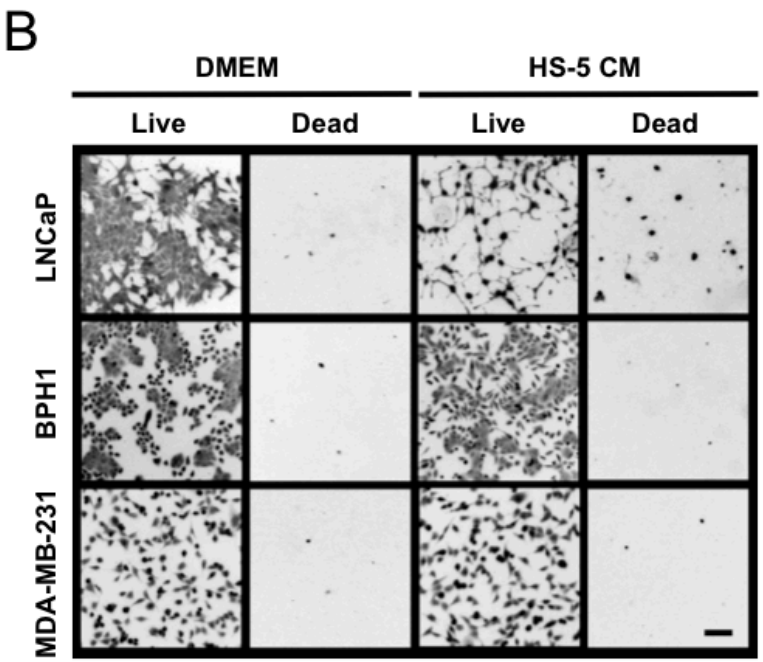

B
DMEM
HS-5 CM
Live
Dead
Live
Dead
LNCaP
BPH1
MDA-MB-231

Figure 4: Conditioned medium from HS-5, but not HS-27a, cells induces death of LNCaP cells, but not other tested cell lines.
Cells were treated with serum-free DMEM as a control, HS-5 CM, or HS-27a CM for 24 hours. A) Graph shows an average estimate of the fold change of dead cells normalized to control medium treatment. Values and error bars represent the mean and standard error of at least three individual experiments performed in duplicate. Statistical analysis was done using Student's *t* test. *, P-value < 0.05.
B) Representative images are shown of LNCaP, BPH1, and MDA-MB-231 cells treated with control medium or HS-5 CM. Scale bar = 100μm.

Table 1: Cell lines used in the study

Cell Line (Reference)	Derivation	Bone Phenotype
HS-5 (Roecklein and Torok-Storb 1995)	Bone marrow stroma	Not applicable
HS-27a (Roecklein and Torok-Storb 1995)	Bone marrow stroma	Not applicable
LNCaP (Horoszewicz, Leong et al. 1983)	PCa metastasis – subclavian lymph node	Non-metastatic
C4-2 (Thalmann, Anezinis et al. 1994)	LNCaP subline	Osteosclerotic
C4-2B (Thalmann, Anezinis et al. 1994, Thalmann, Sikes et al. 2000)	LNCaP subline	Osteosclerotic
DU145 (Stone, Mickey et al. 1978)	PCa metastasis - brain	Osteolytic
BPH1 (Hayward, Dahiya et al. 1995)	Benign prostatic hyperplasia	Not applicable
MDA-MB-231 (Engel and Young 1978)	Breast cancer carcinoma - highly metastatic	Osteolytic
MCF7 (Soule, Vazguez et al. 1973)	Breast cancer carcinoma - weakly metastatic	Osteolytic
ZR-75-1 (Engel, Young et al. 1978)	Breast cancer carcinoma	Osteoblastic
MCF10A (Soule, Maloney et al. 1990)	Normal breast epithelium	Not applicable
NIH 3T3 (Todaro and Green 1963)	Mouse embryonic fibroblast	Not applicable

3.2.3 TGF-β superfamily signaling regulates HS-5 bone marrow stromal-induced cell death or growth inhibition in prostate cancer cells

I investigated the role of TGF-β1, a potent inducer of apoptosis in epithelial cells, in HS-5-mediated cell death. TGF-β1 treatment alone resulted in a 2-fold increase in death in C4-2 cells that was reversible with SB-432541. Co-stimulation with HS-5 CM and TGF-β1 led to an additive effect on cell death in C4-2 cells, as an increase in cell death above treatment with HS-5 CM alone was observed. SB-431542 treatment was intended to demonstrate the dependence upon a type I TGF-β receptor, namely ALK-4, 5 or 7. In LNCaP and C4-2 cell lines, HS-5-induced death was reduced by approximately 3 and 4-fold, respectively, whereas in C4-2B cells, cell death was reduced to a lesser extent, in the presence of SB-431542 (Figure 5A). Stimulation of PCa cells expressing a dominant negative form of TGF-β type II receptor with HS-5 CM had no effect on HS-5-induced cell death, suggesting that the TGF-β subfamily (TGF-β1, TGF-β2 or TGF-β3) was not directly involved in mediating HS-5-induced cell death. In SACF assays, or three dimensional growth, treatment of PCa cells with CM from HS-5 cells, but not HS-27a cells, resulted in a drastic reduction in SACF in LNCaP, C4-2 and C4-2B cell lines. Colony formation was not altered significantly by TGF-β1, and minimal protection from cell death was observed in the presence of SB-431542. In the bone-metastatic C4-2B subline, the number of colonies formed in the presence of SB-431542 was similar to or less than that of HS-5 CM alone, indicating that colony formation is dependent upon signaling through ALK-4, 5 or 7 (Figure 5B). No noticeable change in colony formation was detected when cells were stimulated with HS-27a CM alone or with TGF-β1. However, C4-2B cells again showed a significant reduction in SACF in the presence of SB-431542 (Figure 5C).

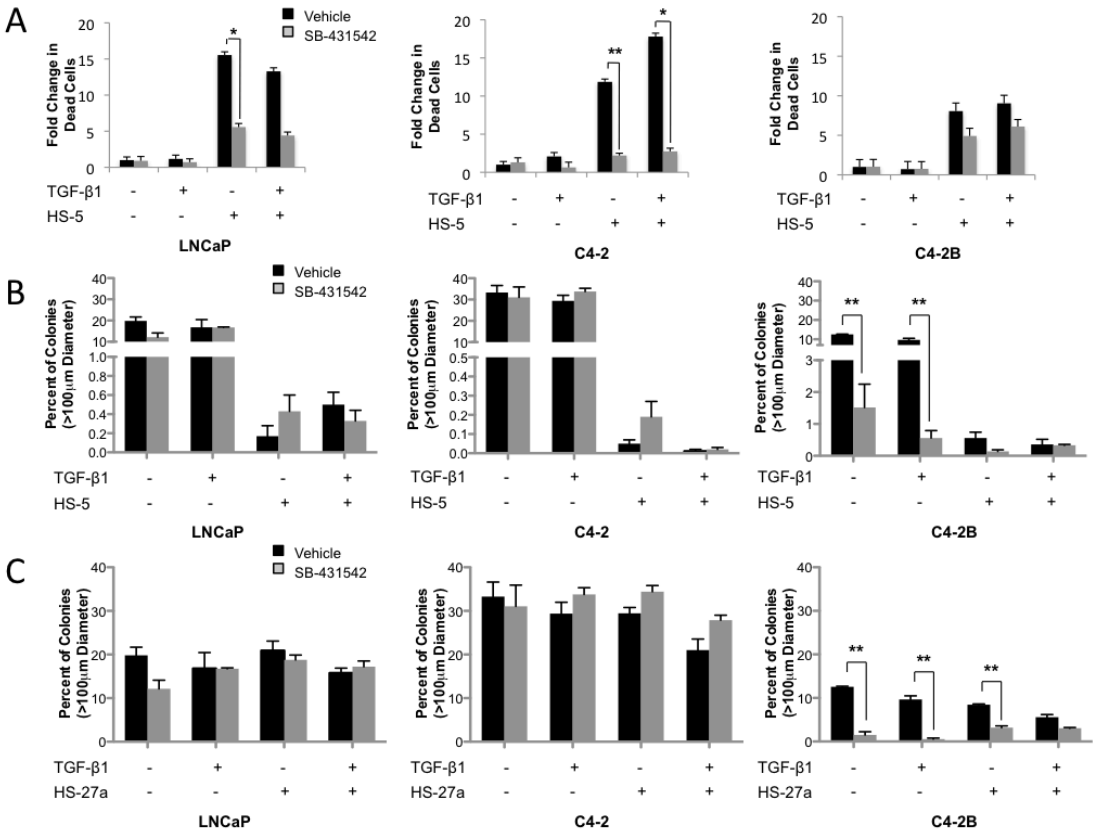
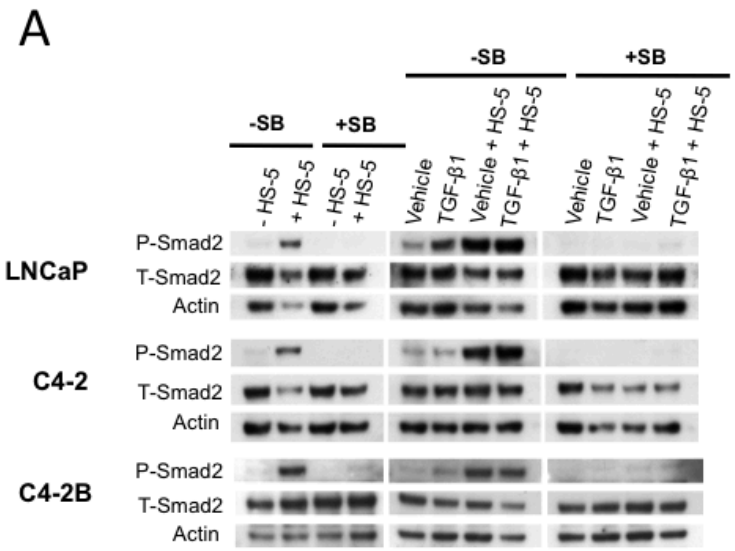


Figure 5: TGF-β superfamily signaling regulates HS-5 bone marrow stromal-induced cell death or growth inhibition in PCa cells.
A) LNCaP (left), C4-2 (middle), and C4-2B (right) cells were conditioned with HS-5 medium, or pre-treated with TGF- β1 or vehicle before conditioning as described, and subsequently stained with Calcein AM/ethidium homodimer for live/dead analysis. Four fields of live and dead cells captured using fluorescence microscopy were counted and averaged to obtain a ratio of dead to live cells. Graphs show the average fold change of dead cells (Data from F. Miles dissertation). B) Soft agarose colony formation assays were performed and prostate cancer cells were cultured for four weeks with HS-5 CM in the presence of TGF-β1 or vehicle, with or without SB-431542. C) Soft agarose colony formation assays were performed by culturing prostate cancer cells with HS-27a CM for four weeks in the presence of TGF-β1 or vehicle, with or without SB-431542. Cells were subsequently stained with p-iodonitrotetrazolium (INT) violet and counted. Graphs show percentage of colonies reaching a diameter over 100μm. Values and error bars represent the mean and standard error of at least three independent experiments performed in duplicates. Statistical analysis was done using Student's *t* test. *P-value < 0.05, **P-value < 0.01.

3.2.4 Smad2 is activated upon treatment of prostate cancer cells with conditioned medium from HS-5 bone marrow stromal cells

Since the phosphorylation of R-Smads such as Smad2 is a typical indicator of TGF-β stimulation, the ability of HS-5 CM to activate Smad2 in PCa cells was examined. Phosphorylated Smad2 (P-Smad2) levels increased dramatically in all PCa cell lines after treatment with HS-5 CM. HS-5 CM-induced increases in P-Smad2 were abrogated completely in the presence of SB-431542, while total levels of Smad2 persisted under all treatment conditions (Figure 6A). Confocal microscopy demonstrated robust nuclear translocation of P-Smad3 after treatment of C4-2 cells with HS-5 CM, in contrast to cells treated with culture medium alone. As expected, nuclear accumulation was inhibited in the presence of SB-431542 (Figure 6B). P-Smad2/3 transcriptional activity was demonstrated using a transcriptional response assay with a p3TP-luc reporter construct. In LNCaP and C4-2 cells transfected with p3TP-luc, p-Smad2/3 transcriptional activity increased upon treatment with HS-5 CM or TGF-β1 compared to control. Higher levels of luciferase activity were noted in LNCaP compared to C4-2 cells. However, there was little to no luciferase activity in C4-2B cells. Statistical analysis was done using a 2-way ANOVA test with specific interest in the interaction term (P-value = 0.16 for interaction of TGF-β1 and HS-5 CM treatment effect). The isolated effect of each individual variable (TGF-β1 or HS-5 CM treatment) on all three cell lines was determined by 1-way ANOVA tests (TGF-β1: P-value = 0.00005; HS-5 CM: P-value = 0.02). Post-hoc pair-wise comparisons were done using Tukey-Kramer multiple comparison test. Based on this test, there is a significant difference between the experimental groups with different letters (Figure 6C).


A
-SB
+SB
-SB
+SB
- HS-5
+ HS-5
- HS-5
+ HS-5
Vehicle
TGF-β1
Vehicle + HS-5
TGF-β1 + HS-5
Vehicle
TGF-β1
Vehicle + HS-5
TGF-β1 + HS-5
LNCaP
P-Smad2
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Actin
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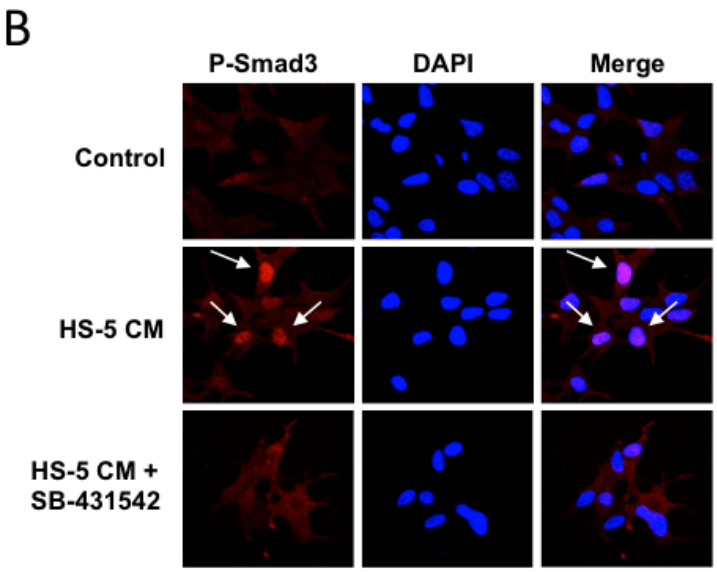
T-Smad2
Actin


B
P-Smad3
DAPI
Merge
Control
HS-5 CM
HS-5 CM +
SB-431542

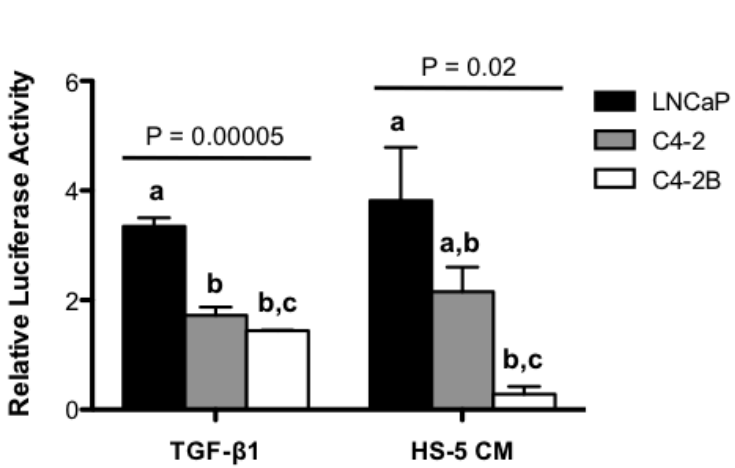

C
Relative Luciferase Activity
6
4
2
0
P = 0.00005
P = 0.02
a
b
b,c
a
a,b
b,c
LNCaP
C4-2
C4-2B
TGF-β1
HS-5 CM

Figure 6: Smad2 is activated after treatment of PCa cells with conditioned medium from HS-5 bone marrow stromal cells.
A) Cells were conditioned with HS-5 medium for 24 hours, or pre-treated with TGF-β1 or vehicle, and lysates were immunoblotted with antibodies to phosphorylated Smad2 (P-Smad2), total Smad2 (T-Smad2), and actin. (Data from F. Miles dissertation). B) Immunofluorescence microscopy was used on cells treated with HS-5 conditioned medium to analyze nuclear translocation of P-Smad3. Cells were labeled with antibodies to P-Smad3 (Rhodamine) and counterstained with DAPI. Arrows show nuclei positive for P-Smad3 with HS-5 conditioned medium treatment only (Data from F. Miles dissertation). C) LNCaP, C4-2 and C4-2B cells were transiently transfected with p3TP-luc and renilla luciferase to analyze Smad2/3 transcriptional activity and treated with HS-5 conditioned medium for 24 hours or TGF-β1 for 4 hours. Firefly luciferase activity was measured using a dual-luciferase kit. Relative luminescence values of firefly luciferase were obtained by normalizing to renilla luciferase. Data are representative of at least three independent experiments performed in duplicates.

3.2.5 TGF-β1 pre-treatment of HS-5 cells largely prevents bone marrow stromal-induced death of prostate cancer cells

Circulating TGF-β1 levels are elevated in sera of PCa patients with bone metastases (Adler, McCurdy et al. 1999). TGF-β1 is also an abundant growth factor in bone and it promotes the vicious cycle of tumor growth and bone destruction by altering the phenotype of cancer cells and stimulating their growth (Hauschka, Mavrakos et al. 1986, Kingsley, Fournier et al. 2007). Because of the high relevance of TGF-β1 in PCa bone metastasis, I was interested in analyzing the effects of TGF-β1-conditioned BMSCs on PCa cells. Specifically, I wanted to determine if such pre-treatment would diminish the HS-5-induced paracrine death effect. Interestingly, stimulation of PCa cells with medium from TGF-β1-pre-treated HS-5 cells had a marked effect on PCa cell death, which was decreased up to 3-fold compared to cells treated with CM from vehicle control HS-5 cells (Figure 7A and B). However, levels of P-Smad2 were not diminished in PCa cells conditioned with medium from TGF-β1-pre-treated HS-5 cells (Figure 7C), suggesting the existence of other active pathways (besides TGF-β superfamily pathways) that signal through Smad2 during HS-5-mediated death.

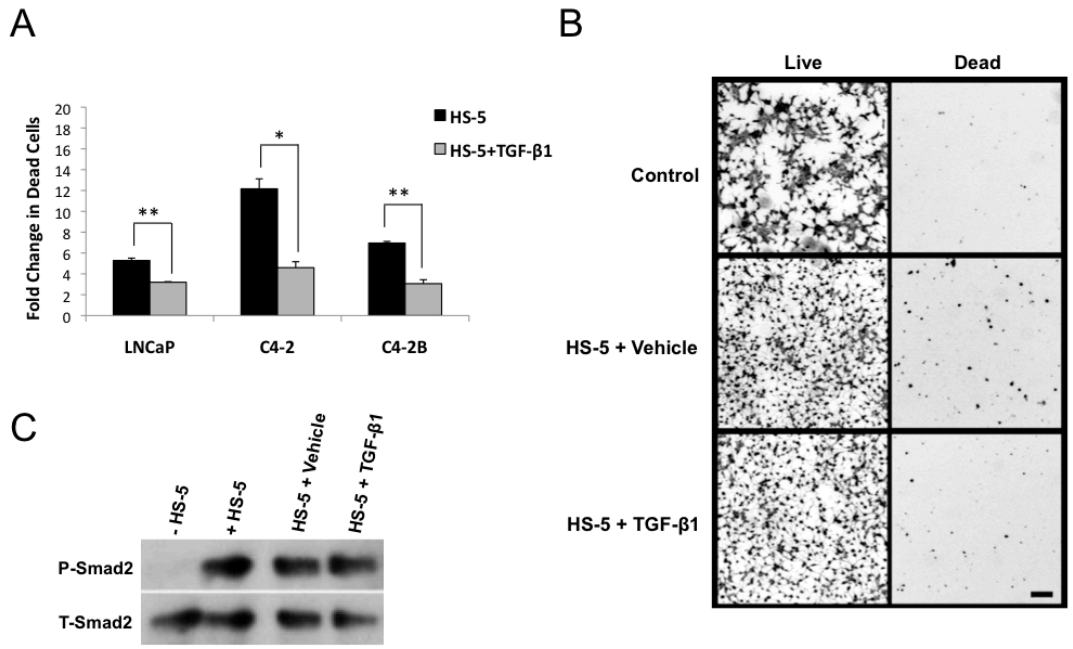


Figure 7: TGF-β1 pre-treatment of HS-5 cells largely prevents bone marrow stromal-induced death of PCa cells.
HS-5 cells were pre-treated with TGF-β1 for four days in DMEM supplemented with 10% FBS, and conditioned medium was collected as described. Prostate cancer cells were treated with HS-5 conditioned medium, or HS-5 conditioned medium collected in the presence of TGF-β1 or vehicle control. A) A live/dead assay was performed as described. Graph shows an average estimate of the fold change of dead cells normalized to control medium (serum-free DMEM) treatment. Values and error bars represent the mean fold change and standard error of at least three independent experiments performed in duplicate. Statistical analysis was done using Student's *t* test. *P-value < 0.05, **P-value < 0.01. B) Representative phase microscopy images are shown of LNCaP cells treated with HS-5 conditioned medium, or HS-5 conditioned medium collected in the presence of TGF-β1 or vehicle. Scale bar = 150μm. C) Subconfluent cultures of LNCaP cells were lysed and immunoblotted for phosphorylated Smad2 (P-Smad2) to examine P-Smad2 levels after treatment with CM from HS-5 cells pre-treated with TGF-β1 (Representative image shown of three replicates) (Data from F. Miles dissertation).

3.2.6 Inhibition of TGF-β signaling in HS-5 bone marrow stromal cells partially reverses the suppression of death of prostate cancer cells induced by the conditioned medium from TGF-β1-pre-treated HS-5 cells

I have demonstrated that exogenous TGF-β1 stimulation suppresses growth in PCa cells and that TGF-β1 stimulation of HS-5 cells before treatment of PCa cells attenuates HS-5-induced death of PCa cells. To further study the role of TGF-β signaling in PCa progression, I used a dominant negative mutant of TGF-β receptor type II (DN-TβRII) to inhibit TGF-β signaling in HS-5 cells. Previous studies have indicated that the loss of TβRII in the prostate stroma promotes PCa initiation, progression, and growth of metastatic PCa in bone (Bhowmick, Chytil et al. 2004, Li, Placencio et al. 2008, Li, Sterling et al. 2012), but the effect of the loss of TβRII in the bone stroma is still unknown. Therefore, I sought to determine the effect of loss of TβRII in HS-5-induced death of PCa cells.

TGF-β1-induced activation of Smad2 as well as basal levels of phosphorylated Smad2 (P-Smad2) were inhibited by the expression of DN-TβRII in HS-5 cells, while total levels of Smad2 persisted and were unchanged under all treatment conditions (Figure 8).

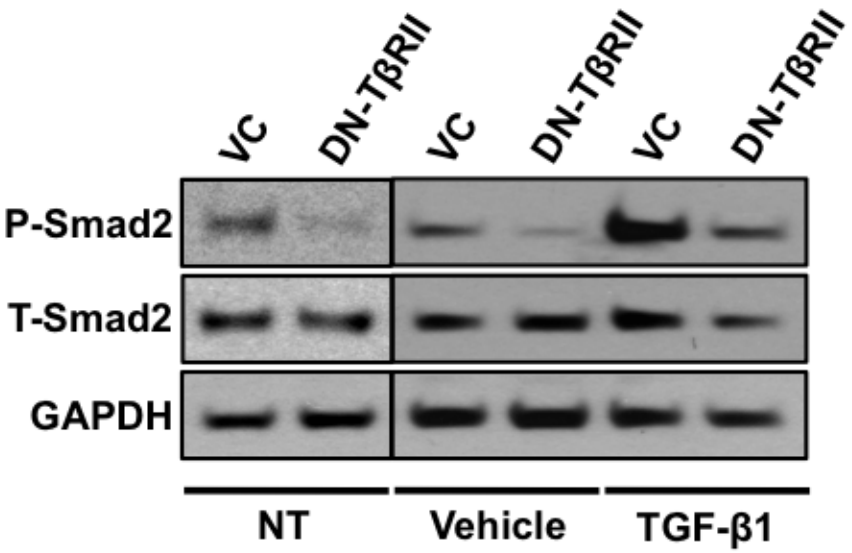


Figure 8: Expression of DN-TβRII inhibits TGF-β1-induced activation of Smad2 as well as basal levels (Non-treated=NT) of phosphorylated Smad2 in HS-5 bone marrow stromal cells.

Whole cell lysates were subjected to SDS-PAGE for Western blot analysis of phosphorylated Smad2 (P-Smad2), total Smad2 (T-Smad2) and GAPDH. GAPDH was used as a loading control. N=3. VC=Vector control (Representative image shown of two replicates).

Results from live/dead assays demonstrated that the CM from TGF-β1-pre-treated HS-5 cells suppresses HS-5-induced death of highly metastatic, castrate-resistant PCa (CRPC) cells to a greater extent (Figure 9A). As expected, the suppression of death of LNCaP cells induced by the CM from TGF-β1 pre-treated vector control and parental uninfected HS-5 cells was reversed by the CM from HS-5 cells expressing DN-TβRII. However, the suppression of death of C4-2 cells induced by the CM from TGF-β1 pre-treated vector control and parental uninfected HS-5 cells was reversed only partially by the CM from HS-5 cells expressing DN-TβRII, and the suppression of death of C4-2B cells induced by the CM from TGF-β1 pre-treated vector control and parental uninfected HS-5 cells was not reversed by the CM from HS-5 cells expressing DN-TβRII. Statistical analysis was done using 2-way ANOVA tests with specific interest in the interaction term for each cell line (LNCaP: P-value = 0.002; C4-2 P-value = 0.0007; C4-2B P-value = 0.005, 2-way ANOVA, for interaction of vehicle and TGF-β1 effect). The isolated effect of each individual variable (vehicle and TGF-β1) was determined by multiple 1-way ANOVA tests for each cell line (LNCaP vehicle: P-value = 0.164, TGF-β1: P-value = 0.013; C4-2 vehicle: P-value = 0.017, TGF-β1 P-value = 0.004; C4-2B vehicle: P-value = 0.013, TGF-β1: P-value = 0.542). Post-hoc pairwise comparisons were done using Tukey-Kramer multiple comparison test. Based on this test, there is a significant difference between the experimental groups with different letters. Student's *t* test was performed further to

determine if there is a significant difference between individual treatments in each cell line (Figure 9A and B).

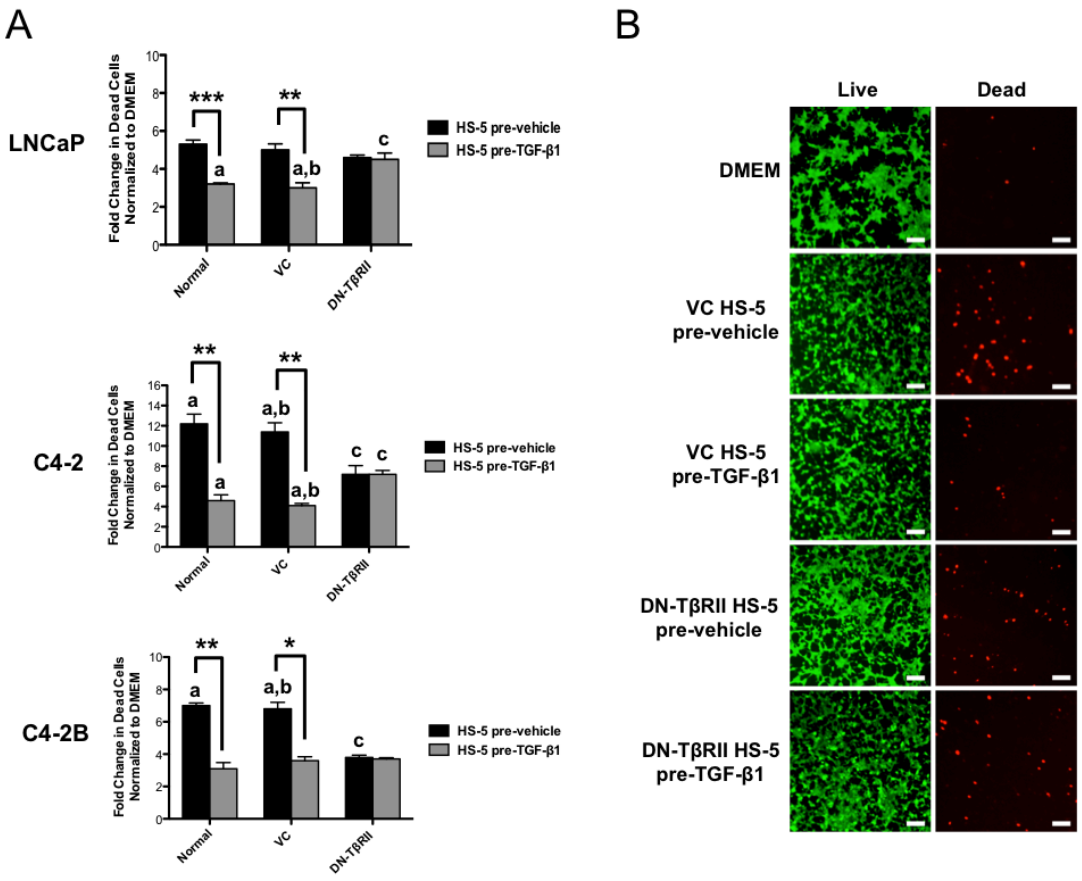


Figure 9: Suppression of death of PCa cells induced by the conditioned medium from TGF-β1-pre-treated vector control and parental uninfected HS-5 cells is partially reversed by the conditioned medium from HS-5 cells expressing DN-TβRII.

A) HS-5, vector control (VC) HS-5, and DN-TβRII HS-5 cells were pre-treated with TGF-β1 or its vehicle for four days in DMEM supplemented with 10% FBS, and conditioned medium was collected for two days in serum-free DMEM. LNCaP, C4-2 and C4-2B cells were treated with control medium (serum-free DMEM), HS-5 conditioned medium, VC HS-5 conditioned medium, or DN-TβRII HS-5 conditioned medium collected after exposure to TGF-β1 or its vehicle as described in Materials and Methods. A live/dead assay was performed as described in Materials and Methods. Graphs show an average estimate of the fold change of dead cells normalized to control medium treatment. Values and error bars represent the mean and standard error of at least three individual experiments performed in duplicate. *, P-value < 0.05; **, P-value < 0.005; ***, P-value < 0.001. B) Representative images are shown of C4-2 cells treated with control medium, VC HS-5 conditioned medium, and DN-TβRII HS-5 conditioned medium collected after TGF-β1 pre-treatment or its vehicle. Scale bar = 150μm.

3.2.7 Conditioned medium from HS-5 bone marrow stromal cells induces neuroendocrine differentiation in prostate cancer cells

In addition to cell death, stimulation with HS-5 BMSC CM induces neuroendocrine differentiation (NED) of PCa cells (Zhang, Soori et al. 2011). PCa cells stimulated with HS-5 CM, which do not undergo apoptosis adopt a neuroendocrine phenotype characterized by long extensions of cellular processes (Figure 10A). LNCaP, C4-2 and C4-2B cells showed a significant increase in the number of branch points per cell (2, 3, and 3-fold, respectively) and the length of processes normalized to cell body size (1.5, 2, and 2-fold, respectively) (Figure 10B). HS-5-induced NED was further characterized by analyzing biomarkers found to be associated with a neuroendocrine phenotype in PCa. Specifically, protein levels of PSA and AR, in addition to NSE were examined using Western blot analysis. Stimulation with HS-5 CM led to dramatic decreases in the levels of PSA and AR in LNCaP and C4-2 cells (Figure 10C).

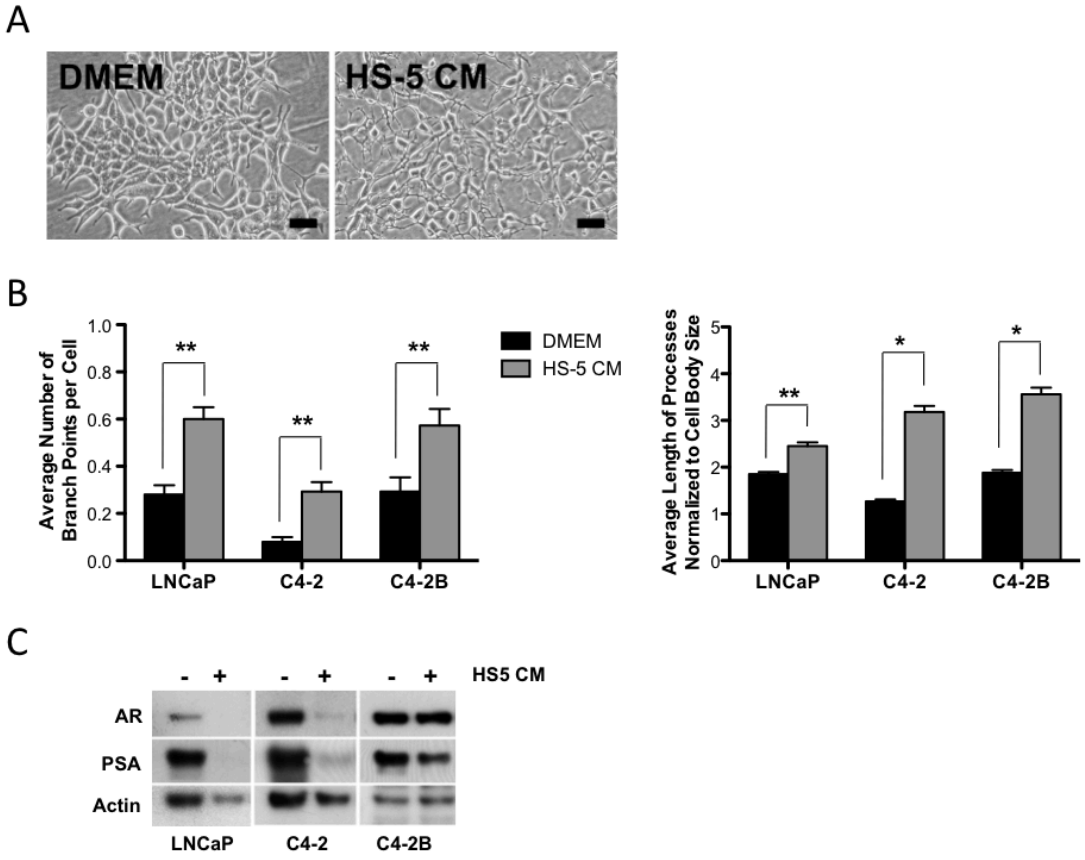


Figure 10: Conditioned medium from HS-5 bone marrow stromal cells induces neuroendocrine differentiation in PCa cells.

A) LNCaP cells were treated for 24 hours with or without HS-5 conditioned medium. Phase microscopy demonstrates HS-5-induced neuroendocrine differentiation as indicated by long extensions of cellular processes and an increased number of branch points. Scale bar = 50μm. B) The number of branch points per cell and the length of processes normalized to cell body size before and after HS-5 treatment were averaged after photomicrographs were taken from five regions of each plate using phase microscopy. Data are representative of at least three independent experiments performed in duplicate. Statistical analysis was done using Student's *t* test for paired data. *P-value < 0.05, **P-value < 0.01 (Data from M. Soori thesis). C) Subconfluent cultures of LNCaP, C4-2 and C4-2B cells were lysed and immunoblotted with antibodies to AR, PSA and actin after treatment with conditioned medium from HS-5 cells (Data from F. Miles dissertation).

3.2.8 Smad2 is activated in prostate cancer cells induced to undergo neuroendocrine differentiation by serum deprivation, independent of conditioning by bone marrow stromal cells

I sought to determine if NED induced independently of BMSC CM could initiate Smad2 signaling in PCa cells. Immunoblotting showed a robust increase in P-Smad2 levels (over 3-fold) in serum-deprived, neuroendocrine differentiated LNCaP, C4-2 and C4-2B cells compared to controls, while total Smad2 was present and unchanged under all treatment conditions. As expected, Smad activity was abrogated in the presence of SB-431542, demonstrating further the ability of SB-431542 to inhibit Smad signaling induced by NED (Figure 11A and B).

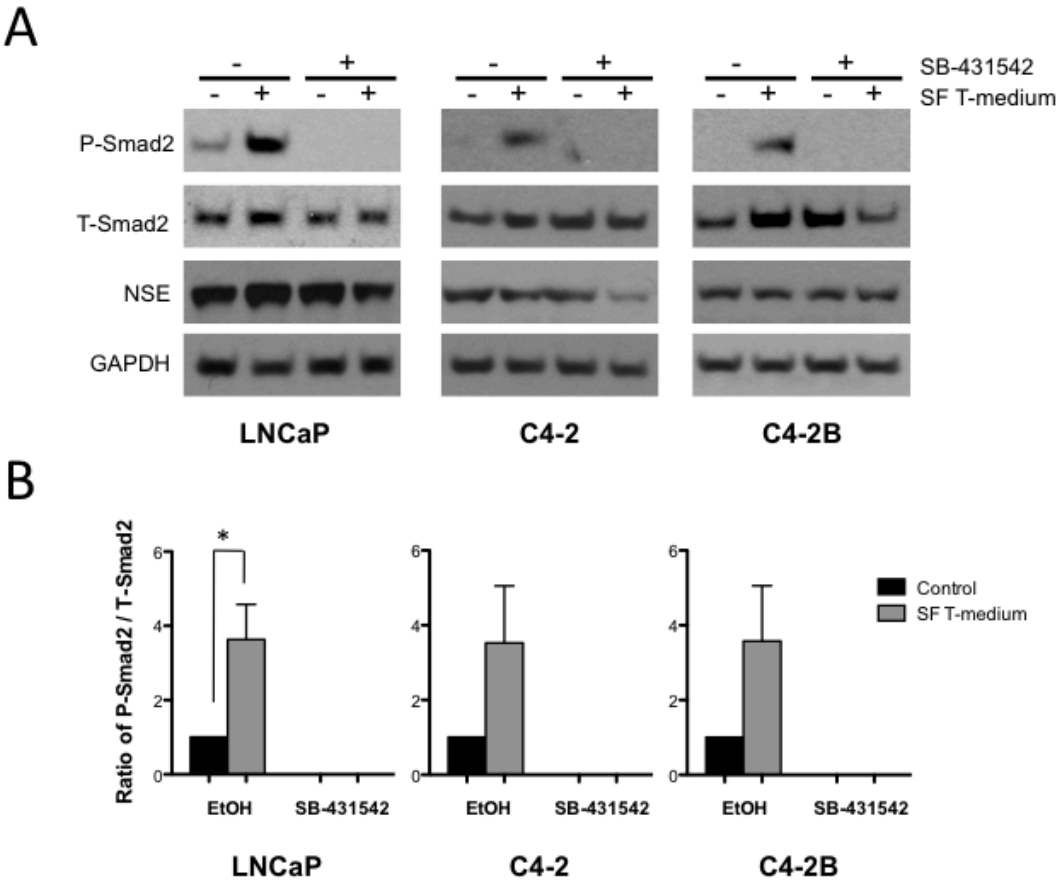


Figure 11: Smad2 is activated in PCa cells induced to undergo neuroendocrine differentiation independent of conditioning by bone marrow stromal cells.
A) Prostate cancer cells were treated with serum-free (SF) medium in the presence or absence of SB-431542 for 24 hours before lysis, and immunoblotted for phosphorylated Smad2 (P-Smad2), total Smad2 (T-Smad2), NSE, and GAPDH (Representative images shown of at least three replicates). B) Quantification of protein levels of P-Smad2 normalized to total levels of Smad2 (T-Smad2) shows neuroendocrine induced increases in P-Smad2. Values and error bars represent the mean ratio and standard error of three independent experiments performed in duplicates. Statistical analysis was done using Student's *t* test. *P-value < 0.05.

LNCaP cells also are known to acquire neuroendocrine characteristics through protein kinase A (PKA) activation upon treatment with the adenylate cyclase activator, forskolin (Fsk) (Cox, Deeble et al. 2000). Therefore, I sought to determine if NED induced by Fsk treatment could initiate Smad2 signaling in PCa cells. Immunoblotting showed no increase in P-Smad2 levels in Fsk-treated, neuroendocrine differentiated LNCaP cells (Figure 12). This finding is not surprising in light of the evidence that cyclic adenosine 3',5'-monophosphate (cAMP)-elevating agents such as Fsk may inhibit TGF-β signaling (Pastorcic and Sarkar 1997, Cox, Deeble et al. 2000). As expected, Smad activity was abrogated in the presence of SB-431542, demonstrating further the ability of SB-431542 to inhibit Smad signaling.

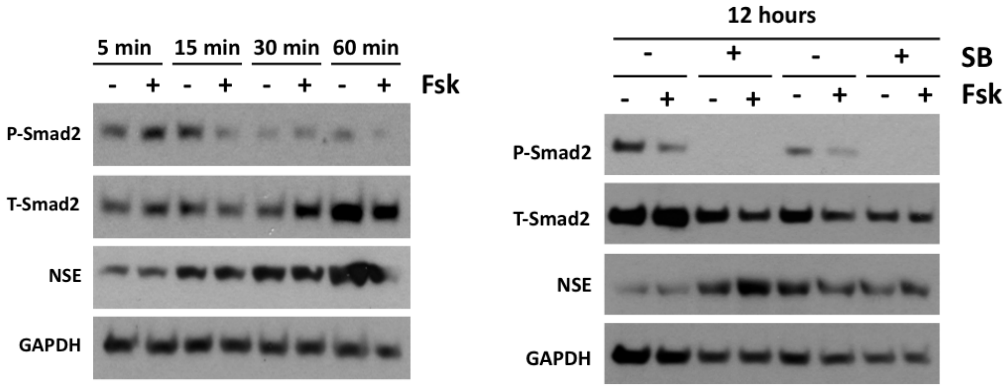


Figure 12: Neuroendocrine differentiation induced by forskolin treatment does not activate Smad2 in PCa cells.
Prostate cancer cells were treated with forskolin (Fsk) for 5 min, 15 min, 30 min, 60 min or 12 hours in the presence or absence of SB-431542 before lysis, and immunoblotted for phosphorylated Smad2 (P-Smad2), total Smad2 (T-Smad2), NSE, and GAPDH (Representative images shown of two replicates).

3.2.9 Prostate cancer cells induced to undergo neuroendocrine differentiation by serum deprivation are resistant to HS-5 bone marrow stromal-mediated apoptosis

The presence of neuroendocrine tumor cells in PCa is associated with aggressiveness, resistance to hormonal therapy, and poor prognosis (Di Sant'Agnese and Cockett 1994, di Sant'Agnese and Cockett 1996, Bonkhoff 2001). Although neuroendocrine cells are withdrawn from the cell cycle, products of neuroendocrine PCa cells have the ability to act as mitogens for PCa (Jongsma, Oomen et al. 2000, Xiao, Qu et al. 2003), but the exact role of neuroendocrine cells in the bone microenvironment is unclear. PCa cells induced to undergo NED by serum deprivation for 72 hours were resistant to HS-5 mediated apoptosis, as apoptosis was reduced to baseline in LNCaP cells, comparable with that of the untreated group (Figure 13), thereby highlighting NED as an adaptive response that increases cell survival.

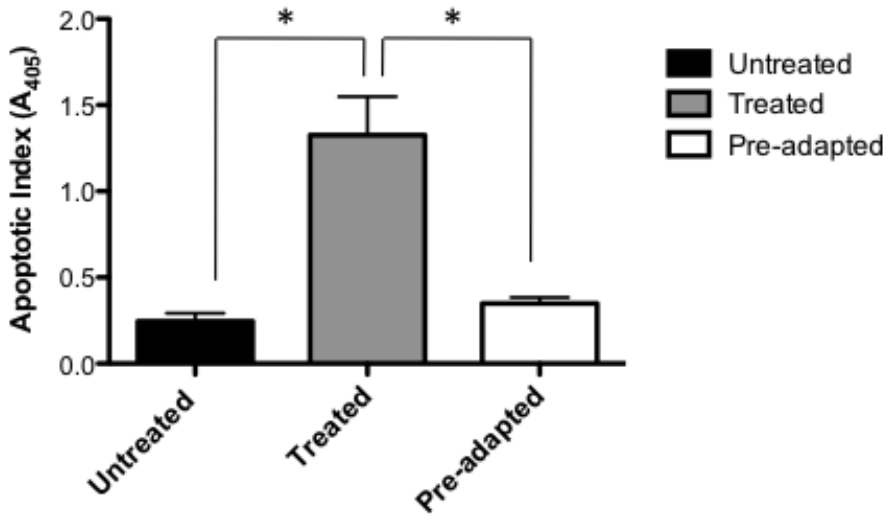


Figure 13: PCa cells induced to undergo neuroendocrine differentiation by serum deprivation are resistant to HS-5 bone marrow stromal-mediated apoptosis.
C4-2B cells were treated with serum-free medium for 72 hours (pre-adapted), followed by stimulation with HS-5 conditioned medium for 48 hours. DNA fragmentation ELISA was used to measure the extent of apoptosis. *P-value < 0.05 (Data from M. Soori thesis).

3.3 Discussion

In this study, I used the isogenic cells of the LNCaP human PCa progression model and the HS-5 and HS-27a bone marrow stromal cells (BMSC) to represent the two major bone marrow stromal cells in the cancer niche (Roecklein and Torok-Storb 1995). The results indicate that conditioned medium (CM) from HS-5 cells, but not HS-27a cells, induces apoptosis of LNCaP lineage cells, but not of any other cell line tested, suggesting the specificity of the apoptotic effect of HS-5 CM for osteoblastic/osteosclerotic PCa cells, although additional osteoblastic/osteosclerotic cell lines should be tested. These results suggest that soluble factors released from BMSCs are initially pro-apoptotic to osteoblastic/osteosclerotic or PSA+, AR+ PCa cells thereby promoting apoptosis of the cells arriving at the bone microenvironment. This is in line with the findings of Lang *et al*. that CM from human red bone marrow does not affect the growth of the PCa cell lines, PC3 and DU145, both of which cause lytic bone degradation (Lang, Miller et al. 1995). This is also in line with the findings of Chang *et al.* that osteolytic PCa cells that escape HS-5 CM-induced apoptosis upregulate p62(sequestome-1)-mediated cytoprotective autophagy (Chang, Morgado et al. 2014).

I have shown that TGF-β signaling is initiated following treatment of PCa cells with BMSC CM. The finding that PCa cell death is suppressed significantly by inhibition of TGF-β superfamily signaling via ALK-4, 5, or 7 implies that a member of the TGF-β superfamily, in part, mediates BMSC-induced cell death, or antagonizes cell survival. This is in line with previous findings that TGF-β1 and other TGF-β superfamily members induce apoptosis or suppress cell growth of PCa cells (Carey,

Sasur et al. 2004, Miles, Tung et al. 2012). Signaling through ALK-4, 5, or 7 is a consequence of TGF-β subfamily members (TGF-β1, TGF-β2, and TGF-β3) as well as activin or nodal signaling, although the specific factor involved in BMSC-induced apoptosis remains to be elucidated. SACF data indicate an enhancement of BMSC growth suppression with virtually no colonies being formed. The inability of SB-431542 to significantly reverse BMSC-mediated colony suppression may be explained by the presence of a positive growth/survival signal that is mediated through ALK-4, 5, or 7. These data illustrate that effects observed in 2D (Miles, Tung et al. 2012) are augmented in three-dimensional conditions. Additionally, signaling through these receptors appears to be fundamental in C4-2B cell growth in three-dimensional conditions, as colony formation was reduced dramatically with SB-431542 treatment alone.

The finding of decreased toxicity of TGF-β1-preconditioned BMSC CM indicates a possible linkage between the observed high serum levels of TGF-β1 in PCa patients having bone metastasis (Wikstrom, Stattin et al. 1998, Shariat, Shalev et al. 2001) and may provide a rationale for increased bone metastasis among special populations, such as African American males, who have overall higher levels of serum TGF-β1 (Eiser 2010). Under this scenario, high TGF-β1 pre-conditions the bone marrow stroma to allow PCa colonization or proliferation. This finding further highlights the context specificity of TGF-β1 and other stromal-secreted factors. TGF-β1 signaling in prostatic stroma is anti-proliferative to prostate cells as loss of TβRII in prostatic stroma leads to PIN lesions and adenocarcinoma *in vivo* (Bhowmick, Chytil et al. 2004, Li, Placencio et al. 2008). Unlike BMSC CM, paracrine factors from

prostatic stromal cells do not appear to induce epithelial cytotoxicity and neuroendocrine differentiation (NED). The mechanism surrounding decreased toxicity in PCa cells after TGF-β1 stimulation of BMSCs is unclear. Nonetheless, it is possible that TGF-β1 signaling in HS-5 cells up-regulates cell survival factors or down-regulates factors responsible for PCa cell death. It should be noted that the ability of TGF-β1 conditioning of HS-5 cells to dampen the paracrine toxic effect of HS-5 CM is consistent with the finding that TGF-β1 is secreted at much lower concentrations from HS-5 cells (4.5-fold), than HS-27a cells, which by itself does not exert the toxic insult on PCa cells ((O'Connor, Farach-Carson et al. 2007) and Figure 3).

The ability of TGF-β1 to modulate intracellular signaling in BMSC is not surprising in light of the role of TGF-β in bone. TGF-β1 facilitates the vicious cycle of bone remodeling that takes place during prostate cancer-bone interactions by coupling bone resorption and formation. Such an action is fundamental in propagating the osteosclerotic nature of PCa lesions. TGF-β1 stimulates osteoclast differentiation and activation through up-regulation of PTHrP in cancer cells (Fox and Lovibond 2005). Whereas TGF-β1 enhances differentiation of hematopoietic cells into osteoclasts (Kaneda, Nojima et al. 2000), it stimulates migration of bone mesenchymal stem cells to the bone surface, and consequently osteoblast differentiation (Tang, Wu et al. 2009). TGF-β1 also stimulates osteoblast activity and inhibits osteoclastic bone resorption by increasing endothelin-1 production by PCa cells (Le Brun, Aubin et al. 1999, Chiao, Moonga et al. 2000).

While the TGF-β signaling pathway suppresses growth when activated in PCa cells, it has an indirect tumor promoting effect on PCa cells when activated in BMSC. Treatment of PCa cells with CM from TGF-β1-preconditioned HS-5 cells suppressed HS-5-induced apoptosis of PCa cells. I wanted to further investigate whether blocking the TGF-β signaling in HS-5 cells by using a dominant negative mutant of TGF-β receptor type II (DN-TβRII) reverses the aforementioned effect. My results suggest that the suppression of apoptosis of highly metastatic CRPC cells induced by the CM from TGF-β1-pre-treated vector control HS-5 cells was not reversed by the CM from HS-5 cells expressing DN-TβRII. The inability of DN-TβRII to reverse TGF-β1-pre-treated HS-5 CM-mediated suppression of apoptosis may be explained by 1) TGF-β1 suppression of apoptosis of highly metastatic CRPC cells occurring independently of TβRII in HS-5 cells, 2) Changes in levels of the pro-apoptotic factor(s) released from HS-5 cells, and/or 3) Increased NED of CRPC cells in response to soluble factors released from HS-5 cells expressing DN-TβRII. This is in line with the findings that PCa cells with neuroendocrine features have resistance to apoptosis ((Fixemer, Remberger et al. 2002) and Figure 13).

The overall implication of these findings is that TGF-β superfamily signaling is activated during NED of PCa and mediates BMSC-induced growth suppression or death of PCa cells, while elevated levels of TGF-β1 in the bone microenvironment induce paracrine signals promoting survival of PCa and perhaps growth. Thus, it is likely that the indirect effects of systemically elevated levels of TGF-β1 on the bone stroma produce an aggressive PCa phenotype in bone, enhancing colonization and remodeling.

Future studies will be necessary to examine the specific role of the TGF-β signaling in PCa cells undergoing NED, as well as the mechanism whereby bone stromal cells in a TGF-β1-rich environment modify signals to promote PCa survival and/or colonization of bone.

Chapter 4

ENDOGLIN IS REQUIRED FOR BONE MARROW STROMAL CELL-INDUCED DEATH OF PROSTATE CANCER CELLS

4.1 Introduction

Endoglin, also known as CD105, is an ancillary TGF-β receptor which binds TGF-β1 and TGF-β3 with high affinity through its association with TGF-β type I and II receptors and thereby modulates the responses to these ligands, but it fails to bind TGF-β2. In addition to TGF-β1 and TGF-β3, endoglin also binds activin-A, and bone morphogenetic proteins (BMP)-2, 7, and 9 by interacting with their respective receptors, suggesting that it is a part of multiple receptor complexes of the TGF-β superfamily (Cheifetz, Bellon et al. 1992, Yamashita, Ichijo et al. 1994, Barbara, Wrana et al. 1999).

Previous studies have indicated that soluble factors released from highly metastatic, castrate-resistant prostate cancer (CRPC) cells, namely C4-2 and C4-2B, attenuate endoglin expression in HS-5 cells and primary bone marrow stromal cells (O'Connor, Farach-Carson et al. 2007). Stimulation of PCa cells with conditioned medium (CM) from TGF-β1-preconditioned HS-5 cells suppressed HS-5-induced death of PCa cells. Due to endoglin's role in regulating TGF-β signaling and the possible implications that its down-regulation may have on the ability of PCa cells to colonize and grow in the bone microenvironment, I sought to examine whether HS-5-induced death of PCa cells is mediated through endoglin regulated TGF-β signaling. Indeed, endoglin levels were decreased upon TGF-β1 stimulation of HS-5 cells suggesting a possible role for endoglin in mediating BMSC-induced death of PCa

cells. Small interfering RNA-mediated knockdown of endoglin in HS-5 cells verified that the effect on death of PCa cells was a direct result of the attenuation of endoglin. Futhermore, the loss of the cytoplasmic domain of endoglin in HS-5 cells attenuated BMSC-induced death of PCa cells indicating the importance of the cytoplasmic domain in maintaining endoglin function and expression of the factor(s) responsible for PCa cell death.

4.2 Results

4.2.1 TGF-β1 treatment of HS-5 bone marrow stromal cells attenuates endoglin expression

Previous studies have indicated that soluble factors released from C4-2 and C4-2B cells attenuate endoglin expression in HS-5 cells and primary bone marrow stromal cells (O'Connor, Farach-Carson et al. 2007). Due to its role in regulating TGF-β signaling and the possible implications that its down-regulation may have on the ability of PCa cells to colonize and grow in the bone microenvironment, I sought to determine whether TGF-β1 treatment of HS-5 cells would alter endoglin levels. TGF-β1 treated HS-5 cells showed decreased levels of endoglin mRNA (Figure 14A) and protein (Figure 14B). Densitometry analysis indicated an approximately 50% decrease in endoglin protein levels upon TGF-β1 treatment of HS-5 cells (Figure 14C).

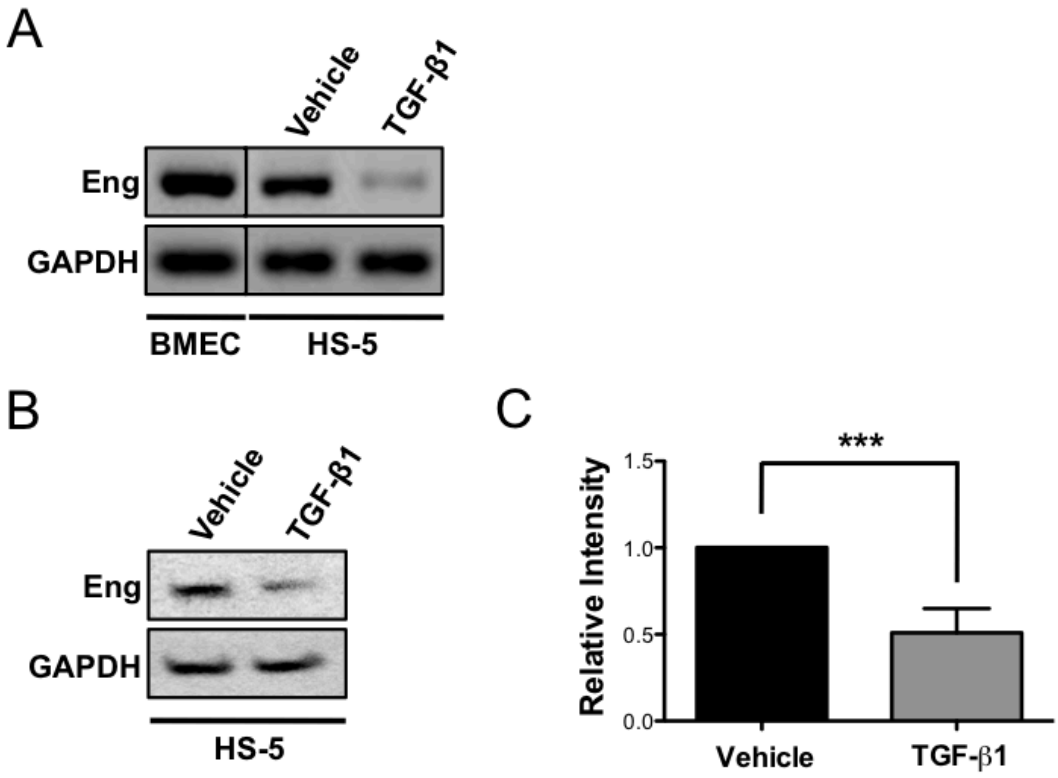


Figure 14: TGF-β1 treated HS-5 bone marrow stromal cells show decreased levels of endoglin mRNA and protein.
A) Endoglin (Eng) mRNA expression was analyzed using RT-PCR. Bone marrow endothelial cells were used as a positive control (Representative image shown of at least three replicates). B) Whole cell lysates were subjected to SDS-PAGE for Western blot analysis of endoglin and GAPDH. GAPDH was used as a loading control and the data were normalized to GAPDH in (C) (Representative image shown of three replicates). C) Graph shows the mean ratio of endoglin signal to GAPDH signal. Values and error bars represent the mean and standard error of three individual experiments performed in duplicate. Statistical analysis was done using one-sample Student's *t* test. ***, P-value < 0.001.

4.2.2 siRNA-mediated knockdown of endoglin in HS-5 cells prevents bone marrow stromal-induced death of prostate cancer cells

I demonstrated that TGF-β1 stimulation of HS-5 cells before treatment of PCa cells attenuates HS-5 CM-induced death of PCa cells. Here, to show that the effect on suppression of HS-5 CM-induced death is the direct result of attenuated endoglin protein levels, I used a small interfering RNA (siRNA) directed against endoglin in HS-5 cells. Endoglin protein levels were decreased by approximately 50% after transfection with an siRNA directed against endoglin compared with HS-5 cells transfected with a scrambled siRNA control, as demonstrated by a densitometry analysis (Figure 15A). Attenuation of endoglin in HS-5 cells resulted in a significant suppression of HS-5 CM-induced death of LNCaP cells and a marginally significant suppression of HS-5 CM-induced death of C4-2B cells, suggesting that endoglin is involved directly in HS-5 CM-induced death of PCa cells. The lower PCa death values reported upon HS-5 CM treatment were due to the shorter assay time. As the persistence of siRNA occurs for only a short period of time, conditioned medium collection was carried out for 24 hours instead of four days (Figure 15B).

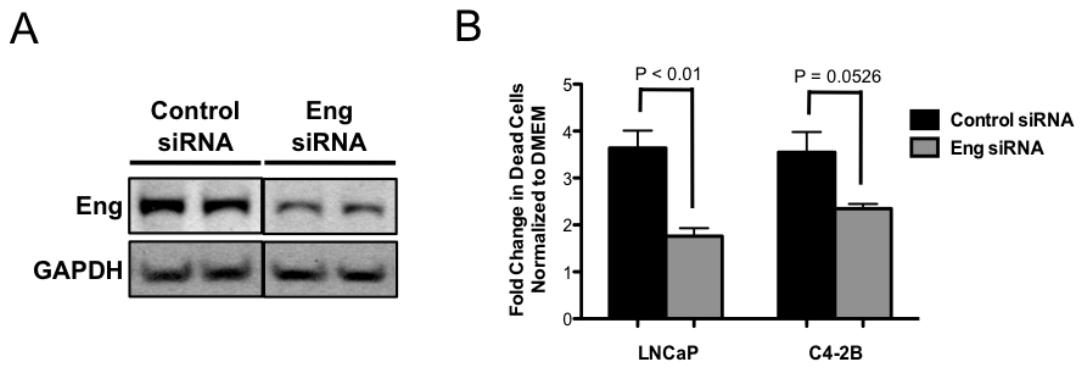


Figure 15: siRNA-mediated knockdown of endoglin in HS-5 cells largely prevents bone marrow stromal-induced death of PCa cells.
HS-5 cells were transfected with an siRNA specific for human endoglin or a scrambled sequence (control), and conditioned medium was collected as described in Materials and Methods. A) Whole cell lysates were subjected to SDS-PAGE for Western blot analysis of endoglin and GAPDH. GAPDH was used as a loading control. Shown are two of three independent experiments. B) A live/dead assay was performed as described in Materials and Methods. Graph shows an average estimate of the fold change of dead cells after treatment with and without conditioned medium from HS-5 cells transfected with an siRNA specific for human endoglin. Values and error bars represent the mean fold change and standard error of at least three independent experiments performed in duplicates. Statistical analysis was done using Student's *t* test.

4.2.3 Conditioned medium from HS-5 cells expressing the C-terminal deleted mutant of endoglin prevents bone marrow stromal-induced death of prostate cancer cells

Since phosphorylation of the cytoplasmic domain of endoglin by TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII) is important in regulating endoglin function (Guerrero-Esteo, Sanchez-Elsner et al. 2002, Koleva, Conley et al. 2006, Ray, Lee et al. 2010), I investigated further the requirement of the cytoplasmic domain of endoglin in HS-5 CM-induced apoptosis of PCa cells. Expression of a truncated construct lacking the cytoplasmic domain (ΔC Eng) in HS-5 cells behaved as a dominant negative and attenuated HS-5 CM-induced apoptosis of PCa cells (Figure 16).

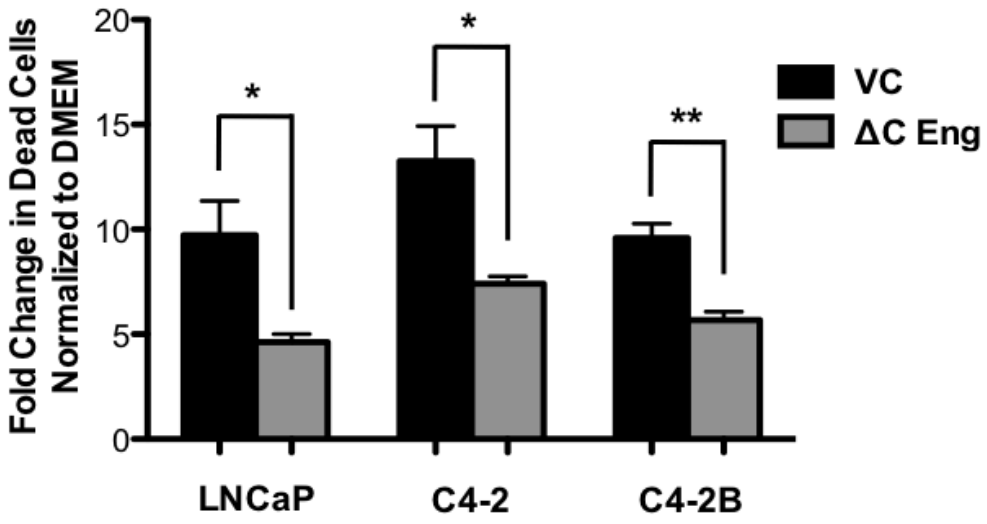


Figure 16: Conditioned medium from HS-5 cells expressing the C-terminal deleted mutant of endoglin largely prevents bone marrow stromal-induced death of PCa cells.

Conditioned medium was collected from vector control (VC) HS-5 or C-terminal lacking (ΔC) Eng transfected HS-5 cells for four days as described in Materials and Methods. A live/dead assay was performed as described in Materials and Methods. Graph shows an average estimate of the fold change of dead cells after treatment with and without conditioned medium from HS-5 cells expressing the ΔC mutant of endoglin. Values and error bars represent the mean fold change and standard error of at least three independent experiments performed in duplicates. Statistical analysis was done using Student's *t* test. *, P-value < 0.05; **, P-value < 0.005.

4.3 Discussion

Endoglin is an ancillary TGF-β receptor that modulates TGF-β-dependent cellular responses. It is a 633 amino acid, 180kDa disulfide-linked, hypoxia-inducible, homodimeric trans-membrane glycoprotein expressed at high levels on vascular endothelial cells (Cheifetz, Bellon et al. 1992, Nassiri, Cusimano et al. 2011). So far, most studies on endoglin have focused on its importance in endothelial cells. Elevated expression of endoglin has been linked to proliferation of endothelial cells and tumor angiogenesis (Burrows, Derbyshire et al. 1995, Miller, Graulich et al. 1999). Here, I provide evidence for a critical role for endoglin in BMSC pro-apoptotic effects on PCa cells. My data suggest that endoglin is required for BMSC-induced apoptosis of PCa cells. Endoglin has a serine/threonine-rich cytoplasmic domain that is phosphorylated by the TGF-β receptors type I and type II and this has been shown to be required for endoglin-mediated endothelial cell adhesion, migration, and growth (Guerrero-Esteo, Sanchez-Elsner et al. 2002, Koleva, Conley et al. 2006, Ray, Lee et al. 2010). My data further suggest the importance of the cytoplasmic domain of endoglin in maintaining its function in cell signaling well beyond co-presentation of TGF-β ligands.

Circulating TGF-β1 levels are elevated in sera of PCa patients with bone metastases (Adler, McCurdy et al. 1999). TGF-β1 also is an abundant growth factor in bone and it promotes the vicious cycle of tumor growth and bone destruction by altering the phenotype of cancer cells and stimulating their growth (Hauschka, Mavrakos et al. 1986, Kingsley, Fournier et al. 2007). Here, I propose a mechanism whereby elevated TGF-β1 in the bone microenvironment permits the survival of PCa cells by down-regulating endoglin in BMSC, which is required for the induction of

apoptosis of PCa cells (Figure 17). Future studies will be necessary to identify the soluble factor(s) that induce PCa cell apoptosis.

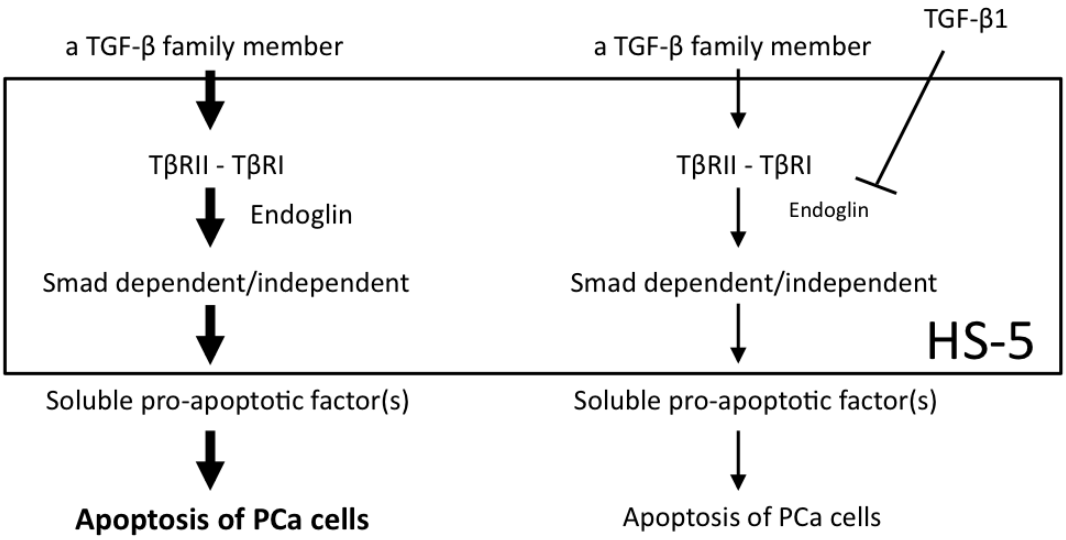


Figure 17: A possible mechanism of HS-5 bone marrow stromal-mediated apoptosis of PCa cells.
In the absence of TGF-β1, a TGF-β family member, which acts through the TβRII-TβRI-endoglin receptor complex induces apoptosis of prostate cancer cells. In the presence of TGF-β1, endoglin is down-regulated which decreases the affinity of the TGF-β ligand to the receptor complex. This leads to decreased production of the pro-apoptotic factor(s) and hence decreased apoptosis in prostate cancer cells. Therefore, elevated TGF-β1 in the bone microenvironment permits the survival of prostate cancer cells by down-regulating endoglin in bone marrow stromal cells.

Chapter 5

DISCUSSION AND FUTURE DIRECTIONS

Paget postulated the important role of the tumor microenvironment in the formation of distal metastases nearly a century ago and this still remains the basic principle of metastasis (Ribatti, Mangialardi et al. 2006). He described the bone microenvironment as a specialized "*soil"* that favors the metastasis of certain cancer cells (or "*seeds"*), including PCa cells. Although the precise mechanisms by which PCa cells preferentially colonize bone still remain unknown, it is well established that the bone microenvironment provides a fertile "*soil"* of cytokines and growth factors, which PCa utilizes to form metastatic lesions (Chung 2003). Notably, TGF-β1, a multifunctional cytokine, has been implicated highly in PCa progression. It has been reported that TGF-β1 signaling in cancer has suppressive effects on normal epithelial and early-stage cancer cells while having tumor-promoting effects on advanced cancers (Bierie and Moses 2006). While the molecular mechanisms underlying the initial process of PCa colonization of bone that contribute to the lethal phenotype are poorly understood, it is well established that there is a dynamic interplay between PCa cells and the bone stroma (Loberg, Bradley et al. 2007). The *in vitro* studies presented in this dissertation were undertaken to simulate the paracrine interactions occurring between PCa cells and the bone marrow stromal cells when they first encounter each other in the bone microenvironment. The results suggest that TGF-β1 exerts tumor-promoting effects indirectly on PCa cells through the bone marrow stroma, while its direct effects on PCa cells remain anti-proliferative and tumor-suppressive. Thus, it is clear that the role of TGF-β1 in PCa is quite complex.

The complex bone microenvironment may initially resist the newly resident PCa cells but PCa cells acquire adaptive changes that allow them to survive and grow in the "hostile" new microenvironment as they co-evolve in their genotypic and phenotypic characters with bone cells (Koeneman, Yeung et al. 1999, Knerr, Ackermann et al. 2004, Sung, Hsieh et al. 2008, Josson, Matsuoka et al. 2010). My studies showed that soluble factors released from immortalized human bone marrow stromal cells induce apoptosis of PCa cells, and the surviving cells undergo neuroendocrine differentiation, characterized by morphological changes consistent with a neuroendocrine phenotype. Although neuroendocrine cells are withdrawn from the cell cycle, products of neuroendocrine PCa cells have the ability to act as mitogens for PCa (Jongsma, Oomen et al. 2000, Xiao, Qu et al. 2003), but the exact role of neuroendocrine cells in the bone microenvironment is understood poorly. The studies presented herein suggest that neuroendocrine PCa cells are resistant to bone marrow stromal induced-apoptosis, thereby highlighting neuroendocrine differentiation as an adaptive response that increases PCa survival.

The major findings of this dissertation are:

1. Soluble factors produced by HS-5 bone marrow stromal cells induce apoptosis or neuroendocrine differentiation of osteoblastic/osteosclerotic PCa cells.

2. TGF-β1 stimulation of HS-5 bone marrow stromal cells before conditioning of PCa cells attenuates bone marrow stromal-induced apoptosis. Inhibition of TGF-β signaling using a dominant-negative mutant of TGF-β receptor type II (DN-TβRII) in

HS-5 cells reverses this effect only in the non-metastatic LNCaP cells which may suggest that 1) TGF-β1 suppression of apoptosis of highly metastatic castrate-resistant prostate cancer cells occurring independently of TβRII in HS-5 cells, 2) Changes in levels of the pro-apoptotic factor(s) released from HS-5 cells, and/or 3) Increased neuroendocrine differentiation of castrate-resistant prostate cancer cells in response to soluble factors released from HS-5 cells expressing DN-TβRII.

3. The mechanism surrounding decreased toxicity in PCa cells after TGF-β1 stimulation of bone marrow stromal cells is unknown but it can be hypothesized that TGF-β1 signaling in HS-5 cells either up-regulates cell survival factors or down-regulates pro-apoptotic factors.

4. TGF-β1 stimulation of HS-5 bone marrow stromal cells attenuates the expression of the ancillary TGF-β receptor endoglin suggesting the importance of endoglin in mediating bone marrow stromal-induced apoptosis of PCa cells. Furthermore, the loss of the cytoplasmic domain of endoglin in HS-5 cells attenuates bone marrow stromal-induced apoptosis of PCa cells indicating the importance of the cytoplasmic domain of endoglin in maintaining its function in cell signaling well beyond co-presentation of TGF-β ligands (Figure 18).

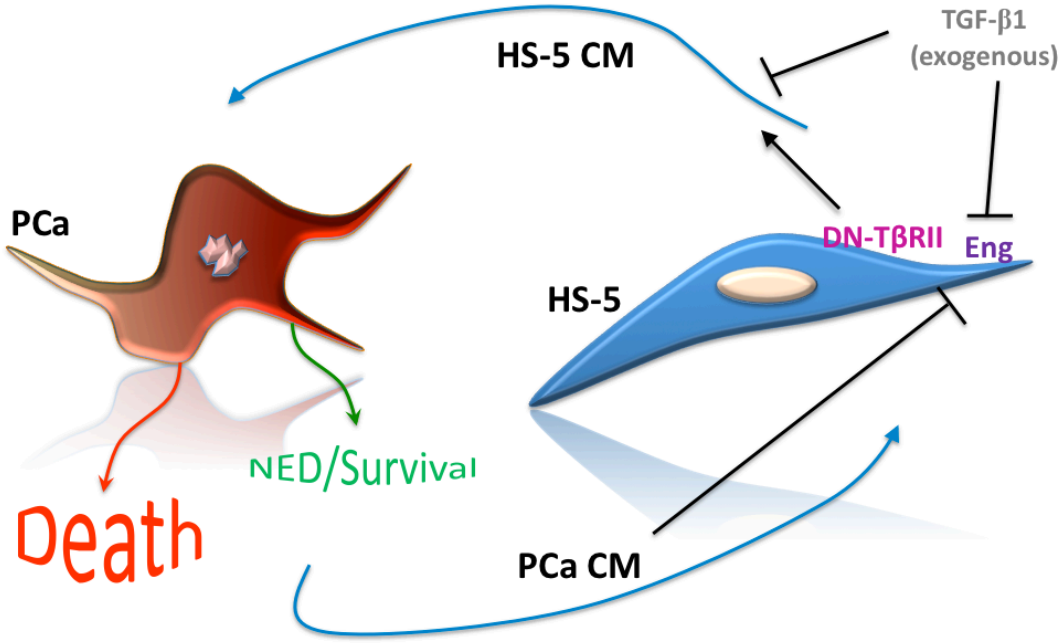


Figure 18: Model of modulation of PCa and HS-5 bone marrow stromal cell interactions by TGF-β signaling.
Soluble factors produced by HS-5 cells induce apoptosis or neuroendocrine differentiation of osteoblastic/osteosclerotic prostate cancer cells. TGF-β1 stimulation of HS-5 cells before conditioning of prostate cancer cells attenuates HS-5-induced apoptosis. Inhibition of TGF-β signaling using a dominant-negative mutant of TGF-β receptor type II (DN-TβRII) in HS-5 cells reverses this effect only in the non-metastatic prostate cancer cells. TGF-β1 stimulation of HS-5 cells attenuates the expression of endoglin suggesting the importance of this ancillary TGF-β receptor in mediating HS-5-induced apoptosis of prostate cancer cells.

An obvious question from my research is the identity of the soluble pro-apoptotic factor(s) secreted from bone marrow stromal cells. Currently, advanced metastatic PCa has no curative therapy, therefore there is a clear need for development of new and effective therapies for advanced PCa. To this end, administration of the pro-apoptotic factor(s) can be used as a means to selectively target and kill metastatic PCa cells and may provide a treatment option for advanced disease.

My findings suggest the requirement of the ancillary TGF-β receptor endoglin in mediating bone marrow stromal-induced apoptosis of PCa cells. The primary focus of further testing should be the dissection of the molecular mechanisms governing this process and the *in vivo* significance.

Endoglin is an ancillary TGF-β receptor that binds TGF-β1 and TGF-β3 but not TGF-β2, with high affinity through its association with TGF-β type I and type II receptors and thereby modulates the responses to these ligands. In addition to TGF-β1 and TGF-β3, endoglin also binds activin-A, and bone morphogenetic proteins (BMP)-2, 7 and 9 by interacting with their respective receptors, suggesting that it is a part of multiple receptor complexes of the TGF-β superfamily (Cheifetz, Bellon et al. 1992, Yamashita, Ichijo et al. 1994, Barbara, Wrana et al. 1999) (Figure 2). Endoglin is expressed at high levels on vascular endothelial cells (Cheifetz, Bellon et al. 1992, Nassiri, Cusimano et al. 2011), and it is required for extraembryonic angiogenesis. This was illustrated by the death *in utero* of endoglin knockout mice from defects in vascular development (Arthur, Ure et al. 2000). In studies done with a MAPKKK, TGF-β-activated kinase 1 (TAK1)-deficient mouse embryos, TAK1-deficient embryos

exhibited defects in the developing vasculature. Such phenotype is strikingly similar to that exhibited by loss-of-function mutations in endoglin, suggesting that TAK1 may be an effector of the TGF-β receptor complex (Jadrich, O'Connor et al. 2006). Furthermore, TAK1 has been shown to function upstream of the TGF-β-mediated activation of JNK and p38 MAPKs (Yamaguchi, Shirakabe et al. 1995, Shim, Xiao et al. 2005). This renders the TAK1-mediated MAPK pathway a potential signaling pathway downstream of endoglin. In light of this information, possible mechanisms of endoglin mediated bone marrow stromal-induced apoptosis of PCa cells are depicted in Figure 19.

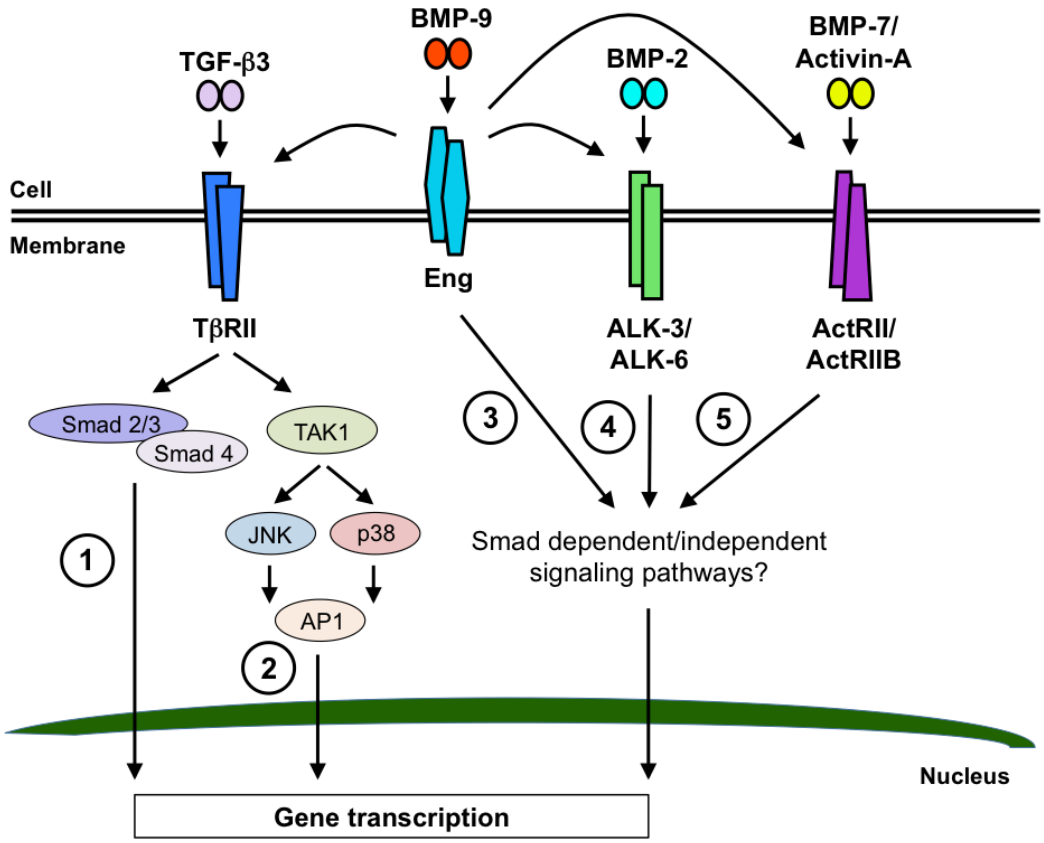


Figure 19: Possible mechanisms of endoglin mediated secretion of pro-apoptotic factor(s) from HS-5 bone marrow stromal cells.
1) Smad-dependent TGF-β3 signaling pathway, 2) TGF-β-activated kinase 1 (TAK1)-mediated TGF-β3 signaling pathway, 3) Smad dependent/independent bone morphogenetic protein (BMP)-9 signaling pathway, 4) Smad dependent/independent BMP-2 signaling pathway, or 5) Smad dependent/independent BMP-7/activin-A signaling pathway. Eng, endoglin; JNK, c-Jun amino-terminal kinase; AP1, activator protein 1; ALK, activin receptor-like kinase; ActRII, activin receptor type II; ActRIIB, activin receptor type IIB.

Based on my data showing that endoglin mediates bone marrow stromal-induced apoptosis of PCa cells, I hypothesize that down-regulation of endoglin in the bone stroma will result in a more permissive environment for PCa cells to survive and grow. Likewise, over-expression of endoglin in the bone stroma will result in an environment where PCa cells are unable to survive. This can be addressed by modulation of endoglin expression in the bone stroma by conditional knock-out or over-expression in transgenic mice.

PCa growth in bone is a function of complex interactions between the cancer cells and the cells in the bone microenvironment. The presence of PCa cells in bone often disrupts the normal RANK/RANKL/OPG signaling in the bone microenvironment, hence proper bone turnover, giving rise to osteosclerotic lesions, which are predominantly osteoblastic in nature (Morrissey and Vessella 2007). TGF-β1 has been shown to be an important cytokine that plays a role in this signaling (Oreffo, Mundy et al. 1989, Murakami, Yamamoto et al. 1998, Takai, Kanematsu et al. 1998, Thirunavukkarasu, Miles et al. 2001, Yan, Riggs et al. 2001, Dallas, Rosser et al. 2002, Liao and McCauley 2006, Bonfil, Chinni et al. 2007, Sato, Futakuchi et al. 2008). Furthermore, several reports have indicated that circulating TGF-β1 levels are elevated in sera of PCa patients with bone metastases (Adler, McCurdy et al. 1999). My hypothesis was that the elevated circulating TGF-β1 in PCa patients reprograms the bone marrow stroma to enhance PCa colonization and survival in bone, and this is mediated through the suppression of pro-apoptotic paracrine factors produced by bone marrow stromal cells. While my *in vitro* findings show that TGF-β1 stimulation of HS-5 bone marrow stromal cells before conditioning of PCa cells attenuates bone

marrow stromal-induced apoptosis thereby suggesting that elevated levels of TGF-β1 in the bone microenvironment induce paracrine signals promoting the survival of PCa, the significance of this phenomenon *in vivo* is unidentified.

Future work should focus on determining the role of elevated TGF-β1 on growth of osteosclerotic PCa lesions in bone**.** The stimulation of osteosclerotic bone metastases may be examined by tail vein injection of TGF-β1 into host SCID mice prior to intra-tibial injection of PCa cells. My hypothesis will be that elevated TGF-β1 levels in mice will increase the ability of PCa cells to form tumors in bone. There will be increased tumor volume and larger/more osteosclerotic lesions with higher bone turnover in TGF-β1 pre-treated mice as compared to vehicle pre-treated control mice. Serum PSA levels, a marker for PCa progression (Mishra, Shiozawa et al.), also are expected to be higher. Vehicle pre-treated mice will have limited ability to form tumors upon intra-tibial PCa injection because of the pro-apoptotic factors produced by bone marrow stromal cells. The pro-apoptotic effect of bone marrow stromal cell conditioned medium on PCa cells may be tested by isolating bone marrow stromal cells from femurs of mice, culturing, and harvesting conditioned medium to be assayed for the ability to induce apoptosis in PCa cells. Here, it is important to note that the pro-apoptotic effect of HS-5 cells will be diluted possibly due to the presence of factors produced by other bone marrow stromal cells. This partially explains why it is still expected to observe colonization and tumor formation in bone by PCa cells upon intra-tibial injection in control animals, although to a lesser extent than in TGF-β1 pre-treated animals. If increasing TGF-β1 levels in mice by administering recombinant TGF-β1 does not increase the ability of PCa cells to form tumors in bone,

this may be due to non-proliferative nature of neuroendocrine differentiated phenotype of surviving PCa cells upon encountering HS-5-like cells in the bone microenvironment. In this case, another alternative to examine how TGF-β signaling exerts its pro-tumorigenic effects indirectly on PCa cells through bone marrow stromal cells *in vivo* would be to use stromal specific *TGFBR2* knockout mice with abrogated TGF-β signaling specifically in the stroma, which was generated previously by Bhowmick *et al*. (Chytil, Magnuson et al. 2002, Bhowmick, Chytil et al. 2004, Li, Sterling et al. 2012).

Identifying regulators of PCa cell survival may lead to new therapeutic strategies for PCa. Undoubtedly, the overall goal of this project is to design therapeutic targeting strategies that can be used to prevent or kill PCa bone metastasis. On the basis of evidence that circulating TGF-β1 is elevated significantly in PCa patients with bone metastases, and its well-established role in cancer progression and bone remodeling, TGF-β1 likely plays a role in PCa colonization of bone (Bonewald and Mundy 1990, Adler, McCurdy et al. 1999). Thus, understanding the role of TGF-β1 in facilitating PCa colonization in the bone microenvironment is essential to designing therapeutic approaches to target bone metastases.

As described, despite intensive research efforts, the mechanisms underlying PCa bone metastases are not yet fully understood. The general understanding of the role of the host microenvironment in PCa survival and growth in the bone evaluated in this dissertation may provide insight into the design of novel therapeutic strategies for cancer metastasis. Since it is well established that there is a dynamic interplay between

the tumor cells and the stroma, it is of pivotal importance to keep in mind both the tumor and the stroma in designing new treatment strategies.

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