CHAPTER V

EPITHELIAL-TO-MESENCHYMAL TRANSITION IN PROSTATE CANCER

The epithelial-to-mesenchymal transition (EMT) was originally defined by developmental biologists as a morphological conversion occurring at specific sites in embryonic epithelia to give rise to individual migratory cells (142). EMT involving modulation of cell-cell adhesion occurs during both physiological and pathological states (143). EMT mediates gastrulation movements, the emigration of neural crest cells from the neural tube, and the formation of cardiac valves (142-145) in many species. EMT has also been associated with the formation of the parietal endoderm in mammals (146). During EMT, cell-cell adhesion is disrupted; tight junctions, adhesions junctions and desmosomes are dissembled and cell substrate adhesion complexes are reorganized (147). Epithelial cell plasticity also occurs in diseases such as fibrosis and cancer. In chronic kidney disease, tubular epithelial cells express fibroblast markers and are associated with interstitial damage (148). In gastric (149) and colorectal cancers (150), epithelial-derived cancer cells take on properties typical of mesenchymal cells during invasion and metastasis. Thus, EMT in tumorigenesis, which allows benign tumor cells to metastasize to distant organs, can be associated with poor prognosis.

The mechanisms that govern EMT are now being unraveled in embryonic development, tissue culture, and in tumors. Several signaling pathways seem to be common to EMTs in development and tumor progression (151), which raises the

hypothesis that tumor progression could be regarded as a reactivation of some aspects of the embryonic program of EMT. E-cadherin, the prototype type 1 epithelial cadherin, has been studied extensively in EMT, and is therefore emerging as a caretaker of the epithelial phenotype. Numerous studies have described a partial or complete loss of E-cadherin during carcinoma progression, which is

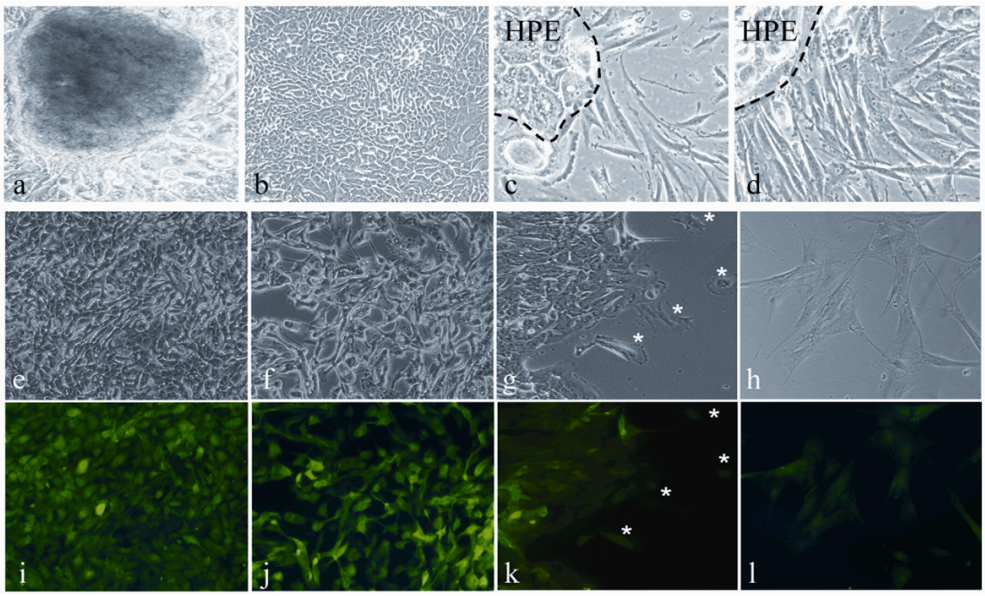

Figure 17. EMT like cells emerge from primary human prostate tissue culture. Spindle like cells emerge under culture conditions that favored epithelial cell growth (panels a-h). HPE cells were labeled with CMFDA tracer dye. CMFDA dye is retained by dividing daughter cells during cell proliferation (panels i and j). EMT-like cells separating from outer edges of epithelial cell colonies retain CMFDA (panels k and l), indicating they arise from HPE cells. * in panels g and k indicate emerging EMT-like cells.

correlated with an unfavorable prognosis (152, 153). Usually, down-regulation of E-cadherin during carcinoma progression occurs by epigenetic mechanisms including transcriptional repression, promoter hypermethylation, and gene mutation (154-160). Along with down-regulation of E-cadherin during EMT formation, occurs the up-regulation of vimentin, which is expressed in the more migratory mesenchymal cells. Vimentin expression is regulated by a variety of cytokines and growth factors such as TGFß1, PDFG, FGF and EGF.

Various signaling cascades have been implicated in EMT formation both in the context of normal development and tumor progression. TGFß1 was first reported to induce EMT in normal mammary epithelial cells by signaling through the Smad family of proteins (161). In response to TGFß1-ligand binding to its receptors, Smad2 and Smad3 are phosphorylated and translocated to the nucleus as a complex with Smad4. These Smad complexes interact with specific response elements in the promoter region of their target genes such as Snail1, which is an E-cadherin repressor and is increased in abundance in EMT cells. The pivotal role played by Smad complexes in EMT formation was further demonstrated when primary tubular epithelial cells derived from Smad3 knockout mice failed to elicit EMT phenotype upon TGFß stimulation (162). While the role played by TGFß ligands signaling through Smad complexes in EMT formation has been well documented, emerging evidence indicate co-operation between Smad-dependent and –independent TGFβ signaling cascades occupies a central role in EMT phenotype (162). Cross-talk between TGFß and Ras/Raf/MEK/MAPK pathways has been reported to be essential to maintain EMT phenotype in various epithelial cell types (162). Activation of Erk1/2 is required to induce disassembly of adherens junction in TGFß induced EMT (163). Induction of EMT in cultured normal mammary epithelial cells (NMuMG) requires rapid phosphorylation of p38MAPK (164). There has been further evidence of cross talk between TGFß and Wnt signaling pathways in the maintenance of EMT phenotype. TGFß has been reported to induce ß-catenin, which is required for the synthesis of α-Smooth Muscle Actin (α-SMA), which is a marker of EMT (165). TGFβ also activates LEF1,

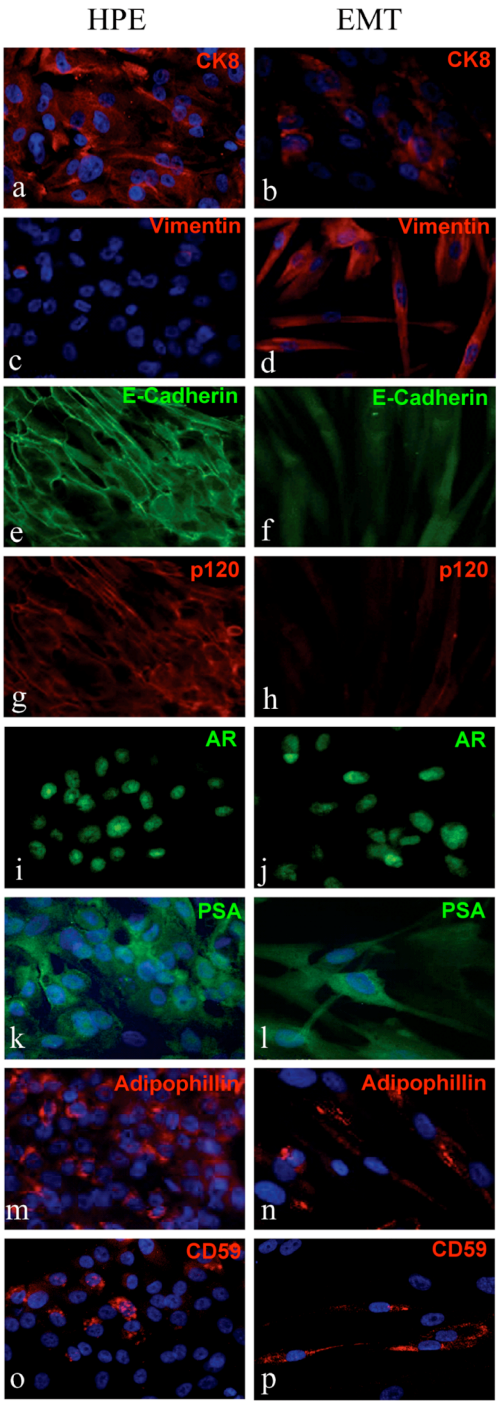

Figure18. EMT cells lost epithelial characters and acquired mesenchymal characters and both HPE and EMT cells retain secretory phenotype. a and b: CK8 (red) expression in HPE and EMT cells. c and d: Vimentin (red) expression in HPE and EMT cells. e and f: E-cadherin (green) expression in HPE and EMT cells. g and h: p120 (red) expression in HPE and EMT cells. Both HPE and EMT cells express AR (i and j,green) and PSA (k and l, green). Adipophilin immunoreactivity (red) is localized in a vacuolar, intracellular compartment in HPE and EMT cells (m and n). CD59 staining (red) shows intracellular granular staining in HPE and EMT cells (o and p).

which is a downstream target of Wnt/β-catenin signaling pathway (166). LEF1 can induce EMT directly in the presence of nuclear β-catenin, which is yet another marker of EMT (167). Cross talk between TGFβ and NF-κB pathway has also been established in the EMT phenotype. Inhibition of NF-κB blocked TGFβ-induced EMT formation in mammary epithelial cells (168). It has also been shown that NF-κB causes up-regulation of Snail (169), which is a target gene of Smad family of transcription factors and is frequently up-regulated in EMT cells. TGFβ signaling cascade also co-operates with the Rho signaling network to activate phosphatidylinositol-3-OH kinase (PI3K) during EMT formation in mammary epithelial cells (170). These studies demonstrate the interaction and cross talk of TGFβ pathways with other pathways such as the MAPK, Wnt, NF-κB and PI3K pathways, in inducing and maintaining the EMT phenotype.

In the context of human malignancies, it is often debated whether the EMT phenotype can explain the progression from a benign non-invasive tumor to a metastatic tumor. EMT-like cells are rarely seen in primary tumor sections and the histological similarities between metastatic tumor in a distant organ and the primary tumor from which it is derived stems such controversies. Although the apparent histological similarity between primary tumors and metastatic tumors derived from them can be explained by a reverse Mesenchymal Epithelial Transition (MET) phenotype, the rarity of EMT-like cells in a primary tumor section calls for models which can be used to track the changes during the EMT phenotype. Unfortunately, it is not possible to follow the EMT process in human tumors *in vivo* due to the diverse cellular organization of neoplasms.

Until now, little was known about EMT in prostate cancer. Cell clones derived from the Dunning R-3327 rat prostate adenocarcinoma exhibit EMT characteristics with loss of E-cadherin expression, increased invasiveness *in vitro*, and lung metastases in the xenograft model *in vivo* (171). In this study, we present a novel model to study the emergence of EMT in prostate cancer.

EMT in primary human prostate tissue culture

In culturing primary HPE cells from prostate specimens obtained from radical retropubic prostatectomy, we observed the emergence of spindle-like cells under culture conditions that favored epithelial cell growth (Fig. 17). The following experiments were designed to determine the origin of these cells. HPE cells were cultured and labeled with 5-chloromethylfluorescein diacetate (CMFDA) tracer dye (Fig. 17 Panel C). To determine if the CMFDA was being released into the medium, culture medium was collected from CMFDA-labeled cells after 24 hours in culture and transferred to new plates of untreated HPE cells. No labeled cells appeared in control dishes, indicating that CMFDA was not released into the medium. CMFDA-labeled HPE cells were subsequently cultured from 3 weeks to 3 months to determine whether they underwent EMT. The green CMFDA tracer dye is retained by dividing daughter cells during cell proliferation (Fig. 17 Panel C). Furthermore, EMT-like cells that separate and migrate out from the outer edges of the epithelial cell colonies retain CMFDA, indicating they arise from HPE cells (Fig. 17 Panel C). Spindle-shaped cells arise in five out of seven Gleason 8 and five of six Gleason 9 biopsies after 3 weeks in culture (Table 2), confirming that EMT occurs in late stage disease. In contrast, this transition is not observed in any HPE

cell cultures derived from benign prostatic hyperplasia (BPH) and prostatic intraepithelial Neoplasia (PIN), as well as low- and intermediate-grade prostate cancer (gleason 5-7) specimens (Table 2), even when cells were cultured up to 3 months.

EMT cells express both epithelial and mesenchymal markers. HPE and EMT-like cells which arose from them were analyzed for expression of E-cadherin, p120, CK8 and vimentin. As expected, expression of epithelial cell markers CK8, E-cadherin and p120 in EMT-like cells are greatly decreased and residual E-cadhein and p120 staining is no longer associated with the cell membrane (Fig. 18a-h). In addition, EMT-like cells express vimentin (Fig. 18a-h), suggesting that they have aquired mesenchymal characteristics. HPE and EMT-like cells were also stained with antibodies to other proteins typically expressed by prostate epithelial cells. Similar to that seen in HPE cells, EMT-like cells express androgen receptor (AR) and prostate specific antigen (PSA), implying they remain androgen-responsive (Fig. 18i-l). Furthermore, they retain a secretory phenotype as evidenced by the expression of adipophillin and CD59 (Fig. 18m-p). Adipiphillin is a protien component of lipid storage droplets associated with cellular differentiation and secretion (172). CD59 is found in prostasomes which are membrane-bound storage vesicles in prostatic acinar epithelial cells (173). Thus, EMT-like cells express both epithelial and mesenchymal proteins and will be referred to as EMT cells in the following experiments.

EMT is thought to promote cell invasiveness during late stage tumorigenesis (174). To determine whether EMT cells had aquired a more invasive phenotype, primary

EMT cells were compared to primary HPE cells, LNCaP prostate cancer cells, which are minimally invasive and PC3 prostate cancer cells which are highly invasive, utilizing the modified Boyden chamber assay (Fig. 19 Panels A, B). HPE cells are minimally invasive, simmilar to that observed in LNCaP cells (Fig. 19 Panels A, B). EMT cells exhibit a greater rate of invasion through Matrigel than either HPE or LNCaP cells, but not as high as metastatic PC3 cells (Fig. 19 Panels A, B). The rate of proliferation of EMT and HPE cells were also compared by Alamar blue assay. EMT cell proliferation is significantly greater than that of HPE cells at the same passage number (Fig. 19 Panel C).

Signaling pathways activated in EMT phenotype in primary human prostate tissue culture

To investigate the possible mechanisms by which this transition occurs, we compared mRNA expression profiles on 5 pairs of HPE and EMT cells using 30K human microarray chips (Fig. 20). We found that the expression of 1946 genes significantly changed (>2 fold), of which 908 genes were upregulated and 1038 genes were downregulated (Fig. 21 Panel A). The observation that signaling pathways, such as the TGFβ, Ras/MAPK and Wnt signaling pathways that have been reported to contribute to EMT phenotype in other models were also misregulated (Fig. 21 Panel B), led further credence to our model. Of the 305 genes representing the TGF-β pathway on the microarray chip, 16 are up-regulated and 8 are down-regulated (Fig. 21 Panel B) (Table 3). Similarly, out of 263 MAPK pathway genes, 7 are up-regulated and 6 are down-regulated (Fig. 21 Panel B) (Table 4). Interestingly, the microarray analysis reveals a significant overlap between TGF-β and Wnt signalling since 15 out of 16 up-regulated

genes are held in common and 8 of the 8 down-regulated genes are held in common in both the pathways (Fig. 21 Panel B) (Table 5).

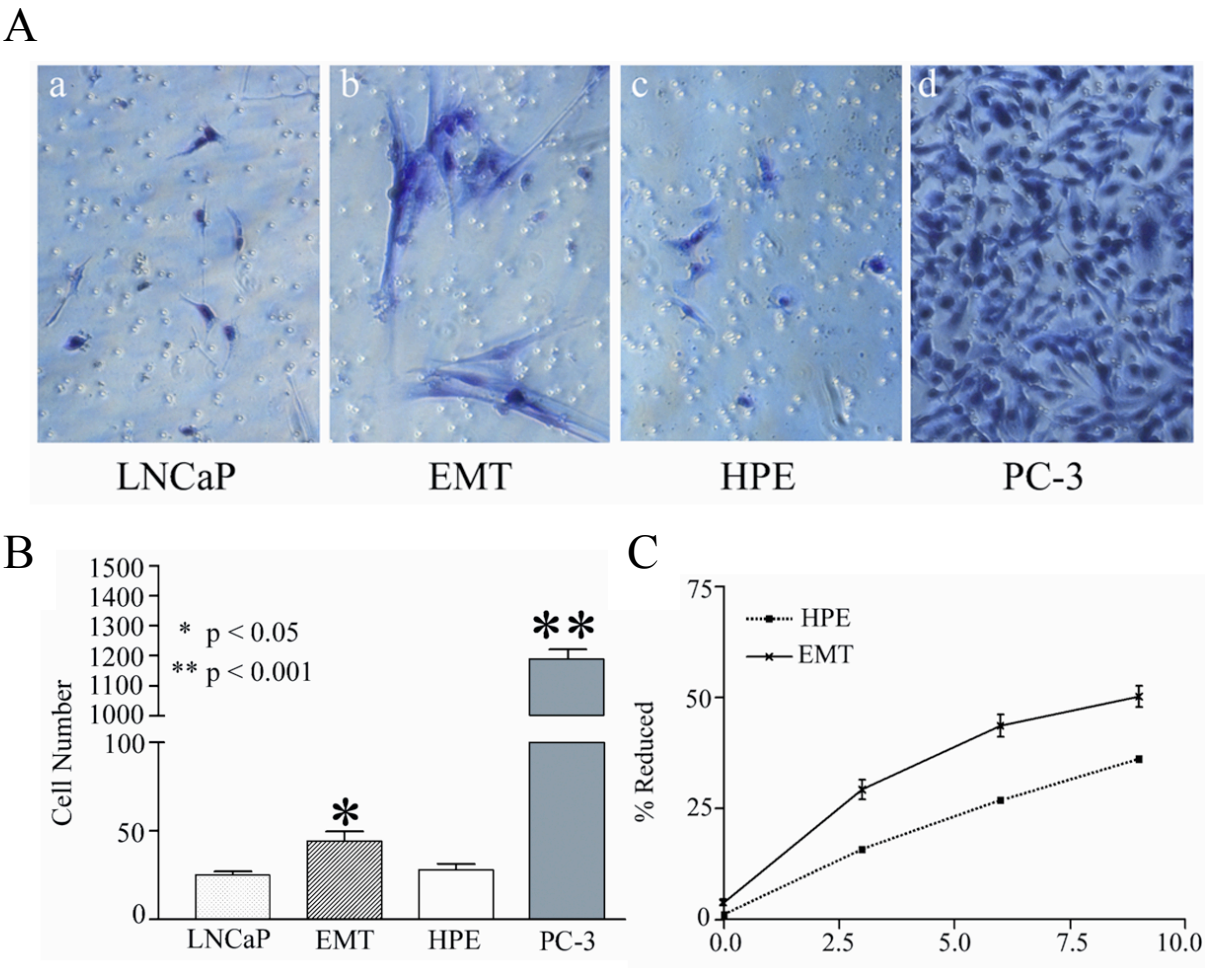

Figure 19: EMT cells are more invasive and have a greater rate of proliferation than HPE cells. Invasive potential of HPE and EMT cells were compared by Boyden Chamber Assay (Panels A and B). HPE cells are minimally invasive (c) similar to LNCaP cells (a). EMT cells (b) are more invasive than either LNCaP or HPE but less than PC-3 (d) cells. Rate of proliferation of EMT and HPE cells were compared using Alamar Blue assay (Panel C). EMT cell proliferation is significantly higher than that of HPE cells at the same passage number.

One of the genes down-regulated during EMT is stathmin, a protein which promotes microtubule destabilization (98). Furthermore, stathmin has been implicated in a number of cancers including prostate cancer (175). Smad2, 3 and 4 have been reported to be bound to microtubules in mouse aortic endothelial cells (78). TGF-β stimulation leads to dissociation of Smads from microtubules and subsequent phosphorylation in these cells. Since, TGF-β signaling has been linked to microtubules, we hypothesized stathmin may be involved in this pathway. To determine which pathway(s) stathmin regulate in TGFβ-induced EMT, an *in vitro* model of EMT will be of paramount importance. The short life-span of primary cell cultures, coupled with the paucity of high Gleason score tumor samples makes it extremely difficult to employ the primary cell culture model to study EMT. Hence, concerted efforts to establish a tissue culture model to study EMT can further our understanding of the molecular events during tumor progression and invasion leading to metastasis.

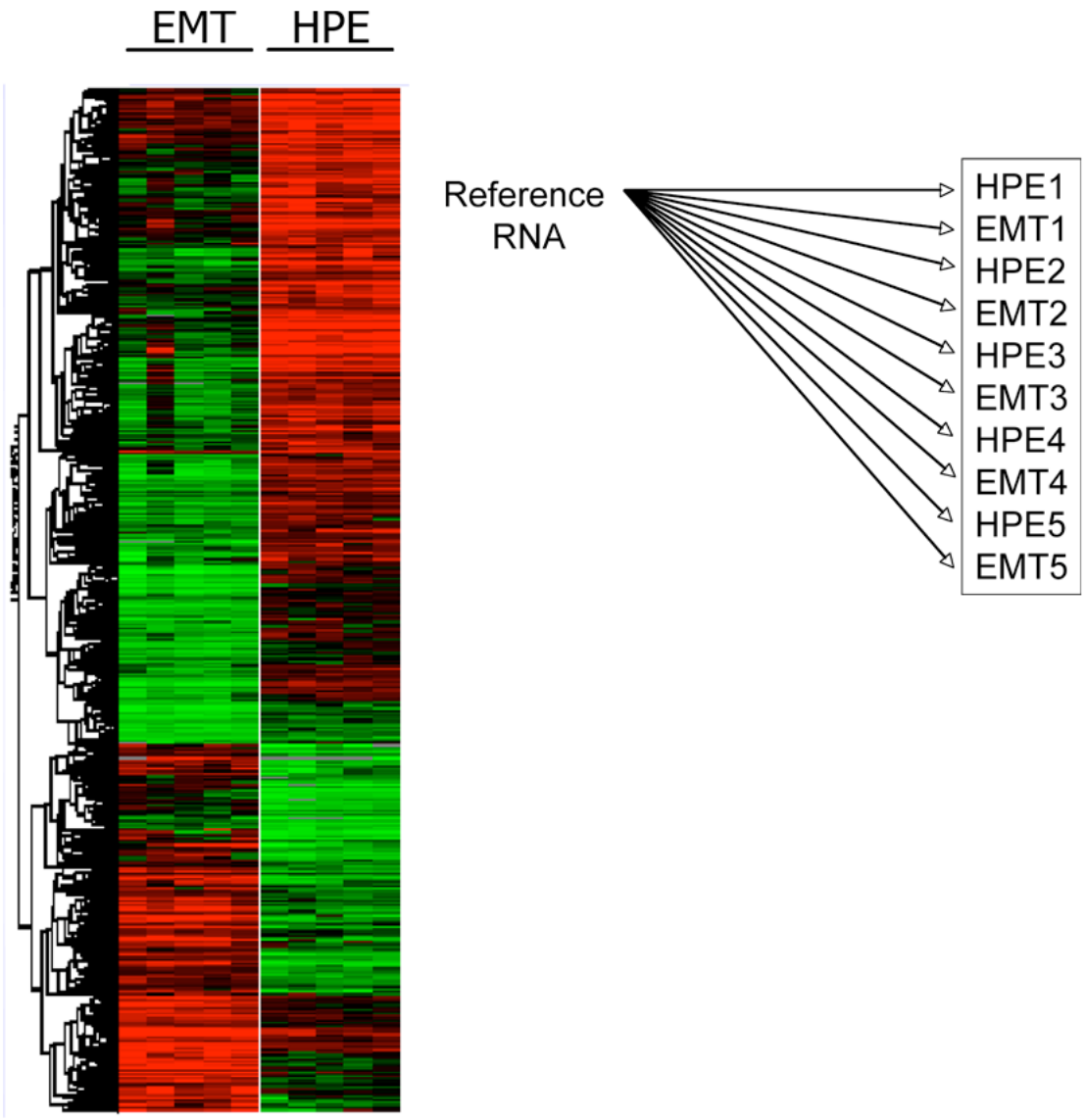

Figure 20: Microarray Analysis to compare gene expression profiles of EMT and HPE cells. mRNA expression profiles of 5 pairs of EMT and HPE cells were compared using 30K human microarray chips.

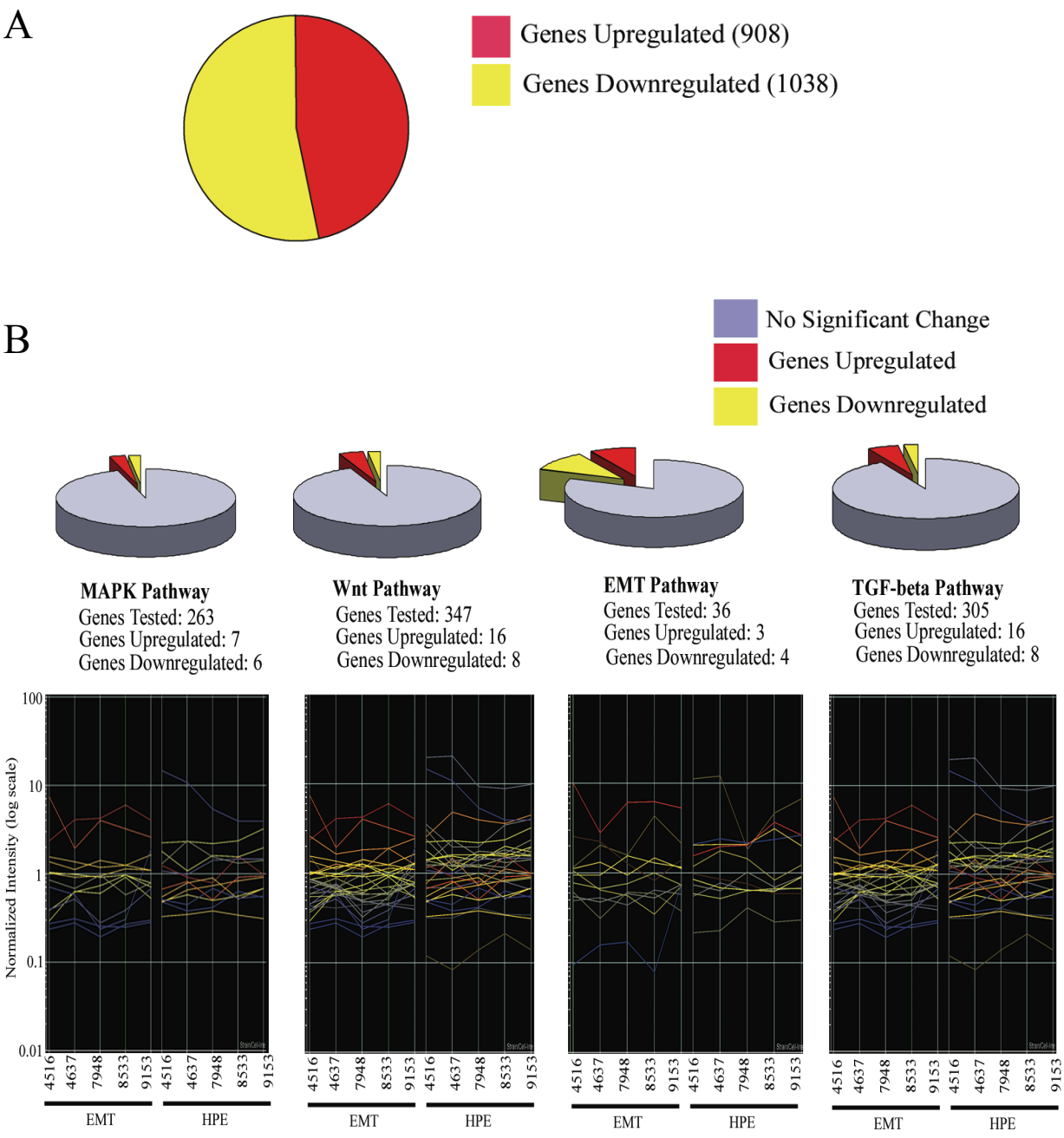

Figure 20: Pathway Analysis to compare gene expression profiles of EMT and HPE cells. Genespring softare were used to analyze the results of the microrray experiment. A total of 1946 genes were differentially expressed in HPE and EMT cells (panel A). A pathway analysis, using the Genespring software (Panel B) exhibits the major pathways modulated during EMT formation with the most prominent ones being the MAPK, TGFβ ans Wnt signaling pathway.

Table 2: EMT in Human Primary Prostate Culture. EMT-like cells do not emerge from tissue samples from BPH, PIN and Gleason 5-7 patients. However, 71.4 and 83.3% of tissue samples from Gleason 8 and 9 patients exhibit EMT phenotype.

Diagnosis	No. of Cases	EMT	%
BPH	23	0	0
PIN	5	0	0
Gleason Score 5	12	0	0
Gleason Score 6	33	0	0
Gleason Score 7	4	0	0
Gleason Score 8	7	5	71.4
Gleason Score 9	6	5	83.3
Total	**90**	**10**	**11.1**

Table 3: Genes Differentially modulated in the TGFβ Pathway during EMT

Gene Description	Gene ID	Status	Fold Difference	P Value
Homo sapiens cofilin 2 (muscle) (CFL2), mRNA.	NM_021914	Upregulated	2.6	0.0469
Homo sapiens phosphoinositide-3-kinase, class 2, beta polypeptide (PIK3C2B), mRNA.	NM_002646	Downregulated	2.2	0.0293
Homo sapiens proteasome (prosome, macropain) activator subunit 1 (PA28 alpha) (PSME1), mRNA.	NM_006263	Downregulated	2.0	0.0288
Homo sapiens phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), mRNA.	NM_006218	Upregulated	2.2	0.0288
Homo sapiens poly(A) binding protein, cytoplasmic 1 (PABPC1), mRNA.	NM_002568	Downregulated	2	0.0229
Homo sapiens G1 to S phase transition 2 (GSPT2), mRNA.	NM_018094	Upregulated	2	0.0209
Homo sapiens rhophilin-2 (RHPN2), mRNA.	NM_033103	Downregulated	5.5	0.0178
Homo sapiens protein kinase, cAMP-dependent, regulatory, type II, beta (PRKAR2B), mRNA.	NM_002736	Upregulated	7	0.0164
Homo sapiens adenylate cyclase 3 (ADCY3), mRNA.	NM_004036	Upregulated	4	0.0147
Homo sapiens proteasome (prosome, macropain) 26S subunit, ATPase, 2 (PSMC2), mRNA.	NM_002803	Upregulated	1.9	0.0147
Homo sapiens ELK1, member of ETS oncogene family (ELK1), mRNA.	NM_005229	Upregulated	2.8	0.0147
Homo sapiens v-ski sarcoma viral oncogene homolog (avian) (SKI), mRNA.	NM_003036	Upregulated	2	0.0133
Homo sapiens endoglin (Osler-Rendu-Weber syndrome 1) (ENG), mRNA.	NM_000118	Upregulated	4	0.0083
Homo sapiens Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB), mRNA.	NM_001175	Downregulated	3.9	0.0062

TGFβ Pathway (Contd..)				
Gene Description	**Gene ID**	**Status**	**Fold Difference**	**P Value**
Homo sapiens v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma) (AKT3), mRNA.	NM_005465	Upregulated	2.9	0.0062
Homo sapiens guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 (GNAI1), mRNA.	NM_002069	Upregulated	2	0.0058
Homo sapiens mitogen-activated protein kinase 7 (MAPK7), transcript variant 1, mRNA.	NM_139033	Upregulated	1.8	0.0052
Homo sapiens integrin, beta 6 (ITGB6), mRNA.	NM_000888	Downregulated	8.6	0.0015
Homo sapiens zinc finger homeobox 1b (ZFHX1B), mRNA.	NM_014795	Upregulated	25	0.0003
Homo sapiens hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), mRNA.	NM_000862	Downregulated	3	1.57E-05

Table 4: Genes Differentially modulated in the MAPK Pathway during EMT

Gene Description	Gene ID	Status	Fold Difference	P Value
Homo sapiens proteasome (prosome, macropain) activator subunit 1 (PA28 alpha) (PSME1), mRNA.	NM_006263	Downregulated	2	0.0401
Homo sapiens phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), mRNA.	NM_006218	Upregulated	2.2	0.0401
Homo sapiens poly(A) binding protein, cytoplasmic 1 (PABPC1), mRNA.	NM_002568	Downregulated	2	0.0341
Homo sapiens G1 to S phase transition 2 (GSPT2), mRNA.	NM_018094	Upregulated	1.9	0.0324
Homo sapiens rhophilin-2 (RHPN2), mRNA.	NM_033103	Downregulated	5.5	0.029
Homo sapiens protein kinase, cAMP-dependent, regulatory, type II, beta (PRKAR2B), mRNA.	NM_002736	Upregulated	7	0.0273
Homo sapiens ELK1, member of ETS oncogene family (ELK1), mRNA.	NM_005229	Upregulated	2.8	0.0253
Homo sapiens adenylate cyclase 3 (ADCY3), mRNA.	NM_004036	Upregulated	4	0.0253
Homo sapiens proteasome (prosome, macropain) 26S subunit, ATPase, 2 (PSMC2), mRNA.	NM_002803	Upregulated	1.9	0.0253
Homo sapiens guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 (GNAI1), mRNA.	NM_002069	Upregulated	2	0.012
Homo sapiens protein kinase C, iota (PRKCI), mRNA.	NM_002740	Downregulated	1.7	0.012
Homo sapiens Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB), mRNA.	NM_001175	Downregulated	3.9	0.012
Homo sapiens hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), mRNA.	NM_000862	Downregulated	3	1.36E-05

Table 5: Genes Differentially modulated in the Wnt Pathway during EMT

Gene Description	Gene ID	Status	Fold Difference	P Value
Homo sapiens cofilin 2 (muscle) (CFL2), mRNA.	NM_021914	Upregulated	2.6	0.0469
Homo sapiens phosphoinositide-3-kinase, class 2, beta polypeptide (PIK3C2B), mRNA.	NM_002646	Downregulated	2.2	0.0293
Homo sapiens proteasome (prosome, macropain) activator subunit 1 (PA28 alpha) (PSME1), mRNA.	NM_006263	Downregulated	2.0	0.0288
Homo sapiens phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), mRNA.	NM_006218	Upregulated	2.2	0.0288
Homo sapiens poly(A) binding protein, cytoplasmic 1 (PABPC1), mRNA.	NM_002568	Downregulated	2	0.0229
Homo sapiens G1 to S phase transition 2 (GSPT2), mRNA.	NM_018094	Upregulated	2	0.0209
Homo sapiens rhophilin-2 (RHPN2), mRNA.	NM_033103	Downregulated	5.5	0.0178
Homo sapiens protein kinase, cAMP-dependent, regulatory, type II, beta (PRKAR2B), mRNA.	NM_002736	Upregulated	7	0.0164
Homo sapiens adenylate cyclase 3 (ADCY3), mRNA.	NM_004036	Upregulated	4	0.0147
Homo sapiens proteasome (prosome, macropain) 26S subunit, ATPase, 2 (PSMC2), mRNA.	NM_002803	Upregulated	1.9	0.0147
Homo sapiens ELK1, member of ETS oncogene family (ELK1), mRNA.	NM_005229	Upregulated	2.8	0.0147
Homo sapiens frizzled homolog 8 (Drosophila) (FZD8), mRNA.	NM_031866	Upregulated	2.6	0.0471
Homo sapiens endoglin (Osler-Rendu-Weber syndrome 1) (ENG), mRNA.	NM_000118	Upregulated	4	0.0083
Homo sapiens Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB), mRNA.	NM_001175	Downregulated	3.9	0.0062

Wnt Pathway (Contd..)				
Gene Description	**Gene ID**	**Status**	**Fold Difference**	**P Value**
Homo sapiens v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma) (AKT3), mRNA.	NM_005465	Upregulated	2.9	0.0062
Homo sapiens guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 (GNAI1), mRNA.	NM_002069	Upregulated	2	0.0058
Homo sapiens mitogen-activated protein kinase 7 (MAPK7), transcript variant 1, mRNA.	NM_139033	Upregulated	1.8	0.0052
Homo sapiens integrin, beta 6 (ITGB6), mRNA.	NM_000888	Downregulated	8.6	0.0015
Homo sapiens zinc finger homeobox 1b (ZFHX1B), mRNA.	NM_014795	Upregulated	25	0.0003
Homo sapiens hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), mRNA.	NM_000862	Downregulated	3	1.57E-05

Table 6: Genes Differentially modulated in the EMT Pathway

Gene Description	Gene ID	Status	Fold Difference	P Value
Homo sapiens dishevelled, dsh homolog 1 (Drosophila) (DVL1), mRNA.	NM_004421	Upregulated	2.12	0.0334
Homo sapiens epidermal growth factor (beta-urogastrone) (EGF), mRNA.	NM_001963	Downregulated	2.1	0.0188
Homo sapiens fibroblast growth factor 1 (acidic) (FGF1), transcript variant 1, mRNA.	NM_000800	Downregulated	1.8	0.0186
Homo sapiens cDNA FLJ37465 fis, clone BRAWH2011823, highly similar to BONE MORPHOGENETIC PROTEIN 7 PRECURSOR.	AK094784	Downregulated	2.9	0.0082
Homo sapiens ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1), transcript variant Rac1b, mRNA.	NM_018890	Downregulated	1.6	0.0056
Human (clone SF1) hepatocyte growth factor (HGF) mRNA, complete cds.	M73239	Upregulated	10	0.0029
Homo sapiens phosphoinositide-3-kinase, regulatory subunit, polypeptide p101 (P101-PI3K), mRNA.	NM_014308	Upregulated	2	0.0005