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Research article

INHIBITOR OF DIFFERENTIATION 1 (ID1) PROMOTES CELL SURVIVAL AND PROLIFERATION OF PROSTATE EPITHELIAL CELLS

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Abstract: Id1 (inhibitor of differentiation 1) is a member of the bHLH protein family. Consistent with its role in promoting proliferation and inhibiting differentiation, Id1 expression is low or negligible in normal prostate epithelial cells but is high in prostate cancer. Ectopic expression of Id1 in normal prostate epithelial cells could therefore provide a model for understanding early events involved in initiation of prostate cancer. Over-expression of Id1 immortalized but did not transform ventral prostate epithelial cells (Id1-RPE). Immortalization was associated with decreased Cdkn2a, Cdkn1a, androgen receptor and increased Tert expression. Gene expression profiling over successive doublings was used to identify transcriptomic changes involved during immortalization (Tieg, Jun, alpha actin, Klf10, Id2) and in maintaining the immortalized phenotype (Igfbp3, Igfbp5, Mmp2, Tgfb3). Network analysis indicated that Id1 promotes cancer/tumor morphology, cell cycle and epithelial to mesenchymal transition by influencing AP1, tnf, tgfβ, PdgfBB and estradiol pathways. During immortalization, the expression of majority of differentially expressed genes reduced over progressive doublings suggesting a decline in transcriptional

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Abbreviations used: bHLH – basic helix loop helix; CDKI – cyclin dependent kinase inhibitor; EMT – epithelial – mesenchymal transition; HGF – hepatocyte growth factor; Id – inhibitor of differentiation; KGF – keratinocyte growth factor; MAPK – mitogen activated protein kinase; NHUC – normal human urothelial cells; PrEC – prostate epithelial cells (human); R1881 – methyltrienolone (non-aromatizable synthetic androgen analogue); RPE – rat prostate epithelial; TERT – telomerase reverse transcriptase

regulatory mechanisms. The associated molecular/gene expression profile of Id1-RPE cells provides an opportunity to understand the molecular pathways associated with prostate epithelial cell survival and proliferation.

Key words: Id1, Prostate, Epithelial cells, Immortalization, Cancer

INTRODUCTION

Inhibitor of differentiation 1 (Id1), together with its other family members Id2, Id3 and Id4 are dominant negative regulators of the basic helix-loop helix (bHLH) family of transcription factors [1]. Id proteins lack the basic domain hence they can form transcriptionally inactive heterodimers with bHLH proteins. In doing so, Id proteins can alter the transcription of genes that are dependent on functional dimerization between bHLH proteins [2]. It is generally believed that Id proteins inhibit differentiation and promote proliferation [1, 3-5]. Consistent with these observations, Id1 expression is low or absent in differentiated epithelial cells but high in proliferating cells and cancer [6-8].

Increased Id1 expression is also observed in prostate cancer [9-11]. In prostate cancer cells, Id1 cross-talks with MAPK [12], NFkB [13], TGFβ and TNFα [14] to promote cell survival, proliferation, metastasis and androgen independence [15-17]. In vitro, Id1 expression is repressed by androgens in normal rat prostate epithelial cells [15] whereas an altered androgen axis in rats leads to increased Id1 expression and prostate cancer [18].

The observations that ectopic Id1 leads to intestinal adenomas in vivo [19] suggests that Id1 expression is associated with cancer initiation. In vitro, Id1 promotes immortalization of epithelial cells [20-22] by down regulating p16/INK4a [23, 24]. However, Id1 immortalized epithelial cells are generally non-tumorigenic. Independent studies have also demonstrated that Id1 can lead to chromosomal aberrations [25] possibly due to stabilization of Aurora A in telomerase immortalized cells [26].

Normal prostate epithelial cells isolated from humans [27, 28] and rodents [29] can be cultured for short periods of time. These normal cells, in early passages maintain androgen response and differentiation markers [30]. Over time (approximately 15-20 passages), these cells exit from cell cycle that is associated senescence, decreased Id1 [31] and increased TERT expression [27]. In general, cells that overcome these senescence barriers re-enter the cell cycle and with progressive cells divisions, accumulate genetic mutations that eventually lead to oncogenic transformation. The molecular pathways associated with sustained cell survival and proliferation are therefore critical to understand the initial stages of prostate carcinogenesis.

In context of these observations, we investigated the hypothesis that ectopic Id1 may sustain cell cycle in prostate epithelial cells. In order to test this hypothesis we ectopically expressed Id1 in normal epithelial cells isolated from rat ventral prostates, a representative model for hormone-dependent prostate hyperplasia [32] and cancer [33]. The epithelial cells from these glands are hormone

dependent and other cell populations such as basal and stromal are essentially hormone independent [34]. In culture, and during initial passages, rat ventral prostate epithelial cells also demonstrate functional androgen dependent gene expression [35-37]. This approach blocked the exit of rat ventral prostate epithelial (RPE) cells from cell cycle that is normally observed after 10 passages and promoted immortalization. Transcriptome analysis lead to the identification of potential Id1 dependent pathways associated with progressive stages of immortalization and cell survival, a key early event in prostate cancer. The results from this study, together with the observations that increased Id1 is associated with prostate cancer, suggests that aberrant Id1 expression may be an early event in prostate cancer.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats bred and raised in the Washington State University vivarium were used. The university animal care committee approved all animal procedures performed in this study.

Culture of ventral prostate cells

The 20-day-old rats were euthanized by cervical dislocation and ventral prostates were removed. The prostate epithelial cells (RPE) were purified according to the published procedure [15, 38], with some modifications [30]. At passage-2 the cells were re-plated in 6 or 24-well culture plates and grown to 80% confluency. The cell purity and response to synthetic androgen analogue R1881 was determined by measuring FGF8 expression as reported previously [30] by our laboratory. The purified rat prostate epithelial cells can be propagated for about 5-7 passages (approximately 20 population doublings) before undergoing apoptosis. During this period the cells loose androgen receptor (measured by RT-PCR) and serum responsiveness (as measured by rate of proliferation). No surviving cells are observed after passage 9-10.

Establishment of stably integrated rat prostate epithelial cell line

Purified rat ventral prostate epithelial cells (RPE) cultured in 24-well plates (10^6 cells/well) were transfected with the human Id1 (in pcDNA3.1 expression plasmid [22]) by Fugene cell transfection kit (Roche) according to manufacturer's instructions in passage-2. Seventy-two hours after transfection the cells were subjected to G418 selection (300 μg/ml). The transfected cells (Id1-RPE) were subsequently maintained in the Ham's F12 medium containing 250ug of G418. At this concentration of G418, all the primary non-transfected ventral prostate epithelial cells failed to survive. The empty vector (EV) transfected cells survived initial G418 selection but failed to survive beyond passage-12. This approach ensured that surviving cells incorporated hId1 and were resistant to G418. The transfected cells were then allowed to grow for at least 10 passages before subjecting to an additional G418 selection to ensure the

absence of any non-transfected cells that escaped first selection and to rule out the presence of spontaneously immortalized cells.
The cells were routinely reseeded at a concentration of $1x10^5$ cells in 100-mm tissue culture dishes and incubated in the medium described above and passaged at 80% confluency. Each passage was of 1 week duration and consisted of 3-4 doublings. All biochemical analyses were performed 15 passages after initial transfection and selection. For analysis, the cells were serum starved for 48 hours in order to synchronize the cell cycle. The serum starved cells were either not treated (control, C) or treated with serum (S) or synthetic androgen analogue R1881 for 2 hours.

Cell proliferation assay

The proliferation rate as reflected by rate of DNA synthesis was performed using ^{3}H thymidine incorporation assays. Briefly, 10^4 cells/well were seeded in 24 well plates. Twenty-four hours after the seeding or when the cells reached ~50% confluency, the cells were incubated with ^{3}H thymidine at 37^{o}C for 5h. Cells were subsequently washed with PBS, 10% TCA and 100% ethanol and then dissolved with 0.55 mM NaOH at 37^{o}C for 30 min. Radioactivity in the samples was determined by liquid scintillation counting. The amount of DNA in the well was assessed by SYBR green assay and data expressed as thymidine incorporation per μg DNA.

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was obtained using the Trizol (Invitrogen) method described previously [15]. The final RNA pellet was washed with 70% ethanol dried and resuspended in diethylpyrocarbonate (DEPC)-treated H_2O at a concentration of 1 mg/ml and stored at -80^{o}C until analysis. RT-PCR using gene specific primers as indicated below was performed using standard protocols.
Gene specific primers:
Nested human Id1 (NM_002165): Forward 5': AAG TAA ACG TGC TGC TCT ACG ACA, Reverse 5': TTC CAA CTT CGG ATT CCG AGT TC.
cMet-receptor (NM_031517): Forward 5': CAG TAA TGA TCT CCA TGG GCA AT, Reverse 5': AAT GCC CTC TTC CTA TGA CTT C.
Cyclophilin (1B15, M19533): Forward 5': CTT GCT GCA GAC ATG GTC AA, Reverse 5': TTG CAA TCC TGC TAG ACT TG
Tert (telomerase reverse transcriptase, NM_053423): Forward 5': GAC ATG GAG AAC AAG CTG TTT GC, Reverse 5': ACA GGG AAG TTC ACC ACT GTC.
Androgen receptor (NM_012502): Forward 5': GTG TCG TCT CCG GAA ATG TT, Reverse 5': GGA ATC AGG CTG GTT GTT GT.
Cdkn2a (p16, NM_031550): Forward 5': CAT CTC CGA GAG GAA GGC GAA CT, Reverse 5': CGC AGT TCG AAT CTG CAC CAT AG.
The published rat hepatocyte growth factor (HGF) [39] and keratinocyte growth factor (KGF) [40] primers were used for the RT-PCR based expression analysis of corresponding genes in RPE and RPE-Id1 cell lines.

Western blot analysis

Western blot analysis was performed on 10μg of total protein as per standard procedures [22] using protein specific antibodies (Id1: BD Pharmingen 556522 - mouse monoclonal ant-human Id1, p53: Santa Cruz sc-6243, rabbit polyclonal, Cdkn2a (p16): Santa Cruz sc-1661, mouse monoclonal and actin: Santa Cruz sc-7210 rabbit monoclonal).

Soft agar assay

10,000 cells (DU145 prostate cancer cell line as positive control and Id1-RPE cells at passage 69) in agar media (Ham's F-12 with respective antibiotics, 10% FBS and 0.35% DNA grade agarose) were plated onto 35mm sterile culture dishes (Nunc) containing agar media. The plates were incubated at 37^{o}C (5% CO_2) for 2 weeks and colonies were analyzed and counted on an inverted microscope (Zeiss).

Nude mice assay

DU145 and Id1-RPE cells (passage 69) were grown to 70-80% confluency in complete media as described above. The cells were then harvested and washed twice in PBS. The cell pellet was re-suspended in PBS. 5 x 10^6 cell were then suspended in 300 μl PBS containing 10% matrigel and injected subcutaneously (s.c.) into the lower flank of the mice. Tumors were allowed to grow until visible and diameters measured with calipers. The tumor volume in mm^3 was calculated by the formula: Volume = $(width)^2$ x length/2.

Karyotyping

Karyotyping of RPE (passage 2) and Id1-RPE (passages 57 and 68) was performed by Dr. Lisa Shaffer, Director Molecular Cytogenetics core, Center for Reproductive Biology, Washington State University.

RNA quality for microarray

RNA quality was determined by both electrophoretic methods using a denaturing agarose gel and absorption readings at 260 and 280 nm (a minimum OD 260/280 ratio of 1.8 was a requirement for microarray hybridization). If excessive degradation or protein contamination of the RNA was observed then the sample was not used for microarray hybridization.

Microarray hybridizations

The Affymetrix gene chip platform as described previously [30] was used to determine transcriptional changes in the epithelial cells. Ten micrograms of total RNA from each of the samples was used to create the target for the microarray. The biotinylated cytosine and uridine triphosphate labeled cRNA was fragmented, hybridized to RAE230A arrays (Affymetrix, Santa Clara, CA), and stained in accordance with the manufacturer's standard protocol. The arrays were stained and washed utilizing the Affymetrix GeneChip Fluidics Station 400 and scanned using a GeneArray Scanner 2500A (Agilent, Palo Alto, CA). The

resulting data were viewed and preliminary assessment was made using GCOS software (Affymetrix). All reactions and microarray hybridization procedures were performed in the Laboratory for Biotechnology and Bioanalysis I (LBBI) at Washington State University.

Absolute and statistical analysis for microarrays

Microarray output was analyzed essentially as described previously [30]. The absolute analysis from GCOS was imported into GeneSpring 7.0 software (Silicon Genetics, Redwood City, CA). The data was normalized within GeneSpring using the default/recommended normalization methods. These included setting of signal values below 0.01 to 0.01, total chip normalization to the 50th percentile, and normalization of each gene to the median. These normalizations allowed for the visualization of data based on relative abundance at any given time point rather than compared to a specific control value.

Data restrictions and analytical tools in GeneSpring were applied to isolate noteworthy and possibly important patterns of gene expression during the course of ventral prostate epithelial cell immortalization. Transcripts expressed differentially at a statistically significant level were determined using a P-value cutoff of 0.05 and using a Benjamini and Hochberg False discovery rate multiple testing correction. This was applied to all time points (and treatments) and considered all transcripts represented on the arrays. Subsequently, expression restrictions were applied to the transcripts expressed in a significant manner. These restrictions were designed so that the remaining transcripts met the following requirements in addition to being expressed in a significant manner: 1) each transcript must have a signal value of $\geq$ 300 in a minimum of 1 out of 3 comparisons 2) Present (P) flag in at least two of the three comparisons 3) the range of the replicates must not exceed 1 (in the normalized scale) and 4) A 3 fold change or greater between any two comparisons were used to identify differentially expressed genes (DEG). Transcripts that passed these restrictions (Top list) were considered for further analysis that included clustering and pathway analysis.

Pathway analysis

Ingenuity pathway analysis software (IPA version 6, Redwood city, CA) was used to understand and infer the relationships, mechanisms, functions and pathways of relevance influenced by the differentially expressed genes. The gene lists were imported into IPA and core analysis performed as per default software settings. The IPA core analysis is used to identify molecular networks and biological processes that are most significantly perturbed in a dataset. The IPA core analysis provided a list of networks and interactions within a dataset and network associated score. The network score reflected the significance of the network and was generated using a p-value calculation, displayed as the negative log of the p-value (score of 2 or higher indicates a 99% confidence of not being generated by a random chance alone).

Quantitative PCR analysis

The relative gene expression levels of selected genes were determined by real-time quantitative PCR based on TaqMan chemistry using Applied Biosystems probes (TaqMan Probes, Applied Biosystems, Foster city, CA). All PCR reactions were performed in a final volume of 50 μl. The cycle threshold (Ct) was used to calculate relative amounts of target RNA. All experiments were performed in duplicates and repeated thrice.

The ΔΔCt method was used for relative quantification of gene expression. The Ct values of the target genes from triplicate PCR reactions were normalized to the levels of β-actin (endogenous control) from the same cDNA preparations. The average Ct for each gene was calculated by subtracting the Ct of the sample RNA from that of the control RNA for the same time measurement. This value, called the ΔCt reflected the relative expression of the treated sample compared with the control. ΔCt was then used as an exponent in the calculation for amplification $2^{\Delta Ct\ cont-\Delta Ct\ sample}$, the equivalent to the fold change in expression.

Statistical analysis

The data were obtained from a minimum of three different experiments unless stated otherwise. The time matched controls were used to rule out any time dependent changes in control/treated sets in microarray analysis. Standard statistical tests for significance (t-test) were used for semi-quantitative and real-time PCR data sets.

RESULTS

Effect of Id1 over-expression on ventral prostate epithelial cell proliferation

The non-transfected primary rat ventral prostate epithelial cells (RPE) responded to serum with increased proliferation at passage 2 (Fig. 1). A significant decrease in proliferation (basal and serum induced) with increasing doubling time suggested that normal RPE cells exit from cell cycle around passage 10. By passage 12 no surviving RPE cells were observed. The life span of normal rat prostate epithelial cells in culture was therefore arbitrarily set to 10 passages.

The transfection of rat prostate epithelial cells with human Id1 allowed them to survive beyond passage 10 (Fig. 1). The rate of proliferation in control (serum starved for 24 hrs) Id1 expressing RPE cells (Id1-RPE) at passage 15 was significantly higher than the un-transfected RPE cells at passage 2, suggesting that Id1 also increased the basal rate of proliferation of RPE cells (Fig. 1). The rate of proliferation in the presence of serum between Id1-RPE (passage 69) and normal RPE (passage2) was comparable. Ectopic expression of Id1 therefore has the ability to promote cell survival by re-initiating cell cycle. No change in the rate proliferation (basal and serum stimulated) between passages 15, 25, and 69 indicated the continuity of cell cycle (Fig. 1).

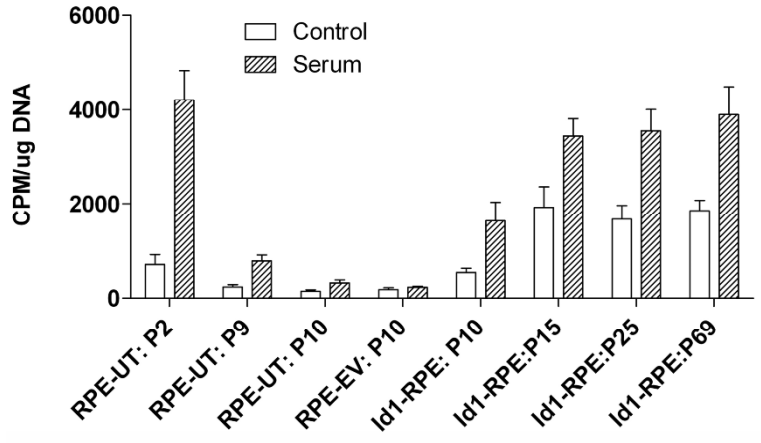

Fig. 1. Cell proliferation of rat prostate epithelial cells (RPE). ^{3}H thymidine incorporation assays were performed to determine the rate of proliferation in the absence (control: serum starved for 48 hrs) and serum as indicated in materials and methods. The counts per minute (CPM) of incorporated ^{3}H –thymidine was normalized to total DNA content measured by SYBR green fluorescent assay. The data is representative of 3 experiments run in duplicate (Abbreviations: UT – untransfected; P – passage number; EV – empty vector (CMV)). Significant differences ($P < 0.001$) between control and serum treated cells were observed in all passages except at RPE-UT:P10 and RPE-EV:P10. Rate of proliferation between control RPE-UT:P2 and Id1-RPE:P10 was also significantly different ($P < 0.001$).

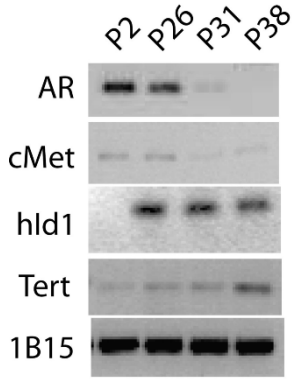

Fig. 2. Reverse transcriptase polymerase chain reaction of androgen receptor (AR), cMet, human Id1 (hId1) and telomerase reverse transcriptase (Tert) and constitutively expressed gene 1B15 (as loading and RT-PCR control) in RPE (passage2) and Id1-RPE cells (passages 26, 31 and 38). The representative RT-PCR of 3 experiments is shown.

Androgen receptor expression and response to androgens

Androgen receptor was expressed as determined by RT-PCR in normal RPE at passage 2 and 4 and in Id1-RPE cells at passage 26 (Fig. 2). However, AR expression was undetectable by passage 31 (Fig. 2). The AR positive normal

RPE cells responded to synthetic androgen analogue R1881 as measured by FGF8 expression in RPE cells at passage 2 as reported previously [30]. The FGF8 expression was insensitive to R1881 stimulation in AR+ve Id1-RPE cells at passage 26 or AR-ve Id1-RPE cells at passage 31 (data not shown) suggesting that normal AR regulated pathways are disrupted. Together these observations suggested that Id1-RPE cells lose their ability to respond to androgens although AR levels were maintained at least till passage 26.

hId1 expression

The human Id1 expression was determined by RT-PCR using human Id1 specific primers. The resultant PCR product was used again as a template in PCR reaction using nested human Id1 primers to confirm that the RT-PCR product was indeed human Id1. The final PCR product was size and sequence validated as human Id1 through a BLAST search. These results demonstrated that ectopic human Id1 was expressed in Id1-RPE cells (Fig. 2).

Rat TERT expression

As expected, rat Tert expression was observed in primary rat ventral prostate epithelial cells (Fig. 2). An increase in Tert expression was evident over progressive doublings suggesting that immortalization of Id1-RPE was associated with an increase in Tert expression.

Prostate epithelial cell markers

The lack of expression of HGF and KGF (data not shown), known prostate stromal markers at all time points (P2, P26 and P69) suggested that the cells were not contaminated with prostate stromal cells. The expression of known prostate epithelial cell markers was also evaluated in the microarray expression database (see below at S2, S26 and S69) to determine if the epithelial phenotype was maintained. The expression of known prostate epithelial cell markers such as acid phosphatase and keratinocyte growth factor receptor remained unchanged during all time points. An increase of over two fold was observed in the expression of epithelial markers E-cadherin (Cdh-1), cytokeratin 8 and prostasin from S2 to S69. The expression database also indicated the loss in CD24, a known luminal prostate epithelial cell marker.

The expression of the HGF receptor *Met* by RT-PCR was then specifically used to monitor epithelial phenotype [41]. The c-Met receptor is expressed primarily by prostate epithelial cells whereas it ligand the HGF is secreted by the stromal cells. The low c- Met expression (Fig. 2) in P31 and P38 as compared to P2 and P26 suggested a change in epithelial phenotype during immortalization process. Taken together, the epithelial marker profile from microarray and RT-PCR (c-met) suggested that some prostate epithelial markers are decreased/lost (c-met, CD24) whereas others increase (Cdh-1, cytokeratin 8 and prostasin) or remain unchanged (acid phosphatase and KGF receptor).

Colony formation in soft agar, nude mice assay and karyotype

Colony forming ability of Id1-RPE cells was used as a measure of anchorage independent growth, a property of transformed cells. The hId1 expressing RPE cells (Fig. 3A) failed to form colonies in soft agar (Fig. 3B) whereas the DU145 prostate cancer cells used as positive controls actively formed isolated colonies (Fig. 3C). These results demonstrated that Id1-RPE cells have not achieved anchorage independent growth and are not tumorigenic/transformed.

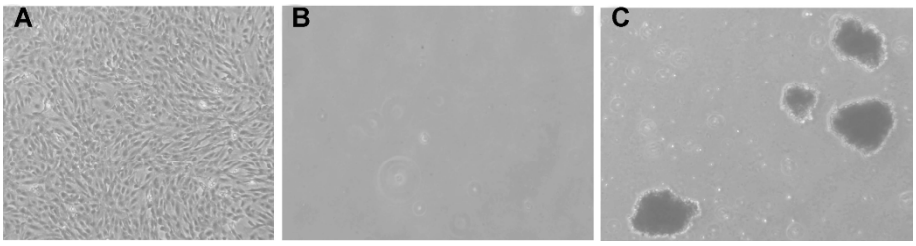

Fig. 3. Rat prostate epithelial cells in culture (A, passage 66) and colony forming assay in soft agar (B). The Id1-RPE cells (A) exhibit a typical fibroblast like morphology. These RPE cells were placed on soft agar and observed for their ability to form colonies. No colonies were observed in RPE cells (B). The metastatic prostate cancer cell line DU145 used as a positive control (C) formed colonies that are indicative of migration.

The tumorigenic potential of Id1-RPE cells was further checked by nude mice assay. The nude mice injected subcutaneously with Id1-RPE cells (passage 69) showed no signs of tumor formation/growth over a 3 month period suggesting the lack of their tumorigenic potential. In contrast, mice injected with the DU145 cells formed measurable tumors at 4 weeks (average: 83 mm^3; range 37-129 mm^3, 3 different sites). These results confirmed the colony formation assay data and supported the absence of metastatic/tumorigenic potential of hId1-RPE cells.

Spectral karyotyping in cells from passage 57 and 68 demonstrated no abnormal karyotype as compared to normal cells (data not shown).

Collectively, the karyotyping, colony formation in soft agar and nude mice assay data demonstrated that hId1 was not able to transform the prostate epithelial cells. These cells were therefore at best considered as immortalized. These results are consistent with earlier reports that Id1 can immortalize but not transform cells of epithelial origin [20-22, 42].

Changes in gene expression during immortalization process - *In silico* analysis of gene expression during immortalization

Microarray analysis was performed to understand transcriptomic changes associated with Id1 mediated immortalization of RPE cells. The following comparisons were performed: 1) Transcriptomic changes between control untreated cells (serum starved for 24 hours) at S2 (RPE), S26 and S69 (Id1-RPE) and 2) transcriptomic changes between serum exposed (2hr) cells at S2 (RPE), S26 and S69 (Id1-RPE). The comparison between all control samples yielded

98 genes that changed during Id1 dependent immortalization process. As expected, a significantly higher number of genes (173) changed following serum treatment. We anticipated that some of the differentially expressed genes in control and serum treated sets could change irrespective of treatment. These genes should therefore be part of both the control (98 genes) and serum list (173 genes). An *in silico* subtraction (Venn analysis) was then used to identify this gene set that changed irrespective of treatment. This approach also allowed us to identify differentially expressed genes that were a) unique in control group (52 genes, Control Only) b) treatment independent that change during progressive passages (46 genes, Common) and c) transcriptionally regulated by serum factors at each passage analyzed (minimal gene set required for serum dependent survival, 127 genes, Serum Only) (Fig. 4).

Control only list. The unique control only list following *in silico* subtraction as described above consisted of genes that were differentially expressed in the absence of any stimulation. This gene set is likely involved in promoting immortalization and is downstream of Id1 or at the least regulated by Id1 initiated events early during immortalization process. The expression of the majority of the genes (81%) decreased and only 10 (19%) increased in immortalized cells as compared to primary non-Id1 transfected cells (Fig. 4). Surprisingly, endogenous rat Id1 expression also increased progressively in immortalized cells as compared to non-transfected primary cells (Fig. 4) that was confirmed by real time PCR at the transcript and western blot analysis at protein (Fig. 5) level respectively. These observations are highly significant and suggest that Id1 expression is independent of serum and is involved in immortalization. Interestingly, increased expression of Id2 was also observed in this set (Figs. 4 and 5), further supporting the role of Id proteins in promoting immortalization.

Common list. The heat maps clearly demonstrated that the expression of genes within the common list was treatment independent but changed as the cells progressed in culture. For example, a strikingly similar expression profile between untreated (control) and serum treated RPE cells (Fig. 4, 2_C and 2_S) and between Id1-RPE untreated and serum treated cells (Fig. 4, 69_C and 69_S). Such a remarkable pattern association suggested that this gene set was truly treatment independent. Another significant observation was that following immortalization, the expression of majority of the genes in this set decreased (93%). Thus down-regulation of these genes may be a common feature of immortalized cells. Most of these down-regulated genes are also tumor suppressors or inhibitors of proliferation. Most notable among this set were TGFβ3, Uch-L1 (ubiquitin thiolesterase L1), Cktsf1b1 (Grm/Gremlin, BMP-antagonist), Dkk-3, Scd (acyl-CoA desaturase), Rgs2 (cell growth-inhibiting

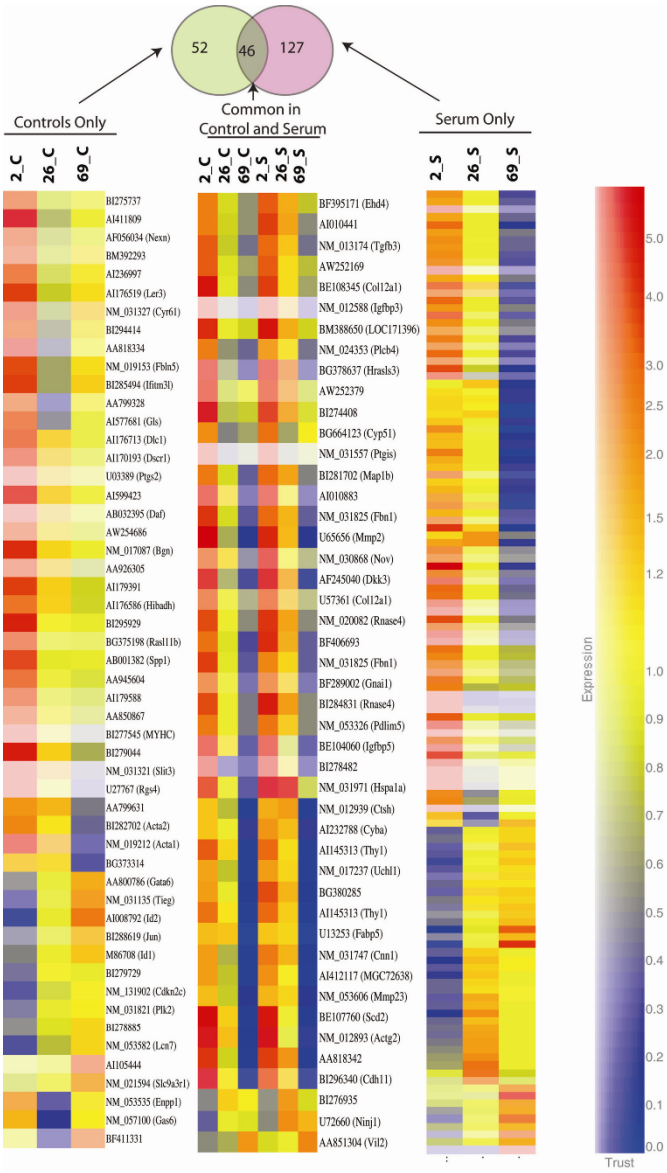

Fig. 4. Gene expression analysis of RPE and Id1-RPE cells during progressive stages of immortalization. The gene expression (Affymetrix RAE230A platform) was analyzed in untreated controls (serum starved, C) and serum treated (2 hrs, 5% BCS, S) RPE cells (2) and Id1-RPE cells (26 and 69). The differential gene expression was analyzed between control cells and serum treated cells. *In silico* subtraction was then performed to identify unique gene sets. Based on this analysis, three sets were identified: Control only (52 gene that are differentially expressed in control cells only during progressive passages), Common in control and serum (46 genes, considered as treatment independent) and Serum only (127 genes, serum regulated). The color in the heat maps indicate relative signal intensity and change in gene expression (with reference to the Expression/trust bar at right).

protein 31), THY1 (cell surface antigen), Actg2 (Smga, actin, gamma 2), Cnn1 (calponin 1) and Hrasls3 (HRAS like suppressor 3). Certain tumor promoters and/or oncogenes were also surprisingly down regulated that include Mmp-2, Igfbp-5 and Adm (adrenomedullin). These genes are usually associated with invasion/metastasis hence their down-regulation could support our observation that RPE-Id1 was not tumorigenic or invasive. The common only genes may represent a minimal gene set required for maintaining the post-senescent cell survival.

Serum regulated genes. Serum regulated genes represented transcriptionally active gene set that was required to maintain serum dependent cell proliferation and survival. In contrast to control only and common gene set, the expression of majority of the genes within the serum set increased (61%) as compared to RPE cells. As expected, the average fold change was also the highest.

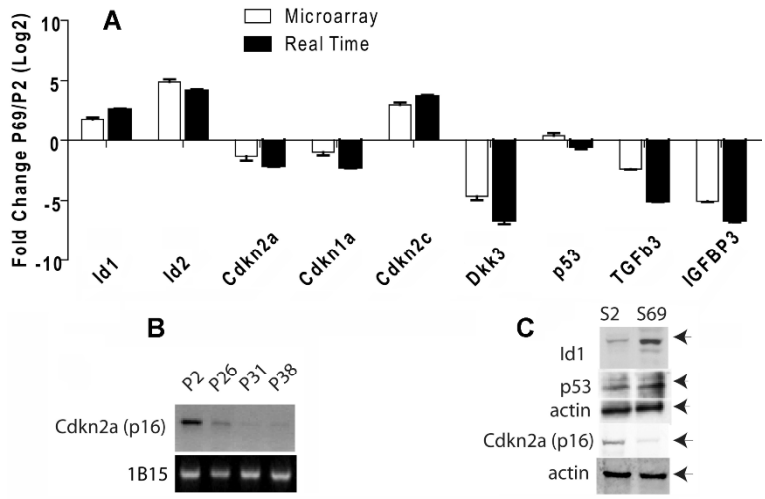

Fig. 5. Validation of microarray data and gene/protein expression of putative Id1 downstream effectors in RPE and RPE-Id1 cells. A – Real time polymerase chain reaction analyses to validate microarray results shown in Fig. 4. The real time PCR was performed using respective Applied Biosystems gene specific probes. Black Bars represent fold change between RPE cells (P2) and Id1-RPE cells (P69) was determined by ΔΔCt method following normalization with β-actin. The white bars represent the corresponding change in the microarray data sets. The data is represented on a log2 scale to highlight increase/decrease in expression. The analysis was performed on 3 different RNA preparations run in duplicate. B – Reverse transcriptase polymerase chain reaction of Cdkn2a (p16) and constitutively expressed gene 1B15 (as loading and RT-PCR control) in RPE (passage 2) and Id1-RPE cells (passages 26, 31 and 38). The representative RT-PCR of 3 experiments is shown. C – Western blot analysis of Id1, Cdkn2a (p16) and p53 expression on total protein isolated from RPE cells (P2) and Id1-RPE cells at Passage 69. Actin was used as a loading and experimental control. The data is representative of 3 experiments.

Effect of Id1 on cyclin dependent kinase inhibitor cdkn2a (p16) and cdkn1a

Id1 promotes cell cycle through down-regulation Cdkn2a (p16) and cdkn1a (p21) expression [23]. A decrease in Cdkn2a expression by real time PCR (Fig. 5A), RT-PCR (Fig. 5B, passages 26, 31 and 38) and western blot analysis (Fig. 5C, passages 2 and 69) and cdkn1a by real time PCR as compared to RPE cells (passage 2) was observed (Fig. 5A). These results suggested that one of the mechanisms by which Id1 promotes and sustains proliferation of prostate epithelial cells is through down-regulation of cyclin dependent kinase inhibitors (CDKI) p16 and p21.

Why then microarray analysis failed to detect CDKI's p16 and p21 as differentially regulated genes during Id1 dependent immortalization of RPE cells. Re-evaluation of raw microarray data demonstrated that at passages 26 and 69, both p16 and p21 had absent calls that did not pass the filtering limits imposed on the analysis. Moreover, the raw expression levels of p16 and p21 were less than 300 at S2 (p16 raw value mean 276$\pm$10.32 and p21 raw value mean 291$\pm$ 36.22) that decreased by over 2.5 fold and 2 fold respectively at S26 (with absent calls) and 4.1 and 4.3 fold at S69 respectively (with absent calls).

Network analysis

Biological network analysis was performed to understand the functional significance of microarray data (Tab. 1). Network analysis indicated that genes in the control set were involved in cancer and tumor morphology (Network 1, score 45, Fig. 6A). In fact Network 1 within this gene set had the highest score of all the networks analyzed across all gene sets (Tab. 1). Most of the genes in this network demonstrated cross talk with Nfkb, Ap1, Gsk3, Pdgf and Pi3k pathways. This gene set may also be required to maintain the prostate epithelial cell phenotype (increase in Lcn7 (Lipocalin 7), an androgen regulated gene), maintain cell proliferation (Id1 and Id2) and block transformation (ptgs2 and Daf). Thus the control set only could represent potential Id1 regulated pathways involved in immortalization.

Majority of the genes in the common gene set associated with Tgfβ, IGFBP5, MMP2, TNF and estradiol regulated pathways (Fig. 6C and D). Consistent with these observations, the major pathways influenced by this set was epithelial to mesenchymal transition (reflected as connective tissue disorders in Network analysis, e.g. decrease in the expression NOV (nephroblastoma over-expressed) and cell-cell signaling (Tab. 1 and Fig. 6C and D). The serum regulated networks involved cell cycle, cancer, cellular growth and proliferation, inflammatory and reproductive system disease (Tab. 1).

Collectively, our results demonstrate that over-expression of Id1 leads to immortalization of prostate epithelial cells that is associated with decreased androgen receptor/response. The early events in RPE immortalization involve down-regulation of p16, IGFBP3, Dkk3 and TGFb and up-regulation of Tert. The microarray analysis, for the first time captured potential Id1 dependent

genes that are involved in initiating and maintaining immortalization of rat ventral prostate epithelial cells.

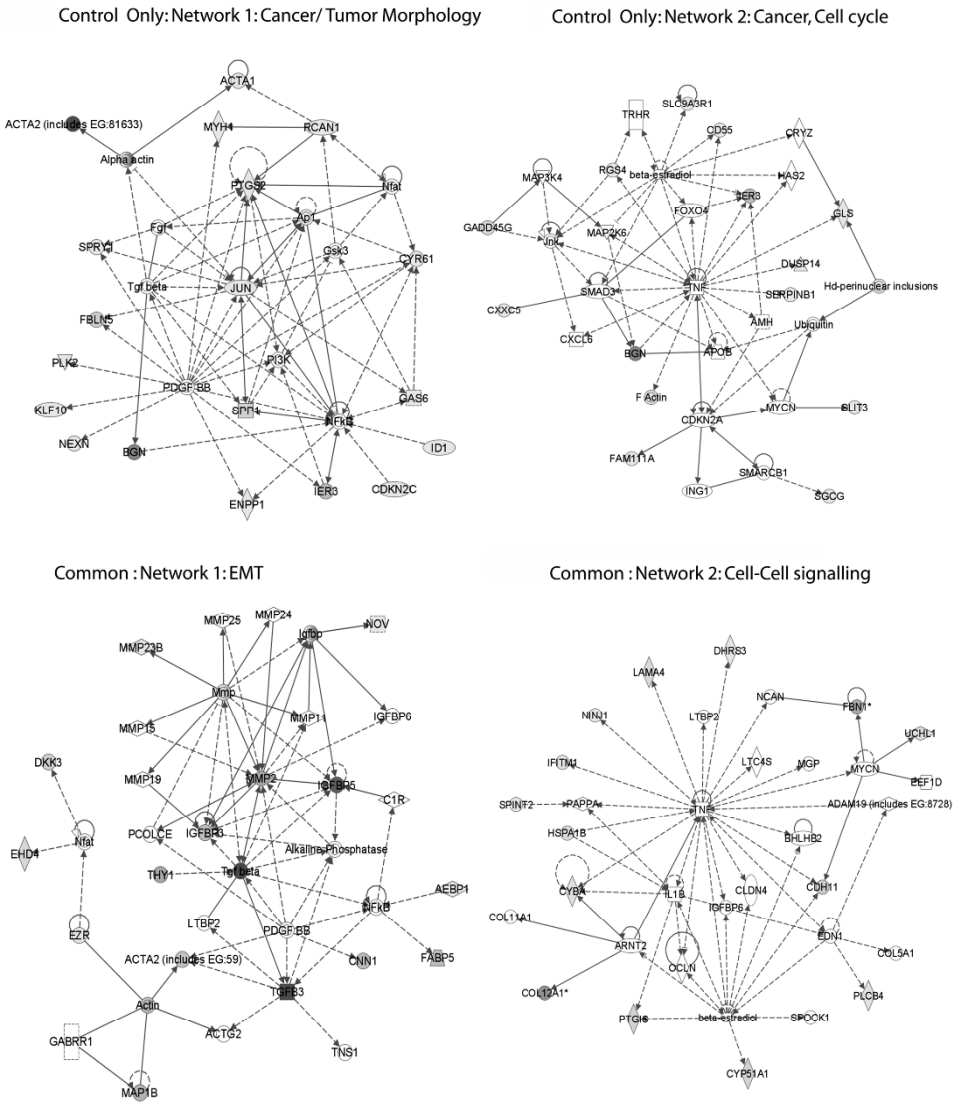

Fig. 6. Gene associations and corresponding Network analysis of differentially expressed genes. The detailed gene lists and all associated networks are included in Tab. 1. Shown in this figure are four representative networks with highest scores in the control only and common lists (2 networks each). The genes on shades of grey background are the differentially expressed genes in the lists whereas those on the white background are associated genes not in the list but are included to understand the molecular/biological interactions and pathways. Solid arrows represent direct interactions and broken arrows indicate indirect interactions. The shapes indicate the type of biological molecule.

Tab. 1. Biological networks influenced by ectopic Id1 in post senescent rat prostate epithelial cells. The differentially expressed genes between primary rat prostate epithelial cells (RPE, passage 2) and Id1 immortalized rat prostate epithelial cells (Id1-RPE) at passages 26 and passage 69 were used to develop networks and associated biological pathways. The comparisons (control versus serum treatment) between cells at P2, P26 and P69 followed by *in silico* subtraction as shown in Fig. 4, was used to identify gene that were differentially expressed in control cells (Control only), serum treated cells (Serum only) and common between the two treatment groups (Common). Each network consists of differentially expressed genes (indicated in bold typeface) and associated genes (normal typeface) that together form a biological network. The weight and significance of each network is represented as a "network score" that indicates the significance of each network (score of 2 or higher indicates a 99% confidence of not being generated by a random chance alone). The focus molecules eventually describe the type of biological pathway influenced by a network.

List	Network score	# Focus molecules
CONTROL ONLY LIST		
Network 1: Cancer and Tumor Morphology		
ACTA1, ACTA2 (includes EG:81633), **Alpha actin**, **Ap1, BGN, CDKN2C, CYR61, ENPP1, FBLN5**, Fgf, **GAS6,** Gsk3, **ID1, IER3, JUN, KLF10, MYH1, NEXN,** Nfat, NFkB, PDGF BB, PI3K**,PLK2**, **PTGS2, RCAN1, SPP1, SPRY1,** Tgf beta	45	19
Network 2: Cancer, Cellular Function and Maintenance, Cell Cycle		
AMH, APOB, beta-estradiol, **BGN, CD55,** CDKN2A, CRYZ, CXCL6, **CXXC5, DUSP14, F Actin**, **FAM111A,** FOXO4, **GADD45G, GLS,** HAS2, **Hd-perinuclear inclusions, IER3**, ING1, Jnk, MAP2K6, MAP3K4, MYCN, **RGS4**, **SERPINB1, SGCG, SLC9A3R1, SLIT3,** SMAD3, SMARCB1, TNF, TRHR, Ubiquitin	28	13
Network 3: **C**ellular Movement, Hematological System, Immune Response		
AGTR1B, CCL2, CCL3, CCL4, CCL18, CCNB1, **CNDP2**, CXCL1, CXCL2, CXCL3, CXCL6, CYP2E1, **GAS6**, GATA3, Histone h3, **IFITM3**, IFNG, IGFBP5, IL8, IL13, ITGA5, JAG1, NOTCH1, PCNA, PML, progesterone, REL, **RIN2**, SCGB1A1, **SPP1**, STAT4, TACR1, TGFB2, TGM2, TP73	8	5
COMMON ONLY LIST		
Network 1: Connective Tissue Disorders (Epithelial-Mesenchymal Transition), Genetic Disorder, Cellular Movement		
ACTA2 (includes EG:59), ACTG2, **Actin, AEBP1,** Alkaline Phosphatase, C1R, **CNN1, DKK3, EHD4, EZR, FABP5,** GABRR1, **Igfbp, IGFBP3, IGFBP5,** IGFBP6, LTBP2, **MAP1B, Mmp, MMP2,** MMP11, MMP15, MMP19, MMP24, MMP25, **MMP23B,** Nfat, NFkB, **NOV,** PCOLCE, PDGF BB, **Tgf beta, TGFB3, THY1,** TNS1	34	15
Network 2: Cell-To-Cell Signaling and Interaction, Lipid Metabolism, Small Molecule Biochemistry		
ADAM19 (includes EG:8728), ARNT2, beta-estradiol, BHLHB2, **CDH11,** CLDN4, COL11A1, **COL12A1,** COL5A1, **CYBA, CYP51A1, DHRS3**, EDN1, EEF1D, **FBN1, HSPA1B, IFITM1**, IGFBP6, IL1B, **LAMA4**, LTBP2, LTC4S, MGP, MYCN, NCAN, **NINJ1,** OCLN, PAPPA, **PLCB4, PTGIS, SPINT2,** SPOCK1, TNF, **UCHL1**	31	14

List	Network score	# Focus molecules
Network 3: Cell Death, Cellular Growth and Proliferation, Cancer		
ATF3, ATF4, BGLAP, CDK2, CEBPD, COL1A1, COL1A2, **CTSH,** CX3CL1, ENG, FASLG, **HRASLS3,** ID1, ID3, IFNG, IGF1R, **IGFBP3,** IGFBP4, IRF9, LGALS3, MIF, **MMP2,** MYBL2, NME1, **PDLIM5, RCAN2, RNASE4, SCD2,** SERPING1, SLC2A1, Sod, T3-TR-RXR, TP53, YBX1	15	8
SERUM ONLY LIST		
Network 1: Connective Tissue Disorders, Immunological/Inflammatory Disease		
ADM, ANKRD1, C8, **C1R, C1S,** CaMKII, **CCL13, CD24, CXCL2, DUSP6, EXPI,** Fibrin, **GREM1, HSPA1A, ICAM1,** IL1, LDL, **LTBP4**, Mmp, **MYLK,** NFkB, NfkB-RelA, **NR4A3**, P38 MAPK, Pdgf, PDGF BB, **PDLIM1, PEA15, Sod, SOD3,** Tgf beta, **TIMP3, UBE2K, UGDH**, Vegf	42	21
Network 2: Cell Cycle, Cellular Development, Cancer		
Alkaline Phosphatase, **APLP2, CALD1, CBX1,** Ck2, **COL8A1,** Creb, Cyclin A, Cyclin E, **DPYSL3, FGFR1,** Histone h3, **HSD11B1, IGF1, Igfbp, IGFBP3, IGFBP6, IL6**, Insulin, **KRT8**, Mapk, Mek, MHC Class I, Myosin, **NEFM, NPDC1, NR4A1,** PLC gamma, **PTTG1, RGS2,** RNA polymerase II, **SMARCA2, SOCS5, TOP2A, VCAN**	42	21
Network 3: Cancer, Skeletal and Muscular Disorders, Hepatic System Disease		
ABCB11, AFP, ANPEP, CTSL2, **DEK, EPHA2**, ESR1, **F11R**, FST, **GPR176, GUSB,** HDC, **HEXIM1, HMGN3,** HPSE, **HSD11B1,** Hsp27, HSPA1B, **LOXL4, MGP,** MT1E, MTTP, **OGN, PEM, PTPRN, SBDS, SERPINB9,** SLC10A1, SLCO1A1, **SOX4,** testosterone, TFAP2C, TNF, TNFRSF11B, **TPST1**	32	17
Network 4: Embryonic Development, Tissue Development, Cancer		
ADAM10, ADCY5, amino acids, **ARC**, BMPR1B ,BMX, CARM1, **CDH3, COL14A1**, CTNNB1, CTSK, cyclic AMP, **DDAH1,** DLG4, **DMPK**, EGFR, **FZD1, GLIPR1, HSPH1**, INDO, KRAS, LRP6, **MSLN,** NF1, **NFIB**, PITX2, **PRKAR2B, PTPN14, SEL1L**, TFF3, TP53, **TSPAN8**, TTK, WNT3, **WNT4**	27	15
Network 5: Cancer, Cellular Growth and Proliferation, Reproductive System Disease		
ANGPTL4, ARG1, **C10ORF58, CALD1,** CDKN3, CIITA, **CITED2,** CMA1, **COL6A3**, **DHCR24,** ENPP2, **GCNT2**, HNRNPC, HNRPK, IL9, IL13, KITLG (includes EG:4254), LAMA4, **MGP, NID2**, PDLIM7, **PGCP**, **PRSS23,** retinoic acid, **SC4MOL**, **SERPINB9**, TERT, TFAP2B, TGFB1, **Timp**, TNNT2, TPM1, **TPM2**, TPT1	22	13
Network 6: Cell Death, Hematological Disease, Immunological Disease		
7-ketocholesterol, Actin, Akt, ANGPT2, Ap1, **CALD1,** Calmodulin, CCR7, **DNAJB1,** DNAJB11, **Dnajb1-Hsp70, EHD4, ENC1,** HNRPK, **Hsp70,** Jnk**, MCAM**, Nfat, Pdgf Ab, PI3K, Pkc(s), **POSTN,** Rar, Ras, **RBP1,** RETNLB (includes EG:84666), TRAF7, **Tropomyosin, WISP1**	12	8

DISCUSSION

We report transcriptomic changes associated with immortalization of ventral prostate epithelial cell line achieved by the over-expression of dominant negative helix loop helix transcription factor Id1 (inhibitor of differentiation 1). Our results are consistent with earlier reports that demonstrated a similar Id1

induced immortalization process in keratinocytes [20, 21], Sertoli cells [22] and human endothelial cells [42]. However selective mutations in the presence of Id1 favouring increased survival cannot be ruled out [21]. Nevertheless, collective observations suggest that ectopic Id1 expression promotes the re-entry of normal differentiated epithelial cells into cell cycle but not sufficient for transformation. The ability of Id1 to promote immortalization of prostate epithelial cells is significant since immortalization is generally considered a pre-requisite for transformation [43, 44].

Decreased proliferation with time in culture observed in normal human prostate epithelial cells (PrEC) is associated with decreased expression of Id1 [31]. Thus over-expression of Id1 could rescue prostate epithelial cells from exiting the cell cycle during extended time in culture possibly by down-regulating cyclin dependent kinase inhibitors Cdkn2a (p16)[45] or Cdkn1a (p21). TERT expression alone is also reported to immortalize human prostate epithelial cells [46]. The TERT mediated immortalization in PrEC was also associated with decreased CDKN2a (p16) [46]. Thus immortalization of RPE cells in our study could be due to persistent down-regulation of p16 and p21 and increased TERT expression. These changes could also reflect the possibility that Id1-RPE cells may acquire growth advantage over a period of time that could select for high Tert expression. Expression of hTERT is also known to bypass Rb and p53 pathway-dependent barriers to proliferation and immortalization in normal human urothelial cells (NHUC) [42]. TERT-NHUC cells exhibit normal karyotypes, are non-tumorigenic and retain p16 [47] expression, a genotype and phenotype remarkably similar to Id1-RPE cells. Although retinoblastoma and p53 expression were not altered at the transcript level but their inactivation at protein level cannot be ruled out in Id1-RPE cells. For example, the Rb could be targeted by Id2 through protein-protein interactions [48, 49]. The treatment independent increase in the expression of Id2 in Id1-RPE cells supports this mechanism. Together with increased Tert expression, these mechanisms may allow Id1-RPE cells to overcome barriers to immortalization. Thus Id1 and Id2 may act synergistically to re-activate DNA synthesis and sustain proliferation in RPE cells.

The gene expression profile demonstrates the potential molecular events associated Id1 mediated immortalization of RPE cells. The treatment independent (control only and common gene sets) unidirectional and progressive decrease in the expression of majority of genes with time in culture in these gene sets are unique observations. The significance of this observation is elusive but it is speculated that Id1, being a known transcriptional repressor may lead to the down-regulation of large number of genes. Overall the data strongly supports progressive changes in gene expression during immortalization process as opposed to a switch that turns on/off the expression of specific gene.

Unexpectedly, the down-regulation is not limited to tumor suppressors (Igfbp3, Igfbp6, Nov, Dkk3, Tgfb3, Tgfb inducible early growth response 1 gene (TIEG/Klf10)) but also tumor promoters (Gas6, Cox2, Mmp2, Igfbp5),

suggesting a complex interplay between the biological networks involved in cell survival and proliferation. Comparison of immortalization associated genes as reported in this study with those of prostate cancer transcriptomes also allowed us to identify potential early events in prostate cancer. These early events could include down-regulation of Igfbp3, Il6 (Igfbp3 and Il6 are also increased in human prostate epithelial cells [31] upon exit from cell cycle), Igfbp6, Dkk3, Tgfb3 and TIEG. For example Dkk3 expression is decreased in prostate cancer [50] and loss of Dkk-3 expression results in the impairment of glandular structure and uncontrolled prostate epithelial cell (PrEC) proliferation, both of which are crucial for prostate cancer progression [51]. The down-regulation in Dkk3 is also observed in human immortalized cells and tumor derived cell lines [52]. Interestingly, over-expression of Dkk3 leads to down-regulation of Id1 and increased apoptosis [53] suggesting a cross talk between Dkk3 and Id1.

The down-regulation of tumor promoters as indicated above also appears to be an early event that is reversed in prostate cancer as a result of transformation. The significance of our results lies in the identification of primary networks such as the cancer/tumor, cell function and maintenance, cell cycle, EMT and cell death influenced by Id1 dependent immortalization. The primary nodes within these networks include established pathways such as NFkB, PI3K, TNF, TGFb and estradiol. The Id1-NFkB [13] and Id1-TGFb [14, 54] interactions have been reported previously in prostate cancer.

Direct Id1 regulated genes are difficult to infer from our analysis because the immortalized transcriptome was analyzed at passage 26 and 69. Our gene sets could therefore represent secondary or even tertiary events that could mask the true Id1 regulated genes. Nevertheless, the gene set that could closely represent the Id1 initiated events is the control only gene set. The increase in the expression of rat Id1 itself and known Id1 regulated genes such as Vegf [55] and alpha actin [56] supports that at least some of these genes may be direct downstream targets of Id1. The increased expression of endogenous Id1 also implies that its expression is associated with immortalization. A carefully executed Id silencing experiments in these cell lines are expected to provide true Id1 dependent genes. Alternatively, direct Id1 targets within the control list could be verified by using transient expression systems.

At the transcriptional level, Id1 acts as a negative regulator of bHLH family of transcription factors that normally promote differentiation. Thus it is not surprising that androgens that normally promote differentiation in normal prostate epithelial cells also down regulate Id1 expression, supporting the role of Id1 in normal prostate epithelial cells as inhibitor of differentiation. The loss of androgen receptor and response associated with immortalization is also not unexpected. Previous studies have also demonstrated that primary prostate epithelial cells in culture loose secretory differentiated function and androgen responsiveness [57, 58]. Moreover, ectopic Id1 expression in androgen sensitive LNCaP prostate cancer cell lines is also associated with loss of androgen response and increased proliferation[16]. Although, AR is expressed at passage

26, the RPE cells however fail to respond to androgens as shown in this study. Evidence therefore suggests that loss of androgen insensitivity is associated with Id1 expression although it could very well be a consequence of increased proliferation. Direct experiments such as Id1 antisense or transient over-expression systems, as suggested above are expected to provide a clear mechanism by which Id1 cross-talks with androgen receptor. The loss of androgen receptor in subsequent doublings (>26) could be an indirect mechanism largely dependent on the transcriptomic changes associated with immortalization.

The absence of chromosomal aberrations in Id1-RPE cells was unexpected. Recent reports have suggested that Id1 is capable of inducing chromosomal aberrations at least in cells of human origin [26]. We speculate that lack of Id1 mediated chromosomal aberrations and ploidy could be due to the use of rodent cells that inherently have long telomeres and at least in this study demonstrated increased Tert expression.

In summary, the studies demonstrated that Id1 alone is capable of immortalizing but not transforming normal prostate epithelial cells. The associated microarray and network analysis provides a comprehensive insight into the molecular events involved in sustained cell survival and proliferation. More importantly, the possible Id1 downstream targets and associated networks revealed novel pathways and mechanism of action of Id1 in promoting immortalization of prostate epithelial cells.

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REFERENCES

1. Norton, J.D., Deed, R.W., Craggs, G. and Sablitzky, F. Id helix-loop-helix proteins in cell growth and differentiation. **Trends Cell. Biol.** 8 (1998) 58-65.
2. Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L. and Weintraub, H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. **Cell** 61 (1990) 49-59.
3. Barone, M.V., Pepperkok, R., Peverali, F.A. and Philipson, L. Id proteins control growth induction in mammalian cells. **Proc. Natl. Acad. Sci. USA** 91 (1994) 4985-4988.
4. Hara, E., Yamaguchi, T., Nojima, H., Ide, T., Campisi, J., Okayama, H. and Oda, K. Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. **J. Biol. Chem.** 269 (1994) 2139-2145.

5. Coppe, J.P., Smith, A.P. and Desprez, P.Y. Id proteins in epithelial cells. **Exp. Cell Res.** 285 (2003) 131-145.
6. Israel, M.A., Hernandez, M.C., Florio, M., Andres-Barquin, P.J., Mantani, A., Carter, J.H. and Julin, C.M. Id gene expression as a key mediator of tumor cell biology. **Cancer Res.** 59 (1999) 1726s-1730s.
7. Norton, J.D. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. **J. Cell Sci.** 113 (Pt 22) (2000) 3897-3905.
8. Perk, J., Iavarone, A. and Benezra, R. Id family of helix-loop-helix proteins in cancer. **Nat. Rev. Cancer** 5 (2005) 603-614.
9. Ouyang, X.S., Wang, X., Lee, D.T., Tsao, S.W. and Wong, Y.C. Over expression of ID-1 in prostate cancer. **J. Urol.** 167 (2002) 2598-2602.
10. Forootan, S.S., Wong, Y.C., Dodson, A., Wang, X., Lin, K., Smith, P.H., Foster, C.S. and Ke, Y. Increased Id-1 expression is significantly associated with poor survival of patients with prostate cancer. **Hum. Pathol.** 38 (2007) 1321-1329.
11. Coppe, J.P., Itahana, Y., Moore, D.H., Bennington, J.L. and Desprez, P.Y. Id-1 and Id-2 proteins as molecular markers for human prostate cancer progression. **Clin. Cancer Res.** 10 (2004) 2044-2051.
12. Ling, M.T., Wang, X., Ouyang, X.S., Lee, T.K., Fan, T.Y., Xu, K., Tsao, S.W. and Wong, Y.C. Activation of MAPK signaling pathway is essential for Id-1 induced serum independent prostate cancer cell growth. **Oncogene** 21 (2002) 8498-8505.
13. Ling, M.T., Wang, X., Ouyang, X.S., Xu, K., Tsao, S.W. and Wong, Y.C. Id-1 expression promotes cell survival through activation of NF-kappaB signalling pathway in prostate cancer cells. **Oncogene** 22 (2003) 4498-4508.
14. Ling, M.T., Wang, X., Tsao, S.W. and Wong, Y.C. Down-regulation of Id-1 expression is associated with TGF beta 1-induced growth arrest in prostate epithelial cells. **Biochim. Biophys. Acta** 1570 (2002) 145-152.
15. Chaudhary, J., Schmidt, M. and Sadler-Riggleman, I. Negative acting HLH proteins Id 1, Id 2, Id 3, and Id 4 are expressed in prostate epithelial cells. **Prostate** 64 (2005) 253-264.
16. Ling, M.T., Wang, X., Lee, D.T., Tam, P.C., Tsao, S.W. and Wong, Y.C. Id-1 expression induces androgen-independent prostate cancer cell growth through activation of epidermal growth factor receptor (EGF-R). **Carcinogenesis** 25 (2004) 517-525.
17. Asirvatham, A.J., Schmidt, M.A. and Chaudhary, J. Non-redundant inhibitor of differentiation (Id) gene expression and function in human prostate epithelial cells. **Prostate** 66 (2006) 921-935.
18. Ouyang, X.S., Wang, X., Lee, D.T., Tsao, S.W. and Wong, Y.C. Up-regulation of TRPM-2, MMP-7 and ID-1 during sex hormone-induced prostate carcinogenesis in the Noble rat. **Carcinogenesis** 22 (2001) 965-973.
19. Wice, B.M. and Gordon, J.I. Forced expression of Id-1 in the adult mouse small intestinal epithelium is associated with development of adenomas. **J. Biol. Chem.** 273 (1998) 25310-25319.

20. Alani, R.M., Hasskarl, J., Grace, M., Hernandez, M.C., Israel, M.A. and Munger, K. Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1. **Proc. Natl. Acad. Sci. USA** 96 (1999) 9637-9641.
21. Nickoloff, B.J., Chaturvedi, V., Bacon, P., Qin, J.Z., Denning, M.F. and Diaz, M.O. Id-1 delays senescence but does not immortalize keratinocytes. **J. Biol. Chem.** 275 (2000) 27501-27504.
22. Chaudhary, J., Sadler-Riggleman, I., Ague, J.M. and Skinner, M.K. The helix-loop-helix inhibitor of differentiation proteins induce post-mitotic terminally differentiated sertoli cells to re-enter the cell cycle and proliferate. **Biol. Reprod.** 72 (2005) 1205-1217.
23. Alani, R.M., Young, A.Z. and Shifflett, C.B. Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. **Proc. Natl. Acad. Sci. USA** 98 (2001) 7812-7816.
24. Zheng, W., Wang, H., Xue, L., Zhang, Z. and Tong, T. Regulation of cellular senescence and p16(INK4a) expression by Id1 and E47 proteins in human diploid fibroblast. **J. Biol. Chem.** 279 (2004) 31524-31532.
25. Hasskarl, J., Duensing, S., Manuel, E. and Munger, K. The helix-loop-helix protein ID1 localizes to centrosomes and rapidly induces abnormal centrosome numbers. **Oncogene** 23 (2004) 1930-1938.
26. Man, C., Rosa, J., Yip, Y.L., Cheung, A.L., Kwong, Y.L., Doxsey, S.J. and Tsao, S.W. Id1 overexpression induces tetraploidization and multiple abnormal mitotic phenotypes by modulating aurora A. **Mol. Biol. Cell** 19 (2008) 2389-2401.
27. Schwarze, S.R., Shi, Y., Fu, V.X., Watson, P.A. and Jarrard, D.F. Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. **Oncogene** 20 (2001) 8184-8192.
28. Peehl, D.M. Primary cell cultures as models of prostate cancer development. **Endocr. Relat. Cancer** 12 (2005) 19-47.
29. Chowdhury, A., Harber, G.J. and Chopra, D.P. Characterization and serial propagation of mouse prostate epithelial cells in serum-free medium. **Biol. Cell** 67 (1989) 281-287.
30. Asirvatham, A.J., Schmidt, M., Gao, B. and Chaudhary, J. Androgens regulate the immune/inflammatory response and cell survival pathways in rat ventral prostate epithelial cells. **Endocrinology** 147 (2006) 257-271.
31. Schwarze, S.R., DePrimo, S.E., Grabert, L.M., Fu, V.X., Brooks, J.D. and Jarrard, D.F. Novel pathways associated with bypassing cellular senescence in human prostate epithelial cells. **J. Biol. Chem.** 277 (2002) 14877-14883.
32. Fujimoto, N., Suzuki, T., Honda, H. and Kitamura, S. Estrogen enhancement of androgen-responsive gene expression in hormone-induced hyperplasia in the ventral prostate of F344 rats. **Cancer Sci.** 95 (2004) 711-715.
33. Homma, Y., Kondo, Y., Kaneko, M., Kitamura, T., Nyou, W.T., Yanagisawa, M., Yamamoto, Y. and Kakizoe, T. Promotion of carcinogenesis and oxidative stress by dietary cholesterol in rat prostate. **Carcinogenesis** 25 (2004) 1011-1014.

34. Prins, G.S., Birch, L. and Greene, G.L. Androgen receptor localization in different cell types of the adult rat prostate. **Endocrinology** 129 (1991) 3187-3199.
35. Rhim, J.S. Research into molecular and genetic mechanisms underlying prostate carcinogenesis would be greatly advanced by in vitro prostate cell models. **Drugs Today (Barc)** 39 (2003) 837-847.
36. Cussenot, O., Berthon, P., Cochand-Priollet, B., Maitland, N.J. and Le Duc, A. Immunocytochemical comparison of cultured normal epithelial prostatic cells with prostatic tissue sections. **Exp. Cell Res.** 214 (1994) 83-92.
37. Ilio, K.Y., Sensibar, J.A. and Lee, C. Effect of TGF-beta 1, TGF-alpha, and EGF on cell proliferation and cell death in rat ventral prostatic epithelial cells in culture. **J. Androl.** 16 (1995) 482-490.
38. Itoh, N., Patel, U. and Skinner, M.K. Developmental and hormonal regulation of transforming growth factor-alpha and epidermal growth factor receptor gene expression in isolated prostatic epithelial and stromal cells. **Endocrinology** 139 (1998) 1369-1377.
39. Shibuki, H., Katai, N., Kuroiwa, S., Kurokawa, T., Arai, J., Matsumoto, K., Nakamura, T. and Yoshimura, N. Expression and neuroprotective effect of hepatocyte growth factor in retinal ischemia-reperfusion injury. **Invest. Ophthalmol. Vis. Sci.** 43 (2002) 528-536.
40. Sugimura, Y., Foster, B.A., Hom, Y.K., Lipschutz, J.H., Rubin, J.S., Finch, P.W., Aaronson, S.A., Hayashi, N., Kawamura, J. and Cunha, G.R. Keratinocyte growth factor (KGF) can replace testosterone in the ductal branching morphogenesis of the rat ventral prostate. **Int. J. Dev. Biol.** 40 (1996) 941-951.
41. Gmyrek, G.A., Walburg, M., Webb, C.P., Yu, H.M., You, X., Vaughan, E.D., Vande Woude, G.F. and Knudsen, B.S. Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. **Am. J. Pathol.** 159 (2001) 579-590.
42. Tang, J., Gordon, G.M., Nickoloff, B.J. and Foreman, K.E. The helix-loop-helix protein id-1 delays onset of replicative senescence in human endothelial cells. **Lab. Invest.** 82 (2002) 1073-1079.
43. Reddel, R.R. The role of senescence and immortalization in carcinogenesis. **Carcinogenesis** 21 (2000) 477-484.
44. Hahn, W.C. Immortalization and transformation of human cells. **Mol. Cells** 13 (2002) 351-361.
45. McConnell, B.B., Starborg, M., Brookes, S. and Peters, G. Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. **Curr. Biol.** 8 (1998) 351-354.
46. Shao, G., Balajee, A.S., Hei, T.K. and Zhao, Y. p16(INK4a) downregulation is involved in immortalization of primary human prostate epithelial cells induced by telomerase. **Mol. Carcinog.** 47 (2008) 775-783.
47. Chapman, E.J., Hurst, C.D., Pitt, E., Chambers, P., Aveyard, J.S. and Knowles, M.A. Expression of hTERT immortalises normal human urothelial

cells without inactivation of the p16/Rb pathway. **Oncogene** 25 (2006) 5037-5045.
48. Iavarone, A., Garg, P., Lasorella, A., Hsu, J. and Israel, M.A. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. **Genes Dev.** 8 (1994) 1270-1284.
49. Lasorella, A., Iavarone, A. and Israel, M.A. Id2 specifically alters regulation of the cell cycle by tumor suppressor proteins. **Mol. Cell. Biol.** 16 (1996) 2570-2578.
50. Zenzmaier, C., Untergasser, G., Hermann, M., Dirnhofer, S., Sampson, N. and Berger, P. Dysregulation of Dkk-3 expression in benign and malignant prostatic tissue. **Prostate** 68 (2008) 540-547.
51. Kawano, Y., Kitaoka, M., Hamada, Y., Walker, M.M., Waxman, J. and Kypta, R.M. Regulation of prostate cell growth and morphogenesis by Dickkopf-3. **Oncogene** 25 (2006) 6528-6537.
52. Tsuji, T., Miyazaki, M., Sakaguchi, M., Inoue, Y. and Namba, M. A REIC gene shows down-regulation in human immortalized cells and human tumor-derived cell lines. **Biochem. Biophys. Res. Commun.** 268 (2000) 20-24.
53. Kashiwakura, Y., Ochiai, K., Watanabe, M., Abarzua, F., Sakaguchi, M., Takaoka, M., Tanimoto, R., Nasu, Y., Huh, N.H. and Kumon, H. Down-regulation of inhibition of differentiation-1 via activation of activating transcription factor 3 and Smad regulates REIC/Dickkopf-3-induced apoptosis. **Cancer Res.** 68 (2008) 8333-8341.
54. Damdinsuren, B., Nagano, H., Kondo, M., Natsag, J., Hanada, H., Nakamura, M., Wada, H., Kato, H., Marubashi, S., Miyamoto, A., Takeda, Y., Umeshita, K., Dono, K. and Monden, M. TGF-beta1-induced cell growth arrest and partial differentiation is related to the suppression of Id1 in human hepatoma cells. **Oncol. Rep.** 15 (2006) 401-408.
55. Ling, M.T., Lau, T.C., Zhou, C., Chua, C.W., Kwok, W.K., Wang, Q., Wang, X. and Wong, Y.C. Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). **Carcinogenesis** 26 (2005) 1668-1676.
56. Li, Y., Yang, J., Luo, J.H., Dedhar, S. and Liu, Y. Tubular epithelial cell dedifferentiation is driven by the helix-loop-helix transcriptional inhibitor Id1. **J. Am. Soc. Nephrol.** 18 (2007) 449-460.
57. Goossens, K., Deboel, L., Swinnen, J.V., Roskams, T., Manin, M., Rombauts, W. and Verhoeven, G. Both retinoids and androgens are required to maintain or promote functional differentiation in reaggregation cultures of human prostate epithelial cells. **Prostate** 53 (2002) 34-49.
58. Grant, E.S., Batchelor, K.W. and Habib, F.K. Androgen independence of primary epithelial cultures of the prostate is associated with a down-regulation of androgen receptor gene expression. **Prostate** 29 (1996) 339-349.