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Human renal tubular cells contain CD24/CD133 progenitor cell populations: implications for tubular regeneration after toxicant induced damage using cadmium as a model

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Abstract

The proximal tubules of the kidney are target sites of injury by various toxicants. Cadmium (Cd^{+2}), an environmental nephrotoxicant can cause adverse effects and overt renal damage. To decipher the mechanisms involved in nephrotoxicity, an *in vitro* model system is required. Mortal cultures of human proximal tubule (HPT) cells have served, as models but are difficult to acquire and do not lend themselves to stable transfection. The immortalized human proximal tubule cell line HK-2, has served as a model but it lacks vectorial active transport and shows signs of lost epithelial features. Recently a new proximal tubule cell line was developed, the RPTEC/TERT1, and the goal of this study was to determine if this cell line could serve as a model to study nephrotoxicity. Global gene expression analysis of this cell line in comparison to the HK-2 and HPT cells showed that the RPTEC/TERT1 cells had gene expression patterns similar to HPT cells when compared to the HK-2 cells. The HPT and the RPTEC/TERT1 cell line had an increased population of stem/progenitor cells co-expressing CD24 and CD133 when compared to the HK-2 cells. The level of expression of cadherins, claudins and occludin molecules was also similar between the RPTEC/TERT1 and the HPT cells. Acute exposure to Cd^{+2} resulted in necrosis of the RPTEC/TERT1 cells when compared to the HK-2 cells which died by apoptosis. Thus, the RPTEC/TERT1 cells are similar to HPT cells and can serve as a good model system to study mechanisms involved in toxicant induced renal damage.

Keywords

Kidney; tubular cells; cadmium; CD24/CD133; stem cells; RPTEC/TERT1

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Conflict of interests

The authors declare that they have no competing interests.

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1. Introduction

The proximal tubules of the human kidney are susceptible to a variety of injuries, both from ischemic episodes and exposure to toxic substances such as heavy metals (Buser et al. 2016). The proximal tubules perform the major component of renal reabsorption and, as such, requires extremely large amounts of energy, rendering this renal segment very susceptible to exposure from nephrotoxins or the loss of nutrients and oxygen from blockage of blood flow. If severe, this results in acute tubular injury where the proximal tubule cell loses polarization, followed by necrosis or apoptosis, cellular detachment and ultimately denuded basal membranes (Racusen et al. 2007). Acute tubular injury, if within defined levels of compromise, are transient and the proximal tubule cells can regenerate following such insults. The ability of proximal tubule cells to regenerate following acute injury has led to efforts to characterize the cells that can regenerate following injury. There appears to be a general agreement that the cells participating in proximal tubule cell renewal are present within the kidney and there is no recruitment from non-renal sources. A number of studies have demonstrated that adult human kidneys contain a population of renal stem cells and more committed progenitor cells. These cells are characterized by the co-expression of cell surface markers CD133 and CD24 (Romagnani and Remuzzi 2014; Smeets et al. 2013; Romagnani et al. 2013; Angelotti et al. 2012; Lindgren et al. 2011; Sallustio et al. 2013; Ronconi et al. 2009). Under cell culture conditions, these cells can be expanded and they retain their phenotype. They also maintain their potential for self-renewal as well as the capacity to differentiate into podocytes and tubular cells *in vivo* and *in vitro* (Romagnani et al. 2013; Angelotti et al. 2012; Lindgren et al. 2011; Sallustio et al. 2013; Ronconi et al. 2009; Sagrinati et al. 2006). During human kidney development, the CD133⁺ renal cells present as a subset of CD24 cells where they constitute the metanephric mesenchyme-derived primordial nephron (Lazzeri et al. 2007). Taken together, these studies suggest that CD24 cells, when co-express CD133, define a putative renal progenitor/stem cell population capable of tubular regeneration in the adult kidney.

Cell culture is used extensively to study the mechanisms underlying normal and disease processes that involve the renal proximal tubule. Until recently, two cell culture models of the human proximal tubule have been used in these studies. The first model is mortal cultures of human proximal tubule (HPT) cells isolated from cortical tissue of human kidneys (Detrisac et al. 1984; Wilson et al. 1985). The second model utilizes HK-2 cells, an immortalized cell line, derived by immortalizing and cloning a cell from a primary culture of the above-described proximal tubule epithelial cells transduced with a construct containing the HPV16 E6/E7 genes (Ryan et al. 1994). More recently, a third model consisting of an immortalized human proximal tubule cell line, RPTEC/TERT1, was derived by immortalizing and cloning a cell from a primary culture of the above-described proximal tubule epithelial cells transduced with a construct containing hTERT (Wieser et al. 2008). The HK-2 cell line, due to its immortalized property, has seen the most usage regarding studies on the proximal tubule, with over 100 citations in the previous 10 years. Primary HPT cells are utilized much less due to the need to secure human tissue and their limited lifespan, although commercial suppliers are now available. The HK-2 and HPT cell models both retain many, but not all, differentiated features of the human proximal tubule. These

properties include proximal tubule markers such as alkaline phosphatase, gamma glutamyltranspeptidase, leucine aminopeptidase, acid phosphatase, and glucose-6 phosphatase (Detrisac et al. 1984, Ryan et al. 1994). An important marker is the enzyme glucose-6 phosphatase that is needed for gluconeogenesis and it is known that the proximal tubule is the only renal segment that can support gluconeogenesis. Functional markers of proximal tubule differentiation also retained are: cAMP responsiveness to parathyroid hormone, but not antidiuretic hormone and, the ability to accumulate glycogen.

There are two major differences between the HPT and HK-2 cells that are reflected in their morphology. One major difference is that the HK-2 cells have lost the capacity for vectorial active transport as noted by the inability to form doming structures in culture (Kim et al. 2002). The formation of domes is a hallmark of cultured renal epithelial cells that retain the *in situ* property of vectorial active transport and appear as out-of-focus areas of the cell monolayer seen upon light microscopic examination. In these raised areas, fluid is trapped underneath the monolayer owing to active transport of ions and water across the cell monolayer in an apical to basolateral direction. This in turn traps a bubble of fluid between the cell layer and the culture dish, forcing local detachment of the monolayer from the plastic surface forming a raised area with an underneath reservoir of accumulated fluid. A second major difference is that, in agreement with the absence of domes, the HK-2 cells do not develop a transepithelial resistance due to the lack of tight junctions (Kim et al. 2002). A corresponding analysis of E- and N-cadherin expression between the cell lines demonstrated a decrease in E-cadherin and an increase in N-cadherin expression in the HK-2 cells when compared to the HPT cells (Bathula et al. 2008; Slusser et al. 2014). These major differences are reflected in the HPT cells having an enhanced epithelial morphology and increased polarization compared to the HK-2 cell line. Although not as well published, the RPTEC/TERT1 cells have been shown to retain gene expression patterns expected for the proximal tubule, as well as to form a polarized monolayer, form domes in culture, and to display a light level morphology indistinguishable from primary cultures of HPT cells (Aschauer et al. 2013). In addition, the RPTEC/TERT1 cell line has been demonstrated to serve as a model for the study of agent-induced nephrotoxicity (Crean et al. 2015).

The similarities in the morphology, doming and polarity of the HPT and RPTEC/TERT1 cells suggest that the RPTEC/TERT1 could be an excellent model system to study alterations in human proximal tubule function. The immortal nature of the RPTEC/TERT1 renders them a far more convenient model system than HPT cells. In this study, we performed a global gene expression analysis to determine the similarity in gene expression patterns between the RPTEC/TERT1, the HPT and the HK-2 cells. The main objective being to determine if the RPTEC/TERT1 cells are similar to the HPT cells and could serve as a model system for human proximal tubule cells. In addition, the presence of progenitor cells in the three cell culture systems was also determined by assessing the expression of CD133 and CD24. As a model toxicant, these cells were compared in their response to the heavy metal, cadmium, a well-known environmental nephrotoxicant that can cause acute tubular necrosis (Alpers and Chang 2015; ATSDR 2012). This metal toxicant is also of concern due to the link between cadmium exposure and chronic kidney disease (Ferraro et al., 2010; Navas-Acien et al., 2009; Sommar et al., 2013), and also due to the accumulation of this

metal within the proximal tubule cells of the renal cortex even within populations thought to be free of metal exposure (ATSDR 2012; Akesson et al., 2005).

2. Materials and Methods

2.1. Cell culture

Stock cultures of HPT cells which have been used previously were grown using serum-free conditions containing a 1:1 mixture of Dulbecco's modified Eagles' medium and Ham's F-12 growth medium (Detrisac et al., 1984). The culture medium was supplemented with selenium (5 ng/ml), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml) as described previously by this laboratory (Detrisac et al., 1984; Kim et al., 2002). HK-2 cells and RPTEC/TERT1 cells were obtained from American Type Culture Collection and grown using identical serum free conditions. The cells were fed fresh growth medium every 2 or 3 days, and at confluence, the cells were subcultured using trypsin-EDTA (ethylenediaminetetraacetic acid). For use in experimental protocols, cells were cultured at a 1:4 (HK-2), 1:2 (HPT) or a 1:3 (RPTEC/TERT1) ratio, allowed to reach confluence and then used in the described experimental protocols.

2.2. Real time PCR analysis

The level of expression of CD133, CD24, CD44, aldehyde dehydrogenase 1 family member A1 (ALDH1A1), N-cadherin (CDH2), E-cadherin (CDH1), P-cadherin (CDH3), Ksp-cadherin (CDH16), claudin 4 (CLDN4), claudin 1 (CLDN1) and occludin (OCDN) genes was assessed using real-time reverse transcription PCR and commercially available primers. The primers for CD24, CD44, ALDH1A1, CDH16, CDH3 and CLDN1 were obtained from Qiagen (Qiagen Company, Valencia, CA) and the primers for CD133, CDH1, CDH2, CLDN4, OCDN, and β -actin were obtained from Bio-Rad (Bio-Rad Laboratories, Hercules CA). Total RNA was isolated using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) as described previously (Garrett et al., 1998). For analysis, 1 µg of total RNA was subjected to complimentary DNA (cDNA) synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules CA) in a total volume of 20 µl. Real-time PCR was performed utilizing the SYBR Green kit (Bio-Rad Laboratories) with 2 µl of cDNA, 0.2 µM primers in a total volume of 20 µl in an iCycler iQ real-time detection system (Bio-Rad Laboratories). The relative levels of mRNA for each of the genes was assessed, and normalized to the change in β -actin expression.

2.3. Microarray analysis of global gene expression

Total RNA was purified from triplicate cultures of HK-2 and RPTEC/TERT1 cells and six individual cultures of human proximal tubule (HPT) cells (single sample per culture) using the RNeasy Mini kit (Qiagen, Valencia, CA). Each HPT cell isolate represents a culture from an individual patient. For global gene expression, total RNA was assessed using the Affymetrix Human Transcriptome 2.0 Array, which contains 44,699 protein coding and 22,829 non-coding transcript clusters. Fragmented biotinylated cDNA was hybridized to arrays, washed and stained using the Affymetrix Fluidics Station 450, and fluorescence intensities determined for each array using the Affymetrix GCS300 7G high-resolution

confocal laser scanner. Signal intensities for all 12 arrays were normalized via the SST-RMA method in Affymetrix Expression Console (build 1.4.1.46). Expression data for 67,528 non-control transcript cluster probes, interrogating known and putative genes in the human genome, was available for gene and sample-based cluster analysis. Gene and sample-based unsupervised hierarchical cluster analysis was executed using the UPGMA algorithm based on Pearson correlation metric while cluster generation was verified via bootstrap and Euclidian distance analysis in GeneMath XT 2.0. All cluster verification was performed using greater than 10,000 permutations and clusters were verified with a cluster stability of 100.

2.4. Western blot analysis

The expression of protein was determined by Western blot analysis using protocols that have been previously published by this laboratory (Cao et. al., 2010). Briefly, twenty micrograms of total cellular protein was separated on a TGX AnyKd SDS polyacrylamide gel (Bio-Rad Laboratories) and transferred to a hybond-P Polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% [wt/vol] nonfat dry milk for 1 h at room temperature. After blocking, the membranes were washed three times with TBS-T and probed with the appropriate primary antibody overnight at 4°C. The antibodies used were; CD133, 1:100 (Miltenyi Biotec Inc., San Diego, CA); CD24, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); CD44, 1:1000 (Cell Signaling Technology, Beverly, MA); ALDH1A1, 1:2000 (Santa Cruz Biotechnology); CDH2, 1:500 (Invitrogen, Carlsbad, CA); CDH1, 1:250 (Santa Cruz Biotechnology); CDH3, 1:800 (Santa Cruz Biotechnology); CDH16, 1:300 (Abcam Inc., Cambridge, MA); OCDN, 0.5 ug/ml (Invitrogen); CLDN4, 1:500 (Invitrogen); CLDN1 1:500 (Life technologies, Carlsbad, CA). After washing three times in TBS-T, membranes were incubated with the appropriate anti-mouse or anti-rabbit antibody (1:2000) in antibody dilution buffer for one h. The blots were visualized using the Phototope-HRP Western blot detection system (Cell Signaling Technology).

2.5. Flow Cytometry analysis

The cells were grown to confluency, washed two times with phosphate buffered saline (PBS) and detached using Accutase (BD Biosciences, San Jose, CA). The cell pellet was washed and re-suspended in BD Pharmingen™ stain buffer (BSA). The cells were counted and adjusted to a concentration of 1×10^6 cells/mL. For every 100 uL of cell suspension, 10 uL of FITC conjugated CD133 (Miltenyi Biotec) and/or PE conjugated CD24 antibody (Miltenyi Biotec) was added to the tubes. The cells were incubated for 30 min on ice in the dark and then washed two times with stain buffer and re-suspended in 700 ul of stain buffer. Cells were counted with a BD LSRII and the data was analyzed using the FlowJo software. For each sample, 10,000 events were acquired. Results were gated to exclude doublets and identify the singlet population.

2.6. Determination of cell viability and fragmentation of nuclei by visualization of DAPI-stained cells

The effect of Cd^{+2} on the viability and fragmentation of nuclei (apoptotic nuclei) was determined by visualization and counting of 4',6-diamidino-2-phenylindole (DAPI)- stained

nuclei as described previously by this laboratory (Garrett et al., 1998; Somji et al., 2004). For this purpose, the HK-2 and RPTEC/TERT1 cells were grown to confluency following which they were treated with the appropriate concentrations of Cd^{+2} . At the indicated time points, wells containing the monolayers were rinsed with PBS, fixed for 15 min in 70% ethanol, rehydrated with 1 ml PBS, and stained with 10 μl DAPI (10 $\mu\text{g}/\text{ml}$ in distilled water). For each time point, a minimum of 20 fields per well and 3 wells per data point were examined. The nuclear counts as well as the number of fragmented nuclei were determined for each field. The percentage of fragmented nuclei were determined for each well and the results presented as the mean $\pm$ SEM for the triplicate wells.

2.7. Determination of LDH release

The release of lactate dehydrogenase (LDH) from Cd^{+2} treated cells was determined by the Cyto Tox 967 assay kit (Promega, Madison, WI) as described previously (Somji et al., 2004). After treatment with Cd^{+2} for appropriate times, 50 μl of the cell culture supernatant was transferred to a 96 well enzymatic assay plate. The reconstituted substrate mix (50 μl) was added to each well containing the sample and the enzymatic reaction was allowed to proceed for 30 min at room temperature in the dark. The assay was stopped by adding 50 μl of the stop solution (1M acetic acid) and the plate was read at 490 nm using an ELISA plate reader.

2.8. Caspase-3 assay

The caspase-3 assay kit from R&D Systems (Minneapolis, MN) was used to determine the activity of the enzyme. The cells were grown as monolayers and after treatment with Cd^{+2} for various time periods, the cells were collected by centrifugation and the pellet was lysed by the addition of the cell lysis buffer provided in the kit. The cell lysate was incubated on ice for 10 min and following centrifugation, the supernatant was transferred to a new tube and the protein concentration was determined using the bicinchoninic acid protein assay.

50 μl of the cell lysate was transferred to a 96 well flat bottom micro plate and 50 μl of 2X reaction buffer and 5 μl of caspase-3 colorimetric substrate (DEVD-pNA) was added to each well. Enzyme activity was determined by enzymatic cleavage of the tetrapeptide substrate DEVD-pNA and the release of the chromophore p-nitroanilide, which was measured in a spectrophotometer at a wavelength of 405 nm. The results are plotted as absorbance units per mg total cell protein.

2.9. Statistical analysis

All experiments were done in triplicates and the data was analyzed using ANOVA with Tukey *post-hoc* testing performed by Graphpad PRISM 4. The data is plotted as the mean $\pm$ SEM of triplicate determinations.

3. Results

3.1. Global gene expression pattern of HK-2, HPT and RPTEC/TERT1 cells

The gene expression profiles were obtained using total RNA from triplicate samples of HK-2 cells and RPTEC/TERT1 cells and six isolates of HPT cells. To determine if the

global expression profile of all non-control transcript clusters could distinguish all three groups of cells, sample-based clustering analysis was performed using the entire 67,528 non-control transcript cluster expression profile without any statistical filtering. The results (Fig. 1) demonstrated that the triplicate samples of HK-2 cells clustered together with 100% accuracy while the HPT and RPTEC/TERT1 cells clustered together with 66% accuracy, respectively. Specifically, four of the six HPT cell samples segregated into a cluster with one sample from the RPTEC/TERT1 cell line while two of the six HPT cell samples segregated into another cluster with two samples from the RPTEC/TERT1 cell line. Sample-based clustering demonstrates three main clusters that segregate the samples from the HPT, RPTEC/TERT1 and HK-2 cell lines. Cluster verification, using bootstrapping, of sample-based clusters revealed all clusters had exceptionally high stability, all equaling 100, indicating the clusters (and segregation of the HK-2 cell line from HPT/RPTEC/TERT1 cells) are representative of authentic structures in the data set. This analysis convincingly indicates that the gene expression profile of the HK-2 cells was distinct from that of either the HPT or the RPTEC/TERT1 cells. The cluster analysis also suggest that the gene expression profile of the HPT and RPTEC/TERT1 cells may be similar because the UPGMA clustering algorithm did not succeed in separating the two groups of cells into distinct clusters. Principal component analysis on the 67,529 non-control transcript clusters confirmed the findings of the cluster analysis (Fig. 2). In terms of separation of samples by component, HK-2 samples separated from HPT/RPTEC/TERT1 samples across the x-component (first component, 36% of variance in the dataset) while HPT and RPTEC/TERT1 samples were slightly separated across the z-component (third component, 14.7% of variance in the dataset).

An increase in the diversity of gene expression profiles from the HPT cells was anticipated due to each culture being derived from an independent patient sample. This is the reason six isolates were used in the current study. As predicted, the samples from the six independent isolates of HPT cells showed the most diversity in gene expression. The samples from the HPT cells segregated with the samples from the RPTEC/TERT1 cell line into two clusters. One cluster was composed of samples from four HPT cell isolates and from one sample from the RPTEC/TERT1 cell line (5.3% similarity). The second cluster was composed of samples from two HPT cell isolates and samples from two RPTEC/TERT1 cell lines (–8.6% similarity). For this second cluster, the sample from the RPTEC/TERT1 cell line segregated under one sub-cluster (60.5% similarity) and the samples from the HPT cells segregated under another sub-cluster (45.8% similarity).

The gene expression patterns from the three groups of cells were subjected to pairwise analysis using parametric methods of differentially expressed genes using an FDR-adjusted p-value of 0.05 or 0.01. Employing a p-value of 0.05 demonstrated 1,427 transcript clusters were induced and 694 were repressed in HK-2 cells compared to HPT cells with 65,407 being of similar expression (Fig. 3A). An identical analysis of samples from the RPTEC/TERT1 cells showed 77 transcript clusters induced and 30 repressed with 67,421 having similar expression when compared to the HPT cells (Fig. 3B). Employing a p-value of 0.01 demonstrated 1,088 transcript clusters induced and 639 repressed with 65,801 being similarly expressed for the HK-2 cell line compared to HPT cells. For the RPTEC/TERT1 cell line, only 63 transcript clusters were induced and 15 were repressed with 67,450

transcript clusters being similarly expressed between the RPTEC/TERT1 cell line and the HPT cells. This analysis shows that the HK-2 cell line has a much greater divergent global gene expression pattern from HPT cell cultures than that of the RPTEC/TERT1 cell line.

The segregation of the gene expression profiles from the three cell culture groups were also subjected to a transposed principal component analysis view after performing discriminant analysis of pre-defined groups (Fig. 4). The results of this analysis shows that the X-component separates the samples from the HPT and RPTEC/TERT1 cells to one side of the axis and the samples from the HK-2 cells to the other side of the axis. This X-component accounts for 73.3% of the variance between the two groups. The Y-component separates the samples of the HPT and RPTEC/TERT1 cells on either side of the axis, while samples from the HK-2 cells are borderline on the Y-axis. The Y-component accounts for the remaining 26.7% of the variance between groups. The fact that the X-component accounts for 73.3% of the variance indicates that the majority of differences in the dataset arise from a comparison of the HK-2 cells to the HPT and RPTEC/TERT1 cells. The differences between the HPT and RPTEC/TERT1 cells are marginal when compared to the HK-2 cells.

To identify differences in gene expression between the HPT, RPTEC/TERT1 and HK-2 cell culture groups, clustering analysis was performed by the UPGMA Algorithm based on Pearson correlation metric. To identify a maximum expression profile distinguishing groups, transcript clusters with FDR-adjusted ANOVA p-value ≤ 0.01 were subjected to sample and gene-based clustering analysis (Fig. 5). The clustering accuracy of the individual groups, HK-2, RPTEC/TERT1 and HPT, segregated into 4 distinct sub-clusters with 100% accuracy. Gene-based clustering analysis identified 6 groups (Cluster ID) of genes with distinct expression profiles (Group A – F, Table 1, Supplemental Materials). Group A contains the vast majority of genes, with a majority being repressed in RPTEC/TERT1 and HPT cells but induced in HK-2 cells. Group B represent genes repressed in RPTEC/TERT1 cells and induced in HK-2 and HPT cells. Group C represent genes repressed in HK-2 and RPTEC/TERT1 cells and induced in HPT cells. Group D represent genes repressed in HK-2 cells, while genes in HPT and RPTEC/TERT1 cells are induced. Group E represent genes induced in RPTEC/TERT1 cells and repressed in HPT and HK-2 cells. Group F genes represent genes repressed in HPT cells, induced in RPTEC/TERT1 cells, with no change expression in HK-2 cells.

3.2. Expression of CD24 and CD133 in the HK-2, HPT and RPTEC/TERT1 cells

The second goal of this study was to determine if the 3-cell culture models contained a population of cells displaying progenitor/stem cell markers. For this purpose, the co-expression of CD24 and CD133 was determined on all 3-cell culture models using flow cytometry (Fig. 6A–D). The results of this analysis demonstrated that CD24 and CD133 molecules were co-expressed in approximately 20% of the HK-2 cells (Fig. 6A). In contrast, 62% of the HPT cells and 79% of the RPTEC/TERT1 cells co-expressed CD24 and CD133 (Fig. 6B and C). Thus, the CD24/CD133 population was significantly increased in both the HPT and RPTEC/TERT1 cells when compared to the HK-2 cells (Fig. 6D). The CD24/CD133 population was also significantly different between the HPT and RPTEC/TERT1 cells (Fig. 6D) with the RPTEC/TERT1 cell culture having a significantly higher percent of

cells co-expressing CD24 and CD133. The expression of CD24 and CD133 mRNA and protein was also determined for each of the cell isolates (Fig 7A–B). The results showed that CD133 mRNA and protein was increased in the RPTEC/TERT1 cells and HPT cells when compared to the HK-2 cells (Fig. 7A). The levels of CD24 mRNA and protein was increased in the RPTEC/TERT1 cells when compared to both the HPT and HK-2 cells (Fig 7B). For the HPT cells, there was an increase in expression in 2 of the isolates, whereas the third isolate had expression levels similar to the HK-2 cells (Fig. 7B). The expression of two additional genes, aldehyde dehydrogenase 1A1 (ALDH1A1) and CD44 that are associated with stem/progenitor cells in other organs, were also assessed in the HPT, HK-2 and RPTEC/TERT1 cells (Fig. 7C and D). As seen in Fig. 7C, the expression of CD44 was increased in the HK-2 cells when compared to both the HPT and RPTEC/TERT1 cells (Fig. 7C), however the expression levels were similar between the RPTEC/TERT1 and the HPT cells. The expression of ALDH1A1 was increased in the RPTEC/TERT1 cells when compared to both the HK-2 cells and HPT cells (Fig. 7D). The HPT isolates had significantly higher levels of ALDH1A1 expression when compared to the HK-2 cells (Fig 7D).

3.3. Expression of Cadherins, Claudins and Occludin in the HK-2, HPT and RPTEC/TERT1 cell isolates

The expression of E-, N-, P- and Ksp-cadherins, occludin (OCDN), claudin 1 (CLDN1) and claudin 4 (CLDN4) genes were determined in the HK-2, HPT, and RPTEC/TERT1 cells. These genes were analyzed since they showed an alteration in expression on the global gene expression array and they are known to be associated with the epithelial to mesenchymal transition (EMT) of cells. The results showed that expression of E-cadherin was elevated in both the HPT and RPTEC/TERT1 cells when compared to the HK-2 cells (Fig. 8A). The level of E-cadherin expression in the HK-2 cells was at or near the level of detection, in contrast, the expression of N-cadherin was elevated in the HK-2 cells when compared to the HPT or RPTEC/TERT1 cells (Fig. 8B). The level of N-cadherin expression in the RPTEC/TERT1 cells was close to the level of detection. The expression of P-cadherin was increased in the HPT and RPTEC/TERT1 cells when compared to the HK-2 cells (Fig. 8C). The expression of Ksp-cadherin was highest in the RPTEC/TERT1 cells when compared to the HPT and HK-2 cells (Fig. 8D). The expression of OCDN was higher in the RPTEC/TERT1 cells compared to the HPT and HK-2 cells (Fig. 9A). However, when the expression levels were compared between the HPT and the RPTEC/TERT1 cells, it was shown that the RPTEC/TERT1 cells had higher expression of OCDN when compared to the HPT cell. The expression of CLDN1 was elevated in the HK-2 cells when compared to both the HPT and RPTEC/TERT1 cells (Fig. 9B), whereas the levels of expression were similar between the HPT and the RPTEC/TERT1 cells. The expression of CLDN4 was opposite to that of CLDN1, with the HPT and RPTEC/TERT1 cells expressing higher levels when compared to the HK-2 cells (Fig. 9C).

3.4. Mode of cell death in RPTEC/TERT1 cells in response to Cd⁺² - induced toxicity

This laboratory has previously shown that HPT cells undergo necrotic cell death when exposed to Cd⁺² while HK-2 cells undergo cell death by apoptosis (Somji et al. 2004). A similar study was performed to determine the mode of cell death in RPTEC/TERT1 cells

following acute exposure to Cd^{+2} (Fig. 10A–D). The results of this analysis showed that the RPTEC/TERT1 cells were more resistant to Cd^{+2} when compared to the HK-2 cells (Fig. 10A vs Fig. 11A). Upon treatment with Cd^{+2} , there was a high level of release of LDH into the growth media (Fig. 10B). Furthermore, there was no increase in the activity of the enzyme caspase-3 (Fig. 10C), and nuclear fragmentation a marker for cells undergoing apoptosis was not observed in DAPI stained nuclei (Fig. 10D). Treatment of HK-2 cells with Cd^{+2} under similar conditions showed changes consistent with cells undergoing apoptosis. The cells were more sensitive to Cd^{+2} (Fig. 11A) when compared to the RPTEC/TERT1 cells (Fig. 10A), and there was lower release of LDH (Fig. 11B) as published previously for HK-2 cells (Somji et al. 2004). In addition, there was an increase in caspase-3 activity (Fig. 11C), with significant numbers of DAPI stained fragmented nuclei visible microscopically (Fig. 11D and E).

4. Discussion

Primary cultures of human-derived renal epithelial cells were first isolated and characterized in the middle to late 1980s (Detrisac et al. 1984; Trifillis et al. 1985; Wilson et al. 1985; States et al. 1986; Kempson et al. 1989). The isolation of these cultures from human renal cortical tissue relied on the use of serum-free, hormone-defined cell culture media. This growth media that was developed supports the growth of animal derived immortal cell lines, such as MDCK and LLC-PK1 cells, as well as primary cultures from animal kidneys (Taub et al. 1979; Taub and Sato, 1979, 1980; Chuman et al. 1982; Chung et al. 1982). The human renal cell cultures so derived from these early animal studies were mortal and displayed a limited life span in cell culture of 10 to 20 generations before senescence. During the time these human renal cell cultures were developed, there was an expectation that the site of origin be identified within the human nephron for the studies to undergo publication. This expectation resulted in most of the reports on the isolation of human renal epithelial cells from human cortical tissue to be identified as retaining differentiated features of the human proximal tubule. Many of these observations were consistent among laboratories and included: epithelial morphology; cell polarization; vectorial active transport; enzyme profiles for markers associated with known *in situ* properties of proximal tubule cells; an increase in cAMP and short circuit current when cells are exposed to parathyroid hormone (PTH) but not arginine vasopressin (VP); the presence of gap and tight junctions; and, the generation of a transepithelial electrical resistance associated with a “leaky epithelium”. There are also features of these cells that are not consistent with an origin and recapitulation of the properties of the proximal tubule. The major feature of the *in situ* proximal tubule not recapitulated in any of the reports on human renal cell cultures is a fully developed brush border, a hallmark of the human proximal tubule (Blackburn et al. 1988). While the putative HPT cell cultures do show increased polarity and apical microvilli when grown on permeable supports, there is no evidence of a brush border approaching that found *in vivo* (Blackburn et al. 1988). Other features of the cultured renal cells not in agreement with *in situ* properties is an increase in the transepithelial resistance of the cultured cells over that noted for the proximal tubule (Blackburn et al. 1984; Todd et al. 1993). While the transepithelial resistance of the cultured cells is still characteristic of a “leaky epithelium”, it is many fold higher than that measured *in situ* for the proximal tubule. Last, the expression

of E-cadherin in the cultured cells appears to be elevated and N-cadherin reduced compared to that noted for the *in situ* proximal tubule (Nouwen et al. 1993; Prozialeck et al. 2004; Bathula et al. 2008; Nurnberger et al. 2010; Slusser et al. 2015). Thus, primary cultures derived as described above from human renal cortex retain some, but not all, features of proximal tubule differentiation and will likely continue to be referred to as cultured HPT cells.

The recent studies defining the progenitor/stem cells of the human kidney impacts on the understanding of the origins of these primary cultures of HPT cells. As detailed in the introduction, in the human kidney the renal epithelial cells capable of regeneration following renal injury are sparsely scattered throughout the kidney cortex and are characterized by the co-expression of CD24 and CD133. The finding that approximately 62 percent of HPT cells co-express CD24 and CD133 suggests that the origin of the majority of these cells are ones that are capable of regenerating the renal tubule cells. The data also showed that the cells not co-expressing CD24 and CD133 do continue to express CD24, in line with studies showing that CD133⁺ cells are a subset of CD24 expressing renal epithelial cells (Romagnani and Remuzzi, 2014). Taken together, these observations would suggest that the HPT cells that co-express CD24 and CD133 are those cells in the culture that are capable of sustaining growth of the cell culture. If the CD24⁺CD133⁺ are the cells sustaining growth of the cell culture, then it is likely that the CD24 expressing cells that do not express CD133 have undergone senescence or have undergone differentiation into a non-dividing CD24 expressing cell type. For the HPT cells, the percentage of cells that co-express CD24 and CD133 do diminish in number as the number of generations increase and the cultures approach senescence. The expression of two other stem cell markers CD44 and ALDH1 was also determined and we found that CD44 was expressed at a very low level in the HPT and the RPTEC/TERT cells when compared to the HK-2 cells. CD44 is normally expressed at a very low level in the kidney and the expression levels increase during renal injury. The expression of ALDH1 was the highest in the RPTEC/TERT1 cells when compared to the HPT cells and the expression was absent in the HK-2 cells. ALDH1 is normally not expressed in the kidney and the expression is upregulated in renal cell carcinomas.

The primary cultures of HPT cells are difficult to use as an experimental model to study proximal tubule structure and function. For many laboratories, human cortical tissue is difficult to obtain, and even for those with tissue resources, the tissue is in limited supply. There is also substantial expertise and cost involved in cell isolation and growth. There is also considerable expense in obtaining these cells from commercial sources. These limitations have led to the wide use of the immortalized HK-2 cell line in research related to defining mechanisms related to human proximal tubule function in health and disease. The HK-2 cell line was derived from a primary HPT cell culture and immortalized by a construct containing HPV16 E6/E7 genes (Ryan et al. 1994). As detailed in the introduction, a major difference between the HK-2 cells and HPT cells is the absence of tight junctions, loss of vectorial transport, and a more mesenchymal morphology compared to the HPT cells. These differences may be important for studies that employ HK-2 cells to study EMT following renal injury (Bathula et al. 2008; Slusser et al. 2014). A second cell line has recently been characterized that is derived from a primary culture of HPT cells and immortalized using a construct containing hTERT (Wieser et al 2008). The morphology of these cultures and the

ability to form domes is similar, if not identical to that shown previously for the primary HPT cells (Wieser et al 2008).

One of the goals of the present study was to determine how closely related the HK-2 cell line and the RPTEC/TERT1 cell line are to the primary HPT cell culture that was the original source of cells used for immortalization. The results from an analysis of global gene expression patterns clearly demonstrated that the primary HPT cell cultures and the RPTEC/TERT1 cell line are closely related, while the HK-2 cell line was substantially different from both the HPT and RPTEC/TERT1 cells. The number of differentially expressed genes in the HK-2 (in comparison to the HPT cells) was about twenty-fold greater than that of the RPTEC/TERT1 cell line. Six independent isolates, each from a different patient, were used in the current study to cover for possible individual variation in culture properties, both phenotypic and genetic, and represent the normal variation in gene expression of this cell type in culture. While there is a clear spectrum of gene expression within this group as seen in the principal component as well as the hierarchical clustering, the RPTEC/TERT1 cell line, while very similar in its overall gene expression, was clearly distinct from the HPT cell group, suggesting that there are fundamental gene expression changes in this cell line that sets it apart from cultures of normal HPT cells.

The array data can further be interpreted in the context of the co-expression of CD24 and CD133 being a marker of a human renal progenitor/stem cell for tubule regeneration following injury (Romagnani and Remuzzi 2014; Smeets et al. 2013; Romagnani et al. 2013; Angelotti et al. 2012; Lindgren et al. 2011; Sallustio et al. 2013; Ronconi et al. 2009). If such is true, then the RPTEC/TERT1 cell line presents as a valuable, easy to use, cell culture system that models the cellular component of the renal epithelium that is capable of renal cell regeneration. The fact that approximately 80% of the RPTEC/TERT1 cell population co-expresses CD24 and CD133 is convincing that it can serve to explore the underlying mechanisms involved in this cells ability to regenerate tubular epithelium. The remaining 20% of the RPTEC/TERT1 cells that do not co-express CD24 and CD133 have likely undergone differentiation to another cell lineage. While speculation, this might indicate that cell culture conditions could be identified to differentiate these CD24 and CD133 co-expressing cell into specific cell types. The HK-2 cells demonstrated a very different pattern of CD24 and CD133 expression and only limited identity with both the HPT and RPTEC/TERT1 cell lines. The lower percentage of HK-2 cells co-expressing CD24 and CD133 (21%) may indicate that these are the cells driving proliferation of the culture with the remaining cells having undergone differentiation into another cell lineage. All the HK-2 cells not co-expressing CD24 and CD133 do express CD24. Precisely what tubule type is modeled by the HK-2 cells has not been resolved by this study. The differences between the two cell lines, HK-2 and RPTEC/TERT1, may simply represent what clonal cell was isolated and immortalized from the primary culture of HPT cells. It would have been informative to compare the global gene expression profiles of the 3 cell cultures to that of the *in vivo* human proximal tubule; however, the authors could not identify the existence of such a study.

One of the main features of the RPTEC/TERT1 cell line that is absent from the HK-2 cells, is the presence of vectorial active transport and the formation of dome structures, a common

feature consistently observed in HPT cells. This feature requires the presence of adherens and tight junctions, cell structures that require the expression of cadherins, claudins and occludin proteins. Particularly important is the formation of the tight junction because this structure forms the boundary of two-dimensional diffusion of the apical and basal-lateral plasma membrane, which enables the polar distribution of uptake and efflux transporters (Cao et al., 2012). The current study assessed the expression of many members of these three classes of proteins. Occludin, a mandatory component of the tight junction showed very low levels of expression in the HK-2 cells when compared to the HPT and the RPTEC/TERT1 cells. The adherens junction cadherins (E-, P- and Ksp-cadherin) were either absent or expressed at a very low level in the HK-2 cells when compared to the HPT and the RPTEC/TERT1 cells. This data is in accord with a previous assessment of these proteins and the development of transepithelial resistance in HK-2 and HPT cells (Slusser et al., 2015 and Bathula et al., 2008). In addition to lacking E-cadherin, the HK-2 line highly expressed N-cadherin, a pattern commonly found in cells that have undergone a transition to a more mesenchymal morphology, also known as EMT. This is also reflected in the loss of vectorial active transport, loss of cell junctions and less polarity. As such, this may render the HK-2 cell line as an appropriate model system for a cell that has undergone part of the process associated with EMT.

The RPTEC/TERT1 cell line was shown in the current study to exhibit a primarily necrotic mode of cell death in response to lethal levels of Cd^{+2} , whereas the HK-2 cell line exhibited extensive apoptosis during lethal exposures of this metal. Cultures of HPT cells respond primarily via a necrotic mechanism as previously reported by this laboratory (Somji et al., 2004 & 2006). Cd^{+2} is a well-known nephrotoxin and a member of a well-known class of nephrotoxins that cause acute tubular necrosis (Alpers and Chang, 2015). Many of the early studies of Cd^{+2} -induced nephrotoxicity both in animal models (Cha 1987; Itokawa et al. 1974; Kawamura et al. 1978; Kotsonis and Klaassen 1978; Prigge 1978) and in case reports (Beton et al., 1966; Chugh et al., 1994), describe necrosis as the morphologically discernable form of cell death (ATSDR 2012). The literature has been replete with studies characterizing the apoptotic mode of cell death in Cd^{+2} -induced nephrotoxicity (Fujiwara et al, 2012, Prozialek et al., 2012), but clearly, necrosis is the predominant mode of cell death during acute exposure and this is similar to that of many acutely toxic nephrotoxins (Sharfuddin et al, 2016) with apoptosis being a contributing component. It is noteworthy that at lower doses of Cd^{+2} that cause low amounts of proximal tubule cell death, apoptosis can predominate (Prozialek et al., 2009, Templeton and Liu, 2010). With the new advancements in the area of programmed cell death, particularly necroptosis, the current study underscores the need for the proper application of an *in vitro* model system to investigate the mechanism of the different modes of cell death.

Overall, the present study provides evidence that the RPTEC/TERT1 cell line could be a valuable model system to determine the effects that nephrotoxins might have on the regenerative capability of tubular epithelium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALDH1A1	Aldehyde Dehydrogenase 1 Family Member A1
ATN	acute tubular necrosis
BSA	bovine serum albumin
Cd⁺²	cadmium
CLDN1	claudin 1
CLDN4	claudin 4
cDNA	complimentary DNA
DAPI	4',6-diamidino-2-phenylindole
EDTA	ethylenediaminetetraacetic acid
CDH1	E-cadherin
EMT	epithelial to mesenchymal transition
HPT	human proximal tubule
CDH16	Ksp-cadherin
LDH	lactate dehydrogenase
CDH2	N-cadherin
OCDN	occludin
CDH3	P-cadherin
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.1% Tween-20

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Highlight

- Human proximal tubular cultures contain CD24⁺/CD133⁺ expressing stem/progenitor cells
- The RPTEC/TERT1 cell line has gene expression patterns similar to primary HPT cells
- Acute cadmium exposure causes the RPTEC/TERT1 cells to die by necrosis

Fig. 1.

Hierarchical clustering of various proximal tubule cell transcriptomes. All transcript clusters from the HTA 2.0 array (67,528) were subjected to hierarchical clustering using the UPGMA algorithm based on Pearson correlation metric without any statistical filtering of transcript clusters. Samples include triplicate RNA samples from three parallel cultures of HK-2 (HK-2-A, -B, and -C), RPTEC/TERT1 (RPTEC/TERT1-A, -B, and -C) and single RNA samples from six independent isolates of human proximal tubule cells (HPT-1, -2, -3, -4, -5 and -6).

Fig. 2. Principal component analysis using all 67,528 non-control transcript clusters. Turquoise dots represents HK-2 cells; grey dots represents HPT isolates, and pink dot represents RPTEC/TERT1 cells. The two outliers of the HPT group are HPT-4 and HPT-5. The X-component accounts for 73% of the variance between groups, and the Y-component represents 26.7% variance between groups.

Fig. 3.

Venn diagram showing differentially expressed genes in HK-2 cells and RPTEC/TERT1 cells in comparison to mortal human proximal tubule cell (HPT) isolates. Pairwise analysis was used to compare the number of differentially expressed genes from HK-2 in comparison to six isolates of HPTs or RPTEC/TERT1 cells in comparison to HPT cells. A differentially expressed gene is defined as one that is an absolute fold change ≥ 2 or ≤ 0.5 , and an FDR-adjusted ANOVA and T-test p-value of ≤ 0.05 or ≤ 0.01 . (A). Differentially expressed genes in HK-2 vs HPT cells at $p \leq 0.05$. (B). Differentially expressed genes in RPTEC/TERT1 vs HPT cells at $p \leq 0.05$. (C) Differentially expressed genes in HK-2 vs HPT cells at $p \leq 0.01$. (D). Differentially expressed genes in RPTEC/TERT1 vs HPT cells at $p \leq 0.01$.

Fig. 4.

Transposed principal component analysis after performing discriminant analysis of predefined groups. Discriminant Principal Component Analysis, selecting for the top 0.015% of discriminating genes that discriminates between groups with 100% accuracy following sample-based clustering by UPGMA. Turquoise dots represents HK-2 cells; grey dots represent HPT isolates, and pink dots represent RPTEC/TERT1 cells.

Fig. 5. Hierarchical clustering of transcript cluster expression after statistical filtering. All 67,528 non-control transcript clusters were filtered to meet an FDR-adjusted ANOVA p-value 0.01, which gave 5,604 transcript clusters. These were subjected to unsupervised hierarchical clustering using the UPGMA algorithm based on Pearson correlation metric. Samples form three clusters, with the HK-2 triplicate samples forming a separate cluster, the RPTEC/TERT1 triplicate samples, and six HPT samples forming two separate sub-clusters. The relative level of expression of each cluster is color-coded and is shown on the right. On the right side of the heat map is designated the gene groupings (A – F) and is listed in Table 1 (Supplementary file).

Fig. 6.

Flow cytometry analysis of CD24 and CD133 populations in proximal tubule cells. Analysis of CD24 and CD133 and CD24/CD133 populations in HK-2 (A), HPT (B) and RPTEC/TERT1 (C) cell cultures. (D). Graphical representation of the number of CD24/CD133 co-expressing cells in the three groups of cell cultures. **** indicates significantly different in the RPTEC/TERT1 cells and HPT cells when compared to the HK-2 cells at p-value of 0.01. # indicates significantly different in the RPTEC/TERT1 cells when compared to the HPT cells at p-value of 0.01.

Fig. 7.

Expression of stem cell markers in cultures of human proximal tubule cells. (A). Expression of CD133 in HK-2 cells, HPT cells and RPTEC/TERT1 cells. (B). Expression of CD24 in HK-2 cells, HPT cells and RPTEC/TERT1 cells. (C). Expression of CD44 in HK-2 cells, HPT cells and RPTEC/TERT1 cells. (D). Expression of ALDH1A1 in HK-2 cells, HPT cells and RPTEC/TERT1 cells. The top graph in figures A–D represent PCR data whereas the bottom graphs represent the IOD of the Western blot analysis. **** indicates significantly different in the RPTEC/TERT1 cells and HPT cells when compared to the HK-2 cells at p-value of < 0.01 . *** indicates significantly different in HPT cells when compared to HK-2

cells at p-value of 0.05. # indicates significantly different in the RPTEC/TERT cells when compared to the HPT cells at p-value of 0.01.

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Fig. 8.

Expression of cadherins in cultures of human proximal tubule cells. (A). Expression of E-cadherin in HK-2, HPT and RPTEC/TERT1 cells. (B). Expression of N-cadherin in HK-2, HPT and RPTEC/TERT1 cells. (C). Expression of P-cadherin in HK-2, HPT and RPTEC/TERT1 cells. (D). Expression of Ksp-cadherin in HK-2, HPT and RPTEC/TERT1 cells. The top graph in figures A–D represent PCR data whereas the bottom graphs represent the IOD of the Western blot analysis. **** indicates significantly different in the RPTEC/TERT1 cells and HPT cells when compared to the HK-2 cells at p-value of < 0.01 .

Fig. 9.

Expression of tight junction proteins in cultures of human proximal tubule cells. (A). Expression of occludin (OCDN) in HK-2, HPT and RPTEC/TERT1 cells. (B). Expression of claudin 1 (CLDN1) in HK-2, HPT and RPTEC/TERT1 cells. (C). Expression of claudin 4 (CLDN4) in HK-2, HPT and RPTEC/TERT1 cells. The top graph in figures A–C represent PCR data whereas the bottom graphs represent the IOD of the Western blot analysis. **** indicates significantly different in the RPTEC/TERT1 cells and HPT cells when compared to the HK-2 cells at p-value of 0.01. *** indicates significantly different in HPT cells when compared to HK-2 cells at p-value of 0.05. # indicates significantly different in the RPTEC/TERT1 cells when compared to the HK-2 cells at p-value of 0.01.

Fig. 10.

Effect of Cd^{+2} on RPTEC/TERT1 cells. (A). Effect of Cd^{+2} on the viability RPTEC/TERT1 cells exposed to 9, 27 and 45 $\mu\text{M Cd}^{+2}$ for 24, 36, 48 and 60 h. Viability was determined by counting the DAPI stained nuclei and is expressed as percent of control. (B). LDH release by Cd^{+2} treated cells. RPTEC/TERT1 cells were exposed to 45 $\mu\text{M Cd}^{+2}$ for 48 and 60 h. The release of LDH by the cells was quantified spectrophotometrically at a wavelength of 490 nm and is expressed as percent of total LDH release. (C). Caspase-3 activation in Cd^{+2} treated cells. RPTEC/TERT1 cells were exposed to 9, 18, 27, 36 or 45 $\mu\text{M Cd}^{+2}$ for 8, 12, 24 and 36 h. Caspase-3 activity was determined by the release of the chromophore, p-nitroanilide, which was quantitated spectrophotometrically at a wavelength of 405 nm. (D). DAPI staining of RPTEC/TERT1 cells exposed to 45 $\mu\text{M Cd}^{+2}$ for 48 h. **** indicates significantly different in Cd^{+2} exposed cells when compared to untreated controls at p-value of < 0.01 . *** indicates significantly different in Cd^{+2} exposed cells when compared to untreated controls at p-value of < 0.05 .

Fig. 11.

Effect of Cd^{+2} on HK-2 cells. (A). Effect of Cd^{+2} on the viability HK-2 cells exposed to 9, 18 and 27 Cd^{+2} μM for 16, 24 and 36 h. Viability was determined by counting the DAPI stained nuclei and is expressed as percent of control. (B). LDH release by Cd^{+2} treated cells. HK-2 cells were exposed to 27 $\mu\text{M Cd}^{+2}$ for 24 and 36 h. The release of LDH by the cells was quantified spectrophotometrically at a wavelength of 490 nm and is expressed as percent of total LDH release. (C). Caspase-3 activation in Cd^{+2} treated cells. RPTEC/TERT1 cells were exposed to 9, 18 and 27 $\mu\text{M Cd}^{+2}$ for 8, 12 and 16 h. Caspase-3 activity was determined by the release of the chromophore, p-nitroanilide, which was quantitated spectrophotometrically at a wavelength of 405 nm. (D). Quantification of nuclear fragmentation for control and cadmium treated cells. The percent of apoptotic nuclei was determined after exposure to 9, 18 and 27 $\mu\text{M Cd}^{+2}$ for 16, 24 and 36 h. Results are expressed as percent of apoptotic nuclei. (E). DAPI staining of HK-2 cells exposed to 27 Cd^{+2} μM for 48 h. Arrows indicate fragmented nuclei. **** indicates significantly different in Cd^{+2} exposed cells when compared to untreated controls at p-value of 0.01.