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Induction of endoplasmic reticulum stress might be responsible for defective autophagy in cadmium-induced prostate carcinogenesis

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Abstract

Earlier, we reported that chronic cadmium (Cd)-exposure to prostate epithelial (RWPE-1) cells causes defective autophagy, which leads to the transformation of a malignant phenotype in both *in vitro* and *in vivo* models. However, the upstream events responsible for defective autophagy are yet to be delineated. The present study suggests that chronic Cd exposure induces endoplasmic reticulum (ER) stress that triggers the phosphorylation of stress transducers [protein kinase R-like ER Kinase- (PERK), eukaryotic translation initiation factor 2- α - (eIF2- α) and Activating Transcription Factor 4 -(ATF-4)], resulting in defective autophagy that protects Cd-exposed RWPE-1 cells. On the other hand, inhibition of the ATF4 stress inducer by siRNA blocked the Cd-induced defective autophagy in transforming cells. While dissecting the upstream activators of ER stress, we found that increased expression of reactive oxygen species (ROS) is responsible for ER stress in Cd-exposed RWPE-1 cells. Overexpression of antioxidants (SOD1/SOD2) mitigates Cd-induced ROS that results in inhibition of ER stress and autophagy in prostate epithelial cells. These results suggest that the induction of ROS and subsequent ER stress are responsible for defective autophagy in Cd-induced transformation in prostate epithelial cells.

1 Introduction

Cadmium (Cd) is a common pollutant of the environment. Several studies demonstrate a close relationship between Cd exposure and prostate cancer (CaP) (Achanzar *et al.*, 2001). The incidence of CaP has been correlated with increased environmental exposure to Cd as well as occupations with high Cd exposure (Armstrong and Kazantzis, 1985; Elinder *et al.*, 1985; Waalkes *et al.*, 1992; Jarup *et al.*, 1998; Pesch *et al.*, 2000; Waalkes, 2003; Sorahan and Esmen, 2004; Adams *et al.*, 2012; Julin *et al.*, 2012). Cigarette smoking is a significant factor for both the causation and severity of CaP (Watters *et al.*, 2009; Zu and Giovannucci, 2009). A clear dose-response relationship between Cd exposure and abnormal PSA levels was found in volunteers working in Cd-polluted areas. Cd exposure causes proliferation abnormalities, cellular necrosis and autophagy in multiple tissues (Templeton and Liu, 2010; Zhao *et al.*, 2017; Sun *et al.*, 2018).

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Reactive oxygen species (ROS) are often implicated in Cd-induced cytotoxicity in a variety of cell culture models leading to mitochondrial dysfunction, inhibition of respiration, and induction of oxidative stress (Bagchi *et al.*, 1997; Mao *et al.*, 2011). On the other hand, inhibition of antioxidant expression facilitates Cd induced ROS which promotes carcinogenesis (Liu *et al.*, 2009). Oxidative stress or inhibition of oxidative DNA damage repair might be possible causes for Cd-induced carcinogenesis (Waisberg *et al.*, 2003). ROS mediated ER-stress has been linked to the induction of pro-survival signaling in multiple cell types (Cheng *et al.*, 2014; Babele *et al.*, 2018; Guo *et al.*, 2018).

ER stress induces self-protective mechanisms, which including the unfolded protein response (UPR). ER transmembrane receptors, such as PERK (Harding *et al.*, 1999), initiate signaling cascades to restore ER homeostasis in the event of ER stress. However, in high ER stress conditions, the UPR engages either ER degradation or selective autophagy to reduce the unfolded protein load (Luo *et al.*, 2016). Cd-induced ER stress induces autophagy via the eIF2 α –ATF4 pathway (Vattem and Wek, 2004), wherein PERK phosphorylates the serine 51 residue of eIF2 α , enabling the selective translation of a set of transcription factors, including ATF4 (Vattem and Wek, 2004). LC3B, Atg-12, and Atg16L are direct transcriptional targets of PERK-dependent ATF4 (Rzymiski *et al.*, 2010). Furthermore, PERK/ATF4 regulation of LC3B has been demonstrated as a critical regulator of autophagy and hypoxic cell survival in tumors (Rouschop *et al.*, 2010).

Autophagy is a highly complex lysosomal-mediated degradation process that removes and recycles damaged organelles to maintain the quality of intracellular components. Autophagy promotes cancer cell survival by protecting them from hypoxia and oxidative damage and promoting chemoresistance (Kroemer and Jaattela, 2005; Kondo and Kondo, 2006; Kroemer and Levine, 2008). Autophagy-related genes (Atg) encode several serine-threonine kinases (including ULK) that comprise an integral part of the Atg/ULK complex, a key regulator of autophagy. This complex is required for the initiation of autophagosome formation, which eventually promotes autolysosomal degradation through the activation of the cytosolic microtubule-associated protein 1A/1B-light chain 3A (LC3A) (Hara *et al.*, 2008; Hosokawa *et al.*, 2009; Jung *et al.*, 2009). In mammalian cells, LC3A can be converted to LC3B through processing by Atg-7, an E1-like activating enzyme, and LC3B is considered a valid marker for autophagy in cancer (Klionsky and Emr, 2000; Wu *et al.*, 2006).

In the current study, we show the Cd- exposure increases expression of ROS causing ER-stress that results in the induction of ER transducers that might be responsible for defective autophagy in prostate carcinogenesis. Overexpression of anti-oxidants, significantly inhibited Cd-induced ROS generation and ER stress that resulted in inhibition of autophagy in prostate epithelial cells.

2 Materials and Methods

2.1. Cell lines and reagents

Human normal prostate epithelial cells (RWPE-1) were purchased from the American Type Culture Collection (Manassas, VA, USA). RWPE-1 cells were treated with Cadmium (10 μ M) for one year which transformed these cells to a malignant phenotype. The transformed

cells were named as Cadmium Transformed Prostate Epithelial Cells (CTPE). RWPE-1, transforming cells (2, 5 and eight months) and CTPE cells were cultured in keratinocyte serum-free medium containing L-glutamine and EGF and BPE, supplemented with 10% fetal bovine serum and 1% antibiotic and antimycotic solution in a humidified atmosphere of 5% CO₂ at 37°C in an incubator. Cadmium was purchased from Sigma (Dallas, TX, USA).

2.2. Protein extraction and Western blotting

RWPE-1, transforming cells (2, 5, eight months) and CTPE cells were seeded in 6-well plates, incubated for 24 h and then treated with cadmium (10 µM) for up to 48h. Cells were collected at specific time points (3, 6, 12, 24 and 48 h), cell lysates were prepared and Western blotting was performed using specific antibodies against Atg3, Atg5, Atg7, Beclin 1, ULK1, LC3A/B (Autophagy antibody sampler kit #4445, Cell Signaling, Danvers, MA), ATF4, PERK, p-PERK (Thr 980), p-eIF2α (Ser 51), (Cell Signaling) SOD1, SOD2, Catalase and β-actin (Santa Cruz Biotechnology, Dallas, TX). Protein-antibody complexes were visualized using enhanced chemiluminescence as previously described (Suman *et al.*, 2013).

2.3. ROS Assay

Cells were seeded at 3×10^4 on a 96-well plate and were allowed to attach overnight. Cells were washed with 1× wash buffer and stained with 25 µM DCFDA (Abcam, Cambridge, MA) for 45 min at 37°C. Cells were washed with 1× wash buffer post-incubation and then treated with 10 µM cadmium for 3, 6, 12, and 24 h. Finally, cells were washed with 1× wash buffer, and the signal was measured at Ex/Em: 485/535 nm. 2',7'-dichlorofluorescein (DCFDA), a fluorescent dye, is used in this assay, which measures hydroxyl, peroxy, and ROS activity within the cell. Once diffused into the cell, DCFDA is deacetylated by cellular esterase to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound (ROS Detection assay kit, Abcam, ab113851).

2.4. Endoplasmic Reticulum Stress Assay

Cells were grown on a coverslip at 80% confluence. Media was removed, and cells were treated with dual detection reagent (Enzo Life Sciences, Farmingdale, NY) and incubated in the dark for 15–30 min at 37°C. Cells were further washed with 1× assay buffer and mounted. Stained cells were analyzed by confocal microscopy. The dual detection reagent contains Calnexin, an ER-resident molecular chaperone that binds transiently to nascent proteins, assisting the folding and assembly, while retaining proteins that persist in a mal-folded or incompletely assembled state. In the ER quality control machinery, calnexin acts as a constituent of the glucosylation/deglucosylation cycle. During this cycle, monoglucosylated glycoproteins bind transiently to calnexin.

2.5. MitoTracker® Assay

Cells were grown on a coverslip at 80% confluence. Media was removed and cells were fixed with 2–4% formaldehyde for 15 min at room temperature. Pre-warmed staining solution containing Mitotracker® probe (Thermo Scientific, M7512, Eugene, OR) was

added to the wells and incubated for 30 min. Post incubation cells were washed and visualized under a confocal microscope. Mitotracker dye selectively accumulates in the mitochondrial matrix where it covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues in mitochondria.

2.6. siRNA transfection

RWPE-1 and CTPE cells were seeded in 6-well plates at a density of 3×10^5 cells/well. After a 24 h incubation, cells were transiently transfected with siRNA specific for ATF4 or a control siRNA or overexpression plasmid specific for SOD1, SOD2 and ATF4, for 48 h. Lipofectamine® 2000 was used as a transfection reagent. 48 h post transfection, cells were collected, the lysate was prepared and Western blotting was performed.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0a software (GraphPad Software, Inc., La Jolla, CA). Results are expressed as mean $\pm$ SEM. Data sets were compared using a two-tailed unpaired Student's t-test. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Induction of ER stress in Cd-exposed prostate epithelial cells.

To gain mechanistic insights into Cd-induced transformation, we analyzed the basal levels of ER-stress by quantitating the expression of Calnexin (a calcium-binding protein embedded in the membrane) as a marker for ER stress in both RWPE-1 and fully transformed prostate epithelial cells (CTPE). A significant ($P < 0.0001$) induction of ER-stress positive cells was seen in CTPE as compared to healthy RWPE-1 cells (Figure-1A). Furthermore, an increased ER stress was persistent in Cd-transforming cells (chronic exposure of Cd in 2, 5, and 8 months), similar to CTPE (Figure 1B). To confirm ER-stress, we measured ER-sensors by Western blot analysis: as seen Figure 1C, phosphorylation of PERK at Thr980 is followed by eIF2 α phosphorylation at Ser51 that, in turn, induced ATF4 expression in Cd-exposed RWPE-1 cells. Similar results were seen in Cd-exposed transforming cells (2, 5, or 8 months) (Figure 1D) and transformed CTPE cells (Figure 1E), suggesting persistent ER stress in Cd-exposed cells.

3.2 Cd induces defective autophagy in RWPE-1 cells.

Acute exposure of Cd upregulates Autophagy-related 5 (Atg5) expression (Figure 2A), however, in chronically exposed RWPE-1 cells (2, 5, 8 Months) and CTPE cells, Atg5 expression was down-regulated (Figure 2B & C). Atg5 is required for autophagosome and autolysosomal fusion, however, in chronically exposed cells, downregulation of Atg5 could have caused defective autophagy during the transformation, which leads the damaged cell to proliferate. Similarly, we have also seen an induction of LC3B during transformation and in CTPE cells (Figure 2B & C). To determine whether ER stress is responsible for defective autophagy, we silenced ATF4 in CTPE cells and then exposed the cells to Cd; in scrambled siRNA-treated cells, Cd induced ATF4, Atg5, and LC3B expression (Figure 3B); but inhibition of ATF4 resulted in downregulation of Atg5 and LC3B expression (Figure 3B),

which confirms ATF4 as a key initiator of the autophagy-signaling axis. To further confirm ATF-mediated Atg5 and LC3B induction, we ectopically overexpressed ATF-4 using various concentrations of ATF-4 plasmid (0–1 µg) in RWPE-1 cells, and Western blot analysis confirmed a gradual induction of Atg5 followed by LC-3 induction (Figure 3A). These experiments confirm the molecular link between ER-stress (ATF-4) and Atg5/LC3B in Cd-exposed prostate epithelial cells. To confirm the blockage of autophagosome and autolysosome fusion in Cd-exposed cells, immunofluorescence analysis was performed against LAMP1 (lysosome marker-red) and LC3B (phagosome marker-green). A fusion of autophagosome and autolysosome were seen in acutely exposed Cd-treated RWPE-1 cells, but a gradual decrease of fusion (merge of both Green -LC3B + Red-LAMP1)-positive cells were seen in transformed cells (Figure 3C) suggesting that sustained ER stress causes defective autophagy, which aids in the transformation of prostate epithelial cells.

3.3 Cadmium increased ROS accumulation in RWPE-1 cells

Next, we dissected the upstream activators of ER stress; we analyzed ROS generation in Cd-exposed cells. RWPE-1 cells were exposed to Cd at different time points, and ROS production was measured by 2',7'-dichlorofluorescein diacetate (DCFDA). A gradual increase of ROS production was seen in Cd-treated cells. However, this increase was not significant in Cd-exposed RWPE-1 cells (Figure 4A). Interestingly, the endogenous ROS levels are significantly lower in CTPE cells as compared to parental RWPE-1 cells (Figure 4B). We also tested if there was any mitochondrial stress when RWPE-1 cells were exposed to cadmium. Chronic exposure of cadmium to RWPE-1 cells significantly ($P < 0.0001$) increased MitoTracker® staining in transforming and CTPE cells (Figure 4C). Next, we analyzed the expression of anti-oxidants in RWPE-1 cells exposed to Cd and fully transformed CTPE cells. A gradual increase of catalase expression followed by decreased expression of superoxide dismutase 1 and 2 (SOD1/SOD2) was seen in Cd-exposed RWPE-1 cells (Figure 4D). Similar results were observed in fully transformed CTPE cells, with low levels of SOD1/SOD2 in fully transformed CTPE cells as compared to the parental RWPE-1 cells (Figure 4E). To ascertain whether decreased expression of SOD1/SOD2 might be responsible for ROS production, we transiently overexpressed anti-oxidant enzymes (SOD1 or 2) and measured ROS levels in Cd-exposed RWPE-1 cells. Decreased levels of Cd-induced ROS were seen in SOD-transfected cells relative to vector-transfected cells (Figure 4H), and the transfection efficiency was confirmed by Western blot analysis (Figure F & G).

3.4 Induction of ROS might be responsible for ER stress in Cd-exposed RWPE-1 cells.

To connect ROS induction and ER stress in Cd-exposed RWPE-1 cells, we overexpressed SOD1 and SOD2 in RWPE-1 cells and then exposed these to Cd. As seen in Figure-4F&G, Cd-induced ATF-4 in vector-transfected cells, whereas significant downregulation of ATF-4 was seen in SOD1 and SOD2 overexpressed cells. Furthermore, to link with defective autophagy, we measured LC3B expression in SOD1 transfectants and Cd-exposed cells; as seen in Figure 4F & G, downregulation of LC3B in SOD transfectants confirms the sequential order of Cd-ROS-ER stress-autophagy in RWPE-1 cells.

3.5 Does Cd induce general autophagy signaling cascade in Cd-exposed RWPE-1 cells.

Conventional autophagy markers, such as the induction of ULK1, Beclin, and Atg family proteins, initiate phagophore formation, followed by autophagosome and autolysosome fusion. Therefore, we determined the expression of autophagy initiators, such as ULK-1, Beclin 1, and Atg-7, in Cd-exposed RWPE-1 cells. Interestingly, downregulation of all three markers in Cd-exposed cells (Figure 5A) suggests that conventional autophagy was not initiated. We then confirmed similar molecular events in Cd-transforming cells (2, 5, or 8 months of exposure) and CTPE cells (Figure 5B& C). Interestingly, acute Cd exposure downregulates p62 expression, but during transformation, a massive accumulation of p62 levels was seen (data not shown), suggesting that autophagosome and autolysosomal fusion would not have occurred; resulting in defective autophagy during the transformation (Figure 5).

4 Discussion

In the present study, we demonstrate that chronic Cd exposure induces constant low levels of ROS production, causing ER stress that, in turn, is responsible for defective autophagy, which protects damaged cells and promotes malignant transformation in prostate carcinogenesis.

A functional link between Cd exposure and CaP has been highlighted in several studies (Sagi *et al.*, 2004; Rodriguez-Serrano *et al.*, 2006); however, the cellular mechanism by which Cd transforms prostate epithelial cells remains largely unknown. Androgen Receptor (AR) is a known driver for prostate cancer. Reports suggest that stimulation of the AR pathway by androgen is actually less effective in the production of AR-related products in cadmium-transformed cells (Benbrahim-Tallaa *et al.*, 2007). In support to their findings Mary *et al.* have reported that cadmium exposure to LNCaP cells lead to decreased AR expression at both transcription and translational levels (Martin *et al.*, 2002). In contrast, Ruiqin *et al.* reported induction of AR expression in LNCaP cells when exposed to cadmium (Wu *et al.*, 2014).

These studies are largely inconclusive about the role of AR in Cd-exposed cells. Long-term exposure to Cd has been widely studied, and kinases, Ca²⁺ fluxes, and de novo synthesis of ROS have been suggested as the leading molecular mechanisms of ROS induction (Sagi *et al.*, 2004; Rodriguez-Serrano *et al.*, 2006). In our studies, an increase in ROS levels was seen in Cd-exposed RWPE-1 cells, although this was not statistically significant, the induction of ROS was persistent in transforming cells, and once Cd-exposed cells were fully transformed, the endogenous levels of ROS decreased. ROS accumulation lead to an increase in mitochondrial stress, another prominent attribute of oxidative damage (Lee *et al.*, 2012). These results demonstrate an increased expression of ROS levels during Cd-induced transformation. Similar studies have reported that ROS levels were high during transformation and after transformation, and that these levels are reduced in Cd-exposed BEAS2B cells (Wang *et al.*, 2018). On the contrary, induction of excessive intracellular ROS can induce cancer cell cycle arrest, senescence, and apoptosis (Chung *et al.*, 2003), while a constant intracellular redox state has been shown to govern crucial steps to allow cancer

cells to abolish anoikis signals and escape apoptotic responses after a loss of cell/ECM contacts (Liou and Storz, 2010).

Radical scavenging enzymes, such as SOD1, SOD2, Catalase, and HO-1, confer cytoprotection against ROS generated oxidative stress (Di Mascio *et al.*, 1991; DeJong *et al.*, 2007; Ferrando *et al.*, 2013). We also measured antioxidant enzymes in Cd-exposed RWPE-1 cells and found that SOD1 and SOD2 expression was downregulated, and an increased expression of catalase was seen. To confirm whether downregulation of SOD1/SOD2 in Cd-exposed cells is responsible for ROS production, we transiently overexpressed SOD1/SOD2, finding that Cd-induced ROS levels were abolished, suggesting that oxidative stress might play an essential role in Cd-induced transformation of prostate epithelial cells.

The ER has been confirmed as a cellular target of Cd toxicity. Cd exposure has been shown to induce apoptotic cell death via the activation of ER stress-related signal transduction pathways in various cell types (Yokouchi *et al.*, 2007; Zhang *et al.*, 2011; Ji *et al.*, 2013). The Cd-induced release of Ca^{2+} from ER stores caused a generalized alteration of Ca^{2+} homeostasis, thereby disrupting the fragile ER environment, and induced ER stress and ER-mediated apoptosis. In our studies, acute exposure of Cd induced ER stress that resulted in growth inhibition in RWPE-1 cells. However, in chronic Cd-exposed RWPE-1 cells, the sustained ER stress induces cell proliferation, which correlated with soft agar colony formation [48]. To restore ER homeostasis, the ER induces several sensors for its recovery. We observed that expression of these markers (pPERK^{Thr980}, pEIF2 α ^{Ser51}) was significantly high in CTPE cells and ATF4 was strongly upregulated in Cd-treated RWPE cells, in fact, a constant increase in ATF4 expression was evident in Cd transforming cells (2, 5, and 8 months). Other metals, such as As and Cr, also elevate the transcriptional and translational activation of ER stress in liver cells via the upregulation of the ATF5 and ATF6 genes and lead to cell death. However, in our studies, we observed only increased ATF-4 levels, which initiates UPR in cells exposed to a toxic environment, and that their functions may be cell type-specific. It is well established that ER stress induces autophagy in many cancer types (Tang *et al.*, 2015; Jia *et al.*, 2016; Xu *et al.*, 2017). Similarly, in our results, Cd-induced ER stress caused oncogenic autophagy in prostate epithelial cells.

The amount of LC3-II and the formation of LC3 puncta are thought to be reliable markers of the autophagosome (Rashid *et al.*, 2015). We measured the autophagosome and autolysosome fusion, (merge of both Green -LC3B + Red -LAMP1)-positive cells in acute and chronically Cd-exposed prostate epithelial cells. A gradual decrease of fusion (merge of both Green -LC3B + Red -LAMP1)-positive cells was seen during transformation (data not shown) but not in acutely exposed Cd-treated RWPE-1 cells, suggesting that sustained ER stress resulted in defective autophagy, which aids in the transformation of prostate epithelial cells. Additionally, silencing of ATF4 in RWPE-1 cells resulted in downregulation of Atg-5 and LC3B expression in Cd-exposed cells. However, the increased expression seen in vector expressing cells suggests that ATF4 initiates the autophagy-signaling axis, which is responsible for Cd-induced prostate transformation. Besides, to mimic the Cd effect, we overexpressed ATF in RWPE-1 cells, which induced Atg-LC3B signaling in RWPE-1 cells, confirming the signaling axis in prostate epithelial cells. Earlier, we have shown that acute Cd exposure induces apoptosis in normal prostate epithelial cells (RWPE-1), while chronic

exposure (>1 year) transforms them to a malignant phenotype (CTPE) that is resistant to apoptosis and continues to proliferate. We have also seen an induction of autophagosome and autolysosome genes (Plac8, LC3B, and Lamp-1) during the transformation of RWPE cells to CTPE cells (Kolluru *et al.*, 2017).

The association between oncogenic transformation and up-regulation of cellular pathways that promote cell death is intriguing. Our study provides a mechanistic link between Cd exposure and the upstream events on ROS generation, activation of ER stress sensors, and the autophagy deficiency process. We also demonstrate that a prolonged period of stress in response to Cd exposure stimulates the eIF2 α /ATF4 pathway, which regulates and replenishes the autophagosome-associated protein pool to allow the cell to maintain selective autophagy and, therefore, facilitate their survival and proliferation.

6 References

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Highlights:

- Increased expression of reactive oxygen species (ROS) is responsible for ER stress in Cadmium exposed prostate epithelial (RWPE-1) cells.
- Chronic cadmium exposure induces endoplasmic reticulum (ER) stress that triggers the signaling cascades of ER stress sensors that causes defective autophagy.
- Defective autophagy protects cadmium exposed damaged cells and leads to transformation.

Figure 1: Cd-exposure to RWPE-1 cells induces ER stress

The basal levels expression of ER stress was quantified in RWPE-1 and CTPE cells, (B) RWPE-1, transforming cells (2, 5, eight months) and CTPE were stained with ER staining and ER-ID®-positive cells (Green) were quantified using fluorescent microscopy at magnification (x20). (C, D, & E) ER Stress markers PERK, p-PERK, p-eIF2α, and ATF4 were measured by Western blotting. Actin was used as an internal control. *P<0.01, ***P<0.0005 and ****P<0.0001.

Figure 2: Defective autophagy is active in Cd-exposed RWPE-1 cells

(A) RWPE-1 cells treated with cadmium (10 μM) for 24 and 48 h; (B) Cd-treated with 10 μM for 2, 5, and 8 months; and (C) RWPE-1 or CTPE cells; expression of autophagy markers was determined by Western blotting.

Figure 3: ATF4 expression regulates autophagy in RWPE-1 cells.

(A) RWPE-1 cells were transfected with either a backbone vector (control) or ATF4. (B) RWPE-1 cells were transfected with either scrambled (control) or ATF4 siRNA and then treated with 10 µM Cd for 24 h, and Western blotting was conducted for the expression of ATF4, Atg5, and LC3BA/B. β-Actin was used as a loading control. (C) Immunofluorescence analysis of LAMP1 (red) and LC3B (green) in RWPE-1, RWPE-1 +Cd, and Cd-transforming cells. The number of fused cells (yellow) are indicated in percentages.

Figure 4: Cd-exposure induces ROS in RWPE-1 cells.

(A) RWPE-1 or CTPE cells were treated with either vehicle or Cd (10 μ M) and labeled with CM-H2DCFDA. ROS generation was measured by a spectrophotometer. (B) Basal ROS generation was measured in RWPE-1 and CTPE cells. (C) RWPE-1, transforming cells (2, 5, and 8 months) and CTPE cells were stained with a mitochondrial stain, and MitoTracker® positive (Red) cells were quantified using a confocal microscope. (D) RWPE-1 or CTPE cells were treated with Cd (10 μ M) and SOD1, SOD2, and catalases expression were determined at the indicated time points. (E) Basal expression of anti-oxidants was measured in RWPE-1 and CTPE cells. (F & G) SOD1 and SOD2 overexpressing cells were treated with Cd; ER stress markers and (H) ROS generation was measured. * $P < 0.01$ and **** $P < 0.0001$.

Figure 5: Defective autophagy is active in Cd-exposed RWPE-1 cells

(A) RWPE-1 cells treated with cadmium (10 μM) for 24 and 48 h, (B) treated with 10 μM for 2, 5, and 8 months, and (C) RWPE-1 or CTPC cells; expression of autophagy markers (ULK1, Beclin 1, Atg3 & Atg7) were determined by Western blotting. β-Actin was used as a loading control.