Punicalagin promotes autophagy to protect primary human syncytiotrophoblasts from apoptosis

Ying Wang^{1,2}, Baosheng Chen¹, Mark S Longtine¹ and D Michael Nelson¹

¹Department of Obstetrics and Gynecology, Washington University School of Medicine, 4566 Scott Avenue, St Louis, Missouri 63110, USA and ²Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, China

Correspondence should be addressed to B Chen; Email: chenb@wudosis.wustl.edu

Abstract

Punicalagin is a prominent polyphenol in pomegranate juice that protects cultured syncytiotrophoblasts from stress-induced apoptosis. Here, we test the hypothesis that punicalagin has this effect by inhibiting the mTOR kinase pathway to enhance autophagic turnover and limit apoptosis in cultured primary human syncytiotrophoblasts. In syncytiotrophoblasts, starvation, rapamycin, or punicalagin all decreased the expression of phosphorylated ribosomal protein S6, a downstream target of the mTOR kinase, and of the autophagy markers, LC3-II and p62. In contrast, in the presence of bafilomycin, an inhibitor of late stages of autophagy and degradation in the autophagolysosome, syncytiotrophoblasts exposed to starvation, rapamycin, or punicalagin all showed increased levels of LC3-II and p62. The number of LC3-II punctae also increased in punicalagin-treated syncytiotrophoblasts exposed to chloroquine, another inhibitor of autophagic degradation, and punicalagin increased the number of lysosomes. The apoptosis-reducing effect of punicalagin was attenuated by inhibition of autophagy using bafilomycin or knockdown of the autophagy related gene, *ATG16L1*. Collectively, these data support the hypothesis that punicalagin modulates the crosstalk between autophagy and apoptosis to promote survival in cultured syncytiotrophoblasts.

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Introduction

The syncytiotrophoblast layer of human placental villi interfaces the maternal and fetal circulations and regulates maternal–fetal exchange of nutrients, wastes, and gases. Syncytiotrophoblasts are exposed to oxidative and nitrate stress and must have mechanisms that minimize nuclear and cytoplasmic injury.

We recently assessed the ability of pomegranate juice to protect cytotrophoblasts and syncytiotrophoblasts from stress-induced damage, with the idea that this dietary supplement has a low risk for adverse effects and may serve as a therapeutic agent (Chen *et al.* 2012, 2013). We chose this juice because pomegranate fruit has been consumed for hundreds of years as a nutrient supplement and is purported to have anti-atherosclerotic, anti-cancer, and anti-oxidant effects (Faria & Calhau 2011). We found that pomegranate juice reduces oxidative stress in human villous trophoblasts both *in vivo* and *in vitro* (Chen *et al.* 2012, 2013). The bioprotective effects of pomegranate juice are commonly attributed to its high concentration of polyphenols. Punicalagin (2,3-hexahydroxy-diphenoyl-4,6-gallagyl-glucose) accounts for more than one-half of

the polyphenols in pomegranate juice (Gil *et al.* 2000), and punicalagin by itself has anti-inflammatory (Jean-Gilles *et al.* 2013), anti-oxidative (Aqil *et al.* 2012), and anti-tumor activities (Wang *et al.* 2013). We tested punicalagin and found that this polyphenol was able to protect cultured human syncytiotrophoblasts from stress-induced apoptosis (Chen *et al.* 2012).

Here, we sought to uncover the mechanism by which punicalagin exerts this protective effect. We targeted autophagy as a pivotal cellular recycling pathway, for several reasons (Klionsky *et al.* 2012). First, autophagy is the pathway by which lysosomes degrade cytoplasmic components, including defective organelles, misfolded proteins, and invading microorganisms. Secondly, the mammalian target of rapamycin (mTOR) kinase pathway triggers autophagy in times of nutrient deficiency to generate amino acids and free fatty acids that pass through the tricarboxylic acid cycle to replenish cellular ATP levels. Thirdly, autophagy is often induced in response to stress as a means of promoting cell survival and reducing apoptosis, and multiple mediators of autophagy also play roles in apoptosis, indicating crosstalk between these pathways. Fourthly, autophagy

is known to occur in trophoblasts of human placental villi (Oh *et al.* 2008). We test the hypothesis that punicalagin modulates autophagy to limit apoptosis in syncytiotrophoblasts.

Materials and methods

Isolation and culture of primary human trophoblasts

This study was approved by the Institutional Review Board of Washington University School of Medicine in St Louis, MO, and all subjects signed written consent forms. Primary human trophoblasts were isolated from placentas of uncomplicated singleton pregnancies delivered by cesarean section at 39 weeks' gestation, as previously described (Kliman *et al.* 1986, Chen *et al.* 2010). Cells were cultured in phenol-red-free DMEM (Sigma) containing 10% charcoal-stripped fetal bovine serum (Life Technologies, Inc., Grand Island, NY, USA), 20 mM HEPES, pH 7.4, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO₂-air atmosphere. Cytotrophoblasts were plated at 300 000/cm², cultured for 4 h to allow cell attachment, rinsed with medium multiple times to eliminate syncytial fragments, and cultured for a total of 76 h, with prominent syncytiotrophoblasts present for 52 h. For starvation experiments, trophoblasts were cultured in Earle's balanced salt solution (Thermo Scientific, Hudson, NH, USA) for the last 2, 4, or 6 h of the 76-h culture period. For the data on punicalagin's effects on autophagy, lysosomes and apoptosis, the syncytiotrophoblasts were treated with punicalagin (33.8 µM; Sigma) at 37 °C in 5% CO₂-air atmosphere for the last 24 h before collection. In some experiments, cells were treated before collection with vehicle control (DMSO), 30 µM chloroquine (Sigma), 10 nM bafilomycin (Alexis Biochemicals, San Diego, CA, USA), or 10 nM bafilomycin and 200 nM rapamycin (Sigma), for the times indicated in the Figure Legends.

Human chorionic gonadotropin analysis

Supernatants from four primary human trophoblast cultures were assayed for Human chorionic gonadotropin (HCG) by enzyme-linked immunosorbent assay (DRG International, Mountainside, NJ, USA) according to the manufacturer's instructions. HCG levels per 24-h interval were expressed as the mean $\pm$ S.E.M.

Western blotting

Primary human trophoblasts were lysed in radioimmunoprecipitation buffer (1% Nonie P-40, 0.5% deoxycholate, and 0.1% SDS in PBS) in the presence of protease and phosphatase inhibitors (Sigma). Proteins were separated by SDS-PAGE (25 µg of protein per lane) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% (w/v) nonfat dry milk in PBST (0.05% Tween 20 in PBS) and incubated overnight at 4 °C with primary antibody in PBST plus 5% (w/v) nonfat dry milk. Primary antibodies used were: rabbit monoclonal anti-phospho-S6 ribosomal protein (Cell Signaling Technologies (CST) #2215, 1:1000, 0.035 µg/ml), rabbit monoclonal

anti-cleaved poly (ADP-ribose) polymerase (CST, #9541, 1:1000, 0.009 µg/ml), mouse monoclonal anti-cleaved cytokeratin 18 (Roche), rabbit monoclonal anti-Atg16L1 (Sigma, SAB2103567, 2 µg/ml), mouse monoclonal anti-p62 (Santa Cruz Biotechnology), goat polyclonal anti-actin (Santa Cruz Biotechnology, SC1616; 0.2 µg/ml), and rabbit polyclonal anti-LC3-II (Novus Biologicals, NB600-1384, 0.5 µg/ml). After incubation with primary antibodies, membranes were washed in PBST and then probed with HRP conjugated secondary antibody (Santa Cruz Biotechnology, 1:5000) for 4 h and then washed in PBST. Signals were detected by enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA), and relative protein levels were determined by using Image J software to measure densitometry of bands on film, with normalization to actin levels.

Immunofluorescence and lysosome staining

After culture and treatment for times as indicated in the Figure Legends, cells were fixed with methanol for 20 min at -20 °C, blocked with 5% BSA in PBS for 1 h at room temperature, and incubated at 4 °C overnight with antibodies to p62 (Santa Cruz Biotechnology, 1:200, 1 µg/ml), E-cadherin (Abcam, Cambridge, MA, USA, 40772, 1:100, 2 µg/ml) or LC3-II (Novus Biologicals, 1:200, 2.5 µg/ml), singly or in the combinations noted in the Figure Legends. After washing with PBS three times, cells were incubated with appropriate anti-mouse or anti-rabbit Alexa-labeled secondary antibodies (Life Technologies, 1:200) and nuclei were counter-stained with Draq 5 (Biostatus Limited, Shepsted, UK, 1:1000) for 1 h at room temperature. The cells were then washed three times and mounted. For staining of lysosomes, trophoblasts were treated with 50 nM LysoTracker Red (Life Technologies, Inc.) for 30 min at 37 °C, and nuclei were counter-stained with Draq 5. To quantify lysosomes, ten random confocal images were obtained from each of four primary cultures, using a Nikon E800 epifluorescence microscope and a total magnification of 600 $\times$, capturing a 512 pixel $\times$ 512 pixel image covering 212 µm $\times$ 212 µm. For each image, lysosomes were counted, defining each lysosome as a LysoTracker-Red positive region encompassing >30 square pixels in area. The lysosomes for each image were then divided by the number of nuclei in that image, providing a lysosome/nucleus measure. Then the ten lysosome/nuclei values were averaged as the final number for each placenta. Finally, the resultant four average numbers from four different placentas were used in the Student's *t* test comparison.

TUNEL

TUNEL staining was performed used the *In Situ* Cell Death Detection Kit (Roche). Briefly, cultured primary trophoblasts were rinsed once in PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100 for 2 min on ice. The cells were incubated in TUNEL reaction mixture containing enzyme solution and label solution for one hour at 37 °C, rinsed with PBS, and nuclei were counter-stained with Draq 5. Images were acquired as described above.

siRNA-mediated gene silencing in primary trophoblasts

ATG16L1 Silencer Select siRNA (no. s30071: UAUCAUG-CAACUGGAUCCTg) and scrambled negative control #1 siRNA were purchased from Ambion (Norwalk, CT, USA). Trophoblasts were plated for 4 h, and transfected for 48 h using DharmaFECT 1 (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions, with punicalagin or vehicle control present in the last 24 h.

Statistical analysis

Each experiment was repeated with the number of primary cell cultures listed in the Figure Legends, each from different placentas. Student's *t* test was performed when comparing between two conditions, and ANOVA with Bonferroni correction was applied when comparing three or more conditions. $P < 0.05$ was deemed significant. Data are mean $\pm$ S.E.M. KaleidaGraph (Synergy Software, Reading, PA, USA) software was used for statistical analyses.

Results

Primary cytotrophoblast cultures differentiate into syncytiotrophoblasts

As diagrammed in Fig. 1A, cultured primary cytotrophoblasts spontaneously undergo fusion, forming multinucleated syncytiotrophoblasts. This differentiation process is also characterized by altered hormone secretion, and we found β -HCG level in the culture medium increased by an average of tenfold as cultured cells transitioned from cytotrophoblasts to syncytia (Fig. 1B). This hormonal differentiation was accompanied by morphological differentiation, with E-cadherin membrane staining of cultures beyond 52 h outlined multiple nuclei in the same cytoplasmic mass, indicating formation of multinucleated syncytiotrophoblasts (Fig. 1C and D). Taken together, these data confirmed our previous findings that the majority of cells in culture for less than 28 h maintained the cytotrophoblast phenotype, while $>85\%$ of the nuclei of cells cultured over 52 h underwent fusion, forming multinucleated syncytiotrophoblasts (Chen *et al.* 2010).

Starvation, rapamycin and punicalagin induce autophagy in cultured syncytiotrophoblasts

The mTOR kinase is expressed in human syncytiotrophoblasts and serves as a nutrient sensor (Roos *et al.* 2007). Inactivation of mTOR kinase activity by rapamycin (Klionsky *et al.* 2012) or by starvation (Desideri *et al.* 2012, Li *et al.* 2013) reduces the level of a downstream target of mTOR, phosphorylated ribosomal protein S6 (P-S6), indicative of mTOR inhibition, which can induce autophagy in multiple cell types (Klionsky *et al.* 2012). We sought to determine if autophagy occurs in syncytiotrophoblasts, and we used both starvation

Figure 1 Differentiation of primary human trophoblasts in culture. Primary human cytotrophoblasts were plated at time 0. Unattached cells and fragments were removed by rinsing at 4 h, and cultures were maintained up to 76 h, during which time cells fused to form syncytiotrophoblasts, as diagrammed in (A). (B) The accumulated content for β -HCG in medium during the immediate 24 h prior to the times of harvest noted on the graph. $*P < 0.05$, ANOVA with Bonferroni correction; $n = 4$ primary cultures. (C and D) Representative images of mononucleated cytotrophoblasts in culture at 28 h and syncytiotrophoblasts in cultures at 52 h. Green staining reflects E-cadherin on the plasma membrane. The arrows show cytotrophoblasts (Cyt; red) and syncytiotrophoblast (Syn; yellow).

(Fig. 2A, B, C and D) and rapamycin (Fig. 2E, F, G and H) to study this phenomenon. As expected, syncytiotrophoblasts showed a time-dependent decrease in the levels of P-S6 under starvation conditions (Fig. 2A and B) or in the presence of rapamycin in unstarved cells (Fig. 2E and F). LC3-II is an autophagosomal-membrane-associated protein that is commonly reduced with enhanced autophagy, as it is degraded in the autophagolysosome. Expression of LC3-II was lower in starvation conditions, but not noticeably with rapamycin alone, compared to control (Fig. 2C and G). Moreover, the expression of a third marker of autophagy, the autophagosome adaptor protein p62, is typically reduced upon induction of autophagy as its degradation in autophagolysosomes, was significantly reduced in syncytiotrophoblasts exposed to starvation (Fig. 2D), but not exposed to rapamycin (Fig. 2H). Autophagic flux can be assessed by treating cells with bafilomycin, which blocks fusion of autophagosomes and lysosomes and thereby, avoids degradation of proteins in the autophagolysosome. In the presence of bafilomycin, increased autophagic flux is expected to result in increased levels of both LC3-II and p62. Indeed, in the presence of bafilomycin both starvation and rapamycin dramatically increased expression of these two proteins (Fig. 2C, D, E, G, H).

Figure 2 Starvation and rapamycin induce autophagy in primary human syncytiotrophoblasts. Cells were subjected to starvation (A, B, C and D) or cultured in nutrient replete conditions (E, F, G and H) and exposed to DMSO, rapamycin (Rap), or Rap and bafilomycin (Baf), for the times indicated. All cells were cultured for a total of 76 h and exposed to the condition tested for the times indicated. (A) Western blot of P-S6, p62, LC3-II, and actin from trophoblasts exposed to starvation conditions. Cells in the last lane were treated for 4 h with bafilomycin before collection. (B, C and D) Densitometric analyses of western blots of the indicated proteins, normalized to actin. (E) Western blot of P-S6, LC3-II, and actin exposed to vehicle control, Rap or Rap and Baf. (F and G) Densitometric analyses of western blots of the indicated proteins, normalized to actin. For all densitometric analyses, * $P < 0.05$; ** $P < 0.01$; ANOVA with Bonferroni correction. $n = 4$ cultures from different placentas. (H) Immunofluorescence for p62 and E-cadherin in trophoblasts exposed for the final 4 h of culture to DMSO, Rap, or Rap and Baf. Red: E-cadherin; green: p62; blue: DNA; arrows: p62 puncta.

As expected because bafilomycin does not prevent inactivation of mTOR, bafilomycin did not prevent the rapamycin-mediated reduction of P-S6 levels (Fig. 2B and F). Collectively, these results are consistent with autophagy occurring in syncytiotrophoblasts.

Having demonstrated autophagy occurs in primary human syncytiotrophoblasts, we next asked whether punicalagin could induce autophagy in these cells. Similar to our observations in starved and rapamycin-treated cells, we found that punicalagin treatment resulted in reduced P-S6, LC3-II, and p62 levels

(Fig. 3A, B, C and D). Treatment with punicalagin in the presence of bafilomycin yielded increased levels of LC3-II and p62 suggesting increased autophagic flux. As expected, bafilomycin had no effect on the reduced P-S6 levels observed in response to punicalagin (Fig. 3E, F, G and H). We next treated cultures with punicalagin or vehicle control in the presence of chloroquine, another agent that inhibits fusion of autophagosomes with lysosomes, and thus, degradation in the autophago-lysosome. Syncytiotrophoblasts treated with chloroquine and punicalagin had substantially more LC3-II puncta (Fig. 3I) than those treated with chloroquine and vehicle (Fig. 3J). Finally, we determined whether or not punicalagin increased the number of lysosomes in trophoblasts, as would be expected if punicalagin resulted in increased autophagic flux. We stained syncytiotrophoblasts cultured in the presence or absence of punicalagin with LysoTracker Red and found that the number of lysosomes per nucleus was indeed higher in syncytiotrophoblasts exposed to punicalagin, compared

Figure 3 Punicalagin induces autophagy in syncytiotrophoblasts. Representative western blots (A and E) and densitometry (B, C and D; F, G and H) of P-S6, LC3-II, p62, and actin from primary human trophoblasts cultured for 76 h, in the presence or absence of punicalagin, DMSO or bafilomycin during the 24 h prior to harvest. * $P < 0.05$; one-tail t test. $n = 4$ primary cultures from different placentas. (I and J) Representative immunofluorescence image of LC3-II in syncytiotrophoblasts treated with chloroquine in the presence or absence of punicalagin (Pun).

Figure 4 Punicalagin increases lysosomes in syncytiotrophoblasts. Representative images of syncytiotrophoblasts treated with vehicle control (A) or punicalagin (B) for the last 24 h with a 30-min (prior to harvest) exposure to 50 nM LysoTracker Red. Nuclei are stained blue with Draq 5. (C) Enlarged image of selected area in A. (D) Quantification of lysosomes. * $P < 0.05$; t test. $n = 4$ primary cultures from different placentas.

to vehicle control (Fig. 4). Together, these findings strongly support the conclusion that punicalagin induces autophagy in syncytiotrophoblasts.

Punicalagin-induced autophagy protects syncytiotrophoblasts from apoptosis

Depending on the cell type and culture conditions, autophagy can either protect or promote cell death (Loos *et al.* 2013). Our previous work showed that punicalagin decreases hypoxia-induced apoptosis in cultured syncytiotrophoblasts (Chen *et al.* 2013). We hypothesized that punicalagin regulates the interplay between autophagy and apoptosis in cultured syncytiotrophoblasts, favoring survival. Using TUNEL staining, we found that punicalagin-treated syncytiotrophoblasts exhibited lower levels of apoptosis than vehicle-control treated cells (Fig. 5A), confirming our previous findings (Chen *et al.* 2013). We then examined the expression of two proteins that serve as markers of caspase-mediated apoptosis, the caspase-cleaved forms of nuclear poly-ADP-ribose polymerase (cl-Parp) and of cytoplasmic cytokeratin 18 (cl-Cyt18). We found that the levels of both cl-Parp and cl-Cyt18 decreased in punicalagin-treated cells (Fig. 5B, C and D), indicating reduced apoptosis. To assess the role of autophagy in this process, we treated

syncytiotrophoblasts with either punicalagin or vehicle control in the presence of bafilomycin. The apoptosis-reducing effect of punicalagin, as measured by expression of cl-Parp and cl-Cyt18, were reversed by inhibition of autophagy using bafilomycin (Fig. 5B, C and D). These data suggest that autophagy is required for punicalagin-mediated protection from apoptosis in syncytiotrophoblasts.

To further test the hypothesis that punicalagin can regulate the interplay between autophagy and apoptosis in cultured syncytiotrophoblasts, we inhibited autophagy using siRNA to knockdown *ATG16L1*, which participates in the elongation step of autophagy and does not directly contribute to regulation of apoptosis (Klionsky *et al.* 2008). Compared to vehicle control, punicalagin modestly enhanced the expression of Atg16L1 (by 22%) and reduced the expression of cl-Parp (by 65%) in syncytiotrophoblasts transfected with scrambled siRNA control (Fig. 6A, B and C). Punicalagin-treated syncytiotrophoblasts transfected with specific *siATG16L1* siRNA showed about 50% reduction in Atg16L1 levels and about 60% more cl-Parp, compared to syncytiotrophoblasts transfected with scrambled siRNA control (Fig. 6A, B and C). These data indicate that punicalagin-mediated protection of syncytiotrophoblasts involves increased autophagy.

Figure 5 Punicalagin decreases apoptosis in syncytiotrophoblasts. (A) Representative TUNEL images of syncytiotrophoblasts treated with or without punicalagin (Pun). (B) Western blots of cl-Parp and cl-Cyt18 in syncytiotrophoblasts in the presence or absence of punicalagin or bafilomycin (Baf). (C and D) Densitometric analyses of cl-Parp and cl-Cyt18. * $P < 0.05$; ANOVA with Bonferroni correction; $n = 4$ primary cultures from different placentas.

Figure 6 Knockdown of ATG16L1 reduces the effect of punicalagin to decrease apoptosis in syncytiotrophoblasts. (A) Representative western blots of Atg16L1, actin, and cl-Parp in primary syncytiotrophoblasts transfected with ATG16L1-specific siRNA or scrambled control siRNA in the presence or absence of punicalagin. (B and C) Densitometric analyses of Atg16L1 (B) and cl-Parp (C), normalized to actin. * $P < 0.05$; ANOVA with Bonferroni correction; $n = 4$ primary cultures from different placentas.

Discussion

The data show that syncytiotrophoblasts undergo autophagy in response to starvation, rapamycin, and punicalagin, the latter being a prominent polyphenol in pomegranate juice. We present multiple lines of evidence that indicate punicalagin-induced autophagy protects syncytiotrophoblasts from apoptosis. First, in the presence of bafilomycin, punicalagin decreases the level of the mTOR downstream target, P-S6, and increases the levels of the autophagy markers, p62 and LC3-II. Secondly, treatment with punicalagin and chloroquine result in accumulation of LC3-II. Thirdly, punicalagin increases the number of lysosomes in syncytiotrophoblasts. Fourthly, punicalagin decreases apoptosis, as indicated by decreased TUNEL staining and decreased expression of cl-Parp and cl-Cyt18. Finally, inhibition of autophagy with bafilomycin or by knockdown of ATG16L1, diminishes the ability of punicalagin to reduce the level of apoptosis in syncytiotrophoblasts. Together, these results support the hypothesis that punicalagin modulates crosstalk between apoptosis and autophagy thereby promoting cell survival in cultured primary syncytiotrophoblasts.

No clinical therapeutic options currently exist to limit injury to human placental trophoblast exposed to stressors *in vivo*. The protective effects of punicalagin on human cultures of syncytiotrophoblasts suggests that this polyphenol may serve as such a therapeutic agent, as no harmful effects of pomegranate juice or its polyphenols have been identified (Cerdeira *et al.* 2003a, Ly *et al.* 2015). Antioxidant properties of phytochemicals in fruits and vegetables generally, and pomegranate juice in particular, are commonly attributed to provide protective effects from injury by reducing the levels of reactive oxygen species and of nitrative species generated within in cells exposed to exogenous stimuli. Such an assumption has recently been challenged (Poljsak & Milisav 2012, Forman *et al.* 2014, Rahal *et al.* 2014), and the beneficial effects of punicalagin actually are likely to result from a plethora of pathways that may or may not

involve antioxidant properties (Adams *et al.* 2006). For example, we reported punicalagin modulates the mRNA levels of p53, p21, and MDM2, while inhibiting p53 activity and decreasing apoptosis in trophoblasts (Chen *et al.* 2013). We also have found that pomegranate juice selectively modulates gene expression in human trophoblasts (Chen *et al.* 2015). Resveratrol is a polyphenol that was recently shown to improve fetal growth in a mouse model of preeclampsia, and this beneficial effect derives, in part, from nitric oxide effects that improve uterine artery blood flow to the fetal-placental unit (Gurusamy *et al.* 2010, Poudel *et al.* 2013). Similar to our observation that punicalagin can induce autophagy and protect syncytiotrophoblasts from apoptosis, resveratrol has been shown to induce autophagy and improve cell survival in cardiac myocytes (Gurusamy *et al.* 2010).

The kinase mTOR is a nutrient sensor that monitors glucose concentrations and growth factors and regulates the activity of placental amino acid transporters in trophoblasts (Roos *et al.* 2007). Moreover, mTOR regulates autophagy to eliminate damaged proteins and organelles and to generate substrates for critical cellular functions when nutrients are limited (Loos *et al.* 2013). Multiple autophagy-related proteins, including Apg9L2, LC3-II, and Beclin 1 (Oh *et al.* 2008, Bildirici *et al.* 2012), are expressed in human placental tissues. Autophagy could likely be valuable to syncytiotrophoblasts, especially during complicated pregnancies, as turnover of cytoplasmic components within the syncytium could occur without breach of syncytial barrier function. Moreover, the substrates generated within the syncytiotrophoblast could potentially supply the fetus with nutrients under conditions of limited supplies. Studies of trophoblast in animal models and villi from pregnancy disorders will help elucidate what role, if any, autophagy contributes to syncytiotrophoblast integrity, function, and survival *in vivo*.

One concern of *in vitro* experiments is whether or not the studies use a physiologically relevant concentration of the agent investigated. In most situations, this is difficult to precisely determine, as *in vivo* metabolic studies are not available for most compounds. Although only a few such studies exist for pomegranate juice (Cerdeira *et al.* 2003a,b, Mertens-Talcott *et al.* 2006, Seeram *et al.* 2006, 2008, Nielsen *et al.* 2008), what is clear is that ingestion of 235 ml/day (about 8 ounces) of pomegranate juice results in detectable metabolites in the circulation (Mertens-Talcott *et al.* 2006, Seeram *et al.* 2006, 2008, Nunez-Sanchez *et al.* 2014). We showed previously that antenatal ingestion of 235 ml/day of pomegranate juice is sufficient to reduce *in vivo* oxidative stress in the placentas of women who underwent labor (Chen *et al.* 2012). In non-pregnant patients on hemodialysis, 100 ml/day of pomegranate juice reduced oxidative stress levels (Shema-Didi *et al.* 2012). Punicalagin is the predominant polyphenol in pomegranate juice (Basu & Penugonda 2009) and,

in juice from POM Wonderful, LLC, Los Angeles, CA, punicalagin is present at $\sim 310 \mu\text{M}$, greater than the $34 \mu\text{M}$ concentration we used in this study. We previously found that $34 \mu\text{M}$ punicalagin is in the middle of a dose-response curve, which we found to protect human cytotrophoblasts from exogenous stressors in culture (Chen *et al.* 2013). Finally, $34 \mu\text{M}$ is similar to the punicalagin concentration used by others in *in vitro* and *in vivo* studies (Yaidikar *et al.* 2014, Peng *et al.* 2015, Xu *et al.* 2015).

Clearly, the metabolism of punicalagin and its interactions with other phytochemicals in fruits and vegetables, like pomegranate juice, contribute to the collective effects of the diet consumed. Future studies will be required to further dissect such interactions and to determine if any agent might serve as a therapeutic option for placental protection.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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