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Molecules and Cells

Status of mTOR Activity May Phenotypically Differentiate Senescence and Quiescence

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SA β-Gal activity is a key marker of cellular senescence. The origin of this activity is the lysosomal β-galactosidase, whose activity has increased high enough to be detected at suboptimal pH. SA β-Gal is also expressed in the cells in quiescence driven by serum-starvation or a high confluency, and it has been hypothesized that SA β-Gal positivity is rather a surrogate marker of high lysosome content or activity. In this study, it was determined how SA β-Gal activity is expressed in quiescence and how lysosome content and activities are differently maintained in senescence and quiescence using DNA damage-induced senescence and serum starvation-induced quiescence as study models. Lysosome content increased to facilitate SA β-Gal expression in both the conditions but with a big difference in the levels of the change. Lipofuscins whose accumulation leads to an increase in residual bodies also increased but with a smaller difference between the two conditions. Meanwhile, lysosome biogenesis was actively ongoing only in senescence progression, indicating that the difference in the lysosome contents may largely be due to lysosome biogenesis. Further, the cells undergoing senescence progression but not the ones in quiescence maintained high mTOR and low autophagy activities. Overall, the results indicate that, although SA β-Gal is expressed due to the elevated lysosome content in both cellular senescence and quiescence, senescence differs from quiescence with high lysosome biogenesis and low autophagy activity, and mTOR activity might be involved in these differences.

INTRODUCTION

Senescent cells not only are irreversibly arrested in cell division, but also experience a notable change in the cell biological properties such as an increase of cell volume as well as in the levels of lysosome, mitochondria, and reactive oxygen species (ROS) (Hwang et al., 2009). Senescent cell also expresses senescence associated β-galactosidase (SA β-Gal) (Dimri et al., 1995). This activity has been detected not only in cells undergoing senescence *in vitro* but also in the tissues from aged animals and human beings (Dimri et al., 1995; Kishi, 2004; Melk et al., 2003; Mishima et al., 1999; Pendergrass et al., 1999), and therefore, has been extensively used as a marker of cellular senescence and aging. However, the molecular mechanism underlying its expression is not fully understood. The origin of SA β-Gal is the activity of lysosomal β-galactosidase, which has increased high enough to be detected at suboptimal pH, 6.0 (Kurz et al., 2000; Lee et al., 2006).

Increased SA β-Gal activity has also been found in other cellular conditions, and its specificity as a marker for senescence has been challenged. It appears in cells after an extended incubation at low serum level (Yegorove et al., 1998) or high confluency (Severino et al., 2000; Yang and Hu, 2005), or after a prolonged cultivation (Krishna et al., 1999). However, whether the same mechanism is underlying the SA β-Gal staining detected in senescent cells and in cells under these non-senescent conditions has not been cleared. Identification of the underlying mechanism of the expression of SA β-Gal activity is important in that, in addition to the required validation for the reliability of the assay, it provides better understanding of the regulation mechanism underlying the cell biological changes in senescence and aging. Further, it would provide information on the mechanisms that regulate the content and activities of lysosomes.

High lysosome content has been reported in senescent cells and cells undergoing senescence progression (Comings and Okada, 1970; Hwang et al., 2009). This increase in the lysosome content may either be an active cellular response to an accumulation of damage products or simply an outcome of the arrest in cell division. Residual bodies, the lysosomes that contain indigestible materials such as lipofuscins, accumulate in aged cells and they would contribute to the increase in the lysosome content (Kurz et al., 2008a; Terman et al., 2003). In fact, an increase of non-functional residual bodies along with a decrease of primary lysosomes is believed to cause a waste of newly produced lysosomal enzymes, which would lead to reduced turnover of damaged organelles, and thereby, augment ROS production and initiate a vicious pro-aging or pro-senescence cycle (Kurz et al., 2008b; Terman et al., 2007). Meanwhile, lipofuscin-loaded lysosomes increase, also, in non-senescent cells under a temporal arrest in cell division (Collins and Brunk, 1976). Along with the presence of SA β-Gal activity in the non-senescent conditions, this suggests that cellular lysosome content may change in the cells where cell cycle has arrested. This also leads to the hypothesis that SA β-Gal activ-

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ity is a surrogate marker for the elevated lysosome content or activity (Lee et al., 2006). Lysosome content and activity would also be regulated by lysosome biogenesis. In cells undergoing senescence, the levels of mRNA of a number of lysosomal proteins is elevated (Johung et al., 2007; Park et al., 2007), suggesting increased lysosome biogenesis during senescence progression. However, the change in the status of lysosome during the progression of senescence or in other cellular conditions has rarely been examined closely. Nor, how cellular status of lysosomes is regulated is not understood well.

Recent studies indicate that the activity of mammalian target of rapamycin (mTOR), a factor that relays growth factor signal and induces cell growth by facilitating the initiation of protein synthesis (Mamane et al., 2006), is critical in determining cellular lysosome status by modulating their biogenesis and turnover. Active mTOR is required for the activity of TFEB (Pena-Llopis et al., 2011), which binds to and activates the promoter of a school of the genes for lysosomal proteins, and therefore, plays a master regulator role in lysosome biogenesis (Sardiello et al., 2009). TFEB may also regulate lysosome clearance by facilitating lysosome exocytosis (Medina et al., 2011). In addition, mTOR appears to play a role in recycling of lysosomes after autophagolysis (or rescue from autophagolysosomes) (Yu et al., 2010), while it attenuates autophagy by blocking autophagosome formation (Abeliovich et al., 2000; Kim et al., 2001). Therefore, for lysosme content as well as activities, mTOR plays a pluripotent role. However, how mTOR activity and its signaling to various activities associated with lysosomes are regulated in cellular senescence and aging has only recently been studied.

In this study, we compared the status of lysosome content and biogenesis as well as the activities of autophagy and mTOR in the cells undergoing quiescence and senescence. The results of our study indicate that, in both the conditions, lysosome content increases to facilitate SA β-Gal expression, but the two conditions are largely different in terms of lysosome biogenesis, autophagy, and mTOR activity. The results also indicate that, for lysosome activity, cellular senescence is not simply a state of cell cycle arrest.

MATERIALS AND METHODS

Cell culture

H460 (ATCC; HTB-177) cells were cultured in medium RPMI-1640 (Lonza, USA) in the presence or absence of 10% fetal bovine serum (FBS; Lonza). To induce senescence, cells were pulsed with 0.25 μM adriamycin (doxorubicin hydrochloride; Sigma-Aldrich Co., USA) for 4 h and chased for five days in fresh medium which was replaced every other day.

In situ staining of SA β-Gal activity and β-galactosidase assay in solution

For the SA β-Gal assay *in situ*, the protocol reported by Dimri et al. (1995) was used. To determine the β-galactosidase activity per cell, cells were lysed in PBS by freeze-thawing. The supernatant was incubated at 37°C for 8 h in reaction buffer [5 mM $MgCl_2$, 0.55 mg/ml chlorophenolred-β-D-galactopyranoside (CPRG) in 0.1 M phosphate buffer (pH 6.0)]. The reaction was stopped by adding 1 M Na_2CO_3, and the transmittance of light at 570 nm was measured.

Flow cytometric determination of autofluorescence, lysosome content, and cathepsin D activity

For measurement of autofluorescence, cells that were collected in PBS containing 1 mM EDTA were applied to flow cytometry with 488 nm excitation and 530 nm emission (BD FACSCanto II, BD Biosciences, USA). For the measurement of lysosome content and cathepsin D activity, the washed cells were incubated with 50 nM of LysoTracker Red DND-99 (Invitrogen/Molecular Probes, USA) or 10 ug/ml DQ™ Red BSA (Life Technologies, USA) for 30 min and then applied to flow cytometry (with 488 nm excitation and 530 nm emission).

Western blotting

Cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with NaF, $NaVO_4$, and a protease-inhibitor mixture (Sigma-Aldrich). Typically 30 μg of proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond ECL; Amersham, USA), and blotted with one of the following primary antibodies: human Lamp1, Lamp2, LIMP2, Cathepsin D, p62 (Santa Cruz Biotechnology, USA), Rab7, LC3B, p-mTOR, mTOR, 4E-BP1, phospho-4E-BP1, p70 S6Kinase, phospho-p70 S6Kinase (Thr421/Ser424) (Cell Signaling Technology, USA), TFEB (Abcam, UK), and β-actin (Sigma-Aldrich). Membranes were further blotted with secondary antibodies, HRP-conjugated anti-rabbit IgG, anti-Mouse IgG antibody (Jackson Immuno Research, USA), or donkey anti-goat IgG-HRP (Santa Cruz), and incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA). Protein bands were visualized by LAS-3000 (Fujifilm, Japan).

mRNA quantification

Total RNA was isolated using RNeasy® Mini kit (Qiagen, USA) following the manufacturer's protocol. A total of 5 μg of RNA was converted to cDNA by using MuLV reverse transcriptase (BEAMS bio, Korea) and oligo(dT) primer (Bionics, Korea), and 1/40 volume of the cDNA reaction was applied to PCR in MyiQ™ (Bio-Rad, USA) using following primers for lysosomal protein mRNA. PCR results were quantitatively analyzed by iQ5 software: β-actin (ACC CCG TGC TGC TGA CC); ASAH1 (ACT TTA TCC TGG GAG GCA ACC AGT); CTSB (GGC CCC CTG CAT CTA TCG); GLB1 (TCC TTG AGC GAA ACA ATG TGA TC); Lamp1 (ACA CCA AGA GTG GCC CTA AGA ACA); Lamp2 (GCC GTT CTC ACA CTG CTC TA); PSAP (TGT ACG GAC CAA CTC CAC CTT TGT); SIAE (CCG TTG GCA TCA AAC AGC AGA CTT).

Confocal microscopy

Cells seeded on a microscope cover slip were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and blocked with 10% FBS in PBS for 1 h. The cover slip was incubated with primary antibody overnight at 4°C and further incubated with anti-rabbit IgG conjugated with either Alexaflour 488 or Alexafluor 633. Cathepsin B activity in cells was visually assessed *in situ* by using Magic Red™ Cathepsin Detection Kit (Metachem Diagnostics, UK) following the protocol provided by the manufacturer. Samples were observed in LSM510 confocal microscope (Carl Zeiss MicroImaging, Germany).

Softwares for analysis

Images obtained in confocal microscopy were analyzed by LSM Image Browser software (Carl Zeiss MicroImaging). Quantitative analysis of the images were done with ImageJ (an open software developed NIH, USA). Densitometric quantification of protein bands in Western blots was done by using MultiGauge (FujiFilm, Japan).

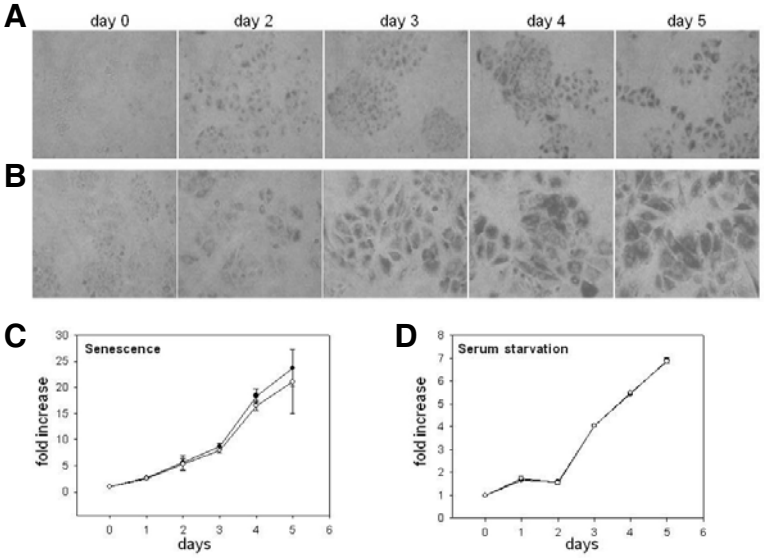

Fig. 1. Changes in SA β-Gal activity in the cells undergoing senescence progression and serum starvation. H460 cells pulsed with adriamycin to induce senescence (A, C) or cultured without FBS (B, D) were incubated for the indicated time points and either stained for SA β-Gal activity *in situ* (A, B) or collected and lysed to measure β-galactosidease activity. An equal number of cells were lysed and assayed either at pH 4.5 (●) or pH 6.0 (○). The activities measured at the two pH conditions changed almost indetically. A mean of three biological repeats were plotted.

RESULTS

Increase of β-galactosidase activity in cells undergoing serum starvation

To study the cellular changes that occur in senescence progression, DNA damage-induced cellular senescence in either normal fibroblasts or cancer cells has frequently been used (Hwang, 2002; Hwang et al., 2009). In this study, H460 cells were pulsed with 0.25 μM adriamycin for 4 h and further incubated in its absence for five days. During the chase period, senescence phenotypes including an increase in SA β-Gal activity, cell size and the contents of mitochondria and ROS were progressively expressed, as has been previously reported (Cho et al., 2011; Song and Hwang, 2005). For a serum starvation-induced quiescence model, H460 cells were cultured for five days in RPMI-1640 medium without serum supplementation.

During the five-day period, SA β-Gal activity increased in both the conditions (Figs. 1A and 1B). This confirms the previous notion that SA β-Gal expression is not limited to cellular senescence as noted by Cristofalo (2005). However, the increase in the intensity of SA β-Gal staining was much smaller in serum starvation as compared to that in senescence progression. In the later, the staining intensity increased dramatically after day three along with a substantial increase in cell size. An estimation of the relative degree of the change in the activity was made through an assay that measured the β-galacto-sidase activity in solution using a chromogenic substrate (Cho et al., 2011; Lee et al., 2006). The activity increased by 24 fold during senescence progression while seven-fold increase was observed in serum starvation (Figs. 1C and 1D).

Increase of lysosome content in senescence progression and serum starvation

Activities of certain lysosomal enzymes and lysosome content have been shown to increase in cells at senescence (Cho et al., 2011; Cristofalo and Kabakjian, 1975; Park et al., 2007). Whether lysosome content also increases during serum starvation was determined. Flow cytometry of the cells stained with LysoTracker Red (LTR) showed near six-fold increase in LTR fluorescence during the five days of serum starvation indicating that lysosome content indeed increases in serum starvation (Fig. 2A). However, this change is relatively small as compared to that occurred during senescence progression where the lysosome content increased by over 13 fold (Fig. 2A). The increase in the lysosome content was confirmed by the gradual increase in the levels of lysosomal proteins, Lamp1, Lamp2, LIMP2, Rab7, and cathepsin D, which changed with kinetics similar to the increase found during the senescence progression (Figs. 2B and 2C).

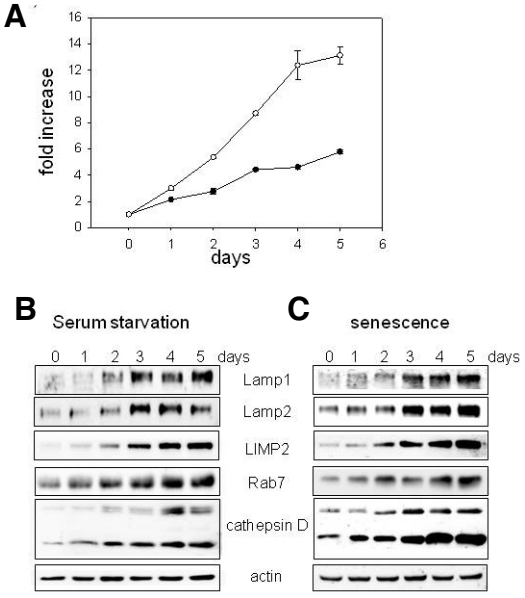

Fig. 2. Changes in lysosome content and the levels of lysosomal proteins. (A) Cells in both conditions were stained with LTR for 30 min and then applied to flow cytometry. Mean values from three biological repeats were normalized against that of the 0 day control and plotted. (-○- senescence; -●- serum starvation). (B, C) Cells undergoing senescence progression (B) or serum starvation (C) were collected at indicated time points, lysed, and applied to Western blotting assay for Lamp1, Lamp2, LIMP2, Rab7, and cathepsin D, and β-acin.

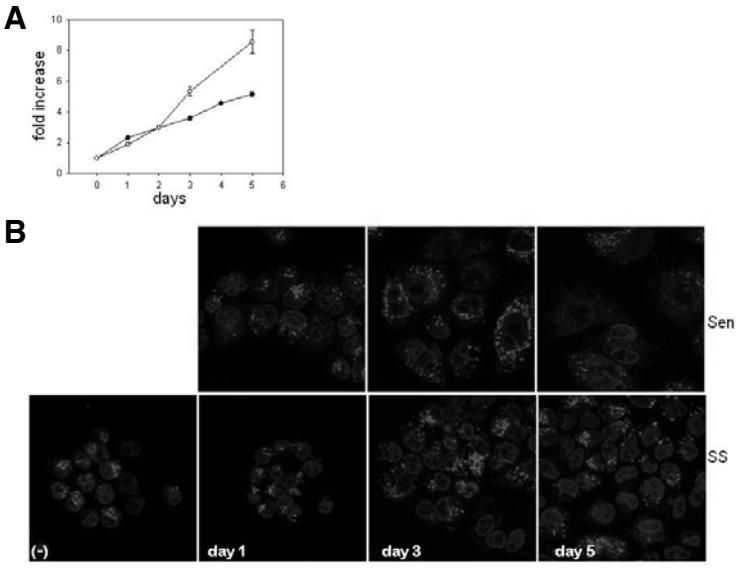

Fig. 3. Changes in lysosomal enzyme activities. (A) After staining with DQ™ Red BSA for 30 min at 37°C, cells were applied to flow cytometry. Mean values from three biological repeats were normalized against that of the 0 day control and plotted. (-○- senescence; -●- serum starvation). (B) To detect the intracellular cathepsin B activity *in situ*, cells were stained with Magic Red™ Cathepsin B (MR-(RR)2) for 1 h at 37°C and observed by confocal microscopy. The brightness of red spots shows the degree of cathepsin B activity within in lysosomes. [(-), day 0 control] [(-), untreated control; top panels, senescence; bottom panels, serum starvation].

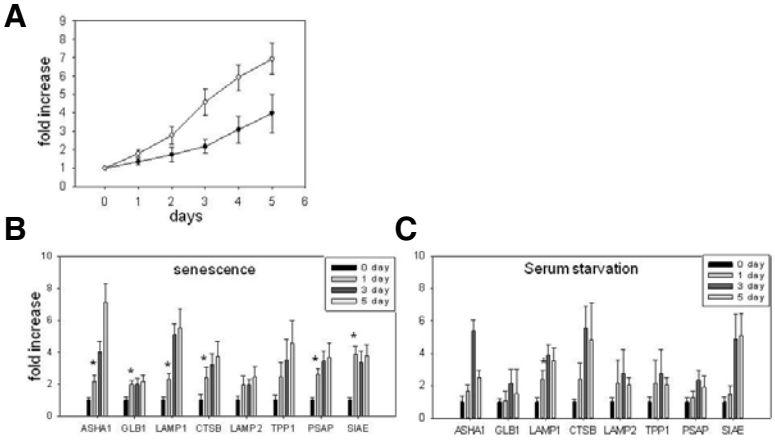

Fig. 4. Changes in autofluorescence and mRNA levels of lysosomal proteins. (A) Cells undergoing senescence progression (-○-) or serum-starved (-●-) were applied to flow cytometry to determine autofluorescene. Mean values from three biological repeats were normalized against that of the 0 day control and plotted. (B, C) mRNA isolated from cells cultured for indicated time points of senescence progression (B) or serum starvation (C) were converted to cDNA and applied for quantitative real-time PCR analysis for the indicated genes of lysosomal proteins (n = 3, *p < 0.05). ASAH1 N-acylsphingosine amidohydrolase; GLB1, β-galactosidase 1; CTSB, cathepsin B; TPP1, tripeptidyl peptidase I; PSAP, prosaponin; SIAE sialic acid acetylesterase.

The gradual increase of the lysosome content was accompanied with a similar change in the activities of the lysosomal enzymes in both conditions. The proteolytic activity of cathepsin D measured *in vivo* by using DQ-BSA, a substrate that emits fluorescence upon degradation, gradually increased during the senescence progression and serum starvation (Fig. 3A). And, the number of punctae that are positive for the activity of cathepsin B, as assessed *in situ* by using a substrate that emits red fluorescence upon degradation, showed an increasing pattern at least during the first three days in both conditions (Fig. 3B). However, cathepsin D activity *in vivo* increased to much higher level in senescence progression and so did the number of the cathepsin B-active punctae (Fig. 3, Sen vs SS). Therefore, during senescence progression, lysosomal activity is upregulated well over the level achieved in quiescence. Interestingly, the red fluorescence representing the cathepsin D activity was not as distinct in many cells at day five of senescence progression (Fig. 3, Sen, day 5). It was present rather diffusively all over the cytosol in a significant number of cells. The diffusive pattern persisted even in the cells where distinctive puntae were present. This cytosolic and diffusive red fluorescence may suggest either cytosolic localization of cathepsin D or a severe lysosomal disruption through which the lysosomal enzymes or their products might have leaked out as previously proposed (Cho et al., 2011). The cells with diffusive fluorescence were also found in the serum-starved population but at much lower frequency.

Increased lysosome biogenesis in senescence progression but not in serum starvation

Lysosome content can increase through an accumulation of residual bodies and an upregulation of lysosome biogenesis. Residual bodies are derived from the lysosomes that contain indigestible lipofuscins (Terman and Brunk, 2004). Therefore, an increase in lipofuscins would lead to an increase in the lysosome content. In fact, high lipofuscin level has been proposed as a reason for the high lysosome content in senescent cells (Brunk and Terman, 2002; Sitte et al., 2001). And, lipofuscins has also been found to accumulate in proliferating cells whose growth is temporarily arrested (Collins and Brunk, 1976). Indeed, in both senescence progression and serum starvation, the levels of autofluorescence, which is an indication of lipofuscin, gradually increased (Fig. 4A). Again, the degree of the change in the lipofuscin level was substantially higher in senescence progression. However, as compared to the difference in the lysosome contents, that in the lipofuscin levels was not as

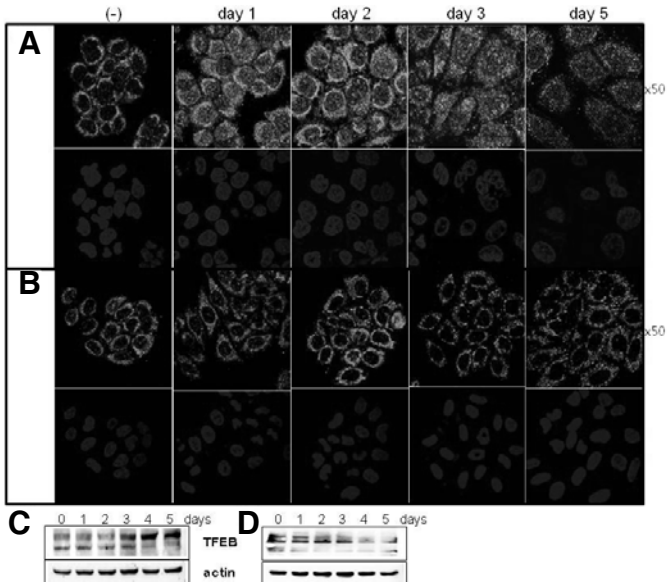

Fig. 5. Changes in TFEB activity. (A, B) Cells were seeded on a cover-slip in 24 wells, and cultured for either senescence progression (A) or serum starvation (B), stained with primary TFEB antibody or DAPI at indicated time point, and observed by confocal microscopy. (C, D) Proteins from either senescence progression (C) or serum starvation (D) were applied to Western blotting for TFEB. TFEB protein appeared in multiple bands due to different phosphorylation status. It has been suggested that one of the fast migrating bands is the active one (Pena-Llopis et al., 2011).

big. During the five day period, autofluorescence increased by 6.9 and 4.0 fold in senescence progression and serum starvation, respectively. This may suggest that the difference in the levels of lysosome content in the two conditions can also be attributed to factors other than the difference in the levels of residual bodies.

Having seen this rather small difference in the lipofuscin levels, we next checked a possible contribution of lysosome biogenesis by examining the mRNA levels of eight lysosomal proteins through real-time PCR analysis. In a previous study with normal fibroblasts which were induced to senescence by the same adriamycin pulse-chase method, the expression of nine (out of 14) genes was found upregulated (Park et al., 2007). In the current study, the mRNA levels of all the eight tested lysosomal proteins increased over two fold in one day during senescence progression (with a significance level of $P < 0.066$ at most) and all further increased thereafter (Fig. 4B). Meanwhile, in serum starvation, only one (LAMP1) of the genes showed an increase over two fold at the same significance level (Fig. 4C). These results suggest that, while senescence progression accompanies lysosome biogenesis as has been previously proposed, serum starvation may not or do only marginally. Taken together, it is suggested that, during senescence progression, lysosome content may increase through the accumulation of the residual bodies and the increased biogenesis, while, in serum starvation, the lysosome content may increase due mostly to an accumulation of residual bodies.

TFEB activation in senescence progression but not in serum starvation

Transcription factor EB (TFEB) has been suggested as a factor that plays a role in the coordinate expression of the lysosomal protein genes (Sardiello, 2009). Upon lysosomal storage stresses, TFEB translocates to nucleus and activates a host of lysosomal protein genes. We checked if TFEB translocates during senescence progression and serum starvation. In normally proliferating H460 cells, TFEB was found exclusively in cytosol [Figs. 5A and 5B, (-)]. Meanwhile, in the cells undergoing senescence progression, total TFEB protein level increased and some of them were found in nucleus starting from day one (Fig. 5A). However, in serum starvation, the status of TFEB did not

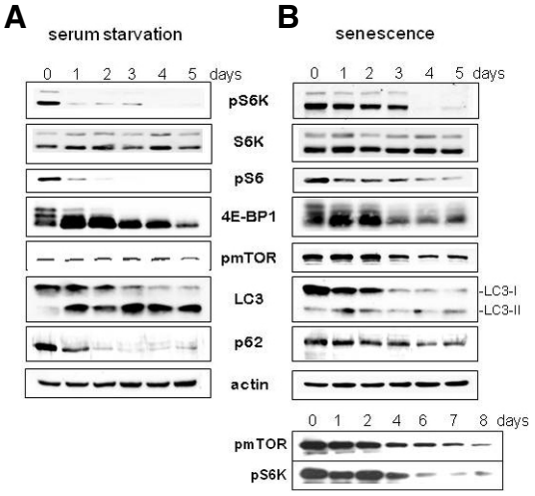

Fig. 6. Cells undergoing either senescence progression (A) or serum starvation (B) were collected at indicated time points and lyzed. Equal amount of proteins were applied to Western blotting for phosphorylated S6K, S6K, phosphorylated S6, 4E-BP1, phosphorylated mTOR, LC3, p62, and beta-actin protein.

change (Fig. 5B). TFEB migrates in gel electrophoresis as multiple bands due to different phosphorylation at multiple sites, and the active protein that localizes in nucleus is believed to be included in those fast migrating ones (Penã-Llopis et al., 2011). In the cells undergoing senescence progression, the fast-migrating TFEB bands were maintained until day three, although slow-migrating ones dominated at later time points (Fig. 5C). In contrast, in serum starvation, the fast-migrating bands rapidly disappeared (Fig. 5D). These results together may support that at least some of TFEB proteins are activated in (the early phase of) senescence progression but not in serum starvation. Although whether the active TFEB protein indeed behaves as suggested in these figures cannot be concluded at this stage, these results at least indicate that TFEB was modified differently in the two conditions.

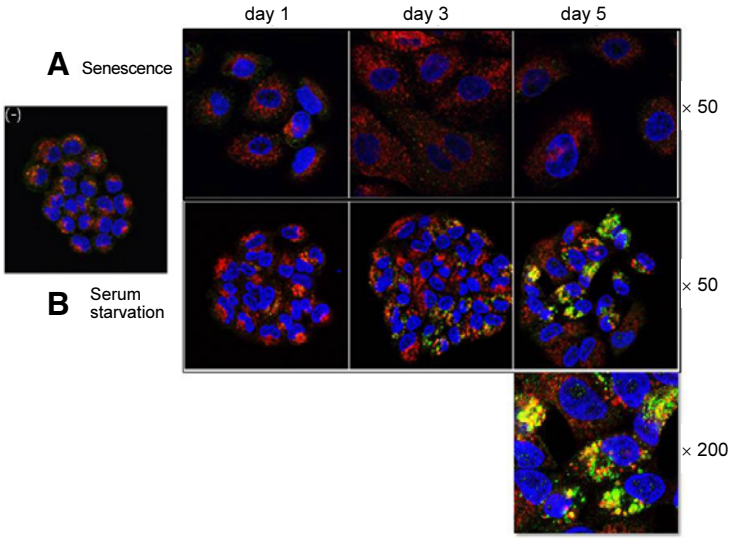

Fig. 7. Changes in LC3 in punctae formation and colocalization with lysosome. H460 cells seeded on a coverslip in 24 wells were treated for senescence (A) or for serum starvation (B), and processed for immunofluorescence staining either LC3 (green) or Lamp1 (red) proteins at indicated time points. Typical field in confocal microscopic fields were photographed. The image at day 5 of the serum starvation was enlarged and presented at the bottom box (× 200).

Large difference in mTOR activity between senescence progression and serum starvation

TFEB nuclear localization has been reported to require active mTOR signaling (Pena-Llopis et al., 2011). Whether mTOR is active in serum starvation or senescence progression was determined by examining the phosphorylaltion status of its substrates, S6K (and its substrate S6) and 4E-BP1 (Sans and Williams, 2002) (Fig. 6). Since mTOR is activated by growth factor signaling, its activity is expected to be low in serum starvation. Indeed, the phosphorylation levels of S6K and its product (phosphorylated S6) as well as 4E-BP1 were severely reduced from day one. And, S6K activity disappeared almost completely after the first day (Fig. 6A). Meanwhile, in senescence progression, phosphorylated mTOR and its activity appeared to be maintained almost intact until day three (Fig. 6B). Even after day three, the level of the phosphorylated mTOR declined steadily (Fig. 6B, low panel). This indicates that, in the cells undergoing senescence progression, mTOR signaling remains intact at least for a while, even though the continued input of growth factors failed in activating cell cycle. Interestingly, however, the mTOR signaling deteriorated after day three in these cells as well.

Downregulation of autophagy during senescence progression

mTOR inhibits autophagy (Corradetti and Guan, 2006). Therefore, cells under serum starvation experience increased autophagy. Most studies on serum starvation reported an immediate (within an hour or so) upregulation of autophagy (Valentin and Yang, 2008). In this study, we not only confirmed the strong upregulation, but also noticed a sustained activation during long-term serum starvation. The levels of LC3 type II molecule, whose increase is a key marker of autophagy activation, was substantially elevated and maintained highly for the five-day period of serum starvation (Fig. 6A, LC3). Furthermore, the level of p62 protein, an autophagy receptor that recruits substrates in autphagosome and gets degraded during autophagolysis (Pankiv et al., 2007), rapidly decreased, indicating a substantial increase in autophagy flux as well. In addition, the number of LC3 punctae, which represent autophagosomes, increased from day one and their size and colocalization with lysosomes also increased later on (Fig. 7B). Meanwhile, in the cells undergoing senescence progression, the level of LC3 type II marginally increased at day one but was maintained more or less at the similar level thereafter (Fig. 6B). Further, prominent LC-3 punctae were rarely seen in these cells (Fig. 7B). Therefore, autophagy was not upregulated significantly during senescence progression. Meanwhile, the level of p62 was maintained for a while but slowly decreased after day 3 (Fig. 6B, p62). Treatment of monensin, which blocks fusion between autophagosome and lysosome, two days prior to cell collection resulted in a slight increase in the level of LC3 type II molecule (SH Cho, data not shown). This and the increase of the ratio of LC3 II/LC3 suggest a small increase of autophagy flux after day 3. These results indicate that, differently from serum starvation, in senescence progression, autophagy is not activated to a significant level. Overall, it is suggested that the activity of mTOR pathway is differently regulated in serum starvation and senescence progression, and this difference might bring in a contrasting status of autophagy in the two conditions.

Finally, in serum starvation, very large structures where the LC3 punctae and lysosome signal overlapped were formed in many cells at day five. mTOR activity has recently been reported to be involved in lysosome rescue after autophagolysis (Yu et al., 2010), and the severe decrease in the mTOR activity at the late stage might be responsible for the attenuated lysosome rescue and the accumulation of these large autophagolysosomes.

DISCUSSION

The results of this study help clarify the nature of SA β-Gal activity expressed in senescence and serum starvation conditions. In both the conditions, SA β-Gal activity and lysosome content increased, but, these two changed were more prominently during senescence progression. This supports our previous notion that SA β-Gal activity is indeed derived from an increased lysosome activity. In addition, this also strongly supports the notion by Cristofalo that SA β-Gal is indeed a surrogate marker of an increased lysosomal status (Cristofalso, 2005). And, the results also show that, differently from quiescence, senescence progression accompanies lysosome biogenesis, which suggests that the higher lysosome content in senescence is largely due to lysosome biogenesis, although the greater accumulation

of the residual bodies could also be attributed to.

A major difference at molecular level between quiescence and senescence existed in the status of mTOR activity. Unlike the serum-starved cells, the ones undergoing senescence progresssion maintained mTOR activity at least for certain period. This indicates that mTOR signaling overrides the block in cell cycle, which is casted within 24 h of the adriamycin-triggered senescence progression (Cho et al., 2011). mTOR controls various cellular activities including cell growth, autophagy, mitochondria metabolism as well as lysosome maintenance (Corradetti and Guan, 2006; Weinchhart, 2012). Importantly, our results suggest the possibility that some of the phenotypes of cellular senescence are also regulated by mTOR. Indeed, the nature of mTOR by itself suggests this as a plausible prediction. One good example would be the increase in cell volume during senescence progression but not in quiescence, which is most likely induced by the mTOR-mediated S6kinase activity which determines cell size (Magnuson et al., 2012).

Whether the difference in mTOR activity indeed is responsible for the senescence phenotype expression would be important not only in the understanding of the mechanisms underlying the expression of senescence phenotypes but also for developing intervention strategies of cellular senescence. It has been reported that rapamycin-mediated mTOR inactivation partially decreased SA β-Gal staining during the adriamycin- or peroxide-induced senescence (Demidenko et al., 2009). The reason for this change has not been sought. However, this result is in good accordance with our hypothesis on the mTOR-mediated lysosome biogenesis during senescence progression. During the senescence progression, lysosome biogenesis is ongoing, and this is likely induced by TFEB activation caused by the active mTOR (Pena-Llopis et al., 2011) [and might be further stimulated by the lysosomal stress caused by a high level lipofuscin as suggested by Sardiello et al. (2009)]. The rapamycin-induced mTOR inactivation would cause TFEB inactivation and attenuate lysosome biogenesis. Therefore, it is possible that the partial decrease of the SA β-Gal activity upon rapamycin treatment could be caused by this nullification of the mTOR-mediated TFEB induction.

mTOR activity underwent a steady decline after day three or four in senescence progression. Since cells are still fed with growth factors, this decline of mTOR activity is unexpected. Currently, no information is available for this change, but it is expected that one or more of the factor(s) in the growth factor-mTOR signaling pathway might be inactivated or desensitized, or certain regulatory factors like TSC1/2 protein might be constitutively activated adding another puzzle to the already complicated network in the mTOR signaling (Wang and Proud, 2011). It may be noteworthy that the increase in the mRNA levels, *in vivo* cathepsin D activity, and TFEB nuclear localization all appeared to peak at day 3 or so and at day 5, they either declined or remained at similar levels. The seemingly coincidental changes in these events and mTOR activity may again point to the possible involvement of mTOR in these cells' biological changes during senescence progression.

Another contrasting difference between senescence progression and quiescence is seen in the status of autophagy. Autophagy was not activated in the cells undergoing senescence progression despite the increase of lysosome content and lipofuscins. This clearly indicates that autophagy is down-regulated in senescence, and this is an analogy to the low autophagy reported in the tissues of aged animals (Cuervo and Dice, 2000; Massey et al., 2006). So far, this low autophagy can be attributed to the high mTOR activity, which negatively regulates autophagy activation (Corradetti and Guan, 2006). Meanwhile, the decline of mTOR activity at later time points during senescence progression might have caused a small increase in autophagy flux. The possibility of this requires validation through a close examination in the machineries involved in autophagy activity and flux.

Overall, cells undergoing senescence progression experience a variety of cell biological changes (Hwang et al., 2009). Intervention of cellular senescence, especially stem cells, can be facilitated by understanding the molecular biological mechanisms underlying these changes. However, other than the destructive effect of ROS, not much is well understood currently. Our results points to the possible importance of mTOR activity for the senescence-specific cell biological changes including not only the increase of cell volume and irreversible arrest of cell cycle, but also SA β-Gal activity and the increase of lysosome biogenesis. This warrants further investigation on the effect of mTOR on various cell biological changes taking place during senescence progression.

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