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SHORT TAKE

Apoptosis resistance of senescent human fibroblasts is correlated with the absence of nuclear IGFBP-3

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Summary

Signaling through the insulin/IGF axis plays a major role in determining the rate of aging in many species. IGF-binding proteins (IGFBPs) modulate the IGF pathway in higher organisms. IGFBP-3 accumulates in conditioned medium of senescent human fibroblasts, suggesting that it may contribute to the senescent phenotype. IGFBP-3 can enhance apoptotic cell death in tumor cells due to its ability to target intracellular regulators of apoptosis, including nuclear transcription factors. Senescent fibroblasts are highly resistant to apoptosis, suggesting that IGFBP-3 fails to induce apoptosis in this cell type; however, mechanisms of apoptosis resistance in senescent cells are poorly understood. To address this question, we studied the production and intracellular localization of IGFBP-3 in senescent fibroblasts. Whereas IGFBP-3 is highly overexpressed by senescent fibroblasts, IGFBP-3 was not detectable in the nucleus of senescent fibroblasts. In tumor cells, IGFBP-3 can be internalized by endocytosis, which is considered as a prerequisite for the intracellular functions of IGFBP-3 and probably also for its transport to the nucleus; we show here that endocytotic uptake of IGFBP-3 does not occur in senescent human fibroblasts. This is correlated with a generally decreased endocytotic activity of these cells, as shown with the model substrate transferrin. The data are consistent with a model where IGFBP-3 accumulation in conditioned medium of senescent fibroblasts contributes to growth arrest of these cells, whereas the failure to endocytose IGFBP-3 and the absence of nuclear IGFBP-3 may contribute to the well-established apoptosis resistance of senescent human fibroblasts.

Key words: Apoptosis; fibroblast; IGFBP-3; senescence.

Signaling through the insulin/IGF axis plays a major role in determining the rate of aging in lower eukaryotes, such as worms and flies (for review, see Hekimi & Guarente, 2003), and there is evidence that the role of insulin/IGF-signaling in aging is conserved in higher organisms, including mammals (Barbieri *et al*., 2003). In contrast to the model organisms mentioned above, genetic analyses of human aging have yielded only very limited information, and most of our current knowledge about molecular pathways relevant for human aging was obtained with cell culture-based systems of *in vitro* senescence (for review, see Campisi, 2001). In higher eukaryotes, signal transduction through the IGF pathway is modulated by a family of six IGF-binding proteins (IGFBPs) and IGFBP-3 regulates proliferation and survival in many mammalian cell types (Firth & Baxter, 2002). It was shown that IGFBP-3 accumulates at senescence in the conditioned medium of human diploid fibroblasts (Goldstein *et al*., 1991), and it was speculated that increased IGFBP-3 may contribute to the senescent phenotype; however, underlying molecular mechanisms have remained unclear.

Most functional studies of IGFBP-3 have been performed with tumor cell lines derived from prostatic cancer or breast cancer and with a few other cancer cell lines. In most instances, IGFBP-3 has been introduced by transient transfection followed by an analysis of the fate of transfected cells. In addition, several studies have applied recombinant IGFBP-3 to cellular supernatants and studied the resulting changes in cellular phenotype. The results from these experiments in tumor cell lines can be summarized as follows (for review, see Firth & Baxter, 2002): one major function of IGFBP-3 is to sequester IGF and modulate its binding to the IGF receptor, thereby blocking a survival signal (most likely mediated via AKT-signaling) and at the same time attenuating mitogenic signaling (via MAP-kinase signaling pathways). This effect requires rather low concentrations of IGFBP-3, which are similar to the concentration needed for IGF. There is a second pathway, which appears to operate efficiently only at higher concentrations of IGFBP-3 (Li *et al*., 1997; Schedlich *et al*., 1998) and this involves uptake of IGFBP-3 from the extracellular space, probably involving endocytosis (Schedlich *et al*., 1998; Lee *et al*., 2004), followed by delivery to the nucleus (Schedlich *et al*., 2000) where it targets nuclear regulators of apoptosis, such as RXRalpha/Nur77 (Lee *et al*., 2005). IGFBP-3 can induce apoptosis in an IGF-independent manner

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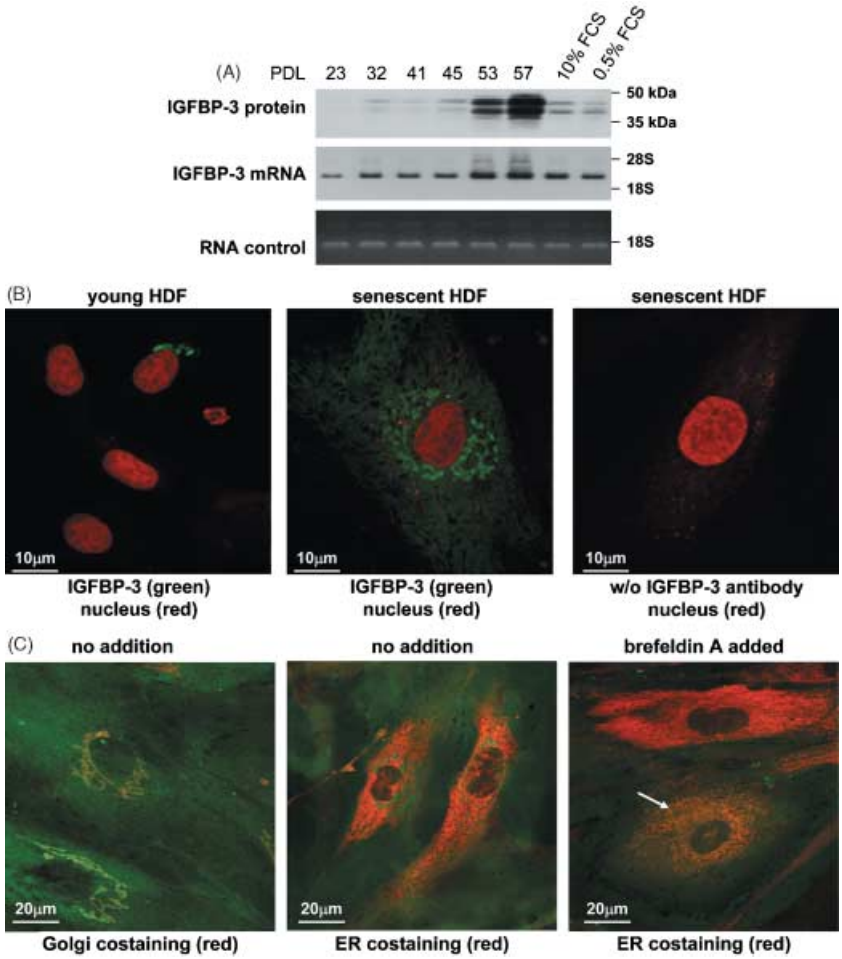

Fig. 1 Accumulation and secretion of IGFBP-3 in senescent fibroblasts. (A) IGFBP-3 expression in young and senescent fibroblasts. Human diploid fibroblasts from human foreskin were grown in monolayer culture until they reached senescence at approximately 56–58 population doublings (PDL), as described (Wagner *et al*., 2001a). The senescent status was verified by *in situ* staining for SA-β-galactosidase as described (Dimri *et al*., 1995). At the population doublings (PDL) indicated, samples were taken. Whole cell extracts were prepared, separated by SDS-PAGE, with loading corresponding to similar cell numbers, and analyzed by Western blot using polyclonal goat antibodies to IGFBP-3 (DSL, Sinsheim, Germany). As further controls, extracts from presenescent fibroblasts (45 PDL) that were kept in the presence of either 10% or 0.5% FCS were loaded. RNA was prepared from parallel samples using the 'RNeasy Kit' (Qiagen, Hilden, Germany) and analyzed by Northern blot using a ^{32}P-labelled cDNA probe, generated by random primed labeling from pX-IGFBP-3 as described (Mannhardt *et al*., 2000). The probe detected a single mRNA species of roughly 2.5 kb. Equal loading of RNA was confirmed by an ethidium bromide stained RNA gel. Size markers for protein and RNA are indicated. (B) Immunofluorescence analysis of IGFBP-3 distribution in senescent fibroblasts. Early passage and senescent cultures of human diploid fibroblasts were stained with antibodies to IGFBP-3, as indicated, and staining detected by secondary antibodies that were labeled with FITC. To control specificity of IGFBP-3 staining and to rule out any contributions from autofluorescence, primary IGFBP-3 antibodies were omitted in the right panel, as indicated. In all panels, nuclei were co-stained with TO-PRO 3 (Molecular Probes, Leiden, the Netherlands) which gave red staining. Cells were seeded onto 14-mm glass slides and grown until they reached 70–80% confluency. Cells were washed three times in PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and stained with antibodies to IGFBP-3 (DSL, Sinsheim, Germany; dilution 1 : 700), as described (Fiedler *et al*., 2004). (C) IGFBP-3 localizes to the secretory pathway in senescent human fibroblasts. Senescent cells were stained with antibodies to IGFBP-3 along with FITC-labeled secondary antibodies and co-stained with antibodies to either giantin (dilution 1 : 1000) to stain the Golgi apparatus, or calreticulin (middle and right panels, dilution 1 : 40) to stain the ER, along with TRITC-labeled secondary antibodies. For co-localization experiments, presenescent cultures (around 50 PDL) were used in which very few (< 20%) cells with low IGFBP-3 expression can be found, which serve as convenient negative controls. Staining and co-localization was analyzed by confocal microscopy as described (Teis *et al*., 2002). In the right panel, cells were treated with Brefeldin A (10 μg mL^{-1}) 60 min prior to fixation to block the export of proteins from the ER to the Golgi apparatus. (D) Pulse chase labeling of IGFBP-3. Young and senescent cultures of human diploid fibroblasts were incubated with ^{35}S-labeled amino acids for 30 min as described (Wagner *et al*., 2001b) and cellular extracts and supernatants prepared. From both extracts and supernatants, IGFBP-3 was immunoprecipitated; the immunoprecipitates were separated by 10% SDS-PAGE and IGFBP-3 detected by autoradiography. (E) Secreted IGFBP-3 is retained at the surface of senescent cells. Senescent cells were stained for IGFBP-3 along with FITC-labeled secondary antibodies; in the left and middle panels, nuclei were counterstained by TO-PRO 3 (red). To determine the dynamics of protein secretion, cells were treated with 2 μg mL^{-1} cycloheximide for 2 h as indicated. The appearance of strong but diffuse green staining observed with cycloheximide-treated cells probably reflects IGFBP-3 retained at the cell surface. In the right panel, actin fibers were co-stained with antibodies to beta-actin (Sigma, Vienna, Austria, dilution 1 : 1000), along with TRITC-labeled secondary antibodies. Extracellular IGFBP-3, which spreads beyond the cell borders as defined by beta-actin staining, is marked by an arrow.

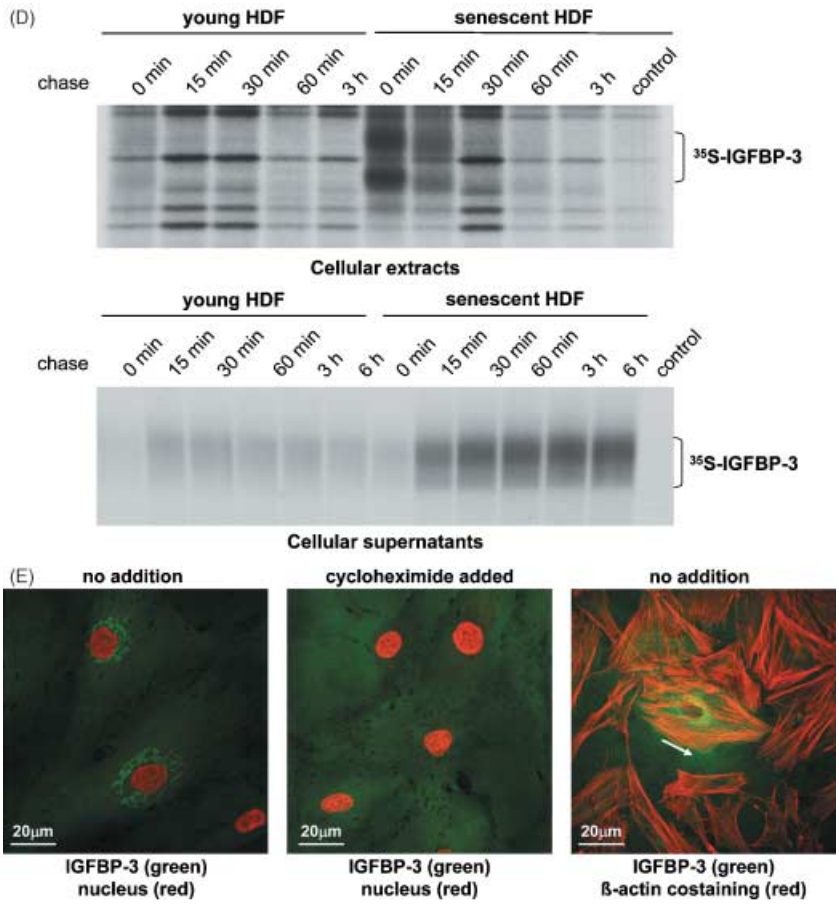

Fig. 1 *Continued*

(Rajah *et al*., 1997; Hong *et al*., 2002) and this property has been associated with intracellular activities of IGFBP-3.

Whereas IGFBP-3 concentrations in conditioned medium are sufficient to attenuate mitogenic signaling through IGF (Grigoriev *et al*., 1995), it has remained unclear why increased IGFBP-3 expression is not associated with increased apoptosis in senescent fibroblasts; rather, senescent fibroblasts are generally resistant to apoptosis induction when compared to young cells (Wang, 1995). Moreover, we have shown that the pro-apoptotic effects of C2 ceramide, well known for its ability to synergize with IGFBP-3 in apoptosis induction (Gill *et al*., 1997), are specifically reduced in senescent cells (Hampel *et al*., 2004). To explore why senescent fibroblasts, albeit producing high levels of IGFBP-3, do not display high levels of spontaneous apoptosis (Wagner *et al*., 2001a), it was of interest to determine the extracellular and intracellular localization of IGFBP-3 in senescent fibroblasts.

To monitor the expression of the IGFBP-3 gene during fibroblast senescence, RNA and protein samples were taken from human diploid fibroblasts at various passage levels and probed for the expression of the IGFBP-3 gene by both Northern and Western blots. The senescent phenotype was confirmed by G1 cell cycle arrest, altered morphology and staining of > 95% of the cells for senescence-associated beta-galactosidase (Dimri *et al*., 1995), as described previously (Wagner *et al*., 2001a). We found an approximately fivefold up-regulation of the IGFBP-3 mRNA with increasing passage number, and this was paralleled by an even stronger up-regulation of IGFBP-3 protein (Fig. 1A). Densitometric analyses of several independent experiments revealed a 60- to 80-fold up-regulation of IGFBP-3 protein in cellular extracts at senescence. Serum withdrawal from pre-senescent cells did not significantly alter IGFBP-3 expression (Fig. 1A), indicating that IGFBP-3 is not up-regulated in quiescent cells. As described by others (Goldstein *et al*., 1991), a significant increase in the IGFBP-3 concentration was also detected in conditioned media from senescent cells (data not shown; see also below, Fig. 1D). However, the pattern of gene expression at the single cell level is unknown, and intracellular accumulation of IGFBP-3 in senescent fibroblasts was not reported before.

To address these points, indirect immunofluorescence experiments were carried out. In fibroblast cultures at early passage, most cells were not stained with IGFBP-3 antibodies (Fig. 1B); whereas such cultures contain a few cells in which substantial IGFBP-3 staining can be detected, staining is still considerably weaker than in senescent cells. With increasing passage number, the number of IGFBP-3 positive cells increase markedly as does the amount of IGFBP-3 per cell. Most IGFBP-3 staining in senescent fibroblasts was found in distinct intracellular structures the nature of which remains to be defined (Fig. 1B,

see also below). In addition, diffuse IGFBP-3 staining was observed in senescent but not young cultures that spread over the entire monolayer, probably reflecting IGFBP-3 associated with the cell surface (McCusker & Clemmons, 1997) (see also below, Fig. 1E). Importantly, thorough examination by confocal microscopy failed to detect any nuclear IGFBP-3 in senescent HDF.

To further address the subcellular distribution of IGFBP-3 in senescent fibroblasts, co-localization studies where carried out, first probing the secretory pathway. For co-localization experiments, presenescent cultures (around 50 PDL) were used in which very few (< 20%) cells with low IGFBP-3 expression can be found and which serve as convenient controls for both types of staining (for example, see rightmost panel in Fig. 1C). When co-localization studies were carried out with antibodies to giantin, a well-established marker for the Golgi apparatus (Linstedt & Hauri, 1993), a clear, albeit partial, co-localization with IGFBP-3 was obtained (Fig. 1C). When IGFBP-3 staining was carried out in parallel with staining for calreticulin, a well-established marker for the endoplasmic reticulum (ER), only a minor part of IGFBP-3 staining co-localized with calreticulin staining, suggesting that only a minor fraction of IGFBP-3 in senescent cells is localized to the ER (Fig. 1C). When senescent cells were treated with Brefeldin A, which blocks protein translocation from ER to Golgi, IGFBP-3 showed nearly complete co-localization with calreticulin, suggesting that a large part of the signal is due to newly synthesized IGFBP-3 that is held back within the ER (Fig. 1C; arrow in right panel). Together, the data suggest that in senescent cells, a large proportion of intracellular IGFBP-3 is present in the secretory pathway.

Our observation that a relatively moderate increase of the IGFBP-3 mRNA level is accompanied by a drastic rise in the protein concentration in senescent cells (Fig. 1A) suggests that differences in the efficiency of translation and/or the metabolic stability may also contribute to the increased abundance of IGFBP-3 in senescent fibroblasts. To address these questions and to determine the kinetics of IGFBP-3 production and secretion, young and senescent cells were labeled by ^{35}S-methionine/cysteine for 3 h, followed by a chase period of variable length. At each time point, cells were harvested and extracted to reveal intracellular IGFBP-3; at the same time, conditioned media were collected and analyzed to detect secreted IGFBP-3. This experiment (Fig. 1D) revealed a significant *de novo* synthesis of IGFBP-3 in senescent cells. The newly synthesized IGFBP-3 disappeared rapidly from the cellular extract (Fig. 1D, upper panel), and this was accompanied by the appearance of radioactively labeled IGFBP-3 in the cellular supernatant (Fig. 1D, lower panel). In young cells, IGFBP-3 was hardly detectable in cellular extracts (Fig. 1D, upper panel), and a low level of IGFBP-3 could be detected in the supernatant (Fig. 1D, lower panel). These data indicate that *de novo* synthesis of IGFBP-3 is strongly increased in senescent fibroblasts and that most of the newly synthesized IGFBP-3 is rapidly secreted. In the conditioned media of both young and senescent cells, IGFBP-3 was quite stable over 6 h (Fig. 1D), suggesting that there is no rapid proteolysis of IGFBP-3 under these conditions.

To further study the dynamics of IGFBP-3 subcellular localization, senescent human fibroblasts were treated with cycloheximide for 2 h, to block protein synthesis. IGFBP-3 disappeared rapidly from the ER and Golgi upon cycloheximide treatment (Fig. 1E), suggesting that most of the protein was actually secreted. Interestingly, part of the protein was apparently retained at the cellular surface (Fig. 1E), as was described by others (McCusker & Clemmons, 1997). Extracellular localization of IGFBP-3 under these conditions was confirmed by our observation that the diffuse IGFBP-3 staining shown in Fig. 1(E) was sensitive to mild trypsinization (data not shown). Extracellular sites of IGFBP-3 accumulation clearly extended beyond the cellular body, as delineated by beta-actin co-staining (Fig. 1E).

It has been demonstrated that IGFBP-3 can be taken up by tumor cells (Li *et al.*, 1997; Wraight *et al.*, 1998), and endocytotic pathways are involved (Lee *et al.*, 2004). Once in the cytoplasm, IGFBP-3 is actively transported to the nucleus (Schedlich *et al.*, 2000), where it may contribute to the regulation of gene expression (Liu *et al.*, 2000); however, genes that are regulated by IGFBP-3 *in vivo* have not been identified so far. To address the possibility that a fraction of IGFBP-3 might be (re)internalized after secretion, which would imply its transient presence in endocytotic vesicles, senescent cells were co-stained with IGFBP-3 antibodies along with antibodies to constitutive components of caveolae, early endosomes, late endosomes and lysosomes, respectively (Fig. 2A). In these experiments, we did not observe any co-localization of IGFBP-3 with any of the markers for endocytotic vesicles, suggesting that in senescent human fibroblasts, significant endocytotic uptake of IGFBP-3 does not occur. This may be due to a generally reduced efficiency of endocytotic uptake in senescent fibroblasts, as senescent fibroblasts, when compared to tumor cells or young fibroblasts, displayed a strongly reduced ability to endocytose fluorescence-labeled transferrin (Fig. 2B), a widely used model substrate for endocytotic uptake (Sager *et al.*, 1984).

The current model of IGFBP-3 action (largely derived from work with tumor cells; Firth & Baxter, 2002) would suggest that extracellular IGFBP-3 plays a key role in modulating the mitogenic response, whereas intracellular (and probably nuclear) IGFBP-3 is largely responsible for the apoptotic response. Concerning IGFBP-3 actions in senescent cells, the literature is quite scarce, and the present study is the first to address the intracellular distribution and dynamics of IGFBP-3 in senescent human fibroblasts. Our finding that IGFBP-3 uptake and nuclear localization are not observed in senescent cells provides a clear basis for the documented inability of this potent pro-apoptotic protein to induce apoptosis in senescent human fibroblasts. This link is further strengthened by our observation that endocytotic uptake of IGFBP-3, known to happen readily in tumor cells, does not occur in senescent fibroblasts. Finally, our demonstration that endocytotic protein uptake is generally attenuated in senescent cells provides an explanation for the latter fact. It will be interesting to investigate further the reasons for the apparent inability of senescent fibroblasts to internalize IGFBP-3 and to explore molecular pathways that may link this observation to their resistance to apoptotic cell death.

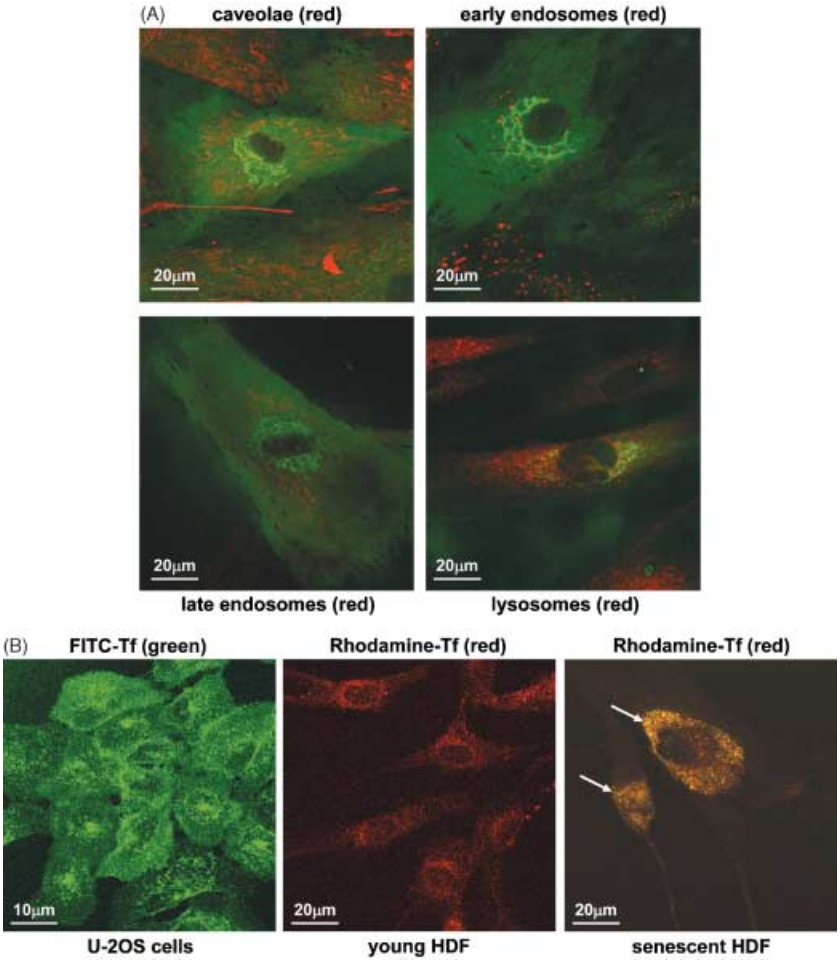

Fig. 2 IGFBP-3 is not endocytosed in senescent fibroblasts. (A) Absence of IGFBP-3 from endocytotic vesicles in senescent fibroblasts. Senescent cells were stained with antibodies to IGFBP-3 along with FITC-labeled secondary antibodies and co-stained with antibodies to either caveolin (dilution 1 : 50) to stain caveolae, EEA1 to stain early endosomes (dilution 1 : 250), LAMP 1 (dilution 1 : 200) to stain late endosomes and antibodies to alpha-beta-Hexosaminidase (dilution 1 : 40) to stain lysosomes, as indicated. For these experiments, presenescent cultures (around 50 PDL) were used in which very few (< 20%) cells with low IGFBP-3 expression can be found, which serve as convenient negative controls. To assess co-localization, TRITC-labeled secondary antibodies were used for the respective antigens. Antibodies and staining procedures were as described by Teis *et al*. (2002). Please note the absence of significant co-localization of IGFBP-3 with any of the other antibodies used for co-staining. (B) Reduced endocytotic activity of senescent fibroblasts. Left panel: Alexa 488-labeled transferrin (Alexa 488-Tf; Molecular Probes, Leiden, the Netherlands) was incubated with U-2OS osteosarcoma cells on ice and uptake initiated by shifting the temperature to 37° for 30 min as described (Teis *et al*., 2002). Alexa 488-Tf could not be used for experiments with fibroblasts since senescent fibroblasts have a very strong autofluorescence at the wavelength required for excitation of Alexa 488, due to the high content of lipofuscin that accumulates in senescent fibroblasts (von Zglinicki *et al*., 1995). To circumvent this problem, rhodamine-labeled transferrin (rhodamine-Tf; Molecular Probes, Leiden, the Netherlands) was used. Young (middle panel) and senescent (right panel) fibroblasts were incubated with rhodamine-Tf on ice, and uptake initiated by temperature shift to 37° for 30 min. Staining was analyzed by confocal microscopy as described (Teis *et al*., 2002). The experiment reveals that uptake of rhodamine-Tf is clearly visible in young cells, but hardly detectable in senescent cells. Greenish autofluorescence of senescent fibroblasts, which was observed even in the red channel, is marked by arrows.

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