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Research Paper

Hyaluronan synthase-2 upregulation protects *smpd3*-deficient fibroblasts against cell death induced by nutrient deprivation, but not against apoptosis evoked by oxidized LDL

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ABSTRACT

The neutral type 2 sphingomyelinase (nSMase2) hydrolyzes sphingomyelin and generates ceramide, a major bioactive sphingolipid mediator, involved in growth arrest and apoptosis. The role of nSMase2 in apoptosis is debated, and apparently contradictory results have been observed on fibroblasts isolated from nSMase2-deficient fragilitas ossium (homozygous *fro*/*fro*) mice. These mice exhibit a severe neonatal dysplasia, a lack of long bone mineralization and delayed apoptosis patterns of hypertrophic chondrocytes in the growth plate. We hypothesized that apoptosis induced by nutrient deprivation, which mimics the environmental modifications of the growth plate, requires nSMase2 activation. In this study, we have compared the resistance of *fro*/*fro* fibroblasts to different death inducers (oxidized LDL, hydrogen peroxide and nutrient starvation). The data show that nSMase2-deficient *fro*/*fro* cells resist to apoptosis evoked by nutrient starvation (fetal calf serum/glucose/pyruvate-free DMEM), whereas wt fibroblasts die after 48 h incubation in this medium. In contrast, oxidized LDL and hydrogen peroxide are similarly toxic to *fro*/*fro* and wt fibroblasts, indicating that nSMase2 is not involved in the mechanism of toxicity evoked by these agents. Interestingly, wt fibroblasts treated with the SMase inhibitor GW4869 were more resistant to starvation-induced apoptosis.

The resistance of *fro*/*fro* cells to starvation-induced apoptosis is associated with an increased expression of hyaluronan synthase 2 (HAS2) mRNAs and protein, which is inhibited by ceramide. In wt fibroblasts, this HAS2 rise and its protective effect did not occur, but exogenously added HA exhibited a protective effect against starvation-induced apoptosis.

The protective mechanism of HAS2 involves an increased expression of the heat-shock protein Hsp72, a chaperone with antiapoptotic activity. Taken together, these results highlight the role of nSMase2 in apoptosis evoked by nutrient starvation that could contribute to the delayed apoptosis of hypertrophic chondrocytes in the growth plate, and emphasize the antiapoptotic properties of HAS2.

Introduction

Sphingomyelinases are a family of enzymes implicated in the catabolism of sphingomyelin, a major sphingolipid present in cellular membranes, rafts and caveolae [1,2]. Several sphingomyelinases have been cloned and characterized, and are known to play a major role in cell biology, *via* the degradation of sphingomyelin, which generates ceramide, a bioactive sphingolipid mediator involved in cell growth arrest, apoptosis, autophagy and cell differentiation [3–6]. A variety of factors, such as nature of the stressors, stress duration, cell and tissue specificity, subcellular localization and metabolism of ceramide can influence its biological effects [6,7]. Ceramide can be catabolized by ceramidases into sphingosine, which can be converted by sphingosine kinases into sphingosine 1-phosphate (S1P), another sphingolipid mediator exhibiting survival and mitogenic properties [6,8].

Several evidences indicate that the balance ceramide/S1P (ceramide/S1P rheostat) is an important determinant of cell fate towards survival or apoptosis depending on the ability of cells to generate S1P from ceramide [9]. The neutral sphingomyelinase 2 (nSMase2), encoded by the *smpd3* gene, is a redox-sensitive [6] enzyme that plays a key role in ceramide generation upon

Abbreviations: nSMase2, neutral sphingomyelinase type 2; HAS2, hyaluronan synthase 2; HA, hyaluronan; LDL, low density lipoprotein

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stimulation by stress-inducing agents, including hydrogen peroxide (H_2O_2) and oxidized LDL [10–13]. We have recently reported that nSMase2 activation by low H_2O_2 concentrations mediates the proliferation and migration of smooth muscle cells (SMC) and fibroblasts, *via* a ceramide-dependent sequential signaling cascade implicating the activation of src, and the subsequent phosphorylation and activation of the PDGF receptor, that is implicated in the activation of sphingosine kinase and the generation of S1P [12]. In contrast, high oxidative stress inhibits sphingosine kinase and triggers apoptotic cell death [14].

The expression of nSMase2 is high in the brain and bones [15,16]. Mice knockout for nSMase2 and nSMase2-deficient *fro/fro* mice (homozygous $smpd3^{fro}/smpd3^{fro}$ with fragilitas ossium phenotype) exhibit bone deformations and neonatal growth retardation [16–18]. A number of studies have been carried out on cells and tissues isolated from these mice, to decipher the physiological role of nSMase2 and its implication in apoptosis evoked by cell death inducers. We recently reported that mutant fibroblasts isolated from *fro/fro* mouse undergo apoptosis similarly to wt fibroblasts, when exposed to stress-inducing agents such as cytokines (TNF-α), H_2O_2 or oxidized LDL [11]. Likewise, the hepatotoxicity resulting from TNF-α injection to mice, is similar in *fro/fro* and wild type mice, indicating that the nSMase2 mutation does not confer any resistance to these acute stress-inducing agents [11]. However, other studies, including experiments done on *fro/fro* cells, indicate an apoptotic role for nSMase2 [19,20]. Recently Kavandhgar et al. [21] reported that the defect in bone mineralization in *fro/fro* mouse is associated with an accumulation of hypertrophic chondrocytes in the growth plate and a reduced number of TUNEL positive cells, indicating a defective apoptosis, which is necessary for bone mineralization [22]. These reports point out the complicated role of nSMase2 in apoptosis, which may differ as function of the state of development, the tissue specificity and the nature of the stress inducer.

The terminal apoptotic differentiation of hypertrophic chondrocytes, is necessary for bone mineralization and involves environmental modifications, including nutrient deprivation [23], which is a known sphingolipid pathway trigger [24]. The present study was carried out to decipher whether nSMase2 is involved in cell death induced by nutrient starvation and to characterize the mechanisms of resistance evoked by nSMase2 mutation in *fro/fro* cells.

Materials and methods

Chemicals and reagents

Anti-HAS2 mouse monoclonal antibody (sc-365263) was from Santa Cruz Biotechnology (Texas, USA); anti-hsp72/73 mouse antibody was from Calbiochem (Merck Millipore, United Kingdom). Anti Akt-phospho (Ser473) rabbit was from Cell Signalling. Secondary antibodies anti-mouse and anti-rabbit were from Cell Signalling Technology (Denver, USA). SYTO-13, propidium iodide, alexa-Fluor 488 (green)- and Alexa-Fluor 546 (red)-conjugated secondary antibody were from Molecular Probes (Invitrogen, Cergy-Pontoise, France). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), GW4869, KNK437 were from Sigma-Aldrich. RPMI 1640, DMEM, fetal calf serum (FCS) were from Invitrogen (France). Dulbecco's Modified Eagle Medium (DMEM) without D-glucose and Sodium pyruvate was from Gibco/Life Technology (Paisley, United Kingdom). Hyaluronan (High Molecular Weight > 950 kDa) was from RD System (Minneapolis, USA). C2-ceramide was from Biomol, Laboratory Research. Acrylamide-4 × /bisacrylamide-2 × solution was from Euromedex (Souffelweyersheim, FR). The ECL chemoluminescence kit was from Amersham Pharmacia (Velizy-Villacoublay, France).

Cell culture

Primary cultures of fibroblasts were obtained by skin biopsies from newborn control and *fro/fro* mice. Briefly, skin samples were minced and put in Petri dishes, dermis facing down. After 15 min of dry contact with the dishes, DMEM culture medium containing 10% FCS penicillin, streptomycin, amphotericin A was added, and the skin preparation was cultured at 37 °C, 5% CO_2. After 1–3 weeks, cells growing around the tissue pieces were expanded.

Control (wt) or *fro/fro* fibroblasts were grown in DMEM Glutamax culture medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) in a 5% CO_2 humidified incubator, at 37 °C. At sub-confluency, this medium was removed and replaced by serum/glucose/pyruvate-free DMEM (nutrient-starvation conditions), or by serum-free RPMI-1640 containing oxidized LDL (200 μg/ml), or H_2O_2 (100 μM), for 48 h.

Animals

The genetic background of *fro/fro* and wt mice was 129/SV. Homozygous mice, harboring a truncating mutation in nSMase2 and fragilitas ossium (fro) phenotype were genotyped by PCR, as previously described [17], using the following primers: 5′-GCCCGCAGCCATGTATAGTA-3′, 5′-CTCAATGGAGGGCACACAG-3′ and 5′-CAGGTTTAGGGACCCTGACG-3′.

TUNEL assay

For detecting apoptosis in cells and tissues, we used the ApopTag® In Situ Apoptosis Detection Kit (Millipore). Apoptotic cells were detected by labeling and by modifying DNA fragments utilizing terminal deoxynucleotidyl transferase (TdT). Total and apoptotic cells were counted after immunoperoxidase and DAPI staining.

Real time quantitative PCR assay

TRI Reagent RT (Molecular Research Center) was added to cell pellets for RNA extraction according to the manufacturer's instructions. RNA was quantified with Xpose (Trinean). One microgram of RNA was used for reverse transcription with a high-capacity cDNA reverse transcription kit (Applied Biosystems/Life Technologies). Fast SYBR green master mix (Applied Biosystems/Life Technologies) and the ABI StepOne+ real-time PCR system (Applied Biosystems) were used to evaluate mRNA levels according to the manufacturer's recommendations. The following primers were used: For HAS2, forward, 5′-GAAACTTCCTTCCACGACCC-3′, and reverse 5′-GCACCGTACAGTCCAAATGAG-3′. For Beclin 1, forward 5'-AATCTAAGGAGTTGCCGTTATAC-3' and reverse 5'-CCAGTGTCTTCAATCTTGCC-3'; for LC3b, forward, 5'-ATTGCTGTCCCGAATGTCTC-3' and reverse 5'-CGTCCTGGACAAGACCAAGT-3'; for HPRT, forward 5'- TTGCTCGAGATGTGATGAAGGA-3' and reverse 5'- CCAGCAGGTCAGCAAAGAATT-3'. Incubation were 95 °C for 20 s, followed by 40 cycles of 3 s at 95 °C and annealing/extension for 30 s at 60 °C. Each sample was done in duplicate and data were analyzed using StepOne+ software version 2.3. Expression was normalized to HPRT.

LDL preparation and oxidation

Human LDL were isolated from pooled fresh sera by sequential ultracentrifugation, dialyzed, sterilized by filtration, and oxidized by UV-C irradiation. Mildly oxidized LDL were obtained by UV oxidation as previously described [11].

Cell viability and apoptosis

The cell viability was evaluated by the MTT assay [11]. Apoptotic/necrotic cells were counted by fluorescence microscopy after staining by fluorescent DNA intercaling agents SYTO-13 and propidium iodide (PI). Cells grown in 6-multiwell plates were incubated with permeant DNA intercalating green fluorescent probe SYTO-13 (0.6 μM) and the non-permeant DNA intercalating red fluorescent probe PI (15 μM), using an inverted fluorescence microscope (Fluovert FU, Leitz). Intact, apoptotic and necrotic cells were characterized on the basis of their morphological features.

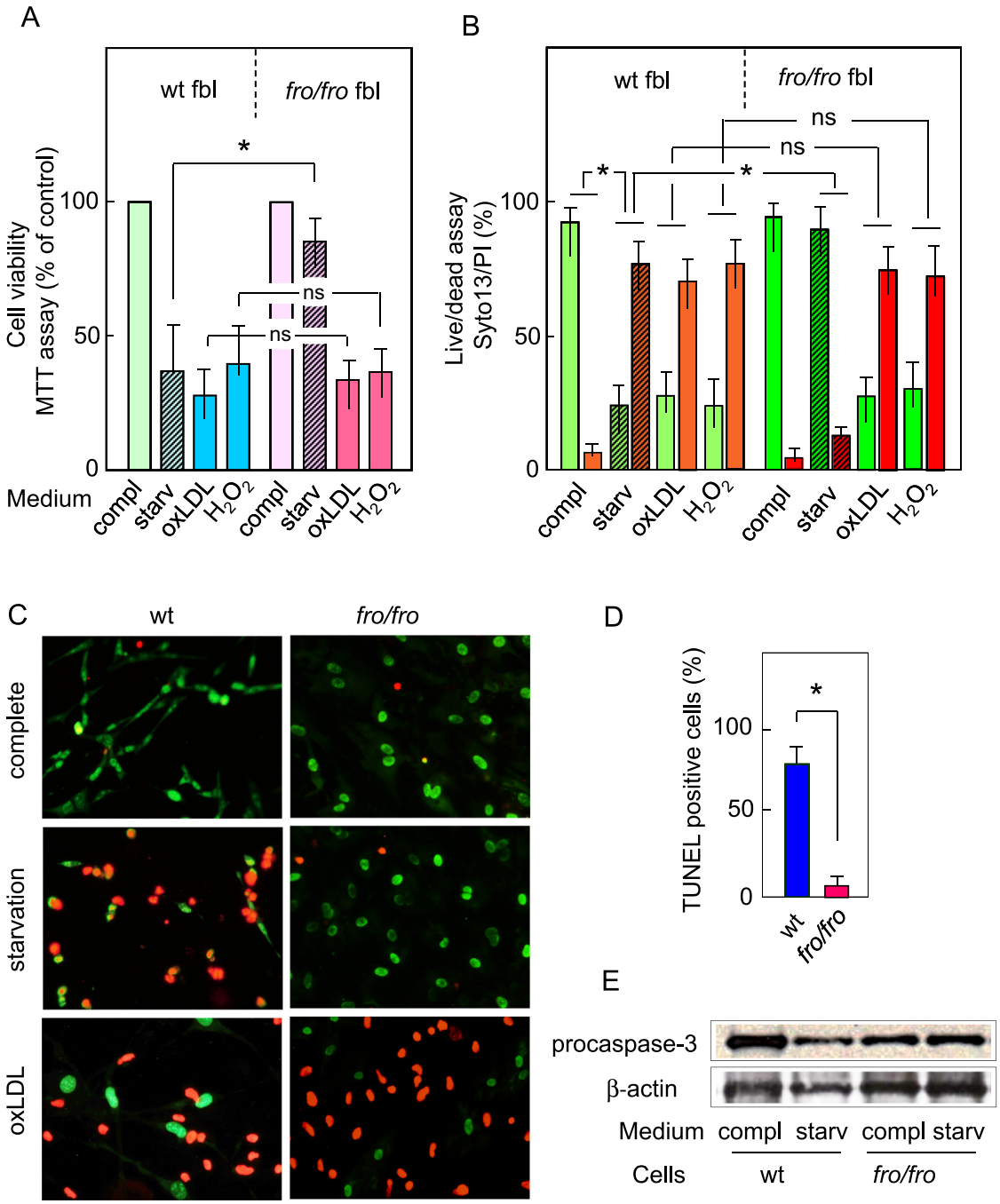

Fig. 1. Resistance of *fro/fro* fibroblasts to apoptosis induced by serum starvation, but not to oxidized LDL and H_2O_2. Sub-confluent fibroblasts from *fro/fro* (fro) or wt mice (wt) were incubated for 48 h in standard medium DMEM containing 10% FCS, 4.5 g/l glucose, pyruvate and GlutaMAX (ref. 61965-026, Life Technologies) (compl) or in serum/glucose/pyruvate-free DMEM culture medium containing L-glutamine (ref. 11966-025, Life Technologies) (nutrient deprivation condition) (starv, stripped bars), or in serum free RPMI, supplemented with oxidized LDL (200 μg apoB/ml) (oxLDL) or H_2O_2 (100 μM). At the end of 48 h incubation, cell viability was evaluated by the MTT assay (A). (B) Counting of apoptotic *vs* living cells after staining with Syto13/PI fluorescent probes which allow to distinguish between living cells (green bars), and apoptotic cells (primary apoptosis and post-apoptotic necrosis) (orange bars for wt, red bars for *fro/fro* fibroblasts). Apoptotic cells were counted, and are expressed as percent of the unstimulated control. C, Representative pictures of syto13/PI-stained wt and *fro/fro* fibroblasts. (D) TUNEL positive wt and *fro/fro* fibroblasts. Cells were incubated for 48 h in serum-free medium, and stained with the ApopTag® In Situ Apoptosis Detection Kit (Millipore), and counterstained with DAPI. The number of TUNEL positive cells is expressed as percent of the total cell number counted as DAPI stained fibroblasts. (E) Western-blot showing the degradation of procaspase-3, indicative of its activation. These data are a mean ± SEM of 5 separate experiments, $^*p < 0.05$.

Western blot analysis

Cultured cells were washed and scrapped in PBS, then disrupted at 4 °C in the extraction buffer (20 mM HEPES, 1 mM EDTA, 1 mM Na3VO4, 250 mM sucrose, 5 μM digitonin, 1 mM DTT, and 1 mM PMSF) for 15–30 min on ice. Cell extracts were centrifuged at 12,000*g* for 15 min (Beckman Optima) and the supernatant was used for Western-blot experiments. Protein concentration was determined using the Bradford reagent (Biorad). 50 μg of protein cell extracts were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membranes (Millipore). Then membranes were probed with the primary antibodies and revealed with secondary antibodies coupled to horseradish peroxidase using the ECL chemoluminescence kit (Amersham). β-actin was used to control equal loading of proteins.

SiRNA transfection

Murine fibroblasts were transfected with murine HAS2 or control siRNA using OptiMEM (Invitrogen) and HiPerFect reagent according to the manufacturer's recommendations.

Statistical analysis

Data are given as mean ± SEM. Estimates of statistical significance were performed by One Way Anova followed by multiple comparison analysis by Holm-Sidak method (SigmaStat software). Values of $p < 0.05$ were considered significant.

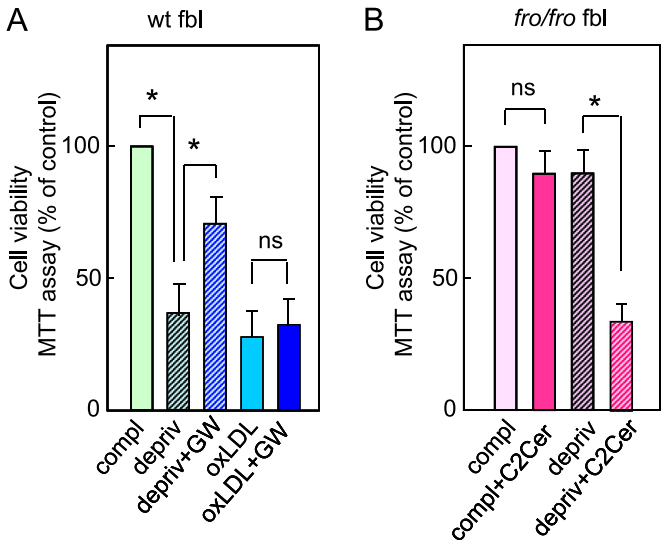

Fig. 2. Modulation of nSMase2 activity by the GW4869 inhibitor and ceramide level by C2Cer alters the cell viability under nutrient depletion conditions. Subconfluent fibroblasts from wt (A) or *fro/fro* (B) mice were incubated in standard medium containing 10% FCS (compl) or in nutrient deprivation serum/glucose/pyruvate-free DMEM culture medium (depriv), or in serum free RPMI, supplemented with oxidized LDL (oxL, 200 μg apoB/ml). In A, protective effect of the nSMase inhibitor GW4869 (GW, 10 μM) on cell death evoked by starvation (depriv) or oxidized LDL (oxLDL). In (B) effect of C2 ceramide (C2Cer, 5 μM). After 48 h, the toxicity was evaluated by the MTT test. The results are mean ± SEM of 6 experiments. *$p < 0.05$.

Results

nSMase2 and Cer are involved in the apoptotic process triggered by nutrient starvation, but not by hydrogen peroxide or oxidized LDL: fro/fro fibroblasts resist to cell death induced by nutrient deprivation

Wt and *fro/fro* fibroblasts were exposed to toxic concentrations of oxidized LDL (200 μg apoB/ml), hydrogen peroxide (H_2O_2, 100 μM) in serum-free RPMI (5 g/l glucose) or to nutrient deprivation in (glucose/pyruvate/serum)-free DMEM culture medium, for 48 h. As shown in Fig. 1A, cytotoxicity experiments, using the MTT assay, indicate that the cytotoxic effect of oxidized LDL or H_2O_2 is comparable in *fro/fro* and wt fibroblasts. In contrast, *fro/fro* fibroblasts strongly resisted to cell death evoked by nutrient starvation, showing more than 90% viability *vs* less than 30% for wt, after 48 h of contact with nutrient-free medium (Fig. 1A). Live-dead experiments using syto13/PI staining confirmed the resistance of *fro/fro* cells to apoptosis evoked by nutrient deprivation, in contrast to oxidized LDL that induced similar apoptosis in wt and in *fro/fro* cells (Fig. 1B and C). TUNEL staining (Fig. 1D) and procaspase 3 cleavage (Fig. 1E), confirmed that nSMase2-deficient *fro/fro* fibroblasts are more resistant to nutrient starvation induced-apoptosis than wt fibroblasts. It is to note that i/ the serum-free RPMI medium, in which oxidized LDL and H_2O_2 were added, was not toxic to wt fibroblasts for the period of the experiment, indicating that glucose and pyruvate starvation is the main trigger of cell death in wt fibroblasts, and ii/ oxidized LDL were toxic for *fro/fro* fibroblasts in the nutrient-free culture medium (data not shown).

The role of nSMase2 in cell death induced by nutrient starvation was confirmed by the effect of the sphingomyelinase inhibitor GW4869, which prevented (or delayed) the apoptotic effect of nutrient deprivation of wt fibroblasts (Fig. 2A). In contrast, GW4869 had no effect on cell death evoked by oxidized LDL in agreement with previously reported data [11]. In the same way, the resistance of *fro/fro* fibroblasts was reversed by the addition of the permeant C2-ceramide (5 μM) to the nutrient-starved medium (less than 25% cell viability) (Fig. 2B). No toxicity of C2-ceramide was observed in complete culture medium (Fig. 2B).

Altogether, these data indicate that i/ nSMase2 and ceramide are involved in cell death of wt fibroblasts evoked by nutrient starvation, but not by oxidized LDL, ii/ nSMase2-deficient *fro/fro* fibroblasts resist to nutrient-starvation induced cell death, but not to oxidized LDL.

Autophagy is not involved in the resistance to apoptosis induced by nutrient starvation in fro/fro fibroblasts

Autophagy and apoptosis processes are often associated, either through a cross inhibitory signaling, or sometimes through interconnected pathways [25,26]. Generally, autophagy is a survival system that blocks the induction of apoptosis, whereas activated caspases inhibit the autophagic process [26]. Moreover, ceramide is a potent autophagy inducer [27], *via* a downregulation of nutrient transporters [28]. This led us to investigate whether Cer generated by nSMase2 plays a role in autophagy induced by nutrient deprivation and in the mechanism of resistance of *fro/fro* fibroblasts.

As reported in Fig. 3, in *fro/fro* and wt cells, nutrient starvation elicited a strong conversion of LC3-I into LC3-II and an increase of Beclin-1 expression, both parameters being characteristic markers of autophagy activation. In contrast, the autophagic machinery was not activated by oxidized LDL, in *fro/fro* and wt cells (Fig. 3A and B). No difference was observed between *fro/fro* and wt fibroblasts, in the induction of autophagy parameters by nutrient starvation, this indicating that autophagy evoked by nutrient starvation does not require the nSMase2 activity.

Finally, autophagy markers are similarly induced by nutrient deprivation in wt and *fro/fro* cells, while these cells exhibit a great difference in their resistance to apoptosis induced by starvation. Thus, it may be concluded that, in this model, i/ ceramide is not required for autophagy marker induction by nutrient deprivation, ii/ autophagy alone plays no major role in the resistance of *fro/fro* cells to nutrient deprivation.

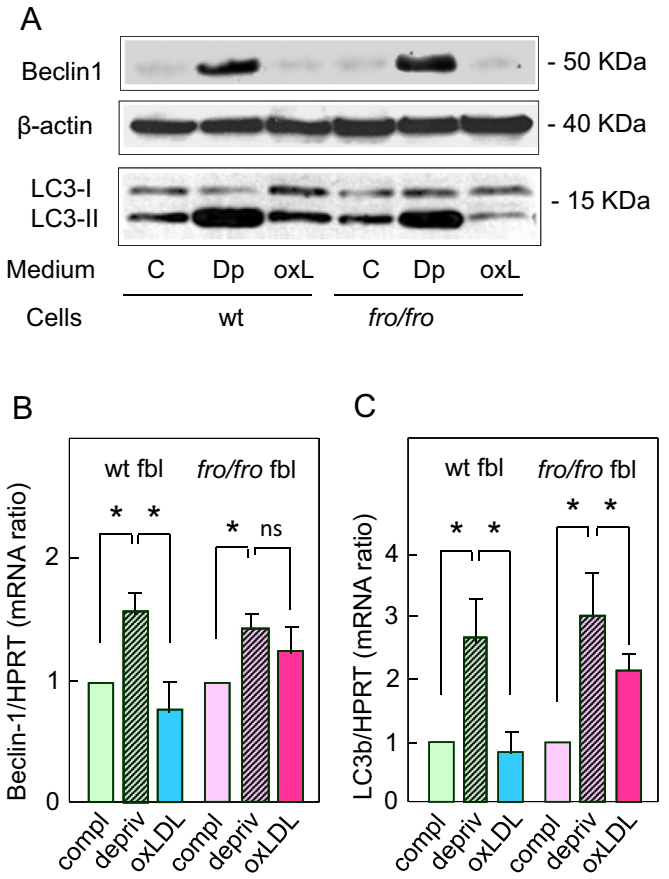

Fig. 3. Autophagy markers are similarly up-regulated in *fro/fro* and wt fibroblasts by nutrient deprivation. Fibroblasts were incubated for 48 h under nutrient starvation condition, or with oxidized LDL, as reported in the legend to Fig. 1. (A) Western-blot experiments showing the expression of Beclin-1 and the conversion of LC3-I to LC3-II, in complete medium (C), nutrient deprivation (Dp) and oxidized LDL (oxL) conditions. B. qPCR experiments showing the expression of LC3b and beclin-1, normalized to HPRT. The results are mean ± SEM of 3 separate experiments. $^{*}p < 0.05$.

Hyaluronan synthase 2 (HAS2) and hyaluronan mediate the resistance to apoptosis induced by nutrient deprivation in fro/fro fibroblasts

Fibroblasts from *fro/fro* mouse secrete high amounts of hyaluronan (HA), due to an increased expression and activity of the hyaluronan synthase 2 (HAS2), resulting from PP2A inhibition and Akt phosphorylation [29]. As HA and HAS2 protect fibroblasts against environmental stress-induced apoptosis [30], we checked whether HA, HAS2 and Akt are involved in the resistance of *fro/fro* cells to apoptosis induced by nutrient starvation.

As expected, HAS2 expression was much higher in *fro/fro* fibroblasts than in wt fibroblasts (Fig. 4A and B), in agreement with Qin et al. [29], but HAS2 expression was not (or only slightly) dependent on culture conditions, since HAS2 was high in *fro/fro* cells grown in complete medium and in nutrient starvation conditions, while HAS2 of wt fibroblasts was low in both culture media (Fig. 4A and B). HAS2 expression was dependent on Cer generated by nSMase2, as shown by treatment with C2-ceramide (5 μM) that decreased HAS2 expression in *fro/fro* fibroblasts (Fig. 4B).

We then checked whether HAS2 plays a role in the mechanism of *fro/fro* fibroblast resistance to nutrient deprivation. HAS2-specific siRNA transfected in *fro/fro* fibroblasts reduced the expression of HAS2 and decreased the resistance of *fro/fro* cells to apoptosis evoked by starvation (Fig. 4C and D). Likewise, methylumbelliferone (MU), a classical HAS2 inhibitor (1 mM), reversed the resistance of *fro/fro* fibroblasts to apoptosis induced by nutrient starvation (Fig. 4D). Conversely, the addition of HA to wt fibroblasts significantly improved their resistance to apoptosis induced by nutrient starvation (Fig. 5A–C). However, HA did not protect wt fibroblasts against cell death evoked by oxidized LDL, in accordance to the lack or resistance of *fro/fro* fibroblasts to oxidized LDL toxicity.

Finally, these data suggest that the increased resistance of *fro/fro* cells to nutrient starvation results from the rise of HAS2 expression and subsequent increased synthesis of HA induced by the deficiency of Cer generated by nSMase2 (deficient in *fro/fro* cells).

Hsp72 is involved in the protective effect of HAS2 in fro/fro fibroblasts

Previous report from Xu et al. [31] had shown that synovial cell death evoked by stress conditions (including serum starvation) in an arthritis model, is suppressed by hyaluronan *via* upregulation of stress-inducible heat-shock proteins of the HSP70 family. In agreement with this report, we found that Hsp72 expression is increased in *fro/fro* fibroblasts both in standard and nutrient-starved conditions (Fig. 6A). In contrast, Hsp72 expression was strongly decreased in cells transfected with the HAS2-specific siRNA, thus confirming that Hsp72 expression in *fro/fro* fibroblasts depends on HA and HAS2 (Fig. 6B). The protective role of Hsp72 was supported by the effect of the pharmacological Hsp70 inhibitor KNK437, which reversed the resistance of *fro/fro* fibroblasts to apoptosis evoked by serum starvation. No increased expression of Hsp72 was observed in wt fibroblasts and in *fro/fro* fibroblasts incubated with oxidized LDL (data not shown).

Taken together, these data indicate that HAS2 expression and HA secretion in *fro/fro* fibroblasts, resulting from Akt activation, are protective against cell death evoked by nutrient starvation.

Discussion

In this article, and as summarized in the graphical abstract, we show that nSMase2 is involved in apoptosis evoked by nutrient starvation, and this is protected in *fro/fro* fibroblasts mutant for nSMase2, *via* an increased expression of HAS2 and of Hsp72.

Role of nSMase2 in apoptosis induced by nutrient starvation

A first important point is that nSMase2 is involved in apoptosis induced by nutrient starvation, but not by oxidized LDL or H_2O_2. A number of studies have been focused on the apoptotic signaling of nSMase2, with controversial responses depending on the cell type, or the nature of the stressors [6]. We recently reported that nSMase2-deficient *fro/fro* mice, characterized by a strong neonatal growth retardation [17], do not resist to TNFα-induced hepatotoxicity, and fibroblasts isolated from these mice, do not resist to apoptosis evoked by oxidized LDL or TNFα ([11] and present article), suggesting that nSMase2 is not involved in acute stress-induced cell death. Here we show that *fro/fro* fibroblasts resist to cell death evoked by nutrient starvation in contrast to wt fibroblasts. Apoptotic cell death of wt cells is evidenced by the increased number of TUNEL positive cells, and by the morphological features of apoptosis observed using Syto13/PI staining, all these parameters being reduced in *fro/fro* fibroblasts. These data point out the different mechanisms of apoptosis evoked by nutrient starvation (protected in *fro/fro* fibroblasts) or by oxidized LDL (not protected in these cells). We previously reported that ceramide is not involved in apoptosis induced by oxidized LDL in endothelial cells, SMC and fibroblasts [32,33], which mainly depends on the deregulation of cytosolic calcium and the subsequent activation of the intrinsic mitochondrial apoptotic pathway [34,35]. In contrast, nSMase2 activation by oxidized LDL, and subsequent ceramide

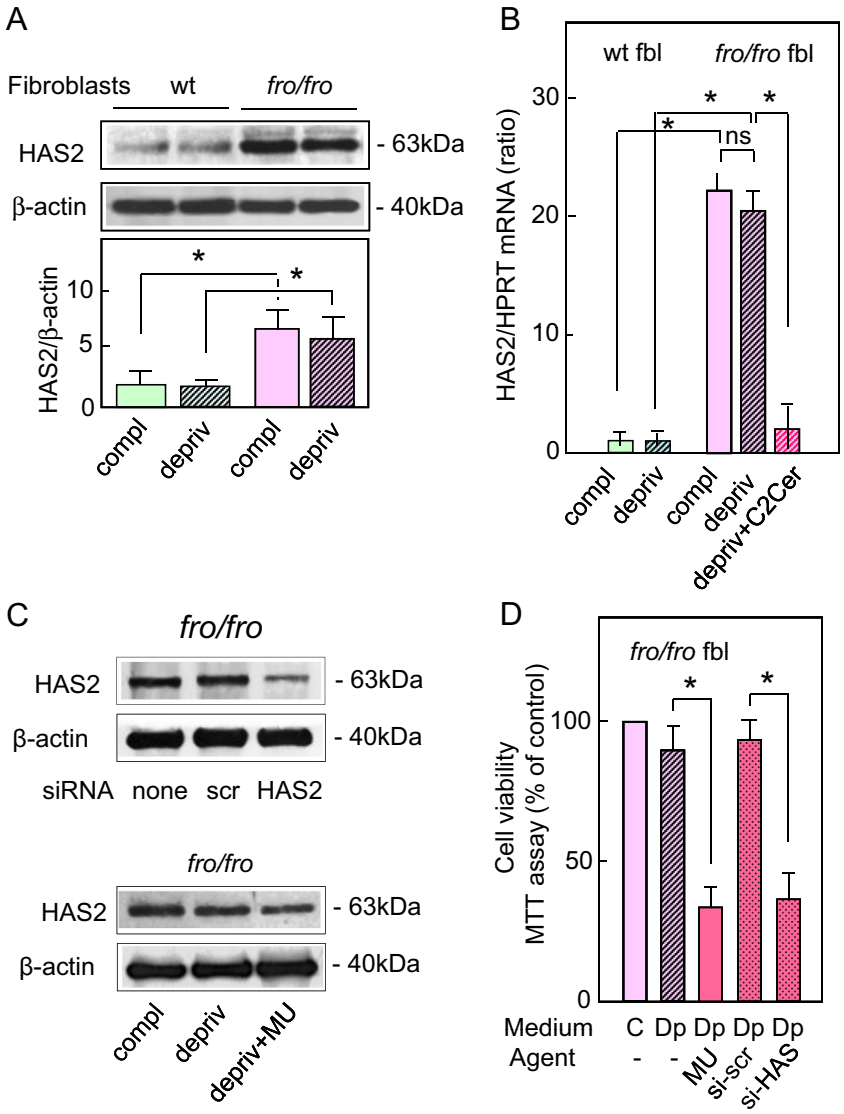

Fig. 4. HAS2 expression is dependent on nSMase2/Cer and is involved in the resistance of *fro*/*fro* to nutrient deprivation-induced apoptosis. (A) Western blot of HAS2 in wt and *fro/fro* fibroblasts grown in complete medium (compl) and nutrient deprivation conditions (depriv). (B) HAS2 mRNA expression evaluated by q-PCR experiments, and normalized to HPRT, in *fro/fro* and wt fibroblasts grown in complete medium (compl), nutrient derivation conditions (depriv). The effect of C2-ceramide (C2Cer, 5 μM), was tested in *fro/fro* fibroblasts, as indicated. (C) Western blot of HAS2 in *fro/fro* fibroblasts grown in complete medium and treated with scrambled siRNA or with specific HAS2 siRNA (upper panel) or in complete (compl) or nutrient deprivation (depriv) medium with or without methylumbelliferone (MU, 1 mM) (lower panel). (D) After 48 h incubation, the cytotoxicity was evaluated by the MTT test. Data are mean ± SEM of 5 separate experiments, $^{*}p < 0.05$.

generation, are involved in SMC proliferation, *via* the activation of a signaling cascade leading to the activation of sphingosine kinase-1 and the generation of the survival and mitogenic sphingolipid mediator, S1P [12]. Indeed, *fro/fro* fibroblasts and nSMase2-silenced SMC do not proliferate in the presence of oxidized LDL [11].

In contrast, our data show that in our model, the mechanism of apoptosis evoked by nutrient starvation involves nSMase2 since i/ *fro/fro* fibroblasts mutant for this nSMase2, resist to apoptosis, ii/ the addition of GW4869, an inhibitor of neutral SMases, protects wt fibroblasts against cell death induced by nutrient privation, but not that induced by oxidized LDL, iii/ the addition of C2-ceramide to *fro/fro* fibroblasts, reverses their resistance to cell death. Thus it can be hypothesized that ceramide released *via* the degradation of sphingomyelin by nSMase2, elicits apoptosis in nutrient-starved conditions.

Our data show that autophagy activation is not deficient in *fro/fro* fibroblasts, thus is probably not involved in the mechanism of cell death mediated by nSMase2. Apoptosis induced by serum or nutrient starvation, is often associated to autophagy, which is a survival mechanism, able to mediate a non-apoptotic cell death, when apoptotic pathways are blocked [36]. Ceramide is a potent autophagic cell death inducer, *via* a downregulation of nutrient transporters [28], thus it was hypothesized that autophagy could be defective in *fro/fro* fibroblasts, in which the ceramide generation is reduced. However, our data do not show any defect in the activation of autophagy markers such as the conversion LC3-I to LC3-II, or beclin-1 mRNA and protein expression, which suggests that ceramide is either not involved in the autophagic process evoked by nutrient starvation in these fibroblasts, or is generated by another SMase, such as the acidic SMase which modulates autophagy in several pathophysiological models for Alzheimer's disease, steatosis or atherosclerosis [37–39].

The resistance of fro/fro fibroblasts to nutrient starvation involves HAS2 increased expression

HAS2 is highly expressed in *fro/fro* fibroblasts, leading to an increased secretion of HA in the extracellular medium [29]. HAS2

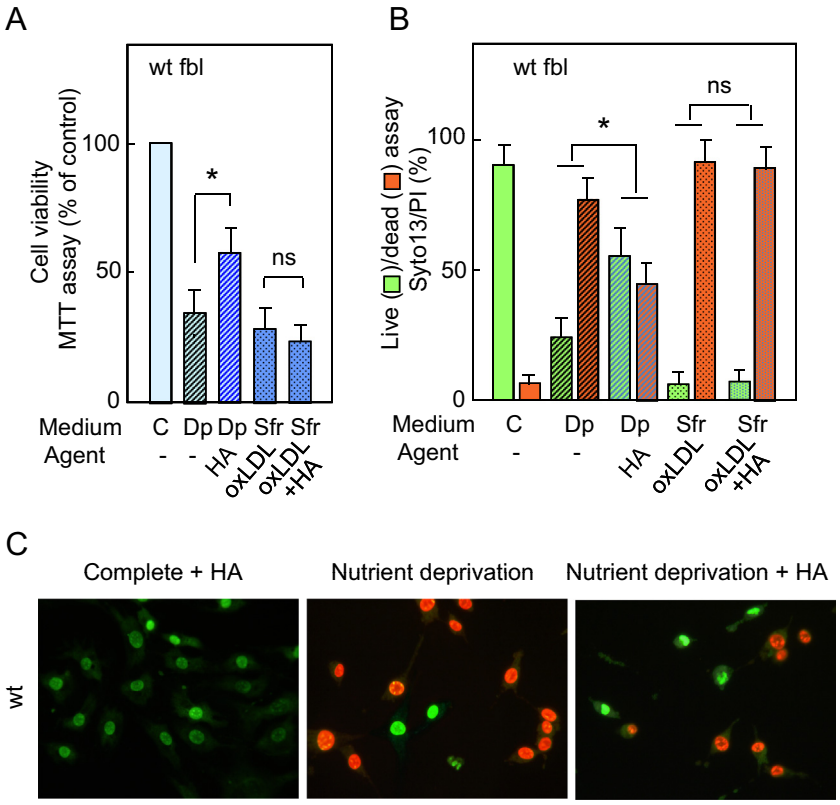

Fig. 5. Protective effect of exogenous hyaluronic acid in wt fibroblast grown under nutrient deprivation conditions. Cell viability of wt type fibroblasts grown for 48 h in complete medium (C), in nutrient deprivation medium (Dp) with or without exogenous high molecular mass hyaluronic acid (HA), or with oxidized LDL with and without HA, in serum free RPMI medium (Sfr). The cytotoxicity was evaluated by the MTT assay (A) or by the live/dead Syto13/PI assay (B), as explained in the Fig. 1. (C) representative pictures of syto13/PI stained cells. Data are expressed as mean ± SEM of 5 separate experiments, $*p < 0.05$.

protects against apoptosis evoked by environmental stress, such as serum starvation [30]. High molecular weight HA are antiapoptotic and protective in various pathophysiological conditions, such as human corneal epithelial cells [40], cornea protection and oxidative stress [41], trophoblasts [42], chondrocytes and synovial lesions [43,44]. We hypothesized that HAS2 contributes to the resistance of *fro/fro* fibroblasts in nutrient deprivation conditions.

In *fro/fro* cells, the high HAS2 expression depends on Akt activation, itself resulting from the decreased activity of PP2A, due to a reduced ceramide content [29]. Indeed, ceramide is a potent activator of PP2Ac/ceramide-activated protein phosphatases [45,46], which in turn inhibits Akt [47]. In *fro/fro* fibroblasts, the nSMase2/Cer deficiency in *fro/fro* cells prevents PP2A activation, thereby impeding Akt inactivation. Thus, Akt activation persists and supports the expression of HAS2 [29]. Under nutrient deprivation conditions, we found that HAS2 expression remains high in *fro/fro* fibroblasts. This high HAS2 expression is required for survival of *fro/fro* fibroblasts under deprivation conditions, as shown by the reversion of this resistance to deprivation by silencing of HAS2 by specific siRNAs and by inhibiting HAS2 with MU, in agreement with Wang et al. [30]. In the same way, the addition of HA protects in part wt fibroblasts against apoptosis induced by nutrient starvation. However this protective effect was not effective against the toxicity of oxidized LDL, which inhibited HAS2 expression in *fro/fro* fibroblasts.

Hsp72 expression is involved in HAS2-induced resistance of fro/fro fibroblasts to nutrient starvation

Heat-shock proteins (Hsps) are induced in response to various stressors including heat stress, toxic chemicals or modifications of cell environment, to suppress apoptosis [48]. HA are known to up-regulate the expression of heat-shock proteins from the Hsp70 family, particularly Hsp72, which may suppress cell degeneration and apoptosis in various models, such as canine arthritis [49], K562 cells exposed to hyperthermia and PC12 cells in serum deprivation conditions serum deprivation [31]. Our results show that Hsp72 expression is increased in *fro/fro* fibroblasts, and its inhibition, either by the specific pharmacological inhibitor KNK437, or by siRNA directed against HAS2, reversed the resistance of *fro/fro* cells, indicating that Hsp72 expression depends on HAS2 and is anti-apoptotic in nutrient-starved conditions, as reported [31].

In conclusion, the reported data show that nSMase2 is involved in cell death induced by nutrient deprivation, through a ceramide-dependent activation of PP2A that negatively regulates Akt activity, thereby reducing HAS2 and Hsp72 expression. In contrast, the data on *fro/fro* cells confirm that oxidized LDL-induced apoptosis occurs through ceramide-independent mechanisms, as previously reported [35,50–52]. Moreover, nutrient starvation triggers a robust increase of autophagy markers, which is independent of nSMase2/Cer and plays no major role in cell death induced by nutrient deprivation.

Finally, the functional link between nSMase2/Cer, Akt and HAS2 suggests that this pathway is involved in the protection against cell death induced by nutrient deprivation, by regulating not only the classical anti-apoptotic mechanisms mediated by Akt, but also the additional protective pathway mediated by HAS2, which is apparently required to prevent the apoptotic effect of nutrient deprivation.

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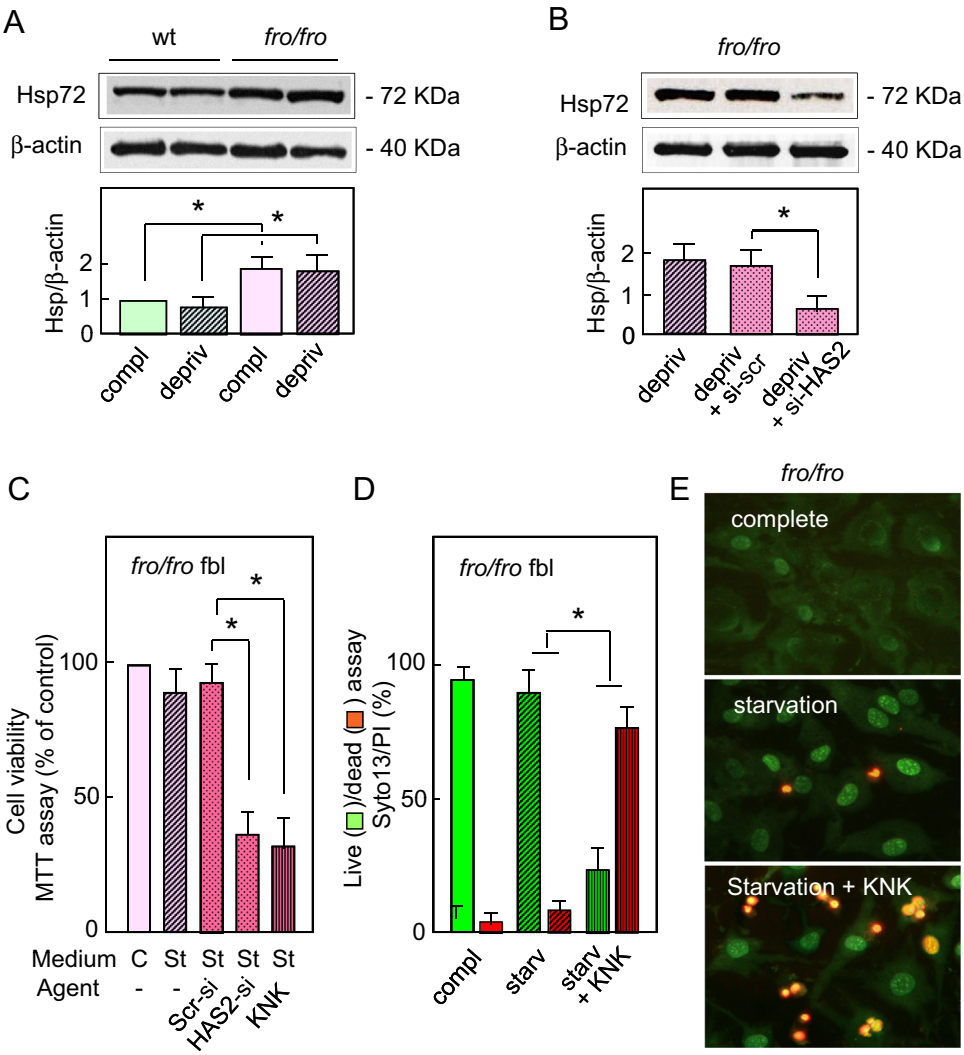

Fig. 6. Hsp72 expression is increased in a HAS2-dependent manner in *fro/fro* fibroblasts and is involved in their resistance to nutrient deprivation. (A) Expression of Hsp72 in wt and in *fro/fro* fibroblasts, after 48 h incubation in complete medium (compl) or in nutrient deprivation conditions (depriv). (B) Effect of scrambled (scr) siRNA or HAS2-specific siRNA on Hsp72 expression in *fro/fro* fibroblasts. (C,D) Effect of the specific Hsp72 inhibitor KNK437 (KNK, 5 μM), on the viability of *fro/fro* fibroblasts, in nutrient starvation conditions (St) evaluated by the MTT assay (C), and by counting the apoptotic cell number, after syto13/PI staining (D). These data are expressed as percent of the results obtained in complete medium (C), and are a mean ± SEM of 5 separate experiments, $^{*}p < 0.05$.

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